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Reprogramming Müller Glia to Regenerate Retinal Neurons

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Abstract

In humans, various genetic defects or age-related diseases, such as diabetic retinopathies, glaucoma, and macular degeneration, cause the death of retinal neurons and profound vision loss. One approach to treating these diseases is to utilize stem and progenitor cells to replace neurons in situ, with the expectation that new neurons will create new synaptic circuits or integrate into existing ones. Reprogramming non-neuronal cells in vivo into stem or progenitor cells is one strategy for replacing lost neurons. Zebrafish have become a valuable model for investigating cellular reprogramming and retinal regeneration. This review summarizes our current knowledge regarding spontaneous reprogramming of Müller glia in zebrafish and compares this knowledge to research efforts directed toward reprogramming Müller glia in mammals. Intensive research using these animal models has revealed shared molecular mechanisms that make Müller glia attractive targets for cellular reprogramming and highlighted the potential for curing degenerative retinal diseases from intrinsic cellular sources.

1. OVERVIEW: RESPONSE OF MÜLLER GLIA TO RETINAL INJURY

Müller glia are a type of radial astroglia present in the retinas of all vertebrates and the predominant non-neuronal cell type in this tissue (Bringmann et al. 2006). Arrayed as radial columns, Müller glia span the thickness of the retina (**Figure 1**). Müller glia somata reside in the inner nuclear layer and extend cytoplasmic processes that ensheath neuronal somata, axons, dendrites, and synaptic complexes. In vascularized retinas, Müller glia also help to form the blood–retina barrier. Similar to astrocytes in other regions of the brain, Müller glia mediate neurovascular coupling and form an intimate metabolic partnership with photoreceptors and other retinal neurons (Bringmann et al. 2006, Vecino et al. 2016).

The response of Müller glia to injury or disease has been intensely studied (e.g., Bringmann et al. 2006, Graca et al. 2018, Reichenbach & Bringmann 2013, Subirada et al. 2018). When retinal neurons are stressed or die, Müller glia undergo a complex cascade of structural and molecular changes that are most frequently described as a gliotic response. In mammals, this reactive gliosis is characterized by cellular hypertrophy, increased expression of intermediate filaments (GFAP, Vimentin, Nestin), and activation of extracellularly regulated kinases 1 and 2 (ERK1/2) (Bringmann et al. 2009). In mammals, in the short term, gliosis acts as a wound repair response to maintain a permissive environment for neuronal function and to prolong neuronal survival through the release of neuroprotective factors (Bringmann et al. 2009). However, chronic gliosis results in changes in Müller glia stiffness, decreased expression of enzymes that maintain retinal homeostasis, marked structural remodeling, secretion of proinflammatory cytokines, abnormal proliferation, and formation of a gliotic scar (Bringmann et al. 2009). Paradoxically, in mammals, chronic gliosis can accelerate neuronal death, thereby exacerbating the original insult. In teleost fish, Müller glia also initiate a gliotic response following neuronal death (Thomas et al. 2016);

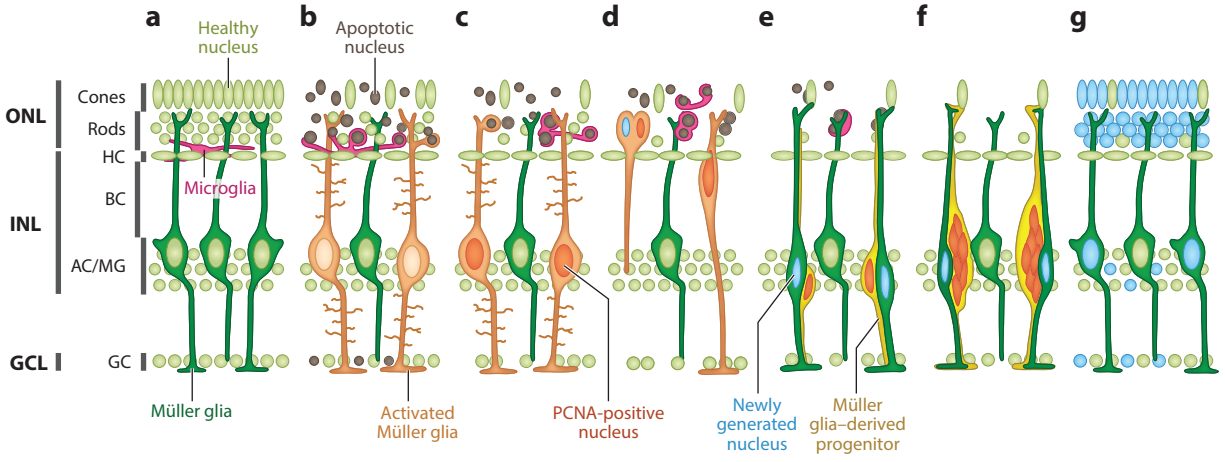


Figure 1

Time course of retinal regeneration in zebrafish. (*a*) Schematic of an uninjured retina. In the injured retina, (*b*) apoptotic cells are cleared by microglia and MG, and a subset of MG dedifferentiate or reprogram (*light orange*), (*c*) resulting in reentry into the cell cycle, indicated by the upregulation of PCNA. (*d*) MG undergo interkinetic nuclear migration to the ONL, (*e*) where they divide into MG-derived progenitors and postmitotic MG that return to the INL. (*f*) MG-derived progenitors continue to proliferate, generating clusters of progenitor cells that (*g*) ultimately differentiate into neurons that replace those that were ablated. Abbreviations: AC, amacrine cell; BC, bipolar cell; GC, ganglion cell; GCL, ganglion cell layer; HC, horizontal cell; INL, inner nuclear layer; MG, Müller glia; ONL, outer nuclear layer; PCNA, proliferating cell nuclear antigen. Figure adapted with permission from Lahne & Hyde (2017).

