Published in final edited form as:

Cell Rep. 2017 April 25; 19(4): 849–862. doi:10.1016/j.celrep.2017.04.009.

Opposing actions of Fgf8a on Notch signaling distinguish two Muller glial cell populations that contribute to retina growth and regeneration

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Summary

The teleost retina grows throughout life and exhibits a robust regenerative response following injury. Critical to both these events are Muller glia (MG) that produce progenitors for retinal growth and repair. We report that Fgf8a may be a MG niche factor that acts through Notch signaling to regulate spontaneous and injury-dependent MG proliferation. Remarkably, forced Fgf8a expression inhibits Notch signaling and stimulates MG proliferation in young tissue, but increases Notch signaling and suppresses MG proliferation in older tissue. Furthermore, cessation of Fgf8a signaling enhances MG proliferation in both young and old retinal tissue. Our study suggests multiple MG populations contribute to retinal growth and regeneration, and reveals a previously unappreciated role for Fgf8a and Notch signaling in regulating MG quiescence, activation and proliferation.

Keywords

Zebrafish; retina; regeneration; reprogramming; Muller glia; Fgf8; Notch

Introduction

Retinal neuron death underlies many blinding eye diseases like macular degeneration and glaucoma. Regenerating lost neurons may restore sight to the blind. Unlike mammals, zebrafish can regenerate retinal neurons (Goldman, 2014). Key to this regenerative response are Muller glia (MG) a cell type that normally contributes to retinal homeostasis and architecture (Reichenbach and Bringmann, 2013). Following retinal injury, zebrafish MG reprogram their genome so they express genes that endow them with stem cell properties (Powell et al., 2013; Ramachandran et al., 2010a). These reprogrammed MG exhibit a transient gliotic response, divide and generate a proliferating population of progenitors that regenerate all major retinal cell types regardless of which neurons are lost (Fausett and Goldman, 2006; Nagashima et al., 2013; Powell et al., 2016; Ramachandran, 2010; Thomas et al., 2016).

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Author Contributions: DG and JW designed and analyzed the research and wrote the paper. JW performed all experiments.

Although a number of secreted factors, genes and signaling molecules regulating MG proliferation have been studied (Conner et al., 2014; Fausett et al., 2008; Hochmann et al., 2012; Nelson et al., 2013; Nelson et al., 2012; Rajaram et al., 2014a; Rajaram et al., 2014b; Ramachandran et al., 2010a; Ramachandran et al., 2011, 2012; Raymond et al., 2006; Thummel et al., 2010; Wan, 2012; Wan et al., 2014; Yurco and Cameron, 2007; Zhao et al., 2014), the molecular logic connecting their activities to uninjured and injured retinal environments remains poorly understood. Here we provide evidence that Fgf8a helps link the retinal environment to signaling molecules and gene expression programs that regulate MG proliferation. Fgf8 is part of an Fgf subfamily that includes Fgf17 and Fgf18 (Ornitz and Itoh, 2015). In the developing retina, Fgf8 regulates neural retina formation and triggers retinal progenitors to differentiate (Martinez-Morales et al., 2005; Nishihara et al., 2012). Interestingly, our studies suggest that Fgf8a differentially regulates Notch signaling in 2 different MG cell populations and that the distribution of these populations changes with age. We demonstrate that the Fgf8a-Notch signaling pathway regulates MG activation and proliferation, and may underlie age-related changes in spontaneous MG proliferation.

Results

Spatial and temporal expression of *fgf8a* following retinal injury

Previous studies indicated that Fgf signaling is important for retina regeneration (Hochmann et al., 2012; Wan et al., 2014). To further investigate this, we surveyed Fgf ligand gene expression following a needle-poke retinal injury and found *fgf8a* and *fgf8b* were transiently suppressed (Figures 1, S1A and S1B). *Fgf8* sub-family members, *fgf17* and *fgf18* were unaffected by retinal injury (Figure S1C). Fgf-responsive genes *dusp6* and *etv5b* were transiently suppressed, while *spry4* and *pea3* were induced (Figure S1D and S1E). Interestingly, injury-dependent expression of *fgf8a* and *pea3* exceeded basal levels around 4-14 days post injury (dpi) before returning at 30 dpi. Because of the robust injury-dependent suppression noted in *fgf8a*, we further characterized its expression and investigated its action on MG proliferation.

In situ hybridization assays showed *fgf8a* expression in the inner nuclear layer (INL) and at the base of photoreceptor outer segments in the outer nuclear layer (ONL) of the uninjured retina (Figure 1B). Within 3 hrs post injury (hpi) this expression was suppressed in a pan retinal fashion (Figure 1). At 4 dpi when *fgf8a* expression had returned to the INL, *in situ* hybridization assays combined with BrdU immunofluorescence showed it is enriched in MG-derived progenitors (Figure 1B and 1C). *fgf8a* RNA is also detected in the ganglion cell layer (GCL), which may reflect expression in MG end feet and/or ganglion cells (Figure 1B).

We used dissociated retinas from *gfap:GFP* transgenic fish to FACS purify GFP+ MG and GFP- non-MG for RNA analysis. RT-PCR confirmed *fgf8a* suppression at 3 hpi and enrichment in MG-derived progenitors at 4 dpi (Figure 1C). This analysis also showed *fgf8a* expression in retinal neurons (non-MG) in uninjured and injured retina. *ascl1a*, a transcript known to be induced in MG-derived progenitors (Fausett et al., 2008; Ramachandran et al., 2010a), served as a positive control for their purification by FACS (Figure 1C). Furthermore, *fgf8a* suppression was not unique to the needle poke injury model as destruction of neurons

in the INL and ganglion cell layer (GCL) using NMDA, or the photoreceptor layer using metronidazole in *zop:nsfb-EGFP* transgenic fish also caused *fgf8a* suppression (Figure S1F-S1I) (Montgomery et al., 2010; Powell et al., 2016).

Fgf8a expression suppresses MG proliferation in the injured retina

Injury-dependent *fgf8a* suppression suggested a role in MG quiescence. To test this, we took advantage of *hsp70:fgf8a* transgenic fish to conditionally express Fgf8a with heat shock (HS) (Kwon et al., 2010). In these fish, a 1 hour (h) HS at 37°C elevates *fgf8a* expression for ~12h (Figure S2A). To maintain elevated Fgf8a levels for multiple days, fish received a 1h HS 3× a day. A needle poke injury through the back of the eye was used to injure the retina and MG proliferation was assayed with an intraperitoneal (IP) injection of BrdU 3h before sacrifice. BrdU immunofluorescence on retinal sections showed Fgf8a inhibits injury-dependent MG proliferation (Figures 2A and S2B). It also inhibited HB-EGF/Insulin-dependent MG proliferation in the uninjured retina (Figure 2B) (Wan, 2012; Wan et al., 2014). Furthermore, daily intravitreal injection FGF2 had no effect on injury-dependent MG proliferation (Figure S2C and S2D). These data suggest Fgf8a maintains MG quiescence and its suppression after injury is necessary for MG proliferation.

