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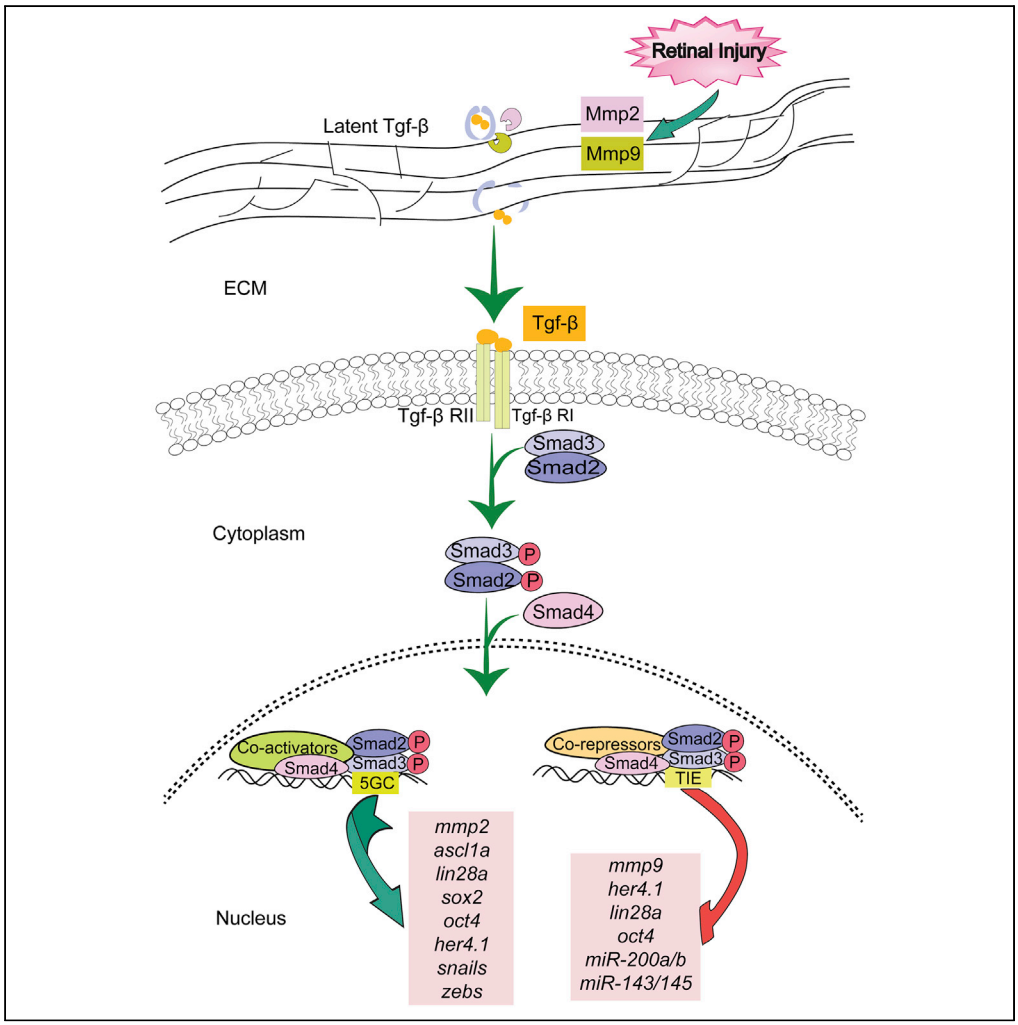
Biphasic Role of Tgf-β Signaling during Müller Glia Reprogramming and Retinal Regeneration in Zebrafish



Poonam Sharma, Shivangi Gupta, Mansi Chaudhary, ..., Mohammad Anwar Khursheed, Navnoor Kaur Saran, Rajesh Ramachandran

rajeshra@iisermohali.ac.in

HIGHLIGHTS

Tgf-β signaling is essential for retinal progenitor proliferation and cell cycle exit

pSmad3 binds to 5GC and TIE elements to cause gene activations and repressions

Tgf-β signaling regulates Zebs and various miRNAs for cellular reprograming

Translation of Tgf-β signaling requires Mmp2/Mmp9 activity

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

Poonam Sharma,[1] Shivangi Gupta,[1,2] Mansi Chaudhary,[1,2] Soumitra Mitra,[1,2] Bindia Chawla,[1] Mohammad Anwar Khursheed,[1] Navnoor Kaur Saran,[1] and Rajesh Ramachandran[1,3,*]

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,

[1]Department of Biological Sciences, Indian Institute of Science Education and Research, Mohali, Knowledge City, SAS Nagar, Sector 81, Manauli PO, Mohali, Punjab 140306, India

[2]These authors contributed equally

[3]Lead Contact

*Correspondence: rajeshra@iisermohali.ac.in

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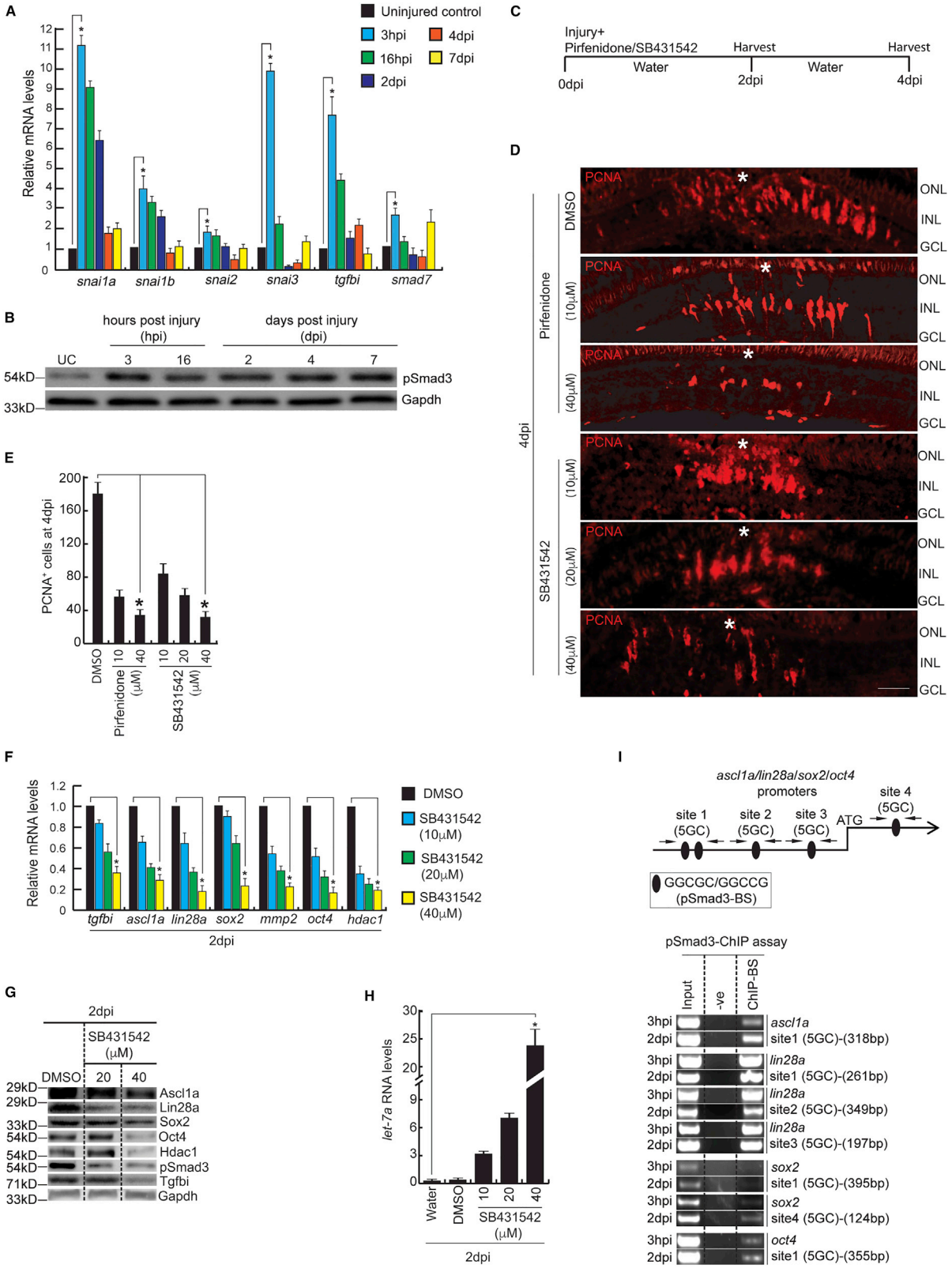

A
Uninjured control
3hpi
4dpi
16hpi
7dpi
2dpi
Relative mRNA levels
snai1a
snai1b
snai2
snai3
tgfbi
smad7
B
hours post injury (hpi)
days post injury (dpi)
UC
3
16
2
4
7
54kD
pSmad3
33kD
Gapdh
C
Injury+ Pirfenidone/SB431542
Harvest
Harvest
Water
Water
0dpi
2dpi
4dpi
D
4dpi
Pirfenidone
SB431542
DMSO
(10μM)
(40μM)
(10μM)
(20μM)
(40μM)
PCNA
ONL
INL
GCL
E
PCNA+ cells at 4dpi
DMSO
10
40
10
20
40
Pirfenidone (μM)
SB431542 (μM)
F
Relative mRNA levels
tgfbi
ascl1a
lin28a
sox2
mmp2
oct4
hdac1
2dpi
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
G
2dpi
SB431542 (μM)
DMSO
20
40
29kD
29kD
33kD
54kD
54kD
54kD
71kD
33kD
Ascl1a
Lin28a
Sox2
Oct4
Hdac1
pSmad3
Tgfbi
Gapdh
H
let-7a RNA levels
Water
DMSO
10
20
40
SB431542 (μM)
2dpi
I
ascl1a/lin28a/sox2/oct4 promoters
site 1 (5GC)
site 2 (5GC)
site 3 (5GC)
site 4 (5GC)
ATG
GGCGC/GGCCG (pSmad3-BS)
pSmad3-ChIP assay
Input
-ve
ChIP-BS
3hpi
2dpi
ascl1a site1 (5GC)-(318bp)
lin28a site1 (5GC)-(261bp)
lin28a site2 (5GC)-(349bp)
lin28a site3 (5GC)-(197bp)
sox2 site1 (5GC)-(395bp)
sox2 site4 (5GC)-(124bp)
oct4 site1 (5GC)-(355bp)

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 , *p < 0.0001, N = 4.