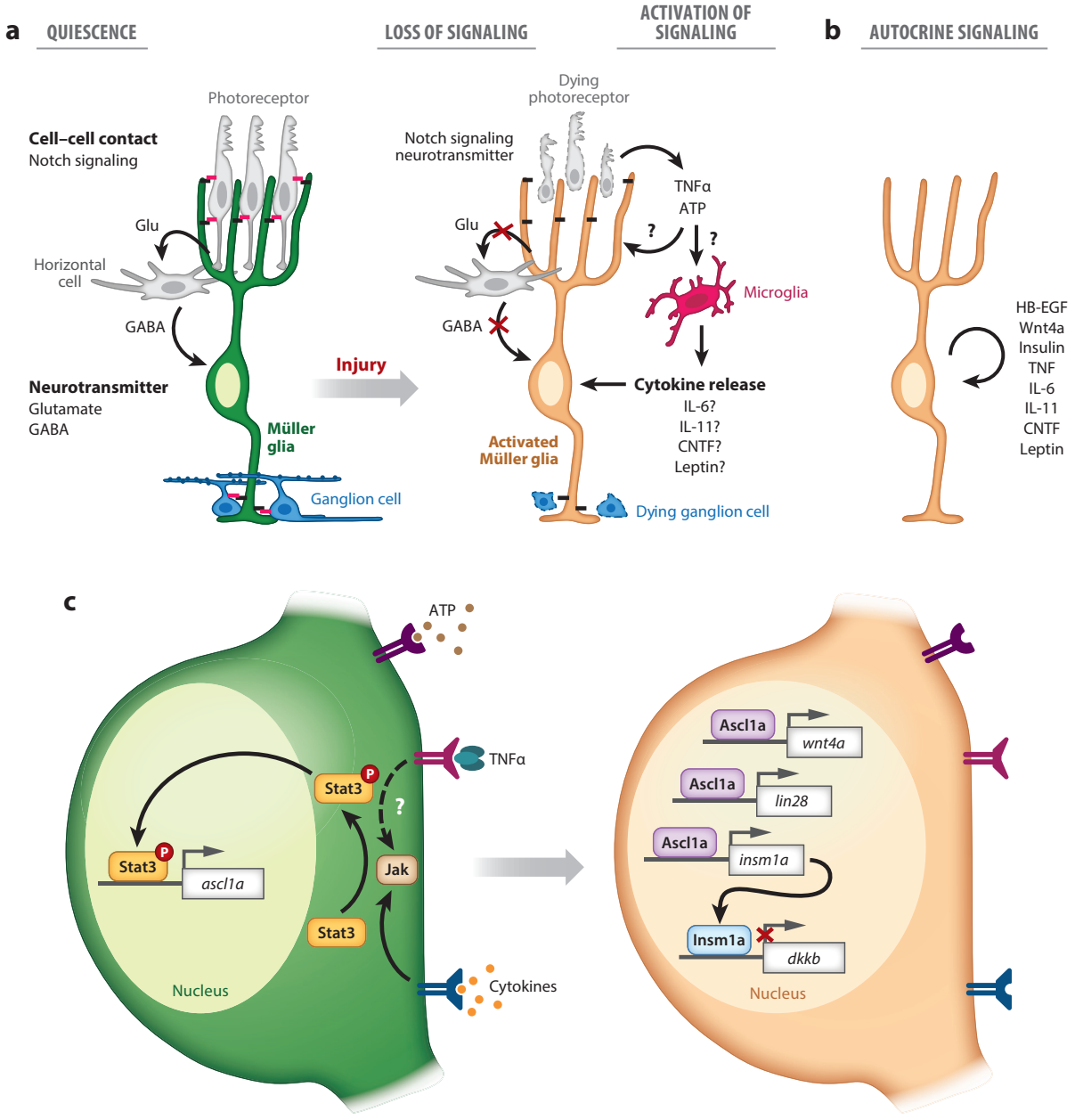
however, in some teleosts, this reactive gliosis is transient and precedes reparative neurogenesis, in which Müller glia acquire characteristics of retinal stem cells and replace ablated neurons that restore functional vision (Bernardos et al. 2007, Fausett & Goldman 2006, Kassen et al. 2007, Sherpa et al. 2008, Vihtelic & Hyde 2000). In this article, we systematically review the literature on retinal regeneration in teleost fish, focusing on the molecular mechanisms that underlie the endogenous reprogramming of zebrafish Müller glia. We then briefly review Müller glia reprogramming in chicks and mammals and report on recent comparative analyses of injury-induced genetic changes in Müller glia in zebrafish, chicks, and mammals.

2. RETINAL REGENERATION IN TELEOST FISH

Teleost fish are a long-standing model for studying retinal development and neuronal regeneration. In teleosts, the embryonic retina is a tiny fraction of its adult size, and retinal growth proceeds both by expansion of the extant tissue and by persistent neurogenesis (Johns 1977, Johns & Fernald 1981). Furthermore, some teleosts have the capacity to fully regenerate ablated retinal neurons from a source intrinsic to the retina (Raymond et al. 1988; see also Maier & Wolburg 1979). Initially, rod progenitors, which give rise to new rod photoreceptors, were suggested as the likeliest origin of regenerated neurons (Raymond et al. 1988). However, it was discovered that proliferating cells in the inner nuclear layer, subsequently identified as Müller glia, give rise to both rod progenitors during persistent neurogenesis and neural progenitors during regenerative neurogenesis (Bernardos et al. 2007, Fausett & Goldman 2006, Hitchcock & Raymond 2004, Kassen et al. 2007, Otteson et al. 2001, Raymond & Rivlin 1987, Raymond et al. 2006, Wu et al. 2001, Yurco & Cameron 2005). These foundational studies led to the immediate realization that unraveling the mechanisms underlying the spontaneous reprogramming in fish Müller glia could provide invaluable insights into approaches to induce reprogramming of mammalian Müller glia and thereby advance the goal of repairing the human retina via intrinsic cellular sources.

In the zebrafish retina, neuronal injury induces Müller glia to dedifferentiate or reprogram and adopt hallmarks of a retinal stem cell, which then reenters the cell cycle and divides asymmetrically to produce a progenitor cell, which continues to divide and differentiate into retinal neurons (Bernardos et al. 2007, Kassen et al. 2007, Nagashima et al. 2013). The response of Müller glia to a retinal injury is a complex, highly regulated, multistep process that requires the expression of specific genetic programs at each step (Gorsuch & Hyde 2014). To undergo reprogramming, Müller glia must sense that neurons are injured or dying. This can occur via molecules released from dying cells or reactive microglia, the phagocytosis of dying neurons by Müller glia themselves, or disruptions in intercellular signaling between Müller glia and nearby neurons (Bailey et al. 2010, Battista et al. 2009, Conner et al. 2014, Nelson et al. 2013, White et al. 2017). The latter could be mediated either by loss of direct cell–cell interactions or contacts or by the absence of soluble signaling mediators such as neurotransmitters (Conner et al. 2014, Rao et al. 2017). Müller glia then integrate these external signaling events into epigenetic and transcriptional changes that accommodate reprogramming (**Figures 1** and **2**). Transcriptional changes include the upregulation of genes encoding pluripotency factors and cell cycle regulators, and the downregulation of genes that maintain Müller glia in a differentiated and mitotically quiescent state (Conner et al. 2014, Gorsuch & Hyde 2014, Gorsuch et al. 2017). Progress through the cell cycle includes interkinetic nuclear migration, where approximately 85% of Müller glia nuclei move to the outer nuclear layer and undergo a single asymmetric cell division to produce a Müller glia–derived progenitor and a postmitotic Müller glia (Nagashima et al. 2013, Lahne et al. 2015). While the phenomenology of retinal regeneration in zebrafish is well described, many questions remain unanswered regarding

the molecular events that underlie reprogramming. Although this is not the topic of this review, many details are also lacking regarding the mechanisms that regulate Müller glia–derived progenitors, e.g., their numerical amplification, fate commitment, differentiation, and integration into extant neuronal circuits.



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Intercellular and transcriptional regulation of Müller glia reprogramming. (*a*) In the uninjured retina, neurotransmitter- and cell–cell-contact-mediated signaling through Notch receptors and their ligands maintain Müller glia in a quiescent state (*left panel*). The loss of neurotransmitter- and cell-contact-mediated signaling, in combination with increased paracrine signaling, initiates the reprogramming of Müller glia (*right panel*). Tumor necrosis factor alpha (TNFα) and nucleotides released from dying neurons directly act on Müller glia and/or stimulate microglia to release cytokines that induce reprogramming (*right panel*). (*b*) Müller glia also release growth factors and cytokines that act in an autocrine fashion. (*c*) Cytokines including TNFα stimulate Jak-mediated phosphorylation of Stat3, which leads to its nuclear translocation and transcription of its target gene, *ascl1a* (*left*). Ascl1a then initiates the expression of *insm1a*, *lin28*, and *wnt4a*, and downstream, Insm1a represses the expression of the Wnt signaling inhibitor, *dkkb* (*right*).

