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Endogenous Induced Cell Fate Change of the Müller Glia in Different Models of Retinal Degeneration

By

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Abstract

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Retinal degeneration are blinding eye diseases that impact millions of lives around the world. Age-related macular degeneration alone is predicted to affect over 250 million people around 2040 as the population ages. The most prevalent form of inherited retinal degeneration, retinitis pigmentosa, has been found to have more than 200 different causative mutations, showing that treatments for retinal degeneration may not have a one-size-fit-all solution and require different approaches and concerted efforts from scientists around the world.

Gene therapy is an emerging and effective treatment option in restoring the vision and the quality of life of a subset of retinal degenerative patients. However, the success of gene therapy faces the limitations of low vector transduction efficiency, low vector carrying capacity, and immutable disease progression. Stem cell therapies face similar problems of immunogenicity and low cell integration efficiency. We show here that, through a combination of these two treatment modalities, endogenous regeneration through the genetic reprogramming of glia may be another effective and more broadly applicable approach in treating retinal degeneration.

Müller cells**,** the primary glia of the retina, have been demonstrated to possess neuroprotective as well as retinal progenitor cell-like regenerative properties in cases of retinal damage in zebrafish. The helix-loop-helix transcription factor Ascl1 is a key factor in determining neuronal cell fate in the nervous system, and it has also been shown to be capable of transdifferentiating astrocytes as well as Müller glia into neurons *in vitro*. The Let7 siRNA regulator Lin28 likewise seems to influence the zebrafish Müller glia cell fate through an Ascl1-dependent manner. While damage-dependent regeneration of the retina in zebrafish relies on the expression of Ascl1 and Lin28, no large scale proliferation and differentiation of the Müller glia have been observed in the mammalian retina, and as such, the role of Ascl1 and Lin28 in determining the mammalian Müller glia cell fate is currently unknown.

In chapter 2, we demonstrate that Müller glia in zebrafish and mice are capable of proliferation and fate change *in vivo* through the approach of forced expression of both Ascl1 and Lin28. Lin28 enhances the Ascl1-dependent proliferation of the Müller glia in zebrafish and mice when retinal damage is induced via NMDA. Notch suppression increases this proliferation in zebrafish further, but curiously not in mice. In chapter 3 we look at the different conditions involved in this Ascl1/Lin28 dependent proliferation through the exploration of different retinal damage models such as cobalt chloride induced hypoxic response and UV light induced photoreceptor apoptosis. We also attempt to induce Müller glia proliferation in retinal degenerative mouse models. Interestingly, while we find some success in inducing Müller glia proliferation in the retinal damage models, we find that genetic mouse models of retinal degeneration do not seem to respond to Ascl1 and Lin28 manipulation.

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Chapter 1: Introduction

The Retina

The retina is a highly organized nervous tissue with very well delineated structure and connectivity. Composed of six distinct neuronal cell types and one glia, it is made of three cell body layers and two connective plexiform layers. During phototransduction, the light enters the pupil and passes through the vitreous humor to reach the retina at the very back of the eye. The light then traverses through the various layers of the retina to reach the photoreceptors. In order, the various layers are: the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), and finally the outer segments of the photoreceptors. The outer segments are nurtured and supported by the retinal pigment epithelium (RPE) and are responsible for the initiation of phototransduction (Fig. 1.1). A light-dependent depolarizing signal is transferred from the outer segments to the cell bodies of the photoreceptors in the ONL, and the photoreceptors pass this signal to the bipolar cells and the horizontal cells of the INL. The bipolar cells, in response, transfer this signal to the ganglion cells with modulation from the amacrine cells. Ultimately, this causes the ganglion cells to send action potentials to V1, the primary visual center of the cerebral cortex (Rodieck, 1973).

Throughout the development of the vertebrate retina, the formation of the different cell types follows a distinctly conserved pattern. For prenatal development, the retinal progenitors first give rise to a population of ganglion cells, which is then followed by horizontal cells, cone cells, and amacrine cells. Postnatal development sees a second wave of differentiation leading to the rise of rod cells, bipolar cells, and finally the Müller glia (MG) cells (Kageyama, 2003). Throughout this process, unique patterns of transcription factor expression determine the final cell fate, creating a constantly changing pool of heterogeneous progenitor cells (Kageyama, 2001). Retinal development involves a complex sequence of transcription factor and signaling factor expression changes, but within this complexity lies the crucial clues to our goal in combating and reversing the damages caused by retinal degeneration.

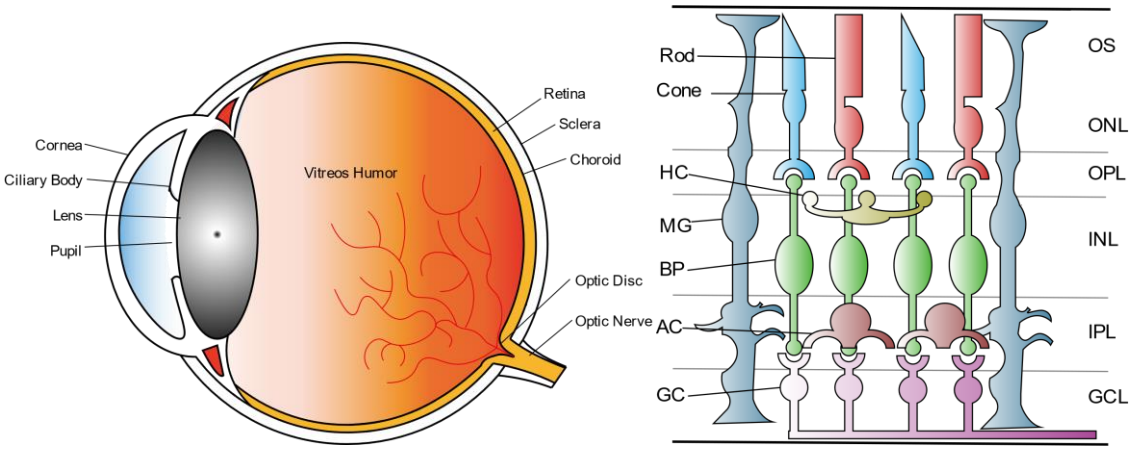


Figure 1.1 The Retina
The retina is situated at the very back of the eye. It is organized into distinct layers with distinct populations of cells. OS: Outer Segment, ONL: Outer Nuclear Layer, OPL: Outer Plexiform Layer, INL: Inner Nuclear Layer, IPL: Inner Plexiform Layer, GCL: Ganglion Cell Layer, HC: Horizontal Cell, MG: Müller Glia, BP: Bipolar Cell, AC: Amacrine Cell, GC: Ganglion Cell

Retinal Degeneration

Retinal degeneration (RD) are blinding eye diseases that involve retinal cell death. It is estimated that more than 250 million people in the world will be affected by age-related macular degeneration (AMD) alone by 2040 (Wong, 2014). The most common form of inherited RD, retinitis pigmentosa (RP) currently affects roughly 1 in 2500 to 7000 people in the U.S (Parmeggiani, 2011). While the severity of RD varies considerably and is entirely dependent on the patient and disease, RD, in general, leads to difficulty in completing everyday tasks and decreases the quality of life of the patients considerably. Even though in recent years, dozens of genes and hundreds of mutations associated with inherited RP have been identified and studied, effective treatments have not yet been forthcoming (Sullivan, 2007). The disease burden associated with RD will only increase as the population ages around the world.

Retinitis pigmentosa, as a major inherited form of RD, result from mutations in critical phototransduction genes. AMD, on the other hand, involves the association of a multitude of factors. While all retinal cell types are potentially affected, both types of RD ultimately leads to the death of the ganglion cells, photoreceptors, or RPE (Miller, 2008)(Juan, 1997). The preservation and restoration of vision, then, depends heavily upon the preservation and restoration of photoreceptors, ganglion cells, and RPE. However, currently available treatment for RD is limited in scope and efficacy. Most involve targeted correction of inherited mutations through gene therapy, and the effect of permanent cell lost isn't addressed. Even clinical stem cell studies that aim to restore lost cells currently only involve the transplantation of RPE, with the transplantation of retinal neurons still being in the animal testing stage (Bainbridge, 2018). Merely halting disease progression requires herculean efforts, and most treatments that strive to restore vision are still many steps away from being applicable to humans.

Figure 1.2 Examples of Vision Under Retinal Degeneration
For patients living with retinal degeneration, normal vision (left) gradually loses its periphery in retinitis pigmentosa (center) as the peripheral cones die off. In macular degeneration, patients lose central vision (right) and much of their visual acuity.

Gene Therapy

Pharmacological agents have found limited success in treating RD through the inhibition of external apoptotic factors. Anti-angiogenin antibodies are effective in treating wet AMD and diabetic retinopathy (DR) through preventing blood-induced damage of photoreceptors (Kim, 2006). Anti-apoptotic and protective agents such as X-Linked Inhibitor of Apoptosis (XIAP) and Rod-Derived Cone Viability Factor (RDCVF) are potentially viable options for a broader range of patients (Flannery, 2014)(Tsilfidis, 2003). The advent of gene therapy treatments, however, has brought forth a solution that aims at the root cause of RD. In gene therapy, viral vectors carrying functional genes could be readily tailored for each patient to correct the disease-causing mutation (Maguire, 1996). As a prime example, clinical trials for the treatment of Leber Congenital Amaurosis 2, which involve Adeno-Associated Viral (AAV) gene delivery of the RPE65 gene to patients lacking functional RPE65 proteins, have demonstrated substantial restoration of vision in patients (Auricchio, 2009). Furthermore, with the development of CRISPR, the option of gene editing instead of gene replacement has been made possible (Bumcrot, 2015).

Nevertheless, photoreceptor and ganglion cell loss are an irreversible process, and due to the restrictive amount of genetic material that can be safely and efficiently delivered into retinal cells, many retinal diseases with mutations in large size genes fall outside the scope of gene therapy treatments (Frizzell, 2008). Viruses that possess larger carrying capacities such as lentivirus are known to either integrate into the genome, potentially causing undesired mutations in the host, or are potentially pathogenic (Naldini, 2001). Gene therapy possesses great potential, but limitations in current technology must be addressed for the mainstream application of these treatments.

Exogenous Cell Replacement Therapy

A distinct and complementary approach to rescue vision is the *in vivo* delivery of functional retinal cells. These cells could potentially be derived from embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). With the relatively simple

expression of a few transcription factors, various mature cell types of the body could be transformed into pluripotent progenitors, creating a seemingly unlimited source of cells for transplantation (Yamanaka, 2006). Using photoreceptors derived from iPSCs to replenish the degenerating retina could potentially lead to significant therapeutic benefit through the restoration of function, as has been seen in mice (Reh, 2008). Stem-cell derived photoreceptor cells have been successfully cultured, showing that neurons of the retina could possibly be replenished (Reubinoff, 2009). Success in animal trials had led to the current clinical trials involving iPSC-derived RPE transplantation (Lanza, 2012). However, while the derivation of retinal cells is possible, the maintenance, purification, and transplantation aspects of cell replacement therapy faces many hurtles especially in the case of neuron transplantation. *In vivo* cell replacement therapies raise concerns with the tumorigenic potential of contaminating, undifferentiated pluripotent stem cells, limited cell integration efficiency, and immunogenicity challenges (Martin, 2011).

The Potential of the Müller glia

An alternative path for cell replacement therapy is *in situ* regeneration, which harnesses endogenous cells for tissue repair. Non-mammalian vertebrates possess an innate ability to regenerate the retina in cases of damage. Zebrafish and chickens have been extensively studied for this process (Reh, 2010). Central to this regeneration is the glia cell of the retina, the Müller glia (MG). The MG is the structural, synaptic, and nutritional maintenance cell of the retina. It is a radial glia that spans the entire thickness of the retina and envelopes most of the synaptic connections. Different water, ion, and neurotransmitter reuptake channels and exchange pumps in the MG maintain not only the osmotic balance of the retina but also the voltage and neurotransmitter equilibriums (Reichenbach, 1996). As the only glia originating from the retinal tissue during development, the MG takes on many jobs and is an essential part of a healthy retina.

In the damaged teleost fish retina, DNA epigenetic and gene expression changes induce MG dedifferentiation through the expression of a variety of signal transduction cascades. Notch, MAPK, PI3K, Jak/Stat, and GSK3β/β-catenin, have all been implicated in the establishment of this injury-induced MG response (Goldman, 2011). These dedifferentiated MG derived progenitors migrate to the various retinal layers and then differentiate into the appropriate types of retinal neurons (Goldman, 2014). This process, however, is severely limited in the mammalian retina, as the activation of the MG brought about by damage only introduces very limited gene expression changes or in the extreme cases, limited proliferation in rat retinas (Takahashi, 2004). The reason for this limitation is not well understood. Mammalian retinas cease growth after maturation while zebrafish retinas experience continuous growth. Presumably evolutionary pressures reduced the amount of aberrant cell proliferation to prevent tumorigenesis. However, as noted previously, retinal development and MG maturation is a well conserved process across many vertebrate species (Kageyama, 2003). Furthermore, the vertebrate MG shares structural and expression similarities with early retinal progenitor cells such as the expression of stem cell markers Sox2 and Nestin (Reh, 2003).

Previous work has already established that, *in vitro,* with the deprivation of mitogens and the supplementation of Notch inhibitors, both mouse and human cultured MGs can adopt neuronal expression profiles that overlap with those of photoreceptors (Broccoli, 2010). However, cultured MG differ in expression pattern and morphology when compared to MG *in vivo* (Khaw, 2002). Since, *in vivo*, MG maintains tight contact with every cell in the retina, it follows that cultured, dissociated, MG experiences an extreme case of activation beyond that of damaged or degenerated retinas. Nevertheless, the conserved developmental process, the expression of progenitor markers, and the ability to proliferate and differentiate *in vitro* all point to the potential of mammalian MG to be a source of lost retinal cells. There may exist a certain set of conditions that would enable mammalian MG to proliferate and enable endogenous regeneration.

Ascl1 and Lin28's Role in the MG Regenerative Response

A variety of transcription factors are responsible for determining the various cell fates in the retina, and a complex cascade of signals are involved in the zebrafish retina repairing process. However, a simple helix-loop-helix transcription factor Ascl1 has been found to be a key player in both processes (Wallace, 2012)(Goldman, 2011). Ascl1 is a general proneuronal transcription factor that plays important roles in determining early neuronal cell fate in the central nervous system. Not only do Ascl1-expressing progenitors follow a neuronal lineage as opposed to a glial one, the overexpression of Ascl1 in glioblastoma progenitors push the tumor cells toward a terminal neuronal fate (Dirks, 2017). Previous work suggests that *in vivo* transgenic expression of Ascl1 can even push mouse MG back into the cell cycle temporarily. In the injured juvenile Ascl1-expressing mouse retina, limited MG proliferation has been observed through BrdU lineage tracing, and the colocalization of BrdU expressing MG with retinal neuron markers has also been observed (Reh, 2013).

As a key player in neurogenesis, Ascl1 seems to be able to reprogram MG to proliferate and adopt neuronal characteristics *in vivo*. However, this process appears to be limited, and the resulting MG-induced neurons lean towards the bipolar and amacrine cell fates. Most importantly, the resulting daughter cells typically do not survive (Reh, 2013). While Ascl1 may be sufficient for mammalian MG proliferation and fate change, the inefficiencies with this process points to other factors that may be needed to fully untap the regenerative potential of the MG. Further research has identified Lin28 as a possible complement to Ascl1-induced MG dedifferentiation. Lin28 is a Let7-binding inhibitor, and Let7 is a microRNA precursor whose family of miRNAs are thought to be associated with terminal differentiation and tumor suppression (Gregory, 2008). Lin28, with Nanog, has even been used in iPSC formation by replacing Klf4 and c-Myc (Rao, 2013). As a general dedifferentiation factor, its expression is highly elevated after zebrafish retinal damage, and is thought to be a part of the zebrafish retinal injury response. In fact, Lin28 expression has been found to be dependent upon Ascl1 expression. The opposing action between Let7 and Lin28 resembles that of embryonic stem cell differentiation versus proliferation (Goldman, 2010).

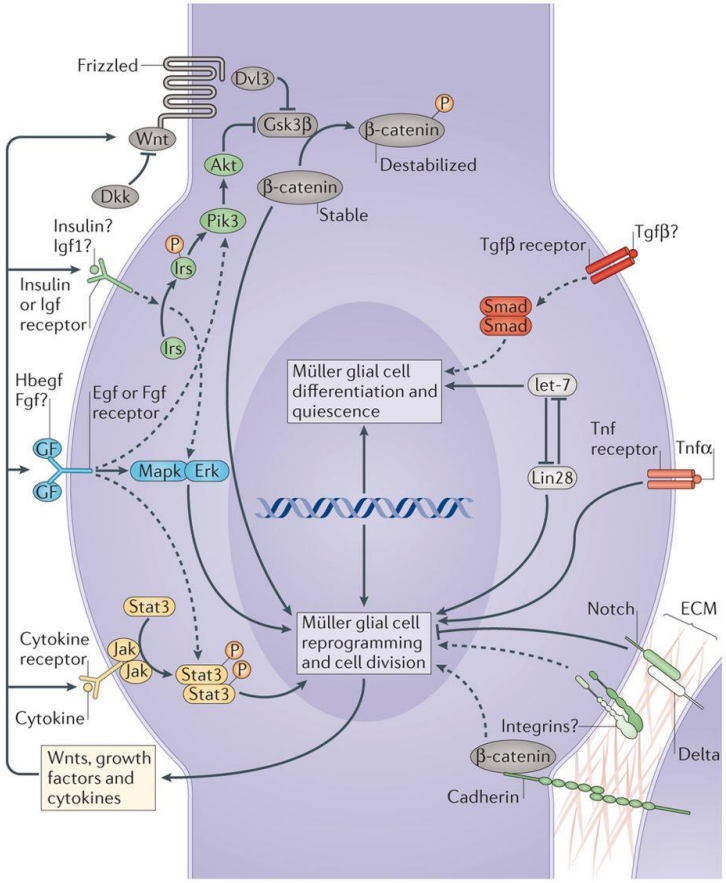


Figure 1.3 The Complexity of the MG Response
(Goldman, 2014) An illustration of the complex signals involved in zebrafish MG quiescence vs dedifferentiation. Note the interplay between Let7 and Lin28 which is under the control of Ascl1 expression.

In chapter 2, we will show that Ascl1 and Lin28 act in a synergistic manner in zebrafish to reprogram MG towards a proliferative state. Furthermore, this effect is also seen in mice in both cases of damaged and undamaged mature retina. This synergistic effect is then further enhanced by the suppression of Notch in zebrafish but not in mice. In chapter 3, we will take this further by exploring the effect of Ascl1 and Lin28 overexpression in photoreceptor ablation models. Afterwards, we will also investigate the effect of Ascl1 and Lin28 in mouse models of retinal degeneration. We find that while photoreceptor ablation lead to MG proliferation that is enhanced by Ascl1 and Lin28 expression, we are unable to elicit a MG response in naturally degenerating mouse models even with Ascl1 and Lin28. Ultimately the mammalian MG proliferation response may be dependent upon the genetic background of the animal and the extend of retinal damage suffered.

References

Rodieck RW. (1973) The Vertebrate Retina: Principles of Structure and Function. Oxford, England: W. H. Freeman.

Hatakeyama J, Kageyama R. (2003) Retinal Cell Fate Determination and bHLH Factors. Seminars in Cell & Dev. Bio. 15(1)83-89. 10.1016/j.semcdb.2003.09.005

Hatakeyama J, Tomita K, Inoue T, Kageyama R. (2001) Roles of Homeobox and bHLH Genes in Specification of a Retinal Cell Type. Development. 128(8)1313-1322

Wong WL, Su X, Li X, Cheung CMG, Klein R, Cheng CY, Wong TY. (2014) Global Prevalence of Age-Related Macular Degeneration and Disease Burden Projection for 2020 and 2040: a Systematic Review and Meta-Analysis. Global Health. 2(2)e106-e116. 10.1016/S2214-109X(13)70145-1

Parmeggiani F. (2011) Clinics, Epidemiology and Genetics of Retinitis Pigmentosa. Curr Genomics. 12(4):236-237. 10.2174/138920211795860080

Daiger SP, Bowne SJ, Sullivan LS. (2007) Perspective on Genes and Mutations Causing Retinitis Pigmentosa. JAMA Ophthalmology. 125(2):151-158. 10.1001/archopht.125.2.151

Jager RD, Mieler WF, Miller JW. (2008) Age-Related Macular Degeneration. New England Journal of Medicine. 358:2606-2617. 10.1056/NEJMra0801537

Santos A, Humayun MS, Juan E. (1997) Preservation of the Inner Retina in Retinitis Pigmentosa. Arch Ophthalmol. 155(4):511-515.

Mehat MS, Sundaram V, Ripamonti C, Gobson AG, Smith AJ, Borooah S, Robinson M, Rosenthal AN, Innes W, Weleber RG, Lee RWJ, Grossland M, Rubin GS, Dhillon B, Steel DHW, Anglade E, Lanza RP, Ali RR, Michaelides M, Bainbridge JWB. (2018) Transplantation of Human Embryonic Stem Cell-Derived Retinal Pigment Epithelial Cells in Macular Degeneration. Ophthalmology. 125(11):1765-1775. 10.1016/j.ophtha.2018.04.037

Ferrara N, Damico L, Shams N, Lowman H, Kim R (2006). Development of Ranibizumab, an Anti-Vascular Endothelial Growth Factor Antigen Binding Fragment, As Therapy for Neovascular Age-Related Macular Degeneration. Retina. 26(8):859-879. 10.1097/01.iae.0000242842.14624.e7

Byrne LC, Dalkara D, Luna G, Fisher SK, Clerin E, Sahel JA, Levillard T, Flannery JG. (2014). Viral-Mediated RdCVF and RdCVFL Expression Protects Cone and Rod Photoreceptors in Retinal Degeneration. J Clin Invest. 125(1):105-116. 10.1172/JCI65654

Petrin D, Baker A, Coupland SG, Liston P, Narang M, Damji K, Leonard B, Chiodo VA, Timmers A, Hauswirth W, Korneluk RG, Tsilfidis C. (2003) Structural and Functional Protection of Photoreceptors from MNU-Induced Retinal Degeneration by the X-Linked Inhibitor of Apoptosis. Inves Ophthal & Vis Sci. 44:2757-2763. 10.1167/iovs.02-0729

Bennett J, Tanabe T, Sun D, Zeng Y, Kjeldbye H, Gouras P, Maguire AM. (1996) Photoreceptor Cell Rescue in Retinal Degeneration (RD) Mice by *In Vivo* Gene Therapy. Nature Medicine. 2:694-695

Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, Rossi S, Marshall K, Banfi S, Surace EM, Sun J, Redmond TM, Zhu X, Shindler KS, Ying GS, Ziviello C, Acerra C, Wright JF, McDonnell JW, High KA, Bennett J, Auricchio A. (2010) Gene Therapy for Leber's Congenital Amaurosis Is Safe and Effective Through 1.5 Years After Vector Administration. Molecular Therapy. 18(3):643-650. 10.1038/mt.2009.277

Gori JL, Hsu PD, Maeder ML, Shen S, Welstead GG, Bumcrot D. (2015) Delivery and Specificity of CRISPR/Cas9 Genome Editing Technologies for Human Gene Therapy. Human Gene Therapy. 26(7). 10.1089/hum.2015.074

Dong JY, Fan PD, Frizzell RA. (1996) Quantitative Analysis of the Packaging Capacity of Recombinant Adeno-Associated Virus. Human Gene Therapy. 7(17). 10.1089/hum.1996.7.17-2101

Kay MA, Glorioso JC, Naldini L. (2001) Viral Vectors for Gene Therapy: the Art of Turning Infectious Agents into Vehicles of Therapeutics. Nature Medicine. 7:33-40.

Takahashi K, Yamanaka S. (2006) Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 126(4):663-676. 10.1016/j.cell.2006.07.024

Lamba DA, Gust J, Reh TA. (2009) Transplantation of Human Embryonic Stem Cell-Derived Photoreceptors Restores Some Visual Function in Crx-Deficient Mice. Cell Stem Cell. 4(1):73-79. 10.1016/j.stem.2008.10.015

Banin E, Obolensky A, Idelson M, Hemo I, Reinhartz E, Pikarsky E, Ben-Hur T, Reubinoff B. (2009) Retinal Incorporation and Differentiation of Neural Precursors Derived from Human Embryonic Stem Cells. Stem Cells. 24(2):246-257. 10.1634/stemcells.2006-0009

Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R. (2012) Embryonic Stem Cell Trials for Macular Degeneration: a Preliminary Report. The Lancet. 379(9817):713-720. 10.1016/S0140-6736(12)60028-2

Bull ND, Martin KR. (2011) Concise Review: Towards Stem Cell-Based Therapies for Retinal Neurodegenerative Diseases. Stem Cells. 29(8):1170-1175. 10.1002/stem.676

Karl MO, Reh TA. (2010) Regenerative Medicine for Retinal Diseases: Activating Endogenous Repair Mechanisms. Trends in Molecular Medicine. 16(4):193-202. 10.1016/j.molmed.2010.02.003

Newman E, Reichenbach A. (1996) The Müller Cell: a Functional Element of the Retina. Trends in Neurosciences. 19(8):307-312. 10.1016/0166-2236(96)10040-0

Ramachandran R, Zhao XF, Goldman D. (2011) Ascl1a/Dkk/β-catenin Signaling Pathway Is Necessary and Glycogen Synthase Kinase-3β Inhibition Is Sufficient for Zebrafish Retina Regeneration. PNAS. 108(38):15858-15863. 10.1073/pnas.1107220108

Goldman D. (2014) Müller Glial Cell Reprogramming and Retina Regeneration. Nature Reviews Neuroscience. 15:431-442

Ooto S, Akagi T, Kageyama R, Akita J, Mandai M, Honda Y, Takahashi M. (2004) Potential for Neural Regeneration After Neurotoxic Injury in the Adult Mammalian Retina. PNAS. 101(37):13654-13659. 10.1073/pnas.0402129101

Fischer AJ, Reh TA. (2003) Potential of Müller Glia to Become Neurogenic Retinal Progenitor Cells. Glia. 43(1):70-76. 10.1002/glia.10218

Giannelli SG, Demontis GC, Pertile G, Rama P, Broccoli V. (2010) Adult Human Müller Glia Cells Are a Highly Efficient Source of Rod Photoreceptors. Stem Cells. 29(2):344-356. 10.1002/stem.579

Limb GA, Salt TE, Munro PMG, Moss SE, Khaw PT. (2002) *In Vitro* Characterization of a Spontaneously Immortalized Human Müller Cell Line (MIO-M1). Inv Ophthal & Vis Sci. 43:864-869

Bassett EA, Wallace VA. (2012) Cell Fate Determination in the Vertebrate Retina. Trends in Neurosciences. 35(9):565-573. 10.1016/j.tins.2012.05.004

Park NI, Guilhamon P, Desai K, McAdam RF, Langille E, O'Connor M, Lan X, Whetstone H, Coutinho FJ, Vanner RJ, Ling E, Prinos P, Lee L, Selvadurai H, Atwal G, Kushida M, Clarke ED, Voisin V, Cusimano MD, Bernstein M, Das S, Bader G, Arrowsmith CH, Angers S, Huang X, Lupien M, Dirks PB. (2017) Ascl1 Reorganizes Chromatin to Direct Neuronal Fate and Suppress Tumorigenicity of Glioblastoma Stem Cells. Cell Stem Cell. 21(2):209-224.e7. 10.1016/j.stem.2017.06.004

Pollak J, Wilken MS, Ueki Y, Cox KE, Sullivan JM, Taylor RJ, Levine EM, Reh TA. (2013) Ascl1 Reprograms Mouse Müller Glia into Neurogenic Retinal Progenitors. Development. 140:2619-2631. 10.1242/dev.091355

Viswanathan SR, Daley GQ, Gregory RI. (2008) Selective Blockade of MicroRNA Processing by Lin28. Science. 320(5872):97-100. 10.1126/science.1154040

Malik N, Rao MS. (2013) A Review of the Methods for Human iPSC Derivation. Pluripotent Stem Cells. Methods in Mol Bio. 997. 10.1007/978-1-62703-348-0_3

Ramachandran R, Fausett BV, Goldman D. (2010) Ascl1a Regulates Müller Glia Dedifferentiation and Retinal Regeneration Through a Lin-28-Dependent, Let-7 MicroRNA Signalling Pathway. Nature Cell Biology. 12:1101-1107

Chapter 2: Notch Suppression collaborates with Ascl1 and Lin28 to unleash a Regenerative Response in Fish Retina, but Not in Mice

Fairouz Elsaeidi, Peter Macpherson, Elizabeth A. Mills, Jonathan Jui, John G. Flannery, and Daniel Goldman

This chapter, in full, is a republication of the material as it appears in F. Elsaeidi et al, (2018). Notch Suppression Collaborates with Ascl1 and Lin28 to Unleash a Regenerative Response in Fish Retina, but Not in Mice. *Journal of Neuroscience,* 38(9): 2246-2261.