(I) The *ascl1a/lin28a/sox2/oct4* promoter schematic reveals the typical 5GC sites (upper), and the retinal ChIP assays confirm the physical binding of pSmad3 at the 5GC 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 Mmp/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, 2015), mesenchymal differentiation (Grafe 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 *snail* 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

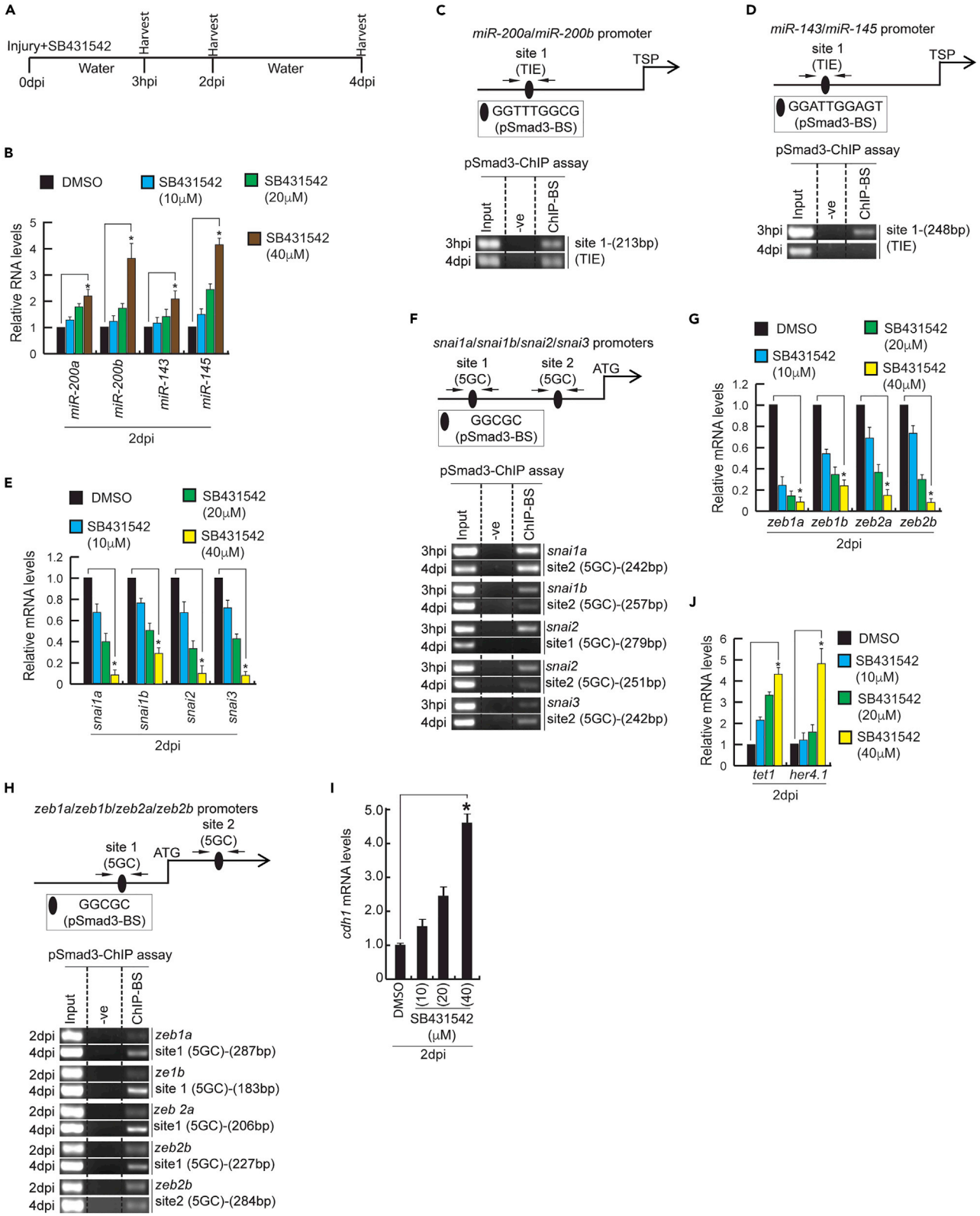

A
Injury+SB431542
Harvest
Harvest
Harvest
Water
Water
0dpi
3hpi
2dpi
4dpi
B
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
Relative RNA levels
miR-200a
miR-200b
miR-143
miR-145
2dpi
C
miR-200a/miR-200b promoter
site 1 (TIE)
TSP
GGTTTGGCG (pSmad3-BS)
pSmad3-ChIP assay
Input
-ve
ChIP-BS
3hpi
4dpi
site 1-(213bp) (TIE)
D
miR-143/miR-145 promoter
site 1 (TIE)
TSP
GGATTGGAGT (pSmad3-BS)
pSmad3-ChIP assay
Input
-ve
ChIP-BS
3hpi
4dpi
site 1-(248bp) (TIE)
E
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
Relative mRNA levels
snai1a
snai1b
snai2
snai3
2dpi
F
snai1a/snai1b/snai2/snai3 promoters
site 1 (5GC)
site 2 (5GC)
ATG
GGCGC (pSmad3-BS)
pSmad3-ChIP assay
Input
-ve
ChIP-BS
3hpi
4dpi
snai1a site2 (5GC)-(242bp)
snai1b site2 (5GC)-(257bp)
snai2 site1 (5GC)-(279bp)
snai2 site2 (5GC)-(251bp)
snai3 site2 (5GC)-(242bp)
G
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
Relative mRNA levels
zeb1a
zeb1b
zeb2a
zeb2b
2dpi
H
zeb1a/zeb1b/zeb2a/zeb2b promoters
site 1 (5GC)
site 2 (5GC)
ATG
GGCGC (pSmad3-BS)
pSmad3-ChIP assay
Input
-ve
ChIP-BS
2dpi
4dpi
zeb1a site1 (5GC)-(287bp)
ze1b site 1 (5GC)-(183bp)
zeb 2a site1 (5GC)-(206bp)
zeb2b site1 (5GC)-(227bp)
zeb2b site2 (5GC)-(284bp)
I
cdh1 mRNA levels
DMSO
(10)
(20)
(40)
SB431542 (μM)
2dpi
J
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
Relative mRNA levels
tet1
her4.1
2dpi

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 RNA 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 5GC sites (upper), and the retinal ChIP assays confirm the physical binding of pSmad3 at the 5GC 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).

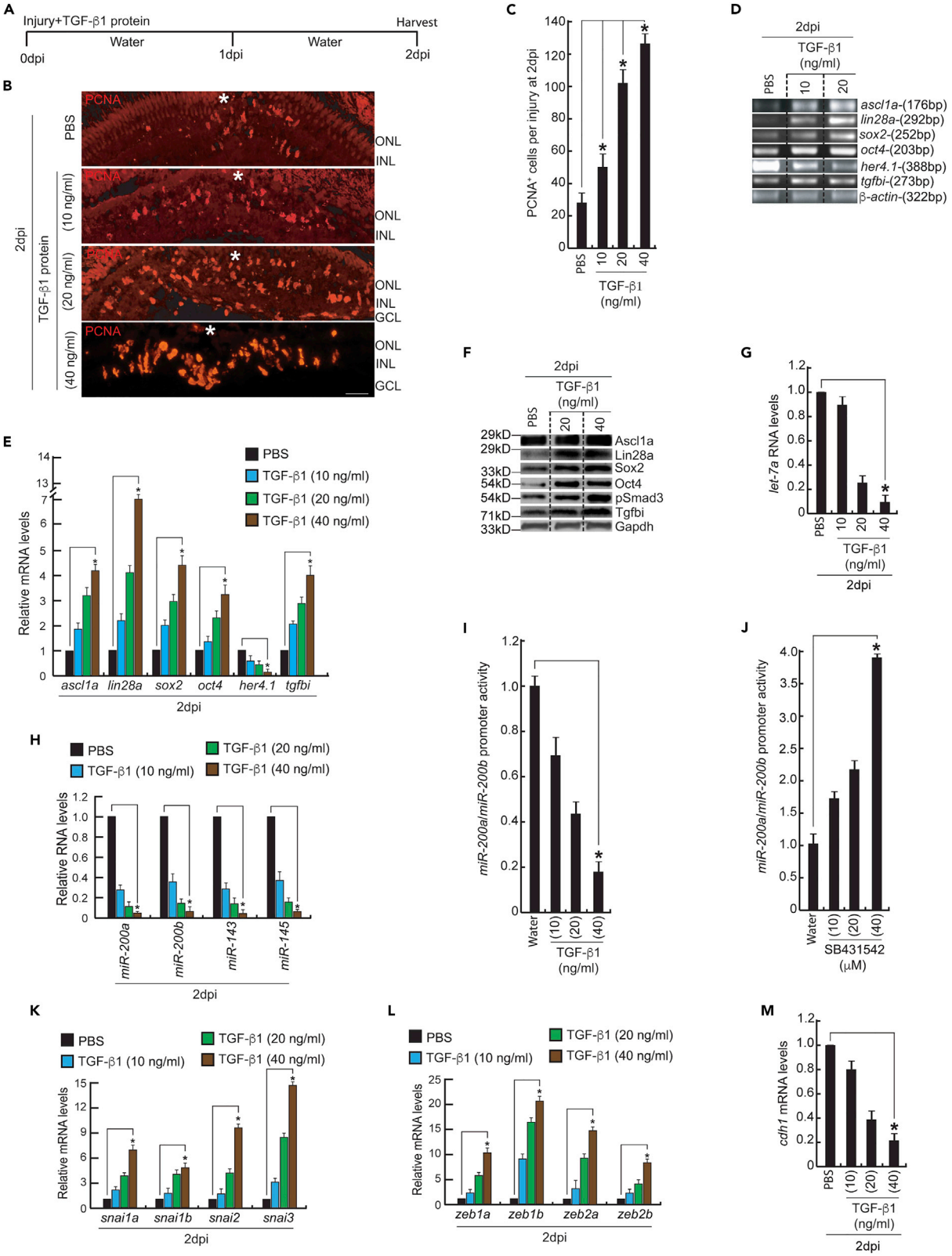
See also Table S1.

after injury using two different pharmacological inhibitors, namely, SB431542 and Pirfenidone (Halder et al., 2005; Stahnke et al., 2017). A representative portion of the injured retina is displayed in all relevant images for marking the injury and regenerative response measured in terms of $PCNA^+$ cells from wild-type fish (Figure S1A) or GFP^+ cells from *1016tuba1a*:GFP transgenic retina (Figure S1B). We found a concentration-dependent decline in the number of MGPCs in the post-injured retina with both these drugs at 4 days post injury (dpi) (Figures 1C–1E). Similarly, we also found a decrease in the number of GFP^+ cells in the *1016tuba1a*:GFP transgenic retina, which marks the actively proliferating MGPCs, at 4 dpi (Fausett and Goldman, 2006), in SB431542 concentration-dependent manner (Figures S1C and S1D).

