

Granulin 1 Promotes Retinal Regeneration in Zebrafish

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PURPOSE. Retinal degenerative diseases can progress to severe reductions of vision. In general, the changes are permanent in higher vertebrates, including humans; however, retinal regeneration can occur in lower vertebrates, such as amphibians and teleost fish. Progranulin is a secreted growth factor that is involved in normal development and wound-healing processes. We have shown that progranulin promotes the proliferation of retinal precursor cells in mouse retinas. The purpose of this study was to investigate the role played by granulin 1 (*grn1*) in the retinal regeneration in zebrafish.

METHODS. We injured the retina of zebrafish with needle puncturing, and the retinas were examined at different times after the injury. We also checked the proliferation and the expression of retinal regeneration-related genes after knockdown of *grn1* by electroporation with morpholino oligonucleotides (MO) and intravitreal injection of recombinant *grn1*.

RESULTS. Our results showed that the level of *grn1* was highly increased after retinal injury, and it was expressed in various types of retinal cells. A knockdown of *grn1* reduced the proliferation of Müller glial cells in zebrafish eyes undergoing retinal regeneration. The knockdown of *grn1* also reduced the expression of achaete-scute homolog 1a (*ascl1a*), an important factor in retinal regeneration. An intravitreal injection of recombinant *grn1* led to a proliferation of Müller glial cells and an increase in the expression of retinal regeneration-related genes, such as *ascl1a* and *lin28*.

CONCLUSIONS. These findings suggested that *grn1* should be considered as a target for stimulating the dedifferentiation of Müller glial cells and retinal regeneration.

Keywords: *grn1*, granulin, Müller glia, retinal regeneration, zebrafish

Many retinal degenerative diseases, such as AMD and retinitis pigmentosa, are progressive,¹ and the changes are permanent. Thus, developing therapies for these diseases could have a significant impact on the quality of life of the affected individuals. Recent clinical trials and basic laboratory studies have shown that transplantation of retinal or RPE cells can restore the vision in some of the eyes with degenerative diseases.^{2,3} The recent use of stem cell transplantation has also had some degree of success; however, the permanence of the degeneration of the retinal cells has made the task of restoring vision very difficult.

The retina of lower vertebrates, such as amphibians and teleost fish, can regenerate after an injury^{4–6}; however, the retina of mammals, including humans, is not able to regenerate.⁷ In amphibians and zebrafish, Müller glia can respond to retinal injury, and then they acquire the function as stem cells that produce neural progenitors that become new neurons.⁸ In zebrafish, retinal injuries trigger signals inactivating glycogen synthase kinase (GSK) and β 3 leading to the stabilization of β -catenin, an activating mitogen-activated protein kinase, extracellular signal-regulated kinase, and Janus kinase (JAK). These activations lead to a reprogramming of the Müller glial cells.^{9–12} The Achaete-scute homolog 1a (*ascl1a*), a member of the basic helix-loop-helix family of transcription factors, is induced by these signaling pathways and plays a key role in retinal regeneration in zebrafish.^{13,14} The level of

ascl1a increases in the Müller cells soon after an injury of the retina, and it regulates genes such as *Notch*, *lin28*, and *myc*.¹⁵ Some growth factors and cytokines, such as fibroblast growth factor 2, heparin-binding epidermal (EGF)-like growth factor (HB-EGF), and tumor necrosis factor (TNF), are induced by retinal injury, and they, in turn, can induce the expression of *ascl1a*.^{10,16}

Granulins are cleaved peptides of progranulin, which is one of the growth factor proteins. Progranulin is highly conserved in vertebrates.¹⁷ This peptide is expressed ubiquitously in neurons, epithelial cells, and immune cells.¹⁸ In the clinic, it has been recognized that loss-of-function mutations of the progranulin-encoding gene, GRN, cause familial frontotemporal dementia.¹⁹ Many studies have shown that progranulin exerts its anti-inflammatory effects through multiple pathways.^{20–22} The results of our laboratory showed that mammalian progranulin has neuroprotective effects on cells of the central nervous system during ischemia and on photoreceptors during exposure to phototoxic levels of illumination.^{23,24} Moreover, progranulin was found to promote the proliferation of retinal precursor cells and the differentiation of photoreceptors in the mouse retina.²⁵ Progranulin was also found to be associated with retinal development in mice.²⁶ These findings strongly suggested that progranulin could be involved in the retinal regeneration processes.

Zebrafish have four subtypes of progranulin: granulin (*grn*) a, *grnb*, *grn1*, and *grn2*. *Grna* and *grnb* have 9 to 10 repeats of the granulin/epithelin motif and are believed to be orthologues of human progranulin.²⁷ Several studies have shown that *grna* is associated with liver morphogenesis,²⁸ muscle growth and regeneration,²⁹ retinal damage,³⁰ and brain and retinal development.³¹ In zebrafish at 48 hours post fertilization, *grna* is exclusively expressed by microglia and/or microglial precursors within the brain and retina. The previous report also showed that *grna* governs neurogenesis by regulating cell cycle kinetics and the transition from proliferation to cell cycle exit and differentiation.³¹ However, the other subtypes of granulins, including *grn1*, have not been investigated in detail, and their functions remain undetermined.

Thus, the purpose of this study was to determine whether the granulins are involved in retinal regeneration. To accomplish this, we injured the retina of zebrafish and conducted time-course analysis of mRNA expressions of four types of granulins. As a result, we detected *grn1* expression upregulated earlier than the other subtypes of granulins and it preceded upregulation of *ascl1a*. We then determined sites of *grn1* expression in the retina. In addition, we examined whether an intravitreal injection of recombinant Grn1 increased the proliferation of Müller cells, and increased the level of expression of retinal regeneration-related genes including *ascl1a*.

METHODS

Animals

Zebrafish from the AB line were raised at 28.5°C under a 14-hour light:10-hour dark cycle. All experimental protocols were approved by University of Michigan and Gifu Pharmaceutical University Committee on Use and Care of Animals and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Methods were carried out in accordance with the approved guidelines.

