Stimulating retinal neurogenesis from Müller glia

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ABSTRACT

Stimulating retinal neurogenesis from Müller glia

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Damage to the retina, through disease or injury, can lead to the permanent loss of neurons and ultimately loss of vision. Non-mammalian vertebrates have a robust ability to regenerate injured retinal neurons from Müller glial cells that activate the gene encoding the proneural factor Achaete-scute homolog 1 (Ascl1) and de-differentiate into progenitor cells. By contrast, mammalian Müller glia have a limited regenerative response and fail to upregulate *Ascl1* after injury. In this work, I explored whether neurogenic transcription factors, including Ascl1, could restore neurogenic potential to mammalian Müller glia by overexpressing these factors in dissociated mouse Müller glial cultures and intact retinal explants. ASCL1-infected Müller glia upregulated retinal progenitor-specific genes and downregulated glial genes. Furthermore, ASCL1 remodeled the chromatin at its targets from a repressive to an active configuration. Müller glia-derived progenitors differentiated into cells that exhibited neuronal morphologies, expressed retinal subtype-specific neuronal markers and displayed neuron-like physiological responses. Additional neurogenic activators potentiated some of these effects. These results indicate that neural-

promoting transcription factors can induce a neurogenic state in mature Müller glia, providing an alternative strategy for repair of the retina after disease or injury.

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Chapter 1:

Introduction

The retina is a 0.5mm thick neural structure that lines the back of the eye. The retina has a common architecture across vertebrates, including three nuclear layers comprised of all the nuclei of the resident neurons and glia (Figure 1.1). The outer nuclear layer (ONL) contains the photoreceptor cells: the rods, responsible for low light level vision, and the cones, responsible for color detection and bright light level vision. The photoreceptors convert light energy into electrical signals through phototransduction and pass these signals on to bipolar interneurons in the inner nuclear layer (INL). Rod bipolar cells connect with rod photoreceptors, and cone bipolar cells are classified as "ON" or "OFF" type depending on whether the circuit they form depolarizes or hyperpolarizes in response to light increments. The bipolar neurons in turn synapse onto "ON" or "OFF" type retinal ganglion cells (RGCs) that reside in the ganglion cell layer (GCL) and send their axons to visual centers in the brain. Two synaptic layers, the inner plexiform layer (IPL) and the outer plexiform layer (OPL), contain all of the connections between these cells. There is one predominant glial cell in the retina, the Müller glia, whose cell body resides in the INL and processes span the width of the retina. Müller glia serve vital support functions in the retina which will be discussed at length in Chapter 2. Finally, the retina is associated with astrocytes, which reside in the nerve fiber layer, and retinal pigment epithelial cells (RPE), which reside in a layer adjacent to the retina and are vital to the health of neighboring photoreceptors. However, neither of these cell types is generated from retinal progenitor cells during development.

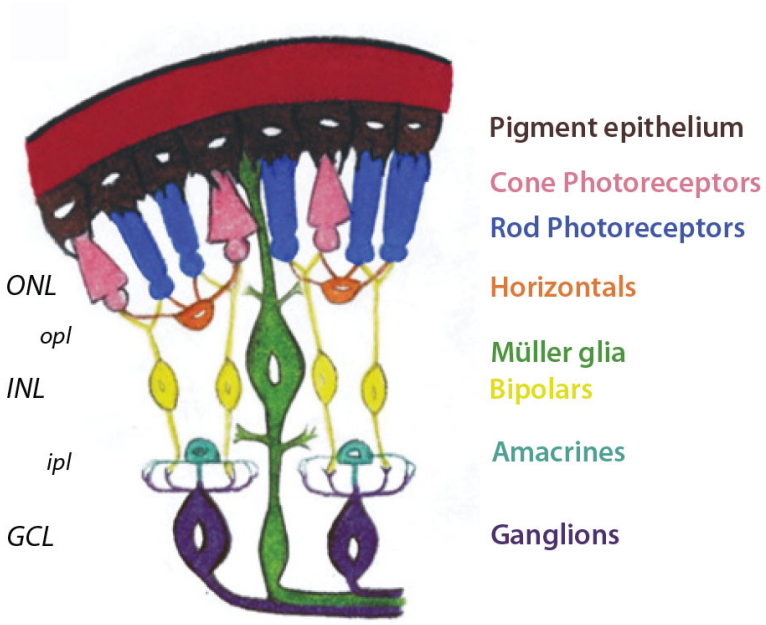


Figure 1.1. Schematic of the architecture of the retina. The Müller glia and the six sub-types of neurons reside in the three nuclear layers of the retina. The retinal pigment epithelial layer lies just outside the retina. ONL, outer nuclear layer; INL, inner nuclear layer, GCL, ganglion cell layer, opl, outer plexiform layer, ipl, inner plexiform layer.

Review of retinal histogenesis

Retinal development in mammals initiates from an evagination of the neural tube, which forms a two-layered structure, the optic cup, lined by the developing retina on its inner surface. The early retina is composed of neuroepithelial progenitor cells, which are multipotent cells that give rise to all of the retinal cell types (Turner et al., 1990). Birth-dating studies have demonstrated that there is a consistent temporal order to this histogenesis. “Early” cell types - ganglion, cone photoreceptor, and horizontal cells - are born first, and “late” cell types – amacrine, bipolar, and rod photoreceptor cells - are born in a second phase (Marquardt and Gruss, 2002). Müller glia are the last cell type generated and are born during the progenitor cell’s terminal cell division.

The specification of these distinct cell types is regulated by both extrinsic cues as well as intrinsic mechanisms, such as transcriptional regulation.

Neurogenesis

Basic helix-loop-helix (bHLH) transcription factors play a central role in regulating neuronal versus glial fates in the central nervous system (CNS) as a whole (Cai et al., 2000; Nieto et al., 2001) as well as in the retina (reviewed in Ohsawa and Kageyama, 2008 and Hatakeyama et al., 2004). Neurogenesis in the retina is driven to a large extent by positively acting proneural bHLH factors, including *Ascl1/Mash1, Neurog2, Neurod1, Neurod4/Math3,* and *Atoh7/Math5*.

Misexpression of bHLH genes in the developing retina promotes neuronal fates at the expense of glia, while loss-of-function studies exhibit increased numbers of Müller glia and a reduction in neurons. For instance, *Atoh7* is required for ganglion cell fate specification; *Atoh7*-null mutants have reduced numbers of ganglion cells and increased numbers of amacrine cells (Brown et al., 2001). Similarly, *Ascl1* misexpression in the embryonic retina augments the number of bipolar (Hatakeyama et al., 2001) or photoreceptor cells (Akagi et al., 2004) at the expense of Müller glia. Although *Ascl1*-null mice do not have profound changes in cell type numbers, *Ascl1-Neurod4* null mice generate fewer bipolar cells and increased numbers of Müller glia (Tomita et al., 2000). Likewise, *Neurod1* and *Neurod4* are required for amacrine fate specification. Although neither is sufficient alone to drive the amacrine program, progenitors in double mutants switch cell fates from amacrine to ganglion fates (Inoue et al., 2002). These studies suggest that each bHLH gene is insufficient alone to drive the neuronal program -

perhaps due to compensation by other proneural fate determinants - but that combinations of proneural genes are instead required for fate specification.

Gliogenesis

Gliogenesis is regulated by an alternative set of extracellular and intracellular molecules (Vetter and Moore, 2001; Jadhav et al., 2009). CNTF, LIF, and EGF (Lillien, 1995) have all been implicated in stimulating Müller glial genesis from progenitors. The Notch pathway also plays a central role in this process. Gain-of-function experiments suggest that Notch can promote the glial fate while repressing neuronal fates (Furukawa et al., 2000), while loss-of-function data suggest a required role for Notch in gliogenesis and subsequent overproduction of neurons (Jadhav et al., 2006; Yaron et al., 2006). Similar results were observed at later periods of development during the phase of Müller glial maintenance; blocking Notch activity at P12 led to a loss of Müller glia while promoting aberrant expression of the proneural gene *Ascl1* (Nelson et al., 2011).

Notch's negatively acting effectors *hairy and enhancer of split* genes, *Hes1, Hes5* and *Hesr2*, also play instructive roles in glial fate determination. *Hes1* misexpression in the developing rat retina increased the number of Müller glia, while knockdown using a dominant negative form of *Hes1* decreased Müller glia genesis (Furukawa et al, 2000). Likewise, forced expression of *Hes5* in the developing retina decreased the number of photoreceptors while increasing the number of Müller glia (Hojo et al., 2000). In embryonic mouse retina, *Hesr2/Hey2* was similarly shown to increase the number of Müller glia (Satow et al., 2001). It is thought that *Hes1* and *Hes5* carry out these roles by repressing proneural bHLH factors, such as *Ascl1*, that normally instruct the neuronal

fate. As a whole, these reports suggest that the balance between proneuronal and negatively-regulating bHLHs regulate the correct ratios of retinal neurons and glia.

The mammalian retina becomes gliotic in response to disease

The retina is susceptible to a number of diseases that lead to a loss of neurons, which are often the susceptible light-sensing photoreceptors (reviewed in Ramsden et al., 2013). Age-related macular degeneration (AMD) affects 1.75 million people in the United States in its advanced form and is the leading cause of irreversible blindness in the world. The degeneration targets the RPE cells, which leads to a loss of support for photoreceptors and their eventual death in the most visually sensitive region of the retina, the central macula (Figure 1.2). Retinitis pigmentosa (RP) and Stargardt's disease are two inherited forms of retinal disease, which both target and lead to the death of photoreceptors. Both progress early and are common in young children, though there are few treatment options.

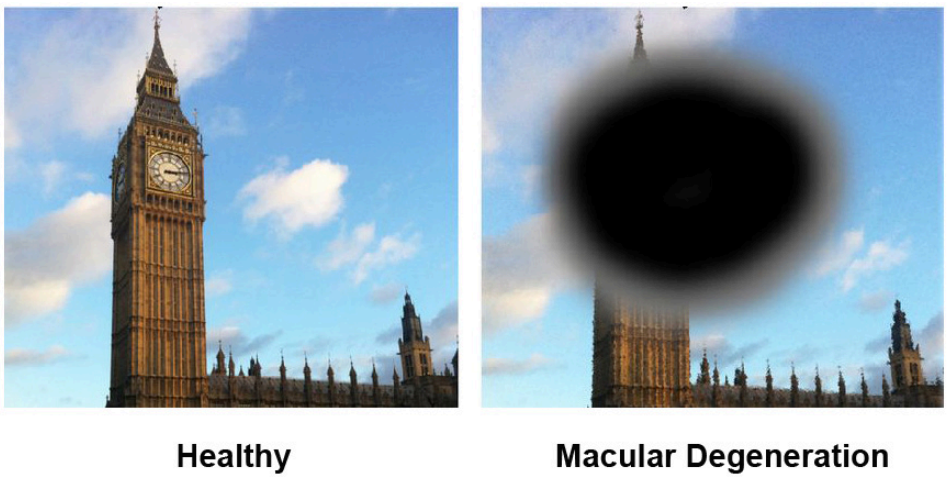


Figure 1.2. Vision loss in retinal disease. Vision is first lost in the central portion of the retina and correlating visual field in patients with age-related macular degeneration (right) compared with healthy patients (left). Adapted from Ramsden et al., 2013.

Unlike non-mammalian vertebrates, mammals do not have an inherent ability to repair the retina after injury from disease. Instead, Müller glia respond to dying and damaged neurons by becoming reactive and undergoing the process of gliosis (Bringmann et al., 2006; 2009; Bringmann and Wiedemann, 2012; Dyer and Cepko, 2000). The gliotic response is defined by a number of distinct characteristics. Initially Müller glia upregulate intermediate filament proteins nestin, vimentin, and GFAP, the latter of which is a "universal early cell marker for retinal injury". Müller glia also undergo hypertrophy and re-enter the cell cycle to divide.

Consensus contends that there are two types of gliosis, proliferative and non-proliferative. Proliferative gliosis is thought to be detrimental and impair tissue repair. It is characterized by uncontrolled proliferation that forms a mass or glial scar. Müller glia also release pro-inflammatory cytokines, such as TNFα, and other substances that contribute to excitotoxicity. Non-proliferative gliosis is associated with the protective functions of Müller glia, and is characterized by GFAP upregulation, hypertrophy, and limited proliferation. Protective effects of gliosis include the release of neuroprotective molecules to shield neurons from death, uptake of excess glutamate that promotes excitotoxicity, potassium buffering, and phagocytosis of harmful substances.

These deleterious processes and the presence of the glial scar are partially responsible for blocking regenerative and repair processes after injury in mammals. Glial processes, which form a fibrotic layer, and hypertrophied Müller glia, which invade the empty retinal space, prevent migration and the integration of new neuronal synaptic contacts. Inhibitory extracellular matrix and cell adhesion molecules are expressed on the surface of reactive Müller glia, and there is some evidence that inhibition of these

molecules allows for a more permissive environment for repair. Müller glia do, however, contribute somewhat to the repair response; their processes form guiding structures for migrating neurons and the sprouting of new neural processes. This suggests that, if we can find ways to stimulate these repair processes while overcoming the gliotic response barriers, mammalian Müller glia could take part in a regenerative response as they do in non-mammalian vertebrates.

Comparison of endogenous retinal repair mechanisms across vertebrate species

In contrast to mammals, many non-mammalian vertebrates, including amphibians, fish, and birds, have the ability to regenerate new neurons after retinal injury (Figure 1.3; reviewed in Karl and Reh, 2010). The processes of neurogenesis during retinal development are recapitulated during endogenous regenerative processes after injury. Here, I will compare these repair processes across non-mammalian species. The sources of these repair responses are the RPE or Müller glial cells. Although the peripheral ciliary marginal zone (CMZ) contributes to the addition of new retinal cells throughout the life of amphibians, fish, and birds, there is no conclusive evidence that it substantially contributes to regeneration in any species and so will not be discussed here.

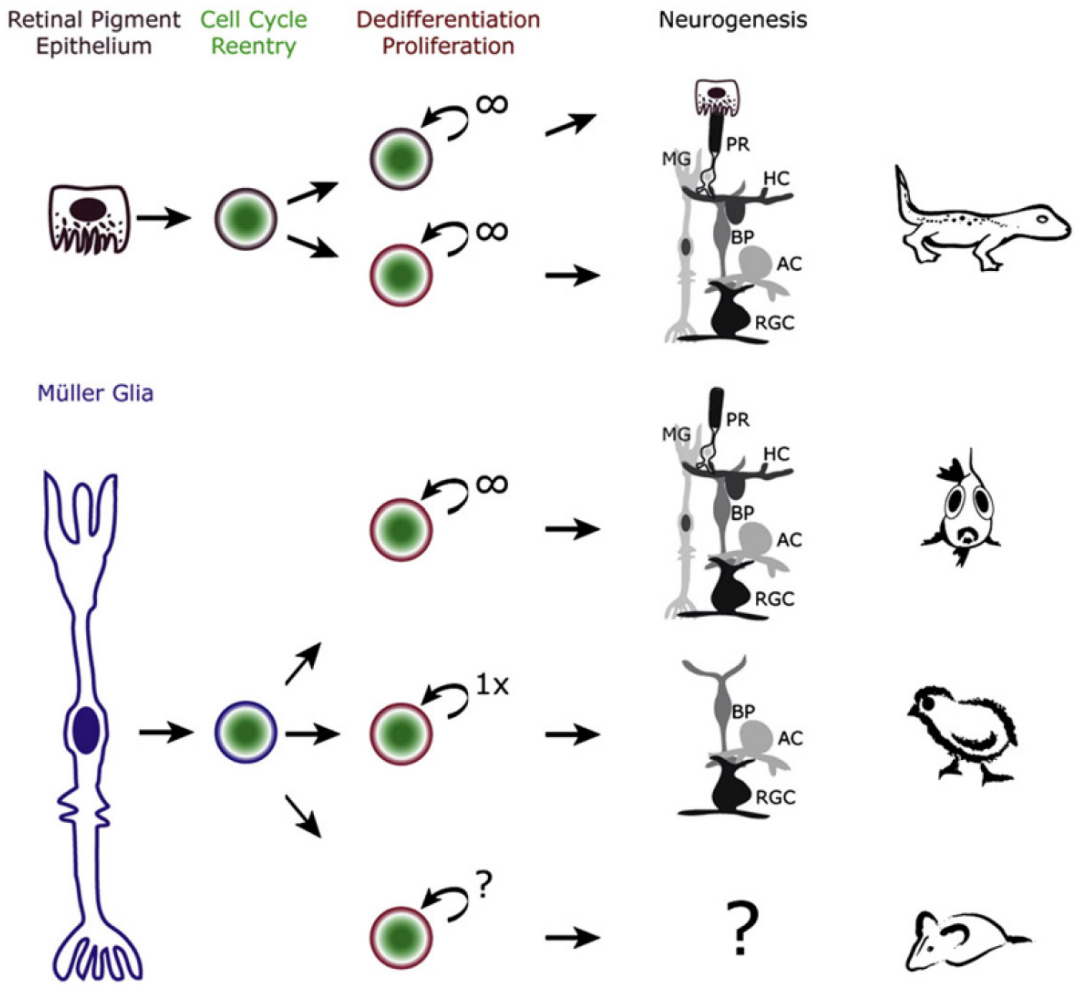


Figure 1.3. Retinal regeneration across vertebrate species. After damage, RPE cells or Müller glia de-differentiate, proliferate, and form new retinal neurons. In amphibians, RPE cells transdifferentiate into all subtypes of retinal neurons while also generating new RPE cells. In fish, mice, and chick, Müller glia serve as the main source of new neurons after injury. Whereas in the fish progenitors proliferate multiple times and generate all retinal sub-types, in the chick progenitors only go through one round of division and generate a restricted subset of retinal neurons. Little regeneration is observed in the mouse without experimental interventions. Lamba et al., 2008.

Amphibians

Amphibians - certain frogs, newts, and salamanders - have a robust ability to regenerate many tissues, including the retina (Chiba, 2013; Moshiri et al., 2004; Barbosa-Sabanero et al., 2012; Araki, 2007). The source of this response is the RPE cells, which transdifferentiate into new retinal cells following removal of the retina. The RPE cells first lose their pigment, re-enter the cell cycle, and form a new pigmented and non-pigmented layer. The cells in the non-pigmented layer de-differentiate to express progenitor markers and in turn differentiate into all of the different types of retinal cells to form a new fully functional retina.

Fish

Fish have a similar capacity for forming a whole newly functional retina, although this process does not involve the RPE cells. Instead, Müller glia (and to some extent rod precursors) serve as the source of new neurons (Raymond et al., 2006; Bernardos et al., 2007; Fausett et al., 2008; Thummel et al., 2008; Ramachandran et al., 2010). After chemical, light, or surgical damage, initially quiescent Müller glia re-enter the cell cycle and de-differentiate to form multipotent progenitors. These progenitors give rise to all retinal neuron sub-types and can position into appropriate retinal layers, integrate into the existing circuitry, and contribute to restored vision (Lindsey and Powers, 2007; Mensinger et al., 2007; Sherpa et al., 2007).

A number of molecular pathways have recently been shown to be required for Müller glial-based regeneration in the fish. Several extracellular signals contribute to initial dedifferentiation, cell cycle re-entry, or differentiation. Lenkowski et al. (2013)

recently demonstrated that repression of Smad2/3-mediated TGFβ signaling was required for the proliferative response of Müller glia after light-induced damage to photoreceptors. This repression was mediated by the corepressors Tgif1 and Six3b, which are upregulated in Müller glia early after injury and are involved in maintaining stem cell identity in other tissues. Another extracellular signaling molecule, tumor necrosis factor α (TNFα), is a pro-inflammatory cytokine that is expressed by dying photoreceptors and in Müller glia later in regeneration (Nelson et al., 2013). TNFα signals Müller glia to proliferate through the activation of Stat3 as well as Ascl1a (discussed below) (Nelson et al., 2012; 2013). Additionally, heparin-binding epidermal-like growth factor (HB-EGF) is necessary and sufficient for the regenerative process (Wan et al., 2012). It acts through the EGFR/MAPK pathway, which induces regeneration-associated genes as well as Wnt, which is involved in the proliferative response of Müller glia (Wan et al., 2012; Das, et al., 2006). Interestingly, TNFα is the only demonstrated extracellular signaling molecule that is expressed by dying neurons, and so it is still unclear what are the "damage signals" that trigger Müller glia regeneration.

Intrinsic factors also play vital roles during retinal regeneration in the fish. Calinescu et al. (2009) found that Müller glia express midkine-a and -b, two factors that are expressed normally during retinal development. Qin et al., (2009) found that two genes required for regeneration in the fin and heart, *hspd1* and *mps1*, were similarly upregulated in Müller Glia following injury; mutants for these genes failed to proliferate and regenerate cone photoreceptors following injury.

Recently, the proneural transcription factor Ascl1a was demonstrated to be required for fish retinal regeneration, and a key point of convergence for many of these

extracellular and intracellular pathways. *ascl1a* was upregulated in proliferating Müller glia within six hours post-injury, and *ascl1a* knockdown blocked Müller glia proliferation and de-differentiation into progenitors (Fausett et al., 2008; Ramachandran et al., 2010; 2012; Wan et al., 2012). It was further demonstrated that Ascl1a acted upstream of the RNA-binding protein Lin-28 and its downstream target for repression *let-7*, which in turn repressed genes associated with retinal regeneration, including *hspd1* and *c-myc* (Ramachandran et al., 2010). This group further demonstrated that Ascl1a is upstream of the repressor *insm1a*; this pathway drove wnt signaling and triggered Müller glia de-differentiation during the initial regenerative phase while triggering cell cycle exit/differentiation during the later phase by repressing cell-cycle genes (Ramachandran et al., 2012). Further evidence has suggested that Ascl1a elicits some of its effects through epigenetic modification. Two cytidine deaminases, *apobec2a* and *apobec2b*, were expressed in dedifferentiating Müller glia after injury and required for Müller glial proliferation (Powell et al., 2012). Interestingly, Ascl1a was required for *apobec2b* expression, although this was independent of the Lin-28 pathway. Taken together, it appears that *ascl1a* is turned on early after damage in Müller glia and a key factor for relieving repression on regeneration-associated genes.

Birds

The avian retina also has an inherent ability to regenerate and can accomplish this from both RPE and Müller glia cells. Similar to amphibians, RPE cells in the chick will transdifferentiate to form new neurons after removal of the retina, and form a new properly laminated retina with the correct ratios of cell types (Moshiri et al., 2004,

Barbosa-Sabanero et al., 2012). However, one major difference from the amphibian is that the retina's orientation is inverted. Many of the factors responsible for the regenerative process in the avian retina have not been elucidated, however FGF, BMP, and hedgehog pathways are involved.

Although RPE cells do serve as a source of new neurons, similar to fish, Müller glia serve as the primary source of regeneration in the chick. Following intravitreal administration of neurotoxins, such as N-Methyl-D aspartic acid (NMDA), Müller glia re-enter the cell cycle and regenerate new neurons (Fischer and Reh, 2001). However, this process differs from the fish in key ways (Karl and Reh, 2010). Müller glia only undergo one round of cell division, as opposed to the multiple rounds that the fish undergoes. Additionally, only a small number of Müller glia go on to give rise to new neurons, whereas most do in the fish. Finally, the types of retinal cells that can be produced are limited to amacrine, bipolar, and ganglion cells. Despite this, there are similarities between the two species; Müller glia de-differentiate to express many of the same neural progenitor genes, including *Chx10, Pax6, Ascl1a, Foxn4, Notch1, Dll1* and *Hes5*. However, most of these new cells remain in a progenitor state and fail to fully differentiate.

Review of current strategies to overcome limits to regeneration and repair the mammalian retina

As discussed earlier, mammals have a limited capacity for regeneration, and instead undergo the process of gliosis. However, recent studies have suggested that a

latent potential for cell replacement does exist. This potential can be strengthened by various experimental interventions.

Cell transplantation

Transplantation of stem cell-derived retinal cells is one route to replace injured cells in the mammalian retina (reviewed in Lamba et al., 2008). Dissociated photoreceptors derived from the immature retina have successfully integrated into the mouse retina (Maclaren et al., 2006). These cells were dissociated from the retinas of GFP-expressing mice in the first postnatal week – much older or younger cells did not integrate properly. GFP+ cells migrated into the outer nuclear layer and developed the morphology of photoreceptors including outer segments, the structure that is responsible for phototransduction. Remarkably, blind mice that received transplantations of these rod precursors exhibited restored vision (Pearson et al., 2012).

Embryonic stem cells (ESCs) have also been explored as a source of expandable numbers of transplantable cells. Many groups have now directed human ESCs towards the photoreceptor fate. Lamba et al. (2009) showed that hESCs could be successfully directed towards the photoreceptor fate and then transplanted back into the subretinal space. These newly-derived photoreceptors could integrate into the appropriate layer and even restore visual function in a blind mouse model. Recent advances have also shown that induced pluripotent stem cells (iPSCs) can be directed to generate photoreceptors and transplanted successfully into the mouse retina (Lamba et al., 2010).

Endogenous repair

Another ongoing strategy to repair the mammalian retina after damage is to stimulate endogenous regenerative processes that are active in non-mammalian vertebrates. These strategies include stimulation through exogenous factors, such as growth factors, or through intrinsic factors, such as transcription factors (discussed in the following section). Many studies have explored ways to stimulate mouse Müller glia to reactivate regenerative processes with extrinsic factors (reviewed in Karl and Reh, 2010).

Müller glia in rodents do have the potential to generate new neurons in response to damage alone, but only in very limited numbers. In certain neurotoxic damage paradigms, Müller glia will re-enter the cell cycle. If subjected to very large amounts of damage by MNU or alpha-Aminoadipate, a number of Müller glia will re-enter the cell cycle and a few will transition into rhodopsin+ or recoverin+ photoreceptors (Wan et al., 2008; Takeda et al., 2008). However, neither of these studies reported the rate of conversion for any cell type i.e. conversion efficiency, and their interpretation is made even more difficult due to the lack of 3-D confocal imaging to confirm that newly derived cells have genuine immunoreactivity to neuron-specific antibodies. It is also noteworthy that these experiments were performed in cultured systems, and these findings may not translate to the *in vivo* retinal environment.

Stimulating the damaged retina with mitogenic factors can further enhance the numbers of new neurons that are generated. Following light damage, injections of EGF into the rat retina increased the number of Müller glial cells in the cell cycle (Close et al., 2006); however this was only during the second postnatal week and little regeneration was seen in the adult. Ooto et al. (2004) and Karl et al. (2008) further found that rodent

Müller glia responded to NMDA-induced neuronal damage and injection of growth factors, EGF, FGF1, or insulin, by re-entering the cell cycle. These Müller glia went on to de-differentiate and express a subset of genes unique to progenitor cells, although many progenitor-specific genes were not re-expressed (Karl et al., 2008). These cells finally differentiated to form new retinal neurons, although the specific types of neurons generated – bipolar and photoreceptor cells (Ooto et al., 2004) or amacrine cells (Karl et al., 2008) - varied between studies.

As in the fish, Wnt signaling is also important for the regenerative response in rodents. Retinal treatment with Wnt3A or GSK3β inhibitors promoted Müller glial proliferation and may have stimulated the differentiation of these cells into Crx+ and Rhodopsin+ photoreceptors after damage (Osakada et al., 2007; Del Debbio et al., 2010). Again, confocal imaging would help to determine whether new photoreceptor cells exhibit authentic immunoreactivity. It is also of note that most of the studies mentioned here were performed in early postnatal, not adult, rodents. To date, there has been little success in stimulating endogenous regeneration from Müller glia in the adult rodent retina.

Review of transcription factor-mediated cell reprogramming

An alternative strategy for cell replacement in the mammalian retina is transcription factor-mediated reprogramming, which will be the focus of this thesis.

Fibroblasts

Recent advances in somatic cell reprogramming towards neural fates have potential application in the rodent retina. In 2010, Vierbuchen et al. demonstrated that fibroblasts could be directly converted into new neurons *in vitro* through the overexpression of three transcription factors, *Ascl1, Myt1l,* and *Brn2*. This conversion was efficient; about 30% of fibroblasts adopted neuronal markers, although they did not transition through a progenitor state. Since this initial finding, numerous reports have demonstrated efficient conversion from both mouse and human fibroblasts to distinct neuronal lineages *in vitro*, including glutamatergic, dopaminergic, and motor neurons, using a minimal number of transcription factors (Vierbuchen and Wernig, 2012). This was recently demonstrated to be feasible *in vivo* as well. Fibroblasts were infected with neural reprogramming factors, transplanted into the brain, induced to express reprogramming factors, and converted into new neurons *in vivo* (Torper et al., 2013).

Glial cells

Glial cells are also ideal candidates for transcription-factor reprogramming, since they share many properties with progenitor cells (Berninger, 2010; Nelson et al., 2011). Forced expression of neurogenic transcription factors in astrocytes promotes neuronal conversion (Heins et al., 2002; Berninger et al., 2007; Heinrich et al., 2010; Blum et al., 2011; Addis et al., 2011; Corti et al., 2012). Berninger et al. (2007) found very efficient conversion of astsrocytes following Ngn2 transduction, with >85% converting to cells with neuronal properties. These cells expressed the neuronal marker Tuj1, adopted neuronal morphology, and exhibited electrical properties similar to cortical neurons including action potential firing. Heinrich et al. (2010) furthered this work by

determining that synapse-forming neurons could also be generated from early postnatal astrocytes if reprogramming factors were carried by non-silencing viral vectors. These cells could further be directed towards glutamatergic or gabaergic lineages by different fate determinants. However, these studies were only carried out in early postnatal astrocytes in dissociated cultures, and it is unclear if these findings can be translated to the adult *in vivo* environment in the absence of damage.

The case for reprogramming mammalian Müller glia towards retinal neurons

Müller glia are ideal candidates for transcription factor-mediated reprogramming to retinal progenitors and neurons. Müller glia retain high levels of many progenitor genes, such as *Hes5*, *Sox9*, and *Lhx2* (Berninger, 2010; Nelson et al., 2011) as well as genes that are necessary for reprogramming to iPSCs, *Sox2*, *Klf4*, and *c-myc*. Most importantly, as discussed above, they retain a neurogenic ability and can de-differentiate after damage in lower vertebrates.

Rodent Müller glia have shown some neurogenic potential *in vitro*. Müller glia grown as neurospheres from postnatal rats could be directed towards ganglion or photoreceptor cells when co-cultured with developing retinal progenitors (Das et al., 2006). Remarkably, Müller glia from the adult human retina have been isolated and display neurogenic potential *in vitro*. Müller glia isolated from adult donor eyes were grown in culture without immortalization and expressed neural progenitor markers (Lawrence et al., 2007; Gianelli et al., 2010; Bhatia et al., 2011). These cells went on to express markers of differentiated retinal neurons and could migrate and integrate appropriately after sub-retinal transplantation. However, it was not conclusive in any of

these reports that these were newly-derived cells and not neurons that had survived initial dissociation.

The neurogenic potential of Müller glia can be enhanced by proneural factors that are normally important during developmental neurogenesis. In rodent retinal explant cultures, the regenerative response of Müller glia after damage was potentiated by these factors (Ooto et al., 2004). Viral infection with transcription factors that are important for amacrine specification, *NeuroD, Pax6,* and *Math3*, could slightly enhance the number of amacrine cells formed; similarly, infection with photoreceptor-specific factors, *Crx* and *NeuroD*, generated higher numbers of new photoreceptor cells.

There is also increasing evidence that neurogenic factors can reprogram non-retinal cells into retinal neurons. Yan et al. (2013) recently demonstrated that RPE cells could be reprogrammed into photoreceptor-like cells *in vivo*. Transgenic mice were generated that expressed neurogenic factors neurogenin1 or 3 under the control of an RPE-specific promoter; by postnatal day 5, a new layer of Recoverin+ photoreceptor-like cells was clearly visible. However, it was less clear whether this transdifferentiation took place at later stages of development and adulthood.