We next investigated if Fgf8a regulated genes known to impact MG proliferation (Fausett et al., 2008; Ramachandran et al., 2010a; Ramachandran et al., 2011, 2012). For these experiments Wt and *hsp70:fgf8a* fish retinas were injured, fish were HS for 2 days and then sacrificed. At 2 dpi MG are beginning to proliferate (Fausett and Goldman, 2006). qPCR revealed that forced Fgf8a expression suppressed *ascl1a*, *ccnd1* and *cdk2* whose products stimulate MG proliferation, and induced *dkk1b* and *p21* whose products inhibit MG proliferation (Figure 2C) (Fausett et al., 2008; Ramachandran et al., 2010a; Ramachandran et al., 2011, 2012; Zhang et al., 2016). Similar results were seen at 6 hpi and 4 dpi (Figure S2E). These gene expression changes provide a molecular link between Fgf8a expression and MG quiescence.

Cessation of Fgf8a expression stimulates injury-dependent MG proliferation

Since Fgf8a is suppressed following retinal injury, we investigated if relief from forced Fgf8a expression impacts MG proliferation. For this analysis, Wt and *hsp70:fgf8a* fish retinas were injured and fish received HS for 2 days; at 4 dpi fish received an IP injection of BrdU 3h before sacrifice. MG proliferation was assayed 2 days later (4 dpi). BrdU immunofluorescence revealed a dramatic increase in MG proliferation in *hsp70:fgf8a* fish (Figure 3A). This was also demonstrated by labelling proliferating cells at 2 dpi with BrdU and then assaying the number of BrdU+ cells still proliferating at 4 dpi with a pulse of EdU (Fig. 2B). When we assayed MG proliferation at different times post HS, we found that forced Fgf8a expression for 2 days delayed the peak in MG proliferation by about 1 day and increased BrdU+ cells by about 3-fold (Figure 3C). Remarkably, a 1h HS at the time of injury (Figures 3D and S3A), just before injury (Figure S3B and S3C) or at 1 dpi (Figure S3D) was sufficient to enhance MG proliferation when assayed 2-4 days later. This enhanced proliferation required Fgf receptor (Fgfr) signaling since it was inhibited by SU5402 (Figure 3D). Importantly, this enhanced proliferation was not associated with increased cell death (Figure S3E and S3F). In contrast, a 1h HS delivered at 2 dpi and

assayed 3 hrs later when Fgf8a levels are still high, suppressed MG proliferation (Figure S3G). Thus, although forced Fgf8a expression for 2 days suppressed injury-dependent MG proliferation (Figure 2A), cessation of this expression stimulated a dramatic increase in MG proliferation (Figure 3A-3C).

The expanded zone of MG proliferation associated with Fgf8a decline was similar to that caused by Insm1a knockdown (Ramachandran et al., 2012). This suggested a link between these signaling molecules. Indeed, a 1h HS at the time of injury in *hsp70:fgf8a* fish suppressed *insm1a* gene expression 24-48 hr later and stimulated *ccnd1* expression (Figure 3E), which is a known consequence of Insm1a suppression (Ramachandran et al., 2012). Thus, insm1a suppression provides a molecular link between Fgf8a decline and the expanded zone of injury-responsive MG.

Fgf8a knockdown and MG proliferation in the uninjured and injured adult retina

The above data suggested injury-dependent Fgf8a suppression is necessary for MG proliferation. To investigate if Fgf8a suppression was sufficient for MG proliferation, we electroporated uninjured retinas with a control or *fgf8a*-targeting lissamine-modified morpholino (MO) that was previously reported to reproduce the *acerebellar fgf8a* mutant fish phenotype (Araki and Brand, 2001). Four days after electroporation, fish received an IP injection of BrdU 3h prior to sacrifice. BrdU immunofluorescence on retinal sections indicated Fgf8a knockdown had no effect on MG proliferation in the uninjured retina (Figure 3F).

Although Fgf8a is suppressed immediately following injury, it returns to ~30% of its basal level by 1 dpi (Figures 1A and S1A). We wondered if knockdown of these returning Fgf8a levels would stimulate MG proliferation similar to that seen when forced Fgf8a expression was ceased (Figure 3A). For this analysis injured retinas were electroporated with control and *fgf8a*-targeting MOs at 1 dpi and MG proliferation assayed 3 days later (Figure 3G). Indeed, suppression of returning Fgf8a levels in the injured retina resulted in increased MG proliferation. These data are consistent with the idea that Fgf8a suppression collaborates with injury-related factors to regulate MG proliferation.

Cessation of Fgf8a signaling synergizes with injury-related factors to stimulate MG proliferation

Relief from Fgf8a signaling expands the zone of injury-responsive MG (Figure 3A). Because pan retinal *fgf8a* suppression only stimulates MG proliferation near the injury site, it is likely to collaborate with injury-related factors. We previously identified secreted factors expressed at the injury site that are capable of stimulating MG proliferation in the uninjured retina (Wan, 2012; Wan et al., 2014; Zhao et al., 2014). We hypothesized that loss of Fgf8a signaling may lower the threshold at which MG respond to these factors and thereby, recruit more MG to proliferate near the injury site. Indeed, a 1h HS in *hsp70:fgf8a* fish followed by daily intravitreal injections of growth factors at concentrations that would not normally stimulate MG proliferation resulted in a robust proliferative response (Figure 3H and 3I). These results are consistent with the idea that cessation of Fgf8a signaling is not sufficient to

stimulate MG proliferation, but may help MG transition to an activated state that exhibit a lower proliferative threshold to injury-related factors.

Fgf8a regulates Notch signaling

Like Fgf8a, Notch signaling activation or repression can inhibit or stimulate injury-dependent MG proliferation, respectively (Wan, 2012). This suggested a connection between these 2 pathways. To visualize Notch signaling we used Tg(*T2KTp1bglob:hmgb1-mCherry*) (abbreviated *Tp1:mCherry*) fish that express a nuclear-targeted mCherry fusion protein under the control of a minimal promoter harboring 12 RBP-Jk binding sites (Ninov et al., 2012; Parsons et al., 2009). In adult retina, mCherry is restricted to glutamine synthetase (GS)+ MG and this expression was suppressed by treating fish with Notch signaling inhibitors, DAPT and RO 4929097 (Figure 4A). Retinal injury suppressed mCherry expression in injury-responsive MG (Figure 4B). However, Notch signaling inhibition was not sufficient to stimulate MG proliferation in an uninjured retina (Figure 4C). These data along with our previous observation that Notch signaling inhibition results in an expansion of the zone of proliferating MG in the injured retina (Wan, 2012), suggested that Notch signaling inhibition, like relief from Fgf8a expression, drives MG into an activated state, that lowers their proliferative threshold for injury-related factors.