RNA Isolation and PCR

All primers used are listed in the Table. Three fish were used for each experiment. Adult zebrafish were overdosed with tricaine, the eyes were enucleated, and the retinae were harvested. Total RNA was extracted from the isolated retinae with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA synthesis and PCR were performed as described.^{13,14} Relative quantitative real-time PCR was performed in triplicate with SYBR Premix Ex TaqII (Takara Bio, Kusatsu, Japan) on the Thermal Cycler Dice (Takara Bio). In each sample, we prepared two retinae from a single fish. In each group, we prepared three fishes as a biological replicate. We conducted qPCR for each biological replicate run in triplicate. In each result, the error bars represent standard error among three biological replicates. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as an internal standard, and relative quantitative analysis was carried out by the $\Delta\Delta C_t$ method. Semiquantitative analysis of RT-PCR was performed using the ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Retinal Lesions and Morpholino-Modified Antisense Oligonucleotide-Mediated Gene Knockdown

Retinas were injured and electroporated with morpholino oligonucleotides (MOs) as described in detail.^{13,14} Briefly, the

zebrafish were anesthetized, and the right retina was stabbed once in each quadrant with a 30G needle inserted through the sclera to a depth of 1.5 mm. Lissamine-labeled MOs (Gene Tools, Philomath, OR, USA) were delivered at the time of injury using the same needle to inject the MOs. Approximately 0.5 μ L MO (1-mM stock) was injected into the eye, and an uptake by the cells was facilitated by electroporation.^{13,14} Custom electrodes were placed across the head of the anesthetized fish with the cathode on the left eye and the anode on the right eye. An ECM 830 Electro Square Porator (BTX, San Diego, CA, USA) was used to deliver five consecutive 50-ms pulses at 70 V with a 950-ms interval between pulses. The following lissamine-labeled MOs were used:

control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'
ascl1a MO^{10,13}: 5'-ATCTTGGCGGTGATGCCATTTCGC-3'
grn1 MO #1: 5'-CATCTTGCTGGCTGGGTTTCCTCCT-3'
grn1 MO #2: 5'-ACATTAACCTCGAGATTCTGTTACCATTGAG
 TAAGGACA-3'

For bromodeoxyuridine (BrdU) incorporation, a solution of 20 μ L of 20 mM BrdU was injected intraperitoneally into anesthetized fish at 4 days after injury.

Recombinant *grn1* Injection and BrdU Incorporation

His-tagged recombinant Grn1 was synthesized with the TnT Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA), and purified with the MagneHisProtein Purification System (Promega) and Zeba Spin Desalting Columns (Thermo Fisher Scientific) according to the protocols of the manufacturer.

The zebrafish were anesthetized, and the right eye was injected with 20 or 200 ng of the recombinant Grn1 that was dissolved in 1 μ L of PBS. The left eye (control) was injected with 1 μ L of vehicle used for the synthesis and purification of the mock plasmids. The solutions were injected into the vitreous through the cornea, being careful not to injure the retina. For BrdU incorporation, a solution of 20 μ L of 20 mM BrdU was injected intraperitoneally into anesthetized fish at 4 days after the recombinant Grn1 was injected.

Quantification of BrdU-Positive Cell Number

At injured condition, the BrdU-positive cells were counted by using sum of 4 or 5 different serial transverse sections from the same fish surrounding an injury. At normal condition, the BrdU-positive cell number was counted by single transverse section including optic nerve.

Tissue Preparation, Immunohistochemistry, and In Situ Hybridization

Adult zebrafish were overdosed with tricaine, and the eyes were enucleated, the lens removed, and the eye cups were fixed in 4% paraformaldehyde. The fixed and frozen eye cups were sectioned at 12- μ m thickness for immunofluorescence as described in detail.^{14,32,33} The antibodies used were diluted in PBS plus 0.1% Triton X-100 as described.^{14,32,33} For BrdU staining, sections were treated with 2 N HCl at 37°C for 20 minutes, rinsed in 0.1 M sodium borate solution (pH 8.5) for 10 minutes, and then processed using standard immunohistochemical procedures. In situ hybridization was performed as described in detail.³⁴ Digoxigenin-labeled RNA probes were prepared using the DIG RNA labeling kit (Roche Diagnostics, Basel, Switzerland). Hybridization was performed by total immersion in a hybridization buffer that

