The Zebrafish Galectin Drgal1-L2 Is Expressed by Proliferating Müller Glia and Photoreceptor Progenitors and Regulates the Regeneration of Rod Photoreceptors

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Purpose. The purpose of this study was to identify secreted proteins in the retina of the adult zebrafish that are induced by the selective death of photoreceptors and to test experimentally the function of these proteins during the regeneration of photoreceptors.

Methods. Induced selective death of photoreceptors in the retina of the adult zebrafish was combined with in situ hybridization and immunocytochemistry to identify the induced cellular expression of the secreted β-galactoside binding protein Galectin 1–like 2 (Drgal1-L2). Electroporation of morpholino oligonucleotides was used to knock down protein synthesis, and regenerated photoreceptors were counted in control and experimental retinas after labeling with cell type-specific RNA probes.

Results. Expression analysis and immunocytochemistry showed that Drgal1-L2 is induced de novo by photoreceptor death and is synthesized by microglia and proliferating Müller glia and their mitotic progeny. Knockdown of Drgal1-L2 expression in Müller glia results in reduced regeneration of rod photoreceptors without affecting injury-induced proliferation or the regeneration of cone photoreceptors.

Conclusions. Based on these data, the authors conclude that Drgal1-L2 is induced by photoreceptor cell death and secreted by stem cells and neuronal progenitors and that it regulates the regeneration of rod photoreceptors. Drgal1-L2 is the first secreted factor shown to regulate aspects of regenerative neurogenesis in the teleost retina. (Invest Ophthalmol Vis Sci. 2010; 51:3244–3252) DOI:10.1167/iovs.09-4879
ation of rod photoreceptors. We conclude from these data that Drgal1-L2 is an injury-induced factor secreted by retinal stem cells and photoreceptor progenitors that is required for the regeneration of rod photoreceptors.

**Materials and Methods**

**Animals**

Three strains of adult zebrafish (3.0–4.0 cm long) were used in this study. Wild-type fish were used for laser capture microdissection and microarray analysis. Wild-type fish and Tg(gfap:GFP) reporter fish\(^{30}\) were used to characterize the cellular expression of Drgal1-L2, and albino zebrafish were used for the loss-of-function experiments. All procedures using animals were approved by the University Committee for Use and Care of Animals at the University of Michigan and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Light Lesions**

To induce photoreceptor death in normally pigmented fish, animals were housed in the dark for 7 days and then exposed to fluorescent light of approximately 27,000 lux for periods between 6 hours to 7 days. To induce photoreceptor death in albino zebrafish, animals were housed in the dark for 10 days and then exposed to incandescent light of approximately 8000 lux for periods between 24 hours and 7 days. These lighting paradigms selectively kill photoreceptors in the dorsal retina, with maximum loss of photoreceptor-specific markers at 4 days after light onset\(^3\) (SELC, PFH, unpublished observations, 2009). Photoreceptors are largely regenerated by 7 days after light onset.\(^{3,9}\) For both pigmented and albino fish, the time course of photoreceptor death and regeneration is similar (SELC, PFH, unpublished observations).

**Real-Time PCR**

Neural retinas were dissected from a cohort of fish, separate from those used for the microarray analysis, at 0, 6, 12, 24, and 48 hours after light onset. Six retinas were collected per time point. mRNA was extracted with an RNA kit (RNaqueous; Ambion, Austin, TX) according to the manufacturer’s protocol and was reverse transcribed into cDNA (Superscript II System; Invitrogen, Carlsbad, CA).

**Histology**

Eye cups were dissected from anesthetized fish, fixed overnight in 4% paraformaldehyde, cryoprotected by infiltration in 20% sucrose in phosphate buffered saline and stored in 0.1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics). The homogenate was spun at 3000 rpm for 3 minutes to pellet nuclei and melanin granules. Protein was quantified with a protein assay kit (BCA; Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE (15% gels), transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and immunolabeled with antibodies against zebrafish Drgal1-L2.\(^{27}\) Horseradish peroxidase-coupled anti-rabbit IgG secondary antibodies were diluted 1:3000, and chemiluminescence (ECL detection system; Amersham Biosciences, Arlington Heights, IL) was used to visualize the proteins. Equal protein loading per lane was verified by stripping the blots with Western blot stripping buffer.
(Restore; Pierce) and reprobing them with a monoclonal antibody against actin (JLA20; Calbiochem, San Diego, CA).

