Biphasic Role of Tgf-β Signaling during Müller Glia Reprogramming and Retinal Regeneration in Zebrafish

HIGHLIGHTS
- Tgf-β signaling is essential for retinal progenitor proliferation and cell cycle exit
- pSmad3 binds to SGC and TIE elements to cause gene activations and repressions
- Tgf-β signaling regulates Zebs and various miRNAs for cellular reprogramming
- Translation of Tgf-β signaling requires Mmp2/Mmp9 activity
Article

Biphasic Role of Tgf-β Signaling during Müller Glia Reprogramming and Retinal Regeneration in Zebrafish

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SUMMARY

Tgf-β signaling is a major antiproliferative pathway governing different biological functions, including cellular reprogramming. Upon injury, Müller glial cells of zebrafish retina reprogram to form progenitors (MGPCs) essential for regeneration. Here, the significance of Tgf-β signaling for inducing MGPCs is explored. Notably, Tgf-β signaling not only performs a pro-proliferative function but also is necessary for the expression of several regeneration-associated, essential transcription factor genes such as ascl1a, lin28a, oct4, sox2, and zebs and various microRNAs, namely, miR-200a, miR-200b, miR-143, and miR-145 during different phases of retinal regeneration. This study also found the indispensable role played by Mmp2/Mmp9 in the efficacy of Tgf-β signaling. Furthermore, the Tgf-β signaling is essential to cause cell cycle exit of MGPCs towards later phases of regeneration. Finally, the Delta-Notch signaling in collaboration with Tgf-β signaling regulates the critical factor, Her4.1. This study provides novel insights into the biphasic roles of Tgf-β signaling in zebrafish during retinal regeneration.

INTRODUCTION

Retinal damage often results in permanent blindness in mammals. Unlike mammals, vertebrates such as fishes and frogs can repair their damaged retina and restore vision. Tissue regeneration is a boon for zebrafish as it can regenerate almost all organs including retina (Gemberling et al., 2013). The regenerative mechanism is a complex phenomenon in which the homeostasis of the damaged part is restored through a series of genetic and epigenetic cascade of events in cells near the injury site (Goldman, 2014; Wan and Goldman, 2016). Soon after the injury, the Müller glia (MG) cells of the retina undergo a cellular reprogramming event to give rise to MG-derived progenitor cells (MGPCs), which differentiate to form various retinal cell types and MG (Bernardos et al., 2007; Ramachandran et al., 2010b). In an injured mammalian retina, the MG cells often fail to elicit an adequate regenerative response to restore vision. It is believed that mammalian retina mounts an inhibitory environment through various cytokines and growth factors to prevent the growth of new nervous tissue (Liu et al., 2008; Schimchowitsch and Cassel, 2006). In zebrafish, the scenario is the opposite, where the vision is restored after an acute injury through retinal regeneration. Several past reports have characterized the molecular events involving various transcription factors, cell signaling networks, and epigenome modifiers in the zebrafish retina, which reveal the complex nature of retinal regeneration (Goldman, 2014; Gorsuch and Hyde, 2014; Wan and Goldman, 2016). It is interesting to note that several such regeneration-associated gene expression events were inadequate or missing in injured mammalian retina accounting for lack of efficient retinal regeneration in them (Wilken and Reh, 2016). Remarkably, the forced introduction of some of these essential transcription factors such as Ascl1a has caused an improved regenerative response in the injured mice retina (Brzezinski et al., 2011; Jorstad et al., 2017). Furthermore, the absence of efficient regenerative response in mammalian models warrants a more in-depth investigation into the circumstances causing MG reprogramming in the zebrafish retina, which would also enable us to connect the cascade of events during retinal regeneration.

In zebrafish, MG reprogramming leads to the induction of progenitors with stem cell-like properties, which is the pivotal step to prevent fibroblast-mediated wound closure and scar formation. Different developmentally essential pathways have been associated with MG reprogramming and retinal regeneration such as Wnt (Meyers et al., 2012; Ramachandran et al., 2011), Delta-Notch (Conner et al., 2014; Mills and Goldman, 2017), Fgf (Hochmann et al., 2012; Qin et al., 2011), and Shh (Kaur et al., 2018) signaling. However,
Figure 1. Tgf-β Signaling Is Essential for Normal Retinal Regeneration

(A–C) (A) The qPCR analyses of Tgf-β signaling-regulated snai gene family and reporter genes of Tgf-β signaling such as tgfbi and smad7 at various times post retinal injury. *p < 0.004, N = 4; hpi, hours post injury; dpi, days post injury. (B) Western blot analysis of phosphorylated Smad3 (pSmad3) at various times post retinal injury. Gapdh is the loading control. (C) An experimental timeline that describes the injury, Pirfenidone or SB431542 drug injection and retina harvest at 2 or 4 dpi.
(D and E) Immunofluorescence (IF) microscopy images of retinal cross sections show decreased PCNA+ MGPCs at 4 dpi in either Pirfenidone or SB431542 drug-injected conditions (D), compared with DMSO control, which is quantified (E), *p < 0.002, N = 3.
(F) The qPCR analyses of tgfbi and several regeneration-associated genes show a decline in mRNA levels in SB431542-treated retina at 2 dpi, *p < 0.005, N = 4.
(G) Western blot analyses of several regeneration-associated proteins along with Tgfbi and pSmad3 reveal an SB431542 dose-dependent decrease in 2 dpi retina.
(H) The qPCR analysis of let-7a microRNA shows an increase in RNA levels in SB431542-treated retina at 2 d, *p < 0.0001, N = 4.
(I) The ascl1a/lin28a/sos2/oct4 promoter schematic reveals the typical SGC sites (upper), and the retinal ChIP assays confirm the physical binding of pSmad3 at the SGC site (lower), in 3 hpi and 2 dpi retina. Error bars are SD. Scale bars, 10 μm (D). The asterisk marks the injury site in (D). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (D). UC, uninjured control in (B). -ve, negative control in (I). BS, binding site in (I).

See also Figures S1–S3 and Table S1.

the significance of Tgf-β signaling during retinal regeneration remained underexplored. The ligand of Tgf-β signaling stays dormant and sequestered in the extracellular matrix, which needs to be activated for its functionality (Barcellos-Hoff, 1996; Maurya et al., 2013; Miyazono and Heldin, 1991). Retinal injury causes an immediate upregulation of the gene encoding the enzyme matrix metalloproteinase 9 (mmp9) near the injury site (Kaur et al., 2018), which has the potential to modify the extracellular matrix (Lu et al., 2011). Mmp9 is also involved in the activation of the crucial cytokine, Tgf-β, from its inactive to active form (Karsdal et al., 2002; Kobayashi et al., 2014; Krstic and Santibanez, 2014; Yu and Stamenkovic, 2000). Conversely, Tgf-β upregulates the transcription of mmp2 (Kim et al., 2007) and mmp9 genes (Han et al., 2001), causing a positive feedback loop (Krstic and Santibanez, 2014). Despite the knowledge on the anti-proliferative involvement of Tgf-β in various model organisms (Close et al., 2005; Lenkowski et al., 2013; Todd et al., 2017), the mechanisms governed by the active Mmp9/Tgf-β axis during retinal regeneration that contributes to the MG reprogramming and MGPCs induction remained unknown.

In this study, we explored the significance of Tgf-β signaling, induced soon after the injury, along with Mmp2/Mmp9 and its interrelationship with various regeneration-associated gene expression events at different phases of retinal regeneration. We found a unique dual role of Tgf-β signaling during MG reprogramming in zebrafish. We mechanistically show that the MG reprogramming is mediated through the Tgf-β/Mmp axis and the Tgf-β signaling regulates some important microRNAs, regeneration-associated transcription factors, and epigenome modifiers, which provide positive and negative feedback to cause the induction of an adequate number of MGPCs. Tgf-β signaling also functions through the regulation of Delta-Notch signaling effector gene her4.1. Furthermore, we demonstrate the contrasting role of Tgf-β signaling toward the later stages, which is necessary for the cell cycle-exit of MGPCs, essential to complete the retinal regeneration.

RESULTS

Tgf-β Signaling Is Essential for Normal Retinal Regeneration

Tgf-β signaling plays critical roles in cellular events such as embryonic development (Liu et al., 2018; Meyers and Kessler, 2017), homeostasis of hematopoietic stem cells (Blank and Karlsson, 2013), mesenchymal differentiation (Graf et al., 2018), aging, and cancer (Papageorgis, 2017). Interestingly, modulation of the Tgf-β signaling in injured mice retina, with little regenerative capability, had a negligible effect on MG activation and regeneration (Kugler et al., 2015). These studies prompted us to explore the significance of Tgf-β signaling in controlling the regenerative response in the zebrafish retina. At first, we decided to see if Tgf-β signaling was activated in the injured retina. For this, we explored the expression patterns of various effector genes regulated by Tgf-β signaling. We analyzed the levels of Tgf-β signaling-induced genes, namely, tgfbi, smad7, and members of the snai gene family, such as snai1a, snai1b, snai2, and snai3. We saw an immediate upregulation followed by the downregulation of these genes at different phases of retinal regeneration (Figure 1A). Furthermore, the phosphorylation status of Smad3 protein (pSmad3), the messenger molecule of Tgf-β signaling to cause target gene expression in the nucleus, also showed an increase in protein levels in the retinal extracts collected at different stages of retinal regeneration (Figure 1B). These observations suggested the active involvement of Tgf-β signaling in an efficiently regenerating tissue such as zebrafish retina. We then decided to explore the significance of the Tgf-β signaling in causing MGPCs proliferation in the injured retina. For this, we blocked TGF-β signaling panretinally soon
Figure 2. Tgf-β Signaling Regulates Müller Glia Reprogramming

(A) An experimental timeline that describes the injury, SB431542 drug injection, and retina harvest at 3 hpi, 2 or 4 dpi.
(B) qPCR analyses reveal that miR-200a, miR-200b, miR-143, and miR-145 genes show an SB431542 dose-dependent increase in their mRNA levels at 2 dpi, *p < 0.01, N = 4.
(C and D) The miR-200a/miR-200b (C, upper) and miR-143/miR-145 (D, upper) promoters’ schematics reveal the typical TIE sequences, and the retinal ChIP assays reveal the physical binding of pSmad3 at these sites (C and D, lower), in 3 hpi and 4 dpi retina.
(E and G) The snail (E, *p < 0.005, N = 4, and zeb (G), *p < 0.002, N = 4, gene family show a dose-dependent decline in mRNA levels in SB431542-treated retina, at 2 dpi.
(F and H) The snail (F) and zeb (H) promoter schematics reveal the presence of typical SGC sites (upper), and the retinal ChIP assays confirm the physical binding of pSmad3 at the SGC sites (lower), in 3 hpi and 4 dpi (F), 2 and 4 dpi (H) retina.
(I) qPCR analysis of cdh1 gene shows an SB431542 dose-dependent increase in the mRNA levels at 2 dpi, *p < 0.002, N = 4.
(J) qPCR analyses reveal increased levels of tet1 and her4.1 mRNAs in SB431542-treated retina at 2 dpi, *p < 0.001, N = 4. Error bars are SD. -ve, negative control in (C), (D), (F), and (H). BS, binding site in (C), (D), (F), and (H). See also Table S1.