Furthermore, the gene expression analysis of various regeneration-associated genes such as *ascl1a*, *lin28a*, *sox2*, *mmp2*, *oct4*, *hdac1*, and *tgfbi* 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, Hdac1, pSmad3, and Tgfbi 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 translatability 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)/CG sequence, also known as 5GC elements. The occupancy of 5GC by pSmad3 is essential for its transcriptional activation function (Martin-Malpartida et al., 2017). We then explored the 5GC-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 5GC sites in 3 hpi and 2 dpi retinal extracts revealed the occupancy of pSmad3 at these sites (Figure 1I). 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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Water
0dpi
1dpi
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PCNA
PBS
(10 ng/ml)
(20 ng/ml)
(40 ng/ml)
TGF-β1 protein
2dpi
ONL
INL
GCL
C
PCNA+ cells per injury at 2dpi
PBS
10
20
40
TGF-β1 (ng/ml)
D
2dpi
TGF-β1 (ng/ml)
PBS
10
20
ascl1a-(176bp)
lin28a-(292bp)
sox2-(252bp)
oct4-(203bp)
her4.1-(388bp)
tgfbi-(273bp)
β-actin-(322bp)
E
PBS
TGF-β1 (10 ng/ml)
TGF-β1 (20 ng/ml)
TGF-β1 (40 ng/ml)
Relative mRNA levels
ascl1a
lin28a
sox2
oct4
her4.1
tgfbi
2dpi
F
2dpi
TGF-β1 (ng/ml)
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29kD
33kD
54kD
54kD
71kD
33kD
Ascl1a
Lin28a
Sox2
Oct4
pSmad3
Tgfbi
Gapdh
G
let-7a RNA levels
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20
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TGF-β1 (ng/ml)
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TGF-β1 (10 ng/ml)
TGF-β1 (20 ng/ml)
TGF-β1 (40 ng/ml)
Relative RNA levels
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miR-200b
miR-143
miR-145
2dpi
I
miR-200a/miR-200b promoter activity
Water
(10)
(20)
(40)
TGF-β1 (ng/ml)
J
miR-200a/miR-200b promoter activity
Water
(10)
(20)
(40)
SB431542 (μM)
K
PBS
TGF-β1 (10 ng/ml)
TGF-β1 (20 ng/ml)
TGF-β1 (40 ng/ml)
Relative mRNA levels
snai1a
snai1b
snai2
snai3
2dpi
L
PBS
TGF-β1 (10 ng/ml)
TGF-β1 (20 ng/ml)
TGF-β1 (40 ng/ml)
Relative mRNA levels
zeb1a
zeb1b
zeb2a
zeb2b
2dpi
M
cdh1 mRNA levels
PBS
(10)
(20)
(40)
TGF-β1 (ng/ml)
2dpi

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 assayed 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 *SNAI1* (SNAIL), 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 silico* 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 *zeb*s 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 *zeb* family genes (Figure 2G), whose promoters also had 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 upregulated 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-β 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-β 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-β 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

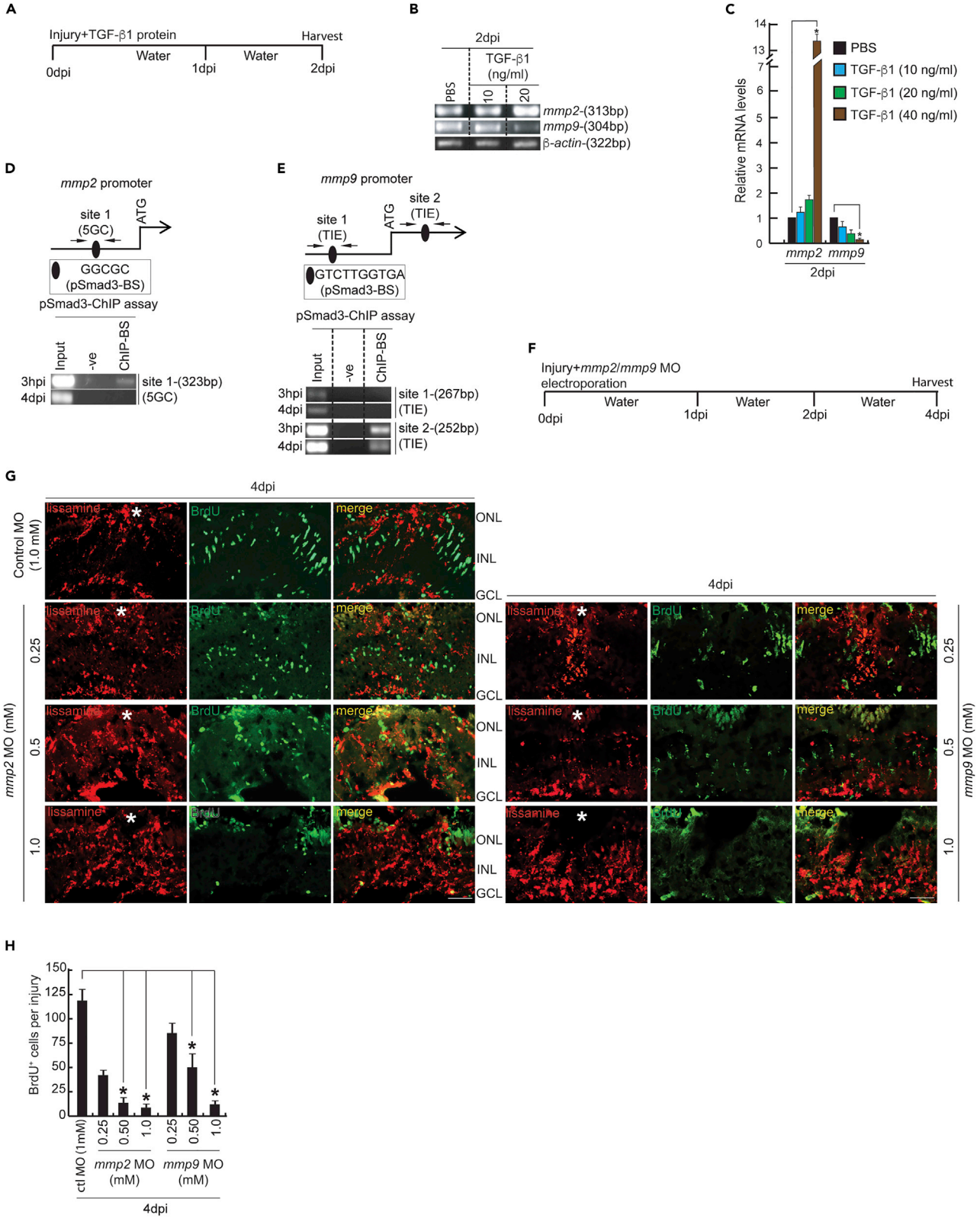
A
Injury+TGF-β1 protein
Harvest
Water
Water
0dpi
1dpi
2dpi
B
2dpi
TGF-β1 (ng/ml)
PBS
10
20
mmp2-(313bp)
mmp9-(304bp)
β-actin-(322bp)
C
Relative mRNA levels
PBS
TGF-β1 (10 ng/ml)
TGF-β1 (20 ng/ml)
TGF-β1 (40 ng/ml)
mmp2
mmp9
2dpi
D
mmp2 promoter
site 1 (5GC)
ATG
GGCGC (pSmad3-BS)
pSmad3-ChIP assay
Input
-ve
ChIP-BS
3hpi
4dpi
site 1-(323bp) (5GC)
E
mmp9 promoter
site 1 (TIE)
site 2 (TIE)
ATG
GTCTTGGTGA (pSmad3-BS)
pSmad3-ChIP assay
site 1-(267bp) (TIE)
site 2-(252bp) (TIE)
F
Injury+mmp2/mmp9 MO electroporation
Harvest
Water
Water
Water
0dpi
1dpi
2dpi
4dpi
G
4dpi
Control MO (1.0 mM)
mmp2 MO (mM)
mmp9 MO (mM)
0.25
0.5
1.0
lissamine
BrdU
merge
ONL
INL
GCL
H
BrdU+ cells per injury
ctl MO (1mM)
mmp2 MO (mM)
mmp9 MO (mM)
4dpi

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 5GC 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 5GC 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 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 *ascl1a*, *lin28a*, *sox2*, *oct4*, and *tgfbi* and downregulation of *her4.1* were observed (Figures 3D and 3E). The dependency of *ascl1a* 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 *ascl1a*, *lin28a*, *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 3I and 3J). 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-200* 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 Wlodarczyk, 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 S3C) 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

