Title: Rebuilding the retina: prospects for Müller glial-mediated self-repair

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Abstract

Retinal degeneration is a leading cause of untreatable blindness in the industrialised world. It is typically irreversible and there are few curative treatments available. The use of stem cells to generate new retinal neurons for transplantation purposes has received significant interest in recent years and is beginning to move towards clinical trials. However, such approaches are likely to be most effective for relatively focal areas of repair. An intriguing complementary approach is endogenous self-repair. Retinal cells from the ciliary marginal zone (CMZ), retinal pigment epithelium (RPE) and Müller glial cells (MG) have all been shown to play a role in retinal repair, typically in lower vertebrates. Among them, MG have received renewed interest, due to their distribution throughout (centre to periphery) the neural retina and their potential to re-acquire a progenitor-like state following retinal injury with the ability to proliferate and generate new neurons. Triggering these innate self-repair mechanisms represents an exciting therapeutic option in treating retinal degeneration. However, these cells behave differently in mammalian and non-mammalian species, with a considerably restricted potential in mammals. In this short review, we look at some of the recent progress made in our understanding of the signalling pathways that underlie MG-mediated regeneration in lower vertebrates, and some of the challenges that have been revealed in our attempts to reactivate this process in the mammalian retina.

Key words

Müller glia, regeneration, stem cells, neurogenesis, endogenous repair, retinal degeneration, photoreceptors.

Introduction

Retinal degeneration caused by loss of the light sensitive photoreceptor cells in the retina is a leading cause of untreatable blindness and encompasses conditions including age-related macular degeneration, diabetic retinopathy and retinitis pigmentosa. While significant advances in gene therapy have raised hopes of a therapy for some forms of inherited blindness, the majority of currently available treatments aims to slow the progression of degeneration. As yet, there are few treatments available that can reverse the loss of vision once photoreceptor death has occurred. Recent advances in stem cell research have raised hopes for photoreceptor replacement by stem cell-derived photoreceptor and/or RPE transplantations [1-3]. We, and others [4-6], have previously demonstrated the successful restoration of rod function and vision by donor-derived photoreceptor transplantation in mouse models of retinal degeneration. Similar findings have been achieved using photoreceptor cells obtained from 3D cultures of pluripotential stem cells (PSCs) from both murine and human sources [7-9]. More recently, however, we, and others [10-12], have shown that when transplanted into models of slow or partial degeneration, where significant numbers of recipient photoreceptors still remain, the donor photoreceptors do not integrate within the host retina, but instead undergo an unusual process of material transfer. This involves the robust exchange of material (RNA and/or protein) between donor and host photoreceptors, rendering the host acceptor photoreceptor cells functional. This has renewed efforts into establishing restoration of function in advanced disease (where no host photoreceptors remain to accept material from donors) and there have been significant steps towards this goal. Nonetheless, conclusive proof of restoration of vision in advanced disease by transplantation of human PSC-derived photoreceptors has yet to be achieved.

Another attractive strategy is to stimulate endogenous self-repair mechanisms in patients earlier in the disease process, by recruiting cells within the retina with stem cell-like properties to generate new neurons following injury or disease. In this context, lower vertebrate models have provided useful insight. In contrast to mammals, in lower vertebrates such as fish and frogs, the eye continues to grow throughout their life and they have a remarkable capacity for repairing the retina after damage [13,14]. Endogenous repair of the retina following tissue damage can take place through different stem cell populations, according to the nature of the damage inflicted and regeneration required. The cellular sources with potential to mediate retinal repair include cells of the CMZ, RPE and the MG [15,16]. Various animal models are used to study retina regeneration, but fish, frogs, chicks and rodents dominate the field.

Below, we will briefly discuss the relative contributions to retinal repair made by different retinal cell populations, before focussing on the regenerative potential of MG.

Endogenous repair mechanisms

CMZ-mediated repair

The CMZ in zebrafish and frogs is well-defined and comprised of the ciliary body and the ciliary zone. The ciliary zone consists of different regions of cells arranged in a developmental gradient. Starting at the most peripheral border is the retinal stem cells, followed by proliferating progenitors and then, coming more centrally, the post-mitotic progenitors [17,18]. In lower vertebrates, such as amphibians and fish, new cells are continuously produced in a highly coordinated manner by the stem cells, which have the capability to self-renew [19,20]. Both neurons and glia are generated by the stem cells of the CMZ, a region of continuous

neurogenesis [21]. Recent lineage tracing performed in zebrafish provides new insights into asymmetric cell division at the periphery of CMZ (peripheral to central axis), showing that one daughter cell remains within the CMZ to maintain the retinal stem cell pool, while the second is pushed centrally and becomes a retinal progenitor cell [22].

In addition to adding new neurons to the margin of the eye throughout life, the CMZ of lower vertebrates can play a major role in the response to injury. In frogs (*Xenopus tropicalis*), the entire retina can be regenerated from the CMZ following retinectomy [23], while the capacity for CMZ-mediated regeneration of the goldfish neural retina following intraocular Ouabain injections has been established for many years [24]. Recent studies in Medaka fish have shown that the preference exhibited by CMZ retinal stem cells for asymmetric division is maintained during the regenerative process, as well as during normal tissue growth, enabling it to generate all retinal cell types whilst retaining the stem cell population [25]. Similar findings have been described for frogs (*Xenopus laevis*) [19,26]. In newts, a slightly different pattern of regeneration has been described; while the CMZ is still crucial for regeneration, the type of injury affects the extent of its involvement. For example, degeneration caused by optic nerve and/or optic blood vessel section is primarily repaired by the CMZ, but regeneration after retinectomy is principally mediated by the RPE (reviewed in [27]).

In birds, the eye is largely formed and functional at the time of hatching but a population of progenitor cells is retained within the CMZ that appears to continue to add new neurons to the retina until around 2-3 weeks post-hatching [28]. However, these are unable to mediate repair in response to injury and exhibited a very low proliferative potential following retinal damage by Kainic acid or stimulation by exogenous insulin [20,29].

The adult mammalian eye lacks a CMZ equivalent to that of lower vertebrates, but it retains the ciliary body, while the cells of the peripheral retina exhibit a more immature phenotype than

central retinal cells. For example, the intermediate filament nestin, which is often expressed in retinal progenitor cells (RPCs), is absent in the majority of mature MG but is retained by the MG located in the peripheral margin.