Further, the gene expression analysis of various regeneration-associated genes such as ascl1a, lin28a, sox2, mmp2, oct4, hdac1, and tgbf showed a decline in their expression levels, which could account for the reduced number of MGPCs with the blockade of Tgf-β signaling (Figure 1F). The mRNA in situ hybridization of these genes also showed a similar trend in their expression patterns in Tgf-β signaling-inhibited retina at 4 dpi (Figure S1E). Moreover, expression analysis of Ascl1a, Lin28a, Sox2, Oct4, Hdad1, pSmad3, and Tgfb1 revealed a significant decline in their protein levels in 2 dpi retina because of compromised Tgf-β signaling (Figure 1G). These results suggest the involvement of Tgf-β-mediated gene regulation that necessitates adequate MGPCs proliferation during retinal regeneration. Notably, in SB431542-treated retina, we found a drastic increase in let-7a microRNA levels (Figure 1H), which is known to maintain the differentiated status of the retinal cells and prevent the translatable status of several regeneration-specific factors (Kaur et al., 2018; Ramachandran et al., 2010a). Furthermore, promoter sequence analysis of ascl1a, lin28a, sox2, and oct4 revealed the presence of GGC/GC/GC sequence, also known as SGC elements. The occupancy of SGC by pSmad3 is essential for its transcriptional activation function (Martin-Malpartida et al., 2017). We then explored the SGC-binding of pSmad3 at 3 hpi when its induction is at the peak (Figure 1B), and also at 2 dpi, a time when MGPCs proliferation begins. Chromatin immunoprecipitation (ChIP) analysis of these SGC sites in 3 hpi and 2 dpi retinal extracts revealed the occupancy of pSmad3 at these sites (Figure 10). This result could explain the decline in expression levels of ascl1a, lin28a, sox2, and oct4 in the SB431542-treated retina. These findings support the view that Tgf-β signaling significantly contributes to the formation of MGPCs as a prelude to retinal regeneration.

Tgf-β Signaling Regulates Müller Glia Reprogramming

The above findings suggested the possibility of Tgf-β signaling participating in the reprogramming of MG to give rise to proliferating MGPCs. Furthermore, Tgf-β is known to cause upregulation of microRNAs such as miR-200a, miR-200b, miR-143 and miR-145, which play essential roles during cellular reprogramming and cancer in various mammalian systems (Davalos et al., 2012; Long and Miano, 2011) and zebrafish retinal regeneration (Sharma et al., 2019). The miR-200 family targets the zinc-finger enhancer-box binding (Zeb) transcription factors (Beclin et al., 2016; Wang et al., 2013), whereas the miR-145 targets Oct4, Sox2, and Klf4 (Cordes et al., 2009; Huang et al., 2012) to inhibit stem cell characteristics. These attributes also made us to explore if Tgf-β signaling influenced the expression pattern of these microRNA family members to mediate MG reprogramming. Unlike that found in mammalian systems, the blocking of Tgf-β signaling elevated the levels of miR-200a/miR-200b and miR-143/miR-145 (Figures 2A and 2B). Promoter sequence analysis of the miR-200a/miR-200b and miR-143/miR-145 gene clusters revealed the presence of a bona fide Tgf-β inhibitory element (TIE), essential for repressive transcriptional events mediated by TGF-β1 (Kerr et al., 1990). Furthermore, the ChIP analysis performed in 3 hpi and 4 dpi retinal...
Figure 3. Tgf-β Signaling-Dependent Gene Regulations Are Essential during Retinal Regeneration

(A) An experimental timeline that describes the injury, TGF-β1 protein injection, and retina harvest at 2 dpi. (B and C) IF microscopy images of retinal cross sections show increased PCNA+ MGPCs at 2 dpi in TGF-β1 protein-injected condition, compared with PBS control, which is quantified (C), *p < 0.001, N = 4. (D and E) RT-PCR (D) and qPCR (E) analyses of several regeneration-associated genes in TGF-β1 protein-injected retina at 2 dpi, *p < 0.005, N = 4. (F) Western blot analyses of several regeneration-associated proteins along with pSmad3, the effector of Tgf-β signaling, reveal a TGF-β1 protein dose-dependent increase in 2 dpi retina. Gapdh is the loading control. (G) The qPCR analysis of let-7a microRNA shows a dose-dependent decline in RNA levels in TGF-β1 protein-injected retina at 2 dpi, *p < 0.003, N = 4. (H) The qPCR analyses of miR-200a, miR-200b, miR-143, and miR-145 reveal a decrease in RNA levels with increasing concentration of TGF-β1 protein in retina at 2 dpi, *p < 0.001, N = 4. (I and J) The miR-200a/miR-200b promoter activity assay in zebrafish embryos co-injected with miR-200a/miR-200b-EGFP-luciferase reporter construct and renilla luciferase mRNA reveals a dose-dependent decrease with TGF-β1 protein injection (I), *p < 0.004, N = 3, and increase when exposed to SB431542 drug (J), *p < 0.001, N = 3. (K-M) The qPCR analyses of snail family (K), *p < 0.01, N = 4, zeb family (L), *p < 0.01, N = 4, genes show an increase and that of cdh1 (M), *p < 0.007, N = 4, reveals a decrease in mRNA levels, respectively, with increasing concentration of TGF-β1 protein in retina at 2 dpi. Error bars are SD. Scale bars, 10 μm (B). The asterisk marks the injury site in (B). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (B). See also Figures S2 and S3 and Table S1.

extracts confirmed that the pSmad3 indeed occupied both these sites at 3 hpi and 4 dpi in miR-200a/miR-200b promoter (Figure 2C). In miR-143/miR-145 promoter, the pSmad3 occupied TIE only at 3 hpi but not at 4 dpi, when the MGPCs proliferation peaks (Figure 2D). This result could explain the cause of elevated levels of miR-200a/miR-200b and miR-143/miR-145 in the retina with blocked Tgf-β signaling.

Cellular reprogramming to form induced pluripotent stem cells (iPSCs) is a physiologically similar mechanism to mesenchymal to epithelial transition (MET). Studies on MET during reprogramming have shown that the knockdown of SNAIL (SNAI1), a facilitator of epithelial to mesenchymal transition (EMT), caused a decline in reprogramming efficiency in human and mice cells (Unternaehrer et al., 2014). Recent findings also show that EMT, apart from imparting cell motility, is also capable of inducing stem-cell properties while preventing cellular apoptosis and senescence (Brabletz and Brabletz, 2010; Mani et al., 2008; Shuang et al., 2014). Here, the reduced MGPCs proliferation seen in SB431542-treated 4 dpi retina is also associated with a significant decline in the expression of Tgf-β signaling-activated snail gene family members (Figure 2E), which are negative regulators of E-cadherin (Saitoh et al., 2016; Yu et al., 2015) and let-7 microRNAs (Unternaehrer et al., 2014). In situ analysis of the snail family gene promoters revealed the presence of 5GC elements, and ChiP analysis in retinal extracts from 3 hpi and 4 dpi confirmed that the pSmad3 occupied all of these sites (Figure 2F), causing their transcriptional activation. EMT is an essential phase in development and disease. ZEB1 and ZEB2 are vital EMT activators, which in turn regulate members of the miR-200 family to establish a stringent, reciprocal feedback loop, with each one controlling the expression of the other (Brabletz and Brabletz, 2010). Therefore, we investigated the levels of zebs in injured retina along with the blockade of Tgf-β signaling. We observed that the inhibition of Tgf-β signaling caused a dose-dependent decline in zebs family genes (Figure 2G), whose promoters also included several pSmad3-binding 5GC elements, which are functionally evaluated by retinal ChiP assay at 2 and 4 dpi (Figure 2H). The blockade of Tgf-β signaling also caused an increase in E-cadherin (cdh1) levels (Figure 2I), which could be because of the decline in the levels of its repressors Zeb (Galvan et al., 2015; Sanchez-Tillo et al., 2010) and Snails (Yu et al., 2015).

It is also interesting to note that tet1 (ten-eleven translocation 1), a gene that stays downregulated in various cancers and is known to be involved in DNA demethylation (Rasmussen and Helin, 2016), gets up-regulated because of SB431542 treatment in 2 dpi retina (Figure 2J). Low Tet1 activity is known to favor pluripotency, whereas in high levels, it promotes EMT (He et al., 2019). Moreover, the early blockade of Tgf-β1 signaling also upregulated her4.1, a negative regulator of cell proliferation (Mitra et al., 2018, 2019), which also explains the cause of the reduction in MGPCs number at 4 dpi (Figure 2J). These findings support the view that normal Tgf-β1 signaling is essential for MG reprogramming to cause the formation of an adequate number of MGPCs during zebrafish retinal regeneration.

Tgf-β Signaling-Dependent Gene Regulations Are Essential during Retinal Regeneration

The results of impaired retinal regeneration in the SB431542-treated scenario prompted us to explore the regenerative response of the retina in enhanced Tgf-β1 signaling conditions. When we injected the recombinant TGF-β1 protein at the time of retinal injury (Figure 3A), we saw a significant increase in the number of PCNA+ cells as early as 2 dpi (Figures 3B and 3C). We also found an increased number of GFP+ MGPCs in...
Figure 4. Effect of TGF-β1 Protein Is Mediated through Active Mmp2/Mmp9

(A) An experimental timeline that describes the injury, TGF-β1 protein injection, and retina harvest at 2 dpi. (B and C) RT-PCR (B) and qPCR (C) analyses of mmp2 and mmp9 in TGF-β1 protein-injected retina at 2 dpi. *p < 0.008, N = 4.

