

405 Retinal Development

Wednesday, May 07, 2014 8:30 AM–10:15 AM
S 310E-H Paper Session

Program #/Board # Range: 4032–4038

Organizing Section: Retinal Cell Biology

Program Number: 4032

Presentation Time: 8:30 AM–8:45 AM

Retinoic acid signaling regulates expression of the tandemly duplicated LWS1 and LWS2 genes in zebrafish.

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Purpose: Differentiation of diverse photoreceptor phenotypes in the vertebrate retina requires multiple signaling pathways that activate cascades of gene expression. The signaling molecule retinoic acid (RA) is known to regulate rod and cone cell fate, differentiation, and survival. The purpose of the current study is to identify photoreceptor genes controlled by RA signaling in the embryonic retina of the zebrafish.

Methods: We treated embryos with RA at 48 hours post-fertilization (hpf) and isolated total RNA from eyes for microarray analysis at 75 hpf in order to identify genes responding to RA over the period of photoreceptor differentiation. Differentially expressed genes were validated by quantitative RT-PCR (qRT-PCR) and in situ hybridization. Wild-type zebrafish and those carrying an RA signaling reporter transgene (RARE:YFP) were used.

Results: We identified 180 genes with significantly altered gene expression. Of interest was the long wavelength sensitive opsin 1 (LWS1) gene, which was upregulated in eyes of RA-treated embryos. LWS1 is the upstream member of the tandemly duplicated LWS gene array, and is normally expressed in red-sensitive cones of ventral retina, but not until larval stages. qRT-PCR verified significant upregulation of LWS1, but not LWS2, in eyes of RA-treated embryos. In situ hybridization using probes specific for the 3' UTR of each LWS gene revealed that nearly no control retinas expressed LWS1, while those of embryos treated with (9-cis or all-trans) RA from 48 hpf to 5 dpf all expressed LWS1, predominantly in the ventral half of the retina. Control embryos all expressed LWS2 throughout their retinas, while RA-treated embryos showed a dramatic reduction in the number of cones expressing LWS2. The expression domain of LWS1 in the photoreceptor layer of RA-treated embryos matched the expression domain of a YFP reporter gene driven by a series of RA response elements.

Conclusions: RA signaling regulates numerous molecular targets in the developing eye, including the tandemly-duplicated LWS1 and LWS2 genes. It is possible that the level of RA signaling within a developing red-sensitive cone acts as a molecular toggle to favor the expression of LWS1 and/or suppress the expression of LWS2. This is the first evidence that an extracellular signal may regulate expression of opsins in a tandemly duplicated gene array.

Commercial Relationships: Deborah L. Stenkamp, None; Craig B. Stevens, None; Ruth A. Frey, None; Shoji Kawamura, None
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Presentation Time: 8:45 AM–9:00 AM

Regulation of Spatial Patterning of Rods and Cones in the Larval Zebrafish Retina

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Purpose: Humans are largely dependent upon cone vision. The highest cone density is found in the fovea positioned at the center of gaze, with comparatively few present in the rod-dominated retinal periphery. Surprisingly, few mechanisms are known that regulate the spatial patterning of rod and cone photoreceptors. The larval zebrafish retina is anatomically and functionally cone-dominated with conspicuously few rods in the central retina and greater numbers in the ventral retina and periphery. Our strategy takes advantage of these regional differences in the numbers of rods to identify fundamental mechanisms regulate their spatial patterning.

Methods: Immunolabeling and expression of cell-specific reporter genes were analyzed by confocal microscopy. Zebrafish embryos and larvae harboring mutations of *lor/tbx2b*, *lak/ath7*, *lep/pct2*, *ljr*, or morpholino knockdown of *six7* or *trb2* were used throughout the study. Transcriptional activity was measured using *luciferase* reporter assays of HEK293 cells co-transfected with transcription factor constructs and opsin promoter-luciferase reporter plasmids.

