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ABSTRACT BOOK



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Glaucoma

GLA1 - Cell Plasticity, Fibrogenic Mechanisms and Glaucoma

The Role of AP-2 β in Anterior Segment Dysgenesis/ Fibrosis and Glaucoma

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Anterior segment dysgenesis (ASD) encompasses a group of developmental disorders in which a closed angle phenotype in the anterior chamber of the eye can occur and 50% of patients develop glaucoma. Many ASDs are thought to involve an inappropriate patterning and migration of the periocular mesenchyme (POM), which is derived from cranial neural crest cells (NCC) and mesoderm. Although, the mechanism of this disruption is not well understood a number of transcriptional regulatory molecules have previously been implicated in ASDs. Here we use a loss of function approach in mice to investigate the function of transcription factor AP-2 β , encoded by *Tfap2b*, which is expressed in the neural crest and its derivatives. Since full germ-line AP-2 β knockout mice are not viable past birth, we employed a knockout approach in which conditional deletion of *Tfap2b* was induced in the NCC using Wnt1-Cre. The resulting AP-2 β NCC mutant mice exhibited postnatal ocular defects typified by loss of opacity and fibrosis. Histological data revealed that the AP-2 β NCC mutants exhibited dysgenesis and fibrosis of multiple structures in the anterior segment of the eye including a lack of corneal endothelium, a hypercellular and thickened corneal stroma, fibrotic cataracts and a disruption in the iridocorneal angle with adherence of the iris to the cornea. We further show that this phenotype leads to a significant increase in intraocular pressure and a subsequent loss of retinal ganglion cells and optic nerve degeneration, features indicative of glaucoma. Overall, our findings demonstrate that AP-2 β is required in the POM for normal development and maintenance of the anterior segment of the eye and that the AP-2 β NCC KO mice may serve as a new and exciting model of ASD and glaucoma that is fully penetrant and with early post-natal onset.

Scleral and Lamina Cribrosa Remodeling in Response to Chronically Elevated IOP

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Glaucoma is a leading cause of blindness in the developed world, yet little is known about the mechanisms underlying its development or progression. Progressive remodeling of the load-bearing connective tissues (lamina cribrosa (LC) and sclera) of the optic nerve head (ONH) is a defining feature of glaucoma. This remodeling alters the microenvironment of the retinal ganglion cell (RGC) axons traversing the ONH and manifests at the macro-, micro-, and cellular scales. While intraocular pressure (IOP) is currently the only known modifiable risk factor for glaucoma, the disease can occur at statistically 'normal' IOPs and can progress despite IOP lowering.

Cell-driven progressive remodeling of the load-bearing ONH connective tissues (cupping and excavation of the LC and sclera) is the defining feature of glaucoma as it compares to other optic neuropathies. It is unknown how eye-specific differences in ONH structural stiffness and/or cellular reactivity increase glaucoma susceptibility or contribute to disease progression. IOP-related stress (force/cross sectional area) and strain (local deformation of the tissues) in the corneoscleral shell and ONH affect astrocytes, glia, LC cells, and RGC axons.

Regardless of the primary insult in glaucomatous injury, IOP-related stress and strain in the laminar connective tissues are key elements in the biomechanical paradigm of glaucoma, and remodeling of the ONH connective tissues play a large role in the ocular mechanical response to IOP. These dynamic changes to the connective tissues are significant, and include progressive, remodeling-driven posteriorization of the lamina within the neural canal that results in regional laminar insertion into the pia, changes in the material properties of the peripapillary sclera and LC, as well as changes in the density and orientation of the laminar beams as they remodel in response to IOP-related strain distributions. These studies show that IOP-driven remodeling is a dynamic process that changes the both the morphology and tissue stiffness of the LC and sclera.

Fibrotic Mechanisms in Glaucoma; Lamina Cribrosa and Trabecular Meshwork

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Glaucoma is an optic neuropathy that affects approximately 60 million people worldwide. There are extracellular matrix (ECM) changes associated with optic disc cupping in the optic nerve head (ONH) and subsequent visual field defects. Fibrosis occurs as a build-up of ECM materials in the trabecular meshwork (TM) at the anterior of the eye, and in the lamina cribrosa (LC) at the ONH. This fibrotic mechanism plays a role in disease progression; ECM materials build up in the TM, the aqueous humor cannot easily exit via its normal pathway and intraocular pressure (IOP) increases. Elevated IOP is one of the main risk factors associated with the development and progression of glaucoma and is the only target for therapies in clinical use. There are however few if any therapies combatting fibrosis.

Here, we describe our studies detailing cellular mechanisms for regulating fibrosis, specifically epigenetics, in age-matched human primary LC and TM cells from normal (N) and glaucoma (G) donors. Previous studies has shown that DNA methylation has been shown to be altered by hypoxia and in turn regulate expression of the pro-fibrotic transforming growth factor (TGF) β 1 and the anti-fibrotic Ras protein activator like 1 (RASAL1). There is also evidence for a hypoxic environment in glaucoma. Firstly, we wished to examine the Global DNA methylation (GDM) profile and expression of DNA methyltransferases in LC and TM cells and the expression of TGF β 1, RASAL1 in GTM cells when compared to NTM cells. Secondly, we wished to determine if hypoxia could induce a glaucomatous-like phenotype in NTM cells. Further, we wished to delineate the association between TGF β 1, RASAL1 and DNA methylation in glaucoma.

Data showed there is a significant increase ($P < 0.05$) in GDM levels between normal and glaucomatous cells and that GTM cells have differential ($P < 0.05$) gene and protein (TGF β 1 & RASAL1) expression profiles compared to NTM cells. Exposure of NTM cells to hypoxia (1%O₂) was shown to induce a similar phenotype to that observed in GTM cells with regard to GDM levels and protein expression (TGF β 1 & RASAL1). Inhibitor (5-azacytidine), siRNA (TGF β 1)

and recombinant TGF β 1 treatment studies indicate that TGF β 1 and RASAL1 have an interacting & dependent role in glaucoma, which could perpetuate the observed fibrosis. This study may provide data required to embark on the use of chromatin modifying intervention in counteracting the fibrotic pathology integral to glaucoma.

Mechanical Homeostasis Mechanisms in Trabecular Meshwork Cells

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As most cell types, trabecular meshwork (TM) cells are mechanosensitive. TM cells respond to dynamic stress as well as to extracellular matrix (ECM) stiffness by adaptation of protein expression patterns. ECM stiffness-dependent modulation of gene expression was reported for various molecules such as smooth muscle actin (SMA), myocilin, cochlin, transglutaminase-2 or fibronectin in TM cells. Furthermore, responses to growth factors such as TGF- β 2 are strongly influenced by cell substratum stiffness. Soft substrates were shown to enhance TGF- β 2-induced PAI-1 and collagen-VI transcription while SMA and collagen-I transcription responses were attenuated. Tissue stiffness is largely determined by ECM structure and composition which are mainly controlled and maintained by the cells inhabiting the tissue. Most ECM components, with the exception of elastin, are subject to life-long turnover. It is thus remarkable that TM stiffness in normal eyes appears rather constant with age. In contrast, primary open-angle glaucoma (POAG) was shown to be associated with increased TM stiffness. Unfortunately, the mechanisms governing TM mechanical homeostasis in health as well as their disturbance in POAG are only poorly understood. Preliminary observations indicate that the expression of regulatory micro-RNAs (miRNA) is governed by ECM stiffness. In equilibrium states, collagen and fibronectin transcription levels in TM cells were similar on substrates of different stiffness. At the same time, expression levels of miR-21, -27b, -29, -100, -125 were increased on stiff substrates, while miR-92a and -423 showed a reverse distribution. MiR-29 targets several ECM molecules, is modulated by TGF- β and has a role as an antifibrotic modulator. Since single miRNAs can alter the expression levels of a multitude of proteins, miRNAs appear well-suited to promote mechanical homeostasis. Mechanosensitive miRNA expression patterns may also govern ECM stiffness-dependent adjustments of responses to growth factors and mechanical challenges.

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Effects of Cytokines on Aqueous Outflow and Fibrogenic Activity of Trabecular Meshwork Cells

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Various cytokines are included in aqueous humor, and the evidences of their functions related to glaucoma pathology have been accumulated. Previously, we reported the elevated levels of multiple cytokines, such as interleukin (IL)-6, IL-8, and monocyte chemoattractant protein(MCP)-1, in aqueous humor of open-angle glaucoma patients. Recently, we also found higher levels of those cytokines in uveitic glaucoma and neovascular glaucoma, and clarified the relations between cytokine levels and other clinical factors. Of those cytokines, MCP-1 increased aqueous outflow facility in the perfusion model, whereas survival analysis showed that MCP-1 was a prognostic factor for surgical success of trabeculectomy.

Of the quantified aqueous cytokines, we found that the production of IL-6 from cultured trabecular meshwork (TM) cells was increased after treatment with transforming growth factor (TGF)- β 2, a well-known fibrogenic factor. Pretreatment with IL-6 before stimulation with 2.5 ng/ml TGF- β 2 did not significantly change the downstream targets of TGF- β 2, such as α -SMA expression, actin polymerization, Smad2 promoter activity, and the phosphorylation levels of Smad2, MLC2, and p38. Additional pretreatment with soluble IL-6 receptor partly suppressed the downstream targets of TGF- β 2 accompanied with increased phosphorylation of STAT3. On the other hand, induction of STAT3 siRNA also attenuated the activity of TGF- β 2 signaling in TM cells. The complicated regulatory mechanisms between TGF- β 2 signaling and IL-6 signaling in TM cells will be addressed.

Trabecular Meshwork Cell Plasticity, Fibrogenic Activity and IOP

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Primary open angle glaucoma, a leading cause of irreversible blindness worldwide is associated with elevation of intraocular pressure (IOP) resulting from increased resistance to aqueous humor (AH) outflow through the trabecular meshwork (TM). To identify the etiological mechanisms responsible for increased resistance to AH outflow, we have been investigating the

hypothesis that dysregulation of TM contractile activity triggered by increased levels of Rho/Rho kinase activators such as TGF- β 2, lysophosphatidic acid (LPA), endothelin-1 or by mechanical strain drives the conversion of TM and Schlemm's canal cells into matrix producing myofibroblast-like cells, through a process involving endothelial-to-mesenchymal transition (EndMT). Acceleration in EndMT may subsequently trigger fibrogenic responses, ECM accumulation, stiffening of the juxtacanalicular tissue and increased resistance to AH outflow through the trabecular pathway. Our ongoing studies using human TM cells revealed that hypercontractility of TM cells induced by TGF- β 2, LPA (lysophosphatidic acid), GDF-15, RhoAV14 and endothelin-1 augments expression of various fibrogenic markers including α -smooth muscle actin, fibronectin, Hic-5, FSP-1 (fibroblast specific protein) and collagen-1. Increased expression of these fibrogenic markers was found to be associated with elevated levels of transcription factors involved in cell fate transition including Twist, Slug and Snail. Moreover, SRF/MRTF, YAP/TAZ and SMAD transcription factors acting downstream of Rho/Rho kinase signaling and F/G actin appear to regulate the fibrogenic activity and cell plasticity of TM cells. Importantly, inhibition of the Rho/Rho kinase pathway suppresses the fibrogenic activity of TM cells. In addition to the studies carried out with TM cells, an in vivo rodent model exhibiting RhoAV14 induced ocular hypertension also demonstrated a Rho kinase-dependent increase in fibrogenic activity in the trabecular meshwork. Taken together, these studies suggest that altered TM cell plasticity leading to EndMT transition and increased fibrogenic activity in the trabecular pathway can lead to elevated IOP, and that targeting the pro-fibrotic pathways in TM may offer a means to lower IOP. Supported by the NIH grants (R01EY018590 & R01EY025096).

GLA2 - Major identified players in glaucoma – genes and signaling pathways

Autophagy in Ocular Hypertension and Glaucoma: More than Keeping the House Clean

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Malfunction of the trabecular meshwork (TM)/Schlemm's canal (SC) conventional outflow pathway is associated with elevated intraocular pressure (IOP) and, therefore, increased risk of developing glaucoma, a potentially blinding disease affecting more than 70 million people worldwide. This TM/SC tissue is subjected to different types

of stress, including mechanical, oxidative, and phagocytic stress. Long-term exposure to these stresses is believed to lead to a progressive accumulation of damaged cellular and tissue structures causing permanent alterations in the tissue physiology, and contribute to the pathologic increase in aqueous humor (AH) outflow resistance. Autophagy is emerging as an essential cellular survival mechanism against a variety of stressors. In addition to performing basal functions, autophagy acts as a cellular survival pathway and represents an essential mechanism by which organisms can adapt to acute stress conditions and repair stress-induced damage. A decline in autophagy has been observed in most tissues with aging and has been considered responsible, at least in part, for the accumulation of damaged cellular components in almost all tissues of aging organisms. Dysfunction in the autophagy pathway is associated with several human diseases, from infectious diseases to cancer and neurodegeneration. Here, we will summarize our current knowledge of the emerging roles of autophagy in outflow tissue physiology and pathophysiology, including novel evidence suggesting compromised autophagy in the glaucomatous outflow pathway.

Homeostatic Regulation of IOP by Nitric Oxide

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Nitric oxide (NO) is potent regulator of conventional outflow facility, a primary determinant of IOP. Hence, NO-donating drugs are in clinical trials for the treatment of ocular hypertension and glaucoma. Like other endothelia, NO production by Schlemm's canal (SC) cells is regulated by shear stress, a consequence of circumferential flow of aqueous humor through SC lumen. Interestingly, SC is a collapsible vessel, such that increasing IOP narrows SC lumen and increases the magnitude of shear stress acting on SC cells. This mechanism allows IOP-dependent production of NO, providing a homeostatic feedback loop to regulate outflow facility and IOP. To investigate this feedback mechanism, we developed NO-sensitive microbeads that we validated using human umbilical vein endothelial cells exposed to physiological levels of shear stress. To investigate NO production in an intact outflow system, we perfused the NO-sensitive microbeads into the trabecular meshwork (TM) of C57BL/6 mice. Results show that there was a concomitant increase in the microbead signal with increasing IOP, consistent with IOP-dependent

NO release and retrograde diffusion into the TM. NO, in turn, likely increases outflow facility by two known mechanisms, decreasing the contractility of TM cells and the volume of SC cells. Theoretically, this feedback mechanism would be disrupted by glaucomatous alterations in TM stiffness that suppress IOP-induced changes in SC caliber. Thus, targeted NO delivery to the downstream TM and inner wall endothelium of SC in glaucomatous eyes may help restore NO-mediated homeostatic regulation of outflow facility.

The Role of Trabecular Meshwork Cadherins in Regulation of IOP

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Purpose: The molecular mechanisms for pathogenic damage to the aqueous humor outflow pathway responsible for IOP elevation in POAG are poorly understood. Expression of both TGF β 2 and the Wnt antagonist SFRP1 are elevated in the aqueous humor and TM of POAG eyes. Increased expression of either TGF β 2 or SFRP1 leads to increased outflow resistance and elevated IOP in ex vivo and in vivo models. However, the molecular mechanisms responsible for TGF β 2 as well as Wnt regulation/dysregulation of IOP are still unknown. The purpose of this study is to determine whether TGF β 2 and Wnt regulation of TM cadherins mediates ocular hypertension.

Methods: Cadherin expression in TM cells and tissues was examined using exon microarrays and by qPCR. Normal human TM cells were exposed to TGF β 2, Wnt3a, SFRP1, or vehicle control and effects on β -catenin translocation to the nucleus and cadherin expression were determined by western immunoblotting and immunofluorescent microscopy. To determine effects on IOP, TM tissues of mouse eyes were transduced with Ad5 vectors expressing activated TGF β 2, SFRP1, and/or specific cadherins.

Results: The major cadherins expressed by TM cells and tissues are cadherins (CDH) 6, 11, 19, 2, and 1. Both TGF β 2 and Wnt3a increased nuclear translocation of β -catenin. TGF β 2 increased the expression of CDH1 in cultured HTM cells. Wnt3a increased nuclear translocation of β -catenin and increased CDH6 expression in HTM cells, while SFRP1 had the opposite effect. Over expression of TGF β 2 or CDH1 significantly elevated IOP in mouse eyes. SFRP1-induced ocular hypertension was significantly inhibited by concomitant expression of CDH6, while overexpression CDH6 alone had no appreciable effect on IOP.

Conclusion: IOP is regulated by expression of specific

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cadherins, with CDH1 increasing IOP and CDH6 decreasing SFRP1-induced IOP elevation. Cadherins may act as mechanotransducers in the TM to regulate aqueous humor outflow.

Neuroprotection and Optic Nerve Regeneration for Glaucoma Therapy: Contribution of Oxidative Stress in Animal Models of Normal Tension Glaucoma

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Glaucoma is the major cause of blindness, and 70% of glaucoma patients suffer from normal tension glaucoma (NTG) in Japan. In addition to high intraocular pressure (IOP), oxidative stress, glutamate neurotoxicity etc. may be involved in the pathogenesis of retinal ganglion cell (RGC) death and therefore, development of therapies based on neuroprotection may achieve promising results. We have previously reported generation of NTG mouse models, which are glutamate transporter (GLAST or EAAC1) knockout mice. We evaluated therapeutic potential of some agents with neuroprotective properties in these mice, in which effects of drugs and gene manipulations can be evaluated in a short period (1~2 months). For example, valproic acid, widely prescribed for treatment of epilepsy, mood disorders and migraines, has antioxidant effects and protects RGCs in NTG models. In addition, spermidine, a polyamine compound found in ribosomes and living tissues, has strong antioxidative effects and delayed the time course of retinal degeneration and stimulates axon regeneration after optic nerve injury. For the stimulation of axon regeneration, we are interested in the function of Dock3, a member of atypical guanine exchange factors (GEFs) that activates the Rho GTPases Rac1. Dock3 is expressed in the central nervous system and we recently showed that Dock3 plays a role in protecting RGCs from neurotoxicity and oxidative stress as well as in promoting optic nerve regeneration (*Prog Retin Eye Res* 43:1-16, 2014). Finally, I would like to introduce some of our recent progress on glaucoma research utilizing marmosets, a non-human primate.

Myocilin: Ocular and Extraocular Functions

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The *Myocilin* (*MYOC*) gene encodes a secreted glycoprotein and is expressed in ocular and non-ocular tissues. Mutations in *MYOC* may lead to juvenile and adult-onset primary open-angle glaucoma. However, no other pathologies beside glaucoma have been reported in patients carrying *MYOC* mutations. Published data suggests that the loss of functional myocilin does not impede normal eye function, nor is it critical for the development of glaucoma. However, careful analysis of *Myoc* null mice demonstrated that the absence of myocilin negatively affects myelination of the optic and sciatic nerves. In addition, cortical bone thickness and trabecular volume, as well as the expression level of osteopontin in the femur were dramatically reduced in *Myoc* null mice compared with wild-type mice. Expression of the human Y437H myocilin mutant in the eye of transgenic mice sensitized trabecular meshwork cells to oxidative stress in comparison to their wild-type littermates. The application of oxidative stress to these transgenic mice through genetic manipulations led to a more severe glaucoma-like phenotype. To gain better insight into the molecular mechanisms underlying myocilin action in different eye structures, a shotgun proteomic approach was used to identify proteins interacting with myocilin. Lysates of adult mouse eyes with removed lenses were used for immunoprecipitation using polyclonal rabbit antibodies against mouse myocilin. Several proteins have been identified that were present in the immunoprecipitates of wild-type but not *Myoc* null mice. One of these proteins was tissue inhibitor of metalloproteinases-3 (TIMP3), a known inhibitor of matrix metalloproteinases (MMP). The C-terminal part of myocilin is sufficient for interaction with TIMP3 and some mutations in this region reduced its interaction with TIMP3. In the eye tissues, myocilin and TIMP3 were co-localized in the retinal pigmented epithelial cells, Bruch's membrane, trabecular meshwork, sclera, and ciliary body epithelium. Inhibitory effects of TIMP3 on MMP2 activity were enhanced by addition of myocilin, suggesting a modulatory role to the myocillin-TIMP3 interaction. In summary, these data demonstrate that myocilin has multiple functions in ocular and non-ocular tissues. Myocilin may play a role not only in glaucoma but in other pathologies including Sorsby fundus dystrophy and age-related macular degeneration through the modulation of metalloproteinase activity.

GLA3 - Restoring Conventional Outflow

Molecular and Cellular Components of Compromised and Restored IOP Homeostasis in Glaucoma

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Elevated intraocular pressure (IOP) is the primary risk factor and currently the only treatable parameter for glaucoma. Previously, we established that most people do not develop glaucoma primarily because of a robust IOP homeostasis mechanism. Sustained pressure increases or decreases are sensed by outflow pathway cells and trigger corrective adjustments in the outflow resistance, which restore IOP to within a narrow safe range. We hypothesized that the ability of the outflow pathway to mount a successful IOP homeostatic pressure response and make these corrective outflow resistance adjustments is the key and central factor in most, if not all types of glaucoma. Alvarado determined that trabecular meshwork (TM) cellularity was significantly reduced in the eyes of persons with glaucoma, but no experimental cause-and-effect relationship between TM cellularity and outflow facility had been shown. Recently, we showed that controlled chemical reduction of TM cellularity by approximately 30% did not directly impact outflow facility, but did obliterate the IOP homeostatic response to pressure challenge. Transplanting TM cells to these depleted anterior segments restores the IOP homeostatic response. This supports the significance of Alvarado's observation, but no direct relationship between IOP homeostasis and glaucoma has yet been established. Here we compared the ability of normal and glaucomatous human outflow pathways, in perfused anterior segment organ culture, to adjust the outflow resistance in response to a homeostatic pressure challenge. Although the outflow pathway from normal eyes was able to respond to this sustained pressure challenge and correctively adjust the outflow resistance, the outflow pathway from glaucomatous eyes failed to do so. This supports the contention that the inability to mount a corrective IOP homeostatic response to sustained pressure fluctuations is a hallmark of the glaucomatous anterior segment. Correction of the IOP homeostasis response to sustained pressure fluctuations may be the ultimate treatment for glaucoma.

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Prevention of Glaucoma Phenotypes through iPSC-TM Transplantation *in vivo*

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Loss and dysfunction of trabecular meshwork (TM) cells frequently accompanies primary open angle glaucoma. It has been hypothesized that replacing TM cells in the tissue may lead to the reestablishment of intraocular pressure (IOP) control. In order to test this prediction, we developed methods to induce a TM cell- like phenotype in induced pluripotent stem cells (iPSC). These cells, designated iPSC-TM, were then transplanted into the TM by injection into the anterior chamber of transgenic mice expressing a pathogenic allele of human myocilin (Tg-MYOC^{Y437H}) (N=20). The IOP was monitored by rebound tonometry and the aqueous humor outflow facility was determined for up to nine weeks after transplantation. Immunohistochemical approaches were employed to determine the number of TM cells as well as that of the surviving retinal ganglion cells (RGC).

Our findings demonstrate that, compared to sham injected controls, transplantation of iPSC-TM results in a significant drop of IOP (16.4 vs 12.2 mmHg, P=0.0006) and increased aqueous humor outflow facility (0.027±0.01 vs 0.011±0.005 µl/min/mmHg, P=0.017). The improved aqueous humor dynamics resulted in enhanced RGC survival in this animal model (2222 vs 1707 RGC/mm², P=0.041). Finally, a dramatic increase in TM cellularity was noted (54.9 vs 25.7 TM/section, P=6.83E-06). However, contrary to our expectation, only very few iPSC-TM were detectable within the TM, suggesting that the increase cellularity results from proliferation of endogenous TM cells. This assumption was supported by gene expression analysis indicating transcriptional activation of cell-cycle genes. Furthermore, *in vitro* experiments indicate that co-culture of primary TM cells and iPSC-TM induces enhanced growth rates in the former.

In vivo these newly derived TM cells appear to be healthy, as revealed by transmission electron microscopy. TM cells

Glaucoma

in iPSC-TM transplanted Tg-MYOC^{Y437H} mice do not display the distorted endoplasmic reticulum that is typically observed in the TM cells of transgenic control mice.

These data demonstrate that restoration of TM cellularity in this mouse model does improve function of this tissue and suggests that cellular therapy for primary open angle glaucoma may be feasible.

From Fistula to Reconstruction

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Glaucoma is a worldwide leading cause of irreversible vision loss. Surgery is an important therapy for glaucoma. Trabeculectomy, a fistula surgery, is still one of the most popular glaucoma surgeries. There are several severe complications related to trabeculectomy. Recently, several micro-invasive glaucoma surgeries were invented, including trabectome, canaloplasty, and iStent. We performed canaloplasty which is an aqueous outflow system reconstruction surgery since 2009. Our clinical data showed that canaloplasty is safer than trabeculectomy. In addition, we found that IOP was still controlled for patients who received canaloplasty without Descemet's window and intrascleral lake. Canaloplasty is also effective for glaucoma patients following failed trabeculectomy. Recently, we designed a relay needle method for canaloplasty and expanded its indication to POAG patients with disrupted Schlemm's canal following failed trabeculectomy.

Therapeutic Effect Analysis on the Treatment of Congenital Glaucoma through Modified Combined Trabeculotomy-trabeculectomy

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Aim: To evaluate the therapeutic effect and the safety of the treatment of congenital glaucoma through modified combined trabeculotomy-trabeculectomy.

Methods: The clinical data of 27 cases (altogether 42 eyes), which included 7 cases of infants (10 eyes) and 20 cases of teenagers (32 eyes), of congenital glaucoma undertook modified combined trabeculotomy-trabeculectomy were analyzed retrospectively. The parameters evaluated include the post operation visual acuity, the anterior

chamber, the filtering bleb, the intraocular pressure, the C/D ratio, visual field, the retinal nerve fiber layer changes and the complications.

Results: The follow-up period was 1 to 29 mo, averaging 13.3±7.7 mo. Upon last visit after the operation, functional filtering blebs developed in all the involved eyes. The intraocular pressure was controlled under 21 mm Hg, which was decreased by 60% when compared with that before the operation, without using any medication. There were no significant changes in the post operation visual acuity and the retinal nerve fiber layer thickness before and after the operation in teenager group ($P>0.05$), and both the post operation C/D ratio and the visual field mean defect (MD) were reduced compared with those before the operation ($P<0.05$). There were no severe complications in any of the patients.

Conclusions: The modified combined trabeculotomy-trabeculectomy can effectively reduce the intraocular pressure and control the development of glaucoma in cases of congenital glaucoma. It is a safe and effective operative method for the treatment of congenital glaucoma.

Keywords: Congenital glaucoma; trabeculotomy; trabeculectomy

Measuring Discrete Outflow Enhancement

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Focal outflow and its enhancement by microincisional glaucoma surgeries has only been modeled mathematically but not been measured directly. We refined recently introduced methods of quantitative canalography and used them to measure conventional outflow enhancement following plasma-mediated ab interno trabeculectomy (AIT). Gonioscopic, plasma-mediated ab interno trabeculectomy was established in enucleated pig eyes. We developed a program to automatically quantify outflow changes using a fluorescent tracer reperfusion technique. Trabecular meshwork (TM) ablation was demonstrated with fluorescent spheres in 6 eyes before formal outflow quantification with two dye reperfusion canalograms in 6 further eyes. Eyes were perfused with a central, intracameral needle at 15 mmHg. Canalograms and histology were correlated for each eye.

The pig eye provided a model with high similarity to AIT

in human patients. Histology indicated ablation of TM and unroofing of most Schlemm's canal segments. Spheres highlighted additional circumferential and radial outflow beyond the immediate area of ablation. Differential canalograms showed that AIT caused an increase of outflow of 17 ± 5 fold inferonasally (IN), 14 ± 3 fold superonasally (SN) and also an increase in the opposite quadrants with a 2 ± 1 fold increase superotemporally (ST) and 3 ± 3 inferotemporally (IT). Perilimbal specific flow image analysis showed an accelerated nasal filling with an additional perilimbal flow direction into adjacent quadrants.

In 41 additional eyes, trabecular micro-bypass (TMB) resulted in insignificant ($p>0.05$) outflow increases of $13\pm 5\%$, $14\pm 8\%$, $9\pm 3\%$, and $24\pm 9\%$ in the inferonasal, superonasal, superotemporal, and inferotemporal quadrants while AIT caused a $100\pm 50\%$ ($p=0.002$), $75\pm 28\%$ ($p=0.002$), $19\pm 8\%$, and $40\pm 21\%$ increase in those quadrants. AIT eyes had a 7.5 ($p=0.01$), 5.7 ($p=0.004$), 2.3, and 1.8-fold greater outflow enhancement than matching quadrants of paired TMB-implanted eyes. Quantitative canalography demonstrated that TMB, when successful, provided focal outflow enhancements, while AIT achieved a more extensive access to outflow pathways including and beyond the surgical site itself. When applied as a glaucoma surgical teaching model, objective outflow data could be correlated to a number of training eyes per surgeon.

Trabecular Meshwork Regeneration by Stem Cells

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Glaucoma is the second leading cause of irreversible blindness worldwide. Elevated intraocular pressure (IOP) and aging are the most important risk factors for most forms of glaucoma. IOP level is highly dependent on aqueous humor dynamics. The resistance of aqueous humor outflow rate is mainly localized within the trabecular meshwork (TM) of the conventional outflow pathway. Reduced cellularity and abnormal extracellular matrix within the TM is observed in glaucomatous conditions and correlates with elevated IOP. Restitution of the TM cell population may improve aqueous outflow facility and lower IOP, hence prevent glaucomatous blindness.

We have successfully isolated and characterized stem cells from trabecular meshwork (TMSCs); shown that TMSCs can home to the TM tissue and laser damaged TM tissue. Other stem cell types, such as induced pluripotent stem cells (iPSCs), adipose-derived stem cells (ADSCs)

and corneal stromal stem cells (CSSCs) were successfully induced to differentiate into TM cells in vitro. These stem cells were able to home to the TM region and reduce IOP in vivo.

All these suggest a potential of autologous and allogeneic stem cell therapy for TM regeneration and controlling IOP, a new treatment for glaucoma.

GLA4 - ONH/NFL imaging in glaucoma

Inner Retinal Layer Imaging in Glaucoma

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Speckle-noise reduction technique in spectral-domain optical coherence tomography (SD-OCT) allows observation of an individual layer of the inner retinal layers, such as retinal nerve fiber layer (RNFL), ganglion cell layer (GCL) and inner plexiform layer (IPL). These layers have vertically symmetrical characteristic structures in normal eyes. In eyes with glaucoma, the RNFL and GCL are selectively diminished frequently in focal regions, and consequently lose their symmetrical structures. The thinning of RNFL and GCL is apparent even in eyes with preperimetric glaucoma, thus providing an opportunity for developing early glaucoma detection methods. Observation of vertical B-scans is a simple, but effective way to find out the asymmetric RNFL and GCL thinning. It is also an effective method to find out the arcuate RNFL defect pattern in the thickness map or deviation map of the macula and peripapillary region. Evaluation of asymmetry of RNFL and GCL thicknesses is another powerful method. Our previous study showed that our asymmetry index had extremely high glaucoma detection ability regardless of the severity of glaucoma. These methods are useful even for the highly myopic eyes that have variably deformed optic disc. It should be noted that selective thinning of the RNFL and GCL also occurs also in the optic nerve and brain diseases, such as anterior ischemic optic neuropathy and cerebral infarction. The selective thinning of RNFL and GCL is necessary, but not sufficient for glaucoma diagnosis.

Glaucoma

Impact of Lamina Cribrosa Morphology in Glaucoma

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The lamina cribrosa (LC), a multi-layered collagenous sieve-like structure at the optic nerve head, is presumed to be the primary site of axonal injury in glaucoma. Posterior displacement of laminar insertion has been confirmed in autopsied and enucleated human eyes with advanced glaucoma. Recently, posterior displacement of anterior laminar insertion has been investigated in *in vivo* human eyes using swept-source optical coherence tomography (SS-OCT). Using this technique, The peripheral LC was displaced more posteriorly in the POAG eyes compared with the age-matched healthy eyes. In the POAG eyes, the peripheral LC was displaced more posteriorly at the vertical meridian than at the horizontal meridian. The peripheral LC in the vertical meridian might have increased IOP-related strain (deformation) compared with horizontal meridian in glaucomatous eyes. LC posterior bowing was increased in POAG eyes, and was significantly associated with structural optic nerve head (ONH) changes but not with functional glaucoma severity. Focal LC defects (FLCD) are more frequently visible in eyes with glaucomatous disc hemorrhage (DH) than in eyes without DH. DH and LC defects, moreover, are often spatially correlated. These findings suggest that DH is associated with focal abnormalities of the LC. The DHs that correspond to FLCD location tend to have larger areas and to be more proximally located than those without correspondence. This suggests that FLCD might affect the topographic characteristics of DH.

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Imaging of Optic Nerve in Eyes with Pathologic Myopia

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Pathologic myopia is the major cause of visual impairment worldwide, especially in east Asian countries. One of the major cause of visual impairment is myopic optic neuropathy or glaucoma. Due to a mechanical expansion, the papillary and peripapillary regions are expanded and deformed.

The recent advance in optical coherence tomography (OCT) has been useful for imaging the optic nerve in eyes with pathologic myopia. The subarachnoid space (SAS) containing the cerebrospinal fluid is not generally observed in normal eyes *in situ*, however in highly myopic eyes with a large conus, the SAS is visible. In the B-scan images of highly myopic eyes, the SAS was visible in 93% of highly myopic eyes, and the SAS appears to be dilated in the highly myopic eyes. The dilated area exposed to the cerebrospinal fluid pressure along with thinning of the posterior eye wall may influence the way in which certain diseases, such as glaucoma, are manifested.

By using swept-source OCT, pit-like clefts are observed at the outer border of the optic disc or within the adjacent scleral crescent. The optic disc pits were associated with discontinuities of the lamina cribrosa, whereas the conus pits appeared to develop from a scleral stretch-associated schisis or emissary openings for the short posterior ciliary arteries in the sclera. The nerve fiber tissue overlying the pits was discontinuous at the site of the pits. Peripapillary intrachoroidal cavitation (ICC) is observed as yellowish-orange lesions located most typically inferior to the optic disc in highly myopic eyes. A full thickness defect in the retina along the margin of the ICC is considered the cause of the visual field defects.

Implications of Optic Disc Tilt in the Progression of Primary Open-angle Glaucoma

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Glaucoma is a progressive optic neuropathy that accompanies the functional loss of vision. Myopia has been classified as a risk factor for the development of glaucoma in numerous studies to date. However, it remains controversial whether the presence of myopia worsens the severity of glaucoma. In other words, some studies have shown that glaucoma patients with myopia progressed

faster, and other publications report that myopia is not associated with glaucoma progression. Some studies even demonstrate that myopia is a protective factor against glaucoma progression.

Regarding these conflicting results, one possible explanation is that myopic glaucoma is a mixture of disorders with different clinical characteristics. Conflicting results may stem from the proportion of these different groups included in each study. This hypothesis is somewhat plausible because "myopia" is a general term used to define the condition that one cannot see far-sighted objects without optical correction. The reason why it has been speculated that myopia may be related to glaucoma is that the axial elongation of the eyeball causes structural changes in the optic disc and/or peripapillary retina, which are the target tissues for this condition. However, the eyeballs do not experience the same changes in the optic disc and/or peripapillary retina according to the elongation of eyeball, and thus myopia does not have the same features in terms of the optic disc and peripapillary retina. In other words, myopic glaucoma demonstrates different configurations in the optic disc and peripapillary retina. These differing anatomic characteristics between myopic glaucomatous optic discs and peripapillary retina may be associated with different clinical courses during long-term follow-up.

In this topic, we will discuss the result of our analysis which involved longterm follow up of myopic glaucoma patients who had a long axial length. We compared the clinical characteristics of the myopic eyes that demonstrated progressive glaucomatous changes with myopic eyes that remained stable during follow-up. We paid particular attention to the relationship between optic disc tilt and torsion, which are frequently observed in myopic optic discs and glaucoma progression.

Bruch's Membrane Opening (BMO) and BMO-minimum Rim Width (BMO-MRW) in a Normal Japanese Population

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Background: Bruch's membrane opening (BMO) has been proposed as the new benchmark aperture through which axons exit the eye, and the minimum rim width from BMO (BMO-MRW) to the internal limiting membrane. Spectral-domain optical coherence tomography (SD-OCT) data acquisition and analysis are proposed to be performed according to the

axis connecting the fovea and BMO center (FoBMO axis) on an individual basis. Although racial differences have been reported for conventional clinical disc and circumpapillary retinal nerve fiber layer thickness (cpRNFLT) centered on the clinical disc center, characterization of BMO, BMO-MRW and FoBMO angle has been reported only for a normal white population. We characterized BMO, BMO-MRW and cpRNFLT centered on the BMO center and FoBMO angle in a non-white Japanese population.

Subjects and methods: Healthy Japanese were recruited . The optic nerve head (ONH), peripapillary RNFL and macular area were measured with SD-OCT (Spectralis, Heidelberg Engineering GmbH, Heidelberg, Germany) as reported previously (Chauhan et al, Ophthalmology 2015) .

Result: 258 eyes of 258 subjects underwent analysis. The subjects' characteristics and measurement results are summarized in Table 1. No inter-eye difference was seen for Global BMO-MRW and cpRNFLT, but BMO area was greater by about 3 % (P=0.000) and FoBMO angle was more negative by about 2° in the left eye (P=0.000). In the temporal, superotemporal and inferotemporal sectors, BMO-MRW showed no inter-eye difference, while cpRNFLT showed no inter-eye difference only in the inferotemporal sector. Pearson's correlation coefficients between the two eyes were > 0.846 for BMO area, global BMO-MRW and global cpRNFLT, and for sectoral BMO-MRW and cpRNFLT > 0.722, while no inter-eye correlation was seen for FoBMO angle (r=0.080). BMO area and global cpRNFLT were greater, but global BMO-MRW smaller than those reported in a normal white population (Chauhan et al, Ophthalmology 2015), while FoBMO angle and its inter-eye difference was similar between normal Japanese and white population.

Conclusion: Normal values of BMO, BMO-MRW, 3.5-RNFL and FoBMO angle were reported.

| | |
|-----------------------------|--------------|
| Age (yrs) | 51.7 ± 18.2 |
| Intraocular Pressure (mmHg) | 14.0 ± 2.3 |
| Refractive error (D) | -0.6 ± 1.8 |
| BMO area (mm ²) | 2.06 ± 0.45 |
| Global BMO-MRW (µm) | 305.5 ± 50 |
| Global 3.5-RNFL (µm) | 101.8 ± 10.9 |
| FoBMO angle (°) | -7.8 ± 3.8 |

[Table 1]

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Glaucoma

GLA5 - Translaminar pressure gradient and glaucoma progression

Intracranial Pressure a New Risk Factor for Glaucoma

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During the past 5-years, it is the iCOP study group first prospectively found that cerebrospinal fluid pressure (CSFP) is relatively low in Normal Tension Glaucoma (NTG) patients. And it is the iCOP study group first find that the subarachnoid space of the optic nerve is narrow in NTG patients, which proved the real trans-laminar cribrosa pressure difference is enlarged by a relatively low CSFP around the optic nerve in NTG patients. Then, it is also the iCOP study group first proved that lowering of CSFP in monkeys could induce glaucoma like optic neuropathy, which demonstrated the causing effect of low CSFP in glaucoma. Based on these findings, iCOP study group developed the non-invasive CSFP measurement way based on MRI and also a formulacould be used to estimate CSFP in large populations. However, new problems arising upon the current works. What are the problems? How could we resolve these problems? Here we would discuss with you and introduce you our new thoughts about iCOP based on the latest data.

Cerebrospinal Fluid Pressure Influence upon the Translaminar Pressure Gradient and Retinal Veins

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The translaminar pressure gradient (TLPG) describes the change in pressure across the lamina cribrosa and determines the forces applied to that tissue. It is probably not linear nor uniformly applied across the lamina, however our current measurement ability tends to give a simplistic notion. Our work demonstrated that the intraocular pressure (IOP) and retrolaminar tissue pressure (RLTP) set the start and end points of the gradient with regression modelling showing that the gradient was close the the pressure difference divided by lamina thickness. Work in dogs shows

the RLTP is largely determined by cerebrospinal fluid pressure (CSFP) modulated by pia mater properties and orbital pressure, which tend to buffer against a low CSFP. Using dog measurements and human anatomic values we estimate that at an IOP of 15 mmHg, a CSFP and orbital pressure of 0 mmHg, the mean pressure difference across the human pia mater will be 4.8 ± 2.2 mmHg and the TLPG will be from 20 to 33 mmHg/mm - one of the highest in the body. Pressure gradients of 45mmHg/mm are known to impede rapid axonal transport, and depending upon the lamina thickness an elevation in IOP to between 20 and 27mmHg will reach this level. Reduction in CSFP and orbital pressure as well as pia mater thinning will amplify this gradient. The gradient exists in an altered form along the central retinal vein leading to haemodynamic changes and a water fall phenomenon with frequent spontaneous venous pulsation. This is likely to be responsible for the arterIALIZATION of venous endothelial cell morphology noted in the lamina region. Retinal venous pulsation is in phase and is driven by CSFP pulsation travelling anteroGRADE to venous flow. Threshold IOP measures required to induce venous pulsation (vein pulsation pressure - VPP) are known to be related to CSF pressure. Additionally, venous resistance alters VPP and this probably accounts for the increased VPP seen in glaucoma. Raised VPP is strongly associated with glaucoma progression ($p = 0.004$) with the added progression risk being 6% per mmHg increase in VPP, independent of IOP and other factors. We have developed a photo-plethysmographic system to map pulsation amplitude across the retina and are using various analytical methods in order to estimate CSFP. We will show some results using extrapolation techniques taking multiple pulsation maps at varying IOP. We hope that similar techniques may allow the CSFP and TLPG to be estimated in glaucoma patients.

The Interaction between IOP and ICP

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Glaucoma is often thought of as a one-pressure disease with the focus on intraocular pressure and the optic nerve. What is behind the nerve is often forgot about. Recently, much attention has been focused on intracranial pressure and it's role in glaucoma. The possibility exists that glaucoma is not a one-pressure disease, but rather a two-pressure disease: intraocular pressure and intracranial pressure. In fact, it may not be the individual pressures that matter, but the difference between the intraocular and intracranial pressures, also known as the translaminar

pressure difference (TLPD). Scuba divers are able to travel down to 800 feet below sea level and experience an intraocular pressure of 19,000 mmHg, and yet do not develop glaucoma. As they travel deep into the sea, the intracranial pressure also increases, and the TLPD remains the same protecting the optic nerve from damage. If the TLPD becomes too great as intraocular pressure is elevated relative to intracranial pressure, glaucomatous damage ensues.

Anatomical Features of the Optic Nerve Head in Relationship to the Trans-lamina Cribrosa Pressure Difference

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The main proven risk factor for glaucomatous optic neuropathy (GON) is an intraocular pressure (IOP) higher than the pressure sensibility of the optic nerve head allows it. Fulfilling Koch's postulates, numerous studies have shown that the presence of high IOP leads to GON, that lowering IOP stops the progression of GON, and that a re-increase in IOP causes again progression of GON. There are however many patients with glaucoma who have statistically normal or low IOP, and despite of the low IOP values, they develop progressing GON. These observations led to findings that the IOP is only one of the two determinants of the trans-lamina cribrosa pressure difference (TLCPD) which is the main pressure-related parameter for the physiology and pathophysiology of the optic nerve head. The second parameter influencing TLCPD is the orbital cerebrospinal fluid pressure (CSFP) as the counter-pressure against the IOP across the lamina cribrosa. Recent experimental and clinical studies have suggested that a low CSFP could be associated with GON in normal-(IOP-)pressure glaucoma. These investigations included studies with an experimental long-term reduction in CSFP in monkeys, population-based studies and clinical retrospective and prospective investigations on patients with normal-pressure glaucoma. Besides the TLCPD, other ocular parameters influenced by the CSFP may be choroidal thickness, retinal vein pressure and retinal vein diameter, occurrence of retinal vein occlusions, and occurrence and severity of diabetic retinopathy.

Impact of Elevated Pressure in the Adenosinergic System in Microglia and Retina

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Elevated intraocular pressure (IOP) is a major risk factor for the onset of glaucoma, the second leading cause of blindness worldwide. It has been claimed that inflammation is involved in glaucomatous damage and microglial cells contribute to disease progression. Extracellular ATP and adenosine have been implicated in inflammation, cell death and neuroprotection and may have a role in the pathogenesis of glaucoma. In this work, we investigated the impact of elevated pressure in the adenosinergic system, namely in CD73/ecto-5'-nucleotidase and adenosine deaminase (ADA), in microglia and retina.

BV-2 microglial cell line and retinal microglia primary cultures were exposed to elevated hydrostatic pressure (EHP; 70 mmHg above the atmospheric pressure). Ocular hypertension was induced by laser photocoagulation in Sprague Dawley rats.

The exposure of BV-2 cells to EHP increased the extracellular levels of ATP and adenosine. Also, in BV-2 cells, EHP decreased the enzymatic activity of ADA, the enzyme that degrades adenosine into inosine, without altering its protein levels. CD73, the enzyme that converts extracellular AMP into adenosine, has not been described in BV-2 cells. Therefore, we assessed CD73 expression in retinal microglia primary cultures. We found that the exposure of retinal microglia cultures to EHP increased CD73 mRNA without changes in ADA expression. In the animal model of ocular hypertension, we detected an increase in ADA mRNA levels and a decrease in CD73 mRNA levels in the retina.

Our results suggest that elevated pressure impacts the adenosinergic system in microglia cell cultures and in the retina, which may contribute to alterations in the formation and degradation of adenosine. Since elevated pressure induced distinct responses in the different models used, other cells beside microglia might contribute

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to increased levels of ATP and adenosine in glaucoma. Understanding the alterations in the adenosinergic system in glaucomatous conditions might help to unravel the mechanisms underlying inflammation and cell death associated with glaucoma.

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Aphakic Glaucoma after Pars Plana-lensectomy with and without Removal of the Peripheral Lens Capsule - A Comparative Study

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Background: The etiology of aphakic glaucoma is yet unclear. A recent hypothesis suggests that remaining lens epithelium releases cytokines transducing trabecular meshwork cells leading to elevated intraocular pressure. To address this hypothesis, we compared two cohorts of children undergoing lensectomy. Both cohorts differed only in the extent of lens removal: in cohort 1 the entire lens including its capsule was removed, in cohort 2 the peripheral lens capsule was left intact, also to facilitate a secondary IOL implantation later on.

Methods: We included children with uni- or bilateral congenital cataract who underwent lensectomy during the first year of life and were subsequently fitted with contact lenses. Cohort 1 had 41 eyes

(11 unilateral, 30 bilateral), cohort 2 had 33 eyes (15 unilateral, 18 bilateral). The median age at surgery was 4.0 months in unilateral and 3.0 months in bilateral cases in cohort 1, and 8.1 months and 2.4 months in cohort 2. The median follow-up was 12.8 years in cohort 1 and 9.3 years in cohort 2. All cases were analysed for the prevalence of aphakic glaucoma (Kaplan-Meier estimation), visual acuity and compliance in visual rehabilitation (contact lens / occlusion therapy).

Results: At 10 years after surgery the prevalence of aphakic glaucoma was 19% in cohort 1 and 14% in cohort 2 ($p > 0.05$). In unilateral cases the median visual acuity was logMAR 0.7 in both cohorts. In bilateral cases it was logMAR 0.4 in cohort 1 and logMAR 0.2 in cohort 2 ($p = 0.05$).

Conclusion: Leaving the peripheral lens capsule intact

had no negative effect on resulting visual acuity or on the incidence of glaucoma. Our data do not support the hypothesis of a negative influence of remaining and proliferating lens epithelial cells on trabecular meshwork function and the induction of aphakic glaucoma. Other causes such as developmental chamber angle anomalies or a loss of zonular tension may play a more important role.

GLA6 - Biomechanics of glaucoma

MRI-based Finite Element Analysis Predicts Large Optic Nerve Head Strains during Horizontal Eye Movements and Validations Using Optical Coherence Tomography

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Purpose:

- (1) To combine finite element (FE) analysis and dynamic magnetic resonance imaging (MRI) to estimate optic nerve head (ONH) strains (deformations) during eye movements, and identify factors influencing such strains;
- (2) To compare ONH strains induced by eye movements with those induced by intraocular pressure (IOP);
- (3) To validate that eye movements induce ONH strains using optical coherence tomography (OCT) and 3D tracking.

Methods: The eyes and orbital tissues of a healthy subject were visualized during visually-guided horizontal eye movements, using dynamic MRI. A baseline FE model of the left eye was reconstructed in the primary gaze position and included details from the orbital tissues (visualized with MRI) and from the ONH tissues (using literature data). The effect of a lateral eye movement of 13° was then simulated, based on the MRI findings. ONH strains due to eye movements were compared with those resulting from an IOP of 50 mmHg. A sensitivity study was performed, in which we varied the stiffness of all connective tissues to understand their influence on the ONH during eye movements. In addition, the ONHs (left eye) of 3 normal subjects were imaged twice with OCT: in primary and in lateral gaze (adduction: 15°) positions. Lamina cribrosa

(LC) strains were then computed using a validated 3D tracking algorithm.

Results: Our models predicted that, during eye movements, the optic nerve had a pulling action on the ONH, which resulted in large strains within the ONH tissues. Specifically, the strains generated within the prelamina, the LC, and the retrolamina following an eye movement were higher than those resulting from an IOP of 50 mmHg. These results held true even when considering variations in connective tissue stiffness. In addition, we found that stiff dura and pia matters significantly increased those strains. In all subjects imaged with OCT, we observed a pulling action (possibly from the optic nerve sheaths) deforming the temporal side of the LC posteriorly. On average, we found that LC effective strains (using 3D tracking) induced by a 15° lateral gaze were $5.85 \pm 2.12\%$.

Conclusions: This study is the first to combine FE with MRI to estimate ONH strains during eye movements, and to provide validations using OCT. Our models predict high ONH strains during eye movements, which were aggravated with stiffer optic nerve sheaths. Further studies are needed to explore a link between gaze-induced ONH strains and axonal loss in glaucoma.

Measuring the Pressure-induced Full-field Deformation of the Human Lamina Cribrosa

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The intraocular pressure produces deformation in the optic nerve head and excessive deformation may lead to glaucomatous axon damage through a variety of mechanisms. We have developed an ex vitro inflation method that uses multimodal nonlinear microscopy and digital volume correlation to map the strains in the lamina cribrosa. Eight human eyes from 6 donors (ages 26-93) were obtained within 48 hours post-mortem. The optic nerve was carefully removed at the myelin line, and the posterior sclera mounted on a custom inflation chamber. The pressure was raised 45 mmHg. A Zeiss LSM 710 NLO microscope was used to simultaneously acquire second harmonic generation (SHG) of collagen and two photon fluorescence (TPF) of elastin. The Fast Iterative Digital Volume Correlation (DVC) algorithm (Bar-Kochba et al. 2014) was used to post-process the SHG stacks to calculate the 3D displacement fields and correlation error. Strains were calculated by fitting a high order polynomial function to the displacement field and taking the gradient. The LC was divided into 8 regions surrounding the central retinal artery and vein (CRAV): four quadrants, nasal,

temporal, inferior and superior defined by 45° and 135° bisectors, in a central region within a 550 micron radial distance from the CRAV and a peripheral region.

The in-plane strain response increased nonlinearly with pressure and stiffened significantly at 10 mmHg. In paired t-tests of the inflation response from 5-45 mmHg, the average strain E_{yy} along the inferior-superior direction was greater than E_{xx} , and E_{xy} was significantly smaller than E_{xx} and E_{yy} . This signifies that the LC on average stretched more along the inferior-superior directions than the nasal-temporal directions and there was little shear (distortion) from its original shape. The LC were elliptical in shape and the strain along the long axis was larger than along the short axis for every specimen. The in-plane principal shear strain, which denotes the largest shape distortion at a given point in the LC, was on average larger in the central than peripheral regions. The maximum principal strain decreased significantly with age, which suggest a significant age-related stiffening of the LC.

Determination of Iris Mechanical Properties Using Image-based Finite Element Modelling

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Introduction: In addition to iris anatomical biometrics (e.g. thickness), clinical studies have identified material properties (e.g. iris compressibility) as one of the angle closure glaucoma risk factors (Ophthalmology, 117:1-2). Thus, quantifying iris material properties are of great importance in early detection of glaucoma. In this study, an inverse finite element (FE) model is used to estimate iris material properties assuming that the in-vivo iris deformation is available.

Methods: An axisymmetric FE model of the iris was constructed based on porcine geometry (IOVS, 53:1188-1194). The model was combined with an optimization technique based on genetic algorithm (J. Global. Optim, 11: 341-359) to calculate the iris shear modulus G (a measure of iris stiffness) and Poisson's ratio ν (a measure of iris compressibility). It was assumed that the iris configuration during dilation was known. The method was verified using two sets of pseudo-experimental data obtained from predefined values of 9 kPa for G and 0.35 for ν with and without addition of $\pm 8\%$ noise. Three different objective functions in each case were chosen as the norm of the differences between the pseudo-experimental and genetically driven FE solution values. The first objective function was designed to incorporate both chord length and concavity. However, the second and third functions were

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designed solely based on the chord length and concavity, respectively.

Results: For the noise-free data sets, all objective functions accurately determined the values of G and ν . However, for the noise-added data only the first objective function predicted the values accurately. The calculated values of G and ν were 6.5-10.5 kPa and 0.31-0.48, respectively. The optimization convergence was independent of our initial guess choices and broader ranges of initial guesses only increased the computational time.

Discussion: Our method could be easily applied to data obtained from clinical imaging modalities such as UBM or AS-OCT. As such, our next step will be to determine iris mechanical properties from ex vivo and/or in vivo experimental data. Since iris stiffness closely affects the iris contour in normal and glaucomatous eyes, quantification of such properties using clinical data will enable us to identify new glaucoma risk factors.

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Estimates of Trabecular Meshwork Stiffness Using Novel Approaches

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Purpose: Human trabecular meshwork (hTM) stiffness, measured by atomic force microscopy (AFM), was markedly elevated in glaucomatous vs. normal eyes (Last et al., 2011). However, there were possible methodological concerns in that study; further, it is not known if the increased TM stiffness causes increased outflow resistance, or is an epiphenomenon. Intrinsic inter-strain IOP differences in mice provide a way to investigate possible correlations between TM stiffness and outflow resistance. However, no data has been published on mouse TM (mTM) stiffness. Our goal was to estimate hTM stiffness in situ and determine mTM stiffness using novel approaches.

Methods: Experiments were carried out with both human and mouse eyes. *Human:* Anterior segments from ostensibly normal post mortem eyes were dissected into corneoscleral wedges (n=5). A cannula was inserted into Schlemm's canal (SC) to control SC luminal pressure by

a variable height reservoir. Optical coherence tomography (OCT) captured tissue displacements and a quasi-3D finite element model (FEM) was created based on the observed anatomy in one cross-section at low SC pressure. TM stiffness was varied in the simulations until the L_2 -norm difference between OCT-observed and computed SC displacement was minimized. *Mouse:* Eyes were snap-frozen and stored at -80 °C. 10-20 unfixed sagittal sections per eye were cut on a cryostat with a thickness of 10-20 μ m. AFM (MFD-3D; 10 μ m spherical tip) indentations were performed on thawed sections, probing the tissue located between SC inner wall and the ciliary body, representing the mTM. Stiffness was measured for at least 3 locations in the TM region per cryosection. The stiffness of the mTM for each eye was defined as the average moduli of all measurement locations in the mTM from at least one cryosection.

Results: *Human:* Estimated hTM stiffnesses were 42-159 (mean=100.8) kPa, with a clear "best match" value for each of the 5 wedges. *Mouse:* mTM stiffnesses were 3.22 ± 1.84 kPa for 3 C57BL/6J mice and 3.84 ± 3.37 kPa for 7 CBA/J mice. Human vs mouse TM stiffness differences likely reflect different measurement approaches and modes of tissue loading.

Conclusions: Combining FEM and OCT has the potential to provide an alternative approach to assess hTM stiffness. Mouse TM stiffness has been measured, and this work establishes the AFM technique for future studies relating mTM stiffness and outflow facility.

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High Throughput Screening for Glaucoma Drugs Using Cellular Contractile Force

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In glaucoma, lowering intraocular pressure (IOP) is neuroprotective and tends to slow or stop progression of the disease. Elevation of IOP is caused by increased resistance in the conventional outflow pathway where the trabecular meshwork and Schlemm's canal (SC) cells interact. Recently, we have found that the drugs that are known to decrease IOP *in-vivo* also act to decrease the stiffness and contractile force generated by SC endothelial cells *in-vitro*. Motivated by these observations, we hypothesize that drugs that modulate the contractile force

generated by SC endothelial cells have the potential to be developed as glaucoma therapeutics. We set up a high throughput screening assay based on Contractile Force Screening (CFS) which utilizes not any surrogate of cellular contractile force but rather the contractile force itself. To quantify cellular contractile force, elastic substrates were constructed using acrylamide gels in 96-well plates. Fluorescent markers and collagen were coated on the gel surfaces to visualize deformation of elastic substrate produced by cellular contractile force and to promote cell attachment, respectively. Upon each gel, we cultured primary human SC endothelial cells to near confluence. Drug effects were quantified as the force response ratio (FRR), namely, the ratio of the average contractile forces before and after addition of drug. To demonstrate proof-of-concept and to potentially repurpose drugs already approved by the US Food and Drug Administration, we screened 447 drugs comprising the NIH clinical collection I (version 2013) at a concentration of 6.7 μ M in quadruplicate. We identified 18 positives that decreased contractile force of SC cells by 20% or more (FRR < 0.8). Among these positives, some were known modulators of SC cell mechanics including beta2 adrenergic receptor agonists, but several were unexpected. The selected positives will be further evaluated to test their cytotoxicity and dose-dependent efficacy. In this study, we demonstrated CFS as a new platform for glaucoma drug discovery based upon a physiological endpoint, namely, cellular contractile force. Also, we found several potential candidates that can be repurposed to use in glaucoma.

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GLA7 - Biology of the TM

Growth Factors and their Modulation of Trabecular Meshwork Biology

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The biological properties of the trabecular meshwork (TM) outflow pathways are under constant influence of secreted growth factors. The molecules are constituents of the aqueous humor (AH) that enter the TM outflow pathways by flow or are endogenously synthesized in the TM microenvironment. Among the most important molecules that have been substantially characterized so far are fibroblast growth factor-2 (FGF2), transforming growth factors- β 1 and β 2 (TGF- β 1 and - β 2), connective tissue growth factor (CTGF), bone morphogenetic factors-4 and

-7 (BMP-4 and-7), and vascular endothelial growth factor (VEGF). FGF2 is likely the most potent survival factor of the TM. It is a potent inducer of TM cell growth and may well modulate TM stem cell proliferation. TGF- β 1 and - β 2 modulate synthesis and turnover of the TM extracellular matrix and the activity of their actin cytoskeleton. TGF- β 1 is secreted in the TM microenvironment, very likely in response to traumatic stimuli, while TGF- β 2 is highly enriched in the AH and elevated in eyes with primary open-angle glaucoma (POAG). TGF- β s' effects are mediated through their downstream mediator connective tissue growth factor (CTGF). High activity of TGF- β signaling causes, via CTGF, a switch to a myofibroblast-like phenotype including the augmentation of the actin cytoskeleton and its directly associated fibrillar extracellular matrix. Taken together, those effects may well increase TM stiffness and induce the increase in TM outflow resistance that is commonly observed in POAG. Quite intriguingly, CTGF expression is also elevated in Schlemm's canal (SC) endothelial cells derived from patients with POAG and may well cause an increase in their stiffness. The TGF- β /CTGF-induced effects on TM and SC biology are antagonized by BMP-4 and -7 signaling. This effect appears to be important to maintain ECM and actin homeostasis in the TM and to prevent the onset of TM stiffness and POAG. VEGF is secreted by TM cells, probably to act on the SC endothelium. AH flow across the SC endothelial layer requires the presence of inter- and extracellular pores and VEGF is the signaling molecule that is required for the generation of leaky vascular endothelial layers. Understanding of the complex growth factor signaling network in the TM outflow pathways and its effects on the homeostasis of the specific microenvironment has significantly contributed to our understanding of the biology of the TM outflow pathways and their changes in POAG.

Steroid-induced Alterations in Trabecular Meshwork

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Steroid-induced increase in intraocular pressure (IOP) has been already reported since 1960s, and prolonged IOP elevation often results in the development and progress of secondary open-angle glaucoma. Since glucocorticoid is widely used for various diseases, steroid-induced glaucoma has been a significant issue in clinical practice. To explain the mechanism of steroid-induced IOP elevation, it was reported that steroid administration leads to abnormal changes in the conventional outflow pathway such as an accumulation of extracellular matrix (ECM) components

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on the trabecular meshwork (TM). Previous studies indicate that steroid administration increases ECM production and decreases phagocytic function in TM cells, which result in the accumulation of trabecular debris. In fact, we recently reported that DEX increased the mRNA of collagen type IV and fibronectin in TM cells, and we also revealed that the DEX-induced changes in TM cells were relieved by the Rho-associated kinase (ROCK) inhibitor Y-27632.

Moreover, it is reported that abnormal DEX-induced changes in the actin cytoskeleton, cross-linked actin networks (CLAN), in cultured TM cells, and that the CLAN-like structure more frequently observed in the TM tissue of glaucoma patients compared to normal control TM tissue. Subsequently, many studies have explored the molecular mechanisms of CLAN formation, and revealed that CLAN formation was related to various molecules in intracellular signaling pathways, such as integrin, PKC, and noncanonical Wnt signaling. However, the functions and dynamics of CLAN in TM cells are not completely clear. To address this issue, we conducted live-cell imaging of the actin dynamics in TM cells and evaluated the relationship between CLAN formation and cell movement, and found that the DEX-treated cells with CLAN-like structures showed less migration than DEX-treated cells without CLAN-like structures. The recent findings including the dynamics of CLAN-like structure will be presented.

Biological and Cytoskeletal Interactions of Glaucomatous Medications in Trabecular Meshwork

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Intraocular pressure (IOP), which is the critical risk factor for glaucoma, is generated and maintained by the aqueous humor circulation. It is reported that the IOP builds up in response to a resistance to aqueous humor outflow in the trabecular outflow pathways. Several studies have suggested that the increase in resistance of aqueous outflow is related to characteristic biological and cytoskeletal changes in the juxtacanalicular connective tissue (JCT) and resident trabecular meshwork (TM) cells, which involve an augmentation of their actin cytoskeleton, cellular contractility and adhesions, and of their surrounding extracellular matrix. Several drugs acting on the cytoskeleton have been shown to increase aqueous outflow by acting directly on outflow tissue. Previously we introduced Rho kinase (ROCK) inhibitors as a potential glaucoma therapy by showing that a selective ROCK inhibitor significantly lowered IOP by direct action

on the TM and Schlemm's canal (SC) and alterations of extracellular matrix, differing from the target sight of other glaucoma drugs. Ripasudil hydrochloride hydrate (K-115), a novel specific ROCK inhibitor, had been newly developed and launched as a clinically available first ophthalmic solution developed for the treatment of glaucoma and ocular hypertension in Japan. There also exists several clinically available glaucomatous drugs which are also reported to enhance trabecular aqueous outflow. For example, parasympathetic agonist increases trabecular outflow, but the main action is to contract ciliary muscles whose tendon connect to trabecular meshwork, which leads to widening the trabecular space. In clinical use, glaucomatous drugs are often used as combination therapy. Thus, it is worth clarifying the interaction of these different mechanisms. We have investigated the interactions of ROCK inhibitor when concomitantly applied simultaneously with other glaucomatous medications. We evaluated the effects of each drugs on cultured human trabecular meshwork cells (HTM) and found that the parasympathetic agonist treatment interfere with the cytoskeletal changes caused by K115. Furthermore, we measured IOP with a micro needle method in mice and confirmed the attenuation by simultaneous concomitant administration with parasympathetic agonist ($p < 0.01$). In the presentation, I would like to summarize our results of evaluations concerning biological and cytoskeletal interactions between glaucomatous medications in trabecular pathway.

The Role of Extracellular Matrices in the Anterior Chamber Angle Development

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During human embryogenesis, neural crest cells migrate to the anterior chamber of the eye, and then differentiate into the inner layers of the cornea, the iridocorneal angle, and the anterior portion of the iris. Developmental glaucoma is highly associated with anomalies in the neural crest-derived tissues such as the corneal endothelium defect (Peters anomaly), iris coloboma (Axenfeld-Rieger syndrome), cleft palate, jaw defect, and ear deformity. Both the orderly migration and differentiation of the neural crest cells are controlled by cues from various cell guidance factors, morphogens, and extracellular matrices. We have shown that heparan sulfate (HS) deficiency in neural crest cells causes anterior chamber dysgenesis, which includes corneal endothelium defect, corneal stroma hypoplasia and iridocorneal angle dysgenesis, all of which are phenotypes

in the human developmental glaucoma, Peters anomaly. Neural crest cell-specific gene disruption of *Ext1*, an indispensable enzyme for HS synthesis, leads to the Peters anomaly phenotype in the anterior chamber via impairment of TGF-beta2-mediated morphogenesis, including reduced phosphorylation of Smad2 and downregulated expression of *Foxc1* and *Pitx2*, transcriptional factors identified as causative genes for developmental glaucoma. Furthermore, impaired interaction between HS and TGF-beta2 induces developmental glaucoma, which manifests as an IOP elevation caused by iridocorneal angle dysgenesis. These findings suggest that HS is necessary for neural crest cells to form the anterior chamber via TGF-beta2 signaling.

Conditional Deletion of Fibronectin in the Trabecular Meshwork Outflow Pathways of the Mouse Eye

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Intraocular pressure (IOP), the critical risk factor for primary open-angle glaucoma (POAG), is maintained by an outflow resistance for aqueous humor in the juxtacanalicular region of the trabecular meshwork (TM) outflow pathways. Outflow resistance is commonly elevated in POAG. The nature of the molecules that generate outflow resistance in normal and glaucomatous eyes has not been identified. Since the amounts of fibrillar extracellular matrix (ECM) in the juxtacanalicular tissue (JCT) of the TM are commonly elevated in POAG, a role of the ECM for maintenance of outflow resistance is likely. Fibronectin (FN) is abundantly present in the JCT ECM and is elevated in POAG and animal models of glaucoma. To identify the specific role(s) of FN in the JCT, we developed a mouse model with a conditional and inducible deletion of FN. Mice with a floxed allele of FN were crossed with CAGG-Cre-ERTM mice expressing Cre-recombinase under control of a tamoxifen-inducible promoter. The mice were treated with tamoxifen eye drops from postnatal day (P) 1 to 5 (three times per day) and studied by light and transmission electron microscopy (TEM), immunohistochemistry and quantitative real-time RT-PCR. To visualize Cre-induced recombination in the TM, Rosa LacZ reporter mice were used.

In tamoxifen-treated CAGG-Cre-ERTM/FN^{fl/fl} mice, the expression of FN mRNA was reduced to 16 % of the levels seen in control eyes. In addition, the intensity of FN immunoreactivity in the TM was massively decreased. The reduced amounts of TM FN did not cause obvious changes in the anterior segment of the eyes. By TEM, the fibrillar ECM in the JCT consisted of fibrils with a diameter of

40.15 ± 7.40 nm (mean ± SD) or 10.67 ± 2.23 nm, and of elastic fibers that contained microfibrils with a diameter of 5.81 ± 1.45 nm. The amounts of all those fibers were markedly reduced in FN-deficient eyes, and optical empty areas without fibrillar ECM were much more prominent. The reduction correlated with a decreased thickness of the space between SC endothelium and JCT cells. The space measured 596.5 ± 310.36 nm in normal eyes, and 320.24 ± 247.0 nm in fibronectin-deficient eyes.

Our data indicate that FN is an important ECM component of the juxtacanalicular region that is required to maintain the structure of the TM outflow pathways. FN-deficient mice will be an important tool to investigate the specific functional role of JCT FN for the generation and maintenance of aqueous humor outflow resistance.

GLA8 - Distal Outflow Resistance: Towards Understanding MIGS

Intrascleral Veins Require Deep Thoughts beyond Superficial Episcleral Veins

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The new glaucoma surgical procedures raise opportunities to re-think the traditional understanding of both conventional and unconventional outflow pathways. With regard to those procedures that target Schlemm's canal, a major assumption of successful outcome is that abnormal resistance resides within the trabecular meshwork, and that once this resistance is by-passed, then outflow through Schlemm's canal and beyond is essentially unimpeded. Previous studies in human enucleated eyes suggest that one third to half of the outflow resistance may lie distal to the inner wall of Schlemm's canal depending upon the perfusion pressure. Success of Schlemm's canal-based surgery intended to restore outflow into the episcleral venous system, which would depend on whether there are some permanent pathological changes in the distal outflow pathway. Canalogram imaging using fluorescent tracers and clinical intraocular pressure outcome data will advance our knowledge and understanding of the intrascleral venous plexus in the distal outflow structures. The intrascleral venous plexus may represent a key structural biomarker that determines the desired outcome of intraocular pressure reduction following Schlemm's canal-based surgeries.

Glaucoma

Measurement and Physiology of Episcleral Venous Pressure

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Episcleral venous pressure (EVP) is an important determinant of intraocular pressure (IOP). Aqueous humor that exits through the conventional outflow pathway passes from Schlemm's canal into the collector channels, the aqueous veins, and then into the episcleral veins and the systemic vasculature. EVP acts as a base pressure below which IOP cannot fall as long as aqueous humor continues to exit through the conventional outflow pathway. While understanding of EVP physiology is crucial to understanding IOP physiology, difficulty in the measurement of EVP has led to a relative lack of information about its physiology in humans.

EVP can be measured non-invasively by placing a clear, inflatable pressure chamber on the eye and estimating the pressure required to compress an episcleral vein to a predetermined endpoint. However, the lack of objective endpoints makes EVP measurement in humans uncertain, and a wide range of mean EVP has been reported in the literature. Our recently developed objective method for non-invasive pressure chamber based venomanometry includes a computer-controlled motor drive to increase pressure automatically, a transducer to record pressure, and a high-definition video camera to record vein collapse. Pressure measurements are synchronized with the video stream to determine the pressure required to just begin the collapse of a vein. Mean EVP is typically about 7 mmHg using this technique. Using this system, we are beginning to gain new insight into EVP regulation, including variations with body position, water drinking, and systemic blood pressure.

Animal models for EVP measurement have provided further evidence that EVP is dynamic, and may be modulated by pharmacotherapy. In rabbits, reduction of EVP has been recorded with topical application of proparacaine and brimonidine, while elevation of EVP has been recorded with topical nitroprusside. In mice, reduction of EVP has been detected with topical latanoprost. It is currently unknown if similar responses occur in humans. However, animal data suggests that reduction of EVP may provide a novel target for reduction of IOP in glaucoma patients.

Role of Schlemm's Canal and Collector Channels in Intraocular Pressure (IOP) Regulation

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The conventional outflow pathway is a dynamic system consisting of the trabecular meshwork, Schlemm's canal, collector channels and the episcleral venous system. Past studies have indicated that increased outflow resistance leading to elevated IOP in primary open angle glaucoma (POAG) is located in the region of Schlemm's canal inner wall endothelium, its basement membrane and the adjacent juxtacanalicular region of the trabecular meshwork. Recent studies suggest that in addition to this proximal outflow resistance, downstream structures including the outer wall of Schlemm's canal and the collector channels may also contribute to outflow resistance and have a role in IOP regulation in both normal and POAG eyes. Here we introduce studies from our laboratory that describes collector channel structures from simple ovals to complex flaps and bridges that connect the inner and outer wall of Schlemm's canal. Collector channel orifices with and without flaps are lined with smooth muscle actin, implying the presence of a contractile function. Variable response to pressure changes in collector channels suggests the presence of a compensatory mechanism that is available for short and transient changes in IOP regulation. Analysis of normal and POAG eyes under low and high pressure confirmed reduced outflow area due to decreased SC volume in POAG eyes. In addition, collector channels in POAG eyes have a smaller orifice diameter and an increased number of occluded collector channels when compared to normal eyes under similar pressure conditions. Together, these data indicate that distal parts of the conventional outflow pathway, particularly collector channels, may have a prominent role in IOP regulation.

Anatomy of Smooth Muscle Cells in Distal Vessels

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Outflow resistance in the aqueous drainage tract distal to trabecular meshwork (TM) is potentially an important

determinant of intraocular pressure (IOP) and success of glaucoma trabecular bypass surgeries. The mechanism by which distal resistance is modulated is unclear. We sought to determine the presence of contractile features in the distal tract with reference to other ocular contractile tissues such as choroidal arteries and aqueous drainage tissues of TM and ciliary muscle. 2-photon excitation fluorescence (TPEF) and second harmonic generation (SHG) imaging were performed in transgenic fluorescent reporter (Prox1-GFP; mT/GFP) and wild-type (C57BL/6 and Balb/c) mice in vivo and ex vivo to view the TM, Schlemm's canal (SC), collector channels (CC) and intrascleral plexuses (ISPs). Lumen of the drainage tract distal to TM appeared as scleral collagen SHG signal voids. The Prox1-positive, LYVE-1-negative endothelium of SC, CCs and ISPs bordered the lumen of these drainage structures. Intrascleral channel walls showed broad filamentous actin (F-actin) labeling corresponding to the location of smooth muscle as F-actin co-localized with smooth muscle epitopes of alpha smooth muscle actin, caldesmon and calponin in these regions. This labeling and co-localization profile mirrored that of smooth muscle in ciliary muscle and arterial walls, but differed from that of TM, a contractile non-smooth muscle tissue. These mural smooth muscle features indicate that the distal aqueous drainage tract has capacity to contract and dynamically alter caliber and resistance in analogous fashion to arteries sharing a similar profile of contractile features.

Aqueous Angiography and Minimally Invasive Glaucoma Surgeries

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Purpose: To present aqueous angiography as an aqueous humor outflow (AHO) imaging method in the eye and its relevance for surgical glaucoma management.

Methods: Enucleated eyes from multiple species (pig, cow, and human) were obtained from abattoirs or the San Diego Eye Bank. Aqueous angiography was conducted via a Spectralis HRA+OCT (Heidelberg Engineering, Germany) after placing an anterior chamber maintainer and perfusion with balanced salt solution (BSS). Tracers (2% fluorescein, 0.4% indocyanine green [ICG], or 3 kD fixable and fluorescent dextrans [0.2 mg/ml]) were introduced. Concurrent anterior segment optical coherence tomography (OCT) was performed. Eyes using fluorescent dextrans for aqueous angiography were histologically sectioned for fluorescent microscopy. In some human eyes,

trabecular microbypass stent or sham was performed in low angiographic flow regions using ICG followed by fluorescein aqueous angiography to interrogate the effects of surgical trabecular bypass. Alternatively, aqueous angiography was performed in living non-human primates using ICG and/or fluorescein with sometimes concurrent OCT.

Results: In enucleated eyes, segmental aqueous angiographic patterns were seen. Concurrent anterior segment OCT in angiographically positive but not negative regions demonstrated intrascleral lumens consistent with AHO pathways. Aqueous angiography with fluorescent dextrans demonstrated trapping in AHO pathways. Sequential aqueous angiography with ICG followed by fluorescein in the same eyes demonstrated very similar patterns. Trabecular microbypass stent but not sham placement in regions initially with low angiographic signal by ICG aqueous angiography could demonstrate recruitment and increased angiographic flow when queried by fluorescein. Aqueous angiography in living non-human primates demonstrated segmental patterns with a pulsatile nature. Dynamic patterns were seen where regions with angiographic signal could recede and regions without angiographic signal could variably and naturally develop angiographic flow.

Conclusion: Aqueous angiography is a real-time AHO imaging modality in model eyes across multiple species that can be used to investigate interventions on the trabecular/conventional AHO pathway. Aqueous angiography in an intact eye in a living organism demonstrates dynamic variability.

GLA9 - Status of glaucoma gene and stem cell therapy

Schwalbe's Line Cells Show Stem Cell Characteristics

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The number of cells in the trabecular meshwork (TM) outflow pathways declines with age and glaucoma. It is unclear, if adult TM stem cells exist and whether they would be able to compensate for the loss of TM cells. Adult

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stem cells in the body typically reside in defined niches, divide rarely, and retain proliferation markers such as 5-bromo-2'-deoxyuridine (BrdU) for a prolonged period of time. Here we searched for cells with long-term BrdU-retention in the primate TM outflow pathways.

To this end, four young adult cynomolgus monkeys were daily treated with BrdU (30 mg/kg body weight) for 4 weeks. Two animals were sacrificed immediately thereafter (group1) while the two other animals were sacrificed four weeks after BrdU treatment (group2, long-term BrdU retention). Following enucleation, the eyes were embedded in paraffin and analyzed for BrdU-positive cells by immunohistochemistry. The number of BrdU-positive cells was quantitatively analyzed for the different parts of the TM, the scleral spur, the operculum region, along the inner surface of the cornea in region of Schwalbe's line, and in the lining of Schlemm's canal (SC). Double labeling experiments were performed to clarify the nature of the BrdU-positive cells.

We found that the number of BrdU-positive cells was evenly distributed throughout the different regions of the TM. In contrast, in the SC endothelium, the number of BrdU-positive cells was significantly higher than in all other areas of the TM. BrdU-positive cells of the SC endothelium had characteristics of differentiated vascular endothelial cells and were immunoreactive for the endothelial marker CD31. In group 2, the intensity of the BrdU signal was markedly weaker, and the overall number of positive cells was smaller. In contrast, BrdU-staining in cells covering Descemet's membrane in the region of Schwalbe's line was as frequent and intense as in group 1 indicating long-term BrdU retention. Double labeling experiments with OCT 3/4 strongly supported the concept that cells with long-term BrdU retention have stem cell characteristics.

There is evidence for the presence of cells with long-term BrdU retention in the primate outflow pathway. The cells are localized in the region of Schwalbe's line. It is tempting to speculate that this specific area constitutes the niche in which neural crest-derived stem cells reside in the eye. The cells might be able to compensate for the loss of TM and/or corneal endothelial cells.

Patient-derived Stem Cells for IOP Control

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Transplanting patient-specific stem cells may be useful in

treating open-angle glaucoma since reduced trabecular meshwork (TM) cellularity in the outflow pathway is associated with this disease. Persistently elevated intraocular pressure (IOP) is the primary risk factor and the only treatable parameter for glaucoma. Previously, we showed that comparable chemical reduction of TM cellularity blocks the normal IOP homeostatic response that keeps IOP within acceptable bounds. Transplantation of human induced pluripotent stem cells (iPSCs) differentiated to a TM-like cell restores normal IOP homeostasis to the chemically reduced TM, suggesting that autologous stem cell therapy may be successful in treating glaucoma. However, approximately 70 genes have been associated with glaucoma, and autologous iPSCs would retain the genetic defect that originally caused the disease. Correcting the genetic defect in that patient's stem cells before transplantation might alleviate this problem. As a step towards eventually implementing this approach, we have been evaluating CRISPR/Cas9 gene editing to create individual gene defects that have been associated with glaucoma and then evaluating their effects in TM cell culture and in anterior segment organ culture. In the first set, we used lentiviral delivery of guide RNAs, Cas9 and specific repair templates from GenScript to trigger homology-dependent repair (HDR) of the Cas9 cleavages targeted by the guide RNAs. We initially used the myocilin (Q368STOP) as our target and an equivalent silent mutation in this codon for a control. In porcine TM cells, we achieved high-level lentiviral delivery and upon sequencing after 72 hours, found specific HDR sequence modifications with all 3 guide RNAs used. This outcome suggests the feasibility of correcting this gene defect in glaucoma patients' own stem cells prior to transplanting the differentiated TM-like iPSCs in vivo. Repopulating the trabecular meshwork of glaucomatous patients with corrected, patient-derived TM-like iPSCs will potentially achieve long-lasting restoration of IOP homeostasis.

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Transplantation of iPSC-derived TM Cells Restores Outflow Facility in an Aged Glaucoma Mouse Model

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Trabecular meshwork (TM) degeneration is associated with the pathology of primary open-angle glaucoma (POAG). We previously demonstrated that transplantation of iPSC-derived TM cells (iPSC-TM) restores TM function in tg-MYOC^{Y437H} mice leading to retinal ganglion cell (RGC) survival. Here we extended these studies to investigate if a similar effect can also be observed in older mice with more established pathology. Mouse induced pluripotent stem cells (iPSC) were differentiated into TM cells for 14 days. 50,000 iPSC-TM cells were then injected into the anterior chamber of 6-month-old Tg-MYOC^{Y437H} mice (N=8). Tg-MYOC^{Y437H} mice receiving PBS injections and age-matched wild type (WT) mice were used as controls (N=14 and 10, respectively). Intraocular pressure (IOP) and aqueous humor outflow facility were measured throughout the study. TM cell and RGC densities were determined after 12 weeks at the conclusion of the experiment. In contrast to our earlier studies using younger mice, older animals only displayed minor improvements in IOP and outflow facility after 6 weeks. However, 12 weeks after transplantation IOP in iPSC-TM recipients was statistically lower (14.72 vs 21.02 mmHg, P=0.019) and outflow facility was markedly improved (0.0074 vs 0.0041 ul/min/mmHg, P=0.04) when compared to PBS recipients. These values are similar to those observed in age-matched WT mice (IOP: 15.13 mmHg, facility: 0.0087 ul/min/mmHg). These data demonstrate that this approach can rescue TM function in older mice, although more time is required for improvements to manifest themselves. 12 weeks after transplantation, eyes were collected and the TM cellularity was determined using immunohistochemical approaches. The TM of iPSC-TM recipient mice, now age 9 months, contained approximately the same number of TM cells than that of WT mice (43.1 vs 37.78 TM number/section), which is significantly higher than that in PBS recipients (26.12, P=0.0028). However, all eyes displayed lower TM cell numbers than those observed in younger mice (e.g. 57.15 in 7-mon-old mice). A rescue effect on RGC was not observed, suggesting that IOP independent damage mechanisms may significantly contribute to the pathophysiology in this mouse model. In summary, transplantation of iPSC-TM is also a viable approach to restore IOP and outflow facility in aged Tg-MYOC^{Y437H} mice. These findings are promising for stem cell-based therapy for glaucoma patients who are predominately of older age.

Hurdles Affecting Successful Therapeutic Transduction of Retinal Ganglion Cells

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Gene therapy holds great promise as a long-term, cell-specific biological treatment to prevent or reverse pathological phenotypes. Retinal ganglion cells (RGCs) are possible targets for gene therapy, especially in pathologies related to optic nerve damage. The genomes of RGCs respond to damage by both up-regulating protective and apoptotic gene expression. Pathways associated with both changes in the transcriptome are amenable to modification by strategic expression of exogenous genes. But just how easy is it to do gene therapy on damaged RGCs? The hurdles are actually quite wide ranging, but mostly stem from nuclear and molecular changes that damaged RGCs undergo that may impair proper activation and transcription of therapeutic genes. We know, for example, that early after axonal damage, RGCs undergo nuclear atrophy that is associated with wide-spread histone deacetylation, heterochromatin formation, and selective transcriptional silencing of many genes. Another potential barrier is the reorganization of cell structure marked by dendritic arbor retraction. It is not known if cell surface molecules, required for viral transduction, are still present and able to allow interaction of the virus with the plasma membrane. Lastly, timing is an issue, especially with the favored use of AAV2 to transduce RGCs, which has limitations associated with payload size and replication efficiency of the complementary strand of genomic DNA. We tested many of these variables in a single experiment. Mice were bred to carry the *Bax* mutant allele (to prevent soma apoptosis after optic nerve damage) and the *Rosa26(LoxP)-tdTomato* reporter. The optic nerves were then crushed, and at times after axonal damage, the mice received an injection of either AAV2-CMV-Cre or AAV2-Pgk-Cre. One month after transduction, retinal cells were evaluated for expression of the Tomato reporter, which would validate that (i) cells could be transduced by AAV2 after damage; (ii) the transgene could be expressed; and (iii) the transgene could modulate the cellular genome to express a quiescent gene. Data will be presented showing that the AAV2-Pgk-Cre virus meets all these criteria, indicating that post-damage gene therapy can be successfully executed.

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Trabecular Meshwork and Ciliary Muscle Gene Therapy for Glaucoma

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Gene therapy for glaucoma is largely aimed at enhancing aqueous humor outflow by targeting the relevant tissues in the outflow pathways: the trabecular meshwork / Schlemm's canal (TM/SC) and the ciliary muscle (CM). An alternate strategy is targeting the ciliary process non-pigmented epithelium (NPE) to reduce AHF. In the first gene transfer to the anterior segment of non-human primates (NHPs) LacZ (B-Gal) was expressed in NPE and TM/SC after intracameral injection of a herpes simplex virus (HSV) vector carrying the gene. Since then various viral vectors, promoters, dose levels, concentrations and delivery methods have been investigated in cells, monkey and human organ cultured anterior segment systems (MOCAS and HOCAS) and live animal models. Genes can be delivered via viral or non-viral methods. Viral vectors have a longer duration of effect and high transduction efficiency. Several viral vectors have been investigated for anterior segment gene transfer. The most studied vector for ocular indications is the adeno-associated virus (AAV), which has serotypes showing preferential transduction for specific ocular tissues. The self-complementary version of the AAV (scAAV) can effectively transduce the TM. Lenti virus vectors, such as feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV), are becoming more commonly used in large animal models, can transduce the TM and can carry a larger payload than the AAVs. Adeno (Ad) vectors have large capacity but are short-lived so the duration of effect is not adequate for glaucoma therapeutics. Cytoskeleton active genes such as C3 and caldesmon can transduce TM cells and modify the architecture of the outflow pathway in MOCAS. Outflow facility increases after intracameral delivery of Ad vectors encoding the caldesmon and C3 genes in the MOCAS system. Intraocular pressure decreases for 5 months in live monkeys receiving a FIV vector encoding prostaglandin F2 α synthase. Viral vectors can be delivered to the target tissue via transcorneal injection or micro invasive glaucoma surgical (MIGS) techniques e.g. the iStent or canaloplasty. Proteasome inhibition by MG132 can enhance transduction efficiency of FIV vectors co-expressing green fluorescent protein (GFP) in human TM-1 cells and MOCAS. There are constraints and challenges in the development of gene transfer, but the resulting therapeutics could benefit many patients, especially those in underserved areas.

Exoenzyme C3 Transferase Lowered IOP in Rats

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Purpose: To evaluate the IOP-lowering effect of C3 exoenzyme(C3)using hydrogel sustained-C3 release systems and lentiviral vector-mediated C3 transduction in rat eyes.

Methods:

(1) C3 was expressed in E.coli and purified by affinity chromatography. Immunofluorescence was performed in NIH 3T3 cells treated with C3 to verify the cellular uptake of the protein. Six μ l of hydrogel (30%) was injected into the anterior chamber (AC) of both eyes of SD rats to induce an acute ocular hypertension, and IOP was measured before and 2, 14 and 24 h post-injection. After 24 h, 6 μ l of the sustained-release hydrogel formulation (containing 15 μ g C3) was injected into the AC of the right eyes, while 6 μ l hydrogel was injected into the left eyes as control. IOP was measured at 2 h and then every 12 h.

(2) Lentiviral vector containing C3 (LV/C3) was prepared. Human trabecular meshwork (HTM) cells in culture were treated with LV/C3 at different MOI, and changes in cell morphology were observed.

Five μ l LV/C3 (8×10^8 TU/ml) or eight μ l LV (5×10^8 TU/ml) was injected into the AC of the right and left eyes, respectively. The GFP was visualized by Micron III Retinal Imaging Microscope and by fluorescence microscope. IOP was measured every day post-injection for 8 days, and then every two days thereafter for another three weeks.

Results:

(1) Intracameral injection of hydrogel raised IOP of rats to 35–47 mmHg. The sustained-release of C3 significantly lowered IOP for about 60 h. Maximal IOP reduction was 41% at 26 h after the 2nd injection

($p < 0.05$).

(2) LV-mediated C3 expression induced changes in HTM cell morphology. GFP expression in the AC angle tissues was observed by frozen-section examination 8 days after injection of LV/C3 or LV.

In the LV/C3 group, IOP was significantly decreased compared to control eyes or baseline by the 3rd day post-administration, and this effect lasted for at least 14 days ($p < 0.05$).

Conclusions: Hydrogel sustained-released C3 reduced IOP in a rat model with ocular hypertension. LV mediated C3

expression induced changes in cell morphology of cultured HTM cells, and was expressed in AC angle tissues and reduced IOP when injected into the rat AC.

GLA10 - Lymphangiogenesis, Lymphatics and IOP Regulation

Paradoxical Roles of Schlemm's Canal Inner Wall

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The inner wall of Schlemm's canal (SC) forms part of the blood-aqueous barrier, preventing reflux of blood products from the venous system into the anterior chamber of the eye. Paradoxically, the inner wall of SC must also be sufficiently conductive to allow aqueous humor to drain from the eye into the venous system without causing an elevation of intraocular pressure. Thus, in coordination with juxtacanalicular tissue cells, the inner wall's second role involves the regulation of aqueous humor outflow resistance, which is the primary determinant of intraocular pressure. To execute both of these critical functions, the SC endothelium uniquely possesses both lymphatic and blood vascular characteristics. This presentation will detail the biological features that enable these responsibilities.

Recent Advances in Ocular Lymphatic Research and Implication in Glaucoma

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Lymphatic research has progressed rapidly in recent years. The eye offers an ideal site for lymphatic study due to its unique features. Our research goal is to elucidate the mechanisms underlying ocular lymphatic formation and development and to identify new targets for therapeutic intervention. This presentation will introduce our recent advances in ocular lymphatic research and implications in eye disorders such as corneal transplant rejection and glaucoma. Our study has provided the first evidence showing that Schlemm's canal expresses Prox-1, the master control gene for lymphatic development, as corneal limbal lymphatics, and this structure shows a differential response to inflammatory insult compared with limbal lymphatics. Our recent efforts on the development of experimental models for further investigation on these findings will also be discussed.

The Effect of Podoplanin Inhibition on Lymphangiogenesis under Pathological Conditions

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Podoplanin has been shown to be a reliable marker of lymphatic endothelium, but its role in the lymphatic system has not been well investigated. Therefore, we investigated the role of podoplanin in lymphangiogenesis under inflammatory conditions. Moreover, whether the neutralizing podoplanin antibody (Pmab-1) was used for suppressing lymphangiogenesis or extend the survival of corneal graft.

Mouse corneal suture, transplantation and ear section models were used to induce lymphangiogenesis. Anti-LYVE-1 antibody was used to visualize lymphatic vessels. Also, the survival rate of graft after corneal transplantation was observed.

Administration of Pmab-1, reduced lymphangiogenesis both in the corneal suture and ear wound healing models. Moreover, administration of Pmab-1 led to a significant suppression of the rejection reaction in the corneal transplantation model.

Podoplanin neutralization resulted in inhibition of lymphatic growth associated with corneal and ear wound healing. Our data suggest that podoplanin is a novel therapeutic target for suppressing lymphangiogenesis.

Schlemm's Canal Is a Unique Vessel with a Combination of Blood Vascular and Lymphatic Phenotypes that Forms by a Novel Developmental Process

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Schlemm's canal (SC) plays central roles in ocular physiology. These roles depend on the molecular phenotypes of SC endothelial cells (SECs). Both the specific phenotype of SECs and development of SC remain poorly defined. To allow a modern and extensive analysis of SC and its origins, we developed a new whole-mount procedure to visualize its development in the context of surrounding tissues. We then applied genetic lineage tracing, specific-fluorescent reporter genes, immunofluorescence, high-resolution confocal microscopy, and three-dimensional (3D) rendering to study SC. Using these techniques, we show that SECs have a unique phenotype that is

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a blend of both blood and lymphatic endothelial cell phenotypes. By analyzing whole mounts of postnatal mouse eyes progressively to adulthood, we show that SC develops from blood vessels through a newly discovered process that we name "canalogenesis." Functional inhibition of KDR (VEGFR2), a critical receptor in initiating angiogenesis, shows that this receptor is required during canalogenesis. Unlike angiogenesis and similar to stages of vasculogenesis, during canalogenesis tip cells divide and form branched chains prior to vessel formation. Differing from both angiogenesis and vasculogenesis, during canalogenesis SECs express Prox1, a master regulator of lymphangiogenesis and lymphatic phenotypes. Thus, SC development resembles a blend of vascular developmental programs. These advances define SC as a unique vessel with a combination of blood vascular and lymphatic phenotypes. They are important for dissecting its functions that are essential for ocular health and normal vision.

JNT1 (GLA+OMG) - Genetics of glaucoma

Recent Advances in the Genetics of Angle Closure Glaucoma

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Primary angle closure glaucoma (PACG) is a major form of glaucoma in Asia. In the past few years, several advances have been made in the genetics of PACG.

1. In 2012, we reported a genome-wide association study (GWAS) identifying three common genetic variants associated with PACG (Vithana EN et al, Nature Genetics 2012). Three new loci for PACG were identified; rs11024102 at PLEKHA7 (per-allele odds ratio (OR) = 1.22, $P = 5.33 \times 10^{-12}$), rs3753841 at COL11A1 (per-allele OR = 1.20, $P = 9.22 \times 10^{-10}$), and rs1015213 located between PCMTD1 and ST18 on Chromosome 8q (per-allele OR = 1.50, $P = 3.29 \times 10^{-9}$).
2. We recently conducted a GWAS underlying anterior chamber depth (ACD), a major risk factor for PACG, on a total of 5,308 population-based individuals of Asian descent. Genome-wide significant association was observed at a sequence variant within ABCC5 (rs1401999; per-allele effect size = - 0.045mm, $P = 8.17 \times 10^{-9}$). This loci was associated with an increase in risk of PACG in a separate case-control study of 4,276 PACG cases and 18,801 controls (per-allele OR 1.13 [95% CI: 1.06 - 1.22], $P = 0.00046$). The association was strengthened when a sub-group of controls with open angles were included in

the analysis (per-allele OR = 1.30, $P = 7.45 \times 10^{-9}$; 3,458 cases vs. 3,831 controls).

3. We recently expanded our GWAS of PACG to >6500 ACG cases/15,000 controls followed by replication in >3500 ACG cases/8,000 controls from 22 countries. This latest work has identified 5 more novel genes for ACG.

Recent research on the genetics of ACG have increased the current understanding of the genes underlying PACG. Nine genes for ACG have now been identified from studying >10,000 ACG cases from >20 countries involved in this huge consortium.

Cacna2d1: A Novel Therapeutic Target for Lowering IOP

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Purpose: Glaucoma is a leading cause of blindness worldwide and intraocular pressure (IOP) is the only modifiable risk factor. Genetic variability is a major contributor to interpersonal differences in responses to IOP lowering therapies. In this study, we combined systems genetics using the aged BXD mice strains with human GWAS and pharmacology to define and validate a genetic modifier of IOP associated with individual genetic variations.

Methods: IOP was measured in 66 BXD strains at 10-13 months using a TonoLab. Genomic regions modulating IOP were identified using Quantitative Trait Locus (QTL) analyses. Stringent refinement based on QTL mapping, correlation analyses and single nucleotide polymorphisms (SNPs) was performed to identify candidate genes. Subcellular localization of candidates in mouse and human eyes was determined by immunohistochemistry. The GLAUGEN/NEIGHBOR consortium database was used to identify SNPs within the candidate genes associated with glaucoma in humans. IOP lowering effects of nimodipine and pregabalin were evaluated as eye drops in C57BL/6J (B6), BXD14 (B parent allele) and BXD48 (D parent allele) strains. The minimum effective concentration was determined.

Results: A single eQTL on Chr 5 was identified with a significant likelihood ratio statistic (LRS) of 19.6. *Cacna2d1* was identified as a *cis*-regulated candidate gene. CACNA2D1 is expressed in the ciliary body and

trabecular meshwork, in mouse and human eyes. GLAUGEN/NEIGHBOR POAG meta-analyses revealed an imputed SNP (7:82011270:AATAC) nominally associated with POAG ($p=9.6E-04$), which is significant at the gene level. This candidate is a component of an L-type voltage gated calcium channel (CACN) regulating ionic transport. Nimodipine³ an antagonist for the CACN pore³ reduced IOP in B6 by $18.4\pm 1.7\%$, BXD14 by $31.3\pm 3.5\%$ and BXD48 by $3.2\pm 2.0\%$ compared to baseline. Similarly, pregabalin, an antagonist specific for the $\alpha 2\delta 1$ subunit of CACN, reduced the IOP in B6 by $20.4\pm 5.5\%$, BXD14 by $28.5\pm 3.5\%$ and BXD48 by $14.2\pm 3.9\%$.

Conclusions: This is the first study to combine systems genetics, bidirectional studies using human GWAS, and pharmacology to identify and validate a genetic modulator of IOP. Both nimodipine and pregabalin lowered IOP significantly in strains with the B parent allele compared to strains with D parent allele. In the future, similar pharmacogenetic studies could pave the way for improved POAG therapies tailored to individual genotypes.

Primary Open-angle Glaucoma: Hereditary and Ethnic Effects

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In familial primary open-angle glaucoma (POAG), rare gene mutations in *MYOC* and *OPTN* have been identified through family linkage analysis and gene sequencing. In sporadic POAG, the common form, a multitude of susceptibility gene loci have been mapped, mainly through gene wide association studies (GWAS) and more recently, genome wide exome sequencing (GWES). To date, more than 120 POAG genes have been reported. Some of them are associated with traits, especially intra-ocular pressure, central cornea thickness and vertical cup disc ratio. Different ethnic populations, including Caucasian, Japanese and Chinese study subjects, have been used as primary study cohorts. Ethnic differences in disease-causing mutation profiles have been demonstrated in our previous work on the *MYOC*, *OPTN*, *NTF4*, *WDR36* genes. In recent studies on multiple polymorphisms in the *SIX1/SIX2*, *CAV1/CAV2* and *CDKN2B-AS1* loci in the Chinese and Japanese populations, we identified ethnic differences in the allele frequencies, effect sizes and population attributable risks.

A Large Scale International Genome-wide Association Study of Exfoliation Syndrome

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Purpose: Exfoliation syndrome (XFS) is an age-related, systemic fibrillinopathy that is associated with increased risk for glaucoma. It is reported that 15 - 40 % of patients with XFS develop XFG. Once converted to XFG, its progression is faster than other open angle glaucoma. We recently reported a genome-wide association study (GWAS) identifying *CACNA1A* as associated with XFS (*Nature Genetics* 2015; 47:387-92). To further dissect the genetic architecture underlying XFS susceptibility, we now expand the GWAS to include a large number of cases and controls from 6 continents around the world.

Methods: We performed an expanded GWAS on 9,700 XFS cases and 98,000 controls from 24 countries in 6 continents. Meta-analysis summarizing the results across all cohorts was performed using fixed effects modeling weighted in an inverse-variance manner.

Results: We confirm strong association at *LOXL1* (multiple SNPs) and *CACNA1A* rs4926244 in this new, expanded GWAS discovery collection. We also note genome-wide significant association ($P < 1 \times 10^{-8}$) at four novel genetic loci. These signals map to Chromosome 6p ($P = 8.5 \times 10^{-9}$), 6q ($P = 1.1 \times 10^{-9}$), 11q ($P = 1.86 \times 10^{-9}$), and 13p ($P = 6 \times 10^{-10}$). We also observed a significant excess of genetic markers surpassing $P < 1 \times 10^{-6}$, suggesting that many of them could represent true positive associations with XFS.

Conclusions: Our expanded GWAS conducted on 9,700 XFS cases and 98,000 controls confirmed earlier observations and identified at least four novel genetic loci for XFS. Replication experiments for robust verification of these new findings are now underway. Our findings will broaden current understanding of the disease pathways that underlie XFS. Moreover, they may provide a new paradigm for further medical therapy of XFS and XFG.

Functional Analysis of the LOXL1 Locus Associated with Exfoliation Glaucoma

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Glaucoma

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Exfoliation syndrome (XFS) is a common, age-related, systemic fibrillinopathy. XFS greatly increases risk of exfoliation glaucoma (XFG), a major worldwide cause of irreversible blindness. Coding variants in the lysyl oxidase-like 1 (*LOXL1*) gene are strongly associated with XFS in all studied populations, but a functional role for these variants has not been established. To identify additional candidate functional variants, we sequenced the entire *LOXL1* genomic locus (~40kb) in 50 indigenous, black South African XFS cases and 50 matched controls. The variants with the strongest evidence of association were located in a well-defined 7kb region bounded by the 3 prime end of exon 1 and the adjacent region of intron 1 of *LOXL1*. We replicated this finding in U.S. Caucasian (91 cases/1031 controls), German (771 cases/1365 controls), and Japanese (1484 cases/1188 controls) populations. The region of peak association lies upstream of *LOXL1-AS1*, an antisense RNA encoded on the opposite strand of *LOXL1*. We show that this region contains a promoter and, importantly, that strongly-associated XFS risk alleles in the South African population are functional variants that significantly modulate the activity of this promoter. *LOXL1-AS1* expression is also significantly altered in response to oxidative stress in human lens epithelial cells and in response to cyclic mechanical stress in human Schlemm's canal endothelial cells. Knockdown (using RNAi) of *LOXL1-AS1* in lens epithelial cells results in modulation of expression of a variety of genes involved in maintenance of the extracellular matrix. Taken together, these findings support a functional role for the long non-coding RNA *LOXL1-AS1* in cellular stress response and suggest that dysregulation of it and *LOXL1* itself plays a key role in XFS pathogenesis.

LEN1 - Lens Development

Mechanism of Ectopic Lens Fiber Differentiation in Response to Early Endosome Trafficking Defects

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Vertebrate lens consists of a spherical lens fiber core and the lens epithelium. The lens epithelium covers the anterior half of the lens fiber core. During development, lens epithelial cells proliferate and move posteriorly, and start to differentiate into lens fiber cells at the peripheral rim of the lens epithelium called the lens equator. Thus, the lens provides a good model for studying spatial regulation of cell differentiation. To understand this mechanism, we use zebrafish as an animal model. Previously, we identified zebrafish VSP45 (vacuolar protein sorting 45) mutant. In VPS45 mutant, monolayer structure of lens epithelium is disrupted to form multiple layers, indicating an epithelial-mesenchyme transition (EMT)-like phenotype. Furthermore, lens epithelial cells express molecular markers of lens fiber cells. Thus, ectopic fiber differentiation occurs in lens epithelium of the VSP45 mutant. VPS45 encodes a SM protein family and regulates early endosome trafficking. These observations suggest that dysfunction of early endosome trafficking causes ectopic differentiation of lens fiber cells, independent of spatial regulation through the lens equator. It was reported that disruption of lens epithelium induces EMT in human secondary cataract patients through the activation of TGF- β signaling. We found that ectopic lens fiber differentiation in VPS45 mutant is suppressed by application of a TGF- β inhibitor, suggesting that ectopic lens fiber differentiation in the VSP45 mutant depends on TGF- β signaling. These data suggest that early endosome trafficking system is important for maintenance of lens epithelial state through the suppression of TGF- β signaling.

Neural Retina Identity Is Specified by Lens-derived BMP Signals

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The eye has served as a classical model to study cell specification and tissue induction for over a century. During the early development of the vertebrate CNS, the

anterior neural domain becomes restricted into different regions giving rise to the telencephalon, the optic cup and the diencephalon. Simultaneously, cells contributing to the PNS, including lens placodal cells, are specified in the neural plate border. The molecular mechanisms that regulate the induction and maintenance of eye-field cells, and the specification of neural retina cells in relation to other forebrain domains have been poorly characterised. In addition, how prospective lens cells and retina progenitors regulate each other's development remains controversial. We have analysed these issues by manipulating signalling pathways in intact chick embryos and explant assays. Taken together, our results provide evidence that BMP signals emanating from the lens ectoderm maintain eye-field identity, inhibit telencephalic character and induce neural retinal cells, which highlights a novel role for BMP signals during the development of the retina that is distinct from previously described roles in dorso-ventral patterning of the retina and fate choice between neural versus retinal pigmented epithelial (RPE) cells. Our findings link the requirement of the lens ectoderm for neural retina specification with the molecular mechanism by which cells in the forebrain become specified as neural retina by BMP activity.

Lens Epithelium Loosing Control - New Mechanisms Underlying Fibrotic Cataract

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The lens is enclosed within the lens capsule, which is made of specialized extracellular matrix (ECM) necessary for physically supporting the lens, and provides an anchoring substrate for ciliary zonules that hold the lens in its correct position. The capsule also serves as a reservoir of growth factors required for correct function of lens epithelial cells (LECs). The ECM of the lens capsule is synthesized and secreted by anterior LECs, and one of its main components is collagen IV. The proper function of LECs during development is critical for the formation of an intact lens capsule and hence for correct lens function.

Using forward genetics in zebrafish we have identified a mutation that disrupts anterior LECs function and collagen secretion. Mutant embryos exhibit a severely reduced lens capsule, disruption of lens epithelium integrity and formation of cellular masses that displace the lens. Molecular analyses show that these cellular masses are

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characterized by hallmarks of a fibrotic reaction. Inhibition of TGF-beta signaling blocks the fibrotic reaction but not the lens dislocation phenotype and other abnormalities in LECs.

Together, our findings identify a new model for fibrotic cataract that provides new insights into molecular genetic mechanisms that can lead to fibrosis and other pathological processes due to abnormal function of LECs.

Systems Biology of Lens Development

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While molecular biology informs on the functional significance of individual genes and proteins in biological processes, systems biology aims to draw a global picture of how these individual components interact with each other to generate a particular biological outcome. So far, lens development in vertebrates has been well studied using the tools of molecular genetics, resulting in the identification of several important transcription and signaling factors that contribute to regulate its morphogenesis. The availability of new systems-level interrogative approaches, especially regarding transcript expression, is now providing unprecedented opportunities to define tissues/cell types in their wild-type or mutant state on a global level. Further, there are now opportunities to integrate these data with other publically available information such as molecular interaction datasets. We have used high-throughput gene expression data on mouse lens development to generate a web-based resource tool called *iSyTE* (integrated Systems Tool for Eye gene discovery). Application of *iSyTE* has impacted the characterization of several new genes such as *Caprin2*, *Mafg/k*, *Pvrl3*, *Sep15*, and *Tdrd7*, that function in lens development and homeostasis, and whose perturbations result in its defects including cataracts. Further, *iSyTE* has also contributed to the understanding of other genes involved in regulating lens development such as *Sip1* (Zeb2), *CBP*, *p300*, *Prox1*, among others. In particular, *iSyTE*-based identification of a novel RNA granule and RNA binding protein (RBP) *Tdrd7* as a cataract linked gene has initiated the investigation of other RBPs that function in distinct post-transcriptional gene expression control mechanisms in the lens. In addition to RBPs, *iSyTE*-based analysis has identified small Maf transcription factors to be involved in the regulation of genes involved in sterol synthesis in the lens, which is clinically important especially in light of recent discoveries from other laboratories on the impact of sterol derivatives

on lens protein aggregation. In this presentation, I will discuss ongoing efforts to expand *iSyTE* by integrating new molecular and systems-level data for its increased efficacy in gene discovery and construction of gene regulatory networks in lens development.

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Regulation of Lens Fiber Elongation by FGF Signaling

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Cell shapes are important for cellular functions, but how they are regulated is poorly understood. Fibroblast Growth Factor (FGF) signaling pathway is known to be required for lens fiber cell differentiation and elongation, but the key mediator(s) of FGF in stimulating lens fiber cell elongation are not clear. We generated a lens specific knockout of *Crk*/*CrkL*, two SH2- and SH3-containing proteins that transduce signals from upstream tyrosine phosphorylated proteins to downstream effectors. Deletion of *Crk* and *CrkL* led to a defect in lens fiber cell elongation without affect lens cell differentiation. It also prevent extra fiber cell elongation caused by overexpression of *FGF3*, indicating an epistatic relationship between *Crk*/*CrkL* and FGF signaling during lens fiber cell elongation. Further studies revealed that the activity of *Crk* and *CrkL* in FGF signaling is controlled by phosphatase *Shp2* and the defect observed in lens fiber cell elongation can be rescued by constitutive activation of GTPases *Ras* and *Rac1* in *Crk* and *CrkL* mutant lens. These findings suggest that *Crk* and *CrkL* play an important role in mediating FGF signaling induced lens fiber cell elongation during lens development.

LEN2 - Oxidative Stress

Oxidative Stress Regulation of Organelle Function by α -Crystallin

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α B-crystallin is important for lens epithelial cell viability, lens transparency and the functions of multiple other tissues. It is a multifunctional protein that prevents protein aggregation through its chaperone-like activity and prevents apoptosis through its direct interactions with multiple pro-apoptotic factors. We have shown that exposure of lens epithelial cells (LECs) to oxidative stress results in translocation of α B to the mitochondria and the nucleus suggesting that it defends LECs against oxidative stress insult at least by regulating the functions of these organelles. Here, we report that α B is induced by oxidative stress conditions and that translocation of α B to the mitochondria and nucleus upon oxidative stress requires its phosphorylation at specific sites. We demonstrate that phosphorylated mitochondrial and nuclear α B is specifically modified by sumoylation. We demonstrate that activation of specific LEC kinases is required for phosphorylation and translocation of α B to these organelles. We demonstrate that α B is required to prevent mitochondrial depolarization/damage upon oxidative stress exposure and that α B interacts with cyt c to prevent its release, LEC apoptosis and LEC death. α B protection of LEC mitochondria appears independent of chaperone activity since the R120G mutant form of α B that lacks chaperone activity translocates to the mitochondria and provides oxidative stress protection. In the nucleus, α B translocates to nuclear speckles. Previous studies suggest that α B interacts with alternate splicing machinery localized to nuclear speckles. We also show that α B interacts with p53 that can regulate chromatin conformation and accessibility. These data suggest that specific signal transduction pathways regulate the expression, phosphorylation and sumoylation of α B that in turn regulate its localization to LEC mitochondria and nuclei upon oxidative stress exposure. These events likely limit LEC apoptosis upon oxidative stress exposure through mitochondrial protection and regulate LEC gene expression upon oxidative stress exposure through control of alternate mRNA splicing and chromatin accessibility.

The Effect of Low Dose Ionising Radiation on the Lens Epithelium

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Cataract is an iconic non-cancer pathology associated with exposure to ionizing radiation (IR). The eye lens is well known to be the most IR sensitive eye tissue and yet the mechanistic basis for this is largely unknown. We established a low-dose IR exposure mouse model. Cell cycle biomarkers (e.g. cyclin D1), EdU labelling of newly synthesised DNA as well as markers of DNA damage response (e.g. MRE11, γ H2AX, RAD51, 53BP1) were monitored to track the process of DNA repair in the lens epithelium. They were compared to those seen in circulating blood lymphocytes. We also tracked the effects of IR upon cell proliferation and cell density in the lens epithelium. Mice were exposed to 0.02-2 Gy X-rays and their lens epithelia analysed. We established that the eye lens showed a linear dose response in terms of DNA damage repair during the first few hours after exposure. Regional differences in the response of the lens epithelial cells related to their proliferation status were confirmed and equatorial epithelial cells were the most sensitive to IR. The most surprising observation was the non-linear responses in cell proliferation and cyclin D1 levels in the cells at the lens periphery in response to low doses (< 1 Gy) X-irradiation. These led to effects on lens geometry months after the initial exposure. These data support a low dose stochastic model in the murine lens epithelium and evidence that the response of the eye lens to IR is very much dose dependent. The lens response would appear not to be deterministic, as there is no low dose threshold, and yet it can not be described as linear, because of non-linear responses at low dose. An important mechanistic question is whether other epithelia show similar low dose IR responses. These comparative studies will help us understand how low dose IR causes cataract.

Lens

A Structure-activity Study for the Inhibition of Matrix Metalloproteinase-9 Production by Polymethoxyflavones**YOSHIKI MIYATA, Hiroshi Kosano***Teikyo University, Tokyo, Japan*

Oxidative stress is one of major contributors to the development of several types of cataracts. Reactive oxygen species (ROS) provide a trigger for many downstream pathways that mediate cataract formation such as matrix metalloproteinases (MMP) production and cell proliferation in lens epithelial cells. MMPs are a family of zinc-dependent endopeptidases that have been shown to participate in fibrotic cataract, such as anterior subcapsular cataract (ASC) or posterior capsular opacification (PCO). PCO is caused by aberrant growth of lens epithelial cells that are left behind in the capsular bag after surgical removal of the fiber mass.

Recent research suggested that flavonoids may be involved in vision physiology and eye health. Flavonoids are a group of polyphenolic compounds ubiquitously distributed throughout the plant kingdom. Several reports have demonstrated that flavonoids exert antioxidant effects in ocular cells through their ability to act as free-radical scavengers, hydrogen-donating compounds, single-oxygen quenchers, and metal ion chelators. We also reported that nobiletin, one of the most abundant polymethoxyflavones in *Citrus species* inhibit the production of the pro-matrix metalloproteinase (proMMP)-9 in lens epithelial cells. In addition, we synthesized novel flavonoids based on nobiletin structure and demonstrated the structural properties leading to augmentation of inhibitory action against proMMP-9 production. These results suggest that nobiletin and the congeners could be useful compounds for therapy of fibrotic cataracts, such as ASC and PCO. The structure-activity relationship study using flavonoids may lead to characterize key structural features of antioxidants based on flavone structure.

Minimising Oxidative Stress in the Anterior Eye: The Role of the Cystine/Glutamate Antiporter in Mediating Extracellular Redox Balance**JULIE C. LIM, Renita M. Martis, Paul J. Donaldson***University of Auckland, Dept. Physiology, School of Medical Sciences, NZ National Eye Centre, Auckland, New Zealand*

The cystine/glutamate antiporter (xCT) is involved in a number of important functions in non-ocular tissues that include uptake of cystine for synthesis of glutathione (GSH)

and the maintenance of extracellular cysteine/cystine (CSH/CSSC) redox balance. However, the function of xCT in the eye is unknown. We have localised xCT to the different ocular tissues of the anterior mouse eye and discovered that xCT is expressed in the cornea and lens. Using a global xCT knockout mouse (KO), we showed the absence of xCT resulted in a significant depletion of GSH in the cornea but not the lens. Given the localisation of xCT in the lens, we have hypothesised that the role of xCT in the lens may be to control CSH/CSSC redox balance in the aqueous humor. Therefore, in this study, we examined whether removal of xCT alters CSH/CSSC levels in the aqueous humor and determined the effects of altered redox balance on the oxidative status and ocular structures of the lens and cornea. We collected plasma and aqueous samples from C57BL/6 wild type (WT) and xCT KO mice ranging in age from 6 weeks-12 months of age and measured CSH/CSSC levels by HPLC. We also collected cornea and lenses from WT and KO mice of the same age range and used several biochemical markers of oxidative stress to measure DNA damage and lipid peroxidation. We also examined the ocular structures of the cornea and lens using slit lamp microscopy and OCT imaging to determine whether KO mice developed ocular pathologies earlier than WT mice. As early as 3 weeks of age, we found that the levels of CSSC increased relative to CSH in the plasma and aqueous humor in the KO compared to the WT mice. At 3 weeks of age, increased levels of oxidative stress markers were detected in the eyes of KO mice compared to the WT mouse with ocular pathologies evident at 6 months of age in the KO mice but not WT mice. Collectively, these studies show that xCT is important in maintaining extracellular CSH/CSSC redox balance in the anterior eye and for minimising oxidative stress to tissues that interface with the aqueous humor. Since oxidative stress plays a major role in the onset of age-related eye diseases such as cataract, elucidating the molecular pathways that are involved in maintaining extracellular redox balance will be important information towards the design of effective strategies for delaying the onset of cataracts for which currently no preventive treatment exists.

The Role of PARp-1 and PAR Polymers in DNA Repair and Cell Death in UVB-challenged Human Lens Epithelial Cells**FRANK GIBLIN, Shravan Chintala, Vidhi Mishra, Caroline Cencer, Daniel Feldmann, Mirna Awrow, Nahrain Putris, Mason Geno, Maria Donovan***Oakland University, Eye Research Institute, Rochester, United States*

The purpose of this study was to investigate the function of the enzyme poly(ADP-ribose) polymerase-1 (PARP-1) in cultured human lens epithelial cells (LECs) exposed to UVB radiation. PARP-1 is known to play a role in both repair of DNA and induction of cell death through the production of polymers of ADP-ribose units (PAR) from NAD⁺. Solar UVB radiation and lens epithelial DNA damage have been linked to the formation of human cortical cataract. Human SRA01/04 LECs were exposed to UV light (280-380 nm wavelength, 312 nm peak) for 2.5 min, and then cultured normally. Two different UVB irradiances were employed, 0.09 and 0.9 mW/cm². At various times after UV-exposure, the following assays were conducted: cell viability (MTT assay), DNA strand breaks (Roche TMR Red assay), reactive oxygen species (ROS) (CellROX reagents), mitochondrial superoxide (MitoSOX Red) and fluorescence immunocytochemistry using antibodies to PARP-1 and PAR. Both UVB doses caused DNA strand breaks to form immediately after UVB exposure, which were rapidly repaired. Surprisingly, the higher UV dose also caused DNA strand breaks to occur again at 90 min, which were again repaired. Similar results were obtained for induction of PAR polymer fluorescence (PAR was not present in control cells). Induced PAR was observed to remain in the nucleus immediately after UV exposure, but at 90 min after the higher dose, PAR polymers were seen to flow from the nucleus into the cytoplasm, possibly to the mitochondria. Levels of PARP-1 fluorescence remained constant in the nucleus of control and UV-exposed cells. ROS and superoxide were detected primarily at 90 min after the higher UV dose. Whereas the lower UV dose had minimal effects on cell viability, the higher dose caused 70-80% cell death after 24 and 48 hrs. The results showed biphasic damage and repair of DNA in LECs at 1 min and 90 min following a lethal dose of UVB radiation. PARP-1 and PAR were demonstrated to play active roles in both protecting the human lens epithelium against UVB-induced DNA damage, as well as inducing cell death.

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LEN3 - Biomarkers in Cataractogenesis

Genetics Variances, Gap Junctions and Fiber Cell Morphogenesis in Cataract Formation

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Lens transparency relies on precisely packed lens fibers with unique surface interlocking structures such as ball-and- sockets (BS) and protrusions to minimize light scattering. Gap junctions formed by *Gja3* (Cx46) and *Gja8* (Cx50) are critical for lens transparency and growth. Our recent results reveal abnormally packed fiber cells, decreased F-actin expression at tricellular vertices, and a loss of BS structures on long-sides of hexagonal shaped fibers in *Gja8* knockout (KO) lenses. Thus *Gja8* gap junctions are essential for BS structures likely by providing structural domains to recruit an array of membrane/cytoskeletal proteins to stabilize BS structures. Loss of *Gja8* communication and BS structures probably decreases metabolic exchanges between fibers, subsequently impairs fiber cell elongation and maturation, and ultimately results in smaller lenses. In contrast, *Gja3* KO mice display severe cataracts in the 129 (129S4 and 129SvJ) strains but mild opacities in the C57BL/6J (B6) strain. We have determined that *periaxin* (*Prx*), encoding a scaffold protein with four variances between 129 and B6 strains, modulates cataractogenesis. The 129-*Prx* variant shows abundantly expressed proteins associating with F-actin at aberrant surface protrusions, impairing fiber cell packing and causing inner fiber degeneration in lenses with severe cataracts. The B6-*Prx* variant shows absent *Prx* protein in inner fibers with mild cataracts. Furthermore, deletion of beaded filament protein CP49 also promotes cataract severity. Therefore, lens transparency is achieved through the coordinative functions of gap junctions and cytoskeletal elements by controlling the morphogenesis of the precise surface architecture of lens fibers during development. Genetic variances of these genes have been detected in the human genomes project, this further indicates that genetic variances in combination with fiber cell surface markers will be important for predicting the development and severity of cataracts.

Lens Peptidomics Unravels Lens Aging and Cataract

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The most notable change observed in the aging lens and with the onset of cataracts is the progressive degradation of crystallins and concomitant increase in crystallin fragments (< 18 kDa). To better understand the crystallin fragments that accumulate in the lens peptides from young

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(20 years old; n=5), aged (70 years old; n=5) and cataract lenses (74 years old; n=5) were measured. In addition, the peptides in WS and WIS fractions of young, middle-aged and aged lenses were analyzed by LC-MS and the peptide profiles were compared. The total peptide level in young lenses was 0.29 mg/lens, whereas in old and cataract (with light brown nucleus) lenses peptide levels were only marginally different (0.9 mg/lens and 1.11 mg/lens, respectively).

Total ion current elution profile in LC-MS of water soluble peptides in young, middle-aged and aged lenses showed a clear age dependent appearance of peptides in the samples: more peptides in the aged lens than in the middle-aged lens. In the young versus middle-aged comparison of water-soluble peptides, 1261 peptides were detected. When filtered to exclude peaks whose abundance is < 1% of the most abundant and for 99.9 differential score (based on t-test < 0.1) only 128 peptides were revealed, 18 unique to young, 102 unique to middle-aged, and only 8 shared. Thus, there was a five-fold increase in the number of distinct peptides in middle aged lenses when compared to young lenses. In the middle-aged versus aged comparison, 1804 peptides were detected. Of these, 78 were unique to middle-aged, 199 unique to aged, and only 74 shared. In the young versus middle-aged comparison of urea soluble peptides, 718 peptides were detected. When filtered to exclude peaks whose abundance is < 1% of the most abundant and for 99.9 differential score (based on t-test, $p < 0.1$), only 31 peptides were detected and all were unique to middle-aged; none shared. In the middle-aged versus aged comparison, 1502 peptides were detected with a bias towards middle aged lens of which 195 filtered peptides consisted of 13 unique to middle-aged, 157 unique to aged, and 25 shared. Thus there is a clear progression of peptide appearance from young to aged lenses both in water-soluble and urea-soluble fractions. The above data of age-dependent increase in the accumulation of LMW peptides is consistent with the earlier studies.

Protein Degradation in the Aging Human Lens

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The human lens is comprised of post-mitotic cells and these cells, and some of the components within them, are present

for life. Over a period of decades, some macromolecules within these cells inevitably breakdown. This breakdown is spontaneous and, in the case of proteins, is due to the intrinsic instability of particular amino acids. The major posttranslational modifications (PTMs) responsible were found to be racemization/deamidation [1], truncation [2] and crosslinking [3].

Many of these reactions have been mapped by proteomic techniques using human lenses across the age range and what has emerged is that the PTMs can be subdivided into two types: (i) those that take place with age but do not appear to contribute to cataract *e.g.* racemisation of Asp 151 in α A crystallin [4] and (ii) those that are consistently greater in all cataract lenses than in age-matched normal lenses *e.g.* deamidation of Asn 76 in γ S crystallin. [5]

Whilst much work had been undertaken to examine racemisation/deamidation of crystallins in the human lens with age and cataract, the mechanism of protein-protein cross-linking is still poorly understood. Using a combination of proteomics and peptide model studies, the instability of phosphoserine and cysteine and the spontaneous formation of dehydroalanine (DHA) was investigated. Once formed, DHA becomes a site for nucleophilic attack by Lys and Cys residues leading to covalent crosslinking. Possible implications in cataract will be discussed.

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Novel Chemistries of Aging and Cataract

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Modifications to lens proteins in aged and cataract lenses have been studied for decades to test the hypothesis that age-related modifications lead to protein crosslinking, aggregate formation, and light scattering. Although crosslinking chemistries have been identified, the lens proteins involved and the sites of crosslinking have, until recently, remained unknown. The goal of this work is to identify lens protein modifications that increase with age and that lead to crosslinking; information that will assist in the elucidation of crosslinking chemistries. Changes in lens

proteins that disrupt the ordered gradient of refractive index may also cause light scattering, e.g. membrane binding of crystallins. In this work, imaging mass spectrometry (IMS) and LC-MS/MS analyses were used to identify cataract-specific protein changes and non-disulfide protein crosslinks in cataractous and aged human lenses. For IMS studies, cataract lenses and age-matched transparent lenses were sectioned, coated with MALDI matrix, and imaged in a MALDI-TOF mass spectrometer. For LC-MS/MS analysis, concentric regions of aged and cataractous human lenses were dissected and homogenized and the proteins were subjected to reduction/alkylation and trypsin digestion in either H₂¹⁶O or H₂¹⁸O. Tryptic peptides were analyzed by LC-MS/MS using a high resolution orbitrap mass spectrometer. Crosslinked peptides were identified using a combination of custom software tools and manual validation.

Imaging mass spectrometry revealed specific gs- and bA4-crystallin peptides localized to cataractous lens regions; peptides that were not observed in transparent lenses. These peptides shared a common Arg-Arg motif reported to enhance membrane binding [1]. In LC-MS/MS analyses, glutathione thioether linkages were quantified in different lens regions revealing that this modification results from a combination of protein age and glutathione concentration. Identification of crosslinked peptides revealed several crosslinking mechanisms including transglutamination, E/D-K crosslinks, and thioether formation via dehydroalanine. Crosslinked peptides identified include: bB2/bB2 crystallin, bA4/bA4 crystallin, bA3/bA4 crystallin, AQP0/AQP0, gS/bB1 crystallin and filensin/bA3 or bA4 crystallin. These results allow us to expand upon known protein aging chemistries that are specific to cataract and to suggest possible mechanisms to therapeutically target.

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Mapping and Quantifying Metabolites in the Aging Human Lens Using MALDI Imaging

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To protect against oxidative-stress induced protein damage, the lens contains high levels of antioxidants, such as glutathione and ascorbic acid. The development of age-related cataract is thought to be related to changes in lens antioxidants and other metabolites in specific lens regions, and leads to the protein damage, insolubilisation and opacification that characterises lens cataract. In this study, lens small molecules and metabolites have been mapped

in the aging human lens to define lens metabolome changes associated with normal lens aging, and a method to quantitatively map the distribution of predominant lens antioxidants developed using glutathione as a model.

Human lenses ranging in age from 35 to 80, and lens homogenates spiked with different concentrations of heavy GSH, were cryosectioned (20µm) and collected onto MALDI targets using methanol landing. Tissue sections were not washed, and dried in a vacuum desiccator for 30 mins. Matrix (7mg/ml NEDC in 90% MeOH) was applied using a TM-Sprayer (4 passes). Lens sections and spiked homogenates were analysed simultaneously by a MALDI-TOF/TOF mass spectrometer (Bruker UltrafleXtreme), in negative ion mode with MALDI IMS spatial resolution of 100µm. Data were imported into SCI LS Lab 2015b for analysis, while standard curves were plotted using Microsoft Excel.

Signals for predominant lens antioxidants glutathione and ascorbic acid were detected in lenses of all ages, in addition to signals for amino acids such as glutamic acid and taurine. Ascorbic acid was detected uniformly throughout the lens and its signal intensity declined with lens age. In contrast, glutathione signal was most intense in the lens cortex and lower in the nucleus. With age, glutathione signal declined, first in the nucleus, then in the cortex. For glutathione quantitation, a standard curve was successfully plotted from the lens homogenates spiked with heavy GSH and used to quantitate GSH signal levels in the human lens sections. This study highlights the metabolic changes that take place in the aging human lens, providing a baseline measure for age-related changes to be compared with metabolic changes associated with cataract formation.

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The Effect of Interaction between *EPHA2* Gene and Environmental Risk Factors on Cataract Development

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Cataract is the leading cause of blindness in the world. *EPHA2* gene encoding a tyrosine kinase receptor has been implicated in both rare hereditary and common age-

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related cataract. Disruption of this gene also leads to aging-related cataract in mice. Hence EPHA2 signalling is important in lens development and for maintaining lens transparency. In this study we investigated the interactive effect of *EPHA2* gene and environmental risk factors for age-related cataract, ultraviolet (UV) radiation and genetic background, on cataract development. To determine the effect of *EPHA2* gene and UV radiation, 5 week old heterozygous *Epha2*-null (Het) and wild type (Wt) mice (n=8) on C57BL6 background were repeatedly treated with below threshold doses of UVB (0.05 - 0.0125 J/cm²) over 4 weeks and cataract development monitored after treatment and a month later using modified LOCSIII (Lens Opacity Classification System III). Repeated exposure to below threshold doses of UVB resulted in dose-dependent anterior polar cataract in Wt and Het mice compared to no cataract in untreated mice (untreated vs 0.025 J/cm² UVB: Wt p=0.051, Het p=0.011; untreated vs 0.05 J/cm² UVB: Wt p=0.007, Het p< 0.001). Interestingly, a month after termination of treatment with 0.05 J/cm² UVB cataract severity in Wt mice increased (p=0.03) and trended towards being higher than that in Het mice (Effect size 0.31, p=0.09) suggesting that *Epha2* deficiency facilitated repair of UVB-induced damage in the lens. To determine the effect of *EPHA2* gene and genetic background, homozygous *Epha2*-null (Homo), Het and Wt mice (n³18) on two backgrounds C57BL6 and 50:50 C57BL6:FVB were monitored bimonthly for cataract progression using LOCSIII. Absence of functional *Epha2* resulted in age-related cortical cataract in mice; the rate of cataract progression depended upon the genetic background. Homo mice on C57BL6 background developed severe cataract earlier (by 4 months of age) than those on the mixed background (by 8-9 months of age). Additionally, Het mice on C57BL6 background developed severe cataract by 8 months of age whereas those on the mixed background exhibited only mild cataract at 14 months of age. These data suggest that genetic background significantly influences *Epha2*-related cataract development. In summary, interaction between *EPHA2* gene and environmental risk factors can accelerate or slow down cataract development in a context dependent manner.

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LEN4 - Lens Regeneration and Evolution

Loss and Gain of the Lens in the Evolution of Cephalopods

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Coleoid cephalopods have an elaborate camera eye whereas nautiloids have primitive pinhole eye without lens and cornea. The Nautilus pinhole eye provides a unique example to explore the module of lens formation and its evolutionary mechanism. Here, we conducted an RNA-seq study of developing eyes of Nautilus and pygmy squid. First, we found that evolutionary distances from the common ancestor to Nautilus or squid are almost the same. Although most upstream eye development controlling genes were expressed in both species, six3/6 that are required for lens formation in vertebrates was not expressed in Nautilus. Furthermore, many downstream target genes of six3/6 including crystallin genes and other lens protein related genes were not expressed in Nautilus. As six3/6 and its controlling pathways are widely conserved among molluscs other than Nautilus, the present data suggest that deregulation of the six3/6 pathway led to the pinhole eye evolution in Nautilus.

The Potential for Lens Transdifferentiation and Regeneration Across Species

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Among adult vertebrates lens regeneration is only restricted to newts. However, lens regeneration is also possible in some fish and pre-metamorphic frogs and axolotls. Lens regeneration is mediated by the process of transdifferentiation. In the case of newts by transdifferentiation of the dorsal iris pigment epithelial cells (PECs). The ability for transdifferentiation in vitro is also prominent in mammals including humans as well. These results will be discussed in light of the potential of achieving lens regeneration in higher species.

Approaching Newt Lens Regeneration by Transgenesis

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The newt is a unique four-limbed vertebrate (tetrapod) that invented a mechanism to regenerate various body parts repeatedly throughout its lifespan. Our current research focus is to uncover the core mechanism, or the

self-regeneration factor that this organism should have attained in evolution. Thus far, to move this project forward, we established in Japanese fire belly newt (*Cynops pyrrhogaster*) efficient gene manipulation protocols and a comprehensive transcriptome database 'IMORI' as well as protocols for animal rearing in both the laboratory and field (the newt stock center 'Imori-no-Sato'). Recently, we started applying our resources and technologies to the issue of lens regeneration. In this system, it is evident that the tip of the dorsal iris dedifferentiates and produces the lens vesicle, giving rise to a new lens. Amazingly, this process of lens regeneration can be repeated many times (at least 19 times). However, it would be impossible to explain such robustness of lens regeneration by only the tip of the dorsal iris unless the cells of the tip were replenished. It remains to be determined whether pigment epithelial cells (PECs) migrate from other areas to the dorsal tip and become competent for lens regeneration, or the dorsal iris tip cells replenish themselves. To solve this question, cell tracking using transgenic animals would be powerful. We transplanted a piece of the upper part of the dorsal iris from an mCherry transgenic newt to the corresponding area in the iris of a wild-type newt, carried out lentectomy and examined if mCherry fluorescence appears in regenerating lens. We repeated lentectomy at least 3 times. We found that a lens regenerated after the second lentectomy contained mCherry+ cells. This observation indicates that mCherry+ cells from the transplanted upper dorsal iris participated in lens regeneration, supporting the idea that migration occurs from other regions of the dorsal iris. Transplanting a piece of transgenic/gene manipulated iris would be useful to investigate the molecular mechanism by which PECs become competent for lens regeneration as they reside the tip of the dorsal iris.

Cell Signaling and Lens Regeneration: Diverse Developmental Mechanisms

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A number of conserved signaling pathways play key roles in the development of eye tissues, including the lens. Some adult animals, including newts and salamanders, are capable of regenerating a lost lens. In those cases the new lens is derived from the iris pigmented epithelium in a process of transdifferentiation referred to as Wolffian lens regeneration. FGF, Wnt and retinoic acid signaling pathways must be activated for successful lens regeneration during

Wolffian lens regeneration. Other animals, including species of *Xenopus* frogs, are also able to regenerate a lens. In *Xenopus* the new lens arises from the basal layer of the cornea epithelium, most likely from epithelial stem cells and their transient amplifying progeny. Like Wolffian lens regeneration, our lab has shown that FGFR signaling is both necessary and sufficient for lens regeneration to take place. However, the key ligand (FGF1) differs from that which triggers Wolffian lens regeneration (FGF2). While retinoic acid signaling must be active during Wolffian lens regeneration, we have shown that it must be inhibited to initiate cornea lens regeneration in *Xenopus*. Furthermore, unlike the situation found for Wolffian lens regeneration, Wnt signaling must also be inhibited during cornea lens regeneration. Taken together, the conditions found during *Xenopus* cornea lens regeneration are more closely related to that occurring early during vertebrate lens development. These findings are consistent with non-convergent, independent evolution of these two basic forms of lens regeneration.

Measuring Eye Lens Biometrics Rapidly - Taking the Pain out of Quantifying Cell Density, Proliferation Rates and TUNEL in Lens Samples

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The lens epithelium establishes the growth plan for the lens, providing the supply of fibre cells and ensuring their correct spatial organization in the form of the meridional rows before these cells exit the epithelium and enter the body of the lens as differentiating fibre cells. To date, gathering data on cell proliferation rates has either involved sectioning, flat-mounting or observing intact lenses distorted by the restraint used to position the lens for whole mount microscopy. The latter is exacerbated particularly for small, more spherical lenses. Here we have developed a conical gel mount system on glass microscopy slides for the mouse and zebrafish lens, allowing its placement and manipulation using standard LSCM (Laser Scanning Confocal Microscopy). Each lens was subsequently imaged with the Leica SP5 confocal microscope through sequential z stacks to measure cell density, cell proliferation and cell apoptosis events in the lens epithelium. We demonstrate the efficacy of this

Lens

approach for mouse and zebrafish lenses, measuring zonal cell densities and how this changes in mutant and environmentally challenged lenses. Using this approach, fixed mouse and zebrafish lenses were stained for nuclear markers of proliferation and apoptosis. Datasets were analysed *in silico* with an in-house processing suite, calculating cell distribution and density across the natural curvature of the tissue. Each lens required 20-40 minutes of processing and data analysis. A lens density peak is detected at the germinative zone in the murine lens, but some species and age-related differences were observed which will be discussed in greater detail.

LEN5 - Channels and Transporters

TRP Channels as Master Controllers of Homeostasis in the Lens

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To function normally, all cells must maintain ion homeostasis and regulate water content. These actions require active Na-K transport provided by Na,K-ATPase. The lens, however, is a multicellular structure made up almost entirely of fiber cells that have little or no Na,K-ATPase activity. Lens ion and water homeostasis relies on Na,K-ATPase activity in a small number of cells at the periphery of epithelium monolayer. Therefore, the function of the epithelium must be integrated with the needs of the fiber mass. We envision there to be a remote control mechanism that adjusts Na,K-ATPase activity to match increases or decreases of ion leakage that may occur a considerable distance away. Studies on the workings of this remote control mechanism revealed critical role of TRPV4 ion channels. In the intact porcine lens, Na,K-ATPase activity in the epithelium increased when TRPV4 channels were activated either by swelling the lens in hyposmotic (200mOsm) medium, by damaging a remote region of the fiber mass, or by a TRPV4-selective agonist GSK1016790A (30 nM). TRPV4 activation initiated a chain of events that included a rise of cytoplasmic calcium and opening of connexin hemichannels in the epithelium, release of ATP from the lens, and receptor-mediated activation of Src family tyrosine kinases. The entire chain of events could be prevented by TRPV4 blockade with RN1734 (10 μ M) or HC067047 (10 μ M). Separate lines of evidence, including studies on responses to the agonist capsaicin and to hyperosmotic shrinkage, point to a functional but different role of another TRP channel TRPV1. We speculate that TRP channels may be points of control for homeostatic feedback mechanisms.

Dynamic Regulation of Lens Volume: Roles for Channels, Transporters and their Signalling Pathways

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Previously we shown that the K-Cl-cotransporter (KCC), and Na-K-2Cl-cotransporter (NKCC) play key roles in regulating of the volume of lens fibre cells, and therefore in the maintenance of lens transparency¹. The activity of the two transporters is coordinated through signalling pathways that modulate their phosphorylation status². In response to cell shrinkage a signalling pathway, comprising of the kinases With no Lysine Kinase (WNK1, 3, 4), Ste-20 like Proline/Alanine rich Kinase (SPAK) and Oxidative Stress Response Kinase 1 (OSR1) phosphorylate the transporters to increase NKCC mediated ion influx, decrease KCC mediated efflux and effect an increase in cell volume. Conversely in response to cell swelling the kinase activity is reduced, the phosphatases PP1 and PP2A dephosphorylate the transporters to decrease NKCC mediated ion influx and increase ion efflux via KCC that reduces cell volume. We have shown that WNK1, 3, 4, SPAK, OSR1, PP1 and PP2A are all differentially expressed in the lens³, a finding that suggests that this pathway actively modulates lens volume. To determine whether regulation of NKCC phosphorylation is involved in the regulation of lens volume, bovine lenses were exposed to either osmotic challenge, growth factors or hyperglycemia and NKCC1 phosphorylation measured using a phospho-specific NKCC antibody. Modulation of the osmotic strength of the culture media revealed that under hypertonic conditions, lens shrinkage is induced and that this results in phosphorylation of NKCC. Furthermore, under hypertonic stress, addition of bumetanide, a NKCC inhibitor, results in even further loss of lens mass, confirming that NKCC1 activity is linked to the influx of ions and water to restore lens volume. Under isotonic culture conditions the exposure to growth factors contained in bovine serum or vitreous humour also elevated NKCC1 phosphorylation levels, increased NKCC1-mediated ion influx and produced increased lens wet weight. Under hyperglycemic conditions lenses exhibited a decrease in NKCC1 phosphorylation. Taken together these results show that a variety of stimuli can dynamically regulate NKCC1 activity through modulation of its phosphorylation status to affect changes in lens volume

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2. Gagnon & Delpire, *Physiol Rev*, 92:1577-1617.
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Unconventional Roles of Connexin 50 in Lens Cell Adhesion and Differentiation

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Connexins play essential roles in lens homeostasis and development. Aquaporin 0 (AQP0) is known to act as a cell-cell adhesion molecule in addition to its role in water transport in the lens. We have shown earlier that the direct relationship between connexin (Cx) 50 and AQP0 enhances gap junction channel activity. However, the impact of this interaction on cell adhesion of lens fibers remains elusive. In this study, we identified a new role for Cx50 that mediates cell-cell adhesion in the lens. We generated Cx50 and AQP0 double knockout mice and compared their lens morphology with Cx50 or AQP0 single knockout mouse models. Loss of both Cx50 and AQP0 resulted in more disruptions of lens structure and integrity, and increased spaces between lens fiber cells when compared to lenses deficient in either Cx50 or AQP0 alone. This observation is partially consistent with data obtained from thin-section electron microscopy analysis. Moreover, the lens size was greatly reduced in Cx50/AQP0 double knockout mice. Cell-cell adhesion studies showed that co-expression with Cx50 enhanced the adhesive capability of AQP0 in CEF cells. Interestingly, the expression of Cx50 alone promoted cell adhesion at a comparable level to AQP0; however, this increase was not observed with other lens connexins, Cx43 and Cx46. Moreover, this adhesive property occurred in both homotypic with Cx50 expressed in both pairing cells as well as heterotypic with Cx50 in only one pairing cell. Treatment with a fusion protein targeting the second extracellular loop (E2) domain of Cx50 inhibited cell adhesion and such inhibitory effect was not observed with the fusion protein containing E1 domain. These data suggest that the adhesive function is likely mediated by the E2 domain. Furthermore, disruption of cell adhesion by incubation with the E2 domain also impaired primary lens cell differentiation indicated by reduced lentoids formation and AQP0 expression. Together, these results suggest that in addition to its role in forming gap junction channels in the lens, Cx50 plays a unique role in mediating cell-cell adhesion function in the lens. The extracellular E2 domain is involved in this adhesive role of Cx50. Moreover, the cell adhesion mediated by Cx50 plays a critical role in lens epithelial-fiber cell differentiation.

Lens

Connexin Specific Lens Signalling

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Connexin mediated coupling and signal transduction pathways have both been shown to play critical roles in lens growth and development, but little is known about possible interactions between these two intercellular communication systems. To investigate how gap junctional communication and signal transduction pathways work together during postnatal lens development, we have looked for interaction between the lens specific connexins (Cx46 and Cx50) and the class 1A phosphoinositide 3-kinases (PI3K). We demonstrate that Cx50 coupling, but not Cx46, increased in *Xenopus* oocytes when co-expressed with a constitutively active p110 α subunit of PI3K. In addition, inhibition of PI3K signaling by blocking p110 α , or Akt, significantly decreased gap junctional conductance in Cx50 transfected cells, with no effect on Cx46. We also interbred lens specific p110 α knockout mice with Cx50 knockout animals to generate double knockouts. Lenses of littermates were dissected, weighed, and lens volumes were calculated from the measured diameters. We have previously shown that deletion of Cx50 resulted in a statistically significant reduction of lens size by $\geq 40\%$ due to reduced postnatal proliferation. Lens specific knockout of PI3K also produced a statistically significant reduction of lens size by $\geq 30\%$. Double deletion of PI3K p110 α and Cx50 caused a high rate of lens rupture (92% at 5 weeks, n=42). The few lenses that had not ruptured were $\geq 60\%$ smaller. These data suggest that the lens growth defects following Cx50 or PI3K deletion are additive. Furthermore, the increased incidence of lens rupture and in vitro regulation of Cx50 coupling by PI3K suggests that these two genes interact in maintaining lens homeostasis. Supported by NIH grant EY013163.

Connexin 46 G143R Mutation on Intracellular Loop Domain Alters its Interaction with Calmodulin and Gating of Hemichannels

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Connexin channels play a critical role in maintaining lens homeostasis and transparency. The G143R missense mutation on connexin (Cx) 46 is associated with congenital Coppock cataracts; however, the underlying molecular mechanism is largely unknown. In this study, we show that G143R mutation results in the impairment of electrical conductance mediated by Cx46 hemichannels, which is likely to be caused by structural changes of the intracellular loop domain leading to the altered interaction with calmodulin. Cx46 hemichannel activities were analyzed using both *Xenopus* oocytes and HeLa cell expression systems. G143R mutation completely diminished the hemichannel conductance in *Xenopus* oocytes. Moreover, this mutant functioned in a dominant negative manner by inhibiting the conductance of wild-type (WT) Cx46. The whole cell currents were determined in HeLa cells expressing WT or mutant Cx46. Whole cell current was detected in HeLa cells expressing WT, but not mutant Cx46. To understand the underlying mechanism of this functional change, we analyzed the secondary structures of intracellular loop domains using circular dichroism (CD) technique. α -helical structural content of the intracellular loop domain of Cx46 was greatly reduced as a result of the mutation. The interaction between intracellular loop domain and calmodulin was assessed by protein pull-down and isothermal titration calorimetry (ITC) assay. Protein pull-down assay showed that as compared to WT control, the interaction between intracellular loop and C-terminal domains was decreased. Conversely, the mutation enhanced the interaction between intracellular domain and calmodulin with a 2-fold increase of the binding affinity using ITC assay. Given that Cx46 G143 residue on intracellular loop domain is located in the potential calmodulin binding pocket with a close proximity to Cx46 C-terminus binding motif, it is likely that competitive binding between calmodulin defines the activity of Cx46 hemichannels.

Structural Role for Aqp0b in the Zebrafish Lens

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Mammalian AQPO is essential for normal lens development and transparency, and mutations in it lead to cataract formation in humans and in animal models. Understanding the normal function of this lens-specific and most-abundant-fiber-cell membrane protein would

shed light on normal lens physiology, as well as highlight potential mechanisms to prevent cataract formation. However, analysis of the mammalian AQP0 reveals multiple functions in one protein, making it problematic to study these functions individually. The zebrafish (*Danio rerio*) genome has undergone duplication millions of years ago resulting in two genes, Aqp0a and Aqp0b. *In vitro* expression in our lab has previously shown that while Aqp0a transports water, Aqp0b need not. The knock-down by morpholino anti-sense causes temporary cataract formation, which can be self-rescued confirming that both Aqp0 copies are essential for transparency but have subfunctionalized roles (Clemens et al., 2013). In this study, we show that the complete loss of Aqp0b by CRISPR-Cas9 directed genome editing leads to increased susceptibility of embryonic lenses to form temporary cataract at 2 days post fertilization (dpf) and a mild loss of tight cell packing evident specifically at the nuclear-cortical interface from 3 days onwards. This disruption becomes more evident at one month, as a localized, polar disruption of fiber cell packing. Knock-down of Aqp0a by morpholino levels that are subthreshold for forming cataract in wild types, leads to a higher frequency of cataract formation in null Aqp0b embryos at 3dpf that persists to 4dpf suggesting that there is some compensation by Aqp0a in Aqp0b null mutants. These data show that despite their functional and structural differences, there may be some overlap in the functions of Aqp0a and Aqp0b *in vivo*. Furthermore, the fact that zebrafish lenses can still form, albeit not perfectly, in the absence of Aqp0b suggests that early embryonic lens development largely relies on Aqp0a. However, the loss of tight fiber cell stacking is consistent with a structural role of Aqp0b in the zebrafish lens.

Clemens DM, Németh-Cahalan KL, Trinh L, Zhang T, Schilling TF, Hall JE (2013) In Vivo Analysis of Aquaporin 0 Function in Zebrafish: Permeability Regulation Is Required for Lens Transparency *In Vivo* Analysis of Aquaporin. *IOVS* 54:5136-5143.

LEN6 - Animal Models of Cataract

Cataract Surgery and Cognitive Function: Epidemiological Evidence from the HEIJO-KYO Cohort

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Circadian biological rhythmicity is regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus, an essential component of the master biological clock. Physiologically, light information is crucial for synchronizing the internal biological rhythm to the environmental rhythm through the SCN. Therefore, cataract may cause circadian misalignment between the internal and environmental rhythms because clouded lens decrease light transmission to the retina, especially shorter wavelength, which is sensitive to the photosensitive retinal ganglion cells. Previous interventional studies with the pretest-posttest design indicated that cataract surgery (the intraocular lens implantation) improved cognitive function and the risk factors of dementia such as sleep disturbances. However, the relationships between cataract surgery, sleep quality, and cognitive function have not been evaluated in large populations.

In the present cross-sectional study of the HEIJO-KYO cohort, we assessed objectively measured sleep quality (n = 1037) and cognitive function (n = 945) as well as best-corrected visual acuity in elderly individuals with and without previous cataract surgery (mean age, 71.9 years). The cataract surgery group showed significantly higher sleep efficiency by 1.3% and shorter wake after sleep onset by 4.9 min when compared with the no cataract surgery group in the multivariable analysis adjusted for potential confounders; however, urinary melatonin excretion, sleep onset latency, total sleep time, and sleep-mid time did not differ significantly between the cataract surgery and no cataract surgery groups. In addition, odd ratio for cognitive impairment (Mini-Mental State Examination score ≤ 26) was significantly lower in the cataract surgery group than in the no cataract surgery group. The significance remained even after adjustment for visual acuity and sleep quality (odds ratio, 0.64; 95% confidence interval, 0.43-0.96; P = 0.031). In summary, cataract surgery was significantly associated with better sleep quality and cognitive function, and the association between cataract surgery and cognitive function was independent of visual acuity and sleep quality in a general elderly population. Future randomized controlled trials are required to confirm these associations.

Lens

Application of Different Rodent Models to Further our Understanding of Fibrotic Cataract Pathology**FRANK JAMES LOVICU***University of Sydney, Anatomy & Histology F13, Sydney, Australia*

Since TGF β was first discovered in vitro to induce an epithelial to mesenchymal transition (EMT) in rat lens explants and whole lenses, many other animal models were established to demonstrate this phenomenon in lens. Most strikingly was the in situ manipulation of TGF β delivery in mice (directly or indirectly) that demonstrated that this EMT could directly contribute to anterior subcapsular cataract (ASC) formation in the intact lens. This onset and progression of ASC, now clearly evident in situ, led to mature fibrotic pathologies that very closely resembled human ASC, and has since been an established model to further manipulate cataract development in situ. Here we present examples of how these rat and mouse models were instrumental in identifying different genes that play a role in cataract formation, and also the discovery of other molecules that are effective in amelioration or prevention of the cataract. While lens cells in situ are subjected to a multitude of inducing and regulatory agents, it is the earlier established in vitro lens explant system derived from rats and/or mice, that allowed us to further examine the direct role and targets of TGF β -induced EMT, that will later translate to in vivo applications. These rodent models, together with the array of different tools, continue to provide important insights into fibrotic cataract pathology, including ASC and posterior capsular opacification (PCO) and will someday help us better understand how to promote and maintain the normal lens cell phenotype, in turn protecting lens cells against the detrimental effects of many factors, including TGF β .