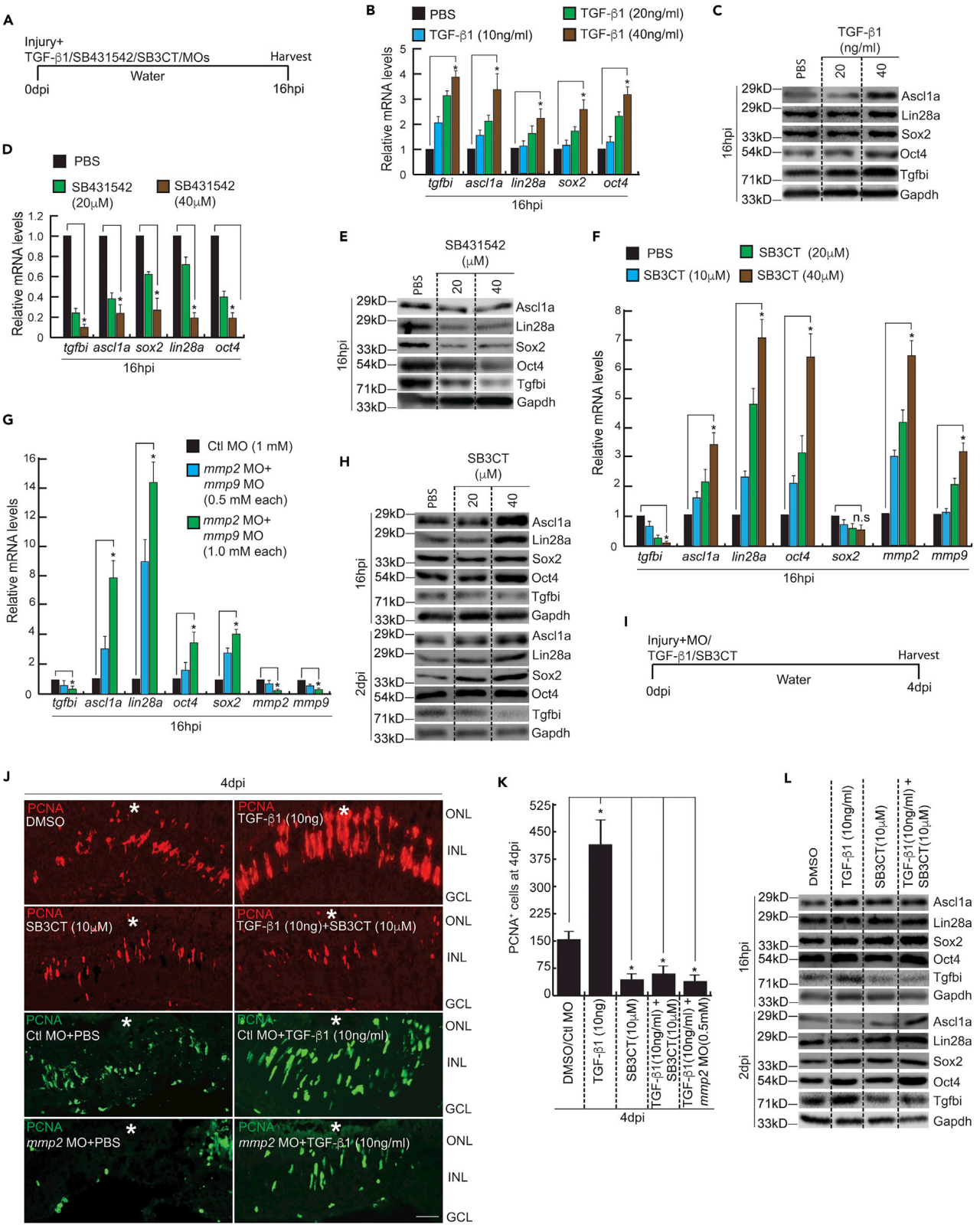
A
Injury+
TGF-β1/SB431542/SB3CT/MOs
Harvest
Water
0dpi
16hpi
B
PBS
TGF-β1 (10ng/ml)
TGF-β1 (20ng/ml)
TGF-β1 (40ng/ml)
Relative mRNA levels
tgfbi ascl1a lin28a sox2 oct4
16hpi
C
TGF-β1 (ng/ml)
PBS
20
40
16hpi
29kD
Ascl1a
Lin28a
33kD
Sox2
54kD
Oct4
71kD
Tgfbi
Gapdh
D
PBS
SB431542 (20μM)
SB431542 (40μM)
Relative mRNA levels
tgfbi ascl1a sox2 lin28a oct4
16hpi
E
SB431542 (μM)
PBS
20
40
16hpi
Ascl1a
Lin28a
Sox2
Oct4
Tgfbi
Gapdh
F
PBS
SB3CT (10μM)
SB3CT (20μM)
SB3CT (40μM)
Relative mRNA levels
tgfbi ascl1a lin28a oct4 sox2 mmp2 mmp9
n.s
16hpi
G
Ctl MO (1 mM)
mmp2 MO+ mmp9 MO (0.5 mM each)
mmp2 MO+ mmp9 MO (1.0 mM each)
Relative mRNA levels
tgfbi ascl1a lin28a oct4 sox2 mmp2 mmp9
16hpi
H
SB3CT (μM)
PBS
20
40
16hpi
2dpi
Ascl1a
Lin28a
Sox2
Oct4
Tgfbi
Gapdh
I
Injury+MO/
TGF-β1/SB3CT
Harvest
Water
0dpi
4dpi
J
4dpi
PCNA
DMSO
TGF-β1 (10ng)
SB3CT (10μM)
TGF-β1 (10ng)+SB3CT (10μM)
Ctl MO+PBS
Ctl MO+TGF-β1 (10ng/ml)
mmp2 MO+PBS
mmp2 MO+TGF-β1 (10ng/ml)
ONL
INL
GCL
K
PCNA+ cells at 4dpi
DMSO/Ctl MO
TGF-β1 (10ng)
SB3CT(10μM)
TGF-β1(10ng/ml) + SB3CT(10μM)
TGF-β1(10ng/ml) + mmp2 MO(0.5mM)
4dpi
L
DMSO
TGF-β1 (10ng/ml)
SB3CT(10μM)
TGF-β1(10ng/ml) + SB3CT(10μM)
16hpi
2dpi
Ascl1a
Lin28a
Sox2
Oct4
Tgfbi
Gapdh

Figure 5. Mmp2/Mmp9 Limit the Early Induction of Regeneration-Associated Genes

(A) An experimental timeline that describes 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* show 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* show 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 protein-injected condition, and a significant decline in the number of MGPCs in SB3CT drug-treated/*mmp2* MO electroporated, in isolation or in combination with TGF-β1 protein, compared with DMSO/ctl MO control, which is quantified (K), *$p < 0.03$, 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 (J). 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 *mmp9*, but not in that of *mmp2*. The *mmp2* promoter analysis revealed the presence of a typical 5GC element. Furthermore, the ChIP analysis performed in 3 hpi and 4 dpi retinal extracts confirmed that pSmad3 bound on to the 5GC 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 retinae, 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 retinae. Interestingly, the pro-proliferative effect of TGF-β1 protein was abolished in the absence of *mmp2/mmp9* (Figures S4A–S4D). 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 S4E). 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, S3C, and S3D), 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, 5F, 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, 5F, 5G, S5A, and S5B). 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 toward 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

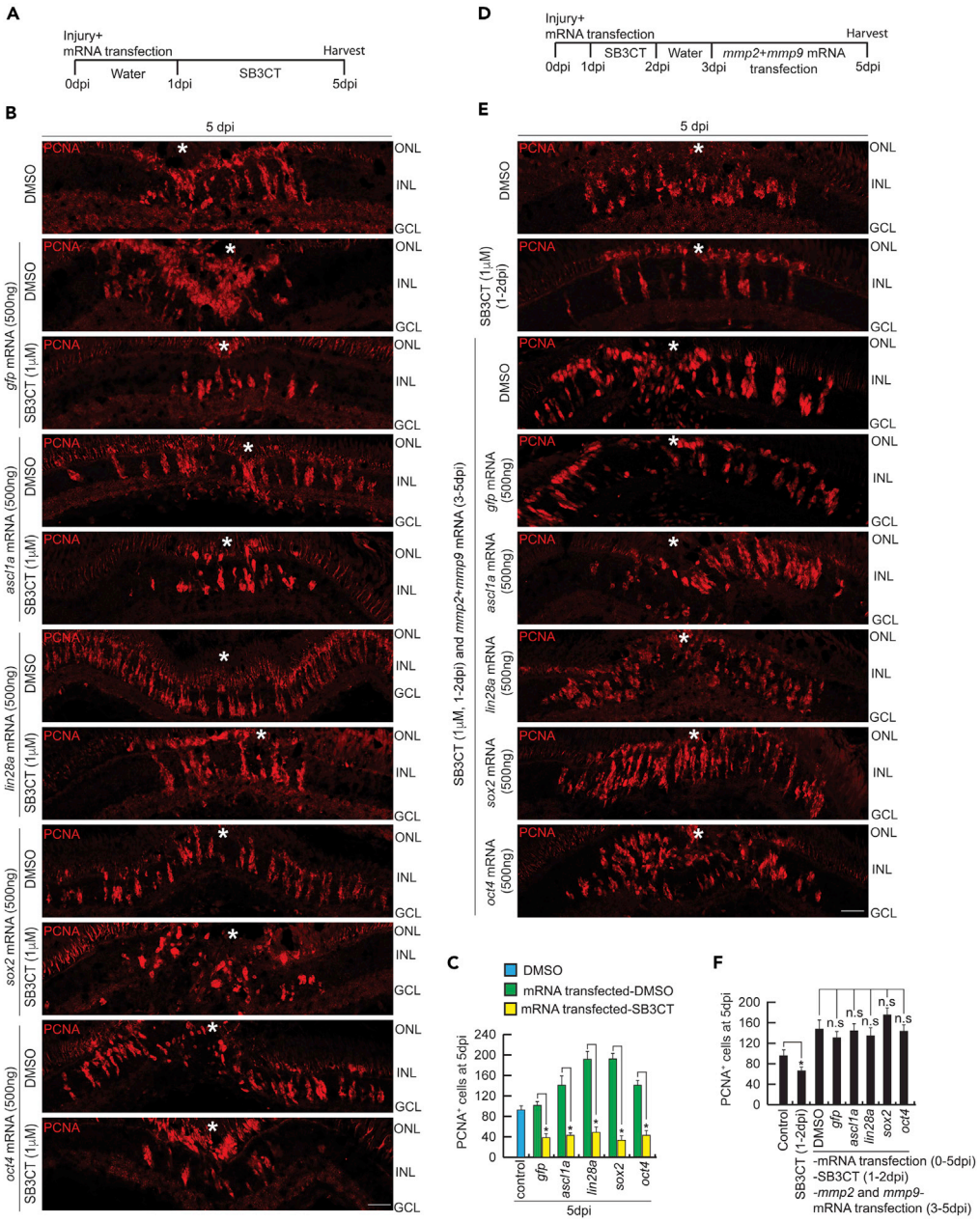

Figure 6. Mmp2 and Mmp9 Are Necessary for the Efficacy of Regeneration-Associated Genes

(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.

