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Turning Müller Glia into Neural Progenitors in the Retina

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Abstract Stimulating neuronal regeneration is a potential strategy to treat sight-threatening diseases of the retina. In some classes of vertebrates, retinal regeneration occurs spontaneously to effectively replace neurons lost to acute damage in order to restore visual function. There are different mechanisms and cellular sources of retinal regeneration in different species, include the retinal pigmented epithelium, progenitors seeded across the retina, and the Müller glia. This review briefly summarizes the different mechanisms of retinal regeneration in frogs, fish, chicks, and rodents. The bulk of this review summarizes and discusses recent findings regarding regeneration from Müller glia-derived progenitors, with emphasis on findings in the chick retina. The Müller glia are a promising source of regeneration-supporting cells that are intrinsic to the retina and significant evidence indicated these glias can be stimulated to produce neurons in different classes of vertebrates. The key to harnessing the neurogenic potential of Müller glia is to identify the secreted factors, signaling pathways, and transcription factors that enable de-differentiation, proliferation, and neurogenesis. We review findings regarding the roles of mitogen-activated protein kinase and notch signaling in the proliferation and generation of Müller glia-derived retinal progenitors.

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Regeneration—the Colder the Blood, the Better the Regeneration

In response to injury, tissue healing can involve regeneration or scar formation. Regeneration entails the re-growth of new and healthy cells to replace those lost to damage with a final outcome of restoration of tissue function. Scar formation entails the mending of damaged tissue with deposition of extracellular matrix and/or fibrotic cells with a final outcome of loss of tissue function. Across vertebrate classes, there is a trend that many cold-blooded species have a greater ability than warm-blooded to regenerate lost or damaged tissue, including limbs and organs. This regenerative ability is also present in the central nervous system, including the retina. Regeneration of the retina in lower vertebrates has been studied for many decades [1, 2] whereas studies into retinal regeneration in higher vertebrates have only gained credence in recent years. Depending on the species, potential sources of regeneration include the retinal pigmented epithelium (RPE), the circumferential germinal zone (CGZ) or ciliary marginal zone (CMZ), and the Müller glia.

Regeneration in Fish

Teleost fish such as zebrafish (*Danio rerio*) have a well-characterized ability to regenerate tissues after injury. They can regenerate damaged fins [3], heart muscle [4] and nervous tissue of the CNS, including the spinal cord [5] and retina [6, 7]. Retinal regeneration in teleost fish has been well studied [8–10]. Throughout life the retina continues to

grow in parallel to overall ocular growth. One source of new neurons is a zone of retinal stem cells, known as the CGZ, that persists after embryonic histogenesis at the peripheral edge of the retina (reviewed by [8]). The retina normally grows by the addition of new neurons, produced by CGZ stem cells, which are integrated into pre-existing circuitry at the retinal periphery. However, the progenitors in the CGZ do not normally produce rod photoreceptors. Instead, rod progenitors that are seeded across central regions of the retina produce rod photoreceptors (reviewed by [8]. The process of on-going genesis of rod photoreceptors persists throughout the animal's adult life so that rod density remains consistent as the retinal area expands [11]. Recent studies have indicated that rod progenitors are derived from the Müller glia [12]. Functional regeneration of the fish retina occurs through progenitors derived from Müller glia [12–15] and possibly through the progenitors in the CGZ [15]. Retinal regeneration in fish has been the focus of several recent comprehensive reviews [16, 17], and accordingly will not be discussed in depth here.

Regeneration in Amphibians

Urodele amphibians, such as newts in the genera *Cynops and Triturus*, have a tremendous ability to regenerate a wide range of body parts: limbs [18], lower jaw [19], portions of the heart [20], ocular tissues including the retina [21, 22], tail and central nervous system tissues, including spinal cord [23]. In newts and salamanders, regeneration of ocular tissues has been known for over a century (reviewed by [2]). Newts and some species of salamander are capable of re-growing the entire neural retina from the retinal pigmented epithelium (RPE). Over the course of several days, these cells lose their pigment granules and undergo several rounds of division to become neural progenitor cells [2, 24], a process that has been termed transdifferentiation [25]. In vitro models have indicated that the choroid must remain attached to the RPE for transdifferentiation to occur. Without the choroid, the RPE may still lose pigmentation, but the cells fail to proliferate or assume a progenitor-like state unless fibroblast growth factor 2 (FGF2) is added [21]. Although there is currently no direct evidence, there is the implication that the choroid provides FGF to stimulate transdifferentiation of the RPE. FGF-mediated retinal regeneration is a common theme across vertebrates, with the exception of the fish retina where there is currently no direct evidence supporting a role for FGF in regeneration. However, a study by Negishi and Shinagawa [26] demonstrated that intravitreal injections FGF1 and FGF2 stimulated the accumulation of clusters of proliferating (PCNA+) cells in the goldfish retina, reminiscent of the neuroblastic clusters of proliferating cells described in regenerating fish retina in subsequent studies (see below). Although the identity of the PCNA-positive cells in FGF-treated retinas remains uncertain, it remains possible that FGFs influence regeneration in fish retinas.