2.1 Summary

Müller glial (MG) cells in the zebrafish retina respond to injury by acquiring retinal stem-cell characteristics. Thousands of gene expression changes are associated with this event. Key among these changes is the induction of Ascl1a and Lin28a, two reprogramming factors whose expression is necessary for retina regeneration. Whether these factors are sufficient to drive MG proliferation and subsequent neuronal-fate specification remains unknown. To test this, we conditionally expressed Ascl1a and Lin28a in the uninjured retina of male and female fish. We found that together, their forced expression only stimulates sparse MG proliferation. However, in combination with Notch signaling inhibition, widespread MG proliferation and neuron regeneration ensued. Remarkably, Ascl1 and Lin28a expression in the retina of male and female mice also stimulated sparse MG proliferation, although this was not enhanced when combined with inhibitors of Notch signaling. Lineage tracing in both fish and mice suggested that the proliferating MG generated multipotent progenitors; however, this process was much more efficient in fish than mice. Overall, our studies suggest that the overexpression of Ascl1a and Lin28a in zebrafish, in combination with inhibition of Notch signaling, can phenocopy the effects of retinal injury in Müller glia. Interestingly, Ascl1 and Lin28a seem to have similar effects in fish and mice, whereas Notch signaling may differ. Understanding the different consequences of Notch signaling inhibition in fish and mice, may suggest additional strategies for enhancing retina regeneration in mammals.

2.2 Introduction

Blinding eye diseases, like glaucoma and macular degeneration result from the death of retinal ganglion cells and photoreceptors. Neural regeneration has the potential to restore sight to the blind by generating replacements for these lost cells. Teleost fish, like zebrafish, exhibit a robust regenerative response to retinal injury that results in

neuron regeneration and restoration of visual function (Lindsey and Powers, 2007; Sherpa et al., 2008; Wan and Goldman, 2016). Unfortunately, mammals have lost the ability to regenerate any retinal cells once they have been lost. Understanding the pathways by which zebrafish regenerate neurons in response to injury may suggest therapeutic strategies for initiating retinal cell regeneration in mammals.

In zebrafish, retina regeneration is initiated in Müller glia (MG) that respond to retinal injury by undergoing a reprogramming event that endows them with stem-cell characteristics and allows them to divide and generate multipotent progenitors for retinal repair (Goldman, 2014; Wan and Goldman, 2016). Many signaling cascades and gene expression programs underlying MG reprogramming and retina regeneration have been identified (Ramachandran et al., 2010b, 2011, 2012; Thummel et al., 2010; Nelson et al., 2012, 2013; Conner et al., 2014; Rajaram et al., 2014; Wan et al., 2012, 2014; Zhao et al., 2014). In particular, Ascl1a and Lin28a have emerged as major regulators of the regenerative program (Fausett et al., 2008; Ramachandran et al., 2010b, 2011; Nelson et al., 2012). Ascl1a is a basic helix-loop-helix transcription factor and Lin28 is an RNA binding protein. Following retinal injury in zebrafish, there is a rapid and dramatic increase in *ascl1a* and *lin28a* gene expression that precedes MG proliferation. Knockdown of Ascl1a or Lin28a reduces the injury-dependent activation of many regeneration-associated genes and this results in a reduced proliferative response by MG (Fausett et al., 2008; Ramachandran et al., 2010b, 2011; Nelson et al., 2012).

Interestingly, these two proteins also participate in somatic cell reprogramming in mammals. Ascl1 can reprogram fibroblasts to adopt neuronal characteristics and Lin28 participates in reprogramming fibroblasts to pluripotency (Yu et al., 2007; Chanda et al., 2014). Furthermore, forced expression of Ascl1 or Lin28 can stimulate some MG proliferation in the injured and uninjured mouse retina, respectively; however, most of these cells do not survive (Ueki et al., 2015; Yao et al., 2016). Although Ascl1a and Lin28a are necessary for MG proliferation and retina regeneration in zebrafish, it is not known whether they are sufficient to drive these processes in the uninjured fish retina.

We hypothesized that proteins that are sufficient to drive MG proliferation and retina regeneration in fish will be the most potent in stimulating these processes in mammals. Therefore, we searched for gene combinations that would stimulate MG proliferation in the uninjured zebrafish retina. We found that coexpression of Ascl1a and Lin28a stimulated a small amount of MG proliferation in the uninjured retina. Furthermore, in the injured retina, this expression expanded the zone of injury-responsive MG. Interestingly, the inhibition of Notch signaling dramatically enhanced the effects of Ascl1a and Lin28a on MG proliferation, resulting in widespread proliferation throughout the uninjured fish retina. Finally, Ascl1 and Lin28a coexpression stimulated a small amount of MG proliferation in the uninjured mouse retina, which was increased with retinal injury. However, unlike in fish, Notch inhibition had little effect on this proliferation.

2.3 Results

Ascl1a and Lin28a stimulate MG proliferation in the uninjured zebrafish retina

Injury-dependent induction of Ascl1a and Lin28a in zebrafish retinal MG cells allows for their reprogramming and proliferation (Fausett et al., 2008; Ramachandran et al., 2010a). To investigate whether their induction is sufficient to drive MG proliferation in the uninjured retina we generated *hsp70:ascl1a* and *hsp70:lin28a* transgenic fish, and also bred these fish to each other to generate *hsp70:ascl1a*;*hsp70:lin28a* double-transgenic fish. These fish allow for conditional Ascl1a and Lin28a expression with heat shock. In these fish, a 1 h heat shock at 37°C stimulates *ascl1a* and *lin28a* expression with RNA levels peaking ~2 h post-heat shock and then returning to basal levels ~10 h later (Fig. 2.1*A*). To maintain transgene expression for more prolonged periods of time, transgenic fish received a 1 h heat shock at 37°C, every 6 h for 1–4 d.

We confirmed that *hsp70:ascl1a* transgenic fish produced a functional protein by injecting embryos with the *4RTK-Luc* reporter that harbors four E-box sites (4R) from the MCK enhancer cloned upstream of the thymidine kinase (TK) basal promoter driving firefly luciferase expression (Weintraub et al., 1990). Ascl1 is a helix-loop-helix transcription factor that activates genes via their E-box sequences and thus regulates *4RTK-Luciferase* expression. Heat shock of *hsp70:ascl1a* embryos injected with the *4RTK-Luc* reporter stimulated Luciferase expression by >15-fold (Fig. 2.1*B*). To test for functional expression of Lin28a in *hsp70:lin28a* transgenic fish we assayed the effects of heat shock on endogenous *let7* miRNA expression in the adult retina. Lin28a inhibits *let7* family miRNA maturation and stimulates their degradation (Rybak et al., 2008; Heo et al., 2009). We found that heat shock reduced *let7g* RNA levels in whole retina by ~ 70% (Fig. 2.1*C*). Quantification of Sox9 and flag-Ascl1 or myc-Lin28a co-immunofluorescence suggested that a 1 h heat-shock treatment was sufficient to stimulate transgenic protein expression in ~ 40–50% of the Sox9+ MG when assayed 8 h post-heat shock (Fig. 2.1*D*). Furthermore, Western blot analysis revealed ~40–50-fold induction of flag-tagged Ascl1a (~30 kDa) and myc-tagged Lin28a (~29 kDa) protein expression after heat shock (Fig. 2.1*E*). Finally, we investigated whether Ascl1a or Lin28a were sufficient to stimulate each other's expression in the uninjured fish retina. For this analysis, *hsp70:ascl1a* and *hsp70:lin28a* transgenic fish received heat shock and 24 h later retinas were dissected and assayed for *lin28a* and *ascl1a*, respectively. We found no significant effect of forced expression of Ascl1a on *lin28a* expression, nor did forced Lin28a expression affect *ascl1a* RNA (Fig. 2.1*F*).

In the injured fish retina, MG divide and generate multipotent progenitors that regenerate all major retinal cell types regardless of which cell type is ablated (Powell et al., 2016). However, in the uninjured retina MG rarely proliferate and when they do they appear to be restricted to a rod progenitor lineage (Bernardos et al., 2007). Therefore, we investigated whether forced Ascl1 and Lin28a expression stimulated MG in the uninjured retina to generate multipotent or unipotent progenitors. For this analysis we used a BrdU-based lineage tracing strategy where uninjured *hsp70:ascl1a;hsp70:lin28a* fish received heat shock every 6 h for 4 d and then an intraperitoneal injection of BrdU.

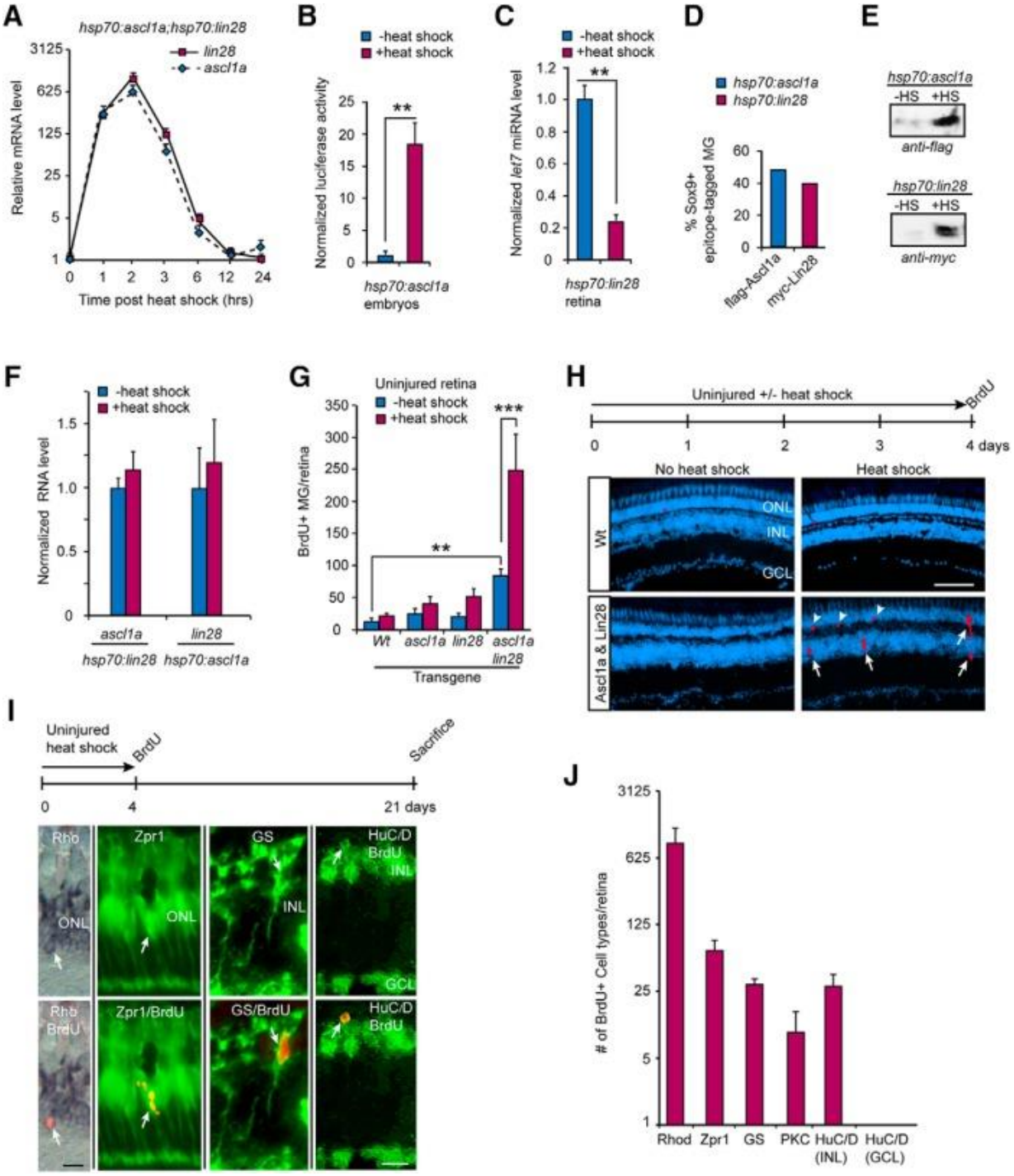


Figure 2.1: Forced expression of Ascl1a and Lin28a stimulates MG proliferation and neuron regeneration in the uninjured retina

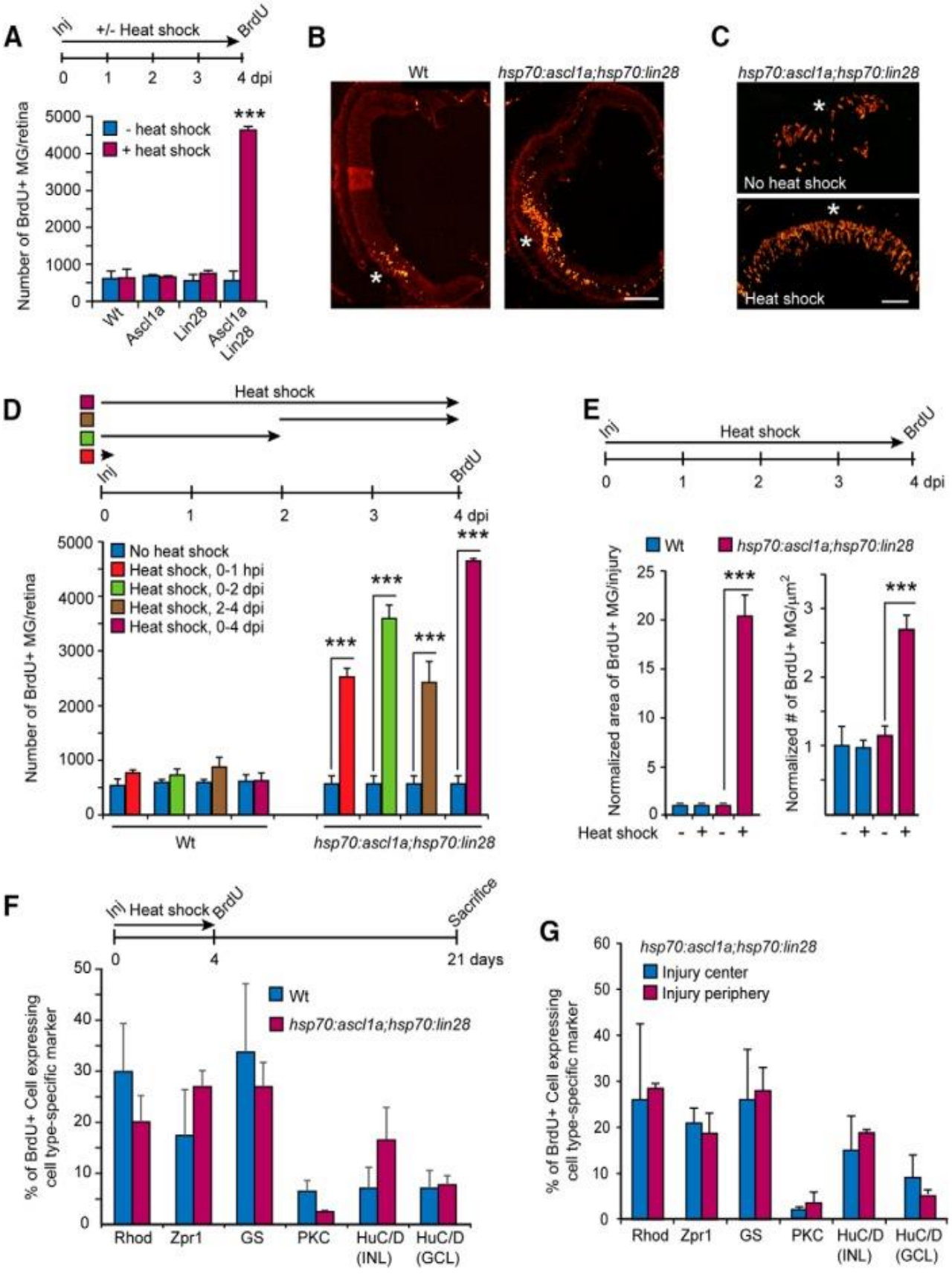
A, Time course of *ascl1a* and *lin28a* RNA expression in retinas of *hsp70:ascl1a;hsp70:lin28a* transgenic fish that received a 1 h heat shock; *n* = 3 different experiments. Error bars are SD. ***B***, Luciferase assays show heat shock of *hsp70:ascl1a* embryos injected with 4RTK-Luc plasmid results in increased expression of the *4RTK-Luc* reporter indicating expression of a functional Ascl1a protein; *n* = 3 different experiments. Error bars are SD. ***p* < 0.009. ***C***, Heat shock-dependent induction of Lin28a in *hsp70:lin28a* adult fish results in reduced *let7g* miRNA expression indicating expression of a functional

Lin28a protein; *n* = 3 different experiments. Error bars are SD. ***p* < 0.01. ***D***, Immunofluorescence quantification of the percentage of Sox9+ MG-expressing flag-Ascl1a and myc-Lin28a 8 h after a 1 h heat shock in *hsp70:ascl1a* and *hsp70:lin28a* transgenic fish. ***E***, Western blot analysis showing flag-Ascl1a and myc-Lin28a protein induction 8 h after a 1 h heat shock in *hsp70:ascl1a* and *hsp70:lin28a* transgenic fish. ***F***, Forced expression of Lin28a in *hsp70:lin28a* does not stimulate *ascl1a* expression and forced expression of Ascl1a in *hsp70:ascl1a* fish does not induce *lin28a* RNA expression. ***G***, BrdU immunofluorescence on retinal sections was used to quantify proliferating MG in the INL of uninjured retinas from transgenic fish expressing the indicated transgenes for 4 d; *n* = 3 different experiments. Error bars are SD. ***p* < 0.01, ****p* < 0.001. ***H***, Timeline of experiment and representative retinal sections showing BrdU immunofluorescence (red) before and after heat shock in Wt and *hsp70:ascl1a*;*hsp70:lin28a* transgenic fish. DAPI nuclear stained cells are blue. Scale bar, 100 μm. ***I***, Timeline of lineage trace experiment and representative retinal sections showing colocalization of either rod rhodopsin (Rho) photoreceptor *in situ* hybridization signal (left, blue), cone (Zpr1), MG (GS), or amacrine cell (HuC/D in INL) immunofluorescence signal (green), with BrdU immunofluorescence signal (red). Scale bar, 20 μm. ***J***, Quantification of data presented in ***I***; bipolar cells (protein kinase C, PKC); *n* = 3 different experiments. Error bars are SD.

Fish were then allowed to survive 21 d before harvesting retinas and assaying progenitor fate. Regenerated rods were identified by colocalizing BrdU immunofluorescence with rhodopsin *in situ* hybridization, whereas other cell types were detected by colocalizing BrdU immunofluorescence with cell-type-specific immunofluorescence. This analysis showed that Ascl1a and Lin28a expression forced some MG to enter the cell cycle and generate a variety of neurons (Fig. 2.1*I*, *J*); however, retinal ganglion cells (RGCs) were not detected. We suspect this reflects the very low number of progenitors that were generated and the relatively small numbers of RGCs normally regenerated from these progenitors (Wan et al., 2012, 2014). The relatively large number of BrdU+ rods after forced Ascl1a and Lin28a expression probably results from proliferating MG-derived multipotent progenitors in the INL (Fig. 2.1*H*, arrows) and rod progenitors normally residing at the base of the ONL (Fig. 2.1*H*, arrowheads). The above data suggest that in the uninjured retina, Ascl1a and Lin28a expression are sufficient to stimulate some MG to proliferate and generate multipotent progenitors.

Ascl1a and Lin28a act in a synergistic fashion to stimulate MG proliferation in the injured fish retina

Although the above studies suggested that forced expression of Ascl1a and Lin28a could stimulate MG proliferation in the uninjured retina, the number of proliferating MG was quite modest. We next investigated whether Ascl1a and Lin28a may collaborate with other injury-derived factors to stimulate MG proliferation. For this analysis we injured retinas in Wt, *hsp70:ascl1a*, *hsp70:lin28a*, and *hsp70:ascl1a;hsp70:lin28a* transgenic fish with a single needle poke. Fish either remained at 28°C or received a 1 h heat shock at 37°C, every 6 h for 4 d. On 4 dpi fish received an intraperitoneal injection of BrdU 3 h before kill. Retinas were then isolated and sectioned, and proliferating MG identified by BrdU immunofluorescence. This analysis revealed that forced expression of either Ascl1a or Lin28a had little effect on injury-dependent MG proliferation (Fig. 2.2*A*).


A
Inj
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BrdU
0 1 2 3 4 dpi
Number of BrdU+ MG/retina
5000
4000
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Wt
Ascl1a
Lin28
Ascl1a Lin28
B
Wt
hsp70:ascl1a;hsp70:lin28
C
hsp70:ascl1a;hsp70:lin28
No heat shock
Heat shock
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% of BrdU+ Cell expressing cell type-specific marker
Wt
hsp70:ascl1a;hsp70:lin28
Rhod
Zpr1
GS
PKC
HuC/D (INL)
HuC/D (GCL)
G
hsp70:ascl1a;hsp70:lin28
Injury center
Injury periphery
% of BrdU+ Cell expressing cell type-specific marker
Rhod
Zpr1
GS
PKC
HuC/D (INL)
HuC/D (GCL)

Figure 2.2: Forced expression of Ascl1a and Lin28a in the injured retina stimulates MG proliferation and retina regeneration
A, Timeline for experiment and graph showing quantification of BrdU immunofluorescence in INL of injured retinas (needle poke) with forced expression of Ascl1a, Lin28a, or Ascl1a and Lin28a; *n* = 3 different experiments. Error bars are SD. ***$p < 0.001$. ***B***, ***C***, Representative images of retinal sections showing BrdU immunofluorescence at the site of injury (needle poke) in Wt and *hsp70:ascl1a;hsp70:lin28a* transgenic fish with heat shock. Scale bars: ***B***, 150 µm; ***C***, 50 µm. ***D***, Timeline depicting retinal needle poke injury (Inj) followed by a 0–1 hpi, 0–2 dpi, 2–4 dpi, and 0–4 dpi heat shock-treatment and BrdU labeling at 4 dpi. Graph shows quantification of BrdU immunofluorescence in the retina's INL of Wt and *hsp70:ascl1a;hsp70:lin28a* transgenic fish; *n* = 3 different experiments. Error bars are SD. ***$p < 0.001$. ***E***, Timeline of experiment, and graph quantifying the retinal area occupied by BrdU+ MG and their density in Wt and *hsp70:ascl1a;hsp70:lin28a* transgenic fish ± heat shock. For this analysis a single needle poke injury was made in each retina. Four days later we quantified the area occupied by proliferating MG by counting the number of 12 µm sections spanning the injury site that harbor nine or more BrdU+ MG. Their density per square micrometer was calculated from the three central section's spanning the injury site. Data are normalized to Wt, no heat shock. Error bars are SD. ***$p < 0.001$; *n* = 3 different experiments. ***F***, BrdU lineage trace experiment shows forced Ascl1a and Lin28a expression in the injured retina does not bias progenitors toward specific fates. Shown is timeline of experiment and graph quantifying the percentage of BrdU+ cells expressing a particular retinal cell-type marker. Markers are as follows: rods (rho), cones (zpr1), MG (GS), Bipolar (protein kinase C-β1, PKC), amacrine (HuC/D in INL), and ganglion cells (HuC/D in GCL); *n* = 3 different experiments. Error bars are SD. ***G***, BrdU lineage trace experiment performed as in ***F*** shows MG-derived progenitors in the central and peripheral regions of the injury site do not exhibit biases in regenerated cell types when forced to express Ascl1a and Lin28a; *n* = 3 different experiments. Error bars are SD.

However, forced expression of Ascl1a and Lin28a together, dramatically enhanced MG proliferation at the injury site and expanded the zone of injury-responsive (BrdU+) MG flanking the injury site (Fig. 2.2*A*–*C*).

Quantification of the proliferative response in Wt and *hsp70ascl1a;hsp70:lin28a* fish with heat shock from 0 to 1 h postinjury (hpi), 0–2 dpi, 2–4 dpi, or 0–4 dpi, revealed that as little as 1 h of heat shock-dependent induction of Ascl1a and Lin28a was sufficient to stimulate MG proliferation (Fig. 2.2*D*). Quantification of the area occupied by BrdU+ MG and the density of BrdU+ MG near the injury site indicated that the forced expression of Ascl1a and Lin28a, expanded the zone of injury-responsive MG (BrdU+) and increased the density of BrdU+ MG within this zone (Fig.2.2*E*). Thus, Ascl1a and Lin28a seem to act very early during the injury response to affect MG reprogramming and proliferation.