EMT-type Cataract in Mice: Roles of TGF β -related Signals**KUMI SHIRAI***Wakayama Medical University, Ophthalmology, Wakayama, Japan*

Human cataract is grouped into several types according to the localization of the opacity. The anterior subcapsular cataract (ASC) contains myofibroblasts and extracellular matrix (ECM) both generated through epithelial-mesenchymal transition (EMT) in lens epithelium. I with colleagues reported that severe alkali burn in a mouse cornea induced EMT beneath the intact lens capsule

and produced a tissue that mimics human ASC. The mechanism underlying the phenotype is considered to be over-activation of transforming growth factor β (TGF β) in aqueous humor and resultant Smad activation in lens epithelium. Development of this type of mouse cataract was blocked by the loss of Smad3. A similar EMT-fibrosis is observed in a mouse lens during healing post-puncture injury. This lesion models human posterior capsular opacification (PCO), that was also reported to be abolished by lacking Smad3. Moreover, crosstalk between Smad and ECM-related signals supports Smad activation. For example, lacking of each of the members of matricellular proteins, i. e., osteopontin, lumican, tenascin C, attenuates Smad activation and the formation of EMT-related lesion inside an injured lens in mice. Not only Smad but also ECM molecules are to be candidates of targets for prevention of human PCO and ASC. The data also provides insights to understanding the pathobiology of EMT-related diseases in other non-ocular tissues.

Identification of Gene Mutations Responsible for Cataract in Mouse and Rat Models**KENTA WADA^{1,2}, Yoshiaki Kikkawa²***¹Tokyo University of Agriculture, Faculty of Bioindustry, Abashiri, Japan, ²Tokyo Metropolitan Institute of Medical Science, Mammalian Genetics Project, Tokyo, Japan*

The animal models of cataract have been contributed to identification of gene mutations and characterization of pathology in human congenital cataracts. To date, many mutant strains which exhibit cataract were established by spontaneous mutation or using mutagenesis. We also have identified causative gene mutations in several cataract mutant mouse and rat strains. Here, we mainly describe about the phenotypic and genetic characters in two kind of cataract models, *rct* mouse and KFRS4 rat.

The *rct* mouse was isolated spontaneously from SJL/J, and shows recessive anterior polar and cortical cataract with mild microphthalmia. Within 3 months of birth, all *rct* homozygous mutants developed a cataract that caused severe opacity throughout the eye. In the lens of *rct* mice, the localization of the nuclei was altered. At postnatal day 5 (P5), the lenses of *rct* mutants contained many nuclei in the posterior compartments. Over the next few days, the lens fibers in the *rct* mice became progressively more disorganized, and huge vacuoles were detected at P28. Based upon linkage analysis, we identified the 22-bp deletion in a putative *cis*-acting element of the *Foxe3* gene, and demonstrated that this mutation leads to lens specific reduction of *Foxe3* transcript during eye development.

Therefore, we suggested that hypomorphic mutation of *Foxe3* which was caused by 22-bp deletion in *rct* mouse leads to lens opacity, and we predicted that putative *cis* element is a lens specific enhancer of *Foxe3*.

KFRS4/Kyo rat was isolated from Kyoto Fancy Rat Stock (KFRS), and develops recessive cataract within 1 month after birth, and shows the opacity of the lens nuclei and the disorganization of lens fiber. Based upon linkage analysis, we identified a 5-bp insertion in exon 1 of *Mip* in the KFRS4. The 5-bp insertion of exon 1 in *Mip* is predicted to cause a frameshift mutation that results in truncation of the peptide chain by generating a stop codon at amino acid position 127. The immunoblot and immunohistochemistry analyses revealed that MIP protein could not be detected in the eye of homozygous KFRS4. Therefore, we concluded that cataract of KFRS4 is caused by 5-bp insertion of *Mip* gene, and completely ablation of MIP protein caused by this mutation was confirmed. The KFRS4 rat represents the first characterized rat model with a recessive mutation in the *Mip* gene.

Is it Possible to Induce Ultraviolet Radiation Nuclear Cataract in the Rabbit?

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Purpose: To create ultraviolet radiation (UVR) nuclear cataract in rabbits, while at the same time keeping the corneas clear for subsequent in vivo photochemical treatment of the cataract.

Methods: In a pilot trial, eight pigmented rabbits were exposed to various doses of UV radiation in 10 nm wavebands centered at 300, 305, 310, 315 and 330 nm. The doses were considerably higher than the ones used by Pitts et al. in pigmented rabbits, creating cortical cataract (IOVS, 1977).

Results: The UVB wavebands 300 to 310 induced slight anterior subcapsular cataract. The 315 nm UVB or the 330 nm UVA exposure did not cause any visible cataract, as judged by slitlamp microscopy. The cataracts became visible within a few days but did not change during subsequent months.

Conclusion: Nuclear cataract could not be induced by radiation in the described UVB or short-wave UVA range. Rabbit species differences might explain the discrepancy between these results and those of Pitts et al. (1977). Further exposures are in pipeline where repeated long-wave UVA exposures will be used to hopefully produce nuclear cataract.

LEN7 - Lens Cytoskeleton

Directed Migration of Lens Fibre Cells - A Two-phase Model

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The spheroidal shape of the eye lens is a prerequisite for its light-focusing function. The convex lens curvature is developed by directed migration of the apical and basal tips of the lens fibre cells to the anterior and posterior poles, respectively. This migration is thought to be driven by formation of actin-based membrane protrusions, namely lamellipodia, as deficiencies of Rho-family small GTPase (Rac1) or WAVE complex component (Abi2), key signalling molecules for actin polymerisation, abrogate this fibre tip migration to the poles. As actively moving cells develop lamellipodia at the migrating front, we should be able to monitor fibre cell movement by examining these structures. To date, no such systematic analysis has been done.

A classical model of lens cell migration is illustrated by proliferating lens epithelial cells being constantly displaced toward the lens equator due to increased cell number. Following their differentiation into fibres at the lens equator, the basal tips of these fibres continue their migration posteriorly. This movement continues until the tips reach the posterior pole. At the pole, each tip meets an opposing fibre tip to form a suture. If this description is correct, we would expect to see lamellipodia at the basal tip of each fibre cell in all locations. Paradoxically, no studies to date have reported or detected membrane protrusions at the basal tips of young fibre cells located just posterior to the equator. In this region the basal tips of the fibres are packed into a hexagonal lattice having straight edges or cell boundaries devoid of membrane protrusions.

Here we demonstrate small actin-based membrane protrusions that emerge at the posterior side of the hexagonal basal tip of fibre cells located approximately a 10 cell-distance from the equator in postnatal-day-26 rat lens. The protrusions extend substantially and the hexagonal lattice becomes loose in fibres posterior to this region. Well-developed lamellipodia were detected at the basal tips of inner fibres, and appear to then regress approaching the posterior pole and sutures.

This observation indicates that the newly differentiated fibre cells are static and that the active migration is induced during the following phase of maturation. We speculate that the lens fibres show two phases of migratory activity, where the cytoskeletal rearrangements required for hexagonal array formation and the development of the migratory mechanism are mutually exclusive.

Lens

Requirement of Aquaporin-0 and Adherens Junctions for the Integrity of Interlocking Protrusions and Transparency of the Lens**WOO-KUEN LO, Sondip Biswas, Lawrence Brako***Morehouse School of Medicine, Neurobiology, Atlanta, United States*

We have previously shown that aquaporin-0 (AQPO) targets interlocking protrusions to control the integrity and transparency of the mouse lens. We have found that complete loss of AQPO specifically causes severe disruption of protrusions which leads to fiber cell separation and cataract formation in the AQPO^{-/-} homozygous lenses at age 1 month and older. Here, we use the AQPO^{+/-} heterozygous lenses to compare the changes of protrusion structures and the N-cadherin-catenin-actin complexes during a slower cataract formation process. In the AQPO^{+/-} lenses of C57B6-J mice (1 to 12 weeks old), AQPO expression in protrusions was detected by immunolabeling using whole-mount samples. Changes in membrane structures and the N-cadherin-catenin-actin complexes were examined with confocal immunomicroscopy, SEM and thin-section TEM. The age-matched wild-type and AQPO^{-/-} lenses were used for control and comparison. The nuclear cataracts were first observed in the AQPO^{+/-} lenses at age 2 months in which interlocking protrusions in the nuclear fibers were significantly disrupted. In contrast, protrusions in the cortical fibers (~240 μm deep) exhibited only minor elongation and deformation which did not cause visible fiber-cell separation and opacification. Furthermore, the enlarged extracellular spaces between cortical fiber cells were rarely observed in the cataractous AQPO^{+/-} lenses (2-3 months old), but they were found extensively in the same cortical regions of the cataractous AQPO^{-/-} lenses (1 month old). Thin-section TEM revealed that many elongated and deformed protrusions were dispersed within the enlarged extracellular spaces in the AQPO^{-/-} lenses. These deformed protrusions were clearly devoid of intact adherens junctions, in sharp contrast with those regularly seen in the intact protrusions in the WT lens. Confocal immunolabeling analysis showed that the N-cadherin-catenin-actin complexes in the cortical fibers were significantly decreased in the AQPO^{-/-} lens, but not in the AQPO^{+/-} lens. It is concluded that the full expression of AQPO in conjunction with the normal distribution of adherens junctions (N-cadherin-catenin-actin complexes) are required for maintaining the integrity of interlocking protrusions and transparency of the lens. Additionally, the changes of the second-type gap junction-associated ball-and-socket interlocking domains due to the absence of AQPO will also be discussed. Supported by NEI/NIH grant R01 EY05314.

Regulation of Caveolar Morphology by the F-BAR Domain Protein PACSIN2/Syndapin II**SHIRO SUETSUGU***Nara Institute of Science and Technology, Nara, Japan*

Caveolae are flask-shaped invaginations of the plasma membrane. Caveolae are supposed to be involved in endocytosis, which is mediated by the constriction followed by the scission of the caveolae membrane invaginations. Caveolae are also involved in buffering of the membrane tension, such mediated by hypotonic or shear stress, through flattening of caveolae from the invaginated structure. However, how these two kinds of deformations, constriction or flattening, are regulated and switched is still unclear.

The BAR domain proteins form crescent-shaped dimers, and their oligomeric filaments are considered to form spirals at the necks of invaginations, such as clathrin-coated pits and caveolae. The subclass of the BAR domain, F-BAR domain, is involved in concave curvature of the membrane. PACSIN2/Syndapin II is one of the F-BAR domain-containing proteins. The PACSIN2 F-BAR domain induces the tubules of membrane *in vitro*, and is localized at the necks of caveolae. PACSIN2 also has the SH3 domain and NPF motif, which bind to dynamin-2 and EHD2, respectively. Dynamin-2 is a mechanochemical GTPase that mediates membrane constriction and scission, and the EHD2 is the protein that links caveolae to the actin filaments. Therefore, PACSIN2 is thought to function in the scission of caveolae through dynamin-2 as well as formation and stabilization of caveolae, presumably with EHD2.

We found that these two functions are considered to be switched by PACSIN2 phosphorylation by protein kinase C (PKC) upon hypotonic stress and sheer stress. The phosphorylation decreases the membrane binding affinity of PACSIN2, leading to its removal from caveolae. The removal of the putative oligomeric spiral of PACSIN2 from caveolar membrane invaginations could lead to the deformation of caveolae. Indeed, PACSIN2 removal from caveolae is accompanied by the recruitment of dynamin-2, suggesting that the removal provides space for the function of dynamin-2. Otherwise, the removal of PACSIN2 decreases the stability of caveolae, which could result in the flattening of caveolae. Accordingly, an increase in the amount of EHD2 restored caveolar stability. Therefore, PACSIN2 at caveolae stabilizes caveolae, but its removal by phosphorylation could induce both caveolar endocytosis and flattening.

Defect of Mitotic Vimentin Phosphorylation Causes Microphthalmia and Cataract via Aneuploidy and Senescence in Lens Epithelial Cells

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Vimentin, a type III intermediate filament (IF) protein, is highly expressed in lens tissue. Vimentin is known to be phosphorylated predominantly in mitosis. We previously found that the expression of phospho-blocking vimentin mutant in T24 cultured cells leads to cytokinetic failure, resulting in binucleation (multinucleation). However, it is not clarified about the significance of mitotic vimentin phosphorylation during organogenesis and homeostasis in lens tissue. Here, we generated knock-in mice expressing vimentin that harbor mutations in mitotic phosphorylation sites. Homozygotic mice (*VIM^{SA/SA}*) presented with microphthalmia and cataract in lens, whereas heterozygotic mice (*VIM^{WT/SA}*) was indistinguishable from WT (*VIM^{WT/WT}*) mice. In *VIM^{SA/SA}* mice, lens epithelial cells reduced in number and exhibited chromosomal instability (CIN) including binucleation and aneuploidy. The electron microscopic analyses revealed that lens fiber cells of *VIM^{SA/SA}* mice exhibited membrane disorganization similar to defects in age-related cataract. Since the mRNA level of senescence (aging)-related gene was significantly elevated in the lens of *VIM^{SA/SA}*, the lens phenotypes in *VIM^{SA/SA}* suggested a possible causal relationship between CIN and premature aging.

The Lens Actin Filament Cytoskeleton: Diverse Structures for Complex Functions

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Lens shape and mechanical integrity relies upon highly patterned morphogenetic differentiation and maturation of fiber cell layers. Age-related changes in lens structure and mechanics are linked to presbyopia, a reduction in the lens' ability to change shape during focusing (accommodation). While mouse lenses do not accommodate, we recently confirmed that mouse lenses show age-dependent stiffening like lenses in primates and humans, and thus mouse lenses provide an excellent genetic model system to elucidate the connection between cytoskeletal

regulation of lens fiber cell architecture, transparency and mechanical integrity. To elucidate the role of the actin filament (F-actin) cytoskeleton, we used biochemistry, immunohistochemistry, live lens microscopy and tissue mechanical testing, to study mouse lenses with genetic deletions or mutations in F-actin-binding proteins, tropomodulin1 (Tmod1), an F-actin capping protein, γ -tropomyosin (γ TM), an F-actin stabilizing protein, and nonmuscle myosin IIA (NMIIA), a force-generating contractile protein.

Genetic deletion of Tmod1 leads to reduced γ -tropomyosin (γ TM) and disruption of the spectrin-F-actin membrane skeleton with abnormal fiber cell packing and fiber cell membrane interdigitations. Tmod1-null lenses are transparent, but display reduced lens stiffness at low mechanical loads. Unexpectedly, genetic depletion of γ TM does not phenocopy loss of Tmod1. Instead, γ TM mutant lenses have subtle and progressive anterior cataracts with reduced lens stiffness under compression at high loads and impaired recovery of lens shape after release of external load (resilience). Mouse lenses with a function-blocking knock-in mutation in the NMIIA motor domain display obvious anterior opacities and defective resilience, similar to γ TM depletion, but unexpectedly, these mutant lenses have normal lens stiffness, unlike loss of Tmod1 or γ TM. In summary, disruption of Tmod1 leads to decreased lens stiffness at low loads and attenuated fiber cell membrane interdigitations, while loss of γ TM causes mild cataracts, and lens stiffness changes at high loads with abnormal resilience. A myosin motor domain mutation causes anterior cataracts and reduces lens resilience without affecting the overall stiffness of the lens. The disparate phenotypes of these mutant lenses imply that the F-actin cytoskeleton non-coordinately regulates the lens tissue level physiological functions of transparency, stiffness and resilience.

Tropomodulin 1 Regulation of Actin Is Required for the Formation of Large Paddle Protrusions between Mature Lens Fiber Cells

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The complex interface between mature lens fiber cell membranes is characterized by interlocking balls-and-sockets along the broad sides, as well as specialized interlocking small protrusions and large paddle

Lens

domains along the vertices of the short sides, which are hypothesized to be important for lens mechanical integrity. Our recent work shows that the spectrin-actin membrane skeleton and beaded filaments play an important role in establishing membrane domains to facilitate the formation of large gap junction plaques that rest within ball-and-socket protrusions on the broad sides. We previously demonstrated that tropomodulin 1 (Tmod1), an F-actin pointed-end capping protein, is important for fiber cell packing and lens stiffness. We hypothesize Tmod1 is needed for formation of complex fiber cell interdigitations that promote cell-cell interactions.

Using Tmod1(+/+) and Tmod1(-/-) mice, we characterized mature lens fiber cell structure using electron microscopy (EM) and immunostaining of single fiber cells for F-actin and F-actin-binding proteins. EM and immunostaining of control lenses reveals rows of fiber cells with coordinated F-actin-rich paddle protrusions that are decorated by smaller protrusions of equal size and spacing. In contrast, Tmod1(-/-) fibers have attenuated paddles with irregular protrusions. In Tmod1(+/+) fibers, Tmod1, β 2-spectrin and α -actinin are localized in large puncta in valleys between paddles, but in Tmod1(-/-) fibers, β 2-spectrin is dispersed while α -actinin is enhanced along the membrane at the base of the small protrusions and rudimentary paddles. At the base of small protrusions, Arp3 and fimbrin are enriched in both control and knockout lens fibers.

These results suggest Tmod1 is required for normal formation/maintenance of large paddle domains between mature fiber cells. Tmod1 may stabilize the spectrin-actin network and α -actinin cross-linked anti-parallel F-actin bundles at the base of large paddles to stabilize their structure. In the absence of Tmod1, expansion of α -actinin-F-actin bundle domains may partially compensate for the disrupted spectrin-actin network. By contrast, formation of small protrusions may be facilitated by Arp3-nucleated actin networks and fimbrin-cross-linked parallel F-actin bundles. This is the first work to reveal distinct requirements for actin cytoskeletal proteins in formation of paddles or protrusions between lens fibers and suggests that paddles are needed for lens mechanical integrity.

LEN8 - Post-translational modification of crystallins

Deamidation, Isomerization, and Racemization in Lens Beta/Gamma-crystallins from Aged, Cataractous Lenses

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Deamidation, racemization, and isomerization are by far the most prevalent modifications found in the crystallins of aged human lenses. Deamidation along with the new Asp and isoAsp moieties introduce destabilizing negative charges and would be expected to further perturb protein structure.

The goal of this study was to precisely define the relative extent of deamidation, isomerization, and racemization at all Asn and Asp residues in β/γ -crystallins from aged cataractous human lenses. We measured peptide masses during the chromatographic separations using the Orbitrap Fusion mass spectrometer in our laboratory at a resolution of 500,000. This has allowed unambiguous assignment of peptides containing deamidation, because, at this resolution, the 19 mDa mass difference between the isotopic peaks of deamidated and non-deamidated forms of the peptides can be resolved.

In a brunescient 85-year-old human donor lens, the intensities of the various deamidated forms of γ S peptide 72-78 were approximately 10-fold higher in the insoluble fraction compared to the soluble one. And, in the insoluble nuclear fraction, sixty-four percent of γ S was deamidated/isomerized/ racemized at N76. When the percentages of deamidation at residues N14, N76, and N143 were added together for this lens, there were an average of 2 deamidated/racemized/isomerized residues per γ S molecule.

The finding of such a strong association between these modifications and water-insolubilization in lens also supports that they are highly detrimental to crystallin structure. These advances will allow the unambiguous assignment of all major deamidated, isomerized and racemized residues in β/γ -crystallins from aged cataractous human lenses.

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Deamidation of Alpha- and Gamma-crystallins: its Effects on Structure and Interactions

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For proper lens transparency, it is crucial that the constituent crystallin proteins maintain their structure, interactions and arrangement, and hence solubility. *In vivo* with age, lens crystallin proteins undergo major modifications. Many of these modifications are non-enzymatically derived. The modifications include racemization of Asn and Asp (i.e. conversion from L- to D-amino acids), deamidation

(e.g. conversion of Asn to Asp) and truncation from the N- and/or C-termini [1]. Two crystallin sites, N76 in gammaS-crystallin and Q147 in alphaA-crystallin, undergo significant deamidation with age. Intriguingly, the amount of deamidation is significantly greater in human cataract lenses than in age-matched controls [1], implying that these two residues contribute to crystallin protein aggregation and hence cataract formation.

In this study, the effects on the structure, function and interactions of these two mutant crystallin proteins was assessed via a variety of complementary spectroscopic, biophysical and protein chemical techniques including NMR, circular dichroism and fluorescence spectroscopy, analytical ultracentrifugation, dynamic light scattering and transmission electron microscopy [2]. The study also involved an examination of the molecular chaperone, i.e. functional, ability of wild type and Q147E alphaA-crystallin. Complementary studies were also undertaken of other deamidated crystallin mutants, e.g. N143D gammaS-crystallin, which are prevalent at significant, but similar, levels in cataract and age-matched, non-cataract lenses. It is concluded that some deamidated crystallin mutants, particularly those preferentially associated with cataract, are more susceptible to aggregation, along with minor structural changes, than their corresponding wild type proteins.

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Age-dependent Isomerization and Racemization at Specific Aspartyl Residues in Lens Crystallins: Analysis and Biological Relevance

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A cataract is caused by clouding of the eye lens that may lead to a partial or total loss of vision. The mechanism of cataract development is not well understood. However, it is thought that eye lens proteins of a cataract are abnormally aggregated, resulting in clumping that scatters the

light and interferes with focusing on the retina. Human lens proteins are mainly composed from the α -, β -, and γ -crystallin superfamily of proteins. Alpha-crystallin is a hetero-polymer of about 800 kDa, consisting of 35-40 subunits of two different α A- and α B subunits, each of 20 kDa. The β/γ -crystallin superfamily comprises oligomeric β -crystallin (2-6 subunits) and monomeric γ -crystallin. The overall structure, stability and short-range interactions of these proteins are thought to contribute to the transparent properties of the lens. Because the lens crystallins are long-lived proteins, they undergo various modifications including isomerization, inversion, deamidation, oxidation, glycation and truncation. Of the modifications, we have proposed that the appearance of the isomers of aspartyl (Asp) residues, that is L β -, D α - and D β -Asp isomers may be responsible for the change in the higher order structure and contributes to the increase in aggregation, insolubilization and disruption of function of lens proteins leading to the formation of cataracts.

In this presentation, we demonstrate a new method for rapidly identifying Asp isomers in proteins based on a combination of LC-MS/MS and isomer-specific enzymes. We found that the isomeric Asp sites precisely, quickly at the femtomole level in lens crystallins. Asp 58, 76, 84 and 151 of α A-crystallin, and Asp 62 and 96 of α B-crystallin were highly converted to L β -, D β - and D α -isomers. The amount of isomerization of Asp was greater in the insoluble fraction at all Asp sites in lens proteins. Furthermore, we also observed the highly inverted Asp sites in the monomeric α B- and β A3-crystallins. This result indicates that the inversion of Asp residues may induce dissociation of polymeric α B- and oligomeric β A3-crystallin. The isomerization of these Asp residues affects the higher order structure of the proteins and contributes to the increase in aggregation, insolubilization, dissociation and disruption of function of proteins in the lens leading to the formation of cataracts. The stereoinversion of Asp may disturb lens protein assembly and induce not only the highly aggregate and but also dissociate to monomer.

Age-related Abnormal Asp Isomers Distribution in Lens Specific α A-crystallin Monomeric and Polymeric State

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Lens

Highly concentrated α -crystallin regarded as molecular chaperone is a major component of human lens. The α -crystallin is composed of two subunits α A- and α B-crystallin (α A-Crys and α B-Crys), which form hetero-oligomeric complex in the lens. Since the aggregates isolated from mature-onset cataracts contain a lot of damaged α -crystallins, one of the major reasons for age-related cataract formation would be due to the loss of molecular chaperone function of α -crystallin. Many covalent modifications, such as oxidation, deamidation and isomerization of aspartate (Asp) residues are accumulated overtime in crystallin, and reported to decrease those stability. Recent studies by LC/MS/MS analysis have shown that huge amount of aspartyl residues (L α -Asp) inverted isomers (L β -Asp, D α -Asp, D β -Asp) in abnormal α A-Crys monomeric fraction from aged lens. In the present study, we examined to clarify the isomerized Asp in dissociated α A-Crys and those in higher molecular weight fraction, which may contribute to decrease molecular chaperone function in aged lens.

We used different aged lenses in this study. Each lens soluble fraction was isolated by size exclusion chromatography and each α A-Crys containing fraction was independently digested by trypsin, followed by applying LC/MS/MS analysis. The ratio of Asp isomers in each fraction was determined by the comparison of the peak area from four Asp isomers containing peptide. As a result, we could identify the polymeric and monomeric state of α A-Crys in the soluble fraction of aged lens. The isomerization of Asp 58 and Asp 151 of α A-Crys was highly detectable in the monomeric fraction, but not in the polymeric one. The results also indicated that LC/MS/MS analysis for Asp isomers could be used for in-gel digested peptide. Non cross-linked polymeric α A-Crys fraction showed small amount of Asp isomers. On the other hand, cross-linked aggregates contain a lot of Asp isomers. We showed that the distribution of Asp isomers is different between in the dissociated α A-Crys and in the aggregated states of α A-Crys in aged lens. Furthermore, age-dependent Asp isomerization in α A-Crys is likely to contribute to the solubility of lens protein in aged lens. The isomerization of Asp would reduce the normal subunit-subunit interaction of α A-Crys with aging, resulting in senile cataract formation.

Role of Deamidated γ -crystallin Proteins in Cataract Formation

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The loss of lens transparency underlying cataract formation is widely attributed to a loss of crystallin protein stability and their subsequent propensity to partially unfold, leading to aggregation and precipitation. Cumulative post-translational modifications, e.g. deamidation, oxidation, glycation and truncation, of the lifelong crystallin proteins contributes to cataract formation. Here, we have investigated the effects of one of the most common modifications identified in aged or cataractous lenses: deamidation of human γ D- and γ S-crystallin at the analogous asparagine 137 and 143, respectively [1]. γ -Crystallins are ostensibly monomeric, two-domain proteins with eight β -strands in each domain arranged in two Greek Key motifs. The site of deamidation is a highly exposed asparagine residue in a flexible loop region in the C-terminal domain. Given the nature of the modification (i.e. polar to negatively charged), deamidation at this site is expected to be highly permissible. Indeed, both γ D- and γ S-crystallin showed no significant changes in their overall structure, thermodynamic stabilities or aggregation propensities following deamidation, consistent with the surface location and nature of the modification. In consideration of the correlation between the frequency of deamidation and solvent-accessibility of asparagine and glutamine residues in γ -crystallins [2], our data suggest that major structural perturbations occurring to γ -crystallins due to deamidation are uncommon in the aging lens.

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LEN9 - PCO/EMT

Regulation of Autophagy in Cyclosporine-A Treated Lens Epithelial Cells

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The current study aimed to clarify the signaling mechanisms involved in Cyclosporine-A (CsA)-induced lens epithelial cell (LEC) autophagy. Inhibition of mTOR potently upregulates autophagy even in the presence of sufficient nutrients and growth factors. Using cultured canine and human LEC, CsA-treatment decreased p-mTOR expression in a dose dependent response. At the top of the mTOR cascade and acting as main proteins involved in autophagy initiation are Akt and adenosine

monophosphate-activated protein kinase (AMPK). *In vitro* CsA-treatment did not change the intracellular AMP/ATP ratio indicating that CsA has a limited effect on the cellular energy status in LEC and may not activate AMPK. As autophagy can also be induced through CaMKK β -mediated activation of AMPK, subsequently leading to inhibition of mTOR, a selective CaMKK β inhibitor, STO-609, was used in conjunction with CsA treatment; autophagy was not prevented. By comparison, following CsA-treatment, expression of pAkt was decreased indicating that this is the more likely upstream mTOR regulator. LC3II expression confirmed CsA activated a functional autophagy response in both canine and human LEC. The signaling adapter p62 protein is a specific target for autophagic degeneration and has been shown to contribute to the formation of ubiquitinated protein aggregates. p62 mediates delivery of these aggregates to the autophagy system, serving as a readout of autophagic degradation. Additionally, p62 can accumulate within a cell and can be considered a marker of autophagic flux inhibition. Exposure to CsA for 24 hours did not result in increased accumulation of p62 in cultured LEC; however, prolonged CsA-treatment resulted in a dose dependent increase in p62. Use of the early stage autophagy inhibitor 3MA, in conjunction with CsA-treatment, abolished this p62 accumulation while treatment with CsA and BafA1, a late stage autophagy inhibitor, increased p62 accumulation. Additionally, treatment with CsA resulted in accumulation of ubiquitinated proteins; co-treatment with CsA and 3MA reduced accumulation while CsA and BafA1-treatment increased ubiquitinated protein accumulation. This data supports the hypothesis that CsA-induces autophagy using the Akt-mTOR pathway and prolonged exposure results in impaired autophagic flux.

Epigenetic Mechanisms Regulating Cell Reprogramming Associated with the Lens Fibrotic Disease PCO

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How cells with a normal function are reprogrammed to become myofibroblasts, the cell type responsible for the lens fibrotic disease Posterior Capsule Opacification (PCO), is not yet understood. Fundamental to the regulation of this cell differentiation process is uncovering how chromatin structure controls access of specific transcription factor (TFs) to DNA to coordinate this cell reprogramming. Using an *ex vivo* lens mock cataract surgery model that recapitulates features of PCO, we

investigated the cell reprogramming events involved in regulating changes in epigenetic markings of chromatin, which result in alterations of chromatin structure and impact gene transcriptional programs to control the emergence of myofibroblasts. Before surgery and until the first day following induction of cell reprogramming, we found that chromatin is characterized by a significant delay in the accumulation of the key repressive histone mark H3K27me3 on the myofibroblast progenitors following DNA replication. This signifies an "open" nascent chromatin structure, revealing a potential window of opportunity for the recruitment of pro-fibrotic TFs, such as MRTF-A, to DNA that are required for the reprogramming of the progenitor cells to a myofibroblast. Following this H3K27me3 rapidly accumulated on nascent DNA after replication, consistent with 'closed' nascent chromatin, reflective of a tight structure of chromatin that would prevent association of unwanted TFs to DNA to maintain the newly acquired differentiated fibrotic phenotype. Changes in the rate of H3K27 modifications associated with the development of fibrosis can be regulated by H3K27 modifying enzymes, which include the H3K27 tri-methylase EZH2 and H3K27 demethylase UTX. Blocking histone-modifying activity has the potential to manipulate chromatin structure, therefore we determined if blocking the histone modifying activity of UTX, which would allow for faster accumulation of H3K27me3 and a closed chromatin structure could prevent myofibroblast differentiation. Inhibition of UTX activity effectively prevented the emergence of α SMA+ myofibroblasts. These findings provide insight into the epigenetic events regulating cell reprogramming to a myofibroblast phenotype as well as reveal the potential to develop therapeutic strategies to modulate epigenetic mediated cell reprogramming to treat fibrotic disease.

Tropomyosin: Its Relationship with Age-related-cataract and Posterior Capsular Opacification

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Posterior capsule opacification (PCO) is still the most significant complication after cataract surgery, which is predominantly caused by remnant lens epithelial

Lens

cell (LECs) proliferation, migration and epithelial-mesenchymal transition (EMT) with resultant fibrosis. The tropomyosin (Tpm) family of cytoskeleton proteins is involved in regulating and stabilizing actin microfilaments. Previously we demonstrated that elevated expression of Tpm1 α /2 β was related to progression of rat PCO after cataract surgery. In this study, we investigated the role of Tpm2 in cataract and EMT during wound healing in mouse lens by generating Tpm2 hetero-knock-out (*Tpm2*^{+/-}) mice, using the clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9) system.

Normally, Tpm2 was not expressed in *Tpm2*^{+/-} or *WT* mice. At 7- and 16-weeks old, lenses were transparent in both *Tpm2*^{+/-} and wild type (*WT*) mice. However, at 40-weeks old, anterior central opacity and vacuole formations at the cortical surface of lenses were observed in *Tpm2*^{+/-} mice but not in *WT* mice. Further, EMT during wound healing in mouse lens was more prevalent in *WT* mice in contrast to in *Tpm2*^{+/-} mice.

The present results indicate that low levels of Tpm2 in the normal lens contribute to lens opacity. Further, Tpm2 is involved in the progression of EMT in wound healing of mouse LECs. Tpm2 may play important roles in maintaining lens transparency and the progression of PCO.

Matrix Metalloproteinase 9 (MMP9) Regulates Cytoskeletal Dynamics during Epithelial to Mesenchymal Transition (EMT) in Lens Epithelium

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Posterior Capsular Opacification (PCO) or secondary cataract is a common postoperative complication after cataract surgery that results in complete blindness. Earlier studies have shown that TGF β -induced EMT is a major contributor to PCO. Previously, we have shown that reduced expression of MMP9 prevents TGF β -induced EMT, through inhibition of fascin, a key protein involved in actin bundling during F-actin polymerization, and stress fiber formation (F-actin polymerization). However, the key signaling molecules and the mechanism/s through which MMP9 regulates cytoskeletal changes during TGF β -induced PCO remain largely unknown. To decipher the role of MMP9 in cytoskeletal dynamics during TGF β -induced EMT, we performed gene expression analysis from whole lenses, and lens epithelial explants (LECs), from wild-type (*WT*), TGF β overexpressing transgenic (TGF β ^{tg}) or TGF β ^{tg} mice on an MMP9 knockout background (TG;M9KO). The analyses show an increase in α -smooth muscle actin (α SMA), an EMT marker and a major contractile

cytoskeletal protein, by approx. 1.5-fold in TGF β ^{tg} ($p < 0.01$) when compared to the *WT* LECs. This increase in α SMA remained unchanged in TG; M9KO ($p < 0.01$) when compared to TGF β ^{tg} LECs. Further, western blot analysis revealed no change in the protein level of α SMA in TG;M9KO when compared to TGF β ^{tg} LECs. However, immunofluorescence analysis revealed an absence in α SMA positive stress fibers in TG;M9KO when compared to TGF β ^{tg} LECs. To investigate into how MMP9 regulates polymerization of α SMA we analyzed proteins and their activated counterparts that play a key role in polymerization of α SMA on samples from *WT*, TGF β ^{tg} and TG;M9KO LECs. Our phospho-cytoskeletal protein array shows a decrease in the phosphorylation levels of focal adhesion kinase (FAK), p-FAK-Ser397 and p-FAK-Ser910; the major p-FAKs involved in relaying signals for cell migration, in TG;M9KO when compared to TGF β ^{tg} LECs. Additionally, we also observed a substantial decrease in p-Lim Kinase (LIMK)-Thr508 and p-myosin regulatory light chain kinase 2 (MLCK2)-Ser18, the major kinases responsible for F-actin polymerization/stress fiber formation, in TG;M9KO when compared to TGF β ^{tg} LECs. The decrease in the activation of major signaling molecules regulating cytoskeleton in TG;M9KO when compared to TGF β ^{tg} LECs indicates a role for MMP9 in regulation of cytoskeletal changes during TGF β -induced EMT in lens. Ongoing studies will reveal how MMP9 regulates these signaling molecules, and thus EMT.

A Role for Nox4 in Mediating TGF β -induced EMT Leading to Cataractogenesis

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Transforming Growth Factor- β (TGF β) can induce an epithelial to mesenchymal transition (EMT) in the lens. In an ocular context, this process gives rise to anterior subcapsular cataract (ASC), impeding the normal refraction of light leading to blindness. Recently, we have shown that the reactive oxygen species (ROS) producing enzyme, NADPH oxidase 4 (Nox4), is upregulated in response to TGF β -signaling in lens. Furthermore, in an in vitro lens epithelial explant model, we showed that pharmacological inhibition of Nox4 (using the *pan* Nox inhibitor, VAS2870) abrogated not only TGF β -induced ROS production, but

also aspects of EMT. To better explore the potential role(s) of Nox4 in lens EMT in situ, the present study utilised a mutant mouse line deficient for Nox4. When first explanted and treated with exogenous TGF β , lens epithelial cells haploinsufficient for Nox4 (heterozygous mutant lines) remained epithelial-like and did not express the myofibroblast, EMT marker, alpha-smooth muscle actin (α SMA). Moreover, when mice overexpressing bioactive TGF β specifically in lens were crossed to the mutant mice deficient for Nox4, interestingly by postnatal day 21, lens from the resultant transgenic progeny were found to be transparent and did not present anterior subcapsular cataracts as normally seen in TGF β overexpressing lines. These results indicate that in mice, Nox4 plays an important role in the development of TGF β -induced EMT leading to cataract in situ. Taken together, these findings now provide a new platform allowing us to delineate the specific targets of Nox4-derived ROS leading to EMT and cataract, and a possible mechanism-based approach to therapy in humans.

LEN10 - The Zonule of Zinn: biology and pathology

Functional Connection of the Key Matrix Is the Intra-capsular Zonule for Lens Accommodation on Monkey Eye

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Accommodation is a visually guided long loop reflex through the visual cortex on ciliary muscle (CM) contraction. The generative system elicits the change of antero-posterior lens axis instantly and sufficiently to minimize the blurring of interest image of object. The CM is innervated by the autonomic nervous system without tendinous ligament. To change the elasto-plastic nature of lens axis, several organelles, i.e. the ciliary process, zonule, lens capsule and lens fiber, coordinate each other for the visual situation accurately. Among them, how the fine fibrous zonule suspends and manipulates the huge lens is the most mysterious.

To proceed the proposed functional organization, the extracellular matrices (ECM) coordinate on each instantaneous and fine configuration changes capable of being stretched and tensile property. The morphological analyses revealed by each molecular components were the *fibrillin* for zonule, *elastin* for ciliary muscle envelope, *collagen-IV* for lens capsule, *fibronectin* for lens epithelium and *laminin* for capsular interspaces.

The ciliary processes synthesized the fibril and fibrils aggregated with neighbors to composite of bundle. The pattern of fixation was disentangled fibrils into capsule. The most critical fixation of anterior zonule brought forth by penetration closely to epithelial layer in central circumferential lamellae. These fixating pattern is critical to transmit the tensile movement of ciliary process finely and instantaneously to lens accommodation.

Another novel finding is the parallel slits in region of central anterior capsule. These slits aligned obliquely in full thickness of capsule about every 100 μ m. A tracer (Cell Tracker[®]) instilled into anterior chamber passed through these slits into the epithelial cell layer suggesting the metabolic route on lens fiber homeostasis. Contrarily, the posterior capsule were monotonous membrane and the tracer instilled into posterior chamber infiltrated in the capsule. As a result, the pathologic lens epithelium brings the deterioration of transparency more in anterior capsular division and decreased plasticity of accommodative alignment. The analyses of anterior capsule of cataract explored the degenerative pathology. These morphological approach on interaction of zonule with lens will be informative to know the functional anatomy of accommodation. For this purpose objective measurement of near triad response by *Trilris*[®] (Hamamatsu Photonics) is useful clinically.

Latent TGF β Binding Protein-2 Is Essential for the Stable Structure of Ciliary Zonule Microfibrils

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Latent TGF β -binding protein 2 (LTBP-2) is an extracellular matrix protein associated with microfibrils. Homozygous mutations in *LTBP2* have been found in humans with genetic eye diseases such as congenital glaucoma and microspherophakia, indicating a critical role of the protein in eye development, although the function of LTBP-2 *in vivo* has not been well understood. We explored the *in vivo* function of LTBP-2 by generating *Ltbp2*^{-/-} mice. *Ltbp2*^{-/-} mice of C57BL/6J background survived to adulthood but developed lens luxation caused by degradation of ciliary zonule after birth without a typical phenotype related to glaucoma, suggesting that LTBP-2 deficiency primarily causes lens dislocation but not glaucoma. In DBA2 background, *Ltbp2* deficiency caused increase in intraocular pressure in addition to lens luxation. Supplementation of recombinant LTBP-2 in culture medium restored unfragmented and bundled ciliary zonules in *Ltbp2*^{-/-} mouse eyes under organ culture. The data above suggest

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that LTBP-2 is an essential component for the formation of microfibril bundles in ciliary zonules.

Next we investigated why *Ltbp2*^{-/-} mice showed only eye phenotype, whereas LTBP-2 is abundantly expressed in other tissues including lungs and arteries. We show that LTBP-4, another microfibril-associating protein that is expressed in lungs and arteries but not in ciliary body, has an overlapping function in the development of stable structure of microfibrils in vitro and in vivo.

Molecular Composition of the Ciliary Zonule and its Role in Regulating Lens Size

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The ciliary zonule is an elaborate system of extracellular fibrils that spans the gap between the non-pigmented ciliary epithelium and the lens equator. Although first described by Johann Zinn two hundred and fifty years ago, many questions remain about the composition and organization of the zonule and its physiological role in the eye.

We used mass spectrometry to investigate the composition of zonular fibers extracted from bovine or human eyes. This provided a semi-quantitative estimate of the type and proportion of matrix components present in the zonule. Zonule composition was similar in the two species. In both cases, many proteins were detected but the majority of the zonule was comprised of two members of the fibrillin superfamily: fibrillin-1 (FBN1) and latent TGF-beta-binding protein-2 (LTBP2).

To investigate the role of FBN1 and LTBP2 in zonule structure and function, we examined the phenotypes of mice deficient in one or other protein. Interestingly, the appearance of the zonule in knockout animals was initially indistinguishable from wildtype but, with time, OCT imaging revealed the breakage of zonular fibers and the eventual dislocation of the lens. We examined in further detail the effect of zonular dehiscence in the *Ltbp2*-null mice. At intermediate time points, when some but not all zonular fibers had broken, we performed EdU-labeling to determine the proliferation rate of epithelial cells in the germinative zone of the lens. These experiments revealed a marked decrease in the rate of cell proliferation in the knockout mice that was most evident in regions subjacent to fiber breaks. Further, measurement of lens diameter indicated that *Ltbp2*-null lenses were significantly smaller than wildtype, presumably due to the measured decrease in cell proliferation.

Together, these observations suggest that Fbn1 and Ltbp2 are necessary for zonular integrity and that attachment of zonular fibers at the lens equator plays an important role in regulating the proliferation of lens epithelial cells and thus the size of the lens.

Investigating the Implications of the Long Anterior Zonule Trait

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The long anterior zonule (LAZ) trait is characterized by zonular fibers that occur more central than usual on the anterior lens capsule. Seen clinically after pharmacologic pupil dilation, LAZ fibers typically appear as radially-oriented pigmented lines resulting from melanin granules being pushed into canal-like channels within aberrant zonules that rub against the posterior iris. Accompanying signs include pigment deposition on the posterior cornea (Krukenberg spindle) and within the trabecular meshwork. LAZ fibers are mostly bilateral and may have distinctive distribution patterns and quadrant predilection. Individual LAZ fibers are frequently "segmental" in nature, whereby they cannot be visually traced all the way to the lens periphery. Central zonule-free zones in LAZ eyes are frequently only 3-4 mm in diameter, which has created concern during capsulorhexis procedures during cataract surgery. Although not well-known and minimally studied, evidence suggests that LAZ fibers are not uncommon and can be easily detected when there is effort to find them. Because the trait is unfamiliar, it may cause diagnostic confusion when related pigment dispersal signs lead clinicians to think of better-known, alternative etiology of anterior segment pigment dispersion. Interestingly, although Krukenberg spindles are widely discussed in the literature, relationship to the trait is rarely mentioned and recent data suggests LAZ fibers could be a leading reason for such corneal pigment deposition. Rarely, the LAZ trait may be caused by a serine 163 arginine mutation (S163R) in the C1q tumor necrosis factor-related protein 5 gene (C1QTNF5/CTRP5) in association with familial late-onset macular degeneration. More commonly, LAZ fibers appear to have different etiology, resulting in a phenotype having predilection for hyperopic females >50 years of age. Studies within a single institution suggest that this LAZ variety may have prevalence near 2%, with other common

features including presence of persistent pupillary iris strands and shorter axial length. Aside from being strongly age-associated, basic mechanisms leading to LAZ formation are unknown. There is suggestion that the trait may have association with both narrow- and open-angle glaucoma, but this has been insufficiently studied. Current preliminary study suggests that intraocular pressure may be slightly higher on average in eyes with LAZ fibers, but this also needs more study.

Cataract Surgery in Exfoliation Syndrome Patients: Errors in the Prediction of Postoperative Refraction and Postoperative Change of Intraocular Pressure

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Exfoliation syndrome (XFS) is associated with complications related to weakened zonules or glaucoma. Zonular weakness could cause refractive errors in cataract/IOL surgery via influences on in-the-bag IOL positioning. We retrospectively examined (1) postoperative refraction and (2) alteration of postoperative intraocular pressure (IOP) in non-glaucoma XFS patients.

(1) Errors in the prediction of post-IOL surgery refraction were significantly greater in the XFS group than in the non-XFS group during the early post-operative stage, but no difference at post-cataract surgery 1 month.

(2) The IOP significantly increased in eyes with XFS at post-cataract surgery day 1 although it was normalized in 1 month postoperatively. Patients with XFS are to be informed of these points prior to surgery. Outcome from a prospective study on the error between one-piece IOL implantation and three-piece IOL implantation in XFS patients will also be discussed.

LEN11 - Alpha crystallins and small heat-shock proteins

Small Molecule Pharmacological Chaperones for Lens Alpha-crystallin

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The molecular chaperone α -crystallin helps to maintain the transparency of the eye lens. The soluble fraction

of this protein decreases with aging, a phenomenon that is associated with age-related cataracts. Moreover, destabilizing mutations in α -crystallin are associated with early-onset, hereditary cataracts. Using computational and experimental high-throughput screening techniques, small molecules ligands for α -crystallin were identified that bound to destabilized α -crystallin. The lead compound (c29) was identified to be an oxysterol that reduced protein amyloid formation in vitro and improved lens transparency in vivo when applied topically to mice with cataracts. Knock-in mouse lenses with cataracts associated with the R49C mutation in α A-crystallin and the R120G mutation in α B-crystallin demonstrated an increase in protein solubility when dosed with c29. The compound also improved protein solubility in ex vivo, aged human lenses. The mechanism of action may involve an increase in stability of cell-cell interaction in the lens epithelium of these mice. These studies suggest that c29 may be developed as a potential pharmacological, non-surgical intervention to treat cataract.

Good Things in Small Packages: The Molecular Chaperone Action of the Small Heat Shock Proteins

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The small heat-shock molecular chaperone proteins (sHsps) are one of the cell's first-lines of defence against protein aggregation and therefore play a vital role in maintaining protein homeostasis (proteostasis). Despite this, the precise mechanism by which they function as chaperones remains unresolved. We and others have shown that the sHsps are potent inhibitors of the (amyloid) fibrillar aggregation of proteins, a process which is associated with diseases that include Alzheimer's disease, Parkinson's disease and Huntington's disease. Moreover, the sHsps interact with a variety of species formed during this aggregation process, from monomers to the mature fibrils. Recently our work has focussed on defining the mechanism by which sHsps interact with mature fibrils. We show that binding of alphaB-crystallin or Hsp27 to fibrils formed by alpha-synuclein involves the N- and/or C-terminal domains as the isolated core domains of the sHsps do not bind. Moreover, through their binding, these sHsps reduce the cytotoxicity of alpha-synuclein fibrils applied exogenously to cells. We have also studied the ability of sHsps to prevent alpha-synuclein aggregation in cells. In doing so we have avoided tagging the sHsps with bulky fluorescent tags as these may interfere with

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their chaperone function. We have done this by using bicistronic vectors for the correlated expression of a (non-tagged) sHsp and a fluorescent reporter (e.g. mCherry protein). Using this approach we show that expression of alphaB-crystallin or Hsp27 prevents the aggregation of alpha-synuclein into cytoplasmic inclusions in cells. Together these findings highlight that sHsps interact with aggregation-prone proteins at various stages along the aggregation pathway and that they are promising therapeutic targets for diseases associated with protein aggregation.

Dynamic Structure and Flexible Functions of sHSPs

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We have developed and applied novel biophysical approaches to overcome the challenges posed by the inherent heterogeneity of sHSPs. This allows to glean simultaneously both structural and dynamical information on these molecular chaperones, and their interaction with target proteins. Here we present detailed biophysical insights into the self-assembly of the polydisperse human sHSPs α B-crystallin and HSP27, and disease-related variants thereof. By combining MS with NMR spectroscopy, X-ray methods and molecular dynamics simulations we uncover the intricate hierarchical dynamics of the sHSPs, and how these regulate interaction with both native and non-native protein targets in the cell.

Investigating the Structure of the Small Heat-shock Protein α B-crystallin under Conditions of Macromolecular CrowdingAIDAN BRADLEY GROSAS¹, Agata Rekas², Jitendra Mata³, Heath Ecroyd⁴, Damien Hall¹, John Adrian Carver¹

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The internal environment of extra lenticular cells contains

high concentrations of macromolecules, 50-400 mg/mL. In the eye lens, the concentrations of crystallin proteins can be up to ~500 mg/mL. Molecular crowding at such high concentrations has many consequences including modulating biochemical interactions such as protein folding, oligomerisation, and chaperone action. In vitro studies have demonstrated that excluded volume effects at high concentrations of inert macromolecular crowding agents impact the structure and function of proteins, particularly those with disordered regions. Such regions of unfolded structure are often found in molecular chaperone proteins, including small heat-shock proteins (sHsps), such as α -crystallin, whose biological function resides in preventing protein aggregation. α -Crystallin exists as a heterooligomer of two subunits named α A- and α B-crystallin although the functional role of this association is not clearly established. Of particular interest is the subunit α B-crystallin which, itself, forms a large, heterogeneous, oligomeric and dynamic assembly. Due to α B-crystallin's dynamic, oligomeric nature and unstructured regions, there are two structural features that may be altered upon molecular crowding:

- (i) the compaction of the unstructured regions leading to an overall smaller monomeric unit and
- (ii) the formation of higher order oligomers due to increased association.

Contrast-matched small angle neutron scattering (SANS) qualitatively displays a significant increase in size consistent with the formation of very large aggregates at >100 mg/mL of the crowding agent Ficoll 400 while quantitative measurement of the Porod exponent (a measure of surface/mass fractal scaling) shows a marked change in the average structure of the heterogeneous ensemble. Spectroscopic measurements including circular dichroism and fluorescence spectroscopy support these changes. While the significance of these results is not yet clear, the formation of very large aggregates of α B-crystallin is unlikely to be biologically favourable. Thus, these results may highlight the need for α B-crystallin to exist as a heterooligomer to remain structurally viable in the crowded cellular environment.

LEN12 - Physiological Optics

Salt and Water Circulation through the Lens: A Role in Controlling Optics?

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Once the embryonic lens has formed, membrane transport becomes dedicated to maintaining a transparent living organ. To avoid light scattering, the lens lacks a vasculature. An internal circulation of salt and water, which performs many functions of a vasculature, is generated by a spatially segregated pattern of transport protein expression. The circulation of water was expected to depend on both transmembrane osmosis and hydrostatic pressure, which is required to push water through gap junctions. We used a microelectrode-manometer system to measure intracellular pressure. Pressures varied from 340 mmHg in central mature fibers (MF) to 0 mmHg in surface cells of mouse lenses, where the gradient could be increased/decreased proportionally by a decrease/increase in gap junctions or by altering salt transport. Pressures in lenses of different sizes from mice, rats, rabbits and dogs were compared, and remarkably they were always 340 to 0 mmHg. The larger lenses appeared to express fewer ion channels in MF, reducing salt and water circulation. The effect of age on transport in mouse lenses showed gap junction coupling was reduced 4-fold between 2 and 14 months of age. The central pressure also increased, but only by about 40%, suggesting intrinsic feedback reduced the circulation to maintain a more constant pressure. In the above studies, pressure in surface cells was 0 mmHg. We found surface pressure is set by a dual feedback control system that uses TRPV1 to sense negative pressure and TRPV4 to sense positive pressure. Either pathway adjusts pressure to zero through regulation of Na/K ATPase activity to adjust osmolarity. In summary, an intracellular hydrostatic pressure gradient drives the internal micro circulatory system, which is important for internal homeostasis and transparency. However, pressure seems to be intrinsically important, as there are feedback systems that maintain a more constant pressure profile at the expense of the micro circulation. Water is generally close to equilibrium: $\Delta p - RT\Delta c = 0$ Thus pressure and concentration gradients are linked. Perhaps elevated pressures in MF reduce water content and increase osmolarity. If so, intracellular pressure could generate the gradients in protein concentration and refractive index. The refractive index gradient reduces spherical aberration and is essential for visual acuity. Supported by ET06391.

MRI Measurements of Physiological Optics of the Lens: Applications to the Human Eye

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Our sense of sight is critically dependent on the optical properties of the ocular lens that enable it to focus light onto the retina. Like a glass window, the lens allows light rays to pass through it with minimal scattering. Furthermore, while the curved surfaces of the lens enable it to focus light effectively onto the back of the retina, the presence of a gradient of refractive index (GRIN) generates an inherent negative spherical aberration, which compensates for the positive spherical aberration introduced by the cornea. Our working hypothesis is that the physiology and optics of the lens are interlinked. In a series of previous experiments using magnetic resonance imaging (MRI), we have shown that the surface curvatures and GRIN of the lens are both actively maintained by a circulating flux of ions and water that is generated by lens's unique cellular physiology. By applying a number of different physiological perturbations in animal models, we have identified the role of several lens's cellular components in maintaining its optical functionality. Currently, we are expanding our understanding of lens physiological optics by using a variety of MRI modalities, such as T1, T2 and diffusion tensor imaging (DTI) in animal models. T1 weighted imaging reveals the water content of the lens and its hydration state. Here we showed that the lens water content is kept at an equilibrium by its internal system of fluid dynamics. T2 weighted imaging is directly related to lens's water/protein varying gradient, which itself creates the GRIN of the lens. We have demonstrated that lens GRIN changes when it's physiology perturbed pharmacologically, although its transparency was unaffected. Finally, DTI has revealed valuable properties of water in the lens, such as internal magnitude and direction of water diffusivity. Recently, we have translated our MRI studies to the clinical environment to better understand the connection between human lens physiology and its optics. We believe that by identifying and measuring appropriate physiological biomarkers in the lens, its optical quality and functionality could be predicted.

Lens

Are Water Channel and Cell-to-Cell Adhesion Functions of AQPO Critical for Eye Lens Biomechanics?

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Proper biomechanics is critical for precise accommodation of eye lens while focusing near or far objects. Aquaporin 0 (AQPO) in lens fiber cells has been shown to function as a water channel and a structural cell-to-cell adhesion (CTCA) protein. Recently, we showed that deletion of 50% of AQPO significantly alters lens biomechanics and refractive index gradient (RING). However, no specific investigation has shown whether both water channel and CTCA functions are equally important, or whether one has precedence over the other in regards to eye lens biomechanics; the current investigation aims to test this distinction. For experiments, mice with the following genotypes were developed in C57BL/6J background, and used along with wild type: heterozygous AQPO knockout (AQPO^{+/-}), AQPO knockout (AQPO^{-/-}), heterozygous AQPO knockout expressing AQP1 (transgenic AQP1) in the fiber cells (TgAQP1^{+/-}/AQPO^{+/-}) and AQPO knockout expressing AQP1 (TgAQP1^{+/-}/AQPO^{-/-}) in the fiber cells. Biomechanical assay revealed that loss of one or both AQPO alleles caused significant reduction in compressive load-bearing capacity of lenses compared to WT. Loss of AQPO significantly altered lens resilience. Previously we reported that expression of AQP1 in fiber cells of AQPO knockout restored fiber cell membrane water permeability and significantly improved lens transparency and RING. However, the present study showed no significant enhancement in compressive load-bearing capacity between AQPO knockout, and AQPO knockout expressing AQP1 even after restoring fiber cell membrane water permeability. Our data suggest that while the water channel function of AQPO is critical for lens transparency and RING, CTCA function of AQPO is vital for lens transparency, RING and biomechanics. Since human lenses require transparency, RING and accommodation, alteration in AQPO fiber cell-to-fiber cell adhesion function could compromise lens transparency, sharp focusing and accommodation, leading to blurred vision, cataract and presbyopia.

Age-dependence of the Crystalline Lens Shape and Power with Stretching Force during Simulated Accommodation

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Purpose: To determine the age-dependence of the shape and optical power changes of baboon crystalline lenses with stretching force during simulated accommodation.

Methods: A lens stretcher (Ehrmann *et al.*, Clin Exp Opt, 2008) was used to simulate accommodation on 38 hamadryas baboon lenses from 32 donors (1.8-28 years, PMT 20.7±15.8 hours). Tissue samples were stretched in a step-wise fashion (0.25mm/step up to 2.5mm radially). At each stretching step, the lens optical power and the force exerted to stretch the lens were measured, and the lens cross-section was imaged using time-domain optical coherence tomography (OCT). The lens contours were extracted from the OCT images to determine the lens diameter and thickness, and the central 6-mm zone was fit with a conic section to calculate the anterior and posterior surface radii of curvature. A linear regression was performed on the changes in lens diameter, thickness, anterior and posterior surface radii of curvature, and power as a function of force during stretching to obtain the corresponding slopes for each lens. Linear regressions were then performed on the force-lens diameter (g/mm), force-thickness (g/mm), force-anterior surface radius of curvature (g/mm), force-posterior surface radius of curvature (g/mm), and power-force (D/g) slopes as a function of age to identify any age-dependent trends.

Results: Linear age-dependencies were observed for all measured parameter slopes: force-lens diameter (0.25 g/mm/year, $p < 0.01$), force-thickness (-0.99 g/mm/year, $p < 0.01$), force-anterior surface radius of curvature (-0.05 g/mm/year, $p < 0.01$), force-posterior surface radius of curvature (0.11 g/mm/year, $p < 0.01$), and power-force (0.35 D/g/year, $p < 0.01$).

Conclusion: The accommodative changes in the baboon lens shape and power for a given stretching force are significantly reduced with age. The older baboon lenses require a greater applied force to achieve the same power and shape changes as a younger baboon lens.

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Ageing Changes in the Refractive Index of the Lens: How the Biological Lens Can Inform Implant Design

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Background: The only viable treatment for cataract is surgical extraction and implantation of an intraocular lens (IOL). As population age increases, the need for surgical correction and the years of life with an IOL rise concomitantly, accentuating the need for better quality IOLs that closely mimic the eye lens. Current IOLs are unable to accommodate and lack a gradient refractive index (GRIN) which is needed to improve image quality.

Methods: Recent advances, using the interferometric techniques at the SPring-8 synchrotron in Japan^{1,2} have provided the most accurate three-dimensional GRIN profiles from human lenses to date. Experiments have been conducted on 66 human eye lenses, obtained from the Bristol Eye Bank in the UK and ranged from 16 -91 years of age.

Results: Subtle discontinuities in the GRIN profiles of all lenses have been found³ indicating that the lens does not have a smooth refractive index profile. Using optical modelling these discontinuities have been linked to the zones of discontinuity seen in the healthy living lens³. With age the overall profile of refractive index shows some general changes with age with a greater definition between the nucleus and cortex but individual variations mask ageing and lenses of a similar age can have varying profiles⁴. There is no age-related change in refractive index profile along the optic axis; statistically significant changes are seen with age in the equatorial aspect, particularly in lenses below the age of 60 years⁴. There is a trend to decreasing central refractive index maxima with age but results are scattered⁴.

Conclusion: The discontinuities in the refractive index are likely to manifest as zones of discontinuity something that

may improve image quality in future intraocular implant designs. Discontinuities in the refractive index gradient may depend on growth and developmental stages of the individual which suggests that a personalised approach may need to be investigated in the design of future implants.

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Cornea and Ocular Surface

COS1 - Corneal Infection

Basic Approach to Viral Corneal Endotheliitis

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Corneal endotheliitis was first reported as presumed autoimmune disease by Khodadoust in 1982. Thereafter, however, herpes simple virus (HSV) was detected as a causative agent, and corneal endotheliitis has been recognized as an infectious disease. In 2006, Dr. Koizumi reported cytomegalovirus (CMV)-DNA detection from a case of corneal endotheliitis patient. Since then, various reports on CMV corneal endotheliitis have been reported mainly from Asian countries, and established as a new clinical entity. CMV corneal endotheliitis occur in immunocompetent patient with male disposition, and characteristic findings are coin-shaped lesions and severe corneal edema with a line of KPs similar to allograft rejection.

Clinically various findings are reported, however the pathogenesis of HSV and CMV corneal endotheliitis is still unknown. We conducted several *in vitro* studies using immortalized human corneal endothelial (HCEn) cells. As results, various findings balancing viral growth and immune reaction were elucidated.

In HSV endothelial infection, NFκB was activated via toll-like receptor (TLR)-9, which was selectively expressed in HCEn cells, and induced various cytokines. On the other hand, HSV-1 utilized this NFκB activation for its own replication as counter action. Also HSV infection stimulated indoleamine 2,3-dioxygenase 1 (IDO1) expression via TLR9 stimulation, and this IDO activity induced regulatory T cells.

In CMV endothelial infection, a certain clinical strain of CMV can actually infect HCEn cells, however viral growth is very slow. Microarray analyses and ELISA revealed CMV infection also induced various inflammatory cytokines and immune regulatory mediators including IDO1.

Also we have investigated the roles of cytotoxic T lymphocyte (CTL) activity in CMV corneal endotheliitis patients. In this *in vitro* study, CD8⁺ T cells from CMV corneal endotheliitis patients or seropositive control donors with HLA-A*2402 were co-cultured and expanded with CMV-infected HCEn cells (HLA-A*2402). When these CMV-expanded CD8⁺ T cells were co-cultured with CMV antigen (IE1 or pp65) epitope-stimulated HCEn cells, seropositive donor-derived CD8⁺ T cells promptly released interferon-γ and granzyme B in a MHC class I-restricted manner, and these reactions were impaired in CMV corneal

endotheliitis patients.

These *in vitro* results may give us various clues to elucidate the *in vivo* pathogenesis of HSV and CMV corneal endotheliitis.

Bacterial Flora on the Ocular Surface

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Bacterial flora in the elderly prior to undergoing cataract surgery has been well studied. However, there is limited published literature on the bacterial flora in children. We investigated normal flora of the ocular surface in both children and adults. The subjects were divided into the following four groups by age: 4 months (4M group), 7 months to 6 years (7M-6Y group), 7 to 15 years (7-15Y group), and adults. The conjunctiva of each subject was swabbed, and the obtained specimens were then cultured in both aerobic and anaerobic conditions. The detection rates of conjunctival bacteria were 33.3% (9 of 27 eyes) in the 4M group, 25.3% (19 of 75 eyes) in the 7M-6Y group, 14.0% (12 of 86 eyes) in the 7-15Y group, and 12.2% (6 of 49 eyes) in the adults. In the 4M and 7M-6Y groups, streptococcus species, especially *S. pneumoniae*, were predominantly detected. In the 7-15Y group, the detection rate of streptococcus species decreased, and *P. acnes* was predominantly detected. In the adults, only *P. acnes* was detected. Our findings indicate that the age of the host has an effect on the type of bacterial flora that exists on the normal ocular surface.

Role of Inflammation on Corneal Nerve Regression Following HSV-1 Infection

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Herpes simplex virus type I (HSV-1) is a leading cause of neurotrophic keratitis, characterized by decreased or absent corneal sensation and blink reflex as a consequence of damage to the sensory fibers innervating the cornea. Our previous results revealed regression of the corneal nerves during acute HSV-1 infection. The mechanism behind this degenerative process is poorly understood. Our aim is to establish the role of the elicited immune response

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in the nerve regression upon HSV-1 infection. To address this question, we infected C57BL/6J mice with HSV-1 or left them uninfected (UI) as controls. Starting at 2 hours post infection (pi), mice were applied a dexamethasone ophthalmic solution (DEX) or artificial tears as controls onto their corneas for up to 8 days pi. Corneas were harvested at 2, 4, and 8 days pi and assessed for viral content by plaque assay, infiltrating leukocytes by flow cytometry (FC), content of inflammatory cytokines by suspension array, and immunohistochemical analysis of nerves. Corneal sensitivity was evaluated using a Cochet-Bonnet esthesiometer. For macrophage cell depletion, Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mice were subjected to systemic treatments with AP20187 dimerizer or vehicle (VEH), infected with HSV-1 or left UI, and their corneas, lymph nodes, and blood assessed for analysis of CD45⁺CD11b⁺ GFP⁺ cells by FC. MAFIA corneas were assessed for sensitivity, nerve structure, and viral content as indicated above, at 6 days pi. DEX significantly preserved corneal nerve structure and sensitivity upon infection despite unchanged viral content at 4 and 8 days pi. DEX significantly reduced myeloid cells (macrophages), and T cells (CD8⁺ T) in the infected cornea while it greatly suppressed IL-6, IFN γ , and IL-1 α . MAFIA mice treated with AP20187 had efficient depletion of CD45⁺CD11b⁺GFP⁺ cells in the tissues analyzed. The decrease of infiltrated macrophages in the corneas of mice treated with AP20187 correlated with preservation of corneal sensitivity and nerve structure despite no changes in viral contents compared to VEH treatment. Our data suggest the nerve regression is caused by the immune response to HSV-1 and not by viral replication in the cornea. DEX treatment of infected corneas results in anatomical and functional preservation of corneal nerves. Our results suggest leukocytes infiltrating the cornea, such as macrophages, are important players in the nerve regression process following HSV-1 infection.

COS2 - Emerging paradigms in stromal regenerative biology

An Investigation in Three-dimensions of Cell-directed Matrix Deposition in the Developing Cornea

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Previously, we used serial block face scanning electron microscopy (SBF-SEM) to show in 3D that collagen fibril bundles in the stroma of the developing chick cornea

midway through development - i.e. from embryonic day 10 (E10) onwards -- are arranged in a predominantly orthogonal manner, mirroring the pattern in the compacted cornea in maturity (Young et al., PNAS 2014;111:687-692). The studies also revealed that the alignment of the fibril bundles corresponded to that of extended cell processes of the presumptive keratocytes, which lay down the collagen at this time supporting the concept of cell-directed matrix deposition. The neural crest-derived keratocyte precursors, however, only invade the developing chick cornea after E5; before this time the primary stroma is devoid of cells and the accepted assumption is that the corneal epithelium deposits the early stromal matrix. Here, we studied the collagen matrix organisation in the early developing chick cornea in the days before the invasion of keratocyte precursors. Chick corneas at E3 through E7 were processed for study by light microscopy, conventional transmission electron microscopy, and SBF-SEM. Data revealed that a clear orthogonality existed between layers of uniformly thin and regularly spaced collagen fibrils at the very early stages of corneal development. In the immediate sub-epithelial stroma these layers were often only a single fibril thick, but an orthogonal inter-layer arrangement in the plane of the cornea was already evident. Also notable in the early stages of development was a series of thin, vertical, electron-dense extracellular structures emanating from the epithelial basement membrane and directed downwards into the primary stroma through (at E6) to the mid-stromal level of the invading presumptive keratocytes. These linear structures tended to be separated by distances of several 10s of μm , and when imaged in 3D there seemed to be some form of regularity about their separation and arrangement. The presumptive keratocytes that invade the primary cornea after E5 in the chick often appeared to interact with these vertical structures, perhaps using them as guides for migration, spatial arrangement, and subsequent secondary matrix deposition.

Extracellular Matrix Regulation of Corneal Fibroblast Patterning during Stromal Wound Healing

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Background: Extracellular matrix (ECM) supplies both physical and chemical signals to keratocytes which can impact their differentiation to fibroblasts and/or myofibroblasts. It also provides a substrate through which they migrate during wound repair. We have previously shown that following transcorneal freeze injury, migrating corneal fibroblasts align parallel to the stromal lamellae

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during wound repopulation. In this study, we compare cell and ECM patterning both within and on top of the stroma following lamellar keratectomy (LK) and photorefractive keratectomy (PRK).

Methods: All studies were performed on New Zealand White rabbits. Rabbits received either LK (24 rabbits) or PRK (12 rabbits) in one eye. Rabbits were monitored using *in vivo* confocal microscopy at 1, 3, 7, 21 and 60 days after injury. A subset of animals was sacrificed at each time point to further investigate cell and matrix patterning. Tissue was fixed and labeled *in situ* with Alexa Fluor 488 phalloidin (for F-actin), and imaged using multiphoton fluorescence and second harmonic generation (SHG) imaging.

Results: Immediately following both LK and PRK, cell death occurred in the native corneal stroma directly beneath the injury. At 7 and 21 days after the procedures, fluorescence (F-actin) and SHG results indicated that fibroblast alignment within this region was highly correlated with the collagen lamellae. In contrast, stromal cells accumulating on top of the native corneal stroma were randomly arranged, contained more prominent stress fibers, expressed alpha smooth muscle actin and secreted a fibrotic ECM. At 60 days, cells and matrix on top of the stroma were co-aligned into lamellar-like structures; cells were elongated but did not express stress fibers. Corneal haze measured using *in vivo* confocal microscopy peaked at 21 days after both LK and PRK, and was significantly reduced by 60 days. Cell morphology and patterning observed *in vivo* was similar to that observed *in situ*.

Conclusions: Based on these results, we hypothesize that the topography and alignment of the collagen lamellae direct fibroblast patterning during repopulation of the native stroma after corneal injury in the rabbit. In contrast, fibroblasts accumulating on top of the native stroma initially align randomly and produce a disorganized ECM. Over time, the ECM is remodeled and/or regenerated to produce a lamellar structure that is similar to the native corneal stroma.

Engineering Stromal Cell Alignment

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Introduction: The increasing interest and effort to create alternative therapies has led to exciting breakthroughs in the attempt to bio-fabricate and engineer live tissues.

This has been particularly evident in the development of new approaches applied to reconstruct corneal tissue. The need for tissue-engineered corneas is largely a response to the shortage of donor tissue and the lack of suitable alternative biological scaffolds preventing the treatment of millions of blind people worldwide. This presentation is focused on recent developments in corneal tissue engineering, specifically on the use of 'tissue-templating' for this purpose.

Methods: We have developed templates to control the spatio-temporal positioning of human corneal stromal cells (keratocytes) using 'smart' coatings. These smart coatings were formed from a self-assembling peptide amphiphile comprising a protease-cleavable sequence contiguous with a cell-attachment and signaling motif. This multi-functional material was subsequently used not only to instruct human keratocytes to adhere and deposit discrete multiple layers of aligned native extracellular matrix, but also govern their own self-directed release from the template solely via the action of endogenous metalloproteases (MMP). The control of MMP production was governed by retinoic acid supplementation in the growth media.

Results: After 90 days in culture the newly-shaped tissues governed their own release from the template via the action of endogenous proteases. Tissues recovered through this physiologically relevant process were carrier-free, and structurally and phenotypically equivalent to their natural counterparts. The cells and *de novo* collagen were aligned and orthogonally stacked lamellae were observed. The tissues were shown to be functional (via contraction assay) and biocompatible following transplantation in to rabbit stroma for 9 months.

Conclusions: The resulting technology contributes to a new paradigm in regenerative medicine whereby materials are able to actively direct and respond to cell behavior. The novel application of materials as a template coating, directing the formation and detachment of complex tissues solely under physiological conditions, will undoubtedly have broad use in future cell and tissue therapies. Moreover we demonstrated that complex, 3D, hierarchical tissue structures can be instructed using a 2D template.

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The Biology and Therapeutic Potential of Limbal Mesenchymal Stromal Cells**DAMIEN HARKIN^{1,2}**¹Queensland University of Technology, School of Biomedical Sciences, Brisbane, Australia, ²Queensland Eye Institute, Ophthalmic Cell Therapies, Brisbane, Australia

Ocular surface failure principally arises from a deficiency of corneal epithelial progenitor cells. Current strategies for the treatment of ocular surface failure are therefore based upon implantation of epithelial progenitor cells. Typically, the epithelial progenitor cells are supplied in the form of a limbal tissue autograft implanted either with or without prior expansion of epithelial cell numbers *ex vivo*. While the implanted epithelial cells are likely to be accompanied by varying amounts of stromal cells, the contribution of these "passenger" cells to clinical outcomes has yet to be fully explored. Moreover, the treatment of patients with bilateral disease is complicated by the inherently high rates of rejection associated with donor limbal tissue. With these issues in mind, we have been developing an adjunct therapy for the treatment of ocular surface failure based upon the co-implantation of limbal epithelial cells and limbal mesenchymal stromal cells. Limbal mesenchymal stromal cells (or L-MSK) are historically the dominant contaminating mesenchymal cell type that arises in cultures derived from the corneal-limbus. While the exact anatomical origin of these cells remains unclear, they can be readily grown from either dissociated or intact samples of limbal stroma using relatively simple growth conditions (i.e. serum supplemented growth medium). Essentially, L-MSK can be regarded as equivalent to "limbal fibroblasts", however, the "MSC" moniker has become widely accepted following consistent observations of shared properties with MSC derived from bone marrow and other tissues. Most significantly with regard to ocular surface disease, L-MSK have been shown to encourage the growth of corneal epithelial progenitor cells and suppress activation of lymphocytes *in vitro*. On this basis we have hypothesised that cultured L-MSK derived from donor tissue might provide a valuable adjunct therapy for the treatment of ocular surface failure. More specifically, we have investigated the potential benefits of allogeneic L-MSK on the retention of human donor epithelial cells when applied to the ocular surface of New Zealand White rabbits. A detailed description of our preclinical model will be presented in conjunction with preliminary data.

Ocular Surface Cell-based Therapies: Opportunities and Challenges in Development and Adoption**MARK ROSENBLATT***Illinois Eye and Ear Infirmary, Ophthalmology and Visual Sciences, Chicago, United States*

There have been tremendous advancements in the application of cell based therapy for the treatment of corneal limbal stem cell deficiency. These include improvements in cell culture techniques which obviate the need for serum or xenogeneic feeder cells, as well as improvements in characterizing the determinants of corneal epithelial cell "stemness". In addition to corneal sources of stem cells, alternative donor cell populations, including oral mucosa, skin, iPS cells, and ES cells have been examined. Our laboratory is working to develop novel nanoengineered silk films which can be used as carriers for ocular surface reconstruction. Despite these advances, significant challenges to widespread adoption of new techniques exist, including scientific limitations to our understanding of corneal epithelial stem cell biology, government regulations, and the current infrastructure of eye banks.

COS3 - Corneal Endothelium: Pathophysiology and Treatment**Comparison in Mean Area between Pentagonal and Hexagonal Cells in Human Cornea Endothelium****MOTOKAZU TSUJIKAWA***Osaka University Graduate School of Medicine, Suita, Japan*

Human corneal endothelium (HCE) is the most inner layer of cornea consisting of monolayer of beautiful hexagonal shape cells.

CECs have two important function, barrier function preventing water to invent corneal stroma, and the pump function.

These functions are quite important to maintain transparency in cornea.

In vivo human, corneal endothelium could not or hardly proliferate, so once they has been damaged, for example, during eye surgery, the number of HCE do not recovered resulting severe corneal edema and the damage of transparency of the cornea, calling Bullous Keratopathy.

So in clinic, the quantitative analysis of viability of HECs are frequently examined by specular microscope, for example before the cataract surgeries.

This is a morphological examination, not functional but

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convenient and reliable because it can be quantitative analyses.

The quantitative analyses by specular microscope basically consists of three parameters.

1. Cell size: Endothelial cell density (ECD), 2. Polymegathism: Coefficient of variation (CV) and 3. Pleomorphism: the ratio of hexagonal cells.

The most important parameter is cell size-ECD.

If this parameter shows under 300-700 cells per mm², corneal edema will occur then HCE could not perform normal function reading bullous keratopathy.

But other two parameters, polymegathism and pleomorphism thought to be more sensitive measurement of HECs in early disease condition.

The reason is explained as following.

Briefly, at the damaged HCE area, surrounding HCEs should move damaged area, changing their morphology and enlarging their shape to cover the damage area.

If this hypothesis are true, the mean area of pentagonal cells should be bigger than that of hexagonal cells.

However, we noticed that in clinical data of pre-cataract surgery patients, some pentagonal cells are much smaller than hexagonal ones in our clinical experience.

Moreover, in several text book, the normal corneal endothelium contains small portion of pentagonal cells which seemed to be smaller than hexagonal cells.

In this paper, we compared the size of pentagonal and hexagonal HCE in a relatively large series of elder normal eyes.

Endothelial Keratoplasty in Asian Eyes

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To date, Descemet stripping automated endothelial keratoplasty (DSAEK) has been the most popularized endothelial keratoplasty technique to treat endothelial dysfunction all over the world. However, there were several technical difficulties to perform DSAEK for Asian eyes; they usually have small eyes with high vitreous pressure which makes endothelial keratoplasty quite difficult. Additionally, Fuchs dystrophy is not so common in Asia, instead, endothelial dysfunction secondary to argon laser iridotomy is more common; they usually have shallow anterior chamber. Especially, donor insertion step using conventional donor taco-folding push-in technique caused severe complications such as iris prolapse, iris bleeding, and in a worst case, a vitreous prolapse with serious endothelial cell damage. To circumvent these

difficulties, donor pull-through technique using IOL sheets glide, Busin glide, Tan endoglide was confirmed safe and quite useful for Asian eyes with minimum endothelial cell damage. Recently, to achieve better visual acuity rapidly compared to DSAEK, selective transplantation of only donor Descemet membrane and endothelium was established, and the procedure is called Descemet membrane endothelial keratoplasty (DMEK). However, DMEK procedure was again quite difficult for Asian eyes in two points. Firstly, to insert Descemet membrane roll into shallow anterior chamber using an implantable collamer lens inserter is difficult. Therefore, we found it quite useful for those eyes to use DMEK glass injector which enabled stable anterior chamber during Descemet membrane roll insertion. Secondary, the background of dark brown iris usually seen in Asian eyes makes the visibility of blue stained DMEK donor graft difficult. To circumvent this difficulty, we used oblique light via endoillumination probe held by assistant surgeon, which significantly improve the visibility of DMEK graft in the anterior chamber with a background of dark brown iris. S-stamp to DMEK donor also helps to prevent donor upside down problem. With these techniques, DMEK as well as DSAEK is now simple, reproducible, safe and effective procedure for most Asian patients with corneal endothelial dysfunction.

A Newly Developed Graft Inserter for Descemet's Stripping Automated Endothelial Keratoplasty

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Although Descemet's stripping automated endothelial keratoplasty (DSAEK) is a promising technique to treat bullous keratopathy (BK), there remains several problems including endothelial cell (EC) loss in early postoperative period. One of the major reasons for EC decrease is intraoperative mechanical stress to graft endothelium. To overcome this problem, we have developed a new graft inserter for DSAEK, *NS endo-inserter*, which can deliver graft only by flow of intraocular irrigating solution without collapse of anterior chamber. Ex vivo examination using experimental donor corneas and artificial anterior chamber revealed that EC damage resulted from our new inserter was significantly less than that from a Busin glide. Additionally, clinical study in patients with BK also shows that EC loss 3 months after DSAEK using our device was significantly less than that with a pull-through technique. Our newly developed *NS endo-inserter* may be a useful device for donor insertion in DSAEK.

Endothelial Keratoplasty Lenticules Prepared from Fresh Donated Whole Eyes: A Visante Optical Coherence Tomography Study

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Purpose: To analyze profile and thickness of endothelial keratoplasty lenticules prepared from fresh donated whole eyes with Visante optical coherence tomography (V-OCT) and comparing with measurements obtained from ultrasound pachymetry (USP) at Central Eye Bank of Iran.

Methods: By using standard eye bank protocol, 316 microkeratome-assisted pre-cut corneas were prepared for Descemet stripping automated endothelial keratoplasty. Central part of pre-cut lenticule on fresh whole eye, before excising corneoscleral disc and transferring to Optisol, was measured by USP. V-OCT was used to measure central, paracentral, and midperipheral thickness of lenticules after transferring tissue to Optisol. Chi-Square and Bonferroni tests were respectively used to compare USP and V-OCT measurements and thickness profile of lenticules. Postoperative reports of all transplanted lenticules were also recorded.

Results: Central measurements of pre-cut lenticules by V-OCT versus USP were statistically different (mean: 136 vs 165 μ m, respectively; $P=0.008$). Thickness profile of the lenticules revealed a slight increase from center to midperiphery (mean increase ranged from 12 μ m to 69 μ m). However, such increase of thickness was symmetric. Postoperative reports of transplanted lenticules were unremarkable.

Conclusion: V-OCT measurements of microkeratome-assisted pre-cut lenticules averaged 29 μ m thinner than USP measurements and revealed a significant but symmetric increase of thickness towards midperipheral parts. However, such variation in thickness profile did not affect attachment or clarity of transplanted pre-cut lenticules.

Understanding Immune Mechanism Of Corneal Transplant Rejection

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Purpose: To study in real time the recruitment and migration pattern of innate inflammatory cells and allo-specific T cells during graft rejection.

Methods: A full MHC mismatch (Balb/c to C57BL/6) murine model of corneal transplantation was used to study immune rejection reaction in real time utilizing in vivo fluorescent intra vital microscopy. To selectively study the fate of T cells, CXCR6-EGFP knock-in mice (i.e. Bonzo/STRL33) mice were used as recipient, in which only CD4 and CD8 T cells express enhanced green fluorescent protein (EGFP). Similarly, to study innate immune cells, the B6-MAFIA mouse, in which only CD11c macrophages express EGFP, were used as recipient. Time lapsed images were obtained at different days after transplantation and real time movies were generated and quantification of EGFP cells and their kinetic pattern was performed. Protein was extracted and chemokine production was assessed. Local delivery of anti-chemokine reagents was tested.

Results: EGFP labeled T cells were identified in corneal allografts as early as day 3 after transplantation and their accumulation peaked by day 17 which correlated with corneal graft clinical rejection. EGFP labeled CD11c cells were detected by day 14 post transplant and continue to accumulate as well. All EGFP T cells and CD11c cells infiltrated the whole corneal graft and their migration pattern was different. T cells moved and meandered faster and interesting were mainly present in the stroma of the corneal transplant. T cells or macrophages did not infiltrate syngeneic transplants. Increased production of CXCL10 and CXCL9 correlated with recruitment of T cells and neutralization of these decreased migration and meandering of immune cells and decreased graft rejection in half of the mice.

Conclusion: Recruitment of T cells is critical for the immune rejection of corneal allografts and these appear to orchestrate innate cells immune attack. Interestingly, this process occurs early after transplantation and immune cells mainly localize to the corneal stroma. This suggests a novel mechanism of tissue damage in corneal graft rejection in which immune cells in the corneal stroma may lead to "endothelial cell exhaustion" by indirect inflammatory reactions. Utilization of anti-chemokine therapy may alter this form of inflammation and may represent a novel form of therapy to prevent corneal allograft rejection.

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COS4 - Corneal Refractive Surgeries

Intralase Enabled Keratoplasty (IEK): Optimal Design and Size

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To compare the outcomes of Intralase enabled keratoplasty(IEK) versus conventional penetrating keratoplasty (C-PKP), 32 consecutive IEK eyes of 32 patients over a 2-year span were compared with age and diagnosis matched eyes of conventional blade trephination PK by the same surgeon. The IEK showed better results in UCVA and BCVA in the early postoperative period compared with the C-PKP. The IEK showed better results in BCVA in the early postoperative period compared with the C-PKP and there was no difference of complication. When considering the donor corneal swelling develops during optisol preservation period and the corneal thickness decreases after corneal transplantation, modified Top-hat configuration would be helpful in IEK.

Comparison of Laser *in situ* Keratomileusis and Photorefractive Keratectomy in Ten-year Follow-up Using a Mixed Effect ModelSHIRO AMANO¹, Yosai Mori², Kazunori Miyata²

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Purpose: Laser *in situ* keratomileusis (LASIK) and photorefractive keratectomy (PRK) have been the most frequently performed refractive surgeries and the results of long-term follow-up up to ten years of the two surgeries have been reported in various studies. However, it is highly likely that these previous studies dealt with patients who completed the long-term follow-up or patients with missing data. When patients with missing data or uncomplete follow-up are excluded, the selection bias likely occurs. On the other hand, when patients with missing data are included, the bias due to missing data is inevitable. Recently, a mixed effect model has been used for analysis of longitudinal clinical data to reduce the bias with selection or missing data. We conducted this study to compare the results of LASIK and PRK at ten-year follow-up using a mixed effect model.

Methods: A comparative retrospective study was conducted on 1127 eyes of 579 patients after LASIK

and 270 eyes of 144 patients after PRK who attended postoperative follow-up of twice or more after three months postoperatively. Uncorrected visual acuity (UCVA), best spectacle-corrected visual acuity (BSCVA), manifest refractive spherical equivalent (MRSE), percentage of eyes within ± 0.5 diopter and ± 1.0 diopter of targeted refraction, and central corneal thickness were compared between PRK and LASIK groups using a mixed effect model.

Results: Compared with the LASIK group, UCVA in the PRK group was significantly worse in initial one year but was significantly better after four years. The average BSCVA was not significantly different between the LASIK and PRK groups after four years. MRSE in the LASIK and PRK groups showed gradual myopic shift until six years after the surgery. After six years, MRSE in the PRK group stayed stable whereas MRSE in the LASIK group continued to shift to myopia. The percentages of eyes within ± 0.5 D or ± 1.0 D in LASIK group were significantly higher than those in the PRK group at three months but were significantly lower than those in the PRK group at ten years.

Conclusions: PRK demonstrated better efficacy than LASIK after 10 years. This is mainly due to continuous myopic shift after LASIK.

Big-bubble Technique with Automatic Depth-sensing Needle for Precision Lamellar Keratoplasty

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Introduction: Lamellar keratoplasty is one of the latest transplantation technologies that has shown to increase the success rate and minimizes subsequent complications. However, lamellar keratoplasty requires additional processes for cornea preparation, and its outcome is highly dependent on the operating surgeon's senses, experiences and hand skills. Thus the result varies widely between doctors and cases.

Purpose: The purpose was to evaluate the effectiveness of an automatic depth-sensing needle system for big-bubble technique in deep anterior lamellar keratoplasty (DALK).

Methods: In this experimental study, the big-bubble technique for DALK was performed with the automatic depth-sensing needle system. Rabbit and human cornea

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was used in this *ex-vivo* study. The automatic depth-sensing needle system was composed of the fiber optic based needle and optical coherent tomography (OCT) system which was developed for sensing the depth between the needle and the endothelial layer of cornea. The needle is aimed to be located at 80-90% of total corneal thickness (about 50-100 μ m above the corneal endothelial layer). The real-time position information of the needle tip in the cornea was displayed on the screen while the needle tip was inserted into the cornea. Simultaneously, the thickness of the cornea (including endothelial layer) below the needle tip was calculated and provided with a numerical value to the surgeon. To verify the feasibility of our device in big-bubble technique for DALK, the thickness of the remaining layer including the corneal endothelial layer was measured with OCT system.

Results: The big-bubble technique with the automatic depth-sensing needle system was achieved in 81.3% and 85.6% for rabbit and human cornea, respectively. The thickness of the remaining layer including the corneal endothelial layer was $103.2 \pm 63.3\mu$ m and $84.2 \pm 28.1\mu$ m for rabbit and human cornea, respectively.

Conclusion: The presented technology using automatic depth-sensing needle showed better results in terms of accuracy and efficiency in big-bubble technique for DALK. With further clinical practices, we believe that our device will break new ground in the field of lamellar keratoplasty including DALK.

The Role of Vitamin D in the Eye: Two Month Oral Vitamin D Supplement in People with Dry Eye/Low Vitamin D

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Background: Dry eye disease is a chronic debilitating ocular condition, affecting more than 20% of adults. It affects vision, quality of life and people's productivity. Deficient vitamin D levels have been linked to dry eye disease. This study investigates whether dryness of the ocular surface is associated with lower vitamin D levels, and whether vitamin D supplementation for 2 months can improve dry eye.

Methods: Participants (n= 36, aged 43 to 69 years, male and female) were recruited from two groups: the Seasonal D study investigating seasonal variations in vitamin D

levels, and the dry eye patients of the Optometry Clinic at the Queensland University of Technology. All participants had their vitamin D serum level analysed at baseline and after taking a vitamin D supplement (Vitamin D, Swisse, 1000 IU) for two months; four (11.1%) did not return for the follow up visit. The degree of ocular surface dryness was objectively assessed using the Oculus Keratograph 5 (OCULUS). Measures included tear film stability (non-invasive tear break up time (NIBUT), tear secretion (Schirmer 1 test), and degree of inflammation of the conjunctiva. Dry eye symptomology was assessed using a validated survey the Ocular Surface Disease Index (OSDI).

Results: Of the 32 participants, 29 (90.63%) had deficient serum vitamin D (defined as a serum level of vitamin D lower than 125 nmol/L) whereas 3 (9.38%) had optimal vitamin D (higher than 125 nmol/L) at baseline. The mean vitamin D levels significantly improved after treatment (vit D prior: M=82.82 nmol/L, SD=34.91; vit D after: M=126.06 nmol/L, SD=125.97; p=0.033). Vitamin D serum concentration increased in 25 participants (78%), with an average 52% increase; although 23 out of 32 participants (72%) remained vitamin D deficient. Dry eye symptoms significantly improved with vitamin D treatment (OSDI prior: M=22.73, SD=15.15; OSDI after: M=10.45, SD=10.44; p=0.001). Tear meniscus height (prior: M=0.28 mm, SD=0.09; after: M=0.31 mm, SD=0.11; p=0.066) and NITBUT (prior: M=12.24 s, SD=6.70; after: M=13.02 s, SD=5.77; p=0.465) were not altered by the Vitamin D treatment.

Conclusions: Vitamin D treatment for 2 months resulted in a statistically and clinically significant improvement in dry eye symptoms. Further study of the effects of longer duration of treatment in a larger participant cohort would determine if dry eye signs were also improved by Vitamin D treatment.

COS5 - Cornea Transplantation and keratoprosthesis

Cell and Gene Therapy of Congenital and Acquired Cornea Diseases

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Umbilical cord mesenchymal stem cells (UMSCs) have unique immunosuppressive properties enabling them to evade host rejection and making them valuable tools for

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cell therapy for treating congenital and acquired cornea diseases. Our previous studies showed that transplantation of human UMSC cure cloudy corneas of Lum knockout mice caused by the loss of Lumican. And mouse bone marrow mesenchymal stem cells can assume keratocyte phenotypes when they are transplanted into corneas of Lum and Kera knockout mice. Interestingly, the xenograft (UMSC) and allograft (mouse bone marrow MSC) modulate host inflammation and suppress the host immune response. We previously showed that human UMSCs can modulate the host immune response enabling them to survive xenograft transplantation via the expression of a glycocalyx. In vitro, UMSCs inhibit the adhesion and invasion and cell death of inflammatory cells, and also the polarization of M1/M2 macrophages and maturation of T-regulatory cells. Moreover, UMSCs exposed to inflammatory cells synthesize a rich extracellular glycocalyx composed of the chondroitin sulfate proteoglycan versican bound to a heavy chain (HC, heavy chains of inter alpha trypsin inhibitors) modified hyaluronan (HA) matrix (HC-HA), which contains TNF α -stimulated gene 6 (TSG6), the enzyme that transfers HCs to HA, and pentraxin-3. Our results, both in vivo and in vitro, show that this glycocalyx confers the ability for UMSCs to survive the host immune system and to regulate the inflammatory cells. Administration of antibodies against the constituents of the glycocalyx, digestion of hyaluronidase and Chondroitinase ABC abolish the UMSC ability to modulate immune responses. Furthermore, treatment anti-CD44 antibodies also greatly diminishes the UMSC ability in modulating formation of M2 macrophages, suggesting that cell surface CD44 is required for the correct assemble of the UMSC glycocalyx for modulating inflammatory cells. Use of CRISPR genome editing, we also show the feasibility of treating lysosomal storage diseases, e.g., MPS VII (mucopolysaccharidosis type VII).

Endothelial Keratoplasty in Japan

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Endothelial keratoplasty (EK), especially Descemet's stripping and automated endothelial keratoplasty (DSAEK), becomes one of the most popular corneal transplant surgeries in Japan as in other countries. However, patients' profile and ocular conditions in our country are considerably different from those in other countries. First, causative diseases for bullous keratoplasty (BK) are unique. While Fuchs' dystrophy is a leading indication

in western countries, BK secondary to argon laser iridotomy (ALI) is most popular in Japan, followed by BK following cataract surgery. Eyes with BK secondary to ALI have shallow anterior chamber and often associated with advanced cataract formation, making EK difficult. In addition, shortage in donor cornea supply in Japan results in delay in surgical intervention, which further makes EK challenging.

We conducted a retrospective study on DSAEK outcomes in the Tokyo Dental College Ichikawa General Hospital, a leading corneal transplant center in Japan. Our results demonstrated that graft clarity rates (91% at 1 year) and visual outcomes (logMAR; 1.18 ± 0.57 preoperatively, and 0.31 ± 0.35 at 12 months) were comparable to other reports, and there were no significant differences among different causative diseases. However, our results showed relatively sharp decline in endothelial cell density (ECD) in early postoperative period (2651 ± 323 cells/mm² preoperatively to 1332 ± 550 at 3 months, and 1244 ± 520 at 6 months).

We found that there were two possible factors that seemed to be associated with decreases in ECD. One is the degree of preoperative iris damage, and the other is the preoperative graft handling. Eyes with higher pre-existing iris damage score had a significantly higher risk of graft failure and lower postoperative ECD ($P < 0.0001$). The results suggest that the changes in aqueous humor may affect the graft survival following DSAEK. We also noted that pre-cut DSAEK grafts transferred from overseas eye banks showed significantly lower postoperative ECD when they were returned to room temperature for endothelial counting preoperatively. Although the exact mechanism is unclear, DSAEK pre-cut grafts may be susceptible to endothelial damage when they had thermal cycling insults.

Deep Anterior Keratoplasty (DALK) for Ocular Surface Disease

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Ocular surface diseases that cause stem cell deficiency also suffer from chronic inflammation that may cause stromal opacification. Deep anterior lamellar keratoplasty (DALK) is ideal for these patients since it does not elicit immunological rejection against the endothelium. Cases such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid (OCP), thermal and chemical burns, trachoma, aniridia, gelatinous drop-like dystrophy (GDLD) are all poor indications for PKP, if not contraindications. Bilateral LSCD

patients also require a limbal allograft as a source of stem cells, unless ectopic tissue such as oral mucosa is used. The most common technique used over the past 20 years in the treatment of bilateral patients is the keratolimbal allograft (KLAL). While careful patient selection is required, DALK with or without limbal transplantation is an effective means to treat patients with stromal opacification following ocular surface disease.

Functional Testing of the Corneal Endothelium before and after DSAEK for Fuchs Endothelial Dystrophy

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Purpose: Posterior lamellar keratoplasty has almost completely replaced the penetrating keratoplasty procedure for treatment of primary or secondary endothelial dysfunction. It is, however, not known whether the normal endothelial pump function is reestablished by the procedure. The purpose of the present study was to evaluate the endothelial pump function in vivo after Descemet stripping automated endothelial keratoplasty (DSAEK).

Methods: In a prospective controlled trial, 17 patients with Fuchs endothelial corneal dystrophy (FECD) eligible for DSAEK surgery and 15 patients with cataract but normal corneas (Controls) were included. A low oxygen-permeable contact lens was used to induce corneal edema. Changes in central corneal thickness measured by Optical Coherence Tomography were monitored as an indirect measure of endothelial cell pump function. Experiments were performed before surgery and repeated 12 months after surgery.

Results: Comparing the FECD and control groups before surgery, there was 24.8% more edema in the FECD group after 2 hours ($P < 0.001$) and 19.9% more edema in the FECD group after 3 hours

($P < 0.001$). One year after DSAEK, there was 15% less edema after DSAEK than before surgery

($P = 0.015$) after 3 hours. Comparing the DSAEK-treated eyes with the control eyes 12 months after surgery; there was 12.8% more edema in the DSAEK group after 2 hours ($P = 0.007$), but after 3 hours, the percentages of edema were similar ($P = 0.11$).

Conclusions: Twelve months after DSAEK surgery, the grafted endothelium cleared the induced edema as fast as the control group, indicating a viable and near-normal endothelial pump function. However, significant differences in the deswelling patterns were detected, which may be caused by the added corneal stroma after DSAEK.

Keratocyte Plasticity and the Potential for Harnessing it for in vivo Regeneration of the Corneal Stroma

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Corneal keratocytes, the cells which populate the middle layer of the cornea, are relatively quiescent and maintain and repair the cornea throughout life via activation into the fibroblast and myofibroblast phenotypes. Injury related changes in growth factor and cytokine signals results in this phenotype switch. We have also shown previously the en-masse conversion of adult corneal keratocytes into a neuronal phenotype when treated with exogenous neurogenic differentiation factors. Those results suggest that in the corneal stroma stem cell-like properties are not limited to a subset of cells. We set out to uncover the potential of adult corneal keratocytes to be directed to produce collagen type II via phenotypic reprogramming with chondrogenic factors and the possibility of using such an approach to regenerate the collagenous stromal matrix. Cells isolated from, and tissue slices of adult rat and human normal and keratoconic cornea were cultured in chondrogenic differentiation factors transforming growth factor beta 3 and dexamethasone. Adult rat corneas were also treated in vivo with the same chondrogenic factors. Immunohistochemical and gene expression analysis revealed that corneal keratocytes in normal and diseased corneas were induced to produce collagen type II upon treatment in vitro and in vivo. Furthermore, the type II collagen deposition resulted in an increase in corneal hardness and elasticity without any scarring or loss in clarity. In situ induction using molecules that effectively provide signaling to stimulate the self-healing potential of the patient's own cells can provide a targeted and novel in vivo approach to tissue engineering. This might be a promising approach for increasing corneal integrity in diseases associated with weakened ectatic corneas such as keratoconus.

COS6 - Keratoconus biology and treatment

Quality of Vision in Keratoconus

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Keratoconus is a bilateral corneal shape abnormality associated with the anterior protrusion of the cornea at the central zone due to the primary thinning of the clear stroma. In this presentation, the quality of vision in patients with keratoconus will be reviewed mainly based on our previous works.

The progression of keratoconus impairs the visual function mostly by the higher-order aberrations (HOAs). The advance in wavefront sensing enabled us to evaluate the quality of vision in keratoconus objectively, and the HOAs in keratoconus were characterized by the increase of vertical coma, negative spherical aberration, and trefoil. The simulated retinal image with these HOAs suggested the comet-like images decreased the spectacle-corrected visual acuity and induced the loss of contrast sensitivity.

The HOAs of the anterior and posterior corneal surfaces can be quantified with corneal tomography. The results of corneal tomography indicated that the residual coma due to posterior corneal surface was the major factor for the insufficient visual recovery with rigid gas-permeable contact lens in patients with keratoconus. The soft contact lens with asymmetric design may be useful to reduce the coma.

For LASIK, phakic IOL, and cataract surgery, it will be important to screen keratoconus before surgery to avoid the complication or deterioration of vision following surgery.

Keratoconus: A Systemic Disease

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Keratoconus (KC) is the most common corneal dystrophy in the US, characterized by corneal thinning and affecting over 1 in 2000 people worldwide. KC is known to be a multifactorial disease with an elusive pathogenesis. Significant difficulties with differential diagnosis cause uncertainty as to its prevalence. In the absence of an animal model, we were the first to introduce a novel 3D *in vitro* model that can mirror the disease. We have also shown that our *in vitro* findings can be directly correlated to *in vivo* studies where sampling of tear film from KC patients is utilized. This study will discuss new clinical findings and will propose a new mechanism for the birth and progression of KC using human saliva screening from healthy and KC donors. In the past decade, the potential use of saliva was found useful not only for detecting various local diseases, including Sjogren's syndrome, oral, head, and neck cancers, through proteomic and

transcriptomic analyses as well as preclinical studies but also for systemic disease detection such as Diabetes type II, lung, pancreatic, breast, and ovarian cancers. The importance of saliva, however, is often appreciated only when it is gone as commonly happens in patients who receive radiation treatments or have oral cancer. In terms of ocular diseases, saliva has never been used before as a diagnostic tool even though it is well known that most of the compounds found in blood are also present in saliva, giving rise to the notion that saliva is a mirror of systemic factors in the human body. Our latest results suggest that KC is a systemic disease driven by altered hormones contributing to stromal thinning in the KC cornea. Further studies are clearly needed with the participation of larger number of KC patients. This work represents a significant step in assisting with KC prognosis and drug development.

Advancements in Early Diagnosis of Keratoconus

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Keratoconus is the most commonly encountered corneal ectasia. About 20% of the patients need corneal transplantation during their lifetime. The introduction of corneal collagen crosslinking can potentially reduce the need for transplantation. However, it is mandatory that crosslinking is performed during the early course of the disease. Traditionally, the diagnosis of keratoconus is made using elevation-based corneal tomography. Newer techniques for early diagnosis include tear proteomics, Fourier Transform Infrared spectroscopy, optical quality assessment, and corneal biomechanics.

Keratoconus: Emerging Disease Genes and Pathways

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Keratoconus is a multifactorial complex disease where the cornea undergoes a degenerative weakening and thinning of the stromal corneal connective tissue, causing high astigmatism, scarring, and vision loss in severe cases. Multiple loci and genetic variants are suspected to contribute keratoconus, but definitive causative mutations or biochemical pathways remain to be identified. We are using a multi-faceted approach to identify underlying genes and altered biochemical-signaling pathways in

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keratoconus. Total proteins extracted from keratoconus transplant corneas analyzed by mass spectrometry indicate endoplasmic reticular stress in stromal cell and widespread decreases in corneal collagens and proteoglycans. Keratoconus corneal stromal cells in culture show poor assembly of collagen fibrils into a cell layer associated extracellular matrix, changes in TGF- β signaling and cell death, mimicking key disease features in culture. Using our cell culture model we are further testing the functional relevance of key candidate genes.

Corneal Regulatory Molecular Networks Driving Keratoconus

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The corneal ectatic disease Keratoconus (KC) is characterised by focal thinning and irregular astigmatism leading to loss of visual acuity. However, the triggering factors and etiology of KC are not yet properly defined, hence drugs for KC management are not yet available. We analysed tears and corneal tissues from KC patients and non-ectatic controls using proteomic, biochemical and gene expression assays to understand the underlying molecular networks driving the disease. Levels of molecular factors were correlated with clinical parameters and biomechanical data from patients and control subjects. Tear proteomic data illustrate elevated oxidative stress, chronic inflammation and protease secretion, but reduced protease inhibitors, collagens and deregulated autophagy associated with KC. Patient tear analyses as well as epithelial gene expression profiling revealed a strong correlation of disease severity with increased MMP9 (matrix metalloproteinase 9), reduced LOX (lysyl oxidase) and elevated inflammatory cytokines (IL6, TNF α , etc). In KC patients undergoing corneal crosslinking procedures, corneal topography mapping was performed, based on which about 4-5 mm diameter of corneal epithelium from the cone area was debrided while peripheral epithelium upto 9mm was debrided separately. Epithelium from the cone apex of KC patients had high levels of TNF α , IL6 and MMP9 but reduced LOX and Collagen 4A1, compared to the periphery, which also demonstrated correlation with corneal curvature and deformity parameters. Stromal gene expression from KC patients showed trends similar to epithelium. Anterior segment OCT (optical coherence tomography) revealed local aberrations and loss of Bowman's layer (BL) integrity in some KC cones. Epithelium collected from cone apex of such patients showed significantly elevated MMP9, TNF α and IL6 levels

but reduced IL10, TIMP1 and Col4A1 expression.

The results suggest that KC is driven by deregulated molecular networks in the cornea which may have been initiated by a local microtrauma at the cone apex. The matrix remodeling factors such as LOX and MMPs are central players in this process, while the inflammatory factors may be the underlying molecular driver suggesting the use of anti-inflammatory drugs for therapy.

COS7 - New Diagnosis and Therapies for Corneal Diseases

Nanomedicine for Corneal Disorders

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Corneal scarring is a leading cause of global blindness. Small molecule drugs and surgical interventions are typically used to treat corneal disorders, however, they provide short-term benefit, cause several side effects, and often fail. We aim to develop novel nanomedicine approaches for treating corneal disorders in people. We have successfully identified potent nanoparticles and clinically-applicable simple vector delivery techniques to introduce therapeutic genes selectively into corneal keratocytes or endothelial cells *in vivo*. In this study we tested the hypothesis that targeted delivery of bone morphogenic protein 7 (BMP7) and hepatocyte growth factor (HGF) genes into the stroma is an innovative nanomedicine approach to treat corneal scars *in vivo*. Donor human corneas were utilized to generate primary corneal fibroblasts (HCF). Human corneal myofibroblasts (HCM) were produced by growing HCF in transforming growth factor- β 1 (5ng/ml) under serum-free conditions. New Zealand White Rabbits were used for *in vivo* studies. Corneal fibrosis in rabbits was produced by topical 0.5N alkali application on 6mm of central cornea. Polyethylenimine-DNA nanoconstruct at nitrogen-to-phosphate ratio of 30 was used for gene transfer in the human cornea *in vitro* and rabbit cornea *in vivo* per previously published protocols (Sharma *et al* Mol Vis. 2012;18:2598-607 and Tandon *et al* PLoS One 2013:e66434). Slit-lamp biomicroscopy, immunofluorescence, western blotting, qPCR and confocal imaging were performed to evaluate efficacy and safety

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of 2-gene combination therapy. The BMP7 gene transfer significantly inhibited conversion of HCF to HCM (89%; $p < 0.001$) by blocking Smad signaling whereas HGF over-expression selectively caused apoptosis in HCM (76%; $p < 0.001$) *in vitro*. The BMP7-HGF combination gene therapy showed significant inhibition of corneal fibrosis in rabbits *in vivo* (Fantès scale 3.3 untreated vs 0.6 treated, $p < 0.001$). Slit-lamp biomicroscopy revealed no significant conjunctivitis or epiphora, and intraocular pressure measurements remained within normal reference ranges. The *in vivo* toxicity testing showed significantly decreased Draize (30 untreated vs 5 treated; $p < 0.01$) and Modified McDonald-Shadduck (4.1 untreated vs 0.8 treated, $p < 0.01$) scores. The results of western blotting, immunofluorescence, and qPCR are underway. Based on our findings to date, the BMP7-HGF nanomedicine approach appears efficacious and safe for treating corneal fibrosis *in vivo*.

Holistic Approach to Keratoconus Diagnosis and Treatment

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Keratoconus (KC) is a progressive, inflammatory, ectatic corneal disease characterized by changes in corneal collagen structure and organization. Expanding the diagnostic strategies and modalities that can predict early changes prior to clinical manifestations or topographical changes would be beneficial in directing favourable outcomes to an otherwise deteriorating corneal disease. In addition to topography maps, clinico-biological prognostic indicators from corneal imaging, tear fluid and epithelium would serve as key indices for monitoring and prevention of KC. *In vivo* confocal microscopy observations revealed changes in corneal sub-basal nerve plexus features in KC patients which can be used as an additional diagnostic marker. Aberrant levels of tear inflammatory cytokines, chemokines and matrix metalloproteinase are observed in KC. Periodic monitoring of these inflammatory factors would provide vital clues about the disease state. Furthermore, envisaging progression kinetics using computational predictive modelling of topography changes is another strategy which is being explored.

Treatment options that are available to manage KC ranges from spectacles, contact lenses, collagen crosslinking, intracorneal ring segment to lamellar or full thickness penetrating keratoplasty. It is necessary to choose the best possible treatment option from a wide spectrum of alternatives for each patient. We have designed a

fivepoint management algorithm for the treatment of KC based on demographics and response to treatments such as contact lenses and collagen cross-linking. Owing to the inflammatory nature of the condition, dampening inflammation is essential to retard the progression of KC and also to improve the outcome of various surgical procedures such as collagen cross-linking. Management of KC by an immunosuppressive agent - Cyclosporine A has yielded promising results. Furthermore, determining the status of vitamin D (a known endogenous immunomodulatory agent) in serum and tears and its subsequent supplementation would also prove to be relevant in the management of KC.

A holistic approach in KC diagnosis and treatment would involve sensitive algorithms for assessing topographical changes, inclusion of tear- and epithelium-based molecular markers, *in vivo* confocal imaging for various corneal cells, managing nutritional deficiencies and personalized or grade dependent treatment strategies.

New Insight into the Diagnosis of Limbal Stem Cell Deficiency

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Clinical presentation of limbal stem cell deficiency (LSCD) varies greatly based on the severity of the stem cell deficiency. Current accepted diagnostic test is impression cytology to detect goblet cells on the corneal surface. However, there are limitations of this approach. *In vivo* confocal laser scanning confocal microscopy can be used to image the ocular surface. Cellular and structural changes in LSCD are characterized according to the clinical severity. A classification of LSCD is developed by incorporating the clinical presentation and microstructural changes.

MicroRNA-184 Downregulation Promotes Corneal Epithelial Wound Healing

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Purpose: MicroRNAs (miRNAs) are endogenous short (~22) nucleotide non-coding RNAs, which inhibit protein translation through binding to target mRNAs. Our previous studies identified differential expression of miRNAs during corneal epithelial wound healing. Specifically, miR-204

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downregulation occurs during epithelial layer regeneration. In the present study, we investigated the function and mechanism of miR-184 in the same process.

Methods: Realtime RT-PCR detected miR-184 expression in mouse corneal epithelium during the wound healing process. Human corneal epithelial cells were transfected with miR-184 using Lipofectamine RNAiMAX reagent. MTS and a wound-healing assay evaluated its effects on human corneal epithelial cell proliferation and migration, respectively. Flow cytometry evaluated cell cycle progression. Bioinformatics, luciferase assay and western blot identified miR-184 target genes. Intrastromal miR-184 injection in mice prior to corneal epithelial wounding evaluated its effect on epithelial layer regeneration.

Results: miR-184 was dramatically downregulated during corneal epithelial wound healing. Transfection of miR-184 into human corneal epithelial cells led to a significant decrease in cell proliferation and induced cell cycle G2-arrest. Furthermore, miR-184 transfection inhibited cell migration. SRC and CDC25A were identified as miR-184 target genes. *In vivo* experiments showed that miR-184 overexpression delayed corneal epithelial regeneration.

Conclusions: Our results demonstrate that miR-184 inhibits human corneal epithelial cell proliferation and migration through targeting SRC and CDC25A. These effects indicate that miR-184 downregulation contributes to hastening wound healing and can be a novel biomarker of this process.

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COS8 - Ocular Surface Epithelial Homeostasis (Conjunctival, Limbal, Corneal)

The Limbal Border: A Stem Cell Niche that Generates Moving Corneal Epithelia

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The simple structural organization of the mammalian cornea, including its accessibility and transparency, renders it the perfect tissue to study cell dynamics particularly stem cell (SC) fate decisions using non-invasive intravital microscopy. Despite more than a century of research devoted to understanding how this tissue is maintained and repaired, many limitations and controversies continue to plague the field, including uncertainties about the specificity of current SC markers, the number of SC within the cornea,

their mode of division, their location, and the programs that govern cell motility. This presentation will discuss historical discoveries as well as recent developments in the corneal SC field highlighting some of the fundamental and fascinating features of the mammalian limbus. In particular, original data will be provided on how and when this transition zone develops in man, the consequences of breaching this barrier, and recent cutting-edge techniques developed in our laboratory to monitor limbal epithelial SC activity and centripetal migration of corneal epithelia.

Developing a Conjunctival Epithelial Replacement Therapy

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The conjunctiva plays a key role in ocular surface immunological defence and the maintenance of a healthy tear film. Many diseases such as mucous membrane pemphigoid and chemical burns lead to irreversible conjunctival scarring, a poor tear film and persistent corneal desiccation that may lead to painful blindness. Corneal and limbal stem cell transplantation will fail in the absence of a healthy conjunctiva. Restoration of the ocular surface could be achieved with an *ex vivo* expanded conjunctival epithelial equivalent. In order to ensure long-term success, conjunctival stem cells, which produce both keratinocytes and goblet cells, must be present and suitable substrates to support these must be developed. We have identified stem cells throughout the human conjunctiva by both colony forming efficiency and expression of the stem cell markers Δ Np63, ABCG2 and Hsp70; with significantly highest levels in the medial canthal area and inferior fornix. We are developing a range of substrates suited to different transplantation requirements: decellularised conjunctiva offers a soft pliable substrate that may suit a range of indications, plasma modified expanded polytetrafluoroethylene (ePTFE) is a synthetic non-degradable substrate more suitable for fornix reconstruction. These both support a stratified conjunctival epithelium containing stem and goblet cells. Substrate coating with cell-secreted extracellular matrix components may further enhance the *ex vivo* adhesion and growth of conjunctival cells. As such we have established the components of the extracellular

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matrix deposited by a human conjunctival cell line (HCjE-Gi) and determined the behaviour of these cells when seeded onto pre-adsorbed proteins modelling the natural ECM composition. Pre-adsorbed fibronectin and collagen IV enhance adhesion, and collagen IV enhances growth of conjunctival cells. Further optimisation and investigation should lead to an improved understanding of the ideal surface and culture conditions required both to eliminate animal products and to promote the development of a stratified conjunctival epithelial equivalent rich in both stem cells and goblet cells.

Development of Stem Cell-based Therapy for Corneal Diseases—from Tissue Stem Cell to iPS Cell

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When corneal epithelial stem cells are lost due to trauma or diseases, the surrounding conjunctival tissue invades the cornea and blood vessels. The cornea then loses its transparency, leading to blindness. Corneal allo-transplantation has been performed with an allograft donor cornea (from an eyebanked eye donated by someone else) to treat serious diseases of the corneal epithelium, but treatment outcomes are limited because of rejection. A shortage of donors is also a problem. To resolve these problems, we have been developing a stem cell-based therapy using iPS cells.

Human iPS cells are pluripotent and can differentiate into any given cell lineage. These cells can also serve as a source of autologous cells that can avoid immunological rejection, so iPS cells could be used in regenerative medicine to treat intractable corneal disease. In the past, there were no techniques to induce human iPS cells to differentiate into corneal epithelial cells and to then isolate those cells to create functional corneal epithelium. Thus, we worked to develop a technique to use human iPS cells to obtain corneal epithelial cells that could be used to reconstruct the cornea. We developed a 2D culture system to promote cell-autonomous differentiation of human iPS cells. The culture system developed in this study can use human iPS cells to generate a 2-dimensional structure (a self-formed ectodermal autonomous multi-zone (SEAM)) consisting of 4 concentric zones of cells. Major groups of cells that comprise the eye during development (e.g. corneal epithelium, the retina, and the epithelium of the lens) are produced at specific locations in the SEAM. The current study successfully isolated corneal epithelial progenitor cells from the 3rd zone of the SEAM, and this study also

successfully generated functional corneal epithelium. Corneal epithelium produced from human iPS cells was transplanted in an animal model, where that corneal epithelium was therapeutically effective.

Results of this study should greatly help to facilitate reconstruction of the corneal epithelium with iPS cells in humans.

The Mechanical Properties of the Human Corneal Limbus and their Influence on Epithelial Stem Cell Phenotype

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The function of the cornea is largely dependent on the maintenance of a healthy stratified epithelium, a process that relies on a population of stem cells residing in the limbus. Limbal epithelial stem cells (LESCs) migrate from the edge towards the center of the cornea, where they undergo differentiation and stratification, eventually shedding from the ocular surface. However, the mechanisms underpinning this homeostatic process are still unclear. Previously, we hypothesized that the biomechanical properties of the surface supporting the corneal epithelium play a fundamental role on the tissue's homeostasis. In this study we address this hypothesis by mapping the bulk modulus of fresh human corneas at an unprecedented resolution using Brillouin spectro-microscopy. This non-contact analysis revealed that the different corneal tissues can be distinguished by their distinctive mechanical properties, with a significantly different pattern of Brillouin frequency shift between central cornea and limbus, in particular in sub-epithelial areas. In the limbus, these areas corresponded remarkably well to the conjunctival matrix directly underlying the LESc niche. We then developed and optimized a method to modulate the modulus of collagen-rich matrices, and test the influence of substrate stiffness on the behavior of human corneal epithelial cells. The results showed that softened tissues supported the growth of slow-moving, proliferative ABCG2+/CK15+/ α 9-integrin+/CK3- corneal epithelial cells capable of depositing a limbus-characteristic basement membrane. Overall, our data supported the existence of a correlation between the mechanical properties of the corneal matrix and the phenotype of corneal epithelial cells. Moreover, it showed that this phenotype can be controlled through the modulation of substrate stiffness, both *in situ* and *in vitro*.

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Glial-like Functions of the Corneal Epithelial Cells Maintain the Subbasal Nerves**MARY ANN STEPP***GWU Medical School, Anatomy and Regenerative Biology, Washington, United States*

The eye is innervated by neurons derived from both the CNS and PNS. While much is known about retinal neurobiology and phototransduction, less attention has been paid to the innervation of the eye by the PNS and the roles it plays in maintaining a functioning visual system. The ophthalmic branch of the trigeminal ganglion contains somas of neurons that innervate the cornea. These small nerve fibers provide sensory functions for the cornea and are referred to as subbasal nerves (SBNs). The density of the SBNs in the cornea is higher than in any other tissue. They project for several millimeters within the corneal epithelium without Schwann cell support. In this review we present evidence supporting the hypothesis that corneal epithelial cells function like Schwann cells to support the SBNs. Much of the data supporting this hypothesis derives from studies of corneal development and reinnervation of the SBNs in the rodent cornea after superficial wounds. Corneal epithelial cells activate in response to injury via mechanisms similar to those induced in Schwann cells during Wallerian Degeneration. Importantly, corneal epithelial cells phagocytize distal axon fragments within hours of axotomy. Corneal SBNs are exposed directly to UV light; their proteins and lipids become damaged over time. SBNs shed aged axon fragments and continuously elongate to maintain their density on the ocular surface. Corneal epithelial cells, like RPE cells, phagocytize shed distal axon fragments. Available evidence points to new unexpected roles for corneal epithelial cells functioning as surrogate Schwann cells for the SBNs during homeostasis and in response to injury.

COS9 - Reimbursement of Ocular Surface Cell Based therapies -Bottlenecks in Bioprocessing**Is Regenerative Medicine Affordable for the Ocular Surface?****JULIE DANIELS***UCL, London, United Kingdom*

The cornea provides a readily accessible target for novel regenerative medicine strategies. Great advances have been made for example in the development of cultured epithelial stem cell therapy for blinding ocular surface

disease (limbal epithelial stem cell deficiency). The scientific dream of personalized autologous stem cell therapy restoring sight is being realised in clinical trials. However, will the potential costs of goods be prohibitive for most? Using our own tissue-engineered product 'RAFT' as an exemplar of an ocular surface therapy in development, this presentation will consider the advantages and disadvantages of personalized medicine for ocular surface reconstruction, together with solutions for bioprocessing and financial bottlenecks in translation into clinical practice.

Molecular Aliasing and Biological Variance**DANIEL GIBSON***University of Florida, Institute for Wound Research, Gainesville, United States*

Reimbursement depends both on the value of the therapeutic technology and its reliability. A lack of confidence in a technology tends to arise from inconsistent results, where there is an obvious very good effect in some patients, but not others. We have learned from lessons in chronic wound care advanced biological products, and from basic research gone awry, that 2 key hurdles affect the treatment and study of disease and they are: molecular aliasing and biological variance.

Many cell-based therapies are wholly dependent upon classification by cellular markers, but these markers may not be as unique as was once hypothesized. Variations in clinical performance are possible if a batch of cells produces the markers of interest, but differ in some other manner which is not resolved by the cell-sorting immunoassay.

In chronic wound care, recombinant growth factors, autologous grafts, and skin substitutes have had a difficult time becoming the standard of care despite their high potential. Clinical studies revealed highly varying results in clinical outcomes which have since been found to be based on the patients' receptiveness to the therapy at that point in time. Point of care assays are being developed to determine the readiness of chronic wounds and corneas to advanced protein, peptide, or cellular therapies to avoid treating those not ready for advanced therapy.

Advanced therapies can be highly beneficial, but are still too expensive to carelessly employ them. Variations in outcomes due possibly to batch variance masked by molecular aliasing can be managed if they are first identified, while variations in patients' readiness for advanced therapeutics can be managed by simple point-of-care assays. These two obstacles must be overcome in

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order for health care payers to have confidence in their use, and therefore confidence in reimbursing their use.

Ocular surface reconstruction using cultivated epithelial cell sheet – Dawning of a new era

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Ocular surface reconstruction using autologous corneal or oral mucosal epithelial cell sheet drastically changed treatment of limbal stem-cell deficiency (LSCD). Morphological and functional characteristics of epithelial cell sheet are similar to those of normal corneal epithelium. The treatment can solve potential problems of limbal transplantation including immune rejection and donor shortage. The treatment has been performed as clinical research so far, and effectiveness and safety were confirmed to some extent. The effort to make it general treatment should be made to spread this treatment widely. The process to make new treatment based on basic research useful for practical applications in the field of health care is called "translational research". We are conducting clinical trials to make cell sheet approved products. Many patients will benefit from approved regenerative and cellular therapeutic products in the near future.

Alginate-encapsulation for the Storage and Therapeutic Delivery of Adipose-derived Stem Cells for Ocular Surface Repair

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In recent years there has been increasing evidence for mesenchymal stem cells (MSCs) improving corneal repair. Mediated by the secretion of a cocktail of factors, MSC-based therapies represent an exciting prospect for new cell therapy products (CTPs) for the ocular surface. A major bottleneck for CTPs, however, is in the short-term storage of cells for distribution and clinical delivery. To meet this need, we developed an alginate-encapsulation system capable of preserving human adipose-derived stem cells (hASCs), MSCs obtained from fat, at hypothermic temperatures (4-21°C). This system yielded a high viable cell recovery (86±6%) and maintained cell phenotype and function following storage for 3 days.

We hypothesized that hASCs stored in alginate could be used for the delivery of therapeutic factors to the ocular surface as a 'storable stem cell bandage'. To investigate this, we examined the effect of conditioned medium from alginate-encapsulated hASCs on wound closure in primary human corneal keratocyte cultures. Serum-free medium was conditioned for 24 hours with alginate-encapsulated hASCs either without storage (NS-CM), or after storage for 3 days (S-CM). Both non-stored and stored cells exhibited a high level of viability after medium conditioning (85±6% and 83±4% respectively). Treatment of keratocyte scratch assays with NS-CM and S-CM accelerated wound closure dose-dependently, with significant increases in closure of 1.8±0.2- and 2.0±0.9-fold of control respectively at the highest concentration ($p < 0.05$). Neither NS-CM nor S-CM significantly affected keratocyte proliferation indicating a pro-migratory effect of hASC-CM on keratocytes: a vital step in stromal wound repopulation and healing. To further understand potential therapeutic effects in corneal wound healing, we examined the effect of encapsulation and storage on the gene expression of selected therapeutic factors. Of these, hepatocyte growth factor (HGF) was the most upregulated in response to encapsulation, an effect maintained after 3 days' storage (almost 30-fold of the control; $p < 0.05$). These levels were further examined in hASC-CM. HGF has been reported to support corneal wound healing and restore transparency making alginate-encapsulation a potential strategy to increase the efficacy of MSC-based therapies at the cornea. Taken together, we present a system integrating cytopreservation during storage, and delivery of hASC-derived therapeutic factors for ocular surface repair.

Second Harmonic Imaging of Corneal Collagen: Insights into Corneal Evolution and Scarless Wound Healing

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Second harmonic generation (SHG) imaging has found an increasingly wide range of applications, particularly in the investigation of the 3D collagen architecture of the corneal stroma. Current strategies for engineering the corneal stroma are limited by incomplete knowledge regarding the mechanisms controlling corneal structure and shape. To provide insights into these mechanisms we used SHG

imaging to first, identify key evolutionary changes in vertebrate corneal structure leading to the development of the mammalian cornea. Second, we evaluated a scarless wound healing model in embryonic chick to assess corneal structural regeneration that may provide cellular and molecular insights into engineering the corneal stroma.

3D data reconstructions of the corneal collagen organization from distinct vertebrate clades revealed that the collagen in non-mammalian vertebrates was organized into stacks of rotating sheets or ribbons. Beyond the similarities in the rotation and orthogonal organization, structural evolutionary changes are present in the form of increasing amounts of organizational complexity. Higher vertebrates exhibit a higher number of collagen fiber branching and anastomosis. Of interest, mammalian corneas have a seemingly random corneal lamellar organizational component with a significant fiber branching, suggesting a distinct evolutionary paradigm on which branching and anastomoses evolved to stiffen the cornea, control its shape and overall facilitate a self-stabilizing stromal structure.

To explore the mechanisms directing collagen organization, scarless wound healing in the embryonic chick cornea was evaluated. To determine whether embryonic wound healing can recapitulate the normal complex avian corneal structure, embryonic day (E) 7 chick corneas were wounded and examined by SHG. No differences were observed between control and wounded corneas at E16 with respect to the rotation and orthogonal organization of corneal collagen suggesting that embryonic chick corneal stroma is capable of regenerating the normal adult structure via a scarless, non-fibrotic process.

These findings have two important implications. First, mammalian corneal structure is unique among vertebrates, suggesting a different corneal development paradigm. Second, regeneration in embryonic tissues are capable of recapitulation complex developmental processes that potentially can be applied to corneal bioengineering.

COS10 - Dry Eye

Comparison of Two Mucin Secretagogues for the Treatment of Dry Eye: Diquafosol Tetrasodium and Rebamipide

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Dry eye is a common ophthalmic disease all over the world. More than 10 million people in Japan are thought

to have dry eye. The 2007 International Dry Eye Workshop report, "Tear stimulation: Secretagogues," which stimulate aqueous or mucous secretion or both, described them as future potential topical pharmacologic agents for treating dry eye, including diquafosol, rebamipide, and other mucous secretion stimulants). Recently, two new secretagogue eye drops were launched in Japan in December 2010 and January 2012, respectively, to treat dry eye: 3% diquafosol and 2% rebamipide. Diquafosol is a P2Y₂ receptor agonist that has stimulatory effects on water secretion, secretion of the gel-forming mucin MUC5AC, and expression of membrane-associated mucins MUC1, MUC4, and MUC16. Rebamipide is a quinolinone derivative that increases conjunctival goblet cells density and gene and protein expression of MUC1, MUC4, and MUC16 and exhibits a protective effect on barrier function. In randomized clinical trials in Japan, corneal and conjunctival staining scores improved significantly from baseline at 2 weeks after treatment with both diquafosol and rebamipide in patients with dry eye.

To clarify the difference in the drugs' characteristics between these two secretagogue eye drops, we compared the short-term effects on the MUC5AC levels and tear volume in normal rabbits. The MUC5AC level and tear volume increased significantly ($P < 0.01$, Tukey's test) 15 minutes after instillation of diquafosol compared with rebamipide. These studies indicated that diquafosol might improve tear fluid stability in the short term with its stimulatory effect on tear fluid and mucin secretion. On the other hand, rebamipide might improve the mucosal epithelia and increase the goblet cell numbers, which cures the ocular surface in patients with dry eye. Further studies are needed to establish the adaptive criteria of these drugs for treating dry eye in clinics.

Mechanism of Visual Disturbance in Dry Eye

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Dry eye has been thought of as a chronic, symptomatic ocular surface disease that affects vision in a limited manner, since it has been difficult to detect visual or optical changes with standard visual acuity testing in dry eye. Recently, quantitative measurements of wavefront aberration or light scattering have shown that the instability of a disrupted tear film over the irregular ocular surface of dry eye is thought to be associated with visual disturbances or degraded optical quality. Clinical application of objective optical sampling in dry eye and

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current understanding of visual disturbance in dry eye will be reviewed in this presentation.

Neuropathic Pain: A Missing Piece of the Dry Eye Puzzle

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Dry eye (DE) is a heterogeneous disease that can include symptoms of ocular pain (e.g. burning, aching, dryness), visual disturbances, and a variety of signs (decreased tear production, increased evaporation, increased osmolarity, and ocular surface damage). Given the various tear film disturbances described in DE, it would make sense that patient reported symptoms would correlate with objective ocular surface findings. Yet, our research, and that of others, has demonstrated that DE symptoms correlate poorly with signs of ocular surface disease. Somatosensory dysfunction and resultant neuropathic pain may explain the discordance between signs and symptoms of DE. In this talk, we will review the data on somatosensory dysfunction (peripheral and central sensitization) in DE, including patients symptoms (spontaneous pain, allodynia, hyperalgesia) and ocular and non-ocular somatosensory morphologic and functional abnormalities. We will further discuss how evaluating for neuropathic ocular pain may impact the diagnosis and treatment of DE.

Glutathione Plays a Novel and Critical Role in Ocular Surface Development

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Purpose: Glutathione (GSH) maintains cellular redox balance and acts as a redox-signaling molecule. Although GSH is actively synthesized in the developing eye at both embryonic and early postnatal stages and is present at high millimolar levels in ocular surface tissues, its role in ocular morphogenesis remains to be elucidated. The aim of this study was to test the hypothesis that GSH plays a critical role in the development of ocular surface.

Methods: We developed *Gclc*^{Le/Le} mice, a strain in which

GSH biosynthesis is selectively abolished in surface ectoderm-derived ocular structures. Gross morphology and histology of eye tissues were performed at embryonic day (ED) 15, and post-natal days (PD) 1, 21 and 50.

Results: *Gclc*^{Le/Le} embryos at ED15 had normal development of ocular surface tissues, including lens, cornea, conjunctiva, and eyelids. At early postnatal stage (PD 1 and 21), *Gclc*^{Le/Le} mice exhibited bilateral small eyes (microphthalmia) by PD21, whereas the eyes of *Gclc*^{w/Le} heterozygotes appeared grossly normal at this same stage. Histological examination revealed both eyes of *Gclc*^{Le/Le} mice to have defective lens featuring poor differentiation and vacuolation of fiber cells. This change was noted only in the right eyes of *Gclc*^{w/Le} heterozygotes. Other ocular surface structures appeared unaffected in either *Gclc*^{Le/Le} or *Gclc*^{w/Le} mice. In adult mice (PD50), *Gclc*^{Le/Le} eyes were completely closed, whereas *Gclc*^{w/Le} heterozygotes showed unilateral small eye on the right side. Anterior ocular structures in affected eyes, including lens, cornea, ciliary body and iris, had histological abnormalities primarily featuring hypercellularity, poor differentiation and apoptotic cell death.

Conclusions: These results demonstrate a novel and critical role for GSH in perinatal lens maturation and postnatal maintenance of the ocular surface. Redox proteomics and tissue imaging mass spectrometry analyses are currently being used to determine the mechanism by which GSH controls eye development.

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Microbiome-oriented Supplements for the Treatment of Dry Eye

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Recently, the microbiome is believed to be related to general health. Dry eye is the condition where unstable tear film and decreased tears affect the patient's discomfort and visual function. We found that lactobacillus combined with lactoferrin had a positive effect on the recovery of dry eye in the mouse model. Next, we developed a combination of supplements based on the microbiome modification, and produced a microbiome-oriented supplement. We further performed the clinical study which showed its efficacy in the treatment of dry eye. Microbiome modification is one of the effective ways to treat eye diseases, and one example, the treatment of dry eye, will be presented.

IMM4 - Innate mechanisms that contribute to RPE pathology**Defective Phagosome Transport in the RPE Activates Complement and Induces AMD-like Pathogenesis****DAVID S. WILLIAMS, Julian Esteve-Rudd, Mei Jiang, Roni Hazim, Ankita Umopathy***UCLA / Stein Eye Institute, Los Angeles, United States*

The degradation of phagosomes, derived from the ingestion of photoreceptor outer segment (POS) disk membranes, is a major role of the retinal pigment epithelium (RPE). Each day, each RPE cell phagocytoses the tips of up to 250 photoreceptor cells. We have found that POS phagosomes associate with myosin-7a in the apical RPE, and, then kinesin-1, as they move into cell body of the RPE. Live-cell imaging showed that the phagosomes moved bi-directionally along microtubules in RPE cells, with kinesin-1 light chain, KLC1, remaining associated in both directions and during pauses. The phagosomes undergo transient interactions with endosomes, which are also transported by molecular motors. Defects in motor proteins resulted in delayed phagosome degradation. Aged *Klc1*^{-/-} mice showed extensive sub-RPE deposits, which stained strongly for markers of oxidative stress and activation of the alternative pathway of the complement system, including antibodies against malondialdehyde, C3, C3d, and C5b-9. The increase in C3 labeling was greater in the center of the retina. No difference was observed in the immunolabeling of C3a or C3b/iC3b, which are elevated under acute inflammatory conditions, suggesting that the observed increase in C3 labeling could correspond to a more chronic inflammatory situation. Therefore, the intracellular trafficking of phagosomes and endosomes in the RPE plays an essential role in phagosome degradation, with defects leading to AMD-like pathogenesis.

Restoration of Organelle Trafficking Protects the RPE from Complement-mediated Damage**APARNA LAKKARAJU^{1,2}, Li Xuan Tan¹, Kimberly Toops^{1,2}**¹*University of Wisconsin-Madison, Madison, United States,*²*McPherson Eye Research Institute, Madison, United States*

The alternative pathway of the complement system has been implicated in the pathogenesis of inherited and age-related macular degenerations. Complement activation terminates in the formation of membrane attack complexes (MAC), which promote inflammation by causing aberrant signal transduction. The retinal pigment epithelium (RPE),

which helps maintain ocular immune privilege, is the first line of defense against unregulated complement activation in the retina. How the RPE counters MAC formation and eliminates MAC pores to preserve cell integrity is not well understood. Here, using polarized primary RPE monolayers and pigmented *Abca4*^{-/-} mice as a model of Stargardt disease, we identify two rapid critical responses to complement in the RPE: accelerated recycling of the complement regulatory protein CD59, which inhibits the final step of MAC assembly; and lysosome-mediated membrane repair, which prevents sustained increases in intracellular calcium and mitochondrial damage. Our data show that cholesterol accumulation, due to vitamin A dimers in *Abca4*^{-/-} mice RPE or oxidized LDL, interferes with both these protective mechanisms. Defective CD59 recycling and lysosome exocytosis allow MAC pores to persist, culminating in mitochondrial damage and oxidative stress. Cholesterol-mediated activation of acid sphingomyelinase (ASMase), which sabotages microtubule-mediated organelle traffic, interferes with CD59 transport to the cell surface and with lysosome-plasma membrane fusion. In agreement with this, drugs that decrease RPE cholesterol or inhibit ASMase restore both protective responses and limit complement-induced mitochondrial injury *in vitro* and in *Abca4*^{-/-} mice. These data identify cholesterol and ASMase as therapeutic targets for preventing RPE dysfunction in Stargardt disease and other retinal dystrophies associated with lipofuscin accumulation.

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Complement Dysregulation in Stargardt Macular Degeneration**ROXANA A. RADU¹, Jane Hu¹, Gayle J. Pauer², Stephanie A. Hagstrom², Mary E. Rayborn², Joe G. Hollyfield², Dean Bok¹, Vera L. Bonilha²**¹*UCLA / Stein Eye Institute, Los Angeles, United States,*²*Cole Eye Institute, Cleveland Clinic, Cleveland, United States*

Stargardt macular degeneration (STGD) is a central blinding disease of children and young adults caused mostly by mutations in the *ABCA4* gene. The pathological hallmark of STGD is deposition of fluorescent vitamin A

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by-products in cells of the retinal pigment epithelium (RPE). The RPE of the *Abca4*^{-/-} mouse, the STGD model, presents with elevated C3 complement fragments and reduced expression of complement negative regulatory proteins. Other phenotypic features of the STGD mouse model include age-dependent RPE lipofuscin-bisretinoid accumulation and loss of photoreceptor cells. Bisretinoid-dependent complement activation was previously reported in cultured fetal human RPE cells but has never been studied in the context of the RPE of a STGD patient. Here, we evaluate the complement system in the donor eyes of STGD patients.

Eyes were obtained through the FFB eye donor program. The patients were clinically diagnosed as STGD based on the symptoms and ocular changes. STGD donor #1 (66 y.o.) had two ABCA4 mutations; ABCA4 genotype for STGD donor #2 (69 y.o.) is unknown. Fixed peri-macular and peripheral tissue samples of STGD and normal eyes (65 and 84 y.o.) were processed for immunohistochemistry using specific antibodies to C5b-9 (membrane attack complex, MAC), C3/C3b/iC3b, and complement factor H (CFH).

STGD eyes showed increased thickness of Bruch's membrane (BM) in the perimacular region in comparison to the control eyes. The deposition of MAC was indicated by significant C5b-9 immunoreactivity between BM and broken RPE basolateral membranes and also in the RPE cells. In contrast, the control eye showed C5b-9 immunoreactivity primarily in the choriocapillaris endothelium beneath an intact BM and little to none in the RPE. Pixel intensity quantification of MAC deposition was about 1.8-fold higher in STGD eye #2 compared to the control eye. C3 breakdown fragments were also observed within the RPE cells of STGD eye and the levels were ~1.3-fold increased compared to the control. CFH levels were two-fold lower in the STGD eye donor in comparison to the control eye.

Our data suggest that, like the RPE of the *Abca4*^{-/-} mouse, STGD human RPE cells are also dysfunctional as they lose their ability to suppress chronic insult by the complement system. Further analyses are directed toward characterization of complement reactivity using STGD patient-derived RPE cells.

Mitochondrial DNA Damage and the CFH Genotype

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More than 34 high-risk loci have been associated with age-related macular degeneration (AMD). The genes at these high risk loci belong to diverse biological pathways, suggesting different mechanisms lead to AMD pathology. Thus, therapies targeting a single pathway for all AMD patients will likely not be universally effective. Recent evidence suggests defects in mitochondria (mt) of the retinal pigment epithelium (RPE) may constitute a key pathogenic event in some AMD patients. To determine if individuals with a specific genetic background have a greater propensity for mt damage, we measured mtDNA damage in macular RPE from 103 human donors with AMD and 54 age-matched controls using a long extension polymerase chain reaction assay. Genotype analyses were performed for five common AMD associated nuclear risk alleles (TNFRSF10A, CFH, CETP, VEGF and COL10A1) representing different biological pathways. Our results show that AMD donors carrying the high risk allele for CFH (C) had significantly more mtDNA damage compared with donors having the wild-type genetic profile. Taken together, these studies provide the rationale for a personalized approach for treating AMD and suggest that patients harboring the CFH high risk allele may benefit from therapies that stabilize and protect the mt in the RPE.

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Distinct RPE and Retina Mitochondrial Respiration Defects in STGD3 Juvenile Maculopathy

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In a mouse model of STGD3 juvenile maculopathy (ELOVL4 TG1-2), cell death is preceded by outer segments disc spacing irregularities and RPE vacuolization, accumulation of undigested phagosomes, loss of mitochondrial cristae. We sought to explore the potential interplay between photoreceptors and RPE pathological events that might lead to photoreceptor loss. While evidence supports that photoreceptors and RPE have one of the highest mitochondrial respiration capacities, both being closely interlinked from the same (choroidal) vascular supply, this physiological aspect has remained essentially unexplored, let alone in pathological contexts.

We measured mitochondrial function with high-resolution respirometry in homogenates from freshly dissected RPE and retina of 12 months old WT and TG mice. A single protocol was used to evaluate the mitochondrial respiratory capacity of Complexes I, II and IV in the LEAK state (before the addition of ADP) and in the oxidative phosphorylation (OXPHOS) state (coupled, after addition of saturating ADP).

Mitochondria were well coupled in both WT and TG, as evidenced by the large increase in respiration in response to ADP. The LEAK state, expressed per mg of tissue, was reduced in retina and RPE of TG, indicating enhanced coupling efficiency in TG. When expressed per tissue mass, both retina and RPE had reduced Complex I OXPHOS capacity, while the RPE had also a reduction in Complex IV respiration; complex II respiration was unaffected. The results were also expressed as a flux control ratio

(FCR; normalized for maximal OXPHOS capacity with complexes I&II substrates), which is independent of mitochondrial content. Complex II FCR was increased in TG retina, suggesting a potential compensation resulting from Complex I defect. Of note, such effect cannot occur in RPE since Complex II is already responsible for the majority of maximal electron transport capacity.

Our results point towards a key pathological role of mitochondrial dysfunction in the progression of STGD3 disease. OXPHOS complex defects are likely contributing to increased production of reactive oxygen species, exacerbating cell death further. Now that we have demonstrated that mitochondrial OXPHOS is compromised at 12 months (lower by a range of 39-62%), with a likely

reduced mitochondrial content, we are in a position to address whether mitochondrial dysfunction (without loss in number itself) might precede photoreceptor loss.

JNT2 (IMM+RPE) - RPE and Inflammation

Fate Mapping Distinguishes Migration Patterns of Microglia versus Monocyte-derived Macrophages to the RPE in Inflammation

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Much of what is known is being revisited concerning the contribution of microglia in the pathobiology of retinal degeneration and AMD, which is owed to recent breakthrough discoveries involving these CNS resident myeloid cells. It is true that photoreceptor degeneration is associated with the irregularly high presence of myeloid cells in the outer retina, and that RPE stress is thought to be contributed by their accumulation in the subretinal space. However, only in the last several years have we begun to fully appreciate the importance of defining whether these myeloid cells represent *bona fide* microglia or monocyte-derived macrophages. This distinction is not without a difference. Microglia are embryonic-derived and maintained locally throughout life. By contrast, monocytes (Mo) are hematopoietic-derived recruits that differentiate into macrophages during CNS disease, including retina. Evidence points to the likelihood for their functionally divergent roles in CNS disease, including in degenerative diseases of the retina. Our group established the use of a fate mapping technique in *CX3CR1-CreER; fGFP-flox* mice to distinguish the two compartments in the retina. In so doing we have identified numerous differences in their phenotypic expression and morphology. Furthermore their distribution and migration patterns toward the RPE are distinct, which will be the focus of this talk.

The RPE Response to Complement Attack

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Polymorphisms in genes involved in the alternative pathway of complement activation are associated with increased (and occasionally decreased) risk of developing age-related macular degeneration (AMD). Retinal pigment epithelial (RPE) cells are strongly implicated in the pathogenesis

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of AMD, and in AMD eyes, RPE cells exhibit a number of abnormalities including accumulation of intracellular and subcellular deposits, mitochondrial dysfunction and disorganisation of organelles. In animal and cell culture models it has been demonstrated that RPE cells are not only a source of complement proteins, they may also be exposed to membrane attack complex, also known as C5b-9.

In previous work we have shown that cultured primary RPE cells are remarkably resistant to C5b-9, and that this is due to the cells being able to rapidly endocytose the complex and target it for destruction by lysosomes. We also showed that if endocytosis is inhibited, so that C5b-9 is retained at the cell surface, this leads to mitochondrial defects (Georgiannakis et al., 2015 J. Immunol. 195:3382-3389). In recent work we have investigated whether RPE cells deal with C5b-9 as effectively when confronted with various challenges, such as inflammasome activation and exposure to indigestible photoreceptor outer segments. In my talk I will present some of our initial findings using this experimental model, in addition to other work in which we are using new mouse mutants to specifically delete complement genes in different retinal cell types.

The Transitional Zone of Geographic Atrophy Lesions: Mononuclear Phagocyte Interactions with RPE and Photoreceptors

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Geographic Atrophy (GA), the untreatable form of late age related macular degeneration (AMD), is characterized by an atrophic zone (AZ) of the retinal pigment epithelium (RPE) and the photoreceptors and leads to central high acuity vision loss. Peripheral to the margin of the AZ, the transitional zone (TZ) is characterized by marked rod degeneration, while cones are preserved but lose their outer segments (CS) despite the presence of RPE. Mononuclear phagocytes (MPs) are invariably found in the AZ but also in contact with RPE and PR in the transitional zone (TZ) of GA patients and are associated with visual dysfunctions and loss of cone segments. Using co-cultures of human MPs and primary porcine RPE and a fully humanized system using human induced-pluripotent-stem-cell (iPS)-derived-RPE, we show that

activated MPs markedly inhibit RPE gene expression of Orthodenticle homeobox 2 (OTX2), a transcription factor that controls essential, homeostatic RPE, in a TNF α depend mechanism. Furthermore using MP/retinal co-cultures *in vitro* and inflammation-prone *Cx3cr1^{GFP/GFP}* mice *in vivo*, we demonstrate that subretinal MPs do not affect cone survival but lead to severe CS degeneration in an IL-1 β dependent mechanism. Our results strongly suggest that subretinal MP accumulation participates in the observed pathological photoreceptor changes in GA, and in particular the TZ. Inhibiting subretinal MP accumulation or MP derived cytokines might protect the CS and RPE function and help preserve high acuity daytime vision in conditions characterized by subretinal inflammation.

Spleen-derived Monocytes in RPE-adjacent Subretinal Inflammation

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Purpose: Hypertension, which is associated with elevated systemic angiotensin II levels, is an important risk factor of Age Related Macular Degeneration (AMD). Angiotensin II type 1 receptor expressing mononuclear phagocytes (AT1⁺MP), originating from the spleen, have been shown to play an important role in the subsequent recruitment of circulating inflammatory CCR2⁺Mo in ischemic myocardial inflammation. We here examined the role of splenic AT1⁺MPs in subretinal inflammation and choroidal neovascularization (CNV).

Methods: Subretinal inflammation and choroidal neovascularisation (CNV) was induced by laser-injury (450mW; 250um; 50ms) in eyes of C57Bl6 treated or not by intraperitoneal AT1 antagonist Losartan (125mg/kg/day), and carrying or not subcutaneous osmotic pumps releasing systemic Angiotensin II (1ug/kg/min) with or without splenectomy. 7 days after the laser impacts IBA1⁺ and AT1⁺ subretinal MPs and CD102⁺CNV were quantified on immune-stained retinal and RPE/choroidal flatmounts.

Results: Our immunostaining revealed 2 distinct subpopulations of subretinal infiltrating MPs, Iba1⁺AT1⁻ and Iba1⁺AT1⁺-MPs. Pharmacological antagonism of AT1 by losartan significantly decreased and AngII osmotic pumps exacerbated the number of both subretinal MP types and CNV. Interestingly, splenectomies significantly decreased

subretinal MP accumulation and CNV and prevented the proinflammatory effect of systemic AngII.

Conclusion: Our study shows that Iba1⁺AT1⁺-MPs participate in subretinal inflammation and that systemic AngII strongly favors their infiltration of the subretinal space. The observation that splenectomy prevented this effect suggests that splenic AT1⁺MPs participate importantly in the process. Our study might help explain why hypertension confers a risk to develop AMD.

JNT3 (IMM+RCB) - Microglia and macrophages as therapy targets for retinal diseases

Dynamics of Microglia and Monocytes in the Mouse Retina in vivo

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Both resident microglia and extravasated phagocytic monocytes contribute to the removal of cellular debris during neurodegeneration. Our lab has assessed the dynamics of both of these cell types in different mouse models of retinal degeneration and damage. *In vivo* AO-SLO time-lapse imaging revealed strikingly little movement of the primary or secondary microglial branches in the retina under normal conditions. In response to damage and degeneration, there was induction of somatic mobility and recruitment of monocytes, but a striking lack of process motility in neighboring ramified microglia. In both genetic degeneration and acute injury models, monocyte recruitment and differentiation proceeded with a time course very similar to that of microglial activation, highlighting the challenges of discriminating the contributions of microglia vs macrophages in disease.

Metabolic Control of Microglial Activation

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Microglia are critically involved in various retinal inflammatory and degenerative diseases. Immune cell activation is known to be regulated by metabolic pathways. How microglial activation is regulated by glucose metabolic pathway remains unknown. Cells can be fueled by two main pathways, glycolysis and the tricarboxylic acid (TAC) cycle. The former converts glucose to pyruvate and generates ATP in the cytoplasm, and the later generates the reducing equivalents nicotinamide adenine

dinucleotide (NADPH) and Flavin adenine dinucleotide (FADH₂), which donate electrons to the electron transport chain to fuel oxidative phosphorylation (OXPHOS) in the mitochondria. Under normal culture conditions microglia express a variety of glucose transporters (GLUTs), and the expression level of GLUT1 was significantly higher than other GLUTs. The GLUT1 inhibitor STF31 significantly reduced glucose uptake by microglial cells. When microglia were activated by LPS + IFN- γ , the expression of GLUT1 was significantly increased. In line with increased GLUT1 expression, microglial cells also showed higher levels of glycolysis. Blocking glucose uptake by SFT31 significantly suppressed glycolysis and reduced the expression of TNF α , iNOS, IL6, and CCL2 in LPS + IFN- γ treated cells. Furthermore, *in vivo* treatment with STF31 suppressed microglial activation and reduced photoreceptor death in a mouse model of light-induced retinal degeneration. Our results show that GLUT1 plays an important role in glucose uptake and metabolism in microglial cells under normal and inflammatory conditions. Pharmacological manipulation of glucose uptake by targeting GLUT1 may be a novel approach to control inflammation.

Complement Factor H Inhibits Inflammation Resolution

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Complement factor H (CFH) variants have been linked to age-related macular degeneration (AMD) susceptibility, but the mechanisms by which CFH impacts the disease remain unclear. We here show that CFH deficiency completely prevented pathogenic, age-related, subretinal mononuclear phagocyte (MP) accumulation in AMD-prone *Cx3cr1^{GFP/GFP}* mice and mice expressing the AMD-risk APOE2 isoform. Mechanistically, we demonstrate that CFH binding to CD11b/CD18 inhibits subretinal MP elimination induced by thrombospondin-1 (TSP-1). The AMD-associated CFH_{402H} isoform markedly increased this inhibitory effect on microglial cells, indicating a causal link to disease etiology. Importantly, CFH also impeded MP clearance during acute sterile peritonitis and TSP-1 accelerated subretinal and

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peritoneal inflammation resolution. Our findings provide important mechanistic insights into how CFH controls inflammation resolution, which may be exploited in the therapy of chronic inflammatory diseases, including AMD.