Anuran amphibians such as the African clawed frog (*Xenopus laevis)* and the northern leopard frog (*Rana pipiens*) lose much of their regenerative abilities once metamorphosis is complete. During larval stages of development, tadpole frogs are capable of regenerating injured hind limb, but limb regeneration no longer occurs in adults [18]. Retinal regeneration from the RPE has been reported for larval frogs of the genus *Rana* [27, 28], and for many years it was assumed that the regenerative ability of the retina was lost in adult frogs. However, in *Xenopus* and some species of *Rana*, grafting a fragment of RPE into the vitreous chamber can stimulate the formation of a new retina [3, 29]. By comparison, the RPE of tadpoles is capable of regenerating retinal neurons in response to FGF2 in vitro [30, 31]. Although compelling evidence indicates that numerous retinal neurons are regenerated, recovery of visual function from regenerated retinas in frogs is currently lacking.

Regeneration in Embryonic Birds

In regards to limb regeneration, birds are perhaps the least plastic of vertebrate classes, lacking the ability to regenerate any portion of a limb at any point in their development [18, 23]. By contrast, the embryonic avian retina is capable of significant regeneration during early stages of embryonic development. Similar to the newt, the RPE of the embryonic chick is capable of transdifferentiating to regenerate all cell types up until embryonic day 4.5 (E4.5) [32, 33]. The ability of RPE cells to transdifferentiate and generate retinal neurons decreases as embryonic development proceeds beyond E4.5 [33–35]. Unlike the newt RPE, which requires choroid to promote transdifferentiation, the transdifferentiation of embryonic chick RPE requires that a fragment of neural retina remain in the eye; if the entire retina is removed the RPE does not transdifferentiate [36, 37]. In place of damaged retina to drive RPE transdifferentiation, exogenous FGF2 or FGF8 can stimulate retinal regeneration from early embryonic RPE [32–34, 37, 38]. In addition, a series of studies has demonstrated that early-stage chick embryos (~E5) can regenerate retinal cells from RPE or from progenitors in far peripheral regions of the optic cup, and this regeneration is stimulated by sonic hedgehog (Shh), bone morphogenetic protein (BMPs), and FGF/mitogen-activated protein kinase (MAPK) signaling [39–41]. Embryonic RPE retains some capacity for retinal regeneration beyond E4.5. A series of studies has demonstrated that ectopic expression of pro-neural transcription

factors, such as *ascl1a*, *ath5*, *neuroD*, *neurogenin1*, and *neurogenin2*, can promote the transdifferentiation of RPE into neuronal cells (reviewed by [42]).

In the eyes of postnatal chicks, the pigmented epithelium in the pars plana of the ciliary body appears to remain plastic with sustained expression of Pax6 and MITF similar to embryonic RPE [43]. These RPE cells can be stimulated by exogenous FGF2 to transdifferentiate and proliferate, but the progeny fail to survive [43]. The CGZ of post-hatched chicks is a source of neural stem cells that persists well into adulthood [44, 45], and, in principle, these cells could be harnessed for retinal regeneration. However, the chick CGZ does spontaneously regenerate neurons; exogenous growth factors must be applied to increase the yield of cells from CGZ progenitors in damaged retina [46]. In addition, the non-pigmented epithelium (NPE) of the par plana of the ciliary body of the postnatal chick can be stimulated by exogenous growth factors, including FGF2, to produce neurons [47]. However, the neurons produced by the NPE cells form neurites that project randomly across the vitread surface of the ciliary body and do not appear to migrate into the neural retina [47]. Thus, the NPE cells are not an endogenous source of retinal regeneration, but hold the potential to be harvested, expanded in culture, stimulated to become neurogenic, and autologously transplanted back into damaged retinas to replaced neurons.

Regeneration in Mammals

Mammals, thus far, has proven to be the least capable of regeneration among vertebrate classes. Embryonic, as well as neonatal, mouse pups are able to regenerate amputated distal phalanges, but not more proximal digits [18]. In vitro studies of early embryonic rat RPE have demonstrated that transdifferentiation can be stimulated by FGF2 to produce cells expressing neuronal markers, but this can only occur within a narrow window of early eye development, between E12 and E15 [35]. Although presumptive retinal stem cells have been described as pigmented cells derived from adult rodent ciliary body [48], this possibility remains controversial [49, 50]. There is some evidence that non-pigmented cells at the peripheral edge of the rodent retina may be analogous to the amphibian and avian CMZ. These cells can proliferate up to at least postnatal day 21, express some markers of neural progenitors, and proliferate at elevated levels with a gain in Shh signaling through the loss of one allele of *patched* [45, 51]. These cells are few in number and are confined to the peripheral-most edge of the retina, and thus do not represent a significant endogenous source of regeneration-supporting cells. The low abundance of these cells and their normally mitotically quiescent state reflects the diminished regenerative capacity in mammals.