3. ACUTE AND CHRONIC INJURY MODELS IN ZEBRAFISH

Various approaches have been employed to induce retinal injury in zebrafish, including (*a*) intense light damage, which kills photoreceptors but spares other retinal neurons; (*b*) exposure to neurotoxins, such as NMDA, 6-OHDA or tunicamycin, which kill specific retinal neurons, or ouabain, which induces widespread neuronal cell death; and (*c*) mechanical injury, such as by inserting a needle through the globe to focally ablate all retinal cell types (Braisted & Raymond 1993, Fimbel et al. 2007, Li & Dowling 2000, Powell et al. 2016, Vihtelic & Hyde 2000). In addition, genetic ablation models have been developed that express the gene encoding *Escherichia coli* nitroreductase under cell-type-specific promoters, thereby enabling the selective ablation of specific cell types. (Ariga et al. 2010, Fraser et al. 2013, Hagerman et al. 2016, Montgomery et al. 2010, White et al. 2017, Yoshimatsu et al. 2016). Importantly, the cellular events leading to regeneration occur in a similar time frame irrespective of the nature of the injury, thereby allowing results from studies using different injury paradigms to be directly compared.

Retinal regeneration has also been studied in mutant and transgenic lines exhibiting chronic cell death. For example, lines have been isolated that display early onset cone (*pde6c*$^{w59/w59}$, *aipl2*$^{oki6/oki6}$) or rod (*Tg[Xla.Rho:GAP-CFP]*^{q13}) photoreceptor cell loss, late onset cone photoreceptor loss (*cep290*$^{fh297/fh297}$, *syu*$^{t4+/-}$), or late ganglion cell death due to increased intraocular pressure (*lrp2*$^{mw1/mw1}$) (Iribarne et al. 2017, Lessieur et al. 2019, Morris et al. 2008, Sherpa et al. 2011, Stenkamp et al. 2008, Veth et al. 2011). The *lrp2*$^{mw1/mw1}$ mutant and the cone degeneration mutants, except for *syu*$^{t4+/-}$, display a low level of proliferation in the inner nuclear layer, whereas, following rod photoreceptor degeneration, proliferation occurs predominantly among rod precursors in the outer nuclear layer (Iribarne et al. 2019, Lessieur et al. 2019, Morris et al. 2008, Sherpa et al. 2011, Stenkamp et al. 2008). Together, these genetic models mimic chronic injuries, similar to some inherited retinal diseases in humans, and may provide important insight into regenerative processes in the context of a continuously injured environment.

4. REPROGRAMMING MÜLLER GLIA IN ZEBRAFISH: NOTCH SIGNALING, GROWTH FACTORS, CYTOKINES, AND INFLAMMATION

Müller glia actively monitor the retina's extracellular environment in support of retinal homeostasis and neuronal function and to maintain the retina's structural integrity (Bringmann et al. 2006, Insua et al. 2008). In addition to sensing the extracellular environment, cell–cell contacts also allow Müller glia to communicate with their neighbors. Notch signaling—one form of contact-mediated intercellular signaling—occurs between neighboring cells when the Notch receptor binds to its ligands, Delta or Jagged (**Figure 2*a***). This stimulates cleavage of the Notch intracellular domain to form a transcriptional complex that regulates Notch target gene expression (Ho et al. 2019). Pharmacological suppression of Notch signaling in undamaged retinas stimulates a subset of Müller glia to proliferate, indicating that active Notch signaling likely functions to maintain Müller glia

in a quiescent state and that decreased Notch signaling is required for reprogramming (Conner et al. 2014). Single-cell RNA-seq analysis of injured zebrafish retinas revealed the downregulation of *notch3* in Müller glia; thus, Notch3 could be the receptor that maintains Müller glia in a quiescent state (Hoang et al. 2019). The identity of the ligand and the cell types that express the interacting ligand have not yet been determined. Following retinal injury, Notch ligands, receptors, and downstream target genes are differentially expressed in Müller glia and their progenitors (Raymond et al. 2006, Wan et al. 2012, Yurco & Cameron 2007), suggesting that Notch signaling is dynamically regulated to control the proliferation and differentiation of Müller glia–derived progenitors and to reestablish Müller glia quiescence (Furukawa et al. 2000, Mizeracka et al. 2013, Wan et al. 2012).

Loss of neurotransmitter signaling has also been suggested as a cue that induces Müller glia programming (**Figure 2*a***). In unlesioned retinas, inhibiting signaling through the $GABA_A$ receptor is sufficient to stimulate proliferation of Müller glia, and increasing signaling through the $GABA_A$ receptor following photoreceptor death suppresses proliferation (Rao et al. 2017). Similarly, inhibition of ionotropic glutamate receptors of the AMPA type stimulates *ascl1a*-dependent Müller glia proliferation in uninjured retinas, and activation of these receptors reduces proliferation following rod photoreceptor death (Rao et al. 2017). Thus, it has been proposed that, following photoreceptor death, GABA release from horizontal cells is disrupted due to a lack of photoreceptor-mediated glutamate signaling, and the resulting absence of GABA signaling initiates Müller glial reprogramming. These results suggest more broadly that ambient neurotransmitter levels, monitored by the Müller glia, may function to maintain these cells in a quiescent state, and altered neurotransmitter levels are also able to initiate reprogramming.

Following an injury, numerous factors are released into the extracellular environment that activate complex networks of signaling cascades to reprogram Müller glia (Goldman 2014, Gorsuch & Hyde 2014, Hamon et al. 2016, Karl & Reh 2010, Lenkowski & Raymond 2014) (**Figure 2*a***). Dying neurons represent one source of extrinsic signaling factors (Kassen et al. 2009, Qin et al. 2011). Tumor necrosis factor alpha (TNFα) was identified as a signaling molecule produced by dying neurons that is both necessary and sufficient to reprogram Müller glia by upregulating the reprogramming factor genes, *ascl1a* and *stat3* (Nelson et al. 2013). Immunoblot analysis revealed that the active, i.e., cleaved, form of TNFα is present at the peak of cell death, and active TNFα induces reprogramming (Conner et al. 2014, Nelson et al. 2013). Additionally, coinjecting TNFα and a gamma secretase inhibitor, to suppress Notch signaling, into an uninjured retina is sufficient to induce nearly 90% of Müller glia to proliferate, a greater percentage than is normally observed following injury, and the resulting Müller glia–derived progenitors generate a small number of retinal neurons (Conner et al. 2014). This demonstrates that a full regeneration response likely requires both the induction of activating signals and the suppression of inhibitory ones. Interestingly, once reprogramming has commenced, Müller glia also synthesize TNFα, which functions to regulate the number of Müller glia–derived progenitors (Nelson et al. 2013).

It was also suggested that dying neurons release nucleotides, such as ATP or ADP, which activate purinergic P2 receptors (**Figure 2*a***). Following retinal injury, inhibiting the metabotropic ADP receptor, P2RY1, reduces the expression of the reprogramming factors *ascl1a* and *lin28* and proliferation of Müller glia via an unknown mechanism (Battista et al. 2009, Medrano et al. 2017). Future studies will have to establish which cell type expresses P2RY1 receptors.