This collective evidence suggests that Müller glia are good candidates for transcription-factor mediated reprogramming and that the retina is an ideal structure to test the feasibility of this approach.

Chapter 2:

Establishment of a reliable method for culturing primary mouse Müller glial cells

Introduction

Müller glia are radial glial cells that span the width of the retina and are the predominant glial cell in the retina. Similar to astrocytes in the CNS, they have many diverse roles (reviewed in Bringmann et al., 2006). During retinal development, they act as a scaffold for young neurons and help to maintain the fledgling architecture of the retina. Müller glial cells are also critical for maintaining homoeostasis in the retina. They offer metabolic support to neurons by supplying lactate/pyruvate and maintain proper extracellular levels of K^+, CO^2, and water by removing excess quantities. Signaling in the retina is also reliant on Müller glia. They uptake glutamate, GABA, and glycine released by neurons into the environment, and in turn release glutamine, which is converted back into glutamate and GABA for synaptic release by neurons. This recycling is required for normal synaptic signaling and prevents excitotoxicity.

As discussed in the introduction, an additional role for Müller glia is to act as a stem-like cell after damage and during regeneration in lower vertebrates. Following damage or injury, Müller glia in the mature retina can re-enter the mitotic cell cycle and divide. In non-mammalian vertebrates, these Müller glia may contribute to a regenerative response (reviewed in Karl and Reh, 2010). In mammals, Müller glia instead undergo a gliotic response and contribute to glial scarring and pathological processes such as proliferative vitreo-retinopathy (reviewed in Bringmann et al., 2006, 2009). However, when immature Müller glia are taken out of the mammalian retina and placed in culture conditions, they may proliferate and even de-differentiate into neurogenic progenitor cells capable of differentiation (Das et al., 2006; Lawrence et al., 2007). Yet, it is unknown what processes guide differences in Müller glial behavior under these various

conditions. Identifying the mechanisms that regulate these processes will give us a better understanding of how to stimulate a regenerative response in mammalian Müller glia.

For these reasons as well as to provide a model for *in vivo* regeneration, much attention has been given to finding a reliable method to grow and expand Müller glia *in vitro*. An *in vitro* mammalian Müller glial model would allow us to 1. access an easily manipulatable system 2. study mechanistic blocks in the regenerative response of mammalian Müller glia 3. test potential avenues to overcome these blocks that have been established in non-mammalian vertebrates. Despite these incentives, few dependable methods have been established.

Due to the difficultly of growing Müller glia from the mature mouse retina, many investigators have turned to immortalization manipulations to generate large numbers of cells for *in vitro* studies. Protocols have generated immortalized cell lines from tumors or from overexpressing oncogenes in primary cells. Most of these lines have been created from Müller glia in the rat retina. Immortalized Müller glial cell lines have been established from both rat retinal cells transfected with SV40 ("rMC-1" line; Sarthy et al., 1998) as well as from temperature-sensitive mutants expressing the SV40 large T-antigen gene ("TR-MUL" line; Tomi et al., 2003) that can be conditionally activated. In the human retina, Müller glia derived from the eye of a 68-year-old donor became spontaneously immortalized ("MIO-M1" line; Limb et al., 2002). These cells could be passaged for at least 45 passages and still retained Müller glial characteristics including glial morphology, expression of appropriate markers, and electrical responses to glutamate. Similarly, in mice, immortalized Müller cells from postnatal day 10 retinas were harvested from the "Immortomouse" and could survive in culture for more than 50

passages (Otteson and Phillips, 2010).

Despite these advances, there are significant drawbacks to the use of immortalized cell lines for studying Müller glial characteristics. Immortalized lines have the potential to form tumors, which is of particular concern for studies in which glial cells will be transplanted back into a host retina. More troubling, these cells are not as reliable as a model for Müller glia in the retinal environment - especially after several passages when cells can become multinucleated and take on non-physiological properties.

Therefore, several groups have focused on strategies to grow and expand primary, non-immortalized Müller cells. Many of these studies have found that primary Müller glia can be successfully cultured at early postnatal ages – the first to second postnatal week - when neurogenesis and gliogenesis are still ongoing. Hicks and Courtois (1990) found that Müller glia derived from postnatal day 8-12 rat retinas could be dissociated and maintained in culture with epitheliod morphology and little contamination from GFAP+ astrocytes. Similarly, untransformed Müller glia from the postnatal day 5-12 mouse retina maintained correct morphology and marker expression and could be maintained in culture for at least 7 passages (Florian et al., 2008). More remarkably, the C57Bl/6 Müller cell line, “C57M10”, derived from postnatal day 10 mouse retina was “spontaneously immortalized” and could be maintained for more than 50 passages (Otteson and Phillips, 2010).

A major drawback to these studies is that they characterized Müller glia from early postnatal rodents, when Müller glia may still retain progenitor characteristics, including the robust capacity for proliferation and potential for neuronal differentiation. Additionally, very few of these studies have analyzed these cell populations for their

purity and whether there was significant growth from other proliferative cell types that reside in the retina, especially astrocytes which share many properties and cell marker expression patterns with Müller glia.

To address these gaps, I investigated whether pure, expandable cultures of Müller glia cells could be derived from the mature mouse retina. Although Müller glia from dissociated adult mouse retinas do not survive *in vitro,* I found that Müller glia could be grown from the mouse retina at postnatal day 12, a time at which Müller glia are mature and progenitors no longer exist in the retina. These cells could be expanded and passaged while maintaining Müller glial morphology, although they could only reliably be passaged once. When examined for glial-specific protein expression, over 95% of cells expressed glial markers. Gene expression analysis confirmed that these cultures had very high levels of Müller glial gene expression and negligible levels of gene expression for contaminating astrocyte or endothelial cells. To my knowledge, this represents the first report to characterize broad-scale gene expression levels in any Müller glial culture system. Furthermore, this system gives us a reliable model to study avenues to stimulate regenerative processes in mammalian Müller glia.

Results

Müller glia from P12 mice can be grown and expanded in dissociated culture.

A previous study from our lab investigated the timecourse associated with the ability of Müller glia to proliferate within the explant system (Ueki et al., 2012). These experiments determined that the ability of these cells to proliferate dramatically declined after postnatal day 12 even with epidermal growth factor (EGF) stimulation. These results suggested that Müller glia derived from mice younger than postnatal day 14 might have the potential to proliferate and expand *in vitro* as dissociated cells.

To test this possibility, I dissociated retinas from postnatal day 11-12 mice and plated the cells in medium containing high levels (10%) of fetal bovine serum and EGF to stimulate cell cycle entry. EGF is a potent mitogen of Müller glial cells both *in vivo* (Close et al., 2006) and in retinal explant cultures (Ueki et al., 2012). Most of the neurons did not survive when dissociated at this age, and dead neurons were removed after the first media change. However, many Müller cells attached to the plate within the first 24 hours. Although cells were rounded up or detached for the first couple of days in culture (Figure 2.1A), over the next few days they elongated and formed spindle-like bipolar-shaped cells. Within 4-6 days, these cells expanded to form confluent monolayers (Figure 2.1B). At confluency, the morphology of these cells was typical of cultured Müller glia, including large flat nuclei, broad processes and an “epitheliod” shape (Hicks and Curtois, 1990).

This first week in culture was characterized by rapid proliferation and expansion. Dividing cells could be labeled with the thymidine analog 5-Ethynyl-2’-deoxyuridine (EdU), which incorporates into the DNA of cells in S-phase. By four days in culture,

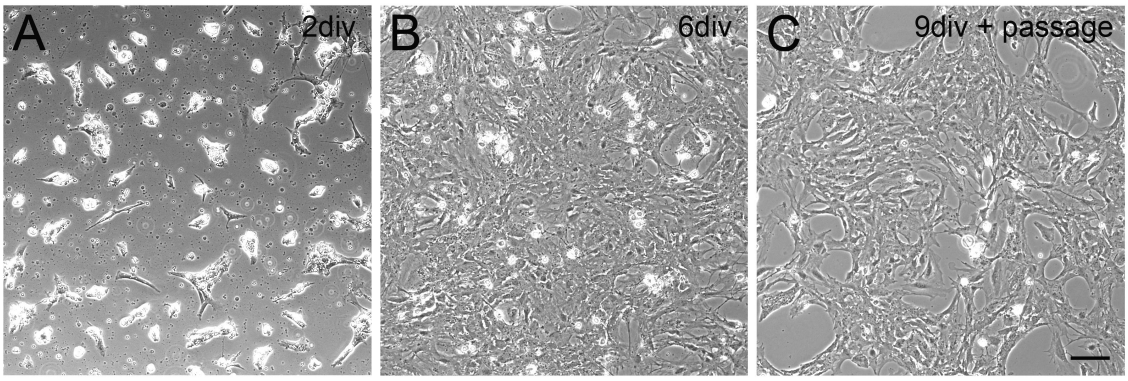


Figure 2.1. Müller glia derived from the P12 mouse retina can be maintained in dissociated cell culture. A. Retinal cultures two days after retinal dissociation. Neurons and glia remain rounded up in clumps. B. By 6 days, most surviving cells are Müller glia, which have attached and formed a confluent monolayer. C. Following passage, 9 days *in vitro*, Müller glia are initially spindle- and bipolar-shaped but quickly extend and spread forming a new confluent monolayer. div, days *in vitro*. Scale bar, 50μm.

most of the Müller glia had re-entered the cell cycle and incorporated EdU (Figure 2.2; 92.4±3.9% EdU+, quantification in Figure 3.1C). EdU labeling also served to track the Müller glia and distinguish them from post-mitotic contaminating neurons that may have survived initial dissociation, which was vital for reprogramming studies described in Chapters 3 and 4.

Müller glial cultures were passaged between 4-7 days *in vitro*, which further reduced the number of surviving non-glial cells and detached cellular debris (Figure 2.1C). However, glial growth slowed down significantly during the second week *in vitro,* and passaging cells a second time did not yield expanded numbers of cells (Ueki et al., 2012). In contrast to these findings, Müller glia cultured from mice older than postnatal day 12 failed to survive initial dissociation in significant numbers, did not proliferate, and could not endure passaging. Thus, it appears that there is a narrow window between postnatal day 11 and 12 when Müller glia have reached maturity and no longer have progenitor properties but can still re-enter the cell cycle and expand when placed in culture conditions.

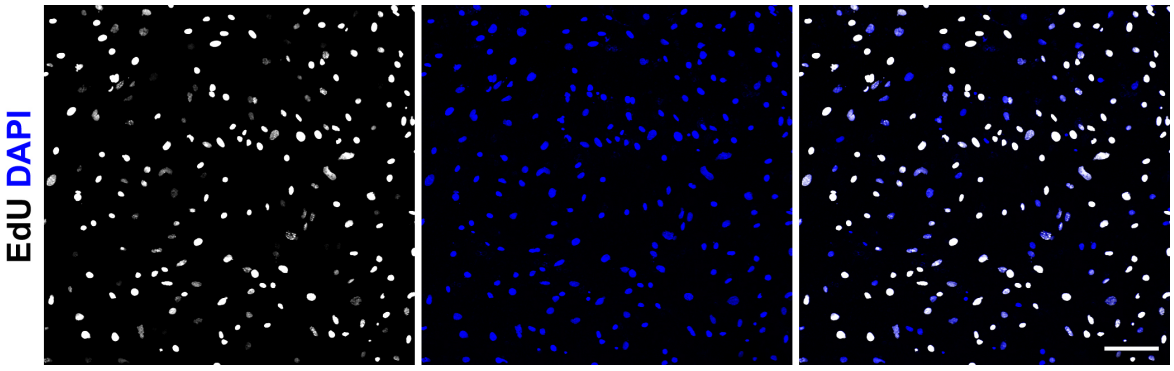


Figure 2.2. P12 Müller glia actively divide in dissociated culture. The majority of Müller glia from P12 retinas at 5 days *in vitro* incorporated EdU. Scale bar, 100 μm.

P12 Müller glial cultures are comparable to mature Müller glia in vivo

Any remaining progenitor properties in P12 Müller glial cultures would interfere with attempts to establish a reliable model to test strategies to stimulate neurogenesis; therefore, it is vital to establish whether these cells have mature gene expression levels and are comparable to adult Müller glia *in vivo*. Gliogenesis is complete in the retina by postnatal day 7 as the last progenitors exit the cell cycle. Müller glial-specific genes, however, are not expressed at mature levels until postnatal day 10 (Nelson et al., 2011). To confirm that cultured P12 Müller glial cells have comparable gene expression levels to mature Müller glia, I used microarray analysis. P12 Müller glia grown in dissociated culture for 10 days were isolated and gene expression patterns were compared to those from freshly dissociated, FAC-sorted Müller glia from mature stages of postnatal retina (P10, P14, and P21) in the Hes5-GFP mouse, which labels Müller glia at these ages (data from Nelson et al., 2011; Figure 2.3). Previous gene expression studies of Müller glia freshly isolated from retina have identified Müller-glial specific markers, including *Glul, Vim, Sox9, Aqp4, Ctsh, Car2, Ccnd3, Dkk3,* and *Apoe* (Hauck et al., 2003; Roesch et al., 2008; Nelson et al., 2011). This gene expression analysis indicated that nearly all of these

Müller glial-specific genes were expressed in dissociated P12 Müller glia at comparable levels to freshly dissociated FAC-sorted Müller glia at P10, P14, and P21 (Figure 2.3).

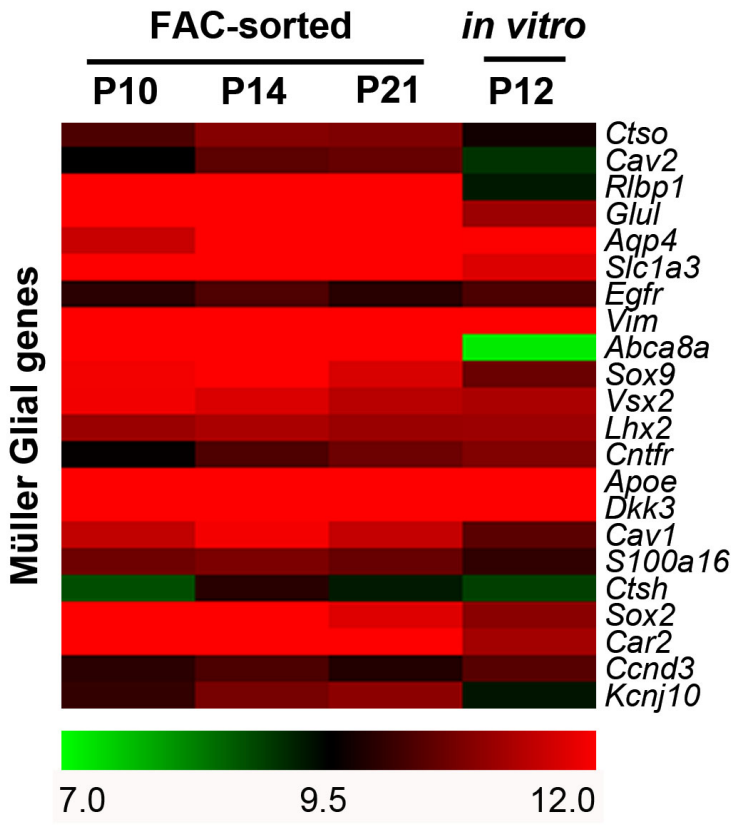


Figure 2.3. Glial genes are highly enriched in cultured P12 Müller glia. Microarray analysis of FAC-sorted Hes5-GFP+ Müller glia from P10, P14, and P21 retinas compared with P12 Müller glia cultures 10div. Expression levels are log transformed normalized values.

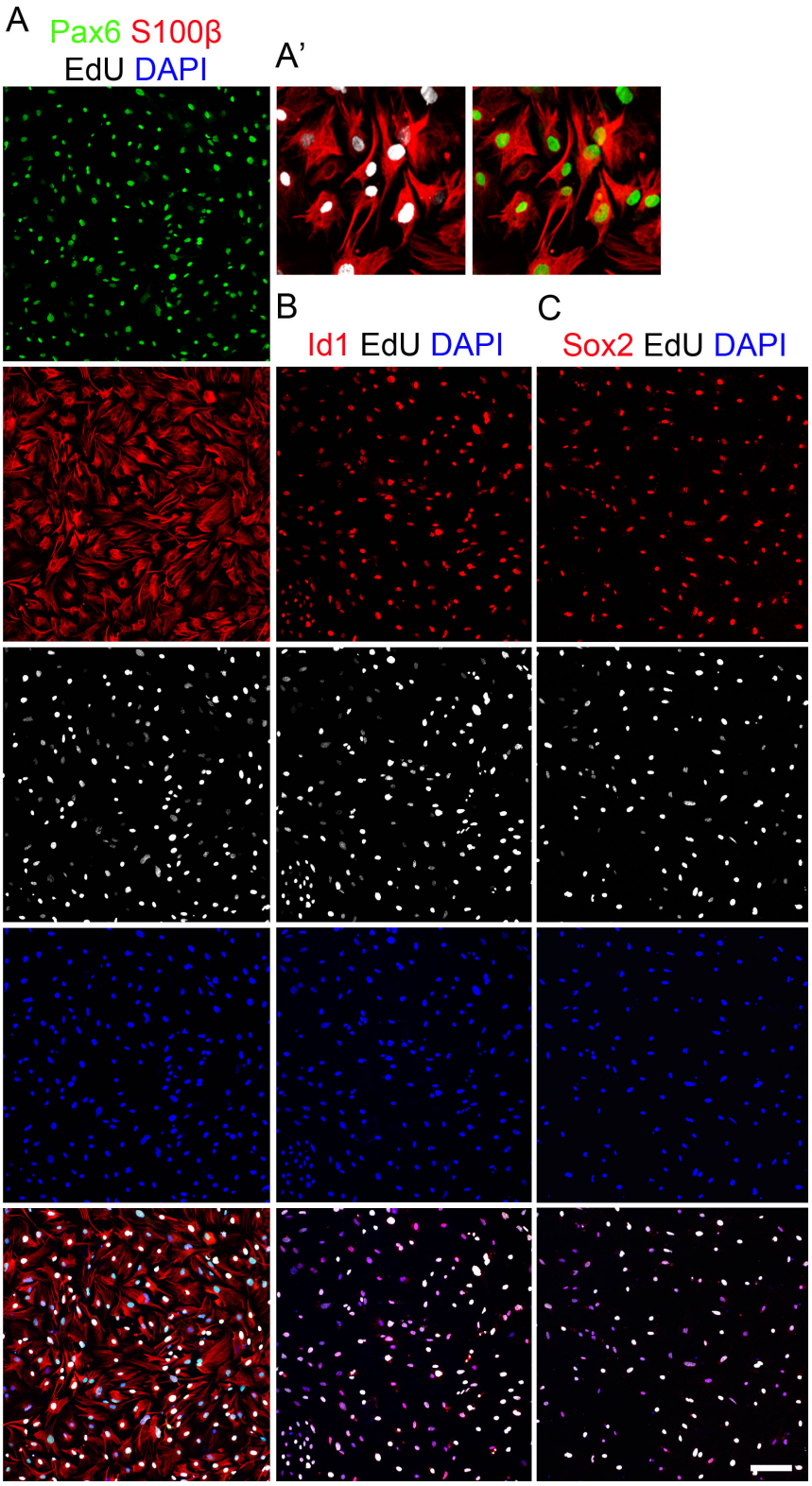


Figure 2.4. P12 Müller glia cultures robustly express glial markers. A. Müller glia from P12 retinas 5 div expressed Pax6 (green) and S100β (red) and incorporated EdU (white, same panel as Figure 2.2). A'. Higher magnification showing colocalization of glial markers S100β/Pax6 and S100β/EdU. B,C. The majority of P12 Müller glia expressed glial markers Id1 (B) and Sox2 (C). Scale bar, 100 μm.

To further validate that these cultures contained large numbers of mature Müller glia, I analyzed the prevalence of Müller glial immunohistochemical markers using several Müller glial-specific antibodies. After 5 days in culture, the vast majority of the cells labeled for the glial-specific marker S100β (Figure 2.4A,A'; Müller glia, astrocytes). Most cells were also immunopositive for Pax6 (Figure 2.4A,A'; Müller glia and progenitors), Id1 (Figure 2.4B; Müller glia, progenitors, RPE, astrocytes), and Sox2 (Figure 2.4C; Müller glia, progenitors, astrocytes). The majority of Pax6+ cells overlapped with those expressing S100β, which is absent from progenitors and RPE cells, indicating that the majority of these cells are Müller glia. Over 95% of the dissociated cells expressed Müller glial markers S100β (99.1±0.36%), Pax6 (97.5±0.63%), and Sox2 (96.8±1.00%) (Figure 2.5). In summary, this analysis establishes that cultured P12 Müller glia express appropriate Müller glial markers and have comparable characteristics to mature glia.

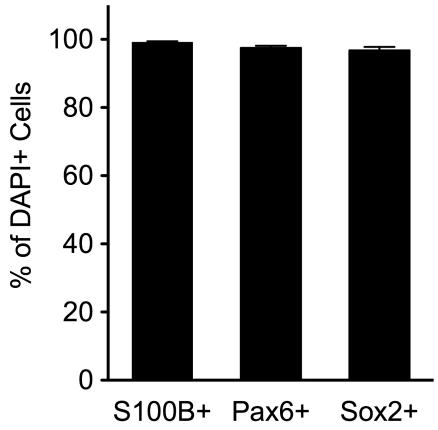


Figure 2.5. Glial markers are expressed by the majority of cells in P12 cultures. Percentage of cells positive for glial markers S100β, Pax6, and Sox2 in dissociated Müller glial cultures from P12 retinas. Bars, mean ± SEM.

Contamination by other cell populations is not substantial in P12 Müller glial cultures

Since the retina contains additional cell types - particularly astrocytes and endothelial cells - that have the potential to expand in cell culture, I investigated whether these cell types meaningfully contribute to Müller glial cultures using gene expression data. Although astrocytes express many of the same genes as Müller glia, we had previously characterized a cohort of genes that are specifically found in astrocytes and not Müller glia (Cahoy et al., 2008). Expression levels of this gene set were evaluated in P12 dissociated cultures and freshly dissociated FAC-sorted Müller glia, as in Figure 2.3. Expression of astrocyte-specific genes was low in dissociated Müller glial cultures, similar to the levels present in freshly isolated Müller glia (Figure 2.6). A subset of genes that are enriched specifically in endothelial cells (Bell et al., 2001) were similarly evaluated in these Müller glial populations; endothelial cell-enriched genes were not present in dissociated Müller glial cultures (Figure 2.6). Immunohistochemistry was also carried out to confirm the absence of endothelial contamination, and fewer than 1% of SIB4+ microglia/endothelial cells were found (data not shown). The distinct morphology of microglia and the pigmented feature of RPE cells also ruled out these cells as a contaminating source. Finally, it was important to establish that pericytes were not a major contaminating population, since they can serve as a source for neural reprogramming (Karow et al., 2012); immunolabeling for the pericyte marker NG2 confirmed that less than 1% of cells were pericytes (Figure 2.7).

In summary, Müller glia derived from the P12 mouse retina express mature Müller glial markers, can be easily grown in culture, and are not substantially contaminated by other cell types.

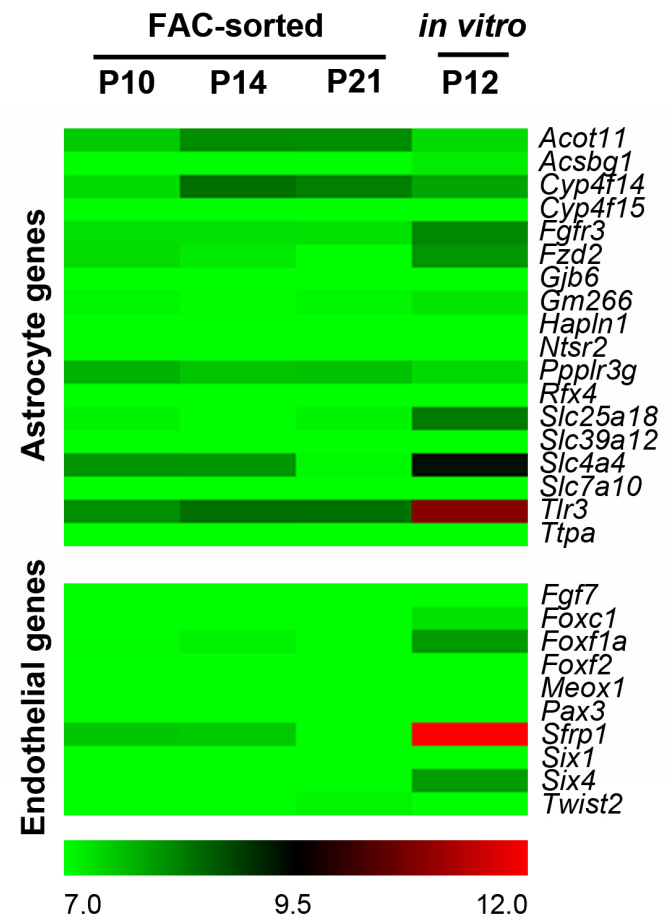


Figure 2.6. P12 Müller glia cultures are free of astrocyte and endothelial gene enrichment. Microarray analysis of FAC-sorted Hes5-GFP+ Müller glia from P10, P14, and P21 retinas compared with P12 Müller glia cultures 10div. Expression levels are log transformed normalized values.

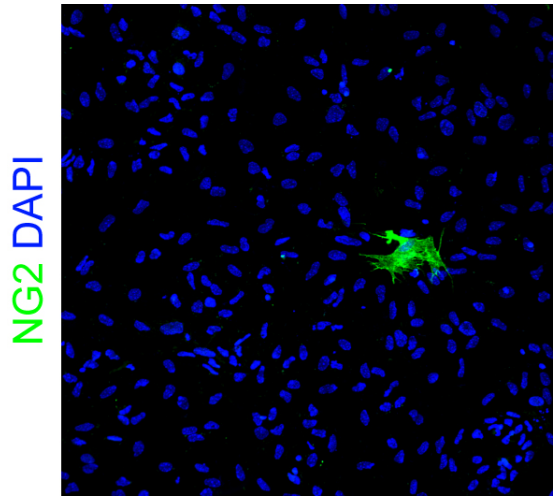


Figure 2.7. Pericytes are not a major cell type found in Müller glial cultures. Less than 1% of P12 Müller glia labeled with the pericyte marker NG2 after 1 week in culture.

Discussion

In this chapter, I established that Müller glia from the P12 mouse retina express Müller glial markers at close to mature levels but can still be expanded through one passage *in vitro*. In contrast, repeated attempts to establish Müller glia from the mature mouse retina have met with relatively little success. Thus, for both dissociated and explant-cultured Müller cells, there appears to be a developmental restriction in the ability to proliferate *in vitro*. Yet, it is unclear what regulates these timing constraints.

Several extracellular signaling molecules have mitogenic effects on Müller glia, although there are complex and conflicting reports regarding the stimulatory or inhibitory effects of many of these factors. One factor may be the decline in responsiveness to the mitogenic factor EGF. Close et al. (2006) demonstrated that EGF is mitogenic *in vivo* during the first two postnatal weeks in the rat retina, although this effect decreases with age. This decrease correlates with a decline in EGFR expression in the Müller glia. Damage to the retina reverses this decline: EGFR expression and EGF-stimulated proliferation increase. Recently, Ueki and Reh (2013) demonstrated that activation of the MEK/ERK1/2, PI3K/AKT, and BMP/Smad1/5/8 pathways is required for EGF's mitogenic effects on Müller glia. HB-EGF was also found to stimulate Müller glia via the MAPK/ERK pathway in the fish (Wan et al., 2012), although these results were not reproduced by another group (Nelson et al., 2013).

TGFβ signaling has also been implicated in the decline in Müller glial proliferation *in vivo* (Close et al., 2005) as well as *in vitro* (Ikeda and Puro, 1995). CNTF signaling via Stat3 is yet another method to stimulate Müller glial proliferation in the undamaged retina (Kassen et al., 2009). Wnt signaling can also stimulate Müller glial re-

entry into the cell signal in the undamaged retina in the fish (Ramachandran et al., 2011) and after injury in the mouse (Liu et al., 2013). Perplexingly, in another report, increased Wnt signaling failed to induce proliferation after damage (Meyers et al., 2012), although the type of damage has been demonstrated to play a role in the response generated by Müller glia.

Some of these mitogenic factors have been linked to downstream cell cycle regulators. $p27^{kip1}$ is critical for maintaining Müller glial quiescence; loss of p27 leads to cell cycle re-entry (Vazquez-Chona et al., 2011) and is required for this transition (Dyer and Cepko, 2000). Knockout of the tumor suppressor p53 has also been shown to relieve the block on cell cycle re-entry, and may be mediated through regulators p21 and c-myc (Ueki et al., 2012). This is consistent with what we observe; p27/*Cdkn1b* is decreased and *Myc* is decreased in P12 cultured Müller glia compared with freshly dissociated P14 Müller glia (not shown). Therefore, manipulating these pathways may be required to stimulate cell cycle re-entry for *in vivo* regeneration.

Despite these developmental restrictions, we recently found that under certain conditions Müller glia from the mature adult retina will retain the ability to grow and expand *in vitro*. If subjected to an intravitreal injection of NMDA, photoreceptor cells in the adult (1-2-month-old) retina will die from excitotoxicity. In response to this damage and presumably signals from dying neurons, Müller glia will be primed to re-enter the cell cycle. If retinas are subsequently dissociated and cultured as described above for P12 retinas, Müller glia will survive and can be expanded through at least one passage at a similar growth rate to that of P12 wildtype Müller glia. Further description of these cells can be found in Chapter 3. The Reh lab also observed similar growth potential in adult

retinas that were subjected to toxic light levels (data not shown). Even more evident, mature Müller glia can be expanded from adult animals that lack the cell cycle genes P53 (Ueki et al., 2012) or ARF (data not shown). Knockout Müller glia could be passaged for at least 16 passages (P53 knockout) (Ueki et al., 2012) or 12 passages (ARF knockout), although longer periods have not yet been tested. Müller glial markers were observed in high numbers in all of these cultures. Although these results are promising, they once again raise the issues discussed above: the potential for tumorigenicity and adoption of non-glial or non-physiological properties. These studies underscore the necessity to find improved methods to culture mature Müller glial cells from the undamaged, wildtype retina. Of particular need is to find approaches that coax these cells to expand over multiple passages while retaining glial characteristics.

Another unclear issue that will be worth addressing in the future is whether Müller glia that can be cultured from the P12 retina represent a subpopulation of Müller glia that retain the ability to proliferate and, perhaps, other stem cell characteristics. It is well established that the age of retinal cells follows a central to peripheral gradient during development, such that cells in the peripheral retina are born later and lag 1-2 days in their development behind center-residing cells. It is possible that Müller glia that reside in the peripheral retina retain the ability to proliferate at P12, whereas those in the central retina have already lost this ability. It would be interesting to test for this possibility by dissecting peripheral and central retinal areas, culturing these Müller glial populations independently, and monitoring their growth potential.

Another possibility is heterogeneity among Müller glial subpopulations within the central retina. It has been shown that only a subset of Müller glia respond to injury by re-

entering the cell cycle in the chick, while those that do not upregulate GFAP instead (Fisher and Reh, 2003). Considering the vast differences in the types of responses, ranging from non-proliferative gliosis to neurogenic de-differentiation, generated by Müller glia after damage in both non-mammalian vertebrates and mammals, it is plausible that certain populations of Müller glia have the capacity for specific responses. However, the molecular signatures or other identifiers that distinguish these populations are unknown.