Müller Glia-Mediated Regeneration

The remainder of this review focuses on recent findings that Müller glia are a potential source of retinal progenitors in warm-blooded vertebrates. The Müller glia are the major type of support cell in the retina, are common to the eyes of all vertebrate classes, and are the only type of retinal glia that is derived from the embryonic retinal neuroepithelial stem cells. Other types of retinal glia originate from extra-retinal sources; these glia can include microglia, astrocytes, oligodendrocytes and non-astocytic inner-retinal glia cells (NIRG cells) [52], also described as astrocytes and diacytes [53]. The Müller glia perform support functions similar to astrocytes in the brain. At a minimum, the functions of Müller glia include providing structural support, synaptic support, osmotic homeostasis, and nutritive/metabolic support to retinal neurons. In several vertebrate classes, the Müller glia are capable of de-differentiating, proliferating and acquiring a progenitor-like state in response to acute retinal injury [12, 13, 54–56] or in response to exogenous growth factors [57].

There is a growing body of evidence that Müller glia can de-differentiate, proliferate, and become neuronal progenitors in acutely damaged retinas. We were the first to demonstrate that Müller glia are a potential source of progenitor-like cells for retinal regeneration [54]. Using the postnatal chick retina as a model system, we found that in response to sufficient levels of excitotoxic damage, elicited by *N-methyl*-D-aspartate (NMDA), numerous Müller glia de-differentiate, re-enter the cell cycle, and express transcription factors found in embryonic retinal progenitors. These transcription factors include ascl1a, Pax6, Chx10 [54], Six3 [46], Sox2 [58, 59], and Sox9 [58, 60]. Similarly, Pax6 [61], ascl1a [62], and notch [63, 64] are involved in Müller glia-mediated regeneration in zebrafish retina. Furthermore, de-differentiating Müller glia express the intermediate filament transitin, the avian homologue of mammalian nestin, which is known to be expressed by neural stem cells [65]. In response to sufficient levels of neuronal damage, numerous Müller glia re-enter the cell cycle and undergo only one round of division in vivo; these cells continue to proliferate and produce some new neurons when dissociated from the intact retina and are grown in culture [54]. The transdifferentiation of pigmented epithelium (PE) during retinal regeneration in tadpoles, salamander, embryonic chicks and embryonic rodents requires that the PE cells de-differentiate (lose pigmentation) and proliferate [43, 66]. Similarly, the proliferation of the Müller glia is an integral step toward becoming progenitor-like cells [46, 66, 67]. In the chicken retina in vivo, the majority (about 80%) of cells that are generated by proliferating new Müller glia remain as un-differentiated progenitor-like cells, while some differentiate into Müller

glia and a few differentiate into amacrine or bipolar neurons. Although few neurons are regenerated, proliferating Müller glia produce tens of thousands of undifferentiated progenitor-like cells that represent a large pool of cells that could be stimulated to differentiate and regenerate the retina to restore vision. Based on our findings in the chicken retina, others have recently reported that Müller glia are the source of retinal regeneration in zebrafish [12, 13, 68] and rodent [55, 56]. In addition, there is some evidence that Müller glia have progenitor-like properties in the primate retina [69]. However, evidence for in vivo neuronal regeneration from Müller glia-derived progenitors in the primate retina is currently lacking.

Functional regeneration from Müller glia requires several key steps. At a minimum, these steps include (1) de-differentiation, (2) proliferation, (3) neural differentiation of progeny, and (4) integration into retinal circuitry. (1) De-differentiation implies that Müller glia have stopped functioning as a glial cell and have acquired phenotypes commonly associated with progenitor cells. For example, de-differentiating Müller glia express filamentous proteins and transcription factors that are normally found in retinal progenitors. It is not precisely clear when Müller glia cease being glia and start being progenitors, and whether the transition into a progenitor-like cells occurs before or after re-entry into the cell cycle. Indeed, during development the distinction between late-stage retinal progenitors and immature Müller glia is somewhat blurry. (2) Proliferation—cell division is required to produce additional numbers of cells, including progeny to differentiate as neurons and new Müller glia to continue supporting neuronal function. Furthermore, proliferation is a function of progenitors and is a symptom of glial de-differentiation; presumably glia must abandon some glial functions to re-enter the cell cycle. However, proliferation can also be a function of reactive glia during scar formation. It is likely that much of the proliferation of Müller glia in damaged avian and mammalian retinas contributes to the formation of a glial scar, and equates to a permanent loss of tissue function. (3) Neural differentiation—the progeny that are produced by Müller glia-derived progenitors must fully differentiate into neurons for meaningful regeneration. Expression of a few neuronal proteins by Müller glia-derived cells is insufficient; there are examples of Müller glia and glia-derived cells expressing a "neuronal" protein, while maintaining many glial phenotypes [70, 71]. For meaningful regeneration, the differentiation of Müller glia-derived progeny must be sufficient to permit neuronal function. The expression of a full-compliment of neuronal proteins is required to obtain proper morphology, make appropriate connections, and function properly within neuronal circuitry. (4) Integration into retinal circuitry—the Müller glia-derived neurons must establish pre- and post-synaptic connections that properly contribute to the image processing functions of the retina. Anatomical evidence has been provided for the formation of synapses by regenerated neurons in the fish retina [72, 73], and the establishment of meaningful synapses is implied by the restoration of visual function. Functional regeneration of neurons in the fish retina has been well established [74–78]. However, unambiguous demonstration of functional integration of Müller glia-regenerated neurons in the retinas of birds and mammals is currently lacking. A further consideration for effectively applying Müller glia-mediated retinal regeneration is whether the activation of neurogenesis from the glia compromises the support functions of these cells. If the neuron-supporting functions of Müller glia are diminished, then the loss of retinal neurons could result. The activation of neurogenesis from Müller glia must occur without causing neuronal damage.