Microglial Phagocytosis of Living Photoreceptors Contributes to Inherited Retinal Degeneration

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Retinitis pigmentosa (RP), a disease characterized by the progressive degeneration of mutation-bearing photoreceptors, is a significant cause of incurable blindness in the young worldwide. While the primary cause of inherited retinal degenerations typically involves mutations in photoreceptor-specific genes, the contribution of non-cell-autonomous inflammatory changes in augmenting photoreceptor degeneration has been implicated. The cellular basis of this contribution however has not been previously well understood. Using the rd10 mouse model, we find that retinal microglia, the principal resident immune cell, demonstrate early and prominent infiltration into the photoreceptor layer during degeneration. These infiltrating microglia interact dynamically with photoreceptors to accelerate their demise by:

(1) primary microglial phagocytosis ("phagoptosis") in which microglia engulf and remove living photoreceptors in a process dependent on the microglial vitronectin receptor and regulated by CX3CL1-CX3CR1 signaling; and
 (2) microglial production of pro-inflammatory cytokines, particularly IL1 β , that potentiates photoreceptor apoptosis. These findings characterize the contribution of retinal microglia to photoreceptor degeneration in RP, and highlight molecular mechanisms (vitronectin receptor signaling, CX3CL1-CX3CR1 signaling, and IL1 β signaling) as potential targets for intervention. Current investigations in our group include a proof-of-concept, Phase I/II clinical trial to demonstrate the potential efficacy of microglial inhibition as a therapeutic strategy for RP.

Interferon-beta Signaling in Retinal Mononuclear Phagocytes Attenuates Pathological Neovascularization

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Age-related macular degeneration (AMD) is a leading cause of vision loss amongst the elderly. AMD pathogenesis involves chronic activation of the innate immune system including complement factors and microglia/macrophage reactivity in the retina. Here, we show that lack of interferon- β signaling in the retina accelerates mononuclear phagocyte reactivity and promotes choroidal neovascularization (CNV) in the laser model of neovascular AMD. Complete deletion of interferon- α/β receptor (Ifnar) using *Ifnar1*^{-/-} mice significantly enhanced early microglia and macrophage activation in lesion areas. This triggered subsequent vascular leakage and CNV at later stages. Similar findings were obtained in laser-treated *Cx3cr1*^{CreER};*Ifnar1*^{fl/fl} animals that allowed the tamoxifen-induced conditional depletion of *Ifnar1* in resident mononuclear phagocytes only. Conversely, systemic IFN- β therapy of laser-treated wild-type animals effectively attenuated microgliosis and macrophage responses in the early stage of disease and significantly reduced CNV size in the late phase. Our results reveal a protective role of *Ifnar* signaling in retinal immune homeostasis and highlight a potential use for IFN- β therapy in the eye to limit chronic inflammation and pathological angiogenesis in AMD.

Effect of Diabetes on Repair of the Inner Blood Retinal Barrier, Microglia and Monocyte Dynamics Following Retinal Ischemia-reperfusion Injury

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Resolving inflammation and reestablishing integrity of the inner blood-retinal barrier (iBRB) are important therapeutic goals for treatment of several sight-threatening retinal diseases, including diabetic retinopathy. We characterized the onset and resolution of innate neuroinflammation and disruption of the iBRB in a mouse intraocular pressure (IOP)-induced ischemia-reperfusion (IR) model of retinal neurodegeneration and examined how diabetes affects these processes. Retinal IR injury was unilaterally

produced by elevation of IOP for 90 min followed by natural reperfusion. The responses to IR injury included inner retinal layer thinning, retinal cell death, leukostasis and leukocyte infiltration, and vascular permeability. Leukocyte populations exhibited an established temporal progression with granulocytes and Ly6C^{hi} inflammatory monocytes predominating at 1 day and Ly6C^{neg} reparative monocytes peaking at day 7. Endothelial tight junction (TJ) proteins were altered with rapid occludin phosphorylation followed by claudin-5, ZO-1 and ZO-2 loss. Microglia responded to IR by proliferating and migrating from plexiform layers toward apoptotic retinal ganglion cells. All pathologies were resolved by 4 weeks. Vessel density, endothelial cell number and perfused vessel area were unaltered, suggesting that restoration of the iBRB was not due to pruning of leaky vessels. Instead, this coincided with a reorganization of TJs at endothelial cell borders. To examine the effects of diabetes on vascular permeability and neuroinflammatory resolution, IR injury was produced after 4 weeks of streptozotocin (STZ-) induced diabetes. Whereas STZ mice exhibited 45% less ($p < 0.01$) vascular leak than normal controls at 2 d following IR, the diabetic mice failed to reestablish the iBRB and thus had 2.4-fold higher ($p < 0.05$) permeability at 4 weeks post IR. There were no significant differences in accumulation of granulocytes or Ly6C^{hi} monocytes between diabetic and controls. Surprisingly, at 2 weeks following IR STZ mice exhibited an exaggerated 2.3-fold microglial population increase ($p < 0.01$) that was not observed in normal controls. Numbers of Ly6C^{neg} monocytes were also more than 2-fold greater ($p < 0.05$) in retinas of STZ mice at both 2 weeks and 4 weeks following IR. Thus, diabetes impaired resolution of inflammation and hindered reestablishment of the iBRB following retinal injury, despite greater numbers of reparative monocytes.

JNT6 (IMM+OPT) - Molecular mechanisms of fibrosis in eye tissues

Ironing out the Wrinkles of Lens Fibrosis

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Strong evidence supports an important role for TGF β in the formation of posterior capsule opacification (PCO) following cataract surgery. Conventional wisdom suggests that TGF β /Smad signaling regulates transdifferentiation, which in turn gives rise to matrix contraction thus contributing to PCO due to increased light scatter. Evidence

to support this link is largely circumstantial; therefore we employed a series of inhibition studies to elucidate the role of Smad-dependent and independent pathways in the regulation of lens cell transdifferentiation and matrix contraction. To achieve this, the human lens cell line FHL 124 was used. TGF β induced contraction was assessed using a patch assay. Transdifferentiation was determined by expression of the marker protein α SMA. Smad4 and α SMA knockdown was achieved using siRNA. MEK, Rho kinase and myosin light chain kinase (MLCK) were inhibited using U0126, Y-27632 and ML-7 respectively. The human lens capsular bag model was also used to study TGF β induced matrix contraction and transdifferentiation. TGF β 2 (10ng/ml) significantly increased expression of α SMA in FHL124 cells. SiRNA against α SMA and Smad4 significantly inhibited both the message and protein levels in the presence and absence of TGF β 2. Addition of 10ng/ml TGF β 2 to patch assays caused a significant contractile event to take place following a 3 day culture period. Knockdown of Smad4 or α SMA did not prevent TGF β 2 induced matrix contraction. Maintenance of cultures in the presence of 10 μ M U0126, Y-27632 or 15 μ M ML-7 when added to control medium had no significant effect on patch area; however, when added in the presence of TGF β 2, contraction was inhibited. In capsular bags, TGF β induced marked contraction (wrinkling) of the posterior capsule and significant elevation of α SMA. Application of 10mM ML-7 to TGF β treated capsular bags inhibited wrinkling. α SMA expression remained strong; but notably exhibited a different distribution pattern to bags maintained in TGF β alone. In summary, TGF β induced transdifferentiation is not critical for matrix contraction. In contrast, Smad-independent signaling pathways do appear to play important roles in TGF β mediated matrix contraction by human lens cells. Moreover, myosin seems to be an important component in the contractile apparatus associated with TGF β mediated contraction.

Myofibroblasts and Non-TGF β Smad Signaling in Corneal Ctroma

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Scarring/fibrosis in corneal stroma disturbs normal transparency and curvature of the tissue and thus impairs vision. Myofibroblasts, the key player of tissue fibrosis, exert excess accumulation of extracellular matrix and tissue contraction. Although a set of Inflammatory/fibrogenic growth factors or cytokines expressed in inflammatory

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cells play a critical role in fibrotic tissue formation, one of the most potent factors, transforming growth factor β (TGF β) is much involved in myofibroblast appearance. Although it is established that TGF β -activated Smad signal is essential to the process of keratocyte-myofibroblast transformation, I emphasize the involvement of non-TGF β molecular mechanisms in modulating Smad activation. I focus on the roles of matricellular proteins (osteopontin, tenascin C and lumican) and, as cellular components, the roles of transient receptor potential (TRP) cation channel receptors will be discussed. My intent is to draw attention to the possibility of modulation of signal transduction cascade (e.g., Smad signal and others involved in tissue fibrosis) by gene transfer and other related technology as being beneficial in a clinical setting to reduce or even prevent corneal stromal tissue fibrosis/scarring and inflammation.

AGE-RAGE Interaction in Fibrosis of Lens Epithelial Cells

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Due to low turnover rate basement membrane (BM) proteins accumulate chemical modifications with age. Glycation is one such modification, which leads to the formation of advanced glycation endproducts (AGEs). The eye lens capsule is a BM secreted by lens epithelial cells. We have observed age-dependent increases in the AGE levels in human lens capsules and higher levels of AGEs in cataractous lens capsules than in age-matched non-cataractous lenses. Our studies have also shown that the AGEs in human lens capsule promote the TGF β 2-mediated epithelial-to-mesenchymal transition (EMT) of lens epithelial cells, and the AGE content of the capsule proteins is directly related to the TGF β 2-mediated EMT in lens epithelial cells. We have now investigated the role of a receptor for AGEs (RAGE) in the TGF β 2-mediated EMT in a human lens epithelial cell line. The RAGE levels were unaltered in cells cultured on either native or AGE-modified BM or upon treatment with TGF β 2. However, RAGE overexpression significantly enhanced the TGF β 2-mediated EMT responses in cells cultured on AGE-modified BM compared with the cells cultured on unmodified BM. In contrast, treatment of cells with a RAGE antibody or EN-RAGE, an endogenous ligand for RAGE resulted in a significant reduction in the TGF β 2-mediated

EMT response and TGF β 2-mediated Smad signaling. N^ε-carboxymethyllysine (CML) is a major AGE in tissues and we have observed that CML enriched BM elicits a TGF β 2-mediated EMT response similar to AGE-modified BM. Together these results suggest that the interaction of matrix AGEs, possibly CML, with RAGE plays a role in the TGF β 2-mediated EMT of lens epithelial cells and suggest that the blockade of RAGE could be a strategy to prevent PCO and age-related fibrosis in other tissues.

 α B-crystallin Regulates Subretinal Fibrosis by Modulation of Epithelial-mesenchymal Transition

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Subretinal fibrosis is a result of a wound healing response that follows choroidal neovascularization in neovascular age-related macular degeneration (nAMD). Although anti-vascular endothelial growth factor therapy has become a standard treatment that improves visual acuity in many nAMD patients, unsuccessful treatment outcomes have often been attributed to the progression of subretinal fibrosis. An initial step of the pathogenesis of subretinal fibrosis is an epithelial-mesenchymal transition (EMT) of retinal pigment epithelium cells. alphaB-crystallin plays multiple roles in age-related macular degeneration, including cytoprotection and angiogenesis. However, the role of alphaB-crystallin in subretinal EMT and fibrosis is unknown. In a review article, we proposed that the late phase of the laser-induced choroidal neovascularization model may be useful for studying the pathophysiology of subretinal fibrosis (*Exp Eye Res.* 2016). In the present study with this model, we showed attenuation of subretinal fibrosis and a decrease in mesenchymal retinal pigment epithelium cells in alphaB-crystallin knockout mice compared with wild-type mice. alphaB-crystallin was prominently expressed in subretinal fibrotic lesions in mice. In vitro, overexpression of alphaB-crystallin induced EMT, whereas suppression of alphaB-crystallin induced a mesenchymal-epithelial transition. Transforming growth factor-beta2-induced EMT was further enhanced by overexpression of alphaB-crystallin but was inhibited by suppression of alphaB-crystallin. Silencing of alphaB-crystallin inhibited multiple fibrotic processes, including cell proliferation, migration, and fibronectin production. Bone morphogenetic protein 4 up-regulated alphaB-crystallin, and its EMT induction was inhibited by

knockdown of alphaB-crystallin. Furthermore, inhibition of alphaB-crystallin enhanced monotetraubiquitination of SMAD4, which can impair its nuclear localization. Overexpression of alphaB-crystallin enhanced nuclear translocation and accumulation of SMAD4 and SMAD5. Thus, alphaB-crystallin is an important regulator of EMT, acting as a molecular chaperone for SMAD4 and as its potential therapeutic target for preventing subretinal fibrosis development in neovascular age-related macular degeneration.

JNT10 (IMM+OPT) - New molecular mechanisms and therapeutic approaches for uveitis

Uveitis: Gaps in Therapy and Understanding

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Despite progress with understanding and with therapy, uveitis remains a major cause of visual loss. Unmet needs include the inability to categorize about one third of patients with uveitis beyond the term idiopathic; the difficulty in excluding infections such as tuberculosis; the inability to predict prognosis or to personalize therapeutic recommendations on the basis of individualized markers; the dearth of medications with minimal toxicity; and the inability to either prevent uveitis or to induce a drug free remission. Regional therapies frequently cause cataract and glaucoma, while systemic therapy can result in serious infections. For those patients without a systemic illness requiring therapy, local therapy has the potential to avoid toxicities associated with systemic immunosuppression.

The Effect of Intravitreal Triamcinolone at the Draining Lymph Nodes

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Purpose: Locally administered corticosteroids are very effective in the treatment of non-infectious uveitis. The primary anti-inflammatory mechanism of intravitreal steroid is presumably via decreasing cytokine production and affecting intrinsic cell populations within the eye. In this study,

we tested whether intravitreally administered triamcinolone acetate (IVTA) travels to the local lymph nodes differentially during periods of quiescence, acute inflammation, and chronic inflammation to affect lymphocyte proliferation.

Methods: Three experimental conditions were examined: (1) non-uveitic, (2) acute inflammation, and (3), chronic inflammation induced by killed *Mycobacterium butyricum* antigen.

Bilateral submandibular (SMLN), deep cervical (DCLN), superficial cervical (SCLN) lymph nodes, vitreous, and aqueous specimens were obtained 1 d after IVTA in New Zealand white albino female rabbits in scenarios (1) and (2), and 28 d after uveitis induction in scenario (3). Quantitation of TA was performed using liquid chromatography-tandem mass spectrometry. Flow cytometry was used to quantitate lymphocyte proliferation using a BrdU assay.

Results: In non-uveitic animals, TA was found in the ipsilateral SMLN and DCLN as well as the contralateral DCLN 24 h after TA injection. In acute uveitis, TA was found in the bilateral SCLNs and the ipsilateral DCLN, 24 h after TA injection. In chronic uveitis, TA was found in the ipsilateral SCLN 28 d after TA injection (Table 1). Lower lymphocyte proliferation rates were significantly correlated with higher concentrations of TA in the lymph nodes (p=0.01). At the concentrations of TA found in the draining lymph nodes, significant direct inhibition of lymphocyte proliferation was demonstrated ex vivo (p< 0.05). At certain lymph nodes, there was increased activation potential in IVTA treated animals compared with saline-injected animals (p< 0.05).

Conclusions: TA is found at ng/mL concentrations in draining lymph nodes after intravitreal injection in uveitic and non-uveitic animals differentially, and inhibits lymphocyte proliferation at the local lymph node. In contrast to untreated uveitic animals, IVTA appears to increase activation potential at the local lymph nodes, which suggests that inherent immune regulatory processes are potentially more effective at the draining lymph node than TA. These findings contribute novel understanding to the mechanism of intravitreally injected drugs used in uveitis.

| | SMLN | | SCLN | | DCLN | | Aqueous | |
|-----------------------|-------|-------|-------|-------|-------|-------|---------|------|
| | Right | Left | Right | Left | Right | Left | Right | Left |
| Normal, 24 h | 0.28 | 0 | 0 | 0 | 0.14 | 0.28 | 158 | 0 |
| Acute uveitis, 24 h | <LLOQ | <LLOQ | 0.323 | 0.199 | 0.286 | <LLOQ | 126 | 0 |
| Chronic uveitis, 28 d | 0 | 0 | 1.86 | NA | NA | NA | 66.6 | 0 |

[TA concentration (ng/mL) at local lymph nodes]

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Local Corticosteroid Treatment of Uveitis

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There are a variety of options available to treat patients with anterior, intermediate, posterior, and panuveitis, and newer options that will likely become available within the next 1-2 years. Corticosteroids, given topically has been the mainstay for anterior uveitis, and corticosteroids given as a periocular, or intravitreal injection, as an intravitreal sustained drug delivery implant (dexamethasone (DDS) and fluocinolone acetonide (FA) delivery systems), or systemically have been the primary therapy for intermediate, posterior, or panuveitis. Topical and systemic treatment requires patient compliance, which may be difficult in chronic uveitis, and while all these agents are very effective, all may cause cataract and elevated intraocular pressure, and systemic treatment has a myriad of side effects that are often treatment-limiting. Currently available shorter-term delivery methods, include intravitreal triamcinolone acetonide (TA) injection, periocular steroid injection, and DDS. However, these methods may not produce a drug effect sufficiently long, without relatively frequent re-injection, to manage these chronic inflammatory diseases. Long-term delivery systems such as the FA implant are typically effective for 3 years or more, but must be surgically implanted. Recently, we have investigated an injectable FA implant that can be placed in the office, and that releases drug for 2-3 years. In an individual investigator-sponsored trial, this agent very effectively controlled inflammation for at least 2 years, improved visual acuity, reduced ancillary anti-inflammatory drug use, and had a favorable safety profile. Furthermore, a phase 3 study of this delivery system met its primary 6-month therapeutic endpoint.

Preclinical and Clinical Aspects of a Locally Injectable mTOR Inhibitor, DE-109, for the Long-term Management of Uveitis

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Introduction: In a preclinical study, New Zealand White rabbits were injected in both eyes with an intravitreal formulation of an mTOR inhibitor, DE-109, to evaluate the pharmacokinetics of this depot formulation. 63 animals were divided into 3 groups receiving 22, 66, or 220 µg sirolimus per eye. At various time points through 162 days post-injection, 3 animals/group were euthanized, enucleated, frozen and dissected to separate retina/choroid

and vitreous humor (VH). Ocular sirolimus concentrations and whole blood were measured using LC/MS/MS and characterized by a gradient of sirolimus concentration of VH >retina/choroid >sclera >whole blood with detectable levels in ocular tissue extending through 60 days. Results from this study were used for dose selection in the clinical studies.

Methods: The SAKURA Study 1 was a phase 3, randomized, multinational, 24-month study assessing the safety and efficacy of DE-109 for the treatment of active non-infectious uveitis of the posterior segment (NIU-PS). 347 subjects (348 eyes) with active NIU-PS (baseline vitreous haze score $\geq 1.5+$ and BCVA ≥ 19 ETDRS letters in the study eye) were randomized 1:1:1 to of 44, 440, or 880 µg administered every 2 months, followed by open-label 880 µg at Months 6, 8, and 10. At Month 12, all subjects who had clinical benefit, determined by the Investigator, could receive DE-109 for an additional 12 months on an as-needed basis, no more often than every 2 months (open-label retreatment period). All AEs reported are based on the entire safety population (346 study eyes) through 24 months.

Results: Efficacy in the double-masked treatment phase was most favorable with the 440 µg dose: 53% of subjects achieved resolution (VH 0 or 0.5+) at Month 5 with 440 µg versus 35% with 44 µg ($p=0.008$). The majority of patients with BCVA $\leq 20/40$ at baseline had at least a 1-line increase in visual acuity at Month 5, regardless of achieving vitreous haze endpoints (51%, 58% and 48% for the 44, 440 and 880 µg groups, respectively). A total of 60.6% (211/348) of study eyes received treatment in open-label treatment or PRN retreatment periods, balanced among the 3 dose groups. Through Month 24, the most common study medication-related AE were increased intraocular pressure (11.3% of subjects), transient drug depot in visual axis, 6.4%) and iridocyclitis (5.5%).

Conclusion: In SAKURA Study 1, improvements in VH and vision demonstrate the utility of intravitreal administration of an mTOR inhibitor for NIU-PS.

PD Ligand Blockade Decreases IRBP-induced Uveitis in Mice

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Introduction: Programmed death-1 (PD-1) binding to its

two ligands is believed to down-regulate autoimmunity. Our previous studies observed a paradoxical decrease in uveitis susceptibility in mice that lacked PD-ligands. To further investigate these findings, we tested the hypothesis that PD-L blockade using an antibody against PD-L1 would decrease susceptibility to uveitis in animal models of experimental autoimmune uveitis (EAU).

Methods: Uveitis was induced in C57Bl/6 and B10.RIII mice using the appropriate IRBP peptides. All experiments were carried out in strict accordance with ARVO guidelines and the Guide for the Care and Use of Laboratory Animals. Three days post EAU induction, mice were untreated (EAU), treated with anti-PD-L1 antibody (BioXCell) (EAU+PD-L1) or IgG control (EAU+IgG). Wild type animals (WT) were not induced for uveitis. Animals were monitored daily for external signs of inflammation and masked clinical assessment by funduscopic examination was performed on day 12 (B10.RIII) and 20 (C57Bl/6) post EAU induction. Retinal inflammation was graded on a four-point scale. At the end of the study, animals were subject to euthanasia, eyes were enucleated, fixed, and evaluated for histological severity by masked observers using a published scale. Splenocytes were evaluated after a short *in vitro* culture using a Luminex multiplex cytokine assay.

Results: Treatment with anti-PD-L1 antibody significantly reduced uveitis severity in both the C57Bl/6 and B10.RIII animals ($P < 0.0001$). A multivariate analysis of Luminex multiplex cytokine results showed significant differences between either EAU+PD-L1 and WT animals compared to EAU or EAU+ IgG treated animals ($P < 0.0001$). More specifically, IL-17A, IL-17E, INF- γ , TNF- α , IL-2, IL-4, and IL-6 were significantly elevated in EAU and EAU+ IgG treated animals. There was no statistical difference in cytokine production between EAU+PD-L1 and WT animals.

Conclusions: Significant abrogation of uveitis occurred in both the animal strains following early treatment with anti-PD-L1. Anti-PD-L1 therapy depresses proinflammatory cytokines, especially IL-17 and INF γ , following uveitis induction. Further studies are required to investigate the mechanisms by which blocking the PD-1 system abrogates ocular inflammation and experiments are needed to help decipher a possible therapeutic potential of PD/L-1 blockade.

Ocular Autoimmunity - a Collusion of Development and Environment

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Non-infectious uveitis of a putative autoimmune nature is responsible for up to 15% of blindness. The disease is thought to be driven by retina-specific T cells which somehow become activated to attack the eye. The reasons why these T cells persist despite self-tolerance mechanisms, and where / how they become activated, is poorly understood. We use mouse models of autoimmune uveitis to provide experimental evidence that retina-specific T cells escape negative selection in the thymus during development and fail to undergo peripheral tolerance due to limited contact with retinal antigens, whose expression outside the immune privileged eye is limited. These cells persist in the body in an ignorant, but not tolerant, state and may become activated by unidentified crossreactive stimuli. Our data suggest that one such stimulus may be a crossreactive antigen(s) derived from commensal microbiota in the intestine, which stimulates migrating retina-specific T cells in the context of innate bacterial signals present in the same environment. We propose that the T cells which become stimulated in the gut reach the eye through the bloodstream, actively penetrate the blood retinal barrier, and elicit autoimmune inflammation. These studies can help to understand the etiology of autoimmune uveitis.

JNT12 (IMM+RPE) - RPE regulation of innate immune activity and functionality in macrophages

RPE Cells Differentiated from iPS Cells Possess Immune Functions Similar to Primary RPE Cells

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Purpose: To determine whether retinal pigment epithelial (RPE) cells from induced pluripotent stem (iPS) cells possess immune functions similar to primary RPE cells.

Methods: Human iPS-derived RPE cells (human iPS-RPE cells) were established from fresh skin fibroblasts obtained from healthy donors after informed consent was obtained. Monkey iPS-RPE cells were also established from skin fibroblasts obtained from healthy adult cynomolgus monkeys (*Macaca fascicularis*). For

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establishment of mouse iPS-RPE cells, retinal progenitor cells were differentiated from mouse iPS cells, and RPE differentiation was enhanced by activation of several inhibitors. The characteristics of these iPS-RPE cells (human, monkey, and mouse) were confirmed by gene expression (quantitative RT-PCR), immunocytochemistry, and flow cytometry. Functions and immunologic features of the iPS-RPE cells were also evaluated.

Results: We obtained these iPS-RPE cells at high purity. The iPS-RPE cells showed apical-basal polarity and cellular structure characteristic of RPE cells. Expression levels of several RPE markers (RPE65, tyrosinase, PEDF, MiTF, and so on) in these iPS-RPE cells were comparable to those of each primary cultured RPE cells. The iPS-RPE cells had formed tight junctions, phagocytose photoreceptor outer segments, express immune antigens, and suppress lymphocyte proliferation, e.g., iPS-RPE cells significantly inhibited T cell proliferation and IFN- γ production by T cells when the target T cells were stimulated.

Conclusions: These iPS-RPE cells can be an attractive tool for functional and morphological studies of RPE cells, possibly replacing the use of rather inefficient primary RPE cultures. Additionally, regenerative medicine techniques using differentiated iPS-derived RPE cells are clinically in progress. Basic research using mouse & monkey iPS-RPE cells would contribute to optimization of regenerative medicine techniques using human iPS-RPE cells.

RPE Regulation of Macrophage Phagocytosis and Antigen Processing Pathways

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Recently, we reported that cultured retinal pigment epithelial cells (RPE) produce soluble molecules that suppress the phagocytic process within macrophages. This is mediated partly by the RPE neuropeptides alpha-melanocyte stimulating hormone (a-MSH) and neuropeptide Y (NPY). In this presentation, we present our recent findings on the molecular mechanisms used by healthy RPE from immune privileged eyes to suppress the activation of the phagolysosome in macrophages. Using a method of magnetic bead isolation of intracellular phagosomes, immunoblots of the isolated phagosome content for OVA degradation, RAB5, RAB7, and LAMP-1 expression demonstrated that the neuropeptides suppress the fusion of the phagosome with the lysosome. While healthy RPE suppressed formation of the phagolysosome this suppressive activity was not mediated by RPE from retinas that have lost immune privilege, such as eyes

with experimental autoimmune uveitis (EAU). There was both a loss in suppressive molecules, and a gain in the expression of IL-6. Neutralization of IL-6 neutralizes RPE enhancement of phagolysosome activation within macrophages. A similar change was seen in isolated retinal microglial cells. Recently, we have demonstrated a strong therapeutic effect of a-MSH to suppress EAU, and to induce regulatory immunity through MC5r, one of its four receptors. The RPE from these a-MSH treated mice had regained the ability to suppress phagolysosome activation; however, it was not mediated through MC5r. The results demonstrate a new mechanism of ocular immune privilege mediated by the neuropeptides produced by the RPE. The mechanism alters uptake and processing of antigens by APC, diminishing presentation of peptide-MHC complexes that are recognizable by T cells. This could be considered a mechanism of antigen sequestration. In autoimmune disease there is a change in the RPE to produce IL-6, and promote APC phagocytic activity. This change would permit presentation of T cell-recognizing peptides of autoantigens. In addition, melanocortin suppression of EAU, also recovers RPE suppression of the phagocytic process in macrophages.

Retinal Microglia Are Critical for Subretinal Neovascularization in a Murine Model of Macular Telangiectasia

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Purpose: Abnormal subretinal neovascularization is characteristic of vision-threatening retinal diseases including macular telangiectasia (MacTel) and retinal angiomatous proliferation (RAP). Subretinal vascular changes and photoreceptor dysfunction are observed in very low-density lipoprotein receptor mutant mice (*Vldlr*^{-/-}). These mirror those observed in MacTel and RAP patients, but the pathogeny is largely unknown. In this symposium, we show that retinal microglia interact extensively with angiomas in *Vldlr*^{-/-} mice, and that ablation of microglia dramatically prevents tuft formation and improves neuronal function as seen by electroretinography.

Methods: Knock-in transgenic mice (*Cx3cr1*^{GFP/GFP}) expressing green fluorescent protein (GFP) under the control of the chemokine receptor 1 promoter, which drives expression in macrophages/microglia, were crossed with

Vldlr^{-/-} mice. Transgenic mice (*Cx3cr1-Cre*) expressing Cre recombinase under the control of the CX3CR1 promoter or the M lysozyme (*LysM*) locus (*LysM-Cre*) were mated with *Vldlr*^{-/-}, *VEGF*^{floxed/floxed}, or *Rosa26*^{IDTR/+} mice to generate conditional gene knockouts and macrophage and microglia ablation. We also used an inhibitor of colony stimulating factor 1 receptor (CSF-1R) to ablate these cells. The phenotypes of the mice were characterized using Ganzfeld electroretinograms (ERGs) and immunohistochemistry.

Results: Myeloid cells are observed in a much higher frequency in the subretinal space of *Vldlr*^{-/-};*CX3CR1*^{GFP/+} mice than in controls (*CX3CR1*^{GFP/+}). Myeloid cells are also consistently observed in very close proximity to neovascular tufts in the deep plexus vascular layer and seem to wrap long processes around the angiomas as they are forming. Using genetic and pharmacological approaches to deplete retinal microglia, we observed significantly fewer angiomas in the *Vldlr*^{-/-} mouse and significantly improved electroretinography recordings.

Conclusions: These findings elucidate the contribution of microglia to pathology and may provide valuable clues about how subretinal neovascularization occurs in MacTel and RAP patients.

Nonclassical Macrophages and Neovascular Remodeling in Neovascular AMD

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Treatment-resistant neovascular age-related macular degeneration (NVAMD) remains a significant clinical problem, affecting up to 40% of patients and increasing risk of long-term vision loss for affected individuals. We have observed that treatment-resistant NVAMD occurs most frequently in patients with choroidal neovascularization (CNV) of the branching arteriolar subtype, characterized by the presence of a large-caliber feeder artery, many branching arterioles, terminal vascular loops, and minimal capillaries. The branching arteriolar subtype reflects the biology of **neovascular remodeling (NVR)**, the transformation of nascent capillaries into branching arterioles with perivascular fibrosis. While NVR appears to be the primary cause of treatment-resistant NVAMD, the mechanisms that mediate NVR remain poorly understood. In this presentation, we will explore NVR pathobiology. Using mouse models of NVR in experimental laser-induced CNV, we will assess the role of blood-derived macrophages of the nonclassical subset, which, upon recruitment to

the incipient new vessel, secrete fibrogenic factors that mediate NVR via recruitment and activation of vascular smooth muscle cells and myofibroblasts. Additionally, we will review specific mechanisms that regulate macrophage effector function in the setting of NVR. Finally, we will consider evidence that nonclassical macrophages mediate NVR in patients with NVAMD.

Abundance of Nonclassical Macrophages in Postmortem Eyes with Dry Age-related Macular Degeneration

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Purpose: To characterize retinal macrophage subpopulations in postmortem human eyes with various stages of AMD, as defined by both histopathologic staging and retinal imaging.

Methods: 62 human postmortem autopsy eyes from patients over age 80 were enucleated and fixed in 4% formaldehyde. The eyes were imaged using SD-OCT (Bioptigen, Morrisville, NC) and scanning laser ophthalmoscopy (SLO)(Heidelberg Engineering, Carlsbad, CA), then paraffin embedded and sectioned for histopathology analysis. H&E and Periodic-acid Schiff stained macular sections were graded according to a new histological classification system for AMD based on the staging by Sarks (1976). Unstained paraffin macular sections were processed for immunohistochemistry (IHC) analysis for CD68 and CD163 macrophage markers, as well as CD45 pan-leukocytic marker. The use of the donor eyes for research was approved by the Institutional Review Board of the Duke University Medical Center.

Results: Analysis of both the macula and retinal periphery revealed that normal, age-matched eyes and AMD eyes contained a large number of CD163+ and CD45+ cells. The number of CD68+ cells in the retina was significantly lower in AMD eyes and nearly negligible in normal aged eyes. In eyes with early AMD, CD163+ macrophages were restricted to the inner retina above the outer plexiform layer. In contrast, in AMD eyes with continuous basal laminar linear deposits, there was a significant increase in the number and size of the CD163+ cells in the outer retina and sub-retinal space. AMD changes observed on SD-OCT and SLO imaging correlated with histopathology findings.

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Subretinal macrophages in AMD eyes with intermediate-severe disease were associated with a targetoid, reticular pseudodrusen pattern on SLO and subretinal hyperreflective deposits on SD-OCT.

Conclusions: We report a new histologic classification system for AMD and novel observations using the macrophage marker CD163. Retinas of normal and AMD eyes contain a significantly larger number of macrophages than previously reported using prior immunohistochemical markers. Importantly, there is a striking change in the retinal distribution of macrophages in postmortem eyes with intermediate-severe AMD as compared to normal or early AMD eyes. AMD pathology including subretinal macrophages could be specifically detected and localized by SD-OCT and SLO imaging in human postmortem eyes.

IMA1 - New advances in ocular imaging - part II

Tear Film Thickness Measured by OCT in Sicca Patients

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In optical coherence tomography (OCT) the axial resolution depends on the coherence length of the light source. Using Ti-sapphire laser technology we set up a prototype OCT system that provides an axial resolution of 1.3 micrometers at the front of the cornea. Using this system the pre-corneal tear film can be measured in vivo. In healthy subjects the tear film is on average 5 micrometers in thickness. In patients with dry eye disease the tear film thickness is negatively correlated with the symptoms of dry eye disease as quantified with ocular surface disease index (OSDI). In patients with severe dry eye disease tear film thickness can be as low as 2.5 to 3 micrometers. Topical lubricants can increase tear film thickness after administration. The residence time of different products may differ ranging from several minutes to several hours. Finally novel applications of OCT in dry eye are discussed including enface tear film maps to estimate evaporation and lipid layer maps as calculated from OCT reflection.

3-D OCT Ocular Biometry

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Ocular biometry is an essential step prior to implanting an intraocular lens (IOL) in a patient's eye, used to select the power of the IOL to be implanted. Standard biometry is generally limited to keratometry and 1-D axial length. IOL power calculation formulas are based on population regressions. We demonstrate the potential of quantitative 3-D Optical Coherence Tomography (OCT) to change the paradigm for IOL selection based on customized patient eye models.

Custom spectral anterior segment OCT was used to capture 3-D eye images (11x11mm; 50 B-scans x 300 A-scans; 0.6 seconds) in patients (n=3;65-72 y.o) before and after IOL implantation, and on ex vivo donor lenses (n=27;19-71 y.o). OCT images were processed using automatic custom algorithms for segmentation, fan and optical distortion correction and surface fitting.

Computer pseudophakic eye models were developed in an optical design software using OCT quantitative data (anterior & posterior corneal elevations, anterior chamber depth, lens tilt & decentration, eccentric focal position, axial length), and used to estimate ocular aberrations. Ocular aberrations were also measured with custom Laser Ray Tracing aberrometry on the same eyes. In addition, OCT images of ex vivo crystalline lenses were used to construct a lens parametric model and algorithms to reconstruct the full lens shape from data available within the pupil. The accuracy of the estimated lens volume (Vol) and equatorial position (EP) was evaluated comparing estimates from the whole lens ex vivo. All experimental protocols met the tenets of the Declaration of Helsinki.

Experimental and simulated aberrations in pseudophakic eyes show a high correlation ($R=0.93; p<0.0001$). On average, the discrepancy in astigmatism, spherical aberration and high order aberrations was 0.15, 0.03 and 0.08 mm (5-mm pupil). The application of full lens reconstruction algorithms in pre-operative eyes allowed estimates of the lens Vol (208mm³) and EP (2.152mm). A validation in donor eyes showed accuracies within 96% (Vol) and < 45 mm (EP), significantly higher than methods based on lens surface intersections or thickness.

The high similarity between optical and geometry-based predictions of high order aberrations in pseudophakic patients indicates that fully OCT-based ray tracing models are excellent platforms for optimizing IOL selection. OCT lens imaging will increase accuracy in IOL power selection and be key in implantation of emerging accommodating IOLs.

Air-puff Swept - Source Optical Coherence Tomography

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Accurate value of intraocular pressure (IOP) is a crucial for screening, diagnosis and management of glaucoma. To provide reliable IOP value the corneal biomechanics has to be taken into account. It is well documented that for stiffened corneas classic tonometers measure a significantly higher IOP even for all other geometric and material properties of the eye remaining constant. In order

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to decouple mechanical properties from IOP we proposed to use air-puff system incorporated with OCT. We have developed a straightforward method of data analysis based on corneal hysteresis representing viscoelastic properties of the cornea. The hysteresis is measured by tracking time dependent OCT signal from corneal apex during deformation-recovery process along with the reference pressure value of the air pulse. In order to test capabilities of new method we performed well-controlled experiments on animal eyeballs *ex vivo* treated with cross-linking procedure. Intraocular pressure was directly measured by invasive application of the pressure detector. Air-puff OCT measurements enabled to calculate corneal hysteresis loop parameters such as the hysteresis curve area or maximum deformation amplitude. In our work we confirmed that the corneal hysteresis area became lower with increasing IOP value. Additionally, viscoelastic behavior of *ex vivo* porcine corneas is manifested as a difference in the hysteresis area measured for ascending and descending IOP values. There is also statistically significant correlation between hysteresis area and maximum deformation amplitude. Finally we demonstrated statistically significant increase of hysteresis area after cross-linking treatment for physiological IOP level.

Towards OCT on Chip

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Optical coherence tomography (OCT) is one of the most rapidly emerging and innovative optical imaging modalities of the last decades enabling *in vivo* cross-sectional tomographic visualization of internal microstructure in biological systems. Recent developments in ultrabroad bandwidth laser as well as OCT technology enable three-dimensional ultrahigh resolution OCT with unprecedented axial resolution, approaching resolution levels of conventional histopathology, enabling optical biopsy of biological tissue. In addition, multimodal extensions of OCT are recently under development that should provide subcellular resolution as well as non-invasive *depth resolved* functional imaging of the investigated tissue, including extraction of biochemical, physiologic or even molecular tissue information.

Despite the fact that optical imaging techniques - especially OCT - offer cost-effective, uniquely performing, flexible, adjunct modalities revealing underlying functional disease information at quasi histologic penetration depths, the vast majority (if not nearly all) commercial and scientific OCT

instruments still have considerable footprints and are in the five to low six digit EURO or USD price range. Although this is still significantly less than established standard clinical imaging techniques, e.g. CT, MR, PET and others, there has been a pronounced trend in OCT towards miniaturized, low-cost, handheld systems for flexible applications in the field of point-of-care or screening.

Although OCT has proven its potential value in several medical fields, wide adoption has not taken place due to size and cost limitations as well as non-existence of handheld devices. Hence recent state of the art miniaturization of OCT technologies will be presented, reviewed and discussed including on chip based OCT and approaches based on integrated optics, enabling a significantly smaller, low-cost, compact and maintenance free alternative. The latter approach promises to develop unprecedented compact, reliable, low-cost, battery operated, wireless handheld OCT device in combination with embedded systems based external console to receive two- and three-dimensional tomograms for instantaneous post processing and visualization as well as immediate diagnosis. In this way instrumentation maintenance requirements should be eliminated and training and healthcare costs should be reduced, paving the way to significantly improve diagnosis for general medicine and primary care.

IMA2 - New advances in ocular imaging - part I

Ophthalmic Surgical Guidance Using Intraoperative Optical Coherence Tomography

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Visual impairment has a prevalence above 3.5% in adults 40 years and older and affects over 3 million people in the United States, and the number of individuals with blindness and low vision is expected to increase markedly as a result of the aging population. Ophthalmic surgical maneuvers are currently limited by the ability of surgeons to visualize and manipulate semitransparent tissue layers as thin as tens of microns. The lack of real-time intraoperative three-dimensional visualization remains the critical barrier to image-guided surgery, which would have a dramatic impact on current clinical practice and lead to novel surgical techniques and instruments that may improve clinical outcomes.

OCT allows non-invasive, high-resolution *in vivo* imaging of tissue microstructure and has become the gold-standard for clinical diagnostics, tracking disease progression,

and treatment planning in ophthalmology. Application of OCT immediately before and during ophthalmic surgery has demonstrated the utility of image-guided clinical decision-making by allowing contrast-free visualization of transparent tissue structures otherwise indistinguishable on conventional widefield microscopy/ ophthalmoscopy. Early iOCT studies used externally mounted scan-heads (perioperative imaging) to visualize epiretinal membrane, macular hole, retinal detachment, vitreomacular traction, and lamellar keratoplasty. Whereas the utility of perioperative imaging was limited because it required interruption of surgery and precluded imaging during surgical maneuvers, several recent studies have demonstrated surgical microscope-integrated iOCT imaging of retinal and corneal procedures using commercial and laboratory prototypes. Here, we describe several iterative advances in iOCT technology, including a second-generation microscope-integrated iOCT system, OCT-friendly surgical instrumentation, optimized dynamic iOCT imaging, and HUD visualization.

Wide Field OCT: Technology and Clinical Application of Retinal Megahertz Imaging over up to 100° Field of View

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The rising interest in imaging the periphery of the eye has triggered substantial technology development efforts. New wide field fundus cameras and scanning laser ophthalmoscopes can visualize the retina beyond the equator. Retinal wide-field imaging plays an increasingly important role for the diagnosis of various types or pathologies, some of which start or prevalently appear in the periphery. Despite the great value of wide field fundus cameras and scanning laser ophthalmoscopes for diagnosis and documentation, these devices only acquire 2-dimensional data in form of en face projections. Obviously, for a layered structure like the retina, optical cross-sections with depth priority can provide a wealth of additional information. This is one of the reasons for the great popularity of optical coherence tomography (OCT). A single OCT image is an optical depth cross-section of the sample, and if many such OCT images are acquired with a dense spacing the resulting 3-dimensional data set can be used to extract cross-sections with any arbitrary orientation. However, to cover a wide area of the retina of 100° or more of viewing angle, 5 - 15 million OCT scans are required. Current commercially available OCT

technology is not able to capture such large data sets and the acquisition time would be prohibitively long.

We recently developed retinal Megahertz OCT (MHz-OCT), an OCT system that can acquire more than 1 million depth scans and more than 2 billion image points per second. It is a swept source OCT working at a center wavelength of 1050nm. It can achieve the high speed by using a new type of laser: the Fourier Domain Mode Locked (FDML) laser. This high speed enables us to acquire a densely sampled 3-dimensional OCT data set in the non-mydiatic eye, covering an area of up to 100° angle on the human retina. Ongoing clinical studies with such a wide-field system reveal the huge diagnostic potential of combining wide-field en face imaging with the power of OCT. We show how choroidal and retinal thickness maps, depth resolved en face projections, angiographic imaging and even eye shape analysis greatly benefit from the ultra-wide field of view of up to 100°. Commercialization of ultrawidefield MHz-OCT will be discussed.

Line Field Optical Coherence Tomography: High Speed Imaging and Digital Aberration Correction

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Despite the large success of OCT in ophthalmology there is still room for performance improvement regarding lateral resolution as well as in speed. The advantage of high speed is the possibility to record large field of views also with the recent functional extension of OCT angiography with minimal patient motion distortion. Another advantage is the possibility of online volume display, which is important for example in intrasurgical OCT guidance. Point-scanning OCT is ultimately reaching its physical detection sensitivity limit. Changing the recording paradigm from point scanning to parallel acquisition relaxes both the system complexity as well as the sensitivity limit. Full field OCT has been demonstrated with several Megahertz equivalent A-scan rate but suffers from multiple scattered light that critically reduces contrast. We introduce instead line field OCT, which scans a line across the fundus, and exhibits both good contrast as well as high sensitivity even at up to one million A-scans per second. Moreover high speed recording opens another new possibility: to perform digital aberration correction, i.e., to enhance lateral resolution in post-processing without the need of expensive adaptive optics hardware. So far, adaptive optics seemed to be

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the only way to achieve cellular resolution for retinal structures. However, OCT gives access to the complex sample field, which carries information about aberration. This information is however highly sensitive to patient motion, and high speed recording of more than 2000 tomograms per second is indispensable to exploit its full capabilities. With line field OCT we are not only able to maintain high sensitivity at high speed, but can retrieve aberration data, that can be used in post-processing to recover high resolution images of retinal structures. The aberration correction is performed non-iteratively in a single step using a novel pupil segmentation algorithm introduced recently by our group. It is straight-forward to perform also OCT angiography with line field OCT. During the presentation the method of line field OCT at a center wavelength of 840nm and up to 1MHz A-scan rate will be demonstrated, showing results for structural OCT as well as OCT angiography. The method of aberration correction will be explained and high-resolution images of retinal photoreceptors, as well as retinal nerve fibers and capillaries with a lateral resolution of 5µm will be shown.

Function Retinal Imaging by Holographic OCT

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Optical coherence tomography (OCT) is one of the most successful imaging technologies in ophthalmology. Two-dimensional tomograms and three-dimensional volume images visualize tissue structures and their pathological changes in the anterior and posterior segment of the eye with a resolution of ten microns or better. Beside the intensity, OCT provides information on the phase of the backscattered light, which enables the measurement of axial changes of morphology or local changes of the refractive index with nanometer precision. The phase sensitivity of OCT is the basis for visualizing and quantifying blood flow by OCT angiography and Doppler OCT. However, in scanned OCT tissue motion dominates the phase and destroys the lateral coherence of the measured OCT data. Completely phase stable OCT imaging of the retina is possible with an ultrafast camera. Using a swept source interferograms of the retina are recorded at up to 1000

wavelengths, which allows the reconstruction of an OCT image. Compared to scanned OCT a significantly increased imaging speed with A-scan rates of up to 1 GHz and volume rates of up to 2 kHz are possible. This opens exciting new opportunities for retina imaging as the acquired data is laterally phase-stable, even when the rapidly moving retina is imaged.

Here, we demonstrate three applications in retinal imaging. First, entirely diffraction-limited 3-dimensional volumetric imaging of the retina was realized by numerical refocusing the structures outside the focus and correction of the aberrations of the eye. Second, using the superior phase stability of FF-SS-OCT we were also able to image heart-beat induced pulse-waves, propagating through the retina and measure their velocity non-invasively. Pulse wave travel in retinal vessels with nearly 1 m/s, orders of magnitude faster than previously measured. Third, we reliably measured intrinsic optical signals (IOS) after optical stimulating the photoreceptors. IOS could even be assigned to single cones by using the computational aberration correction.

In conclusion, establishing lateral phase stability allows high resolution functional imaging of the retina, which may give new diagnostic options.

Optical Coherence Tomography Using Visible Light in Human Subjects

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Purpose: To date, all reported clinical OCT used near infrared (NIR) illumination. While NIR enables deeper penetration into the tissue, it limits the ability to image absorption-related functional parameters, including retinal oxygen metabolism, a key indicator of retinal health. We developed a dual modality OCT/SLO system working in the visible-light spectral range, where hemoglobin molecule has peak absorption, offering the potential advantage of OCT oximetry. We have already demonstrated this capability in rodent studies and are now reporting pilot clinical applications of this device for structural as well as functional applications.

Methods: A supercontinuum laser provided illumination light ranged from 496 to 632 nm for both SLO and OCT. The OCT subsystem used free-space Michelson configuration to avoid optical dispersion associated with fiber optics. When operated in SLO mode, the system has one additional beam splitter inserted within the sample beam of the OCT.

Results: Fig. 1 shows the images from a healthy volunteer. In the SLO image, the macular area is clearly visible. Using both SLO and OCT images, blood vessels can be clearly visualized, with magnified images centered at the fovea showing the fine capillary network within the region. In the OCT B-scan image, a total of 12 retinal layers can be recognized. In addition, the submicron axial resolution permitted us to distinctly resolve Bruch's membrane (BM), which usually cannot be distinguished from the retinal pigment epithelium (RPE) in NIR OCT.

Fig. 2 shows images from the optic nerve head (ONH). In the magnified views, the finer features in the *lamina cribrosa* were observable. The OCT cross-section showed the normal NFL around the ONH, while the detailed scattering textures within the layer was also visible.

We are currently analyzing the human retinal oximetry information, which we will be reported at the meeting.

Conclusions: We have demonstrated the first dual-modality OCT/SLO system for imaging human retina within the visible-light spectral range, with images comparable to existing clinical systems. In addition, visible light illumination provided improved sub-micrometer axial resolution for greater anatomical details. At the meeting we will report the data for human oximetry measurements.

IMA3 - OCT angiography and Doppler OCT

Blood Velocity, Flux and Flow: Objective Measurements of Single Cell Hemodynamics in the Living Retina

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Metabolic Imaging/ Blood Flux and Functional Imaging

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The cells that comprise the neural retina are among the most metabolically active tissues in the body. To serve the energy demands of the retina, a network of capillaries circulates blood to deliver metabolites and remove waste products. This microscopic vascular network is fragile and susceptible to several vascular diseases that collectively give rise to the leading cause of blindness in the developed world. We could far better diagnose, treat and study capillary dysfunction by studying these early-stage changes in the living eye. However until recently, visualizing this network was obscured by optical aberrations of the eye that blurred microscopic capillaries, relied on weak optical

contrast of blood cells, and suffered from motion-blur artifacts of blood cells constantly in motion. Here, I will describe several recent advances in ophthalmoscopy that have surmounted key challenges that now make it possible to study single cell blood flow in the full spectrum of large-to-small vessels in the retina. Using the anesthetized mouse as a model system, we deploy adaptive optics to measure and correct for aberrations of the eye, enabling a detailed study of single blood cells with near diffraction limited imaging. Using adaptive optics with fast camera capture of over 30,000 images per second, we mitigate motion blur of moving blood cells. Additionally, we combine the above strategies with the split-detection imaging approach which compares the differential scatter of light in two phase-locked detectors at the retinal imaging plane. Images captured in this configuration provide exceptionally high contrast of single blood cells based on the differential scatter of near infrared light, to which the eye is relatively insensitive.

Combined, these technical advances now provide new measures of capillary blood cell flux (cells/s), single cell velocity, cell classification (including identification of platelets, white blood cells and red blood cells), a detailed study of blood cell rheology and measures of the intravascular hemodynamics within the largest to smallest retinal vessels in the living eye.

Optical Coherence Tomography Angiography: Split-spectrum Amplitude-decorrelation Angiography

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Introduction: Optical Coherence Tomography (OCT) provides micron-level anatomical information on a micron scale by analyzing reflectance data from retinal structure. However, dynamic information can also be obtained by comparing differences in the reflectance signal from multiple frames obtained at the same retinal cross-section. Consequently, retinal blood flow is itself an intrinsic contrast agent, thus resulting in the technique of OCT Angiography (OCT-A). We sought to maximize angiographic quality without increasing scan time by incorporating signal processing techniques.

Methods: At the level of the OCT detector, the Split-Spectrum Amplitude-Decorrelation Angiography (SSADA) algorithm deconvolves two high-resolution broadband OCT B-scans into 11 pairs of low-resolution frames. Pixels from each of these pairs of frames are then compared to each other to compute the level of decorrelation between them. Finally, the average decorrelation of the sets is determined.

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Results: The resulting averaging of decorrelation within these individual sets utilized in SSADA offers a 4x improvement in signal-to-noise ratio compared to the direct computation of decorrelation from full-spectrum scans. By setting a decorrelation threshold, vessel structure can be resolved cross-sectionally and by scanning across the macula, an OCT-A volume can be produced provide depth-encoded angiographic information. Volumetric OCT-A information can be used to derive useful clinical metrics such as vessel density, capillary nonperfusion area as well as detect and quantify pathologic entities such as retinal and choroidal neovascularization.

Conclusions: OCT-A utilizing SSADA provides a high quality and efficient means for the detection of blood flow *in vivo*. Consequently, OCT-A is now clinically viable, and has been incorporated into commercial instrumentation for use in improving patient outcomes. OCT-A derived from SSADA forms a core technology that additional image processing advances such as projection-resolved imaging can be founded upon. The ability to visualize retinal and choroidal circulation without the use of endogenous contrast agents will allow a more complete assessment of several retinal pathologies and allow direct integration with simultaneously acquired structural OCT data.

Advanced OCT Angiography for Simultaneous Pigment and Vascular Imaging

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Optical coherence tomography angiography (OCT-A) is now widely used in clinical routine. It is a non-invasive and depth-resolved modality for visualization of ocular vasculature. Because of its sensitivity to vascular pathology, it would be a natural direction of development to make it an alternative to conventional angiographies such as fluorescein angiography (FA) and indocyanine green angiography (ICGA). FA and ICGA are powerful diagnostic tool, because it is sensitive not only to vascular pathology but also to some general tissue abnormalities, which includes the abnormalities of retinal pigment epithelium (RPE) and exudates.

In this paper, we present an advanced OCT angiography, so called as pigment and flow OCT (PAF). From the engineering perspective, the PAF device is based on Jones matrix OCT (JM-OCT) technology. JM-OCT measures all optical properties of the eye which includes scattering, small motion (flow), and polarization properties. The

tomography of local polarization uniformity is obtained from the polarization property. And it is known to be sensitive to melanin. From the clinical point of view, PAF is an advanced OCT-A which provides not only the flow but also the abnormality of melanin pigment and RPE.

We will discuss a descriptive case series of exudative macular diseases which are examined by PAF. The vascular abnormality and RPE abnormality are correlatively understood by using this method.

Measurement of Total Retinal Blood Flow by Multi-beam Doppler OCT

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Retinal blood circulation plays an important role in several ocular diseases like glaucoma, diabetic retinopathy, or retinal vein occlusion. While non-invasive, label-free imaging of retinal vessels by optical coherence tomography angiography (OCT-A) is becoming increasingly popular since it is available in commercial OCT systems, quantitative measurements of retinal blood flow are still difficult to achieve. The main reason is that Doppler OCT only provides the blood velocity component in the direction of the sampling beam. To overcome this problem, we are developing new systems that use three sampling beams simultaneously that illuminate the sample at three different angles. This provides three components of the velocity vector from which the absolute velocity and flow of blood within a vessel can be determined, without the need of any a-priori information on the blood vessel orientation.

Circumpapillary measurements provide the flow through all major retinal arteries and veins, from which the absolute flow can be determined. We report on the development of two such three-beam Doppler OCT systems, a spectral domain OCT system operating at 840 nm, and a swept source based system operating at 1050 nm. Results in healthy subjects, achieved with the 840 nm system, demonstrate a precision of ~ 6%, first comparisons between healthy and glaucomatous eyes indicate a statistically highly significant blood flow reduction in the case of glaucomatous eyes.

IMA4 - Functional & contrast enhanced imaging

Jones-matrix OCT for Non-invasive Multi-contrast Imaging

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Jones-matrix optical coherence tomography (JM-OCT) is a generalized OCT and enable to acquire several optical properties more than backscattering intensity. Non-invasive multifunctional imaging will be available by using JM-OCT. This presentation aims to review the current capability of JM-OCT for wide applications in clinical ophthalmology.

Non-pathologic eyes and pathologic eyes of several diseases were scanned by 1- μ m JM-OCT. This system can obtain polarization-artifact-free OCT intensity, attenuation coefficient, OCT-angiography (OCT-A), degree of polarization uniformity (DOPU), and local birefringence images in a single shot. These contrasts are associated with the structure, tissue scattering and absorption, vasculature, melanin concentration, and fibrous structure, respectively. When OCT-A and DOPU are combined, pigment and flow (PAF) imaging is available, which contrast of the vasculature color coded by the relative depth to pigmented tissue.

JM-OCT reveals three-dimensional tissue properties with multiple contrasts. JM-OCT also allows one to quantify the tissue light scattering property by generating the polarization-artifact-free attenuation coefficient image. Polarization artifacts, expressed as alternating dark and light bands in the peripapillary scleral region, are suppressed, while they are evident in the standard OCT image. It shows good contrast between prelaminar tissue and lamina cribrosa. JM-OCT can also produce OCT-A images for the visualization of micro-vasculature and DOPU images for pigment distribution. PAF shows abnormalities of vasculature and retinal pigment epithelium (RPE), simultaneously. Abnormal blood flow which is not blocked by pigmented tissue, such as choroidal neovascularization (CNV) with hypo-pigmented RPE, can be easily interpreted. In addition, JM-OCT enable the clear visualization of choroidal and scleral tissue contrast using local birefringence imaging. Some multi-layered structure

was observed in sclera which is not clear in OCT image. Birefringent tissues were observed around CNV in some patients. These multi-contrast imaging greatly enhances the ability for one to interpret data.

JM-OCT provides non-invasive simultaneous imaging of tissue light attenuation coefficient, pigmentation, vasculature and birefringence, which can provide descriptive information tissue property distribution. It will be useful for comprehensive diagnosis, and investigation of disease progression.

Neurovascular Coupling in the Retina

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It is known that in the brain, neural activity and blood flow are tightly coupled. This so called neuro-vascular coupling is a physiological key feature of neural tissues to adapt its blood flow to local metabolic demands. It has been shown that also in the human retina neurons, glia cells and blood vessels are closely coupled. Increased retinal neural activity, induced for example by visual stimulation, leads to an immediate increase in blood flow in the optic nerve head as well as in the retinal vasculature. Further, it is known that ocular pathological conditions such as glaucoma or diabetic retinopathy lead to a breakdown of this coupling mechanism.

Because of its optical properties and its easy accessibility, the eye offers a unique possibility to non-invasively investigate neuro-vascular coupling in humans. New and sophisticated technologies such as functional optical coherence tomography and non-invasive measurements of retinal oxygen saturation, allow now for a better understanding of the mechanisms behind neuro-vascular coupling in the retina.

The current talk aims to summarize how new imaging technologies can be used for the investigation of neuro-vascular coupling in the human retina. In addition, our current knowledge on the physiological mechanisms behind neuro-vascular coupling will be covered. Further, the role of neuro-vascular coupling and its potential applications in the diagnosis and follow up in neuro-degenerative diseases will be discussed.

Ocular Imaging

Fluorescence Lifetime Imaging Ophthalmoscopy FLIO

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The fluorescence of endogenous organic molecules is not only characterized by the emission spectrum, it has also a specific decay rate or lifetime. Fluorescent lifetime imaging ophthalmoscopy is a new imaging method that allows measurements of fluorescence lifetimes in vivo in the retina. Findings in various retinal diseases such as age related macular degeneration, retinal vascular diseases and retinal dystrophies with distinct fluorescence lifetimes will be presented and discussed. Factors influencing fluorescence lifetimes will be discussed and data from ex vivo measurements of components of the visual cycle will be referred to. Fluorescent lifetime imaging of the retina is a promising new technique that may serve as additional tool to diagnose retinal diseases and may be useful for early detection of metabolic changes in the retina.

Visible-light OCT-based Functional and Multimodal Retinal Imaging

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Purpose: To develop and validate a novel visible light OCT-based technology for functional and multimodal imaging of the retina. The system is able to image the molecular contrast of rhodopsin in vivo and simultaneous OCT and lipofuscin autofluorescence imaging of the RPE.

Methods: Rhodopsin is the functional biomarker of rod photoreceptors, which is responsible for converting light into neuronal signals in a process known as phototransduction. The rhodopsin photo-bleaching provides a contrast mechanism to image rhodopsin with optical imaging modalities with suitable wavelengths. Lipofuscin is a byproduct of the photoreceptor visual cycle. The accumulation of lipofuscin is an important contributor to RPE dysfunction with aging due to its adverse effects on the RPE cells. Lipofuscin can be imaged with fundus autofluorescence by using an exciting wavelength of 488 nm. We developed a novel VIS-OCT technology, which can provide

- (1) molecular imaging of rhodopsin and
- (2) simultaneous OCT and fundus autofluorescence (FAF) imaging.

When imaging rhodopsin the system is tuned to a center wavelength of 520 nm, close to the peak optical absorption wavelength in the dark adapted state. The depth resolution allows the visualization and segmentation of the location where the absorption change occurs and provides an accurate assessment of rhodopsin content. When imaging lipofuscin the system is tuned to a center wavelength of 480 nm. The simultaneously acquired OCT and FAF provides a unique opportunity for quantitative imaging of lipofuscin.

Results: The imaging system was applied to imaging the retina of animals in vivo. We imaged both albino and pigmented rats. The technology has successfully revealed the depth-resolved rhodopsin distribution in the retina, and thus provided direct visualization of the photon propagation pathways. We have designed and successfully performed a pattern bleach experiment, which proved conclusively that the imaging represents rhodopsin distribution. The system was also successfully applied to imaging the age-related lipofuscin accumulation in rats.

Conclusion: The success in animal experiments clearly showed VIS-OCT can be used to image rhodopsin distribution in vivo, and thus assess the distribution of functional rod photoreceptors in the retina. It also provides a unique opportunity for quantification of RPE lipofuscin.

IMA5 - Imaging in glaucoma and myopia

Imaging the Structures of the Optic Nerve Head

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Our goal was to integrate in-vivo and ex-vivo imaging of the same eye for high-resolution visualization of the structures of the optic nerve head (ONH) and to assess the effects of variations in intraocular and/or intracranial pressures (IOP and ICP). Five monkeys were studied. IOP and ICP were controlled through cannulas inserted in the anterior chamber and the lateral ventricle, respectively. IOP and ICP were set to all combinations of IOPs (15 - baseline, 5, 30 and 50mmHg) and ICPs (10 - baseline, 5, 25 and 40mmHg), and allowed to equilibrate for 10min before imaging one eye per animal with SD-OCT 3+ times at each setting. All 10 eyes were perfusion fixed at controlled IOPs, enucleated, cryosectioned and imaged using light microscopy. Histology images were assembled into 3D stacks. For the eyes of two monkeys the main structures of the ONH were delineated in both OCT and histology volumes and analyzed for similarities/differences and repeatability of structure visibility. A recently developed tracking algorithm based on image registration was used on three eyes to extract pressure-induced tissue displacements, stretch and compression from the OCT volumes. We found excellent correspondence between OCT and histology, with lamina cribrosa (LC) thickness of $335\pm 4\mu$ in OCT and $330\pm 30\mu$ in histology. Approximately 50% of the LC was consistently visible across OCT scans. Pressure variations caused substantial LC stretch and compression, sometimes exceeding 30%. Effects differed between LC regions and animals, and depended on whether the pressures were increased or decreased, with larger effects caused by pressure increases. There were strong interactions between the two pressures. Our results demonstrate that highly-detailed effects of IOP and ICP on the LC can be detected in-vivo, even in the most posterior LC. The pressure effects are highly local and nonlinear with strong interactions that vary substantially between individuals. These techniques open a new way to investigate the interplay between IOP and ICP and their dependence on tissue structure.

Jones-matrix OCT for Myopic Posterior Diseases

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Fibrous tissues are widely distributed inside the eyeball and such tissues as nerve fiber layers, lamina cribrosa, and sclera are key organs in developing major ocular pathologies including pathological myopia, glaucoma, uveitis, and choroidal neovascularization. It is critical to identify those tissue location and characteristics to understand the pathogenesis in vivo.

The local measurement of birefringence is an ideal method in order to identify their properties. We recently developed Bayesian estimator for birefringence imaging for precise quantification in tissue. Also the Multi-Functional Jones matrix OCT, shortly, JM-OCT providing scattering, phase retardation, DOPU, Doppler imaging at a same time has been developed. This technology allows additional information, like vasculature and fibrous tissue property.

We review the case series of posterior diseases examined with JM-OCT. JM-OCT gives us more detailed information and profile of the posterior ocular tissues. Additionally this technology is able to depict the deep ocular tissues such as sclera and lamina cribrosa, which is supposed to be helpful to disclose the underlying mechanism.

Anterior segment imaging for glaucoma evaluation

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Although optical coherence tomography (OCT) is widely adopted in clinical practice for imaging and evaluation of the anterior chamber angle, the detection of angle closure has been constrained by the lack of an efficient approach for measurement of the anterior chamber angle dimensions for 360 degrees. With the introduction of anterior segment swept-source OCT, imaging of the entire anterior segment from the anterior corneal surface to the posterior lens surface can now be completed in less than a second. This presentation will focus on the latest development of swept-source OCT for assessment of the anterior chamber angle. Specifically, the application and limitation of a new algorithm, STAAR (Scleral spur Tracking for Angle Analysis and Registration), for automated, circumferential measurement of the anterior chamber angle dimensions will be discussed.

Ocular Imaging

IMA6 - AO in vision sciences

Adaptive Optics for Vision Evaluation

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Adaptive optics (AO) instruments consist of a wavefront sensor to measure the eye's aberrations and a correcting device to modify the eye's optics. Patients view a stimulus (letters or any visual scene) produced by a micro-display. The potential applications of this type of AO instruments in Ophthalmology are enormous. AO will allow optimizing the optical correction for different visual tasks. For example, in some cases, some residual customized amount of spherical aberration could provide some extra depth of focus in presbyopic eyes. Another powerful application will be the pre-evaluation of different optical solutions. In invasive procedures, such as laser refractive surgery, before a definitive ablation of the cornea is performed, the optical profile to be induced could be optimized for each patient. This will open the door to an era of true customized eye treatments. State of the art instruments, including a binocular version will be presented.

Prospects for Two-photon Imaging in the Living Human Eye

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Visualising both structure and function of the *in vivo* retina at a cellular-scale in health and disease provides many advantages for both basic and clinical science. With adaptive optics scanning light ophthalmoscopy (AOSLO) of light reflected from various retinal layers, we can visualize the structure of the photoreceptor mosaic, retinal vasculature and the retinal nerve fiber layer. Single-photon fluorescence techniques have allowed us to see the retinal pigment epithelium cell mosaic and, with the use of extrinsic fluorophores, ganglion cells and Müller cells (Roorda and Duncan, 2015). Two-photon excited fluorescence (TPEF) imaging has two important advantages when applied to the living eye.

First, many of the most important fluorophores in the retina (e.g. retinoids and NADH) are excited by wavelengths below 400 nm, light that is blocked from reaching the retina by absorption in the cornea and lens. Fortunately, TPEF uses long wavelengths, which pass easily through

the spectral window of the eye. Using AOSLO in monkey eyes, we have imaged Müller cells, ganglion cells, vessel walls and photoreceptors, which provide the strongest signal (Sharma et al, 2016).

Second, two-photon imaging can interrogate a whole new range of molecular processes within single cells without the use of extrinsic fluorophores. Vision begins at the level of photoreception in the eye leading to phototransduction and regeneration of photopigment via the visual cycle. Several of the retinoids involved in the visual cycle, including retinol, are fluorescent. The time course of the emitted TPEF intensity in response to visual stimulation is a measure of retinal function. In macaques, we have recently demonstrated a new method to rapidly assess the formation and clearance of retinol in rods and shown that TPEF is directly related to available photopigment.

In humans, many blindness-causing diseases are linked to defects in the visual cycle, such as Stargardt disease. As compared to more gross measures of visual deficits, TPEF imaging has potential to more directly assess functional changes with disease and monitor efficacy of treatment strategies. Concerns about light safety are a major challenge to performing TPEF in people. Using light levels within safety standards, we made TPEF functional measures and assessed ocular safety in macaque. The only notable effect of TPEF imaging was a reduction in IR autofluorescence which slowly recovers and may or may not be harmful.

High-resolution Retinal Imaging in Color Blindness

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Achromatopsia (ACHM) is an inherited cone dysfunction syndrome with an incidence of approximately 1 in 30,000. While there is a complete absence of cone *function*, the degree to which cone *structure* is affected is less understood. With multiple groups having recently initiated gene replacement clinical trials, it is of critical importance that we define the degree to which cone structure is affected and determine the progressive nature (and thus the therapeutic window) of the disease. Recent studies using spectral domain optical coherence tomography (SD-OCT) are conflicting, with some suggesting progression of cone degeneration and others reporting no age-dependent cone loss. This parallels the conflicting results from early histology studies on post-mortem eyes. Part of the difficulty in interpreting these results is that most of the previous studies did not report the genotype of their

patients. As ACHM can be caused by mutations in *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C* and *PDE6H*, it seems likely that the phenotype may be dependent upon the gene(s) involved. For example, we patients having *GNAT2* mutations have been reported to have residual cone function and near-normal foveal cone structure. Here we used two high-resolution retinal imaging tools (SD-OCT and adaptive optics scanning light ophthalmoscopy, AOSLO) to examine residual cone structure in patients with ACHM of known genotype (*CNGA3* and *CNGB3*). Measures included direct measurement of cone density, measuring outer nuclear layer thickness, and evaluating integrity of the photoreceptor bands on SD-OCT. The degree of residual outer retinal structure was similarly variable within the *CNGA3* and *CNGB3* groups. We did not find age-dependent disruption of retinal structure in ACHM, nor any evidence for progression in a longitudinal substudy - suggesting that the window of opportunity for gene therapy intervention may be larger than recently suggested. AOSLO and SD-OCT retinal imaging enable direct assessment of the degree of residual cone structure and should prove to be vital tools in future therapeutic trials, both in identifying good candidates for intervention as well as evaluating therapeutic outcomes.

Adaptive Optics Retinal Physiology

AUSTIN ROORDA

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Adaptive optics ophthalmoscopy, combined with real-time high-resolution eye tracking, enables measures of structure and function in living eyes on a cellular scale. I will present how we use this technology currently to perform cellular-level visual psychophysics to learn about spatial and color vision at the human fovea - a region of the retina that is not well-understood at a physiological level despite its importance for human vision. While subjective testing has tremendous value, the ability to objectively measure functional properties of retinal neurons has noted advantages. I will close my talk with a proposal on how we plan to use in-vivo functional interferometry to accomplish that task.

RPE Choroid Biology and Pathology

RPE2 - Lipid dynamics at the photoreceptor-RPE nexus

Mechanism of Phosphatidylserine Phospholipid Externalization at Outer Segment Tips during Photoreceptor Outer Segment Renewal

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Photoreceptor outer segment renewal is a fundamental retinal process that is essential for life-long preservation of visual function. Its impairment causes severe forms of retinitis pigmentosa. Inefficiency of phagocytic digestion leads to gradual lipofuscin accumulation with age in the RPE in the human eye, which in turn contributes to RPE dysfunction associated with atrophic age-related macular degeneration.

Outer segment renewal involves a diurnal burst of shedding of photoreceptor outer segment tips and their efficient receptor-mediated phagocytosis by the neighboring RPE. We have shown previously that photoreceptors externalize the anionic phospholipid phosphatidylserine (PS) at distal tips of outer segments at light onset. Externalized plasma membrane PS is a universal "eat me" signal recognized by PS receptors and by soluble secreted PS-binding proteins. In the retina, PS on outer segment tips results in ligation of integrin $\alpha v \beta 5$ via its subretinal ligand MFG-E8 and of the tyrosine kinase MerTK via its subretinal ligands Gas6 and protein-S. Downstream signaling pathways of both receptors synergize to promote phagocytosis of shed outer segment fragments. Here, we investigate the contribution of RPE cells to PS exposure by photoreceptor outer segments. We find that RPE-derived glycoproteins localize to the subretinal space in the wild-type mouse eye specifically at light onset. Moreover, adding such glycoproteins to freshly isolated mouse neural retina *ex situ* promotes rapid exposure of PS specifically at outer segment tips. These data suggest that apical glycoprotein secretion by RPE cells synchronizes PS externalization by outer segment tips.

Lipofuscin Fluorophores in Photoreceptors and the RPE

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Lipofuscin is a fluorescent pigment mixture that accumulates with age in the lysosomal compartment of

the RPE. Its major known components are bis-retinoids, the condensation products of two molecules of retinal. Both lipofuscin and bis-retinoids exhibit significant cytotoxic potential, and are thought to play a role in the development of several retinal degeneration diseases. The bulk of lipofuscin originates in the rod photoreceptor outer segments from reactions involving the 11-cis and all-trans isomers of the retinal chromophore of rhodopsin. 11-cis retinal is generated in the RPE from where it flows into the rod outer segments and combines with opsin to form rhodopsin; all-trans retinal is released into the rod outer segments by photoactivated rhodopsin following its excitation by light. Both 11-cis and all-trans retinal can generate lipofuscin-like fluorophores and bis-retinoids when added to rod outer segments. We have used fluorescence imaging to measure the levels of lipofuscin precursors in the outer segments of isolated rod photoreceptors and of RPE lipofuscin from wild type mice. We find that the levels of accumulated lipofuscin are similar in the RPE of cyclic-light- and dark-reared mice. Furthermore, the levels of lipofuscin precursor fluorophores are also similar in the outer segments of dark-adapted rods from cyclic-light- and dark-reared mice. Following light excitation of dark-adapted isolated photoreceptors, lipofuscin precursor levels increased in the outer segments of metabolically compromised rods, but not in the outer segments of metabolically intact rods. Addition of low concentrations of 11-cis- and all-trans retinal to metabolically compromised rod outer segments resulted in increases in lipofuscin precursor fluorescence. However, it was only the addition of 11-cis retinal that resulted in lipofuscin precursor fluorescence increase in metabolically intact rods. The results are consistent with lipofuscin precursors forming in rod outer segments primarily from 11-cis rather than all-trans retinal, even under cyclic light conditions. Thus, 11-cis retinal may be the primary source of lipofuscin in the retina.

Phospholipid Scrambling by Monomeric Rhodopsin

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The light-sensing G protein-coupled receptor rhodopsin is a constitutively active lipid scramblase that equilibrates phospholipids across photoreceptor disc membranes in mammalian retina, a process required for disc homeostasis. The mechanism of rhodopsin-mediated lipid scrambling is not understood. Scramblase activity can be recapitulated in large unilamellar vesicles containing only a few rhodopsin

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molecules, but as rhodopsin dimerizes on reconstitution into vesicles it is not known whether scrambling is facilitated by individual rhodopsin monomers, or whether it requires features of the dimer interface. We now show that a rhodopsin monomer is sufficient for scramblase activity. On replacing four residues in the membrane-facing aspect of transmembrane helix 4 (TM4) with tryptophan we generated a scramblase-active rhodopsin variant that reconstitutes into vesicles as a monomer. Biomolecular docking analyses using the HADDOCK webserver revealed that TM4-mediated dimerization of the quadruple mutant was energetically unfavorable, with a significant decrease in the area of the interaction interface. Our result places an important constraint on possible scrambling mechanisms and provides a method to prevent dimerization of other G protein-coupled receptors during membrane reconstitution.

Retinal degeneration B (RDGB) codes for a lipid transfer protein which maintains lipid homeostasis during PLC signalling in *Drosophila* photoreceptors

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Drosophila phototransduction represents a phosphoinositide based lipid signalling pathway where activation of phospholipase C (PLC) results in depletion of PI(4,5)P₂ on plasma membrane (PM). During acute stimulation the photoreceptors must maintain adequate levels of PI(4,5)P₂ on PM which in turn relies on a series of biochemical events termed as PIP₂ cycle. This cycle operates across two distinct compartments within cell which poses a topological problem for the movement of two key lipid intermediates. Hotta and Benzer did a genetic screen to identify visual mutants in *Drosophila* and had identified a gene called retinal degeneration B (*rdgB*), lack of which resulted in severe retinal degeneration. The gene was found to code for a phosphatidylinositol transfer protein (PITP) and hence was presumed to help in transferring PI from endoplasmic reticulum (ER) to PM where it can be used to synthesize PI(4,5)P₂. However there has been no evidence to prove this hypothesis. We have studied the various phenotypes of *rdgB* mutants in detail and have shown data which shows that RDGB is localized to PM-ER contact sites in photoreceptors where it helps in counter-

transport of two key lipid molecules across the PM-ER interface. This lipid transfer event is critical to maintain lipid homeostasis and is required to support phototransduction. We will also discuss the molecular basis for the action of RDGB within photoreceptors and strategies by which the *Drosophila* photoreceptors maintain very fast time scale of visual response.

Light Mediated Regeneration of Visual Pigments in Rods and Cones: Evidence for a Photic Visual Cycle

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The light absorbing chromophore in most opsins is 11-*cis*-retinaldehyde (11-*cis*-RAL), which is coupled to a Lys residue in opsin through a Schiff-base linkage. Absorption of a photon isomerizes the 11-*cis*-RAL to all-*trans*-retinaldehyde (all-*trans*-RAL), briefly converting rhodopsin to its active signaling state. In the rhabdomeric photoreceptors of insects, all-*trans*-RAL remains covalently coupled to the opsin following activation. Absorption of a second photon isomerizes the all-*trans*-RAL back to 11-*cis*-RAL, restoring light sensitivity to the rhabdomeric opsin through photoregeneration. For this reason, rhabdomeric opsins are called bistable pigments. In contrast, ciliary opsins in the retinas of vertebrates decay following photoactivation to yield unliganded apo-opsin and free all-*trans*-RAL. Light sensitivity is restored when apo-opsin combines with another 11-*cis*-RAL to reform the opsin pigment. Ciliary opsins are hence called bleaching pigments. The conversion of all-*trans*-RAL back to 11-*cis*-RAL is carried out by two enzyme pathways, one in the retinal pigment epithelium (RPE) and the other in Müller glial cells of the retina. Thus, rods and cones rely on thermal reactions in neighboring cells to synthesize visual chromophore, and appear not to benefit from the faster photoregeneration in 'lower' metazoan species. For sustained vision in daylight, ciliary photoreceptors must be supplied with fresh 11-*cis*-RAL at a rate that matches the rate of chromophore consumption by photoisomerization of opsins. In bright light, the rate of photoisomerization may exceed 10⁶/sec per cone cell.

Here, we will present evidence for light-mediated regeneration of visual pigments in vertebrate retinas. In particular, we will show light-stimulated synthesis of 11-*cis*-RAL and regeneration of rhodopsin in bovine retinas and live mice. We will also show light-mediated stimulation

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of cone sensitivity by suction recording from isolated photoreceptors from *gnat1*^{-/-} mutant mice lacking rod transducin. These results suggest the existence of a photic visual cycle in vertebrate retinas for light-dependent regeneration of rhodopsin and the cone opsins.

RPE4 - VEGF and beyond

Therapeutic Targeting of LRG1 Reduces Pathogenic Neovascularisation and Normalises Vessels

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We recently discovered that the secreted glycoprotein, leucine-rich alpha-2-glycoprotein 1 (LRG1), promotes neovascularisation in various models of ocular disease. LRG1, which is found to be up-regulated in many disease conditions, mediates its pro-angiogenic effect in part by modifying the TGFβ signalling network (Wang et al., 2013). Blocking the biological activity of LRG1 results in attenuation of neovascular complications in laser-induced choroidal neovascularisation and oxygen-induced retinopathy. This finding led us to develop a blocking monoclonal antibody that inhibits LRG1 function. We have now observed that in addition to an inhibition of pathological vessel growth, loss of LRG1 results in vessel normalisation suggesting that LRG1 subverts the normal physiological angiogenic process. Early indications suggest that LRG1 interferes with vascular recruitment of pericytes resulting in failure of vessel maturation. Our data shows that therapeutic targeting of LRG1 improves pericyte coverage and vascular function including a reduction in vascular permeability. These findings have important implications in diseases such as diabetic retinopathy where there is a need to prevent abnormal vessel growth whilst promoting the formation of normal functioning vessels. This work has led to the development of a humanised therapeutic antibody that will enter clinical trials in 2018.

Wang, X., Abraham, S., McKenzie, J.A.G., Jeffs, N., Swire, M., Tripathi, V.B., Luhmann, U.F.O., Lange, C.A.K., Zhai, Z., Arthur, H.M., Bainbridge, J.W.B., Moss, S.E. and Greenwood, J. (2013). Lrg1 promotes angiogenesis by modulating endothelial TGFβ signalling. *Nature* 499: 306-311.

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Neuron-derived Factors in Retinal Vascular Disease

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Retinal vasculopathies such as diabetic retinopathy (DR), retinopathy of prematurity (ROP), and age-related macular degeneration (AMD) are the most common causes of vision loss in the industrialized world. Recent studies on DR, ROP and AMD have revealed the paramount role for neurons and neuronal guidance cues in disease progression. During retinal embryogenesis, coordinated interplay between neurons, blood vessels and immune cells is critical for proper retinal development. Although neurovascular and neuroimmune crosstalk shapes vascular development in the retina, it has received limited attention in disease etiology. A better understanding of this crosstalk may provide novel drugable targets to counter vasodegenerative and vasoproliferative eye disease. We will discuss new evidence suggesting that neuronal metabolism and guidance cues secreted by neurons such as retinal ganglion cells and photoreceptors have an inherent ability to influence vascular and immune responses in retinal disease.

VEGF and Myeloid Cells in the Context of ROP

ANTONIA JOUSSEN

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We discuss the role of VEGF in oxygen induced retinopathy in the context of myeloid derived cells.

The discovery of anti-VEGF-directed therapies has been a milestone in the treatment of retinal vascular diseases such as Retinopathy of Prematurity (ROP).

Previous publications have shown that depletion of monocyte lineage cells expressing VEGF leads to the suppression of pathological retinal neovascularization in the ROP mouse model.

Ruhrberg und Coworkers used 2 mouse models of ONV, choroidal neovascularization and oxygen-induced retinopathy, to show that Vegfa is highly expressed by several cell types, but not myeloid cells during ONV. In agreement, the conditional inactivation of Vegfa, Hif1a, or Epas1 in recruited and resident myeloid cells that accumulated at sites of neovascularization did not significantly reduce choroidal neovascularization or oxygen-induced retinopathy (Liyanage et al. 2016).

In this study we demonstrate that myeloid derived cells do not contribute to retinal neovascularization through VEGF-A expression.

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Generating VEGF^{fl/fl} LysMCre^{+/-} mice allowed a myeloid cell-specific VEGF-A knockout in macrophages, microglia and granulocytes. In the oxygen-induced retinopathy (OIR) mouse model, the knockout did not cause alterations in the retinal morphology. There was no reduced relative avascular area and no reduced relative VEGF-A mRNA levels in the retina of the knockout mice after oxygen treatment compared to the control animals. Flow cytometric analysis of the retina revealed an increase in numbers of macrophages and granulocytes on P17 but not on P14 after oxygen treatment whereas there was an increase in microglia numbers both on P14 and P17 after oxygen treatment. Interestingly, there was no reduction in relative avascular area nor neovascular tufts after depletion of circulating macrophages through clodronate liposomes. Müller cell activation was evident after oxygen treatment, independent of VEGF expression in myeloid cells or depletion of circulating macrophages by clodronate. Based on our findings we hypothesize that both circulating and residential myeloid derived cells do not contribute to pathological neovascularization by VEGF-A expression in response to hypoxia, but rather activated residential microglia cells trigger Müller cell activation and VEGF-A expression.

Lipoprotein-associated Phospholipase A₂ (Lp-PLA₂) Is Associated with Breakdown of the Blood Retinal Barrier through a VEGF-independent Pathway

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Breakdown of the blood retinal barrier (BRB) is central to inner and outer retinal edema, such as occurs in diabetic macular edema (DME) and neovascular AMD (nvAMD). In this presentation, the role of the lipoprotein-associated phospholipase A₂ (Lp-PLA₂) has been evaluated in vascular leakage. This enzyme hydrolyses oxidised low-density lipoproteins into pro-inflammatory products such as lysophosphatidylcholine (LPC) which can have detrimental effects on vascular function. As a specific inhibitor of Lp-PLA₂, darapladib has been shown to be protective against atherogenesis and vascular leakage in diabetic and hypercholesterolemic animal models.

We investigated if Lp-PLA₂ and its major enzymatic product LPC are involved in blood retinal barrier (BRB) damage. We assessed BRB protection in diabetic rats through use of species-specific analogues of darapladib. Systemic Lp-PLA₂ inhibition using SB-435495 at 10 mg/kg (i.p.) effectively suppressed BRB breakdown in streptozotocin-

diabetic Brown Norway rats. Since neutralization of VEGF is the current gold-standard treatment for DME and nvAMD, we assessed sub-optimal systemic treatment of an Lp-PLA₂ inhibitor alongside a sub-optimal intravitreal injection with a rat-specific VEGF antibody. The inhibitory effect of Lp-PLA₂ inhibition was comparable to intravitreal VEGF neutralisation and the protection against BRB dysfunction was additive when both targets were inhibited simultaneously. Mechanistic studies in primary brain and retinal microvascular endothelial cells as well as occluded rat pial microvessels, showed that luminal but not abluminal LPC potently induced permeability, and that this required signalling by the VEGF receptor 2 (VEGFR2) independent of VEGF.

This study has identified Lp-PLA₂ as a valid VEGF-independent therapeutic target in BRB dysfunction but also indicated that Lp-PLA₂ inhibition could be combined with that of VEGF. This opens the possibility of combination therapy for retinal edema with the benefits of use of lower drug dosages and lower potential toxicity.

Potential Androgen Receptor-mediated Gene Regulatory Pathways in a Mouse Model of Laser-induced Choroidal Neovascularization

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Choroidal neovascularization (CNV) is a prototypic symptom of exudative age-related macular degeneration (AMD) and its related diseases, which will often threaten central vision. Elucidation of molecular mechanisms underlying CNV will be the first step toward developing therapeutic options for the diseases. Here we performed expression microarray analysis for the posterior pole region of chorioretinal tissues derived from a mouse model of laser-induced CNV. In the model, a laser-induced thermal insult will disrupt Bruch's membrane, which is a barrier between the fenestrated choroid capillaries and retinal pigment epithelium, leading to the development of newly formed leaky choroidal blood vessels into the subretinal space. Laser treatment was performed on the posterior pole region of right eyes of mice with 4 shots each and total RNA was isolated from the posterior pole region of chorioretinal tissues from both treated right and control left eyes 3 days after the event. Comparing

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gene expression levels in laser-treated tissues versus control tissues, several hundred genes were commonly upregulated in a couple of subjects. Bioinformatical analysis reveals that multiple signaling pathways often associated with AMD were substantially activated in the current models, including such as complement pathways, inflammatory responses, proteases and protease inhibitors, chemokines and growth factors, calcium binding proteins, cell adhesion molecules, and lipoproteins. Notably, androgen receptor (AR) is one of the upregulated genes in laser-treated tissues. Public AR occupancy database analyzed by chromatin immunoprecipitation sequencing shows that more than half of the laser-induced genes could be potential AR targets. Because laser treatment is a stressful event for chorioretinal tissues, androgen/AR-mediated gene regulation will potentially contribute to the pathophysiology of CNV model.

RPE6 - The Next Best thing: Making sense of the bestrophinopathies

Insights into Autosomal Recessive Bestrophinopathy from a "Disease in a Dish" Model

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Autosomal recessive bestrophinopathy (ARB) is an inherited form of macular degeneration due to mutations in the gene *BEST1*. *BEST1* encodes bestrophin 1 (Best1) a Ca^{2+} activated anion channel and regulator of intracellular Ca^{2+} signaling. To better understand ARB we have recruited a number of subjects to a clinical study (NCT02162953, Stem Cell Models of Best Disease and Other Retinal Degenerative Diseases). Entry into the study requires that subjects with ARB, their parents, and siblings, provide skin samples for reprogramming to induced pluripotent stem cells (iPSCs). Each subject's iPSCs are differentiated into retinal pigment epithelial (RPE) cells. Successful differentiation of iPSCs to RPE was determined by a rigorous set of morphologic and molecular criteria. Here we report on the results from a 13 year old female Caucasian subject with ARB due to compound heterozygous mutations (R141H & I366fsX384). Western blot of iPSC-RPE lysates for Best1 from the subject and her father (Best1^{+R141H}) were compared to lysates from an unrelated *BEST1*^{+/+} 21yr old female Caucasian control. Best1 expression was reduced, relative to the control in lysates from the father and absent from the subject. All anti-Best1 antibodies only recognize epitopes in the region deleted from the I366fsX384.

However, we have previously shown that this mutant is stable, active as a Ca^{2+} activated anion channel, and that it oligomerizes with wild type Best1 when heterologously expressed in HEK293 and MDCK cells. We used this knowledge to probe for the Best1^{I366fsX384} mutant in RPE lysates from the subject and the Mother (Best1^{+/I366fsX384}) of the subject. Following radiolabeling of iPSC-RPE cells with [³⁵S] cysteine & methionine Best1 was immunoprecipitated from lysates. No Best1 was detected in lysates from the ARB subject. We next performed phagocytosis assays using FITC-labelled bovine photoreceptor outer segments (OS). Both binding and internalization of OS were significantly impaired in iPSC-RPE from the subject versus iPSC-RPE from her father or an unrelated control. Based on these data, we conclude that ARB in this patient results at least in part from absent or severely diminished expression of Best1 leading to a defect in OS phagocytosis.

Unexpected Roles of Best1 in the Brain: The Channel-mediated Release of Gliotransmitters, Glutamate and GABA, and its Role in Excitation/Inhibition Balance

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Bestrophin (Best) is the gene responsible for a dominantly inherited, juvenile-onset form of macular degeneration called Best vitelliform macular dystrophy. It has been shown to encode a functional Ca^{2+} -activated anion channel (CAAC), activated directly by submicromolar intracellular Ca^{2+} concentration in nonneuronal tissues and peripheral neurons. Several studies have suggested the function of Best in various physiological processes such as normal vision and cell volume regulation. In the brain, Best1 was found to be majorly expressed in astrocytes, especially at the microdomains near synaptic junctions. As an ion channel, Best1 shows a relatively high permeability to large anions, including glutamate and isethionate, along with chloride ion. It has been demonstrated that astrocytes release glutamate via Best1, which then targets and activates synaptically localized, GluN2A-containing NMDA receptors in hippocampal CA1 pyramidal neurons to modulate hippocampal synaptic plasticity. In addition to glutamate, it has been demonstrated that Best1 has a significant permeability to gamma aminobutyric acid (GABA) and mediates tonic release of GABA from astrocytes in cerebellum to strongly inhibit neuronal excitability. The possibility of GABA release from astrocytes is a critical finding because tonic inhibition by

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tonic release of GABA has been widely observed in many brain regions under physiological conditions as well as under various pathological conditions. It was reported that tonic inhibition is highly correlated with the presence of cytosolic GABA in cerebellum and hippocampus and other brain regions: tonic inhibition current was high and immunostaining for GABA in astrocytes was high in cerebellum, whereas tonic inhibition current was low and immunostaining for GABA in astrocyte was low in CA1 hippocampus. The astrocytic GABA was shown to be produced via putrescine degradation pathway with monoamine oxidase B (MAOB) as a key biosynthetic enzyme. Thus, under physiological condition, the astrocytic GABA is synthesized by MAOB and released by Best1 when there is sufficient level of cytosolic GABA.

Best1's role in GABA release appears to carry an important weight in neurodegenerative diseases. Future investigations in various neurodegenerative diseases such as Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis as well as retinal degeneration will give us deeper insights on how Best1 by releasing GABA is contributing to each disease.

Trafficking Defects of Mutant Bestrophins: Implications for Calcium Channel Activity

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Several lines of evidence reported that mutant bestrophins fail to traffic to the plasma membrane. Together with its function as anion channel, bestrophin-1 is known as regulator of intracellular calcium signaling by direct interaction with β -subunits of voltage-dependent L-type Ca^{2+} channels. Thus we studied the influence of mutant bestrophins on the activity and surface expression of $\text{Ca}_v1.3$ channels, the predominant subtype of L-type channel in the retinal pigment epithelium.

Using in CHO cells heterologously expressed proteins, the interaction of mutant bestrophins (T6P, F80L, R218C, F305S) with L-type channel β 4-subunits was studied by immunoprecipitation. The surface expression of bestrophin and $\text{Ca}_v1.3$ pore-forming Ca^{2+} channel subunit was studied by co-localization analysis in confocal microscopy. L-type Ca^{2+} channel activity was measured by means of patch-clamp technique.

All four mutant bestrophins showed interaction with β 4-subunits, although the mutant forms T6P, F80L and F305S showed decreased co-precipitation efficiency. Using α 5 β 1 integrin as marker the surface expression of $\text{Ca}_v1.3$ subunits

in the present of mutant bestrophins was measured. The mutant forms T6P, F80L and F305S reduced the $\text{Ca}_v1.3$ surface expression significantly. In concordance to that the maximal density of Ba^{2+} currents through $\text{Ca}_v1.3$ channels was reduced. In addition, the mutant bestrophins F80L and F305S shifted the voltage-dependence towards more positive values.

In summary mutant bestrophins interact with β 4-subunits of L-type Ca^{2+} channels. Since β -subunits of Ca^{2+} channels are regulators $\text{Ca}_v1.3$ surface expression the mutant bestrophins lead to reduced $\text{Ca}_v1.3$ surface expression and, thus, to reduced L-type channel contribution to membrane conductance. Thus mutant bestrophins likely disturb intracellular Ca^{2+} signaling by L-type channels in the RPE.

The First Best Thing: Insights from a Naturally Occurring Animal Model of BEST1-associated Maculopathies

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Canine bestrophinopathy (cBest) is an important translational model for *BEST1*-associated maculopathies in man that recapitulates the broad spectrum of clinical and molecular disease aspects observed in patients. The spontaneously occurring canine *BEST1*-R25X, -P463fs, and -R25X/P463fs-compound mutant models were used to gain new insights into the disease mechanism and for development and testing of AAV-mediated therapeutic strategy.

cBest-affected dogs (age range 2mo - 6yr) were monitored clinically and imaged serially using cSLO/SD-OCT. All examined dogs exhibiting bilateral focal or multifocal lesions were injected unilaterally with rAAV2 expressing either canine or human *BEST1* gene, while the contralateral eyes were used as treatment control. Retinal structure was studied in cryosections by H&E and immunohistochemistry, and assessed using transmitted light microscopy, epifluorescence and confocal microscopy. All *in vivo* and *ex vivo* analyses were carried out in comparison to the age-

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matched wild-type control eyes.

Based on *in vivo* imaging, most early lesions within the treated regions disappeared as early as 8 weeks *post* subretinal treatment while all resolved within 3 months after gene therapy, and the treated areas remained asymptomatic thereafter. A comparable efficacy was noted with canine and human cDNAs, and both, the *in vivo* imaging and immunohistochemical studies, revealed no apparent adverse effects in RPE or retina secondary to the *BEST1* gene augmentation therapy. The untreated regions of the treated eyes or the contralateral control eyes remained unchanged or developed new lesions. Immunohistochemical analysis of untreated retinæ exposed compromised interphotoreceptor matrix and retraction of RPE apical microvilli and the associated cone matrix sheaths. Histological evaluation revealed areas of subtle dissociation of the neural retina from RPE with accumulation of subretinal debris.

Compromised interphotoreceptor matrix and retraction of cone-associated RPE apical microvilli promote loss of retinal adhesiveness and lesion formation in bestrophinopathies. AAV2-mediated *BEST1* gene augmentation therapy shows both a preventive as well as reversal potential in all cBest models tested, and carries a large translational promise as a first specific-treatment for human bestrophinopathies.

The Effect of Premature Stop Mutation in Bestrophin-1 on Calcium Signaling in Retinal Pigment Epithelium

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Mutations in *BEST1* gene cause different types of inherited retinal degenerative diseases, including autosomal recessive bestrophinopathy (ARB). This retinal dystrophy is characterized by loss of central vision, accumulation of subretinal deposits and diminished electrooculogram (EOG). Pathogenesis of ARB is poorly understood and, therefore, patients with ARB do not have effective treatments available. ARB is caused by mutations in *BEST1* gene encoding for Bestrophin-1 (Best1), a membrane protein localized in the retinal pigment epithelium (RPE). Best1 functions as an

anion channel which can co-regulate voltage gated Ca^{2+} channels and intracellular Ca^{2+} homeostasis. However, it is not clear how these functions are disrupted in different disease-causing *BEST1* mutations.

We investigated how RPE Ca^{2+} signaling is affected by a disease-causing nonsense mutation p.R200X *BEST1*. We produced induced pluripotent stem cells (iPSCs) from an ARB patient with p.R200X *BEST1* mutation by reprogramming with episomal vectors, and differentiated these iPSCs into RPE by spontaneous differentiation method. As full length Best1 has been reported to regulate the L-type voltage dependent Ca^{2+} channels (Cav1.3) and affect the ATP-induced changes in intracellular Ca^{2+} levels we investigated the effect of p.R200X *BEST1* mutation on Cav1.3 channel functionality and ATP-induced Ca^{2+} transients. We recorded Ba^{2+} currents through voltage dependent Ca^{2+} channels by patch clamp technique from both ARB iPSC-RPE and human fetal iPSC-RPE, and followed the intracellular free Ca^{2+} concentration by Ca^{2+} imaging.

Our iPSC-RPE formed a pigmented monolayer with classic RPE cobblestone morphology. The p.R200X *BEST1* mutation was confirmed in fibroblast, iPSC and RPE cells. ARB iPSC-RPE expressed classic RPE markers at the protein and RNA level. Full length Best1 protein, however, was undetectable. Ionic currents through Cav1.3 channels were diminished in ARB iPSC-RPE compared to fetal iPSC-RPE, and ATP stimulation induced faster rising and longer lasting Ca^{2+} transients in the ARB iPSC-RPE. Taken together, our results confirm that the ARB p.R200X mutation results in the lack of full length Best1 protein in RPE cells. This disrupts the interaction between Best1 and L-type Ca^{2+} channels and reduces the currents through Cav1.3 channels, possibly by decreasing the number of these channels in the cell membrane. Additionally, the mutation influences the intracellular Ca^{2+} signaling in RPE.

RPE7 - Metabolic Coupling in the outer retina

Metabolic Fate of the RPE's Daily Meal of Photoreceptor Outer Segment Lipids

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An often overlooked source of metabolic substrates is the daily meal of photoreceptor outer segments (OS) taken up by the RPE. Photoreceptors shed approximately

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10% of their OSs shortly after light onset; these are then phagocytosed and degraded by the overlying RPE. In these studies, we explored the hypothesis that the RPE utilizes phospholipids from ingested OS for fatty acid oxidation and ketogenesis, providing the neural retina with metabolic intermediates. HMGCS2, the enzyme catalyzing the committed step in ketogenesis was detected in human and mouse RPE where it co-localized with other mitochondrial specific proteins. Ingestion of OSs in the presence of glucose stimulated ketogenesis resulting in the preferential release of β -HB into the apical medium in both hRPE and to a lesser extent in ARPE19, with no detectable β -HB in the basal media. The extent of β -HB released was dose dependent, with 0.3 \pm 0.028 nmoles of β -HB released with 25 μ M OS and 1.65 \pm 0.148 nmoles released when cells were fed 250 μ M OS. Decreased levels of β -HB were released when hRPE and ARPE-19 were challenged with oxidized OS, from 3.16 \pm 0.29 nmoles with OS to 0.42 \pm 0.54 nmoles (oxOS). β -HB was not generated when the hRPE or ARPE-19 were challenged with latex beads. *In situ* studies documented that the release of β -HB by mouse RPE followed circadian pattern, with secreted β -HB levels declining within 2 hours after light onset -characterized by RPE phagocytic burst. We also found in the *Mreg*^{-/-} mouse model of defective phagocytosis, β -HB release did not follow the same circadian pattern often showing highest β -HB levels 3-4 hours after light onset. Collectively, these results suggest the RPE not only recycles vitamin A and DHA back to the outer retina but also substrates generated from degradation and oxidation of OS including β -HB. Future studies will address the role of fatty acid oxidation in the RPE in supporting health and function of the RPE and photoreceptor cells.

Reverse TCA Cycle Flux through Isocitrate Dehydrogenases 2 Is Dominant in Retinal Pigment Epithelium and Confers Protection from Oxidative Stress

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The retinal pigment epithelium (RPE) is a monolayer of pigmented cells that requires an active metabolism to maintain outer retinal homeostasis and compensate for oxidative stress. Using ¹³C metabolic flux analysis in human RPE cells, we found that RPE has an exceptionally high capacity for reverse TCA cycle flux through isocitrate

dehydrogenase 2, a metabolic pathway also known as reductive carboxylation. The capacity for reductive carboxylation in RPE exceeds that of all other cells tested, including retina, neural tissue, and cancer cell lines. Loss of reductive carboxylation disrupts redox balance and increases RPE sensitivity to oxidative damage, suggesting that deficiencies of reductive carboxylation may contribute to RPE cell death. Supporting reductive carboxylation either by supplementation with an NAD⁺ precursor or by treatment with a PARP inhibitor protects reductive carboxylation and RPE viability from excessive oxidative stress. The ability of these treatments to rescue RPE could be the basis for an effective new strategy to treat retinal degenerative diseases caused by RPE dysfunction.

ATP6AP2/(pro) Renin Receptor Contributes to Glucose Metabolism via Stabilizing the Pyruvate Dehydrogenase E1 β Subunit

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Aerobic glucose metabolism is indispensable for metabolically active cells; however, the regulatory mechanism of efficient energy generation in the highly evolved mammalian retina remains incompletely understood. Here, we revealed an unsuspected role for (pro)renin receptor, also known as ATP6AP2, in energy metabolism. Immunoprecipitation and mass spectrometry analyses identified pyruvate dehydrogenase (PDH) complex as Atp6ap2-interacting proteins in the mouse retina. Yeast two-hybrid assays demonstrated direct molecular binding between ATP6AP2 and the PDH E1 β subunit (PDHB). Pdhb immunoreactivity co-localized with Atp6ap2 in multiple retinal layers including the retinal pigment epithelium (RPE). *ATP6AP2* knockdown in RPE cells reduced PDH activity, showing a predilection to anaerobic glycolysis. *ATP6AP2* protected PDHB from phosphorylation, thus controlling its protein stability. Downregulated PDH activity due to *ATP6AP2* knockdown inhibited glucose-stimulated oxidative stress in RPE cells. Our present data unraveled the novel function of ATP6AP2/(P)RR as a PDHB stabilizer, contributing to aerobic glucose metabolism together with oxidative stress.

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Metabolic Coupling between Müller Cells and Photoreceptors: The Consequences of Genetically Disrupting Energy Metabolism in Müller CellsWEIYONG SHEN¹, Jianhai Du², Michelle Yam¹, So-Ra Lee¹, Ling Zhu¹, James B. Hurley², Mark C. Gillies¹¹The University of Sydney, Save Sight Institute, Sydney, Australia, ²University of Washington, Department of Biochemistry and Ophthalmology, Seattle, United States

Photoreceptors are vulnerable to metabolic derangement since they are amongst the most highly metabolically active cells in the body. We have generated inducible Müller cell-Cre mice to study the metabolic coupling between Müller cells and photoreceptors. Selective Müller cell ablation through expression of an attenuated form of diphtheria toxin led to photoreceptor degeneration. *In vivo* labelling of retinas with ¹³C-glucose found that photoreceptor degeneration was associated with reduced intermediates for glycolysis and the tricarboxylic acid cycle (TCA) cycle. Labelling retinas with ¹³C-serine indicates that selective ablation of Müller cells resulted in disruption of serine/glycine and one carbon unit metabolism. We also studied the impacts on photoreceptor health of selectively knocking down specific metabolic genes in Müller cells. We crossed Müller cell-Cre mice with transgenic mice carrying floxed insulin receptor (IR), hexokinase-2 (Hk-2) or pyruvate dehydrogenase (PDH). Knocking down IR in Müller cells resulted in reduced metabolites for glycolysis and the TCA cycle together with loss of photoreceptor outer segments but not loss of Müller cells. Significant loss of photoreceptor outer segments was also observed after knocking down Hk-2 but less obvious after knocking down PDH in Müller cells. As recent studies indicate that methylene blue (MB) and metformin (MET) are effective in improving mitochondrial function and regulating insulin metabolism, we have further studied the effects of MB and MET on photoreceptor degeneration caused by induced Müller cell ablation. Systemic administration of MB and MET, both alone and in combination, protected photoreceptors and increased expression of glutamine synthetase and glucose transporter 1 in the retina. Further analysis indicates that MB and MET inhibited phosphorylation of p44/42, acetyl-CoA carboxylase and AMP-activated protein kinase- α along with reduced expression of heat shock protein 60. Taken together, our studies suggest that the metabolic support from Müller cells is important for photoreceptor survival and regulation of retinal energy metabolism may offer a novel approach for the treatment of photoreceptor degenerative diseases.

Retbindin, a Novel Retina-specific Protein with a Role in Metabolic HomeostasisMUAYYAD AL-UBAIDI¹, Sinha Tirthankar¹, Ayse Genc¹, J. Du², James Hurley³, Muna Naash¹¹University of Houston, Houston, United States, ²University of Washington, Ophthalmology, Seattle, United States, ³University of Washington, Biochemistry, Seattle, United States

Purpose: Retbindin (Retb), a retina-specific flavin-binding protein, localizes at the outer segment (OS) and retinal pigment epithelium (RPE) interface. Since flavins are cofactors for metabolic enzymes, we hypothesize that Retb is involved in binding/transporting flavins and its ablation will lead to metabolic changes that are prelude to retinal degeneration.

Methods: Developmental structural, functional and biochemical analyses were performed on a Retb knockout mouse (Retb^{-/-}) in which the coding sequence was replaced with eGFP. HPLC was used to assess flavin levels and LC-MS was used to quantify metabolites. Data was analyzed using MetaboAnalyst 3.0.

Results: e-GFP expression was restricted to rod photoreceptors in Retb^{-/-} retinas, consistent with its distribution in WT mice. Levels of flavins were significantly reduced in Retb^{-/-} retinas and were associated with age- and dose-dependent structural and functional decline. Although Retb expression is limited to rods, its absence led to combined rod and cone reduced electroretinographic responses and photoreceptor cell loss. Absence of Retb also led to reduced ability of the retina to bind C¹⁴-labeled riboflavin. Finally, measurements of metabolites revealed that elimination of Retb led to increased utilization of glycolysis associated with higher than normal levels of glucose. To determine the role of metabolic changes in retinal diseases that are not associated with Retb ablation, Retb levels were measured in retinas of mouse models of human retinal disorders and found to be significantly higher.

Conclusion: Our results provide strong evidence to the important role Retb plays in the metabolic homeostasis of the retina. Further investigations into its role in the health of the retina will determine the exact metabolic changes that occur in the Retb^{-/-} retina and lead to photoreceptor cell death. Moreover, upregulation of Retb in several models of retinal diseases suggests a protective role for Retb and points to the potential of Retb becoming a clinically relevant candidate for the treatment of a variety of inherited ocular diseases.

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RPE8 - Exploring the intersection between inflammation and lipid metabolism in age-related macular degeneration (AMD)

Impaired Cholesterol Efflux in Macrophages Promotes AMD

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AMD is the leading cause of blindness in people over 50 in the developed world. A cardinal feature of AMD is the deposition of lipid-rich drusen underneath the retinal pigment epithelium. Recent studies have demonstrated a significant association between abnormalities in cholesterol homeostasis and the development and progression of AMD. Macrophages play a dominant role in cholesterol efflux in tissues such as the eye. The effect of aging on macrophage function and reverse cholesterol transport will be discussed. In addition, the implications of impaired cholesterol efflux on disease pathogenesis will also be reviewed.

Additive Effects of Advanced Age, High Fat Diet and Complement Factor H in Dry AMD

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The pathogenesis of age-related macular degeneration (AMD) is complex and thought to involve cholesterol/lipid removal proteins, diet and the immune system, which are dependent on advanced age and genetic factors including complement proteins. Specifically, the Y402H polymorphism in the complement factor H (*CFH* gene, FH protein) is associated with increased risk for AMD. To test the hypothesis that expression of the risk-associated H402 variant of *CFH* causes an AMD-like phenotype *in vivo* we generated transgenic mouse lines expressing full-length human normal Y402 or risk-associated H402 variants of the FH protein on a mouse *Cfh* knockout background (*CFH-Y:Cfh*^{-/-} and *CFH-H:Cfh*^{-/-}, respectively). Mice were aged over 90 weeks and fed a high fat, cholesterol-enriched (HFC) diet. As we have demonstrated previously in *Cfh*^{-/-} and *Cfh*^{+/-} mice on a HFC diet (Toomey *et al.* PNAS 2015), decreased levels of FH led to increased sub-retinal pigmented epithelium (sub-RPE) deposit formation, specifically basal laminar deposits. Interestingly, despite sub-RPE deposit formation occurring in both *CFH-Y:Cfh*^{-/-} and *CFH-H:Cfh*^{-/-} mice, only old *CFH-H:Cfh*^{-/-} mice fed

a HFC diet developed vision loss and increased RPE cell multinucleation compared to their age-matched, normal diet controls. Taken together, our data suggests that the Y402 variant is protective against AMD-like pathology, and our animal models represent valuable tools to interrogate the molecular mechanisms underlying the association of the H402 variant with AMD risk.

Liver x Receptors (LXRs) as Regulators of Lipid Metabolism and Inflammation in Dry AMD

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Purpose: The early dry sub-type of AMD is characterized by accumulation of cholesterol-rich deposits below the retinal pigment epithelium (sub-RPE). Several genes in the high-density lipoprotein cholesterol pathway have been found to be associated with AMD risk, including the ATP binding cassette transporter 1 (ABCA1). Liver X receptors (LXRs) are nuclear receptors that act as cholesterol sensors regulating not only lipid-metabolism and genes associated with reverse cholesterol transport including ABCA1, but also inflammation. Herein we investigated the impact of LXR activation in AMD-vulnerable cells, and examined the ocular phenotype of aged LXR knockout (^{-/-}) mice.

Methods: LXR expression, activity, and expression of LXR target genes were determined in primary human RPE and macaque derived RF/6A endothelial cells treated with synthetic agonists, antagonists and knockdown of the receptor. Visual function and *in vivo* morphology of aged *LXR*^{-/-} mice were evaluated with electroretinography (ERGs) and optical coherence tomography (OCT). Post-mortem morphological evaluation of retinal sections was performed via electron and light microscopy, and quick-freeze deep etch. The presence of lipids and immune cells were evaluated with the oil red o stain or probed with antibodies to microglial and macrophage markers, respectively.

Results: LXR activation regulates expression of several lipid metabolism and inflammatory genes. Both scotopic and photopic ERG recordings in aged *LXR*^{-/-} (ab>a>b) demonstrated decreased amplitudes, compared to age-matched wildtype mice. Morphological evaluation,

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visualized by OCT and in post-mortem retinal sections, showed thick layers of lipid-rich sub-RPE deposits in *LXR^{-/-}* mice and a. These deposits contain lipoprotein particles. *LXRb^{-/-}* also develop deposits but were relatively thinner and fewer than those observed in the other cohorts of mice.

Conclusions: These findings support the physiological importance of LXR in lipid metabolism and inflammation in the RPE and choroidal endothelial cells and indicate that the LXR signaling pathway may be a potential therapeutic target against cholesterol-rich deposit formation. We also find the absence of LXRs in mice results in phenotypic features of dry AMD. These mice may serve as a model to further understand the pathobiology of lipid-rich sub-RPE deposit formation or as a platform to test therapies targeting deposits.

Functional Implications of Age Associated Alterations in Macrophage Lipids

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Macrophages are critical cells in the innate immune system that participate in the defense against infections and diseases. There is now substantial evidence that associates aging with impaired macrophage functions. Macrophage dysfunction plays a pivotal role during pathologic neovascularization in various diseases of aging including cancers, atherosclerosis and blinding eye disease. In the eye, choroidal neovascularization (CNV) causes blindness in patients with age-related macular degeneration (AMD). Numerous studies have demonstrated the essential role of macrophages in regulating CNV in AMD. Furthermore, it has become increasingly evident that the polarization state of the activated macrophage determines the pathophysiological fate of the neovascular phenotype. However, the programmatic molecular signals that guide macrophage polarization, particularly during aging eye diseases, remain unclear. Although many studies have focused on the functional implication of macrophage aging, little is known about the molecular and cellular basis of this dysfunction. To gain insights into mechanisms underlying the macrophage aging process, we took an unbiased approach and analyzed cellular pathways that are differentially regulated in young vs. old macrophages. Our preliminary findings suggest that cholesterol metabolism is the most relevant cellular process altered during macrophage aging. This is of interest as the interaction between macrophages and sub-RPE lipid rich deposits such

as drusen is thought to be important in disease progression. Next, we investigated the expression profile of 113 genes involved in cholesterol uptake, efflux, biosynthesis, storage and degradation in peritoneal macrophages isolated from young and old mice. Our preliminary data revealed that the expression profile of 39% of the genes studied was significantly different (P<0.05) when comparing old vs young macrophages and differentially expressed genes were involved in different aspects of the cholesterol metabolism pathway. Further exploration of the modulation of cellular cholesterol homeostasis and insights into lipid metabolism in senescent macrophages may enable us to develop potential major strategies in order to prevent macrophage aging and innate immunosenescence.

Cholesterol Crystals Induce Expression of Inflammatory Cytokines by Cells Vulnerable in AMD

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Lipids play a central role in AMD with cholesterol constituting a major portion of the drusen deposits and cholesterol crystals being present in 5-10% of patients with neovascular AMD. Previous studies have shown that esterified cholesterol can induce under certain conditions the inflammasome in immune cells. In contrast to immune cells, cholesterol crystals or oxidized LDL did not induce the nucleotide-binding domain leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) inflammasome since there was no significant mature IL-1 β upregulation, but did increase pro-IL-1 β expression as well as IL-6 and IL-8 expression in ARPE-19 cells by activating the NF- κ B pathway. The inflammatory effects of cholesterol crystals and/or oxidized LDL were reversed by increasing dosages of Atorvastatin. Our study shows that RPE cells can be vulnerable to cholesterol crystals and/or oxidized LDL, by activating the NF- κ B pathway and inducing inflammatory markers, but not overtly activating the NLRP3 dependent inflammasome. Treatment with high dose statins may have beneficial effects beyond their lipid lowering effects.

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RPE9 - Inflammasomes in the RPE

The NLRP3 Inflammasome and RPE Homeostasis

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The NLRP3 inflammasome has come to the fore in recent years as being involved, in some capacity, in the pathophysiology of both dry and wet age related macular degeneration (AMD). The distinct role of NLRP3 in AMD pathology is however still far from clear and simply detecting its presence in AMD donor tissue or indeed in "stressed" RPE cells does not imply a causative role in pathology. Without a doubt, AMD is fundamentally a disease of aberrant clearance mechanisms, highlighted by the presence of drusen between the RPE and choroid, the hallmark pathological feature of the condition. This aberrant clearance is primarily associated with the retinal pigment epithelium (RPE), and inflammatory processes are likely intimately intertwined. Here, we wish to shed light on the role of the NLRP3 inflammasome as it pertains to the maintenance of homeostasis in the retina/RPE as this, while currently unknown, will be critical to our understanding of the disease process. We have recently observed inflammasome components "cycling" in a diurnal fashion in the RPE *in vivo*. In addition, levels of the inflammasome component Interleukin-18 are expressed in the RPE constitutively, yet the role of this inflammatory cytokine in the RPE is not fully understood.

In gaining an understanding of the role of the NLRP3 inflammasome in maintaining homeostasis in the RPE we hope to be in a position to make profound conclusions on its role in the pathophysiology of AMD in general which may lead to more robust therapeutics for patients in the future.

Inflammasome Activation: Evidence in an Animal Model of AMD

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Age-related macular degeneration (AMD) is a neurodegenerative disease of the eye and the most common cause of irreversible vision loss in the elderly in industrialized countries. AMD is a multifactorial disease influenced by both genetic and environmental risk factors. Progression of AMD is characterized by an increase in the

number and size of drusen, extracellular deposits, which accumulate between the retinal pigment epithelium (RPE) and Bruch's membrane (BM) in outer retina. The major pathways associated with its pathogenesis include oxidative stress and inflammation in the early stages of AMD. Little is known about the interactions among these mechanisms that drive the transition from early to late stages of AMD including geographic atrophy (GA) or choroidal neovascularization (CNV). As part of the innate immune system, inflammasome activation has been identified in RPE and proposed to be a causal factor for RPE dysfunction and degeneration. Here, we will review our work on inflammasome activation, then discuss AMD related factors that have been shown to activate the inflammasome in animal models. We will then discuss several novel mechanisms and strategies for regulating the inflammasome activity.

Inflammasomes in the RPE

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Atrophic age-related macular degeneration (AMD) is an irreversible blinding disease characterized by retinal pigmented epithelium (RPE) degeneration. Currently there is no therapy available for this devastating disease, owing to its unclear etiology. Recently our studies provided novel mechanistic insights into the molecular pathogenesis of the disease. We discovered that RPE of atrophic AMD eyes have reduced DICER1 levels and that deficiency of this RNase causes a pathological increase in *Alu* RNA transcripts in the RPE cells. Under healthy conditions, DICER1-mediated processing keeps the *Alu* RNAs levels in check. Surprisingly, the build-up of these endogenous noncoding RNA transcripts lead to RPE cell death via activation of NLRP3 inflammasome and ensuing IL-18 and MYD88-dependent signaling. Interestingly the *Alu* RNA driven RPE degeneration was independent of TLRs and other known intracellular RNA sensors. Overall, these studies have provided an exciting and unexpected convergence of Dicer1, dysregulated *Alu* RNA levels and innate immune receptor NLRP3 in the pathogenesis of AMD. Additional studies aimed at deciphering the mechanism of *Alu* RNA driven NLRP3 inflammasome activation are ongoing.

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Mitochondrial Damage and Inflammasome Signaling in the RPE

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Mitochondria are vital organelles for metabolically highly active retinal pigment epithelium (RPE) cells. Due to its susceptibility to oxidative damage and limited excision repair capacity, the small mitochondrial DNA (mtDNA) is vulnerable to severe damages and able to prime the inflammasome signaling in RPE cells. Inflammasomes are intracellular protein complexes playing a major role in the induction of inflammation. NLRP3 is a pattern recognition receptor capable of forming an inflammasome after recognizing its activating signals. Decayed mtDNA causes dysfunctionality of mitochondria and increases the production of endogenous reactive oxygen species (ROS). Excessive oxidative stress is harmful for the RPE that contains high levels of polyunsaturated fatty acids. Oxidative stress and its effects e.g. on lipids deteriorate the homeostasis of RPE, which subsequently prevents the removal of aged mitochondria by inhibiting autophagy. Increased oxidative stress results direct and indirect inflammasome activation in RPE cells. Also autophagy decline triggers inflammasome signaling when accompanied with proteasome inhibition or at least priming signal through a cytokine receptor. Our recent results show that rotenone, an isoflavone that interferes with complex I in the electron transport chain of mitochondria, does not prime nor activate inflammasome signaling in ARPE-19 cells when administered before or after bafilomycin A that blocks autophagy. This is in line with data obtained with mouse bone marrow-derived macrophages showing that rotenone provides priming signal to NLRP3 inflammasome only when ATP serves as an activator. This is interesting and deserves further investigation but collectively we can already say that mitochondria contribute in many ways to the inflammasome signaling in RPE cells.

Gene Therapy with the Caspase Activation and Recruitment Domain (CARD) Slows the Retina Degeneration of the Sod2 Knock-out Mouse Model of Geographic Atrophy

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Age-related macular degeneration is the main cause of visual impairment among the elderly in developed countries. Geographic atrophy is the advanced form of the disease in which the retinal pigmented epithelium (RPE) has been severely damaged by oxidative stress and inflammation. Studies indicate that the activation of the NLRP3 inflammasome signaling pathway is involved in the parainflammation associated with AMD. Herein, we tested the efficacy of an inhibitor of caspase-1, a critical enzyme required in the inflammasome signaling pathway, in controlling the retinal degeneration observed in both the sodium iodate model of RPE damage and the *Sod2* knock-out model geographic atrophy. Acute RPE-oxidative injury was induced in C57Bl/6J mice one month after intravitreal delivery of AAV2QUAD-T+V viral vector delivering either GFP or a secreted GFP linked to a Tat-fused CARD via a furin cleavage site (known as sGFP-TatCARD). Mice were evaluated by electroretinogram (ERG) at 1 and 4 weeks post sodium iodate injection. Animals were euthanized and flatmounts prepared after the last ERG evaluation. Transgenic C57Bl/6J mice harboring an RPE specific deletion of *Sod2* were injected intravitreally with AAV2QUAD-T+V delivering either GFP or sGFP-TatCARD. Two cohorts of animals were injected at either 2 or 4 months of age and were followed by ERG and spectral domain optical coherence tomography (SD-OCT). Eyes treated with sGFP-TatCARD vector had a recovery of their a-, b- and c-wave amplitudes one month after sodium iodate, which was absent among GFP treated eyes. Flatmounts in these mice showed a partial protection of the RPE layer when compared to eyes treated with the GFP control vector. To test treatment of existing disease, we injected the sGFP-TatCARD vector in the vitreous of *Sod2* knock-out mice at 4 months of age. The treated eyes showed a significant reduction in the rate of ERG decline compared to eyes treated with GFP. These eyes also showed a thicker outer segments than eyes treated with GFP as measured by SD-OCT. To test prevention of disease, we treated *Sod2* KO mice at 2 month of age

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with either sGFP-TatCARD or GFP. We observed a greater protection of the c-wave amplitude in eyes treated with the TatCARD when compared to GFP treated eyes. Intravitreal delivery of AAV2QUAD-T+V/sGFP-TatCARD can protect the retina from both acute and chronic oxidative damage within the RPE. This protection was observed in the *Sod2* deletion model even after the onset of disease.

RPE11 - Aging RPE: Proteostasis mechanisms in health and diseases

Age-related Impaired Trafficking of the Major RPE Proteolysis Regulator Cystatin C

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Major functions of the retinal pigment epithelium (RPE) involve specific proteolytic events, both intra- and extracellularly. The regulation of proteolysis therefore underpins RPE physiology and is implicated widely in pathophysiological processes associated with ageing and disease. One of the most potent regulators of proteolysis, the cysteine proteinase inhibitor cystatin C, is among the top 2% abundantly expressed genes by RPE. The basolateral secretion profile of cystatin C in RPE cells suggests a role in relation to maintaining the structure and function of Bruch's membrane/choroid. Variant B cystatin C is associated with increased risk of developing exudative age-related macular degeneration (AMD) and presents leader sequence-related altered intracellular trafficking, leading to reduced efficiency of processing through the secretory pathway.

Immunoreactivity of cystatin C decreases significantly with age in the posterior, but not peripheral region of the RPE. Furthermore, *in vitro* studies using physiologically relevant RPE monolayers indicate that the expression and secretion of cystatin C, but not that of cysteine proteinases cathepsin B and S, is significantly decreased in response to a common age-related stress, the accumulation of advanced glycation end-products (AGEs). This likely contributes to an altered extracellular proteolytic balance. An additional intracellular role is supported by data showing high aggregation propensity imparted by the leader sequence to the incompletely processed variant retained/delayed intracellularly.

Together the data point out to a role for the wild type cystatin C in regulating the proteolytic homeostasis in the retina/choroid, which declines with age and acts as a compounding factor to the deleterious effects of variant B cystatin C in homozygous carriers of this AMD-associated

variant.

Overall the findings suggest that the RPE has a significant control over extracellular proteolytic events, via the secretion of highly active proteases, and their inhibitors. Molecular stress associated with natural ageing can alter the protease/inhibitor balance in/around RPE, which alongside misfolding of soluble proteins have the potential to contribute to pathological features of AMD, such as breakage of blood-retina barrier, formation of toxic aggregates, and structural abnormalities.

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SQSTM1/p62 a Key Player in the Regulation of Proteostasis Defence during RPE Aging Process

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The pathogenesis of AMD involves impaired protein degradation in RPE cells. The ubiquitin-proteasome pathway and lysosomes including autophagy are the major proteolytic systems in eukaryotic cells. Recently, SQSTM1/p62 has been shown to be a shuttling protein between proteasomal and autophagy clearance. It also regulates antioxidant production via nuclear factor-erythroid 2-related factor-2 (Nrf2).

The RPE is exposed to constant oxidative stress that may lead to tissue damage during aging process. The defence response against oxidative stress involves antioxidant production and proteolysis of damaged proteins both regulated by SQSTM1/p62. The Nrf2 and peroxisome proliferator-activated receptor γ coactivator-1a (PGC-1a) are central transcription factors in the regulation of cellular detoxification. We investigated the role of Nrf2 and PGC-1a in RPE cell proteostasis by using double knockout mice. The Nrf2/PGC1 α ^{-/-} mice evoked RPE degeneration, accumulation of oxidative stress marker 4-HNE, proteasomal ubiquitin and autophagy markers SQSTM1/p62, ELAVL1/HuR, Beclin-1 and MAP1LC3A/LC3 together with visual loss in one year old animals. These results suggest that Nrf2/PGC1 α ^{-/-} mice have the impaired proteasomal and autophagy clearance in the RPE.

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The role of RPE-expressed SIRT1 in the Pathogenesis of Age-related Macular Degeneration

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Age-related macular degeneration (AMD) is a leading cause of blindness in the elderly. The dysregulation of retinal pigment epithelium (RPE) by oxidative stress has been implicated as having an important role in the pathogenesis of AMD. Sirtuin 1 (SIRT1), a histone deacetylase converting enzyme, functions as a NAD⁺-dependent histone deacetylase. SIRT1 regulates cell senescence, DNA damage repair, and apoptosis and can control longevity in response to caloric restriction in many organisms. Recently, SIRT1 has been shown not only to deacetylate histone but also to target a variety of other factors that are related to oxidative stress response. Therefore SIRT1 may be a target in the process of oxidative stress-related disease including AMD. However, it has been still unclear the correlation between pathogenesis of AMD and SIRT1. In the present study, we found that SIRT1 has a strong cytoprotective role through NF-E2-related factor 2 (NRF2) activation and works as an anti-angiogenic factor through an alteration of vascular endothelial growth factor (VEGF) expression in RPE. Our results suggest that the expression of SIRT1 in RPE was strongly associated with the pathogenesis of AMD. Furthermore, we also analyzed the effect of intravitreal administration of an activator of SIRT1, resveratrol, on CNV formation in a laser-induced mouse model, and we found the administration of resveratrol significantly decreased the formation of CNV. Thus, SIRT1 may play an important role in the pathogenesis of AMD, and may be a new therapeutic approach for AMD.

Mechanism of RPE Proteostasis in Health and Disease Using an *in vitro* "Aging" Model

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Retinal Pigment Epithelium (RPE) is a monolayer of polarized, polygonal, pigmented cells that are located between retinal photoreceptors and the choroidal blood supply. Throughout its life, RPE maintains photoreceptor health, functionality and tissue homeostasis, and, thus, is fundamentally critical for vision. During this process RPE faces several proteostatic stressors. Loss of central vision in age-related macular degeneration (AMD) is one

of the main reasons of blindness among elderly in the developed world. It is thought that the primary disease-initiating factor is the dysfunction of RPE. The aged RPE loses its functional capabilities and its ability to maintain sub-cellular and tissue homeostasis. Over time, RPE cell death causes photoreceptor cell death and vision loss. The cellular processes that lead to RPE aging are poorly understood. To study these cellular processes and RPE-aging mechanisms we developed an "aging" assay using RPE derived from Induced Pluripotent Stem Cells (iPSC) from both healthy and AMD patients.

Fibroblast cultures were derived from donor skin biopsies and reprogrammed to pluripotent state using the "Yamanaka factors". Fully-characterized iPSC cells were differentiated to RPE using a developmentally guided differentiation protocol. iPSC cell derived RPE were cultured on semi-permeable membranes for 8 weeks to obtain a functionally mature and polarized monolayer tissue. iPSC cell derived RPE monolayers were stressed with proteostatic 48 hours. Stressed RPE exhibited several features of "aged" RPE including reduced ability to digest photoreceptor outer segments, dampened electrical responses, and increased APOE deposition and protein aggregates towards the basal side of cells. Proteostatic stressor decreased autophagy levels, increased IL-6 secretion, and caspase-1 activation in iPSC cell derived RPE cells. This increased cellular inflammation is associated with the activation of NF- κ B pathway and STAT3 pathway inhibition. We propose that this cell culture model of RPE-aging provides a basis to discover mechanism of disease-related RPE atrophy, a tool to identify the role of AMD genetics in disease initiation and progression, and to discover potential therapeutic interventions for AMD.

Alterations in miR-155 Levels Mediate RPE Physiology & Pathophysiology

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Human RPE cells constitutively maintain relatively low levels of miR-155, but external stimuli monotonically increase RPE miR-155 levels. Physiological stimuli such as phagocytosis of photoreceptor outer segments or cyclic light/dark transitions elevate miR-155 levels (3 to 16-fold). In comparison, pro-inflammatory stimuli produce much larger changes in RPE miR-155 and subsequent physiological and para-inflammatory responses. In a healthy eye, the homeostatic responses to the diurnal alterations of light/dark patterns and photoreceptor

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phagocytosis cause metabolic and circadian fluctuations (eg, miR-155 levels) that are repeated with great fidelity over the decades of our life. Eventually however these responses occur at the expense of elevated RPE oxidative and metabolic stress and the alteration of biochemical synthesis/degradation pathways, perhaps including the baseline levels of miR-155. increased miR-155 expression could activate the RPE inflammasome and the onset of immunosenescence. We hypothesize that monotonic elevation of steady-state miR-155 levels occurs with age and leads to inflammasome formation followed by a host of pathophysiological processes in the retina/RPE/choroid complex.

Effects of Amyloid Beta on Cultured Human Retinal Pigment Epithelial Cells

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Background and aims: Age-related macular degeneration (AMD) is a leading cause of irreversible blindness in patients aged ≥ 50 years. It is known that one of the strongest predictors of AMD is the number and size of drusen. It has been reported that amyloid β ($A\beta$), a peptide associated with the neurodegenerative events in Alzheimer's disease, is an important constituent of drusen. The purpose of this study was to investigate the effect of $A\beta$ on human retinal pigment epithelial (hRPE) cells in culture.

Materials and methods: ARPE-19 cells, a human RPE cell line, were exposed to 0-25 μM $A\beta$ for 48 h. After the exposure, the number of living cells was determined by WST-8 cleavage. The degree of replicative DNA synthesis was measured by 5'-bromo-2'-deoxyuridine (BrdU) incorporation. To investigate the cell death pathways, the number of living cells was determined by WST-8 cleavage after the addition of anti-necroptotic factor, necrostatin-1, and anti-apoptotic factors, caspase-8 and caspase-9 inhibitors. Real-time quantitative RT-PCR of vascular endothelial growth factor (VEGF)-A and pigment epithelium derived factor (PEDF) was performed using $A\beta$ -exposed cellular RNA as a template.

Results: The WST-8 cleavage assay showed that the number of living cells was increased by exposure to 5 μM $A\beta$ exposure ($P < 0.02$), but 25 μM of $A\beta$ decreased the number of living cells ($P < 0.001$). Replicative DNA synthesis by ARPE-19 cells exposed to 25 μM of $A\beta$ was significantly decreased ($P < 0.04$) indicating that 25 μM of $A\beta$ inhibited cell

proliferation. Real-time RT-PCR revealed that the level of the mRNA of PEDF was increased by exposure to 5 μM $A\beta$ ($P < 0.02$), and the levels of the mRNAs of PEDF and VEGF-A were also increased by exposure to 25 μM $A\beta$ ($P < 0.01$). The addition of caspase-9 inhibited the decrease the number of ARPE-19 cells exposed to $A\beta$ ($P < 0.007$).

Conclusion: Low concentrations of $A\beta$ stimulation increases the level of PEDF, and thus inhibit RPE cell apoptosis, resulting in RPE cell proliferation. High concentration $A\beta$ stimulation induces RPE cell death and enhances VEGF-A transcription in RPE cells, suggesting that it may cause the development of choroidal neovascularization.

RPE12 - RPE dysfunction in AMD: From oxidative damage to inflammasome activation

RPE Dysfunction and Cell Death

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In age-related macular degeneration (AMD) -the leading cause of blindness in the developed world-, toxic tissue debris accumulates as drusen beneath the retinal pigment epithelium (RPE), followed by eventual cell death. Although the formation of drusen constitutes an early characteristic of AMD, the mechanisms accounting for its generation and eventual toxicity remain elusive. Prior studies from our group have elucidated that there are two complementary redundant cell death pathways that affect photoreceptors and RPE. Namely caspase dependent apoptosis and receptor interacting protein kinase (RIPK) regulated necrosis. Photoreceptors appear to predominantly die by apoptosis whereas RPE appear to die by necrosis. Another process that is an integral part of cell survival and death is autophagy. Lysosome-associated membrane protein-2 (LAMP2) is a glycoprotein that plays a critical role in the fusion event between lysosome and autophagosome. Here, we explored the role of LAMP2 in RPE and AMD pathogenesis. LAMP2 was primarily expressed in the RPE cells, which was significantly depleted in the proximity of drusen in AMD patients or in aged mice. The decline of LAMP2 was accompanied by

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accumulations of ubiquitinated proteins and autophagy receptor sequestosome 1. Moreover, deletion of Lamp2 gene in mice resulted in massive accumulation of lipofuscin and sub-RPE debris with histopathological features similar to those in AMD, eventually leading to RPE cell death with necrotic features and photoreceptor loss with apoptotic features. Primary RPE cultures from Lamp2 deficient mice demonstrated accumulation of sub-RPE extracellular material when fed with photoreceptor outer segments for phagocytosis. Thus, our study shows that RPE autophagy dysfunction can lead to drusenoid deposit formation via secretion of indigestible autophagic cargo and eventual necrotic RPE cell loss. Stimulation of LAMP2 function on top of cell death inhibition could serve as the basis for future therapeutic intervention in non-neovascular AMD, a disease currently without therapy.

Innate Immune Pattern Recognition Receptor (PRR) Sensing in Retinal Degeneration

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Pattern recognition receptors (PRRs) are the primary initiators of inflammation recognising disturbances or shifts in homeostasis, inducing an immune response to restore the status quo. Persistence of noxious stimuli however, can interfere with the resolution phase of inflammation enabling persistence of pro-inflammatory mediators and local tissue damage. We show that RPE cells express many of the intracellular cytosolic PRRs eg. NLRP3, RIG-I and MDA5 as well as the better-characterised membrane bound Toll-like Receptors (TLRs), responding strongly to the danger associated molecular patterns (DAMPs) that are ligands for these receptors. One of the biological signatures observed in AMD is the marked deposition sub-RPE of complement factor 3 (C3). It is widely accepted that inappropriate activation of the alternative complement cascade is involved in AMD progression however both the cause of aberrant C3 deposition in AMD and the outcome remains elusive. The spontaneous activation of the complement cascade converts C3 to C3b, a component that can serve as an opsonin enhancing phagocytosis by marking antigens and dead cells for recycling. We hypothesized that sterile DAMPs found in the degenerating retina would activate PRR signalling pathways to initiate an immune response directed to aid clearance of the noxious stimuli, including inducing C3 secretion. We find that C3 gene expression is significantly induced in response to TLR 2,3 and 4 in the RPE. Furthermore TLR

activation results in an increase of C3 secretion from both RPE cells and human monocytes. TLR adaptor proteins Mal/TIRAP, MyD88 and TRIF regulate expression of C3. Despite the complement cascade and TLRs both being critical components of the innate immune response little is known about the interaction between these two pathways. We not only demonstrate that TLR activation can promote the expression and secretion of C3, the key factor in all three Complement cascades but also show that complement and TLRs synergize to enhance pro-inflammatory responses. Given the array of possible TLR ligands available in the environment of the degenerating RPE/retina, it is possible that the sheer volume of secretion in response to TLR activation promotes C3 deposition potentially compromising the integrity of the RPE outer blood-retina barrier (oBRB) and contributing to a local inflammatory microenvironment.

Metabolic Control of RPE Maturation and Dysfunction

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A compelling set of evidences indicates that oxidative damage and mitochondrial dysfunctions of the retinal pigment epithelium (RPE) are key early events in age-related macular degeneration (AMD) progression. Despite the significant role of oxidative stress on RPE dysfunction and atrophy, the molecular regulation of RPE oxidative metabolism under normal and pathological conditions remains unclear. The transcriptional co-activators, peroxisome proliferator-activated receptor-gamma coactivator-1alpha and beta (PGC-1a and b) are critical regulators of mitochondrial biogenesis and oxidative metabolism in many tissues. PGC-1 isoforms co-activate numerous transcription factors to orchestrate the expression of hundreds of genes encoding for nearly all aspects of mitochondrial biology. PGC-1 isoforms simultaneously activate an anti-oxidant program that protects cells from the reactive oxygen species (ROS) generated by increased mitochondrial activity, thus coordinating the regulation of oxidative metabolism. The purpose of our study is to characterize the specific roles of PGC-1 isoforms in RPE function and pathophysiology. PGC-1a and PGC-1b are highly conserved isoforms generally considered to share many transcription factor targets and therefore to function similarly. However our work demonstrates that PGC-1 isoforms are not only differentially regulated in RPE, they also exert highly

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divergent functions. Indeed, contrary to PGC-1a whose expression promotes RPE metabolic maturation and resistance to cytotoxic stress, PGC-1b upregulation is detrimental to RPE homeostasis. By elucidating fundamental questions on the roles of PGC-1a and PGC-1b in RPE function, metabolism and response to oxidative stressors under physiological and pathological conditions, these data offer new insights into the mechanisms of AMD progression.

Choroid Endothelium Signals Regulate Outer Retina-Blood Barrier through Modulation of Basement Membrane Assembly

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The survival and function of the retina depends on support functions performed by the retinal pigment epithelium (RPE), including its key role as a selective Outer Retina-Blood Barrier (ORBB) that regulates the transport of nutrients, fluid and waste between the neural retina and the underlying choroid circulation. The RPE sits on top of the Bruch's membrane (BM), an elastin- and collagen-rich basement membrane that separates the RPE from the underlying choroidal capillaries. It has been shown that endothelial cells (ECs) are tissue-specific and constitute instructive niches that secrete key angiocrine factors that promote parenchymal cell differentiation and tissue regeneration. To obtain hints on whether choroid ECs play a regulatory role in the formation of the ORBB, we purified mouse choroid ECs before (P5) and after (P30) terminal differentiation of the retina using a novel protocol that involves intravital staining of the specific EC marker VE-Cadherin followed by flow cytometry sorting. Transcriptome informatics analyses highlighted extensive

changes in ECM-related gene expression. To study the effect of Choroid ECs on terminal differentiation of the ORBB, we performed co-culture experiments on Transwell chambers using primary human fetal RPE (hFRPE) and mouse choroid ECs. We noticed that EC co-culture caused a marked increase in the transepithelial electrical resistance (TER) of hFRPE cultures which was not observed when other cell types were used instead of ECs and an increased frequency of collagen bundles in the basement membrane secreted by hFRPE cells, which correlated with stiffer spots detected by atomic force microscopy. Biochemical and quantitative imaging assays showed that EC-conditioned media induced the activation of beta-1 integrin at the basal surface and the accumulation of the TJ protein occludin specifically along intercellular junctions. Interestingly, all these effects were significantly impaired after chemical inhibition of lysyl oxidase activity, secreted mostly by ECs. In summary our results uncovered a novel EC-RPE regulatory pathway: angiocrine factors produced by choroidal ECs remodel and stiffen the RPE basement membrane; integrin receptors sense these changes triggering Rho GTPase signals that enhance RPE barrier function. The significance of our findings in the context of AMD will be discussed. Supported by NIH, Research to Prevent Blindness, Dyson Foundation, Starr Foundation.

Dissecting Molecular Pathways of HCA2 Signaling in Normal and Aged Retinal Pigment Epithelium

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Oxidative stress, inflammation and dysregulated cholesterol metabolism are involved in AMD pathogenesis. RPE is a key regulator of these processes in retina, however the exact mechanisms by which it does so are not well understood. This gap in knowledge is a major limitation in the development of therapies, especially for dry AMD, a form of the disease for which at present no effective therapy for prevention or treatment exists. Hydroxycarboxylic acid receptor 2 (HCA2) signaling has

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been demonstrated to impact multiple pathways that influence immune cell migration/activation, inflammation, cholesterol homeostasis and aging in a number of tissue and cell types. Here, we evaluate *in vivo*, the age-dependent expression and functional relevance of HCA2 in mouse and human retina and, the potential regulatory effect of the receptor on the expression of genes that mediate the cross-talk of pathways/mechanisms governing RPE cell metabolism and related outer retinal function. Histological assays were used to evaluate changes in morphology, cell viability, reactive oxygen species (ROS) production and cholesterol handling in wildtype (WT; *Hca2*^{+/+}) and HCA2 knockout (KO; *Hca2*^{-/-}) mouse retinas at 2, 5 and 10 months of age. HCA2 expression was evaluated also in control and AMD post-mortem human retina. Microarray/ingenuity pathway analyses (IPA) were used to evaluate age-dependent alterations in gene expression in WT and KO eyes; key findings were confirmed by qPCR, ELISA and/or Western blotting. Retinal function was evaluated by ERG. Cell death, ROS and cholesterol accumulation increased early in HCA2 KO eyes. Similar findings were detected only in aged WT retinas. Pro-inflammatory/pro-oxidant gene expression was upregulated in KO animals compared to WT at all ages while the expression of anti-oxidant genes declined. Scotopic flash responses were reduced significantly in KO compared to WT mice at all ages, and in aged WT compared to young WT mice. HCA2 expression increased AMD human eyes compared to non-AMD controls. HCA2 regulates multiple pathways critical to the regulation of immunity, inflammation and oxidative stress in retina; this is supported by the deleterious effects on retinal morphology, function and gene expression observed in HCA2 KO mice. Given that HCA2 expression is high in human macula and it persists in AMD, therapies targeting HCA2, augmenting its expression and function, could be value in the prevention and treatment of AMD.

A Novel Inhibitor of 5-Lipoxygenase Prevents Oxidative Stress-induced Cell Death of Retinal Pigment Epithelium Cells

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Oxidative stress is implicated in the pathogenesis of several retinal degenerations. Novel therapeutic agents capable of decreasing the oxidative stress-mediated injury of the RPE or retina are desired. 5-Lipoxygenase (5-LOX) oxygenates arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid, which converts into biologically detrimental

leukotrienes, such as LTB₄. The RPE and retina express the *PNPLA2* gene for PEDF-R, a lipase involved in cell survival. The aim is to investigate the relationship between 5-LOX and PEDF-R in RPE under oxidative stress. Synthetic peptides designed from PEDF-R and recombinant His6/Xpress-tagged PEDF-R proteins were used in binding assays by pull-down and peptide-affinity chromatography. Lipoxygenase activity was assayed with soybean and potato lipoxygenase. We found that among five peptides spanning between positions Leu¹⁵⁹ and Met³²⁵ of the human PEDF-R, only two overlapping peptides E5b and P1 bound and inhibited lipoxygenase activity. Human recombinant 5-LOX bound specifically to peptide P1 and to PEDF-R via ionic interactions. Oxidative stress was induced in ARPE-19 and primary pig RPE cells with H₂O₂/TNF- α . Then reactive oxygen species were detected, and cell death and viability rates were measured using respective biomarkers. The two inhibitor peptides E5b and P1 attenuated reactive oxygen species accumulation in a concentration-dependent manner. The inhibitor peptides also promoted cell viability and decreased cell death of RPE cells undergoing oxidative stress in a specific and concentration-dependent fashion. However, a scrambled peptide P1 did not have these effects. We performed RT-PCR of *ALOX5* and *PNPLA2* genes, and immuno-blot to detect respective protein levels. Oxidative stress decreased the levels of *PNPLA2* transcripts and PEDF-R protein, but did not have an effect on *ALOX5* expression. Furthermore, we explored the effects of modulating PEDF-R on 5-LOX activity in RPE undergoing oxidative stress. Leukotriene LTB₄ was measured by ELISA. Silencing the *PNPLA2* gene increased both leukotriene LTB₄ levels and cell death, while exogenous additions of P1 peptide or over-expression of the *PNPLA2* gene decreased both LTB₄ levels and cell death. These results imply that PEDF-R can modulate the detrimental effects of oxidative stress on cell death. Altogether, our findings identify a novel inhibitor of 5-LOX derived from PEDF-R, which in RPE can intersect with cell death pathways induced by oxidative stress.

RPE Choroid Biology and Pathology

RPE13 - Lymphatics and fluid movement in the posterior eye: Recent advances and remaining controversies

Non-invasive Dynamic Tracking of Aqueous Humour Outflow and Cerebrospinal Fluid Drainage

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Introduction: The relationship between cerebrospinal fluid (CSF) and aqueous humor (AH) seems to be relevant for glaucoma and Vision Impairment and Intracranial Pressure (VIIP) syndrome observed in astronauts after long space missions. Using in vivo hyperspectral imaging, we have showed that quantum dot tracers injected into the anterior chamber^{1,2} and the CSF³ in mouse drain to the cervical lymph nodes. However, these imaging techniques could not provide 3D and quantitative information regarding the lymphatic drainage. Non-invasive in vivo imaging techniques such as photoacoustic tomography (PAT) combining tunable near-infrared (NIR) laser illumination and ultrasound detection have the potential to track these fluid pathways in intact animals, and provide 3D and quantitative information regarding the dynamics of CSF and AH.

Purpose: To use photoacoustic tomography (PAT) to map outflow pathways from the eye and from the cerebrospinal fluid *in vivo*.

Methods: Imaging of CD1 mice was performed using a PAT system (MSOT 128; iThera Medical inc.). Imaging of the head and neck of each mouse was performed before and after intracameral or CSF injections of NIR dyes at multiple time points. Quantification of the PA signals was performed using native MSOT view software. Post-mortem tissue sections were examined using near-infrared Image Mapping Spectrometer (IMS) to confirm of the location of the dye in various tissue components.

Results: Following eye injections a 50% decrease in NIR PA signal in the anterior chamber was observed at 2 hours. This was simultaneous with a more than 50% signal increase in the right jugular vein. Additionally, NIR tracer was detected in the right cervical lymph nodes. Following CSF injections, significant decrease in PA signal in CSF was observed with along with increase in jugular veins and cervical lymph nodes on both sides.

Conclusion: This is the first study to track AH outflow from the eye and from the CSF, using non-invasive PAT. Future studies of CSF and AH dynamics using this non-invasive imaging platform may provide insights into relationship

between CSF and AH, and mechanisms of action of potential novel therapies to treat glaucoma and other optic neuropathies.

Acknowledgments: CIHR, CFI, and Henry Farrugia Research Fund

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Evidence of lymphatics and glymphatics in the posterior human eye and retrobulbar optic nerve

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Koyna et al¹ have provided the first structural and phenotypic evidence of lymphatic-like structures in the human choroid. Immunohistochemistry (IHC) and TEM indicated a central-to-peripheral topography of lymphatic formation, with numerous blind-ended lymph sacs, infrequent precollector and collector lymphatic channels. Lymphatic defining ultrastructural feature, anchoring filaments, were identified on human choroidal lymphatic endothelium. The system of blind-ended initial lymphatic segments, external to the fenestrated vessels of the choriocapillaris, is ideally placed for recirculating extracellular fluid and for immune surveillance. We have extended this work to include ultrastructural & IHC characterisation of lymphatics in the dural sheath of the human retrobulbar optic nerve and examined for evidence of glymphatics in the retina. The 'glymphatic' system is a functional waste clearance pathway for the CNS, characterised by AQP4+ astrocytic endfeet in the perivascular space and the movement of fluids through the extracellular space. To examine the optic nerve, we utilized podoplanin (lymphatic marker), and CD34 (blood vessel marker) multiple-marker IHC. To examine the glymphatic system in human we used 11 vascular, lymphatic & glial

RPE Choroid Biology and Pathology

markers¹. Evidence of lymphatics in the optic nerve dural sheath, include detection of anchoring filaments and absence of erythrocytes; fragmented/absent basal lamina; weibel-palade bodies & fenestrae. Retrobulbar dural lymphatics were podoplanin+/CD34-. AQP4+/vimentin+ Müller cells & AQP4+/GFAP+ astrocytes formed a network that completely ensheathed the CD39+/Lectin+ blood vessel system in the retinas; however, many AQP4+/CD39-/lectin- structures with lumens were evident between blood vessels. AQP4+/vimentin+ Müller cells co-localized with podoplanin predominantly around arterioles. This AQP4+/vimentin+/podoplanin+ glial network may be the anatomical correlate of a retinal 'glymphatic' system. In the absence of expressed lymphatic markers, retinal glial cells may play a major role in a glymphatic-like system of the retina. The AQP4+/podoplanin+ network in non-diabetic retinas was enhanced in diabetic retinas, suggesting overactive function of this system in diabetic retinopathy. In conclusion, lymphatic-like channels in the human choroid and retrobulbar optic nerve provides a structural basis for antigen presentation & interstitial fluid exchange in the posterior eye with implications for glaucoma & inflammation.

Organ Characteristics in Lymphatic Drainage

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This presentation reviews histochemical studies of the structural organization of the lymphatic capillary network and lymphatic drainage with regard to organ characteristics in animals and humans. We identified the lymphatics and blood vessels using both an immunohistochemical method as well as an enzyme- histochemical method using 5'-nucleotidase (5'-Nase) and alkaline phosphatase (ALPase) and/or diaminopeptidase (DAPase) staining. The 5'-Nase-positive lymphatics can be distinguished histochemically from arterial and venous vessels through ALPase and DAPase activity, respectively. Comparative histochemical studies in the same specimen using light microscopy and scanning or transmission electron microscopy confirmed the specificity and localization of the enzyme reactions.

In the parenchymal organs, the lymphatics appeared in the connective tissue between lobules, despite the lobule blood capillaries being well developed. In general, the lymphatics forms a loose network a short distance from the blood capillaries and play a role in draining extra tissue fluid from the interstitial tissue. We found that the lymphatics in the

animal thymus were located adjacent to the blood vessels and were partially connected to the blood lumens.