Microglia, the innate immune cells of the central nervous system, are another potential cellular source of extrinsic reprogramming factors (Craig et al. 2008, White et al. 2017) (**Figure 2*a***). The best evidence for this is that injury-induced proliferation of Müller glia depends on the presence of activated microglia (Conedera et al. 2019, White et al. 2017). However, the mechanisms by which microglia detect retinal injury and the factors released by activated microglia are not well

elucidated. Interestingly, molecules released by dying neurons, e.g., TNFα and ATP, can directly act on immune cells (Sieger et al. 2012, Veroni et al. 2010, Yin et al. 2017). Thus, TNFα and/or ATP might not stimulate Müller glia directly, but rather activate microglia that then release factors that induce Müller glia reprogramming. The inflammatory cytokines IL-6, IL-11, CNTF, and leptin play a role in reprogramming Müller glia, although it is currently unknown whether these factors are expressed by microglia (Kassen et al. 2009, Wan et al. 2014). Microglia-mediated phagocytosis of dying neurons likely also modulates the availability of factors released by dying neurons and thus the downstream reprogramming of Müller glia. Finally, acute inflammation is both necessary and sufficient to reprogram radial glia in the forebrain of zebrafish (Kizil et al. 2015, Kyritsis et al. 2012), and immune suppression diminishes microglial activation and proliferation of Müller glia in the retina (White et al. 2017), indicating that inflammation is a required component of reprogramming in Müller glia.

Müller glia themselves rapidly upregulate the expression of both growth factors and cytokines, which activate a core set of signaling cascades, in an autocrine fashion (**Figure 2*b***), that are required for reprogramming and progression through the cell cycle (Goldman 2014; Kassen et al. 2009; Nagashima et al. 2020; Wan et al. 2012, 2014). Binding of the Wnt ligands Wnt4a or Wnt8b to their receptor, Fzd2, inhibits glycogen synthase kinase-3β, thereby preventing β-catenin degradation and allowing the translocation of β-catenin to the nucleus for transcriptional regulation of Wnt target genes (Meyers et al. 2012, Ramachandran et al. 2011). Pharmacological inhibition of glycogen synthase kinase-3β in an unlesioned retina is sufficient to initiate reprogramming of Müller glia, supporting the interpretation that, in an uninjured retina, signals that promote reprogramming of Müller glia are actively suppressed (Ramachandran et al. 2011; see also Conner et al. 2014, Hamon et al. 2019, Rueda et al. 2019). Heparin binding-epidermal growth factor (HB-EGF), which signals through the epidermal growth factor receptor, induces Müller glia proliferation by activating ERK1/2 of the mitogen-activated protein kinase family and the phospho-inositol-3 kinase-Akt signaling pathways (Wan et al. 2012, 2014). These kinase pathways are also activated by insulin, which acts through the insulin receptors Insra and Insrb (Wan et al. 2014). Genes encoding cytokines, such as *m17*, *clcf1*, *crlf1a*, *il-11a*, *il-11b*, and the mammalian leptin homologs *lepa* and *lepb*, are also expressed during Müller glia reprogramming. IL-6, IL-11, and CNTF activate cytokine receptors that contain the signaling subunit Gp130, and leptin acts via its receptor, Lepr (Zhao et al. 2014). CNTF and leptin activate Jak-Stat3 signaling; however, the signaling pathways downstream of IL-11 and IL-6 are unknown (Zhao et al. 2014). These growth factors and cytokines have been suggested to act synergistically, amplifying paracrine signals released by dying cells and microglia (Wan et al. 2014). This synergy is thought to lower the activation threshold of intracellular signaling cascades within Müller glia (Wan et al. 2014, Zhao et al. 2014). Intracellular signaling in Müller glia also exhibits remarkable crosstalk between the different pathways (Goldman 2014, Wan et al. 2012), which may serve to activate multiple signaling cascades simultaneously to initiate the transcriptional changes underlying reprogramming.

Intravitreal injections of recombinant Sonic Hedgehog protein, SHH-N, increase the number of Müller glia that enter the cell cycle following an injury, whereas inhibiting the Hedgehog pathway reduces proliferation (Thomas et al. 2018). It remains to be determined if SHH-N is sufficient to reprogram Müller glia when injected into an uninjured retina. Interestingly, Shh has been suggested to be normally expressed in the nerve fiber layer (Sherpa et al. 2014), which would indicate that mechanisms are in place or that restricted availability of Shh may prevent Müller glia from reprogramming in the uninjured retina. In support of this, the expression of Hedgehog pathway components increases at the time of Müller glia reprogramming; however, the cell-specific expression patterns remain to be established (Kaur et al. 2018).

5. EPIGENETIC CONTROL OF MÜLLER GLIA REPROGRAMMING

The epigenomic landscape of cells is tightly regulated. Epigenetic changes occur during development but also during reprogramming of induced pluripotent stem cells (Gökbuget & Blelloch 2019). In the injured retina, activation and inhibition of gene expression are necessary to mediate the regenerative response, and thus, transcription has to be tightly regulated. DNA methylation and histone modifications control the accessibility of DNA for transcription by regulating DNA compaction. Both DNA methylation and certain histone marks are associated with compacted, inaccessible chromatin (heterochromatin), while unmethylated DNA, together with different histone marks at promoter regions, allows chromatin decondensation (euchromatin) and thereby permits transcriptional machinery to access promoter binding sites. In the regenerating zebrafish retina, the expression of transgenes driven by ubiquitous promoters, which is silenced in the adult retina, is reactivated due to epigenomic changes in the promoter regions of these transgenes (Thummel et al. 2006). This early observation suggested that epigenetic changes likely also occur in genes that facilitate regeneration.

5.1. Function of DNA Methylation in the Regenerating Retina

Methylation of cytosines within CpG contexts in promoter regions is important for gene silencing (Corso-Diaz et al. 2018). DNA methylation is established by DNA methyltransferases (Dnmts), and demethylation can occur either in a passive manner, i.e., loss of methylation sites during replication in the absence of Dnmt1, or in an active manner driven by enzymes (Gökbuget & Blelloch 2019). Active demethylation is a multistep process involving several enzymes such as DNA dioxygenases, cytidine deaminases, and DNA glycosylases (Corso-Diaz et al. 2018).

Injury-induced changes in DNA methylation of cytosine in the context of CpG were observed in Müller glia (Powell et al. 2013). Interestingly, although 73.6% of the analyzed CpGs showed decreased methylation, the promoters of genes required for Müller glia reprogramming (*lin28*, *mycb*, *sox2*, *ascl1a*, *insm1a*; see below) are hypomethylated in zebrafish (and in mice), indicating that these genes are in a poised state (Powell et al. 2013). This raises the question of how the expression of these reprogramming genes is regulated. Furthermore, many bases in the zebrafish retina are differentially methylated, but it is unclear which genes or genomic regions are affected and if these play any role in Müller glia reprogramming. Increased expression of the de novo DNA methylase genes *dnmt3ba*, *dnmt3bb1*, and *dnmt3bb3* (previously named *dnmt7*, *dnmt4*, and *dnmt5*, respectively) suggests that DNA methylation might play a role in regulating Müller glia reprogramming (Powell et al. 2012, 2013). These Dnmt3s may regulate accessibility of genes that keep Müller glia in a quiescent state in the uninjured retina, and these quiescence-maintaining genes have to be downregulated to allow Müller glia reprogramming following injury. It will be important to know whether this dinucleotide methylation occurs in genes that are downregulated as Müller glia undergo reprogramming (Powell et al. 2013). Dnmt1 is also upregulated following retinal injury, and based on its role in maintaining global methylation states during S-phase, it likely also fulfills this role during DNA synthesis in Müller glia (Du et al. 2015).