The key to harnessing the neurogenic potential of Müller glia is to identify the secreted factors, signaling pathways and transcription factors that enable de-differentiation, proliferation and neurogenesis. Not surprisingly, the signaling pathways and transcription factors that drive embryonic retinal histogenesis appear to be re-activated to drive regeneration in a mature retina; many examples are discussed below. The mechanisms that "kick-start" Müller glia-mediated neuronal regeneration, and lie upstream of re-activating a program of neurogenesis, remain somewhat uncertain.

In all published studies, with few notable exceptions, neurogenesis from Müller glia has been stimulated by severe acute retinal damage. One notable exception is the combination of insulin and FGF2, in the absence of retinal damage, to stimulate proliferation and neurogenesis from Müller glia in the chick retina [57]. Another notable exception is the regeneration of photoreceptors in a line of zebrafish that undergoes chronic loss of rods and cones [79]. Another example of regeneration without acute damage is the application of CNTF in zebrafish retina to stimulate the proliferation of Müller glia [80]. Kassen and colleagues demonstrated that CNTF activates the MAPK-pathway in photoreceptors to support survival and activates the Stat3-pathway in Müller glia to stimulate proliferation [80]. However, it remains uncertain whether the progeny of proliferating Müller glia in CNTF-treated retinas differentiate into new neurons or glia. By comparison, CNTF stimulates reactive gliosis in the avian and rodent retina [81–84] and suppresses the de-differentiation and proliferation of Müller glia in the chick retina [85]. CNTF-mediated activation of the Jak/Stat-pathway in the rodent promotes glial reactivity and enhances the neuroprotective functions of the Müller glia [82–84]. Thus, CNTF may not be applied to stimulate Müller glia-mediated retinal regeneration in warm-blooded vertebrates.

It makes no sense to stimulate neural regeneration from Müller glia in slowly degenerating retinas by inducing severe, acute damage. Thus, identification of the secreted factors and signaling pathways that stimulate Müller glia without further damaging neurons is crucial to developing new therapies to treat human diseases of the retina. Significant progress has been made in identifying important roles for notch, insulin/IGF1, FGF2, and MAPK/ERK1/2 signaling in stimulating the de-differentiation and proliferation of Müller glia into proliferating progenitor-like cells. Interestingly, these growth factors and signaling pathways in the retinal glia also have significant impacts upon neuroprotection and neuronal survival.

MAPK Signaling and the De-differentiation and Proliferation of Müller Glia

We have recently begun to reveal the cell signaling pathways that control the ability of mature Müller glia to become progenitor-like cells. We investigated the roles of the MAPK-pathway in regulating the activity of Müller glia in acutely damaged chicken retina [58]. In response to acute retinal damage, Müller glia accumulate phosphorylated ERK1/2 and phospho-CyclicAMP response element binding protein (pCREB), and transiently express immediate early genes, cFos and Egr1, which are known to be downstream of MAPK signaling. Egr1 and pCREB are normally expressed by retinal progenitors in the CGZ whereas cFos and pERK1/2 are not. In addition, small molecule inhibitors of MEK (UO126) and the FGF-receptor (SU5402) suppress the proliferation of Müller glia-derived progenitor-like cells. These inhibitors suppress the accumulation of Egr1 and pCREB whereas levels of cFos are unaffected in Müller glia-derived progenitors. These findings suggest that Egr1 and pCREB are part of the de-differentiation/proliferation signaling cascade activated by FGF receptors and ERK1/2. Collectively, these findings suggest that Egr1 and pCREB may promote the trans-differentiation of Müller glia. We propose that activation of both the FGF-receptor and ERK1/2-pathway is required for the de-differentiation and proliferation of Müller glia. See Fig. 1 for a schematic summary of these findings.