In the tissue that is open on one side with room for abundant material exchange such as skin and gastrointestinal canals, the lymphatics were located at a deeper level, with the blood capillaries near the surface. However, in membranous tissue such as the mesenterium and diaphragm, the initial lymphatics were sinus-like lymph lumens forming a macula cribriformis immediately below the mesothelial cells and the blood vessels were located at the deeper level. In the central nervous system, the dura mater of the meninges extended to form the epineurium of the spinal nerves. The lymphatics were located in the root of dura mater in the brain and spinal cord, originating near the intervertebral foramen and running along the spinal nerve root. Compared to the thoracic or lumbosacral regions, the lymphatics were remarkably well developed in the cervical region, especially in the brachial plexus. This finding confirmed that the cerebrospinal fluid is absorbed not only from the sinus venosus through arachnoidal granulation, but also into the arachnoidal and epidural lymphatic networks from the sheath of upper spinal nerves. In this presentation, the lymphatics in the eye will be discussed in relation to prospective ocular lymphatic drainage.

Evidence of a Glymphatic Clearance Pathway in the Optic Nerve

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The flow of cerebrospinal fluid (CSF) into and out of the brain is critical to the clearance of waste and neurotoxic molecules such as amyloid- β . Recent evidence suggests that there is "Glymphatic" CSF flow through paravascular spaces in the brain, channels that run alongside blood vessels. It is unknown whether a Glymphatic pathway exists in the optic nerve. Here we aim to investigate the presence of a Glymphatic microcirculation in the murine optic nerve. To do so, CD1 mice (n=12) of both sexes (M/F=5/7) were placed in a stereotaxic frame under general isoflurane anesthesia, and the cisterna magna was exposed

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in preparation for tracer injection. 3 μ L of fluorescein isothiocyanate (FITC; Invitrogen, OR, USA) conjugated to dextrans of various molecular weights was injected into the CSF. FITC-dextran sizes included 10 kDa (n=5), 40 kDa (n=3), 70 kDa (n=2), 500 kDa (n=1), and 2000 kDa (n=1). Animals were sacrificed 1 hour after injection by perfusion with phosphate buffered saline and 2% paraformaldehyde. Intraorbital optic nerve segments were frozen and serially sectioned (30 μ m; coronal and sagittal) before staining with isolectin B4-AlexFluor 647 (Invitrogen, OR, USA) to visualize blood vessel endothelium. Optic nerve sections were analyzed by confocal microscopy and compared to non-injected controls (n=2). One hour following tracer injection into CSF, all FITC-dextran sizes were present in the subarachnoid space surrounding the intraorbital optic nerve. In 10 and 40 kDa trials (n=8/8), high intensity fluorescence was found within paravascular spaces of medium sized vessels (10-15 μ m diameter) within the nerve, with minimal to no signal around small vessels and capillaries. Tracers 70 kDa and larger were not found within optic nerve paravascular spaces. Paravascular tracer in all 10 and 40 kDa trials was restricted to the myelinated portion of the nerve and did not continue into the optic nerve head or retina. In conclusion, the distribution of tracers within optic nerve paravascular spaces after CSF injection indicates the presence of a CSF microcirculation consistent with a Glymphatic pathway within the intraorbital optic nerve in mice. Further studies are needed to explore the role of such a CSF microcirculation in health and optic neuropathies including glaucoma.

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Evidence For A Glymphatic System In Human, Primate, Rat And Mouse Retina

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The glymphatic system is a recently defined brain-wide paravascular pathway for CSF & interstitial fluid exchange that facilitates efficient clearance of interstitial solutes. It is a functional waste clearance pathway for the CNS, characterised by aquaporin 4+ (AQP4+) astrocytic endfeet in the peri-vascular space. Evidence for a glymphatic system in the human CNS, especially in the retina, remains to be elucidated. This study investigated the presence of glymphatics in human, primate, rat, & mouse retinas. We studied retinas from 39 human, including 9 from donors with a diabetic history; 10 primates, including 7 with high fat diet-induced Type-2 diabetes; 10 rats, & 10 mice via multimarker-IHC for GFAP, vimentin, AQP4, GS isolectin B4, UEA Lectin, podoplanin, D240, VEGFR3, Prox-1, & LYVE-1. AQP4 was expressed by vimentin+ Müller cells & GFAP+ astrocytes in all retinas, with similar distributions & patterns across species. AQP4+/vimentin+ Müller cells & AQP4+/GFAP+ astrocytes formed a network that completely ensheathed the entire CD39+/Lectin+ blood vessel system; however, many AQP4+ /CD39-/lectin-structures with lumens were evident between blood vessels. AQP4+/vimentin+ Müller cells mostly co-localized with podoplanin (lymphatic marker) predominantly around arterioles. Lymph nodes from rats demonstrated AQP4+ /podoplanin+ lymphatic channels. Lectin+ blood vessels just outside the lymph node were AQP4-. Human diabetic retinas showed: 1) 1.7 fold increased expression of AQP4 on vimentin+ Müller cells ($p < 0.05$) & 1.4 fold increased expression of podoplanin ($p < 0.05$) compared to non-diabetic retina; 2) AQP4+ macrophage-like cells (4/9) that formed networks along large blood vessels in the inner retina adjacent to the vitreous; & 3) AQP4+ vessels that also showed strong podoplanin expression. Similar results were also seen in diabetic primate retina. We suggest that an AQP4+ glial network ensheathes the entire retinal vascular system, including glymphatic-like channels in retinal parenchyma & may be the anatomical correlate of a retinal 'glymphatic' system. The role played by glymphatics in the CNS in fluid & waste removal leads us to suggest that the retinal 'glymphatic-like system' may provide the anatomical basis for posterior outflow, thus having potential clinical relevance for the understanding of glaucoma.

Retinal Cell Biology

RCB1 - New insights on the RPE/Bruchs membrane/choriocapillaris in AMD

Choriocapillaris Dropout in Eyes with Early Age-related Macular Degeneration

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We have previously demonstrated a loss of choriocapillaris (CC) in advanced AMD. In this study, we examined postmortem eyes from donors with clinically documented early AMD (≥ 10 small drusen or < 15 intermediate drusen, or pigment abnormalities associated with AMD) in choroidal whole mounts to determine the area, pattern and severity of vascular changes. Choroids from postmortem aged human eyes without AMD and from eyes with a Grade 2 clinical classification of early AMD were immunolabeled with Ulex Europaeus Agglutinin (UEA) lectin to stain blood vessels. Whole mounts were imaged using confocal microscopy and image analysis was performed to determine the area of affected vasculature, density of vasculature (percent vascular area, %VA), and choriocapillaris (CC) luminal diameters in submacular, paramacular and nonmacular choroid. In aged control eyes, the CC had a homogenous pattern of freely interconnecting capillaries with broad diameter lumen. The %VA area in submacula was 78 +/- 3.5% and CC luminal diameters were 14.7 +/- 1.56 microns in diameter. In eyes with early AMD, CC dropout was observed in the submacular choroid as evidenced by a loss of interconnecting capillaries and a decrease in vascular density to 64.04 +/- 11.8% which was significantly reduced compared to aged controls ($P < 0.0001$). The paramacular and nonmacular %VA were similar in eyes with or without AMD. CC diameters were not significantly different in any region of early AMD choroids compared to controls. The area of choroid affected by CC dropout was 0.04 +/- 0.1mm² in aged control eyes and 7.29 +/- 5.52mm² in eyes with early AMD. In some cases, incipient neovascular buds were observed at the border of regions with CC dropout in early AMD choroids.

Using lectin labeled choroidal whole mounts from donors with clinically documented early AMD has provided a unique opportunity to examine regional changes in vascular pathology associated with small to intermediate drusen. No vascular pathology was observed outside the submacular region. While the affected area in some eyes

was quite extensive histologically, these changes are probably not detectable clinically using standard in vivo imaging techniques.

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Distribution and Quantification of Choroidal Macrophages in Aged Human Eyes and Eyes with Age-related Macular Degeneration

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Macrophages have been implicated in the pathogenesis of age-related macular degeneration (AMD). It is reported that they remove drusen and stimulate the formation of CNV in mouse models of AMD. This study examined choroidal macrophages and their activation in postmortem eyes from aged control subjects and eyes with AMD. Activation was determined by HLA-DR expression (a subunit of MHC-II) and change in their size and shape (sphericity) as determined with image analysis. Choroids from postmortem human eyes with and without AMD were incubated with anti-IBA-1 (ionized calcium-binding adapter molecule 1) to label macrophages and anti-HLA-DR (human leukocyte antigen - antigen D related) as a macrophage activation marker as well as with UEA (*Ulex europaeus agglutinin*) lectin to label blood vessels. Whole mounts were imaged using confocal microscopy. IBA-1 and HLA-DR positive cells were counted in three areas (submacula, paramacula, and nonmacula) and cell volume and sphericity were determined using computer-assisted image analysis and Imaris software.

In aged control eyes, the mean number of submacular IBA-1⁺ and HLA-DR⁺ choroidal macrophages was 433/mm² and 152/mm², respectively. In early AMD eyes, there was a significant increase in IBA-1⁺ and HLA-DR⁺ cells in submacula compared to controls ($p=0.0015$ and $p=0.008$ respectively). In eyes with GA, the numbers of submacular IBA-1⁺ cells was not significantly increased compared to controls but HLA-DR⁺ cells were increased ($p=0.005$). In eyes with neovascular AMD, there was a significant increase in HLA-DR⁺ cells associated with submacular choroidal neovascularization (CNV) ($p=0.001$). Cell volume also

decreased significantly ($p \leq 0.02$) and sphericity increased significantly (change from dendritiform to round) ($p \leq 0.005$) in all AMD groups compared to controls. In Conclusion, the number of IBA-1⁺ macrophages in submacular and paramacular choroid was significantly increased in early/intermediate AMD, compared to aged controls. Activated (HLA-DR⁺) submacular macrophages were significantly increased in all stages of AMD. Macrophage shape became significantly more round and their size decreased in the submacular AMD choroid, also suggesting activation of macrophages in AMD.

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Visualizing RPE Fate in AMD through Validated OCT and Autofluorescence Imaging

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The biophysical basis of reflectivity in optical coherence tomography (OCT) is backscattering towards the detector from organelles (Mie scattering) and via waveguiding, for photoreceptors. The RPE has 3 cushions of light-scattering organelles (melanosomes, lysosome-derived organelles, mitochondria) that make it a triple reflector in OCT. Three possible fates are death, transdifferentiation to a phenotype not recognizable as RPE, and emigration. Under the hypothesis that the RPE exhibits stereotypic stress responses that can be followed in vivo by OCT, we surveyed RPE morphologies in high-resolution histology of 53 late AMD eyes (geographic atrophy, GA and neovascular AMD) and one direct clinicopathologic correlation of a previously imaged patient. We found many fully pigmented RPE cells in the atrophic zones, cells shedding granules basolaterally, consistent with apoptosis, cells sloughing into the subretinal space and migrating into the retina, and cells subducting under basal laminar deposits in atrophic areas and apparently migrating outward under the non-atrophic zone. Using the descent of the external limiting membrane (ELM) towards Bruch's membrane as the border of atrophy (per S.H. Sarks, 1976) we found that RPE phenotypes worsen and the RPE layer thickens towards this landmark, confirming a progressing dysmorphia that underlies variable autofluorescence in geographic atrophy (Rudolf et al 2013). In serial eye tracked OCT of drusenoid RPE detachments, the RPE layer can be seen to thicken in

advance of hyperreflective foci appearing in the overlying neurosensory retina. This is followed by breakup of the layer, collapse of the detachment, and atrophy of the outer retina. Cells resembling migratory RPE phenotypes express inflammatory markers in other studies. These data converge on a new model of how geographic atrophy proceeds via transdifferentiation and migration of RPE, and provides new insights into the microenvironment confronting replacement cells. A comprehensive and quantitative description of late AMD can inform the study of early AMD by enabling accurate natural history through multimodal clinical imaging and focus on relevant precursor processes in model systems.

A Possible Role of Lipid Accumulation in Bruch's Membrane in the Pathogenesis of Age-related Macular Degeneration

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Age-related macular degeneration (AMD) is considered to be associated with chronic inflammation employing oxidative stress and complement activation as well as up-regulation of vascular endothelial growth factor (VEGF). Integrated evidences from basic researches suggested a role of lipid accumulation in Bruch's membrane in the pathogenesis of AMD. The 'lipid wall' in the Bruch's membrane reduces hydraulic conductivity, likely stagnating VEGF secreted from retinal pigment epithelium (RPE) toward choriocapillaris. Oxidized lipid and continuous light exposure may result in oxidative stress and complement activation, against which complement factor H may protect RPE, otherwise RPE is damaged. Using a unique 3D spheroid culture of RPE cells (Sato R, Yasukawa T, et al. IOVS 2013;54:1740), pathophysiology of lipoprotein secretion from RPE cells, Bruch's membranogenesis, and biogenesis of hard drusen can be investigated. In this session, I will discuss physiological functions of RPE, aging changes in RPE and Bruch's membrane, and possible relevance to age-related chorioretinal diseases such as pachychoroidopathy and AMD.

Retinal Cell Biology

Complement Injury to the Choriocapillaris: Lytic and Sublytic ChangesKATHLEEN CHIRCO^{1,2}, Edwin Stone^{1,2}, Budd Tucker^{1,2}, Robert Mullins^{1,2}¹University of Iowa, Ophthalmology and Visual Sciences, Iowa City, United States, ²Stephen A Wynn Institute for Vision Research, Iowa City, United States

Over the last decade, many important studies have provided valuable insight into the role of the complement system in age-related macular degeneration (AMD). For example, AMD-associated polymorphisms in various complement pathway regulators have been identified, including those in the *C3*, *CFI*, and *CFH* genes, with the *CFH* Y402H polymorphism increasing AMD risk by up to sevenfold. The terminal step in complement activation is formation of the membrane attack complex (MAC), which, if unchecked, leads to cell lysis and death. The MAC accumulates in the choriocapillaris with normal aging, and this accumulation is remarkable when compared to other microvascular beds. The high levels of the MAC that accumulate in the choriocapillaris with age may result in bystander injury and eventual loss of endothelial cells (ECs), as direct exposure of choroidal ECs to the MAC results in dose-dependent cytolysis. In support of this idea, our lab and others have observed vascular changes that occur early in AMD progression, including decreased levels of EC-specific mRNA and protein, vascular dropout in the choriocapillaris, reduced choroidal blood flow within the macula, and choroidal thinning in human donors with the *CFH* Y402H polymorphism. Another complement-associated factor that has been gaining recognition for its role in AMD is C-reactive protein (CRP). Elevated levels of CRP in human serum have previously been associated with increased progression rate for AMD as well as overall risk for AMD. Total CRP levels have also been shown to be more abundant in the choroid of donor eyes with wet AMD and those homozygous for the *CFH* Y402H polymorphism. Recently, we found that the pro-inflammatory monomeric form of CRP (mCRP), in contrast to the anti-inflammatory serum-associated pentamer (pCRP), is the more abundant form of CRP in the human choriocapillaris. Moreover, mCRP is elevated in donor eyes homozygous for the *CFH* Y402H allele. Treatment with exogenous mCRP increased EC migration and transendothelial permeability *in vitro*, while also elevating inflammatory response gene expression in human RPE/choroid tissue *ex vivo*. These data suggest a role for mCRP in AMD through its ability to increase inflammation within the choriocapillaris. We propose that complement activation within the choriocapillaris, caused or accelerated by mCRP, leads to EC injury and subsequent

loss of the vasculature required for outer retina function in individuals at increased genetic risk for AMD.

RCB2 - Inflammation in AMD

Degranulation of Choroidal Mast Cells: Possible Involvement in the Pathogenesis of Age-related Macular DegenerationIMRAN AHMED BHUTTO¹, D. Scott McLeod¹, Mercedes Villalonga², Rachel E Silver², Johanna M. Seddon^{2,3}, Gerard A. Luty¹¹Johns Hopkins University School of Medicine/Wilmer Eye Institute, Ophthalmology, Baltimore, United States, ²Tufts Medical Center/Ophthalmic Epidemiology and Genetics Service, New England Eye Center, Boston, United States, ³Tufts University School of Medicine, Ophthalmology, Boston, United States

Purpose: Age-related macular degeneration (AMD) is a complex, multifactorial disease of the eye, resulting in irreversible central vision loss among the elderly population. Inflammation has been found to have an important role in its pathogenesis and progression. Mast cells (MCs) are key effector cells of inflammation and play an important role in immunity. Recent findings have revealed MC direct participation in cardiovascular diseases and metabolic disorders through cell-cell interactions and the release of proinflammatory cytokines, chemokines, and proteases to induce inflammatory cell recruitment, cell apoptosis, angiogenesis, and matrix protein remodeling. The purpose of this study was to understand the role of MCs in AMD pathology. The total number of choroidal MCs (degranulated [DG] and nondegranulated [NDG]) was determined in aged control and AMD eyes. This study compared MC populations in submacular choroid and the relationship of MCs to the AMD-associated pathology.

Subjects and methods: Human donor eyes included aged controls (n=10), clinically diagnosed with early AMD (n=8), geographic atrophy (GA, n=4) and wet AMD (n=11). The choroids were excised and incubated for alkaline phosphatase and nonspecific esterase (MCs) activities. DG and NDG MCs were counted in flat mounts (5 fields/area). Choroids were subsequently embedded in JB-4 and sectioned for histological analyses.

Results: The number of MCs was significantly increased in all choroidal areas in early AMD (p=0.0006) and in paramacular area in wet AMD (139.44±55.3 cells/mm²; p=0.0091) and GA (199.08±82.0 cells/mm²; p=0.0019) compared with the aged controls. DG MCs were also

increased in paramacular ($p=0.001$) and submacular choroid ($p=0.02$) in all forms of AMD. Areas with the greatest numbers of DG MCs had loss of CC. Sections revealed that the MCs were widely distributed in Sattler's and Haller's layer in the choroidal stroma in aged controls, whereas MCs were frequently found in close proximity with CC in GA and wet AMD and in choroidal neovascularization (CNV).

Conclusions: Increased MC numbers and degranulation were observed in all AMD choroids. These results suggest that MC degranulation may contribute to the pathogenesis of AMD: death of CC and RPE and CNV formation. The proteolytic enzymes released from MC granules may result in thinning of AMD choroid.

DICER1 Deficiency Recapitulates Multiple AMD Phenotypes via Innate Immunity

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Insufficiency of DICER1 is implicated in the pathogenesis of geographic atrophy (GA), the advanced form of dry age-related macular degeneration (AMD), due to an accumulation of cytotoxic SINE RNAs and activation of the NLRP3 inflammasome. Here, we report that mice genetically deficient in Dicer1 exhibit features of dry AMD first evident at two months of age and progressing with age to include basal laminar deposits and focal atrophy of the retinal pigmented epithelium (RPE). These pathological changes are accompanied by increases in rodent SINE RNAs and inflammasome activation. Unexpectedly, Dicer1 deficient mice also exhibit hallmark features of neovascular AMD including spontaneous choroidal and retinal neovascularization, which are sensitive to Vegfa blockade. Genetic ablation of the immune adaptor Myd88 dramatically reduced incidence of both RPE atrophy and choroidal and retinal neo-vessels. Finally, reduced DICER1 levels and accumulation of *Alu* RNA were observed in human neovascular AMD specimens. DICER1-deficiency and innate immune activation may therefore underlie both forms of advanced AMD and Dicer1-deficient mice represent a valuable new model of progressive RPE atrophy and aberrant retinal and choroidal neovascularization. These findings also reveal an unexpected anti-angiogenic role for DICER1.

Lipids, Inflammasomes, and Age-related Macular Degeneration (AMD)

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AMD is a pathology of the outer retina that can lead to significant vision loss. While the pathogenesis of AMD is not understood, there is increasing evidence for a role for the mishandling of lipids by the retinal pigment epithelial (RPE) cells. Among the supporting observations are the documentation of lipid accumulation above the RPE layer, lipid deposits within and underneath the RPE themselves and polymorphisms in a number of genes associated with lipid uptake and handling. The hallmark of AMD is the accumulation of drusen, insoluble lipid-rich deposits, beneath RPE and within the Bruch's membrane. We tested the hypothesis that the uptake of oxidized lipids by RPE results in NLRP3 inflammasome activation, leading to the release of IL-1 β and/or cell death. Treatment of primary fetal human RPE cells or ARPE-19 cells for 48 hr with oxidized low-density lipoprotein (ox-LDL), but not native LDL, led to cell death ($P < 0.001$) as measured by the release of lactate dehydrogenase. Exposure of hfRPE to ox-LDL resulted in a decline in transepithelial resistance ($P < 0.001$ at 24 hr and $P < 0.01$ at 48 hr) relative to LDL treated and control cells measured using an EVOM2 Voltohmmeter. Incubation of RPE with function-blocking antisera demonstrated that the uptake of ox-LDL was mediated by the CD36 receptor. Treatment of RPE with ox-LDL significantly increased the levels of CD36 mRNA and protein, whereas native LDL did not impact their levels. Immunolocalization of lysosomal-associated membrane protein-1 (LAMP-1) revealed that ox-LDL taken up by the RPE was accumulating in the lysosomes. Exposure of the RPE to ox-LDL, but not LDL, induced a more than 5-fold increase in NLRP3 mRNA. Moreover, ox-LDL treatment of RPE leads to the activation of caspase-1 and the resulting release of mature IL-1 β . Simultaneous treatment of RPE cells with ox-LDL and isoliquiritigenin (10 μ M), an inhibitor of the NLRP3 inflammasome, significantly blocked ox-LDL-induced cytotoxicity ($P < 0.001$). These findings suggest that ox-LDL could play a role in the pathogenesis of AMD via NLRP3 inflammasome activation, leading to RPE death and the release of inflammatory cytokines.

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Role of Inflammation in Age-related Macular Degeneration**MIHO NOZAKI***Department of Ophthalmology & Visual Science Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

Age-related macular degeneration (AMD) is a leading cause of legal blindness in most developed nations. The blindness in AMD usually arises from invasion of the retina by choroidal neovascularization (CNV). Although the pathogenesis of CNV has not been understood clearly, inflammatory response is one of major cause of CNV.

Among various inflammatory responses, macrophages are thought to play as pivotal role in pathogenesis of CNV. Macrophages were accumulated in human CNV membrane, and macrophages depletion lead to near-abrogation of laser-CNV and suppression of vascular endothelial growth factor (VEGF) in mice. These data supported that infiltrated macrophages are producer and regulator of VEGF, and manipulation of infiltrated macrophages might be a novel therapeutic approach to treat CNV beyond anti-VEGF therapy. This presentation will explore the new in-vivo imaging of inflammation in experimental CNV in mice.

Defending Choroidal Endothelial Cells from Complement-Mediated Lysis in Age-related Macular Degeneration**ROBERT MULLINS¹, Shemin Zeng¹, Meng Wu², Edwin Stone¹, Budd Tucker¹***¹Wynn Institute for Vision Research, University of Iowa, Ophthalmology and Visual Sciences, Iowa City, United States, ²University of Iowa, College of Pharmacy, Iowa City, United States*

Human eyes with early age-related macular degeneration (AMD) are characterized by increased accumulation of choriocapillary membrane attack complex (MAC) as well as choriocapillaris endothelial cell loss. Since the MAC is present at the site of cellular loss in early AMD and is capable of lysing choroidal endothelial cells in vitro, therapeutics that protect choroidal cells from MAC-mediated cell death may be beneficial in stopping or slowing the progression of AMD. We previously characterized a model for complement-induced endothelial cell injury. In the current study, we used this model to screen a library of FDA approved small molecules (Selleck Chemicals) for their ability to reduce cell death. The endothelial cell line RF/6A was plated on 384 well plates and confluent cultures

were incubated with a final concentration of 10 μ M each of 1,080 FDA approved compounds for 4 hours. Medium was removed and 2.5% complement-intact human serum was added. After 4 hours of exposure, cell viability was assessed using Presto blue and lactate dehydrogenase (LDH) assays. All compounds were evaluated in duplicate. A small number of compounds showed a modest protective effect (>50% increased survival). One such compound, the antifungal drug econazole nitrate, was used in replication experiments. This molecule showed a dose-dependent decrease in complement-mediated cell lysis by LDH assay, with 10 μ M pretreatment showing a 6-fold decrease in cell death compared to complement-only controls ($p < 0.05$). These results provide further proof of principle that small molecules can protect choroidal cells from MAC-induced cell death and suggest that FDA approved compounds may be beneficial in reducing vascular loss and progression of AMD.

RCB3 - Retinopathy of Prematurity**The Role of Angiotensin in Modulating the Inner Retina during Retinopathy of Prematurity****KIRSTAN VESSEY¹, Laura Downie², Kate Hatzopoulos¹, Joanna Phipps¹, Jennifer Wilkinson-Berka³, Erica Fletcher¹, Retinal Cell Biology***¹The University of Melbourne, Anatomy and Neuroscience, Melbourne, Australia, ²The University of Melbourne, Optometry and Vision Sciences, Melbourne, Australia, ³Monash University, Immunology, Melbourne, Australia*

It is well known that retinopathy of prematurity is a vascular disease, caused by excessive growth of blood vessels on the surface of the retina in response to the combined effects of extreme immaturity of the retina and high levels of oxygen used for critical care of neonates. Currently, treatment targets the pathological angiogenesis. Despite treatment, many children suffer ongoing vision impairment, especially in scotopic vision. The major aim of our work has been to characterize the relationship between retinal vascular, glial and neural changes in experimental rodent models of ROP. Our results show that during the peak period of angiogenesis, there is significant loss of neurons associated with rod mediated vision, including loss of All amacrine cells and rod photoreceptor changes. In addition, scotopic retinal function was reduced. In order to evaluate the effect of the renin-angiotensin system, we first localized angiotensin II receptors, AT1 and AT2 in the rodent retina, evaluated the influence of angiotensin on the retina and then evaluated how treatment with an

antagonist of AT1 receptors (Valsartan), influenced the development of vascular, neuronal and glial dysfunction in ROP. Overall our data indicate that treatment with valsartan reduced vascular pathology as well as neuronal loss. However, retinal function was not salvaged by treatment with valsartan. These data suggest that there is an intricate relationship between vascular, neuronal and glial changes in ROP. In addition, the finding that valsartan is only partially retinoprotective confirms that while the renin-angiotensin system has a pathogenic role in the vascular changes that develop during ROP, more work is needed to understand the etiology of neuronal dysfunction and loss.

Role of Extracellular Matrix in Retinal Vascular Patterning

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Microglia play an important role in vascular plexus formation; they facilitate vascular anastomosis and regulate endothelial proliferation. We investigated the role of laminins in recruiting and activating retinal microglia. Deletion of *Lamc3*^{-/-} results in a higher density of microglial cells in the superficial plexus of central retina compared to wild type (WT) retina. In *Lamc3*^{-/-} retina, there was an increase both in number and activation of vascular branch-point-associated microglia resulting in a more heavily branched plexus compared to WT retina. As superficial vascular plexus of the *Lamc3*^{-/-} retina exhibit increased endothelial cell proliferation, we assayed TGF- β signaling and saw a decrease in microglial TGF- β expression and endothelial phospho-SMAD3 signaling. Activation of microglia with LPS, replicated the *Lamc3*^{-/-} phenotype in WT retina; inhibition of microglial activation, with minocycline, in the *Lamc3*^{-/-} retina restored WT vascular patterning. In adult retina, γ 3-containing laminins are present in the venous and capillary basement membrane but not arterial basement membranes suggesting a possible differential role in remodeling. During development, γ 3-containing laminins are deposited on both arteries and veins during superficial vascular plexus development. In the *Lamc3*^{-/-} mice, retinal arteries form twice as many branches and they have significantly less peri-arterial vascular pruning than do WT. Arterial endothelial cells express Notch ligand DLL4, whereas the venous endothelial cells do not. As a result, arterial endothelial cells have active Notch signaling, leading to the expression of arterial marker

EphrinB2. Deletion of *Lamc3* disrupts normal Notch signaling; arterial DLL4 expression level is significantly down-regulated and artery-specific EphrinB2 expression is decreased in the *Lamc3*^{-/-} retinas. Vein-specific EphB4 expression is unaffected. These data, together with our published data, suggest that β 2- and γ 3-containing laminins differentially regulate angiogenesis- β 2-containing laminins are pro-angiogenic and γ 3-containing laminins are anti-angiogenic. Furthermore, our findings indicate that γ 3-containing laminins regulate vascular remodeling and arterial morphogenesis via DLL4/Notch signaling pathway. Supported by NIH EY12676 (WJB) and unrestricted funds from Research to Prevent Blindness Inc.

Synergistic Suppression of Retinal Angiogenesis and Oxidative and Inflammatory Damages by Caffeine and Ibuprofen in Neonatal Rats

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Background and objectives: Oxidative injury, inflammation and immature retina lead to retinopathy of prematurity (ROP) via various mechanisms resulting in aberrant angiogenesis. These interacting pathogenetic mechanisms suggest that combined drug therapies are needed to optimize prevention of severe oxygen induced retinopathy (OIR) as shown with Ketorolac and Caffeine in an established intermittent hypoxia (IH) neonatal rat model that simulates neonatal apnea. Using this model, we determined if other NSAIDs (Ibuprofen, IB), given with Caffeine (C) exert synergistic actions preventing severe OIR.

Methods: Neonatal rat pups were randomized to hyperoxia (50% O₂) with brief hypoxia (12% O₂) from day 0 postnatally (P0) to P14 when they had loading doses of Calcein (20 mg/kg IP) and Neoprofen (10 mg/kg IP) on P0, followed by maintenance doses of 5 mg/kg IP/day from P1-P14. Other groups received Calcein or Neoprofen only, or sterile normal saline (NS). Animals exposed to 50% O₂ or room air (RA) served as controls and were treated similarly. Retinas were examined at P14 or following recovery/reoxygenation in RA until P21. Neovascularization was assessed in ADPase stained retinal flatmounts. Ocular biomarkers of angiogenesis (HIF_{1 α} , VEGF, VEGFR-1, VEGFR-3) lipid peroxidation (8-isoPGF_{2 α}); & prostanoids (PGE₂, PGF_{2 α} , 6-ketoPGF_{1 α} & TxB₂) were measured in vitreous fluid (VF), and retina.

Results: Retinal flatmounts showed decreased neovascularization in IB and IB+C treatments in IH

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compared to NS, but punctate hemorrhages+mild neovascularization persisted during recovery from IH. IB+C had greater suppression of retinal angiogenesis biomarkers ($p < 0.01$) at P14 and P21 in IH, but not in RA or 50% O₂. IB alone, and IB+C decreased 8-isoPGF_{2 α} at P14 and P21 in all O₂ conditions ($p < 0.05$), but response was greater in IH ($p < 0.01$). IB decreased all retinal prostanoids in IH at P14 and P21 ($p < 0.05$), but effect was potentiated with C ($p < 0.01$).

Conclusions: IH alters drug responses. Synergism of Ibuprofen and Caffeine occurs mostly during IH, perhaps due to increased drug penetration by IH-induced blood-ocular barrier dysfunction. These two drugs may protect against COX-2 mediated proangiogenic effects of PGE₂. Multi-drug therapies may be needed for complex disease as ROP. (Supported by NIH 1U54HD071594-01)

Studying Effects of Intrauterine Growth Restriction on Retinopathy of Prematurity with Rodent Models

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Intrauterine growth restriction (IUGR) is associated with preterm birth and also to retinopathy of prematurity (ROP). It is difficult to discern effects of IUGR, and not other factors associated with prematurity, on retinal features in ROP. To remove prematurity as a factor and better understand the effect of IUGR on ROP risk, we developed two different models of IUGR involving full-term rodent pups that differed by severity and duration of uteroplacental insufficiency. We tested the hypothesis that IUGR induced by different insults to the placenta affects physiologic retinal vascularization and intravitreal neovascularization (IVNV) in ROP differently. Chronic uteroplacental insufficiency (CUI), analogous to that in pregnancy-induced hypertension, was created through delivery of thromboxane A₂ by micro-osmotic pumps implanted into e12.5 pregnant C57Bl/6 mice through term (E20, ~third trimester). Acute uteroplacental ischemia (AUI) was induced by bilateral uterine artery ligation in pregnant Sprague Dawley dams two days prior to term birth at e19.5. Each model had sham controls. Both models, and not controls, caused IUGR in pups. CUI and sham mice were placed into the 75%/21% oxygen-induced retinopathy (OIR) model at p7 and analyzed at p17 for IVNV and physiologic retinal

vascularization, measured by avascular/total retinal area (AVA). AUI and sham rats were placed into the 50%/10% OIR model at birth and analyzed at p18.5 for IVNV and AVA. IVNV and AVA were determined on lectin stained retinal flat mounts. In rats, retinal angiogenic factors and serum levels were determined at p18 by western blot or ELISA. In the mouse CUI/OIR model, there was no difference in AVA, but IVNV was increased at p17 compared to mouse sham/OIR ($p < 0.05$). In the rat AUI/OIR model, AVA and IVNV were reduced at p18.5 compared to sham/OIR ($p < 0.05$). There were no differences in rat retinal VEGF between sham/OIR and AUI/OIR, but there was a significant increase in retinal erythropoietin (Epo) in AUI/OIR compared to sham/OIR ($p < 0.05$). Serum IGF-1 and VEGF were not different between rat AUI and sham OIR groups. However, serum Epo was increased in male, but not female, rat pups in AUI/OIR compared to sham/OIR ($p < 0.05$). Despite different species, the data suggest that differences in severity and duration of uteroplacental insufficiency lead to different effects on retinal vascularization and features of OIR. The rat AUI/OIR model showed sex-related differences in serum Epo.

Nuclear Localization of Platelet-activating Factor Receptor Controls Retinal Neovascularization in Model of Retinopathy of Prematurity

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Platelet-activating factor (PAF) is a pleiotropic phospholipid with pro-inflammatory, pro-coagulant, and angiogenic actions on the vasculature, that acts via a single PAF receptor (Ptafr). We previously reported that PAF induces retinal vasoobliteration; yet, angiogenic effects have also been documented. Interestingly, we have previously localized PAF receptor (Ptafr) at intracellular sites notably the cell nucleus, which may explain distinct functions based on its cellular localization. Nonetheless, mechanism of localization and physiologic functions of intracellular Ptafr remain poorly understood. We hereby identify importance of the c-terminal motif of Ptafr, and uncover novel roles of Rab11a GTPase and importin-5 in nuclear translocation of Ptafr in primary human retinal microvascular endothelial cells. Nuclear localization of Ptafr is independent of exogenous PAF stimulation as well as intracellular biosynthesis. Moreover, nuclear Ptafr is responsible for upregulation of unique set of growth factors, including vascular endothelial growth factor, *in vitro* and *ex*

vivo; whereas plasma membrane Ptafr controls expression of pro-inflammatory genes. We further corroborate the intracrine PAF signaling, resulting in angiogenesis *in vivo*, using Ptafr antagonists with distinct plasma membrane permeability. Collectively, our findings show that nuclear Ptafr translocates in an agonist-independent manner, and distinctive functions of Ptafr based on its cellular localization point to another dimension needed for pharmacologic selectivity of drugs.

RCB4 - Signal Transduction in the Retina

Activation-induced Conformational Changes in Arrestin

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Arrestins are elongated two-domain proteins that specifically bind active phosphorylated GPCRs. Arrestin binding stops G protein interactions with the receptor and redirects signaling to alternative pathways. Receptor binding is accompanied by global conformational changes. The nature of these changes determines comparative signaling capabilities of receptor-bound and free arrestins. Surprisingly similar conformational rearrangements were observed in crystal structures of arrestin-1 activated by rhodopsin binding and of arrestin-3 activated by IP6-mediated trimerization. The main global conformational rearrangement is the rotation of the two domains relative to each other. This changes both the receptor- and effector-binding sides of the molecule. In addition, on the receptor-binding side, the distal tip of the central finger loop becomes α -helical: it inserts into the cavity between receptor helices in the complex or interacts with the other two protomers in a trimer. Four elements on the effector-binding side always change conformation upon arrestin activation. Therefore we propose to call these elements arrestin switch regions (ASw). ASwI connects the only α -helix in arrestin with the N-domain. In arrestin-3 it has many prolines, including two PPXP motifs usually recognized by SH3 domains. ASwII includes the inter-domain hinge and the first β -strand of the C-domain, which in the active state of arrestin experiences a register shift in the β -sheet, shifting by one or two residues relative to its basal position. ASwIII is an extension of the C-domain lariat loop, which supplies two out of three negative charges to the polar core, the main phosphate sensor of arrestins.

Finally, the C-terminus is ASwIV: it is invariably detached from the N-domain, where it is anchored in the basal state. The C-terminus of non-visual arrestins contains clathrin and AP2 binding sites that mediate arrestin-dependent GPCR internalization. Elements corresponding to ASwII and ASwIII were implicated in the binding of JNK3, one of many arrestin effectors. It appears very likely that arrestin switches play key role in its interactions with other effectors and arrestin-mediated signaling.

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Protein Interactions of Retinal Guanylyl Cyclase in Phototransduction and Congenital Blinding Disorders

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Retinal membrane guanylyl cyclase (RetGC) produces second messenger of phototransduction, cGMP, under control of negative calcium feedback mediated by $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding guanylyl cyclase activating proteins (GCAPs). GCAPs activate RetGC in the light, when free Ca^{2+} concentrations in the outer segment fall and inhibit the cyclase in the dark, when Ca^{2+} concentrations rise. RetGC also binds a retinal degeneration-3 (RD3) protein, which strongly inhibits RetGC and prevents its activation by GCAPs. We identified, by using extensive site-directed mutagenesis, several regions in the RetGC1 primary structure that contribute to the GCAP binding interface. We also identified the location of RetGC-binding interfaces on GCAP1 and RD3. In GCAP1, the RetGC1-binding interface occupies portions of EF-hands 1, 2 and 3, and the GCAP affinity for RetGC1 and Ca^{2+} is further optimized by 'calcium-myristoyl tag' - a helical region connecting the N-terminal fatty acyl group with the C-terminal EF-hand 4. In RD3, the part of its primary structure that most strongly imparts the affinity for the target enzyme locates in the central portion of the molecule containing α -helical region H3. Mutations in RetGC1 isozyme (gene GUCY2D) can cause Leber's congenital amaurosis by suppressing the cyclase catalytic activity and activation by GCAPs, but some can prevent its production and accumulation in the outer segment even if the mutated recombinant RetGC1 retains normal catalytic activity and regulation *in vitro*. Mutation of Arg⁸³⁸ in dimerization domain of RetGC1 linked to cone-rod dystrophy alters calcium sensitivity of RetGC1 regulation and causes progressive degeneration of photoreceptors in transgenic mouse model.

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Organization of the Synaptic Signaling Cascade at the Rod Photoreceptor Synapse

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Signaling in the retina plays essential role in our vision. Light is detected by rod and cone photoreceptors that convert it to the electrical response further propagated through the retina circuitry by means of synaptic communication between neurons. To be able to see at low light levels, highly sensitive rods must faithfully transmit the signal that they generate to downstream bipolar neurons.

Our efforts have been focused on identifying and studying molecular players and signaling events at the first visual synapse formed by rod photoreceptors with ON-bipolar neurons. We have found that the key role in dictating the speed of the signal transmission at this synapse belongs to members of Regulator of G protein Signaling family-RGS7 and RGS11. Affinity purification studies identified that these RGS exist in macromolecular complex with an orphan receptor GPR179. Deletion of GPR179 in mice abolished targeting of RGS proteins to the postsynaptic site. In turn, GPR179 is physically associated with a principle neurotransmitter receptor mGluR6, which detects changes in glutamate released by rods stimulated by light. Our most recent experiments have revealed that mGluR6 is engaged in trans-synaptic interactions with ELFN1, a cell adhesion protein specifically expressed by rod photoreceptors. Deletion of ELFN1 in mice abolished synaptic transmission by rods and resulted in night blindness.

These results indicate that multiple elements of the synaptic signaling machinery are organized and scaffolded together to ensure proper transmission of signal generated by rods enabling vision at low light.

A Novel Mechanism that Drives Retinal Ganglion Cell Oscillation in a Retinal Deafferented Mouse Model

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It has recently been shown in the autosomal dominant photoreceptor degeneration rho Δ CTA mouse model and in the congenital stationary night blindness Nob mouse model that excitatory postsynaptic current (EPSC) oscillation and non-rhythmic inhibitory postsynaptic currents (IPSC) impinge on both ON- and OFF- Starburst amacrine cells (SACs) and lead to oscillation of their membrane potentials. Elimination of the well-documented gap junction network between All amacrine cell (All-AC) and ON-cone bipolar

cell (ONCB) by activating the M-type potassium channels using Flupirtine abolished oscillation in the ON-SACs but not the OFF-SACs, suggesting the existence of a novel oscillation mechanism in the retina. It remains to be determined whether this novel mechanism drives retinal ganglion cells (RGCs) to oscillate or how widely it participates in heightened inner retinal activities in retinas lacking photoreceptors or their inputs to bipolar cells. We are conducting a systematic survey of sensitivity of RGC oscillation to Flupirtine in the Nob mouse background by examining degree of RGC and SAC membrane potential variation before, during, and after Flupirtine treatment. We have found that greater than 50% of RGC oscillation can be completely blocked by and ~15% are insensitive to Flupirtine treatment. Oscillation of the remaining RGCs is partially inhibited by Flupirtine and to a varying degree. These data indicate that the All-AC/ONCB gap junction network imposes wide spread influence in the retina, while a smaller subset of RGCs are uniquely wired to be under the influence of the novel oscillation mechanism. This survey has also revealed a subset of RGC types that are completely insulated from both oscillation mechanisms.

Short Term Adaptations in Rods Triggered by Retinoid Release

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Photoreceptor physiology and biochemistry have been studied extensively for many decades, yet important subtleties remain unexplained especially in the realm of adaptation. Certain stimulus conditions can elicit massive protein translocation over tens of minutes. More relevant to short-term adaptation are the feedback changes that occur in response to changing calcium levels which result in altered phototransduction response kinetics and sensitivity. Here we present a unique form of photoreceptor adaptation that results in an increased sensitivity following sustained illumination.

Using single cell suction electrode and isolated tissue recordings of mouse or monkey photoreceptors, we described a paradoxical, light-history dependent increase in rod sensitivity. Saturating and linear range responses were measured before and after modest conditioning stimuli. Following exposures of sustained illumination of 30-300s, rods exhibit up to a 65% increase in circulating current upon return to darkness. The effect recovers with a time constant of about 7s. A similar enhancement of rod-based human perception was found with comparable

stimulation in experiments using reaction time to test sensitivity. This perceptual hypersensitivity also recovers with a time constant of 7s. Using phosphatase inhibitor and stimulators we ascertained that IGF-1 presented outside the outer segment stimulates dephosphorylation of the CNG channel enhancing its' sensitivity to cGMP. The final piece of the puzzle connecting light stimulation with hypersensitivity is all trans retinol. Exogenously applied retinol, but not retinal mimics the light-stimulated potentiation.

These findings demonstrate both a unique form of light adaptation in photoreceptors and imply a new role for retinoids in reducing in producing this adaptation. Additional psychophysical studies reveal that a similar light history enhances rod driven perception in human subject. The adaptation may enhance sensitivity in dim environments like those found in deep forest, or during specific times of day, like dawn and dusk.

RCB5 - Assembly and Maintenance of the Phototransduction Organelle

Microtubule Organization and the Early Development of Photoreceptors Outer Segments

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Purpose: Intraflagellar transport (IFT) proteins and kinesin motors are required for ciliogenesis in many cell types, including photoreceptors. Nonetheless, emerging evidence challenges the notion that IFT and associated motors are essential for ciliary maintenance even in cases where active IFT can be demonstrated. This led us to return to the earliest stages of photoreceptor outer segment (OS) development in zebrafish to evaluate the role of the homodimeric Kif17 motor. Owing to its postulated parallel role Hedgehog (Hh) signaling, we also evaluated the Costal2 homolog, Kif7.

Methods: We used a previously studied *kif17* mutant allele (*sa0119*) and used TALENs to generate a new 11bp deletion null allele of *kif17*. For *kif7* we used the CRISPR-Cas9 system to generate a 32bp insertion allele. Light microscopy and EM were used to quantify both size and number of OS and axoneme formation. EdU was used to evaluate cell cycle progression. Hh activity was measured by qPCR of *ptch1* and *ptch2*, and modulated by SAG or cyclopamine.

Results: First, we confirmed that *kif17* morphants have greatly reduced OS development at 72hpf. We also found

that both mutant alleles of *kif17* phenocopy the morphants with reduced numbers and size of OS in the central retina until recovering by 6dpf. Loss of *kif7* also reduced OS size and number prior to 6dpf. This was not due to altered Hh signaling as there was only minor upregulation in Hh activity, and drugs modulating Hh had no affect. Volumetric analysis of OS showed that initial OS morphogenesis is delayed, but occurs at a similar rate in control and mutant fish once initiated. *kif7* mutant photoreceptor precursors incorporate EdU for many hours after WT and *kif17* precursors have exited the cell cycle. In contrast, *kif17* mutants form cilia but are delayed at the stage of initiation of OS disc formation.

Conclusions: Loss of either *kif17* or *kif7* causes delayed OS formation, which recovers by 6 dpf. Despite similar phenotypes, the mechanisms underlying the delay are different for the two motors. Loss of *kif7* leads to a prolonged S-phase of the cell cycle of precursors prior to OS formation, while loss of *kif17* delays the early initiation of disc membrane formation at a time that cilia are already present. These results confirm an earlier finding that *kif17* plays a role in embryonic OS formation, but also show that once formed *kif17* is largely dispensable for OS elongation and turnover.

New Insights in the Rod Outer Segment Morphogenesis

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The mechanism of photoreceptor outer segment morphogenesis and particularly disc formation is an intensely debated topic. Evidence have been presented supporting two distinct models, one suggesting that discs are formed upon evagination of the plasma membrane at the outer segment base and another that they are formed upon fusion of intracellular vesicles. The outer segment discs can only be visualized by transmission electron microscopy. However, it is technically challenging to perfectly preserve the ultrastructure of the outer segments since the discs are densely-packed and consists of hydrophobic phospholipid membranes. Previous studies reported reported electron micrographs of the outer segment base displaying either open discs (supporting the evagination model) or closed discs (supporting the fusion of vesicles model). Some of these studies have been conducted in the same species (the mouse), which suggests that observed discrepancies are ought to be explained the differences in tissue preservation procedures.

To achieve better preservation of the outer segment

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ultrastructure, we developed a new tissue processing protocol. Mice were perfused with near isotonic fixative containing paraformaldehyde and glutaraldehyde. Retinal vibratome sections were then obtained and fixed with tannic acid followed by treatment with uranyl acetate. Sections were dehydrated, infiltrated and embedded with resin. The combination of tannic acid and uranyl acetate significantly increased the contrast of membranes. Specifically, the basal part of the outer segments was particularly well-preserved. Their membranes originate from the plasma membrane of the connecting cilia, making several zig-zag folds and then connect to the plasma membrane of the outer segment. This clearly demonstrates the presence of open discs at this location. Typically up to 8 open discs were observed at the outer segment base. No vesicles or tubular cisternae were ever observed in this region. In contrast to these well-preserved, electron-dense open discs, the enclosed discs are less densely stained and show signs of swelling. This indicates that new and old discs differ in their accessibility to fixatives and susceptibility to osmotic variations, once again consistent with the notion that new discs are open and old discs are closed.

Mechanisms of Daily Renewal of Rod Outer Segment Discs

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The specialised light sensing organelle of vertebrate photoreceptors cells is the outer segment. It contains densely stacked disc membranes that, in rods, are enclosed by the plasma membrane and are enriched in the light sensitive pigment rhodopsin. Due to the high metabolic demand of phototransduction, the distal 10 percent of the outer segment is shed daily and replenished at the outer segment base. The mechanism of transport of disc constituents to the outer segment and how they are assembled into new discs has been a subject of intense debate.

Using electron tomography to generate 3D reconstructions of the inner:outer segment interface we have shown that nascent discs form from evaginations of the plasma membrane at the base of the outer segment that mature into discrete discs by membrane fusion. Immunoelectron microscopy labelling confirmed that rhodopsin is transported to nascent discs on the ciliary plasma membrane and the leading edges of these discs are connected to the inner segment by cadherin based junctions.

As the physical dimensions of the flattened nascent discs precludes actin polymerisation as a mechanism driving disc extension, conditions favour a process akin to blebbing. Features that could facilitate this process include, close association of adjacent plasma membrane evaginations; a number of membrane attachment sites that could stabilise the blebbing process; and strong confinement between the outer segment and the periciliary ridge of the inner segment that could promote a flattened shape during blebbing.

Three-dimensional Organization of Photoreceptor Nascent Disk Membranes

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The outer segment (OS) of the vertebrate photoreceptor cell contains an extensive stack of phototransductive membrane disks. The disk membranes are continually renewed to maintain photoreceptor function. In order to gain insight into the process of disk morphogenesis, we used electron microscope tomography (ET) to obtain 3-D visualization of the nascent disks of rod and cone photoreceptors in three mammalian species. Our observations show that nascent disks are continuous with the ciliary plasma membrane, although, due to partial enclosure in certain areas, they can appear to be internal when observed in a single 2-D profile. ET analyses of the basal-most region of rod and cone OS show changes in the shape of the ciliary plasma membrane indicating an invagination, which is likely a first step in disk formation, in both types of photoreceptors. This hypothesis of an invagination as a first step is supported by ET of *Rho^{-/-}* mouse rods, which contain invaginations of the ciliary plasma membrane, but show no evidence of subsequent steps of disk morphogenesis. ET of normal rods shows that, distal to the region of disk initiation, invaginations appear flattened and yield to several lamellae that indicate growth by evagination. The formation of a membrane rim, between adjacent lamellae, was also evident. Our results support the model that the completion of rim formation fully encloses the space between adjacent surfaces of two lamellae, thus forming a mature disk, which is a discrete, internal membrane structure. In cone photoreceptors,

however, many of the lamellae remain open to the extracellular space, but, unlike the rod evaginations, discrete disks can be frequently observed within these lamellae. The present work provides a 3-D analysis of nascent and mature photoreceptor disk membranes at unprecedented z-axis resolution. These findings not only give insight into complex membrane shaping mechanisms, but also provide a basis for addressing fundamental questions ranging from protein sorting and trafficking to the OS, to photoreceptor electrophysiology.

New Approaches to Rod Structure and Disease Mechanisms

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Cilium-associated proteins and complexes and membrane trafficking have emerged as key factors in the normal functions of rod cells and in rod-centric forms of inherited retinal degeneration. Recently developed methods such as electron tomography, superresolution fluorescence microscopy, and multiple approaches to genome manipulation in animal retinas are helping to make mechanistic connections between molecules and their domains, on the one hand, and cellular function and disease on the other. Recent results from applying cryo-electron tomography, superresolution fluorescence, and genetic manipulation of phosphoinositide pathways and markers to mouse rods will be presented.

RCB6 - Oxidative and ER Stress in Retinal Degenerations

Endoplasmic Reticulum Stress in Achromatopsia

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Achromatopsia (ACHM) is an autosomal recessive disorder characterized by absence of cone photoreceptor function and progressive cone photoreceptor loss. Disease-causing variants in genes encoding components of the cone phototransduction cascade cause ~70% of ACHM. Using whole exome sequencing, we recently identified mutations in the endoplasmic reticulum (ER) stress regulator gene, *Activating Transcription Factor 6 (ATF6)*, as a novel cause of ACHM in patients. In response to ER stress, ATF6 undergoes regulated intramembrane proteolysis to

release its cytosolic bZIP transcriptional activator domain to transcriptionally upregulate genes encoding chaperones and enzymes required for ER protein folding. Here, we examined ACHM patients carrying *ATF6* mutations and functionally characterized the molecular defects of ACHM-associated *ATF6* mutations. Recombinant *ATF6* genes carrying different mutations were created by site-direct mutagenesis and expressed in HEK293 cells. Primary fibroblasts were collected from 3 ACHM families with different *ATF6* mutations. ATF6 mutants were assessed for their abilities to traffic from ER to Golgi, undergo proteolytic cleavage, and transcriptionally upregulate downstream target genes (i.e. *GRP78/BiP*). We identified 11 independent families carrying six homozygous and two compound-heterozygous disease-causing variants in *ATF6*. OCT imaging revealed that these patients had foveal hypoplasia with an essentially absent foveal pit and a variable degree of disruption of the cone photoreceptor layer at the macula. Biochemical studies grouped these *ATF6* mutations into 3 functional classes. Class 1 ATF6 mutants show impaired ER to Golgi trafficking and therefore undergo diminished regulated intramembrane proteolysis in response to ER stress. Class 2 ATF6 mutants produce truncated cytosolic ATF6 fragments with intact transcriptional activator/bZIP domains of ATF6 that constitutively upregulate downstream target genes even in the absence of ER stress. Class 3 ATF6 mutants have absent or defective bZip domains, causing loss of transcriptional activity. Fibroblasts from patients with Class 1 and Class 3 mutations showed increased susceptibility to ER stress-induced cell death. Our study identifies a crucial and unexpected role of ATF6 in human foveal development and cone photoreceptor disease. The functional diversity of ATF6 mutants suggests that multiple mechanisms may contribute to the pathology of ACHM caused by mutations in *ATF6*.

Oxidative Stress Studies Using the NaIO₃ Model of Retinal Degeneration

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Sodium iodate (NaIO₃) is an oxidizing agent known to induce preferential degeneration and atrophy of RPE cells with secondary effects on photoreceptors and the choriocapillaris. We used this model to gain mechanistic insights into oxidative stress-related RPE degeneration in a concentration dependent manner. Mice (C57BL/6J and DJ-1 KO) received a single tail vein injection of

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0, 10, 15 and 20mg/kg body weight of NaIO₃; parallel groups of mice were injected with PBS. Histological and immunohistological evaluation of the retinas of mice were performed in toluidine blue section, cryosections as well as whole-mounted retina and RPE/choroid 7 days post injection. Retina/RPE lysates were assayed for oxidation using antibodies. Injection with PBS and 10mg/Kg NaIO₃ did not result in morphological retinal and RPE degeneration. Injection with 20mg/Kg NaIO₃ resulted in significant degeneration of the RPE and photoreceptors; the subretinal space was filled with the presence of immune cells filling the central area of the retina. We further analyzed the progression of the NaIO₃-induced degeneration in westerns from retina/RPE lysates reacted with the antibodies that recognized the C106-SO3H DJ-1, and DJ-1. Immunoblots of these lysates revealed the increased presence of DJ-1 and C106-SO3H DJ-1 in mice injected with 10 and 20mg/Kg NaIO₃. Quantification of the immunoreactivities detected statistically significant increase of C106-SO3H DJ-1 in mice injected with 20mg/Kg NaIO₃. Analysis RPE/choroid flatmounts labeled with phalloidin-TRITC showed that injection of both control and DJ-1 KO mice with 20mg/Kg NaIO₃ resulted in significant RPE degeneration. The increased susceptibility of the DJ-1 KO mice to oxidative stress was confirmed by measurements of the area of RPE degeneration in the RPE/choroid flatmounts using ImageJ software. The mean degenerated RPE area of the NaIO₃-injected control and DJ-1 KO mice was 38.9 and 63.8 μm², respectively; these differences were statistically significant. Our results suggest that injection of mice with increasing concentrations of NaIO₃ leads to progressive signs of retinal/RPE degeneration in association with increased levels of oxidative stress markers. Our data also suggests that lack of DJ-1 renders the RPE susceptible to increased degeneration after exposure to NaIO₃.

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Targeting Oxidative Stress in the Retina Using Gene and Drug Therapies

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Age related damage caused by reactive oxygen species is believed to contribute to the damage to the retinal pigment epithelium (RPE) and choroid leading to the development of age related macular degeneration (AMD). To model this process in a short-lived animal and to test

the hypothesis that mitochondria are a source of RPE oxidative stress, we generated a mouse model in which *Sod2*, the gene for mitochondrial superoxide dismutase, is deleted in the RPE. These mice exhibit some of the salient features of dry AMD including accumulation of lipofuscin, disorganization of Bruch's membrane, atrophy of RPE cells and death of associated photoreceptors. We have used this model to test drug and gene therapies for geographic atrophy, the advanced form of dry AMD. Drug therapies included an orally available 5-hydroxytryptamine 1a receptor agonist that prevented vacuolization of the RPE and increased photoreceptor survival in the *Sod2* deletion model. Gene therapies used adeno-associated virus (AAV) vectors to deliver secreted, cell penetrating peptides to block the activation of the NLRP3 inflammasome or to induce the production of antioxidant enzymes in the RPE. These reduced inflammatory stress in the retina in the endotoxin induced uveitis model and protected the retina in an acute model of oxidative injury caused by injection of sodium iodate. AAV expressing a caspase activation and recruitment domain (CARD) peptide reduced the rate of retinal degeneration in the *Sod2* deletion model of geographic atrophy. We anticipate that the both pharmacological and the gene delivery approaches will have benefit in preventing advanced dry AMD in human patients.

Inhibition of Oxidative and ER Stress by the Mitochondrial-derived Peptide Humanin

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Humanin (HN) is a small mitochondrial-encoded peptide with neuroprotective properties. We have recently shown protection of primary human retinal pigmented epithelium (RPE) cells by HN in oxidative stress (*Invest Ophthalmol Vis Sci* 2016 57:1238-1253); however, the effect of HN on endoplasmic reticulum (ER) stress has not been evaluated in any cell type. HN co-treatment inhibited tert.butyl hydroperoxide (tBH)-induced reactive oxygen species formation and significantly restored mitochondrial bioenergetics in RPE cells. Exogenous HN was rapidly taken up by RPE and co-localized with mitochondria. The oxidative stress-induced decrease in mitochondrial bioenergetics was prevented by HN co-treatment. HN treatment increased mitochondrial DNA copy number and upregulated mitochondrial transcription factor A (mtTFA), a key regulator of mitochondrial biogenesis. HN protected RPE cells from oxidative stress-induced cell

death by STAT3 phosphorylation and inhibition of caspase 3 activation. Polarized RPE demonstrated elevated cellular HN and increased resistance to oxidant-induced cell death. Subsequently, we evaluated the effect of HN on ER stress-induced apoptosis in RPE cells with a specific focus on ER-mitochondrial cross-talk. Confluent primary human RPE cells were treated with tunicamycin (TM) (10 µg/ml) for 12 hours with or without HN (10 µg/ml). We found that HN co-treatment significantly decreased both the number of apoptotic cells, and activation of caspase 3 compared to TM alone. HN co-treatment significantly attenuated the expression of caspase 4 induced by TM. We showed that TM treatment lead to increased mitochondrial superoxide production, and that HN co-treatment resulted in a decrease in mitochondrial superoxide compared to TM treatment alone. We further showed that depleted mitochondrial glutathione (GSH) levels induced by TM were restored with HN co-treatment. These results demonstrate that ER stress promotes mitochondrial alterations in RPE that lead to apoptosis. We further show that HN has a protective effect against ER stress-induced apoptosis by restoring mitochondrial GSH. Thus, HN should be further evaluated for its therapeutic potential in disorders such as age-related macular degeneration that are linked to oxidative and ER stress.

Manipulating ER Chaperones and the UPR for Retinal Neuroprotection

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Endoplasmic reticulum (ER) stress has been linked to neuronal degeneration and vascular injury in retinal diseases such as diabetic retinopathy, age-related macular degeneration, glaucomatous retinopathy and various forms of retinal degeneration. Increased ER stress activates the unfolded protein response (UPR), a set of highly conserved cellular signaling pathways, to restore the homeostasis of the ER in normal cells. In contrast, unmanageable ER stress results in cell death and dysfunction eventually leading to neurovascular degeneration. ER chaperones are a group of proteins in the ER that assist in de novo protein folding/refolding and the timely removal of irreversibly misfolded and aggregated proteins. They serve as the critical components and major downstream targets of UPR to reduce/eliminate ER stress. Major chaperones and folding enzymes in the ER include:

1) chaperones of the heat shock protein family, such as GRP78 and its co-chaperone partners

(e.g., p58^{IPK});

2) chaperone lectins, such as calnexin and calreticulin; and
3) thiol oxidoreductases of the protein disulfide isomerase (PDI) family.

Recent work demonstrates that manipulating the UPR or enhancing ER chaperone activities can protect retinal neurons in a wide range of acute or chronic injury models. Overexpression of GRP78, p58^{IPK} or ERp29 attenuates ER stress and protects retinal cells against apoptosis and cell death. Mice lacking p58^{IPK} are more sensitive to ER stress and RGC loss related to glutamate toxicity or aging. The presentation will discuss the intriguing neuroprotective potential of ER chaperones and UPR manipulation in retinal neurons and RPE cells and highlight their probable implication in the prevention and treatment of retinal degeneration.

RCB7 - Mouse to Human: Modeling AMD

Novel Multigenic Genetic Mouse Model for AMD

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Age related macular degeneration (AMD) is a leading cause of severe visual impairment in the aging population. AMD is a complex disease with many genetic and environmental factors contributing to a pathological disease outcome. Current genetic models of AMD focus on analyzing single gene effects or inducing disease phenotype to recapitulate the neovascularization or drusen deposit formation found in patients with AMD. We identified a novel genetic model, neoretinovascular 2, Nrv2 that exhibits choroidal neovascular disease. A mapping cross was generated to perform a genome scan using >200 F2 animals using the C56BL6/J and AKR/J strains of mice. F2 affected animals segregated in two cohorts with distinct differences in severity of the disease phenotype. Initial mapping identified 4 loci associated with Nrv2 disease phenotype. Comparative gene analysis with human AMD expression data identified the AMD pathways that are affected in Nrv2. This is the first genetic model of AMD with multigenic inheritance pattern.

The Aging Transcriptome in Health and Disease of

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the Retina

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The identification of allele-specific expression (ASE), utilizing genome wide level transcriptomic data of primary tissues combined with genome wide level genotype data, can be a crucial step in elucidating disease mechanism in complex disease. Age-related macular degeneration (AMD) is the leading cause of blindness in the aging population for which there is no cure. We carefully characterized the macular retinal pigment epithelium (RPE), choroid, and neural retina in fresh tissue in both eyes from unrelated donors, using postmortem OCT and/or retinal imaging. Whole transcriptome analysis showed that within the macular RPE tissues, expression was significantly different between different stages of AMD compared to normal age matched controls. The top 10 genes in each of these disease stage comparisons function in neuro-degenerative diseases, Vitamin B12 metabolism, lipid metabolism, and apoptosis. ASE analysis identified 78 single nucleotide polymorphisms (SNPs) that showed significantly different ASE between disease subtypes. Thirty of these SNPs (38%) are located in coding regions of which 22 are synonymous and 8 are non-synonymous changes. Many of the deleterious changes occur in genes primarily involved in lipid transport and metabolism.

Energy Metabolism of Photoreceptors Drive Pathological Angiogenesis

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Tissues with high metabolic rates often use lipid as well as glucose for energy. Current dogma suggests that high-

energy consuming photoreceptors depend exclusively on glucose. We have recently shown that retina also uses fatty acid (FA) beta-oxidation for energy. Very low-density lipoprotein receptor (VLDLR), expressed in tissues with high metabolism, facilitates the uptake of triglyceride-derived FA. VLDLR is present in photoreceptors. In *Vldlr*^{-/-} retina, impaired lipid uptake results in a fuel shortage, HIF1 α stabilization and vascular endothelial growth factor (VEGF) secretion by starved *Vldlr*^{-/-} photoreceptors, attracting neovessels to reinstate energy homeostasis. These aberrant vessels invading normally avascular photoreceptors seen in *Vldlr*^{-/-} retinas are reminiscent of retinal angiomatous proliferation (RAP), a subset of neovascular age-related macular degeneration (AMD). Dysregulated photoreceptor energy metabolism may therefore be a driving force in neovascular AMD and other retinal diseases.

Cholesterol Esterification in Photoreceptors: Retinal and Clinical Significance in Mice and Humans

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Photoreceptor cells, rods and cones, have both unique maintenance and unique distribution of cholesterol, which forms a gradient in these cells with a higher sterol concentration at the inner segment-outer segment junction and a lower cholesterol concentration at the tip of the outer segments. Here we report on the previously unrecognized cholesterol-related pathway in the retina discovered during characterizations of *Cyp27a1*^{-/-}*Cyp46a1*^{-/-} mice. These animals have retinal hypercholesterolemia and convert retinal cholesterol excess into cholesterol esters, normally present in only very small amounts in the retina. We found that in the *Cyp27a1*^{-/-}*Cyp46a1*^{-/-} retina, cholesterol esters are mainly generated by and accumulate in the photoreceptor outer segments, the retinal layer with normally the lowest cholesterol content. Mouse outer segments were also found to express the cholesterol-esterifying enzyme ACAT1, but not LCAT. Mouse retina was compared to human retina for several aspects of cholesterol esterification. Both similarities and differences were found with the latter mainly pertaining to retinal expression of ACAT1. We suggest that activity impairment of ACAT1 in humans may be of pertinence to the biogenesis of subretinal drusenoid deposits, a hallmark of age-related macular degeneration. We generated

and began assessments of *Cyp27a1^{-/-}Cyp46a1^{-/-}Acat1^{-/-}* mice as a possible model for the formation of subretinal drusenoid deposits. Our results provide insight into the retinal response to local hypercholesterolemia and retinal significance of cholesterol esterification.

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RCB8 - Retinoids in vision

New Insights into RPE65's Mechanism

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The retinal pigment epithelium (RPE) retinoid visual cycle isomerase RPE65 is a unique member of the carotenoid oxygenase family that does not oxidatively cleave carbon-carbon double bonds but instead has evolved into an all-trans retinyl ester (RE): 11-cis retinol isomerase. Implicit in this description are both an isomerase activity and an O-alkyl ester cleavage function, suggesting a complex mechanism. To date, the isomerase functionality, supplying 11-cis retinol, is the better characterized aspect, with the ester cleavage aspect less well so. This is despite the fact that the primary bond cleavage activity of RPE65 is the O-alkyl cleavage catalyzed by the non-heme iron cofactor. Previously, we showed that the aromatic side chain-rich environment of the RPE65 substrate-binding cleft is responsible for favoring an 11-cis specific outcome, and mutation of many of these residues decreases 11-cis ROL synthesis but increases formation of 13-cis isomer due to reduced stringency, supporting a carbocation/radical cation mechanism of retinol isomerization. To explore other aspects of the mechanism, we have investigated the role of RPE65 palmitoylation, an area of contention in RPE65 biochemistry. C112 is the only cysteine residue identified as being palmitoylated in RPE65, but different groups confirm or deny the existence of C112 palmitoylation. By acyl-exchange labeling experiments we detect a very low level of RPE65 palmitoylation. Unlike structurally palmitoylated rhodopsin (100% palmitoylated), RPE65 shows incomplete palmitoylation (3-5%). The level of palmitoylation depended on whether RPE65 was co-expressed with LRAT or not, but the functional importance of this difference remains unclear. Our findings negate a putative structural role for palmitoylation, such as in rhodopsin. We conclude

that RPE65 is dynamically palmitoylated with significant turnover of the acylation. Given the apparent ability of RPE65 to be both a cytosolic and a membrane-associated protein, a dynamic palmitoylation may help explain this situation.

Neonatal and Developmental Visual Consequences in the IRBP Knockout Mouse Eye

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Purpose: Interphotoreceptor retinoid-binding protein (IRBP) is the most abundant soluble protein of the subretinal space. It governs levels of free retinoids in the visual cycle, and may play developmental roles due to its early expression and specific binding of morphogens. Illustrating this, we found eye size defects, profound myopia, photoreceptor (PhR) degeneration, and premature development of OPL in IRBP knockout (KO) mice. Here we sought to discover developmental metrics lost without IRBP, contrasting with form-vision dependent emmetropia and normal pupillary responses.

Methods: SD-OCT (Bioptigen R4310) measured axial length (AL), central corneal thickness (CCT), anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD), and retinal thickness (RT) in C57BL/6J (WT) and congenic IRBP KO mice. Pupil diameter was measured using a custom photorefractometer in awake and anesthetized states, with or lacking mydriasis at different light levels. Conventional IHC methods were used. ANOVA with post hoc or unpaired t-tests were used to assess statistical significance among or between groups.

Results: At P30 and P55, the VCD was significantly deeper in IRBP KO by more than 350 microns vs WT, $p < 0.0001$, with parallel increase in AL, $p < 0.0001$. ACD, CCT, and LT were the same in WT and KO. RT was thinner in the KO than WT, $p < 0.01$. There were no notable changes in morphology by immunostaining with CHaT, calretinin, calbindin, beta-III tubulin, or Opn1sw. There was much more GFAP staining in the KO vs WT at P30. The OPL was mature at P5 in the KO, while immature and poorly formed in WT. Melanopsin+ RGCs were normal in number but qualitatively arborization was less extensive in the KO vs. WT. Under anesthesia, pupil diameter differed significantly ($p=0.01$) post-mydriatic

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application. Maximum pupil dilation was less in KO vs WT ($p=0.003$) in response to mydriatic application.

Conclusions: The increase in VCD is the primary contributor to profound myopic shift (14 D) in IRBP KO mice. Loss of PhRs and *Opn4*⁺ RGC arborization contribute to a sluggish pupil. IRBP is essential for normal rates of eye development, maintenance of PhRs, eye size, and pupillary responses. Also, lack of IRBP allows premature development of the OPL, possibly by premature interactions between PhRs and secondary neurons.

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Pineal Photoreception Involving Bistable Pigments, Parapinopsins in Lower Vertebrates

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In non-mammalian vertebrates, the pineal organs contain photoreceptor cells and receive light independently of eyes. The pineal organs of lampreys and teleosts detect the ratio of ultraviolet (UV) light to visible light; that is, they have the ability of wavelength discrimination, similar to the pineal related organs, the frog frontal organ and lizard parietal eye. We found that parapinopsin, which was originally identified in the catfish pineal and parapineal organs, is a UV-sensitive pigment underlying the wavelength discrimination in the lamprey pineal organ [1]. Interestingly, parapinopsin has some unique molecular properties different from those of visual opsins [1-3]. In addition, we identified the parapinopsin gene expression in the pineal and related organs of various non-mammalian vertebrates [1,4,5]. However, a visible light-sensitive opsin involved in the wavelength discrimination has not been still uncovered and it is important to identify it to understand the mechanism and evolution of the pineal wavelength discrimination and compare them with those of eyes. We investigate the visible-sensitive opsin molecular biologically, biochemically, histochemically and electrophysiologically in some lower vertebrates. We found a candidate opsin not only in the lamprey pineal but also in some teleost pineals and revealed they were green-sensitive. On the other hand, we also found a blue sensitive-parapinopsin (PP2) in addition to the UV-sensitive parapinopsin (PP1) in some teleosts [5]. Interestingly, PP2-expressing cells contain serotonin and *aanat2*, the key enzyme involved in melatonin synthesis from serotonin, whereas PP1-expressing cells do not contain either, suggesting that blue-sensitive PP2 is instead involved in

light-regulation of melatonin secretion. Based on these findings, pineal photoreception involving parapinopsins in the lamprey and teleost is discussed.

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The Effects of 11-cis Retinal and Retinal Analogues on Cone Photoreceptor Survival

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Mutations in the protein RPE65 are associated with type 2 Leber congenital amaurosis (LCA2). These mutations result in impaired generation of 11-cis retinal, the retinoid used by visual pigment proteins for light sensitivity. In the absence of 11-cis retinal, LCA2 patients and animal models for the disease display early loss of cone photoreceptor cells. Previous studies supplementing mice lacking RPE65 (*Rpe65*^{-/-}) or both rhodopsin and RPE65 (*Rho*^{-/-}*Rpe65*^{-/-}) with exogenous 11-cis retinal in the dark could preserve cone photoreceptor cell morphology and cell number; however, these improvements were not seen under cyclic light conditions. We have tested a number of truncated retinal analogues and an 11-cis-ring-locked retinal analogue for their effects on improving cone photoreceptor cell survival in the Rhodopsin/RPE65 double knockout mouse. Beta ionone selectively improved M-cone opsin-containing cells; whereas, cyclocitral improved both M- and S-cone cell survival. Mice were treated at postnatal day 9 (P9) with retinoid analogue in Matrigel, which allowed for continual slow release of compound systemically; the eyes were enucleated at P30, and retinas flat-mounted. Cones were probed with antibodies against M- and S-cone opsins and counted. When survival was improved, the cone cell density was about 2-fold higher than mock-treated mice. The 7-membered ring-locked version of 11-cis retinal also improved cone cell survival under cyclic light conditions over mock-treated mice. With this retinoid, cone cell counts improved 2.5 to 3-fold. These results suggest that ligand binding to cone opsins helps stabilize a protein conformation that either favors a folded cone pigment that aids in the proper packaging and transport of cone pigment proteins to the outer segments.

Seeing (Infrared)Red**JOSEPH CORBO***Washington University School of Medicine, St. Louis, United States*

Some vertebrate species have evolved means of enhancing their color vision and extending it beyond the human range. In this talk, I discuss two such evolutionary adaptations. First, I describe how fish convert vitamin A₁ into A₂ to extend their vision into the infrared. This conversion is achieved by the expression of Cyp27c1, a member of the cytochrome P450 family of enzymes. Knockout of Cyp7c1 in zebrafish abrogates production of vitamin A₂, reducing the animal's ability to see and respond to infrared light. In the second part of the talk, I show how birds enhance their ability to discriminate red colors by depositing ketocarotenoids in the oil droplet of their red cone photoreceptors. This clever adaptation is mediated by expression of another P450 enzyme, CYP2J19, which converts yellow yolk carotenoids into red ketocarotenoids specifically in the red cone. Interestingly, we find that this same enzyme mediates the formation of the ketocarotenoids used by red birds, such as the cardinal, to pigment their feathers. Taken together, these studies demonstrate a previously unanticipated role for cytochrome P450 family members in enhancing vision in the red and infrared portions of the spectrum.

JNT4 (RCB+RPE) - Understanding Diabetic Retinopathy and AMD through animal models**Three Dimensional Retinal Cultures for Evaluation of Neuronal Cell Death and Regeneration****TOSHIYUKI OSHITARI***Chiba University Graduate School of Medicine, Chiba, Japan*

Neuronal abnormalities including death are irreversible changes and are directly related to the permanent reduction of vision in diabetic patients. At present, the precise mechanisms underlying the development and the progression of neuronal abnormalities in diabetic retinas remain unknown. We have been studying the mechanisms of diabetic neuropathy in the retina with the expectation that the information will help in developing neuroprotective and regenerative therapies for diabetic retinopathy.

A three-dimensional (3D) collagen gel culture system is a useful method to evaluate neuronal death and regeneration in isolated retinas. High glucose (HG) and advanced glycation end-products (AGEs) in the media can simulate diabetic stress in vitro. In HG or AGEs exposed

retinas, the number of TUNEL-positive cells in the ganglion cell layer is significantly higher than in control retinas, and the number of neurites is significantly fewer than in controls. Immunostaining for apoptosis-related factors indicate that both mitochondria-caspase-dependent and endoplasmic reticulum stress-related cell death pathways are associated with the neuronal cell death in HG- or AGEs-exposed retinas. Several neurotrophic factors such as brain-derived neurotrophic factors, neurotrophin-4 (NT-4), hepatocyte growth factor, glial cell line-derived neurotrophic factor, tauroursodeoxycholic acid, and citicoline have neuroprotective and regenerative effects for damaged neurons in HG-or AGEs-exposed retinas. NT-4 has the most neuroprotective and regenerative effects among these neuroprotectants.

Increased expression of several cell death-related factors such as Bax, caspase-9, caspase-3, c-Jun, and c-Jun N-terminal kinase (JNK) in cultured retinas are also expressed in the degenerated neurons in retinas from diabetic patients. These findings indicate that neuroprotectants which are effective in cultured retinas may be useful for neuroprotective and regenerative therapies for diabetic neuropathy in the retina of diabetic patients.

Characterization and Whole Genome Analysis of Cynomolgus Monkeys with Hereditary Macular Drusen**AKIKO SUGA¹, Mao Nakayama¹, Zai-Long Chi¹, Nobuhiro Shimozawa², Kazuhiro Yoshitake³, Takeshi Iwata¹***¹National Institute of Sensory Organs, Tokyo Medical Center, Laboratory for Molecular and Cellular Biology, Tokyo, Japan, ²National Institute of Biomedical Innovation, Tsukuba Primate Research Center, Tsukuba, Japan, ³Japan Software Management, Yokohama, Japan*

Drusen are extracellular debris located between the retinal pigmented epithelium (RPE) and the Bruch membrane. Accumulation of macular drusen is detected in several macular dystrophies, especially at the early stage of dry type age-related macular degeneration. We have reported about a cynomolgus macaque monkey (*Macaca fascicularis*) pedigree affected by early onset drusen accumulation in the macula (Umeda et al., IOVS 2005). In addition to the similarity of drusen constitution between these monkeys and human, affected monkeys with severe drusen accumulation showed decrease of focal electroretinogram response, suggesting a visual impairment (Umeda et al., J FASEB 2005). To characterize the pathology and genetic cause of this inherited symptom,

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we examined the difference between the primary RPE cells isolated from drusen-affected monkeys and healthy controls, and performed genetic studies using the whole exome/genome sequencing data obtained from 12 affected monkeys and 9 controls. Drusen-affected RPE cells showed poor tight junction formation, decreased phagocytosis, and different expression levels of immune-related genes. Linkage analyses resulted cosegregation of genes with drusen accumulation. Some of these segregated genes had insertions/deletions in their introns, as well as missense mutations.

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Retinal Pathology in a Novel Primate Model of Diabetic Retinopathy

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Diabetic retinopathy (DR) is the leading cause of blindness in the working-age population. Unfortunately there is no cure for this devastating ocular complication. Research towards better understanding of the pathogenesis of DR and development of new therapies has relied heavily on animal models. Currently, the lack of an efficient animal model of DR has presented a major drawback in understanding the pathogenesis of DR and developing novel treatment strategies. Currently the lack of animal models exhibiting full pathology of DR has impeded both our understanding of the complex mechanisms underlying the disease pathogenesis as well as the development of novel therapeutic strategies. Although various animal models of DR seem to respond with comparable patho-anatomical changes to those of human DR under induced diabetes, they still fail in reproducing the human aspects of a full spectrum DR. A further major drawback is the lack of macula in most current animal models for the study of diabetic macular edema. Towards this direction, newer studies in a small non-human primate have demonstrated promising results giving a new perspective to this non-human primate model. The use of primate models of DR could offer a distinct advantage in light of their close phylogenetic relationship with the humans. The common marmoset is a popular new world monkey, which has been used to study new treatments for various diseases but not for DR. To determine the suitability of this primate as a potential model for the study of DR, we have examined

some of the key anatomical and structural characteristics of the retina. The retinal vascular morphology of this primate appears to be very similar to that of the human eye, including the intraretinal branching pattern and the tortuosity index. In addition, quantitative analysis of pericyte (P) and endothelial cell (E) counts from retinal capillary network showed similar E/P ratio to those of human retina. Importantly, we have identified a distinct nonvascular macula in the retina of these primates. Furthermore, in this primate, hyperhexosemic condition induced vascular lesions characteristic of human DR, including retinal capillary basement membrane thickening, pericyte loss, acellular capillaries, and retinal thickening as determined by optical coherence tomography. Overall, the findings indicate that the hyperhexosemia-induced marmosets holds promise as a potential primate model for studying retinal lesions associated with DR.

Restoration of Lysosomal Function in Animals Models of Age-related Macular Degeneration

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Age-related macular degeneration (AMD) is a progressive degenerative disease of the eye and is the most common cause of visual loss among the elderly. Atrophic degeneration of retinal pigment epithelial (RPE) cells is a major contributor to the pathogenesis of AMD. Lysosomes are crucial for proper functioning of the RPE, since these cells are subjected to the daily burden of phagocytosis of photoreceptor outer segments and autophagic degradation of cellular metabolic waste. It has also been shown that mouse models of inherited lysosomal storage diseases show striking signs of AMD, including accumulation of lipofuscin, loss of RPE cells, and degeneration of photoreceptor outer segments. Incomplete lysosomal degradation and concomitant accumulation of undigested or partially digested intracellular material is thought to contribute to lipofuscin formation and RPE cell dysfunction in AMD. We investigated the pivotal role of transcription factor EB (TFEB), a master regulator of lysosomal function in the RPE. TFEB is a member of the basic helix-loop-helix leucine-zipper family of transcription factors that controls lysosome biogenesis and autophagy by upregulation of a family of genes belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) network. In normal RPE cells, we observed that alkalization of lysosomes, starvation and inhibition of the mTOR pathway by treatment with rapamycin resulted in

upregulation of genes in the CLEAR network. Furthermore, we investigated the role of TFEB in *Cryba1* (gene encoding β A3/A1-crystallin) conditional knockout (cKO) mouse model that shows abnormal lysosomal clearance leading to impaired phagocytosis and autophagy in the RPE. These mice show scattered drusen-like lesions, hypervacuolation due to accumulation of undigested photoreceptor outer segments and loss of retinal function. In the *Cryba1* mouse model, reduced activity of TFEB is strongly correlated with impaired lysosomal function, decreased transcription of cellular genes in the autophagy and lysosomal pathway and increased cellular accumulation. Our data also suggest that impaired lysosomal function is associated with induction of apoptotic pathways in the cKO RPE. The *Cryba1* cKO mice has been a valuable model to study the signaling mechanisms that regulate lysosomal function and thereby influence cellular clearance mechanisms in the RPE. In summary, our studies show that lysosomal function is crucial for normal homeostasis of the RPE.

MTORC1 AMD: Insights from a Genetically Engineered Mouse Model

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Age-related macular degeneration (AMD), a leading cause of blindness in the elderly, has few treatment options at present. AMD is a complex and progressive degenerative eye disease involving multiple genetic and environmental factors that can result in severe loss of central vision. It is characterized by early accumulation of cellular waste products in the retinal pigment epithelium (RPE), making rejuvenating impaired lysosome function in these cells a well-justified target for treatment.

It is now clear that amino acids and vacuolar-type H⁺-ATPase (V-ATPase) regulate signaling through the mechanistic target of rapamycin, complex 1 (mTORC1) in lysosomes. Here, we provide evidence that a proton dependent amino acid transporter regulates the amino acid pool in the lysosomes of RPE. In *Cryba1* (gene encoding β A3/A1-crystallin) KO (knockout) mice with abnormal function of the lysosomal machinery, the transcription factor EB (TFEB), is retained in the cytoplasm. Therefore, expression of genes in the coordinated lysosomal

expression and regulation (CLEAR) network that mediate lysosomal function and autophagy is markedly reduced in *Cryba1* KO compared to controls. As these mice age, the RPE cells also lose expression of two vital visual cycle proteins, RPE65 and lecithin retinol acyltransferase (LRAT). This slows down the regeneration of new photoreceptor outer segments, leading to photoreceptor degeneration, reminiscent of human dry AMD disease. For AMD patients, targeting the lysosomal machinery in the RPE may be an effective means of preventing disease or slowing its progression. While effective treatment has been developed for advanced wet AMD and intermediate, dry AMD, currently, there is no treatment or prevention for early dry AMD. A new treatment that targets early disease, at a stage before vision is lost, would have the greatest benefit. In this study, we report a novel function for β A3/A1-crystallin, a protein shown to be essential for normal lysosomal function in the RPE. It is an upstream regulator of the V-ATPase/mTORC1/TFEB signaling axis in the lysosomes of RPE cells. This pathway is currently a prominent target for therapeutic intervention in various age-related diseases. For AMD patients, targeting β A3/A1-crystallin in the RPE may be an effective means of preventing, or delaying the progression, of the disease.

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RND1 - Transcription control of retinal cell identity

Vexin (Vxn) Is a Novel Conserved Protein that Functions in the Nucleus to Regulate Cell Cycle Exit and Neurogenesis

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During development of the retina, neurogenesis depends upon the activity of proneural basic helix-loop helix transcription factors that promote the expression of target genes required for cell cycle exit and differentiation. But which proneural target genes mediate these effects is incompletely understood. We identified *vexin (vxn)* as a novel target gene for proneural basic helix-loop helix transcription factors in *Xenopus laevis*. *Vxn* is expressed by progenitors in the developing *Xenopus* nervous system, is conserved across vertebrate species, and encodes a novel 207 amino acid protein with a predicted SH3-family motif. To assess the function of *vxn* during retinal neurogenesis in *Xenopus*, we targeted retinal progenitors and performed RNA overexpression or knockdown using antisense *vxn* morpholinos (MO). We analyzed cell cycle exit, retinal neuron differentiation and changes in gene expression, and assessed subcellular location of Vxn protein.

Vxn is transiently expressed as retinal progenitors initiate differentiation. Overexpression of either mouse or *Xenopus vxn* in retinal progenitors promoted increased differentiation of early born retinal neurons, and also strongly cooperated with the proneural bHLH factor *Atoh7* to enhance retinal ganglion cell differentiation. Conversely, MO knockdown of *vxn* inhibited retinal neuron differentiation, preferentially maintaining cells as retinal progenitors and reducing expression of terminal differentiation genes. The effects were not due to modulation of Notch signaling. Overexpression of *vxn* caused reduced retinal progenitor expansion resulting in reduced clone size. At neural plate stages, *vxn* overexpression inhibited cell proliferation and promoted increased expression of the cyclin-dependent kinase inhibitor p27^{Xic1}. Both exogenously expressed epitope-tagged mouse or *Xenopus Vxn* protein, as well as endogenous Vxn protein, localized to the cell membrane and the nucleus. By generating forms of Vxn tagged with either nuclear localization or nuclear export sequences

we showed that Vxn must localize to the nucleus to promote cell cycle exit and neural differentiation. Thus *vxn* is expressed in progenitors as they initiate neuronal differentiation, and appears to function in the nucleus to regulate cell cycle exit downstream of proneural bHLH factors during neural development.

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Sox2, Tlx, Gli3 and Her9 Converge on Rx2 to Define Retinal Stem Cells *in vivo*

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Transcriptional networks defining stemness in adult neural stem cells (NSCs) are largely unknown. We studied that topic in the retina load the teleost medaka (*oryzias latipes*) and used the proximal *cis*-regulatory element (pCRE) of the *retina-specific homeobox gene 2 (rx2)* as a starting point to address such a network. We have identified *rx2* by long-term lineage analysis in the fish retina as marker for multipotent NSCs. Strikingly, *rx2* positive cells in the ciliary marginal zone either behave as neuroretinal stem cells, or as stem cells for the retinal pigmented epithelium (RPE). We identified upstream regulators of *rx2* by interrogating the *rx2* pCRE in a *trans*-regulation screen and identified four TFs (Sox2, Tlx, Gli3, Her9) that activate or repress *rx2* expression. We characterized their respective binding sites on the *rx2* pCRE and demonstrated the direct interaction with the four factors *in vitro* and *in vivo*. We applied conditional mosaic gain- and loss-of-function analyses validating the regulatory activity of those factors on *rx2* transcription in particular and the modulation of neuroretinal and RPE stem cell features in general. Finally we have established *rx2* mutant alleles and the mutant phenotype together with the data presented above identify *rx2* as a transcriptional hub for a gene regulatory network balancing stemness of neuroretinal and RPE stem cells in the adult fish retina. Our presentation will be complemented by the analysis of *rx2* downstream factors identified by DAM-ID of the *rx2* fusion protein *in vivo*.

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Toward Understanding Regulation of Middle Wavelength-sensitive Cones and the Opsins in Zebrafish**YOSHITAKA FUKADA***Univ. Tokyo, School of Science, Dept. of Biological Sciences, Tokyo, Japan*

Color discrimination in vertebrates requires multiple cone photoreceptor cells present in the retina, and high-acuity color vision is established by a set of four cone subtypes expressing UV-, blue-, green- and red-sensitive opsins. Previous studies identified several transcription factors governing the cone photoreceptor development in mice, but loss of two genes encoding middle wavelength-sensitive opsins, blue and green, in the evolution of mammals makes it difficult to understand how the high-acuity colour vision was organized during evolution and development. Zebrafish (*Danio rerio*) represents a valuable vertebrate model for studying color vision as it has all the four ancestral vertebrate cone subtypes. We performed fluorescent-activated cell sorting of rod and cone cells from two transgenic lines of zebrafish expressing EGFP specifically in rods and cones, respectively, and our transcriptome analysis identified genes expressed in a rod or cone-specific manner. We found that *sine oculis* homeobox homolog 7 (*six7*), a transcription factor widely conserved in ray-finned fish, is expressed predominantly in the cone photoreceptors in zebrafish at both the larval and adult stages. TALEN-based *six7* knock-out revealed that *six7* is indispensable for the expression of the green cone opsin genes. In addition, the number of blue cones was reduced by half in *six7* knock-out. In this way, we concluded that *six7* is a key player in the development and/or maintenance of the middle-wavelengths-sensitive cones. Further studies are underway to search for transcriptional factors that are involved in the development of the other half of blue cones.

Transcriptional Regulation of Photoreceptor Cell Development and Maturation**TAKAHISA FURUKAWA, Yoshihiro Omori, Shun Kubo, Mayu Furuhashi***Osaka University, Institute for Protein Research, Suita, Japan*

In vertebrate retinal development, five major types of neurons (photoreceptor, bipolar, horizontal, amacrine, and ganglion cells) are generated from common progenitor cells. In this process, combinations of transcription factors create elaborate gene expression regulatory networks to

control cell fate determination, maturation, and survival of neurons and glial cells. Photoreceptor cells develop cell type-specific unique structures, including ribbon synapses and photosensitive outer segments. Thus, photoreceptor cell maturation is important for correct retinal circuit formation as well as photoreception. We have been investigating molecular mechanisms of photoreceptor cell fate determination, maturation, and survival by focusing on functional roles of transcription factors, including Otx2, Crx, Rax, Blimp1, and Mef2d. We will present our recent findings on molecular mechanisms underlying transcriptional regulation of photoreceptor cell development and maturation.

Regulation of Retinal Progenitor Cell Properties by Lhx2 and Vsx2**EDWARD LEVINE***Vanderbilt University Medical Center, Nashville, United States*

Lhx2 and Vsx2 are homeodomain-containing transcription factors expressed in retinal progenitor cells. We have uncovered a strong genetic relationship between the two factors that effectively places Lhx2 upstream of Vsx2. However, each factor has distinct roles in regulating regional identity, proliferation, and neurogenesis in the retinal progenitor cell population. In this talk, I will describe some of our recent findings in this regard and provide an example of how these factors differentially impact important signaling pathways critical for retinal tissue growth and differentiation.

RND2 - Epigenetics in development and disease**Retinal Cell Lineage Specific Modification of Histone H3 during Retinal Development****SUMIKO WATANABE***Institute of Medical Science, University of Tokyo, Department of Molecular and Developmental Biology, Tokyo, Japan*

We are interested in regulation of retinal development and maintenance by Histone modification. Among various Histone modification, Histone H3K27me3 is a negative marker of transcription, and Ezh2 and Jmjd3 are a major methyltransferase and a demethylase of H3K27, respectively. By ablation of Jmjd3 in retinal explants, we previously showed demethylation of H3K27me3 plays pivotal roles for rod-ON-bipolar cell maturation, and

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analysis of *Jmjd3*-retina-specific knockout mice showed that differentiation of retinal ganglion cells was perturbed in addition to the rod-ON-bipolar cells. The retina of *Ezh2*-knockout mice showed severe microphthalmia, but proliferation was suppressed only transiently at perinatal short period in peripheral region, indicating additional mechanisms leading to the microphthalmia. Based on these data, we aimed to analyze cell lineage-specific Histone modification in more details. For that purpose, transitions in global transcriptional and epigenetic changes during retinogenesis was examined by using purified photoreceptors and other retinal cells in terms of *Cd73* expression. In addition, by using *Hes1*-EGFP mice, we performed RNAseq of Mueller glia and other cell population. Genes expressed in the photoreceptor lineage were marked with H3K4me3 in the *Cd73*-positive cell fraction; however, the level of H3K27me3 was very low in both *Cd73*-positive and -negative populations. Subsets of genes expressed in Mueller glia, amacrine- and retinal ganglion cells, which are early born retinal cells, kept high levels of H3K27me3 during late-stage retinogenesis. We performed RNAseq of *Cd73*⁺ and *Cd73*⁻ population of developing *Ezh2*-CKO retina, and upregulation of *Rho*, which was reported to cause degeneration of photoreceptors, was observed in *Cd73*⁺ cells in *Ezh2*-ablated retina, suggesting roles of H3K27me3 for maintenance of proper level of *Rho* in rods. Taken together, our data of the transition of lineage-specific molecular signatures during development suggested that genes, lineage, and stage specific modification of Histone H3 methylation, and they may participate precise regulation of differentiation and maintenance of multiple retinal cell lineages.

The ATRX Chromatin Remodeling Protein Is Required in Bipolar Cells for the Non Cell-autonomous Survival of Amacrine and Horizontal Cells

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ATRX is a chromatin remodeling protein that is mutated in several intellectual disability disorders including the

alpha-thalassemia/mental retardation, X-linked (ATR-X) syndrome. The ATRX protein interacts with the histone chaperone Daxx to load the variant histone H3.3 onto heterochromatin, primarily at G-rich simple repeats. Despite these functions our understanding of how these biochemical activities result in intellectual disability remain poorly understood.

We previously reported the prevalence of ophthalmological defects in ATR-X syndrome patients, and accordingly we find morphological and functional visual abnormalities in a mouse model harboring a mutation occurring in ATR-X patients. The visual system abnormalities observed in these mice parallels the *Atrx*-null retinal phenotype characterized by interneuron defects and selective loss of amacrine and horizontal cells. The mechanisms that underlie selective neuronal vulnerability and neurodegeneration in the CNS upon *Atrx* mutation or deletion are unknown. To interrogate the cellular specificity of *Atrx*, we employed a combination of temporal and lineage-restricted conditional ablation strategies and identified a non-cell-autonomous requirement for *Atrx* in bipolar cells for inhibitory interneuron survival in the retina. *Atrx*-deficient retinal bipolar cells exhibit functional, structural and molecular alterations consistent with impairments in neuronal activity and connectivity. Gene expression changes in the *Atrx*-null retina indicate defective synaptic structure and neuronal circuitry, and suggest the possibility that excitotoxic mechanisms promote interneuron cell loss. Common *Atrx* gene targets in the forebrain and retina suggest that similar neuropathological processes underlie both cognitive impairment and visual dysfunction in the ATR-X patients.

Regulation of Temporal Identity in Mouse Retinal Progenitor Cells

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The complexity of the central nervous system depends on the generation of large numbers of different neuronal subtypes. In the retina, this cellular diversity arises progressively from retinal progenitor cells undergoing temporal transitions in their competence to produce different cell types as developmental proceeds. In

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Drosophila neuroblasts, transcription factor cascades control this process, but how these 'temporal identity factors' act mechanistically remains largely unclear. We have shown previously that Casz1, the vertebrate ortholog of the *Drosophila* temporal factor castor, operates in multipotent retinal progenitor cells to promote the generation of cells born at mid/late stages of development like rod photoreceptors, while suppressing the generation of cells born during the early or latest stages of development, thereby defining a mid/late competence window. In this talk, I will present our most recent results indicating that Casz1 interacts with the polycomb repressor complex in a splice variant specific manner to control cell fate. Both Casz1 and polycomb knock-down in retinal progenitors have similar effects on cell fate. Moreover, while Casz1 overexpression leads to the increased production of rod photoreceptors and decreased production of Müller glia, these effects are lost when polycomb activity is simultaneously blocked. Finally, transcriptomics data indicate that Casz1 overexpression leads to the suppression of some polycomb targets and the upregulation of others, suggesting that Casz1 may switch polycomb targeting in a stage-specific fashion. Generally, these results support a model in which the competence of neural progenitors is controlled by dynamic changes in genome organization mediated by temporal identity factors.

Ronin (Thap11) Regulates Retinal Progenitor Cell Proliferation and Is Implicated in a Novel Variant of Cobalamin Deficiency Syndrome

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We have identified the ESC pluripotency factor *Ronin* (*Thap11*) as a transcriptional regulator that influences the RPC cell cycle. RPC-specific loss of *Ronin* results in a phenocopy of the G1- to S-phase arrest observed in the *Cyclin D1* null retina. However, we did not uncover any genetic or molecular interaction between *Ronin* and *Cyclin D1*. Moreover, CHIP-seq and RNA-seq analyses did not identify *Ronin* target genes within the canonical cell cycle pathways, but instead revealed a cohort of nuclear-encoded mitochondrial genes with significant enrichment of electron transport chain (ETC) components. Recent work in *Drosophila* and mice has implicated the ETC as a regulator of the G1- to S-phase transition. Coincident with premature cell cycle exit, the *Ronin* mutant retinæ have a deficit in ETC complex stability and activity that we ascribe to specific loss of subunits within complexes I, III and IV.

These data implicate *Ronin* as an important regulator of mitochondrial gene expression that coordinates mitochondrial ETC activity and cell cycle progression. In addition to ETC genes, our analysis uncovered *Methylmalonic aciduria and homocystinuria type C protein* (*Mmachc*) as another direct mitochondrial target gene. This gene is mutated in vitamin B12 (cobalamin) deficiency syndrome of the cblC type. CblC is a heterogeneous syndrome that includes severe hematologic, renal, cardiac and CNS developmental defects as well as retinal degeneration. Mutations in the *RONIN* cofactor Host Cell Factor 1 (HCF-1) were previously discovered in patients with an X-linked variant of cblC (termed cblX) and fibroblasts from these patients exhibited a dramatic reduction in expression of *MMACHC*. Recently, a single patient, exhibiting a syndrome similar to the cblX, was determined to be homozygous for a mutation (p.Phe80Leu) in *RONIN*. The identification of human *HCF-1* and *RONIN* mutations raises the intriguing possibility that these proteins jointly regulate *MMACHC* transcription and hypomorphic mutations in either factor result in a cobalamin deficiency-like syndrome. To better understand the complex pathophysiology of this emerging human syndrome, we have used CRISPR/Cas9 genome editing to generate a mouse model with the same p.Phe80Leu mutation in *Ronin*. Phenotyping has revealed several striking CNS defects that are analogous to those observed in cblX patients. Ongoing work is aimed at determining the gene de-regulation contributing to the cblX syndrome affecting the brain and retina.

Integrated CHIP-Seq Analysis and Epigenomic Profiling of Early and Late-stage Retinal Progenitor Cells Identifies a Central Role for Lhx2 in Controlling Developmentally Regulated Modules of Coordinately Accessible Chromatin

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Over the course of neurogenesis, dramatic changes occur in both the proliferative potential and developmental competence of retinal progenitors. To investigate the transcriptional mechanisms underlying these changes, transposase-mediated ATAC-sequencing was performed at subsequent developmental time points: profiles from FACS sorted retinal progenitor cells and post-mitotic murine fractions were compared and classified

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by hierarchical clustering, leading to the identification of modules of coordinately accessible open chromatin, dynamically regulated between embryonic day 14 (e14) and post-natal day 2 (p2).

Lhx2 emerged among the transcription factors enriched in temporally defined chromatin clusters selectively open in early and late-stage progenitors, and two-thirds of its binding sites, hereafter peaks, profiled by ChIP-sequencing at e14 and p2, showed differential occupancy, indicating Lhx2 epigenetic repertoire is renovated along retinal progenitors differentiation. At both developmental time points, Lhx2 consensus was retrieved as the most enriched and focal motif, although stage-specific variations of the same instance were found suggesting cooperative binding with co-factors might affect Lhx2 targets recognition and affinity. Homeobox, Sry, Forkhead and ETS family members were also recalled by motif enrichment analysis, in putative association with genes, profiled by RNA-sequencing, that were differentially expressed in age-matched conditional Lhx2 mutants: retinal ganglion cells, horizontal cells and photoreceptors genes were affected at e14, whereas expression for late-stage progenitors and Muller glia genes was mostly altered at p2.

Lhx2 residence at consensus sites was queried by ATAC-seq footprints analysis: a significant overlap between ChIP-seq identified targets and ATAC-seq open chromatin regions was observed at both time points, with many of the binding events reflected into actual footprints, lost in the Lhx2-deficient retinas and primarily represented in the retinal progenitors sorted fractions.

Complexively, these data identify a central role of Lhx2 in organizing epigenomic changes in retinal progenitors over the course of neurogenesis, provide insight into the transcriptional mechanisms that mediate stage-specific functions of Lhx2 and demonstrate the potential for global epigenomic profiling to unravel transcriptional regulatory networks that control retinal progenitors competence.

Long Range Genomic Interactions Regulate Photoreceptor Gene Expression and Are Affected in Retinal Disease Models

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Precise packaging of genomic DNA into the nucleus is critical for assuring that the cell expresses certain genes and silences others. Photoreceptors offer a powerful

system to determine the role of genomic DNA packaging and how it is affected in disease. By comparing two neuronal subtypes (rods & cones) with a common function, we can investigate the role of genomic organization in cell type-specific gene expression.

To examine the genomic organization of rod and cone nuclei we have used two complementary assays-Circularized Chromatin Conformation Capture (4C) and 3D-Fluorescence In Situ Hybridization (3DFISH). To determine the functional importance of cell type-specific nuclear organization, we also created new mouse lines lacking the Rhodopsin (*Rho*) promoter or enhancer. We tested consequences of these genetic manipulations on rod nuclear organization and expression of *Rho* and other genes with which *Rho* physically interacts. *Crx* null (*Crx*^{-/-}) mice were also compared to understand CRX's role in establishing the organization.

4C assays on P14 WT (rod) vs. *Nrl*^{-/-} (cone) retinas detected a number of long-range interactions of actively transcribed genes in the respective cell type. In rods, *Rho* was found to interact specifically with several active regions on the same chromosome (in *cis*). *Rho* also interacts with several other rod-expressed genes on different chromosomes (in *trans*), despite in lower frequencies than *cis*. In cones, interactions of highly expressed cone genes were detected, while those of *Rho* were not present. 3DFISH on retinal sections confirmed rod-specific *Rho* interactions. Mice lacking *Rho* promoter, but not its enhancer, showed deficiency in *Rho* interactions, which correlated with the loss of *Rho* transcripts and altered expression of *Rho*-interacting genes as detected by qRT-PCR. *Rho* interactions were also lost in the *Crx*^{-/-} retina, suggesting that CRX is essential for these specific contacts. Together, our results suggest that *Rho* genomic interactions in the rod nucleus are important for the appropriate co-expression of a subset of rod-specific genes, which depends on the activity of the *Rho* promoter and the action of key photoreceptor transcription factors. In conclusion, rods and cones have distinct molecular interactions of DNA within the nucleus. This previously unexplored epigenetic mechanism of gene regulation provides a new insight into pathogenic mis-regulation of gene expression in retina disease.

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RND3 - Cell-cell signaling and retinal development

The Hippo/YAP Pathway in Reactive Müller Cells

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The Hippo pathway effector yes-associated protein (YAP) was identified as a major regulator of organ growth during development. Its function in adult tissues and in particular in adult stem cells remains more elusive. YAP appears largely dispensable in a physiological context for the homeostasis of several organs. In contrast, several reports suggest its involvement in tissue regeneration, although its effects are still controversial. Thus, the role of YAP in vertebrate adult stem cells may likely be context-dependent and clearly deserves further investigation. In this talk, I will present some of our recent results investigating the role of YAP in adult mouse Müller cells. Müller cells play a wide variety of roles under physiological conditions. In response to retinal injury, they undergo reactive gliosis, characterized by changes in gene expression and to some extent by cell cycle re-entry. Our recent work indicates that YAP is specifically expressed in Müller cells and is strongly up-regulated in reactive Müller cells alongside photoreceptor loss in several models of retinal degeneration. This reveals for the first time a link between YAP and Müller cell reactive gliosis. I will also provide an overview of our phenotypic analysis of mice lacking *Yap* expression specifically in Müller cells. Altogether, we propose a model where YAP regulates retinal homeostasis and that its up-regulation in Müller cells is required for their cell cycle re-entry. This provides novel insights into the signaling network operating in Müller cells that could serve to identify new therapeutics to trigger retinal regeneration.

Mechanisms of Differential Signaling among Retinal Progenitors during Neurogenesis

BRIAN LINK

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Past studies have demonstrated an important role for interkinetic nuclear migration (IKNM) and polarized signals in regulating neurogenesis within the retina. IKNM is the process where neuroepithelial nuclei oscillate towards

the apical to basal surfaces and in phase with the mitotic cycle. In the zebrafish retina, the depth of nuclear migration correlates with the probability that the next cell division will be neurogenic. Our current research is focused on the mechanisms underlying this relationship between IKNM and neurogenesis. In particular, we have explored the role that IKNM has on endosome biology. Through genetic manipulation and live-imaging in zebrafish, we find that early and recycling endosomes polarize in a dynamic fashion within retinal neuroepithelia and with reference to nuclear position. Functional analyses suggest that dynamic polarization of recycling endosome activity within neuroepithelia modulates the subcellular localization of Crb2a, therefore affecting multiple signaling pathways that impact neurogenesis. From these studies, we propose that the correlations between nuclear position, cell-cycle exit and polarized signaling are regulated in part by differential concentration and activation of endocytic vesicles and their cargo.

A Notch-Gli2 Axis Sustains Hedgehog Responsiveness of Neural Progenitors and Müller Glia

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Neurogenesis is regulated by the dynamic and coordinated activity of several extracellular signalling pathways, but the basis for crosstalk between these pathways remains poorly understood. In the retina, progenitor cells (RPCs) divide and adopt one of two fates, cell cycle exit or cell cycle re-entry. The balance between these two decisions is critical to ensure that the RPC pool is maintained until the completion of histogenesis. Here we investigated the interaction of two pathways that are required for neural progenitor cell maintenance in the postnatal retina; Hedgehog (Hh) and Notch signalling. Hh and Notch activity overlap throughout the period of neurogenesis and we show that both pathways are activated in RPCs in the postnatal retina, based on the co-expression of fluorescent pathway reporter transgenes at the single cell level. Gli transcription factors are the primary mediators of Shh signaling and disrupting Notch signalling, genetically or pharmacologically, induces a rapid downregulation of all three Gli proteins and inhibits Hh-induced proliferation. Ectopic Notch activation, while not sufficient to promote

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Hh signalling or proliferation, increases Gli2 protein. We show that Notch regulation of Gli2 in Müller glia renders these cells competent to proliferate in response to Hh in a Gli2-dependent manner. These data are consistent with a model in which Notch signalling converges on Gli2 to prime postnatal retinal progenitor cells and Müller glia to proliferate in response to Hh.

Fat3-dependent Mechanisms of Amacrine Cell Morphogenesis

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In order to modulate the flow of information from photoreceptors to retinal ganglion cells, most amacrine cells elaborate a single dendritic arbor that is restricted to the inner plexiform layer (IPL). Classic studies suggested that migrating amacrine cells are bipolar and then transition to a unipolar morphology as they approach the IPL. Previously, we showed that in mice lacking the atypical cadherin Fat3, amacrine cells retain their trailing processes and elaborate an extra dendritic arbor. However, Fat3 is localized to processes in the IPL, opposite to where the extra dendrites form. In order to understand how asymmetric Fat3 induces an overall change in cell morphology, we used a time-lapse imaging assay to visualize individual amacrine cell precursors in wild-type and mutant retina. We found that Fat3 orients developing amacrine cells towards the IPL, both as they migrate and as they retract their trailing processes. Additionally, Fat3 acts cell-autonomously and is poised to sculpt the cytoskeleton directly via its intracellular domain, which can bind and localize members of the Ena/VASP family of actin regulators. Moreover, by changing the distribution of Ena/VASP proteins in amacrine cells, we were able to induce formation of extra processes, mimicking the *fat3* mutant amacrine cell phenotype. We propose that Fat3 controls the polarized development of tissues by inducing local changes in the cytoskeleton that are propagated across the cell, echoing the actions of more traditional planar polarity proteins in the larger Fat family.

Combinatorial Actions of the Clustered Protocadherins in Retinal Circuit Patterning

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Proper retina function depends on the precise patterning and assembly of retinal neurons into circuits. The compact and stereotyped organization of interconnected cell-types implies a need for diversification and combinatorial actions of cell-surface recognition proteins. The clustered Protocadherins (Pcdhs) are members of the cadherin superfamily with a remarkable potential for providing a highly diverse cell-surface code for wiring individual neurons. The clustered *Pcdh-alpha*, *Pcdh-beta*, and *Pcdh-gamma* genes are organized in tandem and together encode 58 Pcdh isoforms. Pcdh diversity is amplified because each neuron in a population is thought to express a randomly selected subset of isoforms. Moreover, recognition specificity is conferred through strict homophilic interactions between the diversified extracellular Pcdh domains, and complexes of multiple Pcdhs selectively interact with matching Pcdh combinations. In this way, combinatorial expression of 58 clustered Pcdhs could impart millions of unique cell surface identities and recognition specificities. To determine the role of this extraordinary cell-surface diversity in retina circuit assembly, we are investigating a series of mouse mutants lacking different portions of the clustered Pcdh genes. We have shown previously that the set of 22 gamma-Protocadherins (PcdhGs) regulate two distinct aspects of retina circuit patterning:

- 1) dendrite patterning through self-avoidance and self/non-self recognition; and
- 2) neuronal survival of some cell-types.

Here, we examine the contributions of the 14 Pcdh-alpha genes (PcdhAs), and the combinatorial actions of PcdhAs and PcdhGs in retina circuit development. I will discuss our recent findings that establish critical roles for Pcdh diversity in dendritic patterning and neuron survival. Our studies demonstrate that the highly diverse repertoire of recognition units provided by clustered Pcdhs serve multiple important roles in the formation and function of retinal circuits. Together, our work and those of others suggest that, similar to the immune system, neurons are defined by unique cell surface identities for self-recognition and wiring.

RND4 - Establishment of retinal circuitry and synapses

Starburst Amacrine Cells Orchestrate Assembly of Retinal Direction-selective Circuitry

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During retinal development, ~100 different types of neurons connect into parallel circuits devoted to specific visual processing tasks. Differentiating inner retinal neurons grow their axons and dendrites into the nascent inner plexiform layer (IPL), which at early developmental stages is not even one cell diameter wide. Neurons that share a role in visual processing are able to find each other in this crowded space, establishing selective contacts that eventually become mature circuits. To understand how circuit partners find each other we are using as a model the direction-selective (DS) circuit of mouse retina, which detects the direction of image motion. This circuit is ideal for these studies because its constituent cell types are known and can be prospectively marked prior to circuit formation, allowing us to probe the earliest cell-cell recognition events that establish connectivity. Here we sought to determine which of the three cell types that comprise the DS circuit - the DS retinal ganglion cells (DS-RGCs), DS circuit bipolar cells and starburst amacrine cells - takes the lead in recruiting the others to assemble the circuit. Our data place starburst amacrine cells at the center of this process. We found that starbursts use homotypic recognition mechanisms to self-assemble two dendritic sublayers in the nascent IPL, which subsequently recruit projections from DS-RGC dendrites and DS circuit bipolar cell axons. Perturbing starburst-starburst interactions interferes with formation of the DS circuit sublayers. This was demonstrated through genetic cell ablation experiments as well as by deletion of the *Megf10* gene, which encodes a cell-surface molecule that mediates homotypic starburst recognition. Together, our results indicate that starbursts orchestrate assembly of the DS circuit, first through MEGF10-mediated self-assembly of the two DS circuit IPL sublaminae, followed by recruitment of their circuit partners through mechanisms yet to be determined. The mechanisms and molecules involved may be generally important for coordination of selective synapse formation throughout the nervous system.

Bipolar Cell Dendritic Rearrangements Following Temporally and Spatially Controlled Cone Ablation

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The visual system takes light stimuli as input and processes them via neural pathways that enable the interpretation of photons based on their quantity, location, timing, and

wavelength. This processing begins at the photoreceptor-to-bipolar cell synapse—a critical synapse in the function of the retina, visual, and nervous systems. However, our understanding of retinal dysfunction and disease is incomplete, because current models of disruption in cone and rod function are not suited to investigating the reactions of specific retinal neurons and do not distinguish between retinal insults during development vs. in adulthood. We aim to address these shortcomings, and enhance understanding of retinal disease, by studying the effects of retinal dysfunction with greater precision in space and time than has been done before. We have developed an *in vivo* method for ablating cones under temporal and spatial control using the diphtheria toxin receptor. Here we have used this method to examine how the type 6 ON cone bipolar cells responds to cone death. We find that type 6 bipolar cells lose cone contacts and extend dendrites 14–35 days after cone ablation. Normal center-to-periphery distributions of mGluR6 in the bipolar dendrites are disrupted following cone ablation. Results suggest that type 6 cone bipolar cells are capable of growth following perturbation in the adult retina.

Molecular Mechanisms of Photoreceptor Synaptogenesis

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In retina, two types of photoreceptors, rods and cones, form distinct synaptic connections with different ON bipolar cells (ON-BC). Neural signals from the rods and cones pass through these synaptic connections to the specific downstream ON-BCs. However, the molecule mechanisms of selective synapse formation are still unknown. Recently, we identified a leucine-rich repeat protein, ELFN1, which is specifically expressed in rod photoreceptors, to be essential for the rods and rod ON-BCs synapse formation. ELFN1, expressed in rod axonal terminals, binds *in trans* to the postsynaptic receptor mGluR6 on rod ON-BCs. Elimination of ELFN1 results in the loss of the synaptic contact between rods and rod ON-BCs, but not cones and cone ON-BCs. This selective synaptic disruption results in the loss of rod signals and night blindness. Since ELFN1 plays an important role in synaptogenesis, we further investigated the involvement of ELFN1 in different mouse mutant models with abnormal photoreceptor to ON-BCs synapses which have aberrant axonal retraction, postsynaptic dendrites extension and ectopic synapses. In these mouse mutant models, ELFN1, localized in retracted

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axonal terminals, was found in apposition to postsynaptic receptor mGluR6 in extended postsynaptic dendritic tips. This indicates the trans-synaptic interaction with mGluR6 lead to the recruitment of postsynaptic receptor to the dendritic tips of ON-BCs, allowing the assembly of postsynaptic signaling machinery. In conclusion, ELFN1 is essential for the selective wiring of rods to rod ON-BCs and determine the high sensitivity of rod vision.

Molecular Mechanisms of Retinal Circuit Assembly

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The mammalian retina comprises more than 70 neuronal cell types, which assemble into a variety of circuits that mediate diverse visual functions. I will discuss ongoing studies in my lab, which identified leucine-rich repeat (LRR) proteins that guide the assembly of specific circuits in the outer and inner retina. In addition to elucidating the contributions of these LRR proteins to morphological maturation and synapse development of specific neuron types, we analyze their impact on circuit function and visually guided behaviors.

LIM Code of Light-adaptive Retinal Circuitry

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Paired-homeobox 6 (PAX6) is an evolutionarily conserved master regulator of eye and retinal development. The expression of *PAX6* is controlled by multiple regulatory elements, including an intronic alpha-enhancer that shows biphasic activity in retinal progenitor cells of the embryo and amacrine subsets of the mature retina. However, it has not been studied how the enhancer activity is regulated in the embryonic and mature retina. We identified a LIM domain protein *Hic5* that links LIM domain transcription factors and negatively regulates the alpha-enhancer activity in the post-natal retina. The *Hic5*^{-/-} mouse retina showed a prolonged light response, which became more transient in the auto-stimulation-defective *Pax6*^{ΔPBS/ΔPBS} mice, to interfere with a light-adaptive visual response. Together, we show the antagonistic regulation of the α-enhancer by *Pax6* and a LIM protein complex is necessary for the development of retinal circuitry controlling visual adaptation.

Intrinsically-photosensitive Retinal Ganglion Cells Control Cone Photoreceptor Lamination during Retinal Development

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The strict laminar architecture of the neural retina is conserved across vertebrate species, with photosensitive rods and cones located most distally to the lens and pupil, and Retinal Ganglion Cells (RGCs) located most proximally. Proper localization of primary sensory neurons in such a highly ordered neuroepithelium is critical to vision, yet the developmental mechanisms that establish this order remain largely unknown.

We sought to determine the developmental cues regulating the lamination and apical alignment of cone photoreceptor cells in the mouse retina.

While cone photoreceptors occupy various positions within the apico-basal axis during retinal development, they remain largely restricted to the apical most surface of the retina from post-natal (P) day 14 onwards. We first found that this cone migration is controlled by light: dark-rearing animals from embryonic day 12 onwards resulted in a significant increase in the total number of displaced cones outside of the photoreceptor layer at both P7 and P14. Since rod and cone photoreceptors are not thought to drive conventional visual responses via RGCs until postnatal day 10, this result suggested that light might mediate cone positioning via intrinsically-photosensitive RGCs (ipRGCs). Consistently, we discovered early dendritic contacts between melanopsin RGCs and early cone photoreceptors, and ablation of ipRGCs using an *Opn4*-DTA mouse line phenocopied the dark-rearing effects on cone lamination. To determine whether light-mediated activity is involved, we analysed retinas from mice lacking either cone photosensitivity (*CNGA3*^{-/-}) or ipRGC photosensitivity (*Opn4*^{-/-} and *Opn4*-Cre;*Rosa*-TetanusNeurotoxin). Interestingly, mice lacking intrinsic RGC photosensitivity had cone lamination defects, but not those lacking cone photosensitivity (*CNGA3*^{-/-}), indicating that light triggers an ipRGCs response to control cone lamination. Finally, we believe early cones may provide a UV light component to the neonatal circadian clock, as we found robust cFos induction in the Suprachiasmatic nucleus following UV light exposure in pups. Ensuring the proper lamination of cones may therefore serve to control early UV input to the

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circadian system.

Together this study demonstrates the necessity of early light-evoked activity during development in the establishment of retinal lamination, and the possible contribution of cone responses to early visual behaviour.

RND5 - Retinal circuitry and visual signal processing

Synaptic Connections of S-cones in a Mammalian Retina

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Cone photoreceptors with distinct absorbance spectra form the basis of color vision. Their synaptic connections initiate the retinal processing of the spectral information. To investigate the synaptic output of S-cones in a mammalian retina, we used serial block-face scanning electron microscopy to generate high resolution, three dimensional image series from a piece of the ground squirrel retina. IMOD software was applied to trace and reconstruct the morphology of cone terminals, bipolar cell dendrites and horizontal cell dendrites from 511 series sections. Among 20 cone pedicles reconstructed, two were identified as S-cones. Dendrites of three S-cone bipolar cells were found to make central, invaginating synapses with the two S-cones, which is consistent with their identity of S-cone ON bipolar cells. Other bipolar dendrites connected with S-cones were found to always contact neighboring M-cones, thus are not S-cone selective. ERG experiments provided further support that the S-cone OFF signal is carried predominantly through ON bipolar channels indirectly, but not through OFF bipolar cells directly. For S-cone to horizontal cell synapse, we found that, in addition to the HI type horizontal cell that contacts M- and S-cones non-selectively, there appeared to be an HII type horizontal cell that contacts S-cones exclusively, which may shape the synaptic output of S-cones.

Synaptic Mechanism for Tonic Inhibition of ON Alpha Ganglion Cells in the Mouse Retina

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The ON alpha ganglion cell is perhaps the best-understood ganglion cell in the mammalian retina owing to its accessibility to electrophysiological analysis. Synaptic inhibition is critical to prevent excessive depolarization, thereby maintaining the dynamic range of the ON alpha cell, but the sources and mechanisms of this amacrine cell input are not well established.

Here, we consider the contribution of a genetically-identified population of amacrine cells to synaptic inhibition of ON alpha cells. In the CRH-ires-Cre transgenic line, amacrine cell dendrites co-stratify with those of ON Alpha cells, leading to the hypothesis that some of these amacrine cells might be a source of synaptic inhibition. We characterized potential presynaptic partners for the ON alpha cells using targeted whole-cell recordings and morphological analysis of CRH⁺ cells and found at least three types: two matched those described previously (CRH-1, CRH-2; Zhu et al., 2014; Jacoby et al., 2015), and a third was monostратified with multiple axons and an ON-center, spiking response (CRH-3). We tested for a connection between CRH⁺ amacrine cells and ON Alpha cells using Cre-dependent channelrhodopsin-2 (ChR2) expression (Ai32 line). With photoreceptor-mediated inputs blocked (L-AP4, ACET, DNQX, D-AP5), we observed ChR2-evoked IPSCs in ON Alpha cells. These IPSCs were partially sensitive to TTX, suggesting a contribution from a spiking CRH⁺ cell (CRH-3). ChR2-mediated responses in ON Alpha cells were absent in the nNOS-CreER line, in which the NOS-1 amacrine cell is the CRH-2 cell (Zhu et al., 2014). Paired recordings confirmed a monosynaptic connection between CRH-1 and ON alpha cells.

We examined the dynamics of inhibitory transmission at CRH amacrine cell to ON alpha cell synapses. These synapses appear to be designed to generate a tonic inhibitory input by virtue of loose Ca channel-release site coupling and weak intracellular [Ca²⁺] buffering. ChR2-evoked IPSCs evoked at 5 Hz summed strongly (when compared to IPSCs evoked at 0.5 Hz) and were suppressed quite powerfully by EGTA-AM. By comparison, EGTA-AM had a much weaker effect at a bipolar cell ribbon synapse (Rod bipolar to All amacrine cell) known to have the capacity to encode high frequencies. We conclude that CRH-1 and CRH-3 amacrine cells provide tonic, inhibitory input to ON Alpha ganglion cells in the mouse retina to prevent ganglion cell saturation at elevated backgrounds.

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Cellular and Synaptic Mechanisms Underlying Direction Selectivity in the Retina**WEI WEI***The University of Chicago, Chicago, United States*

Starburst amacrine cells (SACs) are required for direction selectivity in the mouse retina. Precise GABAergic connectivity between individual SAC dendritic processes and direction-selective ganglion cells (DSGCs) enables DSGCs to fire action potentials during motion in their preferred direction, but not during motion in their anti-preferred (null) direction. Although glutamatergic inputs to SACs are non-direction-selective, SAC processes preferentially depolarize and release GABA during motion in the centrifugal direction. Here, we investigated the mechanisms underlying this centrifugal preference using two-photon calcium imaging and whole-cell patch clamp recording from genetically labeled SACs and DSGCs. Our results indicate that direction selectivity of DSGCs and SACs arises from concerted action of synaptic network, cell intrinsic properties and modulatory synaptic transmission.

Inhibition within the Starburst Amacrine Cell (SAC) Network Localizes SAC Dendritic Signaling and Sharpens Direction Selectivity**JEFFREY DIAMOND, Huayu Ding***NIH, NINDS, Bethesda, United States*

Direction selectivity (DS) in the mammalian retina originates in starburst amacrine cells (SACs), which send directionally-tuned inhibitory signals to DS ganglion cells (DSGCs). Previous work has shown that different regions of the SAC dendritic arbor operate independently and respond best to motion in the centrifugal direction (i.e., away from the SAC soma). SACs therefore constitute a powerful model for studying dendritic computations in a physiological context.

DS signaling in SAC dendrites appears to depend on both intrinsic dendritic properties and synaptic interactions within the DS network, but the relative impact of these mechanisms remains unclear. Moreover, the elementary computational unit within SAC dendrites has not been identified. We have addressed these issues in mouse retina by imaging directional calcium signals from individual synaptic varicosities in SAC dendrites. We measured DS by comparing signals evoked by bars moving across the visual field in eight different directions. In addition, we mapped the visual receptive field properties of each varicosity to determine the spatial relationship between light-evoked

inputs and outputs. We found that varicosities immediately adjacent to each other along the same dendritic branch exhibited similar directional preference. In addition, responses in adjacent dendrites arising from the same parent branch were highly correlated and exhibited similar DS. By contrast, responses in adjacent dendrites arising from different parent branches were poorly correlated and exhibited distinct directional preference.

Blocking SAC-SAC inhibition with GABA_Azine reduced the direction selectivity of responses in individual varicosities and increased correlations between adjacent branches, making DS tuning more similar on adjacent dendrites arising from different parent branches. GABA_Azine expanded the visual receptive field properties of individual varicosities, causing greater overlap between the receptive fields of adjacent branches. This effect was primarily due to blockade of SAC-SAC inhibition rather than feedback inhibition onto bipolar cell terminals, as GABA_Azine did not expand the receptive field properties of type 5 cone bipolar cells, which provide excitatory input to ON SACs.

We conclude that SAC-SAC inhibition compartmentalizes DS signaling within smaller regions of SAC dendrites, enabling finer discrimination of motion direction.

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RND6 - RGC axonal targeting and regeneration**Plexin-A1 and Semaphorin-6D Are Involved in Retinal Axon Fasciculation and Targeting****ALEXANDRA REBSAM^{1,2,3}, Delphine Prieur^{1,2,3}, Cedric Francius^{1,2,3}, Carol A. Mason⁴**

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Retinal axons from the same (ipsilateral) and the opposite (contralateral) eyes gather in the optic tract and innervate separate territories in their main target, the dorso-lateral geniculate nucleus (dLGN). The mechanisms regulating axon fasciculation and target innervation are still unclear. Here, we investigated the role of the guidance receptor Plexin-A1 and its ligand Semaphorin-6D (Sema6D) in these processes. After anterograde tracing, Plexin-A1 ^{-/-} mice present fasciculation defect in the optic tract and ectopic retinal projections in the dLGN, without alterations in topographic mapping. A similar phenotype is observed in Sema6D ^{-/-} mice, suggesting that Sema6D is the ligand of Plexin-A1 for the appropriate target innervation by retinal axons. Both Plexin-A1 and Sema6D are expressed in retinal

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ganglion cells and dLGN during development. We used *in utero* electroporation of sema6D-shRNA to downregulate retinal expression of Sema6D before target innervation. Mice electroporated with Sema6D-shRNA present the same targeting defect in the dLGN as Sema6D $-/-$ mice. Thus, Sema6D expression in the retina is essential for retinal axonal targeting. All these results suggest that Plexin-A1 and retinal expression of Sema6D are necessary for fasciculation and positioning of retinal axons as well as for their target innervation.

Reciprocal Connections between Cortex and Thalamus Contribute to Retinal Axon Targeting to Dorsal Lateral Geniculate Nucleus

JIAYI ZHANG, RGC Axonal Targeting and Regeneration

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The dorsal Lateral Geniculate Nucleus (dLGN) is the primary image-forming target of the retina and shares a reciprocal connection with primary visual cortex (V1) that relays and modulates visual signals. Ventral (v) LGN and superior colliculus (SC) receives one-way input from the cortex without efferent projection to cortical regions. Previous studies have demonstrated that cortical and thalamic axons interact and guide each other in the subpallium/pallium boundary around E14.5. Moreover, retinal input regulates the timing of cortical innervation in the dLGN. Using "cortexless" mice, it was shown that corticothalamic axons are essential for retinal input to target the dLGN. However, the timing and mechanism for the corticothalamic axons to affect retinal input to the dLGN remains elusive. Here, we demonstrate a deficit of retinal projections to the dLGN in "cortexless" mice as early as embryonic day (E) 18.5, while projections to the vLGN, SC, and other subcortical visual nuclei were retained. Apoptosis happens along the thalamocortical tract and in some dLGN neurons, but not in the vLGN and SC, between E16.5 and E18.5. RNA sequencing experiments showed that transcriptions of some genes significantly changed in the dLGN. Experiments with NMDA-induced V1 lesions as well as *Fezf2* conditional knockout (cKO) mice that lack corticothalamic projections revealed that the absence of cortical input specifically lead to the disruption of retinogeniculate circuits in the dLGN. Our results provide evidence reciprocal connections between V1 and dLGN are required for the survival of some dLGN neurons, as well as targeting and elaboration of retinal axons in the dLGN as early as E18.5.

Retinal Origin of Direction Selectivity in the Mouse Superior Colliculus

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Visual information is first processed in the retina where different subtypes of retinal ganglion cells (RGCs) encode specific features of the visual scene. The signals encoded by RGC subtypes are further integrated and transformed in various retinal targets in the brain to give rise to specific visual response properties. Recently, mouse has become a useful model in vision research due to the technical advances in genetic, imaging, and physiological methodologies. In mice, more than 70% RGCs project to the superior colliculus (SC), a midbrain structure important for multimodal integration and sensorimotor transformation. The significance of the SC in mouse vision thus makes it an important system to study visual transformation and its underlying mechanisms. In this talk, I will present our recent discoveries of direction selectivity in the SC and how it may be generated by the precise targeting of specific subtypes of RGCs.

Formation of Retinal Ganglion Cell Types

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The retina divides the visual world into distinct channels of visual information and RGCs extend axons to relay this visual information to higher processing centers within the brain. RGCs can be subdivided into classes using a variety of measures including morphology, gene expression, connectivity, and characteristic functional response profiles. Here we show that RGCs can be divided into three classes based on their expression of one of three of the transcription factors: *Isl2*, *SatB2*, or *Tbr2*. Based on their functions in other developmental systems, their onset of expression in post-mitotic RGCs and co-expression with RGC type specific markers, we hypothesize that these three transcription factors mediate RGC type identity, segregating the RGCs into three distinct functional classes: Contrast Sensitive RGCs, Direction Selective RGCs, and non-image forming RGCs.

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Restoration of Visual Function by Promoting Axon Regeneration and Conduction

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Multiple hurdles exist in developing axon regeneration-based strategies of restoring functions after optic nerve injury. Previous studies from us and others showed that a number of repair strategies can promote axon outgrowth following neuronal injury in the mammalian central nervous system (CSN), it remains unclear whether regenerated axons establish functional synapses and support behavior. In an optic tract injury model, we show that either PTEN and SOCS3 co-deletion, or co-overexpression of osteopontin (OPN)/ insulin-like growth factor 1 (IGF1)/ciliary neurotrophic factor (CNTF), induces regrowth of retinal axons and formation of functional synapses in the superior colliculus (SC), but not significant recovery of visual function. Further analyses suggest that regenerated axons fail to conduct action potentials from the eye to the SC due to lack of myelination. Consistent with this idea, administration of voltage-gated potassium channel blockers restores conduction and results in increased visual acuity. Thus, enhancing both regeneration and conduction effectively improves function after optic nerve injury.

RND7 - Retinal regeneration through controlled dedifferentiation

Epigenetic Signatures of Chick RPE Reprogramming

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The chick that has the capacity to regenerate its retina within a small window of its development via the reprogramming of the retinal pigmented epithelium (RPE). This process requires RPE cells to dedifferentiate into cells that can go on to differentiate into retina cells, in other words, these cells undergo a controlled dedifferentiation and differentiation program referred to as transdifferentiation. We have shown that retina removal is sufficient to initiate a molecular reprogramming in the RPE suggestive of retina fate. This dedifferentiation program is not maintained unless growth factors are present such as fibroblast growth factor 2 (FGF2). The molecular switch

controlling this cell fate change must be orchestrated by epigenetic mechanisms. We have analyzed the presence and distribution of epigenetic marks associated with RPE reprogramming including DNA methylation and histone modifications. We have focused on bivalent chromatin (H3K27me3/H3K4me2 or H3K4me3) which has been associated with cellular plasticity and predisposes cells to acquire lineage specific expression upon differentiation cues. By immunofluorescence staining and high resolution imaging, we found the presence of bivalent domains (H3K27me3/H3K4me2 or H3K4me3) and 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5-hmC) in the RPE of chick developing eyes. Interestingly, during RPE dedifferentiation, the nuclear distribution of the marks change dynamically and continues to change as RPE transdifferentiates 3 days post-retinectomy in presence of FGF2. At this time, repression marks such as H3K27me3 and 5- methylcytosine (5mC) decrease in the newly regenerated neuroepithelium, while activation marks persist (H3K4me2 or H3K4me3). Using RT-qPCR, we found that the enzymes involved in epigenetic modifications such as Utx (histone demethylases specific for H3K27me3/me2), Jmjd1 (H3K9me2/me1-specific demethylase) and Tet3 (involved in active DNA demethylation) were up regulated during RPE reprogramming. On the other hand, DNA methyltransferases (DNMTs) were transiently down regulated during RPE dedifferentiation. Our data imply that RPE reprogramming entails a dynamic interplay of chromatin modifications and DNA methylation.

Innate Immune System Regulation of Retinal Regeneration - Enhanced Photoreceptor Replacement Kinetics Following Delayed Immune Suppression

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Müller glia (MG) function as inducible stem cells in zebrafish, repairing the retina after damage. The innate immune system has recently been shown to promote tissue regeneration, where classic wound healing responses predominate. However, a regulatory role for leukocytes in cellular regeneration - i.e., selective cell loss paradigms akin to degenerative disease states - has not been defined. To investigate possible role(s) innate immune cells in retinal cell regeneration, we used intravital microscopy to visualize neutrophil, peripheral macrophage, and retinal microglia responses to induced rod photoreceptor

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apoptosis. Neutrophils displayed no reactivity to rod cell loss. Peripheral macrophage cells responded to rod cell loss, evidenced by ramified to amoeboid morphological transitions and increased migration, but did not enter the retina. Retinal microglia displayed multiple hallmarks of immune cell activation: increased migration, translocation to the photoreceptor cell layer, proliferation, and phagocytosis of dying cells. To test function during rod cell regeneration, we co-ablated microglia with rod cells, or applied immune suppression, and quantified the kinetics of:

- (1) rod cell clearance,
- (2) MG/progenitor cell proliferation, and
- (3) rod cell replacement.

Co-ablation and immune suppressants applied prior to cell loss caused significant delays in MG/progenitor cell proliferation rates and slowed regeneration kinetics. Conversely, immune suppressants applied after cell loss had occurred accelerated regeneration kinetics, possibly by promoting a more rapid resolution of the initial microglia response. Our findings suggest microglia control MG/progenitor cell responsiveness to photoreceptor loss and support the development of immune targeted therapeutic strategies for reversing cell loss associated with degenerative retinal conditions.

Factors that Regulate Müller Glia Reprogramming and Proliferation in the Light-damaged Zebrafish Retina

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Light-induced damage to the adult zebrafish (*Danio rerio*) retina induces photoreceptor cell death that results in the Müller glial cells reprogramming and reentering the cell cycle, which gives rise to neuronal progenitor cells (NPCs) that transiently amplify and migrate to the outer nuclear layer, where they differentiate into new photoreceptors. My lab has been studying the molecular mechanisms underlying Müller glia reprogramming and reentry into the cell cycle. We recently demonstrated that the regeneration process is initiated by dying photoreceptors expressing Tumor Necrosis Factor Alpha (TNF α), which induces Müller glia to reenter the cell cycle. We will present data showing that the soluble processed form of TNF α , and not the transmembrane form, is required to initiate Müller glia proliferation. In addition, TNF α signaling requires the

downstream transcription factor Stat3. At the same time, Notch signaling serves as an inhibitory signal to repress the Müller glia from proliferating and loss of Notch signaling is sufficient to allow the Müller glia to reenter the cell cycle in the absence of retinal damage. We will present data that Notch3 is the receptor that mediates Müller glia quiescence. Furthermore, expression of TNF α and repression of Notch signaling are sufficient to stimulate Müller glia proliferation in the absence of retinal damage and produce NPCs that are capable of differentiating into nearly all the neuronal classes of the retina. Finally, if the Müller glia are reprogrammed in response to retinal damage, then the Yamanaka factors are excellent candidates for regulating the reprogramming process. We determined that Sox2 is both necessary and sufficient to induce Müller glia reentry into the cell cycle, suggesting that the Müller glia are reprogrammed. We will present data on the signaling pathways that are activated by ectopic overexpression of Sox2 in the undamaged zebrafish retina. Through these studies, we are gaining a better understanding of the signals that are essential for Müller glia reprogramming and proliferation to drive regeneration of lost photoreceptors in the adult zebrafish retina.

RB and Hippo Pathway Signaling: Not what you'd Expect

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The Hippo pathway regulates tissue size by inhibiting the pro-survival and pro-growth transcriptional regulators YAP and TAZ. The RB pathway inhibits E2F-mediated induction of genes that drive the cell cycle. The degree to which these pathways cooperate in cancer is unknown. We studied their interaction in the retina. Our initial data suggested that, as predicted, YAP or TAZ could cooperate with RB loss to drive tumorigenesis. However, as the story developed, it became clear that rather than promoting retinoblastoma, YAP and TAZ do the opposite. This result is surprising given the pro-tumorigenic role of these factors in many other solid tumors. We will discuss the findings both in the context of tumor initiation, but also with respect to the relevance of our findings to cell fate and regeneration in the retina.

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Genomic Mechanisms of Lhx2-dependent Control of Reactive Gliosis in Retina

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Astroglia throughout the central nervous system undergo a common series of morphological and molecular changes that occur following injury. These include cellular hypertrophy, characterized by an increase in cell size and stiffness, and upregulation of intermediate filament proteins such as GFAP. Moreover, reactive glia also induce expression of multiple secreted factors, several of which have been shown to be neuroprotective. We have identified the LIM homeodomain transcription factor Lhx2 as a master regulator of multiple different aspects of reactive gliosis in retina. Selective deletion of Lhx2 in mature Muller glia leads to cell-autonomous induction of hypertrophic gliosis in resting glia. Moreover, Lhx2-deficient glia fail to induce expression of secreted neuroprotective factors following injury. To further investigate the mechanisms by which Lhx2 controls retinal gliosis, we have conducted RNA-Seq analysis of wildtype and Lhx2-deficient retina in both uninjured and light-damage conditions, and have used ChIP-Seq to identify Lhx2 target sites in both developing and mature retina. These results of these studies will be discussed.

RND8 - Mechanisms of neuroprotection**The Metabolic and Redox Signaling Controlled by the Rod-derived Cone Viability Gene NXNL1**

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Rod-derived Cone Viability Factor (RdCVF) is an inactive truncated thioredoxin secreted by rod photoreceptors that protects cones. RdCVF is an alternative spliced product of the *Nxn1* gene which also encodes for an active thioredoxin RdCVFL involved in the defense of the retina against photo-oxidative damage. RdCVF is produced by intron retention with the presence of an in-frame stop codon. Through RdCVF, rods control the metabolism of glucose by cones. RdCVF binds to the transmembrane protein basigin-1 (BSG1), a retina specific alternative spliced product of the basigin gene with an additional third extracellular immunoglobulin domain. BSG1 associates at the surface on the cones with the glucose transporter GLUT1. RdCVF interacts with the BSG1/GLUT1 complex and increases glucose entry into cones. This promotes cone

survival by stimulation of aerobic glycolysis. Because the secondary loss of cones in retinitis pigmentosa is leading to blindness, the administration of RdCVF is a promising therapy, independent of mutation in 60 different genes that are known to cause this untreatable neurodegenerative disease.

We have also studied the role of the *Nxn1* gene in cones after its homologous recombination using a transgenic line expressing Cre recombinase under the control of a cone opsin promoter. We show that the cones of these mice are dysfunctional and degenerate over time. This age-related deficit in cones is exacerbated by exposure to high level of oxygen. Accordingly, we found that the cones express only one of the two *Nxn1* gene products, the thioredoxin RdCVFL. The alternative splicing leading to intron retention and the production of the truncated thioredoxin RdCVF occurs in rods, not in cones. Administration of an AAV vector expressing RdCVFL to the mouse carrying a deletion of the *Nxn1* gene in cones or in the *rd10* mouse, a model of retinitis pigmentosa, increases cone function by reducing the damage produced by oxidative stress in a cell-autonomous manner.

While, the non cell-autonomous mechanism of action of RdCVF implies that administration of RdCVF in patients suffering from retinitis pigmentosa could not only stabilize central vision but also ameliorate cone vision by stimulating cone outer segments re-growth, the cell-autonomous mode of action of RdCVFL increases non-functional cone survival by preventing oxidative damage. These results are the rational for a metabolic and redox treatment of retinitis pigmentosa.

The Duality of mTORC1 in Promoting Cone Survival in Retinitis Pigmentosa

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Retinitis pigmentosa is a form of inherited retinal degeneration that remains largely untreatable, and results in blindness due to the loss of rods and cones. Interestingly, mutations in rod specific genes cause rod and cone death, while mutations in cone specific genes do not affect rods. Because cones are essential for high acuity vision in humans, it is their loss that leads to blindness. We have shown that rod mediated cone loss in Retinitis Pigmentosa is caused by a nutrient deficiency in cones induced by the disruption of the retinal architecture as rods die. Consequently, boosting cell metabolism by increasing the activity of the key kinase that regulates cell metabolism, the mammalian target

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of rapamycin complex 1 (mTORC1), through disruption of its negative regulator, the tuberous sclerosis complex, dramatically improves cone survival in the fast progressing retinal degeneration 1 (*rd¹*) mouse model of Retinitis Pigmentosa. In fact, cone death was brought to a halt for about one month, suggesting that secondary cone death in Retinitis Pigmentosa is preventable.

To unravel why after a month of disease stagnation cone death resumed we analyzed the effect of sustained mTORC1 activity on autophagy, another mechanism that is regulated by mTORC1. We found accumulation of autolysosomes suggesting a lack of sufficient lysosomal enzymes causing a shortage of intracellular amino acids. Interestingly, this was not due to the activation of mTORC1 per se but rather to the inability of the cell to intermittently shut off mTORC1, since activation of mTORC1 by disruption of the further upstream regulator, the phosphatase and tensin homolog, did not result in such problem. The data indicates that any increase in mTORC1 activity is beneficial for long-term cone survival and suggests that gene therapeutic approaches with mTORC1 target genes that improve cell metabolism is a achievable strategy to prolong vision in Retinitis Pigmentosa.

Cellular Mechanisms of Cytokine-mediated Neuroprotection in Mouse Models of Retinal Degeneration

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Under stress and disease conditions, retinal photoreceptors and ganglion cells are prone to damages and permanent loss. Unlike gene therapies that target individual monogenic diseases, effective broad-spectrum neuroprotection can promote the viability of different retinal neuron types, thus serving as a useful strategy to rescue vision. Despite the existing endogenous protection potential responding to mild stresses, mammalian retinas lack the capacity to self-repair or the ability to halt degeneration in disease conditions. Treatment with exogenous growth factors, however, can trigger or amplify intercellular signaling events and prevent neuronal death. The cytokine CNTF exhibits potent neuroprotection activity in various photoreceptor and retinal ganglion cell degeneration models, and is currently tested in clinical trials for retinitis pigmentosa, geographic atrophy and glaucoma. Our previous studies have shown that exogenous CNTF initially targets Muller glial cells to activate STAT3 and ERK, and

subsequently promotes rod cell survival through engaging the cytokine signaling pathway in photoreceptors. Using molecular genetic and biochemical approaches, we are currently exploring the cellular events underlying CNTF-induced neuronal viability and the critical links involved in neuron-glia interactions.

An Extracellular Signaling Pathway that Includes Both Leukemia Inhibitory Factor and Endothelin 2 Regulates Both Neuroprotection and Gliosis in the Retina

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Purpose: We and others have shown that stressed photoreceptors release signals that stimulate Müller glial cells to induce the expression of trophic factors such as Leukemia Inhibitory Factor (LIF), and FGF2, and in response, photoreceptors have been reported to induce endothelin 2 (Edn2) expression. Each of these factors has the potential to regulate protection of photoreceptors from inherited mutations and has the potential to induce gliosis in Müller cells. We will report recent studies designed to determine the mechanism of LIF induction in Müller cells and the epistatic relationship between LIF and Edn2.

Methods: All procedures with animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. BALB/cJ mice, gp130 conditional knockout mice, and Edn2 conditional knockout mice were used. Light damage (LD) was mediated by exposing mice to 1300lux light for 4 hours to induce factor expression. ERGs recordings and OCT imaging were used to measure retina structure and function. Chromatin immunoprecipitation (ChIP) was used to measure transcriptional regulation of LIF. Antibodies against RNA polII pS2, H3K4me, H3K4me3, H3K27ac or IgG were used. Immunohistochemistry was used to measure glial fibrillary acidic protein (GFAP) expression. Quantitative PCR (qPCR) was used to measure mRNA levels. Western blotting was used to measure protein expression.

Results: Following induction of light stress our ChIP data suggest that a unique isoform of LIF rather than the conical isoform of LIF is induced by stress. Gp130 knockout mice have reduced induction of edn2 and have increased sensitivity to light damage. Edn2 and EdnrA cKO mice are also more sensitive to light damage. Surprisingly, both gp130 and Edn2 cKO mice have reduced GFAP induction in Müller cells following stress. Edn2 cKO mice also have

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reduced gp130 induction and STAT3 activation with stress. Additionally, when injected with LIF, Edn2 cKO mice fail to induce GFAP in Müller cells, and are not protected from LD.

Molecular Mechanisms Underlying Neuroprotective Effects of PEDF in Retinal Degeneration

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Retinitis pigmentosa (RP) is a major cause for loss of vision during working age. Several genes have been associated with RP. Its genetic heterogeneity has hampered the development of therapeutic interventions, which calls for mutation independent approaches. Neuroprotective factors targeting common molecular pathways activated in degenerating photoreceptors are being sought. They are promising tools because they can overcome the limitations of therapeutic approaches targeting genes specifically linked to this disease.

Previously we and others showed neuroprotective effects of pigment epithelium-derived factor (PEDF) in several rodent models of retinal degeneration. The molecular targets of PEDF have not been identified yet limiting the potential clinical applications of PEDF. We thus analyzed the molecular effects of PEDF in the retinal degeneration 1 (*rd1*) mutant murine retina. This mouse model is well characterized and several molecular events have been linked to the degenerative process caused by mutations in the *Pde6b* gene. We intravitreally injected PEDF (6 pmoles/eye) in *rd1* mutant mice at the age of 11 days. By Fluo4AM staining and cytofluorimetric analysis we found that PEDF significantly decreased intracellular calcium in photoreceptor cells. PEDF neuroprotection correlated inversely with the activation of calpains, Bax and apoptosis inducing factor (Aif). PEDF is able to increase expression of the anti-apoptotic factor Bcl2. Alongside, PEDF also interfered with pathways that are activated by cGMP such as protein kinase G (PKG) and the HDAC Sirtuin 1. Interestingly, PEDF increased nuclear localization of Sirtuin 1 and reduced its cytoplasmic levels. In addition to showing that the neuroprotective effects of PEDF act early on cell death pathways, the findings also emphasize that PEDF is a promising therapeutic tool for retinal degeneration.

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mTORC2 and GSK3 β Are Inhibitory but mTORC1 Is Necessary for AKT3-induced Optic Nerve Regeneration

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Injuries of optic nerve (ON) in optic neuropathies often result in visual function deficits due to the failure of retinal ganglion cells (RGCs) axons to regenerate. Deletion of phosphatase and tensin homolog (PTEN), the negative regulator of phosphatidylinositol 3-kinase (PI3K), induces RGC axon/ON regeneration, potentially through activation of PI3K. AKT is the major effector of the PI3K pathway; it is phosphorylated by PDK1 and mTOR complex 2 (mTORC2) at two different amino acids, T308 and S473, respectively. AKT is also the upstream regulator of mTOR complex 1 (mTORC1) and GSK3 β . Here we molecularly dissect this complicated pathway and elucidate how AKT coordinates the signaling of these two mTOR complexes and GSK3 β in adult mouse RGCs to influence ON regeneration *in vivo*. We found that the predominant AKT isoform in brain and retina, AKT3, induces much more robust axon regeneration than AKT1. By RGC-specific deletion of the key components of mTOR complexes, RAPTOR and RICTOR, we determined definitively that mTORC1 is necessary but mTORC2 is inhibitory for AKT3-induced axon regeneration. Surprisingly, phosphorylation of T308 and S473 of AKT play opposite roles in GSK3 β phosphorylation and inhibition, by which mTORC2 and pAKT-S473 negatively regulate axon regeneration. Finally, we proved that mTORC1 and GSK3 β are two critical parallel pathways for AKT-induced ON regeneration. Thus our study revealed a complex neuron-intrinsic balancing mechanism involving AKT as the nodal point of PI3K, mTORC1/2 and GSK3 β that coordinates both positive and negative cues to regulate adult CNS axon regeneration.

RND9 - Modeling human retinal disease

Exploring the Retinoblastoma Origin with Fetal, Mouse, and hPSC-derived Retina Models

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A number of models have been used to gain insight into human retinal development and disease, with the mouse perhaps the most common despite significant human - mouse differences. Recently, human pluripotent stem cell (hPSC)-derived retinal organoids cultured *in vitro* have been used in efforts to elucidate human-specific retinal features, and have been proposed as a tissue source for retinal cell replacement therapies. However, we currently do not understand how well these organoids portray human retinal development and pathogenesis. Indeed, we have found that early stages of one retinal disease, retinoblastoma, can be simulated in cultured human fetal retina, but not in mouse retina nor in hPSC-derived retina of similar developmental stages. In the fetal retina, pRB knockdown selectively enables cell cycle entry and proliferation of normally post-mitotic cone photoreceptor precursors. pRB knockdown also enables proliferation of prospectively isolated cone precursors, and subretinal xenografts of such cells form retinoblastoma-like tumors. In contrast, pRB-depleted cone precursors in cultured mouse retina or in hPSC-derived retinal organoids fail to enter the cell cycle.

To address the discrepant responses to pRB loss in these systems, we have begun to compare a) the expression of cell proliferation-related proteins that have been implicated in the cone precursor response to pRB loss, and b) single cone precursor transcriptomes both across the normal developmental trajectory and in response to pRB depletion in fetal, mouse, and hPSC-derived retinal tissue. Our data suggest that cone precursors within hPSC-derived retinal organoids retain numerous features that are specific to human retinal development, yet imperfectly model the cone precursor proliferation-related program. Identification of specific cell signaling pathway differences may enable approaches to more accurately simulate human retinal development and disease in mouse and hPSC-derived models.

The Use of Induced Pluripotent Stem Cells to Reveal Pathogenic Gene Mutations and Explore Treatments for Retinitis Pigmentosa

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Background: Retinitis pigmentosa (RP) is an inherited human retinal disorder that causes progressive

photoreceptor cell loss, leading to severe vision impairment or blindness. However, no effective therapy has been established to date. Although genetic mutations have been identified, the available clinical data are not always sufficient to elucidate the roles of these mutations in disease pathogenesis, a situation that is partially due to differences in genetic backgrounds.