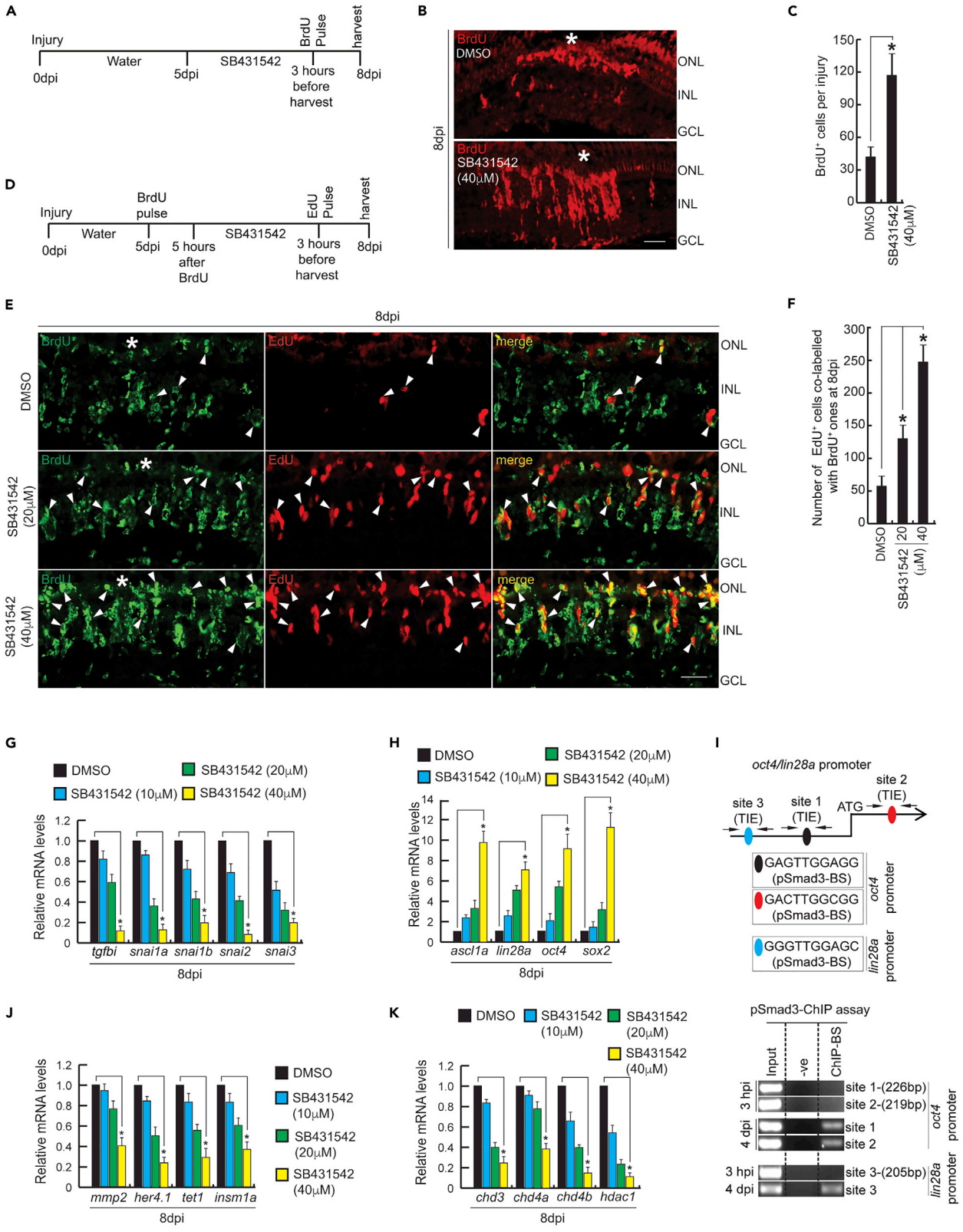
A
Injury
Water
SB431542
BrdU Pulse
harvest
0dpi
5dpi
3 hours before harvest
8dpi
B
BrdU
DMSO
BrdU
SB431542 (40μM)
8dpi
ONL
INL
GCL
C
BrdU+ cells per injury
DMSO
SB431542 (40μM)
D
Injury
Water
BrdU pulse
SB431542
EdU Pulse
harvest
0dpi
5dpi
5 hours after BrdU
3 hours before harvest
8dpi
E
8dpi
BrdU
EdU
merge
DMSO
SB431542 (20μM)
SB431542 (40μM)
F
Number of EdU+ cells co-labelled with BrdU+ ones at 8dpi
DMSO
SB431542 (μM)
20
40
G
DMSO
SB431542 (20μM)
SB431542 (10μM)
SB431542 (40μM)
Relative mRNA levels
tgfbi
snai1a
snai1b
snai2
snai3
8dpi
H
DMSO
SB431542 (20μM)
SB431542 (10μM)
SB431542 (40μM)
Relative mRNA levels
ascl1a
lin28a
oct4
sox2
8dpi
I
oct4/lin28a promoter
site 3 (TIE)
site 1 (TIE)
ATG
site 2 (TIE)
GAGTTGGAGG (pSmad3-BS)
GACTTGGCGG (pSmad3-BS)
GGGTTGGAGC (pSmad3-BS)
oct4 promoter
lin28a promoter
J
Relative mRNA levels
mmp2
her4.1
tet1
insm1a
8dpi
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
K
DMSO
SB431542 (10μM)
SB431542 (20μM)
SB431542 (40μM)
Relative mRNA levels
chd3
chd4a
chd4b
hdac1
8dpi
pSmad3-ChIP assay
Input
-ve
ChIP-BS
3 hpi
4 dpi
site 1-(226bp)
site 2-(219bp)
site 1
site 2
site 3-(205bp)
site 3
oct4 promoter
lin28a promoter

Figure 7. Tgf-β Signaling at Late Phases Is Essential for the Cessation of MGPCs Proliferation

(A) An experimental timeline that describes the injury, late SB431542 treatment, BrdU pulse 3 h before harvest at 8 dpi.

(B and C) IF microscopy images of retinal cross sections show increased BrdU$^+$ MGPCs at 8 dpi in SB431542-treated retina from fifth day onward, which is quantified (C), *p < 0.01, N = 4. (D) An experimental timeline that describes the injury, BrdU pulse, late SB431542 treatment to the retina, and EdU pulse 3 h before harvest at 8 dpi.

(E and F) IF microscopy images of retinal cross sections show increased BrdU$^+$ MGPCs at 8 dpi in SB431542-treated retina from fifth day onward, and a proof of the delay in exiting cell cycle revealed by EdU co-labeling with BrdU$^+$ MGPCs (E), which is quantified (F), *p < 0.001, N = 4. White arrowheads mark BrdU$^+$/EdU$^+$ cells in (E).

(G) The qPCR analyses of *tgfbi* and *snail* family genes' mRNA levels in late SB431542-treated retina at 8 dpi, *p < 0.001, N = 4.

(H) The qPCR analyses of *ascl1a*, *lin28a*, *oct4*, and *sox2* mRNA levels in late SB431542-treated retina at 8 dpi, *p < 0.02, N = 4.

(I) The *oct4* promoter/intron and *lin28a* promoter schematics reveal the TIE sequence (upper), and the retinal ChIP assays confirm the physical binding of pSmad3 at the TIE sites (lower) in 4 dpi but not at 3 hpi retina.

(J) The qPCR analyses of *mmp2*, *her4.1*, *tet1*, and *insm1a* mRNA levels in late SB431542-treated retina at 8 dpi, *p < 0.03, N = 4.

(K) The qPCR analyses of NuRD complex genes *chd3*, *chd4a*, *chd4b*, and *hdac1* mRNA levels in late SB431542-treated retina at 8 dpi, *p < 0.02, 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).

See also Figures S5 and Table S1.

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

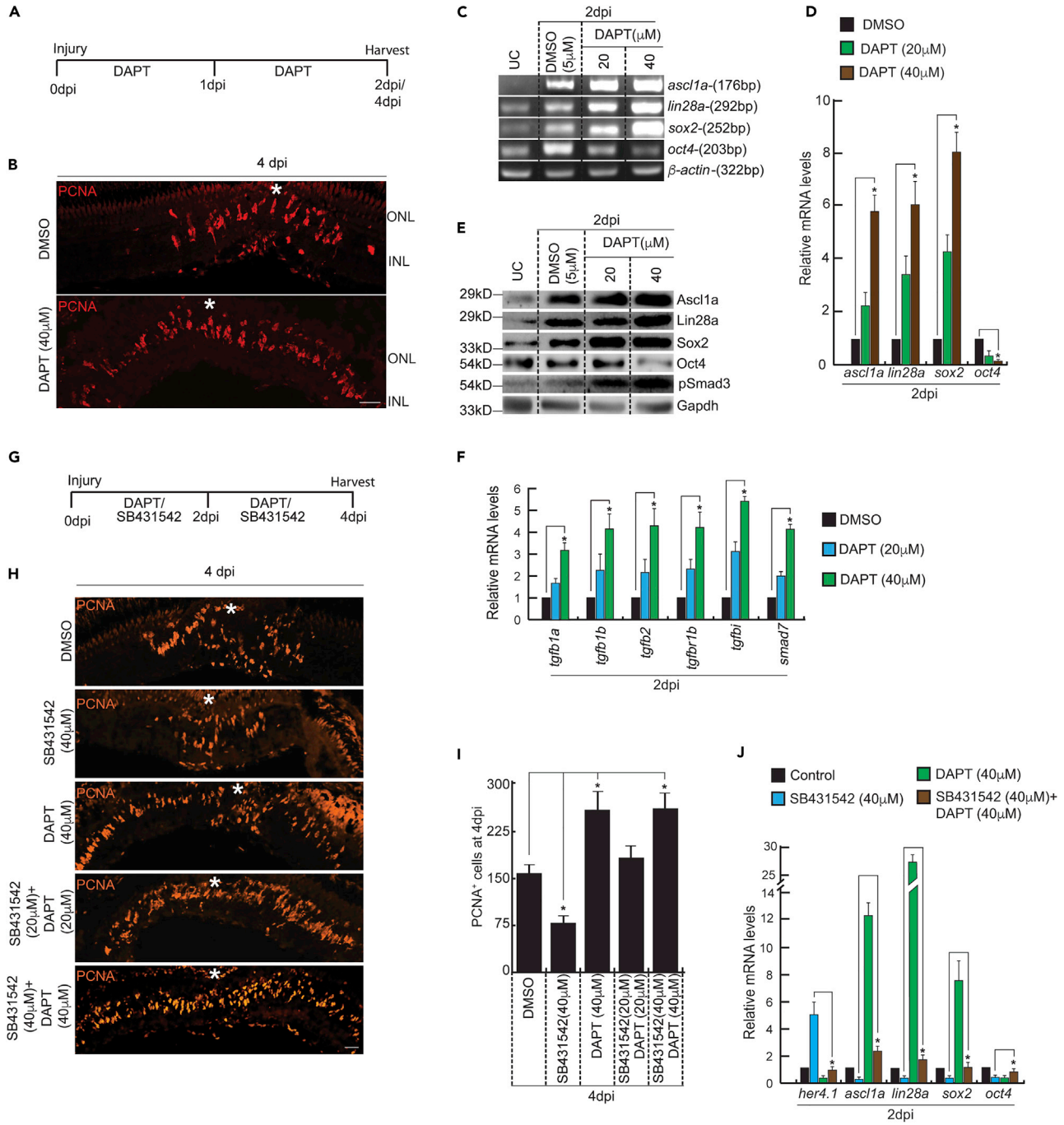

Figure 8. Cross-talk between Notch and Tgf-β Signaling

(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.

Figure 8. *Continued*

(H and I) IF microscopy images of retinal cross sections show differences in PCNA$^+$ MGPCs at 4 dpi in either SB431542 or DAPT drug-injected condition, and also in combination of these two drugs, compared with DMSO control (H), which is quantified (I). *$p < 0.05$, N = 4. Error bars are SD. (J) The qPCR analyses of *her4.1*, *ascl1a*, *lin28a*, *sox2*, and *oct4* genes in DAPT, SB431542-treated retina in isolation as well as in combination, at 2 dpi, *$p < 0.04$, N = 4. Scale bars, 10 μm (B and H). The asterisk marks the injury site in (B) and (H). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer in (B). UC, uninjured control in (C) and (E).