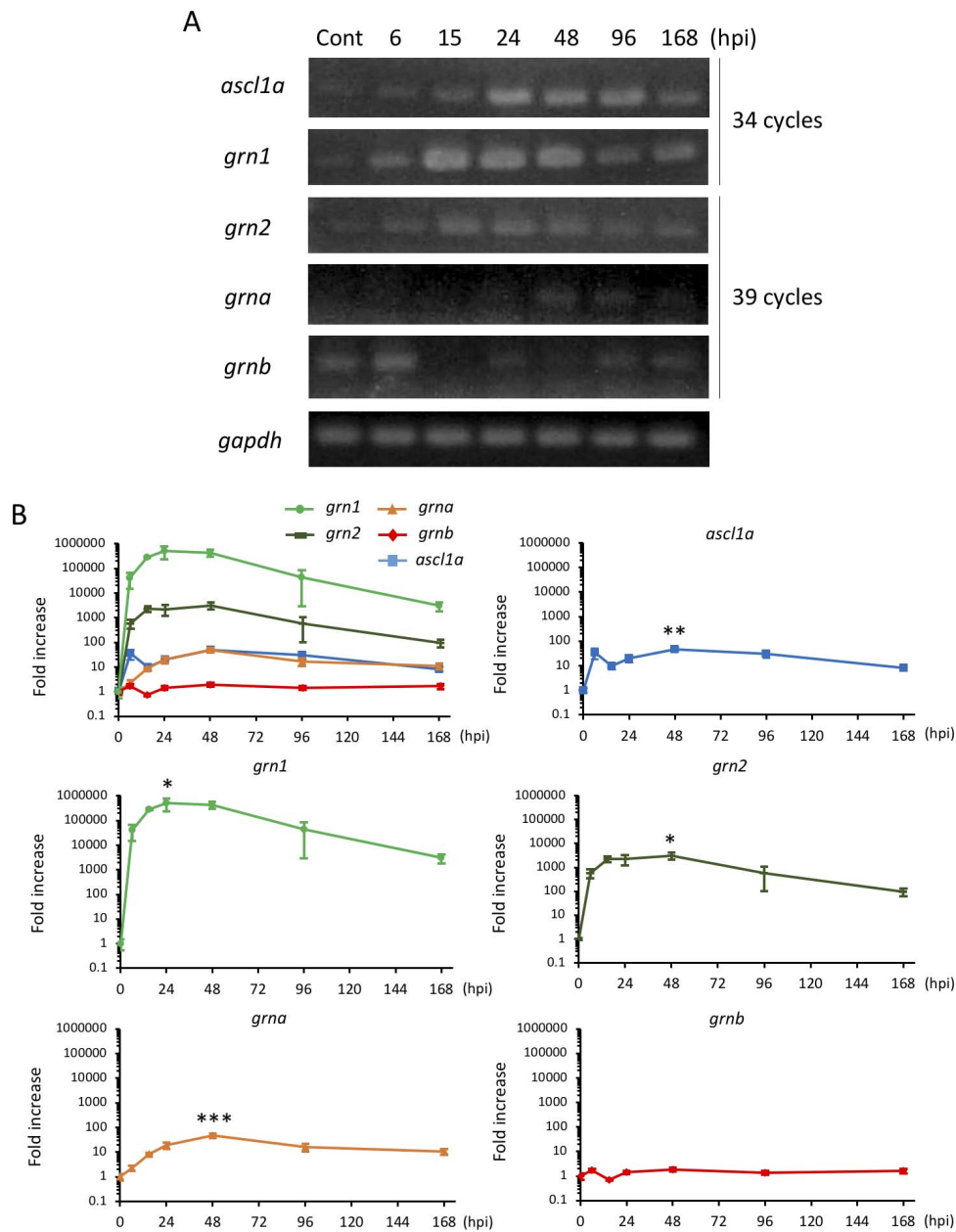


FIGURE 1. Expression of *grn* induced by retinal injury in zebrafish. **(A)** RT-PCR showing the temporal expression pattern of genes encoding the *grn* family and *ascl1a* in the uninjured and injured retinas. **(B)** Quantitative PCR of *grn1*, *grn2*, *grna*, *grnb*, and *ascl1a*. The expression pattern of *ascl1a* is the same as our previous report.¹⁴ Data are the means \pm SEMs ($n = 3$). **** $P < 0.0001$, * $P < 0.05$ versus *ascl1a*. Cropped blots are used in this figure and the full-length blots are presented in Supplementary Figure S5.

contained 50% formamide, 5 \times saline sodium citrate (SSC), 2% blocking reagent (Roche Diagnostics), 0.02% SDS, and approximately 100 ng/mL cRNA probe. Retinal sections on slides were pretreated by proteinase K for 5 minutes. Sections were hybridized at 65°C overnight with a 1-kb DIG-labeled zebrafish *grn1* or *ascl1a* RNA probe. Washing steps included incubations in 2 \times SSC at 65°C. Sections were incubated at room temperature in 1% blocking reagent in maleic acid buffer, then in alkaline phosphatase-conjugated anti-DIG Fab fragments (1:5000 dilution; Roche Diagnostics), and developed 2 hours with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD, USA). Sections were rinsed several times in 100 mM Tris, 150 mM NaCl, 20 mM

EDTA, pH 9.5, and coverslipped with glycerol gelatin (Sigma-Aldrich Corp., St. Louis, MO, USA). The source of the cDNA was a PCR product of adult zebrafish retina. *grn1* cDNA was amplified from zebrafish retina RNA at 4 dpi using primer pairs *grn1* F and *grn1* *isb*. *ascl1a* probe was described previously.¹³

Statistical Analyses

Data are presented as the means \pm SEM. The significance of the differences in the different groups was determined by Student's *t*-tests, Dunnett's tests, or Tukey's tests (GraphPad Prism; GraphPad, La Jolla, CA, USA). A $P < 0.05$ was taken to be statistically significant.