The similar effects of Fgf8a and Notch signaling on MG activation and proliferation suggested they may be part of a common pathway. To investigate if Fgf8a regulated Notch signaling, we generated *hsp70:fgf8a;Tp1:mCherry* double transgenic fish. Retinal injury and HS in these fish showed forced Fgf8a expression from 1-2 dpi or 3-4 dpi stimulates Notch signaling and inhibits MG proliferation (Figures 4D and S4B). Similarly, a 1h HS at the time of injury, which will maintain high levels of Fgf8a for 12h (Figure S2A), prevented the rapid decline in Notch signaling that normally occurs with injury (Figure S4A). In contrast to forced Fgf8a expression enhancing Notch signaling, cessation of forced Fgf8a expression resulted in reduced Notch signaling (Figure S4C). Importantly, the inhibitory effects of forced Fgf8a expression on MG proliferation were overcome by DAPT (Figure 4E). Thus, Fgf8a appears to act upstream of Notch to inhibit MG proliferation.

We previously demonstrated that forced and sustained NICD-Myc expression inhibits MG proliferation (Wan, 2012). Here we tested if relief from Notch signaling enhanced MG proliferation. Indeed, a 1h HS of *hsp70:gal4;uas:nicd-myc* fish (Scheer et al., 2001) was sufficient to enhance injury-dependent MG proliferation 3 days later (Figure S4D).

Notch ligands are a potential link between Fgf8a expression and Notch activity. Although we previously reported *dlA*, *dlB*, *dlC* and *dD*, along with the Notch responsive gene *her4* were induced following retinal injury (Wan, 2012), we found that *dll4* and *hey1* were rapidly and transient suppressed similar to *fgf8a* (Figures 4F and S4E). Furthermore, a 1h HS treatment in *hsp70:fgf8a* fish stimulated *dll4* and *hey1* expression 6h later when Fgf8a levels are still high (Figure 4G). *In situ* hybridization assays suggest that although *dll4* is suppressed by retinal injury in a pan retinal fashion, it is most highly suppressed at the injury site (Figure S4F). Although retinal injury and heat shock in *hsp70:fgf8a* fish resulted in increased *dll4* and *hey1* mRNA expression (Figure 4G), these RNAs were suppressed 2 days after heat shock treatment when MG proliferation is increased (Figure 4H). Thus, *hey1*

expression may be a reporter of Notch signaling in the injured fish retina and Fgf8a-dependent regulation of *dll4* expression may provide a link between Fgf8a and Notch signaling.

Age-dependent switch in Fgf8a signaling

Although Fgf8a inhibits injury-dependent MG proliferation, we noted that forced expression of Fgf8a stimulated a small amount of proliferation in the uninjured retinal periphery of a 6 mo old fish (Figure 5A). Because new neurons are added to the retinal periphery throughout the fish's life, the retinal periphery is younger than the central retina (Johns, 1977; Otteson and Hitchcock, 2003). This raised the possibility that Fgf8a had different effects on MG residing in young and old retina. Indeed, Fgf8a stimulated MG proliferation throughout the 2 mo old fish retina, while in the 6 and 36 mo old fish retina this proliferation was restricted to the retinal periphery (Figures 5A, 5B and S5A). This effect of forced Fgf8a expression was remarkably similar to spontaneous MG proliferation observed in 2 and 6 mo old fish retinas, where proliferation in the central region is reduced with age, while proliferation in peripheral regions is maintained (Figure 5C). In addition, 2 days post HS in 2 mo old *hsp70:fgf8a* fish we observed a ~72% increase in MG proliferation, and in 6 mo old fish this resulted in an expansion of the peripheral zone of proliferating MG (Figure S5A). Thus, forced expression of Fgf8a and relief from this expression both stimulated MG proliferation in young retina. These results suggest 2 different MG populations one of which may be shared with MG residing in the central regions of older retina where relief of Fgf8a signaling also enhanced injury-dependent MG proliferation and forced Fgf8a expression inhibited MG proliferation (Figures 2A and 3A).

We next investigated if we could recreate an environment more similar to that of a 2 mo old fish retina in the central region of an older fish retina by stimulating retina regeneration. For this analysis 6 mo old Wt and *hsp70:fgf8a* fish received a single needle poke injury to their central retina and an IP injection of BrdU at 4 dpi to label proliferating MG. At 10 dpi, when most MG had returned to quiescence (Fausett and Goldman, 2006), fish received HS for 4 days followed by an IP injection of EdU 3h before sacrifice. Quantification of BrdU and EdU immunofluorescence in the INL showed forced Fgf8a expression stimulated BrdU+ MG to proliferate in the absence of an injury (Figure 5D), which is similar to their response to Fgf8a in the 2 mo old fish retina (Figure 5A). This acquired proliferative response to Fgf8a may be the consequences of a new regenerated environment or an intrinsic change in MG as a result of cell division. We also noted some cell division in the ONL (BrdU-/EdU+) that likely represent rod progenitors stimulated to divide by HS, since similar numbers were observed in Wt and *hsp70:fgf8a* fish.

Taken together, the above data suggest an age-dependent switch in how MG respond to Fgf8a signaling. To narrow in on when this occurs, we assayed the consequences of forced Fgf8a expression on injury-dependent MG proliferation in 2-6 mo old fish. We found that MG in the central retina switch their response to Fgf8a from pro-proliferative to anti-proliferative between 3-4 mo of age (Figures 5E and S5B). Consistent with an age-dependent switch in the MG response to Fgf8a, we found that MG in the injured central (older) and peripheral (younger) regions of a 6 mo old fish retina respond in an opposite

fashion to Fgf8a (Figure 5F). Importantly, the different responses of young vs old, or central vs peripheral retina to injury did not correlate with MG density (Figure S5C) (Mack et al., 1998).