Whether the mammalian ciliary region contributes to the development of the neural retina has been the focus of some intriguing recent studies. Live imaging of embryonic murine retina expressing eGFP in the ciliary margin shows that the cells migrate laterally from this region into the neural retina, to the region where differentiated retinal ganglion cells (RGCs) reside, similar to that seen in lower vertebrates. In keeping with these findings, *Cyclin D2*, a cell-cycle regulator that has a proven role in cell proliferation in the lower vertebrate CMZ, is enriched in the ventral ciliary margin in mammals [30]. Herrera and colleagues [30] examined *Cyclin D2*$^{-/-}$ mice to test whether Cyclin D2+ cells within the ciliary margin are a source of retinal cells. They found that neurogenesis is diminished in *Cyclin D2* mutants and leads to a reduction in the number of RGCs, at least in the ventral retina. This implicates the ciliary margin as a site of Cyclin D2-depedent neurogenesis, at least for the production of RGCs. Strikingly, Cayouette and colleagues [31] identified another population of progenitors within the murine CMZ that is distinct from normal RPCs but also involved in mammalian retinogenesis. They demonstrate that *Msx1*-expressing progenitors are functionally and molecularly different to RPCs. *Msx1*-derived lineages contain both neural retina and non-neural ciliary epithelial progenies, but relatively few photoreceptors, compared with classical RPC lineages.

While access to healthy foetal and mature tissue has limited the number of studies examining the ciliary margin of the human retina, there are some indications of a ciliary margin-like region in cadaveric human eyes, which, following culture as explants in the presence of EGF, can go on to express SOX2, CHX10 and SHH [32]. Using human embryonic stem cells (hESCs), Sasai,

Eiraku and colleagues [33] have explored the potential for spontaneous self-organising retinal structures from 3D cultures of hESCs. Strikingly, they reported the generation of a ciliary marginal-like niche within hESC-derived optic cups, and that these continued to add new progenitor cells to the margin of the growing retinal structure in a manner analogous to that seen in lower vertebrates.

Whether progenitor-like cells within the mammalian CMZ can contribute to regeneration remains controversial. Reh and colleagues [34] observed that in *ptc*+/- (patched) adult mice, a population of dividing cells is retained at the retinal margin that bears an expression profile typical of the cells of the CMZ of lower vertebrates. Moreover, these can proliferate in response to injury and show some, albeit limited, potential to regenerate retinal neurons. Patched is a receptor for sonic hedgehog (Shh), indicating a role for the hedgehog pathway in regulating postembryonic ocular growth and the cessation of proliferation at the peripheral margin.

In 2000, two groups [35,36] independently reported that pigmented cells from the ciliary body of adult rodents exhibited proliferative potential when dissociated and cultured *in vitro* in the presence of growth factors. These were identified as retinal stem cells that could proliferate form neurospheres and be differentiated into MG, photoreceptors and bipolar neurons. Since then, however, other studies have found the potential of these stem-like cells to be more limited, exhibiting incomplete differentiation, and suggest that these are de-differentiated pigmented cells that act like progenitor cells, rather than a truly multipotent stem cell [37,38]. Similarly, a recent study confirmed that while cells isolated from the ciliary body of mouse retina can produce neurospheres and exhibit a proliferative ratio similar to neural stem cells, they are unable to fully differentiate *in vitro* into retinal cell types or form retinal organoids [39], as embryonic stem cells can.

RPE-mediated repair

The RPE is situated between the neural retina and choroid and plays a vital role both in supporting visual function and in providing structural support to the retina. RPE cells are quiescent under normal conditions, but when the retina is damaged or undergoes degeneration they can, in some cases, begin to proliferate followed by transformation. In many amphibians, like the frog, retinal injury triggers the RPE cells to de-differentiate, proliferate and finally give rise to retinal precursors that can contribute to partial regeneration. As noted above, certain types of injury, like retinectomy, can induce the RPE of urodele amphibians, like newts, to regenerate the entire retina following retinectomy, although the CMZ also plays a role [40]. Trans-differentiation of the RPE can also regenerate the neural retina in post-metamorphic frogs (*Xenopus laevis*) following retinectomy [41]. However, this retinal regeneration process differs greatly between newts and frogs. Surgical removal of the newt retina results in the proliferation of RPE cells, which can regenerate both RPE and neural retina. In contrast, the RPE cells of frogs (*Xenopus laevis*) do not transdifferentiate at their original site. Instead, when the retina is removed, but the retinal vascular membrane (RVM) is retained, a subpopulation of RPE cells detach from Bruch's membrane, migrate to the RVM, and proliferate to form a new neuroepithelium layer, which subsequently generates all retinal cell types neurons and glial cells. RPE cells that remain in their original location proliferate to renew the RPE itself [26,40,41]. Surprisingly, this ability of the RPE to transdifferentiate seen in amphibians is not observed in fish, which appear to rely more on the CMZ and MG (discussed above and below). The mammalian RPE also lacks the potential to proliferate following injury, although in vitro studies show that these cells can be induced to proliferate and self-renew and can be differentiated into a variety of cell types [42], similar to the pigmented cells of the ciliary body [37].

Müller glial-mediated repair

Müller cells are the major glial cells of the retina and span its entire apico-basal extent. Their main function is to maintain retinal structure and homeostasis [43,44]. MG are one of the last cells to be born during retinal development. Indeed, they share many similarities with late retinal progenitor cells, not only in terms of physical appearance but also, as shown by transcriptomic analyses, their gene expression profile [45,46]. MG thus carry out specialized glial functions but maintain a molecular signature of late stage progenitor cells [47].

As we will discuss below, they can, in some species, return to this progenitor-like state and proliferate to generate new retinal neurons upon injury. Due to their being located throughout the apico-basal extent of the retina, together with extensive lateral branching within the different retinal layers, they are ideally placed to sense retinal damage and hence contribute to a swift response to any change of the retinal microenvironment [43,48]. As MG survive most retinal injuries, they are also ideally placed for retinal regeneration approaches. However, they are also critically involved in other aspects of the injury response: In vertebrates, retinal degeneration typically results in MG entering into a process of reactive gliosis. This involves marked cellular and molecular changes, which can have both supportive and detrimental effects on neuronal function and survival. Immediately after retinal damage, MG release antioxidants and neurotrophic factors, which help to restrict tissue damage. A classic hallmark of gliosis is a marked increase in the expression of Glial Fibrillary Acidic Protein (GFAP) by MG. Indeed, upregulation of this protein often serves as a sign of retinal stress or injury [49] and is thought to contribute to MG hypertrophy and increased stiffness [50]. Together, these changes can contribute to the formation of a glial scar, which is thought to protect the remaining neurons by sealing off the damaged region following retinal injury. Gliosis can trigger the stimulation of extracellular

signal-regulated kinases (ERKs) [48,51]. During degeneration and injury, MG are also stimulated by immune cells and as a result may express TNF-α, IL, interferon, and ICAM-1 enzymes [48,50]. Collectively, prolonged reactive gliosis can lead to proliferative disorders and detrimental scarring that might reduce, cause or exacerbate neuronal degeneration.