The findings from this chapter suggest a new protocol for efficiently generating mouse Müller glial cultures. Although these cells are not grown from the adult mouse, I have shown that they are highly comparable to mature glia in their gene expression patterns. To my knowledge, this is the first report of global microarray analysis on cultured primary mouse Müller glia, as well as the first gene expression comparison between cultured and freshly dissociated Müller glia. Future analysis could compare these results to those from cultured porcine Müller glia (Hauck et al., 2003), since our preliminary analysis indicates that these populations have comparable gene levels. Furthermore, I demonstrated both by gene expression levels and by immunohistochemical markers that these cultures are extremely pure and contain little to no expression of contaminating cell types. This represents the most thorough analysis of contaminating populations in reports of Müller glial culturing.

The establishment of a reliable protocol for generating pure Müller glial cultures is an invaluable tool for studying the barriers to Müller glia-based regeneration and testing mechanisms to overcome these limits. The following chapters will crucially rely on the use of these cultures to test strategies to promote regenerative routes.

Chapter 3:

ASCL1 reprograms mouse Müller glia into neurogenic retinal progenitors

Introduction

The goal of these studies is to find effective strategies to stimulate the neurogenic potential of mouse Müller glia. The proneural bHLH transcription factor Ascl1 is a viable candidate, since it has a known role in promoting the neuronal fate during retinal development. In the developing mouse retina, Ascl1 maintains progenitors by driving expression of components of the Notch pathway (Nelson et al., 2009; Jasoni and Reh, 1996) and specifies them towards the neuronal lineage. Knockout of Ascl1 increases the number of Müller glia relative to other cell types (Tomita et al., 2000; Akagi et al., 2004), while Ascl1 over-expression in progenitors biases cell production towards photoreceptor and possibly bipolar cells in mice (Hatakeyama et al., 2001), amacrine cells in the chick (Mao et al., 2008), and bipolar and amacrine cells in human cells (Gamm et al., 2008).

Importantly, Ascl1a has been shown to be required for retinal regeneration in the fish, as discussed in the introduction. Müller glia upregulated *ascl1a* early after damage was induced, and morpholino-based knockdown of Ascl1a blocked their regenerative response (Fausett et al., 2008; Ramachandran et al., 2010; 2012; Wan et al., 2012). Since Ascl1 is not upregulated in the mouse retina after NMDA-induced damage (Karl et al., 2008), we hypothesized that the limited regenerative capacity of mammalian Müller glia may be due, in part, to their failure to activate Ascl1 after damage.

Moreover, Ascl1 is emerging as a key factor involved in neuronal fate conversion in the CNS. Vierbuchen et al. (2010) demonstrated that viral expression of *Ascl1* along with two other transcription factors, *Brn2* and *Myt1l*, could directly convert fibroblasts into neurons, although *Ascl1* alone was sufficient to induce significant conversion. Conversion of postnatal astrocytes to functional neurons was obtained by forced

expression of Ascl1 as well (Berninger et al., 2007; Heinrich et al., 2010).

In the present study, I tested whether forced expression of ASCL1 could activate a neurogenic program in cultured Müller glia. I demonstrate that viral expression of ASCL1 is sufficient to stimulate neurogenesis from mammalian Müller glia, both in dissociated cultures and in the intact retina. ASCL1 remodels the chromatin at retinal progenitor genes and activates their expression while downregulating glial genes. The reprogrammed Müller glia differentiate into cells that resemble neurons in morphology, gene expression, and their responses to neurotransmitters.

Results

Viral-mediated overexpression of ASCL1 in Müller glia cultures

To study efficient transcription factor-mediated conversion, I needed a reliable and well-characterized cell culture model of mouse Müller glia. In Chapter 2, I demonstrated that Müller glia from postnatal day 11-12 (hereafter referred to as P12) mouse retina have mature glial properties and can expand as dissociated cultures. By this age, all of the progenitors have completed their final mitotic cell divisions and differentiated as retinal neurons or Müller glia. Upon retinal dissociation, most neurons die within one day, and only a small number survive after initial passage. Müller glia proliferate over the next week, and over 95% of cells express common Müller glia markers after 4-5 days *in vitro* (div). Other cell types were not found to be a major source of contamination by immunolabeling and microarray analysis for astrocyte-, neuron-, pericyte-, and endothelial-specific markers (Chapter 2).

To test whether ASCL1 was sufficient to reprogram Müller glia into neurogenic progenitors, I carried out the experimental design detailed in Figure 3.1A. P12 Müller glia were cultured for one week, and EdU was added throughout this period to distinguish Müller glia and their progeny from surviving post-mitotic neurons. Cells were passaged to further remove surviving neurons and then infected with lentiviral particles expressing GFP or hASCL1-GFP in neural medium (1% FBS + B27). More than 85% of P12 Müller glia had incorporated EdU by 4 days post-infection (dpi) (10div), in both GFP and ASCL1-infected cultures (Figure B,C). ASCL1 expression was robust at 4dpi; 85.4 ±2.7% of EdU+ ASCL1-infected P12 Müller glia expressed ASCL1 protein compared

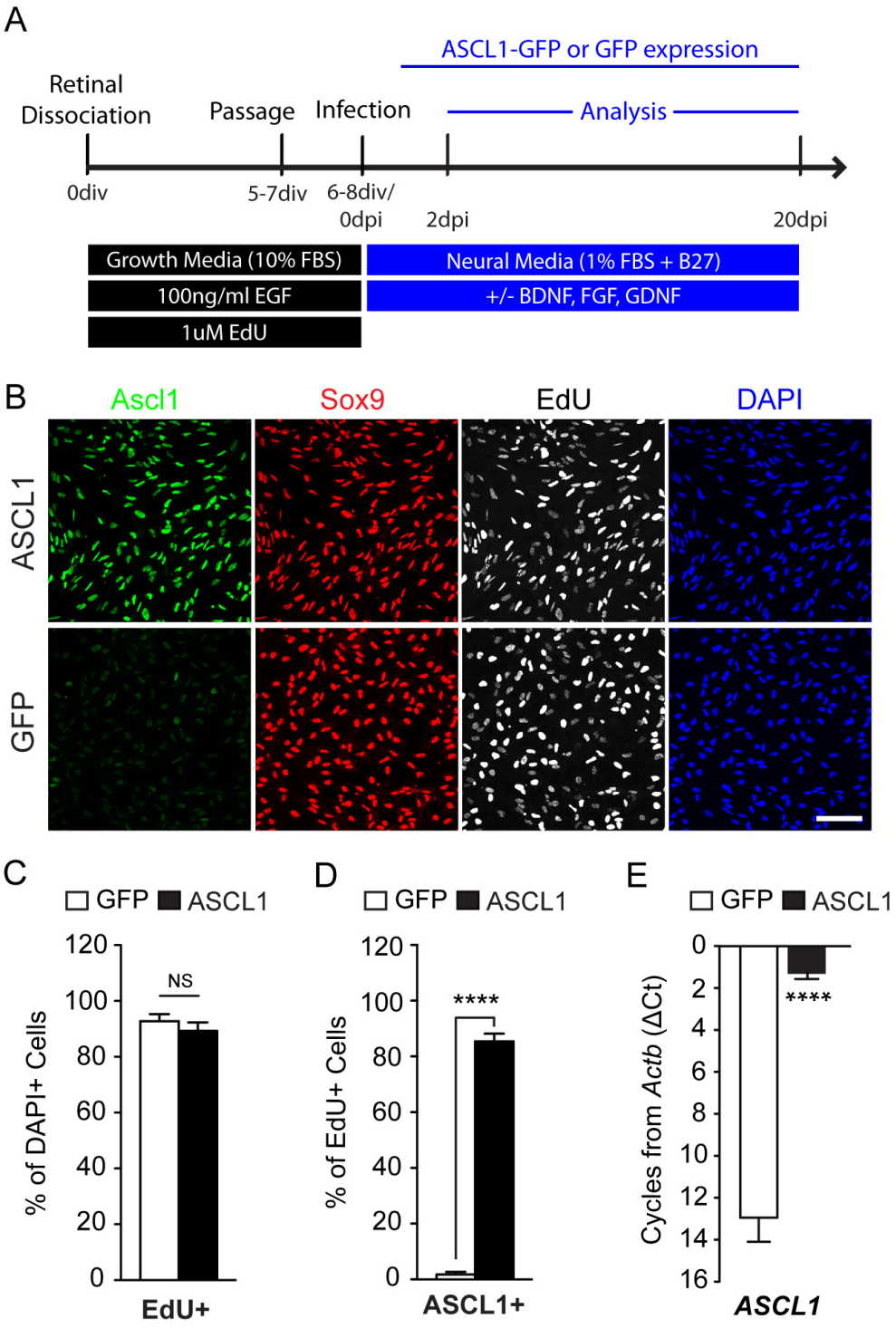


Figure 3.1. Viral-mediated ASCL1 expression in dissociated Müller glia cultures.
A. Experimental design. B. ASCL1-infected P12 Müller glia express Sox9 (red), incorporate EdU (white), and express Ascl1 protein (green) 4 dpi. GFP-infected Müller glia do not label with Ascl1. C. ASCL1- and GFP-infected Müller glia incorporate EdU (4-5div). D. ASCL1-infected EdU+ Müller glia immunolabel with Ascl1, 4-5dpi. E. Human mRNA in ASCL1-infected P12 Müller glia cultures at 4dpi by qPCR. Scale bar, 50µm. Bars, mean ± SEM. NS, not significant. Student's t-test, ****P<0.0001.

with 1.8 ± 0.9% of GFP-infected Müller glia (Figure 3.1B,D). At 4 dpi, *ASCL1* mRNA was highly expressed; Figure 3.1E shows the qPCR for human ASCL1 in ASCL1-infected cultures compared with paired GFP-infected cultures; the ASCL1-infected cells had over 2^{11} (2000 fold) more ASCL1 than the GFP-infected cells.

ASCL1-infected Müller glia express retinal progenitor-specific genes

Since P12 Müller glia do not normally express ASCL1, we initially assessed whether ASCL1 would bind to its predicted targets (Nelson et al., 2009; Castro et al., 2011) in these cells after over-expression. Chromatin immunoprecipitation (ChIP) with an ASCL1 antibody was performed in newborn mouse retina, when endogenous Ascl1 and its predicted targets are highly expressed (Figure 3.2A, gray bars). There was significant pull-down of ASCL1 at the promoters of *Dll3*, *Hes6*, *Mnfg*, *Hes5*, and *Dll1* in P0 retina. Predictably, these targets were not enriched for ASCL1 in GFP-infected P12 Müller glia (Figure 3.3). However, four days after ASCL1 infection, ASCL1 was significantly enriched at these promoters (Figure 3.2A, blue bars), indicating that ASCL1 can bind its developmental targets in P12 Müller glia.

Next, microarray analysis was used to understand the extent to which ASCL1 may activate developmental expression patterns in the infected Müller glia. While many progenitor genes are normally expressed in Müller glia, we recently characterized a subset of genes, primarily proneural factors and Notch pathway components that are highly expressed in Hes5-GFP+ FAC sorted progenitors, but not in Müller glia (Nelson et al., 2011). In Figure 3.2B, I compared the expression levels of these genes in Hes5-

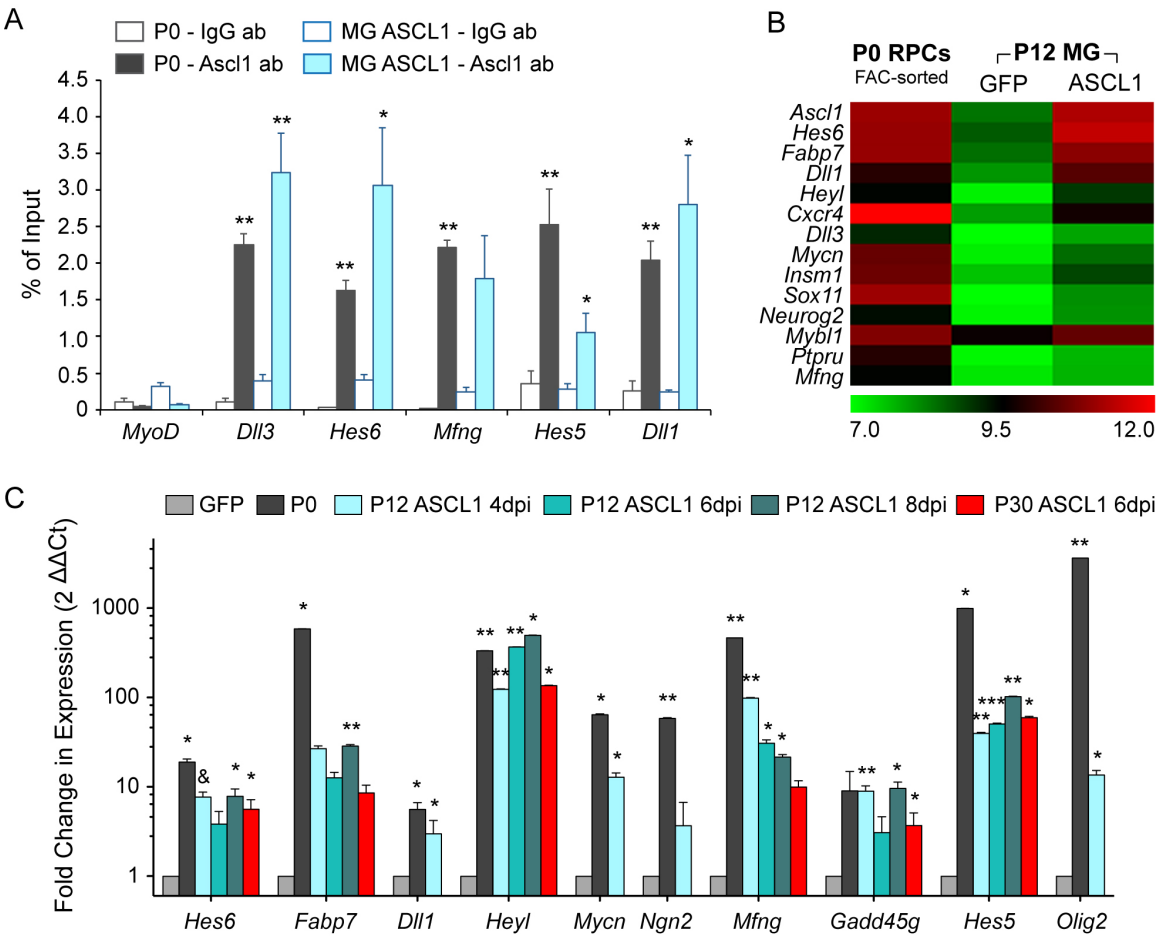


Figure 3.2. ASCL1 induces progenitor gene expression in Müller glia.
A. ChIP, shown as % of input DNA, for Ascl1 or IgG antibodies at the 5' promoter of genes indicated. *MyoD* served as the negative control. B. Microarray analysis comparing *Hes5-GFP* FACS-sorted P0 retinal progenitors (RPCs) to ASCL1- or GFP-infected P12 Müller glia 4dpi. Expression levels are log transformed and normalized. C. qPCR of progenitor genes upregulated in 2B. ASCL1- and GFP-infected Müller glia from P12s at 4, 6, or 8dpi or NMDA-damaged P30s at 6dpi. Bars, mean ± SEM. Student's t-test, *P<0.05, **P<0.01, ***P<0.001, †P<0.0001.

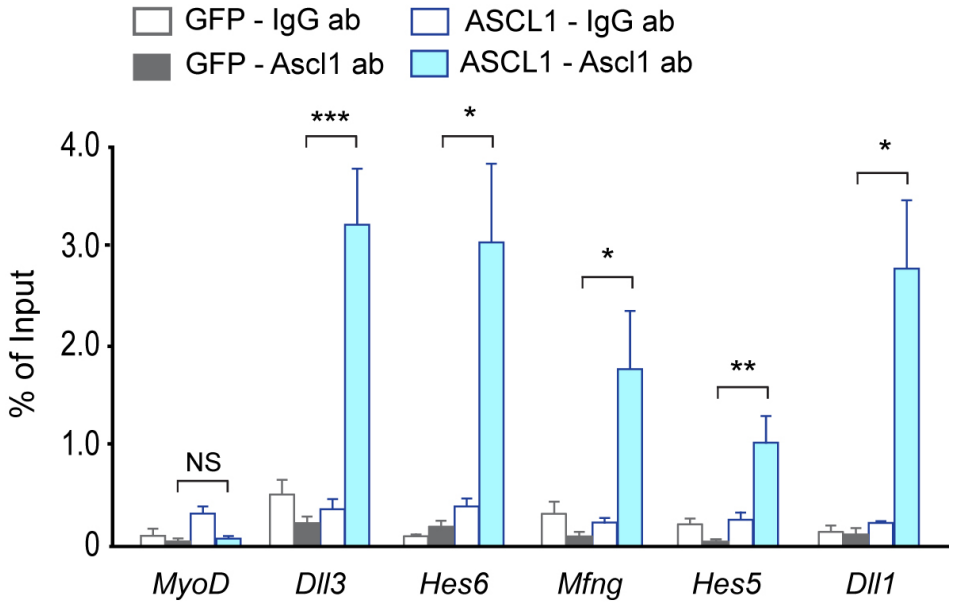


Figure 3.3. Ascl1 does not bind predicted targets in GFP-infected Müller glia.
ChIP for Ascl1 or control IgG antibodies at the 5' promoter region of genes indicated on the x-axis show a statistically significant enrichment of Ascl1 in ASCL1-infected P12 Müller glia compared with GFP-infected P12 Müller glia. Bars, mean ± SEM. Two-way students t-test, *P<0.05, **P<0.01.

GFP+ retinal progenitors (P0 RPCs) to dissociated P12 Müller glia cultures infected with GFP or ASCL1 4 dpi. ASCL1 induced the expression of many of these progenitor-specific genes in P12 Müller glia. Many of the genes most highly upregulated in ASCL1-infected glia are known targets of ASCL1, such as *Dll1*, *Dll3*, *Hes5*, *Hes6 Gadd45g*, *Fabp7*, *Id1*, *Id3, and Mfng*, while others are not known ASCL1 targets, e.g. *Neurog2*, *Mycn*, *Olig2, Fgf15*. The Notch pathway genes, *Heyl*, *Hey1*, *Hes1*, and *Dner,* were also increased. A gene ontology (GO) analysis of the genes that were upregulated in Müller glia after ASCL1 infection (Eden et al, 2007; 2009) revealed that the biological function terms with the highest p-values were those associated with "nervous system development and "regulation of neurogenesis"; terms related to synaptic transmission, conduction of

GO term	Description	*P*-value	FDRq-value	Enrichment
GO:0007399	nervous system development	1.14E-15	1.29E-11	4.95
GO:0060284	regulation of cell development	1.37E-14	7.71E-11	2.48
GO:0045664	regulation of neuron differentiation	1.81E-14	6.81E-11	2.97
GO:0050767	regulation of neurogenesis	3.41E-14	9.63E-11	2.76
GO:0023051	regulation of signaling	8.08E-14	1.82E-10	2.18
GO:0050804	regulation of synaptic transmission	8.42E-14	1.58E-10	5.32
GO:0051969	regulation of transmission of nerve impulse	9.50E-14	1.53E-10	4.98
GO:0010646	regulation of cell communication	9.96E-14	1.40E-10	2.17
GO:0048731	system development	1.08E-13	1.35E-10	3.29
GO:0050793	regulation of developmental process	2.17E-13	2.45E-10	2.04
GO:0051960	regulation of nervous system development	2.89E-13	2.97E-10	2.59
GO:0071840	cellular component organization or biogenesis	3.90E-13	3.67E-10	1.4
GO:0048869	cellular developmental process	1.03E-12	8.97E-10	2.49
GO:0007275	multicellular organismal development	1.26E-12	1.01E-09	2.98
GO:0031644	regulation of neurological system process	1.45E-12	1.09E-09	4.56
GO:0051239	regulation of multicellular organismal process	1.63E-12	1.15E-09	2.03
GO:0032502	developmental process	2.91E-12	1.93E-09	2.02
GO:0016043	cellular component organization	3.94E-12	2.47E-09	1.39
GO:0044708	single-organism behavior	4.43E-12	2.63E-09	3.89
GO:0045595	regulation of cell differentiation	5.65E-12	3.19E-09	2.19
GO:0042391	regulation of membrane potential	5.89E-11	3.17E-08	3.01
GO:0007610	behavior	8.25E-11	4.23E-08	3.42
GO:0022402	cell cycle process	1.94E-10	9.51E-08	1.85
GO:0048856	anatomical structure development	2.82E-10	1.33E-07	1.92
GO:2000026	regulation of multicellular organismal development	4.78E-10	2.16E-07	2
GO:0044057	regulation of system process	5.54E-10	2.41E-07	3.11
GO:0007219	Notch signaling pathway	7.24E-10	3.03E-07	14.14
GO:0048522	positive regulation of cellular process	8.41E-10	3.39E-07	1.57
GO:0030154	cell differentiation	1.34E-09	5.23E-07	2.41
GO:0007268	synaptic transmission	1.36E-09	5.11E-07	4.08
GO:0050806	positive regulation of synaptic transmission	5.72E-09	2.08E-06	8.07
GO:0031646	positive regulation of neurological system process	5.85E-09	2.06E-06	7.44
GO:0048646	anatomical structure formation involved in morphogenesis	6.14E-09	2.10E-06	3.27
GO:0051128	regulation of cellular component organization	6.88E-09	2.28E-06	1.54
GO:0044767	single-organism developmental process	1.17E-08	3.76E-06	1.96
GO:0007049	cell cycle	1.34E-08	4.20E-06	1.8
GO:0051971	positive regulation of transmission of nerve impulse	1.36E-08	4.14E-06	7.63
GO:0048518	positive regulation of biological process	1.67E-08	4.97E-06	1.61
GO:0006260	DNA replication	1.72E-08	4.99E-06	2.7

Table 3.1. GO analysis of the most highly regulated genes after ASCL1 infection of P12 Müller glia.

nerve impulses and cell cycle/DNA replication were also enriched in the genes upregulated in ASCL1-infected Müller glia cells (Table 3.1).

These changes in gene expression were then validated by qPCR by comparing expression levels of progenitor genes in infected Müller glia relative to those present during development. Figure 3.2C shows level of expression in newborn (P0-P1) mouse retina or ASCL1-infected P12 Müller glia relative to GFP-infected P12 Müller glia. At 4dpi, there was very good correspondence between increases in gene expression observed in the microarray data and the qPCR. Almost all genes were significantly increased in ASCL1-infected P12 glia at 4dpi, though in most cases the level of expression did not reach that of the P0 progenitors. At 6 and 8dpi, progenitor genes were still expressed in ASCL1-infected glia; however, by 13dpi, the level of the most highly upregulated genes, *Hes5, Mnfg,* and *Heyl*, were significantly decreased (Figure 3.4). These data suggest that the induction of progenitor genes in Müller glia by ASCL1 infection is transient.

To test whether similar changes could be induced in Müller glia from adult retinas, we used a recently developed protocol to isolate Müller glia from adult (P30) mouse retina after NMDA-induced neurotoxic damage to the neurons. Adult glia from NMDA-damaged retinas proliferated *in vitro*, and the majority of cells were labeled with EdU and the glial marker S100β (data not shown). Typically <1% of the cells in the cultures were isolectin B4+ endothelial cells (Figure 3.5). When adult Müller glia were infected with ASCL1, I found similar levels of activation of progenitor genes as in P12 Müller glia (Figure 3.2C).

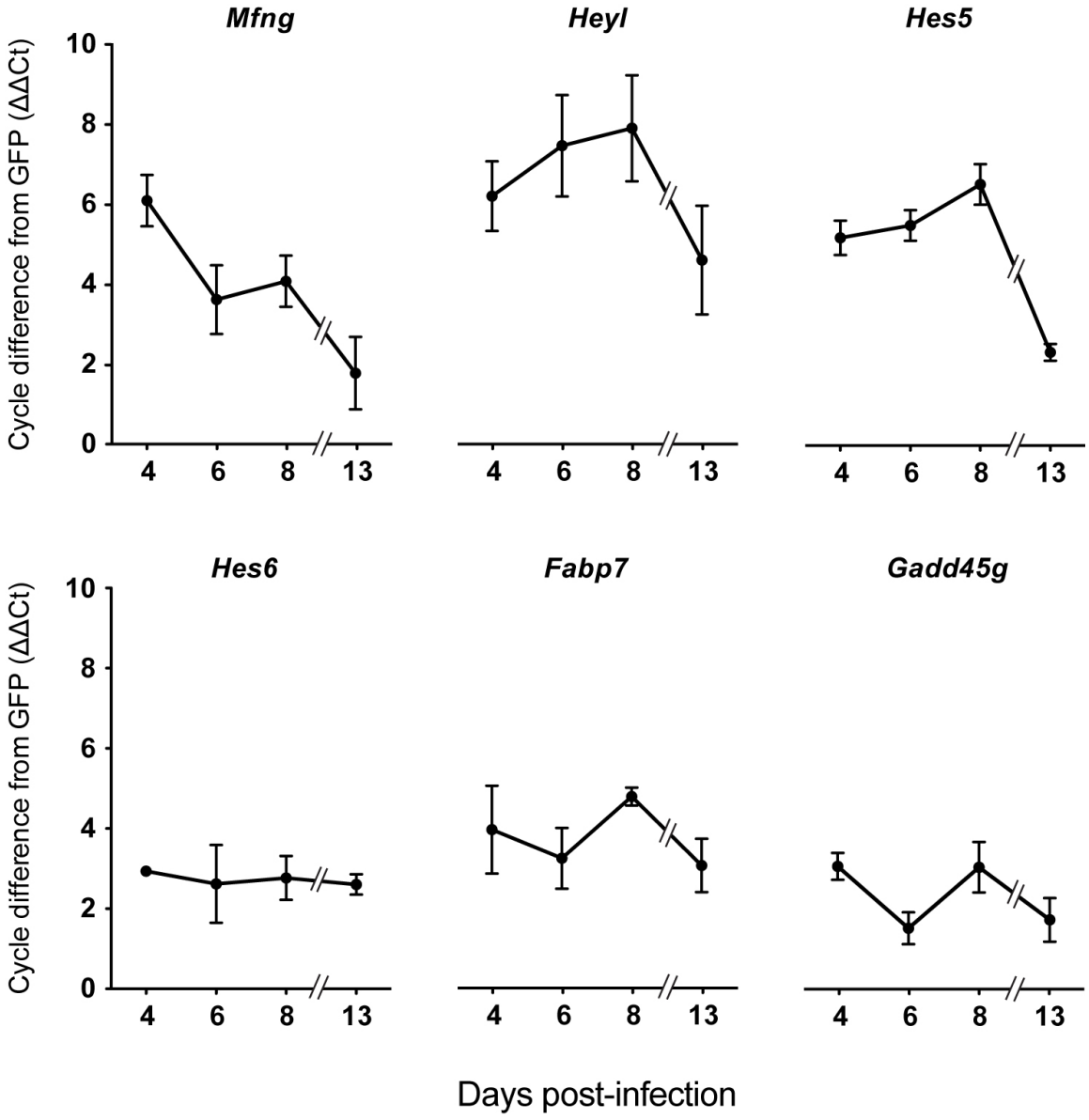


Figure 3.4. Timecourse of expression of retinal progenitor genes.
Progenitor genes are enriched in P12 ASCL1-infected Müller glia at 4, 6, and 8dpi compared to GFP-infected controls from paired data. By 13dpi, *Mfng, Heyl,* and *Hes5* are greatly decreased. Bars, mean ± SEM.

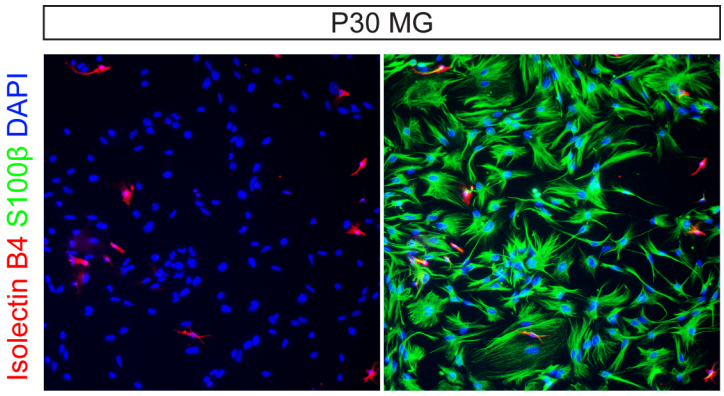


Figure 3.5. P30 Müller glia cultures have low contamination of other cell types.
NMDA-damaged P30 retinas were dissociated 2 days after damage and cultured for one week. The majority of cells immunolabel with glial marker S100β, and low numbers of cells expressing the endothelial marker isolectin B4 were found at this age.

Microarray and qPCR results were further validated using immunofluorescence. *Hes5*, *Insm1*, and *Dll1* are highly expressed in developing retinal progenitors (Nelson et al., 2007; 2009), and are not highly expressed in Müller glia *in vivo* or *in vitro* on our microarray analysis. By contrast, after 4 days of ASCL1 over-expression approximately 15% of EdU+ P12 Müller glia were immunoreactive for Hes5 (Figure 3.6A,B) or Insm1 (Figure 3.6C,D) with a smaller percentage (~10%) of Dll1 (not shown). The percentage of Hes5+ cells was approximately the same over the first six dpi, consistent with the qPCR time course data.

The GO analysis of the microarray data further suggested that ASCL1-infected Müller glia have a higher level of some cell cycle genes. Time-lapse recordings of the Müller glia after ASCL1 infection showed that the infected cells continued to undergo mitotic divisions (Figure 3.7A), and ASCL1-infected Müller glia had a greater number of cells expressing the mitotic marker PH3 (Figure 3.7B,C). Approximately 50% of these PH3+ cells also expressed Hes5 (Figure 3.7D).

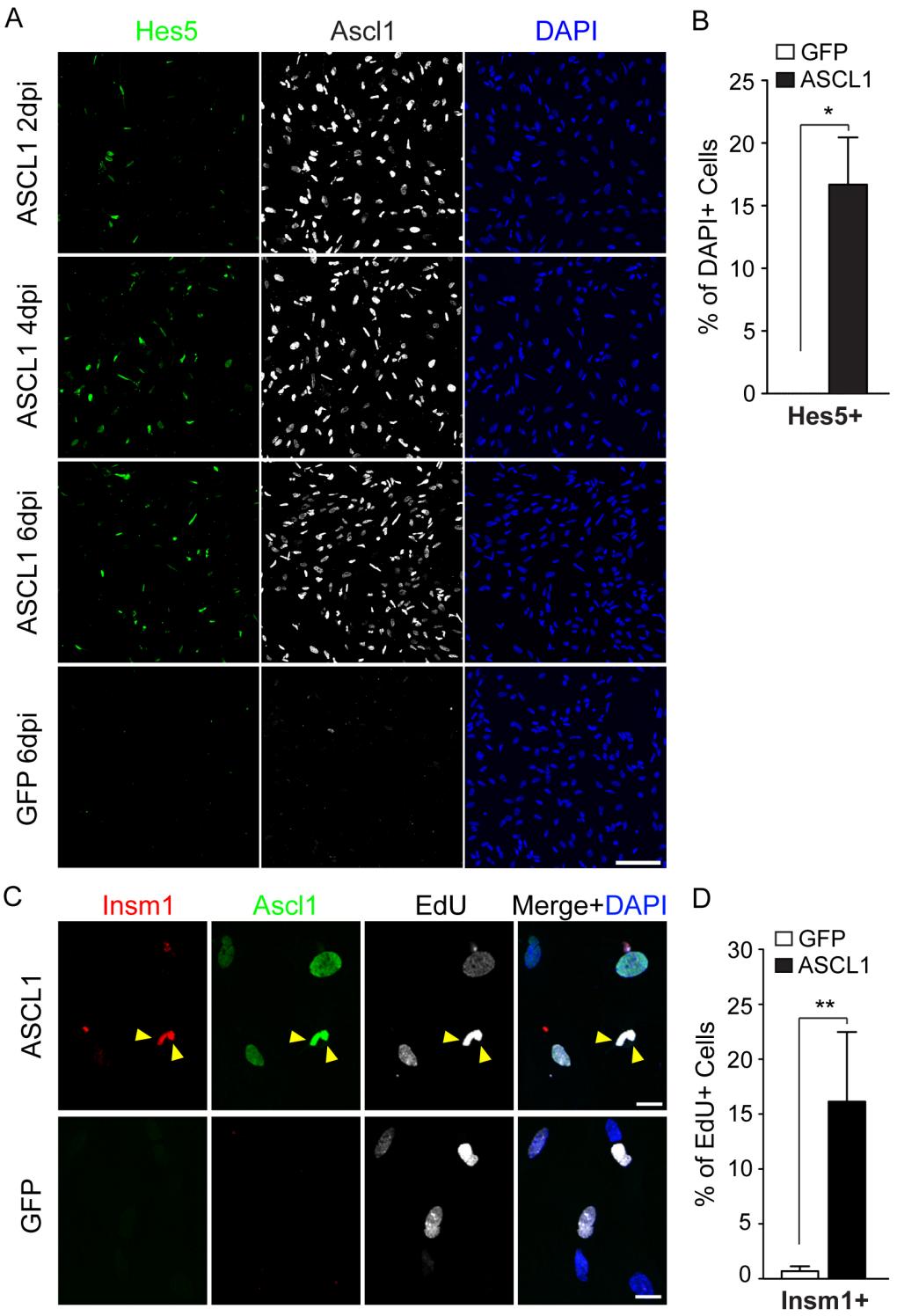


Figure 3.6. ASCL1-infected Müller glia express markers of retinal progenitors.
A. ASCL1+ cells express Hes5 from 2 to 6dpi. Hes5+ cells are absent from GFP-infected Müller glia. B. Graph of Hes5+ cells in ASCL1- and GFP-infected Müller glia 4dpi. C. ASCL1-infected ASCL1+EdU+ Müller glia label with progenitor marker Insm1 4dpi (arrows=triple-labeled). D. Insm1+EdU+ cells are increased in ASCL1-infected Müller glia 4-5dpi. Scale bars, 20 μm (C), 100 μm (A). Bars, mean ± SEM. Student's t-test, *P<0.05, **P<0.01.