In a follow-up study, we investigated the sites and modes of action of insulin and FGF2 [60]. In part, the rationale for this study was to better understand how the combination of insulin and FGF2 stimulate the de-differentiation and proliferation of Müller glia in the absence of retinal damage [57]. Intraocular injections of insulin or FGF2 caused the phosphorylation of ERK1/2, p38 MAPK and CREB, and the expression of immediate early genes, cFos and Egr1. Accumulations of pERK1/2, p38 MAPK, pCREB, cFos, and Egr1 in response to insulin or FGF2 are confined to Müller glia whereas retinal neurons do not appear to respond. Unlike FGF2, insulin-stimulated glia-like cells (NIRG cells, see below) in the IPL to up-regulate the intermediate filament transitin and microglia to up-regulate lysosomal membrane glycoprotein (LMG). With microglia and Müller glia stimulated by insulin or FGF2 there were profound effects upon numbers of dying neurons in response to excitotoxic damage. Although FGF2 significantly reduces numbers of dying neurons, insulin significantly increases numbers of dying neurons. In addition to neuroprotective affects, FGF2 "primes" the Müller glia to proliferate following retinal damage whereas insulin has no effect upon glial proliferation. FGF receptor isoform (FGFR1) and FGFR3 are prominently expressed in the retina whereas FGFR2 is not expressed, or is expressed at very low levels. Insulin receptors were not readily detected in the retina [60], however the PCR primers used to amplify these receptors fail to detect the splice isoform that is expressed by retinal cells (unpublished observations). We conclude that MAPK signaling through FGF receptors stimulates Müller glia to enhance neuroprotection and become more progenitor-like whereas insulin acting on Müller, microglia and NIRG cells had the opposite effect. See Fig. 1 for a schematic summary of these findings.

In the next study we investigated how IGF1 influences the Müller glia and, through serendipity, identified a novel type of glial cell in the avian retina [52]. In normal retinas, we find a distinct cell type that is scattered across the ganglion cell layer (GCL) and IPL; these cells expressed Sox2, Sox9, Nkx2.2, vimentin, and transitin, but not other well-known glial markers such as GFAP, Pax2, or glutamine synthetase. The glia-like cells have a unique immunohistochemical profile, morphology and distribution that are distinct among other known types of retinal glia, including microglia, oligodendrocytes, astrocytes, and Müller glia. Accordingly, we termed these cells non-astrocytic inner-retinal glia-like (NIRG) cells. The NIRG cells may express the IGF1 receptor and respond to exogenous IGF1 by proliferating, migrating distally into the retina, and up-regulating transitin. Similar to the affects of insulin, IGF1 stimulates microglia to acquire a reactive morphology and up-regulate LMG and CD45. With microglia and NIRG cells stimulated by IGF1 there are elevated levels of cell death and numerous focal detachments across the retina in response to excitotoxic damage. Cell death is prominent within areas of detachment which is coincident with a stark loss of Müller glia and accumulation of NIRG cells. We have proposed that NIRG cells are a unique type of retinal glial cell that is sensitive to IGF1 and whose activity impacts the activity and survival of Müller glia. Furthermore, our findings suggest that the activities of the microglia, NIRG cells and Müller glia is somehow