(D and E) The mmp2 (D) and mmp9 (E) promoter schematics reveal the typical SGC element on mmp2 (D, upper) and typical TIE sequence on mmp9 (E, upper) promoters, which in the retinal ChIP assays prove to be functional through physical binding of pSmad3 at the SGC and TIE sequence of respective genes (D and E, lower) in 3 hpi and 4 dpi retina.

(F) An experimental timeline that describes the injury, mmp2/mmp9 morpholino (MO) electroporation, and retina harvest at 4 dpi. (G and H) IF microscopy images of retinal cross sections show the decline in BrdU+ MGPCs with increasing concentrations of mmp2 MO (G, left) and mmp9 MO (G, right) at 4 dpi, which is quantified (H), *p < 0.0002, N = 4. Lissamine is the fluorescent tag on MOs. Error bars are SD. Scale bars, 10 μm (G). The asterisk marks the injury site in (G). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (G). -ve, negative control in (D) and (E). BS, binding site in (D) and (E). See also Figures S3–S5 and Table S1.

The members of the Mmp family, such as Mmp9, are involved in a variety of biological phenomena (Dziembowska and Włodarczyk, 2012; van Kempen and Coussens, 2002), including retinal regeneration (Kaur et al., 2018). Notably, MMP-2 and MMP-9 are known to activate extracellular matrix (ECM)-localized TGF-β1 from its latent to the active form (Santibanez et al., 2018; Yu and Stamenkovic, 2000). TGF-β signaling, in turn, activates MMP-2 and MMP-9, creating a positive feedback loop (Kim et al., 2004). These reports prompted us to explore the interplay between TGF-β signaling and Mmps during zebrafish retinal regeneration. We saw a TGF-β1 protein dose-dependent increase in the expression of mmp2, whereas the opposite was seen with mmp9 (Figures 4A–4C). The TGF-β signaling-dependent regulation of mmp9 was also confirmed through mRNA in situ hybridization in retina treated either with TGF-β1 protein (Figure S2C) or SB431542 (Figure S3D) at 4 dpi, and also by qPCR done in SB431542-treated retina at 2 dpi (Figure S3E). The dependence of mmp2 on TGF-β signaling was also revealed by luciferase assays performed in zebrafish embryos (Figures S3F and S3G). It was interesting to note that, despite belonging to the same family, mmp2 and

The TGF-β1 protein-injected 1016tuba1a: GFP transgenic retina (Figures S2A and S2B) supporting the elevated proliferation seen in wild-type retina. However, no proliferative response was observed in uninjured retina even 4 days post-TGF-β1 protein delivery (Figure S2C). This increased MGPCs proliferation is also associated with the alterations in the expression patterns of regeneration-associated genes wherein an upregulation of asc1α, lin28α, sox2, oct4, and tgfbi1 and downregulation of her4.1 were observed (Figures 3D and 3E). The dependency of asc1α and oct4 on Tgf-β signaling was also revealed by luciferase assays done in zebrafish embryos (Figures S2D–S2G). We also confirmed the elevated levels of asc1α, lin28α, sox2, and oct4 through mRNA in situ hybridization in TGF-β1 protein-injected retina at 4 dpi (Figure S2H). Notably, the high mRNA levels of various regeneration-associated factors are also positively correlated with their protein levels (Figure 3F), which could also be facilitated by the decline in let-7a microRNA levels (Figure 3G), in TGF-β1-protein treated retina. Furthermore, the increased MGPCs seen in TGF-β1-protein-injected conditions were explored until 30 dpi to assess their potential in differentiating to various retinal cell types (Figure S3A). We found that the MGPCs seen in TGF-β1 protein-injected retina were differentiating into retinal cell types (Figure S3B), suggesting a normal regenerative response.

We then probed whether TGF-β1 protein influenced the levels of microRNAs such as miR-200a, miR-200b, miR-143, and miR-145 in regenerating retina. The TGF-β1 protein caused a dose-dependent downregulation of miR-200a/miR-200b and miR-143/miR-145 in the injured zebrafish retina (Figure 3H). Also, the previously confirmed binding of pSmad3 by ChIP analysis in retinal extracts (Figures 2C and 2D) may account for the downregulations of the miR-200a/miR-200b and miR-143/miR-145 in TGF-β1 protein-injected retina (Figure 3H), which is the opposite as seen in the SB431542-treated retina (Figure 2B). These observations were further confirmed by luciferase assay performed in zebrafish embryos co-injected with miR-200a/miR-200b joint promoter driving EGFP-luciferase fusion construct and TGF-β1 protein or incubated with SB431542 in separate experiments (Figures S3I and S3J). Moreover, we also found an increase in the levels of snail (Figure 3K) and zeb family genes with TGF-β1 protein injection (Figure 3L). The decrease in the zeb-repressing miR-200a family and transcriptional upregulation of zeb genes could enhance the Zeb protein, which is a known repressor of cdh1. Concomitantly, we found a drastic decline in cdh1 mRNA with TGF-β1 protein injection in 2 dpi retina (Figure 3M), which could also be contributed by elevated Snail levels. These results suggest that Tgf-β signaling, which has a conventional anti-proliferative role in mammalian systems (Jahn et al., 2012; Li et al., 2014), has a pro-proliferative role during zebrafish retinal regeneration. This disparity perhaps accounts for the differential regenerative response between mammals and zebrafish.

Effect of TGF-β1 Protein Is Mediated through Active Mmp2/Mmp9

The members of the Mmp family, such as Mmp9, is involved in a variety of biological phenomena (Dziembowska and Włodarczyk, 2012; van Kempen and Coussens, 2002), including retinal regeneration (Kaur et al., 2018). Notably, MMP-2 and MMP-9 are known to activate extracellular matrix (ECM)-localized TGF-β1 from its latent to the active form (Santibanez et al., 2018; Yu and Stamenkovic, 2000). TGF-β signaling, in turn, activates MMP-2 and MMP-9, creating a positive feedback loop (Kim et al., 2004). These reports prompted us to explore the interplay between TGF-β signaling and Mmps during zebrafish retinal regeneration. We saw a TGF-β1 protein dose-dependent increase in the expression of mmp2, whereas the opposite was seen with mmp9 (Figures 4A–4C). The TGF-β signaling-dependent regulation of mmp9 was also confirmed through mRNA in situ hybridization in retina treated either with TGF-β1 protein (Figure S2C) or SB431542 (Figure S3D) at 4 dpi, and also by qPCR done in SB431542-treated retina at 2 dpi (Figure S3E). The dependence of mmp2 on TGF-β signaling was also revealed by luciferase assays performed in zebrafish embryos (Figures S3F and S3G). It was interesting to note that, despite belonging to the same family, mmp2 and
Figure 5. Mmp2/Mmp9 Limit the Early Induction of Regeneration-Associated Genes
(A) An experimental timeline describing the injury, TGF-β1 protein injection or SB431542 or SB3CT drug delivery, MO electroporation, and retina harvest at 16 hpi. (B) The qPCR analyses of tgfbi, ascl1a, lin28a, sox2, and oct4 showed increased mRNA levels in TGF-β1 protein-injected retina at 16 hpi, *p < 0.003, N = 4. (C) Western blot analyses of Ascl1a, Lin28a, Sox2, Oct4, and Tgfbi proteins reveal TGF-β1 protein-dependent increase in 16 hpi retina. Gapdh is the loading control. (D) The qPCR analyses of tgfbi, ascl1a, lin28a, sox2, and oct4 showed decreased mRNA levels in SB431542-treated retina at 16 hpi, *p < 0.02, N = 4. (E) Western blot analyses of Ascl1a, Lin28a, Sox2, Oct4, and Tgfbi proteins reveal an SB431542 drug-dependent decrease in 16 hpi retina. Gapdh is the loading control. (F and G) The qPCR analyses of tgfbi, ascl1a, lin28a, sox2, oct4, mmp2, and mmp9 with SB3CT drug treated (F), *p < 0.03, N = 4, and mmp2+mmp9 MO electroporated (G), *p < 0.02, N = 4, retina at 16 hpi. (H) Western blot analyses of Ascl1a, Lin28a, Sox2, Oct4, and Tgfbi proteins reveal an SB3CT drug-dependent increase in 16 hpi and 2 dpi retina. Gapdh is the loading control. (I) An experimental timeline describing the injury, TGF-β1 protein or SB3CT drug injection, and retina harvest at 4 dpi. (J and K) IF microscopy images of retinal cross sections show increased PCNA + MGPCs at 4 dpi in TGF-β1 injected condition, and a significant decline in the number of MGPCs in SB3CT drug-treated/mmp2 MO electroporated retina, *p < 0.03, N = 4. (L) Western blot analyses of Ascl1a, Lin28a, Sox2, Oct4, and Tgfbi proteins in DMSO, TGF-β1 protein, SB3CT drug, and a combination of TGF-β1- and SB3CT-treated retina at 16 hpi and 2 dpi. Gapdh is the loading control. Error bars are SD. Scale bars, 10 μm. The asterisk marks the injury site in (J). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (J). ctl MO-control morpholino (G and J). See also Figures S4 and S5, and Table S1.

mmp9 had contrasting regulation with activated Tgf-β signaling. Closer analysis of these gene promoters revealed the existence of two TIE sequences in the promoter of mmp2, but not in that of mmp1. The mmp2 promoter analysis revealed the presence of a typical SGC element. Furthermore, the ChIP analysis performed in 3 hpi and 4 dpi retinal extracts confirmed that pSmad3 bound on to the SGC element of mmp2 promoter at 3 hpi, but not at 4 dpi (Figure 4D). Conversely, of the two TIE sequences on the mmp9 promoter, one was occupied with pSmad3 at both 3 hpi and 4 dpi, as revealed in ChIP assay (Figure 4E). The ChIP analysis on the mmp2 and mmp9 promoters confirmed the activation of the former and the repression of the latter by Tgf-β signaling. Since in TGF-β1 protein injected conditions, the mmp2 gets upregulated while retaining low levels of mmp9, we explored if the mmp2/mmp9 expression was essential for retinal regeneration. We used morpholinos (MOs) targeting mmp2 and mmp9 to knockdown their expression (Figure 4F). We saw a MO dose-dependent decline in the number of BrdU-labeled MGPCs at 4 dpi in both mmp2 (Figure 4G left) and mmp9 (Figure 4G right) knockdown retinas, which is quantified (Figure 4H). To elucidate this further, we assessed the proliferative effect of increasing concentrations of TGF-β1 protein in mmp2/mmp9 knockdown retinas. Interestingly, the pro-proliferative effect of TGF-β1 protein was abolished in the absence of mmp2/mmp9 (Figures 5A–5D). The decrease in MGPCs was not because of enhanced cell death as evident from the number of apoptotic cells in mmp2/mmp9 downregulated retina (Figure 5E). These observations suggested that the enhancement in the number of MGPCs mediated through TGF-β1 protein could be influenced through the activity of Mmp2 and Mmp9.