Comparative studies of mouse models with different forms of inherited retinal degeneration have indicated that reactive gliosis is highly variable, depending on disease type, even though all exhibit the same final pathway - photoreceptor death [52]. Similarly, in amphibians and fish, the MG respond differently depending upon the type, extent and severity of injury. For example, there is a correlation between the amount of MG cell proliferation and the extent of photoreceptor cell death [53,54], while the MG response is often smaller and slower to initiate after the loss of inner neurons, compared to photoreceptor cell death [55]. Thus, while re-entry into the cell cycle and proliferation can occur, the extent of repair depends on the injury model/paradigm [56].

It is also important to appreciate that a wide variety of injury models and paradigms has been used to study the regenerative potential of otherwise quiescent MG. These include needle poke/mechanical injury, laser ablation, light exposure, surgical removal of the retinal tissue, treatment with Metronidazole (MTZ), injections of Ouabain (Na/K-ATPase inhibitor), and N-Methyl-D-aspartic (NMDA) acid [26,57]. The MG response and extent of gliosis can differ markedly between different types of injury. Since it is becoming apparent that the differences between species in their response to retinal injury, and the ability of their MG to transition beyond the gliotic response to a proliferative one, appears to be key to MG-mediated endogenous repair, such factors must be borne in mind when considering the apparent

differences in MG response between studies and the potential for inducing MG-mediated repair as a therapeutic strategy.

Müller glial-mediated repair in fish and amphibians

In adult fish, small subpopulations of Müller cells are known to generate rod cell precursors at low frequency throughout the life of the fish. In the zebrafish, these cells can also de-differentiate following retinal injury to stem/progenitor cell-like state and divide by asymmetric cell division to both renew themselves and generate a daughter cell that is subjected to consecutive rounds of cell division to reinstate the lost cells. In the uninjured retina, the zebrafish MG typically produces only rod cells but following retinal injury, they possess the potential to generate all types of retinal neurons [58]. In contrast, Medaka MG exhibit a more restricted regenerative potential, generating only photoreceptors following retinal needle poke injury. In contrast to the zebrafish MG, Medaka MG do not divide asymmetrically after cell-cycle re-entry or produce neurogenic clusters [59].

The MG of amphibians are typically quiescent in the uninjured state. However, they too can be induced to proliferate in response to injury. In frogs (*Xenopus laevis*), following either needle poke injury or nitroreductase-mediated photoreceptor ablation, the MG re-enter the cell cycle and generate new photoreceptors. This process is age-dependent, and is to be more effective in pre-metamorphic tadpoles and adult frogs, in comparison to young tadpole stages, in at least two different injury models [57]. This is in marked contrast with MG-mediated regeneration in the mammalian retina, which typically reduces with age (see later).

Although we have yet to establish a complete picture of the extrinsic signals that drive the MG regenerative response to injury in fish, a number of advances have been made. The dying cells represent an obvious potential source of diffusible molecules. Following injury in the zebrafish retina, the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) is produced by dying retinal neurons and has been shown to trigger MG dedifferentiation and proliferation, most likely acting via *Stat3* and *Ascl1a* [60]. Growth factors play an essential role during eye development, variously influencing proliferation, migration and differentiation. Since many of these growth factors are also released following tissue damage, a number of studies have examined the effects of exogenous administration (reviewed in [1]). In zebrafish, introduction of growth factors such as HB-EGF, insulin growth factor-1 (IGF-1), and fibroblast growth factor 2 (FGF2) stimulates MG de-differentiation and proliferation, all acting through the MAPK pathway to upregulate the expression of *Stat3* and *Ascl1a,* amongst others [61]. Conversely, in the MNU-induced injury model of adult zebrafish, transforming growth factor beta (TGFβ) has a negative effect on regeneration and inhibition of this signalling pathway leads to increased proliferation of MG [62], again via *Ascl1a,* as well as *c-myc* and *Pax6* [63]. MG are themselves one source of the growth factors and cytokines that regulate their own regenerative response in an autocrine/paracrine manner [61,64]. These factors appear to exhibit extensive crosstalk and converge through intracellular cellular signalling pathways to promote MG dedifferentiation and proliferation [61,64].

A number of intracellular signalling pathways have been identified as playing important roles in MG-mediated regeneration in lower vertebrate models. These include the MAPK–Erk, PI3K/Akt, and Jak–Stat signaling pathways [22,65], which typically converge on *Ascl1a* and *Stat3* gene regulation [61,64], while recent studies have identified species-specific roles for transcription factors including *Sox2* and *Atoh7*. Injury-dependent expression of the proneural gene *Ascl1a* is

restricted to de-differentiated MG and MG-derived progenitor cells [66]. In zebrafish, *Ascl1a* is rapidly induced in MG after injury and regulates MG dedifferentiation and regeneration through *Lin28/let-7*. *Lin28* is an RNA-binding protein that is important in stem cell renewal, while *let-7* miRNAs are small regulatory RNAs associated with cellular differentiation [67]. They each regulate the other's expression in a complementary manner, such that as *Lin28* increases, so *let-7* decreases. *Ascl1a* is crucial for *Lin28* expression and in turn *Lin28* leads to both *let-7* microRNA suppression and further upregulation of *Ascl1a* [66]. While the role of *Ascl1a* in MG-mediated regeneration is well described, the role of *Stat3* requires further investigation, since Stat3 protein is increased in both quiescent and proliferating MG following retinal injury [66,68] and the retinal cells that express activated pStat3 remain largely uncharacterized. Collectively, in lower vertebrate species that exhibit pro-regenerative MG responses, like the zebrafish, the *Ascl1a/Lin28/let-7* pathway appears central.