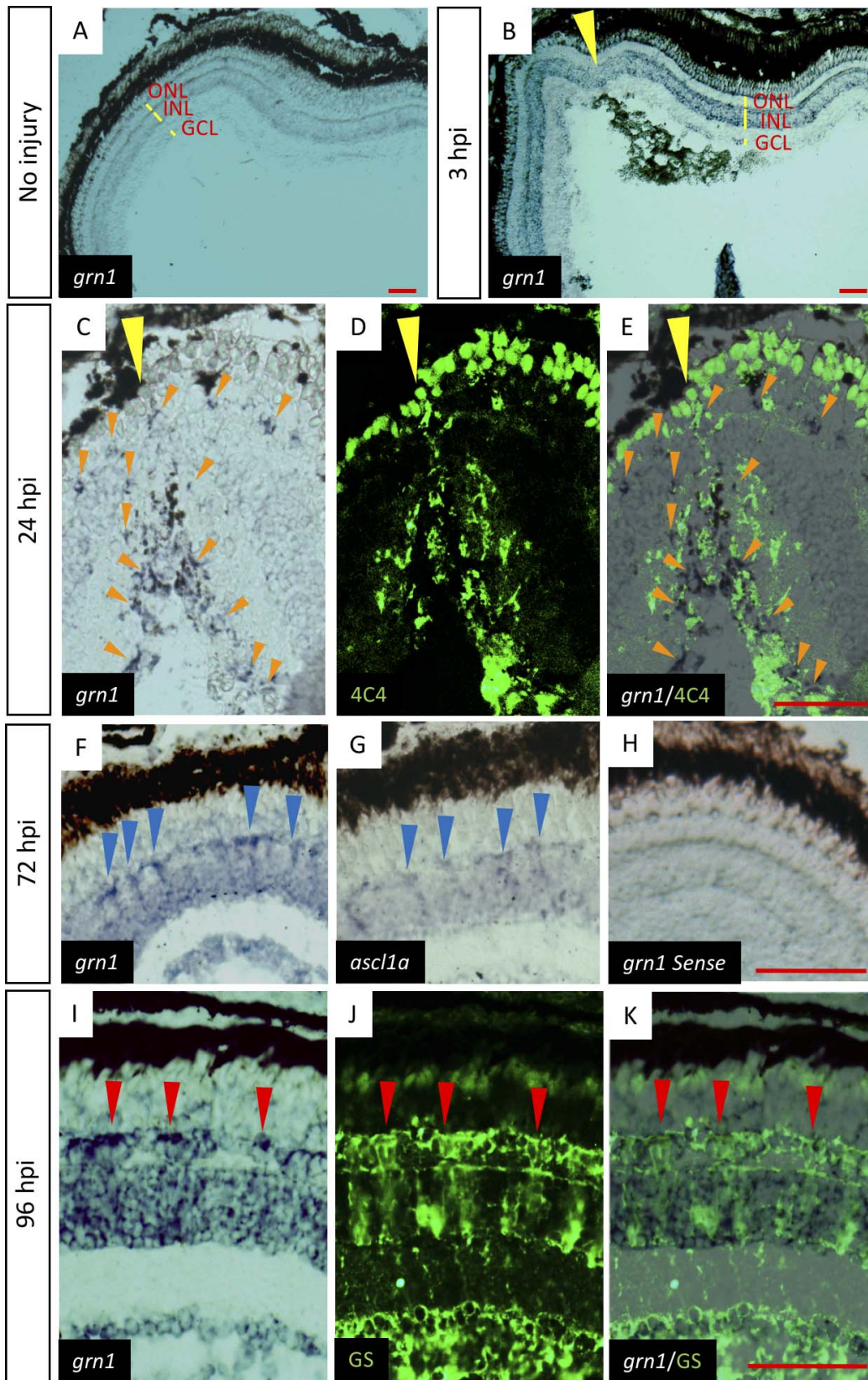


FIGURE 2. Alterations in expression of *grn1* in retinal cells during retinal regeneration. Time-course in situ hybridization analysis shows that *grn1* is expressed in different retinal layers at each time point. In retina, melanin mainly accumulates at the RPE and in situ labeled *grn1* mainly localized inner retinal layer. In situ labeling also shows weaker signal than melanin. (A) In situ hybridization of *grn1* at uninjured control retina. *grn1* is weakly expressed in the INL. (B) In situ hybridization of *grn1* at 3 hpi. Yellow arrowheads indicate the injury site. *grn1* is expressed in the GCL, photoreceptor cells, and INL. The areas that are very close to the injury site express more *grn1*, whereas the injury site has lower expression of *grn1*. (C-E) Retina were stained at 24 hpi. Orange arrowheads indicate expression site of *grn1*. Yellow arrowheads indicate the injury site. (C) In

situ hybridization of *grn1*. (D) Anti-4C4 antibody staining. (E) Merged image. (F-H) Retina were stained at 72 hpi. Blue arrowheads indicate the strong expression site of *grn1* or *ascl1a*. (F) In situ hybridization of *grn1*. (G) In situ hybridization of *ascl1a*. (H) In situ hybridization using *grn1* sense probe. (I-K) retina were stained at 96 hpi. Red arrowheads indicate the colocalization of *grn1* and glutamine synthase (GS). (I) In situ hybridization of *grn1*. (J) Anti-GS antibody staining. (K) Merged image. Scale bars: 50 μ m.