The above data showed Fgf8a stimulates injury-dependent MG proliferation in the periphery of a 6 mo old retina similar to its effects on a young retina. We wondered if the cessation of Fgf8a expression would enhance this injury response similar to what we observe in the central retina. For this analysis we injured the peripheral retina of 6 mo old Wt and *hsp70:fgf8a* fish with a single needle poke injury. Fish then received HS for 2 days and at 4 dpi, fish received an IP injection of BrdU 3h before sacrifice. In addition, another set of fish were left uninjured, but otherwise treated as above to account for Fgf8a-induced injury-independent MG proliferation in the retinal periphery (Figure S5A). Quantification of BrdU immunofluorescence showed that cessation of Fgf8a expression stimulates injury-dependent MG proliferation in the retinal periphery beyond that observed when Fgf8a expression is maintained (Figure 5G). Similarly, cessation of forced Fgf8a expression in the injured 2 mo old fish retina dramatically enhanced MG proliferation (Figure 5H). Furthermore, like the 6 mo old fish retina (Figure 3F and 3G), Fgf8a knockdown in the 2 mo old fish retina had no effect on MG proliferation in the uninjured retina, but did stimulate injury-dependent MG proliferation at 1 dpi when Fgf8a levels are returning (Figure 5I and 5J). The small amount of proliferation noted in uninjured retina electroporated with the control and *fgf8a*-targeting MOs appears to be activation of rod progenitors that are stimulated to divide by electroporation. Thus, forced Fgf8a expression has opposing actions on MG proliferation in the central regions of young and old fish retina, while the cessation of Fgf8a signaling has similar effects. These different effects of Fgf8a expression and relief may best be explained by 2 different MG populations whose distribution changes with age (see Discussion and Figure 7).

Because the cessation of forced Fgf8a expression recruits quiescent MG to a proliferative response and because spontaneously proliferating MG are normally restricted to a rod lineage (Bernardos et al., 2007), we wondered if quiescent MG recruited to a proliferative response following cessation of Fgf8a signaling would be biased towards a rod fate. We used a BrdU-based lineage tracing strategy to compare the fate of MG-derived progenitors in uninjured and injured retina from Wt and *hsp70:fgf8a* HS-treated fish at 6 mo of age (Figure S5D). Although some quantitative differences were noted in the cell types regenerated, all proliferating MG populations were multipotent (Figure S5D), suggesting factors other than Fgf8a restrict MG to a rod progenitor lineage.

Fgf8a-dependent MG proliferation in the uninjured retina utilizes the same signaling systems that drive injury-dependent proliferation

In the uninjured 2 and 6 mo old fish retina, Notch signaling is restricted to MG regardless of their central or peripheral location (Figures 4A, S6A and S6B). Forced Fgf8a expression stimulates Notch signaling and inhibits injury-dependent MG proliferation in the central region of a 6 mo old fish retina (Figure 4D). In contrast, forced Fgf8a expression stimulates MG proliferation throughout the uninjured 2 mo old fish retina and in the periphery of the 6 mo old fish retina (Figures 5A, 5B, S5A, 6A and 6B). This Fgf8a-dependent proliferation is

accompanied by reduced Notch signaling (Figure 6A and 6B) and *dll4* expression (Figure 6C). The reason for a lack of effect of forced Fgf8a expression on *dll4* expression in the central retina of uninjured 6 mo old fish is not known, but may indicate that basal Fgf8a levels are sufficient for maximal *dll4* expression. The expression of other *delta* genes did not correlate with Notch signaling inhibition (Figure S6C). Thus, Fgf8a signaling switches from stimulation to inhibition as the retina ages.

To examined if this age-related switch in Fgf8a signaling is reflected in Fgfr expression, we sorted GFP+ MG from 2 mo and 6 mo old *gfap:GFP* fish and also the central and peripheral regions of 6 mo old fish and assayed *fgfr* RNA expression by qPCR. Although this analysis identified changes in *fgfr3* expression as fish transitioned from 2-6 mo of age, these changes were relatively small and not observed in central vs peripheral retina (Figure S6D and S6E). Finally, we found no evidence that force Fgf8a expression would significantly alter *fgfr* expression in the central or peripheral region of the retina (Figure S6F).

Previous studies demonstrated that in addition to Notch signaling inhibition, injury-dependent MG proliferation requires activation of MAPK, PI3K and Jak/Stat3 signaling (Wan, 2012; Wan et al., 2014; Zhao et al., 2014). Similarly, we found these pathways are necessary for Fgf8a-dependent MG proliferation in the uninjured retina (Figure 6D and 6E). Furthermore, Fgf8a-induced proliferation in the periphery of the 6 mo old retina was accompanied by increased expression of *ascl1a*, *hbegf*, *ins* and *igf1*, and suppression of *dkk1b* (Figure 6F), just as is observed in proliferating MG of the injured retina (Fausett et al., 2008; Ramachandran et al., 2010a; Ramachandran et al., 2011; Wan, 2012; Wan et al., 2014). Thus, the pro-proliferative actions of Fgf8a in the uninjured retina tap into the same signaling and gene expression programs that are activated in response to retinal injury.

Discussion

Our studies have identified a remarkable plasticity in the response of MG to Fgf8a that regulates their proliferation. We found that Fgf8a can either stimulate or inhibit Notch signaling in MG and that Notch inhibition allows MG to adopt an activated state that lowers their proliferative threshold to injury-related factors (Figure 7). These divergent Notch signaling responses to Fgf8a suggest 2 different MG populations (Figure 7). In fish ≥ 4 mo of age, the MG population (MGc) that responds to Fgf8a with increased Notch signaling predominate in the central region of the retina, while the MG population (MGp) that responds to Fgf8a with decreased Notch signaling is found in the periphery and excluded from the central retina. Our data suggest the MGc population responds to retinal injury with increased proliferation when Fgf8a and Notch signaling is relieved. In fish ≤ 2 mo of age, MGc and MGp populations are intermixed throughout the retina. Thus, there is a change in MGc and MGp ratios in the central retina as fish age. Whether this represents a redistribution of these 2 MG populations or a transformation of one population into the other is not clear. This heterogeneity in MG populations allows for differential responses to Fgf8a in central and peripheral regions of the retina that contribute to spontaneous and injury-dependent MG proliferation.

It is well known that MG contribute to the postembryonic growth of the fish retina that occurs throughout life (Johns and Easter, 1977). This growth results from a peripherally restricted stem cell population and also by a balloon-like stretching (Bernardos et al., 2007; Johns, 1977; Johns and Easter, 1977; Julian et al., 1998; Kwan et al., 1996; Otteson et al., 2001; Otteson and Hitchcock, 2003). Importantly, peripheral stem cells do not make rods, rather rod progenitors are generated from spontaneously dividing MG (Bernardos et al., 2007). We found that spontaneous MG proliferation in the uninjured central region of the retina decreases with age similar to the changing distribution of MGc and MGp populations. Thus, in the adult fish retina, pan retinal Fgf8a expression will stimulate Notch signaling in the MGc population concentrated in the central retina and inhibit their proliferation; however, Fgf8a will reduce Notch signaling in the MGp population that is relegated to the retinal periphery and this may be sufficient to lower their threshold response to pro-proliferative factors in the environment.

