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.2–5 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 injury4–6; 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-catelin, 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. Several studies have shown that grna is associated with liver morphogenesis, muscle growth and regeneration, retinal damage, and brain and retinal development. 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. 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 a 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. Relative quantitative real-time PCR was performed in triplicate with SYBR Premix Ex TaqII (Takara Bio). The fixed and frozen eye cups were enucleated, the lens removed, and the eye cups were sectioned at 12-μm thickness. At injured condition, the BrdU-positive cells were counted by single transverse section with 1 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 μL of PBS. The left eye (control) was injected with 1 μL 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 μL 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-μm thickness for immunofluorescence as described in detail. The antibodies used were: MO: 5′-CCITCTACCTCAGTTAATTATA-3′; ascl1a MO: 5′-ATCCTTGCGGTAGTCCCATGCC-3′; grn1 MO #1: 5′-CATCCTGGGTGCTGGTCTCCT-3′; grn1 MO #2: 5′-ACATTAACTCGAGATTTGCAATGAG TAAGGACA-3′.

For bromodeoxyuridine (BrdU) incorporation, a solution of 20 μL of 20 mM BrdU was injected intraperitoneally into anesthetized fish at 4 days after injury.
contained 50% formamide, 5× 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× 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 Fa and grn1 ish. ascl1a probe was described previously.13

**Statistical Analyses**

Data are presented as the means ± 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
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 ± 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.
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 14 dpi of recombinant grn1 (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 ± SEMs; n = 3. P < 0.05 versus vehicle-treated group. N.S., not significant. Scale bar: 200 μm. The cropped blots are used in this figure and the full-length blots are presented in Supplementary Figure S7.

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 ± SEMs; n = 3. P < 0.05 versus vehicle-treated group. N.S., not significant. Scale bar: 200 μm. The cropped blots are used in this figure and the full-length blots are presented in Supplementary Figure S7.

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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 electroperoration (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...
gene cluster is important for development and also tissue regeneration in some organs, including eyes. 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 important for the functioning of Muller glia-derived progenitors after retinal injury. Moreover, the early response genes that can trigger the dedifferentiation of Muller cells during retinal regeneration, such as HB-EGF.10 These findings suggest that grn1 is one of the early response genes that can trigger the dedifferentiation of Muller cells during retinal regeneration, such as HB-EGF. Moreover, various factors can induce retinal regeneration in zebrafish.5,12-22 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-3β-catenin signaling.8,9 Socs3a is also regulated by TNF receptor-associated factor 6 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 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 Muller cells during retinal regeneration, such as HB-EGF. Moreover, various factors can induce retinal regeneration in zebrafish.5,12-22 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-3β-catenin signaling.8,9 Socs3a is also regulated by TNF receptor-associated factor 6 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

### Table. List of Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>Primers for RT-PCR</td>
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<tr>
<td>grn1</td>
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<tr>
<td>grn2</td>
<td>R: 5'-GCGGCGACTGATTTCC-3'</td>
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<tr>
<td>grnα</td>
<td>F: 5'-TTGCGAAAAGATGTTCC-3'</td>
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<td>grnβ</td>
<td>R: 5'-GCGGCGACTGATTTCC-3'</td>
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<tr>
<td>gapdb</td>
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<td>ascl1a</td>
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<tr>
<td>pax6b</td>
<td>R: 5'-GCGGCGACTGATTTCC-3'</td>
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<tr>
<td>c-mycb</td>
<td>F: 5'-ATGTCCTGCTGCGCTGATTTCC-3'</td>
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<td>socs3α</td>
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<td>lin28</td>
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<tr>
<td>Primers for ISH</td>
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<td>grn1 STOP</td>
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F, forward; R, reverse; ISH, in situ hybridization.
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Granulin is associated with hepatocyte growth factor signaling, and it interacts with various proteins, such as the TNF receptor. Thus, grn1 may exert its regenerative effects by coordinating HB-EGF signaling. On the other hand, progranulin is associated with hepatocyte growth factor signaling and it interacts with various proteins.

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 is 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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