Morpholino-mediated knockdown of the cytidine deaminases Apobec2a and Apobec2b reduces the expression of *ascl1a* and *lin28*, suggesting that Apobec2a and Apobec2b regulate Müller glia reprogramming. However, it was not assessed whether knockdown of Apobec2a and Apobec2b affects Müller glia proliferation (Powell et al. 2012). Surprisingly, only 0.023% of analyzed CpG regions were differentially methylated in *apobec2a* and *apobec2b* morphants relative to controls, suggesting that Apobec2a and Apobec2b do not play a role in demethylating DNA during Müller glia reprogramming (Powell et al. 2013). It is unknown which enzymes mediate the

methylation changes in Müller glia. The ten–eleven translocation (*tet*) gene *tet2*, but not *tet1* or *tet3*, is expressed in Müller glia, and none of these three genes change expression following injury (M. Lahne, P. Boyd and D.R. Hyde, unpublished data). Interestingly, TET2 is phosphorylated by the Janus kinase, JAK2, downstream of cytokine signaling (Jeong et al. 2019). Given that Jak signaling plays a role in Müller glia reprogramming (Conner et al. 2014, Zhao et al. 2014), phosphorylation by Jaks might result in rapid activation of Tets that subsequently induce DNA demethylation, allowing effective transduction of a retinal injury into a transcriptional response.

5.2. Function of Histone-Modifying Enzymes in the Regenerating Retina

Methylation, acetylation, and phosphorylation of the N-terminal regions of histones regulate the accessibility of DNA for transcription (Corso-Diaz et al. 2018). The histone modifications at transcription start sites that result in active gene transcription are acetylation of lysine 27 at histone H3 (H3K27ac) together with triple methylation of lysine 4 at histone H3 (H3K4me3). In contrast, triple methylation of lysine 9 and/or 27 of histone H3 (H3K9me3 or H3K27me3, respectively) results in repressive marks. In addition, promoter regions are considered poised in the presence of H3K4me3 and H3K27me3 (Corso-Diaz et al. 2018, Voigt et al. 2013). Acetylation is mediated by histone acetyltransferases [also called Kat for lysine (K) acetyltransferase], whereas deacetylation is facilitated by either histone deacetylases (Hdac) or sirtuins. Lysine methyltransferases (Kmt) and lysine demethylases (Kdm) catalyze the methylation and demethylation of histone N-terminals, respectively (Corso-Diaz et al. 2018).

Following an injury, knockdown of Hdac1 expression reduces proliferation among Müller glia (Mitra et al. 2018). Furthermore, global inhibition of Hdac function results in differential expression of genes associated with Müller glia reprogramming; the expression of *mycb*, *ascl1a*, and *let7* increases, while the expression of the transcriptional repressors *insm1a* and *lin28* decreases. Interestingly, chromatin immunoprecipitation–polymerase chain reaction (ChIP-PCR) established that Hdac binds to Myc binding sites within the *lin28* and *her4.1* promoters (Mitra et al. 2019). While the global acetylation state of histone H4 was assessed following pharmacological inhibition, it was not determined whether H3K27 acetylation was changed in the *lin28* promoter and in any genes that are differentially regulated following these treatments (Mitra et al. 2018). However, downregulation of the *kat2b* gene, encoding an acetylating enzyme, in Müller glia following photo-injury suggests that deacetylation is a necessary step in reprogramming (M. Lahne, P. Boyd and D.R. Hyde, unpublished data).

The Polycomb repressive complex 2 (PRC2) is an enzyme complex that mediates the trimethylation of H3K27 (O'Meara & Simon 2012). Single-cell RNA-seq revealed upregulation of PRC2 complex components *ezh2*, *eed*, and *suz12a/b* in Müller glia following injury (M. Lahne, P. Boyd and D.R. Hyde, unpublished data). The role of the PRC2 complex in the regenerating zebrafish retina is unknown, but trimethylation of H3K27 results in a repressed transcriptional state and is likely correlated with the deacetylation of the same histone mark, which is critical for Müller glial reprogramming (Mitra et al. 2018). Interestingly, the Wnt signaling inhibitor *DKK2* and Notch receptors and ligands (*NOTCH4*, *DLL4*) were identified as Polycomb group target genes in human embryonic diploid fibroblasts, and downregulation of these pathways is required for reprogramming (Bracken et al. 2006, Conner et al. 2014, Ramachandran et al. 2011, Wan et al. 2012). Thus, in the context of Müller glia reprogramming, PRC2 might repress the transcription of genes that maintain Müller glia in a quiescent state.

Phosphorylation of histone H3 at serine 10 and 28 (H3S10, H3S28) provides another level of regulation; this phosphorylation, in combination with the acetylation of nearby H3K14 or H3K27 in promoter regions, respectively, is implicated as active histone marks allowing transcription of

target genes, such as immediate early genes (Sawicka & Seiser 2012). Interestingly, phosphorylation of H3S28 induces the release of PRC2 from trimethylated H3K27 without altering the methylation status and thereby leading to gene activation of immediate early genes or genes involved in differentiation, depending on the upstream stimulus (Gehani et al. 2010). Mitogen- and stress- activated kinases 1 and 2, which are activated by either ERK1/2 or p38 kinases, have been shown to phosphorylate both H3S10 and H3S28 downstream of growth factor or stress signaling (Duncan et al. 2006, Gehani et al. 2010). As ERK1/2 are activated in Müller glia following injury, extracellular signals might be efficiently relayed via these kinases into a response that renders the epigenomic landscape active, thereby allowing for gene transcription (Wan et al. 2012). In addition to the PRC2 components, genes encoding other methylating (*kmt5ab*, *nsd2*, *suv39h1b*) and demethylating (*jarid2b*) enzymes increase expression in Müller glia during reprogramming (M. Lahne, P. Boyd and D.R. Hyde, unpublished data), suggesting that histone modifications are tightly regulated and accommodate both the activation and repression of gene expression.

The differential expression of various histone and DNA modifying enzymes following retinal injury suggests that the epigenetic landscape is tightly regulated to facilitate Müller glia reprogramming. While a few enzymes have been functionally examined for their role in retinal regeneration, further analysis of the specific function of DNA modifying enzymes, their target genes, and the upstream mechanisms that facilitate the remodeling of the epigenetic landscape is necessary to obtain a full understanding of epigenetic changes and their role in regulating Müller glia reprogramming.

6. TRANSCRIPTIONAL CONTROL OF MÜLLER GLIA REPROGRAMMING IN ZEBRAFISH

The bHLH transcription factor Ascl1a was the first transcription factor shown to be required for reprogramming Müller glia in zebrafish. In situ hybridization showed that *ascl1a* is induced in Müller glia by 4 h post-injury, and knocking down Ascl1a expression prevents quiescent Müller glia from entering the cell cycle (Fausett et al. 2008). Ascl1a was identified as a direct transcriptional regulator of the pluripotency factor Lin28 (Ramachandran et al. 2010), an RNA-binding protein highly expressed in embryonic stem cells. In Müller glia, *lin28* expression parallels that of *ascl1a*, and knockdown of Ascl1a reduces *lin28* expression. Furthermore, Lin28 blocks the functional maturation of the *let*7 miRNA, indicating that Lin28 and *let*7 form a regulatory circuit that governs the balance between pluripotency and fate commitment (Rehfeld et al. 2015). As Müller glia enter the cell cycle following injury, the levels of *lin28* and *let*7 change in opposite directions, and knocking down either Ascl1a or Lin28 completely eliminates the injury-induced suppression of *let*7 (Ramachandran et al. 2010). These data suggest that Ascl1a functions through a Lin28–*let*7 network to promote reprogramming, in part by suppressing genetic programs that normally function to promote or maintain cellular differentiation (Goldman 2014). Interestingly, overexpression of either *ascl1a* or *lin28* in an uninjured retina is insufficient to induce Müller glia proliferation, whereas coexpressing these genes activates proliferation (Elsaeidi et al. 2018).

