4531 - 3:00PM
A New Retinoid Signaling Molecule in the Retinal Pigment Epithelium: RPE45
W. Jahng1, A. Ose4, R. Zhang2, J. M. Nolan1, S. Beatty1,3.

Purpose: To investigate the relationship between macular pigment optical density, complement factor H (CFH) and/or LOC387715 genotype. Methods: RPE65 expression levels were assessed in two conditions in vitro (RPE D407), ex vivo (Bovine, mouse), and in vivo (mouse). Proteomics tools such as 2D SDS-PAGE, MALDI-TOF-TOF mass spectrometry analysis and biochemical methods including Western blot, immunohistochemistry, retina binding assay were employed to study functional role of RPE45.

Results: We found that a new 45kDa protein, designated as RPE45, a truncated fragment of RPE65, appears upon light exposure and oxidative stress. RPE45 is formed by recombinate caspasas using a ubiquitination mechanism. We show that RPE45 results from an interaction of specific proteases with RPE65 through ubiquitination induced by light and ROS. RT-PCR revealed that the differentials of stress-induced RPE45 expression are regulated at a post-transcriptional level.

Conclusions: In this study, comparative proteomics using 2D gel electrophoresis and mass spectrometry analysis reveal that RPE65 is one of the major proteins regulated by light and ROS in RPE cells. We find that the level of RPE65 is increased in light compared to dark, and RPE45, a 45kDa fragment of RPE65, is present only in the light or high oxidative stress Therefore, RPE45 can serve as a biomarker for oxidative stress and light exposure in the eye. These findings demonstrate the role of RPE65 as a key regulator that responds to light and ROS in the visual cycle.

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4532 - 3:15PM
Subfunctionalization of a Retinoid Binding Protein Provides Evidence for Two Parallel Visual Cycles in the Cone-Dominant Zebrafish Retina
V. C. Fleisch1, H. B. Schoenthaler1A, J. von Lintig1, S. C. F. Neuhaus3.

Purpose: The vertebrate visual cycle serves the purpose of recycling 11-cis retinal in the retina in order to restore vision after bleaching. In recent years evidence has accumulated that cones might employ an alternative pathway for recycling these retinoids as compared to rods. Retinoid binding proteins (CRBP, CRALBP, IRBP) are known to be essential for the canonical (rod) visual cycle. In order to assess the function of CRALbp in cone visual pigment regeneration, we made use of zebrafish behavioral genetics.

Methods: Two orthologues of the CRALbp gene (CRALBP-a, CRALBP-b) were cloned in the zebrafish. Spatial and temporal expression patterns were investigated. The antisense morpholino technique was employed to create zebrafish larvae lacking CRALbp-a or -b protein. Visual system performance of injected larvae was evaluated in OKR and ERG measurements. Furthermore, a HPLC analysis of retinoids was performed in order to assess 11-cis retinal regeneration capabilities of larvae devoid of CRALbp-a or -b.

Results: The zebrafish genome harbors two orthologues of CRALbp, which we denoted as cralbp-a and -b. These paralogues are differentially expressed in the zebrafish retina. While Cralbp-a is found in the RPE, Cralbp-b expression is restricted to Müller glia cells. Targeted downregulation of Cralbp-a does not have any effect on visual behavior, whereas knockdown of Cralbp-b limited the visual system performance of larvae.

Conclusions: Differential expression of Cralbp paralogues in the zebrafish retina as well as results obtained from behavioral and physiological studies of Cralbp-a/b-deficient larvae provide strong evidence for the existence of two pathways of pigment visual recycling in the zebrafish. We show that Cralbp-a is implicated in the canonical visual cycle located to the RPE, whereas Cralbp-b is part of a cone-specific visual cycle located to Müller glia cells. Moreover, our data suggest the existence of both pathways for recycling 11-cis retinal, however, with a predominant usage of the visual cycle located to Müller glia cells.

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4533 - 3:30PM
The Relationship Between Macular Pigment Optical Density, Complement Factor H, and LOC387715 Genotype
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Purpose: Investigate the relationship between macular pigment optical density (MPOD), and CFH Y402H variant allele, and marginally associated with the LOC 387715 allele. Methods: We recruited 270 healthy subjects for this study. Demographic and health history, and MPOD. However, we await further genotype results, and analysis of a larger subject population.

Results: More than 90% of subjects with family history of AMD had at least one CFH Y402H variant allele, compared with 37.2% of subjects with no family history of AMD. Y402H variant allele; 65.4% of subjects with no family history had at least one CFH Y402H variant allele, compared with 37.2% of subjects with no family history of AMD.

Conclusions: Enhanced water solubility, greater RBP-binding affinity and minimal liver enzyme toxicity of the synthesized NRMs possess increased water solubility and enhanced RBP-binding affinity compared to HPR. The NRMs also showed very little inhibition of key drug metabolizing enzymes of the liver. Importantly, administration of the NRMs to the RPE, whereas Cralbp-b is part of a cone-specific visual cycle located to Müller glia cells. Moreover, our data suggest the existence of both pathways for recycling 11-cis retinal, however, with a predominant usage of the visual cycle located to Müller glia cells.

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4534 - 3:45PM
Development of Non-Retinoid Therapeutics for the Treatment of Lipofuscin-Based Retinopathies
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Purpose: Excessive accumulation of lipofuscin and a toxic vitamin A-based fluorophore (A2E) has been implicated in the death of RPE and photoreceptor cells. Accumulation of these cellular toxins in the aβcr4 null mutant mouse can be completely halted by treatment with 4-hydroxyretinamide (HPR). HPR competes with retinol for binding to serum retinol binding protein (RBP) leading to a reduction in serum RBP-retinol, visual cycle retinoids and, ultimately, A2E. HPR has had extensive human exposure and is generally well tolerated. However, teratogenic properties associated with HPR limit its usefulness in patients afflicted with juvenile-onset retinopathies. To address this unmet need, we have sought to develop non-retinoid modulators (NRMs) which demonstrate the same mechanism of action as HPR and greater potency.