See also Figures S6 and Table S1.

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 *oct4* (Figures 8C–8E and S6A). Furthermore, there was an accelerated expression of various Tgf-β signaling components such as *tgfb1a*, *tgfb1b*, *tgfb2*, and *tgfbr1b* and Tgf-β signaling readout genes such as *tgfbi* and *smad7* (Figure 8F) and a concomitant increase in pSmad3 (Figure 8E). Oct4 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 *tgfb1a*, *tgfb1b*, *tgfb2*, *tgfbr1b*, *tgfbi*, and *smad7*. These observations suggest that the observed increase in MGPCs in the absence of Delta-Notch signaling could be mediated through the Oct4/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

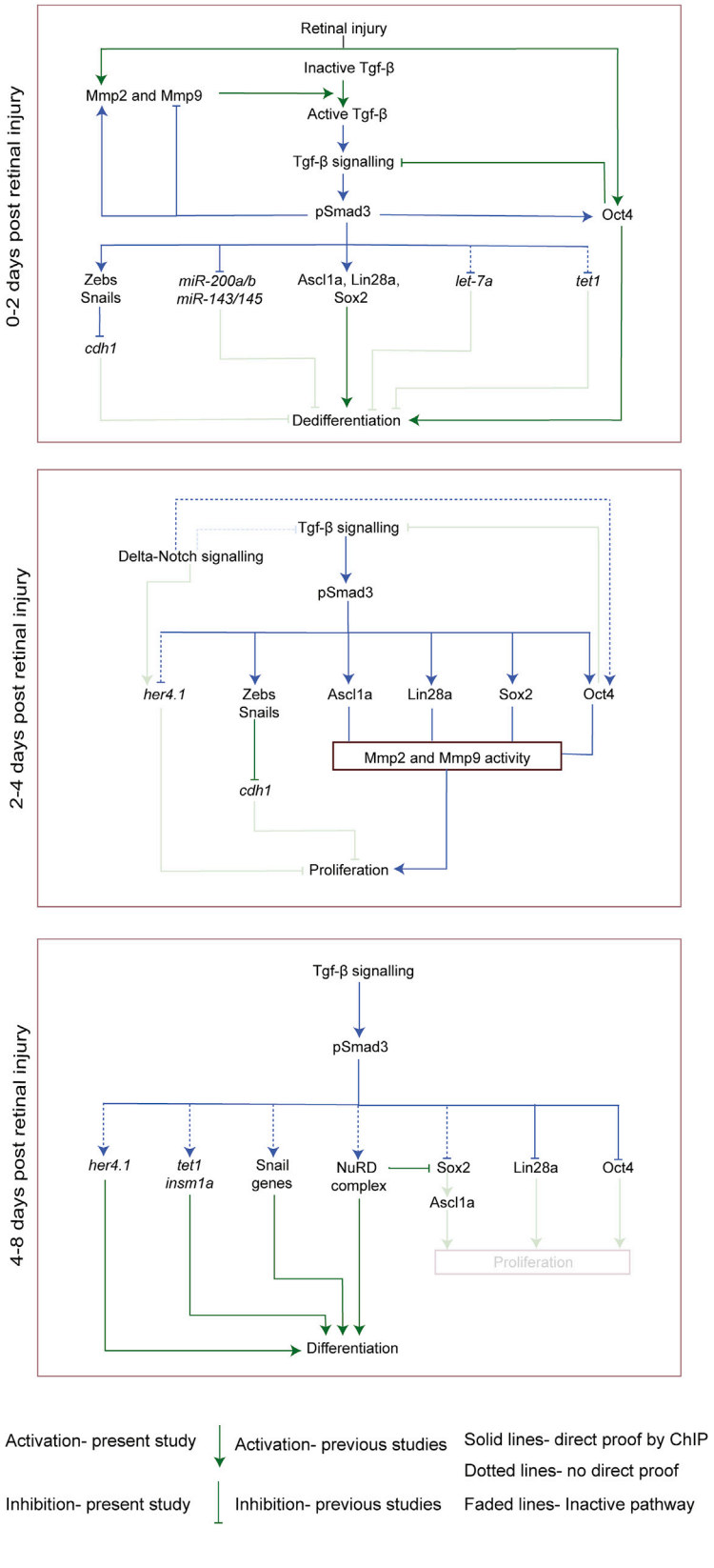
0-2 days post retinal injury
Retinal injury
Inactive Tgf-β
Mmp2 and Mmp9
Active Tgf-β
Tgf-β signalling
pSmad3
Oct4
Zebs
Snails
miR-200a/b
miR-143/145
Ascl1a, Lin28a,
Sox2
let-7a
tet1
cdh1
Dedifferentiation
2-4 days post retinal injury
Tgf-β signalling
Delta-Notch signalling
pSmad3
her4.1
Zebs
Snails
Ascl1a
Lin28a
Sox2
Oct4
Mmp2 and Mmp9 activity
cdh1
Proliferation
4-8 days post retinal injury
Tgf-β signalling
pSmad3
her4.1
tet1
insm1a
Snail
genes
NuRD
complex
Sox2
Lin28a
Oct4
Ascl1a
Proliferation
Differentiation
Activation- present study
Activation- previous studies
Solid lines- direct proof by ChIP
Dotted lines- no direct proof
Inhibition- present study
Inhibition- previous studies
Faded lines- Inactive pathway

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 5GC 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; Krstic and Santibanez, 2014), no mechanistic 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 *tgif1* 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 5GC 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

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.100817.

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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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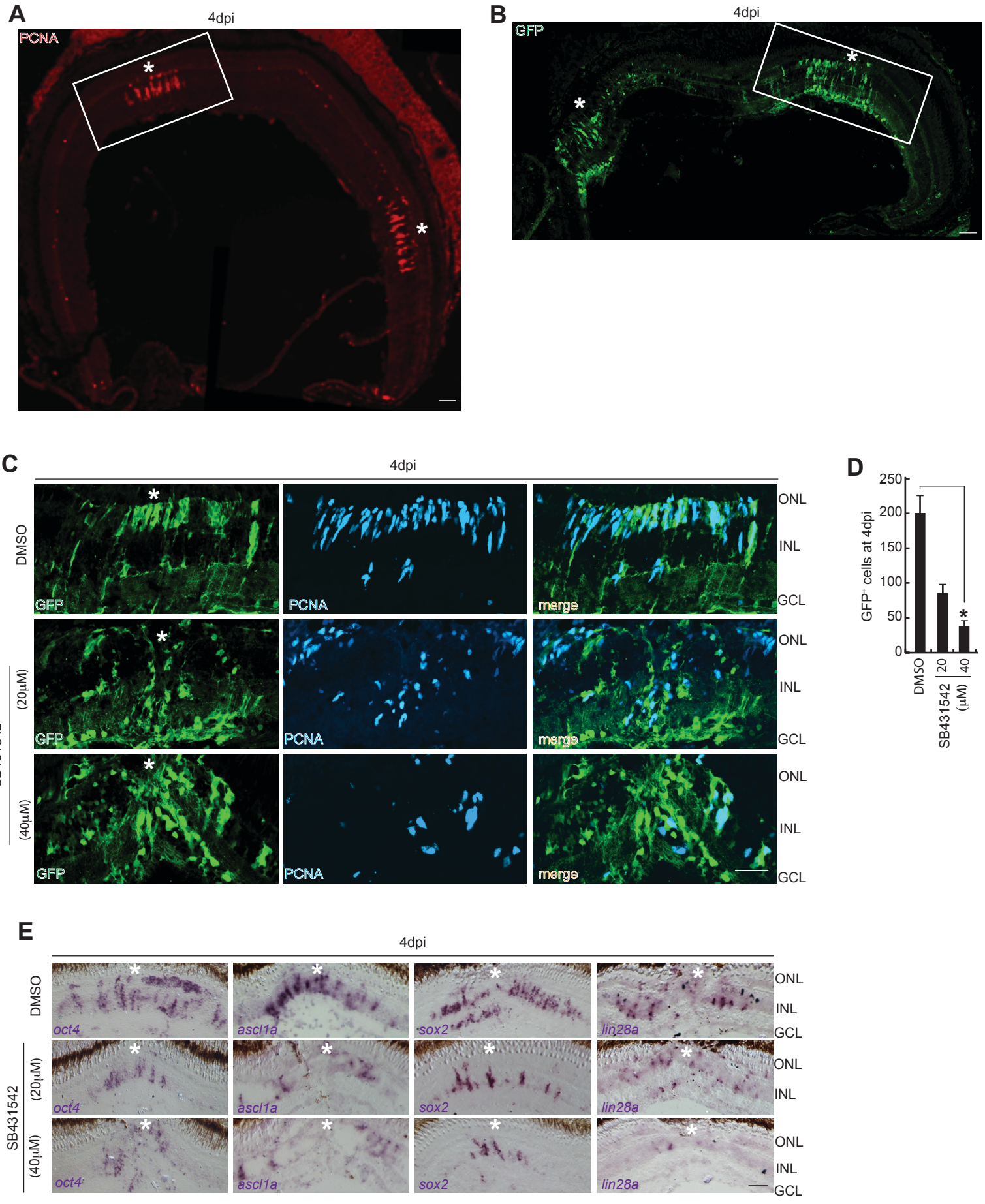
iScience, Volume *23*

Supplemental Information

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

Poonam Sharma, Shivangi Gupta, Mansi Chaudhary, Soumitra Mitra, Bindia Chawla, Mohammad Anwar Khursheed, Navnoor Kaur Saran, and Rajesh Ramachandran