Convincing, indirect evidence shows that the transcription factor gene *insm1a* is a direct transcriptional target of Ascl1a, and knockdown experiments reveal an Ascl1a–Insm1a regulatory loop and an Insm1a autoregulatory loop (Ramachandran et al. 2012) (**Figure 2*c***). In Müller glia, expression of *insm1a* is upregulated by 6 h post-injury, but it is suppressed at 24 h post injury, suggesting a complex functional role for this gene. Insm1a was found to repress the expression of the Wnt signaling inhibitor Dkk, which is required for reprogramming (Ramachandran et al. 2011, 2012). Insm1a likely directly regulates *dkk1b* (**Figure 2*c***). Expression of the Wnt ligand *wnt4*, and to a lesser extent the receptor *fzd2*, is also regulated in an Ascl1a-dependent manner (Ramachandran

et al. 2011). In contrast, the mechanisms that regulate increased expression of the Wnt signaling effector *ctnnb2*, which is required for Müller glial proliferation, are currently unknown (Gorsuch et al. 2017). Together, these data reveal that a complex Ascl1a and Insm1a signaling network plays a role in Wnt-mediated reprogramming of Müller glia.

Stat3 is a member of the family of Stat proteins, which function as activatable transcriptional regulators downstream of cytokine and growth factor signaling pathways. Phosphorylation of Stat3 enables its homodimerization and thereby allows binding of Stat3 to specific DNA binding sites (Huynh et al. 2017). Following retinal injury, Stat3 expression is strongly induced in all Müller glia, although only a subset of Müller glia proliferate (Kassen et al. 2007). While Stat3 phosphorylation levels increase during reprogramming (Kassen et al. 2007), the Stat3 phosphorylation state within individual quiescent and proliferating Müller glia has not been established. Nonetheless, knockdown of Stat3 or inhibition of Jaks, which phosphorylate Stat3, demonstrated that Stat3 is required for Müller glia reprogramming (Conner et al. 2014, Nelson et al. 2012, Zhao et al. 2014). Interestingly, following photoreceptor death, induction of *stat3* expression precedes *ascl1a*, and while Stat3 is present in all Müller glia, Ascl1a is restricted to those Stat3-positive Müller glia that enter the cell cycle (Nelson et al. 2012). In support of Stat3 acting upstream of *ascl1a*, removal of Stat binding sites within the *ascl1a* promoter diminishes *ascl1a* promoter–driven green fluorescent protein (GFP) transgene expression (Zhao et al. 2014). Additionally, Jak inhibition reduces the levels of *ascl1a* and *lin28* expression (Zhao et al. 2014). However, knockdown experiments showed that Lin28 is required for the expression of both Ascl1a and Stat3, and Ascl1a is required for Stat3 expression, but only in a subset of Müller glia that enter the cell cycle (Nelson et al. 2012). Taken together, these data suggest that at least two populations of Müller glia exist in the regenerating retina; both upregulate Stat3, but one remains mitotically quiescent, while the other enters the cell cycle (Nelson et al. 2012). Although this model is incomplete, this study confirmed that not all Müller glia respond similarly to cell death and revealed the complex nature of reprogramming among the population of Müller glia.

Similar to Stat3, there are other transcription factors that are directly regulated by signaling pathways activated by retinal injury. For example, Notch signaling regulates the expression of *her* and *hes* genes, which subsequently control the expression of Notch-responsive genes (Ho et al. 2019). The only Notch target gene known to be downregulated early in response to retinal injury is *hey1*, but it is unknown whether, in an uninjured retina, *hey1* maintains Müller glia in a quiescent state (Wan & Goldman 2017). Expression of another Notch target gene, *her4.1*, is upregulated following injury (Mitra et al. 2018). The promoter regions of the reprogramming-associated genes *lin28*, *il-6*, *il-11b*, *lepr*, *lifra*, *lepa*, *lepb*, and *crlf1a* contain Her4.1 binding sites, and knockdown of Her4.1 expression in Müller glia–derived progenitors increases expression of these genes (Kaur et al. 2018, Mitra et al. 2018). This implies that Her4.1 represses expression of the genes mentioned above, and as such, that Her4.1 could play a role in maintaining Müller glia quiescence.

Sox2 has a well-established association with neural stem cells in both the embryonic and adult central nervous system and is required for neurogenesis in both tissues. Notably, Sox2 is an essential transcription factor, together with c-Myc, Oct4, and Klf4, which facilitate reprogramming of somatic cells into induced pluripotent stem cells (Takahashi & Yamanaka 2006). In zebrafish, Sox2 is also required to reprogram Müller glia (Gorsuch et al. 2017). Interestingly, and in contrast to many genes induced by injury, Sox2 is constitutively expressed in Müller glia, a feature of all vertebrate retinas studied (Fischer et al. 2010, Gorsuch et al. 2017, Surzenko et al. 2013). However, following injury, Sox2 is strongly upregulated in those Müller glia that will enter the cell cycle. Using loss- and gain-of-function approaches, Gorsuch et al. (2017) showed that induction of Sox2 is necessary for Müller glia to enter the cell cycle following injury, and in an uninjured retina, induced overexpression of Sox2 is sufficient to stimulate proliferation. Furthermore,

consistent with its role as a reprogramming factor in somatic cells, Sox2 is near the top of a gene network that regulates the expression of *ascl1a* and *lin28*, but not *stat3*, identifying Sox2 as the first transcription factor that differentially regulates *ascl1a/lin28* and *stat3*. This suggests that independent signaling pathways are likely required for reprogramming Müller glia. It is currently unknown how Sox2 in Müller glia exerts different functions in injured versus uninjured retinas. Interestingly, in human embryonic stem cells, SOX2 interacts with the pluripotency transcription factor OCT4 to regulate the expression of pluripotency genes, while in neural epithelial cells derived from human embryonic stem cells, SOX2 interacts with PAX6 to mediate neuronal differentiation (Zhang et al. 2019). In the retina, a similar switch could explain the context-dependent function of Sox2. In support of this idea, following injury and prior to the onset of proliferation, the expression of *pax6b* declines in Müller glia (Hoang et al. 2019), whereas the expression of *oct4* increases (Ramachandran et al. 2010). Moreover, Oct4 regulates *ascl1a*, *oct4*, and *sox2* expression during reprogramming; however, the effect of Oct4 knockdown on proliferation of Müller glia has not been assessed (Sharma et al. 2019). Similarly, while expression of the zebrafish paralogs of the pluripotency factor *Myc*, *myca* and *mycb*, increases in the injured retina (Mitra et al. 2019), it is also unknown whether Myc regulates Müller glia proliferation. The upstream mechanisms that regulate the expression of the pluripotency factors *sox2*, *oct4*, and *myc* remain to be identified.