Results: In the central retina of larval zebrafish, cones outnumber rods 20:1. Our published data demonstrate that genetic mutation of *tbx2b/lor* leads to an increased number and the uniform distribution of rods due to a cell fate switch of SWS1 cones into rods. However, the increase in rod number following knockdown of *six7* is associated with continued cell proliferation in the central retina. Furthermore, our data show that the effects are additive. Larvae double mutant for *tbx2b/lor* and *six7/ljr* demonstrate twice the number of rods as either mutation alone. Surprisingly, no changes in rod patterning were associated with the increased photoreceptor numbers observed in *lak/ath7* or *lep/pct2* mutant larvae. In *luciferase* reporter assays, *tbx2b* alone had no effect upon transcription from opsin promoter constructs but represses the *Crx/Nrl*-induced transcriptional activation of the *rho*-promoter, and *Crx* activation of the *SWS1* promoter. No effects were observed using *six7*.

Conclusions: Our data show that spatial patterning of photoreceptors can be regulated independently by factors controlling cell fate or cellular proliferation; that *tbx2b* and *six7* act through distinct molecular mechanisms. However the absence of patterning defects in *lak/ath7* or *lep/pct2* mutant larvae suggests that additional factors are also involved.

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Presentation Time: 9:00 AM–9:15 AM

Retina formation requires suppression of BMP and Activin pathways in pluripotent cells

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Purpose: Retina formation requires the correct spatiotemporal expression of key regulatory proteins. Signaling through the bone morphogenetic protein (BMP) pathway represses the formation of neural and retinal fates. It has been shown that Noggin acts as a morphogen to specify neural cell types at low concentrations, and retinal cell types at higher concentrations. The aim of our study is to determine if the higher concentration of Noggin affects signaling pathways other than BMP.

Methods: We treated pluripotent *Xenopus laevis* tissue (animal caps) with chemical inhibitors and function-altering components of the BMP and Activin/Nodal signaling pathway. Animal caps were removed from the embryos at the blastula stage and cultured until neural plate stage. Their effect on retina formation was determined using the Animal Cap Transplant (ACT) assay, in which the animal caps were transplanted into the eye field of sibling embryos. Signaling activity was determined by Western blot and semi-quantitative PCR (RT-PCR) to measure downstream protein and gene target expression.

Results: Overexpressing Noggin in animal caps resulted in a concentration-dependent suppression of both Smad1 and Smad2 phosphorylation, which act downstream of BMP and Activin/Nodal receptors, respectively. This caused a decrease in downstream transcriptional ability, reflected by the reduced expression of mesodermal marker, *Xbra*, and endothelial marker, *Xk81*. However, we also observed that Cerberus was less effective at blocking Smad1/5/8 phosphorylation, yet it can specify retina as efficiently as Noggin in ACT assays. Cerberus has been shown to block the Activin/Nodal pathway, as well as the BMP pathway, suggesting that there is a specific balance between the two pathways that is required to direct a retinal fate. The use of dominant negative BMP and activin receptors revealed that retinal specification was increased when both pathways were inhibited simultaneously. Similar results were observed when the chemical inhibitors Dorsomorphin and SB431542 were used to inhibit Smad1 and Smad2/3 phosphorylation, respectively.

Conclusions: Thus, the dual inhibition of BMP and activin pathways promotes retinal specification in *Xenopus* tissue. Future studies will translate these findings to a mammalian culture assay, in order to efficiently produce a large percentage of retinal cells *in vitro*.

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Presentation Time: 9:15 AM–9:30 AM

HDAC inhibition protects degenerating cones in the cpfl1 mouse

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Purpose: Understanding the mechanisms of cell death during inherited retinal degeneration may allow the definition of novel targets for neuroprotection. The cpfl1 mouse is a model for cone cell death characterized by fast and progressive cone degeneration. Additionally, the Pde6c mutation governing cpfl1 cone degeneration leads to an impaired cone migration during retinal development by unknown mechanisms. The cpfl1 cone degeneration follows a non-apoptotic cell death mechanism and is characterized by cGMP accumulation and increased activities of PKG and calpains (Trifunovic et al., *J Comp Neurol.*, 518(17):3604-17, 2010). In the present study, we asked whether the epigenetic modifications contributing to primary rod degeneration in Pde6b-mutant rd1 mice (Sancho-Pelluz et al., *Cell Death Dis.*, 1:e24, 2010), are also involved in primary cone degeneration.