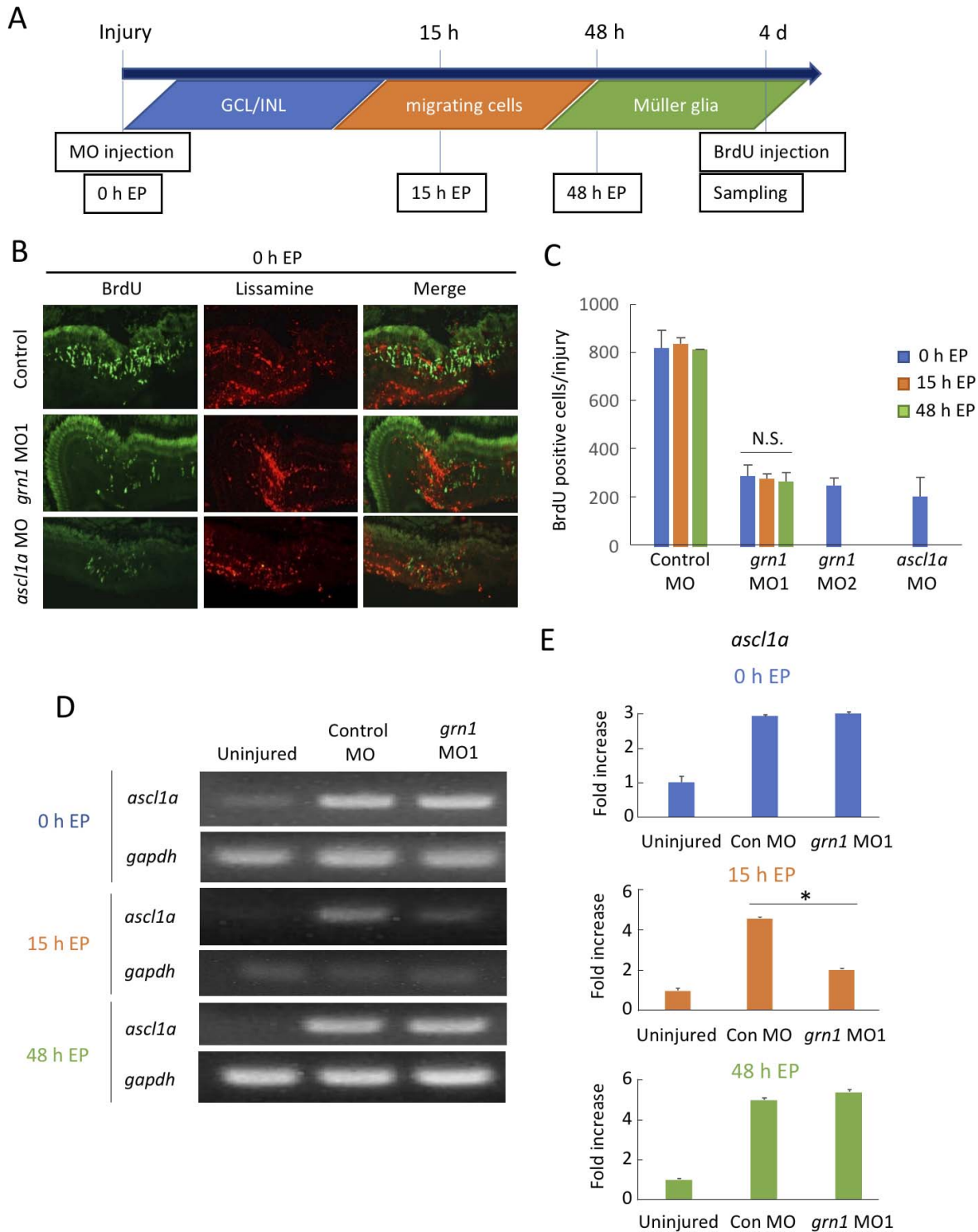


FIGURE 3. Grn1 attenuates proliferation of Müller cells and expression of *ascl1a*. (A) Protocol for antisense MO injection into the zebrafish retina. MOs were injected intravitreally, causing retinal injury. EP was performed at the indicated time. (B) Images of *grn1* MO-, *ascl1a* MO-, and control MO-treated retinas. All MOs were electroporated at 0 hpi. The representative images of BrdU by 15-hour EP or 48-hour EP groups are presented in Supplementary Figure S3. (C) Quantitative analysis of BrdU-positive cells after MO injections. Serial transverse sections were prepared, and the number of BrdU-positive cells per injury were counted. *grn1* MO #2 and *ascl1a* MO were electroporated only at 0 hpi, whereas *grn1* MO #1 was electroporated at 0, 15, and 48 hpi. (D) RT-PCR showing the alteration of *ascl1a* expression by *grn1* knockdown at different times. (E) Semiquantitative analysis of RT-PCR. Data are the means \pm SEMs; $n = 3$. $P < 0.05$ versus control MO-treated group. The cropped blots are used in this figure and the full-length blots are presented in Supplementary Figure S6.