7. COMPARATIVE ANALYSIS

7.1. Chicks

In the chick retina, loss of neuronal cells stimulates the dedifferentiation of Müller glia and their reentry into the cell cycle for a short timeframe after hatching (Fischer & Reh 2001). Proliferating chick Müller glia only undergo a single cell division, thereby producing Müller glia–derived progenitors that do not continue in the cell cycle (Fischer & Reh 2001). While some of the Müller glia–derived progenitors replace a subset of the ablated retinal neurons, the majority of these cells remain in a progenitor state (Fischer & Reh 2001, 2002). In the uninjured chick retina, intravitreal injections of FGF2 are sufficient to induce Müller glia proliferation (Todd & Fischer 2015). A variety of other ligands and their corresponding receptors also play critical roles in mediating proliferation in the injured chick retina, including Sonic Hedgehog, HB-EGF, BMP, retinoic acid, Notch, and Wnt/β-catenin (Gallina et al. 2016; Todd & Fischer 2015; Todd et al. 2015, 2017, 2018). In contrast to FGF2, the aforementioned ligands are insufficient to induce cell cycle reentry in the uninjured retina. Intracellular signaling pathways that are activated in response to injury or exogenous FGF2 include Jak/Stat, ERK1/2, mTOR, and Smad (Fischer et al. 2009, Todd et al. 2016, Zelinka et al. 2016). In addition to activation, repression of signaling pathways such as TGFβ2 is also required for Müller glial reprogramming (Todd et al. 2017). Interestingly, and in contrast to zebrafish, upregulated Notch signaling is required for efficient reprogramming of chick Müller glia (Conner et al. 2014, Ghai et al. 2010, Hayes et al. 2007). In addition to deciphering the similarities and differences in the signaling pathways activated in the chick and zebrafish retina, it will also be critical to understand the road blocks in the chick retina that prevent the amplification of Müller glia–derived progenitors and their efficient differentiation into surviving neurons.

7.2. Mammals

A fundamental insight into the molecular biology of Müller glia in mammals was gained via the discovery that these cells and late-stage neural progenitors share a common gene expression profile (Blackshaw et al. 2004, Gotz et al. 2015). This discovery provided context regarding the

proliferative potential of Müller glia in mammals (Dyer & Cepko 2000, Vetter & Moore 2001) and led to the suggestion that Müller glia may be a form of a late-stage retinal progenitor with a latent ability to generate neurons (Jadhav et al. 2009). In mammals, the ability of Müller glia to reenter the cell cycle is variable and can depend on the species or the nature or severity of the injury (Joly et al. 2011, Karl et al. 2008, Lewis et al. 2010, Ooto et al. 2004). For example, in mice, photoreceptor injury induces the expression of cyclin D1 in Müller glia, but this is not accompanied by BrdU uptake, suggesting that, in mice, Müller glia are unable to progress into the S-phase of the cell cycle (Joly et al. 2011). In contrast, following retinal detachment in rabbits or NMDA-induced toxicity in rats, some Müller glia reenter the cell cycle, as evidenced by either phospho-histone 3 or BrdU labeling, suggesting that Müller glia in these animals can progress through the cell cycle (Lewis et al. 2010, Ooto et al. 2004). However, the majority of these BrdU-labeled cells either remain positive for markers of Müller glia or undergo apoptosis (Lewis et al. 2010, Ooto et al. 2004). In the rat, a few BrdU-positive cells express neuronal markers, but the low number of such cells is far from sufficient to replace the ablated neurons (Ooto et al. 2004).

A variety of strategies have been employed to increase the efficiency of neuronal regeneration in mammals. Exogenous application of growth factors (e.g., EGF, FGF1) can induce Müller glia to proliferate in NMDA-injured retinas, and some of these proliferating cells differentiate into amacrine cells (Karl et al. 2008). Targeted overexpression of *Ascl1* in mice can reprogram Müller glia into progenitor cells that are competent to regenerate retinal neurons in vivo. This action of ASCL1 is due to its capacity to transform chromatin into an active state at genes that are regulated by ASCL1 during retinal development and that induce a neurogenic program (Pollak et al. 2013). How ASCL1 transforms chromatin is currently unknown. However, *Wnt*, a transcriptional target of Ascl1a, regulates the expression of histone-modifying enzymes in retinal development (Aldiri et al. 2013), and thus, an ASCL1–WNT signaling axis could potentially link ASCL1 to chromatin remodeling during reprogramming. Additionally, combining targeted expression of *Ascl1* with a histone deacetylase inhibitor appears to induce Müller glia in adult mice to bypass a progenitor stage and convert directly to a neuronal fate (Jorstad et al. 2017). This could provide an additional mechanism to regenerate retinal neurons from Müller glia in the mammalian retina, although the effect of losing a significant proportion of Müller glia is yet to be determined.

In an uninjured retina, overexpression of *Lin28*, *β-catenin*, and constitutively active *Yap* (*Yap5SA*) also stimulates cell cycle reentry among Müller glia. However, it is unclear whether these Müller glia efficiently divide and produce amplifying neural progenitors (Elsaeidi et al. 2018, Hamon et al. 2019, Rueda et al. 2019, Ueki et al. 2015, Yao et al. 2016). Interestingly, in mice, coexpression of *Ascl1a* and *Lin28* in Müller glia induces a modest amount of cell proliferation in uninjured retinas, and this proliferative response is increased by retinal injury (Elsaeidi et al. 2018). In contrast to the zebrafish retina, suppression of Notch signaling does not increase proliferation when *Ascl1* and *Lin28* are overexpressed (Elsaeidi et al. 2018). Similar to Notch activation in the injured chick retina, exposure of retinal explants to the Notch ligand Jag1 increases Müller glia cell cycle reentry, suggesting that the function of Notch signaling diverges between zebrafish and chicks or mammals (Del Debbio et al. 2010, Ghai et al. 2010, Hayes et al. 2007).

Stat3 and ERK1/2 signaling pathways involved in Müller glia reprogramming in the zebrafish retina are activated by injury in the mammalian retina (Galan et al. 2014, Jiang et al. 2014, Kassen et al. 2007, Kirsch et al. 2010, Nakazawa et al. 2007, Zhao et al. 2014). Following retinal detachment in mice, ERK1/2-dependent upregulation of Cyclin D suggests that ERK1/2 might be required for cell cycle reentry in the mammalian retina in vivo (Kase et al. 2006). In support of this, EGF stimulates Müller glia proliferation in an ERK1/2-dependent manner in retinal explants from juvenile mice (Ueki & Reh 2013). In contrast, the role of STAT3 in reprogramming of mammalian Müller glia is unknown. However, following optic nerve injury, the gliotic response

in Müller glia is regulated by STAT3 (Kirsch et al. 2010). It remains to be determined what regulates reprogramming versus gliosis, and transcription factors like STAT3 need to be examined in this context.

The studies discussed above induced reprogramming of Müller glia by either targeted expression of genes or application of exogenous factors. An alternative approach to reprogramming Müller glia is being pioneered through cell fusion–based techniques (Lluis & Cosma 2010; Lluis et al. 2008; Sanges et al. 2013, 2016). Cell–cell fusion, the merging of plasma membranes to integrate intercellular components, is a tightly regulated process that occurs naturally during vertebrate development (Willkomm & Bloch 2015). Following activation of Wnt signaling, a variety of murine and human stem and progenitor cells, when transplanted into NMDA-injured retinas of adult mice, spontaneously fuse with host retinal neurons and Müller glia (Sanges et al. 2013, 2016). This cellular fusion transiently reprograms the fused host cell into a retinal progenitor that can divide and produce regenerated neurons. If transplantation experiments are performed in *rd10* mice, in which photoreceptors selectively die, the Wnt-activated stem and progenitor cells transplanted into the eye fuse exclusively with and reprogram Müller glia, which give rise to regenerated photoreceptors (Sanges et al. 2016).