Methods: We assessed the correlation of increased HDAC activity and cone degeneration in cpfl1 retina using an HDAC in situ activity assay. The neuroprotective properties of the HDAC inhibitor, Trichostatin A (TSA), were assessed using a cpfl1 ex vivo retinal explant system, followed by immunohistological detection of characteristic cone markers.

Results: Similar to corresponding observations in the rd1 model, cpfl1 cone photoreceptor cell death is associated with increased HDAC activity. TSA inhibition of the HDAC activity in cpfl1 retinal explant cultures resulted in a significant improvement in cone survival. At the same time, TSA treatment did not negatively affect wild-type cones. Notably, HDAC inhibition also significantly improved developmental cone migration compared to non-treated retinas.

Conclusions: Our finding that primary cone photoreceptor degeneration is associated with increased HDAC activity suggests the existence of cell death mechanisms common to both rod and cone degeneration. This raises the possibility that equivalent neuroprotective strategies may be used to prevent both types of photoreceptor degeneration. Indeed, HDAC inhibition emerges as a novel neuroprotective approach for the treatment of primary cone degeneration, and provides an exciting new possibility for a preservation of useful vision in patients suffering from cone dystrophies.

Commercial Relationships: Dragana Trifunovic, None; Blanca Arango-Gonzalez, None; Klaudiaj Masarini, None; Norman Rieger, None; Michelle Dierstein, None; Marius Ueffing, None; Francois Paquet-Durand, None

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Presentation Time: 9:30 AM–9:45 AM

Inhibitor of Apoptosis Stimulating Protein of p53 (iASPP) is required for retinal ganglion cell survival after axonal injury

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Purpose: p53 apoptotic activity is tightly regulated by the apoptosis-stimulating proteins of p53 (ASPP) family members: ASPP1, ASPP2 and iASPP. We previously showed that pro-apoptotic members ASPP1 and ASPP2 contribute to the p53-dependent death of retinal ganglion cells (RGC), however the role of the p53 inhibitor iASPP in the central nervous system is unknown. Here, we addressed the role of iASPP on RGC survival in a model of acute optic nerve injury (axotomy) using loss-of-function and gain-of-function experiments *in vivo*.

Methods: iASPP knockdown was carried out by intravitreal injection of small interference RNA (si-iASPP). Overexpression of iASPP in RGCs was achieved by intraocular delivery of a tyrosine mutant serotype 2 adeno-associated virus (AAV.iASPP). Phosphoserine immunoprecipitation was performed on retinal lysates of intact, axotomized, and iASPP overexpressing retinas. iASPP, Fas/CD95, PUMA, Noxa and Bax protein levels were examined by retinal immunohistochemistry and western blot analysis. RGC immunolabeling was performed with an RBPM5 antibody. RGC densities were assessed by quantification of Brn3a-positive cells on retinal whole mounts followed by statistical analysis using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-test.

Results: Our data demonstrate that iASPP is expressed by intact and injured RGCs, and that iASPP phosphoserine levels, which increase iASPP affinity towards p53, are significantly reduced following axotomy. We show that iASPP downregulation by siRNA exacerbates RGC death, whereas selective AAV-mediated overexpression of iASPP promotes robust RGC survival. iASPP overexpression results

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in an increase of iASPP phosphoserine levels following axotomy compared to retinas treated with a control virus. Analysis of p53 downstream targets demonstrate that increasing iASPP levels in RGCs leads to downregulation of pro-apoptotic PUMA and Fas/CD95.

Conclusions: Our study demonstrates a novel role for iASPP in the death of RGCs, and provides further evidence of the importance of ASPP family in CNS neuronal survival after axonal injury.

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Presentation Time: 9:45 AM–10:00 AM

Neuroprotective effect of KUS121, a VCP modulator, on a rat model of ischemia and reperfusion-induced retinal degeneration
Masayuki Hata¹, Hanako O. Ikeda¹, Munemitsu Yoshikawa¹, Tomoko Hasegawa¹, Noriko Nakano¹, Yuki Muraoka¹, Akira Kakizuka², Nagahisa Yoshimura¹. ¹Ophthalmology, Kyoto University, Kyoto, Japan; ²Graduate school of biostudies, Kyoto University, Kyoto, Japan.