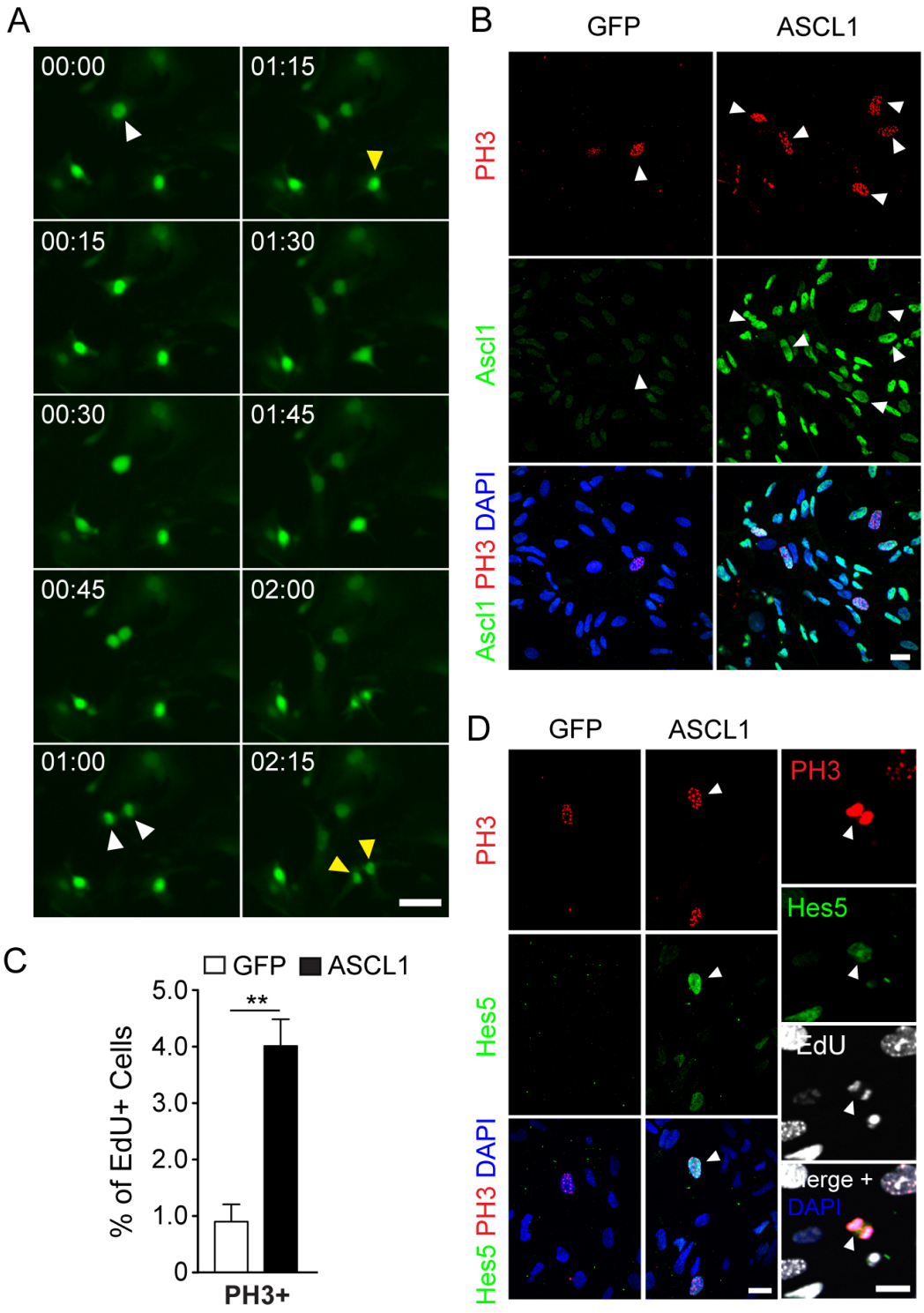


Figure 3.7. ASCL1+ Müller glia-derived progenitors proliferate.
A. Timelapse-imaging of ASCL1-infected Müller glia starting at 7dpi. Two examples of Ascl1-GFP+ cells dividing (arrows). B. Ascl1+ Müller glia label for PH3 (red) at 4dpi. PH3+ cells are found in GFP-infected Müller glia but are not ASCL1+. C. PH3+EdU+ cells are increased in ASCL1-infected Müller glia compared to GFP-infected at 4dpi. D. Hes5+ progenitors label with PH3 and divide (right, inset) 4dpi. Scale bars, 20 μm. Bars, mean ± SEM. Student's t-test, **P<0.01.

ASCL1 remodels target gene chromatin in P12 Müller glia

The fact that ASCL1 is able to directly bind the promoters and/or proximal enhancers of progenitor genes *Hes5*, *Dll1*, *Hes6* and *Dll3* and activate their expression suggests that chromatin at these sites can be reprogrammed to an active state by ASCL1. To directly determine the chromatin status at progenitor genes, we probed the 5' proximal regions of these genes for active and repressive histone modifications in both P0 retinal progenitors and P12 Müller glia. Histone 3 lysine 27 tri-methylation (H3K27me3) represses expression of genes with this histone modification (Cao et al., 2002; Boyer et al., 2006), while H3K27 acetylation (H3K27Ac) is correlated with the promoters and enhancers of actively expressed genes (Karlić et al., 2010). ChIP-qPCR was performed for both modifications at the 5' proximal promoters (or 5' enhancer for *Dll1*). We observed a significant increase in the repressive H3K27me3 modification (Figure 3.8A) and a significant decrease in the active H3K27Ac modification (Figure 3.8B) between progenitors and Müller glia at all four gene loci.

Because repressive chromatin modifications are established at genes that can be subsequently activated by ASCL1, we reasoned that ASCL1 may reprogram the chromatin at these gene loci from a state of repression to activation. We therefore performed ChIP for H3K27me3 and H3K27Ac on dissociated P12 Müller glia infected with ASCL1 or GFP at 4dpi. We observed a significant decrease in H3K27me3, except at *Hes6*, (Figure 3.8C) and an increase in H3K27Ac (Figure 3.8D) at progenitor gene loci in ASCL1-infected Müller glia. These data demonstrate that ASCL1 has the ability to bind

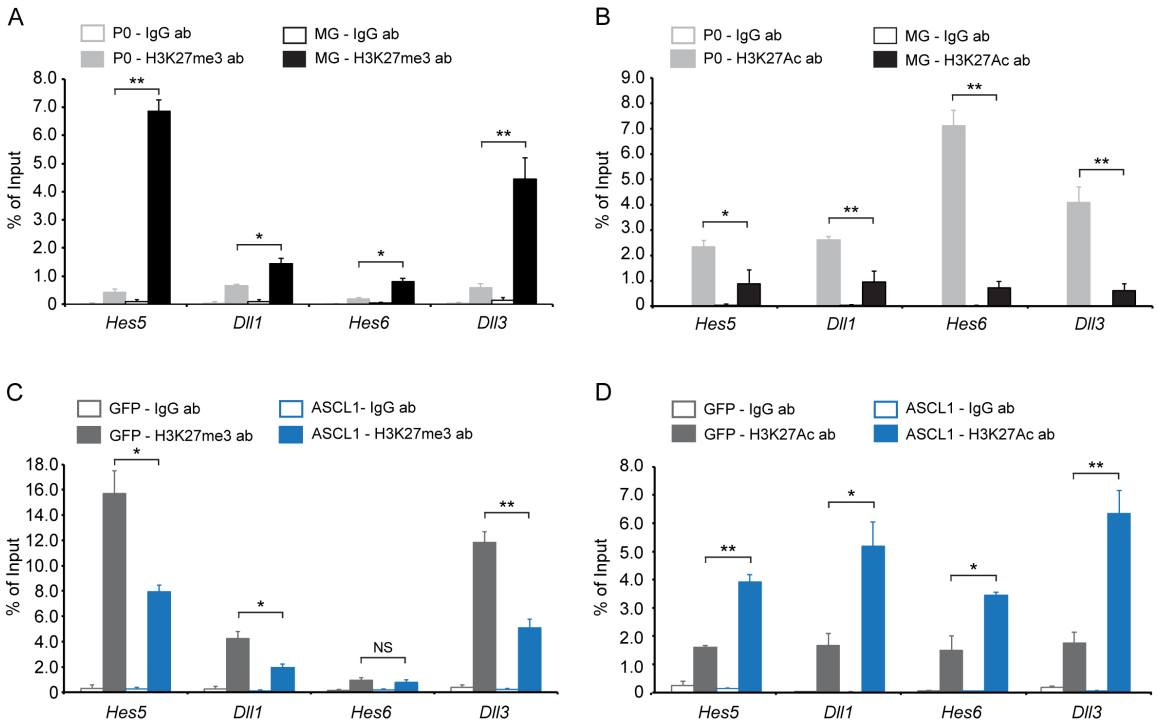


Figure 3.8. ASCL1 remodels Müller glia chromatin.
A, B. ChIP, shown as % of input DNA, versus IgG control antibodies at the 5' promoter of genes indicated. C, D. ChIP at the 5' promoter of indicated genes for H3K27me3 (C) or acetylation (H3K27Ac) (D) between ASCL1-infected and GFP-infected P12 Müller glia at 4dpi. Bars, mean ± SEM. Student's test, *P<0.05, **P<0.01.

genes with repressed chromatin and reprogram the chromatin to an active state. We also assessed the chromatin state of the ASCL1 promoter in Müller glia. We found that like its targets, the promoter of ASCL1 acquires the H3K27me3 repressive histone modification in the Müller glia when compared with P0 retinal progenitors (Figure 3.9A), while at the same time losing the H3K27Ac active modification (Figure 3.9B). Over-expression of ASCL1 in Müller glia was able to partly remodel the ASCL1 promoter, causing an increase in the H3K27Ac modification (Figure 3.9D); however, there was a persistence of the repressive modification H3K27me3 at these loci (Figure 3.9C), which may prevent significant expression of endogenous ASCL1 in the reprogrammed Müller glia.

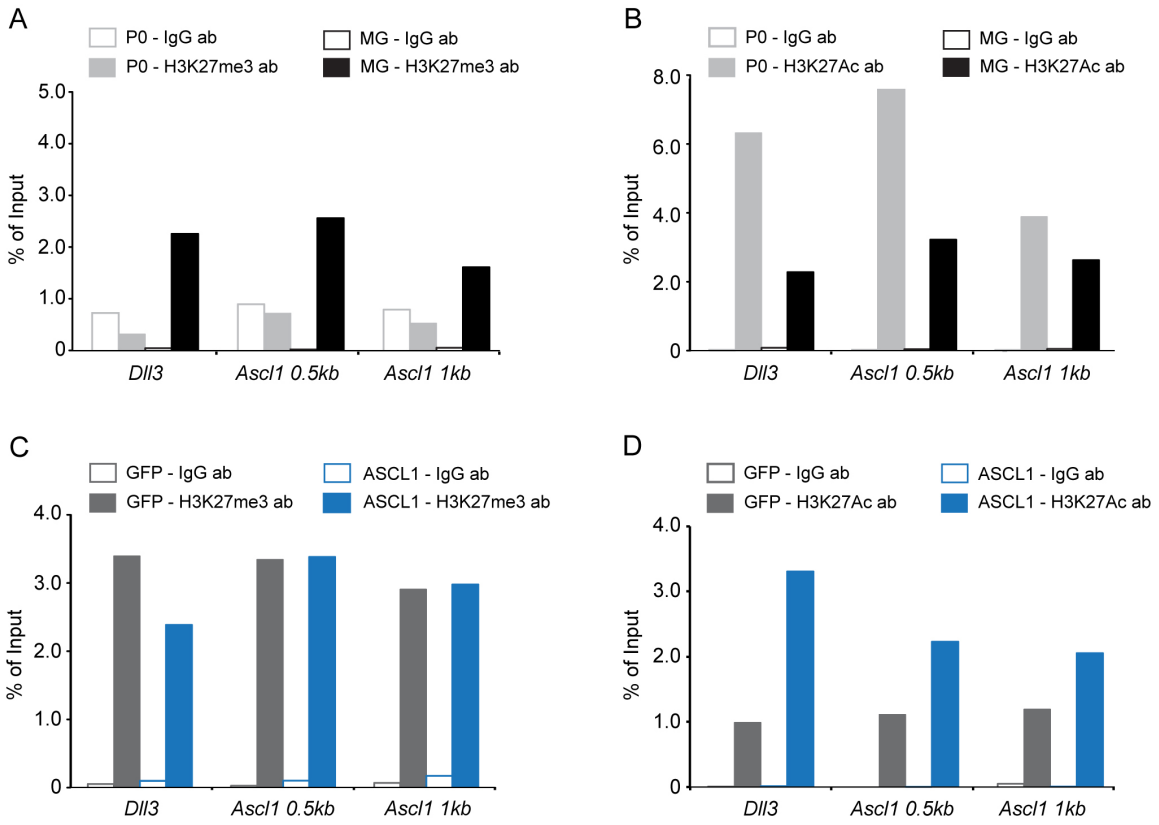


Figure 3.9. The Ascl1 promoter is repressed during development and partially remodeled by ASCL1 expression. ChIP for H3K27me3 or H3K27Ac3 versus IgG control antibodies at two locations in the Ascl1 5' promoter, 500bp and 1kb upstream of the transcription start site. *Dll3* is included as a control progenitor gene locus consistent with Fig. 2. A, B. The Ascl1 promoter shows an increase in tri-methylation (A) and a decrease in acetylation (B) between P0 progenitors and P12 Müller glia. C, D. ASCL1 infection of Müller glia at 4dpi reverses the acetylation (D), but not tri-methylation trend (C) at the Ascl1 promoter.

ASCL1-infected Müller glia lose glial properties

Since ASCL1-infected Müller glia robustly upregulated progenitor genes, I next assessed whether these changes were accompanied by a loss of glial identity. Using microarray analysis, I analyzed a set of Müller glia-specific genes, previously characterized in Müller glia in Ueki et al. (2012) (Figure 3.10A). These genes are highly enriched in dissociated GFP-infected P12 Müller glia at levels comparable to FACS-

sorted Hes5-GFP+ Müller glia. Many of these genes, including *Slc1a3*, *Sox9*, *Rlbp1*, *Aqp4*, and *Glul*, were downregulated following ASCL1 infection of P12 Müller glia. I confirmed that two of these glial genes, *Rlbp1* (Cralbp) and *Slc1a3* (Glast), were downregulated by qPCR following ASCL1 infection at 6, 8, and 13 dpi (Figure 3.10B). Additionally, immunofluorescence of the glial marker S100β was highly reduced by four days after ASCL1 induction (Figure 3.10C). I also carried out Western blots for two Müller glia-specific proteins, Rlbp1 and GS/Glul, and found that both of these proteins were reduced after ASCL1 infection (Figure 3.10D,E).

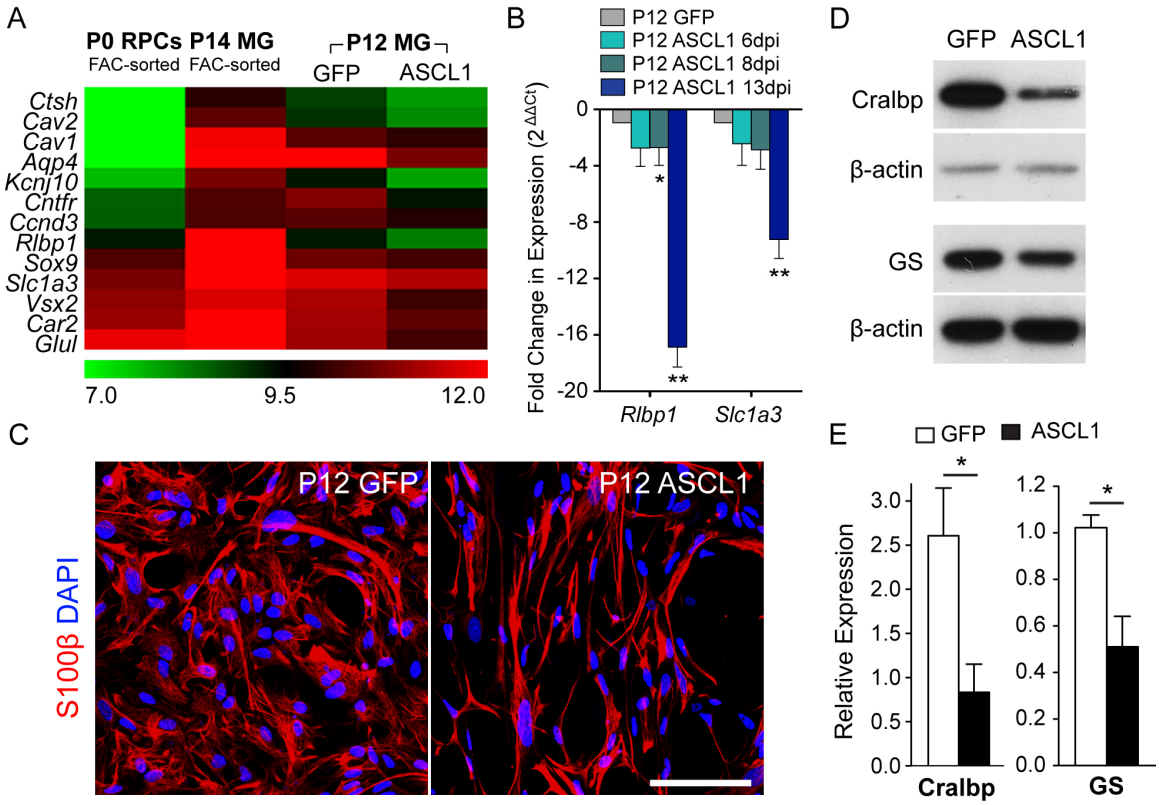


Figure 3.10. ASCL1-reprogrammed Müller glia have reduced glial gene expression.
A. Müller glia-associated genes in Hes5-GFP+ FACS-sorted P0 progenitors, P14 Müller glia and cultured P12 GFP- or Ascl1-infected Müller glia at 4dpi. Expression levels are log transformed normalized values. B. qPCR of Müller glia genes, *Rlbp1 (Cralbp)* and *Slc1a3 (Glast)*, in ASCL1-infected Müller glia compared to GFP-infected Müller glia at 6,8dpi, and 13dpi. C. S100β expression is reduced after ASCL1 infection of P12 Müller glia at 4dpi. D. Müller glia markers Rlbp1 and Glul are reduced after ASCL1 infection at 7dpi by Western blot. E. Quantification of D. Rlbp1 and Glul (normalized to β-Actin). Bars, mean ± SEM. Scale bar, 100 μm. Student's t-test, *P<0.05, **P<0.01.

ASCL1-reprogrammed Müller glia generate cells with neuronal properties

I next assessed the ASCL1-reprogrammed Müller glia for neuronal properties. By 6 days after ASCL1 infection, these cells had lost their glial morphology and adopted a neuronal appearance. Whereas cultured Müller glia have large, flat cell bodies, six days after ASCL1 infection, neuron-like cells with small round nuclei and long, thin processes were observed (Figure 3.11A).

These morphological changes were associated with robust expression of general neuronal markers. By 6dpi, ASCL1-infected Müller glia highly expressed the pan-neuronal markers betaIII-tubulin (Tuj1) (Figure 3.11B) and Map2. qPCR confirmed that *Tubb3* (Tuj1) was significantly enriched in ASCL1-infected Müller glia compared to controls, and that P12 and adult Müller glia upregulated *Tubb3* to similar levels (Figure 3.11C). To confirm that these neurons were newly generated from Müller glia, I cultured Müller glia from *Rlbp1-cre^{ERT2};R26-flox-stop-tdTomato* P12 retinas (Figure 3.12A). These mice express cre-recombinase under the control of the Müller glia-specific *Rlbp1/Cralbp* promoter, and, following tamoxifen administration, Cralbp+ Müller glia expressed tdTomato both *in vivo* and in dissociated P12 cultures (Figure 3.12B,C). Following ASCL1 infection, *Rlbp1-cre^{ERT2};tdTomato*-derived Müller glia adopted neuronal morphology and expressed Map2 (Figure 3.11D, 8A) and Tuj1 (Figure 3.13A).

In addition to the Cralbp-cre lineage tracing experiments, I also followed the progeny of the Müller glia with EdU. The majority of the Müller glia incorporate EdU *in vitro* (Figure 3.1), whereas any neurons that survived from the initial retinal dissociation do not. I found that the majority of Tuj1+ and Map2+ cells in the ASCL1-infected Müller glia cultures were also EdU+ and were, thus, newly generated from proliferating Müller

glia (Figure 3.13A). I quantified the number of EdU+ cells that labeled with these markers and found that over 25% expressed Tuj1 or Map2 in ASCL1-infected Müller glia 9-12 dpi (Figure 3.13C). I also found many Tuj1+EdU+ and Map2+EdU+ double-labeled cells in adult Müller glia at 10dpi (Figure 3.13B,D). Thus, progeny of ASCL1-reprogrammed Müller glia can differentiate into cells with neuronal morphology and expression of pan-neuronal markers.

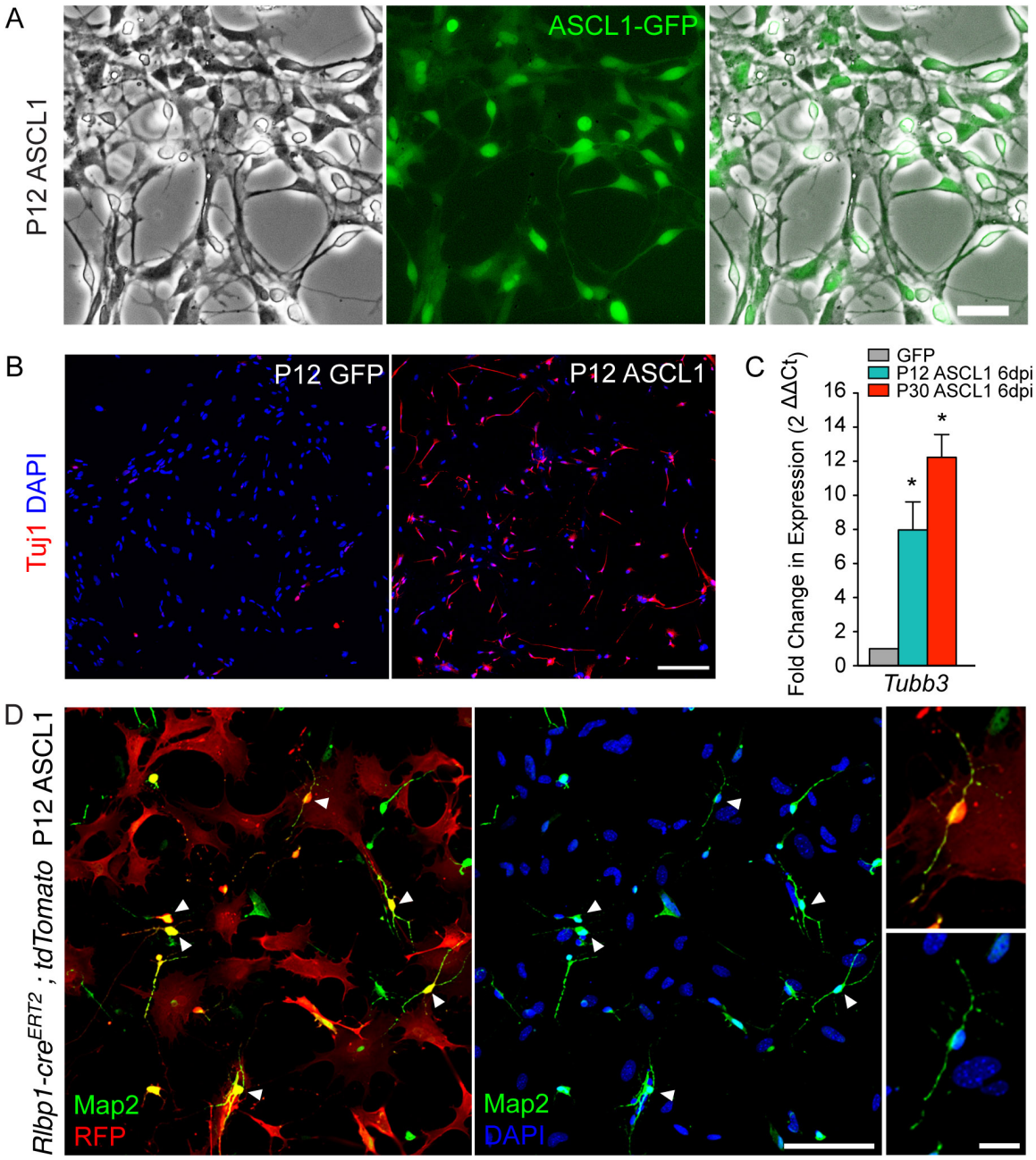
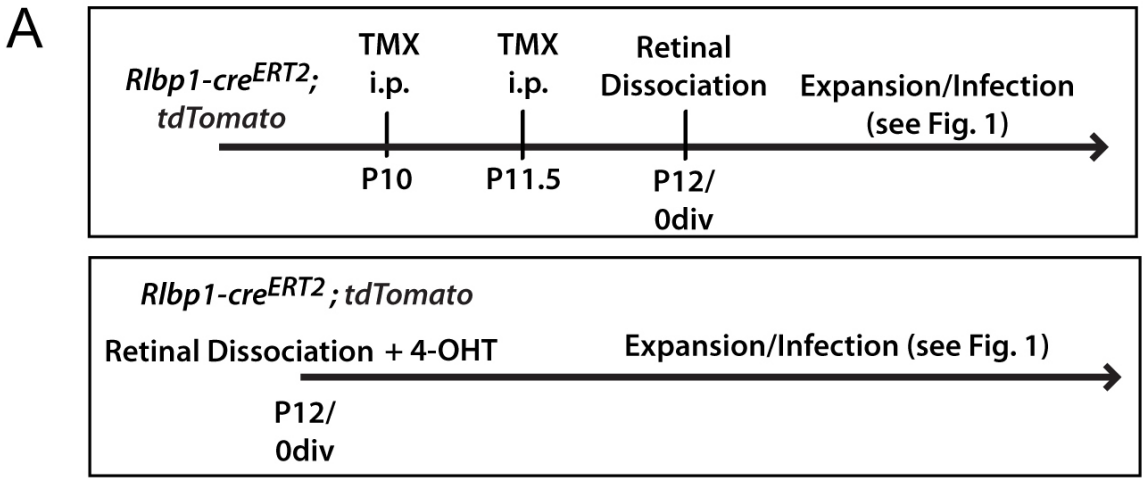
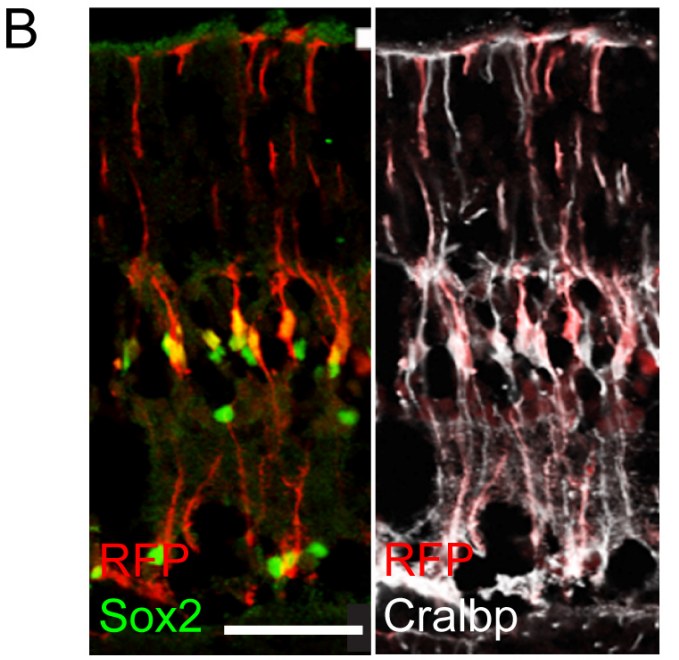


Figure 3.11. ASCL1-reprogrammed Müller glia adopt neuronal properties.
A. ASCL1-infected Müller glia (native ASCL1-GFP) adopt neuronal morphology 18dpi. B. Pan-neuronal marker Tuj1 (ßIII-tubulin) labels ASCL1-infected Müller glia at 10dpi. C. Tuj1 mRNA (*Tubb3*) following ASCL1 infection at 6dpi in P12 or adult Müller glia. D. ASCL1-infected P12 Müller glia derived from glial fate mapped *Rlbp1-cre*^{ERT2}*;tdTomato* retinas (RFP+) express Map2 at 10dpi (arrows). Scale bars, 20 µm (D, high mag), 50 µM (A), 100 µM (B, D). Bars, mean ± SEM. Student's t-test, *P<0.05.





RFP+ of Sox2+	RFP+ of Cralbp+	Cralbp+ of Sox2+	Total #
48.7%	48.8%	100%	566

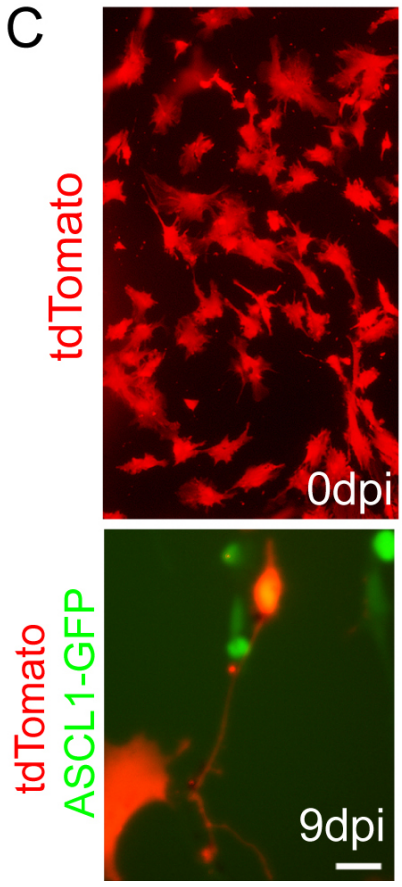


Figure 3.12. Genetic fate mapping of neurons derived from Rlbp1-creERT2;tdTomato P12 Müller glia. A. Experimental diagram. *Rlbp1-cre^{ERT2};R26-flox-stop-tdTomato* retinas express tdTomato in Cralbp+ Müller glia after tamoxifen administration. Tamoxifen was injected at P10 and P11.5 to drive cre into the nucleus. Retinas were then dissociated, grown, and infected as described in Fig. 1A. B. P12 *Rlbp1-cre^{ERT2};tdTomato* retinal cryosections immunolabel with RFP and glial markers Sox2 and Cralbp. C. Dissociated P12 *Rlbp1-cre^{ERT2};tdTomato* ASCL1-infected Müller glia cultures. By 9dpi, ASCL1-GFP+tdTomato+ cells with a neuronal appearance are observable. Scale bar, 20μm (C), 50μm (B).