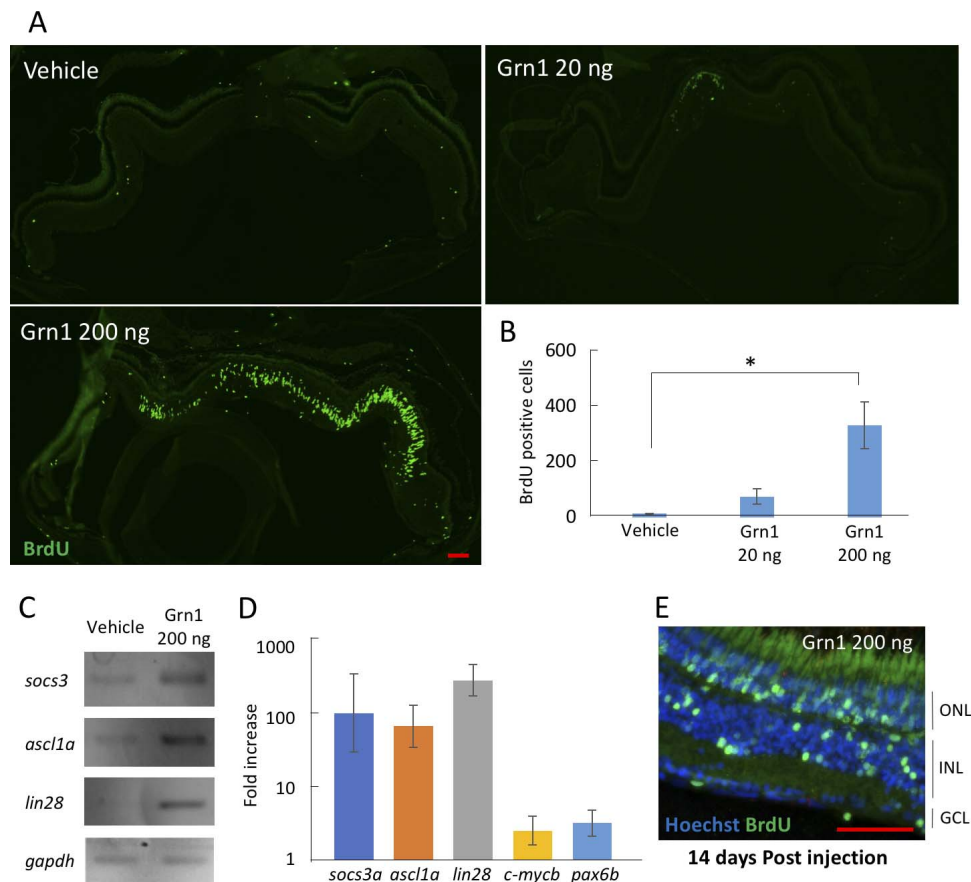


FIGURE 4. Recombinant *grn1* induces retinal regeneration by the induction of retinal regeneration-related genes. (A) Intravitreal injection of recombinant *grn1* induced BrdU-positive cells without retinal injury. BrdU was injected at 4 dpi. (B) Quantitative analysis of BrdU-positive cells on recombinant *grn1* injection. The number of BrdU-positive cells was counted in a single section, including the optic nerve. (C) RT-PCR showing the induction of retinal regeneration-related genes by recombinant *grn1* injection (200 ng). The RNA samples were obtained at 1 dpi of recombinant *grn1*. (D) Quantitative PCR shows recombinant *grn1* highly induced *socs3*, *ascl1a*, and *lin28*, and weakly induced *pax6* and *c-mycb*. (E) BrdU-positive cells have migrated to various retinal cell layers at 14 dpi of recombinant *grn1* (200 ng). Data are the means \pm SEMs; $n = 3$. $P < 0.05$ versus vehicle-treated group. N.S., not significant. Scale bar: 200 μ m. The cropped blots are used in this figure and the full-length blots are presented in Supplementary Figure S7.

RESULTS

Induction of *grn1* in Injured Retina

RT-PCR (Fig. 1A) and quantitative real-time PCR (qPCR; Fig. 1B) showed that *grn1* and *grn2* were induced soon after the retina was injured with the level of expression of *grn1* higher than that of *grn2* (Figs. 1A, 1B). On the other hand, *grna*, which is known to be induced by phototoxicity,³⁰ was induced after *grn1* and *grn2* were induced, and *grnb* was highly expressed at control and unaltered after injury (Figs. 1A, 1B). The peak expression of *grn1* was at 24 hours after the retinal injury, whereas that of *grna* and *ascl1a* were 48 hours (2 days) after the injury (Fig. 1B). Consequently, we found that *ascl1a* induction was preceded by *grn1* upregulation. Therefore, after this experiment, we focused on *grn1*.

Grn1 Changes Expression Location in Retinal Regeneration

In situ hybridization was used to determine the sites of expression of the mRNA of *grn1*. In the uninjured control retina, *grn1* was weakly expressed in the inner nuclear layer (INL) (Fig. 2A). *grn1* was expressed in cells near the injury site at 3 hours post injury (hpi), especially in the retinal ganglion

cell layer (GCL), photoreceptor cells, and INL (Fig. 2B). In contrast, *grn1* was expressed in different retinal layers at 24 hpi (Fig. 2C).

Microglia are known to migrate to the injury site, and sections were stained with anti-4C4 antibody,³⁵ a microglial marker, to determine whether this occurred in the zebrafish (Fig. 2D). To check the migrating cells and *grn1* colocalization, we chose 24 hpi based on the *grn1* expression site. In earlier time points, like 3 hpi, *grn1* expressed in the GCL and INL (Fig. 2B). At a later time point, like 72 hpi, *grn1* expressed in GCL and INL (Fig. 2F) and the migration process is completed. The expression of *grn1* did not colocalize with the 4C4-positive cells (Fig. 2E). Cells double stained by plastin, a marker for microglia and neutrophils, and 4C4 were observed peripheral to the injured site (Supplementary Fig. S1). The pattern of expression of *grn1* was similar to that of *ascl1a* at 3 days post injury (dpi) (Fig. 2F, 2G), and it was also expressed in the GCL and INL, although the migrated cells were not observed at this time. A *grn1* sense-strand probe gave no signal above background (Fig. 2H). Many of the *grn1*-positive cells were colocalized with glutamine synthase-positive cells at 4 dpi indicating the *grn1* was expressed in a subset of Müller cells (Figs. 2I–K).

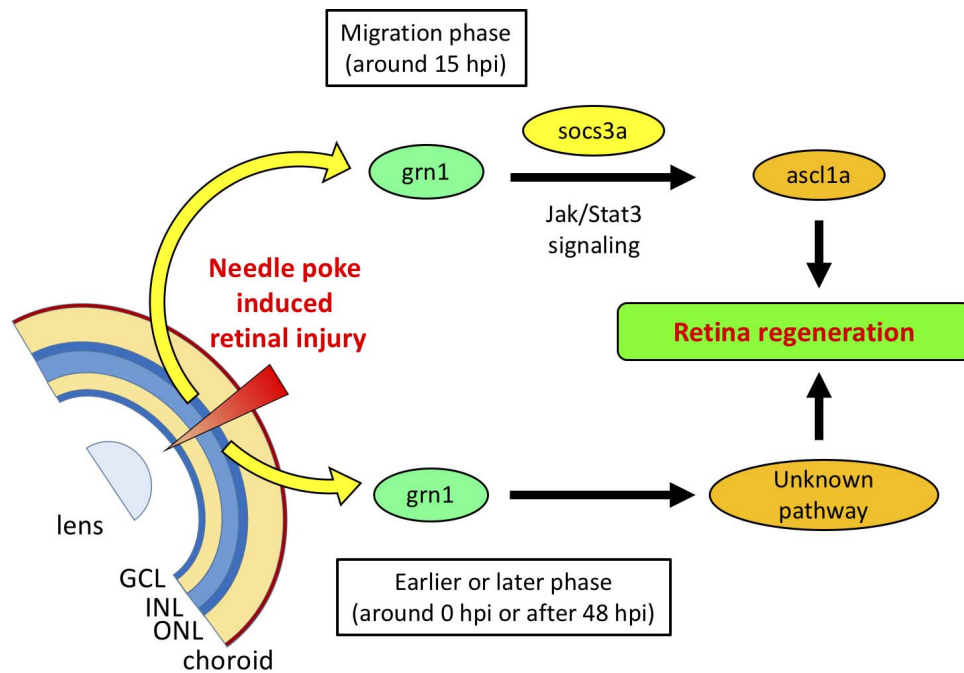


FIGURE 5. Schematic diagram of relationships between *grn1* and retinal regeneration process after needle puncturing.