The molecular mechanism by which Fgf8a elicits opposite effects on Notch signaling in MGc and MGp populations remains unknown. We did not find significant changes in Fgfr subtype expression that correlated with the different MG populations. However, we did find that Fgf8a differentially regulates *dll4* expression in young and old retinal tissue. Interestingly, *fgf8a* RNA is decreased throughout the retina after injury, but *dll4* suppression was greatest at the injury site where Notch signaling was suppressed. This preferential loss in *dll4* at the injury site may reflect the MGc population that predominates there. Additional contributors to this injury-dependent Fgf8a-*dll4* expression pattern might include differences in Fgf8a protein stability in injured and uninjured regions of the retina, and expression of factors locally at the injury site that collaborate with reduced Fgf8a to suppress *dll4* expression. Indeed, consistent with this latter idea, we found that growth factors expressed at the injury site, together with a reduction in Fgf8a levels, resulted in increased MG proliferation locally near the injury site.

In summary, our studies suggest 2 MG populations (MGc and MGp) that impact spontaneous and injury-dependent MG proliferation in the uninjured and injured retina. Although our data suggest Fgf8a signaling may have opposing actions on these 2 MG populations, they remain united in their response to Notch signaling inhibition which stimulates both populations to enter an activated, but quiescent state. Understanding the molecular underpinnings of this activated state may be critical for coaxing mammalian MG to proliferate. Finally, these studies illustrate that even in zebrafish where regeneration is robust, repressive influences from the environment must be overcome. Whether these types of repressive influences also contribute to the regenerative failure of mammals will be an important consideration when attempting to awaken their regenerative potential.

Experimental Procedures

Animals, heat shock and retinal injury

Animal studies were approved by the University of Michigan's Institutional Animal Care and Use Committee. Animals were anesthetized and retinas were injured with a needle poke injury once to each quadrant, intravitreal injection of NMDA, or via transgenic expression of nitroreductase in the presence of metronidazole as previously described (Fausett and

Goldman, 2006; Montgomery et al., 2010; Powell et al., 2016). For gene expression assays retinas received a needle poke injury twice in each retinal quadrant. Heat shock was performed by immersing fish in a water bath at 37.5 °C for 1h before returning to system water at 28 °C. For extended periods of heat shock, this was repeated 3×/day.

Fluorescence-Activated Cell Sorting (FACS)

Briefly, GFP^+ MG were purified from *gfap:GFP* and *1016 tuba1a:gfp* transgenic fish whose retinas received 10 lesions by needle poke. Cells were sorted on a BC Biosciences FACSViDa 3 laser high speed cell sorter.

RNA isolation and PCR

All primers used in this study are listed in Supplemental Methods. Total RNA was isolated using Trizol (Invitrogen). cDNA synthesis and PCR reactions were as previously described (Fausett et al., 2008; Ramachandran et al., 2010a).

Intravitreal injections, MO treatment and BrdU, EdU labelling

Intravitreal injections were as previously described (Wan et al., 2014). The control and *fgf8a*-targeting lissamine-tagged morpholino oligonucleotides (MO) were previously described (Araki and Brand, 2001; Fausett and Goldman, 2006). ~1 μl of MO (0.25 mM stock) were delivered intravitreally with a Hamilton syringe and cellular uptake facilitated by electroporation as previously described (Thummel et al., 2011). For EdU labeling, fish received an IP injection of EdU (10 μl of 10mg/ml stock) 3 hr prior to sacrifice. For lineage tracing, fish retinas were injured and then fish received in IP injection of BrdU at 4 dpi before being sacrificed 10 days later. To assay spontaneous MG proliferation, fish were immersed in fish water containing 5 μM BrdU for 9 days.

FGF receptor, MAPK, PI3K and Jak/Stat3 Inhibitors

Control fish were treated with DMSO (1:200). Mapk/Erk inhibitor, UO126 (Tocris Bioscience); PI3K/Akt inhibitor, LY294002 (Cayman Chemical); FGF receptor inhibitor, SU5402 (Pfizer); PLCγ inhibitor, U73122 (Sigma); and Jak/Stat3 inhibitor, cucurbitacin I (EMD Millipore) were used in this study. Fish were immersed in fish water containing the inhibitor (10 μM) or received 1 μl intravitreally of a 10 μM stock as previously described (Wan, 2012; Wan et al., 2014; Zhao et al., 2014).

Immunohistochemistry and *in situ* hybridization

Samples were prepared for immunofluorescence as previously described (Fausett and Goldman, 2006; Ramachandran et al., 2010a; Ramachandran, 2010). The detection of BrdU and EdU followed the protocol of Click-It (Molecular Probes 1511352) and used BrdU antibody MoBU-1. *In situ* hybridization was performed as described previously (Barthel and Raymond, 2000).

TUNEL

We used an *in situ* Cell Death Detection Kit (TMR red; Applied Science) to detect apoptosis cells.

Cell quantification and statistical analysis

BrdU and EdU immunofluorescence was used to identify and quantify proliferating cells in retinal sections as previously described (Fausett and Goldman, 2006; Ramachandran et al., 2010a; Wan, 2012; Wan et al., 2014). Error bars are standard deviation (s. d.). Individual comparisons were done using unpaired 2-tailed Student *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (NEI grant RO1 EY 018132), a Research to Prevent Blindness Innovative Ophthalmic Research Award and gifts from the Marjorie and Maxwell Jospey Foundation and the Shirlye and Peter Helman Fund. We thank Elizabeth Mills for gRNA; Rose Elsaedi for assaying cell proliferation in Wt fish treated with DAPT; Joshua Kirk for fish care; the University of Michigan Flow Cytometry Core for cell sorting; and the University of Michigan Sequencing Core for DNA sequencing. We thank David Hyde (University of Notre Dame, USA) for *gfap:GFP* transgenic fish, Bruce Riley (Texas A & M) for *hsp70:fgf8a* transgenic fish and Michael Parsons (Johns Hopkins University) for *tp1:mCherry* transgenic fish. We are grateful to the members of the Goldman laboratory for their discussions and feedback on this research and manuscript.