We next investigated whether forced expression of Ascl1a and Lin28a in the injured retina influenced the fate of MG-derived progenitors. For this analysis we used a BrdU-based lineage tracing strategy where Wt and *hsp70:ascl1a;hsp70:lin28a* fish retinas were injured with a needle poke and then received heat shock every 6 h for 4 d before receiving an intraperitoneal injection of BrdU. Fish were then allowed to survive to 21 dpi before harvesting retinas and assaying progenitor fate in retinal sections using BrdU and retinal cell-type-specific immunofluorescence. This analysis showed that forced expression of Ascl1a and Lin28a did not impose a significant bias on progenitor fate (Fig. 2.2*F*). This global analysis of progenitor fate may have minimized differences occurring in the peripheral regions flanking the injury site where normally quiescent MG are recruited to an injury response by Ascl1a and Lin28a overexpression. However, when the fate of progenitors close to the injury site was compared with those at the

periphery of the injury-responsive region no significant difference was noted (Fig. 2.2*G*). Thus, together these data suggest that MG-derived progenitors resulting from forced Ascl1a and Lin28a expression in both the uninjured and injured retina are intrinsically multipotent.

Ascl1a and Lin28a enhance MG responsiveness to injury-related growth factors

The above data showed that forced expression of Ascl1a and Lin28a recruits normally quiescent MG that flank the injury site to mount a proliferative response. This could result from a lowering of the threshold at which MG respond to injury-derived factors. We previously reported that growth factors, like HB-EGF and insulin, are increased at the injury site and capable of stimulating MG proliferation (Wan et al., 2012, 2014). To investigate whether forced expression of Ascl1a and Lin28a lowered the threshold at which MG proliferate upon challenge with growth factors, we divided uninjured *hsp70:ascl1a;hsp70:lin28a* transgenic fish into two groups; one group received heat shock, whereas the other did not. Both groups received 3 daily intravitreal injections of PBS-BSA, insulin, or HB-EGF at concentrations that caused only a small amount of MG proliferation; 2 d later, fish received an intraperitoneal injection of BrdU 3 h before kill (Fig. 2.3*A*). BrdU immunofluorescence on retinal sections was used to visualize and quantify MG proliferation in the INL. This analysis showed a significant increase in MG proliferation in fish with forced Ascl1a and Lin28a expression (Fig. 2.3*A*, *B*), which is consistent with the idea that Ascl1a and Lin28a lowers the threshold at which MG mount a proliferative response to injury-induced growth factor expression. Furthermore, this reduced threshold can explain why the zone of proliferating MG is expanded in injured retinas of heat shocked *hsp70:ascl1a;hsp70:lin28a* transgenic fish.

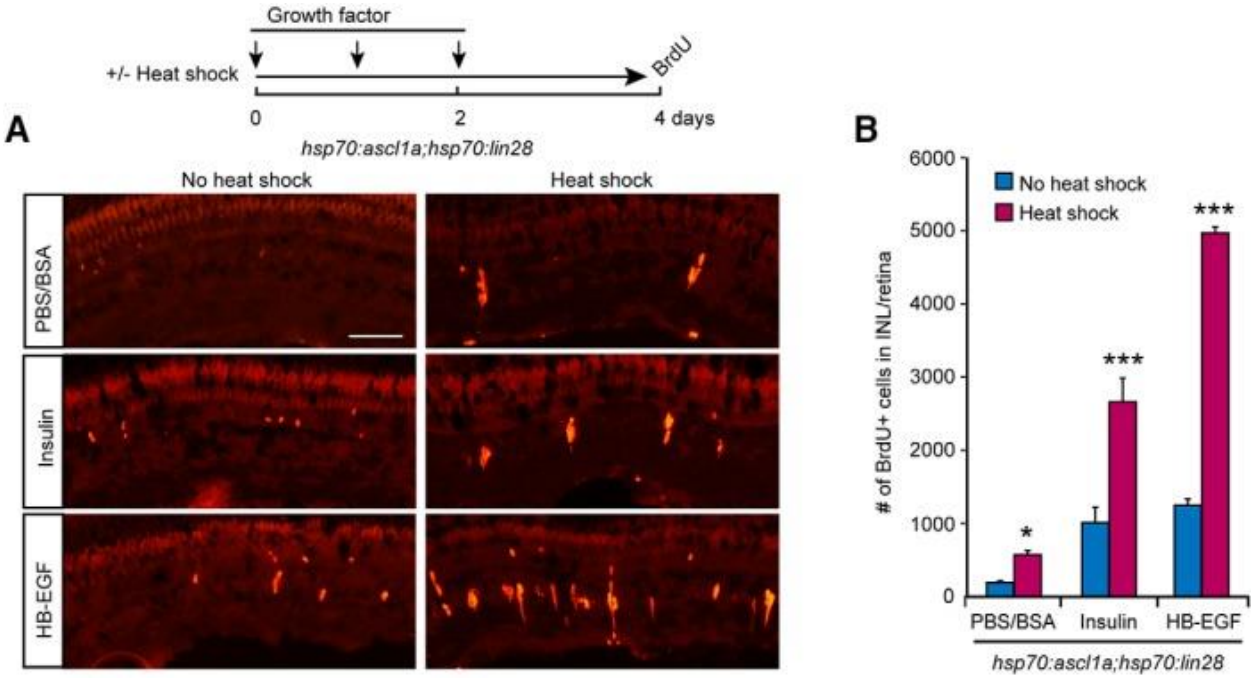


Figure 2.3: Forced expression of Ascl1a and Lin28a in the uninjured retina lowers the proliferative threshold of MG proliferation response to growth factor stimulation
A, Timeline for experiment and representative images of BrdU immunofluorescence in retinal sections from *hsp70:ascl1a;hsp70:lin28a* transgenic fish ± heat shock and growth factor treatment. Scale bar, 50 µm. ***B***, Quantification of BrdU immunofluorescence shown in ***A***; *n* = 3 different experiments. Error bars are SD. **p* < 0.05, ****p* < 0.001.

Notch signaling inhibition collaborates with Ascl1a and Lin28a to stimulate MG proliferation in the uninjured fish retina

In contrast to growth factors and cytokines that stimulate MG proliferation, Notch signaling is associated with MG quiescence and inhibits injury-dependent MG proliferation (Wan et al., 2012; Conner et al., 2014; Wan and Goldman, 2017). Therefore, we wondered whether this Notch-driven inhibitory environment may be contributing to the relatively small effect of Ascl1a and Lin28a expression on MG proliferation in the uninjured retina. Notch signaling was visualized in *Tp1:mCherry* transgenic fish that harbor 12 RBP-Jk binding sites upstream of a minimal promoter that drives nuclear localized mCherry expression (Parsons et al., 2009). In the uninjured retina of *Tp1:mCherry* fish, mCherry expression is restricted to quiescent MG (Fig. 2.4*A*). To investigate whether Ascl1a and Lin28a-dependent MG proliferation is associated with Notch signaling inhibition, we bred *hsp70:ascl1a;hsp70:lin28a* double-transgenic fish with *Tp1:mCherry* transgenic fish. Heat shock of uninjured or injured *hsp70:ascl1a;hsp70:lin28a*;*Tp1:mCherry* fish showed Notch signaling was repressed in proliferating MG regardless of whether the stimulus to proliferate was forced Ascl1a and Lin28a expression, retinal injury, or a combination of both (Fig. 2.4*A*).

Although loss of Notch signaling is associated with MG proliferation, we recently reported that Notch inhibition is not sufficient to stimulate MG proliferation in the uninjured retina (Wan and Goldman, 2017). Indeed, immersing uninjured fish in water containing the Notch signaling inhibitor DAPT for 4 d had little effect on MG proliferation in the uninjured retina (Fig. 2.4*B*, *C*). However, if DAPT-treatment was combined with forced expression of Ascl1a and/or Lin28a, MG proliferation ensued and this was most dramatic in fish expressing both Ascl1a and Lin28a together (Fig. 2.4*B*–*D*). We previously reported that DAPT suppresses mCherry expression in MG of *Tp1:mCherry* transgenic fish (Wan and Goldman, 2017). Consistent with this, we find that DAPT also inhibited *her4.1* gene expression in the uninjured retina (Fig. 2.4*E*). Similar results were obtained with a second Notch signaling inhibitor, RO4929097 that was delivered into the vitreous (Fig. 2.4*C*, *E*). These data, along with our previous studies (Wan et al., 2012; Wan and Goldman, 2017), suggest that MG quiescence is driven/maintained by Notch signaling and that this signaling must be relieved for MG proliferation to occur. Importantly, our data suggest that in conjunction with Notch inhibition, Ascl1a and Lin28a are sufficient to stimulate MG proliferation.

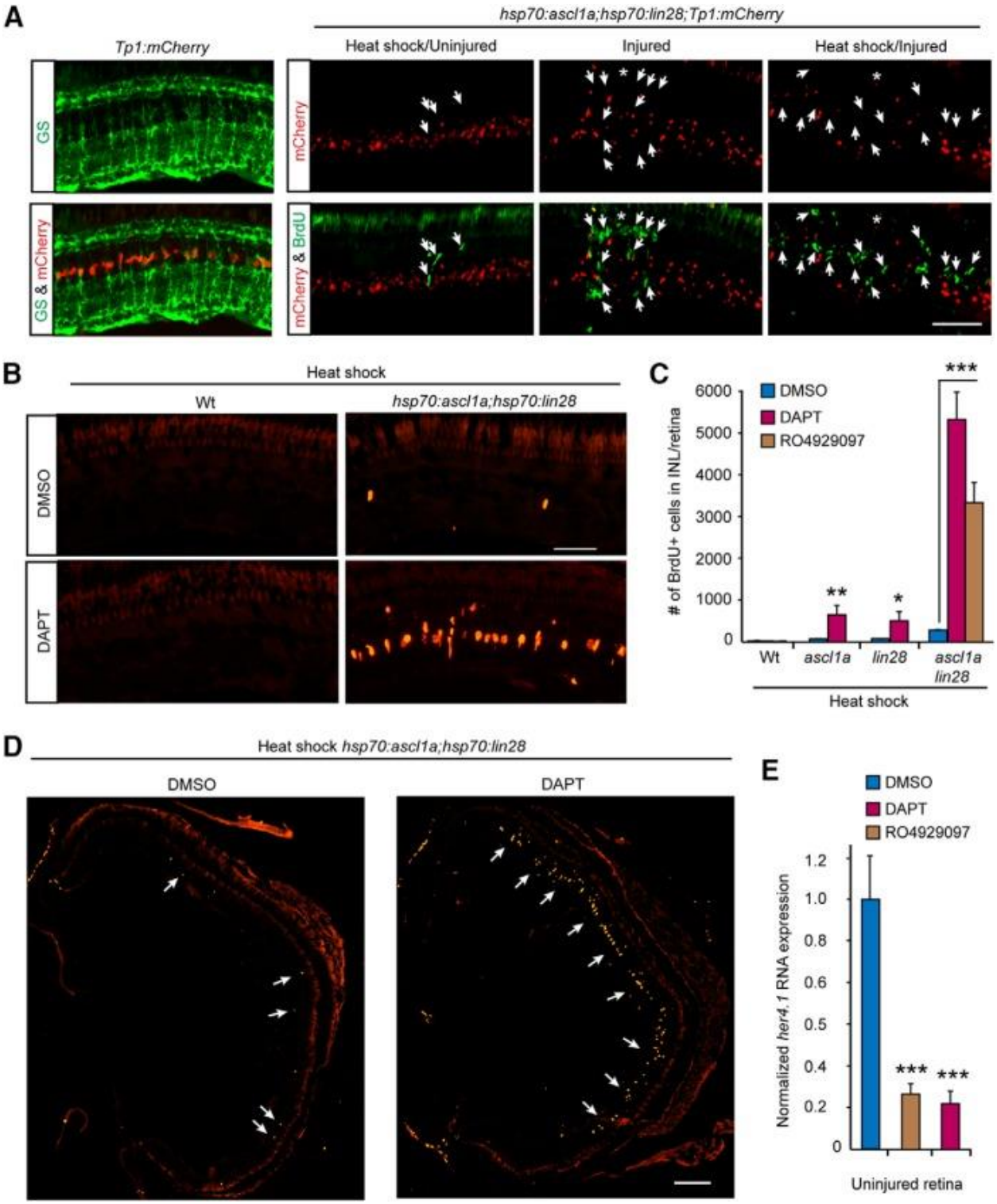


Figure 2.4: Ascl1a and Lin28a expression synergize with Notch signaling inhibition to stimulate MG proliferation throughout the uninjured retina

A, Left, mCherry expression and GS immunofluorescence (green) in uninjured retinas of *Tp1:mCherry* fish and indicate that Notch signaling is confined to quiescent MG. Remaining panels show that MG proliferation (BrdU+; green signal) stimulated by forced Ascl1a and Lin 28 expression in the uninjured and injured retina of *hsp70:ascl1a;hsp70:lin28a;Tp1:mCherry* triple-transgenic fish is accompanied by

reduced mCherry expression (red). Scale bar, 50 μm. ***B***, BrdU immunofluorescence (red) shows that forced Ascl1a and Lin28a expression synergizes with DAPT-treatment to stimulate MG proliferation (BrdU+) in the INL of uninjured retinas. Scale bar, 50 μm. ***C***, Quantification of BrdU+ cells in Wt, *hsp70:ascl1a*, *hsp70:lin28a*, and *hsp70:ascl1a;hsp70:lin28a* fish treated with heat shock, ± DAPT or RO4929097; n = 3 different experiments. Error bars are SD. *p < 0.05, **p < 0.01, ***p < 0.001. ***D***, BrdU immunofluorescence shows that forced Ascl1a and Lin28a expression synergizes with DAPT-treatment to stimulate MG proliferation (BrdU+) throughout the INL of uninjured retinas. Scale bar, 150 μm. ***E***, qPCR quantification of *her4.1* gene expression in uninjured retinas treated with DMSO, DAPT, or RO4929097; n = 3 different experiments. Error bars are SD. ***p < 0.001.

Regulation of regeneration-associated genes following DAPT-treatment and Ascl1a/Lin28a expression

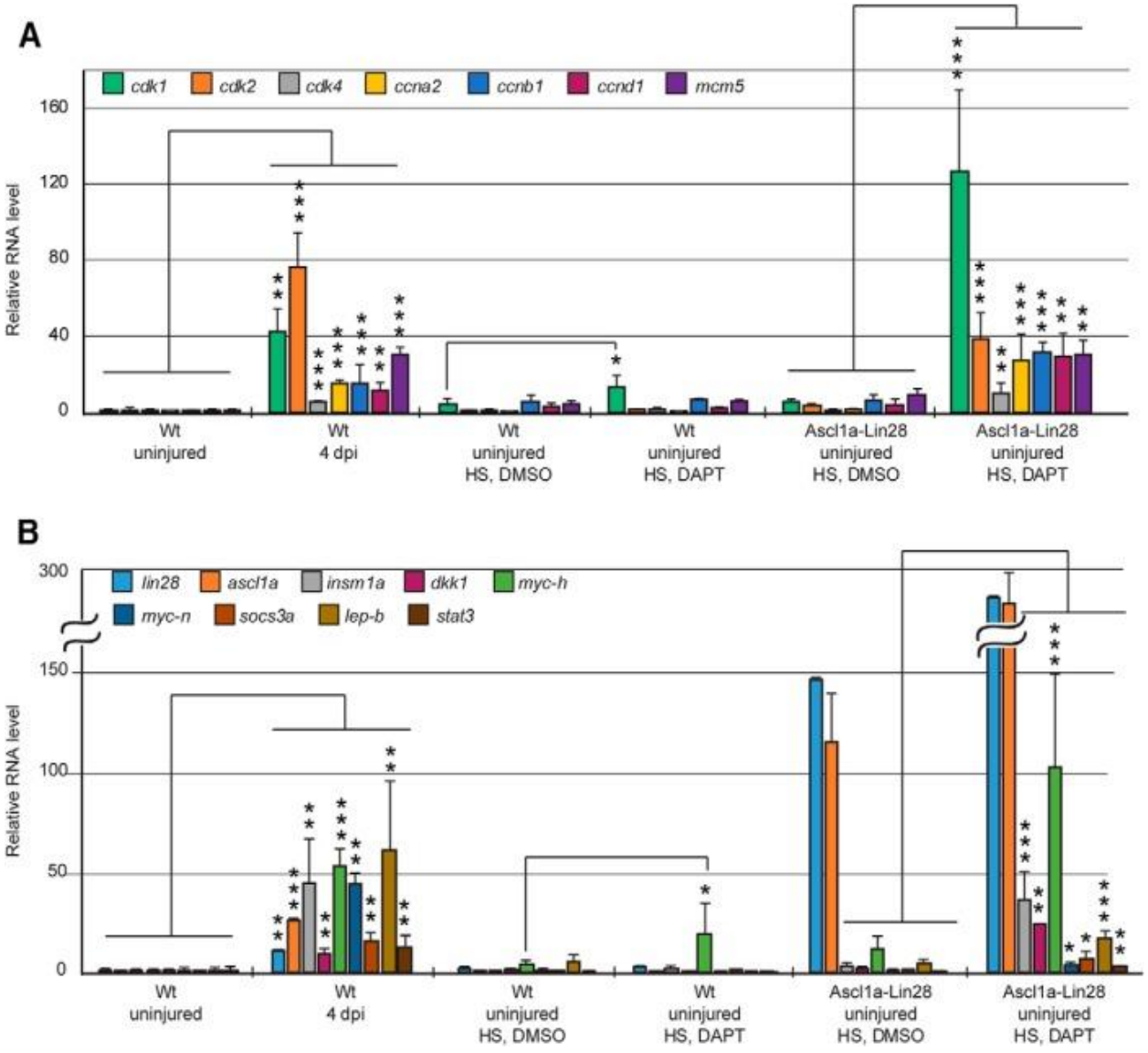
Zebrafish MG respond to retinal injury by acquiring characteristics of a retinal stem cell and this is associated with changes in gene expression (Kassen et al., 2007; Qin et al., 2009; Ramachandran et al., 2012; Sifuentes et al., 2016). These regeneration-associated gene expression changes include those that are associated with somatic cell reprogramming, cell-cycle regulation and other events that are associated with the response of MG to an injured retinal environment (Kassen et al., 2008; Ramachandran et al., 2010b, 2012; Thomas et al., 2016). We wondered whether gene expression programs driving MG proliferation in the injured retina were shared with those driving MG proliferation in the uninjured, DAPT/Ascl1a/Lin28a-treated retina. For this analysis we quantified the expression of select injury-responsive genes whose induction is necessary for MG proliferation in the injured retina (Fig. 2.5). Interestingly, except for *cdk1* and *mych*, all of these regeneration-associated genes retained basal expression levels after either DAPT-treatment or forced expression of Ascl1 and Lin28a; however, when these treatments were combined, a dramatic increase in the expression of these genes was noted (Fig. 2.5). Together with previous studies (Wan et al., 2012; Wan and Goldman, 2017), our data suggest that Notch signaling drives a quiescence program that impinges on regeneration-associated genes and that Notch signaling must be relieved in order for MG to proliferate. Furthermore, neither Notch inhibition alone nor the activation of regeneration-associated genes, like *ascl1a* and *lin28a*, is sufficient to activate a program of gene expression that drives MG proliferation; rather Notch inhibition must be combined with injury-related factors, like Ascl1a and Lin28a, to fully activate a regeneration-associated gene expression program.

Ascl1 and Lin28a synergize to stimulate MG proliferation in the uninjured mouse retina

Because forced Ascl1a and Lin28a expression in the uninjured fish retina was sufficient to drive a small amount of MG proliferation (Fig. 2.1*G*, *H*), we wondered whether they would have a similar effect in mice where MG proliferation and retina regeneration does not occur. Before investigating the consequence of Ascl1 and Lin28a on MG proliferation in the mouse retina, we examined whether *Ascl1* and *Lin28a* RNAs are expressed in the uninjured and injured retina. For this analysis, retinas were injured by intravitreal injection of NMDA, which predominantly ablates RGCs (Fig. 2.6*A*). Unlike their injury-dependent induction in the fish retina, *Ascl1* and *Lin28a* RNAs remain undetectable in the uninjured and injured mouse retina (Fig. 2.6*B*). However, *Ascl1* and

Lin28a were detected in P0 retina and brain, respectively, which served as positive controls (Fig. 2.6*B*). To investigate whether Ascl1 and Lin28a could stimulate MG proliferation in the mouse retina, we took advantage of AAV ShH10 to deliver genes encoding GFP, Ascl1 and/or Lin28a to MG (Klimczak et al., 2009; Byrne et al., 2013). Intravitreal injection of AAV ShH10-GFP efficiently delivered the transgene constructs to MG (Fig. 2.6*C*, *D*) and PCR confirmed expression of *Ascl1* and *Lin28a* RNAs (Fig. 2.6*E*). We determined that AAV ShH10-Ascl1 and AAV ShH10-Lin28a produced functional proteins by infecting HEK 293 cells and assaying their effects on a transfected *4RTK*-

Figure 2.5: Ascl1a and Lin28a expression synergize with Notch signaling inhibition to stimulate regeneration-associated gene expression
A, ***B***, qPCR quantification of cell-cycle related genes (***A***) and reprogramming-associated genes (***B***) in Wt and *hsp70:ascl1a;hsp70:lin28a* fish retinas treated as indicated. HS, Heat shock. *n* = 3 different experiments. Error bars are SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.



Luc reporter construct (Weintraub et al., 1990), and on endogenous *let7* microRNA levels (Rybak et al., 2008; Heo et al., 2009), respectively (Fig. 2.6*F*,*G*). Infection of HEK293 cells with AAV ShH10-Ascl1 resulted in ~25-fold increase in *4RTK-Luc* reporter activity (Fig. 2.6*F*), whereas infection with AAV ShH10-Lin28a reduced *let7g* RNA levels by >60% (Fig. 2.6*G*).

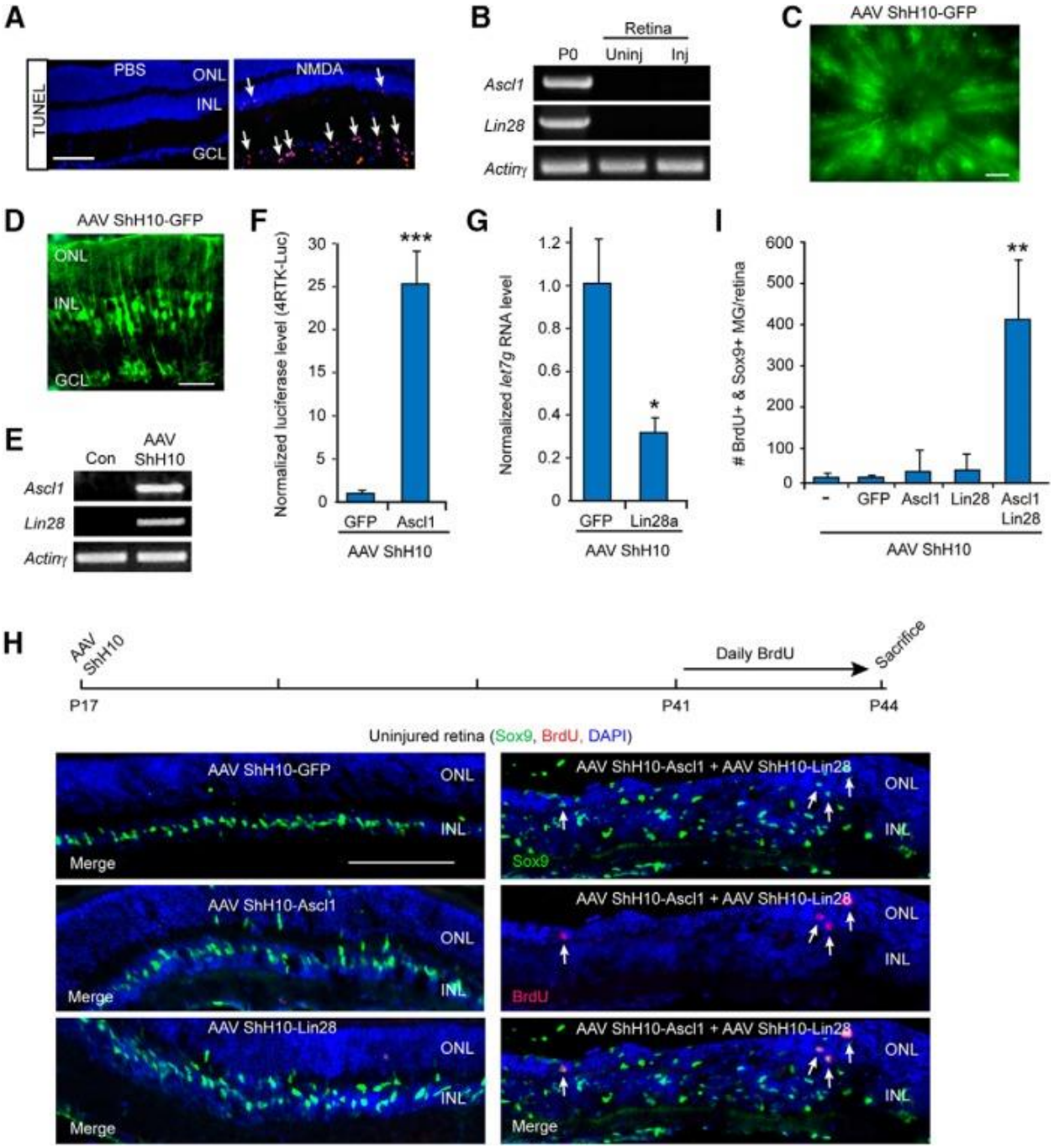


Figure 2.6: Ascl1 and Lin28a expression synergize with each other to stimulate MG proliferation in the uninjured mouse retina
A, TUNEL stain (red) shows NMDA stimulates cell death in the GCL. Scale bar, 100 µm. ***B***, PCR shows Ascl1 and Lin28a are detectable in the P0 retina and brain, respectively, but not in the uninjured or NMDA damaged (Inj) adult retina. ***C***, GFP fluorescence in flat mount retina shows AAV ShH10-GFP expression throughout the uninjured retina. Scale bar, 100 µm. ***D***, GFP immunofluorescence on retinal sections shows AAV ShH10-GFP expression in MG of the uninjured retina. Scale bar, 50 µm. ***E***, PCR shows Ascl1 and Lin28a expression in retinas transduced with AAV ShH10-Ascl1 and AAV ShH10-Lin28a. ***F***, Luciferase assays show HEK293 cells transfected with 4RTK-Luc and transduced with AAV ShH10-Ascl1 result in increased expression of the 4RTK-Luc reporter indicating expression of a functional Ascl1 protein; $n = 3$ different experiments. Error bars are SD. ***$p < 0.001$. ***G***, Transduction of HEK293 cells with AAV ShH10-Lin28a result in reduced *let7g* expression consistent with expression of a functional Lin28a protein; $n = 3$ different experiments. Error bars are SD. *$p < 0.05$. ***H***, Timeline of experiment and representative images of AAV ShH10-Ascl1 and AAV ShH10-Lin28a infected retinas showing Ascl1 and Lin28a synergize to stimulate some MG proliferation (Sox9+/BrdU). Sox9+ MG are labeled green and proliferating BrdU+ cells are labeled red. DAPI labels cell nuclei blue. Scale bar, 100 µm. ***I***, Quantification of data shown in ***H*** shows Ascl1 and Lin28a synergize to stimulate MG proliferation in the uninjured retina; $n = 4$ different experiments. Error bars are SD. **$p < 0.01$.