Results: We generated induced pluripotent stem cells (iPSCs) from an RP patient carrying a rhodopsin mutation (E181K). Using helper-dependent adenoviral vector (HDAV) gene transfer, the mutation was corrected in the patient's iPSCs and also introduced into control iPSCs. The cells were then subjected to retinal differentiation; the resulting rod photoreceptor cells were labeled with an Nrl promoter-driven enhanced green fluorescent protein (EGFP)-carrying adenovirus and purified using flow cytometry after 5 weeks of culture. Using this approach, we found a reduced survival rate in the photoreceptor cells with the E181K mutation, which was correlated with the increased expression of endoplasmic reticulum (ER) stress and apoptotic markers. The screening of therapeutic reagents showed that rapamycin, PP242, AICAR, NQDI-1, and salubrinal promoted the survival of the patient's iPSC-derived photoreceptor cells, with a concomitant reduction in markers of ER stress and apoptosis. Additionally, autophagy markers were found to be correlated with ER stress, suggesting that autophagy was reduced by suppressing ER stress-induced apoptotic changes.

Conclusion: The use of RP patient-derived iPSCs combined with genome editing provided a versatile cellular system with which to define the roles of genetic mutations in isogenic iPSCs with or without mutation and also provided a system that can be used to explore candidate therapeutic approaches.

Development of 3D-retina Cell Models for Understanding Retinitis Pigmentosa Pathomechanisms

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The recent discovery of direct reprogramming of somatic cells into induced pluripotent stem (iPS) cells offers a great potential for regenerative medicine. The iPS technology would not only facilitate stem cell-based replacement therapy, but is also particularly useful in disease modeling. Human iPS cells offer a relatively non-invasive means to study cell types affected by disease from living patients, providing a bridge between clinical and bench research. In theory, any disease can be studied using human iPS

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cells, but this new technology is particularly attractive for disorders with a clear genetic contribution to disease pathogenesis.

Inherited retinal dystrophies such as retinitis pigmentosa (RP) in which photoreceptors and retinal pigmented epithelial (RPE) cells die, are the leading cause of blindness or visual impairment in the young adult population (more than one million people throughout the world). For many subtypes of RP, no appropriated models are available, and a better understanding of the biological process impacted by the mutated genes is required to improve treatment strategies and test efficacy. Patient-specific iPS-derived retinal cells can be used to confirm the clinical and genetic diagnosis of RP and to explore the molecular and cellular mechanisms of specific gene mutations.

Many data have indicated that human iPS cells can be differentiated into RPE cells and photoreceptors after various manipulation of the culture environment. We will discuss the different approaches already reported with RP patient-specific iPS-derived retinal cells. We recently developed a simple and efficient retinal differentiation protocol with the use of minimal exogenous compounds that allows the simultaneous generation of RPE cells and retinal organoids. These 3D structures contain retinal progenitor cells, that can be differentiated into all retinal cell types, including photoreceptors. We will focus on most prevalent forms of autosomal dominant RP in Europe and on the characterization of retinal cells differentiated from these patient-specific iPS cells using 3D retinal organoids.

Generation and Analysis of Induced Photoreceptor-like Cells from Fibroblasts of Patients with Retinitis Pigmentosa

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Direct reprogramming, or redirecting differentiation of somatic cells by overexpression of transcription factors, is a promising, simple, low-cost approach to generate target cells from somatic cells without using induced pluripotent stem cells. My research group successfully generated photoreceptor (PR)-like cells from human somatic cells; iris cells, dermal fibroblasts and peripheral blood mononuclear cells (PBMCs), using a direct reprogramming technique. First, I defined the transcription factor combinations that can determine human PR cell fate. I selected several transcription factors that may contribute to induction of PR-specific phenotypes. A mixture of these genes

was transduced into iris cells, which were examined for inducible expression of PR-specific phenotypes. Expression patterns were dependent on combinations of transcription factors: A combination of *CRX* and *NEUROD* induced rod-specific genes.

I further tested whether human dermal fibroblasts could be converted into PRs. In human dermal fibroblasts, the PR-specific genes were up-regulated by a combination of *CRX*, *RAX*, *NEUROD* and *OTX2*. Global gene expression data by microarray analysis showed that phototransduction-related genes were significantly increased in induced PR-like cells, where a photoresponse was detected using the whole cell patch clamp technique. Finally, I tested whether human PBMCs could be converted into PRs. Retinal disease-related genes were efficiently detected in *CRX*-transduced PBMCs, most of which are crucial to PR functions. By functional studies, a light-induced inward current was detected in some *CRX*-transduced PBMCs. In this study, we generated and analyzed induced PR-like cells from fibroblasts of patients with retinitis pigmentosa associated with defects in *EYS* genes. Global gene expression profiles were virtually similar between induced PR-like cells from healthy donors and patients. However, abnormal transcripts derived from mutated *EYS* genes were detected in induced PR-like cells from patients, which might be associated with pathogenesis of retinitis pigmentosa. These data suggest that our induced PR-like cells might contribute to individualized drug screening and disease modeling of inherited retinal degeneration.

Normal Tension Glaucoma by Optineurin E50K Mutation: Disease Mechanism and Therapeutic

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Optineurin (OPTN) is a multifunctional protein, which mutation in OPTN gene has been associated with normal tension glaucoma (NTG) and amyotrophic lateral sclerosis (ALS). We have previously reported that E50K transgenic mouse (E50K-tg) exhibits thinner retina and retinal ganglion cell (RGC) loss (Chi et al., *Hum Mol Genet* 2010). In E50K-tg retina, E50K mutant protein is accumulated in outer plexiform layer where exhibited the severe cell death and atrophy. The endogenous OPTN and E50K mutant protein dynamics was investigated using neuronal cells derived from E50K-NTG patient iPS Cells (iPSCs) (Minegishi et al., *Hum Mol Genet* 2013). The intracellular localization and protein property of endogenous OPTN in wild-type control

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and in E50K carrier were also examined by iPSCs and iPSC-derived neural cells. To elucidate the precise protein dynamics, FLAG-tagged OPTN and E50K were expressed in HEK293T cells and the anti-FLAG immunoprecipitates were examined by LC-MS/MS proteomics, which resulted with E50K interacting protein as TBK1 protein. The treatment with specific TBK1 inhibitor, BX795 rescued the aforementioned abnormal hydrophobicity of E50K mutant. These results indicate underlying pathoetiology of E50K-NTG originates from the alteration of protein affinity that deteriorates the OPTN/E50K intracellular dynamics. Since nascent protein transition from ER to Golgi is a fundamental physiology for cellular survival, this alteration is the probable cause of other terminal E50K phenotypes such as Golgi deformation, intracellular transport failure and cell deaths. In addition to the E50K-tg mouse, we generated an OPTN E50K knock-in mouse (E50K-ki) by CRISPR/Cas9 genome editing. The thinning of the RGC layer in homozygous E50K-ki mice was observed at 6 months, and the reduction was 20% compared with the wild-type ($p < 0.001$). The E50K-ki homozygous mouse at 12 months showed thinning of the optic nerve fiber compared with wild-type mice. Furthermore, the optic cup increased in depth in E50K-ki homozygous mouse, similar to the changes in glaucoma patients. On the other hand, E50K-ki heterozygous mice showed no significant changes in the retina, even at 12 months. The E50K-ki homozygous mice do not progress to thinning of the entire retina like the E50K-tg mice but rather mimic the phenotype of E50K patients. In this study, TBK1 inhibitor was tested to E50K-ki mice to prevent or delay the retinal ganglion cell death.

RND10 - Noncoding RNA in retinal development and disease

The Role of miRNAs in the Development of the Retinal Pigmented Epithelium

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Normal vision depends on the retinal pigmented epithelium (RPE), cells that reside between the blood vessels of the choriocapillaris and the light-sensitive outer segments of the photoreceptors. To study the in-vivo functions of miRNAs during normal RPE development and their involvement in diseases of the retina, we previously employed a *DctCre* mouse line to inactivate *Dicer1*^{loxP}, an enzyme involved in the biosynthesis of miRNAs in the RPE.

Phenotypic analysis of the *Dicer1* mutant RPE (*Dicer1*-CKO) revealed that RPE miRNAs are not required for acquisition of RPE identity, based on the expression of major RPE markers. Importantly, we observed that the outer segment does not develop in the adjacent photoreceptor layer in which *DctCre* is not active, thus implicating a non-cell-autonomous requirement for miRNAs in the RPE for photoreceptor differentiation. As *DctCre* is already active at the embryonic stage, we analyzed the embryonic stages for primary phenotype. This analysis uncovered a disruption in cell size and polarity based on altered distribution of β -catenin in the *Dicer1*-CKO RPE. These findings reveal that miRNAs are already involved in RPE growth and acquisition of cell polarity at the embryonic stages. Current effort is being invested in identifying the miRNAs and pathways that mediate RPE growth and morphology.

microRNAs in Retinal Progenitor Competence

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The complex structure of the retina -and therefore its ability to convert light information into patterns of action potentials- depends on the generation of different types of cells, at the right proportions and at the right time. Similar to many other areas of the Central Nervous System, the retina is built by the consecutive addition of cell populations in a sequence that is conserved across species. At the same time, lineage-tracing studies demonstrated that retinal progenitor cells are multipotent so that one single retinal progenitor has the ability to differentiate into multiple cell types. Together these observations suggest that retinal progenitor cells change their competence over developmental time. It has been proposed that the retinal progenitor cells have an intrinsic "clock" but the molecular nature of this phenomenon is not completely understood. Increasing evidence shows a crucial role for microRNAs (miRNAs) in the control of developmental timing and cell fate acquisition. In the mouse, a cascade of regulatory miRNAs controls a developmental shift in retinal progenitor competence from a progenitor that has the ability to differentiate into early cell types to a progenitor that no longer can generate these early populations. We have also characterized the miRNA landscape of the developing primate retina as a first important step to elucidate whether miRNAs regulate the developmental timing of the retina across species. It is well documented that the primate retina develops in a centro-peripheral

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gradient where the middle is the fovea centralis. We have investigated the differences in miRNA composition between the fovea and the periphery to uncover region-specific mechanisms.

We propose that the unique characteristics of miRNAs provide a flexible, combinatorial system for gene regulation necessary for the sequential fate-determination events that characterize a cell's temporal identity during development. Additionally, miRNAs manipulation may offer novel strategies to direct stem cells into specific retinal cell fates.

The miR-204/211: Two Micro-regulators of Eye Development and Disease

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Inherited retinal dystrophies (IRD) are one of the major causes of blindness in industrialized countries. They are characterized by progressive dysfunction and death of retinal photoreceptor cells. Although more than 100 IRD responsible genes have been identified to date, there is strong evidence for the presence of many others that still need to be identified. Furthermore, we are still far both from the complete understanding of processes driving photoreceptor cell death progression and efficient therapy. Accumulating evidence suggests that microRNAs (miRNAs), a class of short non-coding RNAs that control the expression levels of their target genes, play a key role in the control of fundamental biological processes in both physiological and pathological conditions. We have recently identified members of the miR-204/211 miRNA family as key players in RPE/retina crosstalk, whose absence causes a progressive death of photoreceptor cells in both KO mouse and medaka models. Moreover, we described the identification of a dominant mutation in miR-204 and demonstrated the functional significance of this mutation in inherited retinal dystrophy (IRD) in humans. In order to gain insight into a better understanding of the role of this miRNA family, by applying gain- and loss-of-function studies we further established that miR-204/211 has a significant impact on the cell proliferation, differentiation and maturation of photoreceptor cells. Our results represent an exhaustive example of the biological relevance of miRNAs in eye development and function, which is expected to have a high relevance to the study of the pathological mechanisms affecting this tissue and to design future therapies to inhibit tissue damage and thus maintain visual function.

The Role of the *miR183/96/182* Cluster in Zebrafish

Retinal Development

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The function and survival of sensory neurons, including photoreceptors, requires the activity of the *miR183/96/182* cluster. In the vertebrate retina, the *miR183/96/182* cluster accounts for almost 70% of all miRNA expression. All three miRNAs are highly conserved among metazoans, with *miR182* and *miR96* sharing identical seed sequences that differ with that of *miR183* only at the first position. Retinal structure or survival, however, is not affected by the loss of single miRNAs from this cluster. We therefore hypothesized that these miRNAs function either cooperatively or redundantly. We used the CRISPR/Cas9 system for multiplex genome editing to generate mutations at all three loci in zebrafish. Using a luciferase reporter system, we demonstrate that each of these mutations result in loss of miRNA function. Furthermore, despite the significant homology between these three miRNAs, we could not detect cross-reactivity between a given miRNA and a non-cognate target site, suggesting functional autonomy. Phenotypic analysis of single, double, and triple mutants will help define the minimal requirements of this miRNA cluster within the context of the vertebrate retina and will provide valuable insight toward the design of miRNA-based therapies.

Identification and Characterization of Long Noncoding RNAs in Retinal Progenitor Cell Competence

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In the developing nervous system, cellular diversification is achieved by progression of a common pool of multi-potent progenitors through a series of developmental competence states, where cells gain and/or lose the ability to generate specific cell types. In the developing retina, retinal progenitor cell (RPC) competence is largely controlled cell autonomously and is hypothesized to result from changes in transcription factor expression and/or activity. Despite extensive RNA expression profiling of the developing

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retina, only a few RPC-expressed transcription factors have been identified whose expression patterns track with changes in, and directly regulate, cellular competence. This suggests that other mechanisms exist to regulate both RPC competence and the transcriptional networks that drive cell-type specification. We hypothesize that dynamically expressed long noncoding RNAs (lncRNAs) are likely candidates to regulate progression through competence states as they can regulate both transcription factor activity and chromatin conformation. Using RNAseq of RPCs and post-mitotic cells at early (E14) and late (P2) competence phases in the mouse retina, we have identified >100 lncRNAs that are enriched in RPCs and display differential expression between early and late-stage RPCs. Using *in situ* hybridization we have characterized the developmental retinal expression patterns of >20 lncRNAs and neighboring protein coding genes. Using *in vivo* electroporation and a suite of moderate to high throughput techniques, we have begun to assess the consequence of changes in candidate lncRNA expression on RPC competence. In particular, we have identified that the intergenic lncRNA *Gm11454* is expressed in the neuroblast layer of the postnatal retina. Overexpression of *Gm11454* results in decreased expression of the neighboring protein-coding gene, *Tox2*, and results in an increase production of photoreceptors at the expense of inner nuclear layer cells. In particular, Müller glia, the last-born cell type in the developing retina, are largely absent from clones where *Gm11454* is overexpressed. Inhibition of *Tox2* expression results in a similar phenotype to *Gm11454* overexpression, suggesting that *Gm11454* regulates RPC competence by regulating *Tox2* expression. Additional experiments are underway to identify the role of additional lncRNAs in RPC competence and to further investigate the mechanisms of *Gm11454* function in the developing retina.

RND11 - Ca⁺⁺ signaling in retinal ganglion cells and outer retinal neurons

Calcium Dynamics and Signaling at the Mammalian Cone Photoreceptor Synapse

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The cone synapse is unique in the CNS because both pre- and postsynaptic neurons use graded responses and because signaling is highly parallel—a cone uses ~20 synaptic ribbons to signal to >12 cone bipolar cell types. Our idea is that the cone signal is re-encoded at

the terminal as a spatiotemporal glutamate gradient that is sampled in different ways by the postsynaptic bipolar cell types. The cone terminal has a ~5 μm dia. base punctuated by ~0.4 μm deep membrane invaginations. Each invagination has an apical synaptic ribbon which is a site of L-type Ca²⁺ channel-dependent vesicle fusion and glutamate release. At steady voltages in the dark, vesicles fuse stochastically at a rates of < 50/s per invagination. After fusion, glutamate flows from the invaginations to the base. We focused on the 5 Off bipolar cell types that express AMPA/kainate receptors. These types differ in the number of dendritic contacts made with a cone (from 1.4 to 7.6) and in contact location relative to the ribbon sites (invaginating vs. basal). To understand how different contact numbers and locations sample the stream of cone vesicles, we counted the number of vesicles released by a cone during a brief depolarization by monitoring the cone glutamate transporter current. Simultaneously, we counted the number of vesicles detected by a postsynaptic Off bipolar cell (types cb1,2,3) by measuring its epsc. Experiments were performed in slices from the ground squirrel retina. Events fluctuated in amplitude from trial to trial. We made a scatter plot of peak bipolar cell vs cone response over an extended range of stimulus strengths. For cb2 cells, which extend dendrites into ~50% of a cone's invaginations, scatter plots were linear with a y-intercept of 0pA. In contrast, plots for basally contacting cb1/3 cells followed a power law with almost no bipolar cell response when 0-10 cone vesicles were released, but increasing responses when more vesicles were released. We conclude that the Off bipolar cell types sample cone transmitter release in different ways. An invaginating cb2 cell dendrite samples its local ribbon and is sensitive to stochastic release at a steady voltage. Basally contacting cb1/3 cells fail to detect stochastic events but can respond to multivesicular release that is coordinated by membrane depolarization and Ca²⁺ influx. A threshold at the cone to cb1/3 Off bipolar cell basal synapse improves the SNR at the expense of sensitivity to small changes in a steady stimulus.

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Regulation of Intraterminal Ca^{2+} at Ribbon Synapses of Rods and ConesWALLACE THORESON¹, Matthew Van Hook¹, Justin Grassmeyer¹, Minghui Chen^{1,2}¹University of Nebraska Medical Center, Ophthalmology and Visual Sciences, Omaha, United States, ²Vollum Institute, Portland, United States

We explored Ca^{2+} handling properties in synaptic terminals of rods and cones from salamander retina. Photoreceptors release glutamate at synaptic ribbons, regulated by Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. Combining whole-cell recordings with confocal Ca^{2+} imaging of synaptic ribbons, we found the voltage dependence of ribbon-localized Ca^{2+} responses in cones were nearly identical from ribbon to ribbon. The many ribbons in a single cone can thus be viewed as a single mechanistically uniform structure. By measuring $I_{\text{Cl}(\text{Ca})}$ and by using the added buffer approach, we found that endogenous Ca^{2+} buffering in rod and cone terminals is weak, equivalent to 0.05-0.1 mM EGTA. With such buffering, depolarization stimulated $[\text{Ca}^{2+}]$ increases that were not constrained to individual ribbons but spread throughout terminals, promoting homogeneity in Ca^{2+} signals among neighboring ribbons. In both rods and cones, weak Ca^{2+} buffering also speeds Ca^{2+} /calmodulin (CaM)-dependent replenishment of vesicles to ribbons. These actions of CaM were blocked by CaM kinase II inhibitors. In rods but not cones, intraterminal Ca^{2+} is boosted by Ca^{2+} -induced Ca^{2+} release (CICR) from endoplasmic reticulum (ER) stores. Enhancing CICR with weak Ca^{2+} buffering boosts non-ribbon release. In cones, elevating $[\text{Ca}^{2+}]$ at non-ribbon sites with weak Ca^{2+} buffering and by inhibiting Ca^{2+} extrusion did not enhance release consistent with prior evidence that exocytosis in cones occurs exclusively at ribbons. How can rods sustain CICR when they remain depolarized in darkness? We measured ER $[\text{Ca}^{2+}]$ changes by loading ER with fluo-5N and then washing dye from the cytoplasm with a dye-free patch pipette solution. Simulating darkness by depolarizing rods to -40 mV depleted Ca^{2+} from terminal ER followed by a slower decline in somatic ER $[\text{Ca}^{2+}]$. Local activation of ryanodine receptors (RyRs) in terminals by a spatially-confined puff of ryanodine caused a decline in terminal ER $[\text{Ca}^{2+}]$ followed by a secondary decrease in somatic ER. Localized photolytic uncaging of Ca^{2+} from NP-EGTA in somatic ER abruptly elevated Ca^{2+} in somatic ER followed by a delayed Ca^{2+} increase in terminal ER. These data suggest that during maintained depolarization, a somato-terminal $[\text{Ca}^{2+}]$ gradient develops within the ER that promotes diffusion of Ca^{2+} ions to resupply intraterminal ER Ca^{2+} stores and thus sustain CICR-mediated synaptic release.

TRP Channels as Multimodal Modulators of Retinal Ganglion Cell Function and Survival

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There has been a long-standing debate whether neuronal output represents an algorithmic 'computation' of inputs or to what extent the output - and indeed, function - can be influenced by intrinsic molecular features characteristic of each neuronal subtype (Llinas, 2014). For example, within the retina, RGCs are characterized by their size, location, spiking patterns and their unique susceptibility to mechanical, osmotic and metabolic stress. What is the relationship between RGC output (cell firing) and sensory overload, and how might transduction of 'nonconventional' stimuli (heat, stretch, pressure, swelling etc) differ from other retinal neurons?

We found that RGCs selectively express TRPV4, a non-selective cation channel which contributes to mechanosensation, thermosensation, volume regulation, together with TRPV1 which is a major mediator of endocannabinoid signaling and TRPC1 that mediates store-operated entry of calcium. The studies combined patch-clamp electrophysiology, optical imaging and molecular analyses in acutely dissociated, immunopanned and intact RGCs from wild type and KO mice to demonstrate that TRP channels uniquely mediate RGC sensitivity to extrinsic sensory information together with the sensing of internal milieu via STIM1/2 proteins. We determined the mechanical thresholds of the cells responding to pressure and osmotic stimuli and characterized the molecular mechanism linking osmotic stress to RGC viability. RGC health depended on a delicate balance between nonretrograde vanilloid (TRPV) and cannabinoid mechanisms. Selective overstimulation of TRP channels recapitulated many of the anatomical/functional deficits observed in retinal diseases (glaucoma, diabetic retinopathy) whereas pharmacological and genetic targeting was neuroprotective in preclinical disease models.

Overall, our results show that TRP activation defines the mechanical, thermal, acidity and lipid messenger thresholds in RGCs. TRPV1, TRPV4 and TRPC1 represent molecular RGC sensors that drive the transient and/or sustained modulation of RGC output and may, under conditions of chronic stress, contribute to retinal disease. Supported by the NIH, DOD, Willard L. Eccles Foundation, Utah Neuroscience Initiative.

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Re-inventing Phototransduction with Ion Channel Photoswitches that Restore Visual Function to Blind Mice**RICHARD KRAMER***University of California, Berkeley, United States*

Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are degenerative blinding diseases caused by the death of rods and cones in the retina, leaving the remainder of the visual system intact but unable to respond to light. We have shown that a synthetic azobenzene-containing photoswitch compound named DENAQ imparts light-sensitive blockade on voltage-gated cation channels, restoring light sensitivity to the retina of blind mice with a degenerative disorder similar to RP. Injection of DENAQ into the eye reinstates light-triggered firing of retinal ganglion cells and restores behavioral responses to light in blind mice *in vivo*. Our long-term goal is to optimize DENAQ and related photoswitches to enable safe, effective, and long-lasting restoration of visual function, first in mice, and eventually in humans. Recent studies have focused on a surprising finding: DENAQ is effective in photosensitizing blind retinas in which the rods and cones have died; but it has almost no effect on healthy retinas that still possess intact rods and cones. New results suggest that the entry of the positively-charged DENAQ into retinal ganglion cells is mediated by large-conductance ionotropic receptors for ATP (P2X receptors) that are up-regulated and tonically active in degenerated retina. Treatment with P2X antagonists or apyrase, an enzyme that hydrolyzes ATP, prevents DENAQ photosensitization. Furthermore, photosensitization is specific for retinal ganglion cells that send dendritic projections to the Off-sublayer of the inner plexiform layer. Only presumptive Off-RGCs have the calcium-permeant voltage-gated ion channels that are the primary electrophysiology target of photoswitches. The degeneration-specific action of DENAQ raises the possibility that this or related compounds might eventually be effective in treating AMD, locally photosensitizing the central region of the retina that has undergone photoreceptor degeneration, while sparing the still-healthy periphery.

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Synaptic and dendritic signaling in the vGluT3 circuit of the retina**Z JIMMY ZHOU, Seunghoon Lee, Minggang Chen, Yi Zhang, Yu Jia, Mingzhao Chen***Yale University, Ophthalmology and Visual Science, New Haven, United States*

Complex receptive field properties of retinal ganglion cells are shaped to a large degree by amacrine cells, which are generally classified as inhibitory interneurons. vGluT3-expressing amacrine cells are a newly identified amacrine type that releases glutamate at functional synapses. Using patch-clamp recording together with optogenetics and calcium imaging in a vGluT3-cre mouse line, we demonstrate that these glutamatergic amacrine cells (GACs) also release glycine. The co-release of this new combination of excitatory and inhibitory transmitters is Ca-dependent and occurs at both ON and OFF sublaminae of the inner plexiform layer, where the GAC makes segregated glutamatergic and glycinergic synapses onto specific postsynaptic target neurons in functionally opposite retinal circuits. Results on the cellular and synaptic response properties of GACs suggest that dendritic signal processing plays an important role in shaping the contribution of GACs to differential visual computation.

JNT5 (RND+RCB) - ES/iPS-based approaches to treating retinal dystrophies**Developing an Autologous iPS Cell Derived RPE Based Cell Therapy for Macular Degeneration****KAPIL BHARTI***NEI/NIH, Bethesda, United States*

Induced pluripotent stem (iPS) cells are a promising source of personalized therapy. iPS cells can potentially provide immune-compatible autologous replacement tissue for the treatment of all degenerative diseases. We are preparing for a phase I clinical trial using iPS cell derived ocular tissue to treat age-related macular degeneration (AMD), one of the leading blinding diseases in the US. AMD is caused by the progressive degeneration of retinal pigment epithelium (RPE), a monolayer tissue that maintains vision by maintaining photoreceptor function and survival. Combining developmental biology with tissue engineering we have developed protocols for manufacturing of a clinical-grade iPS cell derived RPE patch on a degradable scaffold. This patch performs key RPE functions like phagocytosis of photoreceptor outer segments, ability to

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transport water from apical to basal side, and the ability to secrete cytokines in a polarized fashion. Currently, the NEI team is testing the safety and the efficacy of this RPE-patch in animal models in preparation for a Phase I Investigational New Drug (IND)-application. Approval of this IND application will lead to the transplantation of autologous iPS cell derived RPE patch in patients with the advanced stage of AMD. Success of NEI autologous cell therapy project will help leverage other iPS cell-based trials making personalized cell therapy a common medical practice.

Cultivation, Installation and Preservation: Development of Cellular Therapies for Age-related Macular Degeneration

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A promising option for the treatment of ocular disease is to develop cellular therapies using RPE and /or neural retinal cells derived from pluripotent stem cells. Several groups have already initiated clinical trials for the treatment of the non-exudative (dry) form of age related macular degeneration using RPE derived from human embryonic stem cells or induced pluripotent stem cells. The California Project to Cure Blindness has developed an implant consisting of a differentiated, polarized monolayer of hESC-RPE on a biostable parylene scaffold, and has recently initiated a Phase 1/2A clinical trial. We describe the progression from preclinical studies, to IND enabling research, to clinical trial design.

Assessing Authenticity of Human Pluripotent Stem Cell-derived Photoreceptor Precursors

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Inherited and acquired eye diseases that culminate in the degeneration of photoreceptors are a significant cause of visual morbidity. Differentiation of human pluripotent stem cells (hPSCs) toward retinal cell fates provides a potentially valuable source of biological material, including photoreceptor precursor (PRP) cells, for modeling retinal development and disease and devising therapies

for blinding disorders. However, confidence in hPSC technology as a tool for basic and translational retinal research is limited in part by our collective knowledge of the characteristics and capabilities of their progeny. The process of authenticating hPSC-PRPs not only includes an in depth analysis of the final cell products, but also an examination of the path and mechanisms by which they differentiate *in vitro*. I will discuss methods to produce retinal cell progeny from hPSCs and steps we are taking to probe their identity and determine their suitability for modeling disease and developing therapeutics.

Enabling High Throughput Screening in 3-D Retinal Organoids for Drug Discovery

MARIA VALERIA CANTO-SOLER

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The advent of stem cell-derived retinal organoid systems has brought forth new therapeutic promise for retinal degenerative diseases. These systems have the potential to contribute to our understanding of embryonic development as well as disease processes, and are being explored not only as a source for transplantation but as a novel tool for drug development applications. However, several aspects such as the variability of retinal organoid systems and the lack of appropriate 3D high throughput technologies pose severe limitations for their use in this type of applications. In an effort to bring retinal organoids closer to the clinical arena, we recently established a novel strategy to direct hiPSC to differentiate into retinal organoids that faithfully recreate the histoarchitectural organization of the human neural retina *in vivo*, including highly differentiated functional photoreceptors. Building upon this system, we are now developing a platform that enables fluorescence quantification-based large-scale drug discovery in complex human iPSC-derived retinal organoids, while providing the speed, sensitivity and reproducibility necessary for this kind of assays. We expect that this technology will open novel avenues to study the etiopathology of retinal diseases, identify targets for therapeutic interventions, and discover drugs with clinical potential for the treatment of retinal degenerative diseases.

Combining Stem Cells, Genome Editing and Tissue Engineering to Rebuild the Outer Retina

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Inherited retinal degenerative disorders such as retinitis pigmentosa are characterized by death of the light sensing photoreceptor cells of the outer neural retina. Like the rest of the CNS, the endogenous regenerative capacity of the retina is limited, and as a result, photoreceptor cell death causes debilitating irreversible blindness. Although stem cell based photoreceptor cell replacement strategies have been quite successful experimentally, when transplants are performed in hosts with advanced disease, donor cell survival, and in turn functional integration, is often poor. To mitigate this problem, tissue engineering approaches are being developed. In this talk I will demonstrate how we have combined iPSCs, CRISPR-based genome editing and two-photon 3D lithography to create genetically-corrected outer retinal grafts for the treatment of retinal degenerative blindness.

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OPT1 - Translational studies in glaucoma - How do we make basic science studies in glaucoma more clinically relevant: Development of a consensus

What Is the Role of the Basic Science Research Laboratory?

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Experiments in the basic science research lab have led to numerous discoveries on the molecular, cellular, organ and whole animal level. A fundamental question is whether basic science research is essential to advancing our understanding of human disease. The evidence supports this provided one asks the right questions and chooses the best model. The advantages and disadvantages of each model must be clearly understood in order to better interpret findings. Are the cell signaling pathways in animals similar to human? How do animal models of glaucoma differ from humans with glaucoma? Can any animal model be considered the "gold standard"? Can one expect a drug to work in humans in the same manner as animals? How does age, sex, time of day and countless other variables affect the results? When should one terminate a line of research and move in a different direction? Consensus statements developed by experts in the field of glaucoma are sorely needed to aid in designing experiments and answering these questions. With clear understanding of the physiology and pathology of the research model as well as the glaucoma patient, basic science research will continue to blaze the trail towards improved treatments for glaucoma and maybe someday find a cure. This presentation will discuss how the basic science research laboratory has ultimately improved clinical care.

Animal Models of Glaucoma and their Translatability into the Clinic

ANDY WHITLOCK

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Preclinical animal models have played an important role in the field of ophthalmology in order to advance early scientific discoveries to novel therapies. Ocular hypertension is a good example of the translation of preclinical basic science and pharmacology to novel clinical treatments.

Glaucomatous retinal degeneration, however, has been a particularly challenging condition to study pre-clinically. As a result, no treatments to date have been approved for glaucoma retinal neuroprotection. Recent advancements in animal models, endpoint analysis, and drug delivery will hopefully change this in the future. The purpose of this talk will be to describe the current preclinical models used for both ocular hypertension and glaucomatous retinal degeneration, and how these models can best be used to translate early research into the clinical. Animal models should be used to provide information about a potential therapeutic's mechanism of action, efficacious dose range, and safety profile. Many considerations should be taken when choosing the best model to use when evaluating novel glaucoma therapeutics. The first consideration is the target or pathological pathway (i.e. ocular hypertension, ganglion cell death, or optic nerve degeneration). The next major consideration is species. The selection of species will dictate how, where, and how often a therapeutic can be delivered based on ocular anatomy. Species and ocular anatomy will also have an impact on disease pathology and endpoint selection. Careful endpoint selection is the final consideration to take when choosing an animal model. Appropriate and relevant endpoint selection is critical in helping make preclinical animal models of glaucoma more translatable to the clinic.

Clinical Investigations on Rho Kinase Inhibitors-1 Year after Approval

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Rho kinase (ROCK) inhibitor has been shown to lower intraocular pressure (IOP) in animal and human eyes. So far, more than 10 ROCK inhibitors have been subjected to clinical trials, and among them, ripasudil (also known as K-115) is the only one approved ROCK inhibitor as an anti-glaucoma medication. In 2014, it was approved based upon significant IOP-lowering effects and safety profile revealed by phase 1 to 3 clinical trials for primary open-angle glaucoma (POAG) and ocular hypertension (OH). After then, ripasudil ophthalmic solution is used for a variety of secondary glaucoma including steroid-induced, uveitis-related, and exfoliation glaucomas in addition to POAG and OH. Open-label clinical investigations suggested useful IOP-lowering effects of ripasudil for secondary glaucoma, POAG and OH in clinical practices although further clinical studies will be required to elucidate background factors related to IOP-lowering effects of ripasudil.

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Control of Glaucoma by the Brain - Translational Studies

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Purpose: Update on new POAG findings and their importance toward understanding of the mechanisms underlying other age-related neurodegenerative disorders.

Methods: Refined analysis of perimetric data confirmed that as glaucoma progresses, the eyes and brain work together toward maintaining the best possible binocular visual field. First observed in bilaterally severe glaucoma (TVST 2014;3(3):1) this was found to arise early and persist throughout the disease process (TVST 2015;4(2):8 & TVST 2015;4(3):7). These adult human findings reaffirm basic science work showing that the brain meticulously controls glaucomatous neurodegeneration (Calkins DJ. *Prog Retinal Eye Res* 2012;31:702-19).

Results: In 47 consecutive patients with bilaterally severe glaucoma mean HVF 30-2 thresholds were 18.9 dB OD and 19.9 dB OS, but the better of the two concomitant loci provided a mean threshold of 23.4 db ($P=10^{-15}$), greatly exceeding random co-isopteric pairings of the same data ($P=10^{-12}$), again substantially outperforming randomized co-isopteric pairings ($P< 0.0001$). This outcome was affirmed using simultaneous bilateral visual field testing, and again reaffirmed in a masked population of 55 bilaterally moderate to severe glaucomatous individuals from the Rotterdam Eye Study. 44 patients with predominantly mild or moderate bilateral loss were then evaluated using FDT Matrix, bilateral pairings (20.4 dB) greatly exceeding mean unilateral results (17.7 dB) ($P=10^{-12}$), again substantially outperforming randomized co-isopteric pairings ($P< 0.0004$). Spatial characteristics of binocular conservation implicate ocular dominance columns in V1 as likely center for bilateral CNS control of glaucomatous neurodegeneration.

Conclusion: Clinical implications of these findings for the future management of glaucoma are numerous. Moreover, glaucomatous eyes at all stages of disease provide a highly accessible paired-organ study model for developing therapeutics to optimize conservation of function in a range of neurodegenerative disorders.

Neuroprotection and Neuroregeneration of Retinal Ganglion Cells from Basic Science to Clinic

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Glaucoma is a progressive optic neuropathy that leads to irreversible vision loss. Because current therapies, i.e. approaches targeting increased intraocular pressure, in many cases are not sufficient to prevent the progression of the disease, it is necessary to search for new therapeutic options that will protect the retinal ganglion cells (RGC) against damage. Research on neuroprotection and neuroregeneration of RGC became nowadays dynamically developing branch of neuroscience and eye research. While commonly understood term "regeneration" is related to cells proliferation, in case of neurons, i.e. RGC, it express ability to induce outgrowth of axons. Neuroprotection and neuroregeneration processes are linked to each other, since to expect axonal outgrowth, it is necessary to keep cell body, which is energy center of the cell, alive and functional. There are numerous of different studies focusing on neuroprotection and neuroregeneration, however many of them are questionable to be applied in clinical settings due to unsure safety features. There are many barriers identified while applying basic science ideas to the clinic. While experimental settings create possibilities to detect effects of neuroprotective and neuroregenerative therapies in many ways (including detailed histology), in clinical application the evaluation of the effect is limited to *in vivo* methods (i.e. visual fields, optical coherence tomography, electrophysiology). These methods, if imprecisely used, may appear to be not sensitive enough to detect minimal effects of therapy. Recently, the supplementary method - pupilometry, which evaluate function of melanopsin RGC was proposed, however it concerns only about 25% of all RGC. Candidate patients, especially for RGC neuroprotective therapy would have to be selected with a great care. Patients with terminal stage of diseases (i.e. terminal glaucoma) would not be good candidates for such therapy, on the other hand, considering patients with moderate or mild damage may arise ethical questions about benefit/risk ratio. Despite of numerous difficulties, the recent experience of applying basic science ideas in the clinical therapies of other retinal or choroid diseases - choroideremia, Stargardt's disease, indicates that the therapeutic success is possible to achieve with these methods.

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OPT2 - Translational ophthalmology: Novel targets and their development into the clinic

Preclinical Path Forward for a Dry Eye Product that Inhibits Mitochondrial Oxidative Stress

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The advancement and eventual approval of therapeutics for ocular indications depends on a sound preclinical and clinical development plan. This includes early activities including safety, PK, and proof-of-concept in animal efficacy models, as well as clinical trial designs that have been vetted by the appropriate regulatory agency. The goal of the early work can provide insight regarding formulation, dosing regimen, and the best indication for pursuit based on the mechanism of action of the drug under consideration. Animal efficacy models that recapitulate features of the human disease provide a way in which to evaluate the drug in the context of the whole animal, in order to gain confidence that the product under consideration should advance to the next development step. The ability of success in an animal model to predict clinical efficacy depends on how well the animal disease mimics the human condition, and how well the chosen endpoints resemble those used to evaluate success in the clinic.

In this presentation, we will review examples of preclinical studies that were predictive of success in the clinic, focusing on an inhibitor of mitochondrial oxidative stress that was efficacious in the scopolamine-induced model of Dry Eye in mice, and that went on to be validated clinically in a phase 2 study of Dry Eye.

A First-in-Class Oligonucleotide Based Ophthalmic Therapeutic for Vascular Leakage

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Abnormal vascular leak is a major problem in diabetic retinopathy and macular degeneration. A large part of this leakiness is due to the endothelial cells (ECs) that form the cellular interface between the blood and the tissue losing

their integrity and barrier function.

One of the major adhesion molecules specific for EC junctions is VE-Cadherin. VE-Cadherin undergoes homotypic adhesion in the junctions, acting as both a structural and signalling protein. Disruption of the VE-Cadherin adhesion through loss of the protein, its redistribution or through phosphorylation events results in disruption to junction integrity and promotion of EC permeability. Further, antibodies to VE-Cadherin induce vascular leak. Thus, targeting VE-Cadherin to reform stable junctions is a potential mechanism for control of EC integrity.

We have identified VE-Cadherin as a target for the microRNA, miR-27a (Young J et al Blood 2013). Using a new class of oligonucleotide-based inhibitor (Blockmirs) we have developed CD5-2, a drug that selectively targets the miR-27/VE-Cadherin interaction, resulting in an increase in VE-Cadherin expression. CD5-2 also activates the junctional-stabilising pathways, TIE-2 and tight junctions, through a VE-Cadherin dependent mechanism. In a model of retinal vascular leak, the transgenic Muller cell knockout model (Shen et al J Neurosci 2012) CD5-2 inhibits vascular leak and reverses the pathology of the lesions. In the STZ induced model of diabetic retinopathy, CD5-2 also attenuates the pathology. This class of drug is stable, its properties make it suitable for large quantity production and it shows effects 3 weeks after a single intravenous bolus injection.

Transplantation of Autologous iPSC Cell-derived RPE Cell Sheets for Exudative AMD: A Pilot Clinical Study

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Purpose: Recently developed induced pluripotent stem cell (iPSC) s are a promising donor source for regenerative medicine. Since iPSCs can be generated from adult somatic cells, they bypass the need for embryos and can be made in an individual patient-matched manner, which means that each patient could have one's own pluripotent stem cell line. We report the initial 1-year clinical course of the first-in-human case of iPSC therapy in which iPSC-derived retinal pigment epithelium (RPE) cells were transplanted to a patient with exudative age-related macular degeneration (AMD).

Methods: We started an open-label study of the transplantation of autologous iPSC-derived RPE sheets in patients with advanced exudative AMD in whom the

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current standard treatments had not been effective. A small piece of skin was collected from the patient's upper arm and the skin cells were used to generate iPSCs. The iPSCs were differentiated into RPE and formed into the cell sheets suitable for transplantation. The entire process, including regular assessments of safety and quality, requires approximately 10 months for each patient. Once complete, the RPE sheet was subretinally transplanted after surgical removal of the neovascular tissue. The patient was carefully monitored by systemic and ophthalmic examinations for a year after the surgery. The primary outcome to be assessed in this pilot study is the safety of this therapeutic protocol, and the secondary outcome is the clinical efficacy.

Results: The first case of the study underwent autologous iPSC derived RPE sheet transplantation successfully. The submacularly transplanted RPE sheet survives well without any findings of immune rejection nor adverse proliferation, and any significant adverse events associated with the therapeutic protocol were not observed for a year. Retinal imaging examinations showed improvement of the pre-existed exudative change of the macula including retinal edema and serous retinal detachment. Best-corrected visual acuity was maintained at 0.09 (=18/200) without additional anti-VEGF therapy and the score of VFQ 25 (National Eye Institute Visual Function Questionnaire Japanese version) improved from 40.7 to 58.3.

Conclusions: The first-in human iPSC-based transplantation was carried out successfully in a patient with exudative AMD. The primary endpoint, safety of the therapeutic protocol, was attained after a year.

The Translational Research to Qualify Cultured Human Corneal Endothelial Cells for Cell Infusion Therapy as Homogeneous Fully Differentiated Cells

JUNJI HAMURO

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To date, most researchers conceptualize cultured human corneal endothelial cells (cHCECs) only from the aspect that they are derived from corneal endothelium tissue, and disregard details pertaining to the refinement of the biochemical features. Trials pertaining to the in vitro expansion of cHCECs without the cell-state transitioned (CST) and karyotype aneuploidy have been hampered by the lack of a scientifically reliable and semi-quantitative method to evaluate the cellular features of cHCECs. In consideration of the controversy that surrounds the heterogeneity of cHCECs, we first investigated the discrepancy of gene signatures between fresh corneal

endothelium tissues and cHCECs. In this study, we present our novel qualification method to monitor protein products in cHCECs, and describe a quality-control method to ensure the functional characteristics of cHCECs that are indispensable for clinical application. To clarify whether cHCECs, heterogeneous in their differentiation state, exhibit distinctive energy metabolism from the aims of developing a reliable method to sort cHCECs applicable for regenerative medicine. After successfully discriminating SPs in terms of their secretory metabolites, we found that CST SP exhibited disposition to anaerobic glycolysis, instead of mitochondria-dependent oxidative phosphorylation (OXPHOS). Among a variety of morphologically different cHCECs, miRNA expression profiles were distinctively revealed. The one miR capable of discriminating CD44⁻ SP from SPs with CD44⁺⁺-CD44⁺⁺⁺ phenotypes was identified as miR34a. The specified cultured SPs sharing the CD44⁻ surface phenotypes with matured HCECs showed the highest expression of miR-378. Conversely, SPs with upregulated CD44 (+++) showed a reduction of miR-378. Thus, miRNA in cultured cells may serve as an alternative method to qualify cHCECs. The findings demonstrate that cHCECs are composed of a dysregulated expression of a hierarchy of miRs clusters, probably due to the presence of CST and senescence. Isoforms of miR-378 were downregulated and miR146 isoforms, to the contrary, were upregulated in fresh HCE tissues with low ECD, with guttata. The cell-state-transitioned CD44⁺⁺⁺ cHCEC was decisively distinguished from CD44⁻ effector cells by miR-34a. In addition, to this practical purpose, the new findings presented here warrant further investigation to develop a better understanding of the role of the dysregulated expression of miRs in pathogenesis of BK and FECD.

Gene and Drug Based Therapy Prevents Retinal Degeneration in a Mouse Model of Geographic Atrophy

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We established a mouse model of RPE oxidative stress by cre-lox mediated deletion of the *Sod2* gene, which codes for the protective enzyme manganese superoxide dismutase (MnSOD), leading to some of the features of geographic atrophy. The purpose of our study was to test the sub-retinal delivery of an antioxidant gene therapy vector and systemic treatment with a 5HT1a agonist in this model. Specifically, to determine how late in the disease course

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the gene therapy would protect the RPE from oxidative damage. We injected these mice with AAV1-*Sod2* or AAV1-GFP vector into the contralateral eyes of at 6 weeks of age (10 mice), and at 6 months (6 mice) of age. Over a period of 9 months, we monitored retinal degeneration at different time points by electroretinography (ERG) and spectral domain optical coherence tomography (SD-OCT). For testing a 5HT1a agonist, we delivered this drug systemically at 2 months of age and monitored the retinal degeneration up to 6 months of age. Cell culture assays were developed to validate the effect of drug in reducing oxidative stress. One month after subretinal delivery, AAV1-*Sod2* vector resulted in production of MnSOD in the RPE and negligible expression in the neural retina. We did not find any adverse functional and structural effects due to increased expression of *Sod2*. When mice were injected at 6 weeks, electroretinogram (ERG) response and thinning of retinal layers (SD-OCT) was significantly delayed in eyes injected with the *Sod2* vector compared to control eyes injected with GFP. Mice treated with the same vector at 6 months were not similarly protected. Treatment of mice with daily injections of the drug 5HT1a agonist led to increased electroretinogram (ERG) amplitudes and to a slight improvement in visual acuity. Most strikingly, in mice treated with a high dose of the drug (5 mg/kg) the structure of the RPE and Bruch's membrane and the normal architecture of photoreceptor outer segments were preserved. Early delivery of AAV1-*Sod2* vector can be used as a tool to prevent oxidative stress in this mouse model of dry AMD. At later times, damage from oxidative stress may not be preventable. The systemic treatment of 5HT1a agonist may be useful in preventing geographic atrophy, the advanced form of dry AMD, which is characterized by RPE degeneration.

OPT6 - Matching clinical needs and novel drug delivery systems for the posterior segment of the eye

Intraocular Pharmacokinetic Modelling and Drug Delivery

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Intraocular pharmacokinetics is dependent on the clearance processes in the eye (vitreous and anterior chamber). The literature data was analyzed and clearance values calculated. For small molecules, the values in the vitreous span about 100 fold range, while proteins have low and

relatively constant clearance. The approach was modeled to predict clearance based on chemical structure and to predict profiles after drug delivery system injections. The study will illustrate the use of modeling in drug delivery system design.

Materials Inspired by the Challenges in Ophthalmic Drug Delivery

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Drug delivery systems capable of the sustained, local delivery of therapeutic agents provide unique tailor-made solutions for efficient treatment of various ophthalmic pathologies. Here are presented polyesteramides (PEA) to exemplify various benefits obtained by the use of amino acid based degradable polymers and their influence on the material biocompatibility, degradation and performance. By design, PEA predominantly degrade via an enzymatic surface erosion mechanism. Ultimate features of these materials are the remarkable shelf life and versatile process-ability to variety of forms such as particles, injectable fibers, coatings and films. The balance between hydrogen bonding and hydrophobic moieties also provides control over drug release properties for small molecule hydrophobic drugs as demonstrated in drug release case studies including latanoprost, and dexamethasone. Having commercial success in cardiovascular applications, PEA technology is currently explored as a versatile tool in ophthalmic drug delivery. The natural building blocks of the PEA polymers and absence of pH drop in in-vitro degradation studies suggest a high biocompatibility and tissue tolerance. This was confirmed in various in-vitro and in-vivo experimental settings. Furthermore, presented amino-acid based polymers are ideally suited for application in sustained delivery of biopharmaceuticals offering a protein-friendly environment even at advanced stage of biomaterial degradation. The excellent in-vivo biocompatibility is exemplified in a 12 months GLP toxicology study in rabbit model. Placebo and drug eluting implants were administered subconjunctivally and followed up by clinical examinations. In summary: subconjunctival administration of polyesteramide (PEA) microfibers resulted in mild and transient foreign body response confirming the earlier reports about the good safety and tolerability of PEA biomaterial platform in ophthalmic applications.

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The Role of Blood-retinal Barrier Transporters in Retinal Drug Delivery

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There is an increasing need to manage rapidly progressing retinal diseases because of the potential loss of vision. Although systemic drug administration is a promising route for treating retinal diseases, the retinal transfer of therapeutic drugs from the circulating blood is strictly regulated by the blood-retinal barrier (BRB), which consists of retinal vascular endothelial cells (inner BRB) and retinal pigment epithelial cells (outer BRB). Recent progress in the BRB research has revealed that the BRB expresses a wide variety of transporters essential for the blood-to-retina influx transport of nutrients and their analogs. At the same time, the BRB also possesses several transporters responsible for the retina-to-blood efflux transport of xenobiotics and drugs. Verapamil is a cationic substrate of P-glycoprotein (P-gp), which is expressed at the BRB and is involved in the efflux transport of drugs. In P-gp knock-out rats, the retinal uptake index of verapamil have showed the possible involvement of a novel organic cation transporter in verapamil transport from the circulating blood to the retina across the BRB. This information can be exploited to our advantage to establish efficient strategies for optimal delivery of clinically relevant therapeutic drugs into the retina.

Stimuli-responsive Drug Delivery Technologies for the Back of the Eye

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Retinal diseases are currently treated by frequent injections of a drug-containing solution into the eye, an unpleasant procedure that may lead to complications and low treatment adherence. This has been partially addressed by developing implants that once sutured into the sclera or injected into the vitreous can release drug over months to years. However, once these implants are in place, drug release rates cannot be altered based on individual patient needs. Our research within the Buchanan Ocular Therapeutics Unit aims to develop stimuli-responsive implants that are able to slowly release drug over time,

while also allowing for tuneable top-up dosing based on the disease progression.

One such implant is based on porous conducting polymers (PCP) prepared over a template comprising sacrificial beads using either electrochemical or vapour phase polymerization with the anionic drug, dexamethasone phosphate, serving as the dopant. Scanning electron microscopy revealed interconnected PCP structures, with electrical stimulation resulting in increased drug release. However, passive release rates were still quite high thus further sealing layers are currently being investigated to achieve suitable baseline drug levels.

The other two systems are based on photo-sensitive polymers that can either be cured or activated once injected into the vitreous by shining light through the cornea non-invasively. For the light cured implant, various concentrations of methacrylated alginate and photoinitiator were tested with the optimized formulation resulting in liquid-to-solid phase transition within 10 min of UV-light exposure with suitable mechanical properties and in vitro release characteristics. For the light-activated system, azobenzene derivatives were attached onto dye-loaded mesoporous silica microparticles (MSM) and dye release was triggered by application of visible light. Passive dye leak was still observed, especially for small dye molecules, but could be reduced when loading bigger drug molecules and/or extending the azobenzene 'barrier arm'.

Stimuli-responsive drug release was achieved in both the scleral PCP and the intravitreal MSM. Further optimisation will focus on achieving desired release profiles and performance will be evaluated in an ex vivo eye model before moving into animal studies.

Protein Nano-assemblies for Improved Intraocular Delivery

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Translation of intracellularly acting protein drugs for treating eye diseases is limited by their poor cellular delivery, inadequate persistence in the eye, and inadequate physicochemical stability. In order to enhance these aspects of delivery, we developed crystallin protein assemblies of various sizes ranging from nanometers to micrometers and assessed their ability to improve cellular uptake, intravitreal persistence, and stability in the presence of physicochemical stresses. The protein assemblies increased the physicochemical stability of the model

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protein in a nanoassembly size-dependent manner, with the optimal size for stability being about 350 nm. Relative to the native protein, these assemblies exhibited greater cellular uptake and intravitreal persistence. Additionally, the nanoassemblies enhanced chaperone activity relative to the native protein. Preparation of protein assemblies of a nanosize larger than the native protein is one useful approach to enhance and sustain intraocular protein drug delivery.

Novel Biodegradable Oil/Protein Microspheres for the Treatment of Posterior Segment Diseases

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Chronic and multifactorial pathologies affecting the back of the eye represent visual impairment and blindness. Due to the poor accessibility of the active substances to retinal tissues, successive intravitreal injections are needed. However, multiple injections are associated to adverse effects and the risk increases with the number of administrations. Microspheres (1-1000µm size) are able to provide long term delivery of the therapeutic molecules avoiding repeated injections. Contrary to larger devices, administration of microspheres is performed without the need of surgical procedures. Treatments of posterior segment diseases are often performed with biotechnological products. The encapsulation of proteins has been one of the most interesting challenges in the field of pharmaceutical technology as the biological activity of the product must be assured throughout manufacturing, storage and use. Preservation of protein biological activity can be achieved by the use of a novel protein encapsulation procedure based on a solid-in-oil-water (S/O/W) emulsion technique. Biodegradable oil/protein microspheres can be considered as emerging therapeutic tools in the treatment of chronic retinal diseases.

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OPT7 - Novel molecular mechanisms of diabetic retinopathy

Role of Connexin 43 in Human Diabetic Retinopathy

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Connexin 43 (Cx43) is abundantly present in the vascular cells of the retina, and studies have shown that high glucose impairs gap junction intercellular communication (GJIC) activity in endothelial cells and pericytes by altering Cx43 expression. In addition, our previous work has shown that Cx43 ± knockout mouse and diabetic rats exhibit increased vascular cell apoptosis in the retinas. GJIC is essential for cell survival. Cx43 channels facilitate maintenance of tissue homeostasis by allowing the exchange of small molecules and ions between adjacent cells. In diabetic animal models, downregulation of Cx43 expression promotes retinal vascular cell apoptosis. However, its relevance to human diabetic retinopathy has not been established. In this study, we investigated whether diabetes alters Cx43 expression and promotes retinal vascular lesions in human retinas. Diabetic human eyes and non-diabetic human eyes were analyzed in this study for Cx43 protein level by Western blot (WB) analysis and immunostaining of retinal capillary networks. In parallel, retinal capillary networks were stained with hematoxylin and periodic acid Schiff to determine the extent of pericyte loss (PL) and acellular capillaries (AC) in these retinas. Cx43 protein expression was significantly reduced in the diabetic retinas compared to non-diabetic retinas as indicated by WB analysis. Additionally, a significant decrease in the number of Cx43 plaques per unit length of vessel was observed in the diabetic retinas compared to those of non-diabetic retinas. Importantly, a strong inverse relationship was noted between Cx43 expression and the relative number of AC, and between Cx43 expression and number of pericyte loss. Overall, these results show that Cx43 expression is reduced in the human diabetic retinas and that the development of PL and AC is associated with reduced Cx43 expression in human diabetic retinopathy.

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MicroRNAs: Social Network for Diabetic Retinopathy

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Diabetic retinopathy (DR) is a complex disease characterized by disruption of retinal neurovascular integrity. The identification of key molecular mechanisms involved in maintenance of retinal neurovascular homeostasis is key to the development of specific tools to prevent and/or halt DR. Recent evidence has demonstrated that hyperglycemia can promote the occurrence of specific epigenetic changes including altered microRNAs (miRs) expression profiles. We have performed studies in experimental models of DR as well as analyzing human specimens demonstrating the existence of molecular interactions between different retinal cells types sustained by the production and secretion of miRs in exosomes. Here we provide evidence demonstrating the effects of hyperglycemia on miRs production from retinal pigment epithelium and glia cells and affecting retinal vascular integrity as well as exerting autocrine functions. A number of miRs were identified as potential pathogenic candidates including miR34a and miR21 that are contributing to the regulation of pro-inflammatory and pro-apoptotic gene programs in the diabetic retina.

Molecular Mechanism for the Retina-protective Effect of PPARalpha

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Peroxisome Proliferator-Activated Receptor α (PPAR α) is a ligand-activated nuclear receptor and is known to regulate lipid metabolism. Recent clinical studies have shown that fenofibrate, a PPAR α agonist, has robust therapeutic effects on DR in type 2 diabetic patients. The mechanism for the therapeutic effect remains unclear. In diabetic animal models, fenofibrate administration suppressed retinal inflammation, vascular leakage, pericyte loss and neurodegeneration. Fenofibrate also displayed anti-angiogenic effect on retinal neovascularization in the oxygen-induced retinopathy (OIR) model. Further, diabetes knockout exacerbated diabetes-induced retinal vascular leakage, pericyte loss and retinal degeneration and

ischemia-induced retinal neovascularization. In contrast, intravitreal injection of adenovirus expressing PPAR α suppressed retinal inflammation, pericyte loss, retinal degeneration and neovascularization. Fenofibrate and over-expression of PPAR α both decreased ROS production and down-regulated NOX4 expression through blockade of NF- κ B activation in primary retinal pericytes in a diabetic milieu. Furthermore, activation and over-expression of PPAR α both attenuated the oxidant-induced suppression of mitochondrial O₂ consumption. These findings suggest that PPAR α has anti-inflammatory and anti-oxidant effects in the retina. Inhibition of the NOX4 and NF- κ B system and modulation of mitochondrial function may be responsible, at least in part, for the therapeutic effects of fenofibrate on diabetic retinopathy.

Beta-adrenergic Receptor Inhibition of Inflammatory Pathway in the Diabetic Retina

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Diabetic retinopathy is the leading cause of blindness in working age adults. With rates of diabetes reaching epidemic levels, increased understanding of the pathogenesis of the complications of diabetes becomes increasingly critical. We have previously reported that loss of b-adrenergic receptor signaling can significantly contribute to the retinal damage associated with diabetic retinopathy. Subsequently, we developed Compound 49b as a topical eye drop that prevented the functional, neuronal, and vascular retinal damage observed in rodent models of the type 1 diabetes. In an effort to determine the cellular mechanisms responsible for the activity of Compound 49b, we found that Compound 49b can activate both protein kinase A (PKA) and exchange protein activated by cAMP (Epac). Epac1 is a key signaling protein, which can protect the retina against leukostasis and inflammatory mediators. Compound 49b also significantly increased retinal endothelial cell (REC) resistance *in vitro*; however, these actions required active Epac1, suggesting that Epac1 may be key to regulation of retinal endothelial cell (REC) barrier actions. We have also recently observed that use of a Epac1 specific agonist, 8-CPT-2'-O-Me-cAMP, significantly reduced TNF α and IL-1 β levels in REC cultured under high glucose conditions. Additionally, leukostasis, a model of retinal inflammation, was increased in mice with Epac1 knockout of the vascular cells, again suggesting that activation of Epac1 is protective to the vasculature

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of the retina. Because we saw that Epac1 could reduce inflammatory markers in REC and in the retina, we investigated whether Epac1 could inhibit other signaling pathways that can control inflammatory mediators. Recent data demonstrates that Epac1 can significantly reduce HMGB1 levels, as well as TLR4 signaling. Taken together, our data strongly suggest that active Epac1 can reduce inflammatory mediators in the vasculature of the retina. Future studies will focus on the specific mechanisms by which Epac1 mediates this protective effect.

Neuroretinal Changes in Diabetes: Lessons Learned from Post-mortem Human Tissues

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The main goal of this study is to get a better understanding of the mechanistic aspects of diabetic retinopathy (DR) by using tissues from human donors. We have previously demonstrated that intrinsic protective pathways involving the insulin receptor/Akt pathway and crystallin proteins are disrupted in animal models of diabetes. This study was designed to use post-mortem human ocular tissues and perform a targeted approach focusing on those pathways. Disease state information collected by the eye bank was confirmed by fundus photograph and optical coherence tomography (OCT). Tissues were then classified within groups of non-diabetic, diabetic without DR and diabetic with DR (n>7 for each). The left eye was rapidly fixed and used to analyze the regional anatomical changes. Using the OCT images, retinal thickness was measured in each quadrant in relationship to the distance to the macula. Tissues were then processed and analyzed for glial activation (GFAP), microglial activation (Iba1), crystallin proteins expression (alphaA- and alphaB) and inflammatory markers (IDO) by immunohistochemistry. The right eyes of the same donors were rapidly dissected to isolate the different regions of the retina (macula vs peri-macular vs peripheral retina). The impact of diabetes on expression, phosphorylation or activation and subcellular localization of crystallin proteins, apoptosis regulatory proteins (Bax, Bcl-2, caspases), inflammation (cytokine panel) were analyzed either by gene expression, biochemical, and/or proteomic-based methods.

This study clearly demonstrates a specific and progressive thinning of the retina of diabetic patients, especially those with DR. This reduction was correlated with regional changes first detectable in the peripheral retina of diabetic patients and supporting a relationship between the

progression of the disease and increased glial activation, local inflammation and increased expression of crystallin proteins in retinal glial cells. This study also supports that diabetic retinopathy progression is associated with alterations of intrinsic protective pathways in a region- and cell-specific manner.

This work demonstrates how the use of tissues from human donor can complement work performed in animal models of diabetes and extend our understanding of the pathophysiology of diabetic retinopathy.

OPT8 - Prostaglandins for Ocular Hypertension Treatment: A new Era is Dawning

Introduction to Prostaglandins and their Receptors in the Eye

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Prostaglandins (PGs) are endogenous autocoids that are ubiquitously produced and have multiple functions in the body, including in the eye. The many diverse actions of PGs are mediated by at least five bona fide pharmacologically defined receptors named DP, EP, FP, IP and TP. Many of these receptors also have sub-types, for instance EP1, EP2, etc. In general, DP, EP and IP receptors modulate intracellular cAMP to mediate their biological effects, while FP and TP receptor mobilize intracellular Ca²⁺. PG receptors and their sub-types are present in animal and human ocular tissues as determined by receptor cloning, RT-PCR, in situ hybridization, immunohistochemistry, receptor binding and receptor autoradiography. Furthermore, assays involving second messenger detection by ion-exchange chromatography and ELISA assays in isolated ocular cells, production and secretion of cytokines and matrix metalloproteinases, and contraction/relaxation of ocular tissues in organ baths in response to various PGs, with response blockade with selective receptor antagonists, have shown these receptors to be functionally active. Agonists for FP receptors, in particular, are powerful ocular hypotensive (OHT) agents and they lower and control intraocular pressure (IOP) in many animals and in humans. PG FP-agonists such as Latanoprost, Tafluprost, Travoprost, Bimatoprost and Unoprostone are now used clinically as first-line medical treatments for OHT and

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glaucoma. There are other PG and non-PG compounds, and their conjugates and derivatives, under investigation in vitro and in vivo that represent novel potential therapeutic drugs for treating these ocular diseases. These novel entities will be show-cased in this session.

What Are PG-receptor Deficient Mice Teaching us about IOP Regulation?

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PG analogues are in the impregnable position as a 1st drug for glaucoma medication. However, still unknown mechanisms of IOP reduction or current issues for PG-associated periorbitopathy (PAP) should be clarified to develop more effective and safer PG-associated drugs. So far, PG-receptor deficient mice have provided us lots of information about the mechanism of IOP reduction and associated events.

IOP reduction by all current acid types of PG analogues required FP receptor activation, which was clarified by FP knock-out(KO) mice. Interestingly, it was found that bimatoprost itself (non-acid type) may bind to prostamide FP receptor, which is composed by FP and splice variant of FP, products of *FP* gene.

Except FP receptor, EP3 receptor may augment FP-dependent IOP reduction, and this fact leads to the newly developed FP and EP3 dual agonist to reduce IOP. EP2 or EP4 also have a strong potential to reduce IOP, probably by different molecular mechanisms by FP. Although such a lot of studies informed us the important roles of PG receptors to regulate IOP, the true mechanisms to regulate IOP in each glaucoma type is still under investigation.

Aside from IOP regulation, FP may be related to the pathogenesis of PAPs. Major mechanism of PAP is FP-dependent intracellular lipid regulation. Current 4 kinds of PG analogues can completely suppress lipid production in the adipose tissues, which was clarified by KO mice study. PG receptor KO mice will contribute to deep understanding and development of IOP regulation in the eyes.

Latanoprostene Bunod, AN FP Agonist-NO Donor Conjugate Ocular Hypotensive Agent

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Latanoprostene bunod (LBN) is an NO-donating analog of latanoprost which is a PGF₂α analog and one of the

most efficacious ocular hypotensive agents used for the treatment of glaucoma and ocular hypertension. Based on a long history of preclinical and even clinical studies the NO/cGMP pathway has a well-established role in the regulation of intraocular pressure (IOP). The aim was to determine whether conjugating an NO-donating moiety to the latanoprost molecule would improve efficacy. The IOP-lowering activity of LBN was tested in three animal models with transiently or stably elevated IOP. As an indicator of NO-release from the molecule and activity LBN, but not latanoprost, stimulated cGMP accumulation in PC 12 and HEK293 cells. Following topical administration of LBN to rabbit eyes, cGMP was elevated in the aqueous humor and iris-ciliary body. In primates with laser-induced ocular hypertension and in glaucomatous beagle dogs, topical LBN reduced IOP to a greater extent than latanoprost. Peak reduction of IOP from baseline was 35% in primates and 44% in dogs for LBN compared to 26% and 27% for latanoprost, respectively. In the rabbit model, topical pretreatment with LBN significantly blunted the ocular hypertensive response to an intravitreal injection of a hypertonic saline solution. As had previously been reported by others, latanoprost did not affect IOP in rabbits. In summary, topical administration of LBN lowered IOP to a greater extent than latanoprost in three animal models of ocular hypertension suggesting a dual mechanism of action and a contribution of the NO/cGMP pathway to its ocular hypotensive activity. LBN is currently in clinical development for the treatment of glaucoma and ocular hypertension. In clinical studies, a greater reduction of IOP has been reported for LBN in comparison with latanoprost and timolol.

EP₂ Receptor Agonists as Novel Drugs to Treat OHT and Glaucoma

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Prostaglandin (PG) FP-receptor agonist are first-line therapy for the treatment of ocular hypertension (OHT) associated with primary open angle glaucoma (POAG). However, EP₂ receptor agonists such as butaprost, AL-6598, ONO-AE1-259-01 and PF-04217329 also exhibit good intraocular pressure (IOP)-lowering activity in ocular hypertensive animals. We now report on a novel non-prostanoid EP₂-receptor selective agonist, DE-117, whose free acid has a high affinity for the EP₂ receptor (K_i = 10 nM).

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DE-117 significantly and dose-dependently (0.003-0.03%) lowered IOP in normotensive rabbits (by 4 mmHg 6hr post topical ocular [t.o.] dosing using 0.03% DE-117), in beagle dogs, and in OHT Cynomolgus monkeys (by 20 mmHg using 0.01% DE-117). Furthermore, repeated t.o. dosing in dogs out to 28 days, resulted in sustained IOP-lowering efficacy. These ocular hypotensive effects of DE-117 were greater than Xalatan. In a phase-2 study conducted in OHT or glaucoma patients (n = 14-17/group), DE-117 was safe and well tolerated when t.o. applied once daily. DE-117 (0.0003 - 0.003%) produced IOP-lowering in a dose-related manner although the highest dose yielded a slightly lower efficacy. This ocular hypotensive activity was maintained up to 16 weeks, and was significantly better than placebo vehicle, and equivalent to that of Xalatan (0.005%) at most time-points studied. For instance, 0.002% DE-117 induced 30% reduction in IOP from baseline over time, whilst Xalatan lowered IOP by 25% at week 1 and about 30% at weeks 2 and 4. Given that EP₂ receptor agonists raise intracellular cAMP in non-pigmented ciliary epithelial and ciliary muscle cells, relax ciliary muscle, stimulate uveoscleral outflow and thus lower IOP, EP₂ agonists represent another class of powerful ocular hypotensives. Additionally, there is some evidence that EP₂ agonists enhance retinal and choroidal blood flow, and exhibit neuroprotectant activities. Taken together, non-prostanoid and PG EP₂ receptor agonists warrant further clinical studies in order to render them potential future alternative drugs to classical FP receptor agonists for the treatment of OHT and glaucoma. These and other pharmacological aspects of EP₂ agonists will be presented and discussed.

Prostaglandin EP4 Agonists: Potent IOP Lowering Agents that Increase Outflow Facility

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Prostaglandins have long been known to modulate intraocular pressure (IOP). One class of prostaglandins, FP agonists (Travoprost, Latanoprost, Bimatoprost) were approved over a decade ago because of their superior IOP lowering activity. Since then there have been no new glaucoma drugs approved world-wide. Activation of prostaglandin-mediated cAMP signaling via 4 distinct GPCRs, DP1, IP, EP2 and EP4 receptors, is known to lower IOP in preclinical and clinical studies. EP4 agonists (3,7 dithia PGE1, ONO-AE1-329, CP-734432) have shown a lot of promise for their significant ocular hypotensive effects in preclinical species including glaucoma monkey

model. Specifically, these EP4 agonists have also shown to increase conventional outflow facility in mice and monkeys. Data supporting these IOP lowering effects, potential signaling mechanisms by which EP4-mediated IOP lowering may occur and their clinical developability will be discussed.

Ocular Blood Flow Enhancement and Possible Neuroprotective Effects with Topically Instilled Prostaglandins

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The effects of topical FP- agonists on the optic nerve head (ONH) circulation and endothelin-1 (ET-1)-induced constriction of retinal vessels (RVs) were studied in experimental animals. The ONH circulation in the living eyes was monitored using the laser speckle method, adopting the NB_{ONH} value as a quantitative index of the ONH circulation. The RV caliber was measured by fundus photography. In dutch rabbits, 0.004% travoprost (TV), a potent FP-agonist, or 0.12 % unoprostone (UN), a weak FP-agonist, was unilaterally instilled once daily (TV) or twice daily (UN) for 7 days. In one group of rabbits, the NB_{ONH} in the drug- and vehicle-treated eyes was measured just before, 1, 6, 12 and 24 hrs after the last instillation. In another group of rabbits, ET-1 was intravitreally injected in both eyes just after, 30, 60, 90 or 180 min after the last instillation and fundus photographs were taken just before, 30 and 60 minutes after the ET-1 injection to study if difference was seen in the ET-1 induced RV constriction between the drug- and vehicle-treated eyes. The NB_{ONH} was measured in both eyes of cynomolgus monkeys after a single instillation of 0.001% tafluprost (TF), another potent FP-agonist, before and after unilateral induction of laser-induced experimental glaucoma. The NB_{ONH} was significantly increased only in the treated side just before, 1, 6, and 12 hours after the last instillation of TV or UN, and 24 hours after the last instillation of TV. ET-1-induced RV constriction was significantly inhibited only in the TV-treated eyes, when ET-1 was injected 30 or 60 min after the last instillation of TV. Presence of experimental glaucoma showed no significant effects on the TF-induced significant increase of the NB_{ONH}. Indomethacin pretreatment abolished the effects of TV, UN and TF on the NB_{ONH} or the TV's effects on the ET-1-induced RV constriction. These findings indicated that a topically instilled FP-agonist locally penetrated not only to the ipsilateral retrobulbar space, but also posterior retina at concentrations enough to cause

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sustained vasodilating action mediated by endogenous prostaglandins, and support a possibility that topical FP-agonist can cause neuro-protective effects, which were confirmed in vitro using isolated cultured retinal ganglion cells (RGCs), on RGCs in the ipsilateral side in vivo. Effects of a FP agonist on the ONH circulation were independent of the presence of glaucomatous change.

OPT9 - Blue light and circadian system

The Effect of Blue Light for Ocular Health: From Retina to the Ocular Surface

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In addition to the effect of blue light on circadian rhythm, there is a definite influence on ocular health, specifically the retina and the ocular surface. Our research team has found that blue light, not ultraviolet light, has a detrimental effect on the retina because short wavelength light can penetrate the cornea and lens, but ultraviolet does not. Also, blue light has a direct effect on the cornea and conjunctiva. Furthermore, blue light scatters more on unstable tear film in dry eye patients, which disrupts visual function.

Blue Light Reception in Retinal Ganglion Cells and its Role in the Clockwork

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The adaptation of behavior and physiology to changes in the light levels is important for organisms. These adaptations include the light modulation of neuroendocrine function and temporal alignment of physiology and behavior to the day and light cycle by the circadian clock. These non-image forming responses can function independently from rods and cones, but are depend on ocular light reception, suggesting the participation of novel photoreceptors in the eyes. The discovery of melanopsin in intrinsically photosensitive retinal ganglion cells (ipRGCs) and genetic proof for its important role in major NIF responses have offered an entry point to understand how animals adapt to the light environments. I will talk about the updated stories in melanopsin, ipRGCs and NIF research areas and also about the connection to the circadian clock system.

A Blue Light-photopigment Working in the Inner Retina of Vertebrates

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The retina of vertebrates (including birds) contains three types of photoreceptors: typical visual photoreceptor (PR) cones and rods responsible for day/night vision respectively, and intrinsically photosensitive retinal ganglion cells (ipRGCs) mainly involved in the regulation of a number of nonvisual activities. ipRGCs express the blue photopigment melanopsin (Opn4), encoded by two genes: the *Xenopus* (*Opn4m*) and mammalian (*Opn4x*) orthologs respectively responsible for photic responses involved in the regulation of circadian clock synchronization, the pupil reflexes, sleep and melatonin suppression. All opsin photopigments known utilize retinaldehyde as chromophore. The retinoid regeneration process denominated "visual cycle" involves in the outer retina, the retinal pigment epithelium or Müller glial cells. On the contrary, in the inner retina, Opn4 has been characterized as a bistable photopigment, in which a photon of one wavelength isomerizes 11-cis to all-trans retinal (Ral), with a second photon re-isomerizing it back. Nevertheless, it is not known how the chromophore is metabolized, nor is it yet clear whether an alternative secondary cycle may take place in the inner retina. In the chicken retina both Opn4 proteins are found in ipRGCs, and Opn4x is also present in inner retinal horizontal cells (HCs). The aims of this work were to investigate: i) the intrinsic photosensitivity and functioning of HCs and ii) the chromophore recycling in Opn4x (+) cells. Results show that Opn4x (+) HCs are novel photoreceptors requiring Opn4x expression and retinaldehyde as chromophore. In this respect, HCs act through a rhabdomeric-like phototransduction cascade involving Gq protein and PLC activation, Ca²⁺ mobilization, and GABA release. In addition, Opn4x (+) RGCs displaying significant photic responses by Ca²⁺ fluorescent imaging are able to photoisomerize exogenous all-trans to 11-cis Ral and to synthesize other retinoids such as all-trans retinol and all-trans retinyl palmitate. Overall, inner retinal cells (RGCs and HCs) expressing Opn4x are able to metabolize retinaldehyde for further use and store in the inner retina. Moreover, these 2 cell populations display intrinsic photosensitivity that can potentially regulate both non-visual tasks and together with visual photoreceptors, modulate lateral interaction, fine tuning of PR responses and inner retinal processing.

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Effects of Blue Light on the Circadian System and Eye Physiology

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Light emitting diodes (LEDs) are now utilized to provide illumination in industrial and commercial environments. Furthermore LEDs are also employed in TV, computers, smart phones and tablets. Although the light emitted by most of LEDs appears white, LEDs have a peak of emission in the blue light range (400-490 nm). Accumulating experimental evidence indicate that exposure to blue light can affect many physiological functions and can be used to treat circadian and sleep dysfunctions. However blue light can also induce photoreceptor damage. Hence, it is important that the spectral output of LED-based light sources should be considered in order to minimize the danger that may be associated with blue light exposure. In this talk I will summarize the current knowledge on the effects of blue light in the regulation of physiological functions and the possible effects of blue light exposure on ocular health.

Circadian Clocks within the Retina Synchronize to Light: Dark Cycles Using OPN5

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Cell populations within the mammalian retina have the ability to entrain local circadian rhythms of gene expression to light:dark cycles independently of behavioral or SCN phase. In previous work, we demonstrated that OPN5 is necessary for this photoentrainment in cultured mouse retinas. To study this *in vivo*, mice without OPN5 (*Opn5*^{-/-}) and wild-type littermates were behaviorally entrained to light:dark cycles for at least 2 weeks. Retinas and livers were harvested at 3 hour intervals across a 24 hour cycle. RNA transcript levels of the clock genes *Per1* and *Per2* were analyzed by quantitative RT-PCR. The transcripts of *Per1* and *Per2* in wild-type retina displayed predictable differences across the 24 hour day as has been previously reported. However, these transcripts showed variable levels from retina to retina among *Opn5*^{-/-} mice, and the circadian component of *Per1* and *Per2* transcript levels was not observed in the averaged values of *Opn5*^{-/-} retinas. The

rhythms of transcript abundance of *Per1* and *Per2* were not different between the livers of wild-type and *Opn5*^{-/-} mice. OPN5 is expressed in the ganglion cell layer in cells distinct from OPN4-expressing ganglion cells. In conclusion, *Opn5*^{-/-} retinas are deficient in local photoentrainment both *in vivo* and *in vitro* while visual and behavioral photoreception through rods, cones, and melanopsin remains intact.

OPT10 - TBI (traumatic brain injury): Visual dysfunction and treatment

Visual Aspects of TBI

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Traumatic optic neuropathy (TON) describes optic nerve damage and retinal ganglion cell (RGC) death after trauma, causing irreversible visual loss. TON can be direct - where the optic nerve is crushed or cut or - more commonly - indirect, where brain or eye injury is associated with secondary RGC degeneration. Lost central nervous system (CNS) neurons, including RGC, are not replaced. Eye injuries affect both military and civilian populations and TON develops in ~5% of patients after closed head injury, representing 20% of ocular injuries in military personnel. Traumatic optic neuropathy can be induced in a number of different ways in animal models - directly by crush injury and indirectly by blunt ocular trauma and primary blast injury.

The cell death protease caspase-2 has features of both initiator and executioner caspases and active enzyme is detectable by western blotting and immunohistochemistry in rat RGC after injury by optic nerve crush and blunt ocular trauma, when its inhibition is RGC neuroprotective. Caspase-2-dependent RGC apoptosis has been prevented by both pharmacological inhibitors and siRNA. Chemical modification of siRNA confers endonuclease resistance and improves tissue retention, making siRNA knockdown a translatable neuroprotective treatment.

RGC and related glial stress responses after injury and axotomy will be discussed including apoptotic and alternative RGC death signalling pathways such as necrosis and necroptosis, which are also active after blunt ocular trauma, with the potential for neuroprotective interventions.

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Mechanisms and Therapy in Air Blast Induced Eye Trauma

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Purpose: To understand molecular mechanisms underlying blast-induced vision loss and test potential therapies.

Methods: Adult mice were exposed to a single 26psi over-pressure air-wave directed at the left eye. Retinal oxidative stress, neuroinflammation, cell death, and vision were assessed out to 1-month after injury. A subset of mice was treated with erythropoietin, a cytokine currently in Phase III trials for the treatment of neurotrauma.

Results: Blast trauma causes an increase in oxidative stress in the first week followed by cell death and axon degeneration. Erythropoietin exacerbated oxidative stress and cell death early on through increased oxygen and iron delivery to the retina. Prolonged or delayed treatment with erythropoietin was protective due, at least in part, to increased expression of antioxidant enzymes.

Conclusions: Closed-globe eye trauma induces an increase in reactive oxygen species that may contribute to neuronal death. Intraocular treatment with erythropoietin may be more effective in treating blast-induced retinal damage by avoiding an elevation in the hematocrit.

HIOC, a TrkB Receptor Activator, for the Treatment of Blast-induced Vision Loss

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Trauma from explosions or other blunt force injury frequently results in loss of visual function. We hypothesize that drugs that activate the brain-derived neurotrophic factor (BDNF) receptor, TrkB, will slow the progressive loss of visual function following blast injury to the eye. Blast overpressure directed at the mouse eye results in microglial activation in the retina and astrogliosis in the retinal ganglion cell and nerve fiber layers. This is followed by loss of retinal ganglion cells and optic nerve axons, and a decrease in visual contrast sensitivity and acuity thresholds. N-[2-(5-hydroxy-1H-indol-3-yl)ethyl]-2-oxopiperidine-3-carboxamide (HIOC) is a small molecule, potent activator

of TrkB receptors in the mammalian nervous system. It crosses the blood brain barrier and blood retinal barrier following systemic administration. Treatment with HIOC for 1 week following blast injury mitigates the loss of visual function for at least four months. HIOC treatment reduces the loss of retinal ganglion cells and optic nerve axons, and partially prevents the decrease of contrast sensitivity and visual acuity. HIOC is effective when first administered within three hours after blast exposure. The effect of HIOC is blocked by ANA12, an antagonist of the TrkB receptor, supporting the role of the BDNF receptor in its mechanism of action. We conclude that HIOC and similar activators of the TrkB receptor represent useful therapeutic approaches for the treatment of trauma-induced vision loss.

Activation of the Innate Immune System Following Blast Injury to the Eye

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Our group at Emory is characterizing the effects of an 48psi blast injury to the eye. The present study examines the activation of the innate immune system and the chronic infiltration of T-cells into the compromised retina. Blast injury was produced using a modified paintball gun (Hines-Beard et al. Exp. Eye Res 2012, 99:36-41) in the BXD recombinant inbred (RI) strain set. Expression datasets were generated 5 days after blast and compared with normal retinal microarray datasets respectively constructed from 56 mouse strains. The dataset is presented on GeneNetwork (genenetwork.org). The innate immune network and microglia are activated following blast with significant upregulation of C4b ($p=0.01$), Cx3cr1 ($p=0.067$), and Il-10 ($p<0.001$). In addition, there is a clear indication that T-cells are invading the retina by the upregulation of CD4, FoxP3 and CD8 (known markers of lymphocyte subtypes). To investigate the possibility that lymphocytes were invading the retina, we examined a blast eye 10, 21 and 30 days following the initial injury. Surprisingly, we found a significant number of invading CD4 and CD8 positive T-cells. We examined the ligands for these receptors and found that message for Csf and FasL were correlated with both networks. This led us to hypothesize that the activation of the innate immune network as seen 2 days after optic nerve crush goes on to involve the acquired immune system resulting in the infiltration of lymphocytes into the neuronal tissue. Our bioinformatic analysis of the DoD Blast Injury dataset indicates that the cytokines CSF and the membrane protein FASL are likely

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links between the activation of the innate immune system and the infiltration of lymphocytes into the retina. Future studies will focus on targeting this inflammatory cascade to counter long-term detrimental consequences.

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OPT11 - Dry eye diagnosis

Staining of Ocular Surface: Optimizing Diagnosis and Interpretation. Basis of Successful Surgery and Adaptive Therapy of Dry Eye

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Dry eye disease (DED) is a condition of the ocular surface condition that is increasingly recognized to affect not only the patients' well-being but also the surgical outcome of various procedures. Although corneal fluorescein staining (CFS) is the key indicator of ocular surface health, changes in the conjunctiva and sub conjunctival tissue are important actors in DED. Dry eye induced tissue stress and inflammation is linked to pre-fibrotic and fibrotic processes, tissue reactions that do may also determine the future success of ocular surgery. The parameters defining optimized CFS performance and understanding will be presented. The importance of conjunctival staining will be outlined, providing clues for adaptive therapy; both in normal eyes with DED alone as well as in eyes having undergone surgery. This will be demonstrated with cases of patients having undergone glaucoma surgeries with bleb formation. Although CFS is a hallmark of ocular surface health and its used to judge improvement of ocular surface condition during treatments, conjunctival staining apparently persist longer when improvement of DED is considered. Even when the intensity of CFS has normalized during treatment, the conjunctival pathology is not necessarily synchronized with this improvement. In patients undergone surgery intensified staining of the area of surgery or areas thereof does persist a long time after surgery. Accurate staining technique in judgement of CFS is essential. However, the detected delay in decrease of conjunctival staining in DED improvement and after surgery does deserve more attention. This emphasizes the need for proper dry eye care to be adapted to the specific form of dry eye pathology in each patient. Especially in glaucoma patients the structure of the blebs, and their sensitivity to mechanical disturbance does indeed emphasize the urgent

need for appropriate dry eye treatment. Such optimized treatment is of major interest as possible scarring of the filtration site is a known major threat for the persistence of the surgical success. The correct staining of the entire ocular surface is very important in DED. Neglect of conjunctival staining and single focus on CFS may lead to miss-interpretation of the ocular surface condition and premature change of therapy, causing possible relapse of therapy success and possibly contributing to failure of surgery.

Inflammation in the Diagnosis of Dry Eye Disease

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Ocular surface disorders (OSD) constitute a series of complex diseases involving the lacrimal gland and the tear film, meibomian glands and eyelids, and all cellular components of the cornea and the conjunctiva. Whatever the mechanisms initially involved causing dry eye, i.e., allergic, infectious, toxic or environmental aggressions, blepharitis, autoimmunity or steroid hormone imbalance, they stimulate a series of pathological pathways, the main two being inflammation and apoptosis. Tear film instability or hyposcretion can be considered as the central key point of DED. Either will cause local or diffuse hyperosmolarity of the tear film and therefore of superficial epithelial cells of cornea and/or conjunctiva, stimulating epithelial cells and resident inflammatory cells. Cell damage that will result at levels of cornea and conjunctiva, by mean of apoptosis, direct mechanical and/or osmotic stress, will stimulate the reflex neurosensory arc, stimulating lacrimal gland and neurogenic inflammation, with inflammatory cytokine release, MMP activation and inflammatory involvement of the conjunctival epithelium. Goblet cell loss is thus directly related to chronic inflammation and surface cell apoptosis subsequent to cell hyperosmolarity and chronic damage, resulting in further tear film instability/imbalance and actually leading to a vicious circle, characteristic of severe dry eye disease. Inflammation can be assessed by the way of biological, namely proteomics or impression cytology-based expression of biomarkers like HLADR, or morphological techniques, like corneal confocal microscopy. Either primary, and directly cause of tear film impairment, or secondary to corneoconjunctival damage, inflammation has therefore become a major therapeutic target. Various anti-inflammatory strategies based on steroids, topical cyclosporin A, oral doxycycline or other immunomodulating agents have been developed or are under investigations.

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Biomarkers in Dry Eye Disease**ANDREA LEONARDI**

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The identification of biomarkers on the ocular surface may be used in dry eye disease (DED) either to identify a 'disease marker' (diagnostic biomarker), to understand the mechanisms involved in the disease, to correlate the severity of the disease (activity biomarkers), to identify potential targets for therapeutic interventions or as an indicator of treatment outcomes (prognostic, therapeutic biomarker). Limitations of these assays are the lack of extensive validation of candidate biomarkers, the lack of determination of the specificity of the candidate markers and the absence of testing their accuracy as a tool for diagnosis.

Candidate DED biomarkers include some proteins, such as S100A8, S100A9, lipocalin-1, secretory phospholipase A2 and some cytokines and chemokines. Mucin-1 mRNA conjunctival expression levels are shown to be a very sensitive and specific DED diagnosis biomarker; metalloproteinase-9 tear level is confirmed as a good but not specific biomarker; cell parameters such as HLA-DR has been used to assess the efficacy of topical treatments such as cyclosporin A. Recently by proteomic analysis, five biomarkers have been validated: S100A6, annexin A1 (ANXA1), annexin A11 (ANXA11), cystatin-S (CST4) and phospholipase A2-activating protein (PLAA) capable of discriminating among dry eye, meibomian gland dysfunction and control individuals. New technologies such as transcriptome analysis may further identify new and more specific biomarkers for ocular surface diseases. Therefore, protein or peptide analysis can be used as a possible fingerprint for disease biomarkers and pathological molecule identification.

Tear Osmolarity in the Diagnosis of Dry Eye Disease - Recent Findings and the Future**MICHAEL LEMP**

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Osmolarity is an important foundational property of the tear film which affects most other aspects of the lacrimal functional unit. Dry eye disease (DED) is characterized by an unstable and hyperosmolar tear film. Tear hyperosmolarity is regarded as the central mechanism causing direct epithelial damage, ocular inflammation,

symptoms and the initiation of compensatory events in DED. The recent development of an accurate clinic-based tear osmometer has issued in a new era in accurate and precise measurements on very small samples collected from the inferior marginal strip. Tear osmolarity has been shown to be the only single test which parallels increasing disease severity over the entire range as well as distinguishes between normal subjects, those with DED and those with symptoms but a cause other than DED. Tear osmolarity values at or above 308 mOsm/L in either eye are diagnostic and inter-eye differences greater than 8 mOsm/L are confirmatory of the diagnosis of DED. This difference is also an objective measure of tear film instability. Both the absolute values and the inter-eye differences are responsive to effective treatments. Newer biomarker analyses using the same collecting system are in late stage development to complement tear osmolarity in the diagnosis, management of DED and other ocular surface disorders.

Functional Visual Acuity in Dry Eye Disease**ELISABETH MESSMER**

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Functional visual acuity (FVA) reflects an individual's performance in relation to certain daily activities involving visual tasks. It is able to detect masked impairment of visual function in patients who complain of decreased visual acuity despite normal conventional visual acuity. Dry eye patients typically show visual problems when working on a PC, reading or driving. Studies documented slower reading speed and significantly increased reaction time with an increase of non-identified targets in a driving simulator. Several tests have been introduced to measure FVA in Dry Eye Disease including contrast sensitivity, contrast glare test, high order aberrations, FVA tester, FVAM system, Inter-blink-Visual Acuity-Decay, corneal irregularity index, Tear stability analysis system (TSAS) and the Optical Quality Analysis System (OQAS). The evaluation of FVA is mandatory in the understanding of Dry Eye Disease.

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OPT12 - Imaging biomarkers for retinal diseases

In Vivo Imaging of Self-quenched Indocyanine Green-antibody Conjugates in Retinal Disease**ASHWATH JAYAGOPAL***Roche Pharma Research and Early Development, Ophthalmology, F. Hoffmann-La Roche Ltd., Basel, Switzerland*

The goal of this study was to evaluate the utility of self-quenched, indocyanine green (ICG)-based antibody conjugates for imaging retinal disease using preclinical animal models. ICG, a near infrared fluorescent dye used in clinical ICG angiography, is known to exhibit self-quenching of fluorescence emission when conjugated to antibodies. This property was exploited to develop disease-specific internalizing antibodies conjugated to ICG, designed to exhibit fluorescence emission only when specifically internalized within diseased retinal cells. These molecular imaging agents were evaluated in established in vitro and in vivo models of retinal inflammation and neovascular disease.

ICG conjugates of anti-endothelin and anti-ICAM1 exhibited self-quenching of fluorescence in normal conditions, followed by emission of fluorescence upon antibody internalization in cultured retinal microvascular endothelial cells conditioned under hypoxia or inflammatory stimulation (TNF α , LPS), respectively. Nonspecific antibodies functionalized with ICG did not exhibit fluorescence activity due to lack of internalization and maintenance of self-quenching in tissue culture. Self-quenched anti-endothelin targeted conjugates were detectable using in vivo imaging of laser-induced choroidal neovascularization (LCNV) lesions in mice. ICAM1 conjugates were imaged in mouse models of endotoxin-induced uveitis (EIU). Average signal to noise enhancement of these approaches was ~10 compared to age matched control animals using image registration to measure intensities in matched retinal regions of interest. Antibody conjugates were well tolerated as examined by in vitro toxicity assays, ex vivo histopathologic examinations, and ERG analysis.

This imaging approach may facilitate translation of molecular imaging technologies for early detection of disease, disease staging, and assessment of therapeutic response in preclinical models and patients.

Imaging of Retinal Vascular and Oxygen Extraction Responses to Light Flicker in Stages of Diabetic Retinopathy**MAHNAZ SHAHIDI, Anthony Felder, Justin Wanek, Norman Blair***University of Illinois at Chicago, Chicago, United States*

The purpose of the study is to report alterations in retinal vascular and oxygen extraction responses to light flicker stimulation at stages of diabetic retinopathy (DR). Retinal vascular imaging and oximetry were performed with and without light flicker stimulation using our customized optical imaging system. Dedicated image analysis software was developed to measure retinal arterial and venous diameter

(D_A , D_V) and oxygen saturation (SO_{2A} , SO_{2V}). Retinal oxygen extraction fraction (OEF) was calculated from SO_2 measurements. Retinal vasodilatory, SO_2 and OEF responses were calculated by ratios of measurements with light flicker to those obtained without light flicker. Imaging was performed in non-diabetic control subjects, and diabetic subjects diagnosed with no DR, NPDR, or PDR. Retinal D_A and D_V increased with light flicker in all groups and the retinal vasodilatory responses were marginally reduced in no DR and NPDR. Retinal SO_{2A} did not change significantly with light flicker in all groups, while SO_{2V} increased with light flicker in all groups, except NPDR. Retinal OEF decreased with light flicker in all groups, but the response was diminished in NPDR. Combined assessment of retinal vasodilatory and oxygen extraction responses to light flicker stimulation provide promising biomarkers for theranostics of DR.

This study was supported by NIH grants, DK10439 and EY001792, and Research to Prevent Blindness.

In vivo AOSLO Imaging of Changes in Diabetic Retinopathy**STEPHEN A. BURNS, Ann Elsner, Brett J. King, K. A. Sapoznik, H. Othman, Lucie Sawides, TJ Gast***Indiana University, Bloomington, United States*

Diabetic retinopathy (DR) is clinically characterized by retinal lesions including microaneurysms (MA), hard exudates (HE), cotton wool spots (CWS), intraretinal hemorrhages and retinal neovascularization (NV) as well as blood-retinal barrier alterations producing increased retinal thickness. These changes are typically detected using color fundus photography, fluorescein angiography and OCT imaging. However, it is clear that at least in some

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individuals there are widespread changes that are not readily detected by these clinical techniques. We will describe an ongoing study of DR where we use adaptive optics SLO (AOSLO) imaging to both monitor and characterize changes to the retina early in the course of DR. We simultaneously collect both confocal and multiply scattered light images. We have imaged 56 diabetics using a four step protocol. First we image a 4x4 degree region centered on the fovea while focusing on the foveal cones and in most patients an additional 2 degree temporal strip out to 7 degrees temporal. We then focus on the capillary layers and measure a 6x6 degree foveal centered region followed by a 7x3 degree strip again focused on the capillaries along the temporal raphe. The final phase of the protocol is concentrated on patient specific features, such as areas of ischemia, vascular remodeling, or other regions of interest. The entire AOSLO protocol requires about 30 minutes of patient time. We find changes to both the photoreceptors and microvasculature. For photoreceptors in diabetics the confocal imaging provides high contrast images. We find that in approximately 25% of diabetics (14/56) there are localized regions of cones with very low reflectivity. These regions can persist up to 3 years (the maximum follow up to date) and seem to be associated with outer retinal thickening on OCT imaging in many cases. For vascular imaging the multiply scattered light images provide the best visualization. We find widespread clinically undetected changes in the vasculature. These include capillary looping, capillary dropout and ghost vessels and thickening of arteriolar walls, especially for small vessels. Regions of microcystic changes are visualized that seem to be related to some of these microvasculature changes such as capillary looping. Additionally, the wall to lumen ratio (WLR) in the diabetics is increased relative to normal subjects ($p < 0.001$).

Cone Survivability in Aging and Age-related Macular Degeneration and the Neural Economy Hypothesis

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Cones are typically thought to depend greatly upon the retinal pigment epithelium for metabolic support, phagocytosis, and photopigment regeneration. Cones survive and provide good visual acuity despite significant changes to the retinal pigment epithelium (RPE), such as in the early stages of age-related macular degeneration. However, the cones may undergo changes in morphology

or physiology that allow them to survive with fewer resources, called the neural economy hypothesis. In aging and in age-related macular degeneration, the metabolic support from the retinal pigment epithelium declines. Thus, we investigated the distribution and length of cones when the microenvironment is less than ideal compared to a models in younger and older eyes. Using the two-channel Indiana adaptive optics scanning laser ophthalmoscope (AOSLO), we mapped cone distribution with both confocal and multiply scattered light imaging. The scattered light images can be collected simultaneously with the confocal images by displacing the confocal aperture in a second imaging channel. To assess the microenvironment, we performed AOSLO with multiply scattered light imaging to map retinal vasculature and polarization-sensitive *en face* imaging and SD-OCT to map the status of the RPE. Cones are in sufficient numbers to be counted in normal eyes in the peripapillary crescent, and in patients with geographic atrophy, where there is no RPE melanin signal. In a sample of 8 older and 8 younger subjects, 18-35 and 51-65 yr, the older subjects had slightly shorter cones over the central +/- 4.9 deg from the fovea as well as having lower cone densities. The ratio of older : younger cone length is significantly < 1 , but is a small change, i.e. 0.9. Further, the effects of inner segment and total (inner + outer segment) length pooled across all eccentricities and include both rods and cones were more significant than outer segment lengths

($p = 0.0015$ vs. 0.037). When averaging across foveal locations only, measuring cones, the outer segment length was statistically shorter in 5 of 8 older subjects, even though the average was only 6 microns shorter for older vs. younger subjects. Cone lengths in regions of atrophy are sometimes measurable and highly variable. The decreases in cone density and cone length may allow some cones to survive to provide vision.

Dendrimer-based Systemic Therapy and Imaging for Retinal Degeneration

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Inflammation and oxidative stress play a key role in the pathogenesis of retinal and choroidal neovascularization

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in early and late age-related macular degeneration (AMD). Therapies that target the regions of choroidal (CNV)/retinal (RNV) neovascularization and inflammation (microglia/macrophages) would be highly beneficial for delaying the progression of wet AMD. Improving the ability to image inflammation in the back of the eye would be highly beneficial for diagnosis and assessment of the extent of disease progression. In addition, targeted systemic (IV) therapies for CNV would broaden the impact of therapy, improving compliance and reducing costs. The purpose of this study was to evaluate the efficacy and dose dependent response of intravenously administered dendrimer-triamcinolone acetonide (D-TA) conjugates in a lipid-induced CNV rat model, and to image the extent of inflammation, by using the fact that the dendrimers have an intrinsic ability to target inflammation in the back of the eye, from systemic administration.

D-TA conjugates were synthesized with high drug loading. To enable imaging of the D-TA conjugates, these were also labeled with Cy5. The drug release was evaluated in physiological solutions using HPLC analysis. D-TA and Cy5-D-TA conjugates were administered intravenously on day 3 post lipid (HPODE) injection at different doses to:

- (1) image the extent of inflammation in the retina and choroid through dendrimer localization in IBA-1 stained cells, and to assess the effect of therapy on inflammation and dendrimer uptake, and
- (2) to assess efficacy of the systemic D-TA treatment.

Imaging of the Cy5-D-TA conjugates suggested that it selectively targeted the regions of RNV and CNV, and was retained for more than two weeks. D-TA treatment suppressed CNV growth in a dose dependent manner and efficacy was significantly better than that for free TA, with no signs of toxicity or elevated IOP. D-TA attenuated retinal inflammation by suppressing macrophage/microglial accumulation, enhanced adhesion molecule expression and reduced leakage compared to controls dendrimers.

Dendrimers may enable development of targeted theranostics, allowing imaging and therapy for inflammation, oxidative stress and VEGF production, with significant clinical implications. The efficacy of D-TA in the rat CNV/RNV model may have implications for systemic therapy for dry/wet AMD and diabetic retinopathy.

OPT13 - Gene delivery to the eye

Nanoconjugate Gene Therapy of Human Diabetic Limbal Epithelial Progenitor Cells

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Our purpose was to normalize cultured human diabetic limbal epithelial cells (LEC) that have significantly reduced expression of putative epithelial stem cell markers and slow wound healing. To this end, we designed and used novel polymeric acid-based non-toxic nanoconjugates inhibiting MMP-10 and cathepsin F (increased in diabetic corneas) and upregulating proto-oncogene receptor c-met (decreased in diabetic corneas).

LEC cultures enriched in stem cells were obtained from postmortem long-term diabetic donor eyes. Nanoconjugates based on natural-derived polymeric acid scaffold were synthesized as published (1). One nanoconjugate contained a targeting antibody to LEC-expressed transferrin receptor (TfR), morpholino antisense oligonucleotides (AON) to cathepsin F and to miR-409-3p (it targets *c-met* and inhibits its expression), and tripleucine endosome escape unit. Another nanoconjugate of similar composition contained an AON to MMP-10 and a fluorescent tracking dye Alexa Fluor 488. Efficiency of target inhibition (proteinases) or upregulation (c-met) was tested with free AON and then with whole nanoconjugates (at 5 μ M AON) by western blot. Healing of scratch wounds in LEC cultures was monitored microscopically.

Diabetic LEC from several donors were all TfR-positive on western blots justifying the use of respective antibody for cell targeting. LEC internalized nanoconjugates mainly through receptor-mediated endocytosis as revealed by competition with free TfR antibody. By western blot, nanoconjugates were able to reduce the expression of MMP-10 and cathepsin F, and increase the expression of c-met (through inhibition of miR-409-3p). Treatment with nanoconjugates increased the expression of putative limbal stem cell markers ABCG2 and keratin 15. It also accelerated healing of scratch wounds in LEC cultures with no toxicity observed.

In conclusion, non-toxic nanoconjugates appear to be a new viable alternative to viral-based gene therapy in normalizing diabetic limbal epithelial cells with enhancement of stem cell marker expression and wound healing.

1. Proc Natl Acad Sci USA, 2010;107:18143-18148.

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Progress on the Primary Open-angle African American Glaucoma Genetics (POAAGG) Study

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Purpose: To report progress on the enrollment, phenotypic analysis, and genome-wide association study (GWAS) for the Primary Open-Angle African American Glaucoma Genetics (POAAGG) study, a 5-year project designed to elucidate the genetic architecture of primary open-angle glaucoma (POAG) in African Americans.

Methods: African Americans over age 35 were recruited from the Scheie Eye Institute and research sites and from the Penn Medicine Biobank. Glaucoma cases were defined by glaucomatous optic nerve defects with corresponding visual field loss. Clinical features (central corneal thickness, intraocular pressure, cup-to-disc ratio, visual acuity), visual fields, and images (optical coherence tomography, stereo disc photos) were collected to endophenotype cases. Genomic DNA was extracted from peripheral blood or saliva. Genotyping for a discovery GWAS was performed on 5520 DNA samples, using ~2.0 million SNPs on an Illumina Multiethnic Genotyping microarray platform (MEGA-Ex). Targeted resequencing of nuclear and mitochondrial genes was completed, including sequencing of the N-terminal part of the mitochondrial cytochrome c oxidase subunit I gene (*MT-CO1*) for 1308 cases and 849 controls. Masked genotype/phenotype analysis was performed on a subset of cases.

Results: 7409 subjects have been enrolled (2111 cases, 4307 controls, 991 suspects) as of 05/01/2016. 97% of discovery GWAS samples were successfully genotyped, with an average call rate of 99.65% on 5342 samples, with 99.99% reproducibility within replicate pairs. Mitochondrial sequencing targeting the *MT-CO1* gene found that the POAG case group was significantly associated with two *MT-CO1* missense variants, which define a common African mitochondrial haplogroup (L1c2). A subset of L1c2 cases (*MT-CO1* double missense) was matched for age, gender, family history of glaucoma and mitochondrial haplogroups against L1b cases (no missense). L1c2 cases (n=29) had worse visual field loss (6.5 vs 4.3, p=0.009 for PSD; -11-

4 vs -4.5, p=0.006 for MD) and higher cup-to-disc ratio (0.77 vs. 0.71, p=0.04), despite lower intraocular pressure, compared to matched L1b POAG cases (n=29).

Conclusions: Correlating genetic variants with quantitative endophenotypes will help identify subgroups of patients with different features and yield insights into the high prevalence of POAG in African Americans. Mitochondrial haplotype L1c2 may further elevate risk for glaucoma in African Americans and result in more severe disease.

Long Term Results of the Retinal Gene Therapy in LCA2 Patients: A Picture Painted by the Brain

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Recent successes in clinical trials of Leber congenital amaurosis caused by mutations in the gene RPE65 (LCA2) have produced tremendous advances for the scientific community and more importantly for patients suffering from this previously incurable disease. The history of LCA2 gene therapy (GT) research dates back to 1997 when RPE65 was first identified. Ten years later, in 2007 Phase I/II human trials began and now a phase III clinical trial is well underway. The LCA2 Phase III study is the first randomized controlled GT trial for a genetic disease. Preliminary results of this study reveal robust improvements in retinal and visual function and in functional vision as well as a high degree of safety, thereby placing the recombinant adeno-associated virus reagent (AAV2-hRPE65v2) as the frontrunner for being the first approved gene therapy drug in the USA. Due to the success of the RPE65 trials, more than two dozens gene therapy clinical trials targeting retinal disease have been initiated. These studies have enrolled more than 350 individuals and there are excellent safety records reported to date.

Besides leading the way to development of novel treatment, these studies provide the opportunity to learn more about the eye and brain as a visual system. In studies parallel to the clinical trials, LCA2 subjects who received GT and

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a group of controls underwent functional MRI (fMRI) and diffusion MRI (dMRI) to evaluate brain's functional and structural responses to GT. The neuroimaging results reveal enhanced short and long term functional responses for LCA2 patients lasting for at least three years and long-term brain plasticity initiated by visual experiences prompted by gene therapy. These results support the fact that despite severe and long-term visual deprivation, LCA2 subjects have intact visual pathways, which become stimulated and strengthened after GT. Here, we examine the role of brain's structure and function, and its potential as an outcome measure for retinal gene therapy and the unexpected plasticity of the brain in the setting of visual restoration.

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Gene therapy of the corneal endothelium

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The presentation covers both non-viral and viral vector approaches to target corneal endothelial cells. Not only different types of vectors will be demonstrated such as lentiviral, adeno-associated viral vectors, magnetized nanoparticles and calcium phosphate nanoparticles. Moreover, modifications of both vector and plasmid design are discussed to enhance gene expression in this cell type. Moving to functionality, the role of pro- and anti-apoptotic proteins, up- and downstream, are studied. Anti-apoptotic strategies to protect corneal endothelial cells by gene and cell therapy and subsequent translational aspects are presented.

OPT15 - Innovative approaches for retinal degeneration and therapy

Glutaredoxin 2 (Grx2) Gene Knockout Mice: A Novel Model of Age-related Macular Degeneration

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Age-related macular degeneration (AMD) is a leading cause of blindness worldwide. The lack of adequate AMD animal models and poorly understood pathogenesis have greatly hindered our progress in therapeutic development. To address these shortcomings, this project was designed to examine how retinal redox dysregulation leads to AMD and characterize *glutaredoxin 2 (Grx2)*, a mitochondrial thiol redox regulating enzyme, knockout mice as a new animal model for AMD. To determine the effects of age and AMD on Grx2 levels in the retina, the neural retina and RPE layers were isolated from AMD and age-matched donor eyes. Grx2 protein levels were measured by Western-blot analysis. Primary RPE cells were isolated from wild-type (WT) and *Grx2* knockout (KO) mice for the *in vitro* study. The visual function of WT and *Grx2* KO mice were examined by fundus photography and scotopic electroretinography (ERG) at 6, 12, and 16 m. To determine morphological changes, H&E staining was performed. RPE structural changes were assessed by immunostaining of tight junction protein ZO-1. Lipofuscin autofluorescence was examined on cryostat sections. The level of protein glutathionylation (PSSG) was measured by immunoblotting using anti-PSSG antibody. Grx2 protein level and enzyme activities were decreased by approximately 30% in AMD donor eyes compared to age-matched healthy donors. Primary RPE cells isolated from *Grx2* KO mice were more sensitive to H₂O₂-induced oxidative damage than WT RPE cells. *Grx2* KO mice developed age-dependent retinal degenerative pathology. By 12-month of age, *Grx2* null mice showed ~50% decrease in a-wave and ~30% decline in b-wave amplitude (n=8, P< 0.01). Histological analysis revealed extensive RPE lesions, including RPE atrophy, vacuolation, hyper- and hypo-pigmentation, sub-RPE deposits, cellular infiltration, and loss of tight junction integrity. Age-dependent lipofuscin accumulation, a marker of AMD pathology, was also observed in *Grx2* KO mice. Furthermore, *Grx2* KO mice demonstrated increased markers of mitochondrial oxidative damage including PSSG accumulation and ATP

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and GSH depletion. In conclusion, Grx2 plays a critical role in maintaining the mitochondrial redox homeostasis in the aging retina. *Grx2* deficiency causes PSSG accumulation and sensitizes RPE cells to age-related oxidative damage, leading to RPE degeneration and photoreceptor damage. As a new AMD animal model, *Grx2* KO mice will provide new insights into pathogenesis and therapeutics of AMD.

Metabolic Profiling Directed Approach for Therapeutic Development

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Background and aims: The conventional approach of therapeutic development usually is to identify a protein target and discover suitable pharmacological modulators. The advent of high-throughput mass spectrometry allows rapid identification and quantification of metabolites from tiny amount of samples. This enables comparison of metabolic profiles between normal and disease states. The metabolic differences then point to a limited subset of metabolic enzymes associated with the disease state and allow directed therapeutic development. Our aim was to find differences in specific repertoire of lipids and determine the differences in the ocular hypertensive compared to the normotensive state using model organisms. Our aim also included comparison of selected differential lipids in glaucomatous human tissues to determine if they are found to be different in the disease state.

Methods: We used two groups (normo and hypertensive) of DBA/2J mouse (n=40) around 8.5 months of age. We also used cadaveric donor derived human control and glaucomatous trabecular meshwork (TM) tissue and aqueous humor (AH). The Bligh and Dyer method was used to extract lipids. Mass spectrometry and MZmine 2.10 program were used for quantification of Phosphatidylcholine (PC), Phosphatidylserine (PS), Phosphatidylethanolamine (PE) and Phosphatidylinositol (PI) with ratiometric lipid standards for each class. Primary human TM cell culture derived from normal individuals were used for assessment of the effect on elastic modulus using atomic force microscopy. Other assays, such as effect on motility and fluorescein transport were performed as well.

Results: Our investigation has identified a total decrease of PC and PS lipids in both TM and AH while a significant increase in PE lipids was observed. We also found a total decrease of PC22:6/22:6 in glaucomatous TM tissue which is consistent with the finding of a significant decrease of

this species systemically and a strong correlation of the degree of decrease with degree of glaucoma severity (Acar et. al., 2009).

Conclusion: We found PS lipids undergo a decrease with an increased level of PE lipids. Our initial metabolic profiling strongly indicates an altered level of enzymes in this pathway. Thus, metabolic profiling may direct evaluation a smaller subset of proteins for assessment and has the potential for identifying valuable therapeutic targets starting with none or little idea about the target protein.

Optic Nerve Injury-induced Phosphoproteomic Changes in the Retina

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Purpose: Optic nerve (ON) injury is involved in various ocular disorders, such as glaucoma and traumatic optic neuropathy. Tissue injury often affects kinase activities and protein phosphorylation. Characterization of protein phosphorylation changes in disease models is an effective strategy to identify the underlying disease mechanisms. In this study, we evaluated phosphoproteomic changes in the mouse retina induced by ON injury.

Methods: Intraorbital ON crush was performed in adult C57BL/6J mice. Retinas were collected at 0, 6, and 12 h following injury. Retinal proteins labeled with CyDye-C2 were subjected to 2D-PAGE, followed by in-gel ProQ Diamond phosphoprotein staining, and image analysis. The ON crush-induced changes of protein differential phosphorylation were obtained. Proteins with significant changes in phosphorylation (ratios ≥ 1.5) in retinas of the injured eyes compared to the control eyes were spot-picked and tryptic digested. Their peptide fragments were then analyzed by MALDI-TOF (MS) and TOF/TOF (tandem MS/MS). Bioinformatics analysis was conducted using PANTHER Classification System. Identified proteins were validated by western blotting and immunofluorescence staining in separate experiments.

Results: Intraorbital ON crush increased phosphorylation of a variety of retinal proteins in a time-dependent manner. Among them, more than 50 proteins with significantly elevated phosphorylation were identified. PANTHER analysis showed that these proteins fall into several specific biological themes, including kinases, proteins involved in apoptosis and cell survival, glial functions, carbohydrate and protein metabolism, RNA splicing/mRNA

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processing, and others. One example of the identified proteins is hepatoma-derived growth factor, which was previously reported to be up-regulated in adult rat retina 1 d after ON transection and shown to be a potent protective factor for injured RGCs. Other proteins with significantly elevated phosphorylation in ON crushed retinas include protein kinase C alpha subtype, glycogen phosphorylase, and tubulin-folding cofactor B.

Conclusions: This study provides a systemic and temporal picture of ON injury-induced phosphoproteomic changes in the retina, which leads to new insights into mechanisms of retinal degeneration after ON injury. These new insights will lead to novel targets useful for the treatment of retinal and CNS neurodegeneration.

Novel Sphingolipid Mediator for Retinal Ganglion Cell Death and Survival

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Sphingolipids are essential for development and maintenance of neural tissues. Many sphingolipid metabolites, such as ceramide, sphingosine and sphingosine-1-phosphate are bioactive lipids that act as second messengers to regulate cellular functions including apoptosis and inflammation. Acid Ceramidase (ASAH1) is a critical enzyme which converts ceramide to sphingosine in the lysosome. Little is currently known about the role of ASAH1 in retinal neurons. In this study we have characterized a novel mouse line containing a retina-specific deletion of Asah1 gene (Asah1 retinal conditional knock-out), which induces progressive retinal ganglion cell (RGC) death.

The mouse line was characterized using electroretinography, fundus photography, fluorescein angiography, ocular coherence tomography, tonometry, histological and immunohistochemical analyses. Relative levels of ceramide, sphingosine, and sphingosine-1-phosphate were determined using mass spectrophotometry.

We found the ganglion cell layer was predominately affected in the Asah1 conditional knock-out mice. Ganglion cells were significantly reduced with concomitant reduction in inner retinal thickness. RGC death causes irreversible vision loss in glaucoma. Human primary open angle glaucoma (POAG) is highly complex and highly heterogeneous, with significant association with environment and ageing. Sphingolipid metabolism is implicated in lysosomal dysfunction, autophagy, and inflammation in glaucoma

pathology. Alteration in sphingolipid metabolism in the RGCs can lead to RGC death, which could be one of the mechanisms of POAG development and may serve as a potential therapeutic target.

Novel Therapeutics for Age Related Macular Degeneration

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Age-related macular degeneration (AMD) is a leading cause of blindness worldwide. Wet AMD, which affects ~10%-15% of AMD patients, progresses rapidly to blindness if left untreated with severe lesions in Bruch's membrane/retinal pigment epithelium (RPE) layer and concomitant choroidal neovascularization (CNV). The current standard of care for wet AMD is vascular endothelial growth factor (VEGF) antibodies administered through intravitreal route to block VEGF activity, which underlies the CNV. Although this therapy improves visual acuity in a substantial proportion of patients, half of the eyes experience persistent CNV leakage, fibrotic scarring and/or geographic atrophy. Most patients do not achieve substantial visual improvement and a third of treated eyes progress to legal blindness. Thus, a novel therapeutic strategy, which is independent of VEGF signaling pathway and improves outcomes while providing inhibition of angiogenesis with acceptable safety profile, is an urgent and unmet medical need. The discovery of 'aquaporins' (AQPs), a large family of membrane protein channels that facilitate transport of water, has drawn attention to their role in several normal physiological and pathological states that involve water transport. Particularly, more recent evidence from knockout mice studies indicate that AQP1 is involved in cell migration, angiogenesis, and tumor growth. We will discuss some of our recent findings that provides direct evidence for AQP1 in the retinal angiogenesis.

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Adipose stem cell treatment protects against visual deficits of mild traumatic brain injury

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Traumatic brain injury frequently leads to progressing vision problems resulting in blindness. Inflammation due to microglial polarization may play a vital role in the development of visual defects. In this study, we assessed whether mesenchymal stem cells derived from adipose tissue (adipose-derived stem cells ADSC) can limit retinal tissue damage from blast injury and improve visual function. Twelve-week-old C57Bl/6 mice were subjected to 50-psi air pulse limited to a 7.5 mm diameter area on the left side of the head overlying the forebrain resulting in a mild traumatic brain injury. Within 1 hour of blast injury, approximately 1000 ADSC or 1mL CC-101 (ADSC paracrine signal derived biologic) were intravitreally delivered into both eyes. Blast only and no-blast control mice received saline. *In vitro* mouse microglial cells and retinal endothelial cells were used to evaluate the anti-inflammatory effect of ADSC or CC-101. At week 6, a single 50-psi blast produced visual deficits with a significant decrease in visual acuity; a significant increase in the contrast sensitivity (as measured by opto-kinetic tracking); a significant increase in "b" wave amplitude (as measured by electroretinogram), and increased vascular leakage (as measured by fluorescein angiography) compared to control mice. These blast-induced visual deficits were significantly diminished by ADSC and CC-101 ($p < 0.04$). Retinal histological analyses revealed a decrease in gliosis (GFAP; $p < 0.05$) in the retinas of blast injured eyes receiving ADSC. To correlate vascular leakage *in vivo*, retinal endothelial cells exposed to *Staurosporine* exhibited fluorescein leakage in an *in vitro* vascular permeability assay, while cells pre-treated with CC-101 showed significantly attenuated leakage ($P < 0.05$). *In vitro*, mouse microglial cells stimulated with lipopolysaccharide (LPS, 10ng/ml) demonstrated an M1 phenotype with increased nitrite levels, decreased arborization and pro-inflammatory gene transcripts that were significantly abrogated with microglial cells co-incubated with CC-101 ($p < 0.05$). Our findings suggest that both ADSC and CC-101 improve visual deficits of the blast injury through their anti-inflammatory properties on activated pro-inflammatory microglia. Our studies suggest a shelf-stable and battlefield ready regenerative therapy

for immediate delivery at the time of injury that may provide a practical and cost effective solution against the traumatic effects of blast injuries to the retina.

JNT8 (OPT+GLA) - Sustained glaucoma therapy and ocular drug delivery**Liposomes for Sustained Glaucoma Therapy**

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In glaucoma treatment, chronic instillation of daily eyedrops to lower IOP is the primary treatment of choice, although it relies on patient adherence and correct performance. Several sustained drug release platforms are emerging and some already in clinical trials to address the significant unmet need of patient noncompliance in glaucoma medical treatment.

The use of nanoliposomes to provide extended IOP lowering of latanoprost has been recently developed and clinically evaluated. In an open-label, pilot study, the safety and efficacy of a single subconjunctival injection of liposomal latanoprost was evaluated in eyes with either ocular hypertension (OHT) or primary open-angle glaucoma (POAG). Subconjunctival injection of liposomal latanoprost was well tolerated by all the enrolled six subjects. A prolonged and significant IOP lowering was observed through the 3 month study period post injection (≥ 20 % IOP reduction, $P = 0.001$ to 0.049). The nanomedicine reported here is the first nanocarrier formulation that has an extended duration of action in humans, beyond a couple of weeks. The findings in this study open up a new treatment modality that can be extended to other eyedrop medications. This drug delivery platform will greatly enhance patient compliance and improve treatment outcomes.

Development of ENV515 Travoprost XR Therapy with Target Duration of Treatment Effect >6 Months

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Envisia Therapeutics is developing novel ocular extended release medicines by leveraging the PRINT® platform for formulation and manufacturing of nano and micro drug particles with precise control over size, shape and composition. Envisia's lead product is ENV515 travoprost XR, a biodegradable intracameral travoprost formulation which was designed to last for more than 6 months after a single administration. During nonclinical evaluations, ENV515 has demonstrated a sustained ~30% reduction of IOP in ocular hypertensive Beagle dogs for up to 8 months after a single dose. In the first-time-in-human 28-day clinical study, ENV515 has demonstrated IOP lowering at levels similar to timolol and Travatan Z at the low and high doses, respectively. In an ongoing 12-month dose escalation clinical study, the completed interim 3 month analyses of the low dose ENV515 have shown good tolerability and a sustained >7 mmHg reduction in 8AM IOP for 3 months after a single dose.

Semi-fluorinated Alkanes for Topical Ocular Delivery of Cyclosporine A

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Background: Cyclosporine A (CsA) is an anti-inflammatory agent that has been frequently used to treat ocular inflammatory conditions, such as dry eye syndrome. However, the poor water solubility of CsA makes it difficult to formulate into an acceptable ocular dosage form. Semi-fluorinated alkanes (SFAs) are a novel class of inert, non-toxic and amphiphilic liquids that form clear solutions with CsA and have been suggested as efficient carriers for topical administration of CsA.

Purpose: The aim of this study was to assess the corneal bioavailability of CsA from SFAs compared to currently marketed formulations.

Methods: An *ex vivo* porcine eye model was used to study the penetration of

- Restasis® (0.05% CsA ophthalmic emulsion),
- Ikervis® (0.1% CsA ophthalmic emulsion), and
- 0.05% or 0.1% CsA in SFAs.

The amount of drug penetrated per gram of cornea between 0.5 to 4 hours after application was assayed by HPLC and statistically compared using a two-way ANOVA. Drug distribution in different layers of the cornea was also visualized by substituting CsA with a lipophilic fluorescent dye and viewing corneal sections under a fluorescent microscope.

Results: Significant improvement in corneal penetration of CsA could be observed for 0.05% CsA in SFAs (C1hour = 5,844 ± 2,408 ng/g) over Restasis (C1hour = 761 ± 221 ng/g), with the area under curve (AUC) being more than 8-folds greater. The AUC of 0.1% CsA in SFAs (C1hour = 12,556 ± 4,017 ng/g) was 3.6 folds greater than Ikervis (C1hour = 2900 ± 341 ng/g). Microscopic examinations revealed that the dye incorporated into SFAs tended to accumulate mainly in the corneal epithelium.

Conclusion: Overall, this study showed that SFAs can significantly improve the corneal absorption of lipophilic drugs, such as CsA, and could therefore be a promising platform for drug delivery to the eye.

A Nanoparticle Formulation Enhances the Corneal Permeability of Disulfiram and Reduces its Corneal Toxicity

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In treatments for glaucoma, the goal in the search for successful therapies for glaucoma is the reduction of intraocular pressure (IOP), and the search for effective eye drops that reduce IOP is a high priority. We previously reported the potential of a 2-hydroxypropyl-beta-cyclodextrin solution containing 0.5% disulfiram (DSF solution) to provide effective anti-glaucoma treatment in eye drop form. In this study, we designed new ophthalmic formulations containing 0.5% DSF nanoparticles prepared by a bead mill method, and compared the IOP-reducing effects of a DSF_{nano} dispersion with those of a DSF solution. [Methods] The DSF was added in the solution containing 0.001% benzalkonium chloride, 0.1% D-mannitol and 0.5% methyl cellulose, and the dispersions containing DSF nanoparticles (DSF_{nano} dispersion) was prepared by using Bead Smash. The antimicrobial activity was tested by *Escherichia coli*. The transcorneal penetration of ophthalmic dispersions was examined by using rabbits, and the immortalized human corneal epithelial cell line was used for evaluation of cytotoxicity.

[Results] We succeeded in preparing a high quality dispersion containing DSF nanoparticles (particle size, 183 ± 92 nm, means ± S.D.). The high stability of the DSF_{nano} dispersion was observed until 7 days after preparation, and the DSF_{nano} dispersion showed high antimicrobial activity against *Escherichia coli*. In transcorneal penetration experiments using rabbit corneas, only

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diethyldithiocarbamate (DDC) was detected in the aqueous humor, while no DSF was detected. The DDC penetration level (area under the curve, *AUC*) and corneal residence time (mean residence time, *MRT*) of the DSF_{nano} dispersion were approximately 1.45- and 1.44-fold higher than those of the DSF solution, respectively. Moreover, the IOP-reducing effects of the DSF_{nano} dispersion were significantly greater than those of the DSF solution in rabbits (the IOP was enhanced by placing the rabbits in a dark room for 5 h). In addition, DSF_{nano} dispersion are tolerated better by a corneal epithelial cell than DSF solution.

[Conclusions] It is possible that dispersions containing DSF nanoparticles will provide new possibilities for the effective treatment of glaucoma, and that an ocular drug delivery system using drug nanoparticles may expand their usage as therapy in the ophthalmologic field. These findings provide significant information that can be used to design further studies aimed at developing anti-glaucoma drugs.

Maintaining IOP with the InnFocus MicroShunt® 3 Years and Beyond

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There is an unmet need for a simple-to-implant, minimally invasive glaucoma shunt with intraocular pressure (IOP) reduction similar to trabeculectomy. The purpose of this study was to determine the intraocular pressure (IOP) and medications per patient at 3 years using this MicroShunt made of a soft, inert and permanent polyisobutylene-styrene thermoplastic material. A single-site, prospective, nonrandomized study of 23 eyes that had failed maximum tolerated glaucoma medication, was followed for 3 years. A MicroShunt was implanted ab externo through a needle tract under the limbus, draining aqueous from the anterior chamber to the scleral surface. Fourteen patients received the MicroShunt alone and 9 with cataract surgery. At 1 (n=23), 2 (n=22), and 3 (n=22) years of follow-up; the qualified success rate (IOP≤14mm Hg and IOP reduction ≥20%) was 100%, 91%, and 95%; mean medicated IOP was reduced from 23.8±5.3 to 10.7±2.8, 11.9±3.7, and 10.7±3.5mm Hg, and the mean number of glaucoma medications/patient was reduced from 2.4±0.9 to 0.3±0.8, 0.4±1.0, and 0.7±1.1, respectively. The most common complications were transient hypotony (13%, 3/23) and

transient choroidal effusion (8.7%, 2/23), all resolved spontaneously. There were no leaks, infections, migrations, erosions, persistent corneal edema, or serious long-term adverse events. Surgery with the InnFocus MicroShunt transscleral aqueous drainage tube with Mitomycin C achieved IOP control in the low teens in most subjects up to 3 years of follow-up, with no long-term sight-threatening adverse events. The InnFocus MicroShunt® is showing to be safe, effective in maintaining IOP, can be used in eyes that are phakic or that require concomitant cataract surgery and an excellent choice for patients who cannot tolerate medications.

IRB Status: International

Disclosures: Implanting surgeons and data collectors JB, RA and AP have no financial interest in the product. LP, YPK and BAW are employees of InnFocus, Inc. and own stock in the company. J-MP is a co-inventor of the product and may receive royalties

JNT9 (OPT+GLA) - Therapeutic targets for retinal disease: Lessons learnt form bench side

Retinal Vascular Inflammation in Diabetic Retinopathy

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Inflammation is a nonspecific response to injury that includes a variety of functional and molecular mediators, including recruitment and/or activation of leukocytes. Inflammation typically has beneficial effects on an acute basis, but can have undesirable effects if it persists chronically. Diabetic retinopathy (DR) is a circumstance in which inflammation persists chronically. This retinal inflammation, particularly of the vasculature, precedes features of retinopathy that can be observed by fundus examination. We believe it is important to develop therapeutic strategies that target these earliest stages of retinal pathology, which before irreversible damage to the retina has occurred. This presentation will include an overview of the features of retinal vascular inflammation in early stage DR, including identification of which retinal cells are most responsive to diabetes-relevant conditions and the nature of the response of each cell type. Additionally, an overview of the

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consequences of this inflammatory response in the retinal capillary unit will be discussed. The application of this information to animal models of diabetes will be examined. We have investigated the roles of a number of transcription factors in the induction of retinal inflammation by diabetes-relevant stimuli. We will present experimental data from studies targeting these transcription factors in DR-related vascular inflammation, using the results of efficacy experiments conducted in acute and chronic models of retinal inflammation to illustrate this strategy. Specific targets that will be discussed include PPAR- β , GAPDH, NFAT and NF κ B. The presentation will include our hypothesis regarding induction of inflammation under diabetic conditions, the development of experimental models to investigate this induction and its consequences in vitro and in vivo, and our use of those models to test specific therapeutic strategies aimed at inhibiting early DR.

The Ureohydrolase Arginase as a Novel Therapeutic Target for Retinopathy

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Ischemic retinopathies, such as diabetic retinopathy, retinopathy of prematurity and retinal vein occlusion are a major cause of blindness. Neurovascular injury is a common feature in these conditions. However, conventional therapies target clinically significant macula edema and/or neovascularization, which occur relatively late in the disease process. Intra-ocular anti-VEGF injections can be effective in reducing retinal edema, but the effects are usually transient and the need for repeated injections increases the risk of intraocular infection. Laser photocoagulation is effective in controlling pathological neovascularization, but may impair vision and in some patients the retinopathy progresses. Moreover, neither treatment targets early neurovascular injury or promotes repair. Our studies have examined the role of the ureohydrolase enzyme arginase as a mediator of neuronal and vascular injury during ischemic retinopathies. Arginase metabolizes L-arginine to form proline, polyamines and glutamate. Excessive arginase activity reduces the supply of L-arginine needed for proper function of nitric oxide synthase (NOS). This causes NOS to become uncoupled and produce superoxide and less NO. Superoxide and NO combine rapidly and form the highly toxic oxidant

peroxynitrite. Altered levels of polyamines can activate the oxidation pathway and their catabolic products can further increase oxidative stress and DNA damage, exacerbating the cellular injury. Studies indicate that neurovascular injury during ischemic retinopathy is associated with increased arginase expression/activity, decreased NO and increased polyamine oxidation, formation of superoxide and peroxynitrite and dysfunction and injury of both neuronal and vascular cells. Furthermore, data indicate that the cytosolic isoform arginase 1 (A1) is involved in diabetes and hyperglycemia-induced dysfunction and injury of vascular endothelial cells whereas the mitochondrial isoform arginase 2 (A2) is involved in neurovascular dysfunction and death following hyperoxia exposure, ischemia/reperfusion or traumatic optic nerve injury. Thus, we postulate that activation of the arginase pathway causes neurovascular injury by uncoupling NOS and inducing polyamine oxidation, thereby reducing NO and increasing oxidative stress, both of which contribute to the pathological process. Targeting this pathway offers a novel therapeutic strategy for limiting neurovascular injury during ischemic retinopathy.

Is Tiam1-Rac1 Axis a Legit Therapeutic Target for Preventing Retinal Dysfunction in Diabetes? An Outsider's Perspective

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Increased intracellular generation of reactive oxygen species [ROS] has been implicated in the pathology of metabolic [diabetes] and neurodegenerative [Alzheimer's] diseases. Burgeoning evidence suggests NADPH oxidases [Noxs] as the principal source for cellular ROS in humans. Of this class of enzymes, the phagocyte-like Nox [Nox2] has come under intense scrutiny as one of the "culprits" for the induction of cellular damage culminating in the onset of diabetes and its complications. Functional regulation of Nox2 is fairly complex due to its membranous and cytosolic cores [e.g., Rac1], which require specific post-translational modification steps [phosphorylation, prenylation and acylation] for their membrane association. Extant data from our laboratory have suggested key causal roles for oxidative stress in the onset of retinal lesions in diabetes. Along these lines, we observed accelerated Rac1-Nox2 axis and associated intracellular ROS leading to mitochondrial damage and cell demise in retinal endothelial cells exposed to hyperglycemic conditions. We have identified T-cell lymphoma invasion and metastasis-inducing protein 1 [Tiam1] as the guanine nucleotide exchange factor for

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the activation of Rac1 since NSC23766, a specific inhibitor of Tiam1-Rac1 signaling pathway, markedly attenuated Rac1 activation, total and mitochondrial ROS generation, p38 MAPK activation, mtDNA damage and cell apoptosis. Administration of NSC23766 to diabetic mice blunted retinal Rac1 activation and ROS generation. Based on this emerging evidence we conclude that Tiam1-Rac1-Nox2 module represents a "druggable" target for suppressing the cellular dysfunction in target tissues under conditions of glucotoxicity and diabetes.

Chronic Retinal Inflammation in Diabetic Retinopathy

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Diabetes induces chronic inflammation within the retinal tissue. Caspase-1, the enzyme that produces active interleukin-1beta (IL-1 β), seems to be a prominent mediator of this chronic retinal inflammation. Autocrine mechanisms keep this enzyme active throughout the development and progression of diabetic retinopathy. Müller cells are one of the cellular sources for active caspase-1, actively contributing to the process of chronic retinal inflammation. The outcome(s) of consistent caspase-1 activation have not fully been identified. Therefore, the focus of this project was to identify the importance of chronic caspase-1 activation for the development of diabetic retinopathy. Using non-diabetic and STZ-diabetic wild type C57Bl6 mice and Cas-1^{-/-} mice Müller cell loss and formation of acellular capillaries were determined. For *in vitro* studies, human Müller cells (hMC) were treated with either normal (5mM) or high glucose (25mM) media or normal (5mM) glucose media plus 2ng/mL IL-1 β . The effect of IL-1 β and caspase-1 inhibition on VEGF production was determined. Cas-1^{-/-} mice were protected against diabetes-induced Müller cell loss and diabetic retinopathy. *In vitro* results show that high glucose induced VEGF production by two fold in Müller cells. IL-1 β treatment also significantly increased VEGF production from 2.43 \pm 0.5 ng/ml/mg protein to 7.98 \pm 0.1 ng/ml/mg protein. Inhibition of caspase-1 using the specific caspase-1 inhibitor YVAD-fmk (100 μ M) or minocycline (50 μ M) completely prevented VEGF production indicating that caspase-1 activation and IL-1 β production drive VEGF production in human Müller cells. Taken together, caspase-1 activation is crucial for the development of diabetic retinopathy. Therefore, drugs that target caspase-1 might present a new strategy to prevent chronic retinal inflammation and diabetic retinopathy.

Retinal Response Ischemic Injury: Role of Histone Deacetylase and Sphingolipids

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Increased histone deacetylase (HDAC) activity and the resulting dysregulation of protein acetylation is an integral event in retinal degenerations associated with ischemia and ocular hypertension. This study investigates the role of preconditioning and sphingolipids interact to influence protein acetylation and ischemic retinal injury. Rat eyes were unilaterally subjected to retinal injury and retinal neuroprotection induced by 5 minutes of an ischemic preconditioning (IPC) event. Following ischemia, total Class-I HDAC activity increased by 21.2 \pm 6.2%, and this increase resulted solely from a rise in HDAC1/2 activity. Ischemic preconditioning suppressed the rise in Class-I HDAC activity, increased acetylated histone-H3 in the retina. In control animals 7 days post ischemia, ERG a- and b-wave amplitudes were significantly reduced by 34.9 \pm 3.1% and 42.4 \pm 6.3%, respectively. In rats receiving an IPC stimulus, the ischemia-induced decline in ERG a- and b-wave amplitudes was blocked. Ischemic retinal injury and retinal preconditioning response were dependent on the activity acid sphingomyelinases. In the preconditioned retina acid ceramidase upregulation was required for retinal preconditioning and associated hyperacetylation. Multiple HDACs are detected in the retina, these studies provide evidence that hypoacetylation associated with ischemic injury results from the selective rise in HDAC1/2 and acid sphingomyelinases activity. The neuroprotection induced by IPC is mediated in part by suppressing HDAC activity in an acid ceramidase dependent process.

JNT11 (OPT+IMM) - An intersection of receptor signaling pathways with neuroinflammation (inflammasome)

The Blockade of Adenosine A_{2A} Receptor Affords Neuroprotection through the Control of Microglia-Mediated Neuroinflammation in Experimental Models of Glaucoma

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Glaucoma is a progressive retinal degenerative disease and the second cause of vision loss in the world. It is characterized by damage of the optic nerve and retinal ganglion cell (RGC) loss. The degeneration of RGCs in glaucoma is accompanied by a neuroinflammatory response involving retinal microglial cells. The etiology of glaucoma is unknown, but elevated intraocular pressure (IOP) and aging have been identified as main risk factors. Currently, IOP is the only modifiable risk factor and the main target for therapeutic interventions. However, vision loss progresses in up to 45% of glaucoma patients undergoing an IOP-lowering regimen. Therefore, new and more effective treatments are necessary, and neuroprotection of RGCs is considered to offer potential as an alternative therapy.

Adenosine is a neuromodulator in central nervous system (CNS) acting on metabotropic adenosine receptors (A_1 , A_{2A} , A_{2B} and A_3). The blockade of A_{2A} receptors has been demonstrated to confer robust neuroprotection against noxious brain conditions, probably through the control of microglia-mediated neuroinflammation. Taking into account the contribution of microglia in the pathophysiology of glaucoma, we evaluated the effects of A_{2A} receptor antagonists in the control of retinal neuroinflammation and in the loss of RGCs in experimental models of glaucoma (cell and tissue cultures and animal models). Our results demonstrate that increased pressure increases A_{2A} receptor expression and density, mainly in microglia. We found that the antagonists of A_{2A} receptors prevent microglia reactivity and retinal neuroinflammation and confer protection to RGCs in experimental models of glaucoma.

These results open the possibility for the use of antagonists of A_{2A} receptors as therapeutic options to manage neuroinflammation and RGC loss in glaucoma.

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Panx1-mediated Danger Signaling and Caspase Activation in IOP-induced Ischemia and Glaucoma

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In sterile conditions in the retina and CNS neuroinflammation is controlled by the inflammasome, a cytoplasmic macromolecular-complex producing IL-1 cytokines. The activation of the inflammasome is a common response in immune cells, neurons and glia to danger signaling, oxidative stress and dysfunctional autophagy. A persistent inflammasome activation is known to be deleterious has been implicated in ischemic and mechanical stress-induced pathologies in the CNS and retina.

We discovered robust activation of the inflammasome in the mouse retina with IOP-induced ischemia and/or glaucoma. Active inflammasome was localized primarily to the inner retina. The elevated IOP-induced levels of inflammasome convertases caspase-1 and caspase-11, as well as mature IL-1 β were significantly decreased in the retinas of Casp1-null, Panx1-null and WT probenecid-treated mice relative to IR-exposed control retinas. Using gene knockout approach and small drug inhibitors, we have established that blockade of caspase-1 or pannexin1-mediated signaling prevents inflammasome activation. Inactivation of inflammasome resulted in significant protection to retinal ganglion cells (RGCs) in mouse disease models.

These experimental evidence support that injury-induced inflammasome induces major toxicity to RGCs. Our results indicate that pharmacological control of inflammasome activation can protect retina from in various types of retinal pathologies triggered by increased intraocular pressure and endorse inflammasome pathway as a novel target in perspective glaucoma therapies.

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Mechanical Strain Primes the Inflammasome in Astrocytes through the P2X7 Receptor

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Local increases in inflammatory signaling can have pathological influences in many retinal disorders. The cytokine IL-1 β is a master regulator and can trigger the release of other pro-inflammatory cytokines. IL-1 β itself is released following activation of a cytosolic multiprotein complex termed the NLRP3 inflammasome. There are two steps in inflammasome involvement; first the relevant components must be primed and upregulated on a transcriptional and translational level; second, the proteins must be activated to assemble and produce a releasable form of IL-1 β . The P2X7 receptor for extracellular ATP is well known for its ability to activate the NLRP3 inflammasome, but it is unclear whether the P2X7 receptor can also prime inflammasome components. As excess ATP release may accompany the increased IOP of glaucoma, this study asked whether mechanical strain upregulates inflammasome components and whether the P2X7 receptor contributes to this response using *in vivo* and *in vitro* models. The expression of inflammasome components IL-1 β and NLRP3 was increased in rat retinal tissue following the elevation of IOP to a non-ischemic 50 mmHg for 4 hrs. Intraocular injection of the P2X7 receptor antagonist Brilliant Blue G (BBG) prevented the rise, implicating the P2X7 receptor in the response. As the optic nerve head is the focus of mechanical strain in eyes experiencing an elevated IOP, isolated rat optic nerve head astrocytes were investigated further. The astrocytes released ATP when subjected to a 5% equibiaxial stretch or by swelling with a 30% hypotonic solution. This released ATP autostimulated P2X7 receptors on the astrocytes. mRNA message for IL-1 β was increased by swelling astrocytes, and this rise was blocked with P2X7 receptor antagonists 10 μ M BBG, 50 nM A839977, or 5 μ M A740003. The priming of the IL-1 β in astrocytes was also blocked with inhibitor Bay11-7082 (4 μ M), implicating transcription factor NF κ B in the priming. In conclusion, mechanical strain can activate the P2X7 receptor to upregulate the expression of IL-1 β *in vitro* and *in vivo*. This suggests ATP release and the P2X7 receptor may contribute to an increased parainflammatory state with chronic elevation in IOP.

Ophthalmic Genomics

OGM1 - Genetics of multifactorial eye diseases

Genetics of Diabetic Retinopathy

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Diabetic retinopathy (DR) is a polygenic disorder. Twin studies and familial aggregation studies have documented clear familial clustering. Heritability has been estimated to be as high as 27% for any DR and 52% for proliferative diabetic retinopathy (PDR), an advanced form of the disease. Linkage analyses, candidate gene association studies and genome-wide association studies (GWAS) performed to date have not identified any widely reproducible risk loci for DR. Combined analysis of the data from multiple GWAS is emerging as an important next step to explain the unaccounted heritability. Key factors to future discovery of the genetic underpinnings of DR are precise DR ascertainment, a focus on the more heritable disease forms such as PDR, stringent selection of control participants with regards to duration of diabetes, and methods that allow combination of existing datasets from different ethnicities to achieve sufficient sample sizes to detect variants with modest effect sizes.

Genetic Aspects of Chronic Central Serous Chorioretinopathy

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Central serous chorioretinopathy (CSC) is characterized by a serous detachment of the neuroretina from the underlying retinal pigment epithelium (RPE) in the macula due to fluid leakage through a dysfunctional RPE. Clinical evidence from multimodal imaging, such as choroidal congestion, thickening and hyperpermeability of the choroid, implies an important role for choroidal abnormalities as an underlying cause for RPE dysfunction and subretinal fluid leakage in CSC. Two main subtypes of CSC (acute and chronic) can be distinguished. The acute form of CSC generally has a favorable prognosis because the accumulated subretinal fluid often subsides spontaneously within 2 to 3 months,

with (near-)normal recovery of vision. In contrast, chronic CSC (cCSC) is typically not self-limiting and subretinal fluid remains present for >3 months.

Interracial differences in the prevalence of cCSC and the familial occurrence of cCSC suggest strong genetic involvement. However, only a limited number of possible genetic associations have been reported so far.

In a first study we assessed the degree of phenotypic and genotypic overlap between cCSC and age-related macular degeneration (AMD). We used a combination of detailed phenotyping based on multimodal imaging and systematic analyses of single nucleotide polymorphisms at 19 known AMD-associated loci in cCSC patients and found associations with *ARMS2* and complement factor H (*CFH*). Furthermore, a detailed analysis of *CFH* haplotypes in cCSC patients was performed, revealing strong associations. Interestingly, in line with a previous report, we found an inverse direction of genetic associations between AMD and cCSC.

In a second study we further explored the role of the complement system in cCSC by analyzing copy number variations of the *C4* gene. We found that lower copy numbers of *C4B* confer risk for the development of cCSC and higher copy numbers offer protection.

Taken together, these studies and other ongoing work demonstrate that genetic variation in complement genes associate to cCSC and suggest a possible physiological role of the complement system in the etiology of the disease.

Molecular Genetics of Polypoidal Choroidal Vasculopathy

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Polypoidal choroidal vasculopathy (PCV) is a subtype of "wet" age-related macular degeneration (AMD), accounting for about 55% of cases of "wet" AMD in Asian patients. Genetic variants in several genes/loci are significantly associated with both choroidal neovascularization (CNV) subtype and PCV. We found that the rare c.986A>G (p.Lys329Arg) variant in the *FGD6* gene was significantly associated with PCV ($P = 2.19 \times 10^{-16}$, OR = 2.12), but not with CNV ($P = 0.26$, OR = 1.13). Further functional studies, we showed that intracellular localization of *FGD6* Arg329 is distinct from that of *FGD6* Lys329 and *FGD6* could regulate the pro-angiogenic effect, and oxidized phospholipids could increase expression of *FGD6*. Collectively, oxidized phospholipids and genetic variants might act synergistically to increase susceptibility to PCV.

OGM2 - Molecular Genetics of Eye Disease

Mutations in lens-specific genes: Cataract and beyond

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Mutations in lens-specific genes have been frequently reported to play major roles in the development of congenital cataracts. So far, a number of mutations in these genes have been reported, including those identified in our patients with cataract. Based on analysis of data from whole exome sequencing on 1629 probands with different forms of hereditary eye diseases, a numbers of novel variants predicted to be damaging were identified. However, most of such variants were not associated with cataracts. A few mutations reported to cause cataracts were detected in patients without cataracts. Our data imply that many rare variants predicted to be damaging may not necessary pathogenic. Clarifying gene-specific genotype-phenotype is the prerequisite in making precision genetic counseling. International collaborative studies on such kind of data, probably through AEGC or other societies, may be of great help in differentiating rare variants from pathogenic mutations in genes responsible for genetic eye diseases.

Retinal Degeneration (RD) due to the Involvement of *hASRGL1*: Mouse and Zebrafish Models with Mutant ASRGL1 Develop Retinal Degeneration

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Analysis of the exome sequence of a three-generation consanguineous pedigree with early-onset recessive RD with predominant cone abnormalities identified a homozygous mutation (p.G178R) in the *ASRGL1* gene

segregating with disease. The purpose of this study is to evaluate the mechanism underlying RD due to the involvement of *ASRGL1*.

The *Asrgl1* gene ablated mouse model (*Asrgl1*^{mut/mut}) was generated by gene editing using CRISPER/Cas9 methodology. Ocular phenotype of these mice was evaluated by fundus imaging, OCT, ERG, immuno staining with photoreceptor marker and ASRGL1 antibodies and qRT-PCR of *Asrgl1* and photoreceptor marker gene transcripts. Asparaginase activity of ASRGL1 was measured by enzymatic assay. Zebrafish overexpressing *hASRGL1* were generated by injecting 1pg-20pg of wild type (wt) or 1pg-5pg of G178R-*hASRGL1* (mut) mRNA into zero day post fertilization (0dpf) embryos. The ocular phenotype in 6dpf zebrafish was evaluated by morphology, axial length measurements and immunohistochemistry with photoreceptor marker.

The *Asrgl1*^{mut/mut} mice displayed a significant decrease in the levels of expression of *Asrgl1* ($p < 0.0001$), *Opn1mw* ($p=0.003$), *Opn1sw* ($p=0.02$) and *Rho* ($p=0.01$) gene transcripts compared to age-matched control mice. Immunostaining with marker specific antibodies showed a significant loss of M-opsin expressing cones in *Asrgl1*^{mut/mut} mice, near total loss of the ASRGL1 protein and asparaginase activity. Six months old *Asrgl1*^{mut/mut} mice exhibited significant reduction in cone response compared to the response in age-matched controls. Similarly, the zebrafish injected with the mutant *hASRGL1* displayed decreased axial length and life span compared to *hASRGL1*-wt and dye injected fish. No difference in rod and cone antibody staining was observed between the dye-injected and wt-*hASRGL1* mRNA-injected fish. Whereas, fish injected with 4pg G178R-*hASRGL1* mRNA showed smaller eye phenotype (axial length) and loss of blue cone opsins. The fish injected with >5pg of mutant mRNA developed severe retinal pathology and mortality at 1dpf. However, injections with wt-*hASRGL1* mRNA at concentrations over 200pg (40 fold higher) had normal eye morphology.

ASRGL1 gene ablated mouse and zebrafish models illustrated loss of cone photoreceptors, which is consistent with the phenotype observed in patients with *ASRGL1* mutations, suggesting the involvement of mutant *ASRGL1* in causing retinal degeneration.

Ophthalmic Genomics

The role of ubiquitin pathway in maintaining calcium homeostasis and lens transparency

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Precipitation of some lens proteins results in lens opacification, or cataract, the major blinding disease, globally. The K6W mutation of ubiquitin (K6W-Ub) retards proteasomal degradation of polyubiquitin conjugates without interfering their formation and therefore provides a powerful tool to study ubiquitin pathway genetically. There is a dose dependent relationship between expression of K6W-Ub in the lens and the extent of lens aberrations. To initiate elucidation of the role of ubiquitin pathway in cataract development, we compared protein profiles of lenses from transgenic mice that express wild type ubiquitin (WT-Ub) or K6W-Ub. Only ~2% of the proteome was different. Importantly, these included fragmented cataractogenic proteins. In a novel whole tissue assay we observed a hyper activation of calpain in K6W-Ub lenses, consistent with 4-fold elevation of Ca²⁺ and massive elevation of levels of calpain-associated fragmentation of critical lens proteins including vimentin, γ -crystallin, filensin and fodrin (spectrin alpha 2). Two RNA granule proteins Caprin2 and Tdrd7 were also found to be new calpain substrates. Additionally, we observed accumulation of gap junction connexin 43 and its ubiquitinated form, and diminished connexin 46 levels in the K6W-Ub lens as well as in lens cell models in which K6W-Ub is expressed, thus, providing a mechanism by which Ca²⁺ accumulates and calpain is activated. Together, the *in vivo* and *in vitro* findings reveal the first mechanistic association between pathway that a mutation of K6 on ubiquitin alters UPS function, perturbed gap junction function, Ca²⁺ level elevation, activation of calpain and substrates cleavage, that together result in an abnormally differentiated, cataractous lens. The data are critical to understanding roles for ubiquitin in proper organogenesis and for devising new therapies for protein precipitation diseases.

Spatial and Temporal Dissection of Pathogenesis in a Model of Anterior Segment Dysgenesis and Glaucoma Caused by a *Col4a1* MutationDOUGLAS GOULD¹, Mao Mao¹, Yvonne Ou¹, Marton Kiss²

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Mutations in collagen type IV alpha 1 (*COL4A1*) cause anterior segment dysgenesis (ASD) and glaucoma. Because of the widespread distribution of COL4A1 in ocular basement membranes and the complex crosstalk between tissues of different embryological origin during development, dissecting the primary insults of pathogenesis can be difficult. Here, we use conditional expression of mutant COL4A1 to determine the primary location and timing of insults. We used *Actb-Cre* mice (ubiquitous CRE expression) to validate the *Col4a1*^{flex41} allele for CRE-mediated excision. Next, we crossed *Col4a1*^{+/flex41} mice to three different tissue-specific CRE recombinase strains: *MLR10-Cre* (lens), *Wnt1-Cre* (neural crest derived periocular mesenchyme), and *Tie2-Cre* (vascular endothelial cells), to generate tissue-specific mutants. Finally, we used *Rose26-Cre^{ERT}* mice (tamoxifen inducible) to study the timing of pathogenesis. We tested the extent of ASD by performing slit-lamp examination, measuring intraocular pressure (IOP), and analyzing histological sections of anterior segment structures and optic nerves. We found that expressing the mutation after E12.5 did not cause ASD. Lens-specific mutants developed cataracts, mild ASD, IOP elevation, and optic nerve degeneration; however, other cell-type-specific mutants did not. Our results suggest that early developmental events underlie ASD and that the lens is a key site of pathogenesis.