Grn1 Modulates Proliferation of Müller Glia Cells and Expression of *ascl1a* in Regenerating Retina

The sites of the expression of *grn1* changed with time during the retinal regeneration processes. Therefore, next, we conducted time-dependent *grn1* knockdown experiments using *grn1* MO. To determine the time-specific influence of *grn1*, we conducted and compared electroporation (EP) at three different time points in *grn1* or negative control MO-treated groups (Fig. 3A). In all groups, intravitreal MO injection was conducted at the same time as the injury. We considered that (1) 0 hour EP targeted *grn1*, which expressed in GCL and INL (Fig. 2B), (2) 15-hour EP targeted *grn1*, which expressed in migrating cells like neutrophils (Figs. 2C–E), and (3) 48-hour EP targeted *grn1*, which expressed in Müller glia. The MOs targeting *ascl1a* were also injected as positive controls as reported.¹³ The fish then received an intraperitoneal injection of BrdU 3 hours before they were killed on 4 dpi to quantify proliferating Müller glia and other retinal progenitor cells. Treatment with the MO of *grn1* #1 and *grn1* #2 decreased the number of BrdU-positive cells significantly compared with the negative control MO electroporated at 0 hpi (Figs. 3B, 3C). Injection of *grn1* #1 MO by the electroporation at 15 hpi and 48 hpi also reduced the BrdU-positive cells; however, the reductions were not significantly different from that with the *grn1* #1 MO (Fig. 3C). The expression of the mRNA of *ascl1a* showed that the level of *ascl1a* was significantly reduced only in the group that had been electroporated 15 hours after the *grn1* MO injection (Fig. 3D, E). We also confirmed that lissamine-tagged control MO, which was electroporated at 15 hpi, merged with migrating cells that were stained by anti-4C4 antibody (Supplementary Fig. S2).

Recombinant Zebrafish *grn1* Protein Induces Proliferation of Retinal Cells and Retinal Regeneration-Associated Genes

We investigated whether *grn1* will induce retinal regeneration by injecting recombinant zebrafish *grn1* protein intravitreally

and vehicle in the control eyes (Fig. 4A). BrdU-positive cells were increased at 4 days after the injection of the recombinant *grn1* protein in a dose-dependent manner (Figs. 4A, 4B).

The levels of expression of the mRNAs of the genes associated with retinal regeneration were investigated at 1 day after the injection of the protein of recombinant *grn1*. *ascl1a*, *lin-28*, and *socs3* were significantly increased after the recombinant *grn1* injection, and in contrast, *c-mycb* and paired box 6b (*pax6b*) were only slightly increased (Figs. 4C, 4D).

We used a BrdU lineage-tracing strategy to test if recombinant *grn1* injection stimulated proliferating cells, including Müller glia-derived progenitor cells, to migrate to all layers in the uninjured retina. For this experiment, *grn1* was injected into the vitreous, and 3 days later the zebrafish received an intraperitoneal injection of BrdU and were then killed 10 days later. BrdU-positive cells were observed in all layers of the retina, especially in the outer nuclear layer (ONL) and INL (Fig. 4E).

DISCUSSION

The results showed that an intravitreal injection of zebrafish *grn1*, a short form of progranulin, induced retinal regeneration. *grna* is believed to be the orthologue of human progranulin,²⁷ and several studies have reported that *grna* is associated with liver morphogenesis,²⁸ growth and regeneration of muscle tissues,²⁹ and retinal regeneration.³⁰ However, the function of *grn1* is little known. Progranulin is cleaved into 6-kDa granulin peptides by many proteinases, including neutrophil elastase and matrix metalloproteinases.²⁰ It is not clear which single 6-kDa granulin mediates its biological function. *Grn1* has one-half of the granulin motif, thus *grn1* may have functions that are similar to that of single 6-kDa granulin. In our study, we focused on the *grn1* function against retinal regeneration because of the remarkable changes in expression levels of *grn1* mRNA in all granulin subtypes after needle puncturing (Fig. 1). A previous report showed that zebrafish *grn1*, *grn2*, and *grna* are linked to a Hox gene cluster,²⁷ but *grnb* is not linked. Hox

TABLE. List of Primers

Gene	Primer Sequence
Primers for RT-PCR	
<i>grn1</i>	F: 5'-GGAACAACATGCTGTCTGAGTCC-3' R: 5'-GCCGGGCAGTAGAAATTTCC-3'
<i>grn2</i>	F: 5'-TGGCAAATGGACATGTTGTCC-3' R: 5'-GCCGGGCAGTAGAAATTTCC-3'
<i>grna</i>	F: 5'-CTTTCCGCTGTTTACAAATCC-3' R: 5'-CTGCACAACATGCCTTCAGG-3'
<i>grnb</i>	F: 5'-ACATTTTCAGCTGCAGTTTTCC-3' R: 5'-ACCATTGTGTGAATGTTGGC-3'
<i>grnb (qPCR)</i>	F: 5'-ATTACACAATGGTGCCTGC-3' R: 5'-CCATCCGGACAGATCAGAGC-3'
<i>gapdh</i>	F: 5'-ATGACCCCTCCAGCATGA-3' R: 5'-GGCGGTGTAGGCATGAAC-3'
<i>ascl1a</i>	F: 5'-ATCCGCGCGCTGCAGCAGCTTCTGGACG-3' R: 5'-CGAGTGTGATATTTTTAAGTTTCCTTTTAC-3'
<i>pax6b</i>	F: 5'-ATGGCCACTTCTGGCACCCTCCACAGGAC-3' R: 5'-AGTTCCTGGGAGTGTGAAAAGAGCTCGCGCCTC-3'
<i>c-mycb</i>	F: 5'-GTCGGTCTGTCCGCTTCTCCAGGACATG-3' R: 5'-CTCTTCTTCATCTTCATCATCTTCGTCATCGGA-3'
<i>socs3a</i>	F: 5'-CACTAACTTCTCTAAAGCAGGG-3' R: 5'-GGTCTTGAAGTGGTAAAACG-3'
<i>lin28</i>	F: 5'-TAACGTGCGGATGGGCTTCGGATTTCTGTC-3' R: 5'-ATTGGGTCCTCCACAGTTGAAGCATCGATC-3'
Primers for ISH	
<i>grn1</i>	F 5'-GGAACAACATGCTGTCTGAGTCC-3'
<i>grn1 isb</i>	R 5'-TCATATTGGTCAGCTTTAATGAA-3'
Primers for cloning full-length Grn1	
<i>grn1 ATG</i>	F 5'-GGAAACCCAGCCAGCAAG-3'
<i>grn1 STOP</i>	F 5'-TCAGTAAGAGGTGAACCAGTGTA-3'