As noted above, the MG of Medaka and zebrafish exhibit very different regenerative capabilities. Indeed, recent studies into the transcriptional pathways regulating these responses have revealed striking differences [59,69,70]. *Sox2*, an important transcription factor in retinal development, continues to be expressed at low levels in MG and amacrine cells in the adult retina of both zebrafish and Medaka fish [59,69]. After light-induced damage, *Sox2* expression is significantly increased in the zebrafish MG as they undergo proliferation. Gorsuch and colleagues [69] further demonstrated that *Sox2* is necessary, and ectopic expression of *Sox2* is sufficient, to induce zebrafish MG proliferation. This appears to be mediated through actions on the *Ascl1a*/*Lin28*/*let-7* pathway; indeed *Sox2* expression is required for maximal expression of *Ascl1a* by zebrafish MG, most likely through the induction of *Lin28a*-dependent repression of *let-7* miRNA biogenesis and the amplification of MG-derived progenitor cells [69]. Conversely, *Sox2* expression *decreases* in Medaka MG following injury. Hence the loss of Sox2

after injury may limit MG-mediated retinal regeneration in the Medaka fish. Indeed, ectopic expression of *Sox2* in Medaka MG enabled them to generate a variety of retinal cell types, in addition to photoreceptors [59].

Atoh7 is typically considered to be a transcription factor that channels proliferating cells into differentiation. However, in studies of the Medaka fish, *Atoh7* was found to be expressed in proliferating progenitor cells of the CMZ and also in proliferating MG cells after injury [70]. Of several transcription factors examined, only over expression of *Atoh7* was able to induce MG proliferation, causing them to re-enter the cell cycle, proliferate and generate new neurons, preferentially RGCs, even in the absence of injury [70]. *Ascl1a* was amongst those transcription factors that failed to yield a regenerative response, in stark contrast to its actions on zebrafish MG. Further, the authors propose that *Atoh7* acts via Notch signalling; Notch activation induced by introduction of the Notch intracellular signalling domain (NICD) was sufficient to replicate the response induced by *Atoh7* [70].

This requirement for increased Notch signalling in Medaka MG regeneration highlights further differences between pro-regenerative species, such as the zebrafish, and non-regenerative species, including mammals and, possibly, Medaka fish. During retinal development, the levels of Notch in RPCs determines whether they will be retained within the cell cycle or exit and differentiate; a decrease in Notch signalling is necessary for proper differentiation (See [71], for further discussion). However, in the later stages of development the same pathway, if sustained at high levels, stimulates glial differentiation [72,73]. In the uninjured adult zebrafish retina, high levels of Notch signalling serve to maintain the quiescence of MG. Following injury, MG downregulate Notch signalling and upregulate factors such as *Ascl1a* and *Sox2*,

allowing them to re-enter the cell cycle and proliferate. Similarly, inhibition of Notch signalling alone can induce MG cell proliferation even in the absence of injury [74]. Consistent with this, in the uninjured zebrafish *Ascl1a/Lin28*-mediated MG proliferation is potentiated by concomitant inhibition of Notch signalling [75]. Conversely, in the adult retina of non-regenerative species, Notch is low in quiescent MG. If Notch increases endogenously or through exogenous intervention, MG can in some circumstances re-enter the cell cycle and proliferate at low levels. However, sustained Notch results in reactive gliosis and the release of neurotoxins by MG, furthering retinal damage [61].

A key downstream target of the *Ascl1a* pathway is Wnt signalling, which acts via the canonical pathway to promote MG proliferation. In the normal retina, MG are known to express high levels of *Dkks*, which are secreted to sequester Wnt ligands and intracellular components of the Wnt-signalling pathway [46]. Following an acute retinal injury (needle poke) and induction of *Ascl1a* signalling, the expression of *Dkk* is suppressed, while *Wnt4a* expression is induced [63]. Interestingly, even in the uninjured zebrafish retina, the inhibition of glycogen synthase kinase-3β (GSK-3β) (which allows stabilisation of β-catenin and acts to stimulate Wnt signalling), is sufficient to trigger the de-differentiation of MGs and generation of progenitor cells that are competent to produce all retinal cell types [63]. These studies highlight both the complexity and the interplay between the different pathways, with Wnt both stimulating the expression of *Ascl1a* and *Ascl1a* acting to alter Wnt signalling.

Compared to Notch, Wnt, and FGF signalling, the role of the equally developmentally important Shh signalling pathway in MG-mediated regeneration has been relatively underexplored. In the damaged adult zebrafish retina, Sun and colleagues [76] showed that pharmacological inhibition and indirect activation of the endogenous Shh signalling pathway led to reduced and increased rates of MG proliferation, respectively. This was developed further

by Thummel and colleagues [77], who showed that activation of Shh signalling during the early stages of retinal regeneration following light injury, using intraocular injections of recombinant human SHH, resulted in increased Müller cell gliosis, proliferation, and neuroprotection of damaged retinal neurons. Sustained activation of Shh resulted in a greater number of differentiated amacrine and ganglion cells in the fully regenerated retina. Conversely, pharmacological inhibition of Shh signalling resulted in decreased MG proliferation and reduced numbers of regenerated amacrine and ganglion cells. The authors propose pleiotropic roles for Shh in proliferation and differentiation during adult zebrafish retinal regeneration. In an impressive study by Kaur and colleagues [78], the authors were able to identify a complex interplay of Shh/Notch signalling components, transcription factors (*Ascl1a, Zic2b, Foxn4*, and *Insm1a*), the matrix metalloproteinase *Mmp9*, as well as *Lin28a* and *let-7*. *Let-7* was found to tightly coordinate the expression of Shh signalling components in MG-derived progenitor cells and Shh-signalling dependent negative regulation of *Mmp9* in turn regulates Shh levels, as well as genes essential for regeneration including *Ascl1a, Lin28 and Foxn4*.

Müller glial-mediated repair in postnatal chick

In general, adult birds cannot regenerate their retinae following injury. However, a number of studies have shown that chicks do exhibit some regenerative potential, at least for a couple of weeks post-hatching. Fischer and colleagues [79] demonstrated that following damage induced by neurotoxic agents such as N-methyl-D-aspartic acid (NMDA), the chick MG respond with proliferation and generate a limited number of new neurons. This re-entry into the cell cycle is associated with the upregulation of the expression of transcription factors such as *Ascl1, Chx10* and *Pax6* and appears to be stimulated by Notch signalling [80,81],consistent with the role of Notch in Medaka fish. Indeed, Notch has been described to have two distinct roles in avian retinal

regeneration. After neurotoxic damage, Notch signalling components are upregulated in the proliferating MG and activation of the Notch pathway is necessary for the de-differentiation process; inhibiting the Notch pathway soon after injury reduces both the number of MG re-entering the cell cycle as well as the overall proliferative response. Conversely, inhibiting Notch signalling later in the regeneration process, after the MG have already de-differentiated, leads to a significant increase in the extent of neuronal differentiation [80,81].