To investigate whether forced expression of Ascl1 and Lin28a could stimulate MG proliferation in the uninjured adult mouse retina, we injected AAV ShH10-GFP, AAV ShH10-Ascl1, AAV ShH10-Lin28a, or a combination of these viruses into the vitreous of P17 mouse eyes. Three and one-half weeks later, mice received daily injections of BrdU for 4 d before kill. Retinal sections were then prepared and BrdU and Sox9 immunofluorescence was used to assay cell proliferation and identify MG, respectively. Although overexpression of Ascl1 or Lin28a, individually, had no effect on MG proliferation (Fig. 2.6*H*, *I*), they did result in a small amount of MG delamination (Fig. 2.6*H*). In contrast, we noted an increase in MG proliferation and delamination when Ascl1 and Lin28a were coexpressed (Fig. 2.6*H*, *I*). Quantification of BrdU+/Sox9+ MG suggests this proliferation represents <1% of the total Sox9+ MG population (~ 170,000/retina). Nonetheless, these data suggest that similar to their action in the fish retina, Ascl1 and Lin28a synergize with each other to stimulate a small amount of MG proliferation in the uninjured mouse retina.

Ascl1-dependent MG proliferation in the injured mouse retina is enhanced by Lin28a expression

Ascl1a and Lin28a collaborate with injury-derived factors to stimulate MG proliferation in the injured fish retina (Fig. 2.2*A*–*C*). To investigate whether Ascl1 and Lin28a had a similar effect in the injured mouse retina, we infected retinas with AAV ShH10-Ascl1 and/or AAV ShH10-Lin28a at P17; damaged retinas with NMDA at P41; and then gave daily BrdU injections for 4 d to label proliferating cells (Fig. 2.7*A*). Consistent with a previous report (Ueki et al., 2015), Ascl1 expression in the NMDA damaged retina was sufficient to stimulate a small amount of MG proliferation (Fig. 2.7*B*, *C*). Interestingly, this proliferation was further enhanced by Lin28a coexpression (Fig. 2.7*B*, *C*). The effect of Ascl1 and Lin28a on MG proliferation in the injured retina was >10-fold more than that observed in the uninjured retina, suggesting interaction with injury-responsive factors similar to what we noted in the injured fish retina.

We used GFP and mCherry tagged versions of Ascl1 and Lin28a, respectively, to determine whether proliferating cells harbored virally transduced genes (Fig. 2.7*D*). Because AAV ShH10-Ascl1 is sufficient to stimulate MG proliferation (Fig. 2.7*C*), we quantified the number of BrdU+ MG infected with virus. Interestingly, we found that only ~47% of the BrdU+ MG was associated with viral-mediated transgene expression. This might suggest that Ascl1 can act in a cell nonautonomous fashion or, alternatively, may reflect our limits in detecting virus encoded transgenes. Finally, we note that although a previous study indicated that Lin28a expression was sufficient to drive MG proliferation (Yao et al., 2016), in our hands Lin28a infection alone had no discernable effect on MG

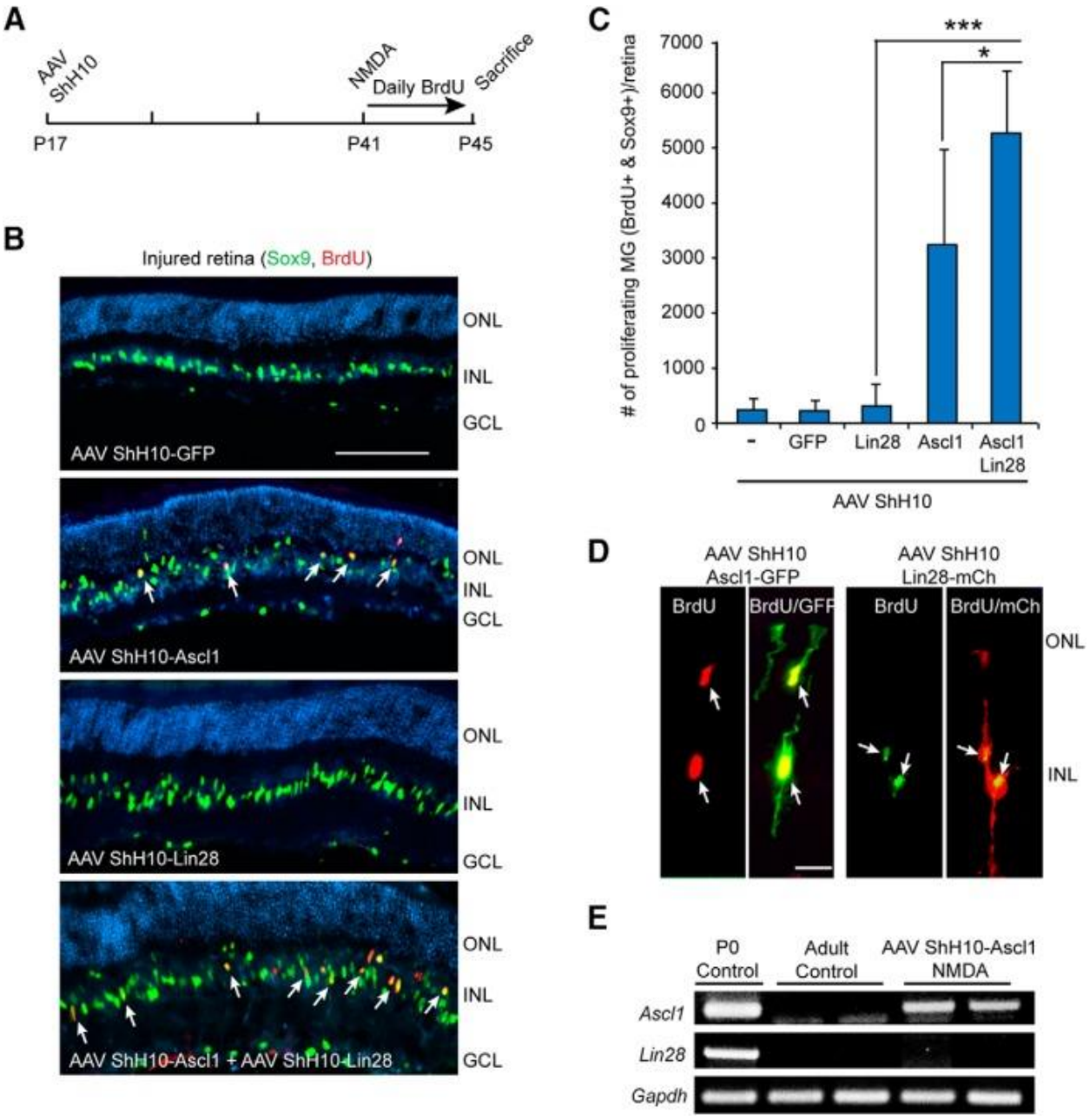


Figure 2.7: Ascl1-dependent MG proliferation in the injured mouse retina is enhanced by Lin28a expression
A, Experimental timeline. ***B***, Sox9 and BrdU immunofluorescence on retinal sections from injured retinas shows forced expression of Ascl1 stimulates a small amount of MG proliferation that is enhanced when combined with Lin28a expression injured retina. Scale bar, 100 μm. ***C***, Quantification of proliferating MG in injured retinas that were infected with the indicated AAV ShH10 virus; *n* = 5 different experiments. Error bars are SD. **p* < 0.05, ****p* < 0.001. ***D***, P17 mouse retinas were infected with either AAV ShH10-Ascl1-GFP or AAV ShH10-Lin28a-mCherry and then treated with NMDA and BrdU, and killed as indicated in ***A***. BrdU/GFP or BrdU/mCherry immunofluorescence on retinal section showed BrdU+ MG expressed Ascl1-GFP or Lin28a-mCherry (Lin28a-mCh), respectively. Scale bar, 100 μm. ***E***, PCR shows retinas infected with AAV ShH10-Ascl1 express Ascl1, but do not activate the endogenous Lin28a gene.

proliferation (Fig. 2.6*H*, *I*). The reason for this difference is not known, but may reflect differences in Lin28a expression levels resulting from the use of different gene promoters.

Because Ascl1 and Lin28a synergize to stimulate MG proliferation in the uninjured fish and mouse retina, we went on to examine whether the effect of Ascl1 expression on MG proliferation in the injured mouse retina resulted from induction of the endogenous *Lin28a* gene. To investigate this possibility, retinas were transduced by intravitreal AAV ShH10-Ascl1 and 3.5 weeks later mice received intravitreal injections of NMDA. Retinas were harvested 4 d later and *Ascl1* and *Lin28a* gene expression assayed by PCR. This analysis indicated Ascl1 expression does not stimulate widespread *Lin28a* expression in the injured mouse retina (Fig. 2.7*E*). We note that two Ascl1 consensus binding sites exist upstream of the mouse *Lin28a* gene's transcription start site, but their functional significance remains unexplored. Thus, consistent with a previous report (Ueki et al., 2015), we found that forced expression of Ascl1 is sufficient to stimulate MG proliferation in the injured mouse retina; however, our data suggest that this can be enhanced by combining Ascl1 with Lin28a expression.

Ascl1 and Lin28a enhance survival of proliferating MG and allow reprogramming to multipotency

Our data indicated that Lin28a expression can enhance Ascl1-dependent MG proliferation. We next investigated whether the survival of MG stimulated to proliferate by Ascl1 expression was altered by Lin28a expression. For this analysis, P17 mice received an intravitreal injection of AAV ShH10-Ascl1 or a mixture of AAV ShH10-Ascl1 and AAV ShH10-Lin28a. At P41, mice received an intravitreal injection of NMDA and BrdU followed by daily intraperitoneal injections of BrdU for 4 d; mice were then killed at either P45 or P59 (Fig. 2.8*A*). Interestingly, when we compared the number of double-labeled BrdU+/Sox9+ MG cells at P45 with those at P59 we found many more persisted when expressing both Ascl1 and Lin28a compared with those expressing Ascl1 alone (Fig. 2.8*B*). However, the fact that most BrdU+/Sox9+ cells remained Sox9+ two weeks after BrdU labeling and were concentrated in the INL suggested that these cells retain MG characteristics (Fig. 2.8*C*). Quantification showed that these cells represent ~76% of the total BrdU population in the INL and ONL (Fig. 2.8*C*, *D*). Although many BrdU+/Sox9− cells were located on the vitreal side of the GCL and expressed the microglia marker 4C4 (Fig. 2.8*C*), only ~16% of the BrdU+ cells in the INL and ONL

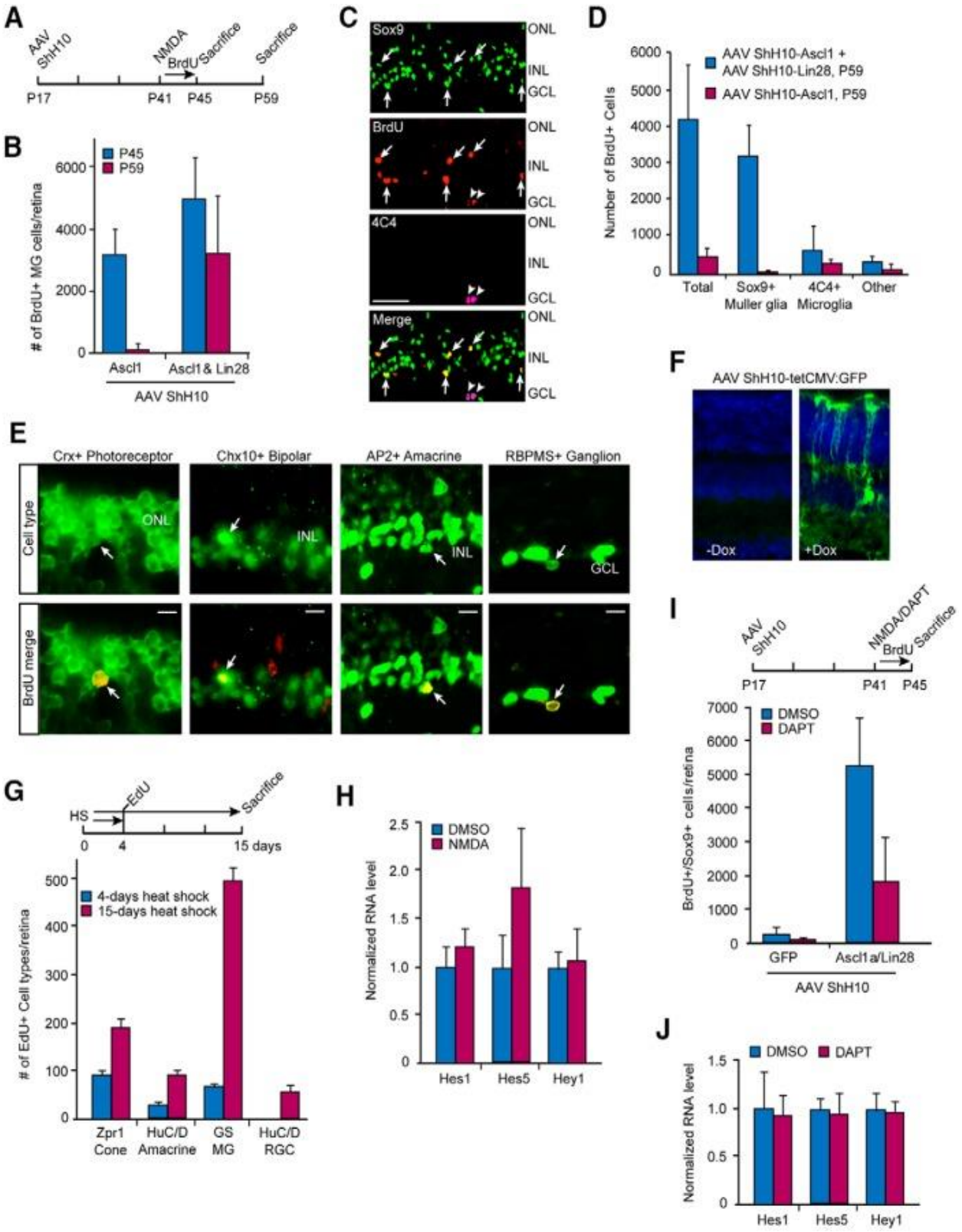
were 4C4+ microglia (Fig. 2.8*D*). Of the remaining (~8%) BrdU+ cells in these retinal layers (Fig. 2.8*D*, “other”), co-immunofluorescence showed some expressed markers for photoreceptors (Crx), amacrine cells (AP2), bipolar cells (Chx10), and RGCs (RBPMS; Fig. 2.8*E*).

Effects of transient versus sustained Ascl1 and Lin28a expression in fish and mice

Our data suggested that constitutive expression of Ascl1 and Lin28a in mice stimulates MG proliferation in the injured retina, but only a very small fraction of these proliferating cells differentiate into retinal neurons (Fig. 2.8*D*, *E*). During development Ascl1 and Lin28a are transiently expressed in stem-cell populations and suppressed as differentiation proceeds (Jasoni and Reh, 1996; Xu et al., 2009). To investigate whether transient expression of Ascl1 and Lin28a facilitate differentiation of MG-derived progenitors, we generated AAV ShH10-tetCMV expression vectors driving Ascl1, Lin28a, and GFP using an optimized Tet-On system (V10; Zhou et al., 2006). These vectors harbor a minimal CMV promoter controlled by rtTA and allow for conditional transgene expression. A mixture of these viruses was injected into the eye's vitreous of P17 mice. At P41, mice received an intravitreal injection of doxycycline (2 µl of 4 µg/µl) 1 d before the standard NMDA injury. A second intravitreal injection of doxycycline was performed 2 d after the NMDA treatment. For these experiments EdU was substituted for BrdU and lineage tracing experiments were performed as described above with progenitor differentiation assayed on P59. Although we confirmed doxycycline-dependent transgene induction *in vivo* in MG (Fig. 2.8*F*), we did not observe any significant effect on progenitor differentiation.

Figure 2.8: Ascl1 and Lin28a expression enhances survival of proliferating MG and regenerate multiple neuron types in the injured retina

A, Experimental timeline. ***B***, Lin28a expression when combined with Ascl1 expression enhances survival of proliferating MG in the injured retina compared with Ascl1 expression alone. Sox9 and BrdU immunofluorescence on retinal sections were used to quantify MG proliferation; *n* = 5 different experiments. Error bars are SD. ***C***, Representative images of lineage traced BrdU+ cells in the injured retina suggests that most proliferating cells in the Ascl1- and Lin28a-expressing retina are Sox9+ MG (arrows). Arrowheads indicate 4C4+ microglia. Scale bar, 50 µm. ***D***, Quantification of data shown in ***C***; *n* = 5 different experiments. Error bars are SD. ***E***, Lineage tracing experiments were performed as indicated in ***A*** and BrdU+ cells (red) with cell-type-specific markers (green) of photoreceptors (Crx), bipolar (Chx10), amacrine (AP2), and ganglion cells (RBPMS) are indicated by arrows. ***F***, Dox-dependent expression of GFP in eyes that received intravitreal injection of a mixture of AAV ShH10-tetCMV:GFP, AAV ShH10-tetCMV:Ascl1, and AAV ShH10-tetCMV:Lin28a. ***G***, Timeline and quantification of lineage trace experiment in *hsp70:ascl1a;hsp70:lin28a* fish treated with heat shock for 4 or 15 d when using EdU labeling at 4 d post-heat shock. Retinal cell-type antibodies were used to quantify the number of Edu+ cells that costain with cone (Zpr1), MG (GS), amacrine cell (HuC/D in INL), and RGC (in RGC layer) antibodies; *n* = 3 different experiments. Error bars are SD. ***H***, NMDA has no effect on Notch-responsive genes in the uninjured retina; *n* = 3 different experiments. Error bars are SD. ***I***, DAPT-treatment inhibits MG proliferation in injured retinas expressing Ascl1 and Lin28a. Top illustration is timeline of experiment and graph quantifies the number of Sox9+ and BrdU+ double-labeled cells/retina; *n* = 5 different experiments. Error bars are SD. ***J***, DAPT treatment has no effect on Notch-responsive genes in the uninjured AAV ShH10 Ascl1 and AAV ShH10 Lin28a transduced retina. *n* = 3 different experiments. Error bars are SD.


A
AAV ShH10
NMDA
BrdU
Sacrifice
Sacrifice
P17
P41
P45
P59
B
P45
P59
# of BrdU+ MG cells/retina
6000
4000
2000
0
Ascl1
Ascl1& Lin28
AAV ShH10
C
Sox9
BrdU
4C4
Merge
ONL
INL
GCL
D
AAV ShH10-Ascl1 +
AAV ShH10-Lin28, P59
AAV ShH10-Ascl1, P59
Number of BrdU+ Cells
6000
5000
4000
3000
2000
1000
0
Total
Sox9+
Muller glia
4C4+
Microglia
Other
E
Crx+ Photoreceptor
Chx10+ Bipolar
AP2+ Amacrine
RBPMS+ Ganglion
Cell type
BrdU merge
ONL
INL
INL
GCL
F
AAV ShH10-tetCMV:GFP
-Dox
+Dox
G
HS
EdU
Sacrifice
0
4
15 days
# of EdU+ Cell types/retina
500
400
300
200
100
0
4-days heat shock
15-days heat shock
Zpr1
Cone
HuC/D
Amacrine
GS
MG
HuC/D
RGC
H
DMSO
NMDA
Normalized RNA level
2.5
2.0
1.5
1.0
0.5
0
Hes1
Hes5
Hey1
I
AAV
ShH10
NMDA/DAPT
BrdU
Sacrifice
P17
P41
P45
DMSO
DAPT
BrdU+/Sox9+ cells/retina
7000
6000
5000
4000
3000
2000
1000
0
GFP
Ascl1a/Lin28
AAV ShH10
J
DMSO
DAPT
Normalized RNA level
1.5
1.0
0.5
0
Hes1
Hes5
Hey1

We next compared the effects of transient (4 d) with sustained (15 d) Ascl1a and Lin28a expression on progenitor differentiation in fish. For this analysis, we exposed fish to either a 4 or 15 d heat shock paradigm with an intraperitoneal injection of EdU at day 4 and then killed 11 d later (Fig. 2.8*G*). This experiment revealed more differentiated progenitors with sustained Ascl1a and Lin28a expression at 15 d of heat shock. Analysis of the fraction of proliferating MG that retain MG characteristics (GS+), indicate ~59% with sustained heat shock and ~34% with transient heat shock, which is consistent with the idea that sustained Ascl1a and Lin28a expression inhibited progenitor differentiation. Nonetheless, this was a relatively small effect and it did not reduce the amount of progenitor differentiation to the very low levels observed in mice. This suggests that additional factors will be required for enhancing MG proliferation and differentiation in mice.

Ascl1 and Lin28a-dependent MG proliferation and differentiation is not enhanced by Notch inhibition in the mouse retina

Our data suggest that forced expression of Ascl1 and Lin28a can stimulate a small amount of MG proliferation in the fish and mouse retina (Figs. 2.1*G*, *H*, ,77*B*, *C*; ;88*D*, *E*). In fish, this proliferation can be greatly enhanced by Notch signaling inhibition (Fig. 2.4). Therefore, we investigated whether Notch inhibition in the mouse retina would also enhance Ascl1 and Lin28a-dependent MG proliferation. Notch signaling is low, but detectable in MG residing in the p21 mouse retina (Nelson et al., 2011; Riesenberg et al., 2018). To determine whether this residual Notch signaling was suppressed following retinal injury, we assayed the expression of Notch target genes, *Hes1*, *Hes5*, and *Hey1*, and found no significant change following NMDA-induced retinal damage (Fig. 2.8*H*). We next investigated whether the residual Notch signaling observed in adult mouse retinas influenced Ascl1/Lin28a-induced proliferation after injury. For this analysis, P17 Wt mice received intravitreal injections of AAV ShH10-Ascl1 and AAV ShH10-Lin28a. At P41 mice received BrdU, ±NMDA as described in the previous section; however, mice also received intravitreal injections of DAPT or vehicle for the 4 d following NMDA treatment (Fig. 2.8*I*). Retinal sections were then assayed for BrdU and Sox9 co-immunofluorescence. This analysis showed that although DAPT had no effect on MG proliferation in the uninjured retina (data not shown), it suppressed MG proliferation in the injured retina (Fig. 2.8*I*). However, this effect was not correlated with changes in the expression of Notch reporter genes *Hes1*, *Hes5*, and *Hey1* (Fig. 2.8*J*). Thus, Notch reporter gene expression may not reflect small changes in Notch signaling in the adult mouse retina where Notch signaling is already at a low level (Nelson et al., 2011; Riesenberg et al., 2018). Furthermore, we cannot rule out the possibility that the anti-proliferative effect of DAPT may be a consequence of inhibiting a γ-secretase unrelated to Notch signaling.

During retina development Notch signaling is increased in progenitors that acquire a MG fate (Furukawa et al., 2000). We wondered whether the reduced number of BrdU+ MG noted following retinal damage and DAPT treatment (Fig. 2.8*I*) reflected a loss of glial identity and enhanced neuronal differentiation. For this analysis, we used DNMAML mice that allow for Cre-mediated expression of a dominant-negative mastermind-like

protein and should allow us to maintain Notch inhibition over prolonged periods (Maillard et al., 2006). DNMAML mice were treated as described in Figure 2.8*A* except that at P27 the left eye received an intravitreal injection of AAV ShH10-Cre, whereas the contralateral control eye received a PBS vehicle injection. At P55, mice were killed and retinas sectioned and immunofluorescence used to assay for BrdU+ cells that coexpress retinal neuron-specific markers. This analysis revealed no significant effect of DNMAML on MG-derived progenitor differentiation. Altogether, our studies suggest Notch signaling inhibitors have little effect on neural differentiation of MG-derived progenitors in the adult mouse retina.

2.4 Discussion

Fish and amphibians have remarkable regenerative powers that provide us with an opportunity to understand this process at the cellular and molecular level. In the zebrafish retina, MG are a source of retinal progenitors used to repair a damaged retina (Goldman, 2014; Wan and Goldman, 2016). It is anticipated that identification of the molecular strategies underlying MG reprogramming and the acquisition of stem-cell properties may suggest strategies for stimulating these events in mammals. Here we report that forced expression of Ascl1a and Lin28a can stimulate sparse MG proliferation and retinal neuron regeneration in both fish and mice. Interestingly, when this expression was combined with Notch signaling inhibition, only in the fish retina did we observe a large increase in MG proliferation.

Ascl1a and Lin28a are potent regulators of zebrafish retina regeneration and appear to coordinate the expression of many genes critical for MG reprogramming and proliferation (Fausett et al., 2008; Ramachandran et al., 2010b, 2011, 2012; Nelson et al., 2012, 2013; Wan et al., 2012, 2014; Gorsuch et al., 2017). Ascl1 and Lin28a are also mammalian reprogramming factors that can convert fibroblasts and astrocytes to neurons and participate in converting somatic cells to pluripotent stem cells, respectively (Yu et al., 2007; Chanda et al., 2014; Liu et al., 2015). Despite these characteristics, forced expression of Ascl1a and Lin28a did not lead to widespread MG proliferation in the uninjured fish retina. We wondered whether this lack of proliferation resulted from antiproliferative signals that MG receive in the uninjured retinal environment. A candidate signaling pathway that might convey these anti-proliferative signals to the MG genome was Notch signaling, which is restricted to quiescent MG and must be suppressed in order for MG proliferation to ensue (Wan et al., 2012; Conner et al., 2014; Wan and Goldman, 2017). Indeed, when Ascl1a and Lin28a expression was combined with Notch inhibition, widespread MG proliferation was noted.

The observation that Ascl1a and Lin28a expression and Notch inhibition together, but not individually, stimulated MG proliferation suggested they act in parallel pathways that converge on genes that drive MG proliferation and retina regeneration. Although Notch signaling generally leads to gene activation, we recently reported that forced activation of Notch signaling in the injured retina represses genes associated with MG reprogramming and proliferation (Wan and Goldman, 2017). This repression might be mediated by Notch-target genes, like the *hes/her/hey* gene family of repressors which

are known to regulate neural stem-cell maintenance (Kageyama et al., 2007; Imayoshi et al., 2010; Imayoshi and Kageyama, 2011). Indeed, we found that *hey1* and *her4.1* gene expression can be activated by Notch signaling in fish MG (Wan et al., 2012; Wan and Goldman, 2017). Furthermore, Hey1 expression has been associated with gliogenesis and inhibition of Ascl1 expression in the mammalian brain (Sakamoto et al., 2003). Thus, our studies suggest that individually, Notch inhibition and Ascl1/Lin28a expression contribute to different aspects of MG activation that must be combined to fully unleash a regeneration-associated gene expression program. Identifying the genetic underpinnings of these activated states may be crucial for devising strategies to stimulate a robust regenerative response in mammals.