Image Analysis

An image analysis approach was used as an indirect method to quantify the number of injury-induced photoreceptor progenitors. This method was used because of the difficulty in distinguishing individual somata within the radial columns enveloping individual Müller glia. Immunostaining and image analysis were conducted identically for each experimental group to minimize the possibility of introducing variability between samples. With the use of ImageJ 1.38x software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), fluorescent images were converted to 8-bit grayscale before thresholding positive staining. Images were captured on the microscope and analyzed using identical settings (see Fig. 5 for example of thresholding). One image per retina was captured in the dorsal retina approximately midway between the retinal margin and the optic nerve head using a 40× objective. This location corresponded to site at which we observed lissamine fluorescence, indicating the presence of labeled morpholinos, and the presence and absence of Drgal1-L2 immunolabeling in control and experimental retinas, respectively. Sections immediately adjacent to those immunolabeled for Drgal1-L2 were immunostained with antibodies against PCNA. The Image J software was then used to calculate the percentage area of the image positive for the fluorescent label. Ten to 13 retinas were evaluated per condition per time point. Values were averaged across similar treated retinas, and statistical analyses were performed using the non-parametric Mann–Whitney U test.

Cell Counts

Rhodopsin-positive and pd66c-positive somata, respectively, were counted in adjacent sections from 10 control and 10 experimental retinas. Cells were counted in the dorsal retina, between the optic nerve head and the retinal margin, and were expressed as number of cells per micrometer of retinal length. Statistical comparisons between values from control and experimental retinas were performed using the nonparametric Mann–Whitney U test.

RESULTS

Transcripts Encoding drgal1-L2 Increase in the Injured ONL 48 Hours after Light Onset

To identify injury-induced changes in gene expression, RNA was isolated from cells within the ONLs of light-lesioned retinas. Based on the occurrence of cell death and proliferation,5,16 five time points—0, 6, 12, 24, and 48 hours—after light onset were selected. Transcriptional changes of twofold or greater were observed for more than 1900 genes, which included genes involved in cell death, stress response, and regulation of DNA transcription.16 The expression of drgal1-L2 mRNA isolated from whole retinas as measured by quantitative real-time PCR (n = 2, mean ± SD).

Drgal1-L2 Is Expressed by Microglia and Regenerative Müller Glia and Their Mitotic Progeny

In situ hybridization and immunohistochemistry were used to determine the cellular expression of Drgal1-L2. No drgal1-L2 mRNA or protein was detected in the unlesioned retinas, in the ventral halves of light-damaged retinas, or in the circumferential marginal zones (data not shown). In contrast, in the light-damaged dorsal retina, in situ hybridization showed that drgal1-L2 was expressed in radial columns of cells in the inner nuclear layer (INL) and ONL, beneath the injured/dying photoreceptors (Fig. 2). Drgal1-L2-specific antibodies27 labeled a similar pattern of cells that were also PCNA positive, confirming that the Drgal1-L2 protein is expressed by injury-induced neural progenitors (Figs. 5a–e). To test whether Gal-Drgal1-L2 is expressed in proliferating Müller glia, PCNA immunostaining was combined with in situ hybridization of drgal1-L2 mRNA on retinal sections from the Tg(gfap:GFP)rm2001 zebratfish. Each signal colocalized to the same cells, confirming that drgal1-L2 is expressed by proliferating Müller glia and their progeny (Figs. 3f–i). A few Drgal1-L2-positive, gfap-GFP-negative cells were also observed. Using the microglial specific antibody 4C4,9 these cells were identified as microglia (Figs. 4f–h). A time course of light damage demonstrated that Drgal1-L2 is expressed as early as 24 hours after light onset in PCNA-positive Müller glia within the INL (data not shown). As evidenced by immunostaining, upregulated expression of Drgal1-L2 persists up to 7 days after light onset (see Fig. 4).

Morpholino Oligonucleotides Targeted against the Translation Start Site of drgal1-L2 mRNA Knocked Down Expression of Drgal1-L2 Protein in Müller Glia

To test the function of Drgal1-L2 protein during photoreceptor regeneration, we electroporated morpholino oligonucleotides directed against the translation start site of either drgal1-L2 mRNA (Drgal1-L2-MO) or a 5-base mismatch control morpholino (drgal1-L2 mm-MO) into retinas immediately before light injury. Both Western blot analysis and immunohistochemistry demonstrated that drgal1-L2-MO efficiently knocked down Drgal1-L2 protein levels compared with the drgal1-L2 mm-MO controls (Fig. 4). This reduction in Drgal1-L2 synthesis was specific to Müller glia and persisted for up to 7 days.
Reducing Drgal1-L2 Protein Does Not Affect Proliferation

Given the expression of Drgal1-L2 in proliferating Müller glia, we evaluated whether this protein functioned in an autocrine manner to regulate proliferation. To test this, control and morphant retinas were immunostained with antibodies against PCNA. Qualitative observation and image analysis showed that at each time point sampled, there was no difference in either the number or the location of proliferating cells in control and morphant retinas (Fig. 5). These data demonstrate that Drgal1-L2 does not regulate the proliferation of Müller glia or their progeny or the migration of the photoreceptor progenitors to the ONL.