Figure S1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4dpi
GFP
C
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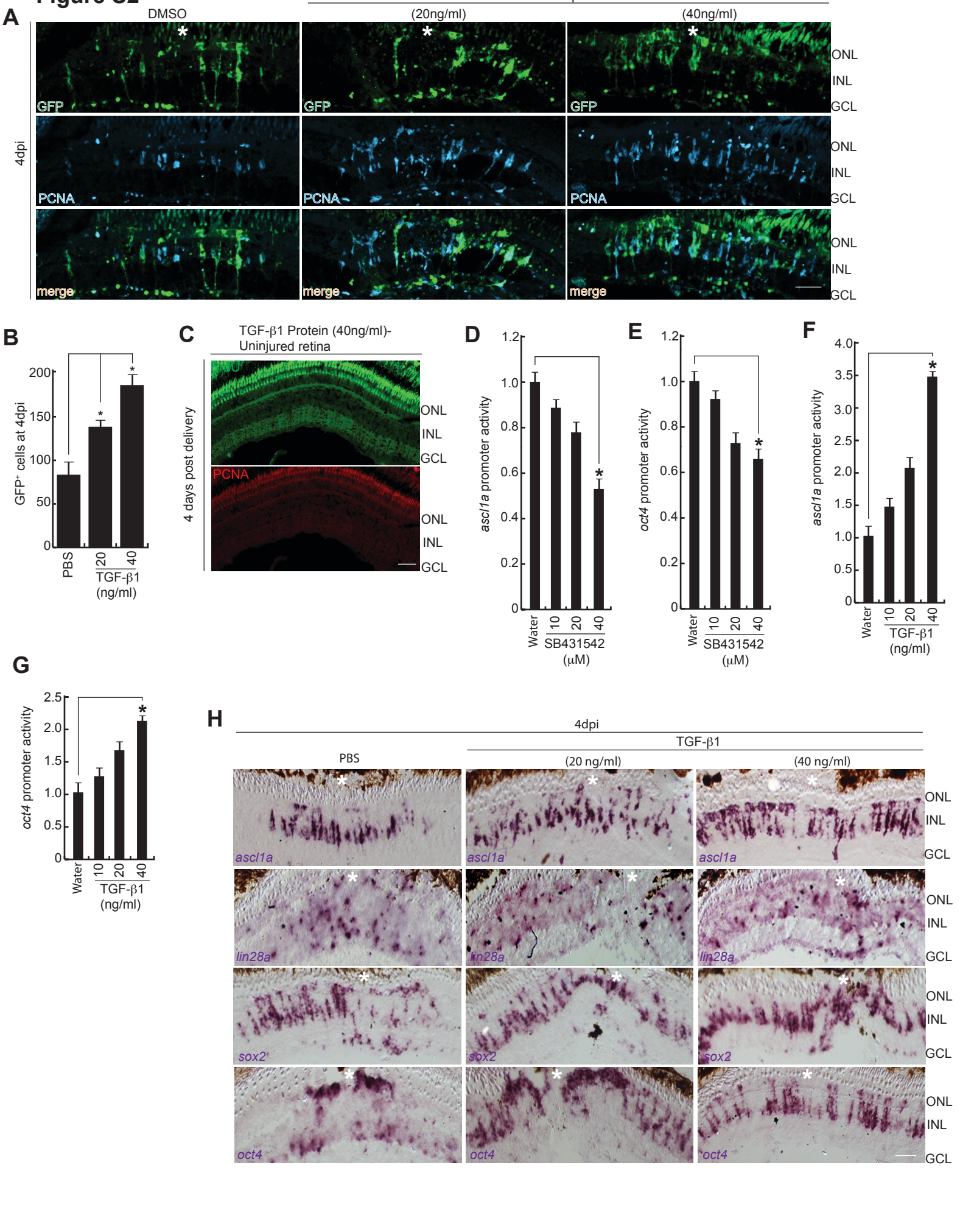
(40μM)
GFP
PCNA
merge
ONL
INL
GCL
D
GFP+ cells at 4dpi
250
200
150
100
50
0
DMSO
20
40
SB431542 (μM)
E
4dpi
DMSO
SB431542
(20μM)
(40μM)
oct4
ascl1a
sox2
lin28a
ONL
INL
GCL

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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(40ng/ml)
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GFP
PCNA
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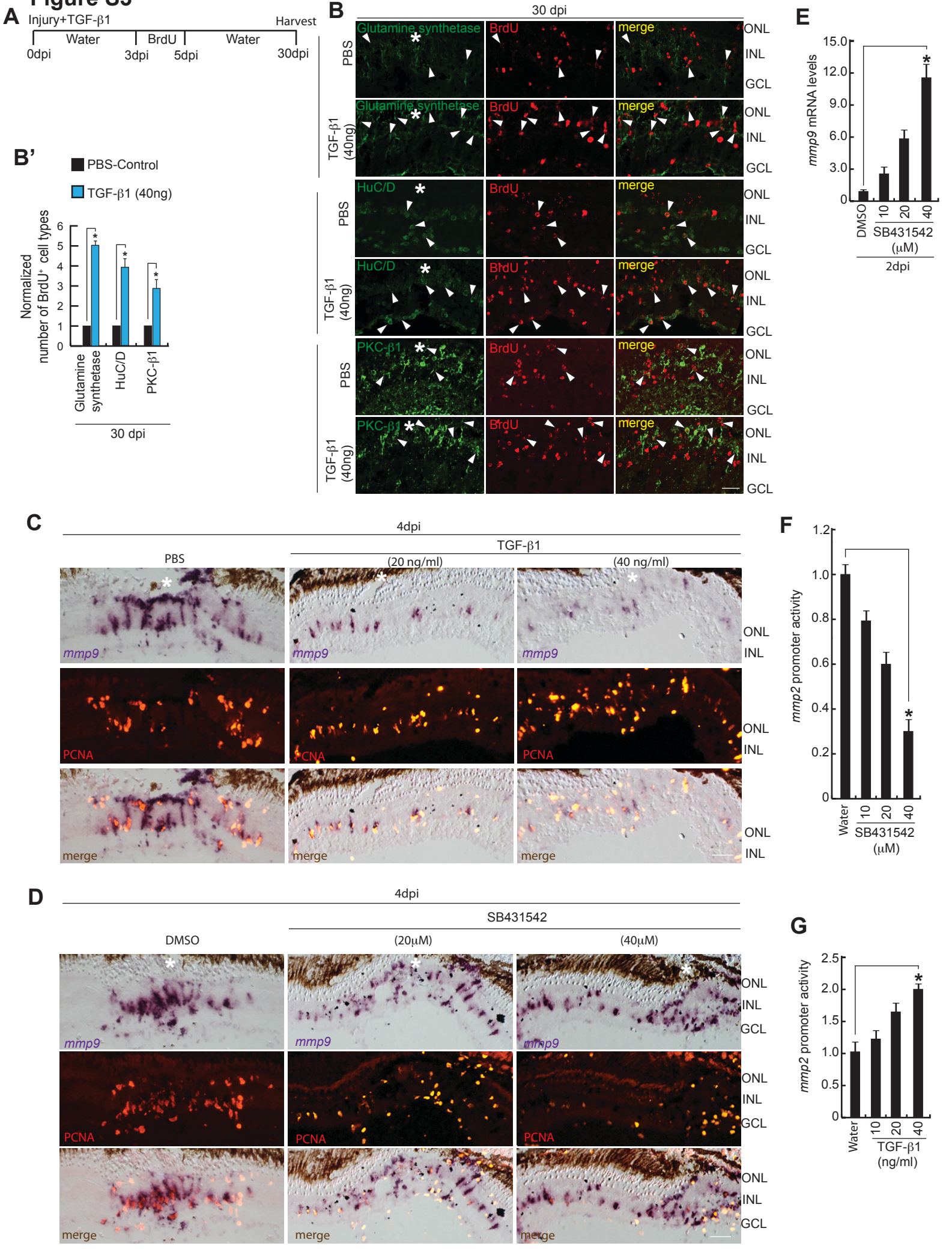
ONL
INL
GCL
B
GFP+ cells at 4dpi
PBS
20
40
TGF-β1
(ng/ml)
C
TGF-β1 Protein (40ng/ml)-
Uninjured retina
4 days post delivery
D
ascl1a promoter activity
Water
10
20
40
SB431542
(μM)
E
oct4 promoter activity
F
ascl1a promoter activity
G
oct4 promoter activity
H
4dpi
TGF-β1
PBS
(20 ng/ml)
(40 ng/ml)
ascl1a
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 4^{th} day of TGF-β1 protein delivery through the cornea. (**D,E**) Zebrafish embryonic luciferase assays reveal the downregulation of *ascl1a* (**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 *ascl1a* (**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 *ascl1a*, *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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BrdU
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3dpi
5dpi
30dpi
B
30 dpi
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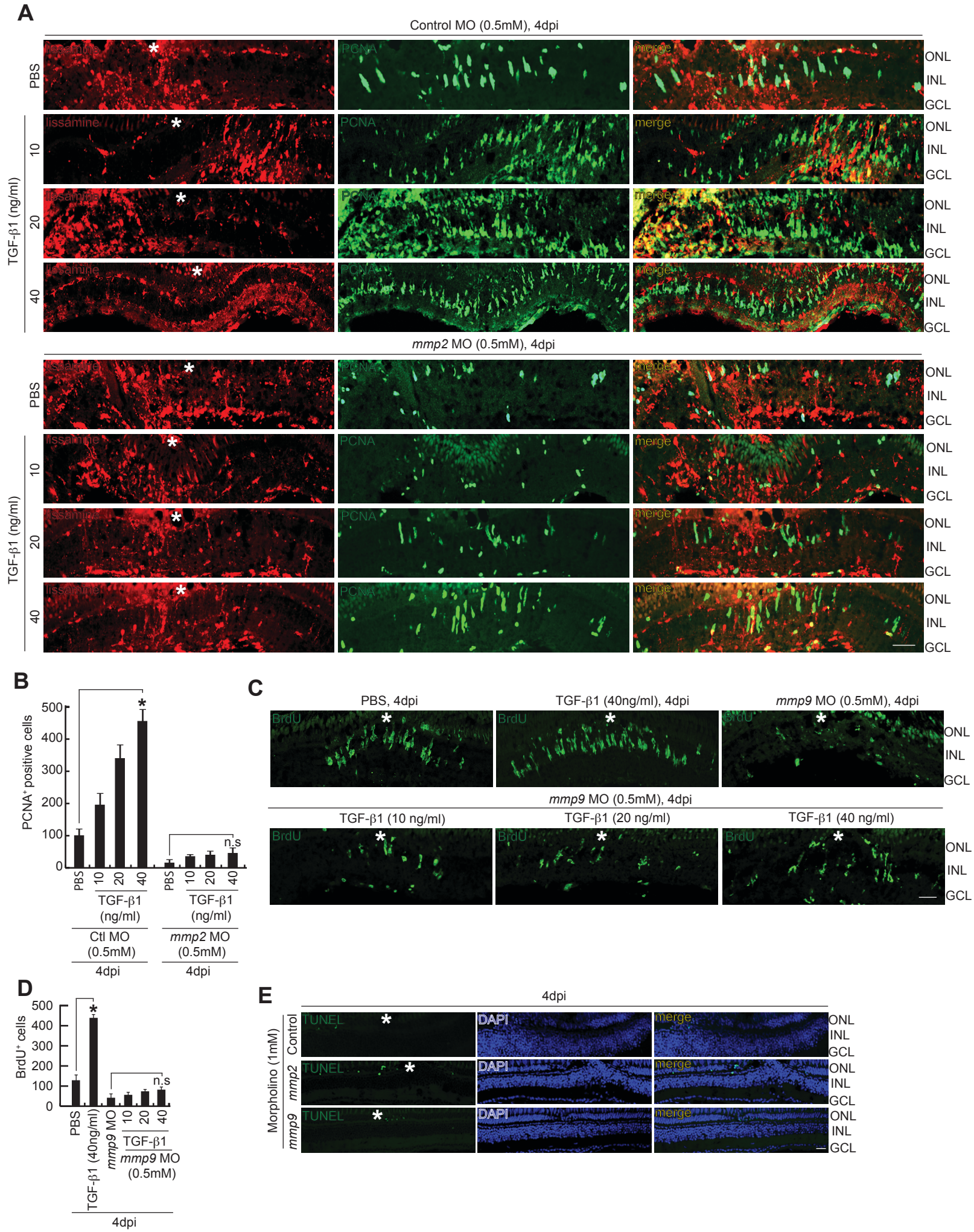
Glutamine synthetase
HuC/D
PKC-β1
BrdU
merge
ONL
INL
GCL
B'
PBS-Control
TGF-β1 (40ng)
Normalized number of BrdU+ cell types
Glutamine synthetase
HuC/D
PKC-β1
30 dpi
E
mmp9 mRNA levels
DMSO
SB431542 (μM)
2dpi
C
4dpi
PBS
TGF-β1
(20 ng/ml)
(40 ng/ml)
mmp9
PCNA
merge
F
mmp2 promoter activity
Water
SB431542 (μM)
D
4dpi
DMSO
SB431542
(20μM)
(40μM)
G
mmp2 promoter activity
Water
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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20
40
Tissamine
PCNA
merge
ONL
INL
GCL
mmp2 MO (0.5mM), 4dpi
B
PCNA+ positive cells
500
400
300
200
100
0
n.s
PBS
10
20
40
TGF-β1 (ng/ml)
Ctl MO (0.5mM)
mmp2 MO (0.5mM)
4dpi
C
PBS, 4dpi
TGF-β1 (40ng/ml), 4dpi
mmp9 MO (0.5mM), 4dpi
BrdU
mmp9 MO (0.5mM), 4dpi
TGF-β1 (10 ng/ml)
TGF-β1 (20 ng/ml)
TGF-β1 (40 ng/ml)
D
BrdU+ cells
TGF-β1 (40ng/ml)
mmp9 MO
mmp9 MO (0.5mM)
4dpi
E
4dpi
Morpholino (1mM)
Control
mmp2
mmp9
TUNEL
DAPI
merge