ASCL1-reprogrammed Müller glia express retinal-specific neuronal markers

Since ASCL1 can induce pan-neuronal markers and morphology, I asked whether more specific retinal neuronal genes could be induced in cells derived from ASCL1-infected Müller glia. I analyzed retinal specification genes that are normally expressed after progenitors have exited the cell cycle: *Neurod4*, *Otx2*, *Neurod1*, *Prox1*, *Crx, Isl1*, and *Atoh7*. All of these early post-mitotic neuronal markers were upregulated by 6 dpi in both P12 and adult Müller glia (Figure 3.13C).

These immature retinal neuronal markers were further validated by immuno-labeling. Otx2 is a marker of early photoreceptor and bipolar cells (Brzezinski et al., 2010; Omori et al., 2011). Many of the progeny (~ 30%) of ASCL1-infected Müller glia developed into cells expressing Otx2 (Figure 3.13F,I). Another early bipolar marker, Islet1 (Isl1), was expressed in ASCL1-infected EdU+ Müller glia (Figure 3.13G, J), although in a much smaller percentage of the cells. Calretinin, a marker of amacrine and some bipolar cells, was expressed by approximately 20% of EdU+ ASCL1-infected Müller glia (Figure 3.13H,K). Although the cells appear to be progressing in their differentiation program, the majority of the differentiating neuronal cells continued to express progenitor markers. Otx2 and Id1 double-labeled cells are shown (Figure 3.13L,M), but I also found Hes5 and Sox9 expression in cells labeled with neuronal markers Tuj1 and Map2 (not shown).

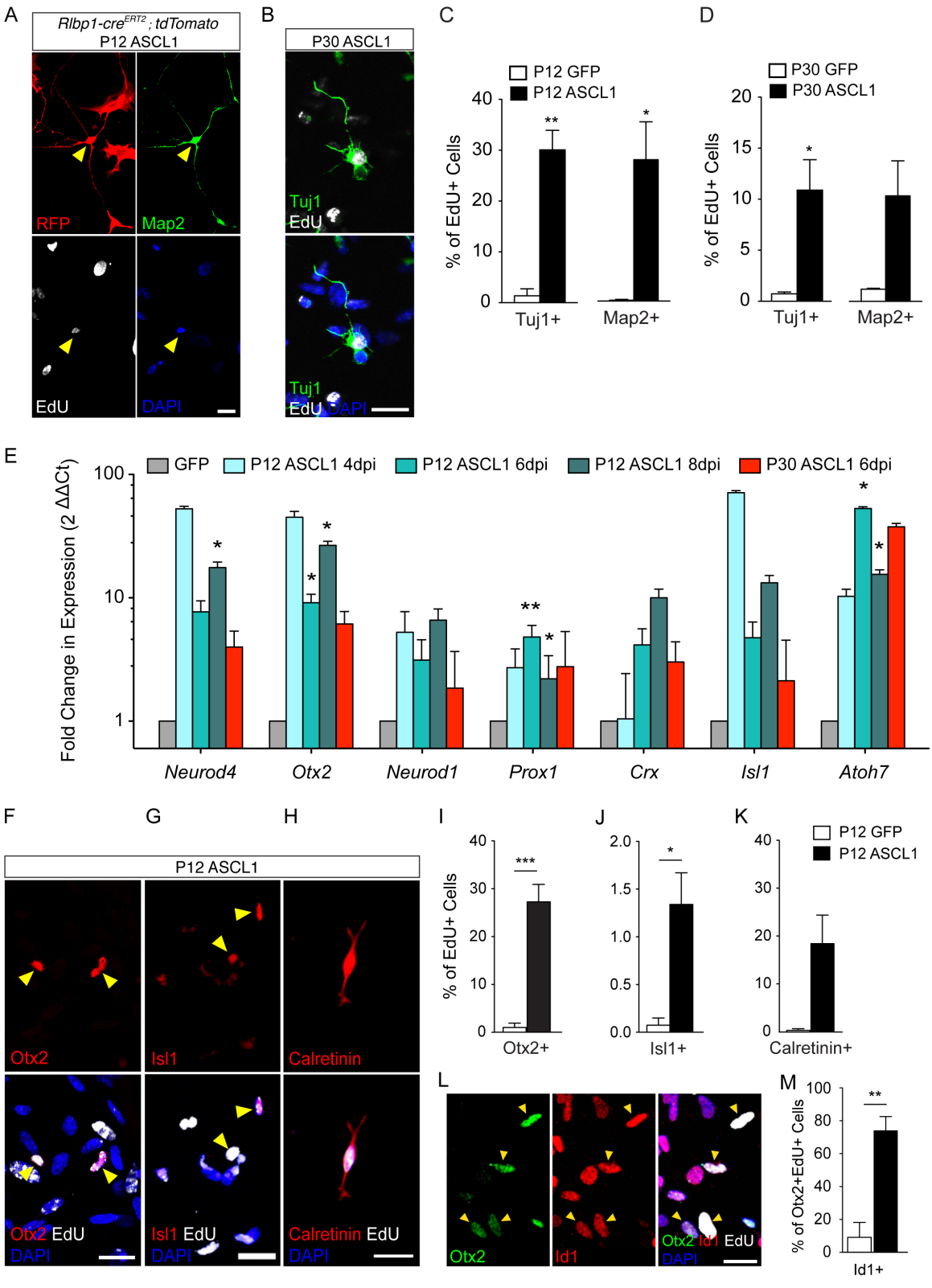


Figure 3.13. Retinal-specific neuronal genes are increased in ASCL1-reprogrammed Müller glia.
A. ASCL1-infected RFP+ P12 Müller glia from *Rlbp1-creERT2;tdTomato* retinas co-express Map2 and EdU at 10 dpi. Yellow arrowheads indicate triple-labeled cells. B. Adult EdU+ Müller glia express Tuj1 10

days after ASCL1 infection. C,D. Tuj1 and Map2 in ASCL1-infected EdU+ P12 Müller glia at 9-12 dpi (C) and P30 Müller glia at 10 dpi (D). E. qPCR for early neuronal markers in P12 and adult ASCL1- and GFP-infected Müller glia at 4-8 dpi. (**F-H**) EdU+ ASCL1-infected Müller glia express Otx2 (F), Islet1 (G) and calretinin (H). Yellow arrowheads indicate double-labeled cells. I-K. Expression of the retinal neuronal markers Otx2, Isl1 and calretinin in ASCL1-infected EdU+ Müller glia at 9-12 dpi. L. Otx2 and the Müller glia/progenitor marker Id1 are co-expressed in P12 ASCL1-infected Müller glia at 10 dpi. Yellow arrowheads indicate triple-labeled cells. M. The majority of Otx2+EdU+ cells are positive for Id1 in ASCL1-infected Müller glia. Bars, mean ± SEM. Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars, 20 μm.

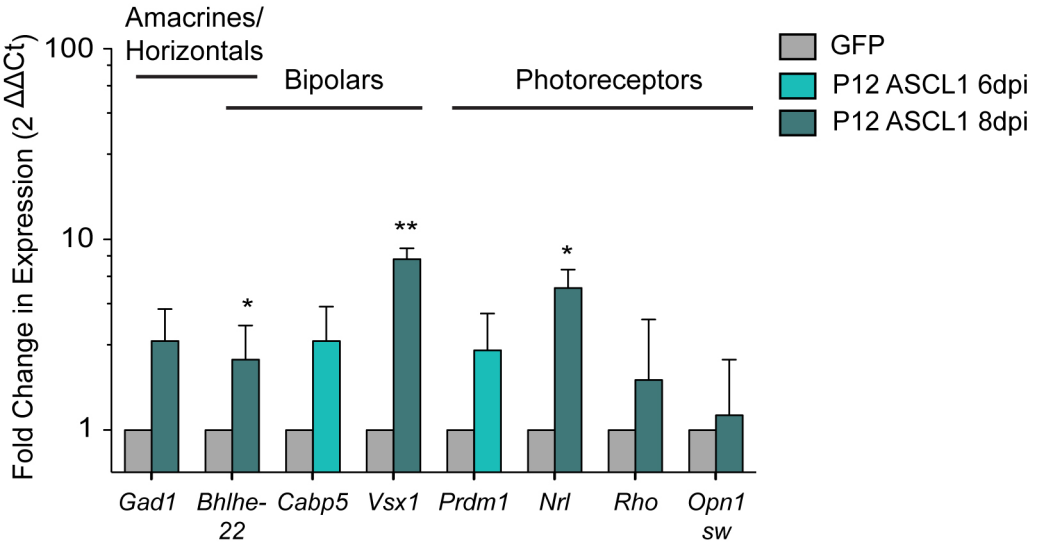


Figure 3.14. Late retinal neuronal genes.
Genes specific to differentiated bipolar and amacrine cells are upregulated in P12 ASCL1-infected Müller glia by qPCR (log transformed normalized values). Bars, mean ± SEM. One-way student's t-test, *P<0.05, **P<0.01.

I also used qPCR and immunolabeling to determine whether mature retinal neuronal markers were expressed in the progeny of the ASCL1-infected Müller glia. I observed small, but consistent expression in genes that are normally expressed in mature retinal neurons (Figure 3.14). The qPCR showed upregulation of *Gad1/Gad67* and *Bhlhb5/Bhlhe22*, genes that are normally expressed in mature amacrine and bipolar neurons, respectively. In addition, ASCL1-infected Müller glia expressed bipolar markers, *Cabp5* and *Vsx1*, and the rod photoreceptor marker *Nrl.* However, the

expression level of most of these genes was very low, and other later-expressed photoreceptor genes *Opn2* (Rhodopsin) and *Opn1sw* (S-opsin), were not detectable in the reprogrammed Müller glia. These results indicate that ASCL1-reprogrammed Müller glia can develop into cells expressing many early markers of retinal neuronal differentiation, but a declining percentage expresses later markers and only a very few develop more mature marker expression. Interestingly, the predominant cell type generated in these cultures expresses markers consistent with the bipolar cell fate.

Glial-derived neurons exhibit neuron-like responses to neurotransmitters.

To assess whether ASCL1-induced neurons could have functional activity, I looked for gene expression changes in neurotransmitter receptors channels (Figure 3.15A). By 4dpi, ASCL1-infected Müller glia upregulated many neurotransmitter receptors, the most prevalent of which were nicotinic cholinergic and kainate receptors. At the same time, ASCL1-infected Müller glia down-regulated P2Y and P2X purinergic receptors, which are normally highly expressed in Müller glia (Wurm et al., 2009; 2011).

I next tested whether cells derived from the reprogrammed Müller glia could respond to pharmacological agonists using Ca^{2+} imaging. At 10-12 dpi, cells were loaded with the ratiometric Ca^{2+} indicator dye Fura-2. NMDA (plus glycine to potentiate responses), Kainate (KA), ATP, and KCl were bath applied to cells, and ΔF340/380 responses were measured. ASCL1-infected Müller glia were derived from *Rlbp1-cre^{ERT2};tdTomato* retinas; tdTomato+ cells with neuronal morphologies (Figure 3.15B, arrow) had a detectable ΔF340/380 response to NMDA and failed to respond to ATP when compared to uninfected cells (Figure 3.15C). In some cases, I was able to locate the

field after fixation, and, in the example shown in Figure 3.15C, the cell was later identified with Tuj1 and EdU immunolabeling (Figure 3.15B).

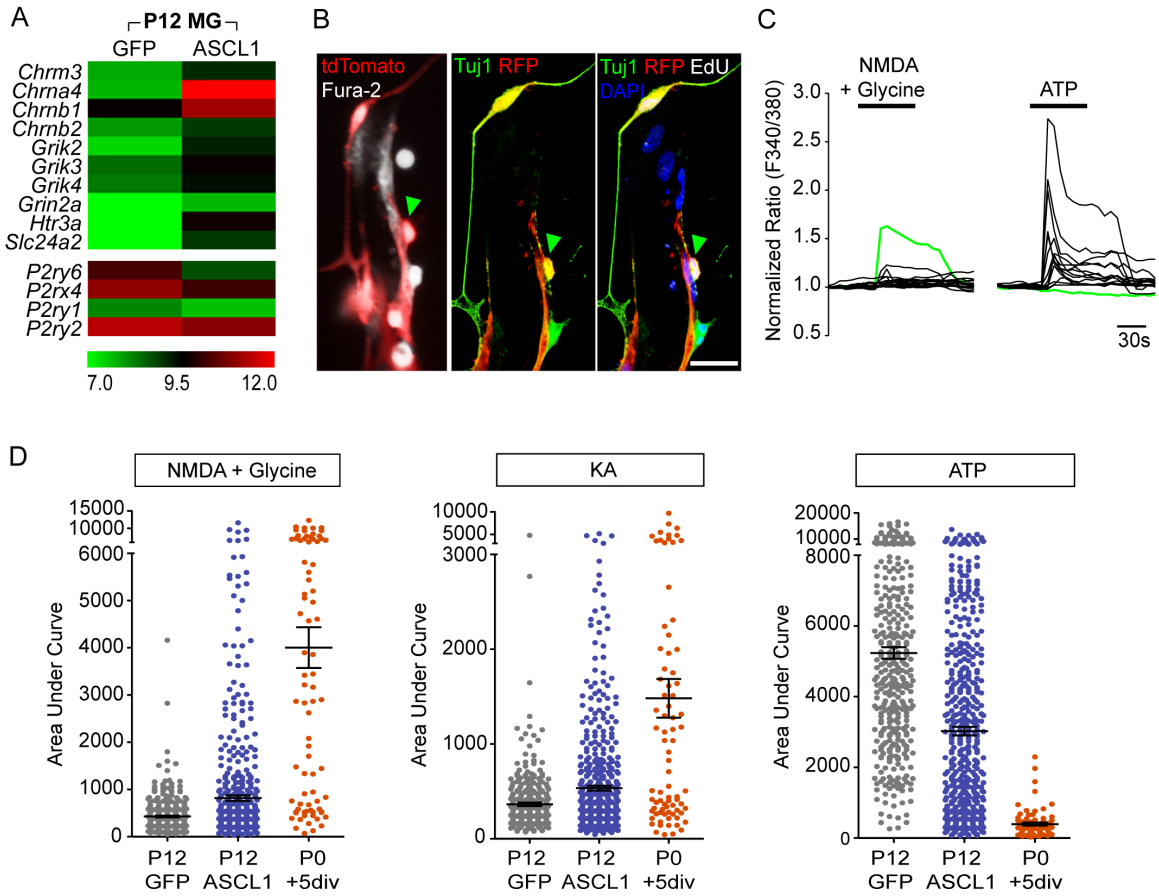


Figure 3.15. ASCL1-reprogrammed Müller glia have neuron-like responses to neurotransmitters.
A. Microarray analysis (4dpi). B. Example of *Rlbp1-cre*^{ERT2}*;tdTomato* ASCL1-infected P12 Müller glia labeled with Fura-2 Ca^{2+} indicator dye, 10dpi. Green arrowhead: tdTomato+ cell with neuronal morphology that was postfixed and colabeled with Tuj1, and EdU. C. Normalized ΔF340/380 responses of cells from field of (B) to NMDA and ATP application. Green traces distinguish unique responses from neuronal-looking tdTomato+ cell in (B). D. Summary of ΔF340/380 responses, plotted as area under curve, for a 120s period following application of NMDA (100μM) + Glycine (10μM), Kainate (10μM), and ATP (100μM) of P12 Müller glia 10dpi compared to P0+12div RPCs. Bars, mean ± SEM for cells in that condition. Student's t-test comparing GFP to ASCL1 for 3 biologically-independent experiments gave ****P<0.0001.

I next assessed overall changes in responsiveness to these agonists in ASCL1- or GFP-infected Müller glia or retinal neurons from newborn mice (P0, 5-12div). Figure

3.15D shows ΔF340/380 signals, plotted as area under the curve, for individual cells responding to NMDA, KA, and ATP. ASCL1 infection caused a significant increase in responsiveness to NMDA and KA and a significant decrease in responsiveness to ATP. These differences were more pronounced in ASCL1-infected Müller glia that had a neuronal or transitional morphology compared to those with a glial morphology or GFP-infected Müller glia (Figure 3.16B). Statistically significant differences were not observed in response to KCl (Figure 3.16A). Thus, neuronal cells derived from reprogrammed Müller glia uniquely responded to ionotropic glutamate agonists while reducing their responsiveness to purinergic receptor agonists. We also confirmed the Ca^{2+} imaging results by whole-cell patch clamp recordings. Injection of 40 pA could elicit action potentials from ASCL1-infected Müller glia cells, application of kainate induced robust inward currents, and when the ASCL1-infected Müller glia were co-cultured with immature P0 retinal neurons, mini-excitatory postsynaptic currents (EPSCs) could be observed (Figure 3.17A-C).

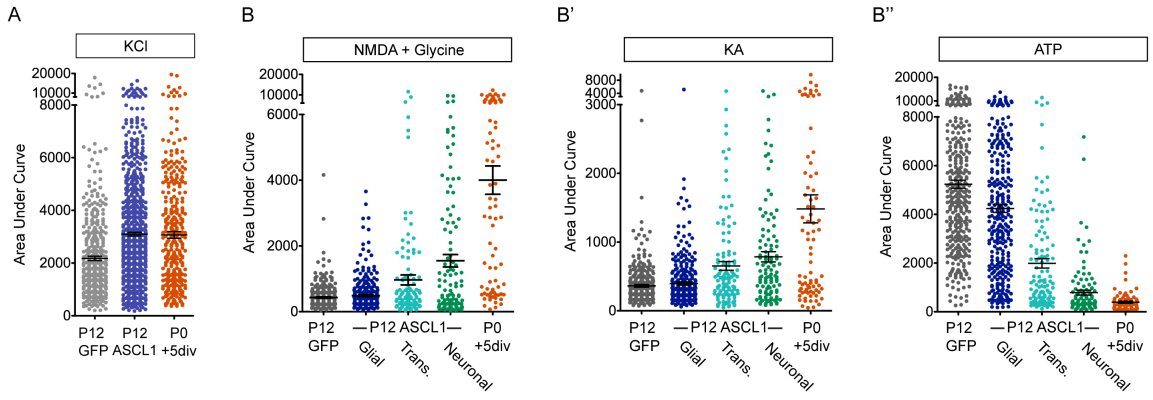


Figure 3.16. Morphology of ASCL1-infected Müller glia is associated with functional responsiveness. A. ΔF340/380 responses to KCl application, as measured by area under the curve, were not statistically different between ASCL1- and GFP-infected Müller glia. B. Classification of ASCL1-infected Müller glia by morphology (Glial, Transitional, or Neuronal). Neuronal-looking cells were more responsive to NMDA and KA compared to glial-looking cells and were less responsive to ATP. Transitional cells (intermediate between glial and neuronal-appearing) responded between glial and neuronal-looking cells.

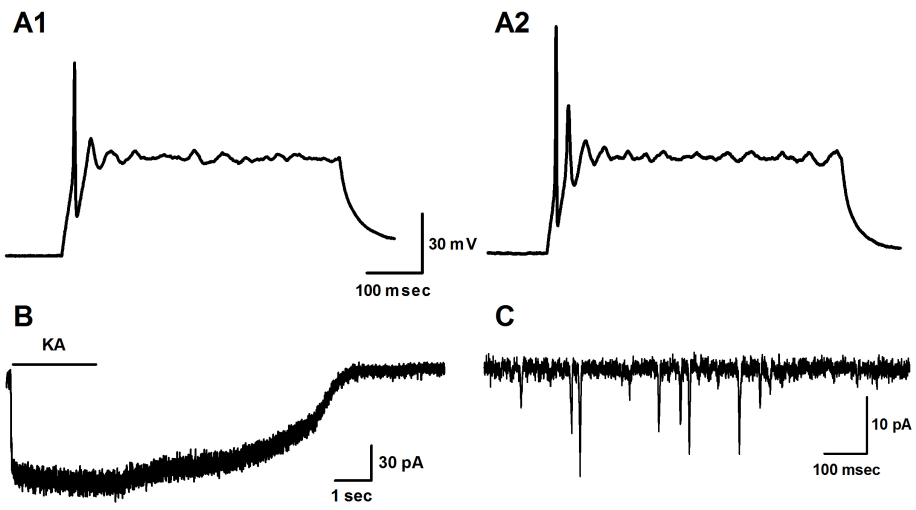


Figure 3.17. ASCL1-infected Müller glia-derived cells have neuron-like electrophysiological responses.
A1,2. Current clamp recordings of action potentials evoked in two different ASCL1-GFP+ cells with neuronal morphology 15dpi in response to a 40 pA current step lasting 500 msec. B. Voltage clamp recording of response (same cell as A1) to 3 sec application of 50 mM kainate (KA). C. Voltage clamp recording of spontaneous miniature postsynaptic potentials in a 3rd ASCL1-GFP+ cell 20dpi co-cultured for 18 days with retinal neurons dissociated from P0 animals.

ASCL1-expressing Müller glia generate new bipolar neurons in retinal explants

To test whether ASCL1 could induce neuronal conversion of Müller glia in the intact retina, explants were infected with ASCL1. We first validated that Müller glia in P12 *Hes5-GFP* retinas cultured as explants re-entered the mitotic cell cycle in response to EGF and incorporated EdU (Figure 3.18A; Ueki et al., 2012), since previous studies in other species suggests that Müller glia have to re-enter the cell cycle prior to their de-differentiation to progenitor cells. Müller glia typically migrate to the ONL during regeneration in those species where this process occurs naturally, and we find the same occurs in the explant cultures of mouse retina when stimulated to proliferate with EGF. To reliably induce ASCL1 expression, we then explanted retinas from *αPax6-cre;R26-stop-flox-rtTA* P12 mice, resulting in rtTA expression in the peripheral retina (Figure

3.18B). EGF was added to promote Müller glia proliferation, and EdU was included to track Müller glia and their progeny. Explants were infected with *tetO-ASCL1-mCherry* lentivirus in neural medium, and doxycycline was added after two days to activate ASCL1 and mCherry expression. ASCL1 and mCherry nearly perfectly colocalized (97-98.4%) in HEK293T cells (Figure 3.19), therefore, mCherry was used as a reliable marker for ASCL1-expressing cells.

Thirteen days after the induction of ASCL1-mCherry, retinal explants were immunolabeled with neuronal markers. Similar to what was observed in dissociated ASCL1-infected Müller glia cultures, there were significant numbers of EdU+/mCherry+ cells (ASCL1-infected Müller glia) that expressed bipolar cell markers (Figure 3.18C-E). Over 80% of ASCL1-infected Müller glia (EdU+/mCherry+) expressed Otx2 (Figure 3.18C, right bar) compared with less than 5% of uninfected cells (EdU+/mCherry-) (Figure 3.18C, left bar). Approximately half of the ASCL1-expressing proliferating Müller glia progeny also expressed Islet1, compared with less than 1% of the uninfected, EdU+/mCherry- population (Figure 3.18D). ASCL1-infected Müller glia also expressed PKC, a marker of more mature bipolar cells. Approximately 5% of ASCL1-infected Müller glia (EdU+/mCherry+) expressed PKC compared with <1% of uninfected Müller glia (EdU+/mCherry-) (Figure 3.18E). Many of the Müller glia-derived cells that express bipolar markers were in the inner nuclear layer, with the existing bipolar cells, though some were also located ectopically in the outer nuclear layer. No significant cell death was observed by DAPI staining (data not shown), suggesting that newborn cells survive in the native environment.

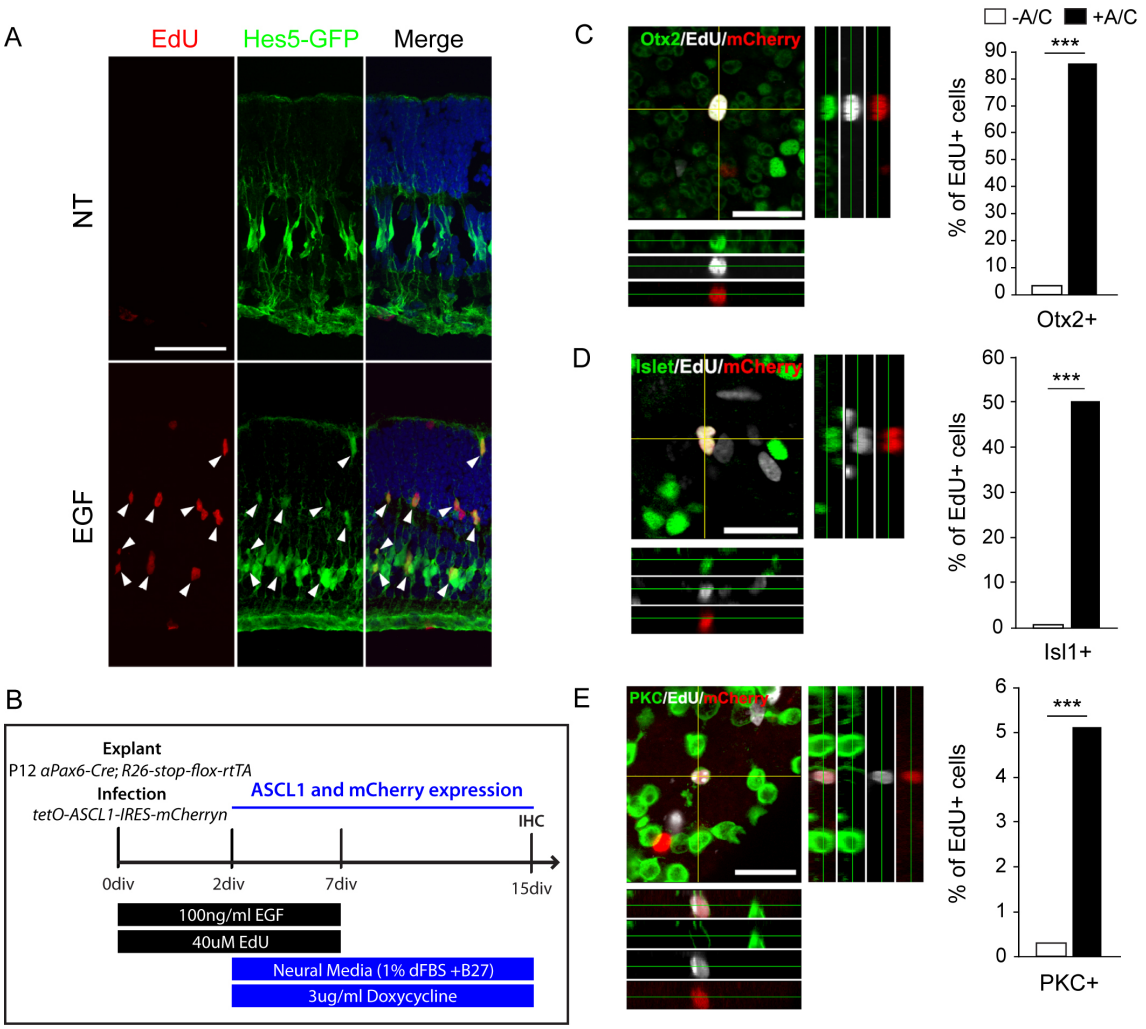
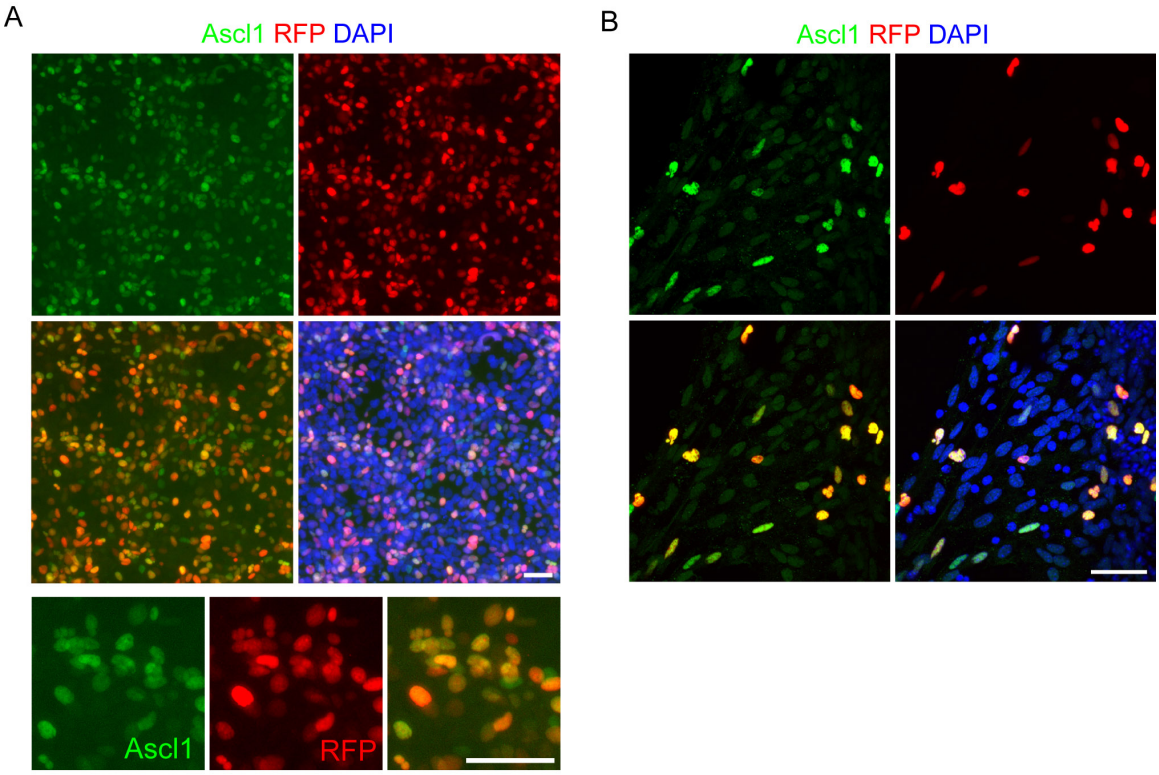


Figure 3.18. ASCL1 promotes reprogramming of Müller glia into bipolar cells in retinal explants.
A. *Hes5-GFP* retinas explanted at P12 with EdU ± EGF, 5 div. Proliferating Müller glia shown (Hes5-GFP+/EdU+, arrows). B. Experimental design. C, D, E. EdU+ (Müller glia-derived) mCherry+ cells label with Otx2 (C), Islet1 (D), and PKC (E). The % EdU+ cells in ASCL1-infected (+A/C) and uninfected (-A/C) Müller glia. 4-8 random fields/explant, 7 explants/marker were analyzed. Scale bars, 50 µm (A), 20 µm (C,D,E). Z-test, ***P<0.001; Z-score = -17.7 (C), -3.9 (D), -18.9 (E).