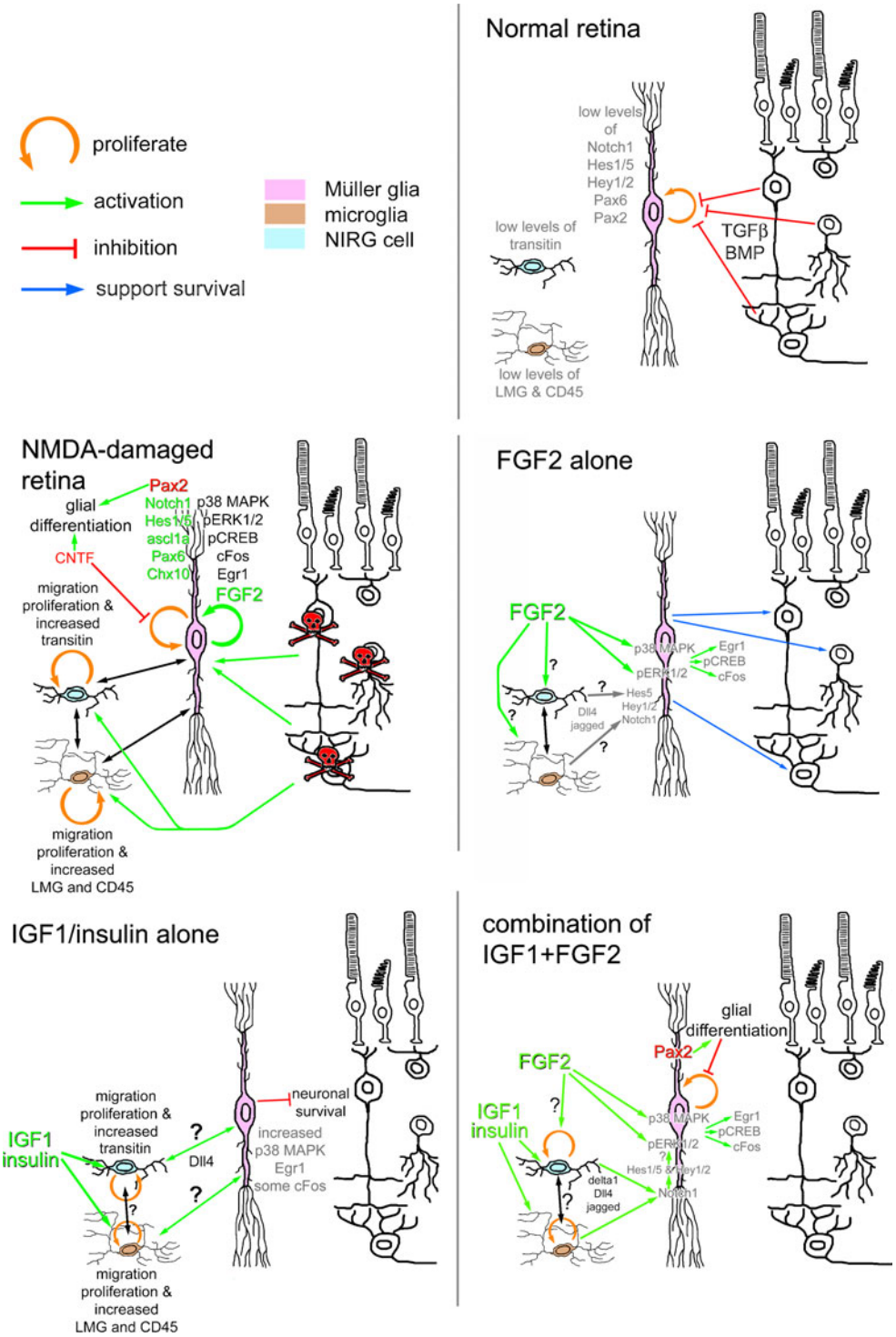

Fig. 1 Schematic diagrams illustrating and summarizing the interactions between retinal neurons and glia in normal retina, NMDA-damaged retina, and retinas treated with FGF2 alone, IGF1/insulin alone or the combination of IGF1 and FGF2. Legend: *orange arrow*, proliferation or transdifferentiate; *green arrow*, activate; *red line*, inhibit or suppress; *blue arrow*, support survival. *Abbreviations*: *FGF2* fibroblast growth factor 2, *IGF1* insulin-like growth factor 1, *CNTF* ciliary neurotrophic factor, *TGFβ* transforming growth factor β, *BMP* bone morphogenetic protein, *ascl1a* acheate schute-like 1a, *MAPK* mitogen activation protein kinase, *LMG* lysosomal membrane glycoprotein, *Dll4* Delta-like 4

coordinated in damaged and IGF1-treated retinas. See Fig. 1 for a schematic summary of these findings.

A recent study from Rompani and Cepko [53] described the lineage of glial cells, derived from extra-retinal precursors, in the chick eye. The authors describe an optic stalk-derived glial progenitor that gives rise to oligodendrocytes, astrocytes and a novel cell type that they termed "diacytes". The authors describe presumptive astrocytes in the IPL and diacytes in the GCL based on morphology; the morphology and lamination of the diacytes and presumptive astrocytes are reminiscent of astro-glial cells. The diacytes and presumptive astrocytes express Olig2 (similar to oligodendrocytes) whereas these cells do not express other well-established markers of astrocytes and oligodendrocytes including GFAP, myelin proteolipid protein, myelin/oligodendrocyte-specific pro-

tein, or myelin-associated glycoprotein. We believe that the astrocytes and diacytes described by Rompani and Cepko are the NIRG cells that we described [52]. Recall that we determined that the NIRG cells/astrocytes/diacytes were negative for glial markers including GFAP, glutamine synthetase, Pax2, Top_{AP}, and transferin-binding protein whereas the NIRG cells were positive for Sox2, Sox9, Nkx2.2, vimentin, and transitin [52, 86]. We propose that the astrocytes/diacytes/NIRG cells in the chicken retina are not a type of astrocyte because these cells are negative for both GFAP and Pax2, these glia are not associated with blood vessels (unlike the retinal astrocytes in mammals), and these cells do not up-regulate GFAP in response to damage [52, 86]. Retinal astrocytes are known to express GFAP and Pax2, and GFAP expression is dramatically increased in damaged tissues [86, 87]. Nevertheless, an unambiguous definition of the astrocytes/diacytes/NIRG cells in the chick retina requires a more thorough investigation into the functions of these glia. A better understanding of NIRG cells is warranted given the impact of these cells upon Müller glia and these cells may be found in the retinas of some mammalian species. NIRG-like cells are present in the retinas of dogs and macaque monkey [88], suggesting that similar cells may exist in the eyes of humans. However, NIRG-like cells are not found in the retinas of mice or guinea pigs [88].

Through the course of our studies into responses of Müller glia to growth factors and damage we found that some of these glias express Pax2 in the mature retina [86]. We found two distinct Pax2 isoforms expressed within the retina and optic nerve. Surprisingly, Müller glia in central regions of the retina expressed Pax2, and levels of expression are gradually decreased in Müller glia in the peripheral retina. In Müller glia the expression levels of Pax2 were increased by acute retinal damage or treatment with growth factors. Based on our findings in the chick, we assayed for Pax2 expression in glial cells in the retinas of mammals. Unlike the chick, Müller glia in the mammalian retina do not express detectable levels of Pax2. In both dog and monkey retinas, Pax2 was detected in astrocytes and putative NIRG-like cells that were scattered across inner-retinal layers and in numerous glia within the optic nerve. Our findings are in agreement with those of Boije and colleagues [89].