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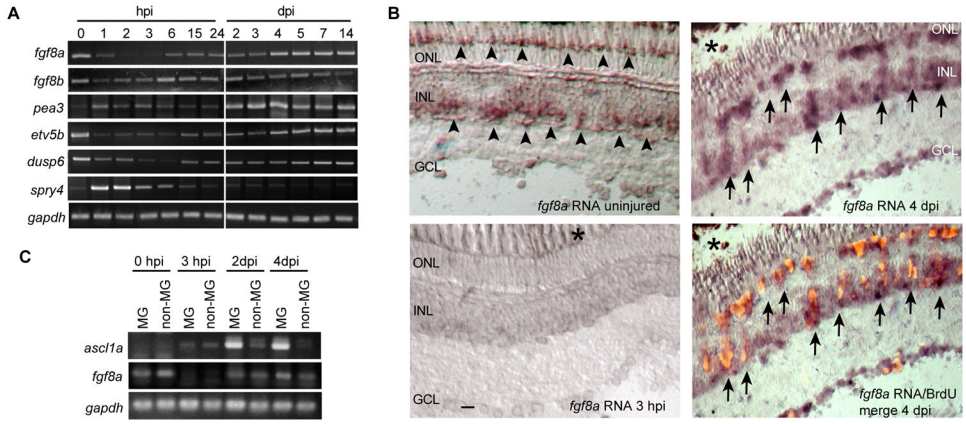

Figure 1. *fgf8a* gene regulation during retina regeneration

(**A**) RT-PCR analysis of *fgf8a, fgf8b* and Fgf-responsive genes in uninjured and needle poke injured retina. (**B**) *In situ* hybridization assays and BrdU immunofluorescence for *fgf8a* expression and MG proliferation, respectively, before and after injury to central retina in 6 mo old fish. Arrowheads point to *fgf8a* RNA in uninjured retina; arrows point to *fgf8a* RNA enriched in proliferating MG-derived progenitors at 4 dpi. Asterisk indicates injury site; scale bar is 50 μm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (**C**) RT-PCR analysis of *ascl1*, *fgf8a* and *gapdh* RNAs in GFP+ MG and GFP- non-MG (retinal neurons) in uninjured and injured retina that were FACS purified from *gfap:GFP* transgenic fish retinas. See also Figure S1.

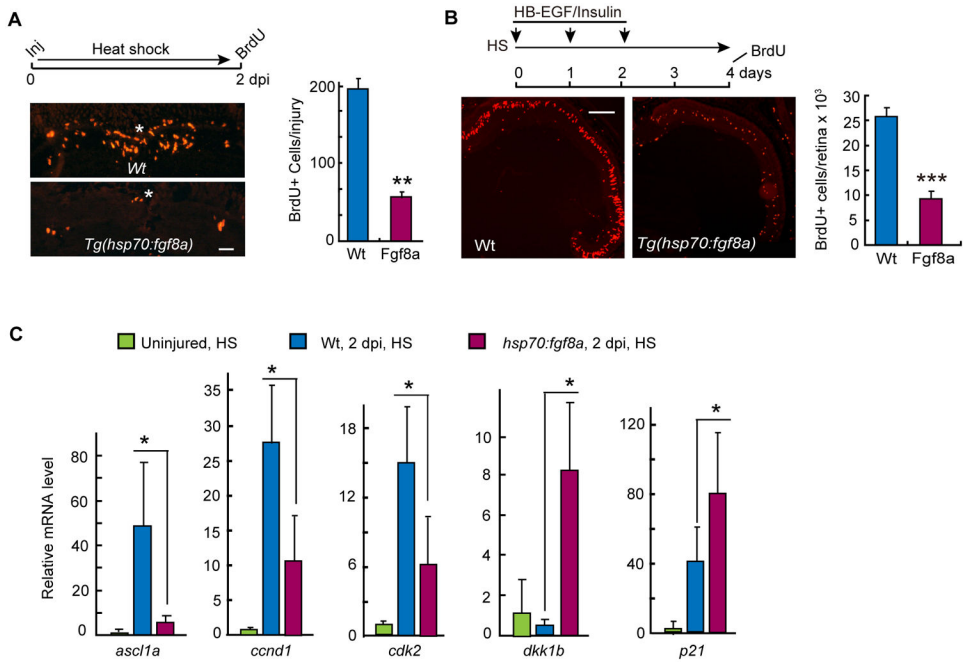

Figure 2. Sustained Fgf8a expression inhibits MG proliferation

(**A**) BrdU immunofluorescence was used to visualize and quantify MG proliferation in injured retinas following 2 days of sustained HS in Wt and *hsp70:fgf8a* fish. Asterisk indicates injury site (central retina of 6 mo old fish); scale bar is 100 μm. Graph shows quantification of BrdU+ cells; n=3 individual experiments, error bars are s. d. ** $P<0.01$. (**B**) BrdU immunofluorescence was used to visualize and quantify MG proliferation in uninjured retinas following intravitreal injection of HB-EGF/Insulin (once/day for 3 days) and HS for 4 days in Wt and *hsp70:fgf8a* fish; scale bar is 150 μm. Graph show quantification of BrdU+ cells; n=3 individual experiments, error bars are s. d. *** $P<0.001$. (**C**) qPCR analysis of gene expression in uninjured and injured (2 dpi) Wt and *hsp70:fgf8a* fish retina with heat shock (HS) n=3 individual experiments, error bars are s. d. * $P<0.05$. See also Figure S2.