Mmp2/Mmp9 Limit the Early Induction of Regeneration-Associated Genes
As we discovered the importance of Mmp2 and Mmp9 in the Tgf-β signaling-dependent induction of MGPCs at 4 dpi, we hypothesized that this mechanism might act as a foundation to the regenerative response. To elucidate if the Tgf-β/Mmp2/Mmp9 axis is pivotal during the early dedifferentiation phase of retinal regeneration, we chose a mid-time point of MG reprogramming at 16 hpi (Figure 5A). We then explored the regulation of regeneration-associated genes through Tgf-β signaling at 16 hpi. For this, we analyzed the levels of ascl1a, sox2, lin28a, and oct4 along with positive control tgfbi mRNAs and their protein in TGF-β1 (Figures 5B and 5C) or SB431542 (Figures 5D and 5E)-treated retina in separate experiments at 16 hpi. Interestingly, we found a dose-dependent increase in the tgfbi and other regeneration-associated genes' expression, both at mRNA and protein levels, in retina treated with TGF-β1 protein (Figures 5B and 5C). We also found an anticipated decline in tgfbi and regeneration-associated gene expression both at mRNA and protein levels in the SB431542-treated retina at 16 hpi (Figures 5D and 5E). These results suggest that Tgf-β signaling-mediated activation of regeneration-associated genes ensue from the dedifferentiation phase of retinal regeneration.

Since we have found a Tgf-β signaling-dependent regulation of mmp2/mmp9 genes (Figures 4B, 5C, and 5D), we speculated that Mmp2/Mmp9 is also required to activate the Tgf-β from its inactive form. If such regulation exists, we would be able to see a decline in the expression levels of tgfbi and possibly that of other regeneration-associated genes as well, in the absence of Mmp2/Mmp9 activity. To check this, we...
employed both pharmacological inhibition and gene knockdown approach to bring down Mmp2/Mmp9 activity in the injured retina. We used an Mmp2/Mmp9 activity blocker drug SB3CT or mmp2/mmp9-targeting MOs at various concentrations in separate experiments soon after the injury to assay its effect in the retina at 16 hpi. We found an anticipated decline in the expression levels of tgfbi indicative of reduced Tgf-β signaling (Figures 5A, SF, and 5G). However, instead of seeing any decrease in the levels of ascl1a, lin28a, sox2, and oct4 mRNA or protein in retinae, either treated with SB3CT or electroporated with mmp2 and mmp9 MOs alone or in combination, we saw an unexpected increase in their expression (Figures 5A, SF, 5G, 5SA, and 5SB). These regeneration-associated genes’ mRNA upregulation also resulted in their elevated protein expression as well, both in 16 hpi and 2 dpi retina treated with SB3CT (Figure 5H). These results indicated that expressions of various regeneration-associated genes are positively regulated by Tgf-β signaling (Figures 3E and 3F), but not limited to the Mmp2/Mmp9-mediated activation of Tgf-β protein. Conversely, the absence of Mmp2/Mmp9 stimulated the regeneration-associated gene expression, probably through a Tgf-β signaling-independent pathway in the injured retina at 16 hpi.

Tgf-β signaling activates mmp2 and mmp9 causing a positive feedback loop in various systems (Krstic and Santibanez, 2014). Also, combined knockdown of mmp2 and mmp9 significantly abolished MGPCs induction in regenerating retina (Figures S5C and S5D). We then explored if the increase in MGPCs seen in 4 dpi retina with TGF-β1 protein injection required active Mmp2/Mmp9. To decipher this, we assayed the regenerative response with TGF-β1 protein injection alone or along with Mmp2/Mmp9 blocker and mmp2 MO in separate experiments (Figure 5I). Interestingly, compared with the control, we saw a significant decline in the number of MGPCs in TGF-β1 protein-injected retina when Mmp2/Mmp9 activity is inhibited with SB3CT drug or mmp2 MO (Figures 5J and 5K). Furthermore, western blot analysis in DMSO, TGF-β1 protein, SB3CT drug, and combination of TGF-β1 protein and SB3CT-treated retina at 16 hpi and 2 dpi revealed the presence of high levels of Ascl1a, Lin28a, Sox2, and Oct4 proteins (Figure 5L). In spite of high levels of these regeneration-associated factors, there was reduced MGPCs proliferation in SB3CT-treated retina with or without TGF-β1 protein at 4 dpi (Figure 5J). These findings suggest that, despite the presence of high levels of Ascl1a, Lin28a, Sox2, and Oct4, the formation of an adequate number of MGPCs requires regular Mmp2/Mmp9 activity.

**Mmp2 and Mmp9 Are Necessary for the Efficacy of Regeneration-Associated Genes**

Since we found high expression levels of various regeneration-associated genes and reduced MGPCs number in Mmp2/Mmp9 inhibited retina, we speculated that Mmp2/Mmp9 activity might be necessary for efficient regeneration mediated by these genes. To address this in depth, we overexpressed the regeneration-associated genes such as ascl1a, lin28a, sox2, and oct4 through mRNA transfection of the retina along with inhibition of Mmp2/Mmp9 activity (Figure 6A). We saw an increased number of MGPCs in ascl1a, lin28a, sox2, and oct4 overexpressed retinae (Figures 6B and 6C). However, the increase in MGPCs number significantly reduced with SB3CT treatment (Figures 6B and 6C), suggesting the necessity of Mmp2/Mmp9 activity for the efficient functioning of these genes. These observations, along with the above findings, wherein elevated regeneration-associated genes were seen in Mmp2/Mmp9-inhibited retina (Figures 5F–5H), could presumably be because of the existence of a negative feedback regulatory loop in controlling the expression of regeneration-associated genes through Mmp2/Mmp9.

To address this further, we decided to perform rescue experiments in which the Mmp2/Mmp9 activity was transiently blocked in retinae overexpressed with regeneration-associated genes, followed by the transfection of mmp2/mmp9 mRNA (Figure 6D). The goal of these experiments was to prove that the blockade of Mmp2/Mmp9 activity, which was sufficient to abolish the effect of regeneration-associated genes’ overexpression, could be alleviated by subsequent transfection of mmp2/mmp9 mRNAs. As expected, the repression of MGPCs number because of SB3CT treatment was rescued in mmp2/mmp9 mRNA-transfected retina (Figures 6E and 6F). These results are suggestive of the idea that Mmp2/Mmp9 activity is essential for the efficacy of regeneration-associated genes in inducing an adequate number of MGPCs.

**Tgf-β Signaling at Late Phases Is Essential for the Cessation of MGPCs Proliferation**

Previous results have indicated a decline in the Tgf-β signaling at 4 dpi, which shows an upregulation towards the late phase of retinal regeneration reflected through snai genes’ expressions and pSmad3 protein levels (Figures 1A and 1B). This result prompted us to speculate that the Tgf-β signaling could play different roles in the late phase of retinal regeneration. To explore this further, we decided to check if the late inhibition of Tgf-β signaling had any effects on MGPCs proliferation. For this, we allowed the regeneration to
Figure 6. Mmp2 and Mmp9 Are Necessary for the Efficacy of Regeneration-Associated Genes

(A) An experimental timeline describing the injury, mRNA transfection, SB3CT drug treatment, and retina harvest at 5 dpi. (B and C) IF microscopy images of retinal cross sections show increased PCNA+ MGPCs at 5 dpi in various regeneration-associated gene mRNA transfections, and a significant decline in the number of MGPCs in SB3CT drug-treated conditions, compared with DMSO/gfp mRNA-transfected control, which is quantified (C), *p < 0.003, N = 4.

(D) An experimental timeline describing the injury, mRNA transfection, SB3CT drug treatment for 1–2 days, water from 2 to 3 days, mmp2+mmp9 mRNA transfection from 3 to 5 days, and retina harvest at 5 dpi. (E and F) IF microscopy images of retinal cross sections show increased PCNA+ MGPCs at 5 dpi in mmp2+mmp9 mRNA transfections, and a decline in the number of MGPCs because of early SB3CT drug treatment that can be rescued with mmp2+mmp9 mRNA transfection, compared with DMSO/gfp mRNA transfected control, which is quantified (F), *p < 0.04, N = 4. Error bars are SD. Scale bars, 10 μm (B and E). The asterisk marks the injury site in (B) and (E). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (B) and (E). n.s, not significant (F).

See also Figure S5.
continue undisturbed until 5 dpi, followed by SB431542 treatment during 5–8 dpi. In an experimental timeline (Figure 7A), when SB431542 treatment was given from the 5 to 8 dpi period, we saw a significant increase in the number of proliferating cells in 8 dpi retina compared with DMSO controls (Figures 7B and 7C). The increased number of MGPCs could be because of two scenarios: (1) accelerated MG reprogramming and cell proliferation and (2) the existing MGPCs continue to be in the cell cycle. To decipher this, we performed double labeling of MGPCs before and after inhibiting TGF-β signaling. We labeled the MGPCs with BrdU at 5 dpi, followed by SB431542 treatment up to 8 dpi with an EdU pulse 3 h before harvest (Figure 7D). The BrdU injected intraperitoneally is metabolically available only for the first 4 h (Ramachandran et al., 2010a), and hence it will get washed off long before the EdU exposure on 8 dpi. At 8 dpi, the presence of more EdU + cells, which are BrdU negative, would indicate the existence of the former scenario (1). In contrast, the co-labeling of EdU and BrdU would suggest the latter (2). Notably, the increased number of MGPCs was the result of these MGPCs failing to exit the cell cycle in the absence of TGF-β signaling, as revealed by the co-labeling of EdU+/BrdU + cells (Figures 7D–7F). In a similar experiment, wherein we injected the TGF-β1 protein into injured zebrafish retina from 5 dpi (Figure S5E) and assayed its effect on MGPCs proliferation (Figure S5F), there was no significant increase in the number of BrdU + cells, but an enhanced number of EdU + cells (Figure S5G). The increased number of EdU + cells could be the newly proliferating cells as evident from discordant BrdU/EdU co-labeling.