Although spontaneous MG proliferation is very low in the uninjured fish retina, it does occur, and is responsible for generating rod progenitors that help maintain a constant rod density as the retina expands throughout the fish's life (Johns and Fernald, 1981; Otteson and Hitchcock, 2003; Bernardos et al., 2007). Our studies suggest that enhancing spontaneous MG proliferation with forced Ascl1a and Lin28a expression stimulates the formation of multipotent progenitors. Although it is not known whether MG that generate unipotent rod progenitors are different from those that make multipotent progenitors, it is noteworthy that we recently identified a heterogeneous population of MG in the zebrafish retina (Wan and Goldman, 2017). The differences in MG responsiveness to forced expression of Ascl1a and Lin28a in the uninjured and injured retina may also reflect MG heterogeneity.

Based on the robust effect that Notch signaling inhibition combined with forced Ascl1a and Lin28a expression had on MG proliferation in the uninjured fish retina, we investigated whether they would have a similar consequence on MG proliferation and neuron regeneration in the mouse where retina regeneration does not normally occur. Similar to the observation that Ascl1 expression can stimulate a small amount of MG proliferation in the injured postnatal mouse retina (Ueki et al., 2015), we found Ascl1-dependent MG proliferation in the injured adult retina. Interestingly, this was independent of Lin28a induction. However, our studies suggested that in the absence of Lin28a, most of these cells do not persist. We did not observe an effect of Lin28a on MG proliferation in the injured mouse retina and this is consistent with what we observed in fish; however, our results are different from a recent study in mice where Lin28a was reported to stimulate MG proliferation (Yao et al., 2016). The reason for this difference is not known, but may be related to the level of Lin28a expression because different promoters were used in these two studies. Nonetheless, like that observed in fish, combined expression of Ascl1 and Lin28a in the mouse retina was sufficient to stimulate a small amount of MG proliferation in the uninjured retina that was enhanced by retinal injury. These similarities in response to Ascl1 and Lin28a are encouraging; however, differences between fish and mice were also noted. First, in the injured mouse retina, Ascl1 stimulated a small amount of MG proliferation, whereas no enhancement of MG proliferation was noted in fish. Second, Lin28a enhanced survival of proliferating MG in mice, whereas it collaborated with Ascl1a to stimulate MG proliferation in fish. Third, Ascl1 and Lin28a-dependent MG proliferation in mice only rarely generated neurons, whereas in fish, neuronal regeneration was more prevalent. Finally, the low

levels of basal Notch signaling in the adult mouse retina appears somewhat resistant to pharmacological or genetic means of reduction, which was indicated by Notch-responsive genes. However, it is possible that these genes remain elevated by intrinsic regulators, like Lhx2 (de Melo et al., 2016a,b). Regardless, attempts to reduce Notch signaling did not enhance Ascl1 and Lin28a-dependent MG proliferation in the uninjured or injured mouse retina; however, in the uninjured fish retina, Notch signaling is relatively high in MG and its inhibition, when combined with Ascl1a and Lin28a expression, dramatically stimulated widespread MG proliferation. This latter effect seems to be a major difference between fish and mice and understanding its roots may help devise strategies for recruiting MG to a regenerative response in mammals.

In addition to generating retinal progenitors through cell division, MG can transdifferentiate into bipolar cells after overexpression of Ascl1 and treatment with the histone deacetylase inhibitor trichostatin-A (TSA; Jorstad et al., 2017). This transdifferentiation process occurs in adult mice and is not dependent on MG proliferation. Although the consequences of reducing the MG population by transdifferentiation is not known, it may have a detrimental effect on retinal structure and function. Regardless, because Lin28a impacts the survival of MG stimulated to proliferate by Ascl1 in the injured retina, it would be interesting to investigate whether Lin28a also affects MG transdifferentiation. Importantly, the combinatorial action of Ascl1 and TSA on MG reprogramming for transdifferentiation highlights the impact chromatin modifying agents can have on stimulating gene expression programs normally suppressed in a differentiated cell type. The potential for these agents to help reprogram MG to generate progenitors for retinal repair is intriguing and is an area for future studies.

In summary, our research identified key components underlying zebrafish retina regeneration that are sufficient to stimulate this process in the uninjured fish retina. Some of these components, like Ascl1 and Lin28a, were also able to stimulate a small amount of MG proliferation and neuron regeneration in mice. Remarkably, Notch signaling inhibition synergizes with Ascl1a and Lin28a expression to stimulate widespread MG proliferation in fish, but not mice. This lack of synergy in mice may reflect our inability to inhibit basal levels of Notch signaling, which are already very low. Although sustained expression of NICD can inhibit MG proliferation (Wan et al., 2012), we recently reported that the cessation of Notch signaling following forced and transient NICD expression stimulates MG proliferation in the injured fish retina (Wan and Goldman, 2017). It is tempting to speculate that cessation of Notch signaling may also be critical for stimulating MG proliferation in the injured mammalian retina; perhaps one will need to enhance Notch signaling in mammals to see an effect of its cessation. Furthermore, Notch signaling may regulate different genes in fish and mice, and identification of these differences may suggest additional strategies for enhancing mammalian MG proliferation. Finally, many gene products and signaling molecules have been found to regulate retina regeneration in zebrafish (Goldman, 2014; Wan and Goldman, 2016) and these provide a rich resource for testing in mammals. It is anticipated that some of these molecules will collaborate with Ascl1 and Lin28a to stimulate retina regeneration in mammals.

2.5 Experimental Procedures

Animals

Animal studies were approved by the University of Michigan's Institutional Animal Care and Use Committee. Zebrafish were kept at 26–28°C with a 10/14 h light/dark cycle. Adult male and female fish from 6 to 12 months of age were used in these studies. *1016 tuba1a:GFP*, *gfap:GFP*, and *tp1:mCherry* fish were previously described (Fausett and Goldman, 2006; Kassen et al., 2007; Parsons et al., 2009; Kwon et al., 2010). We generated *1016 tuba1a:GFP;hsp70:flag-myc-ascl1a* (referred to as *hsp70:ascl1a*) and *1016 tuba1a:GFP;hsp70:myc-lin28a* (referred to as *hsp70:lin28a*) transgenic fish. The *1016 tuba1a:GFP* expression cassette was used to identify fish harboring the various *ascl1a* and *lin28a* transgenes and the flag and myc tags were used to ensure protein expression after heat shock. The *hsp70:ascl1a* and *hsp70:lin28a* transgenic lines were created using standard recombinant DNA techniques using Tol2 vector backbone. Expression constructs were injected into single-cell zebrafish embryos as previously described (Fausett and Goldman, 2006). Retinas were injured with a needle poke injury as previously described (Fausett and Goldman, 2006; Montgomery et al., 2010; Powell et al., 2016). Wild-type C57BL/6J mice were obtained from our breeding colony. DNMAML mice harbor amino acids 13–74 of MAML1 fused to GFP (Maillard et al., 2006).

AAV, intravitreal injection, retinal injury, and BrdU/EdU labeling

A Müller cell-specific AAV capsid variant, ShH10 was used to deliver genes to MG (Klimczak et al., 2009). GFP, mouse Ascl1, human Lin28a, and Cre expression cassettes, under control of the CAG, CMV, or TetCMV promoters were individually packaged into ShH10 AAV vectors. Recombinant ShH10 AAV was prepared as previously described (Klimczak et al., 2009; Flannery and Visel, 2013). Viral titers were between ~0.5×10^{13} to 5×10^{13} vg/ml and ~1 µl was intravitreally injected into isoflurane anesthetized postnatal day (P)17 mice using a Hamilton syringe equipped with a 33-gauge needle. Adult (P41) mice were anesthetized with isoflurane and received ~2 µl intravitreal injections of PBS containing 1 mg/ml BrdU or EdU, ±100 mm NMDA using a Hamilton syringe equipped with a 33-gauge needle. Mice received a second intravitreal injection of BrdU/EdU 3 d later; mice also received daily intraperitoneal injections of BrdU/EdU (50 µg/g body weight) for 3 d following NMDA treatment.

Fish were anesthetized in tricaine and retinas were injured with a needle poke injury as previously described (Fausett and Goldman, 2006). Fish received an intraperitoneal injection of BrdU (20 µl of 20 mm stock) at 4 d postinjury (dpi). For growth factor treatment, fish were anesthetized with tricaine and the left eye (control) was intravitreally injected with ~1 µl of vehicle (PBS, 0.1% BSA) and the right eye was injected with ~1 µl of HB-EGF (50 ng/µl; R&D Systems) or insulin (500 ng/µl; Invitrogen), pH7.6, using a Hamilton syringe equipped with a 33-gauge needle as previously described (Wan et al., 2014). Recombinant proteins were injected once daily for 3 d,

and 4 d after the first injection fish received an intraperitoneal injection of BrdU (20 µl of 20 mm stock).

RNA isolation and PCR

Total RNA was isolated using Trizol (Invitrogen). cDNA synthesis and PCRs were performed as previously described (Fausett et al., 2008; Ramachandran et al., 2010b). Real-time qPCRs were performed in triplicate with ABsolute SYBR Green Fluorescein Master Mix (Thermo Scientific) on an iCycler real-time PCR detection system (Bio-Rad). The ΔΔ*Ct* method was used to determine relative expression of mRNAs in control and injured retinas and normalized to *gapdh* or γ-*actin* mRNA levels. To assay *let7g* miRNA, poly(A) tails were first added to total RNA (Ambion) and then samples were reverse transcribed with Superscript II and a poly(T) adapter. Following cDNA synthesis real-time qPCR was performed as described above. Individual comparisons were done using unpaired two-tailed Student's *t* test. ANOVA with Fisher's PLSD *post hoc* analysis was used for multiple-parameter comparison. Error bars are SD.

Western blots

Samples were boiled in SDS sample loading buffer and fractionated on 10% SDS-polyacrylamide gels. Samples were then transferred to nitrocellulose membranes, blocked with 5% nonfat dry milk, and incubated in primary antibodies mouse anti-myc (1:2000; Sigma-Aldrich, catalog #M4439) or mouse anti-flag (1:2000; Sigma-Aldrich, catalog #F1804) at 4°C overnight. Membranes were then washed in 0.1% Triton-X in PBS three times and incubated with horseradish peroxidase-conjugated secondary antibody goat anti-mouse IgG (1:10,000; Rockland, catalog #610-1302) in 5% nonfat dry milk for 2 h at room temperature and visualized using an ECL kit (ThermoFisher). Western blots were quantified using NIH ImageJ software.

Primers used in this study

The following primers are 5′ to 3′. Unless otherwise indicated primers are for zebrafish.
ascl1a
Forward: ATTCCAGTCGGGCGTCCTGTCA
Reverse: CCTCCCAAGCGAGTGCTGATATTTT
cdk1
Forward: GCTTCACGCTATTCCACACC
Reverse: GCCAGATTCCCAGATTTCCAC
cdk2
Forward: GACTACAAACCCTCCTTTCCC
Reverse: AAACCGATGAACAAGAGCGT
cdk4
Forward: GCAGTATGAGCCAGTAGCAG
Reverse: ATGTTGGGATGGTCGAACTG
ccna2
Forward: ACGAGACTCTTTACCTGGCT

Reverse: GAGAGAACTGTCAGCACCAG
ccnb1
Forward: TGACATGGTCCACTACCCTC
Reverse: GATGCTTAGAAAGGCCCTCG
Ccnd
Forward: AAGTGGGATCTGGCCTCAGT
Reverse: GGCAACTGTCGGTGCTTTTC
dkk1b
Forward: AAGCACAAGAGGAAAGGCA
Reverse: TGGGAGCTGGTGAAAGAAA
Gapdh
Forward: ATGACCCCTCCAGCATGA
Reverse: GGCGGTGTAGGCATGAAC
insm1a
Forward: GCACCACAGTAACCACCAAA
Reverse: TGCACAGCTGACAGACGAAC
Lepb
Forward: TCCCCGTCACCTCCAACTAC
Reverse: TCCTTGCATGTGCCATTGTGT
lin28a
Forward: TAACGTGCGGATGGGCTTCGGATTTCTGTC
Reverse: ATTGGGTCCTCCACAGTTGAAGCATCGATC
mcm5
Forward: CCAGTAGGAGAAGAGACTGT
Reverse: TCTGCATCCGCGGCACTGAA
mych
Forward: CCCGACCGCTTAAAACTGGA
Reverse: CTCATCGTCAAACAGCAACGG
Mycn
Forward: CAGAACAGTCTTCAGTCGCC
Reverse: ATCCTCGTCCGGGTAGAAAC
socs3a
Forward: CACTAACTTCTCTAAAGCAGGG
Reverse: GGTCTTGAAGTGGTAAAACG
stat3
Forward: AGCAGCAAAGAGGGAGGAATCACA
Reverse: GTACAGGTAGACCAGCGGCGACAC
her4.1
Forward: GCTGATATCCTGGAGATGACG
Reverse: GACTGTGGGCTGGAGTGTGTT
Mouse-*Gapdh*
Forward: TCAACAGCAACTCCCACTCTTCCACCTTC
Reverse: ACCCTGTTGCTGTAGCCGTATTCA
Mouse γ-ctin
Forward: AGAAGAAATCGCCGCACTCGTCAT
Reverse: CCTCTTGCTCTGGGCCTCGTCAC

Mouse-*Ascl1a*
Forward: TCTCGTCCTACTCCTCCGAC
Reverse: ATTTGACGTCGTTGGCGAGA
Mouse-*Lin28a*
Forward: CCTTTGCCTCCGGACTTCTC
Reverse: AGGGCTGTGGATCTCTTCCT
Mouse-*Let7 g*
Forward: TGAGGTAGTAGTTTGTACAGTT
Reverse: GCGAGCACAGAATTAATACGACTCACTATAGG(T)12VN
Mouse-*Hes5*
Forward: TAATCGCCTCCAGAGCTCCA
Reverse: GCGAAGGCTTTGCTGTGTTT
Mouse-*Hes1*
Forward: ACACCGGACAAACCAAAGAC
Reverse: ATGCCGGGAGCTATCTTTCT
Mouse-*Hey1*
Forward: TGAGCTGAGAAGGCTGGTAC
Reverse: ACCCCAAACTCCGATAGTC.

Heat shock, Notch inhibition, and TUNEL

For heat shock, fish were immersed in a water bath at 37°C for 1 h before returning to system water at 28°C. For extended periods of heat shock, this was repeated every 6 h. To inhibit Notch signaling in fish, we immersed fish in water containing 40 µm DAPT (Sigma-Aldrich) or RO4929097 (Cayman) prepared in DMSO and diluted 1/200 in fish water. Control fish were immersed in fish water treated with DMSO (1:200). To inhibit Notch signaling in mice, we intravitreally injected DAPT (100 µm) daily into the eye of Wt mice for up to 4 d, or we injected shH10 AAV Cre into the eye's vitreous of DNMAML-GFP mice 1–2 weeks before NMDA injury and/or analysis.

We used an *in situ* Cell Death Detection Kit (TMR red; Applied Science) to detect cells undergoing apoptosis.

Immunohistochemistry, *in situ* hybridization, and microscopy

Zebrafish samples were prepared for immunofluorescence as previously described (Fausett and Goldman, 2006; Ramachandran et al., 2010a,b). Preparation of mouse samples was similar to fish except retinas were fixed for 20–30 min in 2% paraformaldehyde. Primary antibodies used in this study: Zpr-1, Zebrafish International Resource Center (1/500); anti-HuC/D, Invitrogen, catalog #A-21275 (1/500); anti-PKC$_{\beta 1}$, Santa Cruz Biotechnology, catalog #SC-209 (1/200); anti-glutamine synthetase (GS), EMD Millipore, catalog #MAB302 (1/500); anti-BrdU, ThermoFisher, catalog #MA 1-82088 (1/500); anti-CRX, Abnova, catalog #H00001406-M02 (1/1000); anti-SOX9, EMD Millipore, catalog #AB5535 (1/500); anti-Chx10 antibody, EMD Millipore, catalog #AB-9016 (1/300); anti-RBPMS, Phospho Solutions, catalog #1830-RBPMS (1/500); anti-AP2$_{\alpha}$, Developmental Studies Hybridoma Bank, catalog #3B5 (1/1000); anti-4C4 (Gift

from Peter Hitchcock, University of Michigan). Secondary antibodies: AlexaFluor 555 donkey anti-mouse-IgG (H+L), ThermoFisher, catalog #A31570 (1:500); AlexaFluor 555 donkey anti-rabbit IgG (H+L), ThermoFisher, catalog #A31572 (1:500); AlexaFluor 555 donkey anti-sheep IgG (H+L) ThermoFisher, catalog #A21436; Cy3, Jackson ImmunoResearch, catalog #712-166-150 (1:500); AlexaFluor 488 donkey anti-mouse ThermoFisher, catalog #A21202 (1:500); AlexaFluor 488 goat anti-rabbit, ThermoFisher, catalog #A11008 (1:500); Cy5 goat anti-mouse, ThermoFisher, catalog #A10524 (1:500); and AlexaFluor 647 goat anti-rabbit, ThermoFisher, catalog #A21244 (1:500). *In situ* hybridization was performed as described previously (Barthel and Raymond, 2000). Images were captured by a Zeiss Axiophot fluorescence microscope or an Olympus Fluoview FV1000 confocal microscope.

Cell quantification and statistical analysis

BrdU immunofluorescence was used to identify and quantify proliferating cells in retinal sections as previously described (Fausett and Goldman, 2006; Ramachandran et al., 2010a; Wan et al., 2012, 2014). All experiments were done in triplicate with three animals per trial. Error bars are SD. ANOVA with Fisher's PLSD *post hoc* analysis was used for multiple-parameter comparison; two-tailed Student's *t* test was used for single-parameter comparison

2.6 Acknowledgements

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2.7 References

Barthel LK, Raymond PA. (2000) *In situ* hybridization studies of retinal neurons. Methods Enzymol 316:579–590. 10.1016/S0076-6879(00)16751-5

Bernardos RL, Barthel LK, Meyers JR, Raymond PA. (2007) Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. J Neurosci 27:7028–7040. 10.1523/JNEUROSCI.1624-07.2007

Byrne LC, Khalid F, Lee T, Zin EA, Greenberg KP, Visel M, Schaffer DV, Flannery JG. (2013) AAV-mediated, optogenetic ablation of Müller glia leads to structural and functional changes in the mouse retina. PLoS One 8:e76075. 10.1371/journal.pone.0076075

Chanda S, Ang CE, Davila J, Pak C, Mall M, Lee QY, Ahlenius H, Jung SW, Südhof TC, Wernig M. (2014) Generation of induced neuronal cells by the single reprogramming factor ASCL1. Stem Cell Reports 3:282–296. 10.1016/j.stemcr.2014.05.020

Conner C, Ackerman KM, Lahne M, Hobgood JS, Hyde DR. (2014) Repressing notch signaling and expressing TNFα are sufficient to mimic retinal regeneration by inducing Müller glial proliferation to generate committed progenitor cells. J Neurosci 34:14403–14419. 10.1523/JNEUROSCI.0498-14.2014

de Melo J, Clark BS, Blackshaw S. (2016a) Multiple intrinsic factors act in concert with Lhx2 to direct retinal gliogenesis. Sci Rep 6:32757. 10.1038/srep32757

de Melo J, Zibetti C, Clark BS, Hwang W, Miranda-Angulo AL, Qian J, Blackshaw S. (2016b) Lhx2 is an essential factor for retinal gliogenesis and notch signaling. J Neurosci 36:2391–2405. 10.1523/JNEUROSCI.3145-15.2016

Fausett BV, Goldman D. (2006) A role for α1 tubulin-expressing Müller glia in regeneration of the injured zebrafish retina. J Neurosci 26:6303–6313. 10.1523/JNEUROSCI.0332-06.2006

Fausett BV, Gumerson JD, Goldman D. (2008) The proneural basic helix-loop-helix gene *ascl1a* is required for retina regeneration. J Neurosci 28:1109–1117. 10.1523/JNEUROSCI.4853-07.2008

Flannery JG, Visel M. (2013) Adeno-associated viral vectors for gene therapy of inherited retinal degenerations. Methods Mol Biol 935:351–369. 10.1007/978-1-62703-080-9_25

Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL. (2000) rax, Hes1, and notch1 promote the formation of Müller glia by postnatal retinal progenitor cells. Neuron 26:383–394. 10.1016/S0896-6273(00)81171-X

Goldman D. (2014) Muller glial cell reprogramming and retina regeneration. Nat Rev Neurosci 15:431–442. 10.1038/nrn3723

Gorsuch RA, Lahne M, Yarka CE, Petravick ME, Li J, Hyde DR. (2017) Sox2 regulates Müller glia reprogramming and proliferation in the regenerating zebrafish retina via Lin28 and Ascl1a. Exp Eye Res 161:174–192. 10.1016/j.exer.2017.05.012

Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, Yeom KH, Han J, Kim VN. (2009) TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell 138:696–708. 10.1016/j.cell.2009.08.002

Imayoshi I, Kageyama R. (2011) The role of Notch signaling in adult neurogenesis. Mol Neurobiol 44:7–12. 10.1007/s12035-011-8186-0

Imayoshi I, Sakamoto M, Yamaguchi M, Mori K, Kageyama R. (2010) Essential roles of notch signaling in maintenance of neural stem cells in developing and adult brains. J Neurosci 30:3489–3498. 10.1523/JNEUROSCI.4987-09.2010

Jasoni CL, Reh TA. (1996) Temporal and spatial pattern of MASH-1 expression in the developing rat retina demonstrates progenitor cell heterogeneity. J Comp Neurol 369:319–327. 10.1002/(SICI)1096-9861(19960527)369:2%3C319::AID-CNE11%3E3.0.CO;2-C

Johns PR, Fernald RD. (1981) Genesis of rods in teleost fish retina. Nature 293:141–142. 10.1038/293141a0

Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, Wong RO, Rieke F, Reh TA. (2017) Stimulation of functional neuronal regeneration from Müller glia in adult mice. Nature 548:103–107. 10.1038/nature23283

Kageyama R, Ohtsuka T, Kobayashi T. (2007) The *hes* gene family: repressors and oscillators that orchestrate embryogenesis. Development 134:1243–1251. 10.1242/dev.000786

Kassen SC, Ramanan V, Montgomery JE, T Burket C, Liu CG, Vihtelic TS, Hyde DR. (2007) Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish. Dev Neurobiol 67:1009–1031. 10.1002/dneu.20362

Kassen SC, Thummel R, Burket CT, Campochiaro LA, Harding MJ, Hyde DR. (2008) The Tg(ccnb1:EGFP) transgenic zebrafish line labels proliferating cells during retinal development and regeneration. Mol Vis 14:951–963.

Klimczak RR, Koerber JT, Dalkara D, Flannery JG, Schaffer DV. (2009) A novel adeno-associated viral variant for efficient and selective intravitreal transduction of rat muller cells. PLoS One 4:e7467. 10.1371/journal.pone.0007467

Kwon HJ, Bhat N, Sweet EM, Cornell RA, Riley BB. (2010) Identification of early requirements for preplacodal ectoderm and sensory organ development. PLoS Genet 6:e1001133. 10.1371/journal.pgen.1001133

Lindsey AE, Powers MK. (2007) Visual behavior of adult goldfish with regenerating retina. Vis Neurosci 24:247–255. 10.1017/S0952523806230207

Liu Y, Miao Q, Yuan J, Han S, Zhang P, Li S, Rao Z, Zhao W, Ye Q, Geng J, Zhang X, Cheng L. (2015) *Ascl1* converts dorsal midbrain astrocytes into functional neurons *in vivo*. J Neurosci 35:9336–9355. 10.1523/JNEUROSCI.3975-14.2015

Maillard I, Tu L, Sambandam A, Yashiro-Ohtani Y, Millholland J, Keeshan K, Shestova O, Xu L, Bhandoola A, Pear WS. (2006) The requirement for notch signaling at the β-selection checkpoint *in vivo* is absolute and independent of the pre-T cell receptor. J Exp Med 203:2239–2245. 10.1084/jem.20061020

Montgomery JE, Parsons MJ, Hyde DR. (2010) A novel model of retinal ablation demonstrates that the extent of rod cell death regulates the origin of the regenerated zebrafish rod photoreceptors. J Comp Neurol 518:800–814. 10.1002/cne.22243

Nelson BR, Ueki Y, Reardon S, Karl MO, Georgi S, Hartman BH, Lamba DA, Reh TA. (2011) Genome-wide analysis of Müller glial differentiation reveals a requirement for notch signaling in postmitotic cells to maintain the glial fate. PLoS One 6:e22817. 10.1371/journal.pone.0022817

Nelson CM, Gorsuch RA, Bailey TJ, Ackerman KM, Kassen SC, Hyde DR. (2012) Stat3 defines three populations of Müller glia and is required for initiating maximal Müller glia proliferation in the regenerating zebrafish retina. J Comp Neurol 520:4294–4311. 10.1002/cne.23213

Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR. (2013) Tumor necrosis factor-α is produced by dying retinal neurons and is required for Müller glia proliferation during zebrafish retinal regeneration. J Neurosci 33:6524–6539. 10.1523/JNEUROSCI.3838-12.2013

Otteson DC, Hitchcock PF. (2003) Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. Vision Res 43:927–936. 10.1016/S0042-6989(02)00400-5

Parsons MJ, Pisharath H, Yusuff S, Moore JC, Siekmann AF, Lawson N, Leach SD. (2009) Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mech Dev 126:898–912. 10.1016/j.mod.2009.07.002

Powell C, Cornblath E, Elsaeidi F, Wan J, Goldman D. (2016) Zebrafish Müller glia-derived progenitors are multipotent, exhibit proliferative biases and regenerate excess neurons. Sci Rep 6:24851. 10.1038/srep24851

Qin Z, Barthel LK, Raymond PA. (2009) Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish. Proc Natl Acad Sci U S A 106:9310–9315. 10.1073/pnas.0811186106

Rajaram K, Harding RL, Hyde DR, Patton JG. (2014) miR-203 regulates progenitor cell proliferation during adult zebrafish retina regeneration. Dev Biol 392:393–403. 10.1016/j.ydbio.2014.05.005

Ramachandran R, Reifler A, Parent JM, Goldman D. (2010a) Conditional gene expression and lineage tracing of *tuba1a* expressing cells during zebrafish development and retina regeneration. J Comp Neurol 518:4196–4212. 10.1002/cne.22448

Ramachandran R, Fausett BV, Goldman D. (2010b) *Ascl1a* regulates Müller glia dedifferentiation and retinal regeneration through a lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol 12:1101–1107. 10.1038/ncb2115

Ramachandran R, Zhao XF, Goldman D. (2011) Ascl1a/Dkk/β-catenin signaling pathway is necessary and glycogen synthase kinase-3β inhibition is sufficient for zebrafish retina regeneration. Proc Natl Acad Sci U S A 108:15858–15863. 10.1073/pnas.1107220108