# RFP+ /Ascl1-	# Ascl1+ /RFP-	Total #	% RFP+ of Ascl1+	% Ascl1+ of RFP+
14	26	887	97.0	98.4

Figure 3.19. Validation of tet-ON-hAscl1-Ires-mCherry plasmid expression.
A. A stable cell line was created from HEK293T cells expressing rtTA protein. rtTA-expressing 293T cells were infected with *tet-ON-hAscl1-Ires-mCherry* lentiviral particles in the presence of 750ng/ml doxycycline and fixed at 3dpi. 97% of ASCL1+ cells immunolabeled for RFP and 98.4% of RFP+ cells labeled with Ascl1, indicating that mCherry/RFP can reliably be used as a marker for ASCL1 expression. Scale bars, 50μm.

Discussion

Here I report that mammalian Müller glia can be reprogramed to a neurogenic state by forced expression of the proneural transcription factor ASCL1. ASCL1 remodeled repressive chromatin at its targets to an active state and induced expression of progenitor genes, while downregulating Müller glia genes. Reprogrammed Müller glia produce cells that express pan-neuronal markers, neuronal morphology, and up-regulate many retinal-specific neuronal markers. Furthermore, the Müller glia-derived ASCL1-induced neurons exhibit functional activity in response to appropriate neurotransmitter receptor agonists. Finally, ASCL1-reprogramming of Müller glia in the intact retina revealed that newly generated neurons primarily differentiated as bipolar cells that integrated within the pre-existing neuronal population.

ASCL1 was sufficient to induce the re-expression of many progenitor genes, while inhibiting the glial differentiation program. Direct targets of Ascl1, *Dll1*, *Dll3*, *Hes5*, *Hes6*, and *Mfng* (Ueno et al., 2012; Castro et al., 2011) were bound and re-activated in the Müller glia and progenitor genes that are not direct Ascl1 targets, such as *Ngn2* and *Mycn*, were also increased; moreover the Ascl1 promoter was partly remodeled to a more active state. Taken together, these results suggest that ASCL1 initiates extensive reprogramming of the Müller glia.

The response of mouse Müller glia to ASCL1 expression is reminiscent of what occurs in zebrafish after retinal damage, where Müller glia de-differentiate into progenitors after upregulating ASCL1 (Fausett et al., 2008; Ramachandran et al., 2010; Thummel et al., 2008; Raymond et al., 2006). I observed that ASCL1 over-expression upregulates Notch pathway components and Insm1 (Figure 3.2D,F), which are

downstream of Ascl1a and required for de-differentiation in the regenerating fish retina (Ramachandran et al., 2012; Wan et al., 2012).

ASCL1 appears to reprogram Müller glia through a transitional progenitor state, and stimulates proliferation; however, I cannot say whether this is an obligatory step in the process of reprogramming Müller glia, since I did not determine whether the neuronal cells derived from the Müller glia have undergone a mitotic division after infection; moreover, direct neuronal reprogramming of astrocytes (Heinrich et al, 2010) to neurons with Neurog2 does not require mitotic division.

The reprogramming of Müller glia by ASCL1 includes remodeling of the chromatin at the promoters of progenitor genes, including *Ascl1* itself, for at least two important histone modifications. During glial differentiation, progenitors acquire the repressive chromatin mark H3K27me3 and lose the activation mark H3K27Ac at Ascl1 and its targets. However, at targets where ASCL1 was bound by ChIP, ASCL1-expressing Müller glia lost this repressive mark and gained the activation mark (although it is difficult to quantitatively compare the epigenetic state of these chromatin modifications between the progenitors and the reprogrammed Müller glia, due to heterogeneity in the populations). Several models have been put forth to explain reprogramming in contexts where transcription factors are forcibly expressed (Vierbuchen and Wernig, 2012). One model suggests a "permissive enhancer" in which a transcription factor is able to bind at sites of open chromatin at enhancers of activatable genes (Taberlay et al., 2011). An alternative model suggests that these factors can act as "pioneers" and bind at repressed elements to pave the way for other factors (Zaret and Carroll, 2011; Cao et al., 2010).

Müller glia-derived progenitors differentiated to form cells that shared morphological, immunohistochemical, and functional characteristics with retinal neurons. ASCL1-reprogrammed cells adopted a distinctly neuronal appearance by 6dpi, which coincided with their expression of pan-neuronal markers. These cells were responsive to ionotropic glutamate agonists, NMDA and Kainate, and showed decreased responsiveness to the purinergic receptor agonist ATP. These effects are comparable to those from studies of neurogenic reprogramming in postnatal cortical astrocytes (Addis et al., 2011; Berninger et al., 2007; Blum et al., 2011; Corti et al., 2012; Heinrich et al., 2010; Heins et al., 2002) and pericytes (Karow et al., 2012). Berninger et al. (2007) and Heinrich et al. (2010) found that Ascl1 or Ngn2 expression could convert fate-mapped postnatal astrocytes into morphologically distinct neurons by 4dpi. However, astrocyte-derived neurons did not form synapses and mature membrane properties until 2-3 weeks, suggesting that maturation may take significantly longer than initial reprogramming and cell type specification.

Newly generated neurons from ASCL1-reprogrammed Müller glia primarily differentiated as bipolar cells. In dissociated Müller glia, ASCL1 induced early markers of many retinal neuron subtypes; however, later neuronal markers were more restricted to bipolar cells (Otx2, Islet1, Calretinin, Vsx1, Cabp5, Bhlhb5, and PKC,) and, possibly, amacrine cells (Gad67, Bhlhb5, Calretinin). The bias towards bipolar cells was also apparent in retinal explants, where ASCL1-infected Müller glia generated a large number of Otx2+ and Islet1+ cells and a smaller number of PKC+ rod bipolar cells. This is consistent with the role of Ascl1 during normal development, since Ascl1 is expressed in late progenitors which give rise to amacrine cells, bipolar cells, and photoreceptor cells,

but not ganglion cells (Brzezinski et al., 2011), and deletion of Ascl1 in mice leads to a reduction in bipolar and photoreceptor cells (Akagi et al., 2004; Tomita et al., 2000; Brzezinski et al., 2011).

Previous reports have suggested that dissociated Müller glia, from both mouse and human, can act as stem cells by self-renewing and differentiating towards multiple neural lineages (Das et al., 2006; Nickerson et al., 2008; Lawrence et al., 2007; Giannelli et al., 2010). However, many of the stem and progenitor markers used in these studies, such as Nestin and Sox2, are expressed in cultured Müller glia (Karl et al., 2008; Bhatia et al., 2011; Lin et al., 2009), and they are not sufficient to specify the neural stem/progenitor fate. Recently, reports have suggested that Müller glia spontaneously produce neurons *in vitro* (Giannelli et al., 2010) or when co-cultured with hippocampal explants (Das et al., 2006). However, neither experiment distinguished putative new neurons from those that survived the dissociation (e.g. using a thymidine analog or lineage tracing method). Significant numbers of neurons can survive in dissociated retinal cell cultures, even after passage, and thus a method to distinguish surviving neurons from newly generated ones is critical.

Additionally, reports have claimed that Müller glia in mouse and rat retina can regenerate new neurons after damage if treated with exogenous factors. Wnt3a, EGF, FGF, IGF, RA, Notch, MNU, and α-AA have all been shown to stimulate a small number of Müller glia to re-enter the mitotic cell cycle (Ooto et al., 2004; Close et al., 2006; Osakada et al., 2007; Wan et al., 2008; Takeda et al., 2008; Karl et al., 2008; Del Debbio et al., 2010). Some of the progeny of the BrdU+ Müller glia were reported to differentiate characteristics of various types of retinal neurons, depending on the study and the

treatment. However, only one of these studies used confocal imaging and 3D reconstruction to definitively characterize the BrdU+ cells (Karl et al., 2008), and that study was only able to find a small number of new amacrine cells generated from the BrdU+ Müller glia (3.6%).

It is unclear in this report why ASCL1-reprogrammed neurons do not more efficiently differentiate and mature. ASCL1 alone was sufficient to robustly activate pan-neuronal and early markers of retinal neurons, but I found lower levels of genes expressed by more mature retinal neurons, such as Rhodopsin and S-opsin. ASCL1 can activate its direct targets, including *Otx2*, which commits progenitors to photoreceptor or bipolar fates. However, direct targets of Otx2, such as *Nrl* and *Pde6b*, are not robustly expressed, and only 5% of ASCL1-infected Müller glia differentiated to PKC+ bipolar cells, though much higher percentages expressed Otx2 and Isl1. Several potential mechanisms could account for the limited ability of Müller glia-derived neurons to fully mature. Continued expression of progenitor markers, like Pax6, Id1 and Hes5 may limit full differentiation, though this may be also due to continued expression of ASCL1 in the cells. It may also be the case that factors in the retinal environment, not present in the dissociated cultures, are needed for differentiation and/or survival of the new neurons derived from the ASCL1-reprogrmmed Müller glia. Evidence for this comes form the fact that a much higher percentage of the ASCL1-reprogrammed Müller glia express PKC and Isl1 in the explant cultures than in the dissociated cell cultures. Epigenetic restrictions in the Müller glia might also limit full reprogramming. Although repressive histone modifications are remodeled at the Ascl1 targets, other genes may persist in a repressed state. Genes required in photoreceptor cells, eg. *Rhodopsin*, may be repressed

by different mechanisms than progenitor and early neuronal specification genes. A recent report by Powell et al. (2012) found that retinal regeneration in fish required expression of the cytidine deaminases *apobec2a* and *apobec2b*, involved in DNA demethylation.

Viral over-expression of ASCL1 in Müller glia suggests a strategy for stimulating regeneration of the mammalian retina. This approach is complementary to transplantation of stem-cell derived neurons for retinal repair, since it allows for Müller glia to be targeted for reprogramming within their native environment. Viral reprogramming Müller glia cells with ASCL1 predominantly generates bipolar neurons, so other reprogramming factors will likely be needed to direct the Müller glia to photoreceptor and ganglion cells. Nevertheless, our study shows that viral reprogramming may allow Müller glia to serve as a source of new retinal neurons to treat retinal degenerative diseases and one day provide a basis for gene therapeutic approaches.

Chapter 4:

Potentiating Müller glial reprogramming with a combinatorial approach

Introduction

In Chapter 3 I demonstrated that ASCL1 overexpression induced Müller glia to upregulate progenitor and neuronal genes and differentiate as functional bipolar cells. However, a subset of progenitor genes, primarily those present during early retinal development, fail to re-activate in the presence of ASCL1. ASCL1 is also insufficient to induce robust expression of genes that are specific to the development of other retinal neuronal sub-types, e.g. photoreceptor cells. Many factors could be present in Müller glia that block their ability to turn back this developmental clock. Possibilities include the presence of repressors, such as glial genes that may drive a gliogenic program, epigenetic modifications that block neurogenic gene activation, or a lack of neurogenic fate determinants that are normally present and required in the developing retina.

To account for this later possibility, I tested whether a lack of neurogenic activators could account for the inability of Müller glia to fully de-differentiate as retinal progenitors. The aim was to understand whether early progenitor genes could be reactivated in Müller glia – a step that may be required for the specification of additional neuronal subtypes. To test their reprogramming ability, in combination with ASCL1, I infected Müller glia with neurogenic factors that are known to be involved in retinal development or neural reprogramming. Intriguingly, one transcription factor Zic1 partially turned on a subset of progenitor genes that ASCL1 alone did not activate.

Zic1 is a zinc finger transcription factor that is the vertebrate homolog of the Drosophila odd-paired gene. It has important roles in neural developmental processes in a number of species, including the differentiation of ectoderm into neuroectoderm, neural crest development, and formation of the cerebellum (Merzdorf, 2007). In humans,

mutations in the gene have been linked to congenital malformations, including Dandy-Walker malformation (Grinberg and Millen, 2005). However, the role of Zic1 in the development of the retina is less clear. *Zic1* mRNA, along with that of family members *Zic2* and *3*, is expressed in the embryonic retina by E14 and gradually declines postnatally (Watabe et al., 2011). By P12, *Zic1* is no longer expressed in retinal neurons or Müller glia, in my hands (data not shown).

Since *Zic1* is expressed in retinal progenitors, especially in the early phase of neurogenesis [E17: 9.61 log(2) normalized fluorescence by microarray (unpublished data, Reh lab)] but declines in later development [P0: 7.95 log(2) normalized fluorescence (Nelson et al., 2011)], I tested whether Zic1 could reactivate an early developmental program in Müller glia partially reprogrammed by ASCL1. Forced expression of Zic1 in combination with ASCL1 activated a subset of early progenitor genes in P12 Müller glia. However, there was no indication that these expression changes influenced the generation of alternative retinal neuronal fates.

Results

A subset of progenitor genes fails to upregulate in response to ASCL1 overexpression alone.

In Chapter 3, I compared robustly expressed genes in FAC-sorted P0 progenitors to those found in mature Müller Glia to generate a list of progenitor-specific genes. The majority of these genes were activated in Müller glia in response to ASCL1 (Figure 3.2). However, expression levels of a smaller subset of progenitor genes remained unaffected

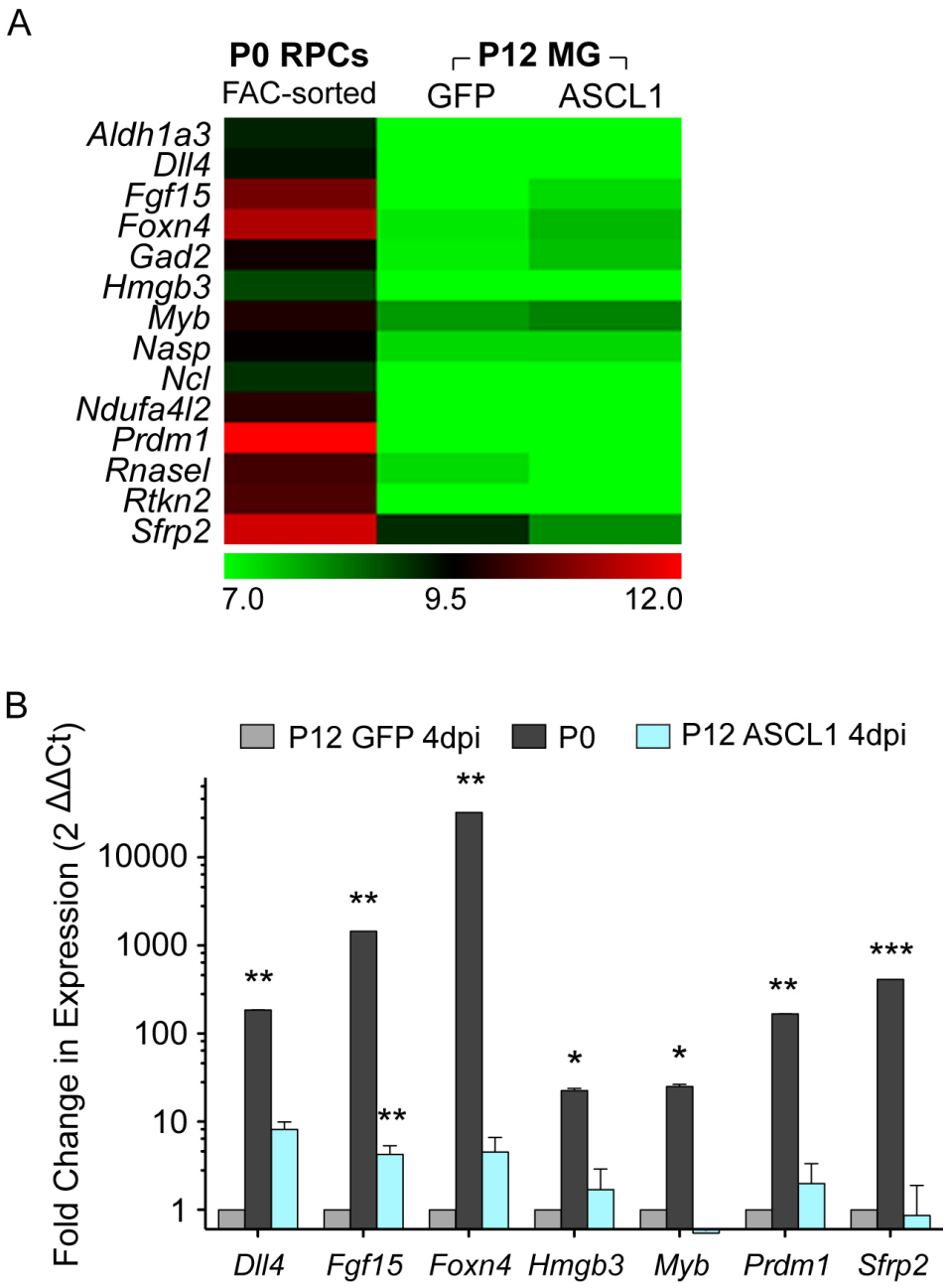


Figure 4.1. A subset of progenitor-specific genes are not reactivated in Müller glia by Ascl1 alone. A subset of genes on microarray analysis at 4dpi are highly expressed (log transformed normalized values) in P0 progenitors but do not upregulate after Ascl1 infection of Müller Glia. B. These expression patterns were confirmed by qPCR. Bars, mean ± SEM. One-way student's t-test, *P<0.05, **P<0.01, ***P<0.001.

in ASCL1-infected Müller glia (Figure 4.1A). Many of these genes are typical of early progenitors (e.g. *Fgf15, Sfrp2, Hmgb3*), which don't express high levels of Ascl1. I confirmed the expression levels of a subset of these genes by qPCR (Figure 4.1B). Early progenitor genes, *Dll4, Fgf15, Foxn4, Hmgb3, Myb, Prdm1,* and *Sfrp2,* were highly expressed in P0 retinal progenitors compared to P12 GFP-infected Müller glia in culture. However, none of these targets were activated following four days of ASCL1 infection; *Fgf15* had a minor increase in expression that was not comparable to levels of robustly activated genes (Figure 3.2).

Neurogenic reprogramming factors activate early retinal progenitor genes in Müller glia

Since early progenitor genes failed to activate in response to ASCL1, I tested whether the addition of transcription factors important to early development or neural reprogramming would force Müller glia to de-differentiate to an earlier developmental state. A set of transcription factors was chosen because they are highly expressed in retinal progenitors and low in Müller glia (*Lin28, Olig2, Brn2, Zic1).* Other factors were chosen because they have a known role in aiding reprogramming towards neural lineages in fibroblasts (*Lin28, Myt1l, Neurod1*; Pang et al., 2011; Vierbuchen et al., 2010). We were also concerned about potential inhibition of ASCL1 function by the repressor Id1, which is present in Müller glia and progenitors and binds to ASCL1's binding partners E12 and E47 blocking some of ASCL1's function. To overcome this, I also tested expression of a construct in which ASCL1 is fused to its binding partner E47.

Candidate reprogramming factors were placed into lentiviral vectors, in some cases in tet-inducible vectors to regulate their expression levels and timing. Cultured P12

Müller glia were infected with these viruses in combination with ASCL1 or as pools of factors. Five days after infection, factors were robustly expressed by qPCR (Figure 4.2).

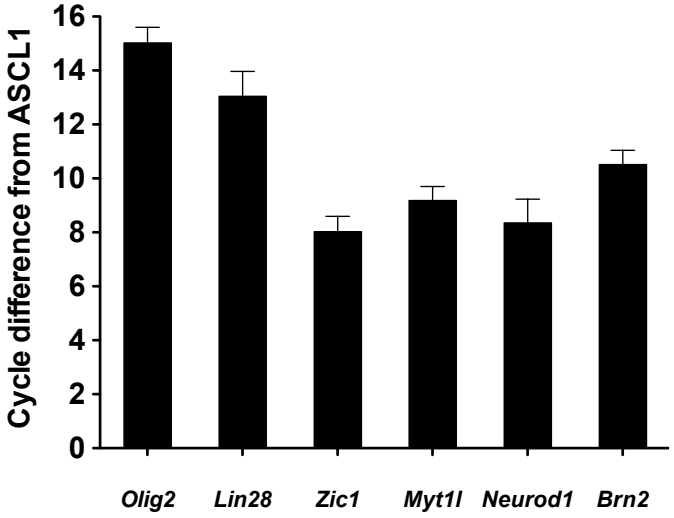


Figure 4.2. Robust exogenous expression of reprogramming factors in infected Müller glia. P12 Müller glia infected with combinations of ASCL1 and one other reprogramming factor for 5 days. QPCR for expression levels of exogenous genes compared to samples infected with ASCL1 alone (deltadeltaCt). Bars, mean ± SEM.

I next tested whether overexpression of these transcription factor combinations in Müller glia could activate a subset of progenitor genes. This subset, *Fgf15, Foxn4, Myb, Prdm1*, was chosen because it encompassed transcription factors that are important for normal retinal development and failed to re-activate in response to ASCL1 infection alone (Figure 4.1). Compared to ASCL1-only infection (value of 1), most combinations of factors did not change the expression levels of the selected progenitor genes (Figure 4.3). However, two combinations, ASCL1+Zic1 (orange bars) and ASCL1+Zic1+Olig2+Lin28 (yellow bars) increased early progenitor genes, *Foxn4* and *Fgf15*, although these levels were modest compared to those found in P0 progenitors (not shown). Levels of the

progenitor genes *Myb* and *Prdm1* were unaffected by any combination of transcription factors.

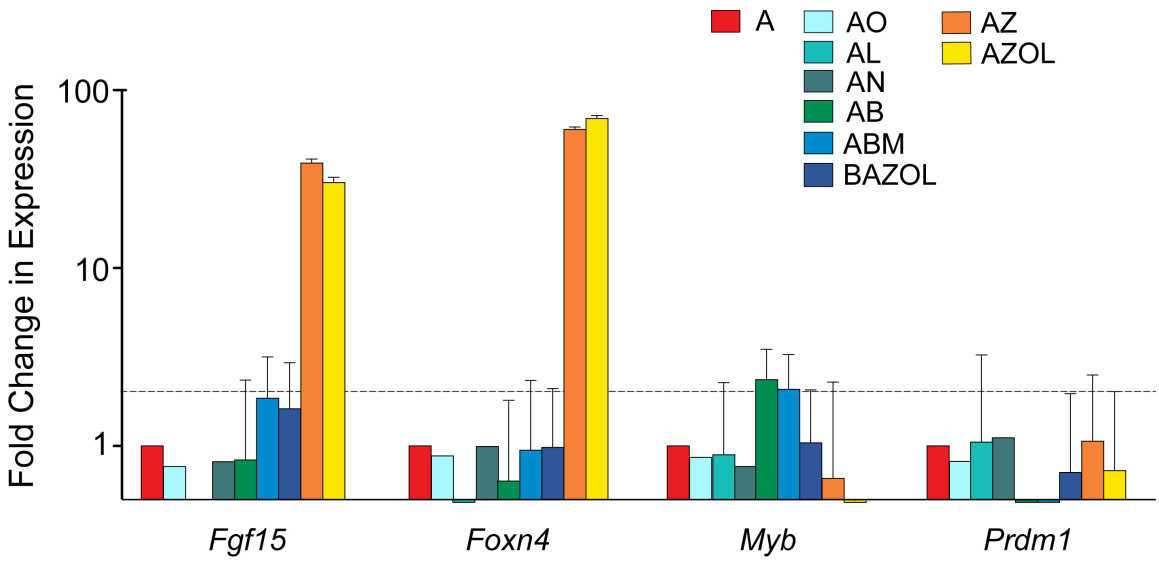


Figure 4.3. Expression of early progenitor genes is induced by lentiviral combinations with Zic1. Expression of progenitor genes (x-axis) in response to combinations of reprogramming factors compared to infection with ASCL1 alone (normalized to a value of 1), P12 Müller glia 5dpi. Combinations with Zic1 activated *Fgf15* and *Foxn4*, which are not activated by ASCL1 alone. A, ASCL1; O, Olig2; N, Neurod1; L, Lin28; B, Brn2; M, Myt1l; Z, Zic1. Fold change, $2^{\text{deltadeltaCt}}$. Bars, mean ± SEM.

To look for the maximum likelihood of reprogramming to early progenitors, I further analyzed the reprogramming potential of the combination of early progenitor genes ASCL1, Zic1, Olig2, and Lin-28 (AZOL). Müller glia were cultured as previously described and infected for five days with ASCL1 or AZOL. To look for broad changes in progenitor gene expression, I isolated RNA from these populations and compared their gene expression profiles by microarray analysis (Figure 4.4). First, the subset of early progenitor genes that failed to upregulate with ASCL1 infection alone was analyzed following addition of AZOL (Figure 4.4A'). As expected from the qPCR data, *Fgf15* was robustly induced; however, *Foxn4*, unexpectedly, was not activated in AZOL compared

to ASCL1-infected samples (Figure 4.4A). *Dll4* was also modestly increased. AZOL did not influence the expression levels of any of the other progenitor genes that were not induced by ASCL1 alone (Figure 4.4A').

Next, I analyzed changes in the broader set of progenitor-specific genes, many of which I had previously confirmed to be downstream of ASCL1. Interestingly, two progenitor genes that were activated by ASCL1, *Fabp7* and *Neurog2*, were further upregulated with the addition of the AZOL combination, suggesting that the induction of some progenitor genes is potentiated by Zic1, Olig2, or Lin-28 (Figure 4.4B). Surprisingly, some of the other genes in this group that are targets of Ascl1, *Cxcr4, Heyl,* and *Mycn,* were reduced by AZOL (Figure 4.4B'). Although it is not clear why AZOL would moderate the expression levels of these genes, it may be that the repressor *Olig2* blocks some of ASCL1's downstream effects. Consistent with this idea, *Otx2*, *Tubb3* (Tuj1), and *Mapt* (Map2), which are normally robustly induced by ASCL1, were no longer activated following AZOL induction (data not shown). The expression levels of other progenitor genes in this subset were not influenced by AZOL (Figure 4.4B").

I next examined the expression of genes that are found in progenitors but failed to make our stringent list of progenitor-specific genes, which was limited to a handful of highly differentially expressed genes between progenitors and Müller glia. *Cdh6* and *Ngfr* are both expressed in progenitors and fail to upregulate in response to ASCL1 infection alone (data not shown). However, both *Cdh6* and *Ngfr* were increased in response to AZOL overexpression compared to ASCL1 alone (Figure 4.4C).

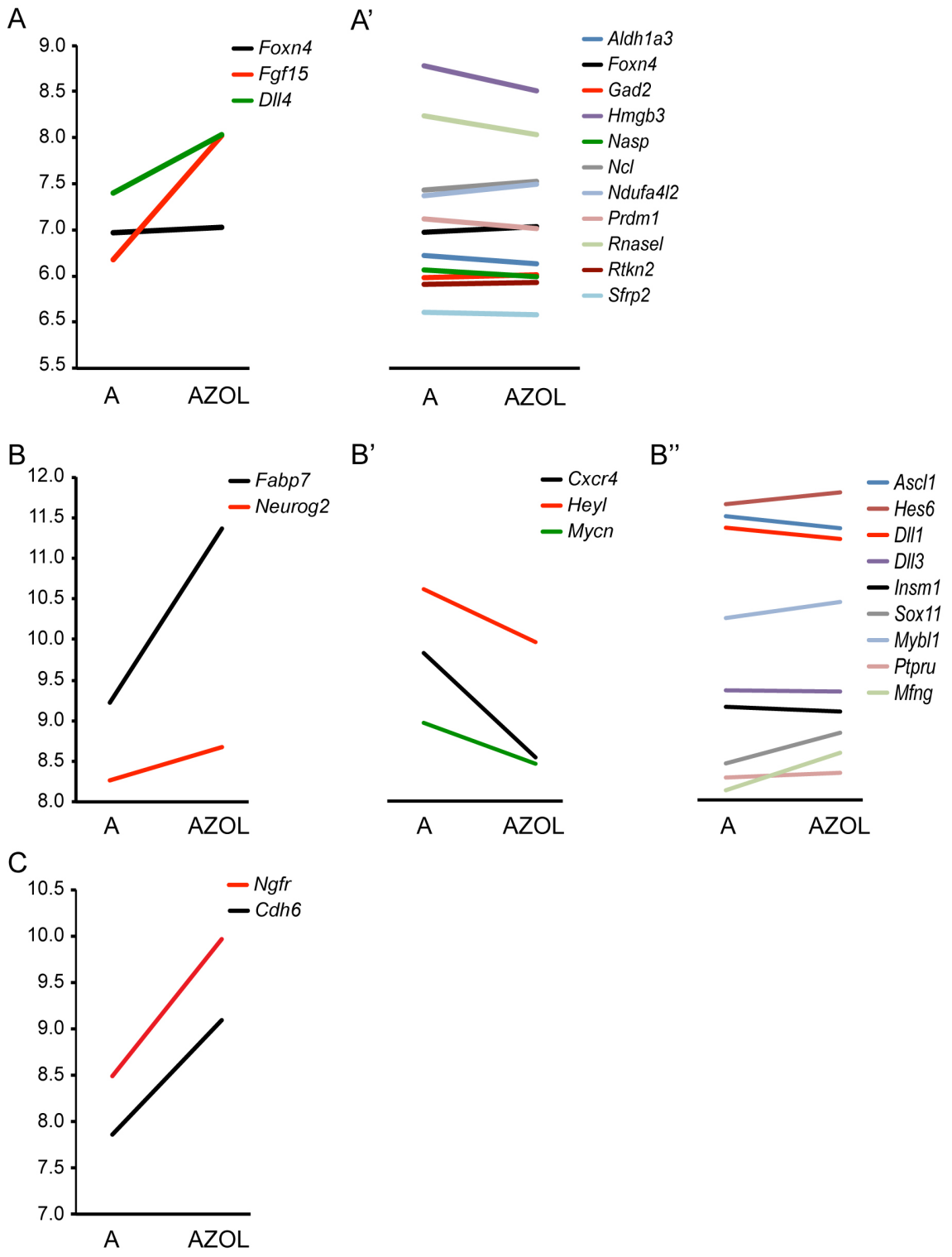


Figure 4.4. Changes in progenitor gene expression in response to AZOL infection. Microarray analysis comparing P12 Müller glia infected with ASCL1 or AZOL (Ascl1, Zic1, Olig2, Lin28) for five days. A. Set of progenitor genes that fail to activate by ASCL1 alone (reference Figure 4.1). A. *Fgf15* and *Dll4* but not *Foxn4* upregulate in response to AZOL. B. *Fabp7* and *Neurog2* increase after AZOL (B), while *Cxcr4*, *Heyl*, and *Mycn* are reduced (B'). Progenitor genes increased after ASCL1 infection are unchanged with the addition of AZOL (B''). C. Additional progenitor genes *Ngfr* and *Cdh6* are strongly induced by AZOL.

Interestingly, expression of BMP pathway components was also altered in response to AZOL. *Bmp4* and *Bmp7* were both increased in response to AZOL, although *Bmp7* showed a much more dramatic increase rising from a level of 8.7 to 11.7 fold. Since the BMP pathway helps regulate the glial fate (Ueki and Reh, 2013), I would expect glial genes to increase in response to increased BMP signaling. However, the expression of glial genes *Rlbp1* and *Gfap* was curbed, albeit modestly.

The transcription factor Zic1 is sufficient to turn on early progenitor genes.

Since Zic1 was the only common factor among viral combinations that led to an upregulation of progenitor genes by both qPCR and microarray, I further analyzed the effects it had on gene expression. First, I tested whether the two early progenitor genes that were induced by AZ and AZOL, *Foxn4* and *Fgf15*, could be activated by Zic1 alone. mRNA levels of these genes in Müller glia infected for five days with Gfp, ASCL1, Zic1, or ASCL1 + Zic1 were examined (Figure 4.5A). *Foxn4* and *Fgf15* were upregulated in response to ASCL1+Zic1, but Zic1 alone was able to induce similar levels of expression at 5dpi or 7dpi (Figure 4.5B). As expected, Zic1 alone or in combination with ASCL1 still had no effect on levels of *Myb* or *Prdm1*.