The predominant expression of Pax2 by Müller glia in central regions of the retina may function to suppress de-differentiation. Little or no Pax2 was seen in Müller glia in peripheral regions of the retina [86], where these glia are known to be more plastic, proliferative and neurogenic compared with glia in central regions of the retina (reviewed by [46]). This correlation suggests that Pax2 may suppress the de-differentiation of Müller glia or promote glial phenotype in central regions of the retina. Consistent with this notion, Pax2 is known to promote glial phenotypes in the developing optic nerve [90–92]. Pax2 may inhibit the acquisition of neuronal phenotypes by suppressing the expression of pro-neural genes and by inhibiting the expression of Pax6, which promotes the multipotency of retinal progenitors (reviewed by [93]). Pax2 may function to maintain glial phenotypes in mature eyes and suppress the neuronal differentiation of Müller glia-derived cells. For example, the majority of cells produced by Müller glia-derived progenitors do not differentiate into neurons, but remains as progenitor-like cells or form new glia [54, 57]. Collectively, these observations suggest that Pax2 may act to maintain glial phenotype and suppresses neurogenic potential in Müller cells in the chick retina.

Notch Signaling and Müller Glia in Normal and Damaged Retinas

Recent studies have investigated the roles of notch signaling in mature retinas that are undamaged, damaged or treated with growth factors that promote glial de-differentiation and proliferation. Studies in the fish retina have demonstrated that notch and notch-associated genes are up-regulated by proliferating/de-differentiating Müller glia in response to acute damage [64, 94]. The notch signaling pathway maintains progenitor cells in an un-differentiated state during early stages of retinal development [95, 96], and notch signaling promotes glial differentiation during later stages of retinal development [97]. Thus, it is not surprising that notch signaling is involved in the proliferation and differentiation of Müller glia-derived progenitors in the retina. Hayes and colleagues reported that notch signaling is up-regulated in proliferating Müller glia-derived progenitors in acutely damaged chick retina [59]. Blockade of notch signaling, with the small molecular inhibitor DAPT, decreases numbers of proliferating Müller glia-derived progenitors; provided that the DAPT is applied shortly after the induction of damage when Müller glia begin to de-differentiate [59]. After progenitors have been generated from Müller glia, blockade of notch signaling caused a significant increase in the percentage of new neurons [59]. Thus, notch signaling appears to play two distinct roles during retinal regeneration. Initially, notch activity promotes the proliferation of Müller glia-derived progenitors whereas after proliferation notch activity inhibits the neuronal differentiation of the progeny of Müller glia-derived progenitors.

We recently conducted studies to test whether notch signaling in normal and growth factor-treated retinas influences glia-mediated neuroprotection and the de-differentiation of Müller glia [98]. We found that notch1 and components of the notch signaling pathway are

expressed by many Müller glia at low levels in normal, undamaged retinas. The expression of notch-related genes varies during early postnatal development, with higher expression in peripheral versus central regions of the retina and consistent with findings that Müller glia in peripheral regions of retina are more plastic or less mature [67]. Inhibition of notch signaling with DAPT prior to acute damage was protective to retinal interneurons, amacrine and bipolar cells, and projection neurons, ganglion cells [98]. In the absence of damage, notch is up-regulated in Müller glia treated with insulin and FGF2, which stimulates proliferation and de-differentiation. Inhibition of notch reduced levels of effectors of FGF2/MAPK signaling, p38 MAPK, and pCREB, in addition to reduced levels of effectors of notch signaling, in Müller glia. Importantly, inhibition of notch activity prevents FGF2-induced proliferation of Müller glia. Taken together, our data suggest that FGF2/MAPK-induced proliferation of Müller glia requires notch signaling to permit proliferation, and that low levels of notch signaling in normal Müller glia reduce the neuroprotective capacity of these glia. See Fig. 1 for a schematic summary of these findings.

The Microenvironment Provided by Damaged, Mature Retina May Inhibit Neuronal Differentiation

Since Müller glia-derived progenitors do not readily differentiate as neurons in mature chick retina, we sought to determine whether embryonic retinal progenitors were capable of neuronal differentiation when transplanted into mature retina. Accordingly, we transplanted dissociated embryonic retinal cells, from GFP-transgenic chick embryos, into the eyes of postnatal chickens where the retinas were damaged [99]. The environment provided by the postnatal eye did not support the proliferation of the embryo-derived cells, unlike the environment provided by culture conditions. Furthermore, the transplanted cells that migrated into host retinas did not differentiate and failed to express neuronal or glial markers whereas those that remained in the vitreous formed semi-laminated aggregates of differentiated cells with widespread expression of neuronal and glial markers. These findings suggest that the environment provided within the mature retina does not support the differentiation and proliferation of embryonic retinal progenitors.