AMD Genetics in Indian sub continent: Trends and Gaps

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Age-related macular degeneration (AMD) is a devastating eye related disease of the elderly, signs of which usually appear after the age of 50. After we showed that the deletion of CCL2 and its receptor in mice could develop cardinal features of AMD pathobiology, an effort to examine the associated SNPs in Indian AMD patients ensued, even though 53 genes were later found to be associated in Caucasian populations. Various genetic loci

involved in maintenance of extracellular matrix (like MMPs and Fibrinogen) were reported to be associated with AMD in South Indian population. HTRA, TLR4 and APOE were also shown to be associated with progression of AMD in South India. Our North Indian AMD study demonstrated association of CFH, CCR3, Eotaxin, VEGF, VEGF2, TLR3, SOD1, CCL2 and its receptor CCR2 with AMD. Additionally, protein levels of monocarboxylic transporter (SLC16A8), TIMP-3 and metabolic protein (LIPC) were also found to be associated with the AMD.

We propose that in Indian AMD population, the inflammatory cascade may be primarily mediated by CCL2/CCR2 pathways, evidenced by decreased basal levels of CCR2 expressing peripheral blood mononuclear cells (PBMCs). Polymorphism in CCR2 may activate CCR3 which may promote choroidal endothelial cell migration, Rac 1 and VEGFR2 which may exacerbate neovascular AMD. It is suggested that genetic variations in the VEGFR2 gene (rs1531289) may also encode the angiogenic processes conferring susceptibility to AMD. The analysis of other gene loci in Indian population is being considered by Department of Biotechnology, Delhi.

In the past few years our knowledge of the genetics of AMD has increased considerably, but the benefits to clinical practice are still catching up. Presently there is no genotype-specific preventive therapy for AMD, neither the genetic profile has a true effect on therapy outcomes. Despite this, it is evident that the risk of AMD of an individual from India carrying all the risk alleles (for : CFH, CCL2, CCR2, CCR3 and VEGFR2) is significant.

OGM3 - Asian eye genetics

Developing International Research Collaborations in Eye Diseases - Asian Eye Genetics Consortium

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There is an increasing interest of the US based investigators/biomedical researchers in pursuing international engagements for bringing new ideas, cross training and other benefits of the international research endeavors in vision research. International research collaborations generate scientific excellence. Citation impact for a publication is typically greater when research groups collaborate, and the benefit strengthens when co-

authorship is international. National Eye Institute (NEI)-NIH in the US is actively involved in global research, seeking scientific opportunities and identifying shared priorities for vision research. NEI supports unique international opportunities in scientific research by partnering with organizations and governments to leverage funds for addressing important questions of human health and biology of eye diseases. There are three key mechanisms that have been used in the most recent times: Parallel funding, Partnering on new initiatives, and Training. Asian Eye Genetics Consortium (AEGC), started in 2014 with a collaboration of NEI with National Institute of Sensory Organs (NISO) in Japan, has an origin at NEI resulting from a meeting of international opinion leaders in late 2013 that identified unique opportunities in which NEI could partner with international groups to support the NEI mission in global vision research. AEGC has developed the following goals:

- 1) Share genetic information in the Asian population to rapidly isolate common disease-associated variants,
- 2) Establish system for accurate diagnosis and grouping of Asian eye diseases,
- 3) Establish system for cost effective genetic analysis,
- 4) Develop a research-oriented database to collect, diagnose and catalog eye diseases in Asia,
- 5) Support and foster collaboration among the Asian countries for the advancement of research that will provide genetic information in the Asian population,
- 6) Collaborate with other international or regional organizations with similar goals, and
- 7) Organize and hold regional congresses and other educational and scientific activities to promote goals of the consortium.

The presentation will describe the recent accomplishments of the AEGC and describe various activities that have taken place in the last two years. The presentation summary will include various scientific opportunities by identifying shared priorities and supporting unique international opportunities in Ophthalmic Genomics.

Association of A69s Missense Polymorphism of Arms2 Gene with Age-related Macular Degeneration in Indian Population

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Introduction: Age related macular degeneration (AMD) is the most common cause of irreversible blindness affecting millions worldwide. Both genetic and environmental factors are responsible to the onset of AMD. Previous studies showed that ARMS2 gene plays a key role in the pathological process leading to wet age-related macular degeneration. Hence, it is fascinating to find out how ARMS2 gene variation influences the pathogenesis of AMD and its correlations in the Indian population. The aim of this study is to investigate the association of ARMS2 gene variation in the development of age-related macular degeneration in Indian population.

Method: Blood was collected from 5 patients diagnosed with wet form of AMD in an EDTA vacutainer. DNA isolation and targeted sequencing of candidate genes, CFH, CFB, ARMS2, HTRA1, ABCA4, TIMP3, C2, CFI, C3, LIPC, CETP, LPL, ABCA1, APOE, HMCN1, CFHR1, CFHR3, CX3CR1, C9, TLR4, ERCC6, FBLN5, RAX2, APOE, and CST3 were carried out at MedGenome Labs Pvt. Ltd, India in a HiSeq Illumina 2000 platform.

Results: The genetic analysis revealed a reported (SNP ID rs10490924) homozygous missense variation (c.205G>T) in exon 1 (Chr10: 124214448; G>T) of the ARMS2 gene in all the five tested samples. Further, this variation is found to result in substitution of Serine for Alanine at codon 69 (p.A69S).

Discussion: The A69S has previously been reported to be a high risk factor for age-related macular degeneration in the Indian population. The homozygous TT genotype has been shown by recessive model to confer an odds ratio of 2.41. This variant is predicted to be damaging by SIFT and PolyPhen and the region is conserved across mammals. Thus, our study highlights that the A69S variant is likely to influence the risk of AMD. Detection of this mutation in subjects would help to predict the individuals who are genetically predisposed to AMD. This would pave way to delay the progression of the disease.

Conclusion: Although genetic testing does not play a role in therapeutic decisions, since an exact link between genotype and response to current therapies for AMD has not been established, it still have a valid application as the outcome of the genetic test may help in delaying the progression of disease by adopting preventive measures. Therefore, genetic testing may be recommended to high-risk patients with family history of AMD and smoking habits for the prediction of genetic predisposition and subsequent prevention.

Genetic Studies on Behcet's Disease and VKH Syndrome in Chinese Population

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Uveitis is a group of inflammatory conditions involving the uvea, retina, retinal vessel and vitreous. Behcet's disease (BD) and Vogt-Koyanagi-Harada (VKH) syndrome are two uveitis entities commonly seen in China. Although the precise pathogenesis remains unclear, accumulating evidences show that complex genetic backgrounds may be implicated in the development of both diseases. Genes encoding for Human leukocyte antigens (HLA) have been shown to be associated with these uveitis entities, including BD (HLA-B51) and VKH syndrome (HLA-DR4, DRB1/DQA1). Genome wide association studies identified two novel susceptibility loci ADO-ZNF365-EGR2 and IL23R-C1orf141 for VKH syndrome in Chinese population. In addition, we have found that a number of genes such as IL-10, STAT4 and miR-146a, miR-182 and FoxO1 are shown to be associated with BD or VKH syndrome. Moreover, copy number variants (CNV) of complement component 4 (C4), IL17F, IL23A, FoxP3, FAS and C5 have been found to be associated with BD or VKH syndrome. In conclusion, our studies support the hypothesis that genetic factors play a role in the pathogenesis of these two uveitis entities.

Genes Associated with Treatment Outcome of Age-related Macular Degeneration in Japanese

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Age-related macular degeneration (AMD) is a leading cause of blindness in industrialized countries, and anti-VEGF drug is often used to treat AMD. Although some patients achieve preferable visual outcome after anti-VEGF treatment, others lose their visual acuity in spite of treatment. To predict the treatment outcome for AMD, genetic variation might be useful. I would like to talk about genetic associations to the treatment outcome of AMD in Japanese after anti-VEGF treatment.

Mutation Spectrum in a Large Cohort of Inherited Retinal Dystrophy Patients Revealed by Next-generation Sequencing

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Purpose: Inherited retinal dystrophy (IRD) is a leading cause of blindness worldwide. Due to an extremely genetic heterogeneity, the etiology and genotypic spectrum of IRD have not been clearly defined, and there is also limited information on genotype-phenotype correlations. The purpose of this study was to elucidate the mutational spectrum and genotype-phenotype correlations of IRD.

Methods: We started by developing a targeted panel of 164 known retinal-disease genes, 88 possible candidate genes, and 32 retina-abundant microRNAs, which were used for exome sequencing. Total 335 Chinese families with IRD were recruited.

Results: In the 335 unrelated families, pathogenic mutations were identified in 191 patients, with a detection rate of 57% (191/335). The total 335 cases consist of 230 sporadic cases (69%, 230/335), 71 autosomal recessive cases (20%, 71/335), 25 autosomal dominant cases (7%, 25/335) and 9 X-Linked cases (4%, 9/335), with a detection rate of 56% (sporadic), 63% (AR), 52% (AD) and 56% (XL) respectively. Among the 230 sporadic patients, 128 cases (57%) were identified to harbor mutations, including 103 autosomal recessive cases (80%), fifteen *de novo* cases (12%) and ten X-linked (8%). In our mutation pool, the top three genes contributing to the IRD were *USH2A*, *EYS* and *CRB1*.

Conclusion: In summary, we have developed a paneled exome sequencing methodology for the molecular diagnosis of IRD and identified pathogenic mutations in 191 unrelated patients among the total 335 Chinese families, with a detection rate of 57% (191/335). Furthermore, novel genotype-phenotype correlations of IRD were uncovered, and we established a novel candidate gene for non-syndromic RP. To the best of our knowledge, this is the largest comprehensive genetic screening of Chinese IRD to date. This study expands the field of genotype-phenotype correlations and the mutational spectrum of the Chinese population with IRD, increasing our understanding of molecular mechanisms of the disease and aiding the clinical diagnosis and personalized treatment of patients with IRD.

Keratoconus: Globally And In The Middle East; Epidemiology, Genetics And Future Research.

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The prevalence of KC is relatively high worldwide, it ranges geographically between Russia; 0.3/105 and USA; 54.5/105. Such variation is mainly due to the variation in both assessment method and criteria in addition to geographic and community specific characteristics.

There is a need for conducting community based rather than hospital based studies to assess the actual prevalence of KC. There are a number of potentially associated risk factors for KC. These factors may include: genetic profile, consanguinity, geographic latitude, exposure to direct light (Especially UV) and personal behaviors (such as eye rubbing). KC is one of the indirect causes of low vision and blindness (as it leads to refractive errors), and hence, it is one of the areas for improvement in reduction of the global magnitude of low vision and blindness. Genetically, there is a number of associated genes and SNPs with KC. Recent studies indicated that it's quite possible that there are more than one gene contributing to the development of KC. This, of course in addition to other factors. The search continues for more genes and SNPs associated with KC. Meanwhile, management of KC is now available and accessible. Such management may include; Corrective Glasses and contact lenses, insertion of intraocular lenses, conduct of Cross linking procedures, using an *Intracorneal Ring Segment*, *Keratoplasty* and *Photorefractive Keratectomy*.

However, some of the cases may either present late to eye care services or even never sought such services, while there is no early detection and referral mechanisms in place yet. In terms of treatment modalities, there is a wide range of available options, however, the crucial issue is that the selection of the most appropriate option should be evidence based and built on specific clinical criteria aiming to increase the probability of success. Meanwhile, specific surgical knowledge and skills in addition to availability of sophisticated instruments are needed to provide such services.

There is a rapid increase in the volume and quality of research in this area. Since the introduction of high quality diagnostic and interventional instruments that can easily, quickly and accurately assess the corneal topography and

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intervene with a very sophisticated and less invasive way at the exact targeted depth of the cornea, the ceiling of expectations for a successful intervention is increasing on daily basis. Improvement in surgical procedures such as cross linking interventions is a concurrent area for clinical and experimental research. Thus, there is almost a new horizon every day in this field. It is anticipated that in the near future, there would be a highly effective resolution of KC which would both correct vision, stop and even reverse the progressions of the disease.

OGM4 - Epigenetic Modifications And Non-Coding RNAs In The Ocular Health and Disease

Is There a Role of Epigenetics in Diabetic Retinopathy?

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Environment of a cell causes stable and heritable covalent modifications that can also alter gene expression without altering the base sequence of the DNA. These modifications are reversible, and are considered to have pathogenic role(s) in chronic diseases, including complex eye diseases such as corneal dystrophy, cataract, glaucoma and age-related macular degeneration. We have shown that diabetes alters DNA methylation status of both nuclear- and mitochondrial DNA-encoded genes in the retina, and this alters mitochondrial homeostasis. This presentation will discuss the role of DNA methylation in the pathogenesis of diabetic retinopathy, especially in the metabolic abnormalities critical in its pathogenesis. Better understanding of the role of DNA methylation in diabetic retinopathy should provide novel therapeutic targets for this blinding disease, which is feared the most by diabetic patients.

Diabetic retinopathy: The microRNA connection

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Dysfunction of endothelial cell causing increased production of vasoactive factors, inflammatory mediators and extracellular matrix (ECM) proteins are characteristic features of diabetic retinopathy. Glucose activates a cascade of signaling pathways converging in the nucleus. Such aberrant signaling leads to activation of transcription factors and transcriptional co-activators. At the post-transcriptional

levels production of such molecules are regulated by epigenetic processes including changes in non-coding RNAs. microRNAs (miRs) are group of non-coding RNAs which regulate gene expression at the post-translational level and controls the downstream effects. These molecules are involved in almost all biologic process and possibly in all diseases, including diabetic retinopathy.

Using array analysis, we identified alterations of multiple miRs in the retina in diabetes, including miR200b, miR146a and miR195. Detailed analyses at various levels of complexities, eg. cultured endothelial cells, mice with endothelial specific miR overexpression with/without chemically induced diabetes and human tissues, were carried out. We found that miR200b regulates retinal VEGF production; miR146a regulates production of several inflammatory mediators and miR195 controls sirtuin1 production in diabetes. In addition, miR200b also was found to be a key mediator in endothelial-mesenchymal transition in the retina in diabetes. Regulatory interactions of miRs with other epigenetic processes such as methylation and acetylation were identified.

Hence, complex webs of pathways, regulated by miRs are involved in the pathogenesis of functional and structural changes in diabetic retinopathy. We and others have identified specific changes mediated by such pathways in the ECs and in the retina in diabetes. Targeting miRs may constitute a potential novel adjuvant treatment strategy for diabetic retinopathy.

Dual Anti-inflammatory and Anti-angiogenic Role of miR-15a in the Retina

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Activation of pro-inflammatory and pro-angiogenic pathways in the retina contributes to pathogenesis of diabetic retinopathy. We identified miR-15a as key regulator of both pro-inflammatory and pro-angiogenic pathways through direct binding and inhibition of the central enzyme in the sphingolipid metabolism, ASM, and the pro-angiogenic growth factor, VEGF-A. miR-15a was downregulated in diabetic retina. Over-expression of miR-15a downregulated, and inhibition of miR-15a upregulated ASM and VEGF-A expression in retinal cells. Diabetic mice overexpressing miR-15a under Tie-2 promoter had normalized retinal permeability compared to wild type littermates. Importantly, miR-15a overexpression

led to modulation toward nondiabetic levels, rather than complete inhibition of ASM and VEGF-A providing therapeutic effect without detrimental consequences of ASM and VEGF-A deficiencies.

The Role of MicroRNAs in Normal and Diseased Corneal Epithelial Homeostasis

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MicroRNAs (miRNAs) are powerful gene expression regulators, but their corneal repertoire and potential changes in normal and diseased limbal epithelial stem cells (LESC) remain unknown. We set to identify microRNAs expressed in the central and limbal compartments of human corneas and to examine their function in regulatory pathways involved in corneal epithelial self-renewal, differentiation and wound healing in normal and pathological conditions such as diabetes mellitus. Previously, using deep sequencing we have identified differentially expressed miRNA in central cornea vs. limbus in both normal and diabetic corneas. Interestingly, principal component analysis and hierarchical clustering showed that the miRNA expression profiles of insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) groups were differentially clustered and separated from each other. Nine miRNAs were upregulated whereas twelve miRNAs were downregulated (2-fold cutoff; p less than 0.05) in IDDM vs. NIDDM limbus. Some miRNAs such as miR-10b that was upregulated in limbus vs. central corneas and upregulated in diabetic limbus also showed significant increase in IDDM vs. NIDDM limbus. Further, miR-10b was more expressed in the LESCs-harboring basal epithelial cell layer suggesting its roles in stem cell maintenance or early differentiation. Overexpression of miR-10b in immortalized human corneal epithelial cells (HCEC) led to an increase in proliferation rate compared to the cells transfected with mimic controls, using both MTS assay and Ki-67 immunostaining. Respective inhibitor (antagomir) significantly decreased proliferation rate. In order to delineate the biological roles of the differentially expressed miR-10b in LESCs function,

mRNA-seq data of HCEC transfected with miR-10b or with mimic control were compared. Pathway analysis of the differentially expressed gene sets identified three major affected pathways (EIF2, mTOR and eIF4/p70S6K signaling) in miR-10b transfected cells, which all play roles in protein synthesis, proliferation and cell survival. In this study, we report for the first time the miRNA signatures of IDDM and NIDDM limbus and show that miR-10b could be involved in the LESCs maintenance or their early differentiation, as its overexpression in HCEC upregulated putative LESCs markers such as keratin 17. Furthermore, miR-10b upregulation may be an important mechanism of corneal diabetic alterations, especially in the IDDM group.

OGM5 - Congenital stationary night blindness from A-Z

Phenotypes in Patients with CSNB

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Congenital Stationary Night Blindness (CSNB) is a heterogeneous group of disorders both clinically and genetically. Fundus examination and electroretinography are critical for a proper diagnosis and classification. We will review distinctive phenotypic features that are subsequently important for appropriate genetic investigation and phenotype/genotype correlations.

Grm6 Missense Mutation Reduces but Does Not Eliminate mGluR6 Expression and ON Bipolar Cell Function

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Background: mGluR6, the primary glutamate receptor of ON bipolar cells, activates a G-protein signaling cascade in response to glutamate released from photoreceptors. Several mouse mutants for *Grm6* have been reported. Each model lacks mGluR6 expression and lacks the b-wave

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component of the electroretinogram (ERG) indicative of ON bipolar cell activity. Here we describe a new model (*nob8*) in which a point mutation in *Grm6* reduces but does not eliminate mGluR6 expression, and in which b-wave amplitude is reduced but not abolished.

Methods: The *nob8* model was identified from an ERG screen of inbred strains at Jackson Laboratory, and a role for *Grm6* was implicated by complementation and confirmed by sequencing. ERGs were used to characterize outer retinal function of *Grm6^{nob8}* mice. Protein expression level of mGluR6 was determined by immunoblotting. Retinal anatomy and localization of mGluR6, TRPM1, GPR179, LRIT3 and RGS7/11 were examined by immunohistochemistry. Retinal ganglion cell (RGC) activity was recorded using multi electrode array (MEA).

Results: A point mutation in *Grm6^{nob8}* mice was identified by complementation and subsequent sequence analysis. The dark- and light-adapted ERGs from *Grm6* mutant mice have a normal a-wave, while their b-wave is significantly reduced, yielding a waveform which is distinct for other *Grm6* mutants and also from other mouse models involving ON bipolar cell proteins. mGluR6 protein expression was reduced at bipolar cell dendrites of *Grm6^{nob8}* mice, while retinal anatomy remained intact. The expression of TRPM1, GPR179, LRIT3 and RGS7/11 were also decreased in *Grm6^{nob8}* ON bipolar cells dendritic terminals. MEA recording of retinal ganglion cells shows the presence of ON pathway signaling in the *Grm6^{nob8}* retina.

Conclusions: Low level expression of mGluR6 on the dendritic terminals of ON bipolar cells is sufficient to support some function of these cells. Reduced expression of other proteins involved in ON bipolar cell signal transduction indicates a key role for mGluR6 in organizing that signaling complex.

Gene Defect Identification in CSNB

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The first steps in vision occur when photoreceptors transform light into a signal, which then gets processed through the inner retina via the bipolar cells. The initial steps described by the phototransduction cascade are well understood, while the downstream transmission from photoreceptors to bipolar cells remains to be dissected in more details. Rods synapse with rod ON-bipolar cells and cones synapse with cone ON- and OFF-bipolar cells. Knowledge about the phototransduction cascade was gained by genetic studies on progressive retinal diseases, in which molecules of this

cascade are mutated. Knowledge about the downstream signaling from photoreceptors to bipolar cells was gained by genetic studies on congenital stationary night blindness (CSNB), in which molecules of this cascade are mutated. Mutations in *CACNA1F*, *CABP4* and *CACNA2D4* lead to incomplete CSNB, which represents ON- and OFF-bipolar cell dysfunction. This can be confirmed by rod and cone photoreceptor synapse immunolocalization of the respective proteins. Mutations in *NYX*, *GRM6*, *TRPM1*, *GPR179* and *LRIT3* lead to complete CSNB, which represents ON-bipolar cell dysfunction. This can be confirmed by rod and cone ON-bipolar cell immunolocalization of the respective proteins. Although gene defect identification in CSNB patients helped to add molecules to this signaling cascade from photoreceptors to bipolar cells, other molecules need to be identified to elucidate this cascade in more detail. Here we will present ongoing studies on CSNB gene defect identification, investigation of the pathogenic mechanism by in vitro and in vivo studies, which will help to better understand this disorder and retinal signaling in general.

Role of TRPM1 Channel in Retinal Circuit Development

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The vertebrate retina, a part of the central nervous system, forms complex and elaborate neural networks to process visual information. Although neural maturation and circuit formation in the brain are known to depend on the neural activity, including the neural transmission from presynaptic terminal and neural firing, contribution of neural activity to those in the retina has not been well understood. A prominent feature of the visual transduction is two retinal pathways in which neurons are excited mainly by increments (ON pathway) or decrements (OFF pathway) from the background light intensity. We previously reported that TRPM1 is the cation channel in retinal ON bipolar cells whose opening is negatively regulated by mGluR6 signaling cascade. *Trpm1*- and mGluR6-deficient mice are the congenital stationary night blindness (CSNB) model mice. In the current study, to evaluate whether neural activity is important for retinal circuit development, we analyzed three types of knockout mice, *Trpm1*-, mGluR6- and VGLUT1-deficient mice, which show the impairment of signal transduction from photoreceptors to ON bipolar cells in different mechanisms. Although these mice show

similar electrophysiological abnormalities, we found that *Trpm1*-deficient mice exhibit the impairment of retinal circuit formation. Our results suggest that neural activity of ON bipolar cells is essential for the normal retinal circuit formation.

Melanoma Associated Retinopathy: A Paraneoplastic Syndrome with CSNB-like Characteristics

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Some people with cutaneous melanoma develop visual abnormalities similar to those associated with CSNB, including poor night vision and a negative electroretinogram. This paraneoplastic syndrome, known as melanoma-associated retinopathy (MAR), is caused by an immune response to the melanoma that produces autoantibodies, which cross-react with retinal ON-bipolar cells. The primary target of MAR autoantibodies is TRPM1, a cation channel normally expressed by ON-bipolar cells and melanocytes, and a major site of mutations causing CSNB1. Case reports of MAR in the medical literature are few, suggesting that production of TRPM1 autoantibodies in melanoma is rare. We are screening sera from melanoma patients with and without reported vision problems to determine the prevalence of TRPM1 autoantibodies in melanoma, and to determine whether TRPM1 autoantibodies from different patients target the same or different epitopes. To better understand the link between TRPM1 autoantibodies and MAR, we are recording ERGs from mice immunized with a TRPM1 MAR antigen.

OGM6 - An omics perspective on pediatric eye diseases

Human Cone Precursor Circuitry Underlying Retinoblastoma Initiation

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Human cone precursor circuitry, including intrinsically high expression of MDM2, high MYCN, and low p27, has been shown to collaborate with *Rb1* mutations and loss of functional pRB to initiate retinoblastoma tumorigenesis. In contrast to human cone precursors, mouse cone precursors lack prominent MDM2 and MYCN and fail to proliferate in response to *Rb1* mutation or to pRB loss, even when MDM2 and MYCN are ectopically expressed. Here we show that maturing human but not mouse cone precursors express an elaborate proliferation-related program that extends far beyond high-level MDM2 and MYCN expression, and additionally includes prominent expression of E2F3, cyclin E, CDK2, and phosphorylated-p27(T187), which likely mediates p27 degradation, in a defined spatio-temporal pattern. To gain insight into the basis of this program, we FACS-enriched human and mouse cone precursors of comparable ages, captured single cells, synthesized cDNA on the C1 Single-Cell Auto Prep System, and sequenced single cell transcriptomes at an average depth of 2×10^6 reads. Mapping of the transcripts to the human or mouse genome identified ~ 3,000-6,000 genes per cell. Cone cell transcriptomes were identified by the expression at >0.1 FPKM of any of 11 cone-specific markers (*RxrG*, *Thrb*, *cArr*, *Opn1lw*, *Opn1sw*, *Gnat2*, *Cnga3*, *Cngb3*, *Otop3*, *Pde6c* and *Pde6h*) and the absence of 15 other cell type-specific markers (*Nrl*, *Nr2e3*, *Rho*, *Cnga1*, *Calb1*, *Calb2*, *Gad1*, *Isl1*, *Lhx1*, *Nefm*, *Tfap2b*, *Gfap*, *Hes5*, *Prkca* and *Vsx2*). In Principle Component Analysis, cone precursor cells formed a cluster distinct from the other cell types. Comparison of human and mouse cone precursor transcriptomes revealed significantly increased expression of a ribosome biogenesis signature in the human cells. These studies are expected to provide a comprehensive catalog of the gene expression and signaling pathway differences between human and mouse cone precursors and may provide opportunities to target genes or pathways that underlie human cone cell sensitivity to *Rb1* inactivation.

A Functional Omics Perspective on the Retinopathy of Prematurity

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ROP a vasoproliferative condition of eye, is a leading cause of blindness among preterm infants with low birth weight

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(BW) and gestational age (GA). The underlying genetic mechanism contributing to ROP are unclear. We aimed to identify gene(s) responsible for ROP by a functional genomics and proteomics based approach. 384 SNPs spanning 27 candidate genes were screened in a cohort of 400 preterm infants (GA \leq 35weeks and BW \leq 1700g) including 200 clinically characterized cases of ROP and 200 No-ROP preterm infants by customized genotyping. Allele and genotype frequencies, linkage disequilibrium and haplotype analysis were done to delineate the ROP-associated variants. Global gene expression profiling was performed in 15 preterms infants with different stages of ROP and 9 No-ROP and 3 full term infants with no retinal disease by using Illumina bead chip array having \sim 47,000 transcripts. The expression profiles were analyzed by the Genome Studio software and fold change was computed. Meta-analysis of differentially expressed genes were performed by Gene Ontology (GO) and pathway analysis using Panther software. The vitreous humor levels of 27 candidate proteins were assessed in patients with severe ROP (n=30) and congenital cataract (n=30) by multiplex bead arrays. Differentially expressed proteins were further validated by western blotting and zymography. Significant differences were noted in the alleles and corresponding haplotype frequencies of few SNPs in *TSPAN12*, *CFH*, *C2/BF*, *IHH* and *MMP9* between cases and controls that withstood Bonferroni correction for multiple testing ($p=1.3 \times 10^{-6}$). Other studied genes did not exhibit any association to ROP. Compared to controls, ROP patients exhibited significant ($p < 0.01$) increased vitreous levels of MMP9, CFH, C3, C4, Prealbumin, SAP, APO A1 and APOC3. These results were further validated by western blotting and zymography. A total of 142 genes were differentially expressed (115 upregulated and 27 downregulated) between the ROP patients and noROP preterm controls. Of these, 19 genes expressed differentially with >2 fold change. Major pathways identified in ROP pathogenesis using differentially expressed genes included inflammation mediated by chemokine and cytokine, endothelin and toll like receptor signaling. Significant genetic associations and differential gene expression observed in ROP suggests the possible underlying mechanisms in the development and progression of ROP.

Investigating Genetic Alterations Causal to Congenital Corneal Anesthesia Using Whole Exome Sequencing

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Congenital corneal anesthesia (CCA) is a rare eye disease where children are born with hypesthetic corneas. The condition usually presents between 8 to 12 months after birth. Early symptoms include clinical history of recurrent redness and photophobia with or without associated absence of tearing and/ or lack of response to touch sensations either localized to face or generalized to the whole body. The accurate diagnosis, recognizing risk factors and a thorough neurological assessment is of utmost importance in lessening the long-term sequel of CCA. We recruited 27 unrelated and clinically well characterized CCA families to identify causal genetic variants in CCA. One of the families was prioritized for whole exome sequencing (WES). This non-consanguineous family had a unique structure, wherein DNA was available from unaffected parents, a dizygotic twin pair with an affected and an unaffected sib and another affected sibling. In this family we have not found previously reported mutations in *NGF* and *PRDM12* genes, observed in patients with pain insensitivity. WES using Illumina HiSeq-2000 platform in these five individuals revealed 99,845 unique variants across these subjects. A total of 504 variants were further sieved based on their status in these five subjects i.e. homozygous in both affected sibs, heterozygous in both parents and not homozygous in the unaffected sib. Among them, 162 were exonic and 71 were non-synonymous DNA alterations. Global minor allele frequency (MAF) was checked for all these 71 non-synonymous variants using data from 1000 Genome Project and similar analyses in different Indian populations. A handful of these variants satisfying the criteria of $MAF < 0.01$ were checked for several mutant protein function prediction tools such as SIFT, PolyPhen2 and PROVEAN, indicating towards a possible involvement of estrogen. A number of reports have already been published on the effect of estrogen in relation to pain modulation. Therefore, we speculate that a deleterious mutation in an early estrogen-inducible gene could potentially be causal to pain insensitivity, which is

primarily observed in patients with CCA.

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Understanding the Development of Anterior Segment Anomalies from an Omics Perspective

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Anterior segment anomalies occur due to a variety of reasons mediated through a complex cascade of activities involving the genome, transcriptome, proteome, metabolome and their corresponding interactions. These spectrum of diseases, also referred to as anterior segment dysgenesis (ASD) involves the malformations in the anterior segment of the eye and may be associated with various forms of developmental glaucomas (congenital glaucomas, aniridia, Axenfeld syndrome and Peter's anomaly etc.). They usually have an early onset and the implementation of prevention initiatives are hampered by the limited understanding of the molecular basis of these diseases. Genetic heterogeneity is well documented and >27 candidate genes, largely comprising of transcription factors have been characterized to harbor mutations across the spectrum of ASDs. Interestingly, the vast majority of patients (>80%) do not harbor mutations in the known genes. Further, the locus and allelic heterogeneity across the entire spectrum of ASDs including developmental glaucomas require a complete identification of the genes and pathways involved in their pathogenesis. It is in this background that we studied the whole repertoire of ASD and developmental glaucoma-related genes in a large cohort of congenital glaucoma, who are devoid of mutation in the known genes (*CYP1B1*, *LTBP2*, *MYOC* and *FOXC1*). Whole exome sequencing (WES) in such cases, using trios, on an Ion Proton platform (Life Tech, CA), led to the identification of ~40 novel genes, that either share known glaucomatous loci, developmental genes, transcription factors implicated earlier in anterior segment anomalies in an animal model and genes involved in autophagy and neurodegeneration. A customized panel including these novel and other ASD-associated genes are currently being screened in additional cohorts of ASD patients along with their expression profiles in the relevant tissues of the anterior segment. These are also being functionally characterized in zebrafish knockdown morpholinos to study gene-gene interactions. Genotype-phenotype correlations based on a regression model (R statistics)

using these disease implicated gene variants along with the quantitative clinical traits at presentation and progression would further help in delineating their precise role in the development of ASDs.

Understanding the Pathogenesis of Developmental Eye Diseases Using Animal Models

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Almost every cell type in our body communicates with the external environment via antennae-like protrusions of the cell membrane, termed primary or sensory cilia. Dysfunction of cilia results in severe developmental and degenerative diseases, collectively termed ciliopathies. Visual impairment at an early age due to defects in the anterior or posterior segment of the eye is commonly observed in ciliopathies. Leber congenital amaurosis (LCA) and early onset Retinitis Pigmentosa (RP) are probably the most common forms of inherited retinal ciliopathies. Here, I will discuss the use of mouse and zebrafish as tools to assess the pathogenesis of retinal ciliopathies in one form of LCA, LCA10 [caused by mutations in the *CEP290* (centrosomal protein of 290 kDa) gene] and two forms of X-linked RP, due to mutations in *RPGR* (RP GTPase regulator) and *RP2*. Our results have revealed crucial information on the variable effects of the complete or partial loss of gene function on ciliary function in the retina. As ciliary function is also critical in the anterior segment of the eye, our studies are expected to unravel common pathogenic mechanisms for selected anterior segment disorders. Lastly, we will discuss the importance and caution associated with the use of zebrafish as a tool for assessing the pathogenesis of retinal degenerative diseases.

OGM7 - Genetics of corneal dystrophies

Mutations in *COL17A1* Cause ERED in the Swedish Population

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Corneal dystrophies represent clinically and genetically heterogeneous group of inherited disorders that bilaterally affect corneal transparency. They are defined according to the affected corneal layer and by their genetic cause. We identified a dominantly inherited epithelial recurrent erosion dystrophy (ERED)-like disease that is common in northern Sweden. The disease is characterized by three phases: recurrent erosions during childhood, alleviated symptoms in the teens and decreased visual acuity after the 40s. Haplotype analysis and whole exome sequencing (WES) on DNA from affected and unaffected family members revealed a novel mutation, c.2816C>T, p.T939I, in the *COL17A1* gene coding for collagen type XVII alpha 1. It appeared to be a founder mutation that segregates with the disease in a genealogically expanded pedigree dating back 200 years to a common ancestor Theodor. Bi-allelic *COL17A1* mutations have previously been associated only with a recessive skin disorder, junctional epidermolysis bullosa, with recurrent corneal erosions reported in some cases. To confirm potential causative role of *COL17A1* in Swedish patients we investigated a unique *COL17A1* synonymous variant, c.3156C>T, previously reported by Sullivan *et al.* (2003) in unrelated dominant ERED-like family linked to 10q23-q24 region encompassing *COL17A1*. We experimentally confirmed that this variant leads to aberrant pre-mRNA splicing of the *COL17A1* transcript *in vitro*, and therefore, we suggest that both dominantly inherited ERED-like corneal dystrophies are allelic and attributed to mutations in *COL17A1*. Our findings improve understanding of the underlying pathology of corneal dystrophies.

Elucidating the Genetic Basis of Posterior Polymorphous Corneal Dystrophy

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Posterior polymorphous corneal dystrophy (PPCD) has been mapped to two different chromosomal loci: chromosome 20 (known as the PPCD1 locus) and chromosome 10 (known as the PPCD3 locus). While the genetic basis of PPCD1 has just recently been identified, PPCD3 was associated over a decade ago with mutations in the zinc finger E-box binding homeobox 1 (*ZEB1*) gene. Four different promoter region mutations in the ovo-like 2 (*OVOL2*) gene have been identified in each of the families previously mapped to the PPCD1 locus. However, the absence of *OVOL2* coding and promoter region mutations in a large number of other families with PPCD in whom a *ZEB1* coding region mutation has not been identified, and in

whom copy number variation in the PPCD1 and PPCD3 loci has been excluded, raises the possibility of another PPCD locus.

ZEB1 encodes a two-handed zinc finger homeodomain transcription factor that is known to repress gene expression via binding to promoter region E2 box motifs, and has been implicated in regulating the epithelial-to-mesenchymal transition (EMT) pathway. To date, each of the approximately 35 frameshift and nonsense *ZEB1* mutations is predicted to result in the truncation of the encoded protein, with subsequent nonsense-mediated mRNA decay or protein dysfunction. While some *ZEB1* truncating mutations have been shown to result in impaired nuclear localization and/or a significant decrease in *ZEB1* protein production, others have been shown to result in the loss of E2 box binding ability, thereby losing the ability to repress transcription of known *ZEB1* target genes.

In summary, *ZEB1* haploinsufficiency is hypothesized to be the cause of PPCD3, with subsequent altered corneal endothelial expression of genes regulated by *ZEB1*. One such gene implicated in the pathogenesis of PPCD is collagen, type IV, alpha3 (*COL4A3*), expression of which is negatively regulated by *ZEB1* binding to E2 box motifs in the *COL4A3* promoter region. Although the mechanisms via which *ZEB1* truncating mutations lead to PPCD3 are unlikely to involve just one gene downstream of *ZEB1*, i.e. *COL4A3*, the demonstration of the ability of *ZEB1* to directly regulate *COL4A3* expression provides the basis for investigation into the ability of *ZEB1* to regulate the expression of other type IV collagens and genes involved in cell adhesion to gain further insight into the role of *ZEB1* in corneal endothelial cell function and dysfunction.

Genomewide Association Analysis Identifies Novel Genetic Loci for Fuchs Endothelial Corneal Dystrophy

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Purpose: Fuchs endothelial corneal dystrophy (FECD) is the most common heritable indication for corneal transplantation in the United States. To identify novel genetic determinants of FECD and explore their potential role in disease etiology, we conducted a genomewide association study followed by replication and meta-analysis.

Methods: We carried out association analysis on a sample of 1404 FECD cases and 2564 controls of European ancestry, by means of logistic regression, using common genetic variants from the Illumina HumanOmni2.5 chip, with imputation of untyped variants from the 1000 Genomes Phase I panel (total of 8,680,745 markers). The association model included adjustments for age, sex and three principal components (to correct for differences in population structure between cases and controls). We genotyped strongly associated variants for replication on a total of 671 FECD cases and 778 controls, and tested for differences in allele frequency by Fisher's exact test. Immunohistochemical analysis was conducted by standard methods.

Results: We identified six strongly associated loci from the initial genomewide scan, and advanced 18 genetic markers from these loci for replication. Association signals in the well-characterized *TCF4* gene and at three new loci, *KANK4*, *ATP1B1* and *LAMC1*, were successfully replicated with a significance level in meta-analysis of $p < 10^{-13}$. These genes are expressed in the corneal endothelium in both affected and normal corneas. Two loci, *LAMC1* and *TCF4*, have sex-dependent effects of FECD risk.

Discussion: We have discovered candidate loci for FECD likely to have essential roles in the corneal endothelium, including water transport, basement membrane maintenance and tissue integrity.

Conclusion: This work supports a multifactorial model for FECD, in which the common theme is the preservation of a barrier to fluid entry into the corneal stroma. Discovery of these novel loci ties many of the attributes of FECD into a comprehensive disease model, with the potential to develop new therapies or preventive measures for this disabling disease.

The Use of CRISPR-Cas9 to Treat Corneal Dystrophy

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Corneal dystrophies are a group of clinically and genetically heterogeneous disorders that cause corneal opacity with impaired vision and painful recurrent erosions. The majority of anterior corneal dystrophies are caused by missense mutations following an autosomal dominant mode of inheritance with accumulation of abnormal protein as the cause of the disease. Genome editing has unprecedented potential for permanent treatment of these genetic diseases. The potency

and specificity of this system has been used to target dominantly inherited conditions caused by heterozygous missense mutations through inclusion of the mutated base in the short-guide RNA (sgRNA) sequence. It can be used to repair an error through the homology directed repair (HDR) pathway or to knockout or damage a mutated allele using the non-homologous end joining (NHEJ) approach. The later being the more common event in cells and a relatively simple and straightforward approach to therapy. We determined that a mutation within *KRT12*, which causes Meesmann's epithelial corneal dystrophy (MECD), leads to the occurrence of a novel protospacer adjacent motif (PAM). We designed an sgRNA complementary to the sequence adjacent to this SNP-derived PAM and evaluated its potency and allele specificity both *in vitro* and *in vivo*. This sgRNA was found to be highly effective at reducing the expression of mutant *KRT12* mRNA and protein *in vitro*. This study highlights the potential for CRISPR/Cas9-based therapeutics for corneal dystrophies.

OGM9 - Genomics of ophthalmic diseases

Profiling of Epigenetic Landscape Changes Reveals Gene Mis-regulation Mechanisms in Mouse Models of CRX-linked Retinopathies

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Mutations in the transcription factor CRX disrupt normal photoreceptor development and survival, causing retinopathies. Genome-wide gene expression studies in mouse models of CRX disease provided insight into CRX target genes, but did not address the molecular mechanism underlying their misregulation. This study was designed to close this gap by investigating the role of CRX in modulating chromatin structure, particularly the gene activation-associated Histone H3 modifications Lysine 4 tri-methylation (H3K4me3) and Lysine 27 acetylation (H3K27Ac). Chromatin immunoprecipitation-sequencing (ChIPseq) was performed for these histone marks in the retinas of wild-type and *Crx* deficient mice before (at P2) and after (at P14) photoreceptor differentiation. The results were analyzed alongside published RNAseq, ChIPseq, and DNaseI hypersensitivity datasets to identify pathogenic epigenetic alterations that correlate with transcription misregulation. During postnatal photoreceptor differentiation, only a subset of CRX-bound target genes showed increases in H3K4me3 and/or H3K27Ac marks together with 'opening' of chromatin (increasing DNaseI

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hypersensitivity), correlating with transcription activation. The DNA in the promoter of these CRX target genes shows significant enrichment for CRX consensus motifs and strong CRX binding. The genes in this set encode cell type-specific proteins essential for photoreceptor structure, function and survival. Importantly, their dynamic chromatin remodeling failed to occur in *Crx* deficient retinas, coinciding with defective transcription. Thus, these genes represent CRX-dependent, differentiation-inducible genes, supporting our hypothesis that graded changes of their expression determine phenotype severity of CRX disease. This study demonstrates the power of functional genomics in understanding mechanisms underlying genetic disease pathogenesis. The findings support CRX's role in dynamic epigenetic remodeling of target genes that are essential for photoreceptor cell identity and function.

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Epigenetic Changes Associated with Age-related Macular Degeneration

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Age-related macular degeneration (AMD) is the leading cause of severe vision loss in people over 60. Genetic association studies have successfully identified genetic variants associated with the disease. However, the genetic variants only explain 40-70% of disease variability, suggesting that factors other than the genetics might contribute to the disease process. Here we attempted to identify the epigenetic factors associated with AMD. We performed epigenetic profiling on retina and RPE layers from postmortem eyes. By comparing the profiles between AMD and normal eyes, we discovered massive and consistent changes in chromatin structures in both retina and RPE. Some AMD susceptibility genes such as *HTRA1* were found to have significant chromatin changes. The epigenetic changes were related to the changes in transcription factor binding in the genome and the alterations in gene expression levels. Our study is the first systematic assessment of epigenetic changes associated with AMD and suggests that epigenetics might play a critical role in AMD.

Epigenetic Regulation of Retinal Development

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During retinal neuron differentiation, expression of cell-type specific inducers must be strictly controlled. Some adult-onset disorders may be linked to dysregulated embryonic gene silencing, yet the mechanisms underlying this association remain poorly understood. We found that embryonic deletion of the histone methyltransferase (HMT) *Ezh2* alone from all retinal progenitors resulted in progressive but selective photoreceptor degeneration throughout postnatal life, via derepression of fetal expression of *Six1* and its targets. Forced expression of *Six1* in the postnatal retina was sufficient to induce photoreceptor degeneration. In contrast, embryonic deletion of the HMT *G9a* alone showed no phenotype; rather, simultaneous deletion of *Ezh2* and *G9a* in Math5-specific cells, primarily retinal ganglion cells (RGCs), caused RGC gene dysregulation and degeneration. *Ezh2* and *G9a*, although enriched in the embryonic retina and RGCs, are not present in the mature retina. Together, these data reveal a complementary epigenetic mechanism that mediates cell type-specific gene silencing and retinal neuron homeostasis. The results suggest novel targets for retinal neurodegeneration therapy.

Altering ocular Disease Phenotypes by RNA Manipulation Approaches

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Although there are a number of potential approaches to treat inherited genetic defects such as gene therapy, stem cell replacement and electronic retinal implants, these are still associated with a number of problems and have not yet reached the clinical setting. RNA therapy is a novel and attractive option as it avoids the problems associated

with standard gene therapy such as poor control of gene expression and the potential for damaging the genome (i.e. cancer). Using this approach we have established RNA suppression therapy for a variety of ocular diseases (e.g., choroideremia, ocular coloboma, aniridia, Leber congenital amaurosis, retinitis pigmentosa). We are now evaluating SMaRT technology (spliceosome-mediated RNA *trans*-splicing) to replace damaged RNA with an artificially engineered molecule to produce normal protein and thereby inhibit disease progression. RNA therapies have the advantage of maintaining endogenous gene regulation and elimination of ectopic gene expression. To test this approach we are applying SMaRT therapy delivered to mouse models of RP (*Pde6b*) and aniridia (*Pax6*). RNA *trans*-splicing modules (RTMs) were designed to intron 1 of mouse *Pde6b* and *Pax6* as this will capture any mutation downstream of exon 1. The RTM consists of (i) a binding domain, that is complementary to 150 nucleotides of pre-mRNA intron 1; (ii) an intron sequence containing a branch point, a polypyrimidine tract and a 3' acceptor splice site; (iii) replacement exons of the *Pde6b* or *Pax6* cDNA without the known mouse mutations and a FLAG tag, cloned into an expression vector. Efficiency of each RTM was tested in mouse fibroblast cell lines expressing the mutant gene sequence. For *in vivo* studies, the RTM is complexed with PVBLG-8, a helical cationic polypeptide, and injected into the *Pde6b* mouse subretinal space or applied to the cornea in *Pax6* mice. Efficacy is tested by histology, electroretinography and optokinetic tracking. Initial results in cell culture suggest that an oligonucleotide is required to block the normal *cis*-splicing to improve efficiency of *trans*-splicing. Our experience with this approach in the two different mouse models will assess the potential of this new therapy for inherited eye diseases.

OGM10 - Genetics of Retinal Degenerations

Discovery of the Underlying Cause of Inherited Retinal Degeneration by Ext Generation Sequencing: Challenges and Opportunities

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To date, mutations in more than 250 genes affecting diverse biochemical pathways have been implicated in causing inherited retinal dystrophies (IRD); nonetheless, mutations in these genes only account for about 60% of the total genetic load of retinal dystrophies. A major proportion of patients with recessive IRD are by large from small pedigrees or are simplex cases. It is challenging to

identify the genetic basis of disease in these patients when mutations are not found in any of the known 250 IRD genes. Next generation sequencing (NGS) methodologies afford an opportunity to generate a comprehensive catalog of rare and novel variants in IRD patients to validate and establish the underlying cause of these diseases. We carried out the analysis of 80 pedigrees with IRD using NGS and established the underlying cause of disease in 48 pedigrees by finding novel and known mutations in genes associated with IRD while the rare variants in novel genes may explain the missing heritability in the remaining 22 pedigrees. Further evaluation of these pedigrees using homozygosity mapping and comprehensive analysis of sequence variants lead to the identification of large sequence re-arrangements in known and novel genes or potentially pathogenic single nucleotide variants in novel genes as the underlying cause of IRD. Experimental evaluation of the candidate variants is needed to establish the underlying cause of IRD in these pedigrees.

Simple and Complex ABCA4 Disease

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The *ABCA4* gene was cloned in 1997 as the causal gene for autosomal recessive Stargardt disease (STGD1). STGD1 usually presents as a juvenile-onset macular dystrophy associated with rapid central visual impairment and the frequent appearance of yellowish flecks, defined as lipofuscin deposits, around the macula and/or in the central and near-peripheral areas of the retina. Subsequently, *ABCA4* mutations were found to co-segregate with retinal dystrophies of substantially different phenotypes, such as autosomal recessive cone-rod dystrophy (arCRD) and atypical autosomal recessive retinitis pigmentosa (arRP, RP19) so, instead of using the term 'Stargardt disease', we now refer to all phenotypes caused by *ABCA4* mutations as '*ABCA4* disease'. Clinical heterogeneity of *ABCA4*-associated phenotypes further complicates the assessment of underlying genetic determinants for variable disease expression. Currently over 1000 disease-associated *ABCA4* variants have been identified, and the finding that 5% (1:20) of the general population carry a disease-associated *ABCA4* allele has enormous implications for the amount of retinal pathology attributable to *ABCA4* variation.

Genetic analyses of *ABCA4*-associated retinal disease have been substantially advanced in recent years. New methods, such as direct sequencing of the entire genomic *ABCA4* locus, have allowed detecting up to 80% of the

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disease-associated *ABCA4* alleles, including 2 (both) mutations in ~65-75% of patients. Of these 75% are in the coding region and 25% in introns, more than half of which are outside of splice consensus sequences, suggesting that many (rare) disease-associated *ABCA4* alleles are yet to be identified and, most importantly, unequivocally confirmed by adequate functional analyses.

Important advances have also occurred in clinical description of *ABCA4* disease which have become possible due to vast improvement in imaging methods, such as OCT, autofluorescence (AF), including quantitative AF, and adaptive optics. As a result, *ABCA4* diseases have been better categorized and disease progression quantitatively measured.

The presentation will summarize most recent genetic and clinical knowledge of *ABCA4* disease and will suggest that a combination of advanced genetic screening coupled with advanced functional analyses of *ABCA4* alleles from both coding and non-coding sequences is necessary to unequivocally determine the *ABCA4*-associated disease load.

Mutations in *CEP78* Define a New Ciliopathy Characterized by Cone-rod Dystrophy and Hearing Loss

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Following the screening by whole-exome sequencing of a cohort of patients with retinal degenerations, we identified biallelic mutations in the orphan gene *CEP78* in three patients, all suffering from cone-rod degeneration and hearing loss. The first patient, a singleton, was a homozygote for the IVS3+1G>T (c.499+1G>T) mutation in intron 3, whereas the two siblings were compound heterozygotes for the nearby mutation IVS3+5G>A (c.499+5G>A) and for the frameshift-causing variant c.633delC; p.Trp212Glyfs*18. Immunostaining revealed the presence of *CEP78* in the inner segments of retinal photoreceptors, predominantly of cones, and at the base of the primary cilium of fibroblasts. Analysis of skin fibroblasts derived from patients revealed abnormal ciliary morphology, compared to control cells. Altogether, our data indicate that mutations in *CEP78* cause a novel clinical entity of ciliary nature, characterized by blindness and deafness but clearly distinct from Usher syndrome, a condition for which visual impairment is due to retinitis pigmentosa.

Genetic Modifiers Interact with *Crb1* to Cause Neovascularization in the Posterior Eye

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Aberrant neovascular growth is a serious complication in many eye diseases causing significant vision impairment and blindness. The diverse spatial and temporal microenvironments in which neovascular factors act result in different forms of neovascularization (NV) and in variable responses to treatments. A better understanding of the origin, timing, molecules and pathways that are activated or suppressed by different neovascular perturbations and their modifiers is critical for identifying the best druggable targets.

Through ocular screens, we have identified mouse models that develop vascular lesions: *Tvrm278*, *Tvrm296* and *rnv3*. In all models, fundus examinations show areas of depigmentation and in some cases, fluorescein leakage from vessels. Hematoxylin and eosin stained slides show blood vessels extending throughout the outer retina. *Tvrm278* and *Tvrm296* were identified in a sensitized chemical mutagenesis screen on a genetic background that includes the *Crb1^{rdB}* mutation. We discovered that *Tvrm278* was caused by a mutation in *Vldlr*, a gene previously implicated in a recessive retinal NV. *Vldlr^{Tvrm278/+}* acts dominantly to induce NV only in the presence of homozygous *Crb1^{rdB}*. Linkage crosses to identify the molecular basis for *Tvrm296* are underway. Linkage crosses indicated that the *rnv3* mutation mapped to Chr 1. Examination of sequences within the critical region revealed a mutation in the *Crb1* gene, the same mutation reported previously for *Crb1^{rdB}*. The *Crb1^{rdB}* defect is, however, insufficient to independently cause NV, as B6/J or B6/NJ strains homozygous for *Crb1^{rdB}* rarely exhibit NV. On the other hand, *Crb1^{rdB}* is necessary for NV, as mice bearing the corrected allele, *Crb1^{corrdb}* in the *rnv3/rnv3* genetic background showed a 90% reduction in neovascular lesions.

Our studies indicate that in the models described, *Crb1^{rdB}* produces a permissive environment that stimulates NV disease development and that the genetic architecture underlying the NV disease is complex, with contributions from multiple players. Identification of the neovascular modifiers may serve as targets for development of therapeutics that can act to delay or ameliorate NV.

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Gene Therapy Rescues Despite Late-stage, Low-efficiency Treatments

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Retinitis pigmentosa (RP) is a progressive neurodegenerative disease and the most common cause of inherited blindness. Most often, RP results from mutations in rod-specific genes, which trigger the cell-autonomous loss of rods that, in turn, cause the non-cell-autonomous loss of cones. Gene therapy is the most promising treatment for RP and other inherited retinal degenerative diseases. In treated RP retinas, the therapeutic gene transduces only a fraction of the target diseased rods - thereby creating a mosaic of untreated and treated mutant rods, as well as cones. In this study, we created two novel RP mouse models to test whether untreated dying rods trigger non-cell autonomous death of rescued rods. The novelty of these mice was that the rescued rods were spatially or numerically controlled. In both models, we demonstrated that rescued rods are not susceptible to cell death triggers from neighboring mutant rods - even in retinas with low-efficiency rescue. This study suggests that monogenic gene therapy can achieve long-term efficacy in RP retinas, even in an overwhelmingly mutant environment.

Identification and Functional Analysis for Novel Gene Mutation Responsible for Autosomal Dominant Macular Dystrophy with Dysfunction of ON-type Bipolar Cells

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Purpose: Macular dystrophy is a hereditary retinal disease, which progress to dysfunction and degeneration of central region of the retina leading to loss of central visual acuity. This study resulted with identification of a mutation in novel gene with autosomal dominant macular dystrophy. Characterization of this novel gene mutation shows novel phenotype of bipolar cells dysfunction leading to photoreceptor degeneration in the macula.

Methods: Genomic DNA was isolated from three generation family and whole exome analysis was performed. An identified candidate *LRRTM* gene was analyzed for retinal expression by western blotting and retinal localization by immunohistochemistry in mouse and monkey retina. Comprehensive ophthalmological examinations including electroretinogram (ERG) were performed from each patient. The *Lrrtm* mutation knock-in mice were generated using CRISPR/Cas9 system and observed the immunostained retina by a inflammation marker, GFAP.

Results: A mutation in novel *LRRTM* gene was identified. *LRRTM* gene is known to code synaptic adhesion molecule and functionally involved in synaptogenesis. Significant increase of the LRRTM protein was observed during P7 to P21 in mouse retina, which overlaps with period of retinal synaptogenesis progression. The LRRTM was localized in the synapses between outer nuclear layer and outer plexiform layer where secondary neurons such as bipolar cells and horizontal cells are located. Further characterization revealed the LRRTM localization in the synapses of ON-type bipolar cell, a secondary neuron connected to rod-photoreceptors. Further examination of affected patients with full-field ERG showed significant decrease of signal from ON-type bipolar cells. The mutation knock-in mice displayed increase of GFAP positive Müller glial cells in comparison with the wildtype at postnatal 7 months, suggesting that the LRRTM mutant caused to retinal dysfunction leading to mildly progressive degeneration of the retina.

Conclusions: We identified a mutation in a novel *LRRTM* gene in Japanese family with autosomal dominant macular dystrophy. Furthermore, we demonstrate that LRRTM protein is specifically localized in ON-type rod bipolar postsynapses and the LRRTM mutant caused to increase of GFAP positive Müller glia in the mutation knock-in mice. The novel phenotype of macular dystrophy with dysfunction of ON-type bipolar cells by the mutation suggests possibility of unique population of macular dystrophy in Asian population.

OGM11 - Genetics of myopia

Genome Cohort Study and Association Study for Axial Length and Refractive Error in Japanese

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Purpose: A prospective cohort study design permits extensive and unbiased characterization of environmental

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exposures. To describe the design of population-based genome cohort study and the distribution and determinants of ocular biometric parameters in the north part of Japanese population.

Material and methods: We set 7 Community Assessment Centers in Miyagi prefecture. Participants were voluntarily recruited from the population older than 20 years. Each subject underwent a screening examination comprised of an interview and ophthalmic examinations, including measurement of axial length (AL), corneal curvature in 2 meridians (K1, K2), spherical equivalent refraction (SER), and fundus photography.

Results: 24,806 participants were recruited in the study. In the right eye, mean AL was 23.98 mm (95% confidence interval [CI], 23.95-24.00) in women and 24.54 mm (CI, 24.50-24.57) in men. In the left eye, mean AL was 23.94 mm (CI, 23.91-23.97) in women and 24.50 mm (CI, 24.46-24.53) in men. There were statistical differences between women and men, and right and left eyes ($p < 0.0001$). From age 40 years or older, a mean reduction in AL with age was observed. We also measured corneal curvature and SER in 7,406 participants. As for the mean K1 of corneal curvature and SER, there were statistical differences between women and men, and right and left eyes ($p < 0.0001$). The SER was negatively correlated with AL.

Conclusions: Our findings serve as an important AL populational reference for multiple purposes for ophthalmic examination. We will also report the latest progress of the association study using genotypes by DNA microarray in this session.

Genetics of Myopia Endophenotypes

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Myopia is highly prevalent worldwide, in particular in East Asia. Epidemiological, clinical, and laboratory research has suggested that the development of myopia is influenced by both environmental and genetic factors, as well as the interaction between them. Recent genome-wide association studies (GWAS) have implicated more than 25 different genetic loci for refractive error and myopia. Many of the genes in these loci are involved in biological pathways known to mediate extracellular matrix composition and regulate connective tissue remodeling. To date, experimental and observational studies have suggested the complex nature of refractive error, including its endophenotypes such as corneal curvature and axial length, in humans. GWAS and other next-generation

genomic technologies have accelerated the discovery of genes contributing to common human myopia. I will present an overview of the genes and genomic regions contributing to the endophenotypes of myopia, and introduce recent research findings from the international Consortium of Refractive Error and Myopia (CREAM).

Trajectories of Future Spherical Equivalent Refraction in Children Using the Longitudinal Data from the Guangzhou Twins Eye

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Objective: The prediction of Spherical Equivalent (SE) Refraction development plays an important role in myopic prevention. In this paper, we characterize individual and sample average growth curves based on longitudinal data and further determine the best set of predictors for Spherical Equivalent (SE) in school-aged children effect from Zhongshan Ophthalmic Center, Sun Yat-sen University.

Methods: The first-born twins ($n=1100$) in Guangzhou Twin Eye Study with at least three annual visit data (baseline age 7-15 years) were used to empirically identify the SE trajectories by a group-based trajectory modelling (GBTM). SE was calculated as the sum of sphere and 1/2 cylinder. The trajectory model was estimated using the user-written program "traj" for STATA. We also evaluate whether indoor reading time, outdoor activity, and 39 candidate SNPs identified from the Consortium for Refractive Error and Myopia (CREAM) affect the SE trajectory.

Results: A 4-class model was selected as the best model on the basis of empirical fit indices ($n=1110$, Bayesian information criterion=-13010.98; Akaike information criterion=12973.39; Maximum Likelihood=-12973.39). The average group posterior probability (AvePP) is 0.971, which is far greater than the recommended value of 0.7. Odd of correct classification (OCC) is 999, which is greater than the recommended of 5 as a general guideline for GBTM. We identified four developmental subtypes of SE trajectories: a "stable" trajectory (36.4%), a "mild declining" (39.4%), a "moderate declining" (22.0%), and a "sharp declining" (2.2%). The sharp declining group have the highest probability to develop myopia and high myopia. Susceptible SNPs do not provide further improvement on the SE trajectories.

Conclusion: This paper highlights the potential importance

of techniques such as GBTM in distinguishing the different response trajectories for SE. The study revealed an additive effect on SE over time.

Keywords: Spherical Equivalent (SE), prediction, group-based trajectory modelling

Genetic and Environmental Influences on Myopia: An Australian Perspective

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In East Asia, there is widespread alarm at myopia rates over 80% in students finishing high school. The prevalence of myopia is increasing in Europe and North America, but there is little evidence of increasing prevalence in Australia. Recently, we set up a study to investigate whether any recent generational change has occurred in the prevalence of myopia among Western Australians. This study also aims to elucidate genetic and environmental determinants of myopia and associated biometry by studying parents-children and sibling pairs. The Raine Study comprises a prospective longitudinal cohort of the offspring born to mothers (n=2900) recruited at 14-18 weeks' pregnancy between 1989-1992, in Perth, Western Australia. During the 20-year eye follow-up, 1344 young adults underwent a comprehensive eye examination. Currently, parents of these individuals are undergoing a condensed eye examination with a focus on myopia and sun-protection behaviors. To date, we have assessed 165 mothers and 105 fathers. We excluded 19 parents who had cataract or refractive surgery and selected 48 Caucasian parents-child trios from the remaining dataset. The mean age \pm SD of mothers, fathers and offspring were 56.21 \pm 4.40 years, 58.33 \pm 5.03 years and 20.13 \pm 0.26 years, respectively. The mean spherical equivalent refraction was -0.39 \pm 1.88 in fathers, -0.07 \pm 1.91 D in mothers and +0.72 \pm 1.28D in the offspring. Forty-two percent of mothers and 50 % of fathers had a university or higher degree. Seventy-nine percent of young adults were continuing on their higher education. The median area of conjunctival UV autofluorescence (CUVAF, a biomarker of sun exposure) was 14.2 mm² in both mothers and fathers while it was as high as 49.2mm² in now young adult children. Parents in this study had more myopia than their contemporaries in other Australian studies. Their now young adult children may become more myopic as they age. While an increase in higher education in this new generation is expected to make the myopic shift larger, it is anticipated that the greater time spent outdoors will cancel out this effect, producing a similar myopia prevalence to their

parents' generation. These preliminary findings suggest the outdoor culture in Australia outweighs the effect of higher education and protects Caucasian individuals from becoming more myopic. Further studies need to be carried out in order to explore the effect of this interaction in other individuals within multicultural Australia.

OGM12 - Current concept in genetics of hereditary ocular developmental anomalies

Novel Factors in Human Ocular Disease

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There is marked genetic heterogeneity in diseases affecting the eye that lead to anterior segment abnormalities and glaucoma, congenital cataracts and microphthalmia/anophthalmia. Next-generation sequencing (NGS) strategies including targeted, whole exome (WES) and whole genome sequencing (WGS) provide unparalleled opportunities for novel variant and disease gene discovery. Use of these strategies provide detection rates in known disease genes in the vicinity of

20 - 70%, depending on the disease grouping under investigation. For novel disease gene identifications, we have developed a discovery and functional genomics pipeline aimed towards improved diagnosis and treatment for patients affected with these disorders. Using genomic approaches and a bioinformatics strategy combining genotype and phenotype information in families and probands, we have identified several novel candidate disease-causing genes encoding proteins with various roles including in signalling pathways, and in cell adhesion and cytoskeletal integrity. We are investigating these proteins using a functional genomics approach including: expression analyses, cell-based assays using shRNA and predicted pathogenic variant transfection, and investigation in zebrafish and mouse models. Using our combined gene discovery and functional genomics approach, we identified *SIPA1L3* as a novel human disease gene leading to congenital cataracts, anterior segment abnormalities and microphthalmia. *SIPA1L3* has a role in the Rap1 signalling pathway. In our *Sipa1l3* knockout mouse model we showed there was abnormal E-cadherin, F-actin and ZO1 staining in anterior epithelial cells of the lens, indicating abnormalities of adhesion and polarity. There was also aberrant α SMA expression indicating a critical role for proper regulation of Rap1 signalling for prevention of epithelial to mesenchymal transition in the lens. Our cell-based assays in conjunction

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with our mouse model work, provide functional validation of our candidate disease-causing genes. This leads to direct translation to improved diagnostic genetic testing for patients. We are also developing relevant cell-based assays from human induced pluripotent stem cells (hiPSCs) and CRISPR-engineered cell lines and mouse models, to provide tractable model systems where treatment approaches can be assessed using genetic, cell-based or pharmacological means.

Non-coding *cis*-acting Defects in Retinal Dystrophies: From Locus Resequencing to Interpretation

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Over 200 disease genes have been implicated in retinal dystrophies (RDs), accounting for 50%-80% of cases. Although most mutations underlying RDs are located in coding regions, part of the missing genetic variation in RD is located in non-coding regions of the genome such as untranslated regions (UTRs), promoters, deep intronic regions influencing *cis*-acting splicing, or in more distant regulatory regions such as enhancers. Here, several types of *cis*-acting non-coding variation in RD, locus-specific approaches for their identification, and tools for their interpretation are discussed.

A first example is *NMNAT1*-associated Leber congenital amaurosis (LCA9), characterized by a typical macular phenotype. In patients with this phenotype and without coding mutations, we identified 5'UTR mutations at adjacent positions c.-70 and c.-69, with loss of heterozygosity, decreased expression of the mutant transcript, and with decreased activity of the mutations.

A second model of non-coding variation is autosomal recessive Stargardt disease in which *cis*-acting deep intronic mutations have been described. In 66 typical Belgian Stargardt patients with one coding *ABCA4* mutation, targeted resequencing of the *ABCA4* gene and its *cis*-regulatory region revealed unique deep intronic variants, a putative regulatory 5'UTR variant and a previously described deep intronic variant c.4539+2001G>A (known as V4) in several patients. For variant interpretation, data

were integrated with *cis*-regulatory datasets generated by ATAC-seq, CHIP-seq, 4C-seq and RNA-seq in adult human retina.

A third example is non-coding structural variation disrupting *cis*-regulatory elements. A homozygous deletion upstream of *EYS* was found in a patient with RD and characterized by Targeted Locus Amplification (TLA), revealing the exact nature and complexity of the structural variation.

In conclusion, *cis*-acting non-coding variation explains a portion of hidden genetic variation in RD. Despite the wide availability of genome-wide approaches, locus-specific strategies are useful in models of non-coding variation such as autosomal recessive RD with strong genotype-phenotype correlations or with a mono-allelic mutation in an RD gene. Finally, *cis*-regulatory datasets generated in human retinal tissues are important for the interpretation of non-coding variants in RDs.

Conserved Genetic Pathways Associated with Microphthalmia, Anophthalmia and Coloboma

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Anophthalmia, microphthalmia and coloboma (MAC) spectrum is characterized by severe congenital ocular defects affecting multiple structures. The overall incidence of MAC has been estimated as 6-13 per 100,000 births, with microphthalmia being most common (2-17) and followed by coloboma (2-14) and anophthalmia (0.6-4.2). MAC conditions are often (in approximately 1/3 of cases) associated with additional non-ocular anomalies, indicating the involvement of the responsible genes in multiple developmental processes. MAC is a highly heterogeneous disorder with *SOX2* and *FOXE3* playing major roles in dominant and recessive pedigrees, respectively; however, the majority of cases lack a genetic etiology. We analyzed ~ 40 MAC patients (without mutations in *SOX2/FOXE3*) by whole-exome sequencing. Analysis of known ocular genes identified novel pathogenic variants in several pedigrees (including mutations in *PAX6*, *OTX2*, *NDP*, *COL4A1*). Further analysis of the remaining samples identified a novel MAC gene, *MAB21L2*, and several additional candidate factors. Studies of the orthologous genes in zebrafish provide support for the involvement of the identified factors in embryonic phenotypes and vertebrate eye development. The generated zebrafish models allow for further investigation of the mechanisms of MAC and integration of various identified genes into common developmental pathways.

Leber's Congenital Amaurosis by *CC2* Compound Heterozygous Mutation and its Phenotypic Analysis in Zebrafish

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Leber's Congenital Amaurosis (LCA) is a hereditary, early-onset severe retinal dystrophy, whose pathoetiology remains unclear. The whole-exome sequencing analysis on a single Chinese LCA family identified the novel and sole gene, *CCT2*, as LCA-causative. The *CCT2* encodes CCT β , one of eight subunits of the molecular chaperone protein CCT. The physiological impact of CCT β mutants, T400P and R516H, was analyzed. Prediction of the 3D structure of both mutant proteins revealed structural decays and their rapid degradation following expression in cell culture corroborates this prediction. LC-MS/MS proteomics demonstrated that the T400P mutant has higher affinity to CCT γ , while the R516H mutant exhibited less affinity to CCT γ , in comparison to wild-type CCT β . Patient-derived iPSCs exhibited slower proliferation compared to the parental iPSCs and siRNA knock down of *Cct2* in 661W cells, a mouse photoreceptor cell line, decreased cell proliferation. Stable expression of CCT β using a lentivirus vector in 661W cells significantly rescued the reduced proliferation mediated by siRNA knock down, while the expression of T400P and R516H mutants exhibited compromised rescue effect and was not significant. Both CCT β and CCT γ were expressed in the retinal ganglion cells and photoreceptors. Mutations in the CCT γ gene of zebrafish leads to defects in retinal ganglion cell development and ultimately, ocular hypoplasia. To validate the developmental impact of CCT β mutation and its physiological role, CRISPR-Cas9 genome editing-mediated zebrafish model of the *Cct2* mutation was generated. The zebrafish *Cct2* genome of wild-type-TL line was sequenced for CRISPR-Cas9 genome editing. The genome was directly sequenced and obtained *Cct2* mutation-carrying fish were used both as experimental animals for the phenotypic analyses, and the rescue study by mRNA injection of wild-type CCT β , T400P and R516H. Here, we present data on novel LCA compound heterozygous mutations in *CCT2* and their function in retinal development. The mutants affect the chaperone complex formation decreasing cell proliferation and implicating it as a fundamental cause of hypoplastic retinal development in LCA patients. The activity of CCT β -associating chaperone function is crucial

for retinal development and homeostasis. The further elucidation of specific clients not only for CCT β but also for other CCT subunits will be beneficial for understanding the retinal molecular biology and the associated human diseases.

Modeling Congenital Ocular Disorders in Zebrafish

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Zebrafish have become increasingly useful as animal models of human visual system disorders owing largely to the ability to perform forward genetic screens and rapidly identify the affected loci, and the ability to utilize genome-editing techniques to generate specific disease models. Research in our laboratory, as well as the laboratories of many others around the world, have taken advantage of these strengths to focus on generating zebrafish models of congenital ocular disorders and to utilize these models to begin to elucidate the molecular and cellular underpinnings of the disorders. In this presentation, I will discuss some of our recent work to model Microphthalmia-Anophthalmia-Coloboma (MAC) disorders and determine the cell biological underpinnings of optic fissure closure, as well as other congenital disorders of the eye.

JNT7 (OGM+GLA) - Genetics of normal tension glaucoma

Normal Tension Glaucoma and TANK Binding Kinase 1 (*TBK1*)

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Some cases of glaucoma are caused by single mutations in one of a few genes. Copy number variations

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(gene duplications or triplications) of the *TBK1* gene cause ~1% of normal tension glaucoma (NTG) cases. Similarly, mutations in the optineurin gene cause another 1-2% of NTG cases. Both *TBK1* and optineurin genes have important functions in a catabolic cellular process (autophagy) that may have an important role in retinal ganglion cell death in normal tension glaucoma. The role of *TBK1* gene mutations (duplications and triplications) and dysregulation of autophagy in retinal ganglion cell death and glaucoma are being explored with transgenic mice and patient-derived cells.

The Role of Cerebrospinal Fluid Pressure (CSFp) in Normal Tension Glaucoma (NTG): Is there a Genetic Contribution?

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The damaging effects of elevated IOP and damage to the optic nerve damage and glaucoma have been known since the mid-19th century, and evidence for a genetic contribution for IOP in glaucoma has been reported (Hysi et al, Nat Genet, 2014). In the early 20th century the questions arose regarding the role of cerebrospinal fluid pressure (CSFp) as a counterbalance to IOP. More specifically, that the pressure difference between IOP and CSFp at the lamina cribrosa (the translaminar pressure (TLP) difference may be a major contributor to optic nerve damage in glaucoma. Growing evidence, provided by retrospective and prospective studies, has shown that patients with POAG and NTG have lower CSFp than non-glaucomatous subjects. Additional factors that influence CSFp, including age, BMI, and blood pressure, similarly support this concept. Taken together, these studies suggest that the balance between IOP and CSFp may be critical for normal optic nerve function. Recently, the NEIGHBOR Consortium reported an association on chromosome 8q22 in a subgroup of patients with NTG in a GWAS (Wiggs et al, PLoS Genet, 2012). The role of CSFp in POAG pathogenesis and early suggestive evidence for a genetic contribution to this mechanism will be presented.

Normal-tension Glaucoma Genome-wide Association Studies

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Optic nerve degeneration caused by glaucoma is a leading cause of blindness worldwide. Patients affected by normal-tension glaucoma are more likely to harbor risk alleles for glaucoma-related optic nerve disease. Genome-wide association studies completed for NTG have identified two significantly associated regions in European Caucasian studies (8q22 and CDKN2BAS) and several interesting associated regions in Japanese (HK2, NCK2, SRBD1, ELOVL5). Genes and regulatory elements located in these regions are involved in a number of different biological processes that could impact the development of NTG. Pathway analyses using genome-wide SNP data from an NTG GWAS has identified significant associations with GABA metabolism and Acetyl-CoA metabolism.

Our recent POAG GWAS identified significant association with SNPs in the 3' regulatory region of TXNRD2, a nuclear gene that encodes a mitochondrial protein that functions to maintain redox homeostasis. To further explore the role of mitochondria in POAG and NTG, we examined the association of 3,146 POAG cases and 3,487 controls with SNPs corresponding to a comprehensive set of nuclear-encoded mitochondrial proteins. We conducted gene-set analyses of mitochondria-enriched biological pathways, examining the association with POAG overall and in the NTG and high-tension glaucoma subgroups. We identified 22 KEGG pathways with significant mitochondrial protein-encoding gene enrichment, belonging to 6 general biological classes. Among the pathway classes, mitochondrial lipid metabolism was associated with POAG overall (P=0.013) and with NTG (P=0.0006), and mitochondrial carbohydrate metabolism was associated with NTG (P=0.030). Examining the individual KEGG pathway mitochondrial gene-sets, fatty acid elongation and synthesis & degradation of ketone bodies, both lipid metabolism pathways, were significantly associated with POAG (P=0.005 and P=0.002 respectively) and

NTG ($P=0.0004$ and $P < 0.0001$ respectively). Butanoate metabolism, a carbohydrate metabolism pathway, was significantly associated with POAG ($P=0.004$), NTG ($P=0.001$) and HTG ($P=0.010$). These results suggest that genes contributing to mitochondrial function are more significantly associated with the NTG subgroup than with POAG overall suggesting a greater role in susceptibility to retinal ganglion cell dysfunction in patients with glaucoma.

Mitochondrial Pathogenic Mechanism in Optineurin E50K Mutation-mediated Retinal Ganglion Cell Degeneration

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Mutations in optineurin (OPTN) are linked to the pathology of primary open angle glaucoma (POAG) and amyotrophic lateral sclerosis. Emerging evidence indicates that OPTN mutation is involved in accumulation of damaged mitochondria and defective mitophagy. Nevertheless, the role played by an OPTN E50K mutation in the pathogenic mitochondrial mechanism that underlies retinal ganglion cell (RGC) degeneration in POAG remains unknown. We show here that E50K expression induces mitochondrial fission-mediated mitochondrial degradation and mitophagy in the axons of the glial lamina of aged E50K^{tg} mice *in vivo*. While E50K activates the Bax pathway and oxidative stress, and triggers dynamics alteration-mediated mitochondrial degradation and mitophagy in RGC somas *in vitro*, it does not affect transport dynamics and fission of mitochondria in RGC axons *in vitro*. These results strongly suggest that E50K is associated with mitochondrial dysfunction in RGC degeneration in synergy with environmental factors such as aging and/or oxidative stress.

Age-Dependent Neurodegeneration And Abnormal Bone With Loss Of Optineurin

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Optineurin mutations have been genetically associated with familial normal-tension glaucoma, in which retinal ganglion cells degenerate despite normal intraocular pressure. Recent patient genetic screens also revealed an association of optineurin with two other diseases – amyotrophic lateral sclerosis (ALS) and Paget's disease of the bone (PDB). Neurodegeneration of motor neurons are observed in ALS, while PDB is characterized by osteolytic bone lesions leading to abnormal bone remodeling and loss. While these three diseases are clinically very different, the age-dependent component of their degenerative phenotypes suggest that optineurin might play a common role in the homeostasis of skeletal and neural tissues. To test this hypothesis, we determined whether loss of optineurin function results in loss of retinal ganglion cells and Pagetoid bone degeneration. Mice with ubiquitous optineurin knockout were generated in our laboratory and aged 12-18 months. Retinal flatmount quantitation showed a 25-30% loss of retinal ganglion cells in aged optineurin knockout mice despite normal intraocular pressure. This is greater than the normal age-related loss observed in wildtype mice ($n = 5$ animals per genotype). More importantly, micro-CT scanning revealed that optineurin knockout mice spontaneously developed polyostotic osteolytic lesions in the skull, femur, tibia, lumbar spine and pelvis, which was not observed in age-matched control mice or younger knockout mice ($n = 5$ per genotype). Dual energy X-ray absorptiometry in optineurin knockout mice showed significantly lower bone mineral density in femur (0.046 ± 0.0033 g/cm²) and lumbar spine (0.039 ± 0.0026 g/cm²) compared to femur (0.056 ± 0.0016 g/cm²) and lumbar spine (0.047 ± 0.0004 g/cm²) in control mice ($p < 0.05$). Taken together, optineurin mice exhibited phenotypes consistent with normal-tension glaucoma and Paget's disease of the bone. Our results suggest the intriguing possibility that a common molecular pathway underlies distinct clinical pathophysiology in age-related neurodegeneration and bone changes.

Independent Session

IND1 - Expanding the functions of ocular surface innervation: photophobia and photoallodynia

Photophobia and other Neuropathic Like Symptoms in Dry Eye

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As defined by the International Association for the Study of Pain (IASP), pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." Based on this broadly accepted definition, some patients with traditional dry eye symptoms (e.g. dryness, fluctuating vision) also have eye pain symptoms. In our study, we asked individuals to rate the intensity of their average eye pain over a 1-week recall period using a numerical rating scale (NRS) anchored at "0," for "no eye pain" and at "10," for "the most intense eye pain imaginable." In a cohort of 154 veterans with mild or greater dry eye symptoms (as defined by a Dry Eye Questionnaire-5 (DEQ5) score of ≥ 6), we found that a large majority (89%) of subjects reported at least mild eye pain (≥ 1) on average over a 1-week recall period. To further characterize pain complaints, we asked the population to evaluate their eye pain using the short form McGill Pain Questionnaire (sf-MPQ) and Neuropathic Pain Symptom Inventory modified for the eye (NPSI-Eye). On the sf-MPQ, 82% of patients with traditional dry eye symptoms (by DEQ5) endorsed at least 1 sensory or 1 affective descriptor for their eye pain. Regarding the NPSI-Eye, the most common neuropathic-like symptoms were allodynia (pain due to a stimulus that does not normally produce pain) and hyperalgesia (increased pain from a stimulus that normally provokes pain) provoked by light or wind. In a study of 220 subjects with mild or greater dry eye symptoms, we found that 161 individuals (73%) reported pain sensitivity to light, with 94 (43%) rating sensitivity to light to be of moderate or greater severity (≥ 4 on scale of 0-10). Similarly, 137 (62%) reported pain sensitivity to wind, with 78 (35%) individuals rating sensitivity to wind to be of moderate or greater severity. A comparison of the rate of endorsement of the NPSI descriptors between those with dry eye symptoms, and data from the literature

in patients with chronic pain conditions, shows that eye pain associated with dry eye is described more similarly to neuropathic pain than non-neuropathic pain conditions, with frequent endorsement of "burning," "pressure," and "tingling." Our results suggest that pain is a significant feature in some patients with dry eye symptoms, and that eye pain characteristics in many dry eye patients overlap with descriptors associated with neuropathic pain conditions outside the eye.

A Potential Role for Melanopsin-expressing Trigeminal Neurons in Photoallodynia and Corneal Function

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Photophobia (photoallodynia) is a significant clinical problem that negatively impacts productivity and quality of life. Previously, we showed melanopsin-expressing neurons mediate innate light aversion, a rodent surrogate for photoallodynia. Mouse preclinical models of corneal surface injury and migraine were used to investigate neural pathways of light aversion.

Corneal surface damage was induced with topical benzalkonium chloride application, and migraine status with nitroglycerin (NTG) injections. Light aversion behavior was assessed in wild type mice (WT), mice lacking melanopsin expressing cells (OPN4^{DTA/DTA}), or mice with a 15 second bilateral optic nerve crush (ONC). Melanopsin expression was analysed by RT-PCR and visualized in the OPN4-EGFP reporter strain. Whole cell patch clamp recordings and calcium imaging of dissociated trigeminal neurons with blue light stimulation was used.

Light aversion is retained in wild type mice with severe bilateral ONC after NTG treatment, but is significantly reduced in mice with the same severe bilateral ONC but lacking melanopsin-expressing neurons. Loss of melanopsin-expressing neurons also reduces corneal surface damage-induced light aversion, and decreases trigeminal-mediated corneal mechanical sensitivity. Therefore, to investigate non-optic nerve mechanisms

of light detection, melanopsin expression was examined in OPN4^{EGFP} mice, identifying EGFP in approximately 3% of small trigeminal ganglion neurons. Consistently, melanopsin mRNA is expressed in trigeminal ganglia and corneal tissue extracts. These isolated melanopsin-expressing trigeminal neurons fire action potentials in response to blue light stimulation.

Melanopsin is expressed in a subset of small trigeminal ganglion neurons, confers intrinsic photosensitivity to these neurons *ex vivo* and may contribute to light aversion independently of the optic nerve. The cornea is likely innervated by these melanopsin-expressing trigeminal neurons. Our results demonstrate the possibility of a novel light-pain neural circuit that may provide the first direct mechanism for light-evoked pain.

Possible contribution of glutamate receptors in ocular hyperalgesia

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Sensory nerve terminals from the trigeminal ganglion are highly distributed in the cornea, contributing to pain and healing in corneal injuries and inflammation. Wounds and inflammation in peripheral tissue cause release of glutamate from nerve terminals and damaged cells for at least several hours. However, the modulatory effects of glutamate elevation on sensory nerves are not yet elucidated. In the present study, we investigated the acute and chronic effects of glutamate on transient receptor potential vanilloid type 1 (TRPV1), a polymodal nociceptive receptor, in sensory neurons. To detect the TRPV1-mediated responses, we monitored intracellular calcium and membrane current responses to capsaicin, a TRPV1 agonist, in cultured dorsal root ganglion neurons. Short-term (5-20 min) treatment with glutamate receptor agonists potentiated capsaicin-induced responses via metabotropic glutamate receptor mGluR5 activation, but did not affect the proportion of capsaicin-sensitive neurons. On the other hand, long-term (4 h) treatment with glutamate receptor agonists increased the proportion of neurons responding to capsaicin through the activation of mGluR1 and only partially through the activation of mGluR5. The increase in capsaicin-sensitive neurons was evident in neurons expressing transient receptor potential ankyrin type 1 (TRPA1). In addition, capsaicin-induced

current was significantly augmented in TRPA1-expressing neurons after the mGluR1/5 stimulation. These results suggest that peripherally released glutamate enhances TRPV1 activity and subsequently increases the number of neurons expressing functional TRPV1 in sensory neurons, which may be associated with hyperalgesia in acute and chronic ocular wounds and inflammation.

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Sensitization of Peripheral Ocular Nociceptors and Central Mechanisms Contributing to Photoallodynia

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Several ocular disorders are often accompanied by a particular type of severe ocular discomfort produced by bright light referred to as photoallodynia, which underlying mechanisms remain unknown. Photoallodynia has been typically associated with inflammatory disorders of the anterior segment (uveitis, iritis, keratitis, ...) but is also developed in normal eyes subjected to intense light. Miosis is a common factor in both cases, that is, ocular inflammation and sudden exposure to intense light.

The firing properties of the different classes of sensory fibers innervating the cornea in response to external stimuli of different modality is reasonably well known. In contrast, information about the functional characteristics of nerve fibers innervating other ocular tissues is very limited. In the present work, neural activity of polymodal nociceptor fibers innervating the iris and ciliary body recorded in the anesthetized cat was analyzed.

Similarly to corneal polymodal nociceptors, polymodal nociceptor fibers innervating the iris (n=17) have large receptive fields, covering almost a quarter of the iris and the adjacent ciliary body. They fired tonically in response to sustained mechanical indentation applied with a servo-controlled device to the iris surface, being their discharge rate proportional to the stimulus intensity. Some units did not respond to indentation but fired in response to traction of iris tissue to the center of the pupil, a movement resembling the tissue displacement produced during miosis. Ongoing activity and mechanical and chemical responsiveness of iris polymodal nociceptors was unaffected during intense light exposure. However, they developed ongoing activity and an increased response to mechanical and heat stimuli after repeated noxious

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stimulation of the iris.

Hyperexcitability of sensitized nociceptors of the iris and ciliary body in response to mechanical stimulation during reflex miosis evoked by bright light may be on the basis of pain sensation evoked by light during ocular inflammation. Convergence of sensory input of these sensitized nociceptors with the light-evoked input from melanopsin-expressing trigeminal neurons and/or retinal ganglion cells along the sensory pathway may also contribute to exacerbate the pain sensation evoked by light in migraine. Supported by SAF2014-54518-C3-1-R and SAF2014-54518-C3-2-R, MINECO, Spain and FEDER, European Union.

IND2 - Electrophysiology of Vision

Clinical Application of Photopic Negative Response to Optic Nerve and Retinal Diseases

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The photopic negative response (PhNR) in response to a brief flash is a negative-going wave following the b-wave of the cone electroretinogram (ERG) that is driven by retinal ganglion cells (RGCs) and their axons. The function of RGCs is objectively evaluated by analysing the PhNR. The PhNR amplitude was well correlated with the visual sensitivity obtained by standard automated perimetry, and morphometric parameters of the inner retina and optic nerve head measured by optical coherence tomography (OCT) in optic nerve and retinal diseases. Moreover, combining the PhNR with focal or multifocal ERG techniques enables the objective assessment of local function of RGCs.

I would like to talk about my experiences in clinical use of the PhNR in glaucoma and macular hole (MH) patients. The PhNR recorded by the focal or multi-focal ERG (focal PhNR) is superior to the PhNR recorded by the full-field cone ERG (full-field PhNR) in detecting early functional loss in glaucoma. In early glaucoma, the sensitivity and specificity of the focal PhNR to detect functional loss of RGCs were nearly 90%. Even in cases with pre-perimetric glaucoma the focal PhNR amplitude is deteriorated corresponding to local loss of the ganglion cell complex (GCC) thickness detected by OCT. The focal PhNR recorded from the macula is more correlated with corresponding GCC thickness than that from the paramacular regions. The full-field PhNR amplitude was reduced after MH surgery combined with internal limiting membrane (ILM) peeling assisted by intravitreal usage of indocyanine green

(ICG). On the other hand, ILM peeling assisted by brilliant blue G (BBG) and triamcinolone acetonide (TA) did not affect the PhNR amplitude after surgery, indicating BBG and TA may be safer than ICG for use during MH surgery. Among components of the focal macular ERG, the PhNR increased more than the a- and b-waves and the Σ OPs after closure of MHs. The function of the RGCs may recover more than that of the other neural elements in the macular area after MH surgeries. Although GCC thinning developed after MH surgery corresponding to the ILM-peeled retinal area, it did not affect the focal PhNR amplitude, suggesting that glial response to ILM peeling contributes to the GCC thinning after MH surgery.

Thus, the PhNR is likely to become established as an objective functional test for optic nerve and retinal diseases involving RGC injury.

Electroretinography of Retino-geniculate Pathways with Relevance for Vision; Implications for Clinical Electrophysiology

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The electroretinogram (ERG) is often used in a clinical environment to assess the functional integrity of the retina. The significance for studying retino-geniculate pathways has been limited. However, in previous work it was demonstrated that an heterochromatic flicker photometric (HFP) paradigm yielded data that resemble those obtained in psychophysical experiments studying the magnocellular (luminance) pathway.

We have shown over the last few years that ERG responses to combined sinusoidal modulation of luminance and chromatic modulation reflect luminance activity at high temporal frequencies (>30 Hz) and red-green chromatic activity at intermediate frequencies (about 12 Hz). This has been demonstrated by multiple different paradigms. From these data we conclude that the ERGs are governed by two fundamentally different mechanisms, which are probably related to retino-geniculate pathways with relevance for vision.

In subsequent studies, we found that ERG responses to instantaneous changes in excitation of only the L-cones or only the M-cones have opposite properties: The responses to L-cone increments ("L-On") and to M-cone decrements ("M-Off") resemble each other. In addition, ERGs to L-cone decrements ("L-Off") and M-cone increments ("M-On") are very similar. These data indicate that the ERGs are to a large extent determined by L/M cone opponent processes.

In a psychophysical experiment, we further found that L-On flashed stimuli are perceived as brightness increases, whereas M-On stimuli are perceived as brightness decreases. This is another demonstration of the close correlation between ERG responses and psychophysical data that rely on selective activities of retino-geniculate pathways. These data also indicate a new dimension in the correlation between the two because this is the first time that a psychophysical experiment was designed based on ERG data instead of the opposite.

We found evidence that the two mechanisms may be differently affected by retinal disorders. Particularly, patients with Duchenne's muscular dystrophy show distinct defects in the ERGs reflecting luminance and L/M-cone opponent mechanisms.

In conclusion, ERG responses are governed by two fundamentally different mechanisms, which are probably related to luminance and L/M opponent retino-geniculate pathways with relevance for vision. The ERGs can be used to study properties of these pathways with non-invasive electrophysiological techniques in human observers.

Basic Research and Clinical Application of RETeval, New Mydriasis-free Full-field ERG Recording Device

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Recently, a new full-field flicker ERG recording system called the RETeval (LKC Technologies, USA) was developed. In this system, a small, hand-held Ganzfeld dome and a special skin electrode array are used. The manufacturer claims that the ERGs can be recorded without mydriasis because the device delivers a stimulus with constant retinal illuminance (photopic Td-s) by adjusting the luminance to compensate for changes in the pupillary area. However, there are very little basic and clinical studies regarding this new ERG recording system.

We studied two basic experiments using this device in 10 normal subjects. (1) the flicker ERG was recorded every 3 minutes after the instillation of mydriatics. (2) The flicker ERG was recorded while the subjects wore soft contact lenses with two different artificial pupil sizes. Results demonstrated that that as pupil size increased, the amplitudes of the fundamental component of the flicker ERG did not change significantly, but the implicit times of

the fundamental component were significantly prolonged for larger pupil sizes. The results suggest that the effective retinal illuminance of the stimulus delivered by the RETeval system decreases for large pupil sizes.

We also wanted to determine whether this new ERG device can be used to screen for DR. To accomplish this, we recorded full-field flicker ERGs with this device from 48 normal eyes and 118 eyes with different severities of DR with diabetes mellitus (DM). Our results showed that there were significant correlations between the severity of DR and the implicit times ($P < 0.001$) and amplitudes ($P = 0.001$). The correlation was stronger for the implicit times ($r = 0.55$) than the amplitude ($r = -0.29$). When the implicit time was used for the index, the area under the receiver operating characteristic curve was 0.84 for the detection of DR, and was 0.89 for the detection of DR requiring ophthalmic treatments. These results suggest that the implicit times of the flicker ERGs recorded by such a mydriasis-free small ERG system can be used to screen for DR with a high degree of accuracy.

Could Birth Asphyxia Impair the Retina More than the Brain?

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Purpose: Neonatal hypoxic-ischemic encephalopathy (HIE), a complication of birth asphyxia in newborns, is frequently accompanied with visual disorders. We have previously shown (Jung et al., IOVS 2015) that the animal model of term neonatal HIE (Vannucci procedure on 10 day old Long-Evans rats) could also yield severe retinal injuries. Given that at P10 the rat brain is more developed than its retina, we examined if this maturational difference could contribute to the relative susceptibility of both organs to HI. We also examined if retinal pigmentation could also play a role given that it was previously shown to exacerbate the oxygen-induced retinopathy phenotype (Dorfman et al., IOVS 2009). **Methods:** The left common carotid artery of albino Sprague-Dawley (SD) and pigmented Long-Evans (LE) pups was ligated (Vannucci procedure; 20 rats) at P10 or P24. Following ligation, the pups were exposed to hypoxia (8%

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oxygen). ERGs were recorded at P30 and P60, and eyes, optic nerves and brains were collected at P70. Data analysis was based on the b/a wave ratio and the left/right (L/R) optic nerve and hemisphere ratios.

Results: In normal rats, the average b/a wave ratio was 2.10 ± 0.07 in SD rats and 2.42 ± 0.11 in LE rats; the L/R optic nerve ratio was 1.00 ± 0.08 in SD and 1.02 ± 0.05 in LE rats, and the L/R hemisphere ratio was 0.96 ± 0.02 in SD and 0.96 ± 0.03 in LE rats. In **HI-SD rats**, we found significantly lower than normal: 1- b/a wave ratio in 3/5 P10 and 5/5 P24 rats; 2- L/R optic nerve ratio in 2/5 P10 and 1/5 P24 rats and, 3- L/R hemisphere ratio in 1/5 P10 and 5/5 P24 rats. In **HI-LE rats**, we found significantly lower than normal: 1- b/a wave ratio in 3/5 P10 and in 4/5 P24 rats, 2-L/R optic nerve ratio in 3/5 P10 and in 4/5 P24 rats and, 3-L/R hemisphere ratio was significantly lower than normal in 2/5 P10 and in 4/5 P24 rats. Out of the 20 experimental animals, 12 had retinal + brain injuries, 4 had retinal injuries only while none had brain injuries only.

Conclusion: Our results indicate that: 1- irrespective of the strain, the susceptibility to retinal and cortical damage following a postnatal HI insult increases with maturity, 2- pigmented LE rats are slightly more affected than the albino SD rats and, 3- the retina appears to be more frequently affected than the brain, thus reinforcing the need to include a complete ophthalmological assessment (including retinal examination and ERG) of term newborns that suffered a perinatal HI episode. Funded by CIHR.

IND3 - Oxidative Stress in Ocular Tissue

ATF-4 Links ER Stress to the Oxidative Stress in Glaucoma

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The pathological mechanisms underlying increased outflow resistance at the trabecular meshwork (TM) that is responsible for elevating intraocular pressure (IOP) have not been fully delineated. We have shown that progressive accumulation of misfolded proteins and endoplasmic reticulum (ER) stress play an important role in the pathophysiology of glaucomatous TM damage. Expression of mutant myocilin or dexamethasone (Dex) treatment activates a protective unfolded protein response (UPR) in the TM that coordinates recovery of ER homeostasis. However, chronic ER stress can activate terminal UPR signals including ATF4 and CHOP, which may drive TM cells to apoptosis. In the present study, we investigated

whether ATF4/CHOP pathway induces ER stress mediated oxidative stress, TM cell death and IOP elevation. Our data shows that both ATF4 and CHOP levels are significantly increased in the glaucomatous TM donor tissues and in anterior segment tissues of *Tg-MYOC^{Y437H}* and Dex-treated mice. Adenoviral expression of ATF4 in the TM significantly elevated IOP in WT mice (15.5 ± 0.33 mmHg in empty Vs 20.1 ± 0.74 mmHg in ATF4 injected mice, $P < 0.0001$, N=8 each). Analysis of oxidative stress using dihydrodichlorofluorescein diacetate (DCF-DA) assay revealed that expression of mutant myocilin in TM-3 cells induced 2.5 fold increase in reactive oxygen species compared to control cells ($n=3$; $p=0.0001$). Furthermore, antioxidant genes including SOD2, catalase and GPx were significantly upregulated in primary human TM cells expressing mutant myocilin. Interestingly, genetic inhibition of ATF4/CHOP pathway significantly reduces elevated IOP in both *Tg-MYOC^{Y437H}* and Dex-treated mice. In addition, knockdown of ATF4/CHOP using CRISPR-Cas9 system dramatically reduced mutant myocilin accumulation and also prevented oxidative stress in TM cells stably expressing mutant myocilin. In addition, treatment of TM cells with sodium 4-phenylbutyrate (PBA) rescued ocular hypertension in *Tg-MYOC^{Y437H}* mice and also reduced oxidative stress in TM-3 cells expressing mutant myocilin. Forced expression of ATF4/CHOP in TM cells stably expressing mutant myocilin further worsened mutant myocilin accumulation and also dramatically increased oxidative stress as well as induced TM cell death. These data indicate that ATF4/CHOP pathway sensitizes TM cells to oxidative stress and ER stress mediated cell death. Genetic or pharmacological inhibition of ATF4/CHOP pathway may provide a novel treatment for glaucoma.

Choroidal Gamma Delta T Cells and Sodium Iodate-induced Oxidative Injury

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Gamma delta T cells (GDTs) are a special type of immune cells that account for a small percentage of the total T cell pool but have important regulatory roles in infection, inflammation and immune responses. They are enriched in certain tissues, such as the intestine and epidermis, that have an epithelial barrier. We have identified the presence of GDTs in the choroid and their activation in response to conditions of RPE and retinal degeneration. The purposes of the current study were to further characterize the functions of the choroidal GDTs and to explore the underlying mechanisms. To induce RPE injury, wild type

C57BL/6J mice and GDT-deficient mice were treated with sodium iodate vial tail vein injection. GDT-deficient mice showed profound RPE and retinal damage at dosage that caused minimal effects in wild type mice, indicating GDTs were protective against sodium iodate-induced oxidative injury. When analyzed for the transcriptome, purified choroidal GDTs were found to mainly express immunosuppressive cytokines such as IL-4 and IL-10. Their cytokine profiles were validated by flow cytometry and immunostaining. Furthermore, we found that ex vivo co-culture of GDTs with RPE explants activated IL-10 production, via an aryl hydrocarbon receptor-dependent mechanism. Putting together, our results showed that the choroid GDTs can maintain immune homeostasis and control cell-cell interactions in the outer retina.

Glutaredoxin 2 (Grx2) Protects Retinal Pigment Epithelial Cells from Oxidative Damage by Regulating Autophagy

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Glutaredoxin 2 (Grx2) is a mitochondrial oxidoreductase that protects the mitochondria from oxidative damage and maintains redox homeostasis. The purpose of this study is to evaluate the cytoprotective effects of Grx2 in human retinal pigment epithelial (RPE) cells and characterize its potential function in regulating autophagy. To establish Grx2 overexpression (OE), human retinal pigment epithelial (ARPE-19) cells were transfected with either a human Grx2 cDNA-containing plasmid (pCR3.1-hGrx2) or an empty vector pCR3.1. To study the anti-apoptotic ability of Grx2, cells were subsequently treated with or without 200 μ M H₂O₂ for 16 h. Cell viability was measured by a colorimetric assay with WST8. The morphology of nuclear chromatin was assessed by staining with Hoechst 33342. Apoptosis was quantitatively analyzed by flow cytometry. The level of protein glutathionylation (PSSG) and autophagy pathway proteins such as LC3I/II, Beclin-1, and ATG3 were measured by immunoblotting. Grx2 protein level in Grx2 transfected cells was significantly increased as compared to vector transfected cells, and enzyme activity doubled in Grx2 OE cells. Grx2 OE protected ARPE-19 cells from H₂O₂-induced cell viability loss with greater cell numbers and fewer blue fluorescent dead cells in Hoechst staining. Assessment of apoptosis indicated that cells transfected

with Grx2 were relatively more resistant to H₂O₂ with fewer cells undergoing apoptosis as compared to vector control cells. Mitochondrial and cytoplasmic PSSG accumulation were also attenuated by Grx2 overexpression with acute H₂O₂ challenge. Furthermore, early autophagy proteins like Beclin-1 and late autophagy proteins such as ATG3 and LC3 II were inhibited in Grx2 overexpressed cells with H₂O₂ treatment in a time-dependent manner. By promoting a healthy redox state, Grx2 rescues RPE cells from lethal oxidative damage, possibly through alleviation of ROS-triggered autophagy and prevention of PSSG accumulation. Funding Support: BrightFocus Foundation for Macular Degeneration (Grant No. M2015180 to Hongli Wu).

In vivo Chemiluminescence Detection of Reactive Oxygen Species in the Mouse Retina

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Oxidative injuries, such as those related to reactive oxygen species (ROS), have been implicated in various retinal and optic nerve disorders. Many ROS detection methods have been developed. Though widely utilized, most of these methods are useful only in post mortem tissues. In the present study, we demonstrated and characterized a chemiluminescent probe L-012 as a noninvasive, in vivo ROS detection agent in the mouse retina. Using optic nerve crush (ONC) and retinal ischemia/reperfusion (I/R) as injury models, we show that L-012 produced intensive luminescent signal specifically in the injured eyes. Histological examination showed that L-012 administration was safe and non-toxic to the retina. Additionally, compounds that reduce tissue superoxide level, apocynin and TEMPOL, decreased injury-induced L-012 chemiluminescence. The decrease in L-012 signal correlated with their protective effects against retinal I/R-induced morphological and functional changes in the retina. Together, these data demonstrate the feasibility of a fast, simple, reproducible, and non-invasive detection method to monitor in vivo ROS in real time in the retina. Furthermore, the results also show that reduction of ROS is a potential therapeutic approach for protection of retinal injuries.

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IND4 - Cell-signaling in anterior segment development and diseases

Programmed Cell Death (PCD) in Anterior Segment Morphogenesis and Diseases

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Programmed cell death (PCD) or apoptosis occurs in the anterior margins of the lens pit during early steps of eye development. This process is precisely controlled temporally and spatially and is essential for normal separation of the lens vesicle from the overlying surface ectoderm (or the prospective corneal epithelium) during normal anterior segment morphogenesis. Disruption of PCD, either by genetic mutations or by environmental insults during human embryogenesis, causes a blinding congenital eye disease, called Peters' anomaly, which is a subtype of anterior segment dysgenesis (ASD). We have been investigating the molecular mechanisms that regulate PCD required for lens-cornea separation. We hypothesize that mitochondrial death pathway activation and retinoic acid (RA, a vitamin A metabolite) signaling both play an essential role in the induction of PCD. We are able to demonstrate in the eyes of apoptosis-defective mutant mice (e.g., *Cytochrome c^{KA/KA}* knockin mice) that a persistent lens stalk is formed and disrupts normal anterior segment morphogenesis. Corneal epithelial cell differentiation was compromised in the region where persistent lens stalk was present in the knock-in mutant mice. The manifestation of developmental abnormalities in the anterior segment tissues in the mutant mouse eye is under examination. We also show that loss-of-function mutation in retinol dehydrogenase 10 (Rdh10), an enzyme in the first step of RA synthesis, inhibits PCD and causes formation of a persistent lens stalk between the lens and cornea. This finding suggests that RA-signaling is essential for induction of PCD. In summary, our findings imply that activation of RA-signaling may trigger PCD via mitochondrial death pathway during early lens and anterior segment development.

Shp2 Is Indispensable for Corneal Innervation and Epithelial Stratification in Mice

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Corneal sensory nerves contribute trophic factors to maintain the anatomical integrity and function of the cornea and particularly of the epithelium. Little is known whether corneal epithelium is mutually important for the corneal nerve function. We, for the first time, test if genetically impaired corneal epithelial stratification due to *Shp2* deficiency can impact sensory innervation during corneal development and nerve integrity in mature cornea. *Krt14-rtTA/tet-O-Cre/Shp2^{fl/fl}/Thy-1-YFP* quadruple transgenic mice administrated without (referring to *Shp2* wild-type) or with (referring to *Shp2* mutant) doxycycline (Dox). Mouse corneas were then dissected and subjected to whole-mount histology examination under confocal microscopy. Thy-1-YFP was served as a fluorescent marker for comparing the pattern of peripheral corneal innervation with or without *Shp2* expression in the corneal epithelium. Transmission electron microscopy (TEM) was also used to examine detail morphology of the corneal nerves. We found that *Shp2* conditional knockout in K14+ cells rendered the failure of corneal innervation during embryonic corneal development. Interestingly, *Shp2* ablation from fully stratified corneal epithelium at P21 could result in the degradation of corneal nerve bundle at P33. TEM investigation of cross-sections from many corneas revealed that single nerve fibers and nerve bundles invaginate deeply into the cytoplasm of the basal epithelial cells and the wing cells and the large single fibers contain many mitochondria, clusters of glycogen particles, and vesicles of different types and sizes of the wild-type cornea. In contrast, however, little of such was found in the *Shp2* mutant cornea. These data suggest that intact corneal epithelial stratification is indispensable for corneal innervation and *Shp2*-mediated receptor tyrosine kinase signaling plays a pivotal role in this corneal epithelium-nerve interaction which is paramount for the corneal morphogenesis and homeostasis.

TRP Channel Receptors and Signal Transduction in Corneal Wound Healing

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We examined whether the loss of transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1), irritant-sensing ion channels, or these antagonist treatment affects the severity inflammation and scarring during tissue wound healing in a mouse cornea injury model. The loss of TRPV1 suppressed post-alkali burn inflammation and fibrosis/scarring, and this was confirmed by histology, immunohistochemistry and gene expression analysis. The absence of TRPV1 attenuated expression of TGF β 1 and other pro-inflammatory gene expression in cultured ocular fibroblasts, but did not affect transforming growth factor β 1 TGF β 1 expression in macrophages. In addition, the effects of the absence of TRPA1 on TGF β 1-signaling activation were studied in cell culture. The lack of TRPA1 in cultured ocular fibroblasts attenuated expression of TGF β 1, interleukin-6, and α -smooth muscle actin, a myofibroblast the marker, but suppressed the activation of Smad3, p38 MAPK, ERK, and JNK. Stroma of the healing corneas of TRPA1 knockout(KO)mice appeared more transparent compared with those of wild-type mice post-alkali burn. Systemic TRPV1 or TRPA1 antagonists reproduced the KO-type of healing. Absence or blocking of these TRP channel receptors suppressed inflammation and fibrosis/scarring during healing of alkali-burned mouse cornea. TRPV1 or TRPA1 are potential drug targets for improving the outcome of inflammatory/fibrogenic wound healing.

Chemokine Signaling Is Required for Proper Neuropatterning during Ocular Development

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Chemokines, a family of small chemotactic cytokines, have recently gained interest for their ability to control cellular processes such as proliferation, migration, and differentiation during embryogenesis and in adults. Although, the chemokine CXCL14 is expressed during embryonic development, its function has not yet been elucidated. In this study, we determined the expression of CXCL14 during ocular development by section in situ hybridization and immunohistochemistry. We performed viral overexpression of CXCL14-shRNA, ocular injection of CXCL14 protein, and tissue culture to identify the function of CXCL14 during ocular development. We show that CXCL14 mRNA and protein are expressed in the trigeminal ganglion, corneal stroma, iris, lens epithelium, and neural retina during development. Knockdown of CXCL14 in vivo resulted in diminished CXCL14 expression

and exacerbated sensory nerve projection into the corneal stroma and epithelium, increased iridial innervation, and also caused miss projection of retinal nerves. We further explored the function of CXCL14 in vitro using isolated trigeminal neurons and we propose its inhibitory role on CXCL12-induced axon growth. Taken together, our study reveals a novel function of CXCL14 in regulating neuropatterning during ocular development and increases our understanding of the potential function of chemokines during wound healing.

Regulation of Ciliary Body Morphogenesis and Secretion: Notch, BMP, Adhesion and Beyond

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The ciliary body (CB) is responsible for secreting aqueous humor to maintain intraocular pressure, which is elevated in the eyes of glaucoma patients. It contains a folded two-layered epithelial structure comprised of the non-pigmented inner ciliary epithelium (ICE) and the pigmented outer ciliary epithelium (OCE), and the underlying stroma. Although the CB has an important function in the eye, how its morphogenesis and secretion are regulated remains poorly studied. Our recent studies show that Notch-RBPJ signaling and N-cadherin-mediated cell adhesion are critical for its morphogenesis and secretion. CB-specific conditional inactivation of Notch2 and its downstream transcription factor RBPJ completely abolishes CB morphogenesis. Notch2 and RBPJ are both required in the CB for promoting its morphogenesis by maintaining active BMP signaling and cell proliferation in the OCE. Surprisingly, Notch2, but not RBPJ, is required for maintaining BMP signaling in the underlying stromal cells. RBPJ, but not Notch2, is required in the CB for promoting the secretion of Collagen IX and Opticin. Since N-cadherin is enriched at the apex junctions between OCE and ICE, its CB-specific deletion causes the defects in CB morphogenesis, cell proliferation and OCE-ICE adhesion, but does not affect Notch2 and BMP signaling. Surprisingly, N-cadherin is also required in the CB for promoting the secretion of Collagen IX and Opticin. Our studies have revealed important roles of Notch-RBPJ signaling and N-cadherin-mediated cell adhesion in regulating CB development and secretion, and have also uncovered RBPJ-dependent and -independent regulation of BMP signaling by Notch2 in the developing CB.

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IND5 - Meibomian Glands and Meibum - From Biochemistry to Physiology to Disease

Anatomy and Physiology of the Meibomian Glands

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The Meibomian glands are an important component of the functional anatomy of the ocular surface because they provide the lipid phase of the precocular tear film that retards evaporation of the aqueous tears. Recent evidence as verified by the TFOS MGD workshop 2011 (see www.tearfilm.org) has shown that Meibomian Gland Dysfunction (MGD) with resulting lipid deficiency represents in fact the underlying reason for the vast majority (ca. 4/5) of dry eye disease cases.

The meibomian glands, named after their first extensive describer Heinrich Meibom (1638-1700), are also termed tarsal glands because they fill the tarsal plates of both the upper and lower eye lids. They are numerous individual long gland bodies in parallel arrangement. Their orifices are located slightly anterior to the posterior lid border still inside the cornified epithelium. Meibomian glands are holocrine glands but lack a direct association with hair follicles although their early embryological development has some similarity with hairs. The lipid is continuously produced in numerous spherical acini that are arranged around the long central duct. This occurs by a process of holocrine secretion in which lipid droplets accumulate inside the cells. The whole acinar cells eventually transform and disintegrate into lipids. Delivery of lipids onto the posterior lid margin appears discontinuous and is maintained by muscular contraction during the blink. The lipids get into the lipid reservoir strip and eventually onto the pre-ocular tear film. With every blink they are drawn out into the very thin (about 100nm) superficial lipid layer overlying the water-mucin gel that represents the main tear film phase.

Several factors in the anatomy can give rise to pathology that contributes to the onset of MGD. This concerns mainly two mechanisms: (1) hyperkeratinisation of the epithelium inside the narrow, delicate ductal system of the glands and on the free lid margin as well as (2) increased viscosity of the lipids. Both of these are influenced by a plethora of internal and external factors. Obstruction of the meibomian glands is the prevailing form of MGD and main reason for dry eye disease. Obstruction does not only lead to a obvious dry eye disease but also to *Non-Obvious* MGD (NOMGD). In any case, obstructed glands appear to

undergo a pressure atrophy with loss of function inside the lids that is undetectable by conventional ophthalmological examination. MGD thus requires early diagnosis and therapy.

Eye on the Lipids: Lipids and the Eye

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Meibomian glands (MGs) are an essential part of the human eye. The main function of MGs is to produce a lipid-enriched secretion - meibum - in a complex, poorly characterized biosynthetic process that we termed *meibogenesis*. Human meibum is a complex mixture of very- and extremely long chain lipids. Deficiencies in both quantity and quality of meibum have been implicated in the onset of dry eye disease (DED) in general, and MG dysfunction (MGD), in particular. However, even basic mechanisms of meibogenesis and the protective effects of meibum remain to be fully understood. The goals of this study were to investigate possible diurnal and seasonal variability in the lipid composition of TF in humans, both qualitatively and quantitatively, and determine if the TF lipid composition can be influenced by the ocular adnexa. The changes in the TF lipids of normal, non-DE/non-MGD volunteers were studied using chromatography and mass spectrometry. The early morning (AM) samples, compared with late afternoon (PM) samples, were found to be highly enriched with meibum (3x to 10x). Simultaneously, a larger molar ratio of shorter lipids (such as cholesterol, squalene, shorter wax esters, etc.) and phospholipids was observed in the AM samples. While the daily intra-donor changes in the TF lipid profiles of the PM samples were found to be rather insignificant (+/-10 % of the basal level for any major lipid species), the quantitative changes were remarkably high: the total meibum concentration in tears varied from 0.2 to >3 µg/µl. Interestingly, no clearly visible seasonal trends in the TF lipid profiles were observed for normal volunteers. The qualitative differences between AM and PM samples seem to be consistent with two mechanisms - lipid degradation and enrichment with metabolites produced by adnexa (e.g., sebocytes) and/or other ocular tissues. To test this hypothesis, the lipid and the gene expression patterns of lipid-synthesizing enzymes in MG were compared with those of lacrimal glands, cornea, conjunctiva, and the eyelid skin. Our current experimental results, taken together with literature data, indicated a highly differential expression of major lipid metabolism-related genes in these tissues, and their noticeably different lipidomes. These analyses

supported a hypothesis about the likely contribution of various ocular tissues and adnexa to the overall lipid makeup of human tears, especially the AM tears, which becomes less of a factor in the PM tears.

Characterization of Meibocyte Differentiation in Human and Mouse Meibomian Glands

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While the cellular and molecular mechanisms underlying meibocyte differentiation and holocrine secretion are poorly understood, recent RNA Seq analysis of young and old mouse meibomian glands point to several genes that may play important roles in this process. The purpose of this study was to validate the protein expression of these identified genes in human and mouse meibomian glands and begin to characterize their potential role in meibocyte differentiation in culture. Mouse and post-surgical human eyelid tissue were obtained with approval from the UCI IACUC and IRB and immunostained for 1) age (DKKL1 and Caspase 14), 2) apoptosis (Caspase 3a, 9) and 3) autophagy (BECN1 and ULK1) related genes. In addition, human telomerized meibomian gland epithelial cells (hTMGE) cultures were stimulated by the PPAR γ agonist, rosiglitazone (10 - 50 μ M), and evaluated for lipogenesis and expression of meibocyte specific proteins. DKKL1 and Caspase 14 both showed strong meibocyte immunostaining, with anti-DKKL1 showing staining of the ductal epithelium while anti-Caspase 14 was specific for the suprabasal acinar meibocytes. Caspase 3a and 9, also showed strong staining specific for suprabasal meibocytes; however, TUNEL labeling was limited to the ductal epithelium and the very distal, disintegrating meibocytes. BECN1 and ULK1 were localized to suprabasal meibocytes. hTMGE cells showed a significant, dose dependent increase in lipid synthesis following treatment with rosiglitazone combined with expression of all meibocyte specific genes. Additionally, protein extracts showed hyper-phosphorylation of ULK1 in rosiglitazone treated cells suggesting induction of autophagy. These results indicate proteins associated with age, apoptosis and autophagy are expressed in human and mouse meibomian glands. Both age-related proteins are known to be involved in epithelial differentiation suggesting that atrophy of meibomian glands may involve loss of meibocyte differentiation potential. The finding that ULK1 is hyper-phosphorylated following stimulation with rosiglitazone suggests that autophagy may play an important role in meibocyte

degeneration and disintegration.

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The Analysis of Meibum Is the Bridge Between Clinical Findings and Basic Science

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Purpose: Meibomian gland dysfunction (MGD) is commonly encountered in ophthalmic clinics. Meibum is a complex mixture of lipids of various classes including wax esters, cholesteryl esters, (O-acyl)- ω -hydroxy fatty acids, acylglycerols, free fatty acids (FFAs), and other polar and nonpolar lipids. Evaluation of changes in meibum lipid composition in patients with MGD as well as examination of the relation of such changes to objective signs and subjective symptoms may provide insight into the pathogenesis of this condition. We measured the components of meibum in patients with MGD and control subjects and then examined the relation between meibum composition and clinical parameters.

Methods: Thirty-eight patients with MGD (13 men and 25 women; 66.9 \pm 15.0 y-o) and 20 control subjects (8 men and 12 women; 64.5 \pm 6.7 y-o) were enrolled. Ocular symptom score, keratoconjunctival staining score, tear film breakup time, and Schirmer's test value were determined. Lid margin abnormalities and meibomian gland morphology were assessed for upper and lower eyelids, and meibum properties were evaluated at temporal, central, and nasal sites of each lid. Free fatty acid (FFA) composition of meibum was analyzed by liquid chromatography-Fourier transform mass spectrometry.

Results: Upper meibum color score was significantly correlated with epiphora and sticky sensation in MGD patients. Meibum grade, color, or viscosity did not differ significantly among the sites evaluated. A total of 103 species of FFA—including very long chain (such as C36 and C37) and odd-numbered chain (such as C17, C19, and C21) FFAs—were detected in meibum. Free fatty acid composition differed between clear and colored (cloudy or yellow) meibum, with unsaturated FFAs tending to be more abundant in colored meibum.

A PCA score plot for meibum samples grouped on the basis of the severity of both telangiectasia and plugging of meibomian gland orifices revealed clear separation of mild and severe groups. This separation of the two groups was largely due to significantly increased linoleic acid content in meibum of the severe group (3.56%, versus 0.70% of

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total FFAs in the mild group).

Conclusions: Free fatty acid composition of human meibum correlates with meibum color as determined with a slit-lamp microscope. The relative amount of linoleic acid in meibum was associated with the severity of telangiectasia and plugging of gland orifices in MGD. These finding may provide insight into the pathogenesis of MGD.

Elovl4 Products and their Relationship to Phenotype in Humans, Mice, and Zebrafish

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Stargardt-like macular dystrophy (STGD3) is an autosomal dominant disease caused by mutations in the ELOVL4 gene. ELOVL4 catalyzes the rate-limiting step in the synthesis of saturated and polyunsaturated fatty acids greater \geq 26-carbons in length (VLC-SFA and VLC-PUFA, resp.). Knock-in mice with the STGD3 mutation do not recapitulate the human disease. However, conditional deletion of Elovl4 in rods and cones leads to reduction in amplitudes of the rod ERG and death of \sim 10% of rods after one year. Ultrastructural analysis of the outer plexiform layer found that the diameter of the synaptic vesicles was smaller in the cKO retinas, suggesting a role for the VLC-PUFA in synaptic transmission. Studies in zebrafish in which *elovl4* was knocked down with morpholinos showed slower retinal and brain development, as well as skin abnormalities. The fish could be completely rescued by co-injecting wild type mouse *Elovl4* mRNA along with the morpholinos. Injecting mouse mutant *Elovl4* mRNA did not rescue the phenotype.

Mice with deletion of *Elovl4* can be rescued from neonatal death by expressing *Elovl4* in the skin under the control of skin-specific promoters. However, at P19, they develop repetitive tonic-clonic seizures and die by P21. Interestingly, this phenotype is consistent with humans homozygous for STGD3 ELOVL4 mutations, who develop epileptic-like seizures and spastic quadriplegia, intellectual disability, ichthyosis, and die within the first decade of life. We found widespread expression of ELOVL4 in the brain at P19-P21, including prominent labeling of pyramidal cells in the hippocampus. We used a MED64 multi-electrode probe with 64 channels to perform extracellular electrophysiology recordings of hippocampal slices, comparing the skin-rescued KO mice with their WT littermates, and found spontaneous and sustained seizure

activity in the mutant but not the WT mice. We cultured E19 hippocampal neurons from WT and mutant mice for FM dye uptake and release studies, and found faster release in mutant synapses (40%) compared to WT. We hypothesize that the VLC-SFA (28:0 and 30:0) in synaptic vesicles alter the biophysical properties of synaptic vesicle membranes in such a way, that they provide a rapid and non-genomic means by which the neuron can control how responsive the vesicles are to a release stimulus. This is supported by their absence leading to increased release rates in response to extracellular potassium stimulation.

IND6 - Autophagy in eye health and disorders

Insights into Membrane Dynamics of Autophagy and Its Implications in Diseases

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Autophagy is an evolutionarily conserved membrane trafficking from the cytoplasm to lysosomes. Although the term "autophagy" (self-eating in Greek), was officially used for the first time in 1963, most of our understanding of the process has come after identification of yeast autophagy-related (ATG) genes in 1993. This break through brought a dramatic expansion of the field. In autophagy, the unique double membrane-bound autophagosomes transiently emerge in the cytoplasm, sequester portion of the cytosol and organelles, and eventually fuse with lysosomes to degrade the contents. In addition to the basic role in nutrient supply under starvation conditions, the process unexpectedly functions in development, longevity, immunity, and suppression of various diseases including infectious diseases, inflammatory diseases, tumorigenesis, neurodegeneration, type II-diabetes, etc.

My group has been investigating the molecular machinery and pathophysiological roles of mammalian autophagy for the last 20 years. We could visualize autophagosome in living cells for the first time by identifying an autophagosome-binding protein and the protein LC3 has been mostly used as a golden marker in autophagy studies until now. This single paper has been cited in over 3,000 papers. Recently, we have provided new insights into biogenesis of autophagosome, which have been topic of longstanding debate. We showed that autophagosome forms at the ER-mitochondria contact site. We also found that autophagy selectively eliminates invading pathogenic bacteria, opening a new field on host-pathogen interaction. Then, we unraveled that autophagy recognizes damage

of endosomal membrane including bacteria rather than bacteria itself. We also found a new role of autophagy; selective elimination of damaged lysosomes. This "lysophagy" suppresses development of nephropathy in hyperuricemic mice. I also discuss about our recent finding that high fat diet increases the amount of a negative regulator of autophagy we identified. Knockout of the gene dramatically improved non-alcoholic fatty liver disease (NAFLD) in mice fed high fat diet.

Autophagy in the Retina, Development, Degeneration and Ageing

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Autophagy is a catabolic pathway that promotes the degradation and recycling of cellular components. This process degrades proteins, lipids, and even whole organelles, which are engulfed in autophagosomes and delivered to the lysosome. Autophagy is a stress-response that mediates the elimination of harmful cell components and recycles them to generate the nutrients and building blocks required to sustain cellular homeostasis. In addition, it plays an important role in cellular quality control, particularly in neurons, in which the total burden of altered proteins and damaged organelles cannot be reduced by redistribution to daughter cells through cell division. The role of autophagy in the retina and eye diseases is started to be investigated. In my talk will review our main studies describing the role of autophagy in retinal development and cell differentiation, and discuss the implications of autophagy dysregulation both in physiological aging and in important diseases such as glaucoma and retinal dystrophies. The findings reviewed here reinforce the essential role of autophagy in maintaining proper retinal function and highlight novel therapeutic approaches for blindness and other diseases of the eye.

Live Imaging and Molecular Dissection of Organelle Degradation in the Lens

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The eye lens is composed of fiber cells that undergo programmed degradation of all organelles during terminal differentiation. However, its underlying mechanism remains largely unknown. We have tested whether this

process depends on macroautophagy, which is mediated by the formation of autophagosomes. We generated mice with lens-specific deletion of either *ATG5*, *PIK3C3/VPS34*, or *FIP200/RB1CC1*, all of which are essential for macroautophagy, and found that the lens organelle degradation was not affected. Thus, we concluded that macroautophagy is not involved in this process. To better understand its mechanism, we performed live-imaging analysis of the organelle degradation in mouse and zebrafish lenses. In the mouse lens organ culture, we observed that lysosomes associate with other organelles such as nuclei and mitochondria immediately before their degradation. The organelle degradation was inhibited by treatment with an inhibitor of the lysosomal acidification. As we essentially observed similar processes in zebrafish lens in vivo, we screened candidate genes responsible for this process using zebrafish. We found that DNase1L3L, a member of DNase1 family, localizes to the lysosome and is essential for nuclear DNA degradation. We also found that several SNARE proteins, which are involved in membrane fusion events, are essential for this process. These findings suggest that the organelle degradation in the lens is achieved by a novel type of autophagy, in which lysosomes directly fuse with target organelles in a SNARE-dependent manner, but not by conventional macroautophagy.

The Dual Protective Role of p62 in the RPE with Aging and AMD

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p62/sequestosome is involved in cell signaling and degradation of misfolded proteins. p62 also is an adaptor protein that mediates autophagic clearance of insoluble protein aggregates in age-related diseases, and possibly including age-related macular degeneration (AMD), which is characterized by dysfunction of the retinal pigment epithelium (RPE). In our studies, we found that all p62 mRNA variants are abundantly expressed and upregulated by cigarette smoke extract (CSE) induced stress in cultured human RPE cells. The most abundant is P62 isoform1. p62 silencing exacerbates the CSE induced accumulation of oxidatively damaged proteins, both by suppressing autophagy and the Nrf2 antioxidant response. Over-expression of p62 isoform1, but not its S403A mutant, which lacks affinity for ubiquitinated proteins, reduces the accumulation of misfolded proteins, but simultaneously promotes an Nrf2-mediated antioxidant response. Thus, p62 provides dual, reciprocal enhancing protection to

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RPE cells from environmental stress induced protein misfolding and aggregation, by facilitating autophagy and the Nrf2 mediated antioxidant response. These changes might be a potential therapeutic target against AMD. In contrast to isoform2, an accumulation of the p62 isoform1 could induce inflammation because it contains the PB1 domain, which promotes Nf-kB signaling. Thus, p62 isoform2, which is devoid of Nf-kB signaling capability, may be a therapeutic target rather than isoform1, to reduce unwanted inflammation.

Effect of Optineurin on Retinal Ganglion Cell Transmitophagy at the Optic Nerve Head

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Recently, we discovered that retinal ganglion cell axons of normal mice pinch-off axonal membranes containing large number of retinal ganglion cell mitochondria at the optic nerve head, and that these mitochondria are degraded by resident astrocytes. This was demonstrated in part by adeno-associated virus 2 (AAV2) mediated expression in mouse retinal ganglion cells of MitoEGFPmCherry, a reporter for mitochondria degradation. Since this appears to be a non cell-autonomous process, we termed it transmitophagy, in order to distinguish it from the more common form of mitochondria degradation, a subtype of autophagy called mitophagy. Also recently, a gene implicated in both glaucoma and amyotrophic lateral sclerosis, Optineurin (OPTN), has been shown to be involved in autophagy in general, and in mitophagy in specific. In order to determine whether OPTN might participate in retinal ganglion cell transmitophagy at the optic nerve head, we used AAV2 to overexpress wildtype and mutant versions of OPTN in mouse retinal ganglion cells together with a new version of the MitoEGFPmCherry reporter. The results demonstrate that glaucoma and ALS associated mutations of OPTN, as well as synthetic mutations predicted to affect OPTN's ability to interact with damaged mitochondria or the autophagy machinery, differentially affect the amount of transmitophagy at the optic nerve head. In addition, tagged versions of OPTN expressed in retinal ganglion can be found within optic nerve head astrocytes. These results suggest that OPTN may participate in retinal ganglion cell transmitophagy at the optic nerve head.

IND7 - Plasticity in the Visual System

Retinal Plasticity in Human Retinitis Pigmentosa and Age-related Macular Degeneration. Implications for Vision Rescue

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Retinal degenerative diseases like retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are progressive, currently irreversible neural degenerative diseases with a number of different initiating events linked to diverse gene defects disrupting the function or architecture of photoreceptors and/or retinal pigment epithelium (RPE). While these diseases are thought to affect the photoreceptors resulting in blindness, it is clear that the inner retina is not spared the degenerative process and becomes progressively disorganized as the outer retina degenerates in a process called retinal remodeling.

The retina is a complex, heterocellular tissue and most, if not all retinal cell classes are impacted or altered by retinal remodeling. Therefore, defining disease and stage-specific cytoarchitectural and metabolic responses in retinal degenerative disease is critical for highlighting targets for intervention.

Much work has been performed in animal models of degenerative disease. Here we present human retinal samples, wherein we demonstrate retinal reprogramming in the earliest stages of the disease in retinal bipolar cells in RP, as well as alterations in both small molecule and protein signatures of neurons and glia in RP and AMD. These alterations reveal neural retinal responses to photoreceptor stress and loss to be progressive and Furthermore, while Müller glia appear to be some of the last cells left in the degenerate retina, they are also one of the first cell classes in the neural retina to respond to stress which may reveal mechanisms related to remodeling and cell death in other retinal cell classes. Also fundamentally important is the finding that retinal network topologies are altered. Our results in both RP and AMD suggest vision rescue interventions that presume substantial preservation of the neural retina will likely fail in late stages of the disease. Even early intervention offers no guarantee that the interventions will be immune to progressive remodeling. Fundamental work in the biology and mechanisms of disease progression are needed to support vision rescue strategies.

Anatomical and Macromolecular Changes in the Retina of the nm3342 Mouse: A Potential Model of Serous Retinal Detachment

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The retinas of the recently identified nm3342 mutant mouse (*aka rpea1*) spontaneously detach similarly to that observed in human central serous retinopathy. The purpose of this study was to identify changes in the retina or retinal pigmented epithelium (RPE) that could account for the loss of retinal adhesion. Optical coherence tomography (OCT) was performed immediately prior to euthanasia at times ranging from P30-P365. Retinal tissue was subsequently prepared for western blotting, immunocytochemistry, light and electron microscopy (LM, EM). By P30, OCT, LM, and EM revealed the presence of shallow detachments that increased in number and size over time. In regions of detachment, there was a dramatic loss of PNA binding to the interphotoreceptor matrix around cones and a concomitant increase in labeling of the outer nuclear layer and rod synaptic terminals. RPE wholemounts revealed a patchy loss in immunolabeling for both ezrin and aquaporin 1. Labeling for tight-junction proteins in RPE cells was disrupted in the mutant. With the exception of remodeling of retinal neurons and glia (e.g. increase GFAP expression in glial cells, retraction of rod synaptic terminals, and neurite sprouting from horizontal cells) all the changes in the retina were specific to the mutant animal and did not occur in eyes with a surgically induced rhegmatogenous retinal detachment. These data indicate that the retinas from eyes with a serous retinal detachment are quite plastic, showing significant remodeling of neurons, glia and epithelial cells. Importantly some of the changes in this mutant mouse may provide clues to the underlying mechanisms of serous retinal detachment in humans. These changes include the production of multiple spontaneous detachments without the presence of a retinal tear or significant degeneration of outer segments, altered expression of proteins involved in adhesion and fluid transport, and a disrupted organization of RPE tight junctions; all changes that may contribute to the formation of focal serous retinal detachments.

Neurovascular interactions: A target for therapeutic intervention in retinal degeneration

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The retina has one of the highest metabolic demands in the central nervous system. This demand is met by an elaborate vascular network providing uninterrupted delivery of oxygen and other nutrients. Two distinct vascular systems supply blood to the retina: choroid and intraretinal vasculature. These two networks are anatomically separated by photoreceptors; however, how the degenerative process affects their structure and function is unclear. In a mouse model of RP, we demonstrate that blood vessels degenerate leading to blood supply disruption and compromised blood-retinal barrier. We hypothesize that both loss of blood vessels and impaired blood-retinal barrier contribute to the progression of photoreceptor degeneration, particularly cones, and also affect the neurons in the inner retina. Understanding how early degeneration of blood vessels contributes to disease progression will provide a new therapeutic approach to prevent blindness in RP.

Remodelling of the Inner Retina Secondary to Photoreceptor Degeneration

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Retinitis Pigmentosa is a collection of inherited retinal degenerations which lead to irreversible blindness through photoreceptor death. Aside from photoreceptors, inner retinal neurons also alter their anatomical and neurochemical structure in this disease through a process termed remodelling. Recently, we and others have observed new remodelling events which occur well after photoreceptor degeneration - specifically, the loss of glutamine synthetase immunoreactivity in Muller cells. In this study, we further investigated late-stage remodelling in the rd1 mouse, an established animal model for photoreceptor degeneration. We assessed changes in ionotropic glutamate receptor function based on permeability of the organic cation agmatine (AGB) following glutamate receptor activation with NMDA or kainate. We found glutamate receptor function of inner retinal neurons

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in 18 month old rd1 mice were abnormal compared to those observed in normal mice and heterozygous rd1 mice of the same age but distinctly different to the neurochemical remodelling events observed in the rd1 mouse in early stages of degeneration (i.e. 3 months old). These changes also appeared to be associated with glutamine synthetase immunoreactivity in Muller cells whereby agmatine entry was reduced in inner retinal neurons where glutamine synthetase was reduced and vice versa. These results suggest late-stage remodelling between glial and neurons may be are closely linked and could have significant implications for current vision restoration strategies for Retinitis Pigmentosa patients which are impacted by neurochemical remodelling.

Ganglion Cell Changes during Retinal Degeneration

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Over recent years there has been increasing interest in restoring vision in those with inherited photoreceptor degeneration using a variety of different methods, including electronic retinal implants. Restoration of light sensitivity with an electronic implant assumes that ganglion cells, the principal output neuron of the retina, remain functionally intact. A central aim of our work has been to evaluate the changes that occur in ganglion cells during retinal degeneration and determine whether inner retinal plasticity influences restoration of light sensitivity from an electronic retinal implant. Our results show that ganglion cell density is reduced to a small extent in a mouse model of retinal degeneration, and that dendritic morphology of some ganglion cell subtypes are altered, particularly in the peripheral retina. Evaluation of synaptic inputs on A-type ganglion cells in mouse models of retinal degeneration revealed a significant reduction in excitatory inputs on ON type ganglion cells. Using the photoreceptor toxin, ATP, to create unilateral retinal degeneration, we next evaluated whether gross anatomical modifications in the inner retina influenced cortical signaling from a subretinal electronic implant. Our results showed that although the cortical threshold associated with a response was higher in retinal degeneration, changes in the inner retina had remarkably little influence on signal propagation. Overall, our results imply that although there are changes in ganglion cells during retinal degeneration, these do not have a large impact on restoration of light sensitivity using a subretinal implant.



POSTERS

Glaucoma

1

A Comparative Study of Intraocular Pressure Change in Different Dosages of Topical Steroid after Pterygium Excision**KOSOL KAMPITAK, Wannisa Suphachearaphan***Faculty of Medicine, Thammasat University, Department of Ophthalmology, Pathumthani, Thailand*

Topical steroids were widely used for suppression ocular inflammation after pterygium excision. Ocular hypertension is a common complication after steroid use. The incidence of ocular hypertension may correlate with the frequency of topical steroid usage. The purpose of this study is to compare intraocular pressure (IOP) change between two groups that received topical 0.1% dexamethasone after pterygium excision in different frequency. A retrospective cohort study was desired. Eighty six cases receiving topical 0.1% dexamethasone eye drops in different frequency after pterygium surgery were reviewed. Forty three cases were administered every 2 waking hours (Group A) and the other 43 cases were given 4 times a day (Group B). IOP was measured at pre and 1 month post steroid use. The number of cases who had increasing IOP more than 5 mmHg was compared between group A and B. The results showed that there were no significant differences ($p > 0.05$) in age and sex of both groups. Mean IOP before steroid use were 13.16 ± 2.87 and 13.47 ± 2.79 mmHg in group A and group B, respectively, which was no statistically significant difference ($p = 0.606$, t-test). After steroid use for 1 month, 8 cases (18.69%) in group A and 2 cases (4.65%) in group B had increasing IOP more than 5 mmHg, and the difference of number was statistically significant ($p = 0.044$, Chi-square test). In conclusion, high frequency of topical steroid use has a more tendency to increase IOP than low frequency.

2

Evaluation of Auditory Nerve in Glaucoma Patients**JAHANGIR AYATOLLAHI¹, Vahid Sanati²***¹Shahid Sadoughi University, Meybod, Iran, Islamic Republic of, ²Shahid Sadoughi University, Yazd, Iran, Islamic Republic of*

Background: Glaucoma is a disease characterized by degeneration of the optic nerve. Elevated intraocular pressure (IOP) has long been thought to be the primary etiology, but the relationship between IOP and optic nerve damage varies among patients, suggesting a

multifactorial origin.

Purpose: To evaluate the auditory nerve in glaucoma patients.

Method: Pure tone audiometry (PTA), impedance, speech discrimination score (SDS) tests were applied on 30 patients who suffered from primary open angle glaucoma (POAG). Results were compared with control group (30 patients).

Results: Significant statistically difference was seen between 2 groups.

Conclusion: With regard to simultaneously involving the optic and auditory nerves, it is concluded that other factors as well as IOP elevation have an influence on glaucoma

3

Morphological Changes of Lateral Geniculate Bodies and Visual Cortex in Patients with Glaucoma and Alzheimer's Disease**VALERY ERICHEV¹, Ludmila Paniushkina¹, Vladimir Tumanov², Anatoliy Fedorov¹***¹Federal State Budgetary Scientific Institution 'Research Institute of Eye Diseases', Moscow, Russian Federation,**²Pirogov Russian National Research Medical University (RNRMU), Moscow, Russian Federation*

Morphological study of the central parts of visual analyzer in patients with neurodegenerative diseases is of particular interest. The relevance of the study in glaucoma cases is determined by the search of new mechanisms of glaucoma progression. In literature, there is evidence of transsynaptic neurodegeneration present during the progression of glaucoma neuropathy. The study of the central parts of visual analyzer in Alzheimer's allows for the cause of visual impairment frequently observed at the onset of the disease to be identified.

The aim of the study is to conduct a comparative analysis of morphological changes of lateral geniculate bodies (LGB) and visual cortex (VC) in glaucoma, Alzheimer's cases and in norm.

Brain autopsy material of one glaucoma, 2 Alzheimer's and 4 control group patients with no ophthalmic or neurologic pathologies was examined. Immunohistochemical analysis for tau-protein and β -amyloid was conducted in addition to morphometry of LGB neurons and VC.

The examination of Nissl-stained specimen in glaucoma and Alzheimer's cases in comparison to control group showed reduction of the neuronal area in magnocellular layers by 33 and 31 per cent and in parvocellular layers by 38 and 39 per cent respectively. The neuronal nuclei

size in VC was also reduced by 35 and 26 per cent. The lowest values of the neuronal nuclei sizes were observed in patients with glaucoma in parvocellular layers of LGB and VC. In Alzheimer's patients the nuclei were greatly enlarged in magnocellular and parvocellular layers of LGB while in the VC they differed insignificantly from control group. The morphological study showed nerve tissue atrophy in LGB and VC in both glaucoma and Alzheimer's patients: shrinkage and death of neurons, pericellular swelling, displacement of nucleus and nucleolus, chromatolysis. Immunohistochemical analysis of LGB slices showed neurodegeneration markers - β -amyloid and tau-protein. This was considered as final confirmation of the involvement of the central parts of visual analyzer in the neurodegeneration process in glaucoma and Alzheimer's patients.

Despite the similarity of the observed morphological changes, they were better expressed in glaucoma cases. While in Alzheimer's LGB and VC injuries are secondary to neuronal death in cognitive centers, in glaucoma the pathological process propagates from retinal ganglion cells to the central parts of visual analyzer as the result of centripetal transsynaptic neurodegeneration.

4

Comparable Retinal Ganglion Cell Loss and Microglia Response in an Autoimmune Glaucoma Model Based on S100 or S100 plus HSP27

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As previously shown, immunization with specific ocular antigens, like heat shock protein 27 (HSP27), leads to retinal ganglion cell (RGC) loss in an autoimmune glaucoma model. Aim of this study was to investigate how immunization with S100 alone or in combination with HSP27 affects neuronal and glial cells in the retina. Therefore, rats were immunized with S100 or S100 plus HSP27 (S100+HSP) and compared to controls ($n=5$ /group). The intraocular pressure (IOP) was measured before and after immunization. 4 weeks after immunization retinas were obtained for immunohistology as well as Western blot analysis. No IOP alterations were noted in both immunized groups throughout the study. About a 30% Brn-3a⁺ RGC loss was observed in both immunized groups at 4 weeks (S100: $p=0.005$; S100+HSP: $p=0.004$). ChAT⁺ cholinergic amacrine cells were also affected (S100: $p=0.02$; S100+HSP: $p=0.05$), while numbers of parvalbumin⁺ All

amacrine cells decreased only in the S100+HSP group ($p=0.02$). Cone and rod photoreceptors remained intact in both immunized groups. An increase in Iba1⁺ microglia cells was noted in both antigen groups at this point in time ($p < 0.001$), especially in the RGC layer. Also, the number of ED1⁺ activated microglia was increased ($p < 0.02$). A slight increase in GFAP⁺ astrocyte reactivity was observed in both immunized groups (S100: $p=0.05$; S100+HSP: $p=0.04$), while vimentin⁺ Müller glia were not altered. In conclusion, immunization with ocular antigens affects mainly the neurons of the inner retinal layers, especially the RGCs and some extent amacrine cells. However, the photoreceptors remain intact. Surprisingly, combining S100 with HSP27 did not lead to additional retinal damage, more severe cell loss or an alteration of the existing microglia response. Both antigens might interact, possibly having inhibitory effects on each other and thus preventing additional damage to the retina at this point in time. This IOP-independent glaucoma model can serve as a tool to study specific neuroprotective agents in the future.

5

Inositol Phosphatase Regulation of Lipid Composition in the Cilia of Trabecular Meshwork

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Dysregulation of aqueous outflow results in elevated intraocular pressure and glaucomatous optic neuropathy. Previously we showed that primary cilia in the trabecular meshwork of the eye play a key role in regulating aqueous outflow. However, the role of ciliary phosphoinositides signaling in the trabecular outflow of the eye is not yet clear. Here we show that the distribution of ciliary phosphoinositides (e.g. PI(4,5)P₂) changes in trabecular meshwork cells under flow conditions. Oculocerebrorenal syndrome of Lowe is a rare X-linked disease that often presents with congenital glaucoma. Lowe syndrome is caused by mutation in OCRL, an inositol polyphosphate 5-phosphatase that dephosphorylates PI(4,5)P₂. We found that ciliary membranes of cells derived from Lowe syndrome patients exhibited increased levels of PI(4,5)P₂, and decreased levels of PI4P. In addition, ciliary accumulation of PI(4,5)P₂ was more pronounced in mouse embryonic fibroblasts derived from a Lowe syndrome mouse model (*Ocrl^{-/-};Inpp5b^{-/-}; INPP5B^{+/+}*),

Glaucoma

and *Ocr1* expression rescued the PI(4,5)P₂ built-up in the cilia. Loss of *Ocr1* in mice resulted in an accumulation of PI(4,5)P₂ within the ciliary axoneme. Further, OCRL loss resulted in decreased outflow facility of aqueous humor, thereby providing a potential mechanism for the glaucoma phenotype of Lowe syndrome. Together, our findings demonstrate that ciliary phosphoinositides, regulated by OCRL, play a critical role in glaucoma pathogenesis underlying Lowe syndrome, and may contribute to other forms of glaucoma and cilia-mediated disorders.

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Overexpression of GLAST Protects Retinal Ganglion Cells Following Optic Nerve Injury

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Glaucoma is one of the leading causes of blindness in the world. Currently, effective therapeutic agents are limited to those that reduce intraocular pressure; however, studies suggest that neuroprotective strategies may also be effective in preventing retinal ganglion cell (RGC) death in glaucoma. The glutamate/aspartate transporter (GLAST) is expressed in Müller glia and it plays a key role in removal of extracellular glutamate. It is also involved in glutathione (GSH) synthesis, in which the antioxidant GSH is synthesized from glutamate taken up by the cell. In mice, deletion of the *GLAST* gene results in development of spontaneous glaucomatous pathology without elevating intraocular pressure and therefore, GLAST knockout mice serve as a mouse model of normal tension glaucoma (Harada *et al.*, *J Clin Invest.* 2007; 117(7):1763-70). In this study, we examined if overexpression of GLAST could promote neuroprotection in an optic nerve injury (ONI) model, which is an experimental model of traumatic optic neuropathy as well as glaucoma. For this, we generated a new mouse line with overexpression of GLAST (GLAST Tg mice) on a C57BL/6J genetic background. The transgene was transmitted in a Mendelian fashion and mice were healthy and viable. Western blot analyses revealed that the retinal GLAST expression was doubled in GLAST Tg mice compared with WT mice. Following ONI, *in vivo* imaging of the retinal layers by optical coherence tomography indicated that the thickness of the ganglion cell complex, a region between the internal limiting membrane and the interface of the inner plexiform layer and the inner

nuclear layer, was decreased in WT mice, but this effect was milder in GLAST Tg mice. Histological analyses demonstrated that the number of cells in the ganglion cell layer, containing RGCs, was significantly reduced in the WT retina, but less cell loss was observed in GLAST Tg mice. These findings indicate that GLAST plays a neuroprotective role in the retina following ONI. Therefore, overexpression of GLAST by gene therapy or upregulation of GLAST by pharmacological agents may be a good strategy for glaucoma therapy.

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Blockade of Adenosine A_{2A} Receptor Prevents Retinal Microglia Reactivity and Oxidative/Nitrosative Stress Triggered by Elevated Pressure

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Glaucoma is a leading cause of blindness worldwide, characterized by progressive loss of retinal ganglion cells (RGCs) and optic nerve damage. One of the main risk factors for the development of glaucoma is elevated intraocular pressure (IOP). The onset of the disease is often accompanied by increased microglia reactivity and neuroinflammation. The blockade of adenosine A_{2A} receptor (A_{2A}R) has been considered a potential therapeutic strategy to treat neurodegenerative diseases since A_{2A}R antagonists provide protection against noxious conditions presumably through the to control microglia reactivity. In this study, we assessed the effects of elevated hydrostatic pressure (EHP) in microglial cells and investigated the potential protective properties of A_{2A}R blockade. Microglia cell cultures (BV-2 cell line), rat retinal primary neural cell cultures and human retinal explants were pre-treated with 50 nM SCH 58261, a selective A_{2A}R antagonist, and then were exposed to EHP (70 mmHg above normal atmospheric pressure), to mimic increased IOP. Control cells were maintained in a standard incubator. The exposure of primary rat retinal cell cultures to EHP increased the expression and the release of TNF and IL-1 β , oxidative stress, iNOS immunoreactivity, NO production, and cell death. Furthermore, the exposure of microglial cells to EHP altered TNF and iNOS mRNA and protein

levels. These effects were prevented by the blockade of A_{2A} R. In human retinal explants, the antagonist of A_{2A} R prevented the increase in oxidative stress elicited by EHP. The pre-incubation of rat retinal cell cultures with antibodies against TNF and IL- 1β to neutralize their action, prevented EHP-induced cell death.

These results demonstrate that the blockade of A_{2A} R prevents microglia reactivity, inflammatory response and oxidative/nitrosative stress elicited by EHP, suggesting that A_{2A} R antagonists can be considered as a promising therapeutic strategy to control microglia-mediated neuroinflammatory response in glaucomatous conditions.

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Lens

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Promoter Hypermethylation Mediated Down-Regulation of Antioxidant Genes in High Myopic Cataractous Eyes

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People with high myopia are always clinically complicated with rapider progressive nuclear cataract at early age. In high myopic eyes, vitreous liquefaction happens earlier than nonmyopic eyes, exposing the lens to higher concentration of O₂, which induces oxidative damages to lens and leads to cataract. However, it's unclear whether the lens has a healthy anti-oxidant system in those high myopic eyes. Given that DNA methylation has been confirmed to participate in cataractogenesis, we chose six antioxidant genes previously reported to be associated with cataract: nuclear factor, erythroid like 2 (NRF2), glutathione S-transferase pi 1 (GSTP1), heme oxygenase 1 (HMOX1), thioredoxin (TXN), thioredoxin reductase 1 (TXNRD1) and thioredoxin reductase 2 (TXNRD2) to investigate the possible epigenetic etiology of high myopic cataract (HMC). The methylation level of one fragment in the promoter of each gene was evaluated with a validated and reproducible method: Sequenom MassARRAY. Real-time PCR, western blot and immunofluorescence staining were used to examine the differences in the expression of differentially methylated genes. Moreover, 5-Aza-2'-deoxycytidine (5-Aza), a demethylating agent was used to treat human lens epithelial cell (LEC) line to further confirm the correlation between DNA methylation and expression.

Of all the antioxidant genes investigated, the methylation levels of CpG units in the promoter of GSTP1 and TXNRD2 were statistically higher in HMC than ARC (Table 1) and the expression of these two genes were detected to be significantly lower in HMC at both mRNA and protein levels. Besides, treatment with 5-Aza led to a significant increase of GSTP1 and TXNRD2 expression in LEC.

| | | ARC Group | HMC Group | P-value |
|--------|-----------|-------------|-------------|---------|
| GSTP1 | CpG 21-25 | 9.80±0.49% | 17.40±2.32% | 0.012 |
| | CpG 31-33 | 10.60±0.98% | 21.13±2.30% | 0.005 |
| TXNRD2 | CpG 34 | 29.50±2.36% | 43.17±3.72% | 0.015 |
| | CpG 36-37 | 7.00±0.91% | 15.17±1.08% | <0.001 |

[Table 1. DNA Methylation level of CpG units.]

The products of both genes have dethiolating capacity for antioxidant protection, which could lessen protein disulfides in lens resulted from oxidative stress. Based on the results of our study, the antioxidant capacity in HMC might have been impaired through DNA methylation. Promoter hypermethylation mediated down-regulation of two antioxidant genes: GSTP1 and TXNRD2 may take part in the cataractogenesis of HMC and partly explain the different clinical features between HMC and ARC.