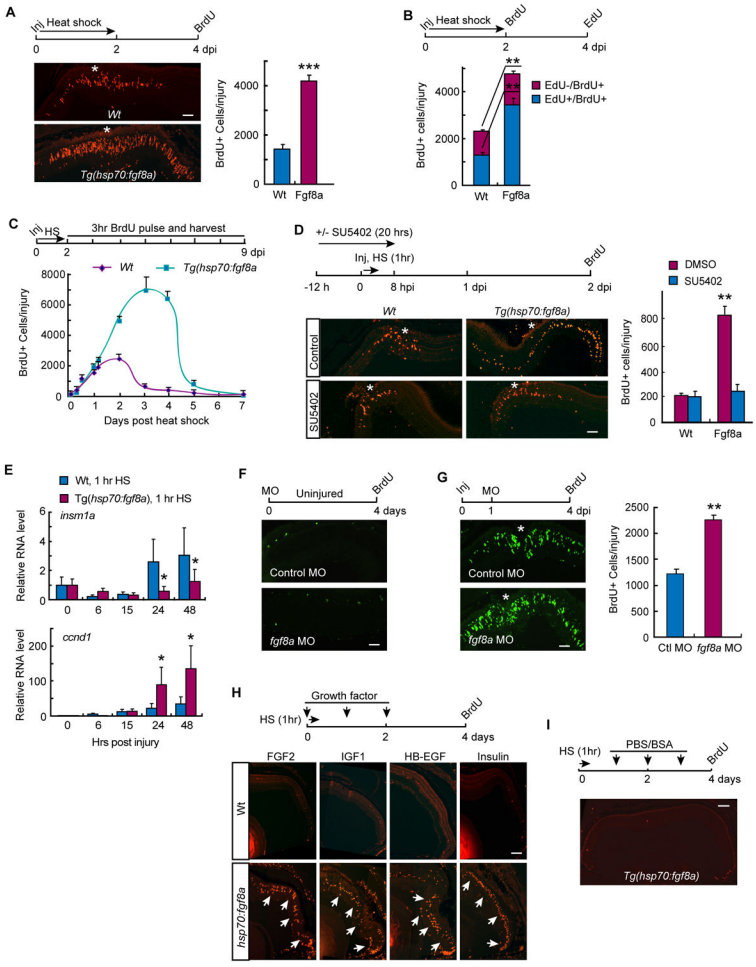

Figure 3. Cessation of forced Fgf8a expression stimulates MG proliferation in injured or growth factor treated retina

(**A**) BrdU immunofluorescence in injured retinas from Wt and *hsp70:fgf8a* fish that received HS for 2 days and assayed for MG proliferation 2 days later; Asterisk indicates injury site (central retina, 6 mo old fish); scale bar is 100 μm. Graph shows quantification of BrdU+ cells; n=3 individual experiments, error bars are s. d. ***P<0.001. (**B**) Graph quantifying BrdU+ and EdU+/BrdU+ double-labelled cells in injured retinas from Wt and *hsp70:fgf8a* (Fgf8a) fish that were heat shocked (HS) for 2 days, and then received an IP injection of BrdU and EdU at the indicated times; n=3 individual experiments, error bars are s. d. **P<0.01. (**C**) Quantification of BrdU immunofluorescence in injured retinas of Wt and *hsp70:fgf8a* fish that were heat shocked (HS) for 2 days and then sacrificed 3h after an IP injection of BrdU at the indicated times post heat shock; n=3 individual experiments, error bars are s. d. (**D**) BrdU immunofluorescence in injured retinas at 2 dpi from Wt and *hsp70:fgf8a* fish that received a 1h HS at the time of injury and immersed in fish water +/- SU5402, for the indicated time; Asterisk indicates injury site (central retina, 6 mo old fish); scale bar is 100 μm. Graph shows quantification of BrdU+ cells; n=3 individual experiments, error bars are s. d. **P<0.01. (**E**) qPCR analysis of *insm1a* and *ccnd1* gene expression at different times post injury in Wt and *hsp70:fgf8a* fish that received a 1h HS at the time of injury; n=3 individual experiments, error bars are s. d. *P<0.05. (**F, G**) BrdU

immunofluorescence in uninjured and injured retina electroporated with control and *fgf8a*-targeting MO at the indicated times. Asterisk indicates injury site (central retina, 6 mo old fish); scale bar is 100 μm. Graph in (**G**) is quantification proliferating MG in injured retina treated with control and *fgf8a*-targeting MO; n=3 individual experiments, error bars are s. d. $^{**}P<0.01$. (**H, I**) BrdU immunofluorescence in uninjured retinas from Wt and *hsp70:fgf8a* fish that received a 1h heat shock (HS) and intravitreal injection of indicated growth factor (HB-EGF, 50 ng/μl; FGF2, 200 ng/μl; IGF-1, 200 ng/μl; Insulin, 500 ng/μl) (**H**) or PBS/BSA (**I**) at the indicated times; scale bar is 150 μm. See also Figure S3.