7.3. Comparative Omics Analysis

The ability to reprogram mammalian Müller glia by overexpressing genes identified in zebrafish represents a significant advance toward developing strategies to repair the injured or diseased human retina. However, some genes required for reprogramming Müller glia in zebrafish are involved in the mammalian gliotic response. To successfully develop therapies in human, it is necessary to fully identify the signaling mechanisms and gene regulatory networks that act in species with and without regenerative capacities. To our knowledge, only one study has compared differentially regulated genes in Müller glia isolated from photo-lesioned zebrafish to genes expressed in Müller glia from mouse mutants that display either early- (*Pde6b*$^{rd1/rd1}$) or late-onset (*Rho*$^{-/-}$) photoreceptor degeneration (Sifuentes et al. 2016). This study revealed that metabolic pathway genes are enriched in both species, whereas genes within cytokine signaling, circadian, and pluripotency pathways that were upregulated in zebrafish were reduced or unaltered in mice. However, this study compared the response of Müller glia in an acute injury in zebrafish to chronic photoreceptor degenerations in mice. Additionally, the data sets for the two organisms were acquired using different techniques (RNA-seq for zebrafish and microarray for mice) that possess different sensitivities.

We recently performed both ATAC-Seq and RNA-Seq analysis on FAC-sorted Müller glia isolated from acutely injured zebrafish and mouse retinas that both experienced light-induced outer or NMDA-induced inner retinal injury (Hoang et al. 2019). Changes in chromatin accessibility resulted either in reduced (i.e., a repressed chromatin state) or in rapidly or slowly induced chromatin accessibility. Chromatin accessibility of genes like *Rlbp/rlbp1a*, *Nfix/nfixb*, and *Pax6/pax6b* were repressed in Müller glia from both mouse and zebrafish, and this correlated with reduced expression of these genes in both species. Additionally, we observed (*a*) genes that upregulated expression in both species, (*b*) species-specific gene expression changes, and (*c*) genes that were differentially expressed but for which an ortholog was not present in the other species.

We also performed single-cell RNA-Seq (scRNA-seq) on control and injured zebrafish, chick, and mouse retinas to obtain specific expression profiles of individual cells (Hoang et al. 2019). In our scRNA-Seq data, zebrafish Müller glia from uninjured and injured retinas separated into distinct clusters based on their RNA expression profiles (Hoang et al. 2019). Heterogeneity was also observed between Müller glia within a cluster. For example, proliferating Müller glia at 36 h of

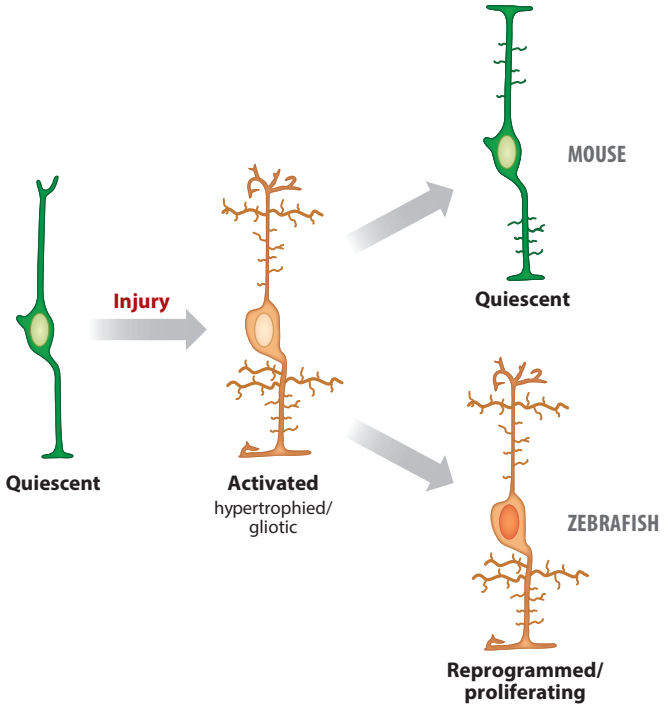


Figure 3

Comparison of the injury response in zebrafish and mice: schematic of a quiescent Müller glia that becomes activated or gliotic following retinal injury, displaying a hypertrophied cell body and processes in both mice and zebrafish. In mice, the activated or gliotic Müller glia returns toward quiescence based on gene expression and morphology, whereas in zebrafish, the activated or gliotic Müller glia reprograms and proliferates.

photo-injury can be distinguished according to the expression of cell cycle stage–specific cyclins (*ccnd1*: G1; *ccnb1*: mitosis). Subcategorizing Müller glia according to cell cycle phase may help identify genes that are necessary for a specific process. For example, genes required to mediate interkinetic nuclear migration would be expected to be expressed in *ccnb1*-positive cells. Müller glia from injured and uninjured retinas of chicks or mice also accumulated in distinct clusters (resting and activated Müller glia). Analysis revealed 325 genes that were differentially regulated in all three species, and there were also a large number of genes that were regulated in a species-specific manner or that overlapped among only two species. Computational modeling that assessed the ATAC-Seq, bulk RNA-Seq, and scRNA-Seq data for differentially expressed transcription factors or regulators in Müller glia at different injury time points predicted transcriptional networks consisting of gene regulatory modules that implicate the progression of Müller glia through different functional states (Hoang et al. 2019). In mice, Müller glia become gliotic and then return toward a transcriptionally quiescent state (**Figure 3**), while zebrafish Müller glia progress from a quiescent to a transient gliotic or neurogenic state and subsequently to a proliferative state (**Figure 3**). In support of this transient intermediate gliotic state, Müller glia persist in a morphologically gliotic state when proliferation is blocked in the damaged zebrafish retina (Thomas et al. 2016). The transcription factors identified in these regulatory modules will require functional testing to determine if they play a role in the predicted cellular processes or functional states in both mice and zebrafish. It will also be of particular interest to examine whether differentially regulated genes

that lack an ortholog in mice are instrumental in reprogramming Müller glia in zebrafish. Additionally, it is important to determine if the mouse genes that lack a zebrafish ortholog function to repress regeneration.

8. CONCLUSION

The studies reviewed in this article establish that injury-induced reprogramming of zebrafish Müller glia involves changes in paracrine and autocrine signaling, which control multiple signal transduction pathways and gene regulatory networks. Retinal injury alters neurotransmitter signaling and stimulates the release of nucleotides and cytokines from dying cells, and cell death itself likely disrupts cell-contact-mediated signaling; these different events can initiate reprogramming. The release of inflammatory cytokines and growth factors from activated microglia also represents a crucial step in the regulatory cascades leading to reprogramming. Additionally, in response to cell death, Müller glia themselves upregulate and release growth factors and inflammatory cytokines, which likely then act in an autocrine manner. Among the proteins regulated by injury, Stat3 and Sox2 reside near the apex of a gene regulatory network, which engages an Ascl1a–Lin28–*let*7 regulatory loop. This loop functions to upregulate the expression of genes that are shared with retinal progenitors and suppress the expression of genes associated with cellular differentiation. The output of this regulatory network allows Müller glia to enter the cell cycle and undergo a single asymmetric cell division that generates a multipotent retinal progenitor. Much less is known about the epigenetic mechanisms that underlie reprogramming in Müller glia, but recent studies show that injury alters the DNA methylation status and initiates the differential expression of histone-modifying enzymes in Müller glia. Importantly, in zebrafish, quiescent Müller glia contain open chromatin for pluripotency and other injury-induced transcription factors. In mice, the same genes contain open chromatin, but injury is insufficient to induce their expression. Numerous gaps remain in our knowledge of the molecular mechanisms that allow zebrafish Müller glia to, but prevent mammalian Müller glia from, reprogramming into stem cells. A recent comparative RNA-Seq study revealed that retinal injury activates shared and distinct genetic programs in Müller glia of zebrafish and mice. Mining these data will doubtless reveal new and important avenues for research that will aid in parsing the injury-induced molecular processes that permit or prevent Müller glia reprogramming.

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