Ramachandran R, Zhao XF, Goldman D. (2012) Insm1a-mediated gene repression is essential for the formation and differentiation of Müller glia-derived progenitors in the injured retina. Nat Cell Biol 14:1013–1023. 10.1038/ncb2586

Riesenberg AN, Conley KW, Le TT, Brown NL. (2018) Separate and coincident expression of Hes1 and Hes5 in the developing mouse eye. Dev Dyn 247:212–221. 10.1002/dvdy.24542

Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R, Wulczyn FG. (2008) A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat Cell Biol 10:987–993. 10.1038/ncb1759

Sakamoto M, Hirata H, Ohtsuka T, Bessho Y, Kageyama R. (2003) The basic helix-loop-helix genes Hesr1/Hey1 and Hesr2/Hey2 regulate maintenance of neural precursor cells in the brain. J Biol Chem 278:44808–44815. 10.1074/jbc.M300448200

Sherpa T, Fimbel SM, Mallory DE, Maaswinkel H, Spritzer SD, Sand JA, Li L, Hyde DR, Stenkamp DL. (2008) Ganglion cell regeneration following whole-retina destruction in zebrafish. Dev Neurobiol 68:166–181. 10.1002/dneu.20568

Sifuentes CJ, Kim JW, Swaroop A, Raymond PA. (2016) Rapid, dynamic activation of Müller glial stem cell responses in zebrafish. Invest Ophthalmol Vis Sci 57:5148–5160. 10.1167/iovs.16-19973

Thomas JL, Ranski AH, Morgan GW, Thummel R. (2016) Reactive gliosis in the adult zebrafish retina. Exp Eye Res 143:98–109. 10.1016/j.exer.2015.09.017

Thummel R, Enright JM, Kassen SC, Montgomery JE, Bailey TJ, Hyde DR. (2010) Pax6a and Pax6b are required at different points in neuronal progenitor cell proliferation

during zebrafish photoreceptor regeneration. Exp Eye Res 90:572–582. 10.1016/j.exer.2010.02.001

Ueki Y, Wilken MS, Cox KE, Chipman L, Jorstad N, Sternhagen K, Simic M, Ullom K, Nakafuku M, Reh TA. (2015) Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. Proc Natl Acad Sci U S A 112:13717–13722. 10.1073/pnas.1510595112

Wan J, Goldman D. (2016) Retina regeneration in zebrafish. Curr Opin Genet Dev 40:41–47. 10.1016/j.gde.2016.05.009

Wan J, Goldman D. (2017) Opposing actions of Fgf8a on notch signaling distinguish two Müller glial cell populations that contribute to retina growth and regeneration. Cell Rep 19:849–862. 10.1016/j.celrep.2017.04.009

Wan J, Ramachandran R, Goldman D. (2012) HB-EGF is necessary and sufficient for Müller glia dedifferentiation and retina regeneration. Dev Cell 22:334–347. 10.1016/j.devcel.2011.11.020

Wan J, Zhao XF, Vojtek A, Goldman D. (2014) Retinal injury, growth factors, and cytokines converge on β-catenin and pStat3 signaling to stimulate retina regeneration. Cell Rep 9:285–297. 10.1016/j.celrep.2014.08.048

Weintraub H, Davis R, Lockshon D, Lassar A. (1990) MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proc Natl Acad Sci U S A 87:5623–5627. 10.1073/pnas.87.15.5623

Xu B, Zhang K, Huang Y. (2009) Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells. RNA 15:357–361. 10.1261/rna.1368009

Yao K, Qiu S, Tian L, Snider WD, Flannery JG, Schaffer DV, Chen B. (2016) Wnt regulates proliferation and neurogenic potential of Müller glial cells via a Lin28/let-7 miRNA-dependent pathway in adult mammalian retinas. Cell Rep 17:165–178. 10.1016/j.celrep.2016.08.078

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920. 10.1126/science.1151526

Zhao XF, Wan J, Powell C, Ramachandran R, Myers MG Jr, Goldman D. (2014) Leptin and IL-6 family cytokines synergize to stimulate Müller glia reprogramming and retina regeneration. Cell Rep 9:272–284. 10.1016/j.celrep.2014.08.047

Zhou X, Vink M, Klaver B, Berkhout B, Das AT. (2006) Optimization of the Tet-On system for regulated gene expression through viral evolution. Gene Ther 13:1382–1390. 10.1038/sj.gt.3302780

Chapter 3: Ascl1 and Lin28 Treatment Induce Different Müller Glia Responses in Different Models of Retinal Degeneration

3.1 Summary

In the previous chapter, the enhanced Müller glia proliferation response to the combinatorial treatment of Ascl1 and Lin28 show that the mammalian retina retains some regenerative capabilities. In this chapter, we investigate the different conditions that lead to the enhanced MG proliferative response through the exploration of different mouse and retinal damage models. We look at transient UV and chemical induced injury where the insult more closely resembles those of natural degeneration. While baseline MG proliferation is observed, the different retinal damage models lead to different levels of MG proliferation. Nevertheless, Ascl1 and Lin28 overexpression appears to enhance MG proliferation across all damage conditions. In order to see whether Ascl1 and Lin28 treatment could elicit the same MG response in cases of natural degeneration, we treat three different naturally degenerating mouse models RD10, RD12, and P23H with Ascl1 and Lin28. However, despite our success in the damage models, we are unable to induce any meaningful MG proliferation in all three RD models. We expected the different speed of degeneration between the RD10, RD12, and P23H mouse models to have different effects on MG proliferation, but this complete lack of response show that natural degeneration may be altogether too slow and the MG activation too mild when compared to transient damage. We also attempt to lineage trace the proliferating MG with fluorescently labeled Ascl1 and Lin28 AAV viruses. Surprisingly, through injections of these fluorescent labeled ShH10 viruses, we discover that the specificity of the ShH10 AAV is lower than previously thought with many different off-target infections. To overcome this problem, we engineer 7m8 AAV

vectors containing a shortened glia fibrillary acidic protein (GFAP) promoter that prove to be more efficient and specific for targeted MG gene expression.

3.2 Introduction

With the approach of forced Ascl1 and Lin28 expression, a strong MG proliferative response could be elicited from both adult zebrafish and mouse retinas. This type of MG proliferation has not been shown in the uninjured mouse retina before, and this development represents an important step towards fully harnessing the potential of MG endogenous regeneration. Nevertheless, uninjured mammalian retinas are still resistant to regeneration, and we were ultimately able to induce meaningful MG proliferation only with transient NMDA damage; a common result as observed from previous studies (Reh, 2015). While NMDA is a potent excitotoxic agent when administered in large quantities in the central nervous system, its effect on the retina is mostly restricted to the ganglion cells (Tamm, 2012). To investigate the effect of Ascl1 and Lin28 in retinal damage models that more closely resemble natural RD, we used cobalt chloride and strong UV light to selectively ablate photoreceptors. While others have utilized light successfully as a retinal damage model, most of the previous studies involve light exposure over an extended period of time (Albert, 2011). We examined whether extreme transient UV light administered over minutes could provide a stronger MG response with Ascl1 and Lin28 due to the fast pace of induced cell death. Cobalt chloride is a hypoxia inducing agent that has been shown to cause strong selective photoreceptor apoptosis when injected intravitreally in mice. To explore the effectiveness of Ascl1 and Lin28 under cobalt chloride damage, we injected increasing amounts of cobalt chloride in order to observe a dose-response relationship between retinal damage and Ascl1/Lin28 induced MG proliferation (Mori, 2006).

Previous studies on MG proliferation *in vivo* mostly utilized mice with wildtype backgrounds such as C57BL/6J mice (Reh, 2015; Goldman, 2018; Chen, 2018). However, it has been shown that different strains of mice may possess MG cells that behave differently *in vitro*. Specifically, the cultured primary MG cells of 129/SVJ mice proliferate significantly faster than the MG cells of the C57BL/6J mice (Takahashi, 2014). Whether the MG of 129/SVJ mice show improved proliferation *in vivo* is not known. To complement our damage models, we looked at the effects of Ascl1 and Lin28 on the damaged 129/SVJ retina *in vivo*. For the purpose of lineage tracing, we also utilized the Aldh1l1-GFP transgenic reporter mouse line. This mouse line has previously been described to express GFP in astrocytes in the central nervous system (Khakh, 2016). We discovered that its GFP expression is MG specific in the retina and that this mouse model is potentially a great tool for visualizing MG proliferation and transformation *in vivo*. Through these two mouse models, we established that photoreceptor damage appears to elicit a general MG proliferative response that is correlated with the strength of the damage. Regardless, with Ascl1 and Lin28 administration through MG-specific ShH10 AAV injection, we were able to improve this proliferative response across the board, showing that Ascl1 and Lin28 remain essential for inducing robust MG proliferation (Schaffer, 2009).

We also investigated methods of identifying pro-proliferative and pro-neuronal factors *in vitro* through establishing primary MG cell lines from 129/SVJ and Aldh1l1-GFP retinas. We hypothesized that 129/SVJ MG would experience a significant change in its expression profile after Ascl1 and Lin28 forced expression *in vitro*. We hoped to utilize these expression changes in order to establish a reliable cell line for the identification of factors that would induce MG fate change. The MG specific GFP expression in Aldh1l1-GFP primary MG cells is also a potentially useful tool in factor identification. However, while 129/SVJ primary MG indeed showed an increase in the expression of neuronal factors and a decrease in the expression of MG factors in response to Ascl1 and Lin28 expression, this response may be dependent on confounding factors such as age of the cells and cell confluency. Furthermore, Aldh1l1-GFP primary MG cells gradually lost their GFP expression over time without any infection or forced expression, casting doubt on their usefulness as a reporter cell line for MG fate change *in vitro*.

In our previous studies, the forced expression of Ascl1 and Lin28 *in vivo* has only been measured indirectly through mRNA or protein expression. While labeling methods for the two proteins exist, we wished to develop a direct and lasting way of detecting AAV delivered Ascl1 and Lin28 *in vivo* without using a transgenic reporter mouse line. We developed ShH10 AAV that express fluorescence coupled Ascl1 and Lin28. With these new viruses, we set out to see whether Ascl1/Lin28 treatment could induce MG proliferation in naturally degenerating retinas. We expressed GFP linked Ascl1 and Lin28 with shH10 in three different RD mouse models: P23H, RD10, and RD12

The P23H mice carry a proline to histidine mutation in the 23rd amino acid codon in their opsin gene. They show progressive retinal degeneration similar to a subset of human patients with autosomal dominant forms of retinitis pigmentosa. The mutated opsin fails to be glycosylated and does not participate in photoreceptor disk formation which leads to the disruption of the outer segment and eventual photoreceptor cell death through toxic protein buildup (Palczewski, 2011). The retinal degeneration involved in the homozygous P23H mutation is relatively fast, leading to a drastic loss of vision and photoreceptors at around 1 month of age. The RD10 mice are a model of autosomal recessive RP. They carry a spontaneous mutation in the rod-phosphodiesterase (PDE) gene, which leads to a defect in rod phototransduction. This disruption eventually leads to rod cell death followed by cone death but is generally mild with mice still retaining some photoreceptors at 2 months of age (Strettoi, 2007). The last form of RD mouse models in this chapter, RD12, involves a nonsense mutation in exon 3 of the RPE65 gene. This mutation prevents retinol regeneration during phototransduction which again leads to cell death. However, retinal degeneration in RD12 is slow, beginning at around 3 weeks of age with most of the photoreceptors still intact at around 2 months of age (Heckenlively, 2005). By treating these three models, we strive to not only promote MG proliferation in RD mouse models that are more relevant to RD in humans, but also to probe the effect that different types and rates of retinal degeneration may have on the MG response.

Unfortunately, we were unable to induce a proliferation response *in vivo* from the RD mouse models that are comparable to those of damage models. Interestingly, we were

unable to elicit any meaningful MG proliferation at all even though in the undamaged wildtype mouse retina, Ascl1 and Lin28 expression lead to minimal MG proliferation. We hypothesize that this may be due to not only the slowness of the degeneration process of these mice when compared to damage models, but also that the MG are more resistant to activation and proliferation. This resistant may arise from the wildly different genetic backgrounds and the slow but constant MG activation from sporadic photoreceptor apoptosis. There's evidence that different retinal degenerative mouse models show different levels of Müller glia activation, further providing evidence for this theory (Pearson, 2015). In addition to these complications, we also discovered that the shH10 virus, when injected in pairs, appeared to elicit strong off-target expression. By utilizing the pan-retinal 7m8 virus and a shortened GFAP promoter, we developed the next generation of MG targeting AAV vectors for induced MG cell fate change.

3.3 Results

UV Light induced photoreceptor cell death leads to Ascl1 and Lin28 dependent MG proliferation

In the previous chapter we established that under the damaging effects of intravitreal NMDA, mouse MG activate and reenter the cell cycle, an effect which is significantly enhanced by the viral delivery of Ascl1 and Lin28a. However, NMDA is a neuron receptor channel agonist that selectively affects ganglion cells in the retina, damaging them at high concentrations through excitotoxicity. As such, this model of retinal damage is far removed from the phenotype seen in retinal degeneration such as retinitis pigmentosa where photoreceptors selectively undergo apoptosis. Whether Ascl1 and Lin28 enhanced proliferation could be observed with mouse models of photoreceptor damage and degeneration has yet to be seen. To investigate, we injected 129/SVJ mice intravitreally at P15 and delivered AAV vectors shH10-CAG-Ascl1 and shH10-CAG-Lin28 while using phosphate-buffered saline (PBS) for control injections. As described previously, shH10 is a MG specific viral vector developed through directed evolution, and the viruses express their genes of interest under the ubiquitous CAG promoter (Schaffer, 2009). 129/SVJ is an albino mouse strain that has been shown to possess MG that are more proliferative *in vitro* (Takahashi, 2014).

Each mouse was injected either with PBS, shH10-CAG-Ascl1, or shH10-CAG-Ascl1 and shH10-CAG-Lin28 together in each of its eyes. After allowing three weeks for protein expression, the mice then underwent 15 minutes of strong xenon UV light exposure to each of their eyes to selectively ablate photoreceptors. This process was then followed by intraperitoneal BrdU injections, and the mice continued receiving daily intraperitoneal injections of BrdU, culminating in their sacrifice 4 days after UV exposure (Fig. 3.1*A*). Initial TUNEL staining revealed that while NMDA damage lead to little cell apoptosis throughout the different retinal layers, transient UV light damage lead to significant amounts of photoreceptor specific apoptosis (Fig. 3.1*B* left, middle). This apoptosis lead to low amounts of MG proliferation (Fig. 3.1*C*) with control PBS exposure and a moderate amount of proliferation with Ascl1 (Fig. 3.1*D*) forced expression. However, it was when the 129/SVJ mice overexpressed both Ascl1 and Lin28 that UV

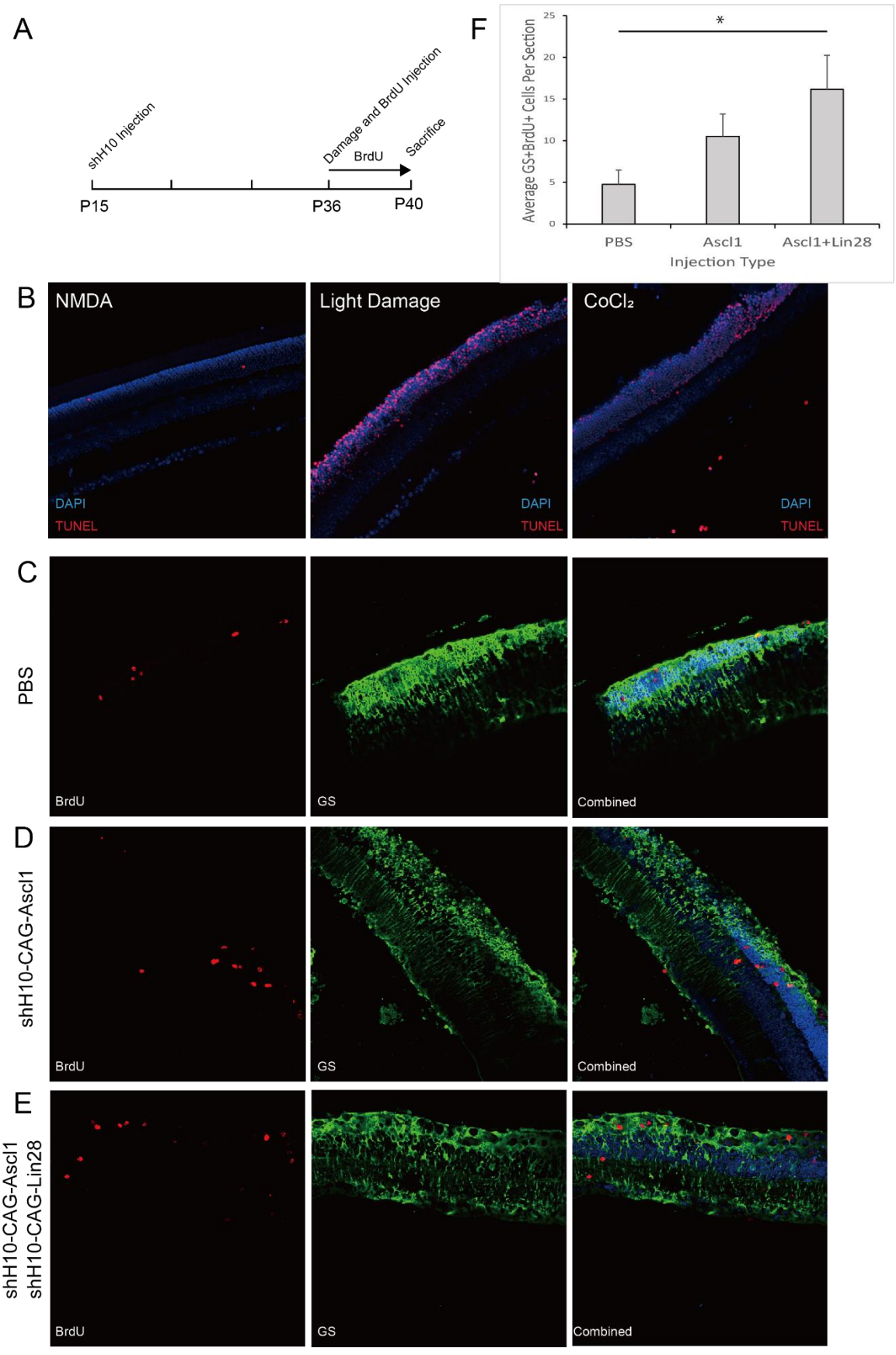

A
shH10 Injection
Damage and BrdU Injection
BrdU
Sacrifice
P15
P36
P40
F
Average GS+BrdU+ Cells Per Section
25
20
15
10
5
0
*
PBS
Ascl1
Ascl1+Lin28
Injection Type
B
NMDA
Light Damage
CoCl₂
DAPI
TUNEL
C
PBS
BrdU
GS
Combined
D
shH10-CAG-Ascl1
E
shH10-CAG-Ascl1
shH10-CAG-Lin28

Figure 3.1: UV light damage induces MG proliferation that is enhanced by Ascl1 and Lin28 expression

A, Experimental timeline. ***B***, DAPI and TUNEL staining of retinal sections of 129/SVJ retinas that were damaged through NMDA, UV light, and cobalt chloride. This indicates that NMDA causes little damage pan-retinally while UV light and cobalt chloride induce apoptosis in many photoreceptors specifically. ***C***, DAPI, glutamine synthetase (GS) and BrdU staining of UV-exposed 129/SVJ mouse retina section previously injected with PBS control. Small amounts of MG proliferation could be seen after UV light damage. ***D***, DAPI, GS, and BrdU staining of UV-exposed 129/SVJ mouse retina sections previously injected with shH10-CAG-Ascl1 AAV. More MG proliferation is seen in the ONL and INL after UV light damage. ***E,*** DAPI, GS, and BrdU staining of UV-exposed 129/SVJ mouse retina sections previously injected with a combination of shH10-CAG-Ascl1 and shH10-CAG-Lin28. Significant amount of MG proliferation could be seen after UV light damage. ***F,*** Quantification of MG proliferation per retina under the different injections conditions. This shows that significant MG proliferation under the UV light damage model could be seen only through Ascl1 and Lin28 expression. n = 3 different experiments. Error bars are SD. *$p < 0.05$.

light damage lead to significant MG proliferation, similar to what was previously observed with NMDA damage (Fig. 3.1*E*, *F*). Unexpectedly, while this model of photoreceptor damage replicated our findings, the combination of both 129/SVJ mice and UV light damage did not lead to higher amounts of proliferation when compared to C57BL/6J mice injected with NMDA (Fig. 3.1*F*). Many factors could have influenced this result. Since this light exposure was short and the light beam concentrated, retinal damage was not pan-retinal and only certain areas of the retina saw a significant loss of photoreceptor cells. This could be observed in Fig. 3.1*C* where the retina shows an ONL of uneven thickness. While the genetic background of 129/SVJ mice lead to more MG growth *in vitro*, the environment is vastly different *in vivo* with different proliferation thresholds. A different approach may be needed in order to establish a more consistent photoreceptor degeneration model for MG proliferation.

Chemical induced photoreceptor cell death leads to Ascl1 and Lin28 dependent MG proliferation

Cobalt chloride is an established inducer of hypoxia-like response that has been shown to target photoreceptors for apoptosis when injected intravitreally (Mori, 2006). Its damaging effect to the photoreceptor layer and its consistency in instilling damage through intravitreal injections make cobalt chloride the ideal candidate for establishing a retinal degeneration-like damage model for studying MG proliferation. To visualize the MG response we utilized the Aldh1l1-GFP mouse strain, which was a generous donation from the Kaufer lab. This transgenic mouse line expresses GFP under the aldehyde dehydrogenase 1 family member L1 promoter, and this expression is astrocyte specific in the brain (Fig. 3.2*A*) (Khakh, 2016). Through fundus imaging and retinal sections, we showed that this Aldh1l1 driven GFP expression is also MG specific in the retina, with GFP outlining the uniquely shaped MG and coinciding with Sox9 expression (Fig. 3.2*B*, *C*).

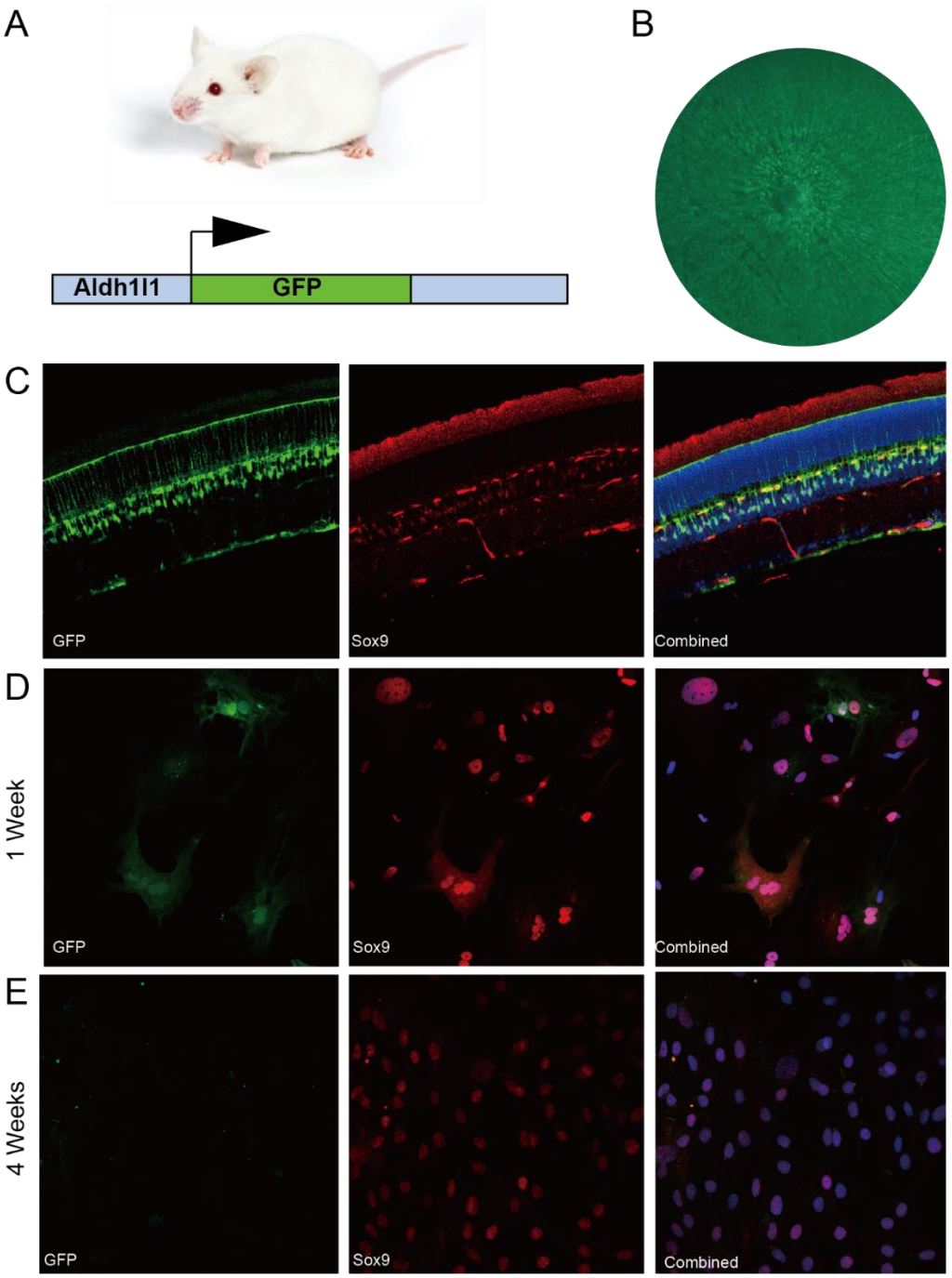


Figure 3.2: Aldh1l1-GFP mice express MG specific GFP *in vivo* and *in vitro*
A, Schematic expression cassette and mouse color (mouse picture is from the Jackson Laboratory website). ***B***, Fundus picture of the Aldh1l1-GFP mouse through a RetCam device. The retina is viewed through the lens, and the picture is centered on the optic disk. GFP expression is seen throughout the retina. ***C.*** A section of the Aldh1l1-GFP retina. The GFP appears MG specific, and the GFP positive cell bodies express Sox9, indicating that they are mature MG. ***D.*** Cultured primary MG cells from the Aldh1l1-GFP mice at 1 week post dissociation. The cells appear to be dividing and mostly express GFP. ***E.*** After 4 weeks of culture, the primary MG cells stop expressing GFP altogether. Even though the cells are dividing and are confluent in the picture, there was little to no GFP expression.