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Is Near Infrared Radiation from Remote Controls and Sensing Systems Induced Lens Damage Cumulative?ZHAOHUA YU¹, Karl Schulmeister², Nooshin Talebizadeh¹, Martin Kronschläger¹, Per Söderberg¹, Gullstrand lab¹Uppsala University, Neuroscience/Ophthalmology, Uppsala, Sweden, ²Seibersdorf Labor GmbH, Seibersdorf, Austria

Early study on the glass and steel workers has implied an association between infrared radiation (IRR) and cataract. The current safety guideline for IRR exposure in the crystalline lens is based on thermal damage. However, two previous findings implied there may be a cumulatively photochemical effect. The recently fast increasing use of near IRR emitting diodes in remote controls and remote sensing presents a potential for accumulation of high doses over long periods of time and therefore has re-created a need for IRR safety estimation. The present study aims to estimate the threshold dose and the time evolution for cataract induction by IRR in seconds exposure time domain; to determine the ocular temperature development during the threshold exposure; to investigate if near IRR induces cumulative lens damage considering irradiance exposure time reciprocity. Before exposure, 6-week-old albino rats were anesthetized and the pupils of both eyes were dilated. Five minutes after pupil dilation, the animals were unilaterally exposed to 1090 nm IRR within the pupil area. Temperature was recorded with thermocouples placed in the selected positions of the eye. At the planned post-exposure time, the animal was sacrificed and the lenses were extracted for measurements of forward light scattering and macroscopic imaging. We found that the in vivo exposure to 197 W/cm² 1090 nm IRR required a minimum 8 s for cataract induction. There was approx 16 h delay between exposure and light scattering

development in the lens. The same radiant exposure was found to cause a temperature increase of 10 °C at the limbus and 26 °C close to the retina. The in vivo exposure to 96 W/cm² 1090 nm IRR with exposure time up to 1 h resulted in an average temperature elevation of 7 °C at the limbus with the cornea humidified and no significant light scattering was induced one week after exposure. Thus, it is indicated that IRR at 1090 nm produces thermal but not cumulatively photochemical cataract, probably by indirect heat conduction from absorption in tissues surrounding the lens.

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αB-crystallin: Possible Role in Signaling and Apoptosis in Ocular Melanoma

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Purpose: Crystallin proteins are major constituents of the vertebrate eye lens and are divided into two primary classes, α-crystallins and β, γ-crystallins. αB-crystallin (CRYAB) is a small heat shock protein and molecular chaperone. CRYAB has been studied extensively in other metastatic cancers, but less is known about its role in ocular melanoma. We provide the first expression patterning of CRYAB and other crystallins in ocular melanoma to help provide insight into its signaling and apoptotic implications in ocular melanoma development and migration in the eye.

Methods: OCM3 malignant melanoma cells derived from human eye tissue were grown to 80% confluence. Protein lysates were prepared and centrifuged to remove cellular debris. Western blotting was performed for CRYAB, αA-crystallin (CRYAA), β-crystallin, and γ-crystallin and the results were analyzed using ImageJ software with retinal pigmented epithelial (RPE) cells as a control. Different OCM3 cell cultures were treated with either 1μg/mL siRNAs against CRYAB, 10uM AKT inhibitor IV, or 10uM Rapamycin, an mTOR inhibition. Western blotting was

performed on these cell lysates for phospho-AKT, cleaved-caspase-9, and CRYAB and results were analyzed with ImageJ.

Results: CRYAB protein expression was present in both RPE cells and OCM3 cells, with a 43 fold actin normalized upregulation observed in OCM3 cells. CRYAA was not detected in RPE cells, but was present as a weak band in OCM3 cells. β and γ-crystallin both had minor expressions in RPE cells and were upregulated in ocular melanoma. AKT and mTOR inhibition significantly downregulated CRYAB expression sevenfold. However, phospho-AKT levels in similar inhibitory experiments were not significantly changed. Cleaved-caspase-9 was upregulated when CRYAB was suppressed.

Conclusions: The findings shed light into the role of crystallin proteins in ocular melanoma. CRYAB expression from western blot analysis shows there is active molecular chaperone activity in ocular melanoma. The data also suggest CRYAB is acting through the AKT/mTOR signaling axis and is active downstream of the proteins comprising the axis. Finally, the expression of cleaved-caspase-9, an activated form of the caspase, indicates that suppressing CRYAB could lead to apoptosis, and heightened CRYAB expression could be helping ocular melanoma bypass apoptosis. These findings raise questions about how crystallins, particularly CRYAB, could be targets for helping halt ocular melanoma metastasis.

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Lifespan of mRNA in the Lens

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The degradation of mRNA during the differentiation of epithelial cells into fiber cells in the lens was studied and the lifespan of mRNA in lens fiber cells was estimated.

Total RNA extracted from epithelial cells of the lens, fiber cells of the shallow cortex of the lens, and fiber cells of the deep cortex of the lens were used. RNA was more degraded in fiber cells of the deep cortex than in those of the shallow cortex. The expression of about 180 genes was more than 3-fold higher in fiber cells than in epithelial cells. Most mRNAs that were present at high levels in epithelial

Lens

cells were also present at high levels in fiber cells of the shallow and deep cortex. Genes encoding crystallins and ribosomal proteins were highly expressed in epithelial cells and fiber cells. In addition, RT-PCR revealed that several genes were expressed with whole size even in the deep cortex. Electron microscopy detected ribosomes in differentiated fiber cells, which contained few membrane organelles. These results suggest that some mRNAs are protected from degradation in fiber cells of the lens and that these mRNAs persist for a long time and continue to be translated into proteins.

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The p110 α Catalytic Subunit of Phosphoinositide 3-Kinase Contributes to Normal Lens Growth

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Signal transduction pathways influence lens growth, but little is known about the role(s) of the class 1A phosphoinositide 3-kinases (PI3Ks). To further investigate how signaling regulates lens growth, we generated and characterized mice where the p110 α and p110 β catalytic subunits of PI3K were conditionally deleted in the mouse lens. Floxed alleles of the catalytic subunits of PI3K were conditionally deleted in the lens using MLR10-cre transgenic mice. Lenses of age-matched animals were dissected and photographed. Postnatal lenses were fixed, paraffin embedded, sectioned and stained with hematoxylin-eosin. Cell proliferation was quantified by labeling S-phase cells in intact lenses with EdU. AKT activation was examined by western blotting. Lens specific deletion of p110 α resulted in a significant reduction of eye and lens size, without compromising lens clarity. Conditional knockout of p110 β had no effect on lens size or clarity, and deletion of both p110 α and p110 β resembled the p110 α single knockout phenotype. Levels of activated AKT were decreased more in p110 α than p110 β deficient lenses. A significant reduction in proliferating cells in the germinative zone was observed on postnatal day 0 in p110 α knockout mice, that was temporally correlated with decreased lens volume. These data suggest that the class 1A PI3K signaling pathway plays an important role in the regulation of lens size by influencing the extent and spatial location of cell proliferation in the perinatal period. Supported by NIH grant EY013163.

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Tob1 and Tob2 Mark Distinct RNA Processing Granules in Differentiating Lens Fibre Cells

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Lens fibre cell differentiation involves a complex interplay of growth factor signals and tight control of gene expression via transcriptional and post-transcriptional regulators. Recent studies have demonstrated an important role for RNA-binding proteins, functioning in ribonucleoprotein granules, in regulating post-transcriptional expression during lens development. Here we have documented the expression of Tob1 and Tob2, members of the BTG/Tob family of RNA-binding proteins, in the developing lens. Both *Tob1* and *Tob2* mRNA were detected by RT-PCR in both epithelial and fibre cells of embryonic and postnatal lenses. By *in situ* hybridisation, *Tob1* and *Tob2* mRNA were most intensely expressed in the early differentiating fibres, with weaker expression in the anterior epithelial cells and were down-regulated in the germinative zone of E15.5 lenses. Tob1 protein was detected from E11.5 to E16.5 and was predominantly detected in large cytoplasmic puncta in early differentiating fibre cells, often co-localising with the P-body marker, Dcp2. Occasional nuclear puncta were also observed. By contrast, Tob2 was detected in later differentiating fibre cells in the inner cortex and did not co-localise with Dcp2. The identity of these Tob2⁺ granules, which often appear as a series of interconnected peri-nuclear granules, is currently unknown. *In vitro* experiments using rat lens epithelial explants treated with or without a fibre differentiating dose of FGF2 showed that both Tob1 and Tob2 were up-regulated during FGF-induced differentiation. In differentiating lens epithelial explants, Tob1 also co-localised with Dcp2 in large cytoplasmic granules. These findings suggest that Tob proteins play important, but distinct, roles in RNA processing during lens fibre differentiation.

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Immunolocation of Aquaporin 8 in Human Lenticular Epithelial Cells

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Aquaporin 8 (AQP8) is known as a diffusion facilitator of hydrogen peroxide (H₂O₂) through cell membranes. This function of AQP8 may be a key to discovering how oxidative stress from the surrounding environment affects cells. We have reported increased superoxide scavenging activity and increased H₂O₂ in the aqueous humor of patients with cataract after intake of a lutein-containing supplement. We had also reported that the changes of glutathione peroxidase mRNA in the anterior capsule were comparable to the changes induced by increased H₂O₂ levels in the aqueous humor of cataract patients after intake of the same supplement. The decrease of AQP8 mRNA expression in human lenticular anterior capsules of patients with cataract after intake of the same supplement has also been reported. AQP8 plays an important role during peroxidation in the aqueous humor, and may influence the lenticular epithelial cells (LECs). The purpose of this study was to confirm and localize AQP8 in human LECs. The lenticular anterior capsule samples, including LECs, were collected during cataract surgery, and after informed consent. The localization of AQP8 was detected by immunohistochemical staining using an antibody to AQP8. Real-time polymerase chain reaction (RT-PCR) was also used to determine the AQP8 mRNA expression levels. The PCR products were analyzed by gel electrophoresis following densitometric analyses of band density. RT-PCR and agarose gel electrophoresis revealed the presence of AQP8 mRNA in the lenticular anterior capsule. Immunohistochemical staining showed AQP8 distribution throughout the whole anterior capsule. AQP8 labeling was observed on the plasma membrane of the LECs. The distribution of AQP8 labeling correlated with the functional diffusion facilitation and influx activity for H₂O₂ in the areas surrounding the LECs. This is the first investigation confirming the presence of AQP8 in human LECs. AQP8 is suggested to play an important role in the oxidative status of the aqueous humor and its subsequent effects on LECs.

IRB Status: Approved

Disclosures: RIJO HAYASHI, MD, PHD: No financial relationships to disclose.

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Frequency of Visit to Eye Clinics by Diabetic Patients in the Kumba Urban Area, Cameroon

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Objective: To study the frequency of visit to Eye clinics by diabetic patients in the Kumba urban area, South West Region, Cameroon.

Materials and methods: The descriptive cross-sectional hospital-based study from which a purposive sampling technique was used on 84 diabetic patients from three hospitals with Diabetic and Eye Clinics in the Kumba urban area, Cameroon. The participants were taken through visual acuity measurement, ophthalmoscopy, retinoscopy, subjective refraction and slit lamp examinations. Ethical approval was taken from the Regional Delegation of Public Health, Buea, Cameroon. Informed concern was taken from study subjects. Data collected was analysed using SPSS v. 20.

Results: A total of 84 diabetic patients from three hospitals in the Kumba Urban Area, Cameroon were examined for the study of which 52.4% were females and 47.6% were males. The total of 78.6% had visited an Eye clinic before while 21.4% had never been to one ($p < 0.05$, 95% CI). The prevalence of ocular conditions among the visually impaired diabetics was 43.3% for cataract, 23.3% for diabetic retinopathy (DR), 16.7% for glaucoma, 13.4% for refractive error and 3.3% for other ocular conditions.

Conclusion: This study revealed that, although a higher percentage of 78.6% of the diabetics had been to an Eye clinic, 59.5% are unaware that diabetes could result to ocular complications, which could eventually lead to visual impairment and blindness. This is very alarming, hence the need for more education and awareness creation by all stakeholders.

| Duration of diabetes (years) | Patients visit to Eye Clinic | | Total (%) |
|------------------------------|------------------------------|------------|-----------|
| | Yes | No | |
| 0-3 | 27 | 14 | 41(48.8) |
| 4-6 | 19 | 2 | 21(25.0) |
| 7-9 | 11 | 0 | 11(13.1) |
| 10-13 | 3 | 1 | 4(4.8) |
| 14 and above | 6 | 1 | 7(8.3) |
| Total/Percentage | 66 (78.6%) | 18 (21.4%) | 84 (100%) |

[Diabetes duration and patients visit to Eye Clinic]

Cornea and Ocular Surface

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Endothelial Keratoplasty Lenticules Prepared from Fresh Donated Whole Eyes: Role of Microkeratome Head and Pass Time**MAHNOUSH REZAEI KANAVI¹, Farzan Nemati¹, Tahereh Chamani¹, Mohammad Ali Javadi^{1,2}**¹Central Eye Bank of Iran, Tehran, Iran, Islamic Republic of,²Ophthalmic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran, Islamic Republic of

Purpose: To compare 400µm versus 350µm microkeratome head in three different pass time intervals in preparation of endothelial keratoplasty lenticules from fresh donated whole eyes.

Methods: Precut corneas were prepared for Descemet stripping automated endothelial keratoplasty according to the standard protocol of the Central Eye Bank of Iran and by using either 400µm or 350µm microkeratome heads. The preparation was performed in three different time intervals: less than 30 seconds (sec), 30-50 sec, or more than 50 sec. Primary full-thickness of central cornea and expected thickness endothelial keratoplasty lenticules (full thickness of central cornea minus size of microkeratome head) were measured by ultrasound pachymetry. Final thickness of endothelial keratoplasty lenticules was measured with Visante-Optical Coherence Tomography. Depth of cut was defined as the difference between primary full thickness cornea and final thickness of the posterior lenticule. ANOVA test and multiple comparisons based on Bonferroni test were used to evaluate the differences of measured thicknesses between different pass times. We used Generalized Estimating Equation to consider the possible correlation of the results between two eyes of a donor.

Results: Three hundred and sixteen precut corneas, prepared between October 2015 and December 2015, were enrolled. Mean expected thickness of the lenticules that were prepared by both 350µm and 400µm microkeratome blades was significantly increased with increasing pass time. Mean depth of cut with the 350µm blades was not significantly different between 3 time intervals; however, the depth of cut with the 400µm blades was significantly different between the time intervals and was meaningfully increased with pass time more than 50 sec ($P < 0.001$).

Conclusion: The results of our study demonstrated that speed of microkeratome pass could affect the depth of cut. Implementation of a single slow pass (more than 50 sec) technique with a 400µm microkeratome head resulted in a higher depth of cut and is recommended for sectioning corneas thicker than 600µm to obtain thin posterior corneal lenticules.

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Primary Conjunctival Malignant Melanoma with Intra Ocular Extension - A Case Report**MANJUNATH KAMATH***Kasturba Medical College Mangalore, Ophthalmology, Mangalore, India*

Malignant melanoma of the conjunctiva is a rare tumor with an unpredictable behavior. It has high risk of local recurrence, metastatic spread and mortality. Intraocular extension of a conjunctival melanoma is rare. We present this case of primary conjunctival melanoma with intraocular extension.

60 years old male patient presented with a pigmented mass close to the limbus with surrounding conjunctival pigmentation and involvement of the cornea since 1 year with rapid progression. Tumor also involved the upper and lower lids. CT showed a heterogeneously enhancing mass lesion (35mmx20mm) arising from extraconal aspect of the right orbit infiltrating into the muscle, extraconal fat, right upper and lower lids.

This patient required exenteration. Histopathology confirmed the diagnosis of malignant melanoma - epithelioid type TNM stage 11B (p T3b Nx Mx) with intraocular extension upto the anterior chamber.

In all cases of pigmented lesions of the conjunctiva malignant melanoma is a possibility. Malignant tumor at an advanced stage should be treated radically. Pictures of the mass, investigations and treatment will be shown.

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Corneal Keratins Aggresome (CKAGG) Formation and Corneal Epithelial Cells Opacification**FAWZIA BARDAG-GORCE, Amanda Laporte, Andrew Makalinao, Imara Meepe, Joan Oliva, Samuel French, Richard Hoft, Yutaka Niihara***Los Angeles Biomedical Research Institute, Torrance, United States*

Purpose: To understand the role played by corneal keratins aggresome (CKAGG) in corneal epithelium opacification. Keratins aggresome are protein aggregates or cytoplasmic inclusions characteristic of several hepatic, muscular and neurodegenerative disorders. Proteasome dysfunction is known to be a major cause of intermediate filaments collapse and aggresome formation. We previously reported proteasome dysfunction and keratins accumulation in

corneal epithelial cells (CEC) following limbal stem cell deficiency (LSCD). It is our hypothesis that, keratins undergo post translational modification and aggregate causing CKAGG formation in injured corneal epithelium, which contributes to CEC haziness and thus opacification.

Method: Rabbit LSCD was surgically induced and diseased CEC and pannus tissue (PT) were collected to investigate K4 and K13 accumulation and CKAGG formation. Rabbit oral mucosal epithelial cells (OMECS) were also used *in vitro* study because, similar to conjunctival epithelial cells (CJEC), they are characterized by K4/K13. Cells were isolated, cultured and treated with high dose of proteasome inhibitor to mechanistically induce CKAGG formation.

Results: Western blot and morphological analysis showed that K4 and K13 were markedly expressed in diseased CEC from the surface of central cornea. Additional higher molecular weight bands of K4/K13 and ubiquitin staining were detected in diseased CEC indicating keratins aggregates and insoluble CKAGG formation. Transglutaminase (TG), a key player in protein cross-linking, was found significantly expressed in diseased CEC. Similarly, LSCD-induced opaque fibrovascular PT dissected from the surface of central cornea showed a significant decrease in proteasome activity and stained positive for K4/K13. PT double staining for K4 and ubiquitin showed co-localization of these proteins indicating ubiquitination of keratins aggregates and CKAGG formation. OMECS proteasome inhibition also showed K4/K13 modification and accumulation similar to diseased CEC, indicating that K4/K13 were posttranslational modified and accumulated due to proteasome dysfunction.

Conclusion: Our results supported the hypothesis that, following LSCD, proteasome dysfunction leads to accumulation of modified K4/K13, which coalesce to form CKAGG in CEC. Consequently, CKAGG formation contributes to CEC opacification. Supported by Emmanuelle Life Sciences.

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Recombinant Fibroin Containing the RGD Motif Enhances Limbal Mesenchymal Stromal Cell Adhesion and Proliferation

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Background: At the periphery of the cornea, in a region called the limbus, exists a population of stromal progenitor cells termed limbal mesenchymal stromal cells (L-MSc). L-MSc likely contribute to maintenance of the corneal stroma. Moreover, L-MScs have anti-inflammatory and immunomodulatory effects. As such, there is widespread interest in exploiting the properties of L-MSc for the treatment of corneal disease. For example, it has been proposed that L-MScs improve wound healing in the cornea by enhancing epithelial cell growth and repair of the stromal tissue. As a consequence, studies have been initiated in our laboratory to optimise techniques for the isolation and cultivation of L-MSc. In parallel studies we have demonstrated that silk fibroin (SF) has potential as a scaffold for supporting the growth and implantation of L-MSc. In the current study we report an attempt to grow L-MSc culture on recombinant silk fibroin containing the RGD motif (RGD-SF), a recognized ligand for cell attachment, provided by National Institute of Agrobiological Sciences (Tsukuba, Japan). We aim to demonstrate that L-MSc display enhanced attachment and growth on RGD-SF compared to SF thus providing a superior scaffold for clinical use.

Methods: Human L-MSc cultures derived from four donors (n=4) were seeded onto either tissue culture plastic, RGD-recombinant fibroin or conventional fibroin for 90 minutes, 6 and 10 days, at a density of 1.5×10^4 cells/cm². Differences in L-MSc adhesion and growth were determined photographically and quantified using the PicoGreen assay (measures dsDNA).

Result: At all time points examined, L-MScs displayed evidence of superior attachment and proliferation on RGD-SF coated surfaces compared with control fibroin as indicated by both morphological examination and PicoGreen assay. Furthermore, morphological examination revealed that while L-MScs cultured on conventional fibroin generally formed sparsely scattered clusters, those cultured on RGD-SF were more uniform in adherence to SF.

Conclusion: The results of the current study suggest that RGD-SF offers superior performance as a scaffold material for L-MSc cultures compared to conventional fibroin. Based on such data we suggest that RGD-SF has potential as a biomaterial for improving strategies for the treatment of corneal disease.

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The role of Mitogen-activated Protein Kinases Pathway in the Cellular Responses of Keratocytes to Cyclic Stretch**HSINYUAN TAN^{1,2}, Chiayi Wang¹, Sungjan Lin^{2,3}, Yunnhwa Ma⁴, Taihorng Young²**

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Mechanical signaling was generally accepted to be crucial for the maintenance of homeostasis in extracellular matrices (ECM). Many studies have suggested common signaling mechanisms are involved in the mechanotransduction pathways in different types of cells. MAPKs are the most prominent kinases activated by mechanical stimuli. In this work, we investigated the responses of corneal fibroblasts to cyclic stretching loads in an *in vitro* cell culture system. Bovine corneal fibroblasts were cultured and subjected to equibiaxial cyclic strain of 15% for 72h at frequency of 0.25 Hz. Bovine skin fibroblasts were paired as comparison. The cellular behaviors including morphological alteration, cell proliferation and metabolic activity following mechanical loads were examined. Gene expression, protein secretion, and enzymatic activity for several major matrix metalloproteinases (MMPs) were determined by Q-PCR, Western blot, and gel zymography. We also investigated the role of mitogen-activated protein kinases (MAPKs) in the regulation of the mechanotransduction. Compared with skin fibroblasts, we found that cell morphology and cell alignment of corneal fibroblasts did not show significant changes following cyclic mechanical loads. And cyclic stretch inhibited cell proliferation of corneal fibroblasts. We found that mRNA, protein secretion and enzymatic activity of MMP-2 expression was enhanced by cyclic stretching. This enhanced MMP-2 activity was regulated by MAPKs, including extracellular signal-regulated kinase (ERK) and p38. In conclusion, cyclic stretching loads induced the upregulation of MMP-2 in corneal fibroblasts through MAPK pathway. It implicates a process of ECM modulation occurs in cornea when being subjected to cyclic stretching loads. Our results may provide a possible mechanism for the disease progression of keratoconus induced by eye rubbing. Supported by Chang Gung Memorial Hospital (CMRPG 3B1071) and Ministry of Science and Technology, Taiwan (NSC 102-2628-B-182A- 009-MY3)

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Creating an in vitro Model of the Cornea Stroma**JIE ZHANG, Aran M.G. Sisley, Charles, N.J. McGhee, Dipika, V. Patel**

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Purpose: We have developed a collagen-based scaffold that has the potential to be used as a corneal substitute for transplantation. The aim of this study was to investigate seeding the scaffold with keratocytes to emulate the corneal stroma *in vitro*.

Methods: Keratocytes were isolated from cadaveric porcine corneas and cultured in a serum-free sphere-forming culture system for 7-10 days. Keratocyte spheres were injected vertically into collagen scaffolds and cultured in serum-free medium supplemented with IGF-II. Scaffolds and keratocyte migration and morphology were imaged using light microscopy. Scaffold contraction and keratocyte migration were measured with NIS Elements, Adobe Photoshop and Leica Application Suite . Keratocyte phenotype was characterised by labelling with antibodies directed against keratocan and alpha smooth muscle actin (α SMA) and imaged with confocal microscopy.

Results: There was no significant difference in contraction of cellularized and cell free scaffolds radially or vertically at each of 12 time points measured across 46 days ($p > 0.05$ for each time point). Keratocytes remained viable in the scaffolds for up to 9 months and migrated radially away from the injection site in sheets, similar to the distribution of keratocytes between collagen lamellae in native cornea. The mean maximum rate of migration was $50 \pm 12 \mu\text{m}/\text{day}$ ($n=10$, range 29- 72 $\mu\text{m}/\text{day}$). Migrating keratocytes had a polar morphology with multiple processes extending in the direction of migration. After at least 9 weeks post-injection, keratocytes took on a quiescent phenotype, characterized by loss of polar morphology, development of a more rounded shape, and increased intercellular processes. Punctate labelling of keratocan was observed and there was no filamentous labelling of α SMA. A similar pattern of labelling was observed in porcine corneal stroma.

Conclusions: We have successfully seeded a collagen scaffold with primary keratocytes and confirmed that the keratocytes can achieve a distribution and phenotype similar to that seen in the human cornea. Further refinement of our model, such as increasing speed and extent of keratocyte migration, is required to fully emulate the corneal stroma *in vitro*.

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Regulation of Vascular Endothelial Growth Factor-C by Tumor Necrosis Factor- α in the Conjunctiva and Pterygium

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Vascular endothelial growth factor-C (VEGF-C) is an important molecule in the development of a pterygium through lymphangiogenesis. We investigated the association between VEGF-C and tumor necrosis factor- α (TNF- α) in the pathogenesis of a pterygium. Cultured conjunctival epithelial cells were treated with TNF- α , and gene expression levels of *VEGFC* were quantified by real-time PCR and VEGF-C protein expression levels were measured by ELISA. In addition, using ELISA, we measured the concentration of VEGF-C protein expression in the supernatants of cultured conjunctival epithelial cells, in which we neutralized TNF- α by anti-TNF- α antibody. Gene expression of *tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A)*, known as TNF receptor 1 (TNFR1), was confirmed using reverse transcription PCR in cultured conjunctival epithelial cells. Immunofluorescence was used to investigate the localization of VEGF-C and TNFR1 in pterygial tissues and TNFR1 in cultured conjunctival epithelial cells. Immunohistochemistry was used to examine the localization of TNFR1 in pterygial and normal conjunctival tissues. *VEGFC* gene expression increased in cultured conjunctival epithelial cells 24 h after the addition of TNF- α . The secretion of VEGF-C protein was significantly increased 48 h after TNF- α stimulation to cultured conjunctival epithelial cells. Increased VEGF-C protein secretion stimulated by TNF- α was significantly reduced by anti-TNF- α neutralizing antibody treatment. In cultured conjunctival epithelial cells, *TNFRSF1A* and TNFR1 were expressed. TNFR1 was immunolocalized in normal conjunctival tissues and VEGF-C-expressing pterygial epithelia. Our data demonstrate that TNF- α mediates VEGF-C expression, which plays a critical role in the pathogenesis of a pterygium.

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A Quantification of Tear O-glycans by 2-CNA Method

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Background and purpose: Mucins, glycoproteins bearing a dense array of O-glycans, are crucial for maintaining a moist ocular surface and therefore the decrease of mucin in the tear fluid is believed to implicate in dry eye disease. Since ocular surface moisture depends on not protein portion, but O-glycans of mucin, a quantification method for O-glycans in tears is of importance to better understand the pathogenesis of dry eye disease; however, it has not been established as yet. The purpose of this study was to establish the quantification method for O-glycans in tears using the technique for fecal O-glycan measurement.

Methods: Fifteen healthy volunteers (5 males and 10 females) and 15 patients (5 males and 10 females) with dry eye were enrolled. Tears were harvested using Shimer strips for 5 minutes and were extracted by phosphate buffered saline added appropriate detergent. After removing low molecular weight contaminants by gel filtration, β -elimination of O-glycans were done under alkali condition. The amounts of O-glycan were quantified by 2-cyanoacetamide (2-CNA) method, commonly used in fecal O-glycan measurement. A major secreted mucin, MUC5AC in tears were semi-quantified by western blotting with MUC5AC monoclonal antibody.

Results: O-glycans in tear samples of the patients with dry eye ($11.0 \pm 13.0 \mu\text{g/ml}$) were significantly decreased in comparison with those of the healthy volunteers ($42.1 \pm 27.6 \mu\text{g/ml}$, $P < 0.05$). MUC5AC were detected in all the samples and the band intensity of MUC5AC significantly correlated with the O-glycan concentration quantified with 2-CNA method ($R^2 = 0.71$, $P < 0.05$).

Conclusion: Modified fecal O-glycan measurement technique was useful to quantify O-glycan concentration in human tear samples obtained from Schirmer strips.

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Disseminated Nontuberculous Mycobacterial Infection with Multifocal Retinochoroiditis in an Immunocompromised Patient with Anti-IFN- γ AutoantibodiesTIAN LOON LEE¹, Rupesh Agrawal², Jackie Yu-Ling Tan², Kiat Hoe Ong², Chen Seong Wong², Su Ling Ho²¹Tan Tock Seng Hospital, National Healthcare Group Eye Institute, Singapore, Singapore, ²Tan Tock Seng Hospital, Singapore, Singapore

Background: Nontuberculous mycobacteria (NTM) are found ubiquitously in the environment. Since exposure to NTM is universal, infection likely represents underlying host susceptibility factors. Anti-IFN- γ autoantibodies have been described previously in patients with NTM. Up to 88% of patients with disseminated NTM or other opportunistic infections have high-titer anti-IFN- γ autoantibodies, compared with 2% of patients with TB and healthy controls.

Case presentation: We report a unique presentation of a patient with anti-IFN- γ autoantibodies with disseminated NTM infection and multifocal retinochoroiditis with vitreo-retinal infiltration. Treatment with systemic anti tubercular therapy without any adjunctive systemic corticosteroid therapy resulted in complete clinical resolution with good visual recovery.

Conclusion: Patients with anti-IFN- γ autoantibodies present with a novel syndrome that links autoimmunity and immunodeficiency. This case emphasizes importance of testing for anti-IFN- γ autoantibodies in patients with disseminated infection including mycobacterial infection.

epidemiology and classification of ocular inflammation.

Methods: Retrospective cohort study of the clinical records of consecutive new cases of ocular inflammation that presented to the Uveitis and Ocular Inflammation subspecialty clinic from 2004-2015 at a tertiary referral eye clinic in Singapore. Data collected included demographics, clinical features, diagnosis, laboratory findings and management. Diagnoses were made based on clinical history, ophthalmological examination and investigations.

Results: 2200 new patients with ocular inflammation were diagnosed from 2004 - 2015. The most common anatomical diagnosis was anterior uveitis (55.9%), followed by posterior uveitis (17.5%), panuveitis (9.6%) and intermediate uveitis (4.7%). In addition, scleritis (6.1%), keratouveitis (2.8%), retinal vasculitis (2.2%) and episcleritis (1.2%) were observed. Etiology was established in 65.1% of patients, with non-infectious etiologies (35.2%) being more common than infectious etiologies (29.9%). The remainder of patients (34.9%) were idiopathic. The most common etiologies was presumed tuberculosis (7.2%), followed by cytomegalovirus infection (6.9%), herpetic infection (6.3%), HLA-B27 associated anterior uveitis (4.2%) and ankylosing spondylitis (3.8%).

Discussion and conclusion: Ocular inflammation represents a significant proportion of patients presenting to the tertiary eye clinic. The pattern of ocular inflammation in Singapore has similarities with both Western and Asian populations. Anterior uveitis was most commonly observed, with presumed tuberculosis being the most common etiology though non-infectious etiologies were more common than infectious etiologies.

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Spectrum of Ocular Inflammatory Disease at a Tertiary Referral Eye Care Institute in Singapore - Report 1ELIZABETH JIAHUI CHEN¹, Amir Md², Helen Mi², Su Ling Ho², Wee Kiak Lim^{2,3}, Stephen Teoh^{2,3}, Rupesh Agrawal²¹National University of Singapore, Yong Loo Lin School of Medicine, Singapore, Singapore, ²National Healthcare Group Eye Institute, Tan Tock Seng Hospital, Singapore, Singapore, ³Eagle Eye Center, Mount Elizabeth Novena Hospital, Singapore, Singapore

Background: We aim to identify the characteristics of ocular inflammatory disease at a tertiary referral eye care centre in Singapore over a 12 year period, in particular the

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Choroidal Structure Determined by Binarization of Optical Coherence Tomographic Images in Children with Anisohypermetropic Amblyopia

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Purpose: To compare the choroidal structure in the eyes of children with anisohypermetropic amblyopia to that of the fellow eyes and of age-matched controls.

Methods: This study was performed at Nara Medical University Hospital, Tokushima University Hospital, and Kagoshima University Hospital, Japan. Thirty amblyopic eyes of anisohypermetropic amblyopic patients (6.1 ± 2.1 years, mean \pm standard deviation) and their fellow eyes, and 100 age-matched controls (6.7 ± 2.4 years) were studied. The total choroidal area, luminal area, and stromal area of the subfoveal choroid were measured by the binarization method of the images obtained by enhanced depth imaging optical coherence tomography (EDI-OCT). The control eyes were divided into myopic, emmetropic, and hyperopic eyes. The luminal/stromal ratio and the axial length of amblyopic eyes and control eyes were compared.

Results: The total choroidal area in the amblyopic eyes ($549124 \pm 84697\mu\text{m}^2$) was significantly larger than that of the fellow eyes ($484683 \pm 99185\mu\text{m}^2$) ($P=0.030$). The luminal area was larger and the stromal area was smaller in the amblyopic eyes ($416137 \pm 90302\mu\text{m}^2$) than that in the fellow ($32599 \pm 76213\mu\text{m}^2$) and the control hyperopic eyes ($365765 \pm 62680\mu\text{m}^2$) ($P < 0.001$). The luminal/stromal ratio was significantly larger in the amblyopic eyes (3.5 ± 1.9) than that in the fellow eyes (2.3 ± 0.8) and the control hyperopic eyes (2.1 ± 0.6) ($P < 0.001$). There was a significant negative correlation between the luminal/stromal ratio and the axial length in the control eyes ($r = -0.30$, $P = 0.001$; Pearson's correlation coefficient) but no significant correlation was found in the amblyopic eyes ($r = -0.02$, $P = 0.39$).

Conclusions: The choroidal structure of amblyopic eyes is different from that of fellow eyes and control hyperopic eyes. Our findings indicate that the choroidal changes are related to the amblyopia.

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Evaluation of the Relationship between the Macular Retinal Thickness in Amblyopic Children and their Control

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Functional amblyopia is a unilateral or less commonly bilateral reduction of best corrected visual acuity that usually caused by abnormal visual experience early in the life during visual system development, resulting from strabismus, refractive errors (ametropia, anisometropia) and visual deprivation. Increased macular thickness accompanied with decreased foveal depression by OCT examination have been reported in amblyopic children in some studies.

Purpose: Comparison of macular retinal thickness in eyes with functional amblyopia and those without amblyopia.

Method: This case control study was conducted on 93, three to ten years old children, 44 with unilateral amblyopia and 49 without amblyopia. Amblyopic eyes were considered as case group and their fellow eyes as internal control and eyes of nonamblyopic children as external control. Retinal thickness of all eyes in these groups were measured by OCT in center (foveola), 1mm ring (fovea), 3 and 6 mm rings of macula to compare with each other.

Results: Although macular thicknesses were not generally different in these groups, there was significant difference of central thickness between moderate to severe amblyopic eyes and external controls ($p=0.037$). Difference of foveal thickness > 10 micron between two eyes were seen in more number of amblyopic children compared to non-amblyopic ones ($p=0.002$). Foveolar and foveal thickness were more in boys ($p=0.037$ and $p=0.033$ respectively), but there was no significant difference among different types of refractive error.

Conclusion: There was not any relationship between thickness of macula and amblyopia, but difference of central thickness (foveola) in moderate and severe amblyopic eyes and external controls was significant. Due to the less number of these cases in this study, we recommend to repeat the study with more moderate to severe amblyopic cases.

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Haemodynamics in the Retinal Vasculature during the Progression of Diabetic Retinopathy

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Introduction: Diabetic Retinopathy (DR) remains a major ocular disease, which can potentially lead to blindness if left untreated. The human retina is a very dynamic tissue, making it difficult to associate any changes with a disease and not with normal variability among people. 96 images from twenty-four subjects were used in this study, including the period of the three years before DR and the first year of DR (4 images per patient, one per year).

Methods: The images were firstly segmented to obtain the vascular trees, selecting the same segments in the entire four-year period, to make a meaningful comparison. The trees, which included a parent vessel and two children branches, were connected using an implemented semi-automated tool. Some hemodynamic features were calculated, using the geometric measurements from the segmentation. At the branching points, the fluid dynamics conditions were estimated under the assumptions of

Poiseuille flow: stiff, straight and uniform tube. Blood flow velocity (v), blood flow rate (Q), Reynolds number (Re), pressure (P) and wall shear stress (WSS) were calculated, both for arteries and veins. Blood viscosity ($\mu=0.04$ P), tube's length (L) and diameter (D), were used to compute fluid resistance to flow ($R=128 \mu L / \pi D^4$) through each vessel. Based on previous studies, the boundary conditions adopted to solve the problem were $P_{CRA} = P_{CRV} = 45\text{mmHg}$. Q_{CRA} and Q_{CRV} were derived from v_{CRA} , d_{CRA} , v_{CRV} , d_{CRV} by using the formula $Q=VA$. WSS was computed as $WSS=32\mu Q/d^3$. Re was calculated as $Re=v d \rho/\mu$, where $\rho=1.0515$ g/mL is the blood density. Each feature (response variable) was analysed by using a linear mixed model, with the levels of the disease being the fixed effects explanatory variable, and the patients being the random effect with a random intercept.

Results: Our study showed that veins were mostly affected during the last stages of the diabetic eye. Furthermore, the blood flow of both children and the Re in the small child branch were mostly affected in the arteries. Table 1 includes only the significant features, with the relevant p-values ($\alpha=0.05$) and Akaike Information Criterion (AIC).

Conclusion: Alongside the already established importance of the retinal geometry, this study showed that the hemodynamic features can also be used as biomarkers of progression to DR. During this four-year period of the disease's progression, retina is adapting to the new underlying conditions.

| Features | p-value (Satterthwaite's approximation) | p-value (comparison with restricted model-Likelihood test) | P-value (wald chi-square) | AIC (with and without fixed effect, the lower the better) |
|---|---|--|---------------------------|---|
| Wssparent_veins , Wsschild1_veins , Wsschild2_veins | 0.02 , <0.000 , 0.001 | 0.02 , <0.000 , <0.000 | 0.017 , <0.000 , <0.000 | 550.7/554.26 , 472.13/484.82 , 490.67/501.21 |
| Vparent_veins , Vchild1_veins , Vchild2_veins | 0.024 , <0.000 , 0.003 | 0.02 , <0.000 , 0.002 | 0.016 , <0.000 , 0.001 | 335.45/339.22 , 231.88/247.24 , 247.5/256.02 |
| Reparent_veins , Rechild1_veins , Rechild2_veins | 0.027 , <0.000 , 0.04 | 0.024 , <0.000 , 0.034 | 0.019 , <0.000 , 0.031 | 693.38/696.82 , 593.87/610.36 , 608.29/610.95 |
| Qchild1_veins , Qchild2_veins | 0.05 , 0.04 | 0.034 , 0.033 | 0.03 , 0.029 | -204.06/-201.45 , -204.01/-201.48 |
| Pressure_veins | 0.02 | 0.017 | 0.014 | 444.03/448.15 |
| Rechild2_arteries | 0.017 | 0.015 | 0.012 | 800.08/804.53 |
| Qchild1_arteries , Qchild2_arteries | 0.012 , 0.011 | 0.01 , 0.009 | 0.008 , 0.009 | -111.48/-106.21 , -110.36/-105.13 |

[The significant features (p-values $\alpha=0.05$).]

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In vivo Molecular Imaging of the Retinal Hypoxia**IMAM UDDIN¹, Stephanie Evans¹, Ashwath Jayagopal^{1,2}, John Penn^{1,3}**¹Vanderbilt University School of Medicine, Department of Ophthalmology and Visual Sciences, Nashville, United States, ²Hoffmann-La Roche Ltd, Basel, Switzerland, ³Vanderbilt University School of Medicine, Department of Molecular Physiology and Biophysics, Nashville, United States

Purpose: Molecular imaging is a powerful tool for the management of ocular diseases, such as early detection, monitoring treatment response and the design of effective treatments. Current successes in development of new imaging modalities enabled clinical screening of ocular tissues and vasculature with detailed morphology. New strategies focusing on early disease detection through molecular imaging and screening protocols in the retina will significantly improve clinical management of ocular diseases. In this research, we have developed a new molecular imaging technology for the detection of retinal hypoxia.

Methods: A new class of hypoxia sensitive fluorescence imaging probes were synthesized and characterized for the detection of retinal hypoxia in animal models of oxygen-induced retinopathy (OIR) and retinal vein occlusion (RVO). The probes were administered systemically and conventional fluorescein angiography equipment was used for *in vivo* imaging. Sensitivity and specificity of these hypoxia sensitive imaging probes were confirmed by *ex vivo* examinations and colocalization with hypoxic retinas as confirmed by pimonidazole-adduct immunostaining. The safety of these imaging probes was confirmed by ERG analysis and TUNEL assays.

Results: Retinal-hypoxia was successfully detected *in vivo* using these new molecular imaging probes in animal models of OIR and RVO, and were colocalized with hypoxic regions of the retinas identified by pimonidazole-adduct immunostaining. These new probes can penetrate deep in to the capillary-free tissues and detect retinal-hypoxia *in vivo*. This new technology will allow overcoming major limitations of existing techniques to measure hypoxic tissues. The imaging probes were well tolerated in the retinal tissues as indicated by ERG analysis and TUNEL assays.

Conclusions: We have developed a facile route for the synthesis of hypoxia sensitive fluorescence imaging probes. Using these new probes, we were able to visualize retinal hypoxia *in vivo* in mouse model of OIR and RVO. These

probes are biocompatible and sensitive, and complement existing technologies for measuring retinal vascular PO₂ and blood flow. This new imaging technique is non-invasive and it promises to be an excellent tool for diagnosis and monitoring retinal hypoxia in preclinical models and patients.

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A Less Invasive Surgical Approach to the Superior Part of the Orbit. A Study of the Orbital Anatomy**DAG KROHN-HANSEN¹, Erling Haaskjold¹, Bjørn Nicolaisen², Lili Zhang³, Torstein R Meling⁴, Ivar Sjaastad⁵**¹Oslo University Hospital, Department of Ophthalmology, Oslo, Norway, ²University of Oslo, Department of Ophthalmology, Oslo, Norway, ³University of Oslo, Institute for Experimental Medical Research, Oslo, Norway, ⁴Oslo University Hospital, Department of Neurosurgery, Oslo, Norway, ⁵University of Oslo, Institute for Experimental Medical Research, Oslo University Hospital, Oslo, Norway

To acquire insight into the topographic anatomy of the superior part of the orbit, and the feasibility of an alternative access corridor for surgery in the upper orbit, we applied various study methodologies of both clinical and laboratory affiliations.

In an anatomy study on human cadaver orbits, including macroscopic gross dissections, sagittal sections and microscopic examinations, we found that the orbital septum extends posteriorly from its junction with the levator aponeurosis. This posterior continuation of the orbital septum encloses the superior orbital fat pad and separates this from the anterior surface of the levator muscle. In-between the orbital septum and the levator, there is a dissection space that provides a minimal invasive corridor to the structures in the upper mid-orbit.

By use of ultra-high-resolution magnetic resonance imaging (MRI) at 9.4 Tesla (isotropic resolution, 20 μm) on six human cadaver specimens, the superior half of the orbit was examined. To visualise the posterior layers of the upper orbit, a dissection of three of the orbits was performed prior to the MRI examination, and a flexible PVC sheet was introduced above the levator muscle. The technique enabled a visualisation of anatomically important landmarks of the anterior and posterior parts of the upper orbit at a resolution near histological levels;

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to our knowledge, this visualisation has not been reported previously. A posterior continuation of the orbital septum, which formed a distinct anatomical structure, was revealed. The posterior aspect of the orbital septum separated the levator muscle from the orbital fat pad.

A series of 18 patients suffering from congenital eyelid ptosis was operated on by use of this anatomical entrance route to surgery on the levator muscle complex, with the advantage of not violate the orbital septum or the retroseptal fat pad during the surgery. Successful results were achieved in 88 % of the cases.

The key-point in our studies is that the orbital septum extends posteriorly from its junction with the levator aponeurosis. This posterior continuation of the orbital septum encloses the superior orbital fat pad and separates it from the anterior surface of the levator muscle. These findings are the topographic rationale for a potential surgical space in-between the septum and the levator, in which the orbital septum and the retroseptal fat pad is not harmed during surgery in the upper mid-orbit.

RPE Choroid Biology and Pathology

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mir-204/211 in Eye Development and Disease: An Intricate Relationship

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In last decade, microRNAs (miRNAs) are emerging as key players in the control of fundamental biological processes in both physiological and pathological conditions. Their role in vertebrate eye patterning is just beginning to be elucidated. We recently identified miR-204/211 miRNA family as a new "key regulator" of vertebrate eye development. We demonstrated they have a fundamental role in eye morphogenesis including lens formation and retinal dorsoventral patterning. We also elucidated their contribution in the control of axon guidance of retinal ganglion cells. Recently, we identified a dominant mutation in miR-204 as the genetic cause of a unique phenotype of photoreceptor degeneration and coloboma and thus highlights the importance of miR-204/211 family as a key regulator of ocular development and normal maintenance. While the role of miR-204/211 in eye development has been deeply characterized, the relationship occurring between their function and photoreceptor development and function is still not completely characterized. To gain insights in this direction, we performed loss of function studies in both mammalian and teleost models. Phenotypic analysis of miR-211 deletion in both mouse and medaka mutants revealed a progressive photoreceptor cell degeneration characterized by an impaired electroretinogram (ERG) response to light stimuli. These studies are allowing us to gain a full comprehension of miR-204/211 function and dysfunction and may provide proof of principle for novel therapeutic strategies to treat inherited retinal dystrophies.

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Compromised Phagosome Maturation Underlies Defective RPE Clearance in an in vitro Model of Smith-Lemli-Opitz Syndrome

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The retinal pigmented epithelium (RPE) in the AY9944 rat model of Smith-Lemli-Opitz syndrome (SLOS), a genetic disorder of cholesterol biosynthesis, exhibits accumulation of phagosomes and other inclusions, compared to controls [Fliesler et al., 2004, *Arch. Ophthalmol.*]. We examined SLOS patient iPSC-derived (SLOS RPE), vs. normal human embryonic stem cell-derived (nhRPE), cells *in vitro* to determine the underlying mechanism of this defect. SLOS RPE (harboring both T93M and IVS8 G-C DHCR7 mutations) and nhRPE were treated with bovine rod outer segments (ROS) for 48 h; rhodopsin levels were quantified by Western blotting/densitometry (WB/D; probed with 1D4 MAb) to assess phagosome clearance. Lysosomal protease (mature Cathepsin-D), and markers of autophagic flux (p62 and LC3-I/II) were assessed by WB/D, normalized to GAPDH levels, with corresponding antibodies. SLOS RPE and nhRPE lysosomal pH was measured using LysoSensor Yellow/Blue DND-160, and lysosomal Cathepsin-D activity was estimated using a BODIPY-Pepstatin assay kit (Molecular Probes). Statistical significance of mean/S.E. values was determined by paired Student's *t*-test (criterion, $p \leq 0.05$). Autophagic marker levels in SLOS RPE (expressed as % change, vs. nhRPE) were as follows ($p < 0.05$): Beclin-1, +62%; p62, +18%; LC3-II, -50%. Mature Cathepsin-D levels were unaltered. Lysosomal pH in SLOS hRPE was slightly more acidic (4.50 ± 0.02 , $p < 0.05$) than in nhRPE (4.56 ± 0.01), but within the normal physiological range; Cathepsin-D activity also was not significantly altered. However, heterophagic clearance of exogenous ROS was defective in SLOS RPE, as evidenced by persistence of 1D4+ material (rod opsin C-terminus), compared to nhRPE. Decreased LC3-II levels, elevated p62 levels, and persistence of 1D4+ material indicate compromised phagosome maturation in SLOS RPE compared to nhRPE. Elevated Beclin1 levels obviate defective initiation of autophagy/heterophagy; also, compromised lysosomal physiology was not involved. However, increased 7-dehydrocholesterol (7DHC) levels (a SLOS hallmark) and/or oxysterols derived therefrom may underlie the SLOS-associated RPE defect.

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Puerarin Protects Human Retinal Pigment Epithelial Cells from All-trans-retinal-induced Oxidative and Nitrosative Stresses

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RPE Choroid Biology and Pathology

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Background: Delayed clearance of free form all-trans-retinal (atRAL) is estimated to be the key cause of retinal pigment epithelium (RPE) cells injury during the pathogenesis of retinopathies such as age-related macular degeneration (AMD). Abnormal accumulation of atRAL can induce RPE cell dysfunction via triggering oxidative stress and nitrosative stress. Puerarin, an important isoflavone-C-glucoside extracted from the root of *Radix puerariae*, has been widely used to treat many ophthalmic diseases by Chinese herbalists due to its strong neuroprotective and antioxidant activities, however, the molecular basis of its application on these eye diseases are not well understood.

Methods: The potential protective effect and underlying molecular mechanism of puerarin against atRAL-induced cytotoxicity in human retinal pigment epithelium ARPE-19 cells was first investigated which targeting at oxidative and nitrosative stresses. MTT assay, flow cytometric analysis, cell adhesion assay and western blot analysis were used in this study.

Results: The results showed that atRAL-induced cell viability loss, cell apoptosis and leukostasis in ARPE-19 were significantly attenuated with the pre-treatment of puerarin. The cellular protective mechanisms of puerarin were likely to be mediated through suppression of reactive oxygen species (ROS) generation, inhibition of mitochondrial-dependent and MAPKs signaling pathways as well as down-regulation of inducible nitric oxide synthase (iNOS), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression.

Conclusion: These findings significantly contribute to a better understanding of the protective effect of puerarin against atRAL-induced RPE dysfunction and form the basis of the therapeutic development of puerarin in treating retinal diseases such as AMD.

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Transplantation of Functional RPE Derived from Bone Marrow Stromal Stem Cells Could Survive and Improve the Retinal Function

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Introduction: Retinal impairment is a most cause of blindness in the world. Production of cells that have function in vitro is the first step to try treatment of disease related to the sensory retina and RPE layer. Adult stem cells due to no ethical and immunological problem are one of the choices for replacement therapy.

Materials and methods: Bone marrow stromal stem cells (BMSCs) extracted from male pigmented hooded rats and after cultivation, differentiated into neural stem cells (NSCs) by using EGF, bFGF and B27 supplement. The NSCs induced into retinal pigmented epithelium (RPE) in two steps with several inducers. After 7-14 days, phagocytosis ability of them investigated with internalization of photoreceptor outer segment (POS) labeled with FITC. Immunocytochemistry (anti-RPE65, anti-CRALBP, anti-OTX2) and real time PCR performed for identification of RPE cells. Then RPE cells labeled with BrdU and injected via trans-scleral approach into the subretinal space of the RPE degeneration model. After 90 days immunohistochemical and electrophysiological test (ERG) performed for tracing cells and functionality of them.

Results: Results showed that NSCs could differentiate into retinal pigmented epithelium that phagocyte labeled POS and internalize it in vitro. Also, immunocytochemistry and real time PCR confirmed the RPE differentiation after 14 days. After transplantation, labeled RPE cells could survive, migrate and integrate into host RPE layer and also rescue the neurosensory retina. b-wave and a-wave increased after 90 days in contrast to control group. Thickness of the neurosensory retina increased and outer nuclear layer cells count showed an increase in the number of ONL cells.

Conclusion: During our investigation, differentiation of BMSCs into RPE cells well had done in vitro. Moreover, these cells could express specific markers and gene expression and also phagocyte and internalized POS in vitro. These cells could migrate and integrate into the RPE layer. These cells could be functional in vivo and are the best choice for autologous cell therapy in retinal degenerative disease.

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Metformin Suppresses Inflammatory Responses *in vitro* and in the Vitreous of Diabetic Retinopathy Patients

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Metformin is the most commonly used anti-diabetic agent in treating type 2 diabetes. This is attributed to multiple cardiovascular benefits beyond glycemic control. We have previously reported that the use of metformin is associated with substantial reduction of the severity of diabetic retinopathy (DR) in patients, and that metformin has significant anti-inflammatory effect *in vivo*. This study was to examine if metformin affects the levels of inflammatory molecules in the vitreous of DR patients and in human retinal vascular endothelial cells (hRVECs).

Patients with type 2 diabetes for more than 15 years and metformin treatment for at least 5 years prior to the onset of DR were included. Patients with similar history of diabetes and DR but had never used metformin were served as control. Undiluted core vitreous were collected for analysis of inflammatory molecules using a Human Cytokine Array. Primary cultured hRVECs were challenged by high glucose with or without metformin co-treatment. The expression of ICAM-1 and phosphorylated NF- κ B in hRVECs were measured by western blot. The production of MCP-1, IL-8, and TNF α by the cell were determined by ELISA.

Among 36 common inflammatory molecules, 21 were detected in the vitreous samples from DR patients. Majority (17/21, 81%) of these, including ICAM-1, MCP-1, IL-8, and etc., had lower levels in the vitreous of metformin users than those of non-metformin users. Non-metformin treatment is associated with significantly positive correlations among the inflammatory cytokines in the vitreous of DR patients, and an extensive reduction of inter-cytokine interactions in metformin users. ELISA verification of ICAM-1, MCP-1, and IL-8 confirmed that the differences between the two groups were statistically significant

($p < 0.05$). *In vitro* study revealed that metformin significantly ($p < 0.05$) suppressed high glucose induced upregulations of ICAM-1 and pNF- κ B expression in hRVECs, as well as the secretion of sICAM-1, MCP-1, IL-8, and TNF α from hRVECs. Metformin's effects on ICAM-1, MCP-1, and IL-8, as well as pNF- κ B and TNF α were completely blocked or partially reversed by compound C, an AMP-activated protein kinase, respectively.

These results confirm that metformin significantly suppresses levels of inflammatory molecules in the vitreous of DR patients. Such strong and broad-spectrum anti-inflammatory effects could be a major mechanism underlying its vascular protection in diabetic retina.

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Angiogenic Role of Netrin-4 in the Retina

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Netrin-4 is a matrix-bound protein functioning in axon guidance. Surprisingly, this molecule is not only involved in development but also in pathological neovascularization (e.g. tumor formation). Unc5B and DCC have been identified as netrin vascular receptors and bind both netrin-1 and netrin-4. Recent data suggest that these receptors are involved in the maintenance of the blood-retina-barrier (BRB). Here we explore the role of netrin-4 and its receptors in a knock-out mouse model under different pathological conditions for retinal angiogenesis and BRB breakdown.

Models employed to compromise the BRB or induce neovascularization in *Ntn4*^{-/-} mice and wildtype (WT) were: laser-induced choroidal neovascularization (CNV), oxygen-induced retinopathy (OIR) and streptozotocin-induced type 1 diabetes (STZ). Vessels were analyzed by fundus angiography (FAG). Immunohistochemistry was applied to retinal sagittal sections and flat-mounts. Gene expression levels were studied using qPCR. Retinal function was performed using Ganzfeld ERG.

Lack of netrin-4 does not dramatically alter retinal development and, in adulthood, retinal function are comparable to that in WT mice. However, FAG occasionally detected a different phenotype including cases of spontaneous leakage, more pronounced vessel tortuosity and presence of hyaloid vessels. *Netrin-4* gene expression levels in the retina were significantly higher than *Netrin-1* expression, and upregulated under the pathological conditions OIR and STZ. In STZ, *Unc5B* and *DCC* expression levels were upregulated only in netrin-4 presence. In OIR, *Unc5B* and *DCC* were differentially expressed and seem

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to be related to changes in retinal glial cells. CNV area after laser was unaltered in *Ntn4^{-/-}* mice compared to WT, although there was a reduction in vascular leakage. Under relative hypoxic conditions in OIR we observed larger avascular areas with faster revascularization.

We demonstrate that netrin-4 plays a role in retinal vascular pathophysiology, especially in hypoxia-driven neovascularization. Analysis of netrin-4 and its receptors Unc5B and DCC might help to better understand vessel stability in the retina and therefore lead to advances in current anti-angiogenesis treatments.

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Galectin-1 Is Associated with Progression of Diabetic Retinopathy

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Purpose: Galectin-1 has been recently shown to increase in the vitreous of proliferative diabetic retinopathy (PDR) eyes independently of vascular endothelial growth factor (VEGF)-A and to co-localize with VEGFR2 in fibrovascular tissues. Galectin-1 application to endothelial cells causes VEGFR2 phosphorylation and in vitro angiogenesis. The purpose of this study was to determine protein levels of galectin-1 in aqueous humor samples with diabetic retinopathy (DR).

Methods: Aqueous humor samples were collected from 25 eyes of 25 patients with DR, including 7 with diabetic macular edema (DME), 9 with PDR and 9 with neovascular glaucoma (NVG). For control, samples were obtained from 8 eyes of 8 age-matched, non-diabetic patients with age-related cataract (CAT) alone. Galectin-1 protein levels were measured by ELISA.

Results: Galectin-1 protein levels in eyes with DME*, PDR* and NVG** showed approximately 2-, 4- and 8-fold increases compared with those with CAT (* $P < 0.05$, ** $P < 0.01$), respectively. Among DR samples, there was a statistically significant difference between DME and NVG in galectin-1 levels ($P < 0.01$), but not between DME and PDR or between PDR and NVG.

Conclusion: The present data demonstrated intracameral protein levels of galectin-1, which we recently identified as the VEGF-A-independent angiogenic factor of PDR, in patients with various clinical stages of DR. Importantly, galectin-1 levels increased with the progression of DR

stages. We revealed for the first time the significant involvement of galectin-1 in the pathogenesis of DR along with the disease severity.

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Preventing pathological Pre-retinal Neovascularization through Modulation of Retinal Metabolism

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Proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP) are leading causes of blindness in adults and children, worldwide.

These diseases are characterized by retinal ischemia and hypoxia, generated by a mismatch between retinal blood supply and metabolic demand (of retinal neurons), which ultimately lead to pre-retinal neovascularization and vision loss.

Current therapies focus on (a) laser-induced thermal retinal destruction, to reduce metabolic demand in the periphery while preserving macular retinal neurons and, thus, central vision; and (b) vascular endothelial growth factor (VEGF) antagonism. These therapeutic strategies are imperfect, as they are either destructive or their effectiveness non-sustainable long-term (or both).

Highly sensitive mass spectrometry (MS)-based metabolomics is a powerful tool that is able to identify characteristic metabolic perturbations of different disease states, in body fluids (including blood and vitreous humor). Applying this technique to ischemic retinopathies could reveal new biomarkers and novel therapeutic targets.

In this work, MS-based metabolomic analyses identified a purine metabolite with putative neuroprotective properties, in the blood serum of "protected" diabetic patients. This metabolite's therapeutic potential was then tested in the oxygen-induced retinopathy (OIR) mouse model, by injecting it intravitreally and assessing its effects on retinal metabolism, retinal neuronal health and retinal vasculature (namely development of vaso-obliteration and pre-retinal neovascularization).

Treated eyes showed a significant reduction in (a) basal metabolism, specifically in ischemic (vaso-obliterated) areas, without compromising mitochondrial fitness or maximal respiratory rates; and in (b) area occupied by vaso-obliteration and pathological neovascularization, at P17. Furthermore, at P30, treated eyes showed significantly

better ERG responses than vehicle-injected eyes, under photopic conditions.

These results suggest that modulation of retinal metabolism, in a non-destructive fashion, could be a promising therapeutic strategy to effectively arrest progression of ischemic retinopathies.

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Effects of NDRG1 Family Proteins on Photoreceptor Outer Segment Morphology in Zebrafish

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Rods and cones are functionally and morphologically distinct. We previously identified N-myc downstream-regulated gene 1b (*ndrg1b*) in carp as a cone-specific gene. In this study, we first examined the localization of the three NDRG1 homologs, NDRG1a-1 and NDRG1a-2, the two splice variants of NDRG1a, and NDRG1b in developing and adult zebrafish retina. In adult zebrafish, all of the homologs were localized mainly in photoreceptors. NDRG1a-1 was localized in the entire cone plasma membranes, and also in rod plasma membranes except at its outer segment (OS). NDRG1a-2 was expressed in the thin process in cones, and NDRG1b was expressed only in cones and in the entire plasma membranes. To understand the functional role(s) of NDRG1 family proteins, we examined the effects of (1) knockdown of two of the dominant homologs, NDRG1a-1 and NDRG1b, at early developmental stages, and then (2) overexpression or ectopic expression of these NDRG1a-1, NDRG1a-2 and NDRG1b proteins in zebrafish rods. Knockdown of NDRG1a-1 or NDRG1b protein with morpholino(s) induced significant reduction of the OS volume in both rods and cones.

Overexpression of NDRG1a-1 in rods and ectopic expression of NDRG1b in rods under the control of *rhodopsin* promoter both induced macroscopic rod OS morphological alterations from cylindrical to tapered shape, which suggested that NDRG1a-1 and NDRG1b are involved in normal rod and cone OS development. Taper-shaped OSs isolated from these rods were not stained with *N,N'*-didansyl cystine that specifically labels infolded membrane structure of cone OS. The result suggested that tapered OS shape is not directly related to the formation of the infolded membrane structure in cone OS. No apparent

alteration was observed in rods where NDRG1a-2 was ectopically expressed. Because NDRG1a-1 and NDRG1a-2 are different in the amino acid sequence only in their N-terminal regions, this region in NDRG1a-1 seems to be important to cause the macroscopic rod OS morphological alterations. This work was supported by Japan Society for the Promotion of Science Grants 23227002 (to S. K.).

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Downregulation of the Liver X Receptor α/β and Sirt1 Signaling Axis Promotes Diabetic Retinopathy Pathogenesis

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Purpose: Clinical trials have demonstrated a strong association between dyslipidemia and the progression of diabetic retinopathy (DR). It has also been shown that activation of LXR α/β prevents diabetes-induced retinal vascular damage. Moreover, SIRT1 mediated LXR α/β activation has been shown to be beneficial in non-retinal systems, but the involvement of this signaling axis in DR remains unknown. The goal of this project was to investigate the role that the SIRT1-LXR signaling cascade plays in the progression of DR.

Methods: Bovine retinal endothelial cells (BRECs) were isolated, validated according to a previously published protocol and treated with pro-inflammatory cytokines and fatty acids. Human retinal endothelial cells (HRECs) were isolated from control or diabetic patients. LXR α/β , SIRT1, CYP46A1, CYP27A1, CYP11A1, ABCA1 and ABCG1 were analyzed by qRT-PCR. Cholesterol esters and oxysterols were analyzed using LC-nanoESI on LTQ Orbitrap Velos. db/db diabetic mice were used to model diabetic retinopathy *in vivo*.

Results: Retinal endothelial cells isolated from diabetic donors had significantly lower expression levels of LXR α/β and SIRT1 when compared to non-diabetic donors (n=6; p< 0.01). Additionally, LXR α was significantly decreased in BRECs treated with IL-1 β or TNF α (10ng/ml) (n=3; p< 0.05 or n=9; p< 0.05, respectively). Cholesterol metabolizing enzymes, CYP46A1, CYP27A1 and CYP11A1 were significantly increased after TNF α treatment (n=9;

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$p < 0.05$). Additionally, ATP binding cassette transporter, ABCA1, was significantly decreased in BRECs following TNF α administration ($n=9$; $p < 0.01$). Oxysterol levels were decreased ($n=9$ $p < 0.05$) and total retinal cholesterol ester abundance was increased in diabetic animals compared to control animals. Finally, retinal levels of SIRT1 and LXR α/β were downregulated in diabetic mice after 8 weeks of diabetes and LXR activation prevented development of acellular capillaries in db/db mice.

Conclusion: The work presented here demonstrates the detrimental effect diabetes has on LXR and SIRT1 retinal levels. Activation of LXR prevents DR-induced pathology in diabetic animals. Hence, these studies suggest that retinal-specific activation of LXR has the potential to be a novel therapeutic target in the treatment of DR.

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Neuroprotective Effect of Tetramethylpyrazine on Glutamate-induced Cytotoxicity in Differentiated Y-79 Cells via Inhibition of ROS Generation and Ca²⁺ Influx

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Background: Glutamate toxicity is estimated to be the key cause of photoreceptor degeneration in the pathogenesis of retinal degenerative diseases. Oxidative stress and Ca²⁺ influx induced by glutamate are responsible for the apoptosis process of photoreceptor degeneration. Tetramethylpyrazine (TMP), one of the alkaloids contained in *Ligusticum wallichii* Franch (*L. wallichii*), has been widely used for decades in China for the clinical treatment of retinal degenerative diseases, however, the detailed molecular mechanism underlying such effect is far from clear.

Methods: Using the approximate in vitro model, the neuroprotective effect and molecular mechanism of TMP against glutamate-induced cytotoxicity in differentiated Y-79 cells were first investigated which targeting at oxidative stress and Ca²⁺ hemostasis. Cytotoxicity assay, indirect immunofluorescence, flow cytometric analysis and western blot analysis were used in this study.

Results: The results showed that pre-treatment with TMP could significantly attenuate glutamate-induced cell viability loss, excessive ROS generation, calcium overload and up-regulated cell apoptosis in a dose-dependent manner. Furthermore, such effect was likely mediated through inhibition of glutamate-induced activation of

mitochondrial-dependent signaling pathway and CaMKII-dependent ASK-1/JNK/p38 signaling pathway.

Conclusion: Therefore, the present study supports the notion that TMP may be a promising neuroprotective agent for the treatment of retinal degenerative diseases.

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Neuronal ROR α Regulates Neurovascular Coupling in Retinopathy via Semaphorin 3E

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Dysregulated crosstalk between the retinal neurons and vasculature contributes to the pathogenesis of proliferative retinopathy, a leading cause of blindness. Class 3 semaphorins (Sema3) are a group of neuron-secreted axonal and vascular guidance factors protective for suppressing disoriented vascular growth in retinopathy. Yet the upstream transcriptional regulators that mediate Sema3 expression in retinal neurons remain poorly understood. Here we investigated the role of retinoic acid receptor-related orphan receptor alpha (ROR α), a lipid-sensing nuclear receptor and transcription factor, in the regulation of Sema3-mediated neurovascular coupling using a mouse model of oxygen-induced retinopathy (OIR). We found that genetic deficiency of ROR α substantially induced expression of *Sema3E* in OIR mouse retinas compared with littermate wild type controls, consistent with decreased neovascularization in ROR α -deficient OIR retinas. Both ROR α and *Sema3E* are expressed in retinal ganglion cells (RGCs), validated with immunohistochemistry and qPCR of laser capture microdissected retinal neuronal layers. ROR α directly recognized and bound to specific ROR α response element on the promoter region of *Sema3E* in the retinas, as revealed by chromatin immunoprecipitation and qPCR assay, showing approximately 2-fold enrichment compared with the positive control *Opn1mw*, a known ROR α target gene. ROR α significantly suppressed *Sema3E* promoter-driven transcriptional activity (>50%) in a dose-dependent manner, when co-expressed with native *Sema3E* promoter-driven luciferase reporter. Treatment with a synthetic ROR α inverse agonist (SR1001) also dose-dependently enhanced *Sema3E* promoter-driven luciferase activity. The transcriptional regulation of *Sema3E* by ROR α is dependent in part on ROR α interaction with its transcriptional co-regulators N-CoR and SRC3. Moreover,

suppression of *Sema3E* in *RORα*-deficient OIR retinas with intravitreal injection of *Sema3E* shRNA expressed from adeno-associated viral vectors promoted disoriented pathologic neovascularization and partially abolished the vascular effects of *RORα*-deficiency. Together, our findings suggest that *RORα* is a novel transcriptional regulator of *Sema3E* in RGCs and a critical regulator of neurovascular coupling in retinopathy via direct transcriptional modulation of *Sema3E*. These findings suggest that the *RORα*-*Sema3E* axis is a potential pathway to target for treating neovascular ocular diseases.

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The Joubert Syndrome Cilia Proteins ARL13B and AHI1 Differentially Modify the Severity of Retinal Degeneration Due to Loss of CEP290

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Ciliopathies refer to a spectrum of inherited disorders resulting from defects in the formation or function of cilia. Joubert Syndrome is a ciliopathy caused by mutations in at least 27 different genes, including genes encoding the cilia proteins *CEP290*, *AHI1*, and *ARL13B*. Joubert Syndrome characterized by a distinctive midbrain-hindbrain malformation and abnormal eye movements, and variable penetrance of other clinical symptoms. Retinal degeneration occurs in approximately 30% of Joubert Syndrome patients. The clinical variance is often attributed to mutations in genetic modifiers, which often encode other cilia proteins. The purpose of this study was to examine the zebrafish *cep290*^{fh297/fh297} and *cep290*^{sa1383/sa1383} mutants, which are a null and hypomorph alleles, respectively, and to determine if loss of *ahi1* or *arl13b* affects the penetrance of retinal degeneration in *cep290*^{fh297/fh297} mutants. The *cep290*^{fh297/fh297} zebrafish are adult viable, but infertile, and exhibit cone degeneration. The *cep290*^{sa1383/sa1383} zebrafish are adult viable and fertile, but also exhibit cone degeneration. No overt retinal phenotypes were observed at 5 days post fertilization (dpf) for zygotic mutants of either *cep290*^{fh297/fh297} larvae or *cep290*^{sa1383/sa1383} larvae. In contrast, maternal-zygotic *cep290*^{fh297/fh297} larvae exhibit both rod and cone degeneration by 5 dpf and die by 10 dpf, suggesting a requirement for maternal Cep290 protein in photoreceptor development. Zebrafish *ahi1*^{-/-} mutants were generated by TALEN-mediated gene editing. At 5 dpf the *ahi1*^{-/-} mutants exhibited mild cone

degeneration but had normal visual acuity. In contrast, the *arl13b*^{-/-} mutants had mild rod degeneration. We found that 5 dpf *cep290*^{fh297/fh297}; *arl13b*^{-/-} mutant larvae exhibited severe rod and cone degeneration, photoreceptor death, disorganized outer segment morphology, and rhodopsin mislocalization. In 5 dpf *cep290*^{fh297/fh297}; *ahi1*^{-/-} mutant larvae, cone outer segments were shorter but no rod degeneration or rhodopsin mislocalization was observed. Our studies demonstrate that genetic interactions between *cep290* and *arl13b* increase the severity of photoreceptor degeneration while genetic interactions between *cep290* and *ahi1* were considerably weaker. These data indicate that *arl13b* could act as a modifying locus affecting retinal degeneration following mutation of *cep290*.

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High Expression of Constitutively Monomeric Arrestin-1 Causes Degeneration of Rod Photoreceptors

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Arrestin-1 binds light-activated phosphorhodopsin and ensures timely signal shutoff. Arrestin-1 of different mammalian species self-associates forming dimers and tetramers. The biological role of this process remains unknown. We previously found that high expression of a mutant arrestin-1-3A with impaired ability to self-associate induces rapid degeneration of rods independently of rhodopsin activation by light. Similarly high expression of wild type (WT) arrestin-1 is not harmful to rods, and co-expression of WT arrestin-1 actually offers protection against toxicity of arrestin-1-3A. Since oligomerization is the only known way for WT arrestin-1 to affect arrestin-1-3A, these data suggested that WT arrestin protects rods by recruiting oligomerization-deficient mutant into mixed oligomers, and that monomeric arrestin-1 at high concentration is toxic. These findings suggested that oligomers of arrestin-1 serve as a non-toxic storage form in the dark, whereas monomeric arrestin-1 binds rhodopsin upon light activation.

However, arrestin-1-3A differed from WT in a number of other functional characteristics, in addition to its self-association deficiency. To directly test for the biological role of arrestin-1 self-association, we created several transgenic lines expressing arrestin-1 mutant deficient in self-association but otherwise unchanged. The expression level of the transgene ranged from 77% (M10 line) of the

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WT level to 292% (M5 line). The low expressing lines M10 (77%) and M4 (111%) have normal retinal morphology up to 1 year of age and displayed normal recovery kinetics in ERG. In contrast, higher expressors M9 (271%) and M5 (292%) demonstrate age-dependent degeneration of the outer nuclear layer (ONL) and reduced length of the photoreceptor outer segments. Retinal degeneration in these lines is more pronounced in the middle and peripheral than in the central retina. Dark rearing of these mice does not prevent the loss of photoreceptors suggesting that the mechanism of degeneration is independent of light-induced signaling. The loss of photoreceptors in M5 and M9 lines is accompanied by reduced amplitude of ERG a-wave and slower recovery kinetics. These data support the conclusion that monomeric arrestin-1 at high expression level is toxic for photoreceptors and that oligomerization serves to prevent this toxicity.

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The Molecular Mechanisms Leading to Alternative Splicing of the NXNL1 Gene: The Origin of the RdCVF Metabolic Signaling

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Photoreceptor cell death is a common feature in many blinding eye diseases. The number and localization of rods and cones in the retina vary widely among species. In the course of retinitis pigmentosa (RP), the progressive degeneration of rods is followed by a secondary loss of cones ultimately leading to total blindness. A neuroprotective agent RdCVF, produced by rods in order to maintain cone survival has been identified. We have recently revealed that, in cones, RdCVF protective mechanism is mediated by its receptor Basigin 1. It is now well accepted that administration of RdCVF is a promising therapeutic strategy for untreatable neurodegenerative diseases including RP, independently of the causative gene. In addition to RdCVF, generated by alternative splicing, *Nxn1* gene also encodes RdCVFL, an active thioredoxin enzyme. Interestingly, this alternative mRNA processing that led to the production of the trophic factor RdCVF only occurs in rod photoreceptors.

In an evolutionary standpoint, cone opsins were present before rhodopsin suggesting that cones preceded rods. The key regulatory event that leads to the trophic factor production is a splicing inhibition with intron retention. Our objective is to unravel the molecular mechanisms

responsible for *Nxn1* gene's alternative splicing and determine whether the process occurred in an ancestral gene or appeared contemporarily to the appearance of rods during evolution. Our preliminary findings indicate that the phenomenon of splicing inhibition of *Nxn1* is present in lampreys, which are ancient aquatic vertebrates and also the first animals to possess photoreceptors functioning as rods. We also found a similar mechanism in animals that predated the lampreys such as *Hydra Vulgaris*, which is sensitive to light despite its lack of visual eye. Although RdCVF is expressed in both lamprey and *Hydra Vulgaris*, its receptor Basigin 1 seems to be only present in lampreys. All together, our data indicate that the splicing inhibition of *Nxn1* that resulted in RdCVF production preceded the emergence of rods.

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Amelioration of Amyloid β Induced Retinal Inflammatory Responses by a LXR Agonist TO901317 Is Associated with Inactivation of the NF- κ B Signaling and NLRP3 Inflammasome

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Background: Retinal inflammation is a key pathogenic process in age-related macular degeneration (AMD). Amyloid β ($A\beta$) peptide, a major component of drusen which is the hallmark of AMD, is known to facilitate inflammatory responses. The purpose of this study was to investigate whether activation of liver X receptors (LXRs) ameliorates retinal inflammatory responses induced by $A\beta$ 1-40 and to explore the underlying mechanism.

Methods: Retinal inflammatory responses were induced with intravitreal injection of $A\beta$ 1-40 peptide in C57BL/6J mice. A synthetic LXR ligand TO901317 (TO90, 50 mg/kg/d) or vehicle was intragastrically administrated from 3 days before to 4 days after $A\beta$ 1-40 injection. The expressions of pro-inflammatory genes TNF- α and IL-6 were examined by real-time PCR. The levels of LXR α , LXR β and their target gene ABCA1, as well as NLRP3, caspase-1 and IL-1 β in the neuroretina and the RPE/choroid complex were detected with real-time PCR and western blotting. The changes of phosphorylated transcription inhibition factor- κ B α (p-I κ B α) in the neuroretina and the RPE/choroid complex were detected with western blotting. Retinal function was assessed with electroretinogram (ERG).

Results: The mRNA expressions of LXR α and LXR β decreased in the neuroretina of the $A\beta$ 1-40-injected

mice. No significant difference was found on the protein expressions of LXRs and ABCA1 in both neuroretina and RPE/choroid complex between the A β 1-40-injected group and the control group. TO90 enhanced the expressions of LXR α and ABCA1 at both mRNA and protein levels in the A β 1-40-injected mice, while the LXR β expression was unchanged. TO90 preserved ERG a- and b-wave amplitudes in the A β 1-40-treated mice. Meanwhile, compared with the A β 1-40 plus vehicle-treated group, the mRNA levels of the pro-inflammatory cytokines TNF- α and IL-6 were significantly decreased in the A β 1-40 plus TO90-treated group. Furthermore, TO90 downregulated the phosphorylation of I κ B α as well as the expressions of NLRP3, caspase-1 and IL-1 β in the neuroretina and the RPE/choroid complex of the A β 1-40-injected animals.

Conclusion: Activation of LXR α and ABCA1 with TO90 inhibits retinal inflammatory responses induced by A β 1-40 in mice. It appears that the anti-inflammatory effect is mediated mainly by LXR α . Furthermore, the beneficial effect is associated with inhibition of the NF- κ B pathway and the NLRP3/caspase-1/IL-1 β axis. LXR agonist may become a new class of anti-inflammatory agent for AMD.

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Over-expression of Angiotensin-converting Enzyme 2 (ACE2) Ameliorates Amyloid β -induced Inflammation in Human Retinal Pigment Epithelium Cells

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Purpose: Retinal chronic inflammation is implicated in the pathogenesis of some ocular diseases including age-related macular degeneration (AMD) which is a common cause of irreversible vision loss in the elderly. Amyloid- β (A β), a major constituent of drusen, is a hallmark of early AMD. The purpose of this study was to investigate whether enhancement of ACE2, an important component of the protective ACE2/Ang-(1-7)/Mas axis of renin angiotensin system (RAS), ameliorated A β -induced inflammatory response and whether the underlying mechanisms is associated with the mitogen-activated protein kinase (MAPK) and the nuclear factor- κ B (NF- κ B) pathways.

Materials and methods: Cell counting kit-8 (CCK-8) assay and Annexin-V FITC/PI assay were used to determine the optimum concentration and incubation time of A β 1-42. ACE2 plasmid was transfected into primary cultured human retinal pigment epithelia (hRPE) and ARPE-19 cells

for 6 hours before stimulated with 1 μ M of A β 1-42 for 48 hours. The gene expression was detected by real-time PCR and the protein levels were determined by Western blotting or ELISA. Inhibitors of BAY 11-7082 for NF- κ B and MAPKs including SB203580 for p38 MAPK, SP600125 for c-Jun N-terminal kinase (JNK) and PD98059 for extracellular signal-regulated kinase 1/2 (ERK1/2) were used to validate the involvement of these pathways. A779, a selective antagonist of Ang-(1-7), was added to confirm the involvement of ACE2/Ang-(1-7)/Mas axis.

Results: A concentration of 1 μ M A β 1-42 and an incubation time of 48 hours were verified with flow cytometry and CCK-8 analysis. ACE2 plasmid significantly up-regulated the expression of ACE2 and Ang-(1-7) in both human RPE cell lines. Activation of ACE2 reduced the over-production of inflammatory cytokines IL-1 β and MCP-1 induced by A β 1-42. While a selective antagonist of Ang-(1-7), A779 reversed the anti-inflammatory effect. Moreover, JNK, ERK1/2 and NF- κ B inhibitors decreased the overproduction of the inflammatory cytokines induced by A β 1-42.

Conclusion: Over-expression of ACE2 ameliorates A β -induced inflammatory response by upregulating the ACE2/Ang-(1-7)/Mas axis in human RPE cells. The beneficial effect is associated with inhibiting of the MAPK and the NF- κ B pathways. The ACE2/Ang-(1-7)/Mas axis may be a promising target for developing novel therapies for chronic inflammatory ocular diseases such as AMD.

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The Effect of Epoxygenated Fatty Acids on Cytokine-induced Inflammation in Retinal Müller and Endothelial Cells

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Multiple pro-inflammatory cytokines are elevated in the vitreous of diabetic patients early in the pathogenesis of diabetic retinopathy (DR), including TNF α , IL-1 β , IL-6, and IL-8. In the present study, we evaluated the relative potency of these inflammatory mediators *in vitro* in order to establish experimental platforms for assessment of therapeutic strategies. Using this approach, we determined the effectiveness of a family of endogenous anti-inflammatory epoxygenated lipids, the epoxyeicosatrienoic acids (EET) and epoxydocosapentaenoic acids (EDP), against select inflammatory stimuli in human Müller cell

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(HMC) and human retinal microvascular endothelial cell (HRMEC) cultures.

HMC were treated with 1ng/ml TNF α , IL-1 β , IL-6, or IL-8 and the auto-amplification of cytokine expression was measured by qRT-PCR. IL-1 β was the most potent inducer of all cytokines, stimulating expression of TNF α by 100.4-fold, IL-1 β by 499.6-fold, IL-6 by 47.4-fold, and IL-8 by 1182.2-fold. IL-1 β co-treatment with 11,12-EET inhibited expression of IL-1 β (35.7%; $p=0.01$), IL-6 (30.1%; $p=0.012$), and IL-8 (27.3%; $p=0.014$). IL-1 β co-treatment with 19,20-EDP also inhibited expression of IL-1 β (22.8%; $p=0.031$), IL-6 (37.2%; $p=0.004$), and IL-8 (24.1%; $p=0.011$). However, IL-1 β co-treatment with 11,12-EET or 19,20-EDP counter-intuitively induced TNF α expression by 2-fold ($p=0.001$) and 1.8-fold ($p=0.015$), respectively.

HRMEC were treated with 1ng/ml TNF α , IL-1 β , IL-6, or IL-8; cell adhesion molecule expression was assessed by qRT-PCR and leukocyte adhesion was measured using a parallel plate flow chamber assay. TNF α was the most potent cytokine in both assays; TNF α stimulated expression of VCAM-1 by 478.6-fold and ICAM-1 by 120.6-fold and leukocyte adhesion by 4.3-fold. 11,12-EET with AUDA

(a soluble epoxide hydrolase inhibitor to prevent epoxide hydrolysis) inhibited TNF α -induced VCAM-1 (32.5%; $p < 0.001$) and ICAM-1 (31.9%; $p < 0.001$) expression. 19,20-EDP with AUDA also reduced TNF α -stimulated VCAM-1 (36.1%; $p < 0.001$) and ICAM-1 (20.3%; $p=0.003$) expression. 11,12-EET or 19,20-EDP with AUDA inhibited TNF α -induced leukocyte adhesion by 47.9% ($p=0.016$) and 49.9% ($p=0.012$), respectively.

In this study, we demonstrated that IL-1 β and TNF α are the most potent stimulators of inflammatory behaviors in retinal glial-vascular cells. Additionally, EET and EDP significantly reduce both production of and response to cytokines, suggesting they might be ideal therapeutic candidates.

hemorrhage are associated with increased risk of retinal detachment and poor prognosis. Retinal angiogenesis is preceded by patterning of astrocytes, which provide guidance cues for endothelial cells. Current surgical and pharmacologic interventions of ROP are mainly targeting the aberrant vessels to prevent disease progression, but the involvement of the neuroretina has been overlooked. In this study, we investigated the retinal proteoglycans in astrocyte migration and vascularization. Ablation of glycosaminoglycan side chains of proteoglycans in neuroretina led to impaired astrocyte migration, compromised retinal angiogenesis, and hyaloid vessel persistence, features shared in some infants with ROP. Proteoglycan-deficient pups undergoing oxygen-induced retinopathy (OIR) developed vitreous hemorrhage that never resolved. Notably, inner limiting membrane (ILM), the basement membrane of the retina, was breached in those mutant retinae prior to the formation of astrocytic network. We propose that neuroretinal cell surface proteoglycans are essential for the initial assembly of ILM, and this cannot be compensated by secreted ECM proteoglycans. In support of this, after removal of ILM in retinal explant by Collagenase digestion, wild type retina can establish a new ILM when incubated with exogenous laminin-supplemented Matrigel. This basement membrane reconstitution failed, however, on proteoglycan-deficient retina or in wild type samples pre-treated with a combination of Heparitinase/Chondroitinase in addition to Collagenase. Taken together, our study reveals a novel function of neuroretinal cell surface proteoglycans in the initial assembly of basement membrane, which subsequently serves as a permissive substratum necessary for astrocyte migration and retinal angiogenesis. These results suggest that assessment of ILM integrity may be of prognostic and therapeutic significance in OIR patients.

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Regulation of Astrocyte Migration and Retinal Angiogenesis by Cell Surface Proteoglycans

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Retinopathy of prematurity (ROP) is one of the leading causes of blindness in children. Though mostly mild and can resolve on its own, ROP of advanced stages leads to complete vision loss if left without medical treatment. In ROP patients, retinal vessel malformation and vitreous

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Selection of Human iPSC-derived Photoreceptors by Targeting of a Cell Surface Antigen

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Rescuing the degenerated retina is a major challenge for which specific cell replacement is one of the most promising approaches. Pluripotent stem cells could be used as an unlimited source of retinal cells since it has been demonstrated they are able to be committed into the retinal lineage and further differentiated into cells expressing photoreceptor markers. Starting from the protocol developed by our group (Reichman *et al.* 2014) for the generation of retinal organoids from human induced pluripotent stem cells (hiPSCs), our work aims to characterize the surface antigen CD73 as a specific marker of hiPSC-derived photoreceptors and as a target for the separation of transplantation-competent photoreceptor precursor population.

Analysis of CD73 expression in our hiPSC-derived retinal organoids indicated that CD73 is specific of cells committed into the photoreceptor lineage, as all of the CD73+ cells co-localized with a well-established marker of photoreceptors, RECOVERIN. Cytofluorometric analysis indicated that the percentage of CD73+ cells in dissociated retinal organoids increased with maturation, with CD73+ cells representing more than 60% of cells at day 180 of differentiation. Dissociated retinal cells expressing CD73 could be sorted by Magnetic-Activated Cell Sorting (MACS), leading to enrichment to 90% of CD73+ cells in the positive sorted fraction. RT-qPCR analysis on sorted CD73+ cells showed over-expression of the most significant photoreceptor-specific genes compared to dissociated retinal cells before CD73 MACS. Re-plating of the CD73+ sorted cells showed that cells were mostly viable, even several days after the separation process, and positive for CRX and RECOVERIN by immunostaining. Freeze-thawing of both whole retina organoids and retinal cells obtained by previous dissociation of the retinal structures resulted in viable cells with unaffected expression of CD73 on the cell surface of photoreceptors.

To conclude, our results support the use of CD73 as a marker of hiPSC-derived photoreceptors, since MACS of CD73+ cells resulted in a significant enrichment of photoreceptor precursors. We also achieved an efficient protocol to bank both retinal organoids and dissociated retinal cells, which will be of a great utility for a clinical translation of pluripotent stem cell-derived neuro-retinal cells.

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Retinal HIF-1 α and VEGF Levels Correlate with Ocular Circulation Measured by the Laser Speckle-micro in an Oxygen-induced Retinopathy Rat Model

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Retinopathy of prematurity (ROP) is a leading cause of childhood blindness worldwide. The neovascularization, retinal vascular dilation and tortuosity observed in ROP are hypothesized to be caused by an increase in the oxygen concentration and other factors. In animal models of ROP, vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1) were shown to be mechanistically involved in the intravitreal neovascularization. VEGF and hypoxia inducible factor (HIF)-1 α are thought to be closely related. Here we used the Laser Speckle Flowgraphy (LSFG)-Micro system (Softcare, Fukuoka, Japan) to examine the relationship between ocular blood flow and various factors in oxygen-induced retinopathy (OIR) model rats. Ten OIR rats were compared with 10 rats reared in room air (Controls). Neonatal Sprague-Dawley rat pups and their dams were placed in an Oxycycler chamber that cycled oxygen between 50% and 10% every 24 h for 14 days (i.e., the 50/10 OIR model). Pups and dams were moved to room air at postnatal day (P) 14. On P18, the rats were anesthetized. On the cornea of each rat, we set a viscoelastic material and a cover glass, and then we measured the ocular blood flow (mean blur rate; MBR) with the LSFG-Micro system. We analyzed the MBR by setting a rubber band on a circle with a radius of 50 pixels (0.25 mm) centered on the optic nerve head center, using LSFG Analyzer software. The recorded MBR values at each time point were the averages of three successive measurements. At P18, the rat pups were sacrificed, and the eyes were collected. The retinal VEGF, HIF-1 α and IGF-1 values were measured by ELISA. We excluded outliers that are considered abnormal when compared with other relevant data. No significant differences in MBR were observed between the control and OIR rats, but compared to the Control rats, significantly higher values of retinal VEGF ($p=0.0002$), retinal HIF-1 α ($p=0.0002$) and IGF-1 ($p=0.0036$) were revealed in the OIR rats. Significant correlations were seen between the MBR and VEGF values ($r=0.77$, $p=0.0095$) and between the MBR and HIF-1 α ($r=0.75$, $p=0.0116$) in the P18 OIR rats. These results suggest that the MBR could serve as an indicator of OIR severity. The LSFG-Micro system provided reproducible blood flow measurements in neonatal rats.

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From Progenitors to Neurogenesis: Multiple Roles for Semaphorin 3f SignalingRAMI HALABI¹, Sarah McFarlane²¹University of Calgary - Hotchkiss Brain Institute, Neuroscience, Calgary, Canada, ²University of Calgary - Hotchkiss Brain Institute, Cell Biology & Anatomy, Calgary, Canada

The developing eye requires the coordination of multiple processes from different contributing cell types to establish tissue functionality. Specifically, during early eye morphogenesis, fetal vasculature is establishing with subsequent waves of neural differentiation proceeding thereafter. Our objective is to understand whether coordination of such processes occurs through concerted molecular mechanisms during embryonic eye development. Here we identify the secreted guidance molecule, Semaphorin 3f (Sema3f), as a novel factor involved in multiple, concomitant processes throughout eye development. *sema3fa* is expressed in the presumptive temporal neural retina during optic cup formation and remains expressed in a spatially restricted manner around the choroid fissure and temporal retina prior to neural differentiation. This expression then resolves to the retinal ganglion cell (RGC) layer and inner nuclear layer during the final stages of retinal neurogenesis. This dynamic expression, both spatially and temporally, is suggestive of multiple roles for Sema3fa signaling during retinal development. To understand these roles, we use zebrafish embryos injected with either antisense morpholino or CRISPR interference guide-RNA to block Sema3fa production. We find that Sema3fa deficient embryos present with microphthalmia and coloboma of the eye by 48 hours post fertilization (hpf), specifically the nasal retina tissue is thicker than the temporal, suggesting that Sema3fa is acting within the domain in which it is expressed. Interestingly, while in control embryos the rate of proliferation is equivalent across the nasal-temporal axis of the eye, we find a 60% reduction in the number of mitotic cells within the temporal retina of deficient embryos. This deficiency in proliferation, and no increase in apoptosis, is the likely cause of coloboma resulting in an asymmetry of tissue during fissure closure. In addition to the early progenitor role, we observe delayed differentiation of RGCs in the temporal retina, characterized by an absence of *islet 2b* expression by 48 hpf. Later in development, when expression of *sema3fa* resolves from progenitor cells to the nuclear layers of the retina, deficient embryos present with aberrant sprouting off the hyaloid artery and

optic nerve. Overall, these data support the possibility that a single signalling ligand can function differently on multiple cell types undergoing coordinated development to establish tissue functionality.

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Cell Cycle Reentry and DNA Damage Response of Müller Glia after Retinal InjuryKAORI NOMURA-KOMOIKE¹, Fuminori Saitoh¹, Yuta Komoike², Hiroki Fujieda¹¹Tokyo Women's Medical University, Anatomy, Shinjuku, Japan, ²Tokyo Women's Medical University, Hygiene and Public Health I, Shinjuku, Japan

Müller glia, the principal glial cell type in the retina, have the potential to dedifferentiate, proliferate, and redifferentiate into neurons after retinal damage. However, unlike fish and birds, this capacity of Müller glia is extremely limited in mammals. Therefore, to gain new insights into the mechanisms that hamper retinal regeneration in mammals, we examined the cell cycle progression and DNA damage response in Müller glia after retinal damage. Expression of cell cycle-related proteins and DNA damage response were analyzed in the adult rat and mouse retinas after N-methyl-N-nitrosourea (MNU) or N-methyl-D-aspartate (NMDA)-induced retinal damage. The adult zebrafish and postnatal rat retinas were also investigated for comparison. Analysis was conducted by using immunofluorescence, western blotting, and quantitative real-time polymerase chain reaction. In the rat retina, most Müller glia reentered the cell cycle after MNU-induced photoreceptor damage and showed DNA damage response including H2AX phosphorylation and upregulation of p53 and p21. The DNA damage response was also observed in rat Müller glia after NMDA-induced loss of inner retinal neurons, but not in zebrafish Müller glia or rat retinal progenitor cells. In the mouse MNU model, Müller glia proliferation and the DNA damage response were not observed. However, in the mice deficient in two cell cycle inhibitors p27 and p21, Müller glia reentered the cell cycle and showed DNA damage response after MNU treatment similar to the rat models. Our findings suggest that the DNA damage response induced by unscheduled cell cycle reentry may be one of the mechanisms that limit the proliferative and regenerative capacity of Müller glia in the mammalian retina.

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Impaired Retinal Synaptic Transmission in Simvastatin Fed MiceAMANY MOHAMED¹, Sarah Samuelson¹, John Dimopoulos², Monika Sharma³, Yves Sauve^{2,4}, Ian MacDonald², Elena Posse de Chaves¹¹University of Alberta, Department of Pharmacology, Edmonton, Canada, ²University of Alberta, Department of Ophthalmology and Visual Sciences, Edmonton, Canada, ³University of Alberta, School of Dentistry, Edmonton, Canada, ⁴University of Alberta, Department of Physiology, Edmonton, Canada

The cholesterol synthesis inhibitor, Simvastatin, is highly effective in preventing cardiovascular disease. In addition to reducing cholesterol level, Simvastatin also decreases isoprenoid production. Isoprenoids are required for post-translational modification of small GTPases (prenylation). Many retinal diseases have been associated with impaired prenylation of small GTPases. Our goal is to examine whether simvastatin-induced inhibition of protein prenylation might have a detrimental effect on retina function.

Methods: C57BL6 mice were fed a simvastatin containing diet (10mg/kg/day) for 6 months. Blood samples were obtained along with electroretinogram recordings before the start of the treatment and throughout the course of treatment. At the end of the treatment, eyes were collected, a subset was dissected to separate the neural retina from the eyecup (RPE) for biochemical analysis of cholesterol mass using Amplex red kit and protein prenylation using Rab GDI capturing method; the remaining eyes were processed for flat mount preparation and cryosectioning.

Results: Simvastatin treatment slightly decreased the amplitude of both scotopic a- and b-waves. Interestingly, by applying the Naka-Rushton equation, a significant drop was revealed in the scotopic b-wave sensitivity to the light impulses (k value) in retinas of simvastatin-treated mice. This indicates inefficient retinal signal transmission. Moreover, cryosections of simvastatin-treated mouse retinas showed bipolar cells sprouting, a previously reported potential compensatory mechanism in response to reduced photoreceptor pre-synaptic modulation. RPE flat mounts of simvastatin-treated mice showed the presence of larger RPE cells, a feature that is observed in retinal degenerative diseases upon RPE cell loss. Levels of plasma and retinal cholesterol mass as well as retinal Rab protein prenylation did not differ between treated and untreated mice.

Summary and conclusions: These experiments suggest that simvastatin treatment might have detrimental effects on retinal function. This may constitute a possible side effect

common to all statins and could be particularly relevant to patients with pre-existing retinal diseases.

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Amyloid β 1-42 Activates the Complement System and Induces Retinal Inflammatory Responses and Malfunction in Mouse

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Purpose: To investigate whether an oligomeric peptide amyloid β 1-42 (A β 1-42) induces retinal inflammatory responses through the complement system.

Methods: To determine the optimal concentration and times, different concentrations of an oligomeric peptide A β 1-42 (0.125 mM, 0.25 mM, 0.5 mM, 1 mM in 2 μ l) or PBS were injected intravitreally into C57BL/6J mouse eyes. The expressions of the major inflammatory cytokines IL-6, TNF- α and the key components of the complement system, including C1qa, C3, complement factor B (CFB), complement factor D (CFD), complement factor H (CFH) and CD59a, were analyzed by real-time PCR at days 1, 4 and 7 after A β 1-42 injection. The fold changes of cytokine and the complement component genes in the A β 1-42 groups were compared with those treated with a reverse peptide A β 42-1 at days 4 and 7. Retinal function was assessed by dark- and light-adapted electroretinography (ERG) at the same time.

Results: Compared with the PBS group, the mRNA expressions of C1qa and C3 in the A β 1-42 treated retina increased from day 1 to day 7. The level of CFB, CFD or CFH increased from day 4 to day 7. A regulator of membrane attack complex (MAC) CD59a increased from day 1 to day 7. The retinal gene expression of IL-6 in mouse treated with A β 1-42 increased from days 1 to 7, and the expression of TNF- α increased at days 1 and 4. At days 4 and 7 after intravitreal injection, the amplitudes of the dark-adapted ERG b- and a-wave and light-adapted b-wave significantly decreased in the A β 1-42 treated eyes when compared with the PBS and the A β 42-1 treated eyes.

Conclusion: It is found that A β 1-42 induced a time-dependent activation of the complement pathways. We confirm that exogenous A β 1-42 activated the classical and the alternative pathways of the complement system and induced inflammatory responses and malfunction of the retina. Our data support the notion that over expression of A β may be involved in the pathogenesis of age related macular degeneration.

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Biocompatibility of a Novel Biopolymer Scaffold for Retinal Cell Transplantation in the Subretinal Space of Pigs

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Purpose: Photoreceptor cell replacement has demonstrated potential for restoring vision in retinal degenerations, but only a small fraction of transplanted cells survive and integrate into the host retina. This failure is largely due to lack of physical support for the cells after injection. We tested the biocompatibility of a hydrogel-based polymer scaffold, poly(caprolactone) (PCL), suitable for patient-specific iPSC-derived retinal cells, in the subretinal space of a large animal model.

Methods: Methacrylate-functionalized PCL scaffolds were polymerized at 50 wt% with Irgacure 651 by exposure to UV light. Complex prototype structures with varying pore sizes were created using two-photon polymerization with Irgacure 369 as the initiator. Compressive modulus was measured using dynamic mechanical analysis. Four month old Yucatan mini pigs (n=5 eyes from 5 animals) had 23G three-port vitrectomy and hyaloid induction. A subretinal bleb was raised with BSS, followed by placement of a 1.25 x 4mm PCL scaffold in the subretinal space, then fluid-air exchange. Five pig eyes underwent control surgery (i.e. same as experimental arm except no subretinal polymer). Animals were sacrificed 1 month after surgery at which time ophthalmoscopy and spectral-domain OCT (SD-OCT) were performed; eyes and other tissues were collected. Histological analysis was performed on paraffin sections of control and experimental retinas.

Results: At 1 month after surgery, ophthalmoscopy revealed all 10 eyes had complete, spontaneous retinal reattachment. PCL polymer implants were detectable by ophthalmoscopy in 5/5 treated eyes, and their anatomic location in the subretinal space was confirmed *in vivo* with SD-OCT. On SD-OCT the surface of the polymer was hyper-reflective while the body was hypo-reflective compared to the retina. No eyes had evidence of intraocular inflammation or vitreous opacities. Hematoxylin-eosin stained sections through a subset of injected eyes demonstrated subretinal location of the implanted polymer, and preservation of retinal layers adjacent to the implant.

Conclusions: PCL polymer can be successfully delivered to the subretinal space and is well-tolerated

ophthalmoscopically. This knowledge will be fundamental to the development of effective autologous cell-based sub-retinal transplantation grafts for retinal degenerative diseases.

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Immunohistochemical and Transcriptome Analyses of the Developing Human Fetal RetinaAKINA HOSHINO¹, Rinki Ratnapriya², Chi Zhang¹, Rachel Wong¹, Anand Swaroop², Thomas Reh¹¹University of Washington, Seattle, United States, ²National Eye Institute, NIH, Bethesda, United States

The retina is composed of 6 different types of neurons and 1 glia that are derived from a common progenitor pool in an organized temporal order. While the specific order of cell generation is highly conserved among species, the duration of retinal development varies. For example, retinogenesis in the mouse is complete within a few weeks whereas in the human it takes months. Furthermore, human retinas have a unique area called the fovea, which develops faster than the rest of the retina. Previous studies of the human retina have focused primarily on photoreceptors and opsin expression in the fovea. To better understand the spatial and temporal gene expression patterns affecting the development of the human fetal retina, we performed immunohistochemical (IHC) and RNA-seq analyses on human fetal retinas from 52-150 days (d) post-conception. We found that by 59d, the incipient fovea could already be distinguished from the surrounding regions as an area of the central retina temporal to the optic nerve that had fewer mitotic cells, a thick layer of ganglion cells, and a neuroblastic layer containing cones, horizontal cells, and amacrine cells. Surprisingly, by 73d, the fovea expressed markers of all the major cell types, including Müller glia. For retinas older than 96d, the fovea was identified as an area with a single layer of cones in the outer nuclear layer that was devoid of S-Opsin and Nr2E3 expression. By this time, cells had organized into the appropriate lamina. We also observed expression of synaptic markers in the inner plexiform layer as early as 67d and in both plexiform layers from 96d.

Our RNA-seq data correlated well with our IHC data and captured onset of gene expression in the fovea. Principle component analysis revealed that age was the major driving factor driving the differences across samples. In addition, we performed unsupervised hierarchical clustering analysis to identify four gene clusters. Interestingly, a subset of genes that was downregulated over time was

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related to ganglion and progenitors cells. Accordingly, at 59d by IHC, the retina was composed mostly of these two populations, and the proportion of these declined over time as other cell types were generated.

These data suggest that neurogenesis in the fovea is complete by 96d and is developmentally ahead of the peripheral retina by as much as 6 weeks. This is an important consideration for future developmental studies or when staging the development of hESC-derived retinas.

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A Novel Approach for the Determination of Retinal Temperature Based on ERG Photoresponses

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Heating of the retinal pigment epithelium (RPE) offers a potential treatment for several retinal diseases. However, the lack of effective methods for measuring the temperature rise of retina and RPE hinders the development and testing of treatment procedures. In this study we lay the foundation for a retinal temperature determination method which is based on two mutually independent temperature-dependent properties of photoresponses recorded by electroretinography (ERG): Firstly, temperature elevation leads to acceleration of response kinetics. Secondly, relative photoreceptor sensitivity to long wavelength (low photon energy) stimuli increases when temperature rises. Scotopic ERG responses to dim flashes (amplitudes ~15% of b-wave maximum, stimulus wavelengths 532 nm and 780 nm) were recorded from thirteen isolated mouse (C57BL/6J) retinas at temperatures 35.0 - 44.0 °C. The temperature-dependence was determined for 21 different features extracted from the responses with the feature value at 37.0 °C as a reference. Cross validation of the data was utilized to select the best feature combination and a multivariable linear regression model was fitted between the feature values and the temperatures. Similar *in vivo* ERG responses were recorded from five mice to obtain preliminary information on the applicability of the method. The resulting temperature determination model based on *ex vivo* ERG consisted of two features: time-to-80% of the b-wave trailing edge, and the amplitude ratio of responses to 780 nm and 532 nm flashes (representing long-wavelength relative sensitivity). Testing the performance of the model with a separate dataset of *ex vivo* responses showed RMS temperature prediction error of < 0.60 °C for predictions based on one dim flash response pair.

Additionally, a model consisting only of a single kinetics feature (time-to-peak of b-wave) was constructed using *ex vivo* responses. Testing this model with the feature values extracted from *in vivo* responses led to RMS prediction error of < 0.80 °C (2-10 responses averaged). The feature representing the long-wavelength relative sensitivity was not used in the latter model since, for an unknown reason, it was not functional at *in vivo* recordings.

Retinal temperature determination accuracies obtained by the models described above would be sufficient in various retinal and RPE heating applications as well as in other cases where retinal temperature is of interest and ERG recording is feasible.

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Protection Against Retinal Degeneration in the RCS Rat by a Traditional Chinese Medicine, BSYJ FormulaLINA LIANG¹, Xueli Li¹, Youzhi Tang¹, Kai Xu¹, Jiping Fan^{1,2}¹Eye Hospital, China Academy of Chinese Medical Sciences, Beijing, China, ²China Academy of Chinese Medical Sciences, Beijing, China**Background:** In China, retinitis pigmentosa has been treated by acupuncture and Chinese medicinal herbs for decades.**Objective:** To investigate the effect of a traditional Chinese medicine, BSYJ formula on retinal degeneration in RCS rats.**Methods:** Royal College of Surgeons (RCS) rats were used as an animal model of retinal degeneration. RCS rats were randomly divided into two groups: the distilled water group and the BSYJ group, while SD rats were used as normal control group. The mice of BSYJ group were gavaged with BSYJ solution(8.8 g·kg⁻¹), and the mice of distilled water group were gavaged with distilled water, while the SD mice were fed normally. At 7 days, 14 days and 28 days after treatment, the effect was assessed respectively. The flash electroretinogram (F-ERG) was examined to detect the function of the retina. HE staining was used to evaluate the retinal histopathological changes and the number of photoreceptor nuclei in the outer nuclear layer (ONL) was also counted.**Results:** ERG results showed that compared with normal control group, the amplitude of RCS rats was significantly decreased, and almost disappeared when the RCS rats were 7 weeks old. At 7 days after treatment, compared with the distilled water group, the amplitude of photopic ERG, scotopic ERG and Ops of the BSYJ group was increased slightly, but the difference was not statistically significant ($P > 0.05$). At 14 days after treatment, b-wave amplitudes of scotopic ERG in the BSYJ group was significantly higher than the distilled water group ($P < 0.05$). At 28 days after treatment, the amplitudes of scotopic ERG, photopic ERG, and OPs were all markedly higher than the distilled water group ($P < 0.05$). It was shown by pathological examination that compared with SD mice, the ONL layer in the RCS got thinning gradually, and the number of nucleus decreased either. Compared with the distilled water group, the pathological change in the BSYJ group was been improved and more nuclei were preserved ($P < 0.05$).**Conclusion:** BSYJ has protective effect on retinal degeneration lesion in RCS rats, and it may delay the

development process of retinal degeneration.

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Vitrectomy with Simultaneous Intravitreal Triamcinolone Injection versus Vitrectomy with Simultaneous Dexamethasone Intravitreal Implant for the Treatment of Diabetic Macular Edema

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*Catholic University of Korea, Department of Ophthalmology, Incheon, Korea, Republic of***Purpose:** This study was performed to compare the effect of dexamethasone intravitreal implant 0.7 mg (dexamethasone delivery system [DDS], Ozurdex) and triamcinolone (4 mg/0.1 mL) for the treatment of persistent diabetic macular edema (ME), which were injected during phacoemulsification and vitrectomy surgery.**Methods:** Treatment and outcomes data were collected retrospectively for 32 eyes from 32 patients who underwent phacoemulsification and vitrectomy for the treatment of persistent diabetic ME. Intravitreal Injection of Triamcinolone (IVTA) or DDS was performed simultaneously after vitrectomy and outcome measures included visual acuity, central retinal thickness (measured by optical coherence tomography) at 1, 3 and 6 month after the surgery.**Results:** Best-corrected visual acuity was significantly improved at 1, 3, 6 months after injection in both the IVTA and DDS injected groups. Patients who received DDS injection appeared to have quicker visual recovery and improved central macular thickness at 1 and 3 months compared with those who received IVTA treatment ($P < 0.05$). The mean central macular thickness (CMT) in eyes with DDS was significantly thinner than in the IVTA eyes at 3 month ($246 \pm 58.4 \mu\text{m}$ and $288.4 \pm 68.5 \mu\text{m}$, respectively; $P < 0.05$) and 6 months ($254.2 \pm 49.4 \mu\text{m}$ and $289.5 \pm 79.4 \mu\text{m}$, respectively; $P < 0.05$) after injection.**Conclusions:** Simultaneous injection of DDS during phacoemulsification and vitrectomy surgery can effectively improve best-corrected visual acuity and reduce central macular thickness in patients with persistent diabetic macular edema (ME) compared to intravitreal triamcinolone injection.

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Perfluoro-n-octane Toxicity: A Sanitary Alert

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Recent several acute cases of blindness after vitrectomy proceeded to circulate alerts by various sanitary product monitoring agencies. It was suspected perfluoro-n-octane (PFO) to produce acute toxicity and current testing methods to fail in detecting this toxicity. Hence, several lots of PFO (manufactured in 2013, 2014 and 2015) were tested (n=9) in human retinal pigment epithelium (RPE) cell line ARPE-19 (ATCC® CRL-2302™) and porcine neuroretinal organotypic cultures (n=3). Culture plates prepared following ISO norms (UNE EN ISO 10993-12:2007 and 5:2009) were exposed to suspected PFO (experimental groups), non toxic PFO (negative controls; PFO of same manufacturer (lots 2013 and 2015); PFO of other two manufacturers) and phenol (positive control). It was performed MTT assay for cell cultures (30 and 60 minutes exposure followed by 24 and 72 hours cultures) and microscopic study of toluidine blue stained of epoxy resin embedded neuroretina explants (time zero explants, 30 minutes exposure followed by 72 hours cultures) followed by qualitative grading (grade 0: normal, 1: light, 2: mild, 3: moderate and 4: severe) depending on anatomical structure damages. Results showed in unexposed and control negative groups (lot 2015 and PFO of other two manufacturers), cultures were confluent without any significant morphological changes (>70 viability; non toxic according to ISO norms) however, control positive groups were completely dead (< 70% viability; toxic according to ISO norms). The OD reading was >0.2 and difference between left and right columns and mean of both of unexposed groups was < 15% confirming quality of experiments followed ISO norms. A lot of 2013 which was provided as a positive control by manufacturer failed to retain cell morphology although viability was >70. All tested lots of 2014 showed < 70% culture viability and damage severity of grade 2, 3 and 4 in explants structure, thus confirming toxic nature of lots, and also found toxicity increased over time. Lots packaged later were more toxic and showed severe damages. This confirmed failure of previous methods applied to detect PFO toxicity by different certification authorities although they were performed following ISO norms. This study concluded in proposing a suitable and more sensitive method to detect PFO toxicity, which is in test with L929 fibroblast cell line

(used in previous methods widely). Physical/chemical study is in progress to identify the cause which generated toxicity.

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Clinically Compatible Human Embryonic Stem Cells-derived Retinal Pigment Epithelium Cells Grafted as an Epithelium Potentiates Vision Rescue in Dystrophic Rodent

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Replacement of defective retinal pigment epithelium (RPE) by new RPE cells derived from human pluripotent stem cells provides a novel rational approach for treating forms of blindness that affect the RPE. First attempts in clinical trials demonstrated safety for the delivery of such cells as a suspension. Transplanting a functional monolayer of RPE cells is the next challenge to effectively cure patients. We developed, under clinically compatible conditions, a tissue-engineered product (TEP) consisting of RPE cells derived from human embryonic stem cells (hESCs) disposed on a biocompatible substrate: the human amniotic membrane. We transplanted this TEP into the subretinal space of Royal college of Surgeons (RCS) dystrophic rats and evaluated visual preservation using multiple approaches (electrophysiology, optokinetic behaviors, histology and optical coherence tomography). This treatment was compared to SHAM and to classical RPE cell suspension injections.

Through a new surgical approach to engraft the TEP into the subretinal space of Royal college of Surgeons (RCS) dystrophic rats, we demonstrated that TEP transplantation improved photoreceptor rescue and the visual phenotype compared to RPE injected as a cell suspension.

We demonstrated the proof of concept in rodent and for the first time the benefit of RPE epithelium engraftment compared to cell suspension injections in visual function. These results are supportive for the initiation of a phase I/II clinical trial to treat RP patients.

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Diverse IL-6 Signaling in Human Retinal Müller and Endothelial Cells under Hyperglycemic Conditions

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Chronic inflammation seems to be involved in the induction and progression of diabetic retinopathy. Interleukin-6 (IL-6), a pleiotropic cytokine, is known to participate in acute and chronic inflammatory events. Its actions depend on cellular environment, cell type, and receptor expression and exert protective as well as detrimental effects. Previous studies by us have shown that IL-6 protects Müller cells from hyperglycemic insult. In contrast, effects of IL-6 on the vasculature have been associated with angiogenesis, one of the detrimental events in diabetic retinopathy. Therefore, this study aimed to better understand the effects of IL-6 on different retinal cell types and to identify the diverse signaling pathways mediating these different actions. Primary human Müller cells (hMC) and retinal endothelial cells (HREC) were treated with normal (7.8mM) and high (25mM) glucose for 48 hours in the presence or absence of recombinant IL-6 and the IL-6/IL-6 receptor complex (1-10 ng/ml). Cell death was assessed by trypan blue exclusion. Flow cytometry was used to determine expression levels of receptors such as glycoprotein 130 (GP130) and the membrane-bound interleukin-6 receptor (mIL-6R). Treatment of hMCs with IL-6 (2ng/ml) reduced high glucose-induced cell death from $18.6 \pm 3.6\%$ to $9.4 \pm 3.2\%$ ($p < 0.05$; $n=6$). hMCs express GP130 and expression levels of GP130 were significantly increased by $78 \pm 15\%$ under high glucose conditions ($P < 0.05$, $n=3$). hMCs express the membrane bound IL-6R. In contrast, HRECs expressed GP130 but lack mIL-6R expression indicating that IL-6 action on these different cell types is mediated by differences in receptor expression. Our results suggest that IL-6 exerts diverse effects by activating different receptor combinations depending on the cell type affected. Therefore, in order to develop therapies for diabetic retinopathy targeting IL-6, a more complete understanding of IL-6 signaling in the diabetic retina is necessary.

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Glycyrrhizic Acid Rescues Retinal Degeneration Induced by Blue Light-emitting Diode Exposure in Mice

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The root and rhizomes of licorice (*Glycyrrhiza*) have been used as an herbal medicine, because they have various therapeutic effects, such as anti-inflammatory and antioxidative activities. Glycyrrhizic acid (GA) is a major component in the root and rhizomes of licorice. In this study, we examined the effect of GA in an animal model for retinal degeneration (RD), which is the leading cause of blindness and characterized by the irreversible and progressive degeneration of photoreceptor cells in the retina. RD was induced in BALB/c mice by exposure to a blue light-emitting diode (LED) (460 nm) for 2 hours. To examine retinal functions, electroretinography (ERG) was performed. To assess histopathological changes, hematoxylin and eosin (H&E) staining were conducted. Apoptotic cell death was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. In addition, changes in proinflammatory cytokines were detected by real time RT-PCR and retinal stress and inflammation were evaluated by immunohistochemistry with anti-ionized calcium binding adaptor molecule 1 (Iba-1) and anti-glial fibrillary acidic protein (GFAP). Scotopic ERG showed that both a- and b-waves were significantly reduced in RD mice, while amplitudes of both waves were significantly increased in GA-treated RD mice, compared to those in non-treated RD animals. H&E and TUNEL assay showed that the outer nuclear layer where photoreceptors reside appeared to be more preserved and less apoptotic cells were observed in GA-treated RD retinas than in non-treated RD retinas. GA reduced expression of proinflammatory cytokines, such as TNF- α , interleukin (IL)-6, IL-1 β , CCL2 and 6, iNOS, and Cox-2. In addition, GA reduced expression of Iba-1 and GFAP, indicating decreased glial response, retinal stress and inflammation. These results demonstrate that GA reduces retinal inflammation and prevents photoreceptor cell death from experimentally induced RD, suggesting that GA may have a potential for the treatment of RD as a medication.

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Effects of the Choroidal Blood Flow in Eyes with Macular Edema Secondary to Branch Retinal Vein Occlusion**MASAHIRO OKAMOTO, Mariko Yamashita, Nahoko Ogata**
*Nara Medical University, Ophthalmology, Kashihara, Japan***Purpose:** To determine the effect of the choroidal blood flow in eyes with macular edema secondary to branch retinal vein occlusion (BRVO).**Methods:** Thirty-two eyes of 32 patients with macular edema secondary to BRVO were treated with a single injection of intravitreal injection of ranibizumab (IVR) and were followed for 2 months. The best-corrected visual acuity (BCVA) and the central retinal and subfoveal choroidal thicknesses were measured. The retinal and choroidal blood flows were determined by laser speckle flowgraphy. After the IVR, patients were classified into a recurrent group and a resolved group.**Results:** The BCVA improved and the central retinal and subfoveal choroidal thickness were significantly reduced after the IVR. The retinal blood flow was significantly decreased by 13.6% ($P < 0.001$) and the choroidal blood flow by 10.6% ($P < 0.001$) at 1 week after the IVR but no significant differences thereafter. The retinal blood flow was significantly reduced by 17.4% ($P < 0.001$) in the resolved group at 1 week after IVR but a reduction was not found in the recurrent group during the follow-up period. The subfoveal choroidal thickness and the choroidal blood flow were significantly reduced during the follow-up period in the resolved group, whereas the significant reduction was found only after 1 week in the recurrent group.**Conclusions:** The reduction of the subfoveal choroidal thickness and choroidal blood flow after IVR indicates that the macular edema secondary to BRVO is not entirely a retinal disorder.

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Evaluations of Eyes with Good Visual Acuity after Anti-VEGF Therapy for Neovascular Age-related Macular Degeneration**MASASHI KOJIMA, Tadanobu Yoshikawa, Kimie Miyata, Nahoko Ogata**
*Nara Medical University, Department of Ophthalmology, Kashihara, Japan***Purpose:** To evaluate eyes that had good visual acuity after

anti-VEGF therapy for neovascular age-related macular degeneration (nAMD).

Methods: This was a retrospective analysis of 31 nAMD patients who were followed for at least 12 months after ranibizumab and/or aflibercept injections and had best-corrected visual acuity (BCVA) $\geq 20/20$ at the last visit. The BCVA at baseline and at the last visit, the type of anti-VEGF drug, the frequency of application, and type of nAMD were evaluated.**Results:** Thirty-two eyes of 31 patients with nAMD were analyzed. The mean age was 71.1 years, and the mean follow-up period was 34 months. The mean BCVA improved significantly from 0.18 logarithm of the minimum angle of resolution (logMAR) units at the baseline to -0.026 logMAR units at the last visit. There were 25 eyes with typical age-related macular degeneration, 19 eyes with the occult type of choroidal neovascularization (CNV), 6 eyes with the classic type of CNV, and 7 eyes with polypoidal choroidal vasculopathy. None of the eyes had retinal angiomatous proliferation. The mean number of injections was 4.17 with ranibizumab, 5.27 with aflibercept, and 8.56 with ranibizumab and aflibercept.**Conclusion:** The eyes that had good visual acuity at the last examination at 34 months had good visual acuity at the baseline, and the main site of the nAMD lesions was under and not above the retinal pigment epithelium.

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Lutein/Zeaxanthin Isomers (L/Zi) May Enhance Exercise by Effective Utilization of Lipids and Decrease Oxidative Stress: In vivo model**VIJAYA JUTURU¹, Mehmet Tuzcu Tuzcu², Cemal Orhan Orhan³, Ragip Pala Pala⁴, Nurhan Sahin Sahin³, Oguzhan Ozdemi Ozdemi², Kazim Sahin³**¹*OmnActive Health Technologies Inc., Scientific and Clinical Affairs, Morristown, United States*, ²*Firat University, Department of Biology, Faculty of Science, Elazig, Turkey*, ³*Firat University, Department of Nutrition, Faculty of Veterinary Medicine, Elazig, Turkey*, ⁴*Firat University, Faculty of Sport Science, Elazig, Turkey***Objective:** Lutein/Zeaxanthin isomers (L/Zi) are the major xanthophyll carotenoids naturally occurring in fruits, vegetables and fish species. We recently demonstrated that L/Zi inhibit cholinesterase enzyme, upregulate eNOS and increase catalase enzymes. L/Zi protect against UV rays and filter blue light. Therefore, this study is to investigate L/Zi supplementation on exercise (Ex) performance, exhaustion time, and changes in lipids, oxidative stress

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(OS) and total antioxidant capacity (TAC) in rats after exhaustive Ex.

Methods: Eight week old male Wistar rats were divided into four treatment groups

(i) control [no Ex, Group I (CTL)]

(ii) CTL + L/Zi [no Ex, 100 mg L/Zi, Group II]

(iii) CTL + Ex [Group III] and

(iv) CTL + Ex+ L/Zi [Ex, 100 mg L/Zi, Group IV].

The Ex protocols were performed on a motor-driven rodent treadmill (TMR). The animals in the chronic Ex groups were habituated by treadmill Ex over a 5-d period such as: 1st day 10 m/min, 10 min, 2nd day 20 m/min; 10 min, 3rd day 25 m/min, 10 min, 4th day 25 m/min, 20 min and 5th day 25 m/min, 30 min. Animals were exercised at 25 m/min, 45 min/d, 5 d/ week for 8 wks. Blood analysis for triglycerides (TG) and cholesterol (CHOL), muscle analysis for lactate, muscle TAC and muscle oxidative stress (OS) were estimated and protein in the gastrocnemius muscle was evaluated in each group of rats by real time RT-PCR and Western blotting.

Results: Group IV significantly increased running performance and exhaustion time. In Group IV a significant decrease in TG and CHOL were observed compared with other treatments. A significant decrease in lactate, muscle oxidative stress and increase in muscle antioxidant activity were observed in Group IV. Protein levels of phosphorylated AMP-activated protein kinase (pAMPK), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and *heme oxygenase-1* (HO1) increased and nuclear factor (NF- κ B) and Interleukin-10 (IL-10) decreased in Group IV. There were no significant differences in any of the end points in Group I and II. No significant changes in liver and kidney functions were observed in any of the treatments.

The present data suggests that L/Zi enhance running performance and decrease OS. These results suggest L/Zi with Ex may enhance the effect of exercise by effective utilization of lipids by increasing pAMPK and Nrf2 an emerging regulator of cellular resistance to oxidants.

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Neuroprotective and Regenerative Approach for Diabetic Retinopathy (in vitro Study)

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Purpose: To invent the combined therapeutic agents with neuroprotective abilities for diabetic retinopathy.

Methods: All of the procedures were performed in

accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinal explants of 7 adult SD rats were three-dimensionally cultured in collagen gel, and incubated in serum free media, AGEs, AGEs+100 μ M citicoline, AGEs+10ng/ml NT-4, AGEs+100 μ M TUDCA, AGEs+100 μ M citicoline + TUDCA (doublet), AGEs+100 μ M citicoline+TUDCA+10ng/ml NT-4 (triplet). The number of regenerating neurites was counted under a phase-contrast microscope after 7 days of culture. After counting, retinal explants were fixed, cryosectioned, and stained by TUNEL and DAPI. The ratio of TUNEL-positive cells to the number of DAPI-staining nuclei in the ganglion cell layer was calculated. Immunohistochemical examinations for the active-form of caspase-9 and JNK were performed. Statistical analyses were performed by one-way ANOVA.

Results: In retinas incubated with triplet, the number of neurites was significantly increased more than other groups and the number of TUNEL-positive cells, caspase-9-, JNK- immunopositive cells was fewer than other groups. In retinas cultured in doublet, the number of neurites was more than that of single treatment groups and the number of TUNEL-positive cells, caspase-9-, JNK- immunopositive cells were fewer than of single treatment group.

Conclusions: Doublet (without NT-4) and triplet (with NT-4) significantly increased the number of regenerated neurites and decreased the number of TUNEL positive cells, caspase-9-, JNK- immunopositive cells, thus they may be considered as promising agents for neuroprotective and regenerative therapy for diabetic retinopathy.

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Early Applications of Granulocyte Colony-stimulating Factor (G-CSF) Can Stabilize the Blood-optic Nerve Barrier and Further Ameliorate Optic Nerve Inflammation in a Rat Model of Anterior Ischemic Optic Neuropathy (rAION)

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G-CSF was reported that had neuroprotective effects in a rAION model. However, the therapeutic window and the anti-inflammatory effects of G-CSF in the rAION model remain poorly understood. Thus, this study aimed to determine the therapeutic window of G-CSF and to investigate the mechanisms of G-CSF via regulation of optic nerve (ON) inflammation in the rAION model. Rats were treated with G-CSF on day 0, 1, 2, and 7 post-rAION inductions for 5 consecutive days, control rats were treated with PBS. Visual function was assessed by flash visual evoked potentials (FVEPs). The survival rate and apoptosis of retinal ganglion cells (RGCs) was determined by FluoroGold labeling and TUNEL assay. ON inflammation was evaluated by staining of ED1 and Iba1. ON vascular permeability was determined by Evans blue extravasation. The type of microglial activation was evaluated by using qRT-PCR. The levels of TNF- α and IL-1 β were analyzed by Western blotting. The therapeutic window of G-CSF in rescuing visual function and RGC survival was demonstrated within 2 days post infarct. Macrophage infiltration was reduced by 3.1- and 1.6-fold by G-CSF treatment starting on day 0 and 1 post-rAION induction, compared with the PBS-treated group ($p < 0.05$). This was compatible with 3.3- and 1.7-fold reductions in ON vascular permeability after G-CSF treatment compared with PBS treatment ($p < 0.05$). Microglial activation was increased by 3.8- and 3.2-fold in the early G-CSF-treated group compared with the PBS-treated group ($p < 0.05$). Immediate treatment with G-CSF also induce M2 microglial activation. The cytokine levels were lower in the immediately G-CSF-treated group compared with the later G-CSF-treated group ($p < 0.05$). Early treatment with G-CSF stabilized the blood-ON barrier to reduce macrophage infiltration into the ON and to induce M2 microglial activation, further decreasing the expressions of pro-inflammatory cytokines for reducing the cytokine-induced injury after rAION induction.

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The Apstatin Analog, ST-115, Reduces Retinal Ganglion Cell Loss after Ischemia/Reperfusion Injury

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Visual impairments and blindness are common comorbidities after stroke, which remains the fifth leading cause of death and the leading cause of disability in the United States. Previous studies have shown that reperfusion injury is positively associated with the extent of stroke damage both in the retina and in the brain. Currently, there is a paucity of efficacious and safe drugs that target the deleterious effects of reperfusion injury in the central nervous system.

Apstatin is a selective aminopeptidase P2 blocker that has previously been shown to be highly effective in preventing cardiac reperfusion injury. Aminopeptidase P2 is localized at the luminal plasma membrane in endothelial cells, where it inactivates the peptide hormone bradykinin through hydrolysis. Here we tested the hypothesis whether the apstatin analog, ST-115, can prevent ischemia/reperfusion injury in the retina in an experimental model for stroke and retinal ischemia. Male Sprague-Dawley rats (2 months of age) were subjected to experimental stroke surgery by transient (45 min) bilateral occlusion of the common carotid artery and unilateral occlusion of the middle cerebral artery, followed by 2 weeks of reperfusion. Animals received either saline, or the apstatin analog ST-115 (16 $\mu\text{g}/\text{kg}$), which is 1000-times more potent than apstatin ($\text{IC}_{50} = 3.7 \text{ nM}$) by intravenous injection immediately prior to initiation of reperfusion.

ST-115 reduced the number of apoptotic retinal ganglion cells (RGCs) from 62.1% to 40.5% ($P < 0.01$), as assessed by fluorescent TUNEL staining on frozen sections. Similarly, ST-115 improved retinal thinning and significantly reduced expression of astrocytic and microglial markers, glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1).

Our data provide strong proof-of-concept and feasibility data supporting the development of apstatin analogs for targeted therapy against ischemia/reperfusion injury after stroke.

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Animal Models for Safety and Function of IPS Cell-derived RPE Tissue

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Previous work suggests replacing diseased RPE with healthy autologous RPE sheet can provide vision rescue for AMD patients. Following *in vitro* evaluation, we have tested the RPE tissue in animal models for safety and function.

iPS cells are differentiated into RPE using a directed-differentiation protocol that generates RPE in three phases: neuroectoderm/RPE progenitors, committed RPE, and immature RPE. RPE cells at the immature RPE stage are purified and seeded onto either electrospun poly(lactico-glycolic acid) (PLGA) scaffolds or non-biodegradable polyester cell-culture inserts. The RPE tissues are evaluated *in vitro* during the maturation stage for biodegradability, alteration in mechanical properties, and biocompatibility. *In vitro*, PLGA and polyester substrates produce functionally similar tissues. Electrophysiological and histology experiments show that RPE cells grown on PLGA scaffolds and polyester membranes form a single cell layer, develop transepithelial resistance of more than 200 ohms/cm², and show typical electrophysiological responses to [K⁺] and ATP changes, previously observed in native RPE tissues.

To assess function, the tissues were implanted into an acute RPE injury-model pig eye and later evaluated using optical coherence tomography (OCT), multifocal electroretinography (mfERG), and histology. Following transplantation into pig subretinal space, PLGA scaffold continued to degrade over the course of 35 days. Functional evaluation of biodegradable scaffold rescue properties showed notable improvement in support of retina function. These PLGA scaffolds showed comparable integration results to polyester implants. Subretinal injections in RNU rats showed no adverse reaction to the vehicle or test article. We confirmed that biodegradable substrates are suitable support for RPE transplantation in degenerative eye diseases. We found that RPE cells behave similarly on biodegradable and non-biodegradable substrates, forming a tissue which is similar to native RPE in its molecular and functional properties. Biodegradability and biocompatibility data from a pig model demonstrated that PLGA scaffold is safe, with the added benefit of being resorbed by the body over time, leaving no foreign material in the eye. Studies in rats demonstrated safety of implant. Taken together, this body of evidence demonstrates the suitability of our implant for a Phase-I clinical trial.

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The Effects of Connexin43 Mimetic Peptide Loaded Nanoparticles on Reducing Acute Photo-damage of the Chorio-retinal Complex

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Background: Connexin43 mimetic peptides (Cx43MP) have been successfully applied for the treatment of central nervous system conditions and trialled in *in vitro* and *in vivo* models of ocular disease. Cx43MP bioavailability after intravitreal or intraperitoneal injections is relatively short when delivered in an aqueous solution. In the light-damaged rat retina, double doses of Cx43MP were therefore required to decrease the light-induced chorio-retinal damage. This current study investigates the effect of delivering Cx43MP incorporated within nanoparticles (NP) for sustained slow-release of Cx43MP offering long-term bioavailability and efficacy in the treatment of light induced chorio-retinal damage used to model changes seen in AMD.

Objectives: To evaluate the effect of Cx43MP-NP in the prevention of structural and functional damage in a light-damaged rat eye.

Methods: Light damage was created by exposure of adult albino Sprague-Dawley rats to constant intense light for 24 hours. Within 2 hours of onset of the light damage procedure, 4 µl of Cx43MP-NP at concentration of 280 µM was injected as a single dosage intravitreally. Electroretinogram recordings were performed at various time points following the cessation of light damage procedure up to 2 weeks post light-damage. The retinal structure was analysed using optical coherence tomography (OCT) and the inflammatory response that accompanies light-induced injury was assessed using antibodies against glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (IBA-1), leukocyte common antigen (CD 45) and Cx43 via immunohistochemistry.

Results: Cx43MP-NP significantly preserved retinal photoreceptor a-wave and inner retinal b-wave electroretinogram function. Functional improvement was seen at 1 week and 2 weeks post-light damage compared with light-damaged untreated animals. The thinning of the retina photoreceptor layer that characterises the light damaged model was significantly ameliorated by

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Cx43MP-NP ($p=0.01$). Inflammation was reduced and was paralleled with down-regulation of Cx43 expression in the chorio-retinal complex.

Conclusion: These results suggest that Cx43MP loaded into NP minimises drug administration procedure and provides prolonged therapeutic effects to the degenerating retina following light damage.

Ophthalmic Genomics

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Expressions of MyoD, IGF Binding Protein, Thioredoxin and p27 in Overacting Inferior Oblique Muscle

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Background: Previous study showed inferior oblique muscle overaction (IOOA) was not related with muscle circumference. However, we wanted to know whether there was any difference in expressions of some proteins between inferior oblique muscle (IO) with overaction and normal function. Muscles respond to mechanical burdens with two ways, hyperplasia and hypertrophy. In extraocular muscles, satellite cells, as adult stem cell, carry out hyperplasia by activation, division, and differentiation. We selected some proteins, for example MyoD, thioredoxin and p27, which carried out important roles in satellite cell cycle, and IGF which was proven as intrinsic factor of direct cardiac muscle hypertrophy. MyoD is necessary for satellite cell differentiation. Thioredoxin decreases as an antioxidant in the redox imbalance state. P27 functions as cell cycle arrest and decreases before cell division and in oxidative stress (redox imbalance). We checked any difference in their expressions between overacting and normal IO.

Methods: We gained 20 inferior oblique muscle samples from superior oblique (SO) palsy patients with inferior oblique muscle overaction $\geq +3$ who received inferior oblique myectomy (IOOA group) and 20 inferior oblique muscle samples from cadavers whose inferior oblique muscle had functioned normally by their recent ophthalmologic chart review (Control group). By using immunofluorescent assay, we identified and compared expressions of MyoD, IGF binding protein, thioredoxin, and p27 between the two groups.

Results: MyoD and IGF binding protein expressed similarly in both groups. However, expressions of thioredoxin and p27 was significantly decreased in IOOA group. In cell cycle, p27 was decreased and satellite cell would cell division in overacting IO, but MyoD for differentiation did not any difference between overaction and normal IO

Conclusion: Based on these findings, the overacting IO of patients with superior oblique palsy had a redox imbalance status. Further study is needed to investigate whether this imbalance in antioxidant capacity is present in the extraocular muscles of patients with other strabismus.

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Fine Genomic Analysis of Deletion Mutations in the Locus Control Region of OPN1LW/OPN1MW Genes in 2 Japanese Families with Blue Cone Monochromacy

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Blue cone monochromacy (BCM) is a rare congenital color vision disorder characterized by the absence of cone sensitivity to long (red) and medium (green) wavelengths in the retina. BCM is caused by discrete mutations in each of *OPN1LW* and *OPN1MW* genes or a single mutation within the locus control region (LCR). We performed a fine mutation analysis of the genomic organization at the *OPN1LW* and *OPN1MW* gene region in 2 Japanese families with BCM (BCM1 and BCM2).

This study was approved by the Institutional Review Board for Human Genetic and Genome Research at the Hamamatsu University School of Medicine and Nagoya University Graduate School of Medicine. All study procedures adhered to the tenets of the Declaration of Helsinki. Written informed consents were obtained from the patients of all cases before any study procedure or examination was performed. Affected males were examined clinically, and their genomic DNA was extracted from the peripheral lymphocytes of patients using standard procedures. To identify disease-causing mutations, we performed a mutation analysis according to the following 3 steps: 1) PCR amplification and sequencing the amplified products for various regions of the *OPN1LW* and *OPN1MW* genes to verify their existence and to detect possible sequence alteration; 2) "Junction PCR" to amplify and sequence the distant regions connected with each other after a possible large deletion for the region in which PCR amplification was unsuccessful; 3) Further analysis by genomic walking and cloning the unknown genomic fragments when the junction PCR was unsuccessful.

Consequently, in BCM1, a 16,856-bp deletion and 53-bp insertion were found. The proximal boundary of the deletion was 8,899 bp upstream of the *OPN1LW* translation initiation codon, and the distal boundary was 1,290 bp downstream of *OPN1LW* exon 2. While, BCM2 harbored a complex mutation with an 87,682-bp deletion, an inverted re-insertion of 328 bp that was a part of the deleted sequence, and a 3-bp insertion. The proximal and distal boundaries of the deletion were 28,144 bp upstream of the *OPN1LW* initiation codon and 7,764 bp downstream of the *OPN1MW1* translation stop codon, respectively. Breakpoints of both mutations were within Alu repetitive sequence.

In conclusion, the disease-causing mutation in 2 Japanese families with BCM was a long deletion that included the LCR. This is the first single base-level mutation analysis of Japanese BCM cases caused by a deletion that includes the LCR.

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Aggregate Effects of Type 2 Diabetes Genetic Variants on Diabetic Retinopathy in a Multi-ethnic Asian Population

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Purpose: Genetic association studies to date have not identified any robust risk loci for diabetic retinopathy (DR). We hypothesized that individuals with more diabetes genetic risk alleles have a higher risk of developing DR.

Methods: We evaluated the aggregate effects of multiple type 2 diabetes-associated genetic variants on the risk of DR among 1528 participants with diabetes (480 Malays, 750 Indians and 298 Chinese) from the Singapore Epidemiology of Eye Diseases Study, of which 547 (35.8%) had DR. Participants underwent a comprehensive ocular examination, including dilated fundus photography. Retinal photos were graded using the modified Airlie House classification system to assess the presence and severity of DR following a standardized protocol. We identified 76 previously discovered type 2 diabetes-associated single

nucleotide polymorphisms (SNPs) and constructed multi-locus genetic risk scores (GRSs) for each individual by summing the number of risk alleles for each SNP weighted by the respective effect estimates on DR. Two GRSs were generated: an overall GRS that includes all 76 discovered type 2 diabetes-associated SNPs, and an Asian-specific GRS that includes a subset of 55 SNPs which were previously found to be associated with type 2 diabetes in East and/or South Asian ancestry populations. Associations between the GRSs with DR were determined using logistic regression analyses. Discriminating ability of the GRSs was determined by the area under the receiver operating characteristic curve (AUC).

Results: Participants in the top tertile of the overall GRS were 2.56 times (95% CI: 1.92-3.40, $P = 1.5 \times 10^{-10}$) likely to have DR compared to participants in the bottom tertile. Participants in the top tertile of the Asian-specific GRS were 2.00 times (95% CI: 1.51-2.65, $P = 1.3 \times 10^{-6}$) likely to have DR compared to participants in the bottom tertile. Both GRSs were associated with higher DR severity levels ($P < 0.001$). We did not observe any inter-ethnic differences. Addition of the GRSs to traditional risk factors improved the AUC modestly by 3-4% (AUC difference = 0.02-0.03, $P < 0.05$).

Conclusions: Type 2 diabetes-associated genetic loci were significantly associated with higher risks of DR, independent of traditional risk factors. Our findings may provide new insights to further our understanding of the genetic pathogenesis of DR.

Glaucoma

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Comparison of iPad Based Visual Field Testing with Humphrey SITA in Glaucoma**ELIZABETH GRAHAM^{1,2}, Angela Schulz¹, Yuyi You^{1,2}, Alexander Klistorner^{1,2}, Stuart Graham^{1,2}**¹Macquarie University, Faculty of Medicine and Health Sciences, Sydney, Australia, ²Save Sight Institute, University of Sydney, Sydney Medical School, Sydney, Australia

Purpose: A hand held iPad visual field testing system (Melbourne Rapid fields - MRF) has been developed to potentially facilitate testing in the community. We examined its performance in untrained users with glaucoma and controls to assess its accuracy and utility for clinical testing.

Methods: 53 glaucoma patients including 36 with manifest field defects and 17 pre-perimetric - mean age 64.6 +/- 10.5, HVF mean deviation (MD) range +2.5 to -20DB, and 18 unmatched controls (mean age 47.8 +/- 19.6) were tested. No subjects in either group had performed iPad based perimetry before, but all glaucoma patients had recently undergone Humphrey SITA 24-2 (HVF) testing on multiple occasions. The majority had both tests performed on the same day, and all but 5 patients had their last HVF test < 6 months earlier and had stable HVFs. The controls were new to perimetry testing. One eye per patient was analysed, random choice if both eyes normal, worst eye in manifest glaucomas, using only reliable HVFs as reference. Correlations and Bland Altman analysis were performed between global indices, and visual field index (VFI) was compared to MRF VC. Visual fields were then divided regionally into 9 sectors to examine corresponding test areas since the two tests have different test locations (temporal sector was then excluded).

Results: Global indices were highly correlated between the 2 tests - Mean Deviation $r^2 = 0.79$, Pattern standard deviation $r^2 = 0.75$, while VFI vs VC $r^2 = 0.84$ ($p < 0.0001$). For the regional sectors, when only those 36 patients with manifest glaucoma were examined correlations for mean sectoral thresholds were still significant ranging from $r^2 = 0.49-0.78$, despite the fact that there were varied numbers of tests points between the devices. Using a definition of a cluster of 3 points at < 1% or 2 at 0.5% to define a scotoma on HVF, the MRF detected 35/46 abnormal hemifields (various MRF definitions tried based on threshold depth since MRF does not provide probability levels). Patients found the MRF easy to perform, but holding the iPad and touching the screen presented functional issues.

Conclusions: The MRF shows reasonable performance characteristics to HVF in terms of detection of defects and

correlation of regional mean threshold values and global indices, despite using a completely different test paradigm. Further testing on reproducibility is needed. Reliability and ease of use should be improved with a stand and blue-tooth keyboard.

Biogen Idec

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Aqueous Humor MicroRNAs as Potential Biomarkers of Outflow Function in Primary Open Angle Glaucoma: A Pilot Study**HARI JAYARAM^{1,2}, Jay I. Phillips³, Elaine C. Johnson¹, John C. Morrison¹, Devin M. Gattley¹, Julie Saugstad³, Kate E. Keller¹**¹Oregon Health & Science University, Casey Eye Institute, Portland, United States, ²Glaucoma Service, NIHR Moorfields Biomedical Research Centre, London, United Kingdom, ³Oregon Health & Science University, Anesthesiology & Perioperative Medicine, Portland, United States

Primary Open Angle Glaucoma (POAG) is a leading cause of worldwide blindness, characterized by progressive loss of retinal ganglion cells and optic nerve damage secondary to raised intraocular pressure (IOP). IOP is established by resistance to aqueous humor (AH) outflow through the trabecular meshwork (TM) and Schlemm's canal endothelium. AH has been proposed to contain potential molecular biomarkers and modulators of aqueous outflow function. Identification of such biomarkers may potentially help characterize and stratify both the severity of outflow dysfunction and responses to treatment. MicroRNAs (miRs) are small, endogenous non-coding RNAs that modulate post-transcriptional gene expression. Several miRs have been implicated in aspects of TM function in cell culture, while other preliminary reports have detected miRs within human AH. To determine whether miR expression within AH warrants further investigation as a potential biomarker of aqueous outflow function, we performed a pilot study to test the hypothesis that miR expression within AH differs between patients with POAG and normal controls.

We collected 6 AH samples (100-200µl) from patients with stable, clinically-diagnosed POAG and 8 samples from age-matched controls, following informed consent at routine cataract surgery. The application of high throughput screening techniques to study miR expression in AH is restricted by the quantity of RNA required and usually requires pooling of samples. To overcome this, we profiled each individual patient sample on paired low-

density PCR array cards through targeted pre-amplification following reverse transcription, to study 754 mature miRs. Differential expression was identified by the $\Delta\Delta\text{Ct}$ method after normalization. The analysis was stratified for fold changes larger than ± 2 with significance considered for values of $p < 0.05$ (2-tailed t-test). miR-518d (12x) and miR-143 (4x) were significantly upregulated, and miR-660 (4x) significantly downregulated within AH of POAG patients compared to controls. Computational prediction of gene targets of the differentially expressed miRs identified pathways associated with reduced cell proliferation, endocytosis, Wnt signaling and cell adhesion.

Our findings demonstrate that AH miRs exhibit potential as biomarkers of aqueous outflow function and merit further investigation in a larger case-controlled study. This technique provides a cost-effective approach to assay individual AH samples without the need for pooling.

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Cofilin-1 Mediated Neuroprotection after α -synuclein Antibody Injection in a Glaucoma Animal Model

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Introduction: Glaucoma is a neurodegenerative disease, leading to the loss of retinal ganglion cells (RGC) and axons in the optic nerve. The pathogenesis includes alterations in autoantibody (Aab) profiles, as altered Aab levels were observed in sera of glaucoma patients. One of those candidates is α -synuclein Aab. The purpose of this study was to identify the molecular mechanisms underlying the neurodegeneration in glaucomatous eyes as well as the neuroprotection observed after injection of α -synuclein antibodies (Ab).

Methods: Intraocular pressure (IOP) was elevated in Sprague Dawley rats ($n=14$) by occlusion of 3 episcleral veins. Intravitreal injection (IVI) was performed after successful rise of the IOP. Fellow eyes served as

(1) normotensive controls ($n=14$) and were compared with IOP elevated eyes of
 (2) controls ($n=3$, no IVI),
 (3) buffer ($n=6$, IVI of buffer),
 (4) α -synuclein Ab ($n=5$, IVI of 25 μg α -synuclein antibody). IOP was recorded weekly. Animals with 7 weeks elevated IOP were sacrificed and optic nerves and retinae were collected for PPD- and Brn3a staining. Retinal tissue was used for mass spectrometric analysis using ESI-MS/MS technology. Analysis was performed double-blind and data

is presented as mean \pm SD.

Results: Episcleral vein occlusion increased IOP significantly to 17.8 ± 1.1 mmHg compared to fellow eyes with 11.3 ± 0.4 mmHg ($p < 0.01$). Axon density/mm² showed a decay to 322477 ± 37055 ($p < 0.01$) in controls, 307787 ± 28399 ($p < 0.01$) in buffer group, and 399818 ± 16529 ($p=0.19$) in the α -synuclein Ab group compared to fellow eyes with 439529 ± 18161 axons/mm². The RGC density/mm² was reduced to 1076 ± 132 ($p < 0.05$) in controls, 1068 ± 163 ($p < 0.01$) in buffer group and 1252 ± 95 ($p=0.08$) in α -synuclein Ab group compared to fellow eyes with 1516 ± 186 RGC/mm². Mass spectrometric analysis revealed downregulated levels of coiled-coil domain-containing protein 93 (-2.6x), cofilin-1 and reticulon 4 (both -2.5x) in IOP elevated eyes and upregulated levels of peripherin-2 (2.4x) and cofilin-1 (3.5x) in α -synuclein Ab injected eyes.

Discussion: The results of this study indicate that neurodegeneration in the retina of experimental glaucoma animals is influenced by a cofilin-actin rod stress response. Furthermore, a neuroprotective effect on the retina has been observed after injection of α -synuclein antibodies, which might originate from modulated cofilin-1 expression levels. Concluding, injection of α -synuclein antibodies could be a new approach to glaucoma therapy.

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Optical and Electron Microscopic Study of Ex-PRESS Device Blockages of Neovascular Glaucoma Patients

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Purpose: The Ex-PRESS glaucoma filtration device is a bypass for the trabecular meshwork, and acts as a more effective filtration surgery than a trabeculectomy. Neovascular glaucoma (NVG) has a high risk of hyphema from the iris and angle neovascularization. There is a possibility that blood from the angle's neovascularization may block the tip and fibrin adjacent to the scleral flap may obstruct the inside of the device. Out of 14 patients we studied with NVG, 12 benefited from the Ex-PRESS device. However, two patient's devices were blocked. We studied three removed devices from them to try to determine the cause of the blockages.

Glaucoma

Methods: After fixation by a 20% formalin solution, we cut a device into cross sections along the coronal section from the tip up to the blockage around the cylindrical wire. We photographed the materials and then did Energy Dispersive X-Ray Spectroscopy (EDS). The aggregation was placed in a centrifuge to separate the materials. We collected the material by Cytospin onto a glass-slide, and then observed under a light microscope after the Gimsa staining.

Results: Optical investigation revealed a translucent aggregation adhered to the tip of one device and to the sides of the cylindrical wire of the other devices. The Scanning Electron Microscope (SEM) investigation revealed the blockage material adhered to the surface of the outflow pathway. Compared to the surface of the cylindrical wire which was smooth, the surface of the outflow pathway on either side of the wire appeared rough. The cross section of one device was filled with aggregation on both sides of the cylindrical wire. EDS revealed the content ratio of the material on both sides of the cylindrical wire was inconsistent with metal. The substances collected by Cytospin were not cellular, but consisted of various shaped debris lightly stained by Gimsa.

Conclusion: Without knowing the exact content of the blockage my assumption continues to be that the fibrin formed in the anterior chamber may easily adhere to the less smooth surface of the outflow pathway of the EXPRESS device.

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Characterization of Optineurin E50K Mouse Model with Normal Tension Glaucoma

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Glaucoma is a group of diseases that damage the eye's optic nerve and result in progressive visual field loss caused by death of retinal ganglion cells (RGC) and degeneration of neural axons known as the optic nerve fiber, leading to glaucomatous cupping. Mutations in optineurin (OPTN) gene has been well characterized to associate with glaucoma and Amyotrophic lateral sclerosis (ALS). The role of E50K mutant of OPTN in normal tension-glaucoma (NTG) is well accepted. Our previous studies have shown wild-

type OPTN localized to the tips of Golgi ribbons, while the E50K OPTN mutant accumulation in the ER and Golgi body of neuronal cells derived from NTG patient iPS cells. We also determined OPTN binding of TBK1 is enhanced by the E50K mutation (Minegishi et al., Hum Mol Genet 2013). The transgenic E50K mice generated with the CAG promoter showed reduction of retinal thickness in entire retina by 18 months (Chi et al., Hum Mol Genet 2010). In this study, we generated an OPTN E50K knock-in mouse by CRISPR/Cas9 genome editing. we performed fundus and retinal imaging using a small animal retinal-imaging microscope (Micron IV, Phoenix Research) in E50K knock-in homozygous mice (n = 20), E50K knock-in heterozygous mice (n = 10), and wild-type mice (n = 20). We also scanned the mouse optic nerve by optical coherence tomography and analyzed the average thickness of the optic RGC layer using Insight software (Micron IV, Phoenix Research). The thinning of the RGC layer in E50K knock-in homozygous mice was observed at 6 months, and the reduction was 20% compared with the wild-type (p < 0.001). HE staining of retina and β -III tubulin-stained nerve fiber in E50K knock-in homozygous mouse at 12 months showed thinning of the optic nerve fiber compared with wild-type mice. Furthermore, the optic cup increased in depth in E50K knock-in homozygous mouse, similar to the changes in glaucoma patients. On the other hand, E50K knock-in heterozygous mice showed no significant changes in the retina, even at 12 months. The E50K knock-in homozygous mice do not progress to thinning of the entire retina like the transgenic E50K mice but rather mimic the phenotype of E50K patients. This is the first NTG mouse model in which the exact same mutation of a patient was genetically introduced into mice, which developed a NTG-like phenotype. This homozygous E50K knock-in mouse will be a useful mouse model to understand NTG and for development of NTG therapeutics.