F, forward; R, reverse; ISH, in situ hybridization.

gene cluster is important to development and also tissue regeneration in some organs, including eyes.^{36,37} Possibly, these features are reflected as the different expression pattern in our results. However, further studies are needed to uncover the underlying mechanism. Grn1 was expressed in various cell layers, and the amount varied at different times (Figs. 1, 2). The number of BrdU-positive cells was decreased by *grn1* knockdown even though EP was done at 2 days after the injection (Figs. 3A, 3C, Supplementary Fig. S3). Thus, *grn1* may be associated with the proliferation of Müller glia-derived progenitors after retinal injury.^{38,39} Moreover, the *grn1* knockdown study using MO showed that *grn1* strongly relates with the migration process of neutrophil and the blockage of *grn1* at this time point only attenuated the expression of *ascl1a* (Figs. 3D, 3E). In Supplementary Figure S1, we conducted double staining using 24 hpi retina by 4C4 and plastin antibody. Plastin is expressed in both microglia and neutrophils, but 4C4 is expressed in microglia. Thus, we could detect neutrophils as plastin-positive and 4C4-negative cells (green in the merged image of Supplementary Fig. S1). The expression patterns and morphologies of these cells closely resembled the expression pattern of *grn1* mRNA at 24 hpi (Figs. 2C, 2E). Therefore, in our view, *grn1* possibly expressed in neutrophils. In our needle puncturing injury model, choroid is also punctured by needle. We considered the choroid vessels mainly as the source of the neutrophils. Actually, we found slight hemorrhage when sampling eyeballs at the early time points, such as 6 to 24 hpi. We also observed punctured cells that expressed plastin and mpeg at 3 hpi (Supplementary Fig. S4). Thus, amebocytes, such as neutrophils, may migrate into the injury site. Neutrophils secrete neutrophil elastase that can cleave progranulin protein. Hence, proteolytic modifications

may be important for the functioning of *grn1* on *ascl1a* expression. Several studies have reported that neutrophils are important for tissue regeneration.^{40,41} At present, no reports have shown the relationships between *ascl1a* and migrating cells in detail. In our experiments, we showed the importance of *grn1* function at the specific time point (15 hpi) by *grn1* knockdown using MO on *ascl1a* induction (Figs. 3D, 3E). This result indicated that *grn1* acts in an autocrine fashion in migrating cells to regulate *ascl1a* induction; however, at present, the mechanism of this interaction is almost unclear. Therefore, more detailed studies on neutrophils and retinal regeneration will be necessary. Moreover, *grn1* mRNA was also expressed in photoreceptor cells at 3 hpi. However, this expression was attenuated at later time points (Fig. 2). We cannot provide appropriate explanation about the roles and reasons of *grn1* expression at photoreceptor cells. It also needs further investigation.

An intravitreal injection of the recombinant *grn1* protein induced a proliferation of retinal cells including progenitor cells and an upregulation of the genes involved in retinal regeneration (Fig. 4). These findings suggest that *grn1* is one of the early response genes that can trigger the dedifferentiation of Müller cells during retinal regeneration, such as HB-EGF.¹⁰ Moreover, various factors can induce retinal regeneration in zebrafish.^{5,12,42-44} Grn1 induces *socs3a*, which is downstream of Jak-Stat3 signaling, but *grn1* had little effect of the induction of *pax6b*, which is downstream of GSK 3 β -catenin signaling.⁸ *Socs3a* is also regulated by TNF receptor-associated factor 6⁴⁵ or insulin signaling.⁴⁶ However, this signaling is also linked to Jak-Stat3 signaling pathway-associated retinal regeneration.^{16,47} Therefore, we considered *grn1* also associated with Jak-Stat3 signaling like these factors and is less involved in the

GSK3 β signaling pathway. Rat progranulin and its single 6-kDa peptide, granulin A, interact with the EGF-like domain of cartilage oligomeric matrix protein, and they promote cell proliferation.⁴⁸ Thus, *grn1* may exert its regenerative effects by coordinating HB-EGF signaling. On the other hand, progranulin is associated with hepatocyte growth factor signaling,^{24,25,28} and it interacts with various proteins, such as the TNF receptor,⁴⁹ which are associated with retinal regeneration.¹⁶ However, the effect of *grn1* knockdown on the expression of *ascl1a* was limited. Thus, *grn1* may have a supportive effect on the initiation factors of the retinal regeneration. The association of *grn1* with other factors involved in the retinal regeneration are important areas of future investigations. The BrdU-positive cells induced by *grn1* migrated to all retinal cell layers at 14 dpi (Fig. 4E), suggesting they may differentiate into various retinal cells. Further studies will determine whether *grn1* can promote cell differentiation and the fate of these cells.

In conclusion, we have determined that *grn1* is involved in both the dedifferentiation of Müller cells and proliferation of progenitor cells. Our results suggest that *grn1* may play different roles depending on the cells types and the timing of its expression (Fig. 5). Determination of *grn1* signaling will be necessary to understand its roles in retinal regeneration and its application to mammals.

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