Decreased levels of tgfbi and snai gene family members confirmed the downregulation of TGF-β signaling in late inhibition conditions (Figure 7G). Furthermore, the increased cell proliferation observed with the late blockade of TGF-β signaling is also associated with increased expression levels in several regeneration-associated genes such as ascl1a, lin28a, sox2, and oct4 (Figure 7H). Of these genes, oct4 and lin28a gene promoters had TIE sequences that enable TGF-β signaling-mediated gene repression. Interestingly, the ChIP analysis confirmed that the pSmad3 did not bind on to the TIE sequences present in the promoter and first intron of oct4 gene at 3 hpi but was able to bind at 4 dpi (Figure 7I). Similarly, the TIE present on the lin28a promoter was seen to bind with pSmad3 at 4 dpi, unlike seen at 3 hpi (Figure 7I). These observations suggest that TGF-β signaling has a direct inhibitory role on oct4 and lin28a expression towards later phases of retinal regeneration.

It is also important to note that the late inhibition of TGF-β signaling had a repressive effect on genes such as mmp2, her4.1, tet1, and insm1a (Figure 7J). The mmp2 is regulated through TGF-β signaling, whereas the her4.1 regulation seemed to be perplexing, because TGF-β signaling activation downregulated her4.1 expression from 0 to 2 dpi (Figure 3E) and its blockade with SB431542 from 5 to 8 dpi did the opposite (Figure 7J), unlike seen in 0 to 2 dpi blockade (Figure 2J), which needs further in-depth evaluation. Notably, either the injection of TGF-β1 protein in the early phase or blockade of TGF-β signaling at a later time point caused an enhancement in MGPCs proliferation. The observed downregulation of her4.1 is supportive of the increased MGPCs seen at 8 dpi. Furthermore, the reduced expression of insm1a (Figure 7J) that facilitates the cell cycle exit during zebrafish retinal regeneration (Ramachandran et al., 2012) could contribute to the persistence of the MGPCs in cycling stage for a prolonged time with late blockade of TGF-β signaling. It is interesting to note that tet1, a gene that stays upregulated with early SB431542 treatment of the retina (Figure 2J), showed a downregulation because of late inhibition of TGF-β signaling (Figure 7J). These...
Figure 8. Cross-talk between Notch and Tgf-β Signaling

(A) An experimental timeline that describes the injury, DAPT treatment, and retina harvest at 2/4 dpi.

(B) IF microscopy images of retinal cross sections show increased MGPCs in DAPT treated retina, compared with DMSO control at 4 dpi.

(C and D) RT-PCR (C) and qPCR (D) analyses of ascl1a, lin28a, and sox2 show an increase and oct4 shows a decrease, in DAPT-treated retina at 2 dpi, *p < 0.009, N = 4.

(E) Western blot analyses in retinae at 2 dpi reveal a DAPT dose-dependent increase of Ascl1a, Lin28a, Sox2, and pSmad3 and decrease in Oct4 levels. GAPDH is the loading control.

(F) The qPCR analyses of Tgf-β signaling component genes and its targets tgfbi and smad7 show increased mRNA levels in DAPT drug-treated retina at 2 dpi, *p < 0.001, N = 4.

(G) An experimental timeline that describes the injury, SB431542, DAPT drug treatment alone or in combination, and retina harvest at 2/4 dpi.
observations suggest the existence of Her4.1, Insm1a, and Tet1-mediated gene regulatory events, governed through Tgf-β signaling, which bring about the cell cycle exit of MGPCs toward the late phase of retinal regeneration.

Nucleosome remodeling and histone deacetylation (NuRD) complex-mediated gene silencing is inevitable for the pluripotency of embryonic stem cells and is also essential for the commitment of cells to various developmental lineages (Kaji et al., 2006). Furthermore, the NuRD complex mediates the cellular reprogramming of neural stem cells into induced pluripotent stem cells (dos Santos et al., 2014). However, it is demonstrated that the downregulation of the NuRD complex is also required for efficient reprogramming (Luo et al., 2013). These studies suggest the existence of a fine-tuned balancing of NuRD complex-mediated gene expression events that enable the balance between pluripotency and self-renewal states of the cells (Hu and Wade, 2012). Here, during retinal regeneration, the analysis of the gene family members of the NuRD complex also showed a decline in the expression levels because of the late blockade of Tgf-β signaling by SB431542 treatment (Figure 7K). These results suggest the involvement of NuRD complex-mediated gene regulations that are necessary for the cell cycle exit of MGPCs. These observations suggest the involvement of Tgf-β signaling during later stages of retinal regeneration in cell cycle exit that is governed through epigenetic modifiers such as Tet1, NuRD complex, and transcriptional repressors, namely, Insm1a and Her4.1.

Cross-talk between Notch and Tgf-β Signaling

Based on the above observations wherein the her4.1 level decline because of the late inhibition of Tgf-β signaling, which in turn keeps the MGPCs in the proliferative phase, we speculated the existence of cross-talk between Her4.1 and Tgf-β signaling. Earlier studies have shown that blockade of Notch signaling by DAPT in 2 and 4 dpi retina (Conner et al., 2014; Wan et al., 2012), as well as late inhibition of her4.1 (Mitra et al., 2018), results in an increased MGPCs proliferation. To explore this further, we followed an experimental timeline (Figure 8A), where fish were exposed to DAPT post retinal injury up to 2/4 dpi. Along with the increase in MGPCs number in DAPT-treated retina (Figure 8B), we found a dose-dependent increase in the expression of proliferation-associated marker genes such as ascl1a, lin28a, sox2 (Figures 8C–8E and S6A), with a drastic decline in occt4 (Figures 8C–8E and S6A). Furthermore, there was an accelerated expression of various Tgf-β signaling components such as tgfβ1a, tgfβ1b, tgfβ2, and tgfβr1b and Tgf-β signaling readout genes such as tgfbi and smad7 (Figure 8F) and a concomitant increase in pSmad3 (Figure 8E). Occt4 is a negative regulator of Tgf-β signaling components in various systems (Li et al., 2010; Sharma et al., 2019; Tan et al., 2015), and its downregulation in DAPT-treated retina could have caused the upregulation of tgfβ1a, tgfβ1b, tgfβ2, tgfβr1b, tgfbi, and smad7. These observations suggest that the observed increase in MGPCs in the absence of Delta-Notch signaling could be mediated through the Occt4/Tgf-β signaling axis. To explore this further, we adopted a double blocker strategy (Figure 8G) in which the injured retinas were exposed to increasing concentrations of DAPT and SB431542 (Figure 8H). To our surprise, we found a concentration-dependent increase in the number of MGPCs and probably rod progenitors as well, from double blocker experiments compared with either DMSO control or SB431542-treated retina (Figures 8H and 8I). Notably, many of the regeneration-associated genes were upregulated in both Delta-Notch and Tgf-β signaling inhibited conditions, compared with Tgf-β signaling compromised scenario (Figure 8J), which could be the cause of increased MGPCs proliferation in the retina from double blocker experiments (Figure 8H). These results, where the DAPT almost wholly nullified the inhibitory effect of SB431542 in MGPCs proliferation, suggest that a common factor, Her4.1, could hold the answer to the regulation of MGPCs proliferation (Figure 8J). Both activation of Tgf-β signaling and repression of Delta-Notch signaling brings the Her4.1 level (Mitra et al., 2018, 2019) down to accelerate MGPCs proliferation in regenerating retina. This scenario presumably shows the existence of Her4.1, regulated either by Tgf-β or Delta-Notch signaling, as a nodal factor to control MGPCs proliferation. These observations
Figure 9. The Gene Regulatory Network Mediated through Tgf-β/Mmp Regulatory Axis in Different Phases of Retinal Regeneration

The model schematically describes gene regulatory mechanisms of various regeneration-associated factors discovered in this study, along with already reported ones. Activation and repression functions of Tgf-β signaling is through binding of pSmad3 on to SGC and TIE elements, respectively, of various gene promoters. See also Figures S1–S6.

and assumptions suggest the presence of a Tgf-β/Delta-Notch/Her4.1 regulatory network to control the MGPCs proliferation in regenerating retina.

DISCUSSION

Several research studies have elucidated the roles of various genetic and epigenetic factors that are pivotal to retinal regeneration in zebrafish. However, the importance of the evolutionarily conserved Tgf-β signaling remained underexplored. In the present study, we delved into the significance of Tgf-β signaling during various phases of regeneration. Importance of Tgf-β signaling during zebrafish retinal regeneration was intriguing mainly because this pathway was known to be anti-proliferative during neural development (Meyers and Kessler, 2017), cell differentiation (Moustakas et al., 2002), and cancer (Deng et al., 2013; Suzuki et al., 2010). In this study, we wanted to elucidate if the popularly known anti-proliferative function of Tgf-β signaling exists during zebrafish retinal regeneration. This idea is mainly because some developmental pathways such as Delta-Notch signaling behave differently during zebrafish retinal regeneration. Unlike the substantiated pro-proliferative role of the Delta-Notch signaling pathway in various systems (Azizidoost et al., 2015), in the context of zebrafish retinal regeneration, this pathway is known to restrict the zone of proliferation and number of MGPCs (Conner et al., 2014; Wan et al., 2012). Interestingly, we found that during zebrafish retinal regeneration, the Tgf-β signaling is pro-proliferative during initial stages, whereas it is anti-proliferative toward the later stages. The increased number of MGPCs in early TGF-β1 protein-injected conditions were also capable of differentiating into retinal cell types, which were traceable even at 30 dpi, supporting their functionality similar to earlier reports (Ramachandran et al., 2010a; 2012b). In short, at different phases of retinal regeneration, we found opposing roles played by Tgf-β signaling in regulating various transcription activators and repressors. The detailed findings from this study are summarized and organized in a model (Figure 9).