Methods: A proprietary high-throughput screen was used to identify small molecules that can replace RBP with high-affinity binding to Lαβ. Structure-activity relationships among these small molecules were compared in order to identify bioactive pharmacophores. Based on this comparison, new chemical entities (NCEs) were synthesized and submitted for screening. NCEs which demonstrated efficacy in vitro and minimal cytotoxicity were administered to abcr4 null mutant mice to assess efficacy in vivo.

Results: Biochemical characterizations showed that the synthesized NRMs possess increased water solubility and enhanced RBP-binding affinity compared to HPR. The NRMs also showed very little inhibition of key drug metabolizing enzymes of the liver. Importantly, administration of the NRMs to abcr4 null mutant mice produced dose-dependent reductions of RBP-retinol in serum and A2E in the RPE.

Conclusions: Findings of the present study validate modulation of serum RBP-retinol levels for treatment of lipofuscin-based retinopathies. This approach offers a new target for development of orally available therapeutics.

CR: K. B. Phan, Sirion Therapeutics, E; Sirion Therapeutics, P; T. V. Bui, Sirion Therapeutics, E; Y. Han, Sirion Therapeutics, P; Y. Han, Sirion Therapeutics, E; N. L. Mata, Sirion Therapeutics, E; Sirion Therapeutics, P.

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The Relative Distribution of Free and Bound Matrix Metalloproteinase (MMPs) Enzymes in Human Bruch's Membrane


**Purpose:** Matrix metalloproteinases (MMPs), released by the retinal pigment epithelium (RPE) enter Bruch's to form the degradative arm of a remodeling process for maintaining the structural integrity of the membrane. However, aging is associated with accumulation of inactive MMPs and increased thickness of Bruch's suggests impaired degradation and remodeling. The present investigation was designed to assess the possibility that these MMP enzymes may become sequestered and therefore unable to carry out their functional role (activation and degradation) leading to the observed ageing of Bruch's membrane.

**Methods:** Freshly isolated Bruch's-choroid preparations (14 eyes, age range 45-86 years) were mounted in Ussing chambers and MMP elution was instigated by application of a hydrostatic pressure of 14.16mmHg. The ensuing fluid transported across the preparation was collected at timed intervals over a 6-8 hour period and the amount of released MMPs quantified by gelatin zymography and densitometry. At the end of the experiment, the bound/trapped MMP pool was extracted with SDS-sample buffer and quantified.

**Results:** Most of the free MMP pool (MMPs 2&9) could be eluted from Bruch's within 5-6 hours representing an elution volume of 350-1000ul. Surprisingly, 60-80% of the total endogenous pool of MMPs remained bound to the matrix and could only be released by harsh SDS extraction. Furthermore, polymeric forms of MMPs 2&9 (indicative of oxidative damage and cross-linking) were routinely observed in the membrane extracts.

**Conclusions:** The high degree of sequestration and/or entrapment of MMPs may explain the reduction in degradation capacity of ageing Bruch's membrane leading to the accumulation of denatured collagens. The relevance of this finding to functional deterioration of Bruch's in normal ageing and age-related diseases will be discussed.

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4537 - 4:30PM

Functional Analysis of Mouse Embryonic Stem Cells-Derived Retinal Pigment Epithelium Cells Transplantation in Rpe65<sup>Rd12/Rd12</sup> Mice


**Purpose:** To induce stem cell differentiation into RPE cells in vitro and if these cells can rescue retinal function in the mouse retinal degeneration model Rpe65<sup>Rd12/Rd12</sup>.

**Methods:** To induce stem cell differentiation into RPE, mESCs were cultured on PA6 cells media containing bFGF, dexamethasone, and cholera toxin. mESCs were cultured for six days and observed for morphological changes, such as pigmentation and epithelial-like shape. In addition, these cells were assessed for expression of known RPE specific markers (RPE65 and Bestrophin) by immunocytochemistry staining. The specificity of anti-RPE65, and anti-bestrophin antibodies were tested on the human RPE cell line (ARPE-19), using immunocytochemistry and western blotting. To assess the function of PA6-treated mESCs cells in vitro, 2 x 10<sup>5</sup> cells were injected into the subretinal space of postnatal day 5 (P5) Rpe65<sup>Rd12/Rd12</sup> mice. Frozen retinal sections were analyzed to determine the location and morphology of transplanted cells. Finally, electroretinogram (ERG) was performed on injected mice to evaluate the functional outcome one, two, and four months after injection.

**Results:** After six days culturing on PA6 cells, mESCs showed signs of morphological change, but no pigment expression was detected. Observations of any additional morphological changes, after long-term culturing, will be presented. However, anti-RPE65 and anti-bestrophin antibodies stained PA6-treated mESCs and ARPE-19 cells, while secondary antibodies alone failed to stain. Frozen retinal sections showed transplanted cells were in the subretinal space 10 days after injection. Data from serial ERGs experiments are in progress and will be presented.

**Conclusions:** Our data indicate that PA6-treated mESCs express RPE65 and bestrophin, suggests that mESCs have the potential to differentiate morphologically and functionally to RPE. If retinal function can be rescued, the use of stem cell-based therapy may represent a future option in treating some forms of retinal degeneration. Otherwise, further studies are required to identify optimal conditions for generating functional RPE cells from mESC, such as improved cell lines, culture conditions, and transplantation techniques.

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