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

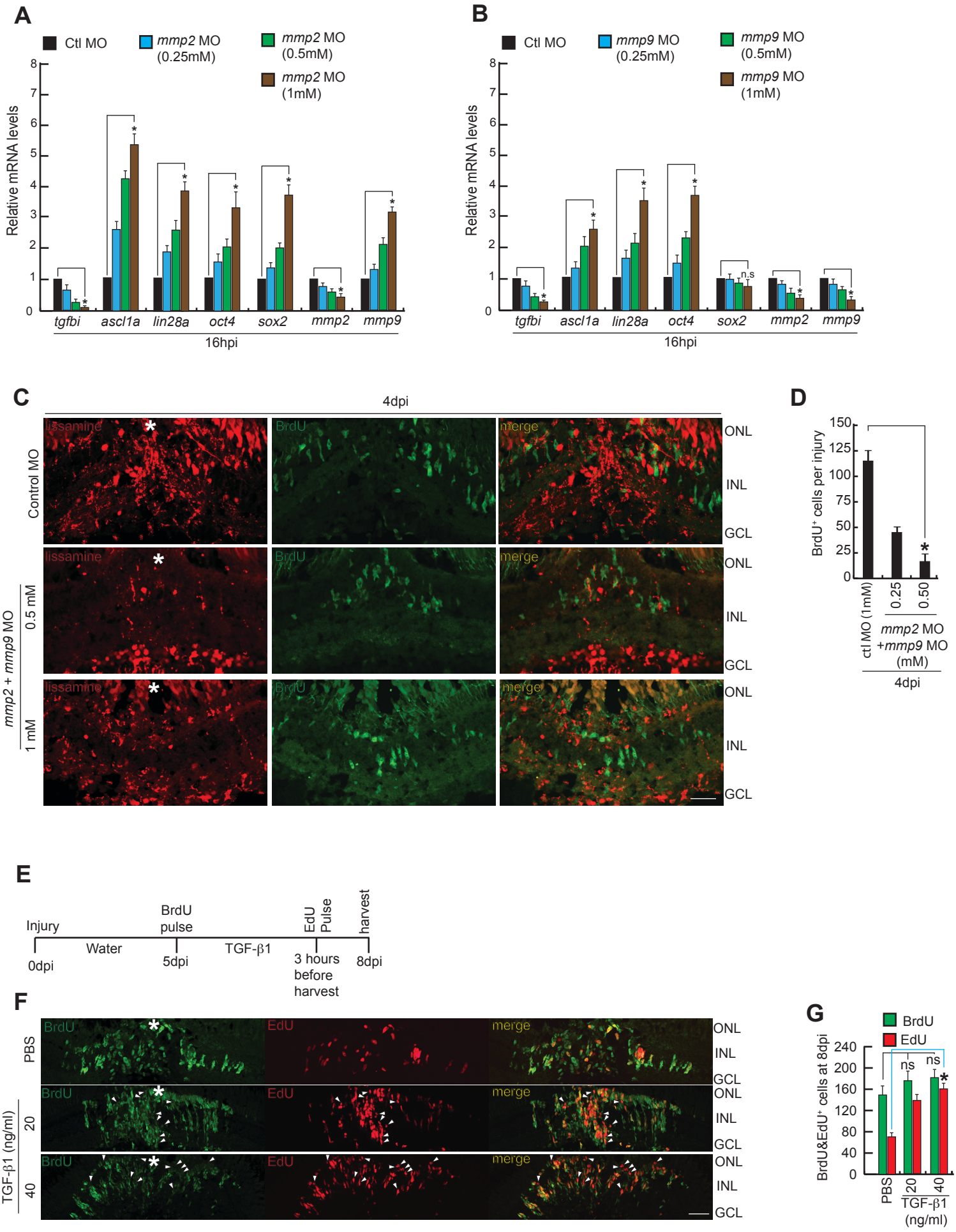
A
Ctl MO
mmp2 MO (0.25mM)
mmp2 MO (0.5mM)
mmp2 MO (1mM)
Relative mRNA levels
tgfbi
ascl1a
lin28a
oct4
sox2
mmp2
mmp9
16hpi
B
Ctl MO
mmp9 MO (0.25mM)
mmp9 MO (0.5mM)
mmp9 MO (1mM)
Relative mRNA levels
n.s
16hpi
C
4dpi
Control MO
mmp2 + mmp9 MO
0.5 mM
1 mM
Dissamine
BrdU
merge
ONL
INL
GCL
D
BrdU+ cells per injury
ctl MO (1mM)
0.25
0.50
mmp2 MO +mmp9 MO (mM)
4dpi
E
Injury
Water
0dpi
BrdU pulse
5dpi
TGF-β1
EdU Pulse
3 hours before harvest
harvest
8dpi
F
PBS
TGF-β1 (ng/ml)
20
40
BrdU
EdU
merge
ONL
INL
GCL
G
BrdU
EdU
BrdU&EdU+ cells at 8dpi
ns
ns
PBS
20
40
TGF-β1 (ng/ml)

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 5^{th} 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

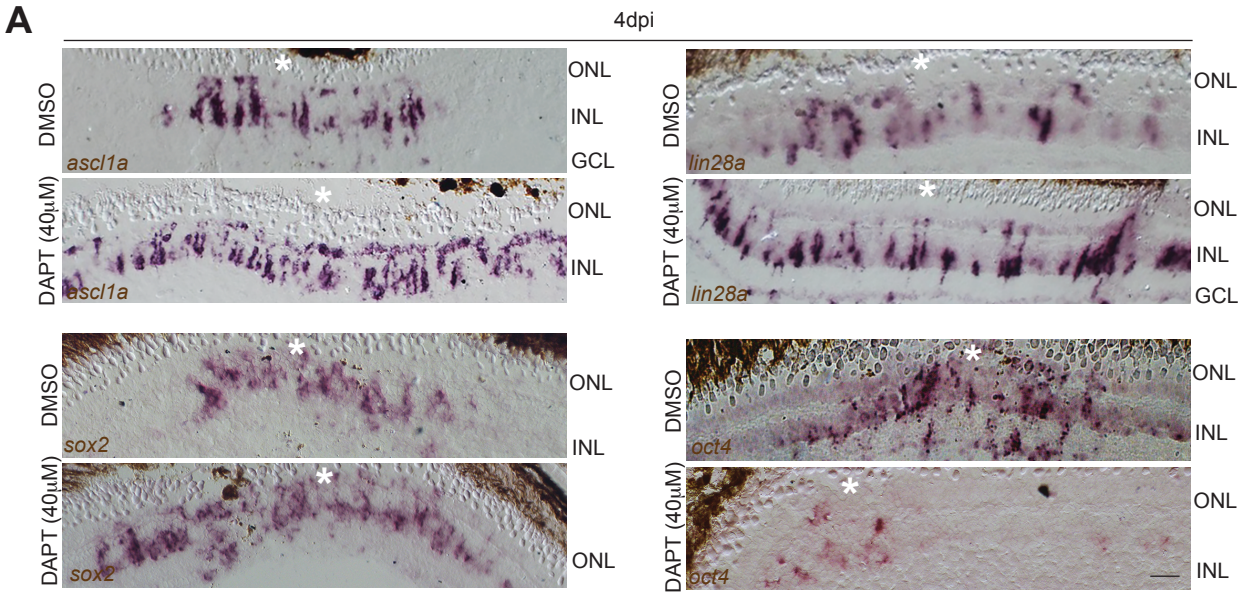

Figure S6. Expression analysis of various genes in Delta-Notch signaling-inhibited condition. Related to Figure 8. (**A**) BF microscopy images of retinal cross sections show the DAPT-dependent increase in the expression levels of *ascl1a*, *lin28a*, *sox2*, and decrease in that of *oct4*, as revealed 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 retinae 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 electroporationand *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 retinae 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 retinae were normalized to *β-actin.*

Western blotting and antibodies.
Western blotting was performed using 6 retinae 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 N*i*-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 retinae were used per experiment and all the proliferating cells at each injury spot were counted. In case of TGF-β1 and DAPT treated retinae, 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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