Similar to the previous experiment, we injected Aldh1l1-GFP mice intravitreally with either PBS, the shH10-CAG-Ascl1 virus, or the shH10-CAG-Ascl1 and shH10-CAG-Lin28 viruses together. After allowing 3 weeks for viral protein expression, the mice were injected intravitreally with either 1mM, 5mM, or 8mM of cobalt chloride in PBS with BrdU. They then received daily intraperitoneal injections of BrdU for 4 days and were sacrificed afterwards. Interestingly, even though the amount of photoreceptor apoptosis was dependent upon the concentration of cobalt chloride injected (Fig. 3.1*B* right; Fig. 3.2*A*), we found proliferating MG as long as photoreceptor damage was observed. With control PBS injections, MG proliferation was low at 1mM concentration of cobalt chloride, but this proliferation was significantly increased at 5mM. Increasing the concentration of cobalt chloride injected past 5mM did not seem to affect the amount of proliferating MG, perhaps signifying an intrinsic cap on chemical damage induced MG proliferation without influence from exogenous factors (Fig. 3.3*A, B*). However, with the expression of Ascl1 alone and Ascl1 and Lin28 together, the MG was capable of proliferating at a rate beyond this cap (Fig. 3.3*C, D*). Thus, we found that cobalt chloride induced retinal damage lead to a proliferative MG response in the Aldh1l1-GFP mice. This response was dependent upon the amount of damage induced, and it was also further enhanced by Ascl1 and Lin28 forced expression.

Attempt to establish an *in vitro* system to identify MG fate-change factors

While forced expression of Ascl1 and Lin28 is sufficient to induce mouse MG to reenter the cell cycle, the fate of the proliferating MG remains unchanged. Unlike the zebrafish retina, the mouse retina remains unable to produce new retinal neurons at a significant quantity. New and unexplored factors may be needed in order to further push the proliferating MG towards a neuronal fate. In order to identify such factors, we set out to establish a stable primary MG reporter cell line with dissociated MG from the 129/SVJ and Aldh1l1-GFP mouse lines. Eyes from both mouse lines were enucleated at 10 days of age and the retinas extracted and dissociated with trypsin. The dissociated cells were left to grow in culture media for a week. Cells that did not attach to the cell culture dish were washed away, and the final attached cells were allowed to grow further. After the cells had grown to confluency, they were split and allowed to be tested further.

Ascl1, Lin28, and other factors of interest such as NeuroD4 (ND4) were packaged into tetracycline/doxycycline responsive lentiviral vectors separately. The primary MG from the 129/SVJ mouse line were infected with individual lentiviral vectors and were allowed four days for protein expression. Puromycin was then added to the culture media over ten days to screen for infected cells as the lentivirus carried puromycin resistance. Doxycycline was added after selection to allow the factors of interest to be expressed. We extracted the RNA from the selected cells and did reverse transcription and quantitative polymerase chain reaction (RT-qPCR) on the mRNA to look for retinal glia and neuron markers. The quantified expression levels were divided by the expression levels of control cells that never received doxycycline. Worryingly, the control uninfected cells saw a decrease in expression levels of both neuron specific markers βIII-tubulin (B3T) and Atoh7 and MG specific markers glutamine synthetase (GS) with only the

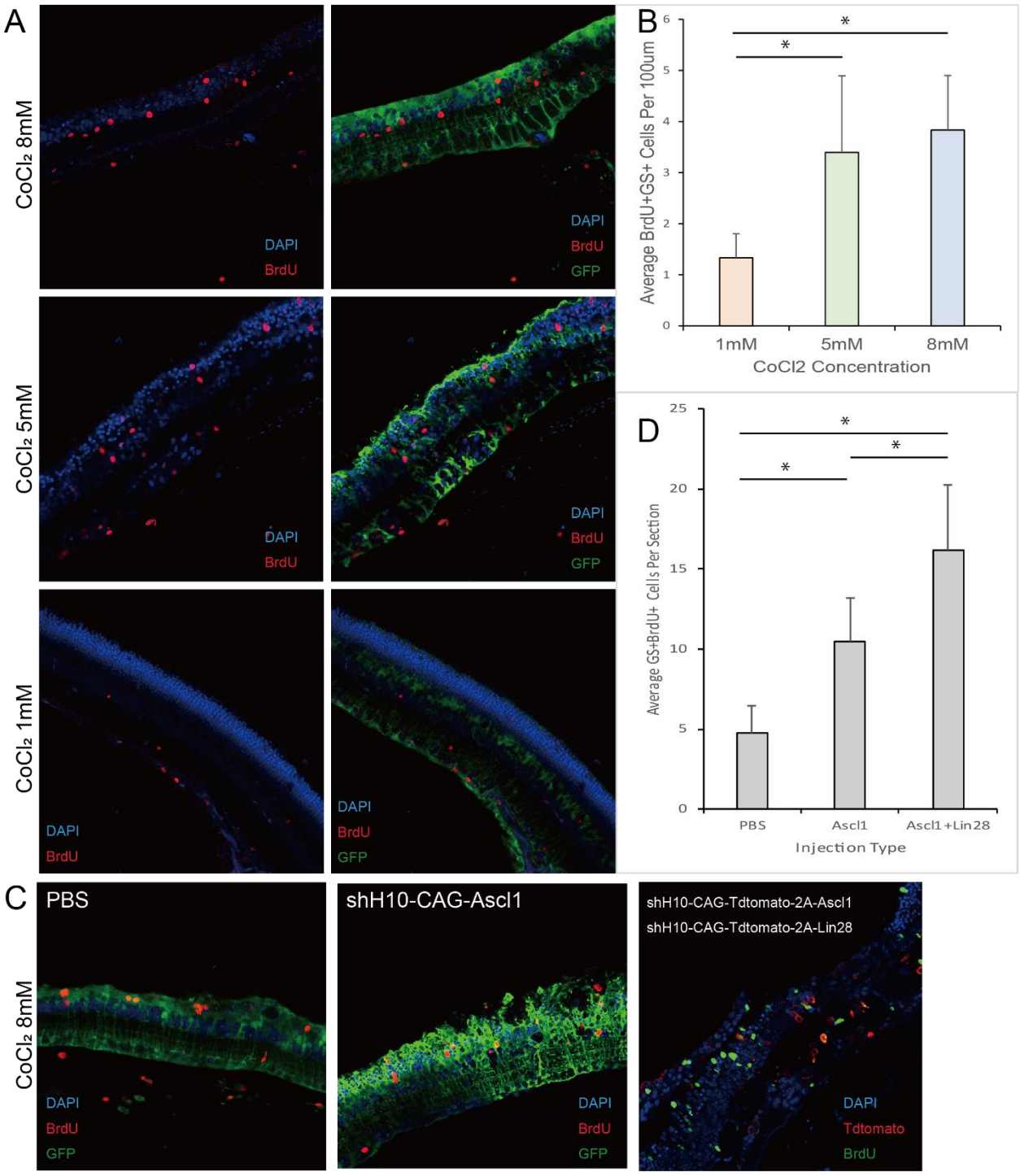


Figure 3.3: Cobalt chloride induced retinal damage leads to MG proliferation
A, Retinal sections of Aldh1l1-GFP mice injected with different concentrations of cobalt chloride. With increasing concentrations of cobalt chloride, GFP-expressing MG increase their proliferative response. ***B.*** Quantification of MG proliferation with cobalt chloride damage. There is a significant increase in proliferation going from 1mM to 5mM of cobalt chloride, but with 8mM of cobalt chloride no difference is observed. ***C.*** Using 8mM of cobalt chloride as a baseline damage model, forced expression of Ascl1 using the shH10 AAV lead to more MG proliferation while expressing both Ascl1 and Lin28 lead to the

highest amount of proliferation. ***D.*** Quantification of MG proliferation with 8mM cobalt chloride under different treatment regimes. Significant difference was found between PBS control and Ascl1 expression. Combined expression of both Ascl1 and Lin28 lead to a significantly higher amount of MG proliferation even when compared with Ascl1. *n* = 3 different experiments. Error bars are SD. *$p < 0.05$

addition of doxycycline (Fig. 3.4*A*). However, Ascl1 infected cells saw a significant increase in βIII-tubulin and Atoh7 expression with doxycycline exposure in contrast to the control cells (Fig. 3.4*B*), and the Lin28 infected cells saw a decrease in Nestin expression accompanying an increase in βIII-tubulin (Fig. 3.4*C*). These differences in response were significant and were unexplainable with simple doxycycline exposure as they ran counter to the responses of control cells. Nevertheless, a second round of quantification saw the trends reversing with many of the different markers across the different cell lines, with the only significant constant being the accompanying increase in βIII-tubulin expression when Ascl1 was expressed (Fig. 3.4*D*). We hypothesized that different factors such as cell confluency and cell age may have affected these changes in expression, and we looked at the expression profile of ND4 infected cells at 2 weeks vs 1 month. Even though the cells were of the same lineage except in age, the expression levels of the different MG and neuron factors were wildly different (Fig. 3.4*E*). Thus, the 129/SVJ primary MG were determined not to be a reliable reporter cell line. Unfortunately, Aldh1l1-GFP primary MG cells gradually lost their fluorescence as they remained in culture (Fig. 3.2*D, E*), showing that the MG specific GFP expression is not a reliable marker *in vitro* as well.

Ascl1 and Lin28 do not stimulate MG proliferation in naturally degenerating mouse models

We have shown previously that a combined delivery of Ascl1 and Lin28 to the MG in the mouse retina by AAV pushed MG into a proliferative state. While this proliferation was increased by NMDA, UV light, and cobalt chloride damage, we wished to investigate whether the same treatment would elicit a similar response in the retinas of natural RD mice. For this experiment, we looked at three different transgenic retinal degenerative mouse models: P23H, RD10, and RD12. The three different RD models allowed us to look at the effects of different rates of degeneration on the MG proliferation response. P23H showed the fastest rate of degeneration, losing most of its photoreceptors at 1 to 2 months of age. RD10 mice showed intermediate degeneration and retained small amounts of functional photoreceptors at 2 months of age. RD12 mice did not lose a significant amount of photoreceptor function until 4 to 6 months of age (Palczewski, 2011; Strettoi, 2007; Heckenlively, 2005).

Similar to the previous experiments, we injected, intravitreally, ShH10 AAV viruses containing Ascl1 and Lin28 under the CAG promoter into the eyes of P23H, RD10, and RD12 mice. However, in order to lineage trace Ascl1 and Lin28 infected cells without the use of a transgenic reporter mouse line, we inserted GFP into the vectors and connected its expression to Ascl1 and Lin28 with P2A peptides. The 2A peptides allowed for equal expression of both GFP and Ascl1/Lin28 under the CAG promoter (Jethwa, 2015). After waiting three weeks for viral expression, we injected each mouse

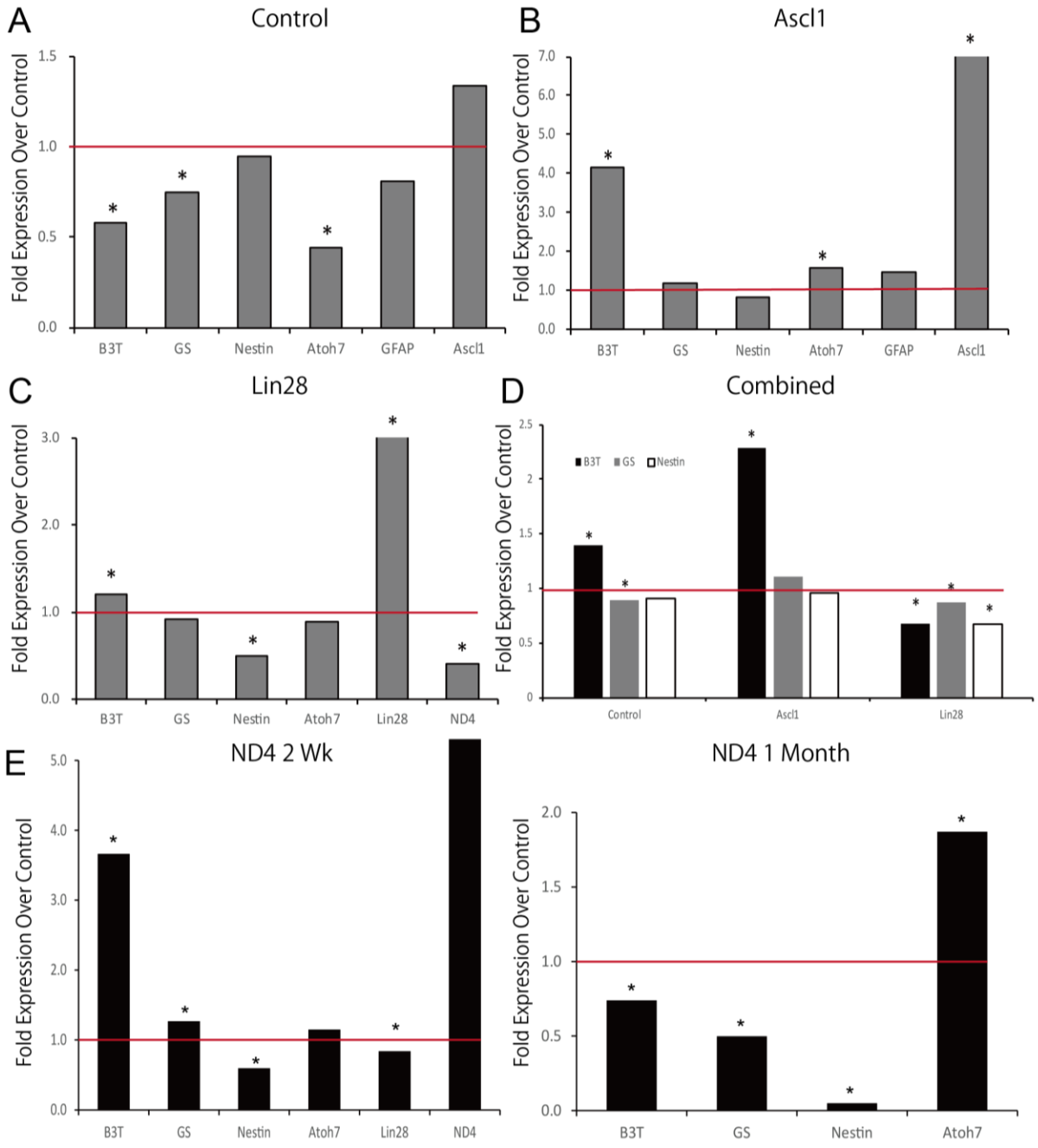


Figure 3.4: 129/SVJ primary MG is an unreliable reporter cell lines for detecting MG fate change
A, Expression levels of βIII-tubulin (B3T), glutamine synthetase (GS), Nestin, Atoh7, glial fibrillary acidic protein (GFAP), and Ascl1 of control uninfected cells cultured with doxycycline were measured through RT-qPCR. The quantified results were then divided over the baseline expression of the cell line cultured without doxycycline. The red line signifies expression levels over baseline. ***B***, The same measurements were made with Ascl1 lentivirus infected cells. ***C,*** Similar measurements were taken with Lin28 lentivirus infected cells. NeuroD4 (ND4) was measured. ***D,*** A second experiment was conducted measuring only B3T, GS, and Nestin over all of the cell lines. ***E,*** Measurements of NeuroD4 lentivirus infected cells taken after 2 weeks of NeuroD4 expression and after 1 month of NeuroD4 expression. n = 3 different cell plates. Error bars are SD. *$p < 0.05$

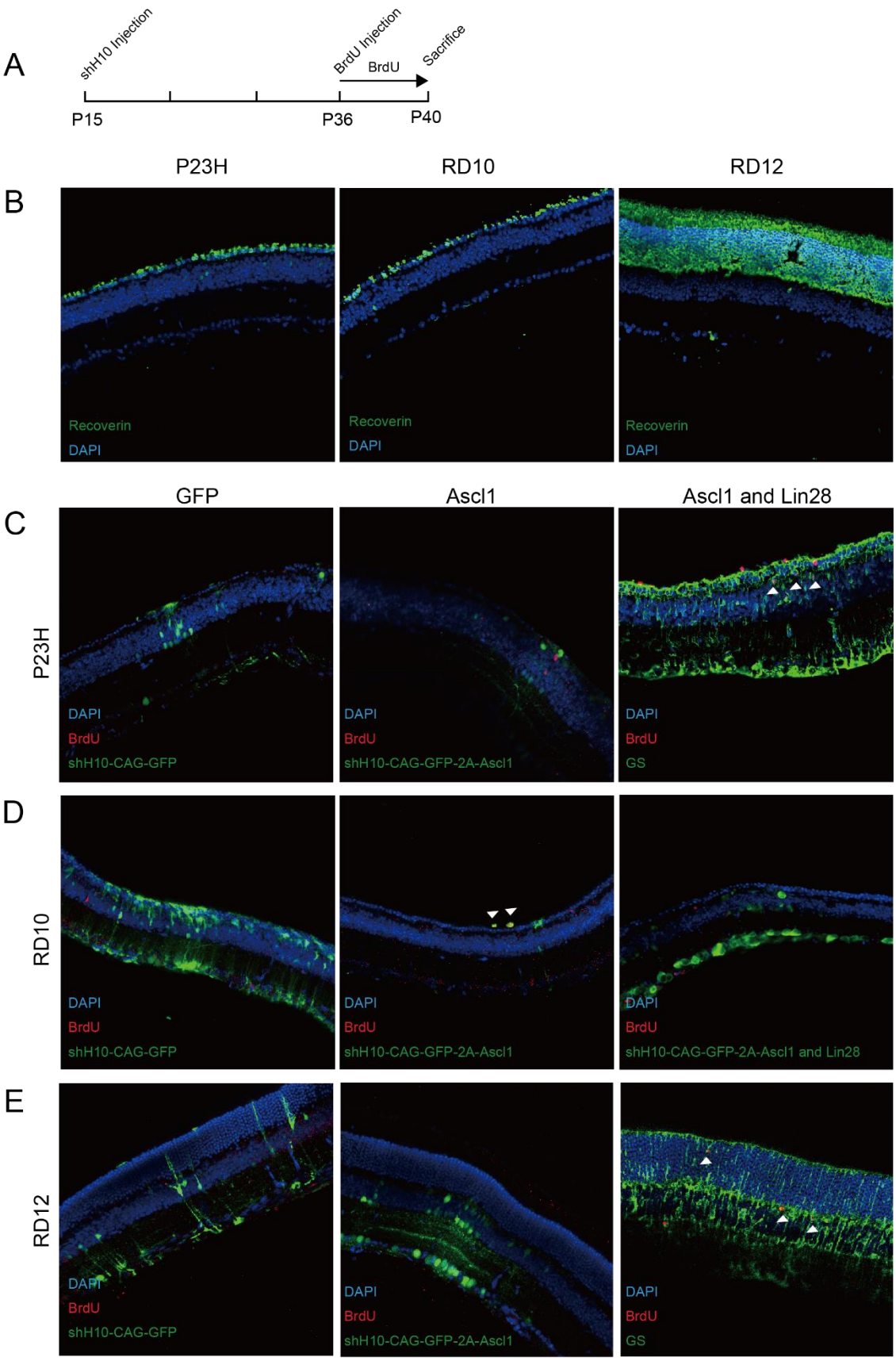
with BrdU intravitreally at 1mg/mL. The mice then received peritoneal injections of BrdU at 50ug/g of body weight daily and were sacrificed after four days (Fig. 3.5*A*). At the time of sacrifice, P23H mice retained a single layer of photoreceptor cell bodies in the ONL, RD10 mice retained 1 to 3 layers, and RD12 mice retained all but a few of its photoreceptor cells (Fig. 3.5*B*). Unfortunately, across all three different mouse models, MG proliferation was rarely seen (Fig. 3.*5C, E* arrows). The vast majority of BrdU labeling occurred with cells outside of the retinal layers (Fig. 3.5*D* arrows) that were mostly likely invading immune cells. Since co-labeling of GFP and BrdU was such a rare event, the expression of Ascl1 and Lin28 appeared to have no effect on MG proliferation in these natural RD mouse models. The slow rate of degeneration compared to transient damage may not be enough to activate the MG to the extent where it reenters the cell cycle, and the constant baseline damage and activation experienced by these retinas may have rendered the MG unresponsive to Ascl1 and Lin28.

Establishing new AAV vectors for lineage tracing the MG

Through our experiments on the retinal degenerative mice, we found that the GFP labeled shH10 AAV viruses were not as specific to the MG as we once thought. We had shown in a previous study that the shH10 virus appeared to be greater than 95% specific to the MG when injected into the mouse eye (Schaffer, 2009). In this study, we found that the shH10 virus remains efficient *in vitro*, but has inconsistent expression *in vivo*. Seemingly unexplainable off-target expression occurred despite the maintenance of consistent DNA and viral quality. This off-target expression also seemed to be exacerbated with the coinjection of two viruses in a single eye. With the expression of a single fluorescent protein, we found a significant amount of off-target expression in the ganglion cell layer (Fig. 3.6*A* arrows). Strangely, with the expression of both shH10-CAG-GFP-2A-Ascl1 and shH10-CAG-mCherry-2A-Lin28 in a single eye, the off target expression appeared to spread to other cells such as amacrine cells (Fig. 3.6*B*) and even photoreceptor cells (Fig. 3.6*C*). On average, the shH10 virus expressing a single factor appeared to be less than 80% specific for the MG, while a double infection with the shH10 virus dropped this specificity to less than 30% (Fig. 3.6*D*). This difference in specificity may stem from the amount of total virus injected. With double injections, the total amount of each virus injected were invariably halved in order to allow room for the other virus. Nevertheless, the specificity of the AAV is largely determined by the capsid and promoter, as such, differences in titer and expressed DNA ultimately fail to explain this discrepancy.

Figure 3.5: P23H, RD10, and RD12 MG do not proliferate with the overexpression of Ascl1 and Lin28

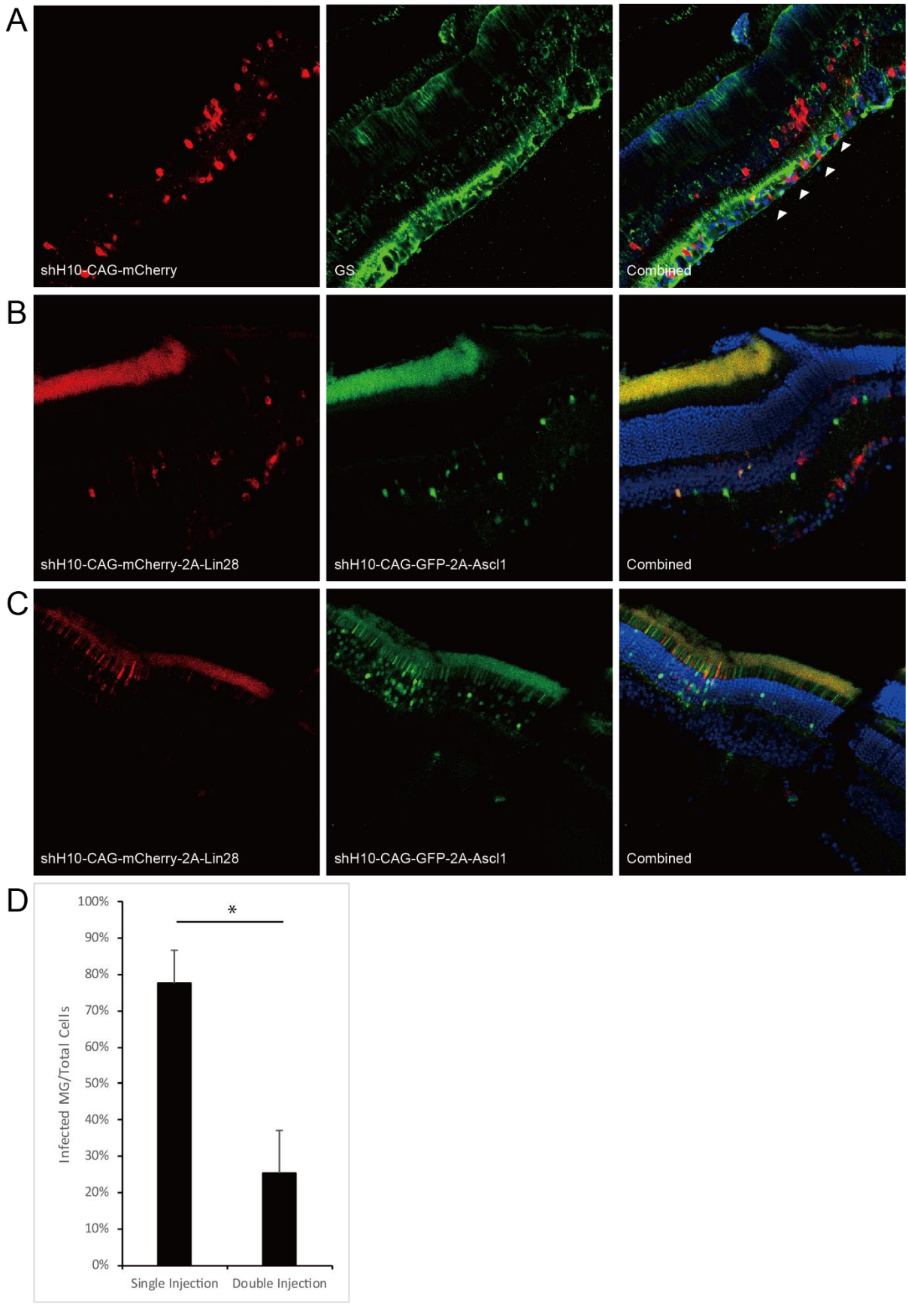
A, Experimental timeline. ***B,*** Recoverin labeling of the photoreceptor cell layer at the date of sacrifice. 1 layer of photoreceptors remain in the P23H retina while 1-3 layers remain in the RD10 retina. The RD12 retina appears to have retained most of its photoreceptors. ***C,*** Retina sections of P23H mice injected with shH10-CAG-GFP, shH10-CAG-GFP-2A-Ascl1, or shH10-CAG-Ascl1 and shH10-CAG-Lin28. Extremely small amounts of MG proliferation are visible in a few of the injected mice (arrows). ***D,*** Retina sections of RD10 mice injected with shH10-CAG-GFP, shH10-CAG-GFP-2A-Ascl1, and shH10-CAG-GFP-2A-Ascl1 and shH10-CAG-GFP-2A-Lin28. BrdU labeling mostly occurred in cells outside of the retinal layers (arrows). ***E,*** Retina sections of RD12 mice injected with shH10-CAG-GFP, shH10-CAG-GFP-2A-Ascl1, and shH10-CAG-Ascl1 and shH10-CAG-Lin28. Again, small amounts of proliferation are visible (arrows).