During late stages of retinal development, signals are provided to "shut-down" neurogenesis; numbers of proliferating progenitors in the retina decrease dramatically during late stages of development. Close and colleagues [100] demonstrated that TGFβ signaling inhibits the proliferation of late-stage progenitor cells and/or immature Müller glia in the rodent retina. Evidence is provided that TGFβ2 is produced by inner-retinal neurons and acts at TGFβ-receptors that are expressed by late-stage progenitor cells and/or immature Müller glia. In P10 rat retina, inhibition of TGFβ signaling combined with exogenous EGF-stimulated proliferation of Müller glia that would otherwise be quiescent [100]. These results suggest that during late stages of retinal development levels of TGFβ become sufficiently elevated to "shut-down" mitosis of progenitors in the postnatal rat retina. It is possible that anti-neurogenic signals persist in the mature retina and these signals diminish neurogenesis from Müller glia-derived progenitors; TGFβ2 may be one of these signals.

Speculation

It remains uncertain why the capacity for retinal regeneration is diminished in birds and mammals compared with that of fish and amphibians. This raises the question whether there is an evolutionary advantage to retain the capacity to regenerate the retina. In short, perhaps, if vision is lost in one eye from a retinal injury, a one-eyed animal should still be able to find food, avoid predators, and propagate genetic materials, albeit with reduced efficacy. If vision is lost because of damage to both retinas, a no-eyed animal should encounter difficulty finding food, avoiding predators, and finding a mate to pass along genes. Furthermore, the chances of damaging the retina without damaging other ocular tissues seem slim. Ocular damage would require regeneration of extra-retinal ocular tissues for retinal regeneration to be meaningful. Collectively these arguments suggest that the pressure to evolve the ability to selectively regenerate the retina should not be significant. Since a selective advantage for retina-specific regeneration seems unlikely, we propose that the ability of retinal regeneration is merely an extension of the ability for generic organ regeneration. Alternatively, an "evolutionary bottle-neck" through primitive rodent-like mammals, which do not rely heavily upon vision for survival, may have excluded traits of retinal regeneration.

Clearly, there is no evolutionary selective advantage to maintain vision into old-age, beyond the years of propagation. Thus, progressive vision loss during old-age would not be selected against, and diseases such as glaucoma and age-related macular degeneration can remain prevalent among humans. It should be noted that there is currently no evidence to indicate that slow, progressive retinal degeneration is repaired by Müller glia-mediated neuronal regeneration. Nearly all reported examples of retinal regeneration occur following acute retinal damage. Thus, it remains uncertain whether functional regeneration from Müller glia can occur in adult retinas that are slowly degenerating.

Future Directions and Conclusions

For Müller glia-mediated retinal regeneration to become a viable strategy to treat human disease many important steps must be accomplished. These steps, at a minimum, should include: (1) Identification of the secreted factors that stimulate glia-mediated neurogenesis, inhibit glial differentiation while supporting the neuroprotective functions of the Müller glia. In the chick retina, the majority of Müller glia-derived progeny do not differentiate as neurons even when notch is inhibited [59], suggesting that mechanisms in addition to notch signaling suppress neuronal differentiation. Promising strategies have been applied to enhance neural versus glial differentiation. As discussed above, inhibition of the notch pathway is a promising therapeutic target; inhibition of notch promotes neural differentiation, but inhibits proliferation of glia-derived progenitors [59] and promotes neuronal survival [98]. Furthermore, inhibition of TGFβ-mediated signaling [100], combined with activation of FGF/MAPK [58, 60, 100] or Wnt signaling [101] may prove to be an effective combinatorial approach to enhancing glia-mediated regeneration. (2) Identification of the transcription factors that stimulate glia-mediated neurogenesis, inhibit glial differentiation, while supporting the neuroprotective functions of the Müller glia. In mature retinas, it seems likely that epigenetic silencing of progenitor and pro-neural factors, or activation of pro-glial factors such as Pax2, act to dampen the plasticity of Müller glia-derived cells. A potential strategy is to promote neural differentiation of Müller glia-derived cells is to over-express neurogenic factors [71]. Similarly, Ooto and colleagues have reported that misexpression of combinations of bHLH and homeodomain factors enhance glia-derived neurogenesis [56]. (3) Identification of mechanisms to stimulate the de-differentiation and proliferation of Müller glia in retinas that are degenerating slowly. The reactivity of Müller glia in retinas suffering from slow progressive degeneration likely influences the ability of these glia to become neurogenic. There is likely to be a balance between reactive gliosis, that occurs during progressive degeneration, and activation of glial de-differentiation and neuronal regeneration. (4) Identification of the mechanisms that coordinate the activity of the different types of retinal glia and how these interactions influence the neurogenic capacity of the Müller glia. The reactivity and functions of Müller glia is coordinated with those of microglia and astrocytes in the retina. The nature of the coordination among different types of glial cells is poorly understood. An understanding of the mechanisms that permit retinal regeneration in cold-blooded vertebrates, combined with an understanding of the mechanisms underlying the muted retinal regeneration in chicks and rodents, should act as guide-posts to develop new therapies to stimulate retinal regeneration in humans.

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