Although we lack a complete picture of the extracellular signals that mediate avian MG proliferation, it is likely to include many of the same players identified for lower vertebrates and include growth factors. For example, administration of growth factors such as FGF2, insulin and insulin-like growth factors can induce de-differentiation and proliferation of MG, not only after injury but even in the uninjured retina [82]. These growth factors appear to act on a variety of downstream pathways, including Notch [81], the FGF receptor, mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (Erk) signaling pathways [81,83]. Following NMDA-induced retinal injury in chick retina, Hedgehog signaling stimulates the de-differentiation of MG and proliferation of MG-derived progenitor cells. Shh is normally present in the axons of retinal ganglion cells but becomes associated with MG and MG-derived progenitor cells following retinal damage. Activation of Hedgehog signaling with recombinant human SHH or smoothened agonist (SAG) increased the expression of several genes including *Ptch1, Gli 1-3, Hes1, Hes5, Pax6*, *Klf4* (transcription factor), and *cFos* (MAPK effector) and stimulated the formation of MG progenitor cells, but only in the injured retina and not in the uninjured [84].

Notably, the effect of Hedgehog signalling on MG proliferation is potentiated by FGF2/MAPK signaling, as is Wnt/β-catenin, the activation of which is also necessary for formation of MG-

derived progenitors in chick [85]. Todd and Fischer propose that FGF2/MAPK signalling recruits Hedgehog signalling into the network that drives the formation of proliferating MG-derived progenitor cells [84].

Similarly, both CNTF and FGF2 stimulate Jak/Stat-signaling [86] and the production of MG-derived progenitor cells is prevented following inhibition of components of the Jak/Stat pathway, including glycoprotein 130, Jak2 and Stat3. Notably, administration of FGF2 and CNTF together appeared sufficient to increase proliferation of MG-derived progenitor cells, even in the undamaged retina. Zelinka and colleagues [87] investigated the role of Mammalian target of rapamycin (mTOR) signaling in NMDA-induced retinal injury in post-hatched chick. In this context, components of the mTOR signaling pathway are upregulated in MG when MG-derived progenitor cells are generated. In injured retinas, the actions of Wnt, Hedgehog and glucocorticoid signaling in promoting MG de-differentiation and proliferation were all effectively prevented following the inhibition of mTOR, although mTOR signalling alone is insufficient to induce MG to enter cell cycle and generate progenitor cells.

Very recent findings reported in BioRxiv indicate a role for Nuclear Factor kappa B (NF-κB), a molecule that is important in managing inflammation following injury and also governs cell survival, proliferation and differentiation. In chick retina, components of the NF-κB pathway (*Nfkbia, Nfkbib, Nfkbiz, Chuk*) and TNF-related ligands (which stimulates NF-κB signalling) are expressed in MG and are dynamically regulated after neuronal damage or treatment with growth factors, including FGF2. Inhibition of NF-κB increases, while its activation suppresses, the formation of proliferating MG-derived progenitors, in a process that appears to rely upon the presence of reactive microglia [88]. The authors propose that NF-

κB-signalling is an important signalling "hub" that suppresses the reprogramming of MG into progenitor cells and acts to coordinate signals from the reactive microglia.

Müller glial-mediated repair in mammals

Compared to fish, amphibians and even postnatal chicks, the regenerative potential of mammalian MG is remarkably limited. Rather than initiating the process of de-differentiation and proliferation exhibited by the MG of lower vertebrates, mammalian MG typically enter reactive gliosis after retinal injury, leading to changes in the morphology of the MG (hypertrophy, increased stiffness) and the deposition of inhibitory ECM molecules, such as chondroitin sulphate proteoglycans (CSPGs). Collectively, this can lead to the formation of glial scars and, if extensive, retinal detachment.

While the default response is one of gliosis, changes within the MG during this process still point to attempts to enter a more regenerative state. During reactive gliosis, MG downregulate the expression of the tumour suppressor protein, p27Kip1. This appears to permit a limited re-entry into the cell cycle during the first 24 hours after acute retinal injury. However, this is shortly followed by the upregulation of genes typical of gliosis and a downregulation of the proliferative gene, *Cyclin D3*. Notably, *p27Kip1* knockout mice present with a constitutive form of reactive gliosis and retinal dysplasia [89]. Together, these findings indicate that *p27Kip1* may be important for the initiation of the mammalian MG proliferative response, but other pathways are important for the initiation/prevention of gliosis.

One of the first studies to show that the murine retina may retain some regenerative potential was that by Karl, Reh and colleagues. By destroying amacrine and retinal ganglion cells

using intraocular injections of NMDA, and combining this with administration of the growth factors EGF, FGF1 and insulin, they were able to elicit a limited proliferation of MG, which appeared to generate a small number of amacrine cells [90]. Further similar reports demonstrated that rat MG can apparently show a limited proliferative response to injury, as assessed by the incorporation of BrdU [91,92], although others have provided conflicting reports, instead reporting an absence of MG proliferation and neurogenesis in the mouse retina after injury [93,94]. Whether this reflects a specific species difference requires further direct comparative analysis.

In an effort to increase MG proliferation a number of different cellular pathways previously identified to play a role in lower vertebrate regeneration have been tested. These include modifications, either *in vivo* or *ex vivo*, of Wnt [95-97], *Ascl* [98,99], EGF [90] (see Figure 1), Shh [100] and BDNF [101] pathways, some of which are consider further below.

[Figure 1 near here]

As noted above, the *Ascl1a/Lin28/Let-7* pathway appears key to the MG regenerative response in lower vertebrates. Importantly, *Ascl1* is not upregulated in mammalian MG following retinal injury [90] leading to the hypothesis that activating this pathway might unlock their regenerative potential. Reh and colleagues [98] over-expressed *Ascl1* in mice and showed that, following NMDA-induced inner retinal cell death, the transduced MG could indeed re-enter the cell cycle and generate new neurons, including bipolar cells, amacrine cells and, to a limited extent, new photoreceptors. However, this response was only seen when combined with injury and then only in relatively young mice; the MG of adult mice did not proliferate, with or without injury.