A
shH10 Injection
BrdU Injection
BrdU
Sacrifice
P15
P36
P40
P23H
RD10
RD12
B
Recoverin
DAPI
GFP
Ascl1
Ascl1 and Lin28
C
P23H
DAPI
BrdU
shH10-CAG-GFP
shH10-CAG-GFP-2A-Ascl1
GS
D
RD10
shH10-CAG-GFP-2A-Ascl1 and Lin28
E
RD12

In order to not only validate our previous findings with fluorescent tagged proteins but to also allow for a more consistent way to lineage trace the proliferating MG in the future, we set out to construct AAV that are more specific to the MG *in vivo*. Our interest first settled on the glia specific GFAP promoter that has been extensively utilized in the central nervous system. However, its large size of 2.2k base pairs prohibited its inclusion in the size limited AAV vector. However, a truncated version of the GFAP promoter named gfaABC$_1$D was determined to be efficient in expressing genes of interest in astrocytes (Brenner, 2008). In order to determine if the gfaABC$_1$D is efficient at expression in the retina *in vivo*, we constructed 7m8-gfaABC$_1$D fluorescent vectors for future study. The 7m8 AAV capsid variant was found to be efficient at expressing factors pan-retinally. With the 7m8- gfaABC$_1$D vector, we were able to observe seemingly 100% MG specificity *in vivo* (Fig 3.7*A*) not only with simple fluorescent vectors, but also with 2A peptide viruses expressing Ascl1 and Lin28 along with GFP (Fig 3.7*B*).

Figure 3.6: ShH10 fluorescent vectors show nonspecific expression in single and double infections

A, Retinal sections of 129/SVJ mice injected with shH10-CAG-mCherry virus. While specific mCherry expression was observed with GS staining, nonspecific ganglion cell expression was observed (arrows). ***B,*** Retinal sections of 129/SVJ mice injected with both shH10-CAG-GFP-2A-Ascl1 and shH10-CAG-mCherry-2A-Lin28 viruses. Nonspecific expression was observed in amacrine-like cells at the bottom of the INL. ***C,*** Similar retinal sections, but nonspecific photoreceptor expression was observed in the ONL, complete with GFP and mCherry expression in the long and thing outer segments. ***D,*** Quantification of MG specific expression. Single infection averaged 78% MG specificity while double infection averaged 25% specificity. $n = 3$ different mice. Error bars are SD. $*p < 0.05$


A
shH10-CAG-mCherry
GS
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B
shH10-CAG-mCherry-2A-Lin28
shH10-CAG-GFP-2A-Ascl1
Combined
C
shH10-CAG-mCherry-2A-Lin28
shH10-CAG-GFP-2A-Ascl1
Combined
D
Infected MG/Total Cells
100%
90%
80%
70%
60%
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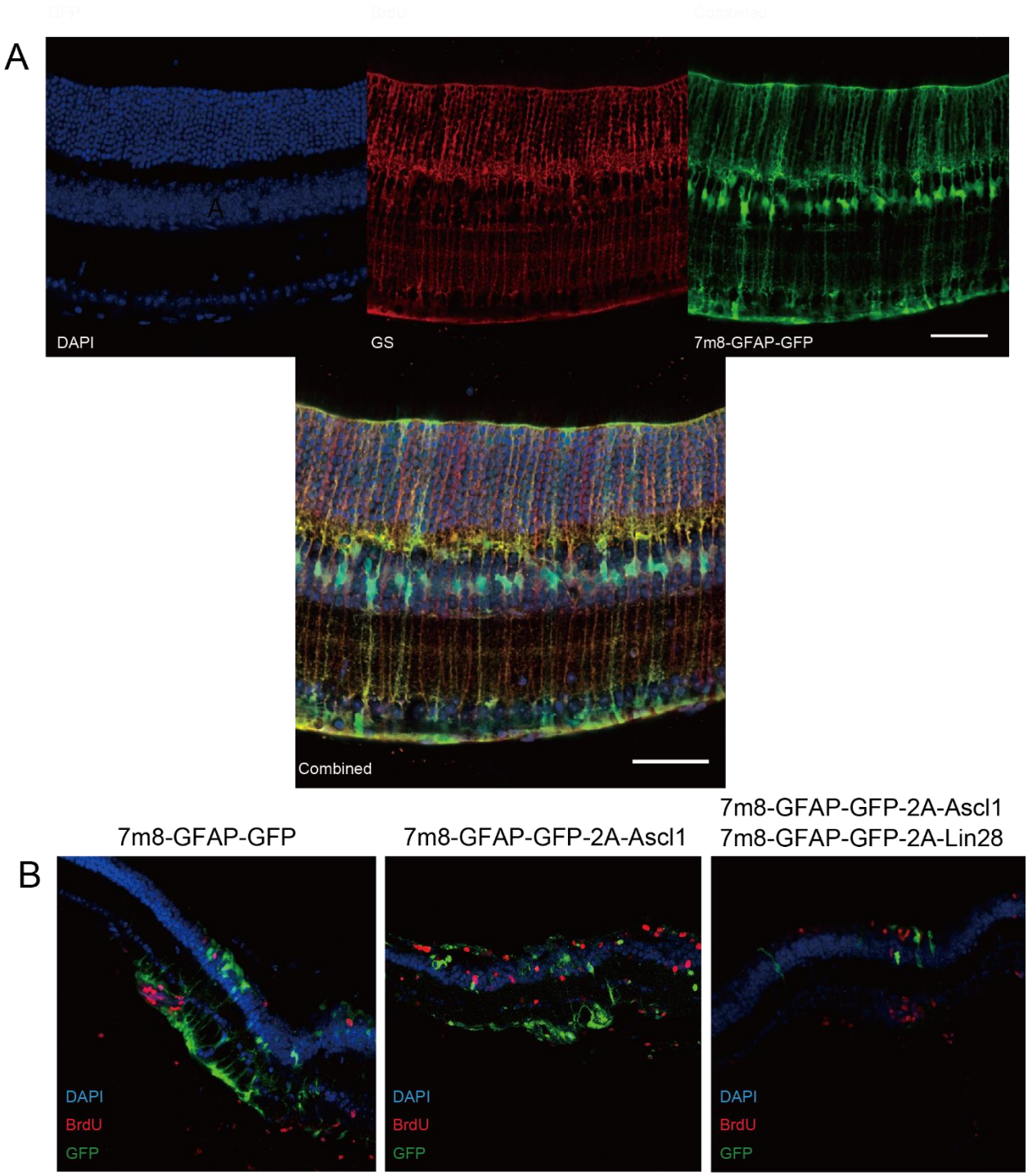

Figure 3.7: 7m8- gfaABC$_1$D viruses are MG specific *in vivo*
A, Retinal sections of wildtype C57BL6/J mice infected with 7m8- gfaABC$_1$D-GFP colabelled with GS. Viral expression appears 100% MG specific. ***B,*** Retinal sections of Aldh1l1-GFP mice (cobalt chloride damaged) expressing 7m8-gfaABC$_1$D-GFP, 7m8-gfaABC$_1$D-GFP-2A-Ascl1, and 7m8-gfaABC$_1$D-GFP-2A-Lin28 shows viral specificity even with 2A peptides and double infections. Strong BrdU labeling shows the effectiveness of these new viruses in enhancing damage-induced MG proliferation response.

3.4 Discussion

We have previously established an uninjured mouse model of MG proliferation brought about by a combinatorial treatment of Ascl1 and Lin28 overexpression. This proliferation was enhanced by NMDA damage and improved further through Notch suppression. Even though significant MG proliferation was only seen with retinal damage, this marked the first time that MG proliferation was observed in an uninjured mature mouse retina. Nevertheless, the NMDA induced ganglion cell specific damage model appeared incomparable to retinal damage brought about by natural degeneration. In response to this observation, we established retinal damage models that specifically target the photoreceptors. Using xenon lamp UV light as a damage source, we injured the albino 129/SVJ mouse retina to test if its MG's capability to proliferate *in vitro* also translated to a more active MG *in vivo.* While we were successful in establishing a MG proliferation response, the amount of proliferation was unexpectedly low. We hypothesized that the inconsistent damage brought about by the concentrated UV light was a limiting factor and attempted to use cobalt chloride as a source for pan-retinal photoreceptor damage.

Using a transgenic MG reporter mouse line, Aldh1l1-GFP, and increasing amounts of cobalt chloride, we showed that differing amounts of photoreceptor damage lead to different levels of MG proliferation without exogenous factor expression. This proliferation, however, seemed to reach an inherent limit before total photoreceptor ablation, and this limit was breached by Ascl1 and Lin28 overexpression. Unlike our previous experiments with NMDA, photoreceptor damage by itself induced the MG to reenter the cell cycle in a limited fashion. However, we were only able to test the damage models on the albino 129/SVJ and Aldh1l1-GFP mouse strains. Since 129/SVJ mice MG appeared to possess higher proliferative capabilities in *in vitro* dissociated cell cultures and in *ex vivo* retinal explants, it remains possible that both strains of mice possess certain expression profiles that result in MG that were more responsive to photoreceptor cell damage. We wish to explore this difference further in future studies through identifying the differences in MG expression patterns between different strains of mice and through including different retinal damage models.

Even though, conceptually, 129/SVJ mice along with photoreceptor apoptosis brought about by UV light and cobalt chloride exposure seemed primed to maximize the MG activation response, the amount of proliferation found ultimately fell short of what was previously observed with NMDA damage. With Ascl1 and Lin28 as the only factors responsible for facilitating a strong MG proliferation response, we believe that there are potent pro-proliferative factors yet to be identified. In addition, the ultimate glial fate of the daughter MG remained unchanged, and in order to promote endogenous regeneration, identification of new factors to push the proliferating MG towards a neuronal cell fate is necessary. We attempted to establish primary reporter MG cell lines with the dissociated MG of 129/SVJ and Aldh1l1-GFP mice. However, through RT-qPCR analysis, we found that cultured MG expression profile fluctuated dramatically over time and appeared to be an unreliable reporter system. In addition, Aldh1l1-GFP cultured cells eventually lost their GFP expression, further revealing the expression inconsistencies inherent with cultured MG. We hypothesize that, since MG proliferation

in vitro relies heavily on cell to cell contact, the dissociated state represents an extremely damaging/activating environment whereby MG cell expression becomes far removed from those of *in vivo* MG.

Disappointingly, we also found that mouse models carrying retinal degenerative mutations did not respond to exogenous Ascl1 and Lin28 treatment. Even though these mouse models carry mutations found in human RP, the degeneration associated with the P23H, RD10, and RD12 mouse line may be too slow compared to the damage models we explored earlier. While the P23H and RD10 mice eventually lost all photoreceptor function, due to photoreceptor apoptosis taking place over a period of months, retinal degenerative cell death at any one point of time was relatively low even when compared to cases of more rapid degeneration such as in RD11 mice (Pang, 2017). Despite the fact that we were able to elicit small amounts of MG proliferation in the C57BL/6J mice without damage, we weren't able to do so in the RD mice. Another explanation for this discrepancy would be the inherent genetic differences between mouse strains impeding the MG from reentering the cell cycle. Perhaps the low baseline damage experienced by the RD retina inhibited the MG from proliferating in response to Ascl1 and Lin28, or the baseline activation experienced by the MG instilled resistance to genetic reprogramming. We find that further experiments are warranted, and we plan to include different retinal degenerative models in the future.

Through our experiments with fluorescently tagged AAV vectors we found that the shH10 virus exhibited more off-target effects than previously thought. This off-target expression was dramatically increased by the addition of an extra virus within a single injection. A combination of 2A peptide interference and decreased viral titer may be at play. With the addition of an extra virus, the viral titers of the new and original viruses were invariably lowered due to volume constraints. However, it was not clear how the 2A peptide and a lower titer affected the specificity of an AAV virus. An additional theory is the existence of interference between .the different injected viruses. Perhaps the vectors themselves interacted with each other in some fashion to affect the specificity. Nevertheless, we constructed 7m8 pan-retinal vectors carrying MG specific truncated GFAP promoters to bypass this problem, and further experiments showed that the new viruses were not only more specific but also appeared to have higher infection rates. There may be additional studies to be done in order to elucidate the exact mechanism at play with the low specificity observed, but going forward, we will be utilizing this new suite of viruses for MG targeting experiments.

In a recent study, Crx, Otx2, and Nrl transcription factors had been shown to be effective in pushing proliferating MG towards a photoreceptor specific fate (Chen, 2018). After Wnt pathway induced MG proliferation, the combination of these three vectors appeared to change fluorescently labeled MG into photoreceptors. However, as previously mentioned in our experimental results, a combinatorial injection of shH10 viruses, which the study utilized, lead to off-target expression in not only amacrine cells and ganglion cells, but also photoreceptors. Worryingly, there appeared to be no transitional state between an infected mature MG and an infected mature photoreceptor, which raises doubts as to whether or not the fluorescence seen was fate

change or simple off-target infection. Nevertheless, the study has identified interesting avenues to pursue. By combining photoreceptor damage and Wnt activation while also using the 129/SVJ mouse line, it seems possible to push MG proliferation further. In addition, by utilizing the new 7m8 GFAP viruses, we could validate the effectiveness of the Crx, Otx2, and Nrl factors in pushing for photoreceptorigenesis.

3.5 Experimental Procedures

Animals

Animal studies were approved by the University of California Berkeley's Institutional Animal Care and Use Committee. Wild-type 129/SVJ mice were obtained from the Jackson Laboratory. Aldh1l1-GFP mice were a kind gift from UC Berkeley professor Daniela Kaufer. Rd10, Rd12, and P23H mice were from our breeding colonies.

AAV, intravitreal injection, retinal injury, and BrdU labeling

A Müller cell-specific AAV capsid variant, ShH10 was used to deliver genes to MG (Klimczak et al., 2009). GFP, mouse Ascl1 and human Lin28a under control of the CAG promoter were individually packaged into ShH10 AAV vectors. Recombinant ShH10 AAV was prepared as previously described (Klimczak et al., 2009; Flannery and Visel, 2013). Viral titers were between ~0.5×10^{13} to 5×10^{13} vg/ml and ~1.5 µl was intravitreally injected into ketamine and xylazine anesthetized postnatal day (P)15 mice using a Hamilton syringe equipped with a 33-gauge needle. Adult (P40) mice were anesthetized with ketamine and xylazine and received ~2 µl intravitreal injections of PBS containing 1 mg/ml BrdU, ±1, 5, or 8 mM cobalt chloride using a Hamilton syringe equipped with a 33-gauge needle. Mice received a second intravitreal injection of BrdU 3 d later; mice also received daily intraperitoneal injections of BrdU (50 µg/g body weight) for 3 d following cobalt chloride treatment.

UV light damage was induced via xenon arc lamp. Mice were anesthetized via ketamine and xylazine intraperitoneal injection and were put in an isolated chemical hood on heat pads. A xenon arc lamp was placed inside the chamber with a fiber optic cable and a focuser attached. The focuser was placed 10 inches from the head of the mouse and aligned with one eye. The UV light exposure was kept at 15 minutes. After exposure and allowing time for cooldown, the other eye was exposed for 15 minutes. Proper protection was used including UV shields, face masks, tinted goggles, and extra layer PPE.

The pan-retinal 7m8 AAV capsid variant was used to deliver genes to MG as well (Dalkara, 2013). GFP, mouse Ascl1, human Lin28a, GFP-2A-Ascl1, and GFP-2A-Lin28 under the control of the shortened GFAP promoter gfaABC$_1$D were individually packaged into 7m8 AAV vectors prepared as previously described. Viral titers were between ~0.5×10^{13} to 5×10^{13} vg/ml and ~1.5 µl was intravitreally injected into ketamine and xylazine anesthetized postnatal day (P)15 mice using a Hamilton syringe equipped with a 33-gauge needle. Adult (P40) mice received daily intraperitoneal

injections of BrdU (50 μg/g body weight) for 3 days. The gfaABC$_1$D promoter is a kind gift from Sergey Kasparov (Addgene plasmid #19974).

RNA isolation and PCR

Total RNA was isolated using a total RNA minikit (Qiagen). Samples were reverse transcribed with Superscript III (Invitrogen). Real-time qPCRs were performed in triplicate with ABsolute SYBR Green Fluorescein Master Mix (Thermo Scientific) on an iCycler real-time PCR detection system (Bio-Rad). The ΔΔ*Ct* method was used to determine relative expression of mRNAs in control and injured retinas and normalized to *gapdh* or γ-*actin* mRNA levels. Individual comparisons were done using unpaired two-tailed Student's *t* test. ANOVA with Fisher's PLSD *post hoc* analysis was used for multiple-parameter comparison. Error bars are SD.

Primers used in this study

The following primers are 5′ to 3′. Unless otherwise indicated primers are for mice.
Ascl1
Forward: GCAACCGGGTCAAGTTGGT
Reverse: GTCGTTGGAGTAGTTGGGGG
Lin28
Forward: TGCGGGCATCTGTAAGTGG
Reverse: GGAACCCTTCCATGTGCAG
NeuroD4
Forward: AGGAGCAAGAGAGAAGACCG
Reverse: CTTGGGACCTCTTCTTTTAGGT
Beta Actin
Forward: GGCTGTATTCCCCTCCATCG
Reverse: CCAGTTGGTAACAATGCCATGT
Tuj1
Forward: TCTTTGGTCAGAGTGGTGCT
Reverse: ATTCTCACACTCTTTCCGCAC
Glutamine Synthetase
Forward: GGATAGCCCGTTTTATCTTGC
Reverse: GTGGTACTGGTGCCTCTTGC
GFAP
Forward: ACCTCCAGATCCGAGAAACC
Reverse: CCTTAATGACCTCACCATCCCG
Nestin
Forward: AGTTCTACTGGTGTCCTCCC
Reverse: TAGAGTGGTGAGGGTTGAGG
Atoh7
Forward: GCTGTCCAAGTACGAGACAC
Reverse: AGGTAGACCCTGAGCCCTA
GFP
Forward: AGTCCGCCCTGAGCAAAGA

Reverse: TCCAGCAGGACCATGTGATC

TUNEL staining, Immunohistochemistry, and microscopy

We used an *in situ* Cell Death Detection Kit (TMR red; Applied Science) to detect cells undergoing apoptosis.

Mouse retinas were surgically removed from enucleated eyes and fixed for 12–18 hours in 4% paraformaldehyde. Afterwards, the retinas were embedded in 5% agarose and sectioned via vibrotome to 100 µm thickness. For staining, the sections were first immersed in 0.5% Triton-X100 in PBS, washed, and then incubated with 10% goat serum, 1%BSA, and 0.1% Triton-X100 in PBS for an hour. The sections were then incubated with the primary antibodies overnight at 4°C. For secondary antibody incubation, the sections were washed and incubated with the secondary in PBS for an hour. The sections were then mounted with DAPI for imaging. The Primary antibodies used in this study: anti-glutamine synthetase (GS), EMD Millipore, catalog #MAB302 (1/500); anti-BrdU, Abcam, catalog #ab6326 (1/500); anti-SOX9, EMD Millipore, catalog #AB5535 (1/500); anti-Recoverin, Abcam, catalog #ab85292. Secondary antibodies: AlexaFluor 488 goat anti-rat-IgG (H+L), ThermoFisher, catalog #A11006 (1:1000); AlexaFluor 594 goat anti-rat IgG (H+L), ThermoFisher, catalog #A11007 (1:1000); AlexaFluor 488 goat anti-rabbit IgG (H+L) ThermoFisher, catalog #A11036 (1:1000); AlexaFluor 594 goat anti-rabbit IgG (H+L) ThermoFisher, catalog #A27016 (1:1000); AlexaFluor 488 goat anti-mouse IgG (H+L), ThermoFisher, catalog #A28175 (1:1000); and AlexaFluor 633 goat anti-rat IgG (H+L), ThermoFisher, catalog #A21094 (1:1000). Images were captured by a Zeiss LSM710 confocal microscope courtesy of the UC Berkeley Bioimaging Facility Core and analyzed via Zen (Zeiss) and ImageJ.

Primary MG Culture and lentiviral selection

Mice were euthanized with carbon dioxide according to UC Berkeley animal use protocol. The eyes were enucleated and the retinas extracted surgically. The retinas were submerged in 0.05% Trypsin-EDTA at 37°C and triturated lightly over 15 minutes. The dissociated cells were then spun down and suspended in 1:1 DMEM/F12 with 10% FBS. The cells were plated onto poly-D-lysine coated plates and left for one week in an incubator. None-adherent cells were washed and the cells were left to grow to confluence.

The lentiviral packaging system were a gift from the Schaffer lab. The packaging plasmids were transfected via PEI into HEK293T cells. The resulting supernatant were harvested over 4 days, and the mixture was spun down by ultracentrifugation at >24,000 RPM. The lentivirus pellet was washed and suspended in PBS for infection.

Primary MG cells were infected with the purified lentivirus over 3 days. Afterwards, puromycin was added to the media at 5µg/ml. The selection took place over 10 days, at the end of which the cells were split and allowed to grow. After confluency, doxycycline

was added at 1µg/ml. The cells were cultured in doxycycline conditions for three days before harvest.

Cell quantification and statistical analysis

BrdU immunofluorescence was used to identify and quantify proliferating cells in retinal sections as previously described (Fausett and Goldman, 2006; Ramachandran et al., 2010; Wan et al., 2012, 2014). All experiments were done in triplicate with three animals per trial. Error bars are SD. ANOVA with Fisher's PLSD *post hoc* analysis was used for multiple-parameter comparison; two-tailed Student's *t* test was used for single-parameter comparison

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3.7 References

Ueki Y, Wilken MS, Cox KE, Chipman L, Jorstad N, Sternhagen K, Simic M, Ullom K, Nakafuku M, Reh TA. (2015) Transgenic Expression of the Proneural Transcription Factor Ascl1 in Müller Glia Stimulates Retinal Regeneration in Young Mice. Proc of the Nat Acad of Sci. 112(44):13717-13722. 10.1073/pnas.1510595112

Seitz R, Tamm ER. (2012) N-Methyl-D-Aspartate (NMDA)-Mediated Excitotoxic Damage: A Mouse Model of Acute Retinal Ganglion Cell Damage. Methods in Molecular Biology: Retinal Degeneration. 935:99-109. 10.1007/978-1-62703-080-9_7

Youssef PN, Sheibani N, Albert DM. (2011). Retinal Light Toxicity. Eye (Lond). 25(1):1-4. 10.1038/eye.2010.149

Hara A, Niwa M, Aoki H, Kumada M, Kunisada T, Oyama T, Yamamoto T, KOzawa O, Mori H. (2006) A New Model of Retinal Photoreceptor Cell Degeneration Induced by a Chemical Hypoxia-Mimicking Agent, Cobalt Chloride. Brain Res. 1109(1):192-200. 10.1016/j.brainres.2006.06.037

Yao K, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, Liu X, Chang B, Zenisek D, Crair MC, Demb JB, Chen B. (2018) Restoration of Vision After de Novo Genesis of Rod Photoreceptors in Mammalian Retinas. Nature. 560:484-488. 10.1038/2Fs41586-018-0425-3

Suga A, Sadamoto K, Fujii M, Mandai M, Takahashi M. (2014) Proliferation Potential of Müller Glia After Retinal Damage Varies Between Mouse Strains. PLOS One. 10.1371/journal.pone.0094556

Srinivasan R, Lu TY, Chai H, Xu J, Huang BS, Golshani P, Coppola G, Khakh BS. (2017) New Transgenic Mouse Lines for Selectively Targeting Astrocytes and for Studying Clacium Signals in Astrocyte Processes In Situ and In Vivo. Neuron. 92(6):1181-1195. 10.1016/j.neuron.2016.11.030

Klimczak RR, Koerber JT, Dalkara D, Flannery JG, Schaffer DV. (2009) A Novel Adeno-Associated Viral Variant for Efficient and Selective Intravitreal Transduction of Rat Müller Cells. PLOS One. 10.1371/journal.pone.0007467

Sakami S, Maeda T, Bereta G, Okano K, Golczak M, Sumaroka A, Roman AJ, Cideciyan AV, Jacobson SG, Palczewski K. (2011) Probing Mechanisms of Photoreceptor Degeneration in a New Mouse Model of the Common Form of Autosomal Dominant Retinitis Pigmentosa Due to P23H Opsin Mutations. Journal of Bio Chem. 286:10551-10567. 10.1074/jbc.M110.209759

Garqini C, Terzibasi E, Mazzoni F, Strettoi E. (2007) Retinal Organization in the Retinal Degeneration 10 (Rd10) Mutant Mouse: A Morphological and ERG Study. J Comp Neurol. 500(2):222-238. 10.1002/cne.21144

Pang JJ, Chang B, Hawes NL, Hurd RE, Davisson MT, Li J, Noorwez SM, Malhotra R, McDowell JH, Kaushal S, Hauswirth WW, Nusinowitz S, Thompson DA, Heckenlively JR. Retinal Degeneration 12 (Rd12): A New, Spontaneously Arising Mouse Model for Human Leber Congenital Amaurosis (LCA). Molecular Vision. 11:152-162

Hippert C, Graca AB, Barber AC, West EL, Smith AJ, Ali RR, Pearson RA. (2015) Müller Glia Activation in Response to Inherited Retinal Degeneration Is Highly Varied and Disease-Specific. PLOS One. 10(3):e0120415.

Lewis JE, Brameld JM, Hill P, Barrett P, Ebling FJP, Jethwa PH. (2015) The Use of a Viral 2A Sequence for the Simultaneous Over-Expression of Both the VGF Gene and Enhanced Green Fluorescent Protein (eGFP) IN Vitro and In Vivo. J Neurosci Methods. 256:22-2

Lee Y, Messing A, Su M, Brenner M. (2008) GFAP Promoter Elements Required for Region-Specificity and Astocyte-Specific Expression. Glia. 56(5):481-493. 10.1002/glia.20622

Zhang H, Li X, Dai X, Han J, Zhang Y, Qi Y, He Y, Liu Y, Chang B, Pang JJ. (2017) The Degeneration and Apoptosis Patterns of Cone Photoreceptors in Rd11 Mice. Journal of Ophthalmology. Vol. 2017, Article ID 9721362, 13 pages. 10.1155/2017/9721362

Flannery JG, Visel M. (2013) Adeno-Associated Viral Vectors for Gene Therapy of Inherited Retinal Degenerations. Methods in Molecular Biology. 935:351-369. 10.1007/978-1-62703-080-9_25

Dalkara D, Byrne LC, Klimczak RR, Visel M, Yin L, Merigan WH, Flannery JG, Schaffer DV. (2013) In Vivo-Directed Evolution of a New Adeno-Associated Virus for Therapeutic Outer Retinal Gene Delivery from the Vitreous. Science Translational Medicine. 189(5):189ra76. 10.1126/scitranslmed.3005708

Fausett BV, Goldman D. (2006) A Role for Alpha1 Tubulin-Expression Müller Glia in Regeneration of the Injured Zebrafish Retina. J Neurosci. 26(23):6303-6313. 10.1523/JNEUROSCI.0332-06.2006

Ramachandran R, Fausett BV, Goldman D. (2010) Ascl1a Regulates Müller Glia Dedifferentiation and Retinal Regeneration Through a Lin-28-Dependent, Let-7 MicroRNA Signalling Pathway. Nat Cell Biol. 12(11):1101-1107. 10.1038/ncb2115