Reducing Drgal1-L2 Protein Diminishes Regeneration of Rod Photoreceptors

Given that the induction of Drgal1-L2 expression coincides with the proliferation of Müller glia but does not precede it (see above), a tenable hypothesis is that Drgal1-L2 protein regulates later events in regeneration. We took two approaches to determine the effect of the loss of Drgal1-L2 on the regeneration of photoreceptors in retinas sampled 7 days after light onset. First, image analysis was used as an indirect estimate of the number of nuclei in the ONL in DAPI-stained sections from experimental and control retinas. This analysis showed there were fewer nuclei in the ONLs of experimental retinas than in controls (mean percentage areas of the ONL labeled with DAPI for control and drgal1-L2 morphant retinas were 6.8% ± 1.7% and 4.6% ± 1.1%, respectively; Mann–Whitney U test, \( P < 0.05 \)). Second, rod and cone photoreceptors were labeled with cell type-specific RNA probes, respectively, and the number of each cell type in adjacent sections from control and experimental retinas was counted. For these experiments, adjacent sections were stained with antibodies against Drgal1-L2, which showed loss of protein in the experimental retinas (data not shown). Variability in the average number of labeled cells was high, but comparisons revealed that there was no statistically significant difference in the values for cone photoreceptors in control and experimental retinas (57.0 ± 10.1 vs. 49.1 ± 18.1, respectively), whereas there were significantly fewer rod photoreceptors in experimental retinas than in controls (26.4 ± 13.0 vs. 57.1 ± 20.7, respectively; Figs. 6, 7). To determine whether the reduction in the number of rod photoreceptors was a consequence of reduced regeneration or differential survival, we immunolabeled retinal sections with the photoreceptor-specific antibodies from eyes at 48, 72, and 96 hours after light onset. By 96 hours after light onset, maximum photoreceptor death was observed, as indicated by the reduction of photoreceptor-specific immunolabeling. At each of the time points, there were no differences in the number of red/green double cones (Fig. 8) or rods (Fig. 9) in control and drgal1-L2 morphant retinas (Fig. 5).

The data indicate that Drgal1-L2 protein regulates later events in regeneration.
showed there were no differences in the number of apoptotic cells in control and morphant retinas (data not shown). In sum, these data demonstrate that reduced Drgal1-L2 protein does not affect the death of photoreceptors or the regeneration of cone photoreceptors but that it does result in a reduction in the number of regenerated rods.

**DISCUSSION**

The results of this study demonstrate that the expression of Drgal1-L2 is induced by the death of photoreceptors and is expressed by microglia and regenerative Müller glia and their progeny and that this molecule functions to regulate the regeneration of rod photoreceptors. We used laser capture mi-
crodissection to harvest cells from the ONL but found subsequently that Drgal1-L2 is expressed by Müller glia. At early time points after the selective death of photoreceptors, nuclei of Müller glia migrate to the ONL, and we infer that the laser capture microdissection harvested nuclei and mRNA from these Müller glia. Although our goal was to identify genes specific to photoreceptors and photoreceptor progenitors, we consider it fortuitous to have identified a gene also expressed by Müller glia, which for teleosts are the retina’s intrinsic stem cell and the immediate antecedent to photoreceptor progenitors (e.g., see Ref. 6). We found that photoreceptor death also induces Drgal1-L2 expression in microglia. However, given that these cells are refractory to protein knockdown by the Drgal1-L2-targeted morpholinos (Figs. 4f–h), we infer that Drgal1-L2 synthesized by Müller glia plays the predominant role in regulating the regeneration of photoreceptors.

For several reasons, the observations regarding the expression and function of Drgal1-L2 are novel. First, Drgal1-L2 is the first secreted factor shown to regulate neuronal regeneration in the teleost retina. Second, our results suggest that distinct molecular mechanisms control the regeneration of rod and cone photoreceptors. Third, Drgal1-L2 is the first molecule identified in the teleost retina that functions during retinal regeneration, but it appears not to have a role in retinal development. Fourth, these data add to the functional roles of galectins in biological systems and expand the list of candidate molecules and signaling pathways expressed in stem and pro-
genitor cells during retinal injury and regeneration.33 Finally, these data add Drgal1-L2 to the molecular signature of Müller glia as they adopt the features of neural stem cells (see also Refs. 9–12, 14, 34–36).