Temporal evaluation of various genes’ expression regulated by Tgf-β signaling gave us important clues about its involvement during zebrafish retinal regeneration, after a mechanical injury damaging all retinal layers equally. Furthermore, the blockade of Tgf-β signaling causing a significant decline in MGPCs formation at 4 dpi, unlike reported in exclusive photoreceptor injury (Lenkowski et al., 2013; Tappeiner et al., 2016), substantiated our hypothesis that Tgf-β signaling is pro-proliferative during retinal regeneration. Apart from this, blockade of Tgf-β signaling caused a substantial decline in various regeneration-associated factors, which confirmed its importance in MGPCs formation. Several of these transcription factors are directly regulated through pSmad3 binding onto their regulatory DNA sequences. Furthermore, the Tgf-β signaling was essential for the regulation of several transcription repressors such as Zebs/Snails and microRNAs, namely, miR-200a, miR-200b, miR-143, and miR-145, that contribute to cellular reprogramming. Notably, Tgf-β signaling could reversibly methylate miR-200 gene promoters to cause their downregulation (Davalos et al., 2012; Gregory et al., 2011). The miR-200 family members target zeb1 and zeb2 mRNAs to block their translation. Zeb1 and Zeb2, in turn, are transcriptional repressors of E-cadherin.

E-cadherin is a molecule that enables cellular adherence, which is essential during MET. Similar results were found with activation of Tgf-β signaling through TGF-β1 protein injection. Therefore, these two sets of experiments involving repression or activation of Tgf-β signaling confirmed that this pathway is essential for adequate MG reprogramming and MGPCs induction during zebrafish retinal regeneration. These results are contrary to what is seen in mice retina after an acute injury wherein no MG reactivity could be found with accelerated or decelerated Tgf-β signaling (Kugler et al., 2015). This scenario could probably be because of the lack of robust regenerative capability in mice retina.

In this study, we found the involvement of Tgf-β signaling to cause the expression of various reprogramming and pro-proliferative transcription factors, which contributed to Müller glia reprogramming. We found a fascinating correlation between Tgf-β signaling and Mmps. Despite the knowledge on the positive feedback regulation of Tgf-β signaling and Mmps (Han et al., 2001; Kim et al., 2007; Krsic and Santibanez, 2014), no mechanism links were established in a regeneration context until now. Our earlier study has reported the importance of Mmp9 during zebrafish retinal regeneration (Kaur et al., 2018). In this study, we
saw a differential regulation of mmp2 and mmp9 by Tgf-β signaling. Tgf-β signaling acted positively on mmp2 expression and showed a negative impact on mmp9 regulation. Interestingly, the Tgf-β signaling-mediated upregulation of mmp2 was essential for the formation of an adequate number of MGPCs. It is also important to note that the downregulation of mmp9 with or without TGF-β1 protein injection also had a negative effect on the MGPCs number, which suggested the importance of Mmp9 similar to that of Mmp2 in translating the Tgf-β signaling during retinal regeneration. These findings indicate the existence of significant gene regulatory events governed by the Tgf-β signaling-Mmp2/Mmp9 axis during retinal regeneration. In spite of having the regular expression of various regeneration-associated factors, in Mmp2/Mmp9 alone or both Mmp2 and Mmp9 activity-deprived retina, there was a significantly lesser number of MGPCs. Furthermore, the absence of Mmp2/Mmp9 significantly abolished MGPCs proliferation in the injured retina either with TGF-β1 protein injection or with overexpression of regeneration-associated genes. These results suggest that various regeneration-associated factors alone may not be able to carry out the entire cascade of events leading to the induction of adequate MGPCs in the absence of Mmp2/Mmp9. These observations strongly support the roles of Mmp2/Mmp9, whose expression is fine-tuned by Tgf-β signaling during retinal regeneration in zebrafish.

We further elucidated the influence of Tgf-β signaling on other parallel pathways, such as interplay with Oct4, as seen in mammalian cellular reprogramming (Radzisheuskaya and Silva, 2014). Oct4 is recently shown to be important in Müller glia reprogramming during retinal regeneration (Sharma et al., 2019). Although Oct4 is known to have adverse effect on the components of Tgf-β signaling (Radzisheuskaya and Silva, 2014; Sharma et al., 2019), recombinant TGF-β1 protein caused an upregulation of oct4 along with a few other regeneration-associated genes such as ascl1a, lin28a, and sox2 in an injured retina, creating an injury-dependent enhancement of MGPCs proliferation. It is also interesting to note that, in the uninjured retina, the Tgf-β1 protein injection was inadequate to cause any proliferative response. However, during retinal regeneration, the Tgf-β signaling is a contributory pathway that keeps the MGPCs proliferation within desired limits. Once turned on, the Tgf-β signaling upregulated Oct4 that in turn could block the components of Tgf-β signaling to potentially establish a regulatory negative feedback loop between Tgf-β signaling and Oct4.

The earlier studies that reported anti-proliferative properties of Tgf-β signaling were based on indirect blockade of this pathway through tgfif or six3b mutants (Lenkowski et al., 2013), along with light-mediated ablation methods that selectively damages photoreceptors. These could be the reasons for not finding a pro-proliferative response in activated Tgf-β signaling conditions and the opposite with its inhibition, as reported in this study. Another study wherein retinal damage was made using N-methyl-N-nitrosourea in fish (Tappeiner et al., 2016) indicated the presence of pSmad3 in the nucleus of MGPCs suggestive of activated Tgf-β signaling in them. In contrast to the fish model, NMDA-mediated retinal damage in the chick (Todd et al., 2017) demonstrated the presence of Smad2/3 in the nucleus of MG, which localizes to cytoplasm after the chemical damage, suggestive of anti-proliferative roles of Tgf-β signaling in avian retina, a model that lacks robust regenerative capacity. Furthermore, the retinal injury made in these studies target specific cell types and probably the cellular damage does not occur uniform across all the three retinal layers. This scenario could have affected the functioning of Tgf-β signaling in those experiments and hence resulted in reduced number of MGPCs in the injured retina, unlike reported in this study. In our study where a needle poke is used to uniformly damage all the three retinal layers, the Tgf-β signaling played a pro-proliferative role on MGPCs in the initial stage and facilitates the cell cycle exit toward the later phase of retinal regeneration. At the early stages of retinal regeneration, the pSmad3 bound to the SGC elements in the promoters of regeneration-associated genes caused an upregulation of their expression. At later stages, Tgf-β signaling switches its activation function on genes that facilitate cell proliferation to their suppression. The NuRD complex is known to repress pluripotency inducing factors during cell differentiation phase of embryonic stem cells (Reynolds et al., 2012) as well as in zebrafish retinal regeneration (Sharma et al., 2019). In the present study also, suppression of pluripotency factors such as oct4, sox2, and lin28a may be because of activated NuRD complex in the late phase of retinal regeneration. The downregulation of ascl1a could be regulated by Sox2, which is known to positively regulate ascl1a and lin28a genes during the zebrafish retinal regeneration (Gorsuch and Hyde, 2014). Apart from this, the TIE sequences present on oct4 and lin28a promoter sequences bound with pSmad3 at 4 dpi but not at 3 hpi, supporting a mechanistic involvement of Tgf-β signaling in gene repression towards later stages of retinal regeneration. It is interesting to note that late induction of Tgf-β signaling through TGF-β1 protein injection caused a fresh proliferative response in the retina probably through reprogramming of MG that did not participate in the earlier injury response.
Furthermore, the late inhibition of Tgf-β signaling is associated with decreased her4.1, a situation similar to blockade of the Delta-Notch signaling using DAPT. Inhibition of the Delta-Notch signaling and associated decrease in her4.1 is deemed to enhance both the number and the zone of MGPCs proliferation in regenerating retina (Kaur et al., 2018; Mitra et al., 2019; Wan et al., 2012). Notably, (1) the decreased her4.1 in DAPT-treated condition probably enhances Tgf-β signaling component genes and (2) the double blockade of both Tgf-β and Delta-Notch signaling caused an increase in MGPCs proliferation. Based on these, our finding supports the view that the Tgf-β signaling acts upstream or parallel to Delta-Notch signaling to regulate her4.1 and influence MGPCs proliferation. In conclusion, our study opens up new vistas of exploration in similar lines that would enable designing therapeutic strategies to cure mammalian retinal blindness.

Limitations of the Study
Absence of well-characterized Müller glia specific dedifferentiation markers during the immediate-early phase of retinal regeneration limited a few of our experimental explorations.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
R.R. conceived the study and designed experiments. P.S. performed full sets of experiments. S.G., M.C., S.M. gave critical inputs in some experiments and also in the preparation of the manuscript. M.A.K. designed a few primers. B.C. made a few gene clones. N.K.S. did some cell counting.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Biphasic Role of Tgf-β Signaling
during Müller Glia Reprogramming
and Retinal Regeneration in Zebrafish

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Figure S1

A 4dpi

PCNA

*

GFP

ONL

INL

GCL

DMSO

SB431542

(40 μM)

(20 μM)

4dpi

B 4dpi

GFP

ONL

INL

GCL

DMSO

C 4dpi

DMISO

GFP

PCNA

merge

SB431542

(40 μM)

(20 μM)

4dpi

D 4dpi

GFP+ cells at 4dpi

DMSO

SB431542

E 4dpi

DMISO

oct4

ascl1a

sox2

lin28a

GFP

ONL

INL

GCL

SB431542

(40 μM)
Figure S1. Effect of blockade of Tgf-β signaling on cell proliferation and gene expression. Related to Figure 1. (A,B) IF microscopy images of retinal cross sections showing the entire retina with PCNA+ MGPCs in wild-type (A), and GFP+ ones in 1016tuba1a:GFP transgenic retina at 4dpi. The box around the cells represents the part of the retinal image that is used in rest of the figures. (C,D) IF microscopy images of retinal cross sections show a SB431542 dose-dependent decrease in GFP+ and PCNA+ MGPCs in 1016tuba1a:GFP transgenic retina during regeneration at 4dpi (C), which is quantified (D), *P<0.0002, N=4. (E) BF microscopy images of retinal cross sections show the SB431542 dose-dependent decline in expression of oct4, ascl1a, sox2 and lin28a mRNAs as revealed by mRNA in situ hybridization. Error bars are s.d., Scale bars, 10 µm (A,B,C,E). The asterisk marks the injury site in (A,B,C,E). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (B,C,E).
Figure S2