Indeed, age and the extent of injury both appear crucial in determining the extent of MG reprogramming to the proliferative state. Ooto and colleagues [91] reported that very few MG de-differentiate following retinal injury in adult mice, in contrast with young mice. Similarly, and consistent with studies in the chick, in their study injecting EGF, FGF1 and insulin, Karl and Reh [14,90] showed that this could induce MG de-differentiation following inner retinal injury by NMDA neurotoxicity, but only in explants derived from young mice. This reduction in regenerative capacity exhibited by older animals led Reh and colleagues [99] to consider whether it might be due to epigenetic factors and decreased accessibility of the necessary genes in adult chromatin. To address this, they combined administration of trichostatin-A (TSA), a histone deacetylase inhibitor, with overexpression of *Ascl1*. Encouragingly, this did improve MG proliferation and induced a limited neurogenic response even in adult mice following NMDA induced retinal injury [99].

Despite the known importance of the microRNA, *let-7*, in *Ascl1/Lin28/let-7* mediated MG-reprogramming, relatively little is known about miRNA expression in MG and how this relates to their neurogenic potential (or lack of). Reh and colleagues [102] sought to address this by comparing miRNA expression in both RPCs and MG. Three miRNAs of significance were identified; *miR-25*, *miR-124* and *let-7*. Specifically, over-expression of *miR-25* and *miR-124* and antagonism of *let-7* induced *Ascl1* expression and conversion of just under half of mature MG into a neuronal/RPC phenotype in culture, suggesting that manipulations in miRNAs may provide a new tool to reprogramme MG for retinal regeneration.

Wnt/β-catenin signaling has received significant recent attention in the attempt to reactivate mammalian MG. The pathway has been shown to be upregulated and involved in a limited MG proliferative response after laser induced retinal injury in mice [96]. *Wnt3a* has been shown to

stimulate MG proliferation *in vitro* and has been introduced by intravitreal injections into adult mice following NMDA-induced inner retinal damage, leading to an increase in the number of BrdU+ cells. Consistent with these results, inhibition of GSK-3β yielded a similar effect. Introduction of *Wnt3a* into the murine model of rapid retinal degeneration, *Rd1*, had a similarly positive effect, increasing the number of proliferating MG [95]. Strikingly, MG were shown to possess the potential to proliferate in adult mice even without any form of retinal damage, driven only by the activation of Wnt signalling. Chen and colleagues [97] showed that gene transfer of β-*catenin* or deletion of *GSK-3*β is sufficient for inducing proliferation of MG. β-*catenin* is known to bind *Lin28* promoters, and removal of *Lin28* prevented β-catenin-induced MG proliferation, highlighting the interplay of the Wnt and *Lin28/Let7* pathways in the proliferation of MG, even in the uninjured retina [97]. In recently published data, the same group applied a similar approach to a model of retinal dysfunction, the $Gnat1^{rd17}$:$Gnat2^{cpfl3}$ mouse, which lacks cone and rod function (but is non-degenerative). As exemplified in the studies described elsewhere in this review, the majority of the new neurons generated by MG-mediated proliferation typically appear to adopt an inner retinal identity, and the generation of new photoreceptors is even more rare. To address this, Chen and colleagues [103] first introduced virus-mediated gene transfer of β-*catenin*, and then also introduced viruses carrying the photoreceptor transcription factors *Otx2, Nrl, Crx*, which are important for rod photoreceptor commitment, to drive the differentiation of any newly generated cells towards a rod fate, as well as *Gnat1*, the gene missing in the host rod photoreceptors (see Figure 2). This complex experiment required the transduction of individual MG by each of 7 different viral vectors. Nonetheless, the authors were able to show MG-mediated proliferation and rescue of visual function.

[Figure 2 near here]

A recent addition to the repertoire of signalling pathways known to be involved in MG-mediated regeneration is that of Hippo and its terminal effector proteins, YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif). The Hippo-YAP pathway plays a vital role in the control of organ size during development, and coordinates proliferation in a cell-cell contact manner, with Hippo suppressing YAP and cell proliferation. Perron and colleagues [104] recently demonstrated that, following a variety of injury paradigms including needle poke or selective rod photoreceptor ablation, YAP is upregulated and is necessary for MG cell-cycle re-entry and proliferation in the frog *(Xenopus laevis)*. Strikingly, in mice, which do not normally regenerate in response to injury, targeted over-expression of YAP in MG was sufficient to induce their reprogramming into highly proliferative cells. They also found that the mitogenic functions of YAP and its ability to drive MG out of their quiescence rely on an interplay with the EGF receptor. In a parallel study, Poche and colleagues [105] showed that MG-specific deletion of Hippo pathway components, or transgenic expression of a Hippo non-responsive form of YAP (YAP5SA) resulted in upregulation of *Cyclin D1* and reprogramming to a proliferative, progenitor-like state. It is not yet clear what the neurogenic potential of these proliferative MG is and lineage tracing studies are required to determine whether bona fide retinal regeneration occurs. It will also be of significant interest to establish whether there is crosstalk between the Hippo and the Wnt pathways in MG-mediated retinal regeneration, as has been described for other systems [106-108].

To date, most experimental approaches to induce MG reprogramming have either introduced recombinant protein (e.g. FGF, EGF) or have used viral vectors to overexpress a particular component of one of the key signalling pathways (*Ascl1, β-catenin*). A recent and novel alternative has been the use of extracellular vesicles (EVs). EVs are nanoscale vesicles that can carry an array of proteins and RNA, including mRNAs and miRNAs, and are increasingly being

recognised as a key player in intercellular signalling. Almost all cell types can generate and release EVs in the extracellular space and these are taken up by binding specific cell surface receptors on recipient cells. The contents of the EVs are specific to the cells of origin (but are not a simple sampling of cytoplasmic contents). Farber and colleagues [109] have investigated whether stem cell derived-EVs can stimulate a stem cell-like profile in acceptor cells, specifically MG; when cells from a human MG cell line (MG were exposed to embryonic stem cell-derived EVs (ESEVs), they increased the expression of some pluripotency markers, including *Oct4*, as well as a downregulation in the expression of some extracellular matrix molecules and inhibitory scar components (aggrecan, Gfap). Whether ESEVs can mediate a similar effect *in vivo* remains to be determined.

Gliosis and Müller glial-mediated repair

A key issue that remains poorly understood is how the nature and extent of injury incurred affects the regenerative capacity of that retina. To our knowledge, there are few, if any, comparative studies examining the regenerative response of MG to different injury types in the same species. The majority of studies, not unreasonably, compare the effects of manipulating signalling pathways on a single injury type and within a single animal model. Perhaps more surprising is the frequent omission of a wildtype, uninjured control, where MG are in a completely non-reactive state. Each injury model varies in the extent of damage and the cell types affected and the magnitude of the MG-mediated response: for example, higher numbers of MG were reported to re-enter the cell cycle (as assessed by BrdU incorporation) in adult rats following widespread damage caused by MNU injection [92], compared to more modest levels of BrdU incorporation reported following the introduction of milder injuries using NMDA [91].