Partial knockdown of Drgal1-L2 protein resulted in a significant reduction in the number of regenerated rod photoreceptors, and these data are interpreted to show that Drgal1-L2 is required for the regeneration of rods. Further, because the number of proliferating Müller glia and their progeny was unchanged after knockdown of Drgal1-L2 expression, we propose that reduction of this protein in the injury-induced photoreceptor progenitors selectively diminishes the regeneration of this cell type. Although no differences were observed in the number of PCNA-positive or TUNEL-positive cells at the time points sampled, we cannot exclude the possibility that the absence of Drgal1-L2 results in subtle changes in cell proliferation or apoptosis among rod progenitors and nascent rods. A small change in either of these events, not detected here, could lead to the reduced regeneration of rod photoreceptors. For example, given that rods are regenerated after cones,4 the absence of Drgal1-L2 could produce a subtle slowing of proliferation that would result in a reduction in the number of photoreceptors regenerated, which, over the intervals studied here, would result in a reduced number of regenerated rods. In contrast to the paucity of rods in experimental retinas, knocking down Drgal1-L2 protein did not affect the regeneration of cone photoreceptors. Although the same caveats apply, these data provide additional evidence that in the teleost retina different molecular mechanisms control the regeneration of rod and cone photoreceptors, consistent with a recent observation that rods and cones are regenerated from distinct populations of progenitor cells.37

Contrary to the assumption that regeneration recapitulates development (see Ref. 38), our expression data suggest that Drgal1-L2 is uniquely an injury-associated molecule. Drgal1-L2 is absent in retinal progenitors in the embryonic and larval retina (SELC, PFH, unpublished observations, 2009; see also Ref. 27), and it is absent in rod progenitors in the ONL and multipotent retinal progenitors in the adult circumferential marginal zone. Consistent with these observations, brain development is largely normal in mice that are null for galectin-1 (see Ref. 39), suggesting that in mammals this molecule has no profound role in early brain development. However, loss of galectin-1 diminishes stem cell proliferation within the subventricular zone of adult mice,22 and perturbating galectin-1 function with neutralizing antibodies reduces stem cell proliferation in the subventricular zone after stroke injuries to the overlying cerebral cortex.26 In vitro, galectin-1 is highly expressed in neural stem cells derived from embryonic stem cells and downregulated in neurons, suggesting a role for this molecule in regulating neuronal differentiation.25 Both Müller glia in the teleost retina and stem cells in the murine SVZ serve as a reservoir of adult stem cells that sustain persistent neurogenesis. This raises the possibility that galectin-1 functions only in the stem cells of the mature nervous system and is a component of the response to neuronal injury or death.
We propose three potential molecular mechanisms to explain the function of Drgal1-L2 during photoreceptor regeneration in zebrafish. First, the selective binding of Drgal1-L2 to glycoproteins on the surfaces of rod progenitors could determine the fate or differentiation of these cells. Galectin-1 can bind β1 integrin,\(^{19,40}\) which inhibits the growth of cell lines that model epithelial cancers by repressing the Ras-MEK-Erk pathway and increasing Sp1-mediated transcription of p27 and p21.\(^ {41}\) Because p27 promotes the cell cycle exit of retinal progenitors,\(^ {42}\) the Drgal1-L2-induced upregulation of p27 in rod progenitors could control the timing of cell cycle withdrawal and, secondarily, control their fates or differentiation. A role for β1 integrin in neuronal differentiation has been described in mouse cortical radial glia in which β1 integrin expression is necessary for morphologic differentiation of neurons.\(^ {43}\) β1 Integrin expression in the telocast retina has not been characterized, but it or other glycoproteins could be putative receptors for Drgal1-L2, leading to lattice formation and signaling.\(^ {44}\)

Second, Drgal1-L2 could regulate the incorporation of cell adhesion molecules into the ECM. In vitro studies show that soluble galectin-1 inhibits the binding of vitronectin and chondroitin sulfate B to ECM proteins, thus regulating the assembly of the ECM.\(^ {18}\) The composition of the ECM is critical to photoreceptor development. Severe defects in photoreceptor differentiation are observed in the zebrafish mutants, grumpy and sleepy, which have mutated forms of the laminin b1 and c1 chains, respectively,\(^ {45}\) demonstrating a selective role for cell adhesion molecules in regulating photoreceptor differentiation.

Third, Drgal1-L2 could anchor rod-specific growth factors in the ECM. For example, in the neural stem cell niche of adult mammals, heparin sulfate proteoglycans bind many growth factors known to stimulate neurogenesis, including bone morphogenetic proteins, sonic hedgehog (shh), Wnts, FGF, and IGF-II.\(^ {44}\) Drgal1-L2 could sequester rod-specific growth factors in the regenerating ONL.

Of the three potential mechanisms, the latter two are best supported by our data, which show the reduction of Drgal1-L2 protein results in a decrease in the number of regenerated rods but not their complete loss. We favor a modulatory role for Drgal1-L2, whereby it influences the ability of (yet unidentified) rod-specific cue(s) to function. The experimental paradigm we used in this study results in the selective death and regeneration of photoreceptors, and we have interpreted our data in light of the regeneration of these two cell types. It remains to be determined whether Drgal1-L2 is indeed a rod-specific signal or whether it also regulates the regeneration of other retinal cell types.

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References


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