A

PCNA
BrdU
TGF-β1 Protein (40 ng/ml)-
Uninjured retina
4 days post delivery
C
GCL
ONL
INL
A

GFP
PCNA
merge

TGF-β1 (20 ng/ml) (40 ng/ml)

DMSO
4 dpi

B

GFP+ cells at 4 dpi

D

ONL
INL
GCL

PCNA

merge

TGF-β1 (ng/ml)

B

Water

10
20
40

SB431542 (μM)

D

asc11a promoter activity

50
100
150
200

asc11a promoter activity

E

oct4 promoter activity

0
0.2
0.4
0.6
0.8
1.0

oct4 promoter activity

F

TGF-β1 (ng/ml)

H

TGF-β1

4 dpi

PBS

asc11a

lin28a

sox2

oct4
Figure S2. Effect of TGF-β1 protein on the MGPCs of 1016tuba1a:GFP fish and gene expressions in injured retina, and various gene promoter activity assays in zebrafish embryos. Related to Figure 3. (A,B) IF microscopy images of retinal cross sections show a TGF-β1 protein dose-dependent increase in GFP+ and PCNA+ MGPCs in 1016tuba1a:GFP transgenic retina during retina regeneration at 4dpi (A), which is quantified (B), *P<0.003, N=4. (C) IF microscopy images of uninjured retinal cross section showing no BrdU or PCNA positive cells even on 4th day of TGF-β1 protein delivery through the cornea. (D,E) Zebrafish embryonic luciferase assays reveal the downregulation of asc11a (D), *P<0.04, N=3, and oct4 (E), *P<0.03, N=3, promoter activity because of blockade of Tgf-β signaling using SB431542. (F,G) Upregulation of asc11a (F), *P<0.005, N=3, and oct4 (G), *P<0.002, N=3 promoter activity in zebrafish embryos injected with TGF-β1 protein. (H) BF microscopy images of retinal cross sections show the TGF-β1 protein dose-dependent increase in the expression of asc11a, lin28a, sox2 and oct4 mRNAs as revealed by mRNA in situ hybridization. Error bars are s.d., Scale bars, 10 µm (A,C,H). The asterisk marks the injury site in (A,H). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (A,C,H).
Figure S3

A) Injury + TGF-β1

| Time (dpi) | PBS | Water | BrdU | Water |
|-----------|-----|-------|------|-------|
| 0         |     |       |      |       |
| 3         |     |       |      |       |
| 5         |     |       |      |       |
| 30        |     |       |      |       |

B) Normalized number of BrdU cell types

- PBS-Control
- TGF-β1 (40ng)

C) 4dpi

- PBS
- TGF-β1 (20 ng/ml)
- TGF-β1 (40 ng/ml)

| PBS | TGF-β1 (20 ng/ml) | TGF-β1 (40 ng/ml) |
|-----|-------------------|-------------------|
| mmp9 |                   |                   |
| PCNA |                   |                   |
| merge |                  |                   |

D) 4dpi

- DMSO
- SB431542 (20μM)
- SB431542 (40μM)

| DMSO | SB431542 (20μM) | SB431542 (40μM) |
|------|-----------------|-----------------|
| mmp9 |                 |                 |
| PCNA |                 |                 |
| merge |                |                 |

E) mmp9 mRNA levels

F) mmp2 promoter activity

G) mmp2 promoter activity

- SB431542 (μM)
- TGF-β1 (ng/ml)
Figure S3. Cell-fate determination in TGF-β1 protein injected retina, expression dynamics of various Tgf-β signaling-dependent genes, and mmp2 promoter activity assays. Related to Figure 1, Figure 3 and Figure 4. (A) An experimental timeline that describes the injury, TGF-β1 protein injection, BrdU delivery, and retina harvest at 30dpi. (B) IF microscopy images of retinal cross sections show BrdU+ cells that co-labeled with retinal cell-types of Müller glia (Glutamine synthetase), Amacrine cells (HuC/D) and bipolar cells (PKC-β1) in TGF-β1 protein injected retina at 30dpi. White arrowheads mark BrdU+ retinal cell types, which is quantified (B’), *P<0.02, N=3. (C,D) BF microscopy images of retinal cross sections showing the decreased mmp9 expression and increased PCNA+ cells with TGF-β1 protein-treatment (C), and the opposite was seen with SB431542 exposure (D), as revealed by in situ hybridization and immunofluorescence. (E) qPCR analysis of mmp9 mRNA levels based on experiments from (D) *P<0.0002, N=3. (F,G) Downregulation because of blockade of Tgf-β signaling using SB431542 (F), *P<0.0004, N=3, and upregulation with TGF-β1 protein injections (G), of mmp2 promoter activity, revealed in luciferase assay done in zebrafish embryos. Error bars are s.d., Scale bars, 10 µm (B-D). The asterisk marks the injury site in (B-D). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (B-D).
Figure S4

A

Control MO (0.5mM), 4dpi

PBS

TGF-β1 (ng/ml)

10 20 40

* n.s

mmp2 MO (0.5mM), 4dpi

B

PCNA positive cells

PBS, 4dpi

TGF-β1 (ng/ml)

10 20 40

C

TUNEL

DAPI

merge

mmp9 MO (0.5mM), 4dpi

TGF-β1 (40ng/ml), 4dpi

PBS, 4dpi

D

BrdU + cells

PBS, 4dpi

TGF-β1

mmp9 MO

(0.5mM), 4dpi

4dpi

E

TUNEL

DAPI

merge

mmp9 MO

(0.5mM), 4dpi

4dpi

* n.s
Figure S4. Cell proliferation and TUNEL assay in mmp2/mmp9 knockdown, along with TGF-β1 protein injection. Related to Figure 4 and Figure 5. (A,B) IF microscopy images of retinal cross sections show PCNA+ MGPCs along with standard control morpholino (MO) or mmp2 MO injected with TGF-β1 protein during retina regeneration at 4dpi (A), which is quantified (B), *P<0.0002, n.s-not significant, N=4. Lissamine is the fluorescent tag on MOs. (C) IF microscopy images of retinal cross sections show BrdU+ MGPCs along with TGF-β1 protein and mmp9 MO during retina regeneration at 4dpi, which is quantified (D), P<0.003, N=4. (E) Cell death analysis through TUNEL assay in control MO, mmp2 MO and mmp9 MO electroporated conditions, do not reveal an increase in apoptotic cells compared to control MO electroporated retina. Error bars are s.d., Scale bars, 10 µm (A,C,E). The asterisk marks the injury site in (A,C,E). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (A,C,E). n.s-not significant in (B,D).ctl MO-Control morpholino in (B).
Figure S5

**A**

Relative mRNA levels as assessed by qPCR at 16hpi with different MO concentrations:
- **Ctl MO**
- **mmp2 MO (0.25mM)**
- **mmp2 MO (0.5mM)**
- **mmp2 MO (1mM)**

| Gene   | Ctrl MO | mmp2 MO (0.25mM) | mmp2 MO (0.5mM) | mmp2 MO (1mM) |
|--------|---------|------------------|-----------------|---------------|
| tgfbi  |         |                  |                 |               |
| ascl1a |         |                  |                 |               |
| lin28a |         |                  |                 |               |
| oct4   |         |                  |                 |               |
| sox2   |         |                  |                 |               |
| mmp2   |         |                  |                 |               |
| mmp9   |         |                  |                 |               |

**B**

Relative mRNA levels as assessed by qPCR at 16hpi with different MO concentrations:
- **Ctl MO**
- **mmp9 MO (0.25mM)**
- **mmp9 MO (0.5mM)**
- **mmp9 MO (1mM)**

**C**

Immunofluorescence images at 4dpi for different treatments:
- **Control MO**
- **mmp2 MO (0.5mM)**
- **mmp2 MO (1mM)**

**D**

Bar graph showing BrdU+ cells per injury at 8dpi with different MO concentrations:
- **Ctl MO**
- **mmp2 MO (0.25mM)**
- **mmp2 MO (0.5mM)**

**E**

Timeline of BrdU and EdU pulse at 3 hours before harvest:
- 0dpi
- 5dpi (BrdU pulse)
- 8dpi (EdU Pulse)

**F**

Immunofluorescence images for different TGF-β1 concentrations (ng/ml):
- **PBS**
- **20 ng/ml**
- **40 ng/ml**

**G**

Bar graph showing BrdU & EdU+ cells at 8dpi with different TGF-β1 concentrations (ng/ml):
- **PBS**
- **20 ng/ml**
- **40 ng/ml**

* ns: not statistically significant
Figure S5. Cell proliferation assay and expression analysis of various genes in mmp2/mmp9 downregulated conditions, and effect of late injection of TGF-β1 protein in regenerating retina. Related to Figure 4, Figure 5 and Figure 7. (A,B) The qPCR analyses of tgfbi, ascl1a, lin28a, sox2, oct4, mmp2 and mmp9 genes in mmp2 MO (A) and mmp9 MO (B) electroporated retina at 16hpi, *P<0.03, N=3. (C,D) IF microscopy images of retinal cross sections show the decline in BrdU+ MGPCs with increasing concentrations of mmp2 and mmp9 MO in combination (C) at 4dpi, which is quantified (D), *P<0.003, N=4. Lissamine is the fluorescent tag on MOs. (E) An experimental timeline that describes the injury, BrdU pulse 5 hours prior to late TGF-β1 protein-injection to the retina, and EdU pulse 3 hours before harvest at 8dpi. (F,G) IF microscopy images of retinal cross sections show marginal increase in BrdU+ MGPCs at 8dpi in TGF-β1 protein-injected retina from 5th day onwards, and a proof of fresh cells entering cell cycle revealed by EdU+ cells that do not have BrdU label (F), which is quantified (G), *P<0.02, N=4. n.s-not significant in (B,G). White arrowheads mark BrdU- but EdU+ cells in (F). ctl MO-Control morpholino in (A,B,D).
Figure S6
Figure S6. Expression analysis of various genes in Delta-Notch signaling-inhibited condition. Related to Figure 8. (A) BF microscopy images of retinal cross sections show the DAPT-dependent increase in the expression levels of *ascl1a*, *lin28a*, *sox2*, and decrease in that of *oct4*, as revealed by mRNA *in situ* hybridization at 4dpi. Scale bars, 10 µm (A). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (A).
**Transparent Methods**

**Animal husbandry, retinal injury and pharmacological agents.**
Zebrafish (Danio rerio) were maintained on a 14:10-hour, light: dark cycle at 26-28.5 °C in circulating aquatic habitats. The 1016 tuba1a:GFP transgenic fish used in this study have been characterized previously (Fausett and Goldman, 2006). Zebrafish embryos used for microinjection in Luciferase assays were obtained by natural breeding of wild type fish. For retinal injury, 6-12 months old zebrafish were anaesthetized with Tricaine methanesulfonate and mechanical injury was made with a 30G needle, as described previously (Fausett and Goldman, 2006). All zebrafish injury experiments were performed in anesthetized conditions using tricaine methanesulphonate and euthanized by deep anesthesia in the terminal phase of the experiment according to the recommended protocols. All experiments were carried out on animals according to established and accepted protocols of institutional biosafety and ethics committees. All animal experiments were approved by Institute Animal Ethical Committee (IAEC).