Moreover, how the MG respond to injury, with respect to reactive gliosis, is also likely to play a crucial role in determining their ability to progress to a regenerative state. Indeed, it is becoming increasingly apparent that pathways that might typically yield MG proliferation in the lower vertebrate instead stimulate glial reactivity when activated in the mammalian retina; CNTF/Jak/Stat-signalling is one such example [110]. It is well established that the extent of reactive gliosis can vary markedly between injury models and even within injury categories. For example, we have previously shown that different forms of inherited retinal degeneration, which all present with progressive photoreceptor loss, can elicit very different gliotic responses in the MG population [52]. We also know from studies of lower vertebrates that the repair mechanisms activated (MG-mediated, RPE-mediated, CMZ-mediated) depend on the nature of the initiating injury. Despite this, there are very few studies looking at MG-mediated regeneration in models of progressive degeneration. It would appear crucial to consider the sorts of injury models used and how these relate to the clinical disorders we seek to treat. Moreover, we may need to delve deeper into the mechanisms that initiate and maintain gliosis and whether these can be modulated to allow the regenerative pathway to proceed with greater efficacy.

Lineage tracing and material transfer

Collectively, the studies above show exciting promise in the potential for endogenous MG-mediated repair and the progress made in the past decade certainly warrants our continued enthusiasm. However, there are also some technical issues that must be taken into consideration as we seek to move the field further forward. As noted above, the vast majority of studies have relied upon the incorporation by MG of BrdU (or a recent derivative, EdU) during S-phase as a marker of proliferation. While incorporation of thymidine analogous strongly indicates that MG are capable of entering the cell cycle, it does not provide direct evidence that the labelled

neurons are derived from MG. While genetic lineage tracing is routinely used in the retinal development field, and even in lower vertebrate MG regeneration studies, it has featured in only a handful of mammalian MG regeneration studies. Ueki, Reh and colleagues [98] used a *Glast*-CreERT2 mouse line to drive Müller cell-specific expression of GFP in conjunction with overexpression of *Ascl1*. As described above, when combined with injury at a young age, *Ascl1* expression yielded GFP-labelled bipolars, amacrines and a few rods, which appeared correctly integrated within the retina. Conversely, no GFP+ neurons were detected when the injury was induced at adult stages. In the studies by Löffler et al., [111] and Yao et al., [103] most of the tracing utilised BrdU incorporation, but a limited number of genetic lineage tracing experiments were performed using retinae from *GFAP-Cre;Rosa-yellow* and *GFAP-Cre;Rosa-tdTomato* lines, respectively, to verify the findings from BrdU incorporation. The addition of lineage tracing is an important step towards our understanding of the neurogenic potential of MG-mediated regeneration and should be considered a central experimental tool going forward.

That said, even lineage tracing is not without its limitations. We, and others, have shown that transplanted donor photoreceptors can exchange material, including fluorescent reporters such as GFP and TdTomato, with host photoreceptors by a process we have termed material transfer [10,11]. The cellular mechanisms by which this process occurs remain to be elucidated but it demonstrates the potential for a wide array of proteins or their mRNA to be passed between photoreceptors and possibly between other retinal cell types (see [112,113] for discussion). Whether MG can engage in similar mechanisms of intercellular exchange, which could confound the interpretation of lineage tracing experiments, remains an important question to be addressed.

Conclusions

The eye continues to be at the forefront of novel therapeutic approaches for the treatment of neural degeneration, with the past decade seeing the first clinical trials in both ocular gene and cell therapy. While endogenous repair is still at the preclinical stage, it too has seen remarkable progress over the same period. Many of the intrinsic and extrinsic signals that regulate MG-mediated regeneration in lower vertebrates have been identified, and modulation of these same pathways in the mammalian retina is yielding improved levels of MG-mediated regeneration. However, there are many challenges remaining. The extent of regeneration is still very limited and typically only studied in models of acute injury. Definitive proof of MG-mediated regeneration and restoration of vision in models of progressive neurodegeneration, as seen in most forms of blindness, has yet to be achieved. Indeed, the challenges presented by progressive degeneration should not be underestimated. We require a much greater understanding of the differences between species in what governs the transition of MG between gliotic and regenerative states, and how the extent of degeneration affects the ability of MG to respond in a regenerative capacity. Finally, exploration of the full potential of mammalian MG-mediated regeneration will require careful use of lineage tracing, and cautious interpretation in the light of recent findings from the transplantation field regarding material transfer. Despite these challenges, the future looks exciting with endogenous MG-mediated repair being a realistic addition to the approaches we seek to apply in the treatment of retinal degenerations.

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Figure captions

Figure 1. MG-derived proliferation and generation of retinal neurons in an NMDA-injured mammalian retina. Schematic representation summarising finding from [90,95,98,99]. Briefly, application of *Wnt3a* stimulates a limited MG proliferation response (Osakada et al., 2007) [95], while a combination of growth factors induces proliferation and the generation of a small number of amacrine cells (Karl et al., 2008) [90]. Overexpression of *Ascl1-GFP* by gene transfer in young mice leads to the generation of amacrine, bipolar and photoreceptor cells (Ueki et al., 2015) [98]. By combining the Histone Deacetylase inhibitor Trichostatin-A with gene transfer of *Ascl1*, it is possible to induce MG proliferation even in adult mice. These went on to generate new amacrine and bipolar cells (Jorstad et al., 2017) [99].

Figure 2. MG-derived rod photoreceptors following gene transfer in an uninjured mammalian retina. Schematic representation summarises findings from Yao et al., 2016 [97] and 2018 [103]. In brief, in the absence of injury, gene transfer of β-catenin can still trigger *Wnt-Lin28-let7* signalling and induce MG de-differentiation and proliferation. Subsequent gene transfer of rod transcription was used with aim of directing newly generated cells towards a rod fate.