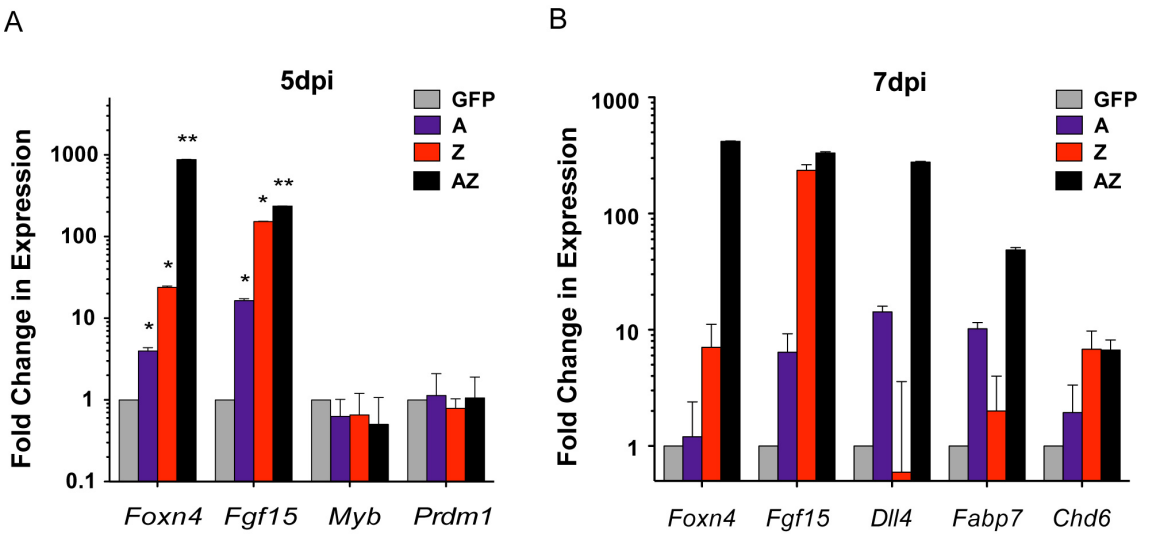


Figure 4.5. Zic1 is sufficient to activate a subset of progenitor genes. Expression of progenitor genes by qPCR after P12 Müller glial infection with ASCL1, Zic1, or in combination at 5dpi (A) or 7dpi (B). A, ASCL1; Z, Zic1. Fold change, $2^{deltadeltaCt}$. Bars, mean ± SEM. Student's t-test, *P<0.05, **P<0.01.

I next validated our microarray findings by qPCR and tested whether the observed changes in progenitor gene expression were contributed by Zic1 induction rather than Lin-28 or Olig2. At 7dpi, Zic1 potentiated the effect of ASCL1 on the expression of *Dll4, Fabp7, and Cdh6,* progenitor genes that upregulated in response to AZOL on the microarray analysis (Figure 4.5B). However, only *Cdh6* was activated by Zic1 alone at comparable levels to AZ; *Dll4* and *Fabp7* were not substantially induced by Zic1 alone, although Zic1 did potentiate the effects of ASCL1. I also tested whether Zic1 induced changes in genes expressed during early development that were not activated by ASCL1. Expression changes in early progenitor genes, *Six3, Pax6, Hmgb3, Prtg*, *Onecut2* (Figure 4.6) and *Rnasel, Sfrp2* (data not shown) were examined by qPCR, but were not altered in response to Zic1 or the AZ combination. These data suggest that Zic1 alone is sufficient to activate some early progenitor genes in Müller glia, but, in corroboration with the

microarray data, fails to activate other progenitor genes needed for full de-differentiation to a retinal progenitor state.

Since a subset of early progenitor genes are activated in response to Zic1, it is possible that these progenitors have a greater capacity to differentiate towards alternative retinal neuronal fates. I tested whether early specification markers for amacrine and ganglion cells, two cell types that are not generated in ASCL1-expressing Müller glia, were increased following Zic1 overexpression. Early ganglion (*Cdh6*) and amacrine genes (*Dll4, Foxn4*) were upregulated in response to Zic1 on microarray analysis (Figure 4.4B) or qPCR (Figure 4.5B). However, Zic1 did not activate other early genes specific to ganglion (*Six3, Pax6, Onecut2*) or amacrine cells (*Six3*) (Figure 4.6).

ASCL1 overexpression produced Müller glia that turned on a partial set of developmental genes; yet, these "partial" progenitors were still capable of differentiating

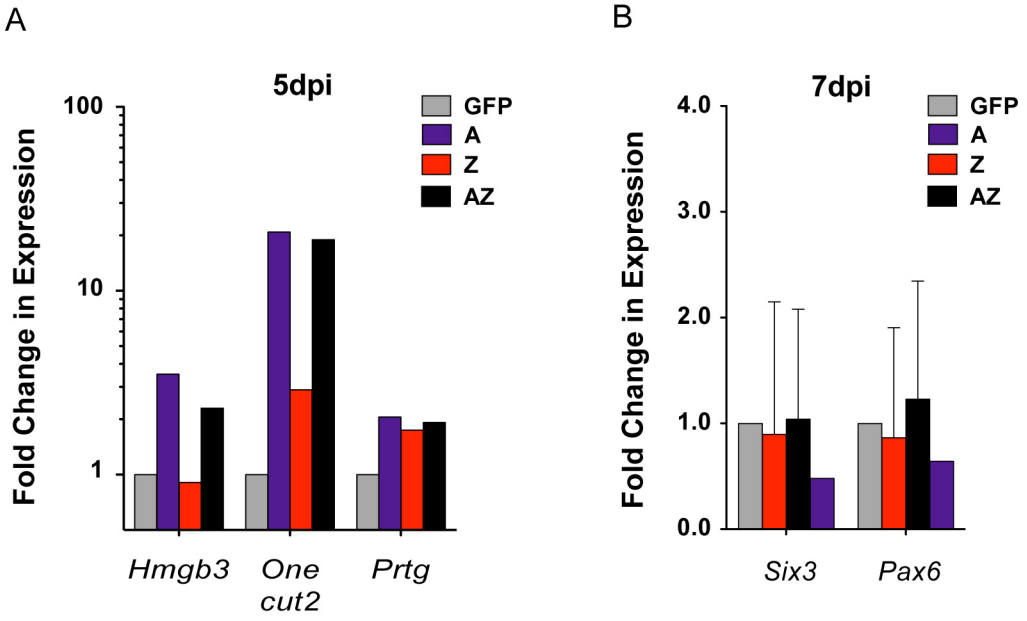


Figure 4.6. Zic1 does not activate additional early progenitor genes. Expression of progenitor genes by qPCR after P12 Müller glial infection with ASCL1, Zic1, or in combination at 5dpi (A) or 7dpi (B). A, ASCL1; Z, Zic1. Fold change, $2^{\text{deltadeltaCt}}$.

towards neurons. Therefore, the ability of Zic1 to potentiate expression of early progenitor genes may be sufficient to turn on mature neuronal genes and drive cells towards non-bipolar cell fates. I looked for expression of early and late neural differentiation genes that are found in ganglion, amacrine, and other retinal cell types, *Neurod4/Math3, Neurod1, Bhlhe22/Bhlhb5, Gad1/Gad67, Atoh7/Math5, Thy1, Nrl, Otx2, Tubb3,* by qPCR. Zic1 failed to activate these genes in Müller glia at either 5 or 7dpi (Figure 4.7). Furthermore, I did not detect immunolabeling of ganglion or amacrine markers in Zic1 or AZ-infected Müller glia (preliminary data, not shown). Compared with ASCL1 or GFP-infected controls, Zic1 or AZ-infected cells did not change morphology or appear more "neuronal". Finally, Zic1 did not affect Müller glial-specific gene expression (data not shown), suggesting that the glial identity of these cells was not mutable in the presence of Zic1.

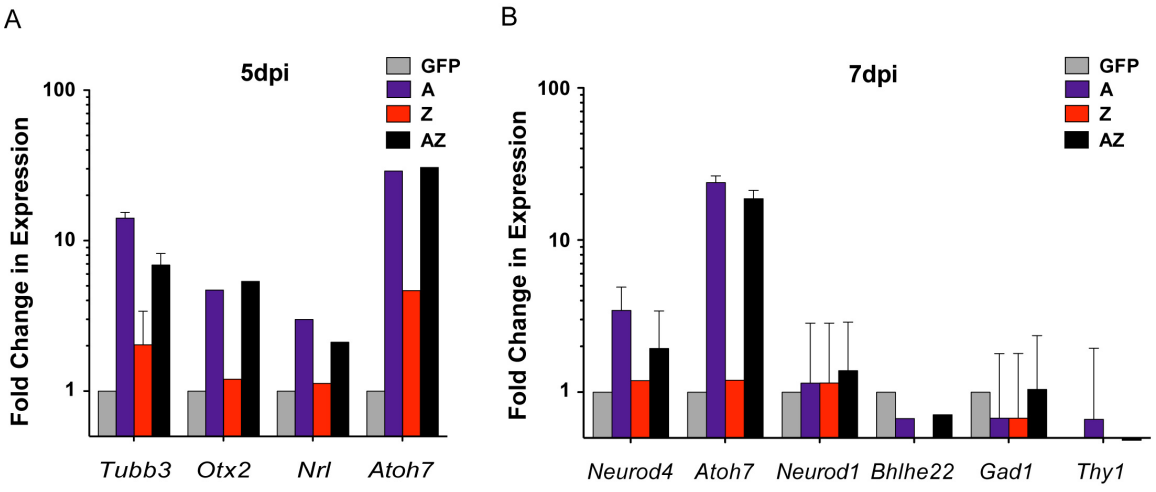


Figure 4.7. Neural differentiation genes are not induced by Zic1. Expression of genes found in developing and post-mitotic retinal neurons. QPCR after P12 Müller glial infection with ASCL1, Zic1, or in combination at 5dpi (A) or 7dpi (B). A, ASCL1; Z, Zic1. Bars, mean ± SEM. Fold change, $2^{deltadeltaCt}$.

Prdm1/Blimp1 is not sufficient to direct reprogrammed Müller glia towards photoreceptor fates.

To direct reprogrammed Müller glia towards more differentiated states, especially photoreceptor fates, I looked towards factors that are known to be involved in photoreceptor specification. ASCL1 was able to induce expression of Otx2, which is required for both bipolar and photoreceptor specification. These cells can further turn on later bipolar cell markers such as PKC and Islet1, but they fail to activate photoreceptor-specific genes *Nrl, Rhodopsin,* and *S-opsin* (Figure 3.14). This suggests a lack of photoreceptor specification/differentiation genes that may need to be included to generate these cell types. Therefore, I tested whether overexpression of a factor that mediates photoreceptor fate determination, Prdm1/Blimp1 (Brzezinski et al., 2010) could direct Müller glia towards photoreceptor fates.

Prdm1/Blimp1 is a zinc-finger transcription factor that recruits repressive machinery to target genes. In the retina, Prdm1 is transiently expressed in developing photoreceptor cells and is necessary to shift Otx2+ precursors towards the photoreceptor fate and away from the bipolar fate. *Prdm1* mutants generate increased numbers of bipolar cells at the expense of photoreceptor cells, and Prdm1 binds to the enhancer of the bipolar-inducing factor Vsx2/Chx10 to repress it (Brzezinski et al., 2013).

Prdm1 was cloned into a tet-inducible lentiviral vector and transfected into P12 Müller glia, as previously described, either alone or in combination with ASCL1. Müller glia were cultured for 7 days after infection; aside from a modest increase in the number of surviving Müller glia compared to ASCL1-infected cultures, there were few gross differences in the numbers or morphology of reprogrammed cells.

To look for enhanced reprogramming toward photoreceptor fates, Prdm1/ASCL1-infected Müller glia were isolated for RNA analysis and qPCR (Figure 4.8). Compared to uninfected and ASCL1-infected controls, Prdm1- and Prdm1/ASCL1-infected Müller glia expressed the *Prdm1* gene, although not as highly as ASCL1 in ASCL1-infected conditions. I examined expression of early photoreceptor specification genes *Thrb*/Trβ2 and *Nrl*. *Nrl* is expressed early after rod formation and is required for their specification; *Nrl* deletion leads to a loss of rods. *Thrb*/Trβ2 is an early specific marker of cones. Neither gene was affected by Prdm1 or ASCL1 overexpression compared to uninfected controls at 7dpi. *Nrl* also regulates *Rho* (Rhodopsin) expression, which is a later rod-specific marker; as predicted from *Nrl* expression, *Rho* was not induced by Prdm1. Finally, I tested whether the bipolar maker Vsx2/Chx10, which is necessary for bipolar cell specification, was repressed by Prdm1, as predicted by Brzezinski et al. (2013). *Vsx2* levels were not reduced by Prdm1; however Vsx2 is normally expressed by cultured Müller glia at reasonable levels and is not induced by ASCL1 alone, though other bipolar genes are.

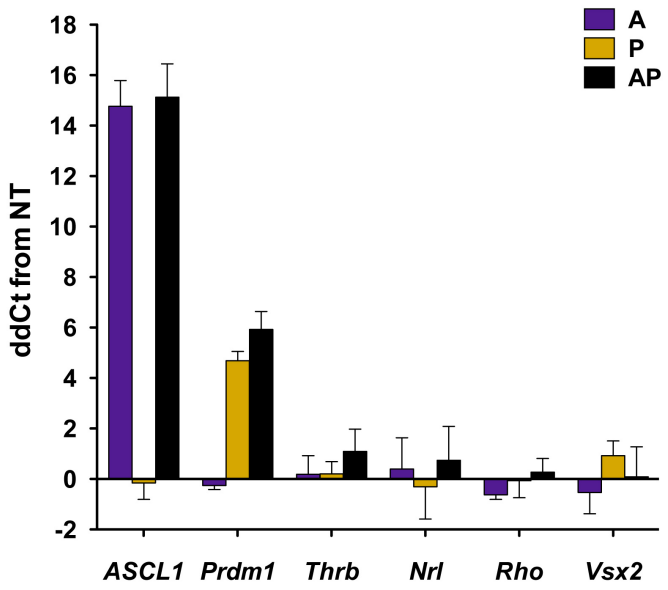


Figure 4.8. Photoreceptor genes are not induced by Prdm1/Blimp1. Although Prdm1 overexpression was induced, early cone gene *Thrb* and rod genes, *Nrl* and *Rho*, were not upregulated. QPCR after P12 Müller glial infection with ASCL1, Prdm1, or in combination at 7dpi. A, ASCL1; P, Prdm1. Bars, mean ± SEM. DeltadeltaCt from non-treated.

Discussion

The findings in this chapter suggest an improved protocol for generating neural progenitors from cultured Müller glia. Several combinations of transcription factors were overexpressed in Müller glial cultures, but only one factor, the zinc finger transcription factor Zic1, influenced the ability of these cells to de-differentiate. Zic1 activated a subset of genes that are involved in the early stages of retinal development, but had no effect on neural genes necessary to generate alternative retinal cell fates.

These studies focused on reactivating the set of genes that are expressed in early progenitors. We believe that efficient reprogramming to an early retinal progenitor state will be vital for successful regeneration of the rodent retina. First, this reprogramming recapitulates the processes during normal development of the retina. Although fibroblasts

can reprogram to neurons without reversing through a neural progenitor state, it would be advantageous to generate proliferative progenitors that could replenish lost Müller glia while generating the myriad of cell types. Secondly, Müller glial-based regeneration in the fish retina does undergo a progenitor phase when neurogenic clusters of progenitors are formed that generate new neurons. Since this is likely to be a latent potential of rodent Müller glia, it would be advantageous to stimulate these naturally regenerative processes. Finally, ASCL1 reprograms Müller glia primarily towards the bipolar fate, a less than ideal cell type to target for the treatment of retinal disease (which primarily affect photoreceptor and ganglion cells). Generating early retinal progenitors may allow for the normal process of neurogenesis that would give rise to the diversity of retinal cell types, including early-born ganglion cells, cones, and horizontal cells.

Zic1 had a modest but reproducible effect on the expression levels of early progenitor genes. Although ASCL1 failed to activate the early genes *Cdh6, Dll4, Foxn4,* and *Ngfr,* the combination of ASCL1+Zic1 increased their expression substantially. In the case of *Cdh6*, Zic1 over-expression alone could induce these changes; however, although neither ASCL1 nor Zic1 modified *Foxn4* expression levels, the combination of the two factors dramatically induced its expression. This suggests some synergistic effect of Zic1 and ASCL1 at the *Foxn4* locus. Overexpression of Zic1 in combination with ASCL1 also potentiated ASCL1's activation of the early genes *Fgf15, Fabp7,* and *Neurog2*. Although Zic1 alone could induce *Fgf15* expression, it had no effect on the expression level of *Fabp7* alone, again suggesting synergistic effects. Furthermore, other early progenitor genes *Six3, Pax6, Rnasel, Hmgb3,* and *Sfrp2* were not activated, suggesting the need for additional activators or other interventions to fully stimulate the

progenitor program. Additionally, identification of Zic1 targets in the retina through ChIP experiments would help generate a clearer picture of the relationships represented here.

The ability to proliferate in a controlled manner is another hallmark of progenitor cells. Evidence has shown that Zic1 allows progenitors to proliferate beyond the normal window; Zic1 over-expressing retinal progenitors were able to sustain proliferation (Watabe et al., 2011). It is unclear how Zic1 might influence cell cycle maintenance; cell cycle genes were not upregulated following AZOL expression in the studies presented here. It is possible that Zic1 extends the progenitor window through Id expression. Ids maintain progenitors during retinal development by repressing proneural bHLH factors. During retinal development, the Ids have overlapping levels and timing of expression with the Zic proteins. Additionally, Watabe et al. (2011) noted that Zic2 overexpression led to an increase in Id3 expression. Since it will be valuable to understand whether Zic1 exerts these effects on Müller glia, the Reh lab will test whether Müller glia are more likely be found in the cell cycle after Zic1 overexpression by EdU window labeling.

Although we had expected that expressing combinations of neurogenic activators would induce earlier progenitors capable of more extensive differentiation, I did not find much evidence for this. ASCL1 overexpression alone pushed Müller glia towards bipolar neurons, although early neural genes of most retinal lineages were activated. Zic1 did not induce expression of any neural determination genes, such as *Neurod4, Neurod1, Atoh7*. However, evidence suggests that Zic1 may in fact prevent neural differentiation. Zic1 is downregulated in mature neurons, which may allow for their differentiation. Overexpression experiments have shown that Zic1 blocks the ability of progenitors to differentiate as photoreceptor cells, although other cell types were not affected (Watabe

et al., 2011). If Zic1 sustains the proliferation potential of progenitors it may contribute to maintaining the progenitor state and account for delaying their differentiation.

Although Zic1 does not appear to affect cell fate specification, it does upregulate two progenitor genes, *Cdh6* and *Ngfr*, that are involved in regulating axonal processes. Cdh6, the adhesion molecule cadherin-6, is expressed by a subset of ganglion cells and their axons. It is critically involved in ganglion cell neurite pathfinding or target recognition during development; *Cdh6*-null mice innervate targets incorrectly in visual centers in the brain (Osterhout et al., 2011). *Ngfr* is similarly involved in axon targeting and is also known as p75 neurotrophin receptor (p75NTR), which interacts with tyrosine kinase receptors (Trk). Ngfr/p75 is expressed in ganglion cells and the optic nerve during development and is necessary for proper axon pathfinding; *p75*-/- mice mistarget axons to inappropriate layers within the superior colliculus, although the development and numbers of ganglion cells remain unaffected (Lim et al., 2008). p75 is also associated with cell survival and the apoptotic cascade, although I did not observe any gross increases in cell death after Zic1 infection. There is no direct evidence that either *Cdh6* or *Ngfr* are regulated by Zic1 (or Lin-28 or Olig2) in the retina or elsewhere in the CNS. Zic1 ChIP studies could address these relationships. It is additionally unclear whether over-expression of Zic1, and the associated upregulation of these axon guidance genes, affects neurite outgrowth in reprogramming Müller glia. Although I did not observe any gross changes on neurite outgrowth, these changes may become more apparent during reprogramming in the *in vivo* retinal environment where neurite targets are present.

Unexpectedly, the majority of reprogramming factors that were tested did not affect gene expression. However, this is not surprising in the field of somatic cell

reprogramming. Many studies test the reprogramming potential of large numbers of reprogramming factors, but very few of these combinations lead to meaningful cell fate changes (Ambasudhan et al., 2011; Vierbuchen et al., 2010). Therefore, additional neurogenic transcription factors may be needed to induce an early progenitor state and additional retinal cell fates. During retinal development, combinatorial misexpression of bHLH proneural activators was required to drive the neuronal program (Tomita et al., 2000; Inoue et al., 2002). Since many bHLH factors work synergistically by dimerizing to activate common targets, it is likely that multiple factors will be required to activate some developmental targets. Several reports have found that additional reprogramming factors, such as *Myt1l* and *NeuroD1*, are needed for fuller morphological maturation of reprogrammed neurons (Vierbuchen et al., 2010; Pang et al., 2011). It is possible that these activators are already expressed at sufficient levels, but that repressive elements, e.g. epigenetic silencing, are active to dampen their effects and need to be targeted for suppression.

On the other hand, specific determinant factors required to direct cells toward specific cell lineages may not be present in Müller glia. *Prdm1*/Blimp1 is required for commitment to the photoreceptor lineage (Brzezinski et al., 2010), but is not induced in Müller glia by ASCL1 overexpression. However, in my results Prdm1 overexpression in combination with ASCL1 failed to increase photoreceptor genes in Müller glia. There may be additional repressive mechanisms, such as DNA methylation, that are responsible for blocking the activation of these downstream photoreceptor genes. Knockdown of Vsx2 may be a viable method to repress the bipolar fate and push cells towards photoreceptor fates. Additional candidates for overexpression are early progenitor genes

Myb and *Foxn4*. Neither gene was induced by ASCL1 or Zic1, and they may generate further expression of developmental genes in Müller glia cultures.

Chapter 5:

Conclusions and Future Directions

Conclusions

Many of the most prevalent causes of blindness worldwide are due to diseases of the retina that lead to the irreversible loss of retinal neurons. Despite the prevalence of these diseases, there are limited treatments and even fewer cures.

Strategies to repair the retina have had encouraging but modest successes. The stimulation of endogenous repair processes presents an elegant solution to the problem of replacing lost and damaged cells. Approaches to promote endogenous cell replacement have focused on the delivery of extracellular growth factors, but with limited success. A promising alternative is the delivery of intrinsic factors, which has had few attempts in retinal cells. The work presented in this thesis establishes that neurogenic transcription factors ASCL1 and, to a limited extent, Zic1 can reprogram mouse Müller glia to a progenitor state capable of giving rise to neuronal fates.

My studies indicate that it is feasible to coax mammalian Müller glia back to a progenitor state capable of producing new neurons *in vitro*. These results suggest that stimulating neurogenesis in Müller glia by ASCL1 could provide an alternative strategy for repair of the retina after disease or injury. Future work will be needed to determine if these processes can be stimulated *in vivo* and whether these new cells are capable of contributing to functional circuitry and vision restoration.

These studies further bridge what is known about the regeneration process in the fish retina and the latent potential that exists in the mouse. These investigations were largely informed by findings in the fish, and there were many parallels as well as disparities between the regeneration processes in the fish retina and ASCL1-based

reprogramming in mouse cells. I will discuss similarities between these two systems as well as places of divergence that future studies will need to address.

Unlike current observations of direct reprogramming to neurons, ASCL1-reprogrammed Müller glia transition through a progenitor state before converting into neurons, as demonstrated by the upregulation of progenitor genes and the remodeling of these sites. This may be due to the close relationship that Müller glia still share with retinal progenitors; Müller glia retain high levels of many progenitor genes, such as *Hes5*, *Sox9*, and *Lhx2*, as well as known iPSC reprogramming genes, *Klf4* and *Sox2*. This unique transition through a progenitor state may be advantageous for the generation of large numbers of new neurons during retinal repair.

There is large overlap between progenitor genes that upregulate during fish regeneration and those that change after Ascl1 reprogramming. Notch pathway ligands, receptors, and effectors are reactivated in the fish after injury, and I observed similar changes in these studies. *Notch1* upstream and downstream components *Dll1, Dll3, Heyl, Hes5, Hes6,* and *Insm1*, were all increased in response to ASCL1 expression in mouse Müller glia. *Midkine*, which is upregulated in fish Müller glia after injury (Calinescu et al., 2009), was not upregulated by ASCL1 but was increased in response to AZOL in our system by microarray analysis. *Ascl1a* also induces expression of Lin-28 and its downstream repressed target *let-7*, which represses regeneration-associated genes, *hspd1* and c-*myc* in fish (Ramachandran et al., 2010; Qin et al., 2009). None of these components were changed in response to ASCL1 in the mouse, except *Hspd1,* which was upregulated on the microarray.

Additionally, unlike the fish, newly-derived mouse progenitors only turn on a portion of genes that are involved in early development. Many of these early progenitor genes, such as *Fgf15, Sfrp2,* and *Hmgb3*, fail to upregulate after ASCL1 infection, and may account for the failure of these cells to fully differentiate to all types of retinal neurons. It is unclear why this developmental program is only partially re-activated, but a few ideas will be presented here.

A second distinction in the fish is that new neurons downregulate expression of glial and progenitor markers. Although progenitor/neuronal markers are co-expressed as cells adopt neuronal fates (Bernardos et al., 2007), progenitor genes are eventually downregulated as more mature neuronal markers are expressed. Although I detected downregulation of Müller glial genes *Cralbp* and *Glast*, I observed continued strong expression of other glial-specific and progenitor markers, Id1, Hes5, and Sox9, within cells that expressed neuron-specific markers Otx2 and Tuj1. It is possible that these markers take longer to downregulate within the proliferative *in vitro* environment than in the fish; however, it is also plausible that the continued expression of these genes may account for the failure of these cells to fully differentiate.

The differentiation and maturation of newly born neurons is also partially limited in mammalian reprogramming. After damage in the fish, the architecture of the retina is completely restored and all subtypes of retinal neurons, including early-born neurons, are re-established. ASCL1-reprogrammed Müller glia primarily generate bipolar neurons, although some amacrine and photoreceptor genes are increased. Since Ascl1 is expressed by "late" progenitors that give rise to late-born neuron sub-types, ASCL1 may not be sufficient to generate early-born neuronal lineages. Although early neural genes, such as

Neurod1, Neurod4, and *Otx2*, are induced, later genes are not; Otx2's downstream targets, *Crx* and *Pde6b,* were weakly increased if at all. Zic1 and other developmental genes were not sufficient to induce these early-born lineages or more mature genes. Modulation of the early progenitor program or downstream effectors may be required to induce these cell types.

Additional information can be derived from comparing reprogramming mechanisms during fibroblast conversion to those in Müller glial reprogramming. Recently, RNA-seq was used to identify the set of genes that are activated in fibroblasts in response to Ascl1 overexpression alone (Wapinski et al., 2013). I analyzed the genes that were most highly regulated in response to Ascl1 in fibroblasts to levels found in Müller glia after ASCL1 overexpression by microarray analysis (Table 5.1). Genes that uniquely responded during reprogramming in fibroblasts are highlighted in yellow. Similarly, the set of genes most highly activated in Müller glia were compared to levels found in fibroblasts, and genes that uniquely responded in Müller glia are highlighted in purple. The set of genes common to both cell types during Ascl1 reprogramming include: *Ankrd1, Btbd17,Chrna4, Ctrb1, Dll3, Dner* (*among most highly activated in both datasets), *Fabp7, Frmpd1, Gadd45g, Gal*(*)*, Hes6, Kcnc4, Kcnq4, Kcnh6, Lrrn1, Luzp2, Ppp1r16b, R3hdml, Sct, Slit1,* and *Tnr*(*). These comparisons may give us unique insight into the genes that are universal reprogramming factors and those that may need to be targeted for re-expression in Müller glia.

It will be vital to continue to employ the mechanisms observed in the fish, as well as those from other reprogramming approaches in the CNS, to guide our strategies for improving mammalian reprogramming. Future studies will be needed to overcome these

reprogramming barriers through neurogenic activation, blocking the glial program, and modulating the epigenome.

Most highly activated in fibroblasts

Gene Symbol	MEFs rtTA	MEFs Ascl1	MEFs ratio (Ascl1-rTTA)	MEFs ratio (Ascl1/rTTA)	MG GFP	MG ASCL1	MG ratio (ASCL1/GFP)
Actc1	0.000	6.711	6.711	na	8.827	8.881	**1.006**
BC020535	0.000	2.060	2.060	na	7.819	8.004	**1.024**
Btbd17	0.000	2.750	2.750	na	8.969	10.242	**1.142**
Calb2	0.000	1.719	1.719	na	6.616	6.507	**0.984**
Cox8b	0.000	19.403	19.403	na	7.288	8.042	**1.104**
Ctrb1	0.000	6.724	6.724	na	7.821	8.909	**1.139**
Fabp7	0.000	41.833	41.833	na	9.045	11.431	**1.264**
Fbp2	0.000	1.639	1.639	na	7.166	7.182	**1.002**
Kcnc4	0.000	6.124	6.124	na	7.641	8.735	**1.143**
Kcnh6	0.000	3.219	3.219	na	7.614	10.385	**1.364**
Kirrel2	0.000	6.218	6.218	na	7.507	7.762	**1.034**
Krtap3-3	0.000	2.794	2.794	na	6.183	6.433	**1.040**
Mixl1	0.000	2.521	2.521	na	7.263	7.607	**1.047**
R3hdml	0.000	88.157	88.157	na	7.744	13.392	**1.729**
Sct	0.000	13.197	13.197	na	8.031	9.099	**1.133**
Sln	0.000	2.290	2.290	na	6.856	6.920	**1.009**
Ptchd2	0.001	0.323	0.322	468.996	7.286	7.566	**1.038**
Crtac1	0.002	3.961	3.959	1737.809	7.565	7.946	**1.050**
Chrna1	0.004	3.247	3.243	905.647	6.804	6.711	**0.986**
Ppp1r16b	0.004	4.153	4.149	1130.261	7.860	9.987	**1.271**
Lmod3	0.005	2.360	2.354	443.831	6.688	6.579	**0.984**
Gal	0.009	5.875	5.867	690.645	6.783	12.718	**1.875**
Mybpc1	0.009	6.548	6.539	720.416	7.294	6.704	**0.919**
Dll3	0.015	26.016	26.001	1766.565	7.303	9.109	**1.247**
Tnr	0.022	25.303	25.280	1148.501	6.778	11.488	**1.695**
Oasl1	0.031	15.464	15.434	502.202	8.701	9.550	**1.097**
Gal3st2	0.038	49.539	49.501	1317.848	7.015	6.807	**0.970**
Bmp7	0.051	57.713	57.661	1126.540	6.615	6.240	**0.943**

Most highly activated in Müller glia

Gene Symbol	MEFs rtTA	MEFs Ascl1	MEFs ratio (Ascl1-rTTA)	MEFs ratio (Ascl1/rTTA)	MG GFP	MG ASCL1	MG ratio (ASCL1/GFP)
Lrrn1	0.381	27.981	27.600	**73.450**	6.048	12.132	2.006
Gal	0.009	5.875	5.867	**690.645**	6.783	12.718	1.875
Chrna4	0.013	4.028	4.015	**299.875**	7.720	13.093	1.696
Tnr	0.022	25.303	25.280	**1148.501**	6.778	11.488	1.695
Mest	103.081	146.495	43.414	**1.421**	7.869	12.742	1.619
Mpzl2	0.140	0.413	0.273	**2.948**	6.805	10.824	1.591
Rgs13	0.000	0.123	0.123	na	5.560	8.803	1.583
Neurod4	0.000	0.000	0.000	na	6.677	10.173	1.524
Slit1	0.011	0.071	0.060	**6.373**	7.417	11.272	1.520
Otx2	0.000	0.000	0.000	na	6.804	10.056	1.478
St8sia5	0.000	0.058	0.058	na	6.891	10.170	1.476
Dner	0.513	54.442	53.929	**106.137**	7.586	11.165	1.472
Htr3a	0.000	1.227	1.227	na	6.658	9.673	1.453
Frmpd1	0.014	4.990	4.976	**367.461**	7.376	10.651	1.444
Bmp2	0.742	1.142	0.400	**1.540**	7.724	10.915	1.413
Nrxn3	0.015	0.009	-0.006	**0.601**	7.284	10.251	1.407
Kcnq4	0.034	1.536	1.502	**45.199**	9.308	13.039	1.401
Snai2	43.058	37.408	-5.650	**0.869**	8.890	12.291	1.383
Gadd45g	8.389	54.899	46.510	**6.544**	8.696	11.889	1.367
Ankrd1	7.867	26.809	18.942	**3.408**	8.508	11.625	1.366
Luzp2	0.009	0.016	0.007	**1.781**	8.667	11.743	1.355
H19	2547.138	1412.772	-1134.366	**0.555**	9.257	12.372	1.337
Hes6	7.256	214.043	206.787	**29.498**	8.943	11.884	1.329
Mtap2	20.999	7.261	-13.738	**0.346**	9.863	12.796	1.297

Table 5.1. Comparison of genes activated in response to Ascl1 in fibroblast and Müller glial reprogramming. Top, List of genes most highly activated following Ascl1 overexpression in murine embryonic fibroblasts (MEFs) derived from RNA-seq dataset published in Wapinski et al., 2013. These levels are compared to those found during Ascl1 reprogramming in Müller glia. Yellow indicates genes that are uniquely upregulated in fibroblasts during reprogramming. Bottom, List of genes most highly activated following Ascl1 overexpression in Müller glia. Purple denotes genes that are uniquely upregulated in Müller glia; green denotes those that may be unique to Müller glia.