Pharmacological inhibitors and TGF-β1 protein were delivered by injecting into the eye using a Hamilton syringe of 10μL capacity. All the drugs namely, TGF-β signaling inhibitor, SB431542 and pirfenidone; Mmp2/Mmp9 blocker, SB-3CT; Notch signaling blocker, N-[N-(3,5-difluorophenylacetyl)-L-alanyl]- S-phenylglycine t-butyL ester (DAPT), were from Sigma-Aldrich and their stock of 1mM were made in DMSO for all experiments. TGF-β1 protein (Abcam, Catalogue number ab50036) was made to a stock of 4mg/ml in 10mM citric acid. Working concentrations of TGF-β1 protein in ng/ml, were made in PBS, while that of other drugs to a final concentration of μM, were made in autoclaved deionized water. All the drugs and protein, used in experimental setups were injected into the vitreous at the time of injury, unless mentioned specifically.

**Primers and plasmid construction.**
All primers used in study are listed in Supplementary Table S1. The promoters of mmp2, miR-200a/b and oct4 were amplified from zebrafish genomic DNA using their respective primer pairs, HindIII-mmp2-P-F, XhoI-miR-200a/b-P-F, EcoRI-oct4-P-F and AgeI-mmp2-P-R, BamHI-miR-200a/b-P-R, AgeI-oct4-P-R (~3 kb). The digested PCR amplicons were cloned into a pEL luciferase expression vector to create mmp2:GFP-luciferase, miR-200a/b:GFP-luciferase and oct4:GFP-luciferase constructs. The ascl1a:GFP-luciferase construct was described previously (Ramachandran et al., 2012). Coding regions (CDS) of ascl1a, oct4, lin28a, sox2, mmp2 and mmp9, were cloned by PCR amplification of complementary DNA, prepared from RNA of 24 hours post fertilization zebrafish embryos, using primer pairs given in cloning primers list (Supplementary Table S1). Post-digestion, PCR amplicons were cloned in pCS2+ plasmid and linearized with NotI/KpnI (NEW ENGLAND Biolabs, R3189/R3142) for *in vitro* mRNA transcription using Sp6 RNA polymerase (mMESSAGE mMACHINE SP6 Transcription kit, Thermo Fisher Scientific, AM1340). Full CDS clones of ascl1a, sox2, and mmp9, were obtained by TOPO cloning in pCR2.1 TOPO vector (Thermo Fisher Scientific) for making RNA probes.

**Embryo micro-injection and ChIP assay.**
For luciferase assay experiments, single-cell zebrafish embryos were injected with a total volume of ~1nl solution containing 0.02 pg of Renilla luciferase mRNA.
(normalization), 5 pg of promoter-GFP-luciferase vector. TGF-β1 protein was injected along with the above mixture in respective experiments, while embryos were exposed to SB431542 solution in 6-well plates. To assure consistency of results, a master mix was made for daily injections and ~300 embryos were injected at single cell stage. 24 hours later, embryos were divided into 3 groups (~ 70 embryos/group) and lysed for dual luciferase reporter assays (Promega, catalogue number E1910).

Chromatin immunoprecipitation (ChIP) assays were done in adult retina at different time points using ~20 adult retinas after dark adaptation. Chromatin was isolated as described previously (Lindeman et al., 2009). After sonication, a part of chromatin was kept as input and remaining was divided into two halves. A part of which was pulled down with anti-pSmad3 antibody (described below), while the other half was pulled down with Rabbit IgG (Sigma Aldrich, I5006) as negative control. Primers used for ChIP assays are described in Supplementary Table S1.

**Morpholino electroporation and in vivo mRNA transfection.**

Lissamine-tagged MOs (Gene Tools) of approximately 0.5 µl (0.25 to 1.0 mM) was injected at the time of injury using a Hamilton syringe of 10 µl volume capacity. MO delivery to cells was accomplished by electroporation as previously described (Fausett et al., 2008). The sequence of control MO has been previously described (Wan et al., 2012). Morpholinos targeting mmp2 and mmp9 are:

- mmp2: 5'-ATCATGCTGGTCCGATTCCGAGATG-3'
- mmp9: 5'-AACGCCAGGACTCCAAGTCTCATCT-3'

In vivo overexpression of ascl1a, oct4, lin28a, sox2, mmp2 and mmp9, is achieved by mRNA transfection in retina. For this transfection reagent containing mRNA and lipofectamine in combination with 2X HBSS was prepared as described previously (Mitra et al., 2018; Sharma et al., 2019). Around 1 µl of this transfection mixture was injected into the retina either alongside injury (ascl1a, oct4, lin28a, sox2) or through cornea for late (mmp2 and mmp9) overexpression experiments. Transfection mixture containing GFP mRNA was injected parallelly into the control retinae. mRNA injection was followed by electroporation with five pulses at 70v of 50 milliseconds duration with a gap period of 950 milliseconds between the pulses.

**RNA isolation, RT-PCR and qPCR.**

Total RNA was isolated from dark-adapted zebrafish retinas of control, injured and drug treated/MO electroporated groups, using TRIzol (Invitrogen). A combination of oligo-dT and random hexamers were used to reverse transcribe approximately 4µg of RNA using RevertAid (Thermo Fischer Scientific, K1622) or SuperScriptIII (Thermo Fischer Scientific, 18080051) First Strand cDNA synthesis kit to generate cDNA. PCR reactions used Taq or GoTaq (Promega, M4021) DNA polymerase and gene-specific primers (Supplementary Table S1) with previously described cycling conditions (Ramachandran et al., 2010a). Quantitative PCR (qPCR) was carried out in triplicate with Power SYBR Green Master mix (Thermo Fischer Scientific, 4367659) on a real-time PCR detection system (Eppendorf MasterCycler RealPlex4). The let-7a miRNA levels were determined with Taqman hsa-let7-a probe (Applied Biosystems) as per manufacturer’s instructions. The relative mRNA levels in control and injured retinas were normalized to β-actin.

**Western blotting and antibodies.**

Western blotting was performed using 6 retinas per experimental sample, lysed in
Laemmli buffer, size fractioned in 12% denaturing acrylamide gel before transferring on to Immuno-Blot PVDF membrane (Biorad, 162-0177), followed by probing with specific primary antibodies and HRP-conjugated secondary for chemiluminescence assay using Clarity Western ECL (Biorad, 170-5061). Primary antibodies and secondary antibody used have been previously described (Mitra et al., 2018; Mitra et al., 2019). Other primary antibodies used in study are, Rabbit polyclonal antibody against Oct4 (Merck, AB3209); Rabbit polyclonal antibody against Sox2 (Abcam, ab59776); Rabbit polyclonal antibody against Lin28a (Cell Signalling Technologies, 3978), Rabbit polyclonal antibody against pSmad3 (Abcam, ab52903), Rabbit monoclonal antibody against Tgfbi (Abcam, ab170874).

**BrdU/EdU labeling, tissue preparation, Immunofluorescence, TUNEL assay and mRNA in situ hybridization.**

BrdU labeling was performed by intraperitoneal injection of 20 µl BrdU solution (20 mM), 3 h before euthanasia and retina dissection, unless mentioned specifically. EdU labelling was done by intravitreal injection of 10mM EdU solution as described earlier (Mitra et al., 2018; Mitra et al., 2019). Fish were given higher dose of tricaine methane sulphonate and eyes were dissected, lens removed, fixed in 4% paraformaldehyde and sectioned as described previously (Fausett and Goldman, 2006). TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) was done with commercially available kit (In situ cell death detection kit, Fluorescein, Roche Diagnostics, 11684795910) following manufacturer’s instructions. The mRNA in situ hybridization (ISH) was performed on retinal sections with fluorescein or digoxigenin-labelled complementary RNA probes (FL/DIG RNA labeling kit, Roche Diagnostics), as described previously (Barthel and Raymond, 2000). Immunofluorescence protocols and antibodies were previously described (Mitra et al., 2019; Ramachandran et al., 2010b; Wan et al., 2012). EdU staining was performed with Click-iT EdU Alexa Fluor 647 Imaging kit (Thermo Fischer Scientific, C10085), following manufacturer’s instructions.

**Microscopy, cell counting and statistical analysis**

After the completion of staining experiments, the slides were examined with a Nikon Ni-E fluorescence microscope equipped with fluorescence optics and Nikon A1 confocal imaging system. The PCNA+ and BrdU+ cells were counted by observation of their fluorescence in retinal sections. A minimum of three retinas were used per experiment and all the proliferating cells at each injury spot were counted. In case of TGF-β1 and DAPT treated retinas, where proliferation increases with a large retinal span, all the cells per injury spot in total span were counted. Proliferating cells in all the retinal sections per injury site were taken into consideration for calculation of average and standard deviation. Data obtained were analyzed for statistical significance, wherever applicable, by comparisons done using a two-tailed unpaired Student’s t-test to analyze data from all experiments. Error bars represent s.d in all histograms.

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