Purpose: Valosin-containing protein (VCP) is a ubiquitously expressed ATPase that is reported to be involved in several physiological activities as well as neurodegeneration. Newly synthesized compounds (Kyoto University Substances, KUSs) that inhibit VCP ATPase activity have been shown to protect cells under stress conditions. This study aimed to confirm whether KUS121, one of KUSs has a neuroprotective effect on a rat model of ischemia and reperfusion (I/R)-induced retinal degeneration.

Methods: KUS121 was orally administered to adult Thy1-GFP rats (Magill CK et al. Arch Facial Plast Surg 2010) before I/R injury. Retinal I/R injury in the rats was induced by elevating the intraocular pressure for 60 minutes with subsequent reperfusion. Spectral-domain optical coherence tomography (SD-OCT) examinations (Multiline OCT, Heidelberg Engineering) were performed to evaluate the inner retinal thickness (IR: RNFL + GCL + IPL+INL) around the optic nerve head 1, 4, and 7 days after I/R injury.

Results: The IR thickness of untreated I/R rats (n = 9) changed from 126.6 ± 14.7 µm at baseline to 133.9 ± 9.6 µm at day 1, 96.3 ± 14.0 µm at day 4, and 84.3 ± 15.3 µm at day 7 after I/R injury. In contrast, the IR thickness of I/R rats treated with KUS121 (n = 9) changed from 125.6 ± 9.7 µm at baseline to 126.0 ± 4.8 µm at day 1, 104.7 ± 21.9 µm at day 4, and 93.0 ± 29.8 µm at day 7 after I/R injury. The mean IR thickness of the rats treated with KUS121 was greater than that of the control rats 7 days after I/R (P = 0.008).

Conclusions: KUS121 has a neuroprotective effect on an I/R injury rat model, suggesting the potential usefulness of the compound for the treatment of human ischemic ocular diseases.

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Presentation Time: 10:00 AM–10:15 AM

MANF in Retinal Therapies: Improving Regenerative Therapies by Promoting Tissue Repair

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Purpose: Stem cell based therapies, have been shown to hold real promise in the treatment of degenerative diseases of the retina. However, the efficiency of such strategies is still considerably low. Tissue repair mechanisms are conserved at the organism level and enhance the regenerative process. We hypothesized that promoting tissue repair may also enhance the efficiency of cell engraftment in the retina. Key components of the retinal repair network have been identified in *Drosophila* involving interactions between the damaged retina and hemocytes. We have used the *Drosophila* to identify hemocyte derived factors that can promote tissue repair in the retina and have tested the conservation of their function in the mammalian retina. Our work focused on Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF).

Methods: We have used UV induced retinal damage in flies and light induced retinal damage in mouse as model systems to test the effects of MANF. MANF was overexpressed in flies using the UAS/Gal4 system. In mice, recombinant protein was delivered by intravitreal injection.

Results: We have identified MANF as a hemocyte derived protein in *Drosophila* that can promote tissue repair in the fly retina, using RNAseq. We show that MANF is expressed in hemocytes of *Drosophila* larvae, it is secreted to the hemolymph and induced in response to stress in a Pvf-1/PvR dependent manner. Hemocyte specific MANF expression is sufficient to reduce tissue loss after UV and genetically-induced photoreceptor apoptosis. Moreover, stress induced MANF results in changes in the hemocyte population correlating with increased lamellocyte differentiation. We have tested the conservation of the pathway in mammalian retinal repair. As in flies, MANF is induced in microglia/macrophages invading the retina following light damage and this correlates with reduced tissue loss. Importantly, intravitreal delivery of MANF recombinant protein is sufficient to limit cell death following light damage and promotes alterations in macrophages/microglia.

Conclusions: MANF is a conserved neuroprotective factor in the retina. MANF acts as an immune-modifying factor to limit cell loss following acute damage. This work will serve as a proof of concept to the use of tissue repair promoting factors as co-adjuvants in stem cell regenerative therapies.

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