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Pigment Epithelium Central Limit-Inner Limit of the Retina Minimal Distance Integrated over 2Pi (PIMD-2Pi), a Promising Morphometric Variable for Follow up of Glaucoma Derived from 3D-OCT Volumes of the Optic Nerve Head

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Conclusion: A newly developed algorithm for estimation of PIMD-2Pi from OCT volumes of the optic nerve head (ONH) was associated with sufficiently low measurement variation to allow resolution of a significant change within a glaucoma suspect patient faster than reported for Humphrey visual field (2.5-4 yrs.) and Heidelberg Retinal tomography (4-5 yrs.).

Methods: 3D volumes of the ONH were recorded with a Topcon OCT 2000 system in one eye of each of 18 glaucoma suspects at 2 visits within 6 weeks. At each visit, 3 volumes were captured and PIMD-2Pi was segmented 3 times in each volume with a semi-automatic algorithm developed by us. The semi-automatic algorithm includes automatic detection of the inner limit of the retina and user-assisted location of the central limit of the pigment epithelium in 500 angles. A full segmentation typically was achieved in 30 min/ONH volume. The variation among subjects, visits, volumes and segmentations, respectively was estimated with an analysis of variance. Finally, the consequences of the estimated variances on the possibilities to detect a change over time was derived assuming a loss of PIMD-2Pi of 0.1 of base line/year, using an algorithm derived by us and setting the significance limit to 0.05 and the required power to 0.8.

Results: The variance for subjects was found to be 20 times larger than that for visits and the variance for visits 5 time larger than that for volumes. The variance for segmentations was insignificant in relation to the variance for volumes. The large variation among subjects reflects inter-individual variability of the ONH among subjects and makes cross-sectional comparison of level between independent groups of subjects unsuitable. However, detection of significant difference of change from baseline can be compared between two independent groups with a sample size of 30 per group. Finally, if one patient is followed over time with four visits per year, a significant change from baseline can be detected after 20 months.

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Mechanical Strain Regulates Transcription, Translation and Calcium Levels in Retinal Neurons and Glia - Relevance for Disease

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Purpose: Mechanical deformations of the eye can lead

to neuronal damage, inflammation and may contribute to optic neuropathies. The molecular mechanisms that transduce strain into dysfunction of retinal neurons and glia remain to be identified but are likely to involve activation of specialized mechanosensors. To gain insight into strain-dependent plasticity we investigated transcription, protein expression and calcium signaling in retinal ganglion cells (RGCs) and Müller cells (MCs) exposed to uniaxial or biaxial mechanical strains. Our focus was on recently identified putative mechanosensitive calcium-permeable ion channels together with the expression of key cytoskeletal proteins.

Approach: In vitro studies using acutely dissociated and immunopanned retinal cells were conducted by seeding them onto silicon membranes. RGCs and MCs were stimulated with uniaxial and biaxial strains (Strex, Flexcell apparatus) at different % stretch and exposure (10 min-7 hours, 0.5-15% stretch). mRNA levels encoding mechanosensitive channels were detected by qRT-PCR. Proteins were measured with Western blots. Calcium responses in stretch-stimulated Fura-2 loaded MCs, RGCs and rods were measured with optical imaging.

Results: Mechanical strain had profound, dose-dependent and time-dependent effects on transcript levels of multiple TRP channel isoforms, but also modulated mRNA levels encoding Piezo1 & Piezo2 channels and 2-pore potassium (K2P) channels from TREK/TRAAK families. Stretch-evoked responses were reduced or absent in cells exposed to selective blockers or isolated from KO mice. Likewise, RGCs and MCs responded to increased % radial stretch with increased amplitudes of $[Ca^{2+}]_i$ elevations that were suppressed by pharmacological antagonists of TRP channels and mimicked by TRP agonists. Changes in gene/protein expression and $[Ca^{2+}]_i$ were correlated with concomitant modulation of cytoskeletal proteins.

Conclusions: Our results show that strain is a profound modulator of RGC and glial physiology, driving functional changes at every level from genes, proteins, 2nd messengers and the cytoskeleton. We propose that the extraordinary sensitivity of retinal cells to mechanical stress plays key functions under normal conditions, in force-associated diseases such as glaucoma and diabetic retinopathy, as well as during remodeling associated with retinitis pigmentosa and macular degeneration.

Glaucoma

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Molecular Pathogenesis of Early Glaucomatous Optic Neuropathy in a Spontaneous Feline Model

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The lamina cribrosa region of the optic nerve head (ONH) is considered an important site for initial axonal damage in glaucoma. However, early molecular mechanisms in glaucomatous optic neuropathy (GON) remain poorly understood. The purpose of this study was to identify early changes in the ONH transcriptome in a spontaneous feline congenital glaucoma model (FCG). Eleven 10-12 week old homozygous *LTBP2* mutant cats with FCG and 8 age-matched normal cats were studied. Clinical glaucoma phenotype was confirmed by rebound tonometry, electrophysiological testing, and SD-OCT imaging. Optic nerve was processed routinely from all cats for semi-automated targeted counting of axons in PPD stained sections and qualitative assessment of axon morphology by light microscopy. Total RNA was extracted from carefully dissected ONHs and cDNA libraries constructed for RNA-seq (Illumina HiSeq2000). Differentially expressed genes (DEGs) were identified using DESeq2. Additionally, weighted gene co-expression network analysis (WGCNA) was performed. For functional analysis, goseq and Blast2Go were applied. Results of these bioinformatics analyses were validated by qPCR of selected DEGs and by immuno-fluorescence labeling of ONH tissues. For these confirmatory studies, data from one ONH per cat were compared between groups by unpaired t-test, with $p < 0.05$ considered significant. Significant optic nerve axon damage or loss was not observed in FCG cats at this early stage of disease. 274 DEGs were identified in the ONH of 10 cats with FCG from which high quality RNA was isolated ($FDR < 0.05$ and $Fold\ change > 1.5$ relative to 6 normal age-matched feline ONH). On functional enrichment analysis, $>75\%$ of up-regulated DEGs were ascribed to cell proliferation, response to wounding, inflammatory response. WGCNA identified 39 distinct gene modules. MyD88 dependent toll-like receptor signaling and NF- κ B signaling pathway

were over-represented in a gene module that significantly correlated with glaucoma ($R=0.68$, $p < 0.01$). Ki67 positive proliferating cells were more abundant in glaucomatous ONH and this marker for cell proliferation co-localized with IBA1 or SOX2 in ONH cells. Our findings support initial activation and proliferation of resident cells of glial lineage, and immune/inflammation responses as early events in GON in this spontaneous large animal model. Support: NIH grants EY018609, EY0016665 and UL1TR000427; a Fight for Sight Grant in Aid, Research to Prevent Blindness

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VPS45 Mutation Induces Ectopic Lens Fiber Differentiation in the Lens Epithelium through the Activation of TGF- β Signaling

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Vertebrate lens consists of the lens epithelium and lens fiber core. The lens epithelium is monolayer which covers the anterior half of the lens fiber core. During development, lens epithelial cells move posteriorly and start to differentiate into lens fiber cells at the lens equator. However, it remains to be elucidated why lens fiber differentiation occurs only at the equator. Recently, we identified zebrafish VPS45 (vacuolar protein sorting 45) mutant. In the VPS45 mutant, the lens epithelium loses monolayer structure but forms multiple layers, indicating an epithelial-mesenchyme transition (EMT)-like phenotype. Furthermore, lens epithelial cells express molecular markers of lens fiber cells. Thus, ectopic fiber differentiation occurs in lens epithelium of the zebrafish VPS45 mutant. VPS45 encodes a SM protein family and regulates early endosome trafficking. Thus, dysfunction of early endosome trafficking induces ectopic lens fiber differentiation. It was reported that disruption of lens epithelium induces EMT in human secondary cataract patients through the activation of TGF β signaling. We found that ectopic lens fiber differentiation in VPS45 mutant depends on TGF β signaling. These data suggest that VPS45 and early endosome trafficking system are important for maintenance of lens epithelial state through the suppression of TGF β signaling.

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Analyzing Anterior Epithelial Cell Division in Whole Pig Lenses

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Background: The percentage of cells in the germinative zone lens epithelium (GZLE) that are mitotically active at any given time is needed information to better understand their role in the development of presbyopia as well as their possible manipulation to slow the progression of this

accommodative loss. The goal of this study is to assess the location and percentage of GZLE that are mitotically active in whole pig lenses, a model for laser manipulation of lenses to change its morphology.

Methods: Fresh pig eyes are obtained from a local abattoir and dissected to extract whole lenses. Lenses are kept warm and bathed in 3mg/mL chymotrypsin to remove excess ciliary body and then exposed to 0.6mM 5-ethynyl-2'-deoxyuridine (EdU), a DNA precursor analogue. All samples are then processed using ClickIt chemistry and counterstained with a nonspecific DNA marker (Hoechst 33342) to define cell nuclei as landmarks for co-localization with mitotic cells that have incorporated EdU. Whole lenses are secured to a stage so that the anterior lens surface is flat and images are taken in each lens quadrant using a Nikon AR1 multi-photon microscope. Cell quantification is completed using Fiji imaging software to automatically count the total cells in a Z-stack obtained. Then cells that show EdU uptake were counted by hand using a threshold of 30% above localized background to indicate cells that were mitotically active.

Results: From our preliminary analysis, the pig GZLE appears to be approximately 500 μ m wide and located about 500 μ m from the equator. In each Z-stack 1292 \pm 159 cells were analyzed. Across all lenses, the average number of cells that had EdU labeling was 0.99 \pm 0.49%. The range of double-labeled cells was 1.86% with some regions analyzed showing no staining.

Conclusions: Dividing cells are not equally distributed within the germinative zone. The observed staining pattern may support a concept of spiral or bidirectional lens fiber development. Methods to analyze the entire surface of the lens are being investigated to provide a more accurate number.

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A Conserved RNA Binding Protein Celf1 Regulates Lens Development through Distinct Post-transcriptional Mechanisms

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Functional characterization of Tdrd7 and Caprin2 has indicated the importance of RNA binding protein (RBP)-mediated post-transcriptional gene expression control mechanisms in lens development. Using *iSyTE*, we have identified another conserved RBP Celf1 (CUGBP, Elav-like family member 1, also known as Cugbp1) as a potentially important post-transcriptional regulatory factor in

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vertebrate lens development. *Celf1* deficiency results in eye defects in zebrafish, *Xenopus* and mouse. In non-lens tissues or cell types, *Celf1* has been shown to regulate its target mRNAs through distinct mechanisms such as alternative splicing, mRNA decay and translational repression. We find that *Celf1* is necessary for nuclear degradation in differentiating lens fiber cells. *Celf1* null mouse lenses exhibit reduced levels of *Dnase2b* mRNA and abnormally increased levels of p27 protein, which may contribute to the fiber cell nuclear degradation defect. RNA immunoprecipitation (RIP) and cross linked immunoprecipitation (CLIP) assays demonstrate that *Celf1* protein directly interacts with *Dnase2b* and p27 mRNAs. While binding of *Celf1* to *Dnase2b* mRNA may increase its stability, *Renilla* luciferase reporter assays with p27 5' UTR suggest that *Celf1* negatively regulates p27 in the lens by translational repression. To investigate *Celf1* function in regulating mRNA decay and alternative splicing in the lens, we analyzed mouse *Celf1* conditional knockout (*Celf1^{ckO}*) lenses by RNA sequencing and identified 306 down-regulated and 584 up-regulated genes. Interestingly, majority of the down-regulated genes are found to be highly lens-enriched in *iSyTE*. To identify *Celf1* mRNA targets that are potentially regulated through mRNA decay, we analyzed the 3'UTRs of the top 50 up-regulated genes and identified a *Celf1*-binding motif termed GRE (GU rich element) in nearly half of these candidates. RIP assays suggest that the candidates *Ell2* and *Rgs8* may be direct targets of *Celf1*. Our initial findings suggest that *Celf1* may also function in regulating alternative splicing in the lens. For example, splice isoforms of a lens structural gene *Spbt* are differentially expressed in *Celf1^{ckO}* lens. Together, these findings provide evidence that a single RBP mediates distinct post-transcriptional gene expression control mechanisms in lens development.

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A Role for Spred Proteins in Lens Development

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The transparent and refractive properties of the ocular lens are dependent on its precise cellular architecture

that is established and maintained through coordinated proliferation and differentiation of its cells. The ERK/MAPK signaling pathway plays a crucial role in regulating lens cell proliferation and differentiation, and in turn is regulated by inhibitory molecules, including the Spred family of proteins. We hypothesized that Spred proteins play a role in tightly managing lens cell proliferation and differentiation via ERK1/2 signaling.

Embryos deficient for both *Spred1* and *Spred2* in lens (both germline, and conditional lens mutants using *MLR10-Cre*) were compared to control embryos at various developmental stages (E12.5-E16.5). Embryonic tissue was collected and processed for histology, examined with PAS staining and immunohistochemistry. The lens phenotype was characterized relative to changes in cell proliferation, epithelial cell numbers, lens fiber cell length and differentiation, as well as overall lens size.

Mice deficient for all alleles of *Spred1/2* in the lens exhibited microphthalmia and severe lens epithelial defects (increase in epithelial cell numbers leading to cell multilayering) from E12.5 to E16.5, when compared to lenses that retained an allele of both *Spred1/2* during early lens development. *Spred1/2* deletion compromised lens fiber cell length, resulting in a significant reduction in total lens size at E12.5 to E14.5; however, this was not the case at later stages of development (E16.5 onwards). The deficiency of *Spred1/2* correlated with increased immunolabeling for phosphorylated (active) ERK1/2 in areas of lens cell proliferation and differentiation. Interestingly, our preliminary studies also revealed elevated levels of *Sprouty* (a related negative regulator of ERK1/2 signaling) expression in mutant embryonic lens when compared to control lens.

From our studies, *Spred* proteins appear to be important for tightly regulating lens cell behaviour at earlier stages of lens morphogenesis through to foetal growth. This research provides a greater understanding of the molecules that may help refine the process of lens growth, potentially leading to the development of strategies to preserve and regenerate normal lens cell structure.

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Zebrafish as a Model for Studying the Effects of Ionizing Radiation on the Eye Lens

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Introduction: The lens of the eye is recognized as one of the most radiosensitive tissues in the human body, and it is known that cataracts can be induced by acute dose of the ionizing radiation (IR). IR effects are classified as either deterministic (ie there is a threshold dose of IR) or stochastic effects, such as cancer and heritable effects, where the risk increases with any dose, no matter how small, according to a linear-no-threshold (LNT) model. The eye lens is well known to be the most IR sensitive eye tissue and yet the mechanistic basis for this is largely unknown, but recent analyses using a mouse model suggest that at low dose (< 1 Gy) the effects are stochastic, but non-linear, supporting a hormetic response by the eye. To investigate the mechanistic detail of this response, we started to develop a Zebrafish model, given the genomic similarities between human and zebrafish, especially DNA repair-related genes, and the technological advantages of this system over the mouse eg; easy gene manipulation, *in vivo* cell tracing and high throughput screening of living animals.

Method: The present work used zebrafish to study *in vivo* effects of the ionizing radiation upon lens development. Embryos at 24h were dechorionated and exposed to IR in an X-ray irradiator at single doses of 0, 0.1, 0.3, 1 or 10 Gy. Embryos were analyzed 1h or 24 h after irradiation.

Results: Morphometric analysis of the zebrafish animal showed a reduction of the eye size of the embryos as single IR doses increased. This pattern was correlated with increased numbers of TUNEL positive cells. Low dose and high dose IR effects are different. Using real time PCR the activation of DNA repair, cell cycle, stress and toxicity genes was investigated. Once again, the gene pattern expression was different between low and high dose irradiated groups.

Conclusions: The zebrafish eye lens is a radiosensitive tissue. The *in vivo* response after irradiation shows IR doses dependency.

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Corneal Cross-linking in Children with Progressive Keratoconus: A 5-year Follow-up**BEATRICE E. FRUEH***University of Bern, Ophthalmology, Bern, Switzerland*

Methods: Prospective study of cross-linking (CXL) for keratoconus performed in patients younger than 18 years of age. Inclusion criteria were complete follow-up of 5 years or progression (i.e. increase in KMax of ≥ 1 D in 1 year). 21 eyes were included in the study, plus 2 cases of progression. Topography and tomography were performed.

Results: Mean age at surgery was $14.3 \pm$ years (range, 4-18). CDVA remained unchanged in 13 eyes, improved (≥ 2 lines) in 7 and worsened (≥ 2 lines) in 1. Topographic indices remained stable or improved. KMax flattened considerably (53.8 ± 5.7 D preop to 51.8 ± 5.4 D at 5 years, $p < 0.001$) and mean cornea thickness decreased continuously throughout the 5 years (from $479 \pm 40 \mu\text{m}$ to $439 \pm 38 \mu\text{m}$, $p < 0.001$).

Two cases of progression occurred at 2- and 3 years postoperatively. Re-CXL was performed.

Conclusion: CXL in children is as safe as in adults. Corneas thinned continuously after CXL. At 5 years, the corneas were flatter than preoperatively. Although progression is rare, children need to be followed up regularly, and re-CXL has to be considered when progression is confirmed.

Methods: This study included 8 eyes with limbal dermoids that underwent LKP. A total of 24 normal eyes served as controls. Using the anterior segment OCT (SS-1000, Tomey Corp., Japan), the main outcome measurements were corneal total HOAs with a 4-mm and 6-mm pupil at >12 months postoperatively. Topographic indices, including cylinder power, total HOAs, coma, spherical aberrations (SA), coma-like, and SA-like, were compared. The value for the coma magnitude and axis was calculated by Zernike vector analysis.

Results: The comparison of corneal astigmatism pre- and postoperatively showed changes of < 2.0 diopters (67%). The mean magnitude of each Zernike vector terms, including total HOAs, coma, coma-like, and SA-like [root mean square (μm)] indices, was significantly higher in limbal dermoids (0.49, 0.39, 0.28, and 0.26) than control eyes (0.09, 0.05, 0.06, and 0.07) respectively. ($P = 0.001$). The HOAs, coma, and coma-like RMS values were found to be positively correlated with the preoperative dermoid size ($P = 0.001$). The corneal HOAs were dominant of coma aberration and decreased 6 months after removing the graft sutures. By vector calculation, preoperative (279.3°) and postoperative (170.8°) mean axis for the anterior coma were in the opposite directions. Postoperatively, the coma aberrations were approximately normalized in the center of the cornea; however, the peripheral coma remained.

Conclusions: Preoperatively, the changes in the shape of the cornea were affected by the dermoid size. Postoperatively, the changes were affected by the sutures in or out of the eye.

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Evaluation of Changes in Higher-order Aberrations after Lamellar Keratoplasty in Patients with Limbal Dermoid Cyst Using Swept-source Optical Coherence Tomography**RYOTARO TODA, Sousuke Inokawa, Taiichiro Chikama, Yoshiaki Kiuchi***Hiroshima University Graduate School of Biomedical & Health Sciences, Department of Ophthalmology and Visual Science, Hiroshima, Japan*

Purpose: Lamellar keratoplasty (LKP) for limbal dermoids is a useful cosmetic procedure. However, it only offers a mild reduction of corneal astigmatism. There have been no studies investigating the optical characteristics of this disease, such as corneal higher-order aberrations (HOAs). Therefore, the aim of the study was to investigate the characteristics of the HOAs after LKP [Editor1] in patients with limbal dermoid, using swept-source optical coherence tomography (OCT).

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Proteome Changes in Human Tears Associated with Aqueous-deficient and Evaporative Dry Eye Syndrome**FRANZ GRUS, Natarajan Perumal, Sebastian Funke, Norbert Pfeiffer***University Medical Center, Experimental Ophthalmology, Ophthalmology, Mainz, Germany*

Dry eye syndrome (DES) is a common yet deplorable ocular pathology. Despite the high prevalence of DES worldwide, the underlying proteomic anomalies associated with this disease remain largely unexplored. Therefore, this study investigated in-depth the tear proteome of DES patients employing different mass spectrometry (MS)-based proteomic strategies. Eighty patients were recruited and categorized into three major DES subgroups, which are the aqueous-deficient (DRYaq), evaporative (DRYlip) and a

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combination of the two (DRYaqlip) DES, as well as healthy subjects (CTRL). Discovery proteomics strategy identified 22, 58 and 67 significantly differentially expressed tear proteins in DRYlip, DRYaq and DRYaqlip, respectively. For the first time, biological functional analysis demonstrated that proteins responsible for various metabolic processes were highly expressed in both DRYaq and DRYaqlip subgroups in addition to other known processes, namely the immune and inflammatory processes. Targeted proteomics strategy verified that 13 major proteins were differentially expressed in specific DES subgroups, comprising PRR4, ZG16B, SCGB2A1, DMBT1, PROL1, LACRT, ALDH3A1, ENO1, TF, S100A8, S100A9, PEBP1 and ORM1. In conclusion, this study explored in-depth the proteome of DES by unravelling various novel functional processes and more insight the key role proteins for the maintenance of tear film stability.

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Topical Ozone Application Augments Corneal Collagen Crosslinking

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Purpose: Corneal collagen crosslinking (CXL) is the contemporary treatment of keratoconus. In CXL, riboflavin and ultraviolet A is applied to the cornea. The oxygen radicals that are formed as the product of this reaction leads the cross links at the collagen fibril surfaces and proteins surrounding the collagen and increases the biomechanical strength. It was shown that the tissue oxygen is the key element for the reaction. Ozone is an unstable trioxylene molecule. Its break-down to oxygen gives rise to oxygen free radicals, which are highly reactive.

This study is designed to assess the effect of topical ozone application on CXL procedure.

Methods: Enucleated fresh cadaver yearling lamp eyes (n:28) were studied. Eyes were divided into 5 groups: control (K, n:6), sham (A, n:6), ozone only (B, n:6), CXL only (C, n:5), Ozone+CXL (D, n:5). In group A, only the epithelium layer was removed mechanically. In group B 20 µg/ml liquid ozone was topically applied to the de-epithelized cornea. In

group C, CXL was performed as a total energy of 5,4 J/cm² in accelerated pulse mode. In group D, both CXL and ozone treatment was done. Group K was not touched at all. Pre and post-interventional AS-OCT, confocal microscopy, immediate post-interventional corneal stromal oxygen levels were achieved. After the procedure, each cornea was dissected from the globe and specimens were taken for light and electron microscopic evaluation.

Results: Corneal stromal oxygen levels were higher in ozonated groups (B and D). There were increased tissue reflectivity in stroma, in groups B, C and D. Straight lines of collagen bundles were prominent especially in group D. Pre-interventional pachymetry was thickest in control group and similar in the remaining groups. Post-interventional pachymetry was thinnest in ozonated groups (B and D). The pre- and post-interventional pachymetric difference (decrease) were present in ozonated groups (B and D). The hyperreflective stromal area was forming a demarcation, which was similar in groups B and C. In group D there were multiple demarcations extending to deeper stroma.

Caspase activity was higher in cross-linked groups (C and D). Group D showed more regular, tight and parallel located less undulating collagen fibers.

Conclusion: Ozone increases corneal oxygenation and it seems to create CXL like effect, and augment CXL. Future experimental studies are needed to investigate the safeness and feasibility.

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Next-generation Comprehensive Diagnosis of Suspect and Keratoconus Eyes Using High Resolution Tomography and Biomechanics: Thinking Beyond Topography

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Purpose: To evaluate the precision of a recently developed indices of Bowman's layer structure and corneal biomechanics for early detection of keratoconus.

Methods: 44 normal, 205 keratoconus and 62 keratoconus suspect eyes underwent Corvis-ST, Pentacam (OCULUS Optikgeräte GmbH, Germany) and Envisu C2300 (Bioptigen Inc, USA) examination. A „suspect eye“ was the term

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assigned to an eye with no topographical abnormalities in a patient with topographical changes confirming KC in the other eye. Total deformation data from Corvis-ST was analyzed after separating corneal and extra-ocular tissue deformation curves. A biomechanical model was developed to derive corneal stiffness (kc) parameters [linear (constant kc) and non-linear (mean kc)] from the deformation data. Bowman's layer edge was traced on OCT images and the undulations of the layer were quantified as the Bowman's roughness index (BRI). Demographic data such as keratometry, corneal thickness (CCT) and cone location magnitude index (CLMI) were obtained. Analysis of covariance (ANCOVA) was applied to the computed constant kc, mean kc, BRI and CLMI with intraocular pressure (IOP) and central corneal thickness (CCT) as covariates. After covariance correction, the area under the curve (AUC), sensitivity (%) and specificity (%) were reported for the same.

Results: Normal eyes had significantly different constant kc, mean kc and BRI as compared to keratoconus ($p < 0.001$) and suspect eyes ($p < 0.001$). However, no difference was observed between keratoconus and suspect eyes. AUC \pm SEM (sensitivity, specificity) for constant kc, mean kc and BRI were 0.98 ± 0.01 (97%, 90.2%), 0.97 ± 0.01 (95.9%, 88.1%) and 0.79 ± 0.05 (73.8%, 70%), respectively. AUC \pm SEM (sensitivity, specificity) for axial CLMI anterior, axial CLMI posterior, tangential CLMI anterior and tangential CLMI posterior were $0.99 \pm 2.67 \times 10^{-3}$ (96.5%, 100%), $0.99 \pm 5.25 \times 10^{-3}$ (93%, 100%), $0.86 \pm 20.0 \times 10^{-3}$ (77.4%, 100%) and $0.99 \pm 4.31 \times 10^{-3}$ (98.5%, 92%), respectively.

Conclusion: BRI and corneal stiffness were found to have a reliable diagnostic ability in detecting both suspect and keratoconus eyes, comparable to CLMI. A combined use of these indices could possibly aid in bridging the lack of suitable tools in early detection of keratoconus.

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Corneal Surface Temperature in the Guinea Pig: Influence of Age, Tearing and Inflammation

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The purpose of this work was to study corneal surface temperature (CST) of the guinea pig, analyzing its changes with age and the influence of tear secretion volume and ocular inflammation. CST was measured in guinea pigs of both sexes and different ages (1-18 months), under different

conditions: 4-8 weeks tear deficiency after surgical removal of the lachrymal gland (DE), allergic keratoconjunctivitis in animals sensitized to ovalbumin (AK), UV-induced photokeratitis, ocular instillation of warm saline drops, and absorbing tears with a sponge. Video images were recorded with an infrared camera and analyzed off-line with dedicated software to calculate CST at the center of the cornea immediately after eye opening. Tearing rate was measured with commercial phenol red threads. TBUT and blinking frequency were also determined.

Tearing rate was significantly increased ($p < 0.001$, Pearson's coefficient) and TBUT significantly decreased with age ($p < 0.05$). The initial CST after eye opening was not dependent on the age.

In DE animals, tearing was significantly decreased (21.5 ± 0.8 vs 8.5 ± 1.4 mm; $p < 0.001$, t-test), and the initial CST was higher than control (37.1 ± 0.13 vs $36.7 \pm 0.1^\circ\text{C}$; $p < 0.05$). Under AK, tearing (26.3 ± 3.0 vs 36.7 ± 2.0 mm, before and after AK, respectively; $p < 0.05$, paired t-test) and the initial CST (36.8 ± 0.1 vs $36.5 \pm 0.1^\circ\text{C}$; $p < 0.05$, paired t-test) were increased significantly. During the mild ocular surface inflammation developed by UV-irradiation, no significant changes were observed in tearing or CST.

Tear volume was artificially increased applying 5 or 10 μl drops of warmed saline and decreased by absorbing the tears in control animals. A negative correlation was found between tear volume measured in these conditions and the initial CST values ($p < 0.001$, Pearson's coefficient).

Altogether, the data show that corneal surface temperature increase under different inflammatory conditions where tearing is either increased or decreased. This higher CST is not present in healthy eyes when tear volume is increased or decreased experimentally, suggesting that CST increase found in inflamed eyes is due to the increased blood flow usually developed in ocular surface inflammation.

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Towards Good Manufacturing Practice: Comparing the Suitability of Collagens to Produce Tissue-equivalents for Ocular Surface Reconstruction

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The cornea, on the front surface of the eye, is our window to the world. The World Health Organisation estimates 10 million people in the world are blinded by corneal disease

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and 1.5 million new cases occur annually. For many conditions, transplantation of a donor cornea may restore vision for up to 5 years. However, there is a global shortage of suitable donor tissue and a high risk of rejection.

Stem cell therapy, together with tissue engineering approaches, has the potential to address this significant unmet clinical need.

'RAFT' collagen tissue equivalents (TE) have been extensively investigated by our group using research grade collagen for ocular surface reconstruction. Now clinical manufacturing quality standards should be considered prior to patient transplantation. This study compared our research First Link collagen with clinically relevant Koken collagen in order to trace any physical property changes in the TE that might affect cell behaviour. RAFT TE were prepared with First Link collagen (2mg/ml) or Koken collagen (2mg/ml and 3mg/ml).

Scanning electron microscopy was performed to inspect the surface topography and to quantify collagen fibril diameter as this dictates the surface area available for cell attachment. The diameter of the Koken (3mg/ml) collagen fibrils was significantly greater than First Link fibrils [$p < 0.0001$]. This might have an effect on cell attachment as the surface area available is smaller.

The wettability of the TE was verified by the measurement of the contact angle of the surface with water. There was no significant difference between the contact angle of the three TE preparations so should not affect protein adsorption and consequent cell behaviour.

Break-stress tests showed that Koken TE (3mg/ml) were significantly stronger [$p < 0.05$] than First Link and Koken TE (2mg/ml).

TE thickness was accessed by optical coherence tomography (OCT) and light transmission was obtained by absorbance readings using a spectrophotometer.

Translation from bench to clinics is always a challenge and this work highlights the importance of considering clinically accepted reagents early in the process of development. This study provides us with quantitative comparative data against which other collagens could be compared if required.

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Basal Tear Production and Corneal Surface Temperature in Awake and Anaesthetized Restrained Mice

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Activity of corneal cold thermoreceptors activated by environmental temperature reductions is involved in maintenance of ocular surface wetness. In addition, increased basal tear rate (BTR) was reported in older mice. However, most available data were obtained in anesthetized animals. This work is aimed to explore BTR and corneal surface temperature (CST) in restrained mice of different ages, both awake and under general anesthesia (GA).

A custom-made restrainer was developed to allow both manipulation of the eye and continuous recording of infrared images with an infrared video camera. Under constant environmental conditions ($\sim 24^{\circ}\text{C}$, 45-65% partial humidity), BTR (expressed in mm of wet phenol red thread placed during 30s under a stereomicroscope) was measured in restrained C57BL/6J mice of both sexes and different ages: 2-month old (Young) and 15-month old mice (Old). CST was determined off-line from infrared thermography images of the cornea. Each animal was explored while restrained in two consecutive experimental situations. First, under general anesthesia with 2% isoflurane and then awake, 15min after full recovery from anesthesia.

BTR was significantly lower under general anesthesia compared with awake situation, both in young and old mice (Young: 0.92 ± 0.42 vs 8.29 ± 1.87 mm in anesthetized and awake situation, respectively. Old: 5.33 ± 1.38 vs 17.50 ± 2.93 mm. $P < 0.05$, Kruskal-Wallis; $n = 6$, 3 male/3 female). In contrast, CST was significantly lower in awake animals in both age groups (Young: 30.84 ± 0.40 vs $29.04 \pm 0.37^{\circ}\text{C}$ in anesthetized and awake situation, respectively. Old: 30.63 ± 0.23 vs $29.35 \pm 0.37^{\circ}\text{C}$. $P < 0.05$). No significant correlation was found between BTR and CST for any age group or experimental situation.

We have developed a preparation for measuring tear production and corneal surface temperature in anaesthetized and awake mice under controlled environmental conditions. CST was lower in awake than in anesthetized restrained mice, being this difference unrelated to environmental conditions. Results suggest that anaesthetizing the animals caused a profound reduction of BTR. Consequently, data coming from experiments in which animals had been anaesthetized should be interpreted cautiously.

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In vitro Effects of Benzalkonium Chloride on Human Meibomian Gland Epithelial Cells**ULRIKE HAMPEL^{2,3}, Anca Gavrilut¹, Michael Eichhorn¹, Friedrich Paulsen¹**¹Friedrich Alexander University of Erlangen-Nürnberg, Erlangen, Germany, ²Department of Ophthalmology, Mainz, Germany, ³Department of Anatomy II, Erlangen, Germany**Purpose:** The aim of this study was to get deeper insights of benzalkonium chloride (BAC), a preservative used in most ophthalmic topical solutions, on the meibomian gland.**Methods:** A human meibomian gland epithelial cell line (HMGEC) was used and cells were cultured in the absence or presence of serum. Sudan III lipid staining and real-time PCR (cornulin, involucrin) were performed. Cell proliferation, viability and impedance sensing were evaluated. The results after *in vitro* stimulation with BAC or Lumigan were compared.**Results:** In absence of serum, the proliferation of HMGECs decreased starting with 0.1 µg/ml BAC. At 50 µg/ml BAC or higher concentration the cell viability dropped down after only 10 min exposure and the cells changed their shape. Toxicity of Lumigan was even greater than BAC alone. Confluence, cell-cell-contacts and serum-containing medium appeared to facilitate meibocyte survival. Expression of involucrin and cornulin declined after exposure to Lumigan and BAC.**Conclusion:** Our *in vitro* experiments show that BAC is toxic to HMGECs in the concentrations used in eye drops and that the addition of prostaglandins as in Lumigan even aggravate this effect.

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The Effect of Proparacaine on the Corneal Epithelium and Neuropeptides**YONG-SOO BYUN¹, Ji-Young Kwon², Hee-Jung Ju², Young-Sik Yoo¹, Choun-Ki Joo¹**¹Catholic University of Korea, Seoul St.Maty's Hospital, Ophthalmology and Visual Science, Seoul, Korea, Republic of, ²Catholic University of Korea, Seoul St.Maty's Hospital, Catholic Institute of Visual Science, Seoul, Korea, Republic of**Purpose:** We evaluated the corneal toxicity and neurotoxicity of topical proparacaine (PPC) and identified proper range of its concentration with the purpose of eye pain control.**Materials and methods:** Cell proliferation/toxicity assay and scratch wound healing assay were performed using human corneal epithelial cell in two-fold serial diluents of PPC0.5%. Primary neuronal cells from rat trigeminal ganglions (TGs) were used to assess the neurotoxicity of PPC. For *in vivo* wound healing assay, three-milimeter epithelial wounds were made in SD rats before PPC0.5%, 0.1%, 0.01%, and vehicle were topically applied every 4 hours. To investigate the effect of PPC on intact corneal epithelium and innervation, immunohistochemistry and ELISA assay of neuropeptides in rat corneas and RT-PCR for those in rat TGs were performed after two-week treatment of PPC0.5%, 0.1%, 0.01%, and vehicle.**Results:** Lethal dose 50%(LD50) of PPC on corneal epithelial cells was found in range of 0.1-0.125%. *In vitro* scratch assay showed the significant delay in gap closure in PPC0.5%-treated cells. PPC0.5% inhibited the epithelial wound healing *in vivo* and PPC 0.1%, 0.01% did not show the difference compared to vehicle. Substance P and CGRP protein level and RNA expression decreased in cultured TG neuronal cells treated with PPC whereas those were not changed in rat corneas and trigeminal ganglions after PPC treatment.**Discussion:** Topical PPC has been used as topical anesthetics for ophthalmic surgeries or examination. Our data showed the evidences that proper concentrations of PPC, if not abused, may be useful to control the eye pain.

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Correlations of Fleischer Deposits Location with Topographical Parameters within Various Deformations of Cornea**ANATOLY SERGEEVICH MISTRYUKOV^{1,2}, Sergei Igorevich Anisimov^{1,2}, Svetlana Yurievna Anisimova²**¹State Medical and Dental University named after A.I. Evdokimov, Department of Ophthalmology, Moscow, Russian Federation, ²OOO Eye Center «East Sight Recovery», Moscow, Russian Federation**Summary:** Topographic and tensiotopographic maps of 30 patients' eyes after various surgical interventions, including RK, CK, PTK, hyperopic and myopic LASIK, ICRS, ortho-lenses, and keratoconus were evaluated and correlations of those with location of Fleischer deposits were analyzed. There was a strong connection between zones of maximum mechanical stress gradient and Fleischer deposits arrangement due to changes of corneal biomechanical parameters. Authors assess the reliability of different theories and give their opinion on emergence of

corneal sediments.

Materials and methods: Topographic indications of 30 patients after various refractive surgical interventions (RK, CK, PTK, hyperopic and myopic LASIK, ICRS, ortho-lenses) and keratoconus were assessed. Patients undergone routine ophthalmic examination complemented by photoregistration of Fleischer deposits, corneal topography and then calculating keratensiotopography (KTT) - stress distribution map. Subsequently, there was made a comparison of sediments location with the distribution of mechanical stress on the cornea.

Results: Fleischer deposits are pathognomonic sign of corneal biomechanical changes, of either natural or iatrogenic genesis. Their correlation is directly proportional to the type of (surgical or therapeutic) intervention. KTT is effective in primary diagnosis and observation of the dynamic process. Though there are no incisions during corneal refractive therapy, a theory of tear flow and dispersion of microelements in corneas with keratoconus can be confirmed by sediments after usage of ortho-lenses.

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Keywords: Keratoconus, corneal ectasia, radial keratotomy, ortho-lenses, LASIK, Fleischer ring, keratensiotopogram.

this difference was more obvious in mixed astigmatic eyes. Anterior asphericity showed differences in myopic and hypermetropic excimer laser surgery carried out eyes ($p=0.001$ and $p< 0.001$). Posterior asphericity was the same in all groups. Central corneal thickness was thinner in excimer laser surgery carried out myopic eyes ($p< 0.001$). Corneal volume was different in excimer laser carried out hypermetropic eyes ($p=0.026$). Anterior chamber volume and depths were different in myopic, hypermetropic and excimer laser carried out myopic eyes (between $p=0.003$ and $p< 0.001$).

Conclusions: Compared with emetropic eyes there were differences in topographic anterior and posterior astigmatism, central corneal thickness, cornea and anterior chamber volume and anterior chamber depths. Anterior and posterior keratometry and posterior asphericity were same in all groups.

Financial interest: None

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Scheimpflug Evaluation of Emetropic, Ametropic and Excimer Laser Operated Eyes

SIDIQE ALIYEVA

National Centre of Ophthalmology Named after Academician Zarifa Aliyeva, Baku, Azerbaijan

Purpose: Evaluation of Pentacam Scheimpflug images of emetropic, myopic, hypermetropic, astigmatic and excimer laser operated eyes.

Setting: National Centre of Ophthalmology named after academician Zarifa Aliyeva.

Methods: The purpose of this study is to evaluate keratometry at the anterior and posterior corneal surface, astigmatism, asphericity, cornea and anterior chamber volume and anterior chamber depths. Patients were grouped based on their refractive figures and excimer laser surgery carried out on them.

Results: On average, anterior and posterior keratometry was the same in all groups ($p=0.250-1.0$). Mixed and combined astigmatic eyes showed differences with respect to emetropic eyes in anterior topographic astigmatism, while in posterior topographic astigmatism

Ocular Immunology

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Cytokine Profiling in Patients with Age Related Macular Degeneration and Polypoidal Choroidal Vasculopathy**RUPESH AGRAWAL¹, Praveen Kumar Balne¹, Bennett Lee², Venonice Bijin Au², John Connolly²**¹Tan Tock Seng Hospital, Singapore, Singapore, ²Institute of Molecular and Cell Biology, Singapore, Singapore

Purpose: To investigate the distinctive cytokine profiles in plasma and aqueous humor of patients with age related macular degeneration (AMD) and polypoidal choroidal vasculopathy (PCV) and its comparison with age and gender matched controls.

Methods: In this prospective case-control study, undiluted aqueous humor (up to 200 μ l) and blood samples were collected from 24 AMD/PCV patients and 39 controls and only blood samples were collected from 9 controls. Forty one cytokine levels were measured in the clinical samples using Luminex bead based multiplex assay and the differences in the cytokines levels in plasma and aqueous humor in each group was analysed.

Results: The mean age of the patients with AMD/PCV was 72.5 \pm 10.5 (mean \pm SD) years and the control group was 62.8 \pm 10.7 (mean \pm SD) years. Male and female ratio was 15:9 and 29:19 in AMD/PCV group and control group respectively. Plasma levels of GRO, PDGF-AA, Fractaline and IL-9 and aqueous humor levels of GRO, MDC, MIP-1a, MCP-1, IP-10, IL-6, IL-8, IL-15, and IFN α 2 significantly differed ($p < 0.05$) between the control and AMD/PCV patients. When compared between AMD and PCV patients plasma levels of PDGF-AA, IL-1RA, MCP-3, sCD40L, IL-8, MIP-1 β and aqueous humor levels of IL-12p70 significantly differed ($p < 0.05$) between the two groups.

Conclusions: In AMD/PCV patients, the pathological changes in the eye are mainly due to deregulation of local immune factors and the aqueous humor cytokine levels (except GRO) cannot be correlated with their counterparts in the plasma. Aqueous humor IL-12p70 can serve as an ideal diagnostic biomarker for differentiation between AMD and PCV.

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Adenosine A_{2A} Receptor Blockade Prevents Microglia-mediated Retinal Neurodegeneration Triggered by Elevated Hydrostatic Pressure**INÉS DINIS AIRES^{1,2}, Catarina Rodrigues Neves^{1,2}, Raquel****Boia^{1,2}, António Francisco Ambrósio^{1,2,3}, Ana Raquel Santiago^{1,2,3}**¹Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal, ²CNC.IBILI, University of Coimbra, Coimbra, Portugal, ³Association for Innovation and Biomedical Research on Light and Image (AIBILI), Coimbra, Portugal

Elevated intraocular pressure (IOP) is a major risk factor for the development of glaucoma, a retinal degenerative disease characterized by damage of the optic nerve and degeneration of retinal ganglion cells (RGCs). Microglia reactivity and neuroinflammation contribute to the loss of RGCs and may have a role in the pathophysiology of glaucoma. Evidence shows that the blockade of adenosine A_{2A} receptor (A_{2A}R) affords protection against several noxious conditions. In this study, we assessed whether blocking A_{2A}R is able to control microglia response and neurodegeneration elicited by elevated hydrostatic pressure (EHP), which mimics elevated IOP.

Microglia cell cultures (BV-2 cell line) and retinal primary neural cell cultures were exposed to EHP (70 mmHg above normal atmospheric pressure). Control cells were kept in a standard incubator. Cells were pre-treated with 50 nM SCH 58261, a selective A_{2A}R antagonist. BV-2 cells were also transfected with A_{2A}R siRNA, to decrease the expression of the A_{2A}R gene. Microglial cells were depleted from the primary neural cell cultures with clodronate-loaded liposomes.

The exposure of retinal primary cell cultures and BV-2 cells to EHP increased the mRNA and protein levels of A_{2A}R. In primary cultures, EHP increased cell death, changed microglia morphology to a reactive phenotype and increased microglia proliferation. EHP also increased phagocytosis and migration of BV-2 cells. These effects were prevented by the pharmacological blockade of A_{2A}R. Furthermore, the silencing of A_{2A}R decreased microglia migration, and the depletion of microglia from retinal primary cultures prevented EHP-induced cell death.

Our results demonstrate that the blockade of A_{2A}R affords protection to retinal cells through the control of microglia reactivity and identify microglia cells as major contributors of EHP-induced cell death. These results suggest that A_{2A}R antagonists could be envisaged as a therapeutic strategy for the management of retinal neuroinflammation in glaucoma.

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Cellular Cone Photoreceptor Imaging and OCT Angiography in Geographic Atrophy

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The purpose of this study was to visualize and compare cone photoreceptor and choriocapillaris differences at the edge of geographic atrophy (GA), in drusen regions, and areas without GA or drusen, in patients with age-related macular degeneration (AMD).

We imaged cone photoreceptors and choriocapillaris using confocal adaptive optics scanning laser ophthalmoscopy (AOSLO) and optical coherence tomography-angiography (OCT-A). Cone photoreceptor and choriocapillaris images from 2 AMD patients with GA were compared with 3 normal subjects. The AOSLO cone photoreceptor montage was superimposed upon OCT-A microvasculature images using retinal blood vessels as landmarks. Regions of interest (ROIs), 200 μm x 200 μm , were selected in the superimposed AOSLO and OCT-A images captured from the same eye. ROIs at the edge of well-defined areas of GA, drusen areas, and in areas without GA or drusen were selected in the 2 AMD patients, and were compared with normal appearing regions at comparable eccentricities in the AMD patients and the normal subjects. Cone spacing was calculated in each of the ROIs and choriocapillaris perfusion was assessed.

Confocal AOSLO images showed areas where cones were not visible and irregular cone packing at the edge of GA and drusen regions in the AMD patients. Cone spacing increased at the edge of GA and in some of the regions over drusen, but not in normal-appearing areas that were not associated with GA or drusen. Choriocapillaris perfusion was reduced but not completely absent in some of the ROIs at the edge of GA and under drusen compared with ROIs in normal-appearing regions at similar eccentricities in normal eyes.

Cone photoreceptor spacing measures were abnormal at the edge of GA and over drusen. Choriocapillaris perfusion at the edge of the GA and under drusen appeared reduced, compared with areas without GA and drusen, and compared with normal eyes. The abilities of AOSLO

and OCT-A to noninvasively visualize alterations in photoreceptor structure and choriocapillaris perfusion make them promising tools for assessing AMD with GA. Cone photoreceptor spacing and choriocapillaris perfusion changes can be distinguished and assessed in AMD patients with GA and normal eyes. The combination of AOSLO and OCT-A imaging and evaluation is useful for elucidating disease development and progression.

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An Automated System that Remove Outliers from Key Feature Points and from Multi-model Retinal Images Registration

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Purpose: To introduce an automated system that removes outliers from key feature points from multi-modal retinal images and mosaics them.

Methods: We develop an automated method to extract key features points and remove outliers and match them. In the method, we first extract salient feature points using the scale-invariant feature transform (SIFT) algorithm. Then compute the partial intensity invariant feature descriptor (PIIFD) for each salient feature point. Putative matching of the PIIFDs is then applied using a nearest-neighbor criterion. Outlier elimination and affine transformation parameter estimation is finally adopted to perform image registration

Results: The system was tested using color fundus images and corresponding fluorescein angiographic images from 100 subjects from the National Healthcare Group Eye Institute. We run comparative experiments between 4 algorithms, comparing our proposed with 3 other methods: Harris-PIIFD, RANdom SAmple Consensus (RANSAC) and M estimator SAmple and Consensus (MSAC). The best success rates are from our method (83%), compared with Harris-PIIFD (22%), RANSAC (32%), MSAC (34%).

Conclusions: An automated method that can remove outliers from key feature points for multi-modal retinal images is tested. Experimental results show that its performance is promising, showing good potential for the system to be used to mosaic multi-modal images.

Ocular Imaging

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Autofluorescence in Stargardt Disease as Predictor for Disease Progression**NATALIA PALARIE^{1,2}, Tatiana Pasenco³***¹State University of Medicine and Pharmacy, Tissue Engineering and Cell Cultures Laboratory, Chisinau, Republic of Moldova, ²International Clinic, Ophthalmology, Orhei, Republic of Moldova, ³State University of Medicine and Pharmacy, Ophthalmology, Chisinau, Republic of Moldova*

Current project seeks to investigate different fundus autofluorescence patterns in Stargardt disease in order to use this information to predict future progression of the disease.

Purpose: To examine the association between fundus autofluorescence (FAF) subtypes and macular atrophy progression in patients with Stargardt disease.

Approach: There were 16 patients (32 eyes) with diagnosis of Stargardt disease included in the study. Follow up period consisted 4 years. We have identified three main FAF subtypes: type 1 had a low signal at the macula surrounded by a homogeneous background, type 2 had a low signal at the macula surrounded by a heterogeneous background with numerous flecks of abnormal signal and type 3 had multiple low signal areas at the posterior pole with a heterogeneous background. There were 3 patients with type 1, 8 with type 2, and 5 with type 3 disease. The areas of reduced FAF signal were measured by IMAGEnet data system (Topcon) and rate of atrophy enlargement (RAE) was calculated as the difference of the atrophy size over time (mm²) divided by the follow up interval (years).

Results: The fastest progression was observed in subtype 2 group - 1,89 mm²/year and the lowest in subtype 1 group - 0,92 mm²/year. Patients subtype 3 FAF had the RAE of 1,63 mm²/year. At the end of observation period 1 patient from the 1 subtype group progressed to subtype 2.

Conclusions: FAF pattern at the baseline can serve as biomarker for prediction of RPE atrophy progression in patients with Stargardt disease. Patients with localized central atrophy have milder progression compared to subjects with multiple atrophic lesions who have more rapid loss of macular retinal structure over time.

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Cloud-based Automated Software for Diabetic Retinopathy Screening and Monitoring in a National Screening Program**DANIEL TING¹, Gavin Tan², Wynne Hsu³, Mong Li Lee³, Carol Cheung¹, Tien Yin Wong²***¹Singapore Eye Research Institute, Singapore National Eye Centre, Singapore, Singapore, ²Singapore National Eye Center, Singapore, Singapore, ³National University of Singapore, Singapore, Singapore*

Purpose: Several automated diabetic retinopathy (DR) grading software have been developed and tested, but few have been used in "real-life" settings in screening programs. We evaluated the diagnostic performance of a cloud-based automated software for DR in a national Singapore screening program.

Methods: We developed a "cloud-based" DR screening software (the Singapore Eye LEsion Analyzer, SELENA) that utilizes deep learning technology, a machine-learning technology based on learning representations of data, and applied this to images captured from the Singapore Integrated Diabetic Retinopathy Screening Programme (SiDRP), a national tele-medicine screening program for DR in the primary care setting based on centralized assessment of retinal photographs at a remote reading center of patients with diabetes seen at the primary care setting. We tested images captured from 381 consecutive patients (n=762 eyes) over a 1-month period from SiDRP. We calculated the area under curve (AUC), sensitivity, specificity, repeatability (same image analyzed twice) of SELENA, in detection of referable DR (moderate NPDR and above, including 'ungradable' images) and vision-threatening DR (severe NPDR and above), with reference to the retinal specialists' grading.

Results: Amongst the 762 eyes, 587 (77.0%) had no DR, 42 (5.5%) had mild non-proliferative DR (NPDR), 93 (12.2%) had moderate NPDR, 39 (5.1%) had severe NPDR and 1 (< 0.1%) had PDR. For the detection of referable DR (n=133), the AUC of SELENA was 0.92 whereas for vision-threatening DR (n=40), AUC was 0.94. When the sensitivity level for detection of 'referable DR' was set at 95% and 90%, the specificity level of SELENA was 54% and 62%, respectively, whereas for specificity level of 95% and 90%, SELENA had a sensitivity level of 60% and 70%, respectively. The detection rate of vision-threatening DR (n=40) and repeatability of SELENA (n=762) were 100%.

Conclusions: We tested and validated the performance of an automated DR screening software using retinal photographs from a national tele-medicine DR screening

program in the primary care setting in Singapore. With this performance, the software can be utilized as a DR detection tool to help reduce the manual assessment of retinal photographs for DR screening in Asia.

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Optical Coherence Tomography Angiography for Detection of Carotid Artery Stenosis: A Pilot Study

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We performed a pilot study to investigate changes of the superficial and deep capillary plexus within the retina in patients with a carotid artery stenosis > 50%. Stenosis of afferent vessels such as the carotid artery may lead to ocular ischemic lesions. We hypothesized that a decreased blood flow in patients with a moderate to severe carotid artery stenosis might lead to changes in the superficial and deep capillary plexus which may be quantified by optical coherence tomography angiography (angioOCT; ZEISS AngioPlex OCT Angiography).

We included four patients with moderate to severe homolateral carotid artery stenosis. Five healthy controls were assessed. Demographic data are summarized in table 1. All participants underwent a standard ophthalmological examination to exclude any concomitant ocular diseases. The retinal vasculature of the macula on the homolateral side of the carotid stenosis was analyzed with an angioOCT. To optimize the resolution and to avoid larger vessels, we limited the analysis to the central 3 x 3 mm volume. The percentage of the vessels area and the total number of junctions were analyzed using the NCI's AngioTool software.

There was a significant decrease of apparent vascular density in both the superficial and deep vascular plexus ($p=0.044$ and $p=0.014$, respectively) in patients with carotid artery stenosis. In addition, there was a significant decrease in the number of vessel junctions ($p=0.04$ for the superficial and $p=0.0034$ for the deep vascular plexus, respectively). A positive correlation was observed between severity of the stenosis and vascular density in the deep vascular plexus ($r^2=0.5$).

AngioOCT may be a useful tool to detect moderate to severe carotid stenosis.

| Demographic features | Patients with carotid stenosis (n = 4) | Control Group (n = 5) |
|-------------------------------|--|-----------------------|
| Sex | M:4 | M:3, F:2 |
| Age | 74.00 +/-7.3 | 47.60 +/-6.6 |
| BCVA (Snellen) | 0.9 ±0.2 SD | 1.0 ±0 SD |
| Moderate carotid stenosis (n) | 1 | 0 |
| Severe carotid stenosis (n) | 3 | 0 |

[Table 1]

RPE Choroid Biology and Pathology

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p27 Behaves as a Regulator of Phagocytosis and Epithelial-mesenchymal Transition in RPE Cells after Photoreceptor Damage**NORIHIRO SUDOU¹, Reeshan Ul Quraish^{1,2}, Kaori Nomura-Komoike¹, Hiroki Fujieda¹**¹Tokyo Women's Medical University, Tokyo, Japan, ²Toho University, Tokyo, Japan

p27KIP1 (p27) is a cell cycle inhibitor, but little is known about its roles beyond cell cycle regulation. To elucidate the function of p27 in the retinal pigment epithelium (RPE), we explored whether p27 depletion affects the response of the RPE to photoreceptor damage induced by N-methyl-N-nitrosourea (MNU) treatment.

MNU treatment caused severe photoreceptor damage in wild-type (WT) and p27 knockout (KO) mice retinas to the same degree. We found that the number of bromodeoxyuridine (BrdU)-labeled RPE cells were significantly increased in the p27 KO in comparison to the WT after the MNU treatment. We also showed that p27 KO RPE cells exhibited enhanced phagocytosis of dead photoreceptors compared to WT. In these mutant RPE cells, phosphorylation of myosin light chains was extremely up regulated, indicating the changes in cytoskeletal dynamics. Furthermore, WT RPE cells underwent epithelial-mesenchymal transition (EMT), including multilayered appearance, induction of α -smooth muscle actin expression, and attenuated expression of tight junction protein ZO-1, but these changes were not observed in the KO RPE. During EMT in the WT RPE, p27 localization was seen in both nuclear and cytoplasm, while the RPE cells in the intact retina show only nuclear staining.

Taken together, these results may indicate that p27 in RPE cells is not only involved in cell cycle regulation, but also prevents excessive phagocytosis and promotes EMT in response to retinal damage.

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Diurnal Processing of NLRP3 Inflammasome Components in Retinal Pigment Epithelium (RPE)**LUCIA CELKOVA¹, Natalie Hudson¹, Sarah Doyle², Matthew Campbell¹**¹Trinity College Dublin, Department of Genetics, Dublin, Ireland, ²Trinity College Dublin, Department of Clinical Medicine, Dublin, Ireland

It is now widely accepted that age-related macular degeneration (AMD) is associated with a chronic low-grade inflammatory profile in the retina and the underlying retinal pigment epithelium (RPE). It has also recently been established that NLRP3 inflammasome components are associated with the RPE in geographic atrophy (GA), the end stage of „dry“ AMD. Although the inflammasome and its components have been suggested to be the cause of RPE atrophy, their exact function in the disease progression remains unclear. Therefore, we aimed to examine the role of the NLRP3 inflammasome in the regulation of RPE homeostasis, focusing on its relationship with phagocytosis of photoreceptor outer segments (POSs) and subsequent processing of this phagocytosed material by the RPE diurnally. Wild-type C57BL/6J mice (10-12 weeks) were euthanised at either an 8am or 8pm time point, when lights were turned on or off in our animal unit, respectively. Eucleated eyes were immediately dissected to remove anterior parts of the eye (cornea, iris and lens) and subsequently the retina was gently detached from the posterior eye cup. RPE/choroid was scraped off the underlying sclera and protein and mRNA isolated to analyse levels of inflammasome and autophagy related components, including NLRP3, ASC, pro-caspase-1, pro-IL-1 β , pro-IL-18, LC3, p62, ATG5, ATG7 and beclin-1, by Western blotting or qRT-PCR. We observed that mRNA and protein expression profiles of NLRP3, ASC, pro-caspase-1, pro-IL-1 β and pro-IL-18 were significantly different between 8am and 8pm. At the same time, levels of mRNA and protein expression of all major autophagy machinery components (Atg5, Atg12, cathepsin B, etc.) varied between the two time points, with higher levels observed in the morning. To mimic this situation *in vitro*, primary hRPE cells were stimulated with bovine POSs and protein and mRNA expression analysed to reveal expression patterns matching the *in vivo* data. In summary, our findings show that autophagy activation in the RPE peaks in the morning as a result of phagocytosis and subsequent degradation of POSs. Moreover, NLRP3 inflammasome components are regulated in a distinctly diurnal fashion in the RPE, suggesting that the phagocytosis of POSs by the RPE and the regulation of NLRP3 inflammasome are intimately linked processes that function as part of normal RPE physiology to maintain RPE homeostasis.

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The Contribution of Insulin-receptor Mediated Signaling in the RPE to the Development of Diabetic RetinopathyIVY SAMUELS^{1,2}, Alecia Cutler², Matthew Tarchick^{1,2}, Bela Anand-Apte²¹Louis Stokes Cleveland Veterans Affairs Medical Center, Research Service, Cleveland, United States, ²Cole Eye Institute, Cleveland Clinic, Ophthalmic Research, Cleveland, United States

Analysis of the Wisconsin Epidemiological Study of Diabetic Retinopathy demonstrated that type 2 diabetic patients undergoing insulin treatment for ≥ 10 years exhibited significantly higher risk of diabetic macular edema compared to patients without insulin treatment within 6 months of the time of analysis. It has been suggested that RPE dysfunction is a precursor of and contributes to the development of diabetic retinopathy and diabetic macular edema. We have demonstrated abnormal light-induced responses of the RPE and retina (i.e., electroretinograms, ERGs) in diabetic mice at very early times following onset of hyperglycemia. Here, we investigate the role of insulin-receptor (IR) mediated signaling specifically within the RPE of a mouse model of Type 1 diabetes through the use of an IR conditional knockout mouse. The contribution of insulin-receptor mediated signaling to ERG defects, RPE and retinal structure, and tight junctions was evaluated. We identified that loss of RPE-specific IR-mediated signaling compromised the integrity of the RPE and affected light-evoked responses in normal mice. Early defects in the ERG of diabetic mice were also exacerbated by the loss of insulin-mediated signaling. These results demonstrate that IR-mediated signal transduction is required for maintenance of RPE health and function. Furthermore, modulation of insulin receptor-mediated signaling in the RPE may be a valid therapeutic target for diabetic retinopathy and/or diabetic macular edema.

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Human Retinal Pigment Epithelial Cell - Extracellular Matrix Interactions: Relevance to Age-related Macular Degeneration (AMD)

STEVEN EAMEGDOOL, Michele Madigan

*Save Sight Institute, University of Sydney, Sydney, Australia***Purpose:** Geographic atrophy, or dry AMD pathogenesis

is associated with retinal pigment epithelial (RPE) cell and photoreceptor outer segment death, soft drusen and basal laminar deposits, disruption to Bruch's membrane, complement activation and macrophage infiltration. The RPE-BM interface is critical for maintaining retinal homeostasis within the eye. In this study, we examined the effects of different extracellular matrix (ECM) proteins on RPE cell function, and the cellular effects from ECM nitrite modification.

Approach: Primary human RPE cells were cultured onto coated surfaces with: collagen I (COL1), collagen IV (COL4), elastin, fibronectin, or laminin; and cell attachment, spreading, migration, and proliferation, as well as mechanism of interaction, was assessed. MMP-2/-9, MCP-1 (CCL2), collagen I/IV secretion was assessed (zymography, ELISA). RPE-derived ECM modified by sodium nitrite was prepared for cell attachment and spreading assays. Immunohistochemistry (paraffin sections) of Iba1⁺/MHCII⁺ macrophage/microglia, and nitro-tyrosine (oxidation) from normal and AMD donors was analysed.

Results: COL1 or COL4 provided a 2-3 fold increase in cellular attachment and spreading, and cells cultured on COL1 or COL4 had a higher migratory capacity and proliferation index respectively. Adhesion to COL4 was magnesium and manganese-dependent, indicating an integrin-mediated interaction. Nitrite modification of RPE-derived ECM (analogous to smoking-induced oxidative stress) reduced cell attachment to the ECM by 15%. This effect was circumvented through COL4 addition. Preliminary examination of COL4 expression by AMD-derived RPE cells was found to be 20% lower compared to normal patients. In AMD donor eyes there was an increase in Iba1⁺/MHCII⁺ macrophage/microglia, and nitro-tyrosine expression.

Conclusions: Primary RPE cells demonstrated enhanced interactions with collagen I and IV evidenced by increased cellular attachment, spreading, migration and proliferation, mediated by integrins. Increased nitro-tyrosine expression was observed in AMD donor eyes. Nitrite modification of the ECM hindered cell attachment and addition of COL4 onto nitrite-modified surface restored RPE attachment, suggesting potential means for therapeutic intervention. Taken together, we have established a comprehensive assessment into the effects between ECM and RPE cellular function, and indicate how disruption of this synergy may implicate a pathologic role in AMD progression.

RPE Choroid Biology and Pathology

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MicroRNA-21 Regulates Prorenin Receptor (PRR)-mediated Induction of Vascular Endothelial Growth Factor (VEGF) Expression in ARPE-19 Cells**RASHIDUL HAQUE, P Michael Iuvone, Elizabeth H Hur, Kimberly Su Chung Choi, Ashley Ngo, Daniel Park, Annie N. Farrell, Li He, Madiha Aseem, Samantha Gokhale, Bhavna Kumar***Emory University, Ophthalmology, Atlanta, United States*

The prorenin receptor (PRR), a component of the renin-angiotensin system, plays an important role in the physiologic and pathophysiological regulation of blood pressure and fluid/electrolyte homeostasis. Previously, we demonstrated that miR-152 interacting with the PRR regulates VEGF, VEGFR2, and TGF β 1 expressions in human retinal endothelial cells in hyperglycemic (HG) conditions (Haque et al., 2015). In our current study, PRR-mediated induction of VEGF expression in human retinal pigment epithelial cells (ARPE-19) was shown to be regulated by a different miRNA, miR-21, via targeting *Smad7*, *Pten*, *Spry*, regulators of MAPK and other signaling pathways. We demonstrated that HG in cells treated with the angiotensin converting enzyme (ACE) inhibitor perindopril significantly induced PRR, VEGF, VEGFR2, and miR-21 expression, but suppressed the expression of SPRY1/SMAD7/PTEN transcript and protein levels. However, knockdown of PRR expression abolished HG-induced VEGF, VEGFR2, and miR-21 expression levels. Also, silencing of the small GTPase Rac1 significantly attenuated the level of miR-21 in HG, suggesting the involvement of both PRR and Rac1 in miR-21 regulation. A miR-21 mimic dramatically increased the activity of pERK, and HIF-1 α and VEGF expression, which was antagonized by knocking down the miR-21 level by a miR-21 antagomir, indicative of the involvement of miR-21 in the regulation of VEGF through ERK signaling. We conclude that the pleiotropic action of miR-21 induced pERK, HIF-1 α , and VEGF expression in HG condition by simultaneously targeting SPRY1, SMAD7, and PTEN. Therefore, miR-21 may serve as a potential therapeutic target for diabetes-induced retinal pathology, including diabetic retinopathy.

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A Novel Erythropoietin Derived Peptide Has Significant Neuroprotective Efficacy in Diabetic Retinopathy

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Exogenous delivery of erythropoietin (EPO) has been demonstrated to have anti-inflammatory and tissue protective effects in a variety of disease states. ARA290, a novel EPO-derived peptide, has been shown to ameliorate vascular leakage and capillary dropout in the retina of diabetic rodents. Since diabetic retinopathy has a significant neurodegenerative component, we assessed the potential of ARA290 to prevent deficits in neural retinal integrity and function, linked to suppression of inflammatory pathways. C57BL/6J mice were rendered diabetic by streptozotocin (STZ) injection; whilst control mice received citrate buffer. Diabetic animals were randomly assigned into treatment groups and received thrice weekly intraperitoneal injection of; vehicle, 30, or 60µg/kg ARA290 for 12 weeks. At the 12 week endpoint, retinal function was assessed by scotopic electroretinography (ERG). Retinal thickness was assessed by optical coherence tomography (OCT), and compared to baseline pre-treatment values. Post-mortem retinal analysis of retinal integrity and activation of immune cells was conducted alongside expression of a panel of pro and anti-inflammatory cytokines.

HbA_{1c} levels across diabetic treatment groups were similar and significantly elevated compared to non-diabetic control animals (***) $p < 0.0001$; One-Way ANOVA) and ARA290 had no effect on the haematocrit in any treated groups. Rod a-wave amplitude was significantly decreased at higher light levels in vehicle-treated diabetic animals compared to non-diabetic controls (** $p < 0.01$; two-way ANOVA, Bonferroni post-test). This decrease in rod a-wave amplitude was attenuated in the 60µg/kg ARA290-treated diabetic animals. The decrease in ON bipolar cell function (b wave amplitude) observed in vehicle-treated diabetic mice compared to non-diabetic mice, was also significantly attenuated in the 60µg/kg ARA290-treated diabetic mice (***) $p < 0.0001$; two way ANOVA). ARA290 treatment reduced the retinal thinning observed in the vehicle-treated diabetic animals measured at 12 weeks diabetes duration, with retinal thickness approaching that observed in non-diabetic animals. Diabetes mediated increases in

pro-inflammatory TNF α , IL-1 β , and ICAM-1 retinal mRNA levels were partially attenuated with ARA290 treatment. ARA290 is a potent neuro-protectant in the diabetic retina, preventing neural retinal degeneration mediated by increased pro-inflammatory stimuli, whilst preserving retinal functionality.

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Histopathological Findings in Eyes from Patients with Stargardt Disease

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To define the retinal histopathology in donor eyes from two donors with Stargardt Disease, eyes were obtained through the FFB eye donor program from a 69 year-old male who died from myocardial infarction. The donor was clinically diagnosed with Stargardt disease with an unknown mutation. The eyes were fixed within 15 hours of death and examined with macroscopic fundus photography (MF), confocal scanning laser ophthalmoscope (cSLO) and spectral-domain optical coherence tomography (SD-OCT). Small areas from the fundus macula and periphery were processed for electron microscopy and indirect immunofluorescence, using specific antibodies to retinal proteins and inflammatory cells. These donor eyes were compared to a matched normal eye (84 y.o.) and to eyes from a 66 y.o. female STGD donor previously characterized and known to carry compound mutations in the ABCA4 gene.

MF images of both STGD donor eyes showed a normal-appearing optic disc, prominent choroidal vessels, and areas of retinal degeneration in the perifoveal region. cSLO autofluorescence (AF) of the 69 year-old STGD donor identified the optic disk and hypofluorescent macula region. cSLO-AF of donor carrying the ABC4 mutations displayed a large well-defined area with weak AF signal when compared to the control. SD-OCT identified retinal changes including disorganization of retinal lamina and absence of the photoreceptor layer in both STGD eyes as compared to the control eyes. Histology analysis showed a severely degenerated fovea with little evidence of any retinal layering or remaining RPE in the STGD donor carrying the ABC4 mutations. The other STGD donor displayed inner nuclear layer disorganization in the perifovea with preservation of some of the RPE. In contrast, retinal nuclear

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layers were present in the periphery of both STGD donors. The perifoveal region contained few cones labeled with cone specific antibodies; some rhodopsin-labeled cells in the STGD donor carrying the ABC4 mutations. However, the perifovea of the other STGD donor displayed several stubby cones and very few rhodopsin-labeled cells. Cone synapses were not observed in either STGD donor eyes. This comprehensive examination of postmortem eyes from donors with STGD disease revealed a highly degenerated perifoveal region with preservation of the peripheral retina. Support: Research to Prevent Blindness, Wolf Family Foundation, National Eye Institute (EY014240-08) and Foundation Fighting Blindness.

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Characterization of Müller Cells Isolated from the Central and Peripheral Human Retina

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Since Müller cells are critical for retinal homeostasis; their dysfunction is likely to contribute to the pathogenesis of many retinal diseases. Müller cell loss has been detected in the macula of patients with Macula telangiectasia type 2 (MacTel), whereas peripheral Müller cells in these patients appear to be unaffected. This suggests that there may be a functional specialization that is specific to macular Müller cells that may make them vulnerable to disease, thus contributing to the pathogenesis of MacTel. In order to identify the differences between central and peripheral Müller cells, we successfully established Müller cells in primary culture from the central and peripheral regions of 22 pairs of normal adult human retinas. The morphology of the macular Müller cells was significantly different from the peripheral Müller cells in 18 out of the 22 pairs of eyes. To confirm the characteristics of the Müller cells derived from both retinal regions, we assessed expression of typical Müller-cell markers, GFAP and carbonic anhydrase II by immunocytochemistry by both cell types. Both sub-populations were decorated by

antibodies against all three proteins. We further extracted RNA samples from the primary cells from both regions for RNA sequencing. We found that both sub-populations strongly expressed most Müller cell markers including vimentin, glutamine synthetase, CRALBP, clusterin, GFAP and carbonic anhydrase II. By contrast, there was little expression of cell-specific markers for photoreceptors, bipolar cells, amacrine cells, microglia, endothelial cells or RPE. Interestingly, the two populations otherwise had significantly different transcriptome profiles. We also conducted Seahorse XF Analysis to study the functional difference between central and peripheral Müller cells by assessing their glycolytic and mitochondrial functions. We found that the central Müller cells possess significantly higher glycolytic capacity and reserve than peripheral Müller cells. We also found that the macular Müller cells had significant higher capability for basal mitochondrial respiration and ATP production but lower spare respiratory capacity than the peripheral Müller cells. These differences we report may partially explain why the human macula is more susceptible to disease, a phenomenon which is yet to be adequately explained.

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RCircadian regulation of the Inner Retinal Vasculature: A Paradigm for Geographic Atrophy Development

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Circadian rhythms are biological processes that occur in 24 hour oscillations which enable an organism to anticipate and prepare for regular environmental changes, such as feeding and sleeping. These processes can be regulated by external cues such as light and temperature. One process that occurs upon onset of light daily is the phagocytosis of photoreceptor outer segments (POS). The involvement of circadian rhythms in retinal function, however, are still not fully elucidated. Here, we wished to study the role of circadian clock components in the regulation of processes directly related to the replenishment of shed POS'. For *in vivo* analyses wild-type C57BL6/J mice (10-12 weeks) were sacrificed at 8AM, 2PM, 8PM and 2AM (8AM and 8PM corresponding to 12 h lights on; 12 h lights off cycle). Retinal protein and mRNA was extracted and tight junction (TJ) and circadian clock components analysed

by western blotting and qRT-PCR, respectively. To re-establish circadian rhythms *in vitro* primary human retinal microvascular endothelial cells were subjected to serum shock experiments.

We found that TJ component claudin-5 and clock component BMAL1 cycled throughout the day in the retinal vasculature and cycling of claudin-5 was dependent on BMAL1. In addition, we found that claudin-5 transcript and protein levels also cycled throughout the day in a circadian-dependent, rather than diurnal, manner. Levels of claudin-5 were found to be lower at 8PM when compared to 8AM. The retinal vasculature was evidently more permeable in the evening compared to the morning as observed by fundus fluorescein angiography (FFA) and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) analyses. This correlated with changes in protein expression observed and similar effects were seen in a range of mouse strains. Circadian regulated changes in retinal vascular permeability was not evident in BMAL1^{FL/FL}-Tie-2 mice, where the clock gene BMAL1 was knocked out of the retinal vasculature. Persistent suppression of claudin-5 in tandem with exposure of mice to a high fat diet led to rapid onset of a geographic atrophy (GA) like phenotype in mice.

This suggests that the inner blood-retinal barrier is highly dynamic and plays a critical role in replenishing POS. Circadian regulation of claudin-5 facilitates exchange of material between blood and the neural retina. Thus, regulating claudin-5 or circadian clock components may represent a novel therapeutic target for treating GA.

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Characterization of HTRA1 Regulatory Element in Patients with Exudative Age-related Macular Degeneration

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Age-related macular degeneration (AMD) is a leading cause of vision loss and blindness in the elderly. The dry form is more common and accounts for about 85-90% of AMD patients in US, while Japanese AMD patients predominantly progress to wet-form or polypoidal choroidal vasculopathy (PCV).

ARMS2/HTRA1 mutation is known to major risk factor for AMD (De Wan et al., Science 2006, The AMD gene consortium, Nat. Genet. 2012). Furthermore, we reported that the Japanese typical wet form AMD patients showed

significant association with ARMS2/HTRA1 (Goto, Akahori et al., JOBDI 2009). But, AMD pathogenic mechanism which derived from ARMS2/HTRA1 gene mutation is still unclear.

The purpose of this study is to elucidate the function of ARMS2/HTRA1 gene region in AMD patients. In this result, the promoter sequence experiment showed that a great number of AMD patients had specific indel mutation in 3.8 kb upstream of HTRA1 gene. 2-3-fold increase of promoter activity was observed in indel HTRA1 promoter compared to control sequence (Iejima et al., JBC 2015). Furthermore, we created transgenic mice ubiquitously overexpressing mouse Htra1 using the chicken actin in promoter, of Htra1 *in vivo* was shown to lead to choroidal neovascularization (CNV), similar to wet AMD patients (Nakayama, Iejima et al., IOVS 2014).

These results suggest that human HTRA1 expression is enhanced by AMD specific indel mutation in the promoter region of HTRA1 gene, and this enhanced HTRA1 may be concerned with induce retinal neovascularization.

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Inflammasome Activation in Photoreceptor Cells

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Purpose: Photoreceptor death is the ultimate cause of vision loss in various retinal degenerative diseases. Inflammasomes are the key signalling platforms that detect pathogenic microorganisms and sterile stressors and convert the pro-inflammatory cytokines interleukin (IL-1b and IL-18 from latent form into activate cytotoxic form. In this study, we investigated the role of inflammasome activation in photoreceptor death.

Methods: The expression of inflammasome components including NLRP1, NLRP3, AIM2, ACS, caspase 1 and caspase 11 by the mouse photoreceptor cell line 661W under clinical relevant stimulation (including TNF α , IFN γ , cytosolic DNA and mitochondrial DNA) were investigated using western blotting and qRT-PCR. The production of IL-1b and IL-18 in the supernatants was measured by ELISA. Cells were also pre-treated with caspase inhibitors including vx-765, Z-VAD-FMK and Ac-YVAD-cmk. Cell viability was assessed using Alamar Blue assay and RealTime-Glo MT cell viability assay.

Results: Under normal culture conditions, 661W cells express low levels of NLRP3, AIM2 and pro-caspase 1. The expression levels of AIM2 ($p < 0.001$) and NLRP3 ($p < 0.01$) were increased following TNF α /IFN γ , cytosolic

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DNA and mitochondrial DNA treatment. Western blot revealed increased caspase 1 and caspase 11 activation. The treatment also induced IL-18 expression and cell death, which can be prevented with the pre-incubation with caspase inhibitors.

Conclusions: Our results suggests that inflammasome activation related cell death can be induced in photoreceptor cells under inflammatory conditions. Inflammasome activation may play an important role in photoreceptor damage under disease conditions.

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The Retinal Light Response Altered by ChR2 Activation in Müller Cells

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Müller cells are the principal glia cells of the retina. Responsible for homeostasis, waste removal, and nutrient delivery, these macroglia cells support their neuronal neighbors. Beyond these maintenance roles, Müller glia can potentially augment neuronal communication through their role in neurotransmitter reuptake. Müller glia take up excess, and potentially toxic, neurotransmitters (e.g. glutamate) via ion exchange transporters. Müller glia maintain a high membrane potential of -80 mV to support their exchange transporters GLAST and GLT-1. We hypothesize that biasing this membrane potential can inhibit neurotransmitter reuptake, modulating the surrounding retinal neuronal light response. Since these ion exchange transporters are sensitive to membrane potential, optogenetic ion channels should be able inhibit them when activated. We've expressed the canonical ChR2 or a bi-stable point mutant, C128S, in Müller cells of mouse retina via ShH10, an Müller cell specific AAV capsid. Electroretinogram (ERG) recordings demonstrate a depressed B-wave from retinas expressing ChR2 and ChR2(C128S) in Müller cells. The ERG measures radial currents passing through the retina in response to light. There is a characteristic negative A-wave, attributed to the hyperpolarization of the photoreceptors, and then a much larger positive B-wave which is thought to be generated by bipolar cell depolarization in response to photoreceptor signaling. We hypothesized that if ChR2 attenuates the membrane potential of Müller cells and inhibits glutamate uptake, we should observe this as a depression of the B-wave amplitude. When compared to a GFP control, the

B-wave for the ChR2- and ChR2(C128S)-Müller retina to be reduced, however, the A-wave remained unaltered. The ChR2(C128S) 'bistable' point mutant, displays different channel kinetics than the canonical ChR2. ChR2(C128S) remains open for ~5 minutes after light stimulation. Since ChR2(C128S) remains open longer, it was expected for that construct to have a greater difference than the regular ChR2. Indeed, there was a greater difference in the B-wave between GFP-ChR2(C128S) (135µV) than GFP-ChR2 (49µV). ChR2(C128S) was also used for multi-electrode array recordings and showed show a recoverable decreased OFF response after ChR2 stimulation.

This electrophysiological data shows changes to normal retinal neuronal responses due to ChR2 stimulation in Müller glia, demonstrating the glial capability to modulate its neuronal neighbors.

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Visual Restoration Effect by Ectopic Expression of Channelrhodopsin in the Murine Retina Using Tet System

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Purpose: Ectopic Induction of optogenetic actuators such as channelrhodopsin (ChR) is a promising approach to restore vision in the degenerating retina. It is not easy to obtain an efficient gene expression in a specifically targeted cell population by transgenic approach. To gain both highly specific and sufficient gene expression simultaneously, we utilize a improved tetracycline transactivator-operator bipartite system (tet system) in which the amount of gene expression have been much enhanced (KENGE-tet system). In the present study we establish a retinal ganglion cell (RGC)-specific gene expression murine model with high efficiency using this system and confirmed a visual restoration effect by an ectopic expression of the ChR.

Methods: We employed a transgenic mouse line which expresses the gene encoding tetracycline transactivator (tTA) protein under the control of a cell-type-specific promoter, muscarinic acetylcholine receptor type 4 (M4) control region. The mice were further crossed with another transgenic mouse line which contains the yellow cameleon (YC) fluorescent gene connected into the downstream of the tet operator (tetO) promoter. The YC gene expression

was induced only by the presence of tTA protein in the double transgenic mice (M4-tTA::tetO-YC). The expression of YC was observed in the double transgenic mouse retina with a fluorescence microscope. As a visual restoration model, M4-tTA line was crossed with tetO-ChR to induce ChR expression in RGCs and we evaluated the function of the ectopic expression of the ChR using multielectrode array recording system (MEA).

Results: In the M4-tTA::tetO-YC mouse retina we identified the expression of YC mainly in RGC and in a few population of amacrine cells. In the M4-tTA::tetO-ChR mouse retina showed a sustained light response independently from photo-bleaching in the naïve photoreceptor cells.

Conclusion: Using the tet system, RGCs-specific gene expression was validated. Furthermore, we induced an ectopic ChR expression in RGCs using same system, and demonstrated a visual restoration effect. These results indicate that the tet-system mediated sufficient actuator induction in RGCs accelerates visual restoration research.

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Potential Contribution of SIK2 to Müller Cell Transdifferentiation

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Müller cells are the main glial cells of vertebrate retina and function to maintain retinal homeostasis and integrity. In fish, a group of Müller cells proliferate, gain stem cell characteristics and generate new neurons upon injury for retinal repair. However, this regeneration ability of Müller glia is constrained in mammalian retina. Growth factors can stimulate transdifferentiation of mammalian Müller cells to a limited extent. FGF2 is one of the factors that enhance the neurogenic potential of Müller glia. MIO-M1 cells, a spontaneously immortalized Müller cell line isolated from human retina, upon FGF2 treatment form neurospheres, express progenitor and neuronal markers. In this context, we aim to provide clues for the potential contribution of SIK2 as a negative regulator of FGF2 dependent Müller cell proliferation to the transdifferentiation process. In our experiments, MIO-M1 cells were treated with FGF2 for 0-7 days. In agreement with the literature, Pax6 transcript level reached maximum 12 hours post treatment, continued to remain high for 4 days and turned back to basal level after 7 days. Decrease in vimentin level after 4 days suggests that some cells lose their glial characteristics. Calretinin levels increased after 6 days indicating transdifferentiation of glial cells to

neurons.

In the same time frame, increased numbers of proliferating cells were observed after 1 hour of FGF2 treatment, reaching peak by 4 hours and turned back to basal level after 48 hours. There was another proliferation peak at 6 days of FGF2 treatment. In 0-24 hours treatment window, increase in ERK activity and decrease in SIK2 level were evident. At 24 hours post treatment, though ERK activity and SIK2 level returned to basal levels, cell proliferation continued to be higher than basal level. Thus it is conceivable that in this late time frame the proliferation is independent of ERK activation. To analyze the potential link of SIK2 to transdifferentiation process, SIK2 was overexpressed in MIO-M1 cells. Cell proliferation and ERK activity remained at basal levels in 0-24 hours in SIK2 overexpressed MIO-M1 cells. In addition, no significant change in marker gene expressions was observed in SIK2 overexpressed MIO-M1 cells throughout the 7 days of FGF2 treatment. All this data suggests that SIK2 did not permit Müller cells to transdifferentiate into neurons via blocking proliferation through ERK activation in the early phase of Müller transdifferentiation.

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Regulatory Role of microRNA-184 in Diabetic Retinopathy

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MicroRNAs (miRNAs), small non-coding RNA molecules, are known to participate in gene expression at post transcriptional level and are involved in multiple physiological and pathogenic processes. It was recently reported that the some miRNAs expression changes during the onset and progression of various diseases including diabetes. The purposes of this study were to identify the miRNAs with differential expression in the retinas of oxygen-induced retinopathy (OIR) mice, a model of proliferative retinopathy, and to study the potential role of miRNAs responsible for the pathophysiology of diabetic retinopathy (DR).

MiRNA expression changes in the retina of OIR mice were analyzed by quantitative reverse transcription-PCR (qRT-PCR). The potential candidate genes of identified miRNAs were predicted by bioinformatics analysis and validated by luciferase-based assay. The mRNA and/or protein levels of identified downstream targets of miRNA and associated signaling molecules were examined by qRT-PCR and

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Western blot analysis. Furthermore, we evaluated the *in vivo* functional role of miRNA in the mouse retinas of OIR. The qRT-PCR analysis showed that miR-184 was significantly down-regulated in the OIR mouse retinas. Bioinformatics analysis predicted that miR-184 may regulate the expression of some components of Wnt signaling. Therefore, we demonstrated that transfection of miR-184 mimic significantly inhibited Wnt signaling activity, whereas transfection of miR-184 inhibitor significantly enhanced Wnt signaling activity compared to negative control miRNA mimic/inhibitor. Furthermore, we identified that frizzled-7 (Fzd7) is a downstream target gene regulated by miR-184 using dual luciferase assay. Finally, delivery of miR-184 mimic in the retina of OIR mice showed significant inhibition of Wnt signaling activity as well as down-regulation of Fzd7, identified downstream target of miR-184, and Vegf-a, a downstream target of Wnt signaling.

It has been shown that aberrant activation of Wnt signaling in the diabetic retina may implicate in pathogenesis of DR. The present study demonstrates that down-regulation of miR-184 in the retina with ischemia-induced retinopathy may induce activation of Wnt signaling, which plays an important role in the pathogenesis and progression of DR. Our studies also suggest that over-expression of miR-184 in diabetic retina may have a therapeutic benefit to prevent inflammatory responses and/or neovascularization in DR.

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IL-1B Inhibition Reduces Chemokine-mediated Inflammation in Retinal Degeneration

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Purpose: Interleukin-1 β (IL-1B), a pro-inflammatory cytokine, has been implicated in the progression of both neo-vascular and atrophic age-related macular degeneration (AMD). Retinal degeneration increases IL-1B production, which stimulates a strong pro-inflammatory response, the secretion of chemokines, the activation of retinal microglia and recruited macrophages, and complement system activation, all of which culminates in photoreceptor-specific degeneration. Here, we investigate the effect of IL-1B inhibition in the diseased retina using a rat model of photo-oxidative stress and inflammation.

Methods: To inhibit IL-1B activity in the retina, IL-1B siRNA

or IL-1B IgG was intravitreally injected in adult Sprague-Dawley rats. Control siRNA or IgG injected animals were utilised for comparison. Animals were exposed to photo-oxidative damage (1000 lux light) for 24 hours. Following light exposure, eyes and retinas were collected to assay photoreceptor cell death (TUNEL), to localise microglia/macrophages (IBA1 immunohistochemistry), to detect IL-1B protein levels (ELISA) and to determine expression and localisation of IL-1B, Ccl2, Cxcl1 and Cxcl10 genes (qPCR and *in situ* hybridisation).

Results: After 24 hours of photo-oxidative damage, elevated levels of IL-1B levels ($P < 0.05$) were detected primarily in infiltrating IBA1+ microglia/macrophages in the retina. Additionally, there was an increased expression of Ccl2, Cxcl1 and Cxcl10 following light exposure ($P < 0.05$). An injection of IL-1B siRNA significantly suppressed IL-1B gene expression in the retina ($P < 0.05$). Treatment with either IL-1B siRNA or IgG reduced Ccl2 expression in Muller cells ($P < 0.05$), Cxcl1 expression in RPE cells ($P < 0.05$) and Cxcl10 expression (IgG only, $P < 0.05$). In both treatments, there was a decrease in microglia/macrophage infiltration into the outer retina ($P < 0.05$), accompanied by a significant reduction in photoreceptor cell death in the ONL ($P < 0.05$).

Conclusion: Inhibition of IL-1B activity significantly modulates microglia/macrophage recruitment and reduces photoreceptor loss in a model of photo-oxidative stress and inflammation. The use of IL-1B inhibitors, or strategies to manage detrimental microglia/macrophage activity could be useful therapeutic avenues to reduce IL-1B expression and consequent photoreceptor damage in retinal degenerations such as AMD.

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Dark Rearing as a Means of Mimicking 'Physiological Hypoxia': A Rationale for Non-invasive Treatment of Retinopathy of Prematurity

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The initiating event in ROP is *delayed retinal vascularization*

- an adverse side-effect of high oxygen levels in the retina, resulting from O₂ supplementation treatment. Because photoreceptors are depolarized and consume more oxygen in the dark, we hypothesized that this side-effect of oxygen supplementation would be mitigated by keeping neonates in total darkness. We propose that dark rearing creates a 'metabolic sump' that depletes oxygen in the retina through continuous depolarization of photoreceptors. Four groups of SD rats were examined at P14 & P18:

- 1) Room Air + normal light reared (NLR);
- 2) Room air + dark rearing (DR). 3) 50/10 OIR + normal light; 4) 50/10 OIR + DR.

Retinas were analysed to determine the vascular density index (VDI) and to estimate vaso-oblivation, neovascularization and astrocyte ensheathment. We used hypoxyprom-1 (HP1) to identify areas of hypoxia, TEM, and gene expression via qPCR. Electroretinograms (ERG) were recorded at P18 & P25 in OIR, & in rats dark reared for 30 days followed by 0, 30, or 60 days in normal light conditions. DR rats had a higher capillary density than NLR rats in room air (VDI=43.0±1.0 vs. 37.98±1.1 p< 0.05 - P7) and on return to room air following O₂ supplementation (hypoxic phase), DR reduced pre-retinal neovascularization (4.1% ± 0.9 v 15.8% ± 1.3 p< 0.05) and tissue hypoxia as evidenced by reduced HP1. DR also protected astrocytes and pericytes from hypoxic damage, and preserved retinal ultrastructure, inner segment mitochondrial integrity and protected rod outer segments from damage under OIR, as assessed by TEM. DR prevented upregulation of HIF1 α (-20.4% ± 4.6 v 65.5% ± 27.2 p< 0.05), VEGF164 (291.2% ± 60.1 v 496.6% ± 11.1 p< 0.05) and AP1/Jun (v -51.7 ± 16.7% v 179.8% ± 26.9, p< 0.05) in peripheral retina at P14. DR had no detrimental effects on retinal function as evidenced by ERG at P18 and P25 in OIR, & after 30 days DR. The data indicate that DR precludes initiation of ROP by maintaining retinal vascularization during oxygen supplementation (Phase 1). This protects the retina from hypoxia-induced vaso-proliferation on return to room air (Phase 2). This cost-effective, non-invasive intervention can reduce disease severity, may be used to supplement current therapies, and may negate the need for invasive treatments including anti-VEGF therapy.

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Systematic Analysis of the Effects of Diabetes-relevant Stimuli on Human Retinal Cell Expression of Extracellular Matrix Constituents

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One of the earliest structural abnormalities of diabetic retinopathy (DR) is basement membrane (BM) thickening of the retinal microvasculature. Recent studies suggest that excessive extracellular matrix (ECM) deposition may cause alterations in BM composition, however much remains to be understood about how ECM deposition or BM thickening contributes to DR pathology. Despite a focus in the literature on endothelial cells, a variety of retinal cell types are part of the glial-vascular unit and therefore could be involved in changes in the architecture of the BM. The purpose of this study was to develop an *in vitro* experimental platform to investigate the role of various retinal cell types in ECM deposition in DR. Under normalized growth conditions, human retinal microvascular endothelial cells (HRMEC), human retinal pericytes (HRP), and human Müller cells (HMC) were treated with a variety of diabetes-relevant stimuli. Included were inflammatory cytokines (10 ng/mL TNF α , IL1 β , IL-6 or IL-8 or 0.1%BSA in H₂O vehicle) and high glucose conditions (5 mM or 25 mM D-glucose, with L-glucose as an osmotic control). Concentrations, treatment times, and growth conditions were systematically optimized for induction of two primary BM components, collagen IV and fibronectin, which was measured by qRT-PCR.

HRMEC exposure to IL1 β caused a 1.8-fold (p< 0.005) and 1.2-fold (p=0.05) increase in collagen IV and fibronectin, respectively. TNF α caused a 2.2-fold (p< 0.005) in collagen IV in HRMEC; IL-6 and IL-8 did not cause significant changes. IL1 β and TNF α increased collagen IV expression in HRP by 1.8-fold (p< 0.005) and 2.6-fold (p< 0.005), respectively. High glucose treatment in HRMEC or HRP produced no significant changes in fibronectin or collagen IV expression. HMC showed no significant changes in collagen IV or fibronectin expression in response to high glucose or TNF α exposure. These results demonstrate the effect, or lack thereof, of a variety of diabetes-relevant stimuli on the expression of ECM proteins by HRMEC, HRP, and HMC. Inflammatory cytokines were shown to be more potent inducers of ECM expression than conditions designed to simulate hyperglycemia. Additionally, HRP demonstrate similar levels of ECM induction in response to diabetes-relevant stimuli as HRMEC, indicating that endothelial cells should not be the only focus in understanding BM thickening. Future studies will employ this experimental platform to test cellular mechanisms underlying BM thickening.

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Humanin G Is Protective against Mitochondrial DNA-mediated and Amyloid- β -induced Cell Stress in Age-related Macular Degeneration (AMD) ARPE-19 Cybrid CellsSONALI NASHINE¹, Marilyn Chwa Chwa¹, Kelvin Yen², Pinchas Cohen², Baruch D. Kuppermann¹, M. Cristina Kenney^{1,3}¹University of California, Gavin Herbert Eye Institute, Irvine, United States, ²University of Southern California, Davis School of Gerontology, Los Angeles, United States, ³University of California, Pathology and Laboratory Medicine, Irvine, United States

Mitochondrial (mt) DNA damage increases with ageing and contributes to retinal pigment epithelial (RPE) cell death in AMD. Moreover, deposition of amyloid- β , a constituent of drusen, has been associated with AMD progression. Recently, Humanin G (HNG), a mitochondrial derived peptide that is encoded from the 16S rRNA of the mitochondrial genome, has been demonstrated to be cytoprotective against amyloid- β -induced neuronal cell death *in vitro* and *in vivo*. However, the role of HNG in AMD ARPE-19 transmitochondrial cybrid cells has never been demonstrated. Therefore, in the current study we tested the hypothesis that HNG protects AMD cybrid cells against amyloid- β -induced cell death. Cybrid cultures were created by fusing platelets obtained from AMD patients or age-matched normal (NL) subjects with RhoD (lacking mtDNA) human ARPE-19 cells. All cybrids had identical nuclei and differed only in mtDNA content. The AMD and NL cybrids (n=3-5) were incubated with either HNG alone, amyloid- β ₁₋₄₂ (active form) peptides alone or a combination of HNG and amyloid- β . The amyloid- β ₄₂₋₁ peptide (inactive form) served as a scrambled control. The cellular metabolic activity, representing viability, was measured with the MTT assay. Untreated AMD cybrids showed significantly lower cell viability compared to untreated NL cybrids (P< 0.05). AMD cybrids treated with HNG alone had a 17% increase in cell viability compared to untreated AMD cybrids (P< 0.05), suggesting that HNG was protective in AMD cybrids. Treatment with amyloid- β ₁₋₄₂ alone reduced cell viability by 26% and 14% in NL and AMD cybrids, respectively, compared to untreated cybrids. The amyloid- β ₁₋₄₂ treated AMD cybrids had a significant decline compared to the amyloid- β ₄₂₋₁-treated cybrids (79.5%, P< 0.01). Furthermore, as hypothesized, HNG increased cell viability by 16% in amyloid- β ₁₋₄₂-treated NL cybrids, and 20% in amyloid- β ₁₋₄₂-treated AMD cybrids (P< 0.05) compared to the amyloid- β ₁₋₄₂-treated cultures. In conclusion, our

cybrid model demonstrates that damaged mitochondria from AMD subjects have lower levels of metabolism and cell viability, compared to age-matched NL mitochondria. In addition, HNG provides protection against mitochondrial DNA-mediated and amyloid- β -induced cell stress in AMD ARPE-19 cybrids. This research is significant because it identifies HNG as a cell survival factor and suggests its role as a potential therapeutic target for the treatment of dry AMD.

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Variable Cone ERG in a Multigenic Canine Model of Cone-rod Dystrophy with RPGRIP1 and MAP9 Mutations

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Background: Genetic modifiers are considered to play major roles in the expression of the phenotypically heterogeneous inherited retinal diseases. Clinical disease expression may vary dramatically even among patients harboring identical gene mutations. Canine population of a given breed typically shows high genetic uniformity allowing isolation of genetic effects. Canine model of cone-rod dystrophy (CRD) was previously associated with a *RPGRIP1* mutation, and a modifier mutation corresponding to the disease onset has more recently been found in *MAP9*. However, phenotype-genotype discordances still seemed to remain and thus are further investigated herein.

Methods: Phenotypic data and DNA/retinal samples were collected from dogs of a purpose-bred, canine research colony segregating both *RPGRIP1* and *MAP9* mutations. To assess the retinal function, visually-guided behavior test was carried out under different light intensity environment. Additionally, photopic single flash and flicker ERGs were recorded to evaluate the cone-derived ERG response. Changes in retinal structure associated with the cone ERG phenotype were examined by histology and immunohistochemistry using retinal sections from *RPGRIP1* mutant dogs with and without cone ERG responses

Results: In the *RPGRIP1* mutant dogs, cone-derived ERG responses varied extensively ranging from normal to complete absence. Strikingly, visually-guided obstacle course was normal at photopic and scotopic conditions in all the dogs tested including those that show no cone ERG response. Immunolabeling of cone markers revealed short cone outer segment and L/M opsin mislocalization in

the absent cone ERG retina. Notably, the *MAP9* mutation identified as the modifier for canine CRD did not correlate with the cone ERG phenotype.

Conclusions: Based on the lack of association between the cone ERG phenotype and the *RPGRIP1* or *MAP9* mutations, involvement of another genetic factor that primarily affects cone photoreceptor function is indicated. While neither of the mutations causes CRD on its own, these genetic factors seem to partly contribute to the phenotypic expressivity in canine CRD. Studying the complex molecular basis of the genetically and clinically heterogeneous retinal disease is facilitated by using the genetically homogeneous canine model that enables isolation of the multiple genetic factors that contribute to disease expressivity.

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Cytochrome P450 Oxidase 2C Inhibition Augments the Protective Effects of Omega-3 Long-chain Polyunsaturated Fatty Acids on Pathological Ocular Angiogenesis

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Objective: Pathological ocular neovascularization is a major cause of blindness. Increased dietary intake of ω -3 long-chain polyunsaturated fatty acids (LCPUFAs) reduces retinal and choroidal neovascularization, but ω -3 LCPUFA metabolites of a major metabolizing pathway, cytochrome P450 oxidase (CYP) 2C, promote ocular pathological angiogenesis. We hypothesized that inhibition of CYP2C activity will augment the protective effects of ω -3 LCPUFAs on neovascular eye diseases.

Approach and results: The mouse models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV) were used to investigate pathological angiogenesis in the retina and choroid respectively. The plasma levels of ω -3 LCPUFA metabolites of CYP2C were determined by mass spectroscopy. Aortic ring and choroidal explant sprouting assays were used to investigate the effects of CYP2C inhibition and ω -3 LCPUFA derived CYP2C metabolic products on angiogenesis *ex vivo*. We found that inhibition of CYP2C activity by montelukast augmented the protective effects of ω -3 LCPUFAs on retinal and choroidal neovascularization by 30% and 20% respectively. In CYP2C8 over-expressing mice fed a ω -3 LCPUFA diet, montelukast suppressed retinal and choroidal neovascularization by 36% and 39% and

reduced the plasma levels of CYP2C8 products. Soluble epoxide hydrolase inhibition, which blocks breakdown and inactivation of CYP2C ω -3 LCPUFA-derived active metabolites, increased OIR and CNV *in vivo*. Exposure to selected ω -3 LCPUFA metabolites of CYP2C significantly reversed the suppression of both angiogenesis *ex vivo* and endothelial cell functions *in vitro* by the CYP2C inhibitor montelukast.

Conclusion: Inhibition of CYP2C activity augments the protective effects of ω -3 LCPUFAs on pathological ocular angiogenesis.

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The Potential Contribution of Endoplasmic Reticulum Calcium Depletion to Endoplasmic Reticulum Stress and Cone Death in CNG Channel Deficiency

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The cone photoreceptor cyclic nucleotide-gated (CNG) channel is essential for cone phototransduction. Mutations in genes encoding the cone channel subunits CNGA3 and CNGB3 account for about 80% of all cases of achromatopsia, and are associated with progressive cone dystrophies. We previously showed that the cone channel-deficient mice display endoplasmic reticulum (ER) stress-associated cone death and increased phosphorylation of the ER calcium channel 1,4,5-inositol triphosphate receptor (IP₃R). The present work investigates the role of ER calcium channels in ER stress and cone death. We used pharmacological inhibitors to block ER calcium channels IP₃R and ryanodine (RyR) in *Cnga3^{-/-}/Nrl^{-/-}* mice with CNGA3 deficiency on a cone-dominant background. Postnatal day 7 (P7) mice received 2-APB (10.0 μ g/g body weight/day) or U-73122 (8.0 μ g/g body weight/day) by daily intraperitoneal injection to block IP₃R, and received tetracaine (10 mM, 1.0 μ l) or dantrolene (30 mM, 1.0 μ l) by a single intravitreal injection to inhibit RyR. Mice were analyzed at P15 for cone death using TUNEL labeling on retinal cross sections and for expression levels of ER stress markers by immunoblotting. Cellular calcium levels were measured *in vitro* from isolated *Cnga3^{-/-}/Nrl^{-/-}* and *Nrl^{-/-}* photoreceptors loaded with Fura-2 (10 μ M). Treatment with ER calcium channel inhibitors resulted in a 30% reduction of TUNEL positive

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cells in *Cnga3^{-/-}/Nrl^{-/-}* retinas. The levels of the ER stress marker proteins, including phospho-IRE1 α , phospho-eIF2 α , and cleaved form of ATF-6, were reduced by about 40-50% in the drug-treated mice, compared with vehicle-treated controls. Baseline $[Ca^{2+}]_i$ levels were reduced by about 50% in P15 *Cnga3^{-/-}/Nrl^{-/-}* photoreceptors, compared with that in age-matched *Nrl^{-/-}* mice. We show that the inhibition of the ER calcium channels reduces ER stress and cone death, supporting the view that ER calcium depletion/cytosolic calcium reduction contributes to ER stress/cone death in CNG channel deficiency. Our findings suggest that suppressing ER calcium channels may represent a novel strategy for photoreceptor preservation.

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Macular Carotenoid Supplementation Increases Macular Pigment Optical Density and Reduces Symptoms Associated with High Screen Time Exposure: A Randomized, Double-blind Placebo-controlled Study**JAMES STRINGHAM, Nicole Stringham***University of Georgia, Physiology and Pharmacology, Athens, United States*

The increased dependency of modern society on accessing the internet, coupled with the advent of smart phones and computer tablets, has led to a dramatic rise in "screen time" (the amount of time spent each day in front of a screen, whether a phone, tablet, computer, or television). Relative to other groups, college students are a demographic that maintains very high screen time, and often complain of symptoms associated with Computer Vision Syndrome such as eye strain and headache. The dietary carotenoids lutein and zeaxanthin, along with the zeaxanthin isomer mesozeaxanthin, are deposited in the macular retina (as macular pigment), where they have been shown to protect vulnerable neural tissues via antioxidant activity and high-energy blue light filtration. In college students with at least 6 hours of daily screen time exposure, we tested the hypothesis that increasing the optical density of macular pigment (MPOD) via supplementation with macular carotenoids for 6 months will lead to lower levels of eye strain, eye fatigue, blurry vision, eye irritation, and headache frequency. 48 normal, healthy subjects participated in this trial. 35 subjects were assigned to receive 24 mg of macular carotenoids (20 mg lutein + 4 mg zeaxanthin isomers), whereas 13 subjects were assigned to the placebo group. All subjects ingested one pill daily with a meal, for 6 months. MPOD, and data for each symptom were obtained at baseline, 3 months, and 6 months. At baseline, there were no significant differences between treatment and placebo groups for any variable tested ($p > 0.05$ for all). In the experimental group, a repeated-measures ANOVA revealed significant increases in MPOD ($p = 0.021$), and improvements in subjects' reported headache frequency ($p < 0.001$), eye strain ($p = 0.048$), and eye fatigue ($p < 0.001$) after 3 months of supplementation. After 6 months, these results were maintained, or became stronger. Subjects' reports of blurry vision and eye irritation was not affected by the carotenoid supplement. The placebo group exhibited no change for any variable measured across the 6-month study period ($p > 0.05$ for all). Supplementing with a relatively high amount of the macular carotenoids increases MPOD, and reduces eye strain, eye fatigue,

and headache frequency in those with high screen time exposure. Possible mechanisms for these effects include the macular carotenoids' ability to filter blue light, and increase the metabolic efficiency of the visual cycle.

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The Role of Brain Aromatase in Eye Development of Zebrafish**ZULVIKAR SYAMBANI ULHAQ, Mitsuyo Kishida***Kumamoto University, Kumamoto, Japan*

Aromatase is the key enzyme for aromatization of testosterone to estradiol (E_2). Previous studies in zebrafish have shown that brain aromatase (*cyp19a1b*) is expressed in the eye. Therefore, in this study we aim to analyze the role of brain aromatase (aroB) in early development of zebrafish eye. Since our recent study showed that knockdown of aroB impairs serotonin (5-HT) expression in brain, we also analysed the function of 5-HT signaling in developing eye. Immunohistochemistry using the specific antiserum to zebrafish aroB showed that immunoreactivity of brain aromatase (irAroB) was localized in retina layers and lens epithelial cells (LEC) of adult fish, and this result was also confirmed by RT-PCR. The irAroB in retinal layers was decreased in 120 hpf larvae, when injected with aroB MO but not with inverted AroB MO (negative control). Injection of 5 ng/nl AroB MO decreased the eye/body length ratio at 48 hpf, and the effect was partially reversed by co-incubation with 1 μ M of E_2 . Apoptosis in the eye detected by acridine orange staining at 24 hpf was significantly increased by AroB MO. Immunohistochemistry for acetylated tubulin demonstrated that AroB injection decreased the diameter of optic nerve, which was reversed by incubation with 1 μ M of E_2 . Injection of AroB MO at 5 ng/nl resulted in decreased response in visual background adaptation (VBA) in 5 dpf larvae. When embryos were exposed to 10 μ M ritanserin (5-HTR2 antagonist) to investigate the role of serotonin signaling in eye development, the results of the eye/body length ratio, optic nerve diameter, apoptosis and VBA in were similar to those obtained in the aroB MO injected embryos. The effects of ritanserin were reversed by co-incubation with 50 μ M 5-HT, except the reversal was partial in VBA experiment. Taken together, our data indicate that estradiol produced by brain aromatase in the eye may be required for development of the eye. Further investigation is needed to confirm whether the function of estradiol is mediated through 5-HTR2 signaling.

Keywords: brain aromatase, serotonin receptor 2, eye development, zebrafish.

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Macular Pigment Augmentation Promotes Light Filtering, Biochemical, and Neuromodulatory Effects that Improve Visual Performance**NICOLE STRINGHAM, James Stringham***University of Georgia, Physiology and Pharmacology, Athens, United States*

The macular carotenoids (MC) lutein (L), zeaxanthin, and meso-zeaxanthin comprise the diet-derived macular pigment (MP). The optical density of MP (MPOD) has been found to correspond significantly to aspects of visual performance such as contrast sensitivity (CS), disability glare (DG), and photostress recovery (PSR). The purpose of this study was to determine effects of MC supplementation on MPOD, repeated-exposure PSR, CS and DG thresholds, and a measure of lateral inhibition. This was a randomized, double-blind, placebo-controlled trial. 48 young (mean age = 21.9), healthy volunteers participated in the study. Subjects supplemented daily their diet with 20 mg L + 4 mg zeaxanthin isomers (n = 35), or placebo (n = 13) for 6 months. MPOD was assessed with customized heterochromatic flicker photometry. PSR times to an 8 cycle / degree, 15% contrast Gabor patch target were determined after each of five successive exposures to intense LED lights. DG was defined as the intensity of a ring of lights through which subjects were able to maintain visibility of the aforementioned Gabor patch target. Thresholds for detection of illusory shadows in the Hermann grid (heretofore referred to as lateral inhibitory sensitivity (LIS)) were determined with a computer-based, user-adjustable Hermann grid. Standard CS thresholds (at 8 cycles / degree) were determined with a computer-based, 2-alternative, forced-choice procedure. Measures of all parameters were conducted at baseline, 3 months, and 6 months. Repeated-measures ANOVA revealed that MPOD increased significantly versus placebo at both 3- and 6-month visits ($p = 0.021$ and $p < 0.001$, respectively). PSR times and DG thresholds improved significantly from baseline compared to placebo at 3- and 6-month visits ($p < 0.001$ for all). Additionally, LIS and CS increased significantly in the treatment group between baseline and 6 months ($p < 0.05$ for both). The placebo group did not change appreciably for any measure throughout the study period. As a function of MPOD, the repeated-exposure PSR curves became more asymptotic, as opposed to linear. Changes in CS and LIS over the 6-month study period were found to be significantly related ($r = 0.41$; $p = 0.0014$). Increases in MPOD lead to substantial improvements in PSR times, DG thresholds, enhanced LIS,

and improved CS. Results suggest that the mechanism for these effects may involve optical filtering, biochemical (antioxidant), and neuromodulatory properties of MP.

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An ER-resident BH3-only Protein, BNip1, Induces Apoptosis in Response to Excessive Vesicular Transport during Photoreceptor Differentiation**YUKO NISHIWAKI, Miyuki Suenaga, Masato Araragi, Ichiro Masai***Okinawa Institute of Science and Technology Graduate University, Onna-son, Japan*

Intracellular protein transport is mediated by budding and fusion of transport vesicles on intracellular membrane organelles and often linked to photoreceptor degenerations in human. However, it is unclarified how protein transport defects are monitored and links to photoreceptor degenerations. We reported that genetic mutations of beta-SNAP, a vesicular fusion regulator, cause apoptosis in zebrafish photoreceptors, and found that this apoptosis depends on BNip1. BNip1 is a t-SNARE component of syntaxin18 (stx18) SNARE complex, which normally regulates retrograde transport from Golgi to ER. BNip1 also contains BH3 domain, which activates Bax-dependent apoptosis. The absence of beta-SNAP compromises the disassembly of stx18 cis-SNARE complex, which is normally generated by the fusion of retrograde transported vesicles to ER membranes. We found that the accumulation of stx18 cis-SNARE complex on ER membrane activates Bax-dependent apoptosis through its BH3 domain (Nishiwaki et al., 2013). Thus, it is likely that BNip1 induces apoptosis in response to vesicular fusion defects. To understand physiological roles of BNip1 for photoreceptor apoptosis, we examined a critical period of beta-SNAP in photoreceptor maintenance. In the beta-SNAP mutant, photoreceptor degeneration largely occurs in the period of 2-3 days post-fertilization (dpf) when protein transport to the outer segment is active. We found that over-expression of beta-SNAP only in this early stage is sufficient to suppress photoreceptor apoptosis in the beta-SNAP mutant. These data indicate that depletion of beta-SNAP can activate BNip1-dependent apoptosis only when protein transport to the outer segment is active. Furthermore, we found that the inhibition of protein transport to the outer segment rescued photoreceptor apoptosis in beta-SNAP mutant. From these data, we propose that BNip1 functions as a safe guard mechanism that inhibits excessive vesicular transport during photoreceptor differentiation.

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Expression of Quaking RNA-binding Protein in the Mouse Retina**CHIEKO KOIKE^{1,3}, Takahiko Suiko¹, Kensuke Kobayashi², Kentaro Aono¹, Togo Kawashima²**¹Ritsumeikan University, College of Pharmaceutical Sciences, Kusatsu, Japan, ²Ritsumeikan University, Graduate School of Life Sciences, Kusatsu, Japan, ³Ritsumeikan University, Center for Systems Vision Sciences, Kusatsu, Japan

Quaking (QKI), which belongs to the STAR family of KH domain-containing RNA-binding proteins, functions in pre-mRNA splicing, microRNA regulation, and formation of circular RNA. QKI plays critical roles in myelinogenesis in the central and peripheral nervous systems and has been implicated neuron-glia fate decision in the brain; however, neither the expression nor function of QKI in the neural retina is known. Here we report the expression of QKI RNA-binding protein in the mouse retina. QKI was strongly expressed by Müller glial cells in the adult and developing retina. Neuronal expression was uniformly weak in the adult, but during development, QKI was expressed in horizontal and amacrine cells, and subsequently in later differentiating bipolar cells, but not in photoreceptors. QKI was expressed in progenitor cells during embryonic stages. Among QKI isoforms (5, 6, and 7), QKI-5 was the predominantly expressed isoform in the adult retina.

We conclude that QKI is expressed in developing and adult Müller glia. QKI is additionally expressed in progenitors and in differentiating neurons during retinal development, but expression weakened or diminished during maturation. Among QKI isoforms, we found that QKI-5 predominated in the adult mouse retina. Since Müller glial cells are thought to share properties with retinal progenitor cells, our data suggest that QKI may contribute to maintaining retinal progenitors prior to differentiation into neurons. On the other hand, the expression of QKI in different retinal neurons may suggest a role in neuronal cell type specific fate determination and maturation. The data raises the possibility that QKI may function in retinal cell fate determination and maturation in both glia and neurons.

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Dissociation between Functional and Anatomical Non-crossing Visual Response in Mice**HIROMITSU KUNIMI^{1,2}, Yusaku Katada^{1,2}, Kazuo Tsubota¹,****Toshihide Kurihara^{1,2}**¹Keio University School of Medicine, Department of Ophthalmology, Tokyo, Japan, ²Keio University School of Medicine, Laboratory of Photobiology, Tokyo, Japan

Purpose: It is important to evaluate the functional viability of retinal ganglion cells (RGCs) in pathological states to investigate the mechanism of optic nerve disorders such as glaucoma, optic neuritis, and traumatic optic neuropathy. Visual evoked potentials (VEPs) are widely utilized to evaluate the electrophysiological visual response especially from RGCs to the visual cortex in the brain experimentally and clinically. It has been reported that the anatomical ratio of non-crossing optic nerve fiber at chiasma is approximately 0.5 to 10% in mice. In this study, we evaluate the functional non-crossing ratio of visual response in a murine model of optic nerve crush (ONC).

Methods: ONC for 5 seconds was performed in 8 week-old C57/B6J mice unilaterally or bilaterally (n=4). Flash VEPs were measured 7 days after ONC for each group. The rate of the functional non-crossing visual response was evaluated by comparing the amplitudes of VEPs between ipsilateral and contralateral recordings.

Results: In unilateral ONC mice, the VEP amplitudes were 82.6 ± 29.1 and 22.4 ± 10.0 μ V at the ipsilateral and the contralateral side, respectively. The functional non-crossing ratio was estimated to be $20.9 \pm 4.5\%$. No significant responses were detected in bilateral ONC mice.

Conclusions: Dissociation between the anatomical and the functional optic nerve non-crossing rate was observed. This result is consistent with the visual pathway compensation according to plasticity as previously reported. The bilateral model would be appropriate to evaluate genetic or pharmacological intervention against the ONC functionally.

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Role of Circadian Clock Genes Bmal1, Per2 and Thyroid Hormone Activating Enzyme Type II Deiodinase (Dio2) in Photoreceptor Development**ONKAR SAWANT¹, Banumathi Tamilselvan¹, Amanda Horton¹, Ivy Samuels², Sujata Rao¹**¹Cole Eye Institute, Cleveland Clinic, Ophthalmic Research, Cleveland, United States, ²Louis Stokes Cleveland Veterans Affairs Medical Center, Ohio Department of Ophthalmic Research, Cleveland, United States

Recent findings from our group demonstrated that cyclic light-dark (LD) exposure during early postnatal period is crucial for rod photoreceptor development and

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maturation. We also reported that these light dependent developmental processes in the eye could be mediated by alterations in thyroid hormones (TH). In the dark reared (DD) animals the expression of retinal clock genes as well as retinal thyroid hormone components were altered. Based on these observations we hypothesized that the clock genes control thyroid hormone signaling mediated photoreceptor development. To test this hypothesis, Crx Cre line (*Crx^{Cre/+}*) was used to achieve conditional deletion of core circadian clock genes *Bmal1* and *Per2* from developing photoreceptors. Deletion of the clock gene *Bmal1* from the photoreceptors resulted in ectopic expression of S-opsin cones in the dorsal retina and loss of a dorsal-ventral S-opsin gradient. Conversely, loss of *Per2*, a component of negative feedback loop of circadian clock assembly exhibited overall decrease in S-opsin expression. Dark and light-adapted ERGs were performed to assay for visual function. Despite having more number of S-opsin positive cones, *Bmal1* mutant animals demonstrated more than 40% decrease in light-adapted b-wave amplitudes. The type II deiodinase (*Dio2*) null mice exhibited similar phenotype as the conditional *Bmal1* mutants. *Dio2* is required for converting prohormone thyroxin (T4) into biological active triiodothyronine (T3). Loss of *Bmal1* also resulted in decrease in *Dio2* levels suggesting that *Dio2* expression could be regulated by BMAL1. These results shed insight on the role of circadian clock components in regulation of cone photoreceptor development, distribution of cone opsin gradient and link between circadian clock and thyroid hormone signaling components.

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The Mouse Model for Human Peroxisome Biogenesis Disorders: Characterization and Treatment of the Associated Retinopathy

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Purpose: Peroxisome biogenesis disorders (PBD) are autosomal recessive disorders caused by mutations in any one of 14 PEX genes whose protein products are required for peroxisome assembly. Retinal degeneration leading to blindness is among the major handicaps faced by affected individuals, but has not been well characterized. To facilitate studying the disease pathophysiology in

vivo, a knock-in mouse model was generated bearing the equivalent to a common human mutation (Pex1-G844D). This study examined, from eye opening, the progression of retinopathy in this mouse model. Drug therapy was also piloted using betaine and diosmin, two chaperones previously shown to effectively recover peroxisomal import in PEX1-G843D patient cell lines.

Methods: Retinal electrophysiology and histology were performed in Pex1-G844D mice of various ages, ranging from eye opening (2 weeks) to 25 weeks. Non-mutant littermates served as controls. At 5 weeks of age, a group of Pex1-G844D mice received orally either 800mg/kg/day of betaine or 100mg/kg/day of diosmin, or a combination of the two drugs, for 7 consecutive weeks.

Results: In contrast to control mice, retinal function was non-recordable in mutant mice at 2 weeks. The cone response, which was first recorded at 4 weeks, was less than 5% of that in controls, and remained residual with age. Rod function was first detected by 3 weeks, but the amplitude was significantly smaller than that in control mice [30% and 20% of the a- and b-wave values in control mice, respectively]. Maximal scotopic ERG amplitudes were reached between 4-6 weeks, and then progressively decreased with age. Histological analysis revealed that the photoreceptor outer segment layer (OSL) was shorter and disorganized in mutant compared to control animals, while the remaining retinal layers were normal. A partial recovery of both rod and cone systems was noted with drug therapy.

Conclusion: At eye opening, the Pex1-G844D mouse model is already characterized by a severe cone-rod malfunction. Our histological data suggest a possible defect at the OSL (i.e. shorter and disorganized) and thus impaired phototransduction. Interestingly, drug therapy efficiently enhanced rod and cone function. A better understanding of the mechanisms of both the retinopathy and drug action will be instrumental in our quest to adequately manage this disease and hopefully accurately determine critical endpoints for drug therapy in PBD patients. Funded by CIHR.

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Amyloid- β_{1-42} Oligomers-induced Retinal Damage: Role of TGF- β 1

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Purpose: To set up a retinal neurodegenerative model in rat that mimics pathologic conditions such as age-related macular degeneration (AMD) using amyloid- β ($A\beta$) oligomers, and assess the effect of TGF- β 1.

Methods: Sprague-Dawley male rats were used. Human $A\beta_{1-42}$ oligomers were intravitreally (ITV) injected (10 μ M) in the presence or in the absence of recombinant human TGF- β 1 (1ng/ml ITV injected). After 48h, the animals were sacrificed and the eyes removed and dissected. The apoptotic markers Bax and Bcl-2 were assessed by Western Blot analyses in retina lysates. Gene-pathway network analysis was carried out in order to identify which pathways are involved in AMD.

Results: Treatment with $A\beta$ oligomers induced a strong increase of Bax protein level (about 4-fold; $p < 0.01$) and a significant reduction of Bcl-2 protein level (about 2-fold; $p < 0.05$). Co-injection of TGF- β 1 triggered a significant reduction of Bax protein induced by $A\beta$ oligomers. The Bcl-2 and the PI3K-Akt are the most connected nodes, for genes and pathways respectively, in the enriched gene-pathway network common to AMD and Alzheimer's disease (AD).

Conclusions: These data indicate that ITV injection of $A\beta_{1-42}$ oligomers in rat induces molecular changes associated with apoptotic neuronal death in retina consistent with a potential pathogenetic role of $A\beta$ oligomers in AMD. Bioinformatics analysis confirms that apoptosis pathways are impaired in AMD. Further, these findings suggest that human recombinant TGF- β 1 can prevent retinal damage elicited by $A\beta$ oligomers.

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The Anti-oxidative Effects of Natural Alkaloids on MIO-M1 and ARPE-19 CellsFANFAN ZHOU¹, Wei Zhou², Xiaoxi Lu¹, Ling Zhu³¹The University of Sydney, Faculty of Pharmacy, Camperdown, Australia, ²Guizhou Medical University, Guiyang, China, ³The University of Sydney, Save Sight Institute, Sydney, Australia

Mammalian eyes are continuously exposed to sunlight as well as high oxygen concentration, which lead to the high oxidative stress burden in the eye. It is known that oxidative stress is a leading cause of many aging diseases, such as the Age-related Macular Degeneration (AMD). Various traditional herbal medicines have long been used in treating retinal diseases in Asian countries, particularly *Dendrobii Caulis* and *Isatidis Radix*. However, the molecular mechanism underpinning the therapeutic effects of these herbal medicines remains unknown. Various alkaloids have been found to be the main constituents of these herbal medicines and it is known that alkaloids may have antioxidant property. In this study, we assessed the anti-oxidative effect of five alkaloids that are enriched in these herbal medicines (dendrobine, matrine, tryptanthrin, chelerythrine and oxymatrine), on human MIO-M1 (a Muller cell model) and ARPE-19 cells (a retinal pigment epithelial cell model). Our H2DCFDA and MTT assay showed that glutamate treatment induced significant ROS elevation and mitochondrial dysfunction in both cell lines. We then assessed glutamate-induced cell death with or without the pre-treatment of these alkaloids. We found that tryptanthrin can significantly reverse the glutamate-induced ROS elevation and mitochondrial dysfunction; while the other alkaloids has no or mild effect on MIO-M1 cells. In contrast, neither of these alkaloids impacts on the glutamate-treated ARPE-19 cells. Overall, our study suggests that tryptanthrin may protect mitochondrial under the oxidative condition in a cell type specific manner. It could be potentially used to counter ROS elevation and thus prevent the progression of retinal degenerative diseases.

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Increasing the Rate of Homology Directed Repair for Inherited Retinal Diseases Using the CRISPR/Cas9 System

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The current advancements in the understanding and utilization of the CRISPR/Cas9 system have provided a new and powerful tool for gene therapy. The applications of this system to specifically knock-out an exon, in vivo, have recently been demonstrated in two liver disease mouse models [1, 2]. While the approach to knockout a specific gene or exon is an invaluable asset, many genes such as peripherin-2 or rhodopsin (RHO), can result in disease through many different known mutations and are also necessary for photoreceptor survival [3]. Gene knock-in by the CRISPR/Cas9 system has one significant drawback; its reliance on homology directed recombination (HDR). The rate of HDR, even in dividing cells, was seen to be as low as 1-2% [1, 2]. For undividing cells, such as photoreceptors, this would allow for gene correction in only a small percentage of treated cells. Therefore, it is the aim of this project to test three methods for increasing CRISPR/Cas9 induced HDR in a developed mouse retina.

Three methods for increasing HDR include inhibition of ligase IV, activation of the mTOR pathway, and earlier time point delivery. Each of these methods will be tested by delivery of a Cas9 nickase, mCherry cDNA with homology flanking arms, and sgRNAs targeting the rhodopsin tagged with green fluorescent protein (GFP), will be achieved via combined Adeno-associated virus (AAV) and liposome-protamine-DNA complexes (LPD). LPDs were tested through transfection of 2.5 ug of plasmid containing control cytomegalovirus promoter driven GFP DNA.

Here, we have confirmed transfection, of CMV-GFP plasmid, by LPDs, into HEK293, RGC-5, and 661W cultured cells by GFP expression. These results confirm that a ratio of 1:2:3:15, DNA, nuclear localization signal and trans-acting transcription peptide, protamine sulfate, and lipids, respectively, provided the best DNA transfection rates in tissue culture and will be used moving forward in mouse models.

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Investigation and Characterization of Ex-vivo Transscleral Diffusion of Dexamethasone Sodium Phosphate and Triamcinolone Acetonide in Solution and a Dry Formulation in Rabbit and Porcine Sclerae

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Purpose: To evaluate and compare the ex-vivo transscleral diffusion permeability profile of dexamethasone sodium phosphate (Dex SP) and triamcinolone acetonide (TA) in solution and lyophilized dry form.

Method: The sclerae were harvested from fresh porcine and albino rabbit globes. The effect of solution and a lyophilized formulation on the transscleral permeability was investigated using Franz-type vertical diffusion cells after delivery of Dex SP and TA at 6 and 9 mg/ml concentrations in the donor side and its quantification by High Pressure Liquid Chromatography in the receptor side. Data samples were collected over time intervals. The transscleral flux and permeability coefficient were calculated. Permeability parameters were determined and compared with ANOVA ($p < 0.05$).

Results: This experiment had demonstrated that both solutions and lyophilized formulations of Dex SP and TA at 6 and 9 mg could diffuse across the sclera and that permeation decreased over time in both species. The flux of Dex SP in solution and lyophilized formulation at 6 mg were 668.37 and 154.35 $\mu\text{g}/\text{cm}^2\text{Hr}$ respectively in porcine. After 48 hours, in an average of 51.75% and 36.70% of Dex SP 6 mg solution and dry formulation, respectively had been recovered in the recipient side ($p = 0.0006$). The flux of Dex SP 6 mg in solution and lyophilized formulation were 1048 and 28.19 $\mu\text{g}/\text{cm}^2\text{Hr}$ respectively in rabbits. The flux of TA in porcine sclera after exposure to 6 mg in solution and lyophilized formulations was 0.16 and 0.0089 $\mu\text{g}/\text{cm}^2\text{Hr}$, respectively, and the permeability coefficients were 7.26×10^{-9} and 4.12×10^{-10} cm/s. After 48 hours of diffusion, the majority of the lyophilized dry form was found in the donor side with a 60.9% of total recovery of TA 6 mg. There was 26.57% remain in the donor side and a total recovery of 38.59% in TA 6 mg solution. The average cumulative recovery of TA 9mg in solution and lyophilized formulation were 0.61% and 0.20% in rabbits respectively.

Conclusion: We have shown that transscleral diffusion of hydrophilic and lipophilic steroidal anti-inflammatory drugs in solution and lyophilized formulations. Both formulations were able to provide drug permeation through the sclerae in different species. It indicated that soluble and insoluble drugs can benefit from the use liquid and dry formulations for episcleral delivery. Furthermore, the clinical application will enhance the veterinary ocular therapeutics for domesticated, aquatic and wild life animals.

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The Approach to Modify Müller Cells -photoreceptor Interactions: The Treatment of 670 nm Red Light prevents Activation of Microglia in the in vitro Model of Light Damage

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Introduction: Müller cells (MCs) are the principal macroglia of the retina providing protection to retinal neurons. Following photoreceptor (PR) damage, MCs respond by releasing pro-inflammatory cytokines (ie.CCL2) to recruit microglia into retinas and later undergo gliosis to cause further damages on retinas. Previously, we demonstrated that 670 nm red light ameliorate a rate of gliosis and reduced PRs degeneration *in vivo*. However, underlying mechanism is still unclear. The aim of this study was to investigate the interaction between MCs and PRs damaged by bright *in vitro*, to study direct effects of 670 nm light.

Methods: Primary MCs, dissociated from rat retinas (P10-12), were placed in a 24-well-plate. Mouse photoreceptor-derived cells (661W) were seeded in an insert of a transwell. After two types of cells respectively reached confluency, an insert containing PRs was either put above MCs or placed in a well with medium only. For the *in vitro* light damage (LD) model, PRs co-cultured with or without MCs were exposed to bright light (BL) (15,000lux) for 4 hours followed by a 24hours recovery. Some MCs were treated with 670 nm light 3 times (9J/cm²) over a recovery of 24 hours (treatment group). After LD, supernatant was collected from the co-culture transwell, and transferred to plates containing microglia cell line, N11, to test inflammatory response from co-cultured system.

Results: PRs, co-cultured with MCs displayed increased

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viability and ATP production, and decreased γ production of H_2O_2 following BL. MCs treated with 670 nm light protected PRs *in vitro*, however to a lesser extent than untreated MCs following LD. A significant increase of CCL-2 was shown in the supernatant after LD compared to control, however, an increase was significantly reduced in the treatment group. Supernatant collected after LD induced the morphological change of N11 cells, as they became amoeboid. Supernatant of the treatment group did not cause a morphological change of N11 cells.

Conclusion: MCs displayed neuroprotective effects on compromised PRs. The interaction between damaged PRs and MCs activated microglia after LD. Modification of MCs with 670 nm red light did not diminish the neuroprotective effect of MCs, and moderated the secretion of pro-inflammatory cytokines. This study supports that non-invasive treatment of 670 nm red light may have a direct effect on the activation of MCs that can reduce an extent of inflammatory leading to damages in retinal diseases.

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Vector Engineering to Improve Transgene Expression in a Non-viral Nanoparticle Gene Therapy

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Gene therapy has been the most promising treatment approach for inherited retinal degeneration. Among the challenges that gene therapy faces are levels and duration of expression of therapeutic genes. The *purpose* of this study is to investigate the use of DNA curved enhancer elements to boost transgene expression.

Methods: Vectors used for this study contained eGFP and either photoreceptor-specific (S-arrestin and interphotoreceptor retinoid binding protein (IRBP)) or ubiquitous (CMV) promoters. The curved enhancers were added 5' proximal to the promoter. For ectopic expression, HEK293 cells were transfected with the plasmid containing CMV and analyzed by flow cytometry and immunoblotting. For initial *in vivo* testing, naked DNA was subretinally injected into postnatal day 1 wild type (WT) mice followed by electroporation. Plasmids with successful transgene expression were compacted into CK30PEG10 and injected subretinally into adult WT mice. Pattern of expression was analyzed at different time points post-injection by fundus imaging then tissues were collected for protein and RNA

analyses.

Results: *In vitro* expression showed that the curved sequences increased transgene expression from all promoters. Fundus imaging of eyes transfected with vectors constructed for *in vivo* analyses showed expression with varied levels and distribution. Assessment of GFP protein and RNA showed that levels of transgene expression significantly improved in a promoter-dependent fashion, whereby S-arrestin has much higher expression levels than IRBP. Of the two curved sequences (named T20 and T36) tested, T36 increased, by two fold, protein levels with S-arrestin and IRBP promoters, while the T20 showed a repressive action. Furthermore, expression from the IRBP promoter persisted at significant levels for 60 days post-injection. Interestingly, the use of the two curved sequences resulted in exactly the opposite when tested with the CMV promoter *in vitro*, with T20 increasing the expression and T36 repressing it. Preliminary results with a third photoreceptor-specific promoter, RK (rhodopsin kinase), shows robust expression *in vivo* as well.

Conclusion: These experiments demonstrate that the addition of DNA elements to cell-type specific promoters can improve levels of expression. This improvement will increase the utility of gene therapy to combat human retinal degenerative diseases while eliminating off-target expression and associated adverse effects.

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Genome Editing and Disease Modeling of RPGR-associated Retinitis Pigmentosa

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Purpose: Retinitis Pigmentosa (RP) is a genetically heterogeneous disease that causes death of the light sensing photoreceptors and underlying retinal pigment epithelium. Mutations in the gene *RPGR* are responsible for a majority of cases of X-linked RP. Interestingly, mutations in *RPGR* can result in rod selective disease, cone selective disease, or in both cone and rod disease. The focus of this study was to develop genome-editing strategies and to model the disease phenotype in retinal organoids derived from patient-specific iPSCs.

Methods: Patient-specific iPSCs were generated from dermal fibroblasts of 7 patients with molecularly confirmed *RPGR*-associated XLRP. Pluripotency was confirmed using

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the TaqMan Scorecard Assay. CRISPR/Cas9 constructs were generated to target mutations in *RPGR*. The CRISPR/Cas9 system and homology directed repair constructs were delivered to iPSCs via electroporation. Corrected cells were enriched using either drug or color selection and confirmed via Sanger sequencing. Patient-specific iPSC-derived retinal organoids were generated from corrected, uncorrected cell lines, and control lines and characterized via immunocytochemistry and confocal microscopy.

Results: Seven iPSC lines were successfully generated with varying mutations and photoreceptor phenotype. Genome editing of patient-specific iPSCs was achieved with transfection efficiencies of approximately 20 percent in hiPSC, and the resulting modified iPSC clones were isolated and expanded via drug or color selection. Patient-specific iPSC-derived retinal organoids were generated and displayed photoreceptor-specific markers PAX6, OTX2, RCVRN, CRX and NRL. *RPGR-ORF15* was successfully characterized in 120 day control retinal organoids.

Conclusions: iPSC technology allows for the unique opportunity to investigate patient-specific pathophysiologic mechanisms and therapeutic interventions in human cells. We have shown that genome editing via the CRISPR/Cas9 system can correct patient-specific mutations in iPSCs. Moreover, corrected versus uncorrected lines will serve as a valuable tool for characterizing the disease phenotype.

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NOX2 Blockade Prevents Diabetes-induced Retinal Endothelial Cell Senescence by Normalizing Arginase Expression/Activity and Restoring no Availability

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In diabetes, reduced production or availability of nitric oxide (NO) in endothelial cells (EC) plays a major role in EC dysfunction. Diabetes induces elevated activity of arginase (ARG), which can compete with NO synthase (NOS) in EC for their common substrate L-arginine, thereby reducing NO production. Decreased L-arginine also uncouples NOS causing it to produce superoxide (O₂⁻) which reacts with

NO to form peroxynitrite. Peroxynitrite and O₂⁻ are potent reactive oxygen species (ROS) that can further elevate ARG activity. ROS. Decreased NO can cause premature EC senescence, leading to defective vascular repair. Our previous work has demonstrated the role of the O₂⁻-generating enzyme NOX2 in diabetes-induced oxidative stress and retinal vascular injury. Here we investigated the involvement of NOX2 in diabetes-induced elevation of ARG, NOS uncoupling and EC senescence.

Studies were performed in diabetic and control NOX2 knockout (NOX2 KO), EC-specific ARG 1 KO and wild type (WT) mice and in retinal ECs treated with 25 mM glucose with or without the NOX2 blocking peptide gp91phox. Other ECs were treated with H₂O₂, NOS inhibitor L-NAME (L-NG-Nitroarginine Methyl Ester), NO donor SNAP (S-nitroso-N-acetylpenicillamine) or arginase inhibitor ABH (2(S)-amino-6-borono-hexanoic acid). NOX2 and ARG expression was assessed by western blot. Arginase activity was assayed by measuring urea formation. Formation of ROS and NO were assayed by dihydroethidium and DAF2-DA (diaminofluorescein diacetate) imaging, respectively. Cellular senescence was determined by assaying SA- β -gal (senescence associated β -galactosidase) activity.

Retinal cells from diabetic mice and high glucose-treated retinal ECs exhibited significant increases in ROS formation along with elevated ARG expression/activity, decreased NO formation and increased SA- β -gal activity. Deletion of NOX2, specific blockade of NOX2 activity or EC ARG1 KO prevented increases in ROS and normalized ARG activity and expression, restored NO formation and reduced SA- β -gal activity. L-NAME, H₂O₂ also induced retinal EC senescence which was prevented by treatment with the NO donor or the arginase inhibitor.

These results indicate that decreased NO levels accelerates EC senescence. NOX2-generated ROS importantly contribute to diabetes/high glucose induced activation of ARG and decreased NO. Blockade of NOX2 or ARG represents a strategy to prevent diabetes-induced premature EC senescence and promote healthy vascular repair.

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Gene Therapy Using Short Hairpin RNAs to Slow Retinal Degeneration in Autosomal Dominant Retinitis Pigmentosa

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Introduction: Autosomal dominant RP (adRP) caused by mutant rhodopsin (RHO) remains incurable. Tandem delivery of a short hairpin RNA (shRNA) to degrade endogenous RHO and a degradation-resistant RHO cDNA with recombinant adeno-associated virus (rAAV) preserves vision in a P23H transgenic mouse model of adRP.

The purpose of this study was to design rAAV vectors containing shRNAs with higher knockdown efficiency of RHO to extend our approach to rapidly degenerating models of adRP with mutant RHO.

Methods: We designed siRNAs to target human RHO and measured the percent knockdown of RHO mRNA and protein *in vitro* with qRT-PCR or flow cytometry. Four of the shRNAs were cloned in to a rAAV2 vector plasmid, packaged in to the rAAV5 capsid serotype and tested for preservation of vision via electroretinography and spectral-domain optical coherence tomography in transgenic mice expressing human T17M RHO.

Results: The siRNAs demonstrated knockdown efficiencies of RHO of up to 70% *in vitro*. We selected siRNAs 131, 134, 765, and 820 for further analysis. When the sequences of these siRNAs were incorporated in to a H1 promoter-shRNA cassette and cloned in to the rAAV2 vector plasmid, they were capable of targeting the degradation of wild-type and mutant RHO mRNA *in vitro*. Preliminary experimentation suggested a trend towards preservation of the outer nuclear layer in the T17M mouse after intravitreal (n=3) injection of the rAAV5/shRNA820 vector.

Conclusions: We generated rAAV vectors containing shRNAs with superior knockdown efficiency that will be tested in rapidly degenerating animal models of adRP expressing mutant RHO.

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The Monthly Eye Drop: Preclinical Testing of Long-term, Hydrogel/Microsphere Eye Drops for Glaucoma

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IOP reduction in patients with glaucoma is typically accomplished through the administration of medicated eye

drops several times daily, with compliance rates as low as 30%. The purpose of this study was to develop and test a novel formulation that provides one month of glaucoma medication from a topical depot. We hypothesize that this treatment will address the issues of compliance and poor bioavailability while avoiding more invasive techniques. Poly(lactic-co-glycolic) acid (PLGA) microparticles containing brimonidine tartrate (BT) were fabricated according to standard methods and mixed into a poly-(N-isopropylacrylamide) (pNIPAAm)-based hydrogel. The properties of the materials were characterized, including long-term stability, cytotoxicity, and *in vitro* release of BT. For *in vivo* studies, a single drop was administered to the inferior fornix of New Zealand white rabbits and compared to twice daily BT drops. BT-loaded microparticles release sufficient drug over 28 days *in vitro*, which is unaffected by the presence of the gel. Intraocular pressure (IOP) was measured throughout the study, and histology was used to characterize bacterial adhesion and cellular infiltration into the depot. Cytotoxicity testing shows no significant effect on cell viability. The gel/microparticle drops are easily administered and form a non-degradable, opaque gel upon reaching body temperature. IOP reduction for a single administration of the hydrogel drops was comparable to twice-daily BT drops, with significantly lower systemic uptake. There was no evidence of irritation or inflammation, and neither bacterial adhesion nor cellular infiltration was observed to be abnormal upon removal of the depot. The BT-loaded microparticles presented in this study are capable of releasing drug for four weeks and the materials are shelf stable over long periods of time. IOP reduction is achieved using 56 times fewer doses than the current clinical standard, representing a vast improvement in bioavailability and dosing frequency over current topical methods.

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Caffeine and A_{2A} Receptor Antagonism Control Microglia Reactivity and Afford Protection against Transient Retinal Ischemia

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Retinal ischemia is a clinical condition that remains a common cause of visual impairment and blindness in diseases such as diabetic retinopathy and glaucoma due to relatively ineffective treatments. Cell death and inflammation have been documented after retinal ischemia and mounting evidence suggests that selective A_{2A}R antagonists or chronic caffeine administration afford robust protection against noxious conditions, including in the retina. The aims of this work were to investigate whether caffeine consumption is able to prevent neuroinflammation and cell death elicited by retinal ischemia-reperfusion (I-R) injury and to investigate the therapeutic potential of A_{2A}R selective antagonism for the treatment of I-R injury.

Wistar rats were administered with caffeine in the drinking water (1 g/L) during two weeks before ischemia and until the end of the experiment (7 days of reperfusion). Caffeine intake prevented microglia reactivity and the expression and release of the pro-inflammatory cytokines TNF and IL-1 β elicited by I-R. Moreover, the number of retinal apoptotic cells was significantly reduced in the retina of caffeine-treated animals, compared with animals drinking water.

The selective A_{2A}R antagonist, KW6002 (istradefylline; 3 mg/kg, p.o.), was administered in Wistar rats 2 h after I-R injury and then daily until the end of the experiment (7 days of reperfusion). KW6002 administration decreased the number of reactive microglia and inhibited the production of IL-1 β induced by I-R injury, without alterations in TNF. Moreover, KW6002 treatment decreased retinal cell death induced by I-R injury.

These results demonstrate that caffeine consumption and blockade of A_{2A}R control neuroinflammation and affords protection to the retina against damage induced by I-R injury, and prompt A_{2A}R blockade as a therapeutic strategy for the treatment of retinal ischemic diseases.

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Targeting Thyroid Hormone Components Locally in the Retina to Protect Cone Photoreceptors

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Thyroid hormone (TH) signaling regulates cell proliferation, differentiation, and metabolism. In the retina, TH signaling plays a central role in the cone opsin expression and patterning. We previously showed that suppressing TH signaling by anti-thyroid treatment preserves cones in mouse models of retinal degeneration. This work investigates the therapeutic significance of targeting intracellular TH components locally in the retina. The cellular TH level is mainly regulated by the iodothyronine deiodinases, DIO2 and DIO3. DIO2 converts the prohormone thyroxine (T4) to the bioactive triiodothyronine (T3), which binds to the TH receptor (TR), initiating gene expression alterations; whereas DIO3 degrades T3 and T4. We used three strategies to suppress TH signaling in the retina:

- 1) blocking TR by TR antagonists,
- 2) inhibiting DIO2 by the enzyme inhibitor, and
- 3) overexpressing DIO3 by transgene delivery.

The Leber's Congenital Amaurosis model *Rpe65*^{-/-} mice and the achromatopsia model *Cpfl1* mice at postnatal day 5 (P5) were treated with the TR antagonist NH-3 or 1-850, or the DIO2 inhibitor iopanoic acid by intravitreal and topical delivery, or the viral vector AAV5-IRBP/Gnat2-*hDIO3* by subretinal injection, and were evaluated for cone death and survival at P25. We found that the intravitreal and topical delivery of TR antagonist or DIO2 inhibitor increased cone density by about 30% in the *Rpe65*^{-/-} mice, and reduced the number of TUNEL-positive cells. Subretinal delivery of AAV5-IRBP/Gnat2-*hDIO3* also significantly improved cone survival in *Rpe65*^{-/-} and *Cpfl1* mice. We show that ocular delivery of TH inhibitors protects cones in mouse models of cone degeneration. Our findings suggest that targeting TH components locally in the retina represents a novel strategy for cone protection.

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iPSc-derived Pericytes Integrate into Developing Retinal Vasculature

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Background: Diabetic retinopathy (DR) results in the progressive loss of vision and remains the leading cause of blindness in working-age Americans. After 10 years of disease, 80% of diabetic individuals will exhibit some characteristics of DR. The elevated glucose concentrations lead to damage of pericytes and neurons, resulting in a variety of microvascular changes. The loss of pericytes in early diabetes appears to be a pivotal step in the development of microangiopathy and their replacement would be expected to block and/or reverse its progression. The goal of our work was to establish pericytes differentiation protocol from mouse induced pluripotent stem cells (iPSc) in defined chemical conditions and test their ability to integrate into developing retinal vasculature after syngeneic transplantation.

Methods and results: A 2D monolayer vascular differentiation protocol was adapted to derive pericytes from dsRed-mouse iPSc. The mesoderm was induced for three days using the combination of ascorbic acid, activin A, BMP4, VEGF and a GSKb3 inhibitor, followed by seven-day vascular specification with ascorbic acid, VEGF and a small molecule TGFβ inhibitor (SB431542). The pericytes were separated from undifferentiated iPSc and endothelial cells by their adhesive properties and then expanded in bFGF-containing medium. Three independent pericyte cell lines were established, each expressing markers characteristic of mural cells: PDGFRβ, PECAM1, smooth muscle actin, NG2, PDGFRα, CD34, Kcnj8, CD248, desmin, vimentin, CD44, RGS5, PSA-NCAM, CD144 and Kir6.1. The homogeneity of cultures was confirmed by flow cytometry. A low-level expression of vascular progenitor/EC marker CD31 was observed and this was downregulated by

PDGFB treatment. The ability of cells to integrate into vasculature as tested by intravitreal injection of 5 to 15 x 10³ cells in 1 ul of HBSS into p4 mouse pups (C5Bl/6J). Three weeks later dsRed+ve, NG2+ve cells were identified as colocalized with retinal vasculature.

Conclusions: We have demonstrated that mouse pericytes can be efficiently differentiated and expanded from iPSc in the defined conditions. The iPSc-derived pericytes can integrate into developing retinal vasculature following intravitreal transplantation and provide proof of principle that may form the basis for pericyte replacement in DR.

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High-precision Anti-microvascular Therapy via the Synergy of Light and Sound

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Antivascular therapy represents a proven strategy to improve the prognosis of a variety of pathological conditions, including cancer and many eye diseases. By synergistically applying laser pulses and ultrasound bursts, we developed a novel photo-mediated ultrasound therapy (PUT) technique as a localized antivascular method. PUT takes advantages of the high native optical contrast among biological tissues, and has the unique capability to self-target microvessels without causing unwanted damages to the surrounding tissue. As demonstrated in the experiments on animal models, PUT can treat microvessels in target tissue via different mechanisms, which include blocking microvessels by inducing blood clots or disrupting microvessels by causing local hemorrhage, each with its value in the clinic. Moreover, PUT working at different optical wavelengths can selectively treat veins or arteries by utilizing the contrast in the optical spectra between deoxy- and oxy-hemoglobin. As a novel antivascular method with the capability to precisely target blood vessels and precisely control the treatment effects, PUT holds the potential to impact clinical management of cancer and eye diseases by delivering improved treatment outcomes with minimized complications.

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The Frequency of Alu Repetitive Elements in Angiotensin-converting Enzyme in Jordanian Diabetic Retinopathy Patients

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Diabetic retinopathy is a major cause of vision loss in working age adults worldwide and is a common and progressive microangiopathic complication of diabetes mellitus. Angiotensin-converting enzyme converts the angiotensin I to the active vasoconstrictor angiotensin II. Inhibition of ACE has been suggested to have retinoprotective actions in diabetic patients. In vitro, angiotensin II stimulates the expression of the angiogenic factor vascular endothelial growth factor (VEGF). Induction of angiogenesis has been implicated in the pathogenesis of diabetic retinopathy in humans and in experimental animals. An insertion sequence, Alu element, in intron 16 of the human ACE gene has been linked with diabetic retinopathy. The Alu element upregulates the transcriptional activity of ACE promoter by approximately 70%. In our study, we aim to find out the frequency of ACE insertion sequence in diabetic retinopathy patients and compare it to that in type II diabetic patients without diabetic retinopathy and normal controls of Jordanian population. In addition, an association between the presence of this element and the severity of diabetic retinopathy will be determined. A total of 176 blood samples were withdrawn from the three patient groups, 48 had diabetic retinopathy, 100 had diabetes without diabetic retinopathy and 28 were normal controls. Diabetes and diabetic retinopathy were diagnosed by standard means. DNA extraction was performed using standard methods and subsequently polymerase chain reaction (PCR) was done. The following results were obtained, diabetic retinopathy (29.17% normal, 52.08% heterozygous and 18.75% homozygous), diabetic (48% normal, 39% heterozygous and 13% homozygous), control (57.14% normal, 53.97% heterozygous and 0% homozygous). The odds ratio was 2.4286, confidence interval (CI) was from 1.1912 to 4.9513 and p-value was 0.0146. A significant increase in diabetic retinopathy risk was detected in patients with homozygous and heterozygous genotypes. Similar studies on different populations show both correlational and no correlational relationships. More sample collection is ongoing until comparative numbers in each group and larger sample size are achieved to reach final conclusions.

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Genomic Disruption of VEGF-A Expression in Human Retinal Pigment Epithelial Cells Using CRISPR-Cas9 Endonuclease

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Purpose: To explore the use of the type II clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 endonuclease for suppressing ocular angiogenesis by genomic disruption of vascular endothelial growth factor (VEGF-A) in human retinal pigment epithelial (RPE) cells.

Methods: CRISPR sequences targeting exon 1 of human VEGF-A were computationally identified based on predicted Cas9 on-target and off-target probabilities. Single guide RNA (gRNA) cassettes with these target sequences were cloned into lentiviral vectors encoding the *S. pyogenes* Cas9 endonuclease (SpCas9) gene. The lentiviral vectors were used to infect monolayers of ARPE-19 cells, a human RPE cell line, for 14 days with puromycin to select for transduced cells. Frequency of insertion or deletion (indel) mutations was assessed by T7E1 mismatch detection assay and Sanger sequencing. Protein levels were determined by enzyme-linked immunosorbent assay (ELISA) for secreted VEGF-A. Angiogenesis was evaluated *in vitro* using a tube formation assay with human umbilical vascular endothelial cells (HUVECs). Cells infected with lentivirus expressing SpCas9 only without gRNA were used as controls.

Results: A total of 5 gRNAs targeting VEGF-A were selected based on the highest predicted on-target probabilities, lowest off-target probabilities, or combined average of both scores. Lentiviral delivery of all 5 gRNAs with SpCas9 resulted in indel formation in the VEGF-A gene, with frequencies ranging from $33.5 \pm 3.8\%$ to $37.0 \pm 4.0\%$. There was a corresponding reduction in secreted VEGF-A protein of up to $61 \pm 1.4\%$ compared with SpCas9-only control ($P < 0.05$). Conditioned media from infected RPE cells also showed reduced levels of angiogenesis using an endothelial tube formation assay. No significant indel formation in the top 3 putative off-target sites tested

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were detected, suggesting that the CRISPR-Cas9-mediated knockdown of VEGF-A is highly specific.

Conclusions: The CRISPR-Cas9 endonuclease system may reduce VEGF-A secretion from human RPE cells, supporting the possibility of employing gene editing for anti-angiogenesis therapy in ocular diseases.

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