Future Directions

Despite the major advances that this work encompasses, there are many avenues to be pursued to advance the feasibility of translation to the clinic and to gain a better understanding of the general mechanisms of neural regeneration. As outlined above, there appear to be several areas where Müller glial reprogramming falls short of recapitulating retinal development and mirroring the processes of retinal regeneration that the fish undergoes after damage. Several potential paths to overcome these barriers are outlined below.

Activating upstream pathways to initiate Ascl1 expression in the mouse

The studies presented in this work clearly demonstrate Ascl1's unique reprogramming potential. No other neurogenic transcription factors, either alone or in combination, elicited as dramatic effects on the ability of Müller glia to de-differentiate and form new neurons. However, it is unknown how Ascl1 is regulated during development, and why its expression is not reactivated after injury in the mouse. Therefore, it will be informative to understand the mechanisms that control the regulation of Ascl1, which would allow us to activate its expression after injury in the mouse to stimulate regeneration.

Possibilities include a lack of transcription factors that are required to activate Ascl1 normally during development. For instance, Brn2 acts synergistically with Ascl1 at

targets in the CNS, and potentiates the ability of Ascl1 to reprogram fibroblasts into neurons (Vierbuchen et al., 2010; Wapinski et al., 2013). Although Brn2 overexpression did not potentiate Ascl1's effect on reprogramming in Chapter 4, it is possible that Brn2 alone may be sufficient to drive Ascl1 and Müller glia reprogramming. An alternative is the presence of transcriptional repressors that inhibit Ascl1's activation, and may normally function to turn down Ascl1 expression at the end of neurogenesis. Current studies in the Reh lab to identify the regulatory sites at the Ascl1 locus and corresponding transcription factor binding sites, will help address these possibilities.

Many extracellular signaling pathways have been identified in the fish that are upstream of Ascl1a and required for the regenerative response of Müller glia. Tnfα signaling activates Ascl1a and stimulates the response to injury by Müller glia (Nelson et al., 2013); however, this relationship has not been tested or made known in the mouse retina. Additional regulation of Ascl1a in the fish has been demonstrated by the HB-EGF/ERK1/2 pathway (Wan et al., 2012), although EGF itself has not affected Ascl1 expression levels in any studies in the mouse retina. TGFβ signaling is also involved in the response to injury in the fish, and its downstream corepressor Tgif1 represses Ascl1a's expression during regeneration (Lenkowski et al. 2013). Additional repression of Ascl1a has been found in negative feedback loops involving Let-7 and Apobec2a/b repressors (Ramachandran et al., 2010; Powell et al., 2012). These relationships have not been established in the mouse retina, although overexpression of the Let-7 repressor Lin-28 did not affect Ascl1 levels, and ASCL1 overexpression did not affect Let-7 or Lin-28 levels in my studies (data not shown).

Suppressing the program that drives glial fate

Although a large focus of my studies has been on strategies to reactivate a neurogenic program in Müller glia, evaluated by changes in progenitor and neuronal genes, a smaller focus has been on understanding the extent to which the factors that drive glial fate determination have been silenced. Many Müller glial genes were downregulated in ASCL1-reprogrammed cultures; however, many were not and may have interfered with the re-activation of neuronal genes. Therefore, it may be necessary to suppress the glial-promoting program to initiate complete reprogramming.

Sox8 and Sox9 are required for Müller glial production and may inhibit rod photoreceptor production (Poche et al., 2008; Muto et al., 2009). Sox8/9 could suppress neuronal fates within immature Müller glia once neurogenesis is complete, although this has not been directly tested. Knockdown of *Sox8/9* by shRNAs in ASCL1-reprogrammed Müller glia could test this possibility, and may accomplish more robust reprogramming and neuronal maturity. Another strategy, which is currently being pursued in the Reh lab, is forced expression of the microRNA mir-124, which targets *Sox9* for repression (Cheng et al., 2009). Although miRNAs are difficult to work with experimentally, it may be more feasible to lentivirally deliver mir-124 than rely on the potential instability and inefficiency of *Sox8/9* RNA knockdown.

Additional repression may come from other glial fate determinants and influences, as discussed in the introduction, such as Egfr, Cntfr, Bmpr, Chx10, Notch, Nfi-a/b/c/x, and Id1/3. Particularly, Notch may have considerable influence in this area. Its downstream effectors *Hes5*, *Hes1*, and *Hesr*, repress proneural bHLH factors to drive the glial fate. In ASCL1-reprogrammed Müller glia, these factors may repress proneural

bHLH factors that are needed in combination with Ascl1 to promote neuronal fates. Additional influence may come from Notch's activation of the transcription factors Nfi-a/b/c/x, which indirectly drive de-methylation of glial genes, such as *Gfap* (Namihira et al., 2009). Manipulating these epigenetic changes may help to suppress the glial program.

Containing these gliogenic elements could drive neuronal fate determination in Müller glia, however, there are drawbacks to manipulating some of these elements. Based on the studies mentioned above, Notch drives the glial program and is a tractable candidate for knockdown. However, Notch is up-regulated in Müller glia after retinal damage and during regeneration in both fish and birds, and inhibition of the Notch pathway blocks regeneration in birds. Notch signaling improves reprogramming in other neural systems, possibly through inhibition of the repressive chromatin-modifying complex, Polycomb repressive complex 2 (Prc2) (Ntziachristos et al., 2012). Notch may regulate retinal cell fate through multiple avenues. Notch levels are high in Müller glia during their specification and early maturation but decrease as they mature. High Notch levels may be required during reprogramming to reactivate this immature progenitor/glial state; later phases of reprogramming during neuronal specification may require the combination of low Notch levels and proneural fate determinants.

Manipulating the epigenetic landscape may relieve restrictions on reprogramming

Multiple silencing mechanisms have been implicated in remodeling loci during somatic cell reprogramming to stem cells and iPSCs. The relevance of these epigenetic modifications, such as DNA methylation and histone modification, has just started to be explored in retinal regeneration. Epigenetic changes may silence neurogenic genes in

mouse Müller glia, but not in fish, thereby restricting their ability to reprogram fully. After ASCL1-reprogramming, epigenetic repression may account for the failure to reactivate early developmental genes as well as later downstream targets necessary for neuronal maturation.

We observed that progenitor genes acquire the repressive H3K27me3 mark on key genes as they develop into Müller glia, suggesting a mechanism by which progenitors repress the neuronal fate. In these studies, ASCL1 bound to progenitor genes and reversed some of these histone marks to activate gene expression. However, it is likely that ASCL1 alone is not able to reverse H3K27me3 on all neurogenic genes, as suggested by their failure to re-activate. The H3K27me3 modification is added to histones by the PRC2 complex. Future studies will benefit from manipulating components of the PRC2 complex or blocking histone deacetylation with small inhibitors, e.g. VPA, to test whether the restrictions on Müller glial reprogramming are relieved.

It is also unclear whether DNA methylation of CpG elements is involved in ASCL1-induced reprogramming in mammals here. In the fish, the cytidine deaminases, *apobec2a* and *apobec2b*, which help remove methylation marks from cytosine residues, are required for retinal regeneration (Powell et al., 2012). Although these factors were induced by Ascl1a in this study, ASCL1 failed to induce *Apobec2* expression in mouse Müller glia in my studies (data not shown), suggesting that some level of DNA demethylation is required and lacking in our reprogramming studies. Interestingly, in my studies ASCL1 did induce expression of the DNA demethylation agent *Gadd45y*, which was also reactivated after injury in the fish (Powell et al., 2012). DNA demethylation could be required to overcome the repression on late-activating neuronal genes, and

should be studied with DNA methyltransferase blockers, e.g. 5-azacytidine, or de-methylation agents, e.g. *apobec2a/b.*

Translating these findings to the in vivo retinal environment

The studies in this work suggest a viable method for reprogramming Müller glia *in vitro*, however it is unclear whether this strategy is tractable *in vivo*. Explant studies in this work suggest that ASCL1 can reprogram Müller glia in the intact retina, and the *in vivo* environment may promote this process. Therefore, the Reh lab has generated a transgenic mouse model to inducibly overexpress ASCL1 in Müller glia. *TetO-ASCL1* mice were generated that express *ASCL1* under the control of the tet-responsive element and crossed to animals that express the Müller glial promoter *Rlbp1* driving cre recombinase. Müller glia in the retinas of these mice express robust levels of ASCL1 when exposed to doxycycline. This strategy offers a tightly controlled system for testing whether ASCL1-reprogramming is viable within the mouse retina. This approach is similar to a recent study that demonstrated this principle in the brain. Lentiviruses carrying inducible neural fate determination genes, *Ascl1, Brn2*, and *Myt1l*, were injected into the striatum. Since astrocytes were targeted through transgene expression of the astrocyte-specific promoter *Gfap*, only astrocytes reprogrammed into new neurons *in vivo* (Torper et al., 2013).

An unclear question at present is whether newly reprogrammed neurons will migrate to appropriate retinal layers, grow neural processes, and wire precisely to their synaptic partners. Currently, there is no evidence for these processes following any regeneration strategy in the rodent retina. My data suggest that ASCL1-reprogrammed

neurons can establish appropriate neurotransmitter receptors and transporters and may synaptically connect to retinal neurons *in vitro.* In contrast, new neurons in the fish retina locate their targets and establish synaptic connections. This may be a large area of future research that will need to be addressed once we understand how to regenerate large enough numbers of neurons to be clinically relevant.

Overcoming the in vivo barrier to Müller glia cell cycle re-entry

It is likely that successful reprogramming of Müller glia *in vivo* will require cell cycle re-entry. We have not tested this possibility yet, since ASCL1-induced reprogramming *in vitro* occurs in cultures of proliferating Müller glia. However, cell cycle entry is dispensable in some *in vitro* reprogramming strategies (Heinrich et al., 2010; Vierbuchen et al., 2010). Regardless of the necessity for reprogramming, cell cycle entry will likely be vital to replenish the population of glia that are lost from cell type conversion *in vivo*. In the fish, regenerating Müller glia are replenished at normal numbers in a tightly controlled manner (Bernardos et al., 2007).

Misexpression of Ascl1 alone may be sufficient to induce cell cycle re-entry. Results from these data indicate that ASCL1 increases the number of dividing Müller glia in dissociated cells as well as in explant culture (unpublished data). This is not surprising, since Ascl1 regulates cell cycle genes in the CNS (Castro et al., 2011), promotes Müller glia proliferation in the fish retina (Nelson et al., 2012), and upregulates cell cycle genes after overexpression in mouse Müller glia in my studies (data not shown).

As discussed in Chapter 2, knockout of the cell cycle regulators P27, P53, and ARF are potential avenues for stimulating cell cycle re-entry that the Reh lab has been

pursuing. Manipulating these pathways *in vivo* has the problematic potential for uncontrolled proliferation in the form of tumors or proliferative diseases. Nevertheless, it is worth exploring these possibilities.

Future application of this research to the treatment and repair of retinal disease

Applying transcription-factor mediated reprogramming to the treatment of retinal disease is a challenging, but feasible, prospect. Transcription factors are difficult and costly to deliver to target tissues. Nevertheless, gene therapy techniques hold promise. Intravitreal or subretinal delivery of genes carried by Adeno-associated viral (AAV) vectors has been successfully implemented in the retina to restore gene function in photoreceptor cells (Mancuso et al., 2009). Although this technique has not yet been used to target Müller glia for gene delivery, it likely will shortly in the future as methods get more advanced. There are, however, potential pitfalls to gene therapy approaches. Reprogramming factors could have oncogenic effects – a concern of the iPSC field – and contribute to retinal tumors following delivery. Secondly, these techniques are costly; finding small molecule replacements for these reprogramming factors may translate into low-cost treatments that can be distributed more widely in the retina.

Many retinal diseases would not be ideal targets for this type of therapy. Many degenerative diseases, such as retinitis pigmentosa, involve mutations in essential visual function genes. Since the Müller glia in the retinas of these patients would still have the mutant gene, they would not be good candidates for gene therapy-based transcription factor delivery unless the mutated gene was targeted simultaneously. Secondly, my data suggest that ASCL1 induces reprogramming towards the bipolar cell fate, although there

is some evidence for amacrine and photoreceptor cells. Although there are no degenerative diseases that target bipolar neurons, bipolar cells are selectively killed off with other inner retinal neurons in transient retinal ischemia (Daugeliene et al., 2000; Goldenberg-Cohen et al., 2008). Our lab is currently testing whether ASCL1 reprograming of Müller glia can restore lost bipolar cells after this type of injury. In order to find ways to repair more prevalent photoreceptor degenerations, upcoming studies will need to address the future directions mentioned above to stimulate the generation of photoreceptor cells and other retinal fates *in vitro*. Non-inherited forms of photoreceptor degeneration such as AMD might be ideal targets for this repair.

Implications of this work

The studies in this work outline an effective and simple protocol for stimulating neurogenesis in mouse Müller glia. I described a highly efficient protocol for generating cultures of mature wildtype Müller glia that serve as a convenient and faithful model for retinal regeneration studies. Further, I illustrated how the overexpression of neurogenic transcription factors induced significant reprogramming of Müller glia, especially from a single transcription factor, ASCL1. Although many groups have modestly stimulated the neurogenic potential of Müller glia through growth factor administration, this represents the first study to demonstrate that mouse Müller glia can be reprogrammed into progenitors and new retinal neurons with intrinsic factors alone. With the aid of future studies that will help to overcome some of the barriers discussed, these studies collectively indicate a tractable path towards stimulating endogenous regeneration in the mammalian retina.

Chapter 6:

Materials and Methods

Animals. Animals were housed at the University of Washington, and protocols approved by the University of Washington IACUC. C57BL/6J mice (Jackson) were used except where indicated. *Hes5-GFP* mice (Basak and Taylor, 2007) have been previously characterized as a Müller glia reporter line *in vivo* and for FACS-sorting (Nelson et al., 2011). *Rlbp1-cre*^{ERT2} mice (gift of Ed Levine, University of Utah, USA; derived from plasmid described in Vázquez-Chona et al., 2009) were crossed to *R26-stop-flox-CAG-tdTomato mice* (Jackson). α*Pax6-cre* mice (R. Ashery-Padan, Tel-Aviv University, Tel-Aviv, Israel; Marquardt et al., 2001) were crossed to *R26-stop-flox-rtTA* (Jackson; Belteki et al., 2005). NMDA injury model was performed as previously described (Karl et al., 2008). Briefly, mice were deeply anesthetized with ketamine (130 mg/kg) and xylazine (8.8 mg/kg), and a single intravitreal injection of NMDA (Sigma) was performed to induce retinal damage. Tamoxifen (Sigma) was administered i.p. at 100 mg/kg in corn oil.

Plasmids and viral production. PLOC-hASCL1-IRES-turboGFP(nuc) (Open Biosystems) was used to generate *PLOC-IRES-turboGFP*(nuc). A *hASCL1-IRES* sequence was inserted into the *pTRE3G-mCherryn* vector (TetOn3G, Clontech), and inserted into the *pLVX-tight-puro* vector (Clontech) using In-Fusion Cloning (Clontech). *pLVX-Tet-ON Advanced* (Clontech) was used to express rtTA protein *in vitro*. *Tet-O-FUW-Olig2, Tet-O-FUW-Neurod1, Tet-O-FUW-Brn2, Tet-O-FUW-Myt1l*, and *Tet-O-FUW-Zic1* were purchased from Addgene. *Ef1α-Lin28* and *pLVX-tight-Prdm1* were cloned similarly as described above. Lentiviral particles were packaged using Lenti-X HTX Packaging

System (Clontech) in HEK293T cells (Clontech), and concentrated with Amicon Ultra Centrifugal Filter Units (Millipore).

Dissociated Müller glia culture. Postnatal day 12 wildtype or postnatal day 30-60 NMDA-damaged Müller glia cultures were derived from pooled retinas of C57BL/6 littermates. Retinas were dissected after sacrifice and dissociated by placing into papain with 180 units/mL DNase (Worthington) and incubating at 37°C for 8–10 min. Cells were then briefly triturated, added to an equal volume of ovomucoid (Worthington), and spun at 300 g for 10 min at 4°C. Cells were plated in Neurobasal media, with 10% FBS (Clontech), 1 mM L-glutamine (Invitrogen), N2 (Invitrogen), 1% Penicilin Streptomycin (Invitrogen), and 100 ng/mL EGF (R&D systems) at a density of two retinas per 10 cm^2 at 37°C in 5% CO^2. 1 μM EdU (Invitrogen) was included during this period. Half of the media was changed every 2–3 days. 4-7 days after dissociation, cells were passaged with TrypLE (Invitrogen) onto tissue culture plates for RNA analysis or onto coated [Poly-D-lysine (Sigma) and Matrigel (BD Biosciences)] glass coverslips for ICC or Ca^{2+} imaging. Lentiviruses were added to cells in Optimem (Gibco) or neural medium (Neurobasal + N2, B27, 1% tet-free FBS), and 3-6 hours later medium was replaced. hBDNF (10 ng/ml, R&D Systems), bFGF (100ng/ml, R&D Systems), and rGDNF (10 ng/ml, R&D Systems) were added for longer cultures.

Retinal explants. Retinas of *αPax6-Cre;R26-stop-flox-rtTA* mice were cultured as explants at P12. Retinas were isolated in cold HBSS and placed onto a 0.4 μm pore tissue culture insert (Millipore) in a 6-well plate. Half the media (DMEM/F12 supplemented

with 1% dialyzed fetal bovine serum (FBS), 0.6% D+ glucose, 0.2% $NaHCO_3$, 5 mM HEPES, 1mM L-glutamine, B27, and N2) was changed daily, and recombinant mouse EGF (100 ng/mL; R&D systems) or vehicle (PBS) was also supplemented during each media change. EGF was added to promote Müller glial proliferation, and BrdU (10 μg/mL; Sigma) or EdU (10 μg/mL; Invitrogen) was added to label proliferating cells throughout the culture period. Explants were infected with *TetOn-Ascl1-ires-mCherryn* lentivirus, and 3 μg/mL doxycycline was added at 2 div.

Reverse transcriptase quantitative PCR and Microarray analysis. Cells were lysed in Trizol (Invitrogen), and RNA extracted, followed by DNAse-1 (Qiagen) digestion and RNA cleanup (Qiagen RNA mini-cleanup kit). Microarray data were generated using GeneChip Mouse Gene 1.0 ST Array (Affymetrix) at the Institute for Systems Biology (Seattle) (see Nelson et al., 2011). cDNA was synthesized (iScript; Bio-Rad), and qPCR was performed (SsoFast EvaGreen Supermix; Bio-Rad) on a Bio-Rad thermocycler. Reactions were performed in triplicate, and values normalized to *Actb*/β-Actin (ΔCt). Differences were calculated between Ascl1-infected and uninfected or GFP-infected Müller glia within the same experiment (ΔΔCt). Means were plotted as the fold change in expression from Ctrl ($2^{\Delta\Delta Ct}$). S.E.M. was determined from ΔΔCt values and log transformed; one-way student's t-test was performed on ΔΔCt values compared to 0 (Yuan et al., 2006). qPCR primers are listed in Table 6.1.

Chromatin Immunoprecipitation. Ascl1 ChIP: P0 retinas or cultured P12 Müller glia were digested with papain into a single cell suspension and fixed with 1% formaldehyde, 10

minutes, at room temperature (RT). Cells were sonicated at 4°C. Ascl1 IP was performed with 40 μL anti-mouse IgG magnetic beads (Invitrogen) and 4 μg mouse anti-MASH1 antibody (BD Pharmingen) or 4 μg mouse IgG against chromatin from $1x10^6$ (P0) or $2.5x10^5$ (cultured Müller glia) cells per IP according to LowCell # ChIP Kit (Diagenode). IP and wash buffers as described in Castro et al., 2006. DNA sequences were quantified by qPCR as above. Values were averaged from 3-6 biologically independent experiments and expressed as a % of input DNA. For histone ChIPs cell suspensions were fixed with 0.5% formaldehyde (10 minutes at RT) and sonicated. IP was performed with 20 μL anti-rabbit IgG magnetic beads (Invitrogen) and 2 μg rabbit anti-H3K27me3 (Active Motif) or rabbit anti-H3K27Ac (Abcam) antibodies or 2 μg rabbit IgG. Values were averaged from 3-5 biologically independent experiments. See Table 6.1 for ChIP primers.

Western Blot. Cells were collected in lysis buffer and processed for western blotting. Each lysate was mixed with 5x sample buffer and boiled for 5 minutes prior to SDS–PAGE using 4–15% Tris-Glycine gradient gel (BioRad). Proteins were transferred to a PVDF membrane, and Western blots were performed using a standard protocol. Signals were quantified with ImageJ. Antibodies used: mouse anti-Cralbp (1:5000, Abcam), Glutamine Synthetase (1:10000, Millipore), β-Actin (1:10000, Abcam), goat anti-mouse HRP (1:10000, BioRad).

Immunofluorescence. Retinas and dissociated cells on coverslips were fixed in 2% paraformaldehyde (PFA) for 20-25 minutes at 4°C; explants were fixed in 2% PFA for 40 minutes at RT. Fixed explants or retinas were cryoprotected in 30% sucrose/PBS at 4°C

overnight, embedded in OCT compound (Sakura Finetek) and cryo-sectioned at 12 μm. Immunohistochemistry (IHC) was carried out using standard protocols. EdU staining was carried out following IHC with Click-iT EdU Alexa Fluor 555 or 647 detection kit (Invitrogen). Primary antibodies: anti-Insm1 (1:100, Genway), anti-Mash1 (1:100, BD Biosciences), anti-Ascl1 (1:250, Gift of J. Johnson, UT Southwestern), GsiB4 Lectin (1:500, Vector), anti-Calretinin (1:1000, SWANT) anti-Recoverin (1:1000, Millipore) anti-Cralbp (1:1000, Gift of J. Saari, U. of Washington), anti-RFP (1:500, Clontech), anti-RFP (1:500, Abcam), rabbit anti-Tuj1 (1:500, Covance), mouse anti-Map2 (1:200, Sigma M9942), anti-S100β (1:1000, Sigma), anti-GFP (1:500, Abcam), anti-Otx2-biotin (1:100, R&D systems), anti-Islet1 (1:50, DSHB), anti-Sox2 (1:250, Abcam), anti-Sox2 (1:250, Santa Cruz), anti-PKC (1:250, Sigma), anti-Id1 (1:200, BioCheck), anti-Pax6 (1:600, Covance), anti-Hes5 (1:100, Santa Cruz), anti-Sox9 (1:500, Millipore), anti-PH3 (1:500, Millipore), anti-NG2 (1:100, Chemicon). Secondary antibodies from Invitrogen, Molecular Probes and Jackson Immunology were used at 1:400 or 1:500.

Microscopy and cell counting. Imaging was performed using an Olympus Fluoview confocal microscope or Zeiss Observer D1 with AxioCam. 3-6 20x random fields/coverslip were counted. 1 μm single slice images were counted from 4-8 random fields/explant. EdU+ or EdU+/mCherry+ cells expressing a neuronal marker (Otx2, Islet1, or PKC) were counted. Seven explants were analyzed for each neuronal marker. 0.5 μm stack images were also captured to analyze colocalization of signals in 3D.

Electrophysiology. Whole-cell voltage clamp and current clamp recordings were made from GFP-expressing cells using an Axopatch 200B amplifier (Molecular Devices). Electrodes (2 – 4 MΩ) were filled with 148.5 mM potassium gluconate, 9 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 0.2 mM EGTA (330 mOsm, pH 7.2). The extracellular solution contained 119 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 30 mM glucose, 20 mM HEPES, and 1 μM glycine. Action potentials were elicited by injecting current into cells in whole-cell current clamp mode. Responses to 50 mM kainate (in extracellular solution) were recorded in response to 3-8 second application of drug, delivered with a local puffer pipette controlled by a Picospritzer (Parker Hannifin) while holding the membrane at –60 mV under voltage clamp. Spontaneous miniature postsynaptic currents were collected during continuous 10s sweeps while holding the membrane at –60 mV under voltage clamp.

Ratiometric Ca^{2+} imaging. Dissociated cells on coverslips were incubated in a solution of Fura-2 (Invitrogen) and Pluronic F-127 (Invitrogen) in Hank's balanced salt solution (HBSS+) (Gibco) for 30 minutes at 30°C, then washed in HBSS+ for 30 minutes. Chemicals (Sigma: NMDA, 100 μM; Glycine, 10 μM; Kainate, 10 μM; ATP, 100 μM; KCl, 50 mM) were diluted in HBSS and applied by bath perfusion at constant flow rate for 60s at 30°C before 60s washout with HBSS. Images were taken every 4 or 6s at 340 and 380 nm alternating excitation, and fluorescence intensity signals were ratioed (Metafluor; Molecular Devices). F340/380 values were normalized to baseline activity. Cells were excluded from analysis if they had high ratioed values at baseline, excessive spontaneous activity, or did not have a >10% response to any agonist. Each experiment

was conducted on multiple coverslips over multiple days, and at least three experiments were conducted for each agonist.

Statistics. In dissociated cells, biological replicates were treated as pooled retinas from littermates. Student's t-test was performed to test significance between means of biological replicates where indicated. For qPCR, one-way t-test was performed on ΔΔCt values compared to 0 (see PCR methods above). For Ca^{2+} imaging experiments, t-test tested the significance between means within each experiment. In retinal explants, statistics were performed using Z-test, with ***P<0.001.

qPCR Primers

Gene Name	F Sequence 5' to 3'	R Sequence 5' to 3'
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
ASCL1 (human)	CATCTCCCCCAACTACTCCA	CAGTTGGTGAAGTCGAGAAGC
Atoh7	ATCACCCCTACCTCCCTTTCC	CGAAGAGCCTCTGCCCATA
Bhlhe22	TGAACGACGCTCTGGATGAG	GGTTGAGGTAGGCGACTAAGC
Cabp5	ATGAGAACGATGGGTTACATGC	CACGGCCACCAAGGTTCATT
Cralbp	GGCACTTTCCGCATGGTTC	CCGGGTCTCCTCCTTTTCAT
Crx	TCTCTCACCTCAGCCCCTTAT	ACCCACTGAAATAGGAACTTGGA
Dll1	CAGGACCTTCTTTCGCGTATG	AAGGGGAATCGGATGGGGTT
Dll4	TTCCAGGCAACCTTCTCCGA	ACTGCCGCTATTCTTGTCCC
Fabp7	GGACACAATGCACATTCAAGAAC	CCGAACCACAGACTTACAGTTT
Fgf15	TCACATGGACCCTGTTGTGT	CAGCAGCCTCCAAAGTCAGT
Foxn4	CATGAAGGAGCACTTCCCCTA	TTTCCGGGCGGTCTGAGAT
Gad1	CACAGGTCACCCTCGATTTTT	ACCATCCAACGATCTCTCTCATC
Gadd45g	GGGAAAGCACTGCACGAACT	AGCACGCAAAAGGTCACATTG
Hes5	AGTCCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
Hes6	ACCACCTGCTAGAATCCATGC	GCACCCGGTTTAGTTCAGC
Heyl	CAGCCCTTCGCAGATGCAA	CCAATCGTCGCAATTCAGAAAG
Hmgb3	AGGTGACCCCAAGAAACCAAA	GCAAAATTGACGGGAACCTCTG
Isl1	TATCCAGGGGATGACAGGAAC	GCTGTTGGGTGTATCTGGGAG
Mfng	ATGCACTGCCGACTTTTTCG	CCTGGGTTCCGTTGGTTCAG
Myb	GAGCACCCAACTGTTCTCG	CACCAGGGGCCTGTTCTTAG
Mycn	ACCATGCCGGGGATGATCT	ATCTCCGTAGCCCAATTCGAG
Neurod1	ATGACCAAATCATACAGCGAGAG	TCTGCCTCGTGTTCCTCGT
Neurod4	AGCTGGTCAACACACAATCCT	GTTCCGAGCATTCCATAAGAGC
Neurog2	AACTCCACGTCCCCATACAG	GAGGCGCATAACGATGCTTC
Nrl	TCCCAGTCCCTTGGCTATGG	CACCGAGCTGTATGGTGTG
Olig2	GCTCACCAGTCGCTTCATCT	GCGCGAAACTACATCCTCAT
Opn1sw	CAGCATCCGCTTCAACTCCAA	GCAGATGAGGGAAAGAGGAATGA
Opn2	CCCTTCTCCAACGTCACAGG	TGAGGAAGTTGATGGGGAAGC
Otx2	TATCTAAAGCAACCGCCTTACG	AAGTCCATACCCGAAGTGGTC
Prdm1	TTCTCTTGGAAAAACGTGTGGG	GGAGCCGGAGCTAGACTTG
Prox1	AGAAGGGTTGACATTGGAGTGA	TGCGTGTTGCACCACAGAATA
Sfrp2	CGTGGGCTCTTCCTCTTCG	ATGTTCTGGTACTCGATGCCG
Slc1a3	ACCAAAAGCAACGGAGAAGAG	GGCATTCCGAAACAGGTAACTC
Tubb3	TAGACCCCAGCGGCAACTAT	GTTCCAGGTTCCAAGTCCACC
Turbo-Gfp	GACCAAGACTGGGGAGATCA	ACAGCCACAATGGTGTCAAA
Vsx1	GAGGCACAGGACGGTTTTCA	AGCTCTGTTTTCGCAGCCA

ChIP Primers

MyoD	GGCTTTTAGGCTACCCTGGAT	TGGTGAAGAAAGCAGTCGTG
Hes5	TTCCCACAGCCCGGACATT	GCGCACGCTAAATTGCCTGTGAAT
Dll1	AGCTCTTTCTCTCCGCATTG	CTGTTATTGTGCGAGGCTGA
Hes6	CATGTCAATGCACCGATTGGC	GCCTAAGTGGCAGGAGGTC
Dll3	TGCCCGAAGACTGAAGACTAATT	TGGGCTCAGGAAGGTGTGA
hAscl1 0.5kb	GCCACTCCTCTGAAAGATGC	TTTATTCCACACAGCCCACA
hAscl1 1kb	CAGGGAAGGGTTTAGGCAGA	CTCTCCCCTCCTACCTTCCT

Table 6.1. Primer sequences used for qPCR and ChIP analyses.

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PUBLICATIONS

Pollak J., Wilken M., Ueki Y., Cox K., Sullivan J., Taylor R., Levine E., Reh T. (2013) ASCL1 reprograms mouse Müller glia into neurogenic retinal progenitors. *Development* 140(12).

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