Figure 1

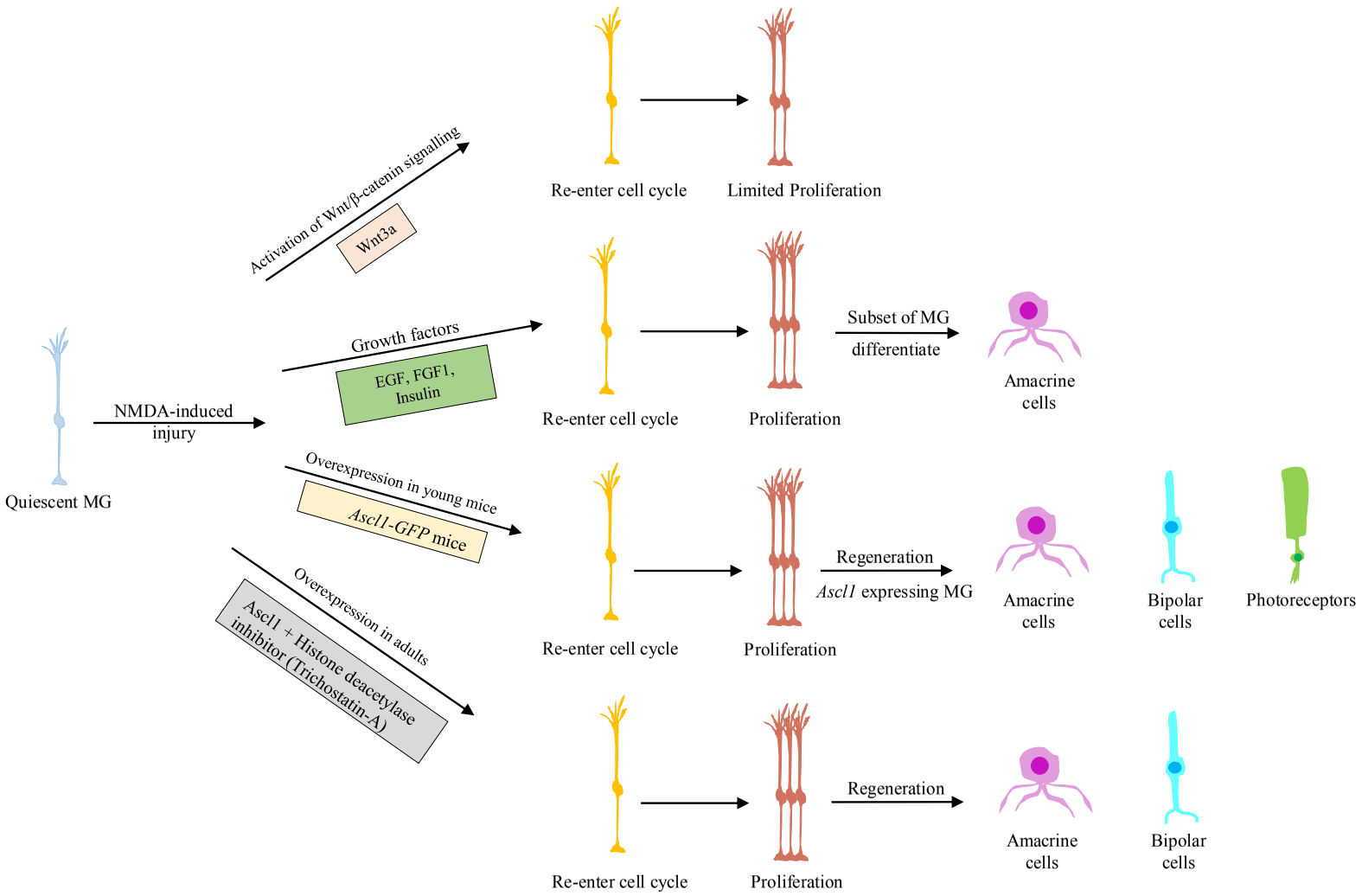
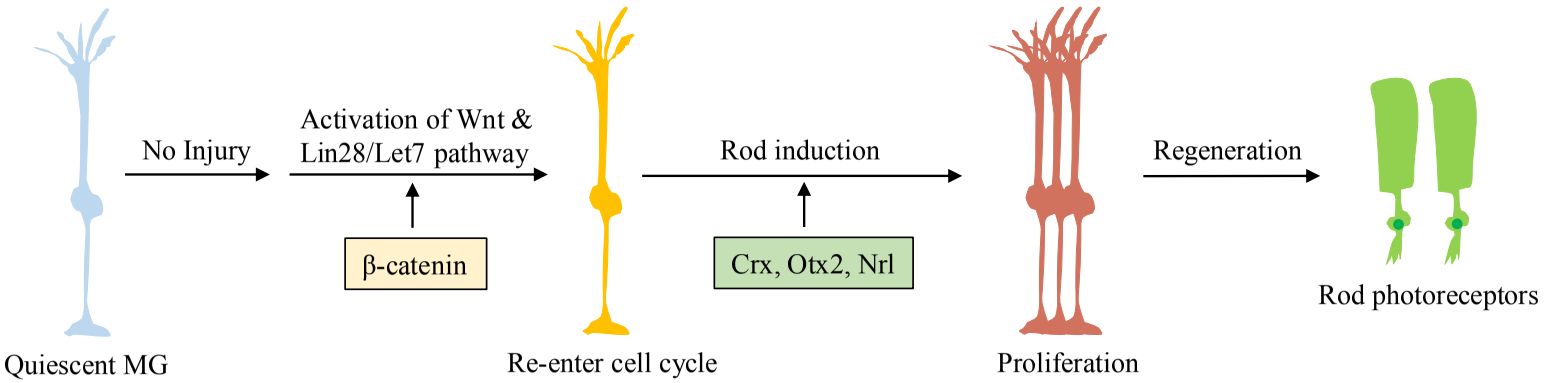

Quiescent MG
NMDA-induced injury
Activation of Wnt/β-catenin signalling
Wnt3a
Re-enter cell cycle
Limited Proliferation
Growth factors
EGF, FGF1, Insulin
Re-enter cell cycle
Proliferation
Subset of MG differentiate
Amacrine cells
Overexpression in young mice
Ascl1-GFP mice
Re-enter cell cycle
Proliferation
Regeneration
Ascl1 expressing MG
Amacrine cells
Bipolar cells
Photoreceptors
Overexpression in adults
Ascl1 + Histone deacetylase inhibitor (Trichostatin-A)
Re-enter cell cycle
Proliferation
Regeneration
Amacrine cells
Bipolar cells

Figure 2


No Injury
Activation of Wnt &
Lin28/Let7 pathway
β-catenin
Rod induction
Crx, Otx2, Nrl
Regeneration
Rod photoreceptors
Quiescent MG
Re-enter cell cycle
Proliferation