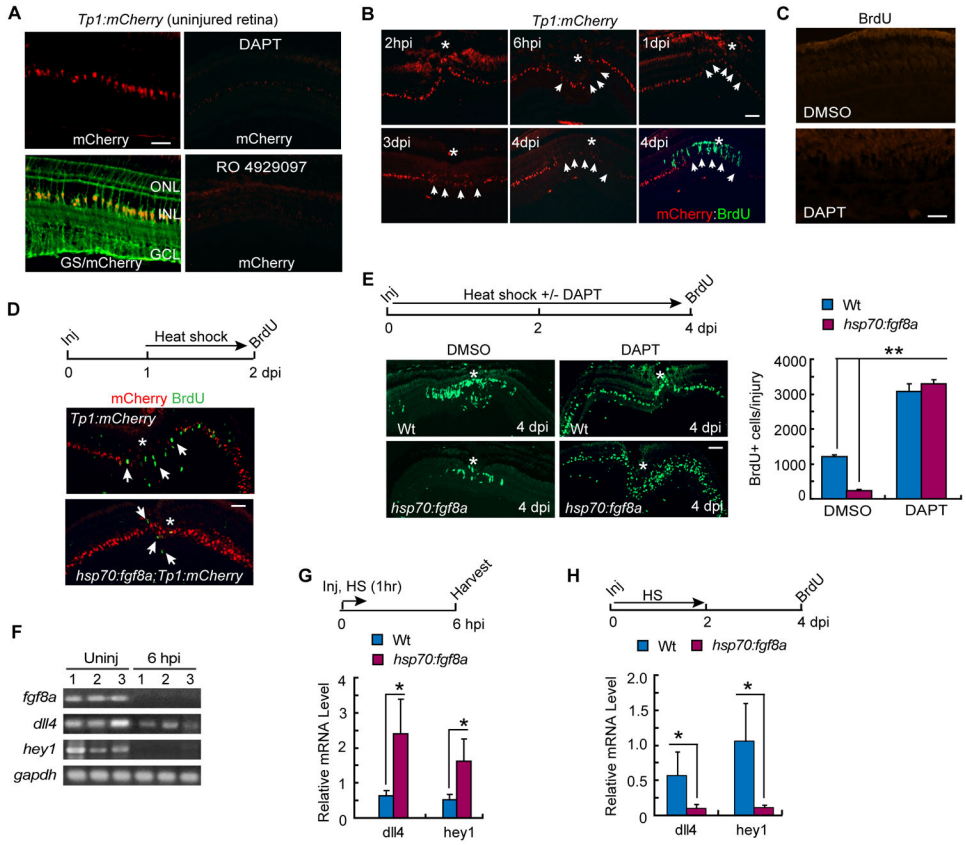

Figure 4. Fgf8a stimulates Notch signaling and MG quiescence

(**A**) mCherry and glutamine synthetase (GS) immunofluorescence in *tp1:mCherry* transgenic fish treated +/- the Notch signaling inhibitors DAPT, or RO 4929097; scale bar is 100 μm. (**B**) mCherry and BrdU immunofluorescence in *tp1:mCherry* transgenic fish at various times post retinal injury. Arrows point to areas of reduced mCherry expression in the inner nuclear layer. Asterisk indicates injury site (central retina, 6 mo old fish); scale bar is 100 μm. (**C**) BrdU immunofluorescence in Wt fish retina 4 days after intravitreal injection of DMSO or DAPT. Scale bar is 100 μm. (**D**) mCherry and BrdU immunofluorescence in injured retinas from *tp1:mCherry* and *hsp70:fgf8a;tp1:mCherry* transgenic fish that received HS from 1-2 dpi. Asterisk indicates injury site (central retina, 6 mo old fish); arrows point to BrdU+/ mCherry- cells; scale bar is 100 μm. (**E**) BrdU immunofluorescence at 4 dpi in Wt and *hsp70:fgf8a* that were immersed in fish water +/- DAPT and received HS over 4 days. Asterisk indicates injury site (central retina, 6 mo old fish); scale bar is 100 μm. Graph shows quantification of BrdU+ cells; n=3 individual experiments, error bars are s. d. ** $P<0.01$. (**F**) RT-PCR analysis of indicated RNAs in uninjured and injured (6 hpi) retinas from Wt fish. (**G**) qPCR quantification of *dll4* and *hey1* gene expression in Wt and *hsp70:fgf8a* fish that received a 1h HS at the time of injury and sacrificed 5 hrs later; n=3 individual experiments, error bars are s. d. * $P<0.05$. (**H**) pPCR as in (**G**), but HS was for 2d and gene expression assayed 2 days later; n=3 individual experiments, error bars are s. d. * $P<0.05$. See also Figure S4.

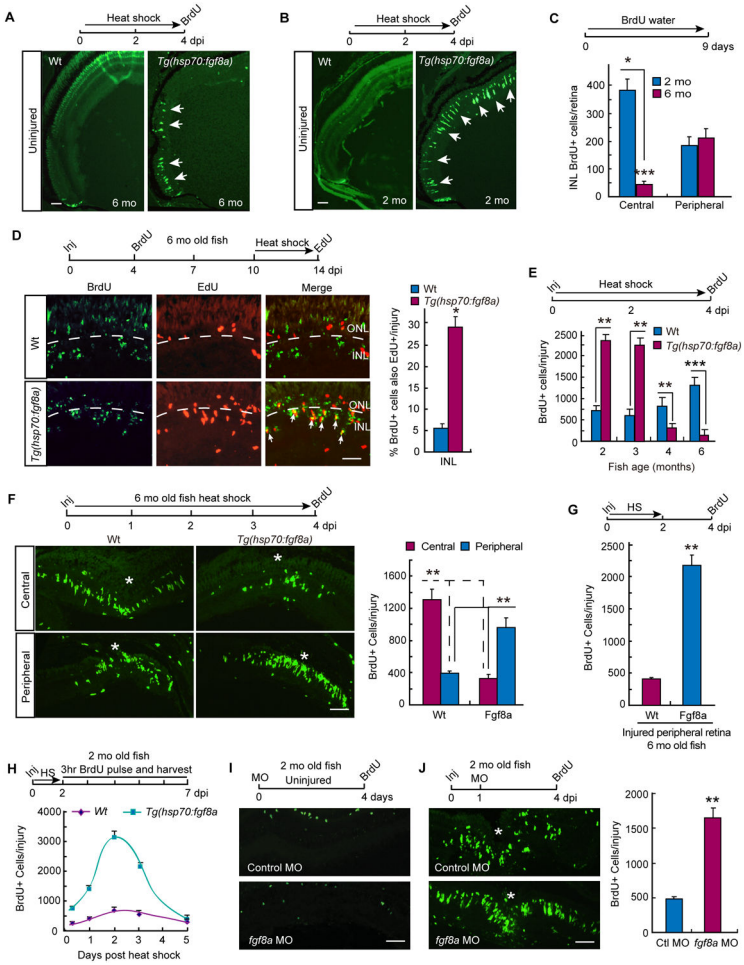

Figure 5. Age-dependent switch in Fgf8a signaling

(**A-B**) BrdU immunofluorescence in uninjured retinas from 2 and 6 mo old Wt and *hsp70:fgf8a* fish that received HS for 4 days before sacrifice; scale bar is 100 μm. (**C**) Quantification of spontaneous MG proliferation in the central (2/3) and remaining periphery of a 2 mo and 6 mo old fish retina isolated from Wt fish immersed in BrdU-containing water for 9 days; n=3 individual experiments, error bars are s. d. ***P<0.001. (**D**) BrdU and Edu immunofluorescence in the injured central retina of Wt and *hsp70:fgf8a* fish that received IP injections of BrdU and EdU at 4 and 14 dpi, respectively, and received HS from 10-14 dp. ONL, outer nuclear layer; INL, inner nuclear layer. Graph is quantification of the % of BrdU + cells that co-label with EdU in the INL; scale bar is 100 μm; n=3 individual experiments, error bars are s. d. *P<0.05. (**E**) Quantification of the number of BrdU+ cells/injury site in retinas from Wt and *hsp70:fgf8a* fish of different ages that were heat shocked for 4 days; n=3 individual experiments, error bars are s. d. **P<0.01, ***P<0.001. (**F**) BrdU immunofluorescence in central and peripheral regions of injured retinas from Wt and *hsp70:fgf8a* fish that received HS for 4 days. Asterisk indicates injury site; scale bar is 100 μm. Graph is quantification of BrdU+ cells/injury site; n=3 individual experiments, error bars are s. d. **P<0.01. (**G**) Quantification of BrdU+ cells/injury site in peripheral retinas from Wt and *hsp70:fgf8a* fish that received HS for 2 dpi and then BrdU at 4 dpi. Values are

the difference between injured fish and uninjured fish; n=3 individual experiments, error bars are s. d. ***P*<0.01. (**H**) Quantification of BrdU immunofluorescence in injured 2 mo old Wt and *hsp70:fgf8a* fish retinas that were heat shocked (HS) for 2 days and then sacrificed 3h after an IP injection of BrdU at the indicated times post HS; n=3 individual experiments, error bars are s. d. (**I, J**) BrdU immunofluorescence in uninjured (**I**) and injured (**J**) 2 mo old Wt fish retina electroporated with control and *fgf8a*-targeting MO at the indicated times. Asterisk in (**J**) indicates injury site; scale bar is 100 μm. Graph in (**J**) is quantification of proliferating MG in injured retina treated with control and *fgf8a*-targeting MO; n=3 individual experiments, error bars are s. d. ***P*<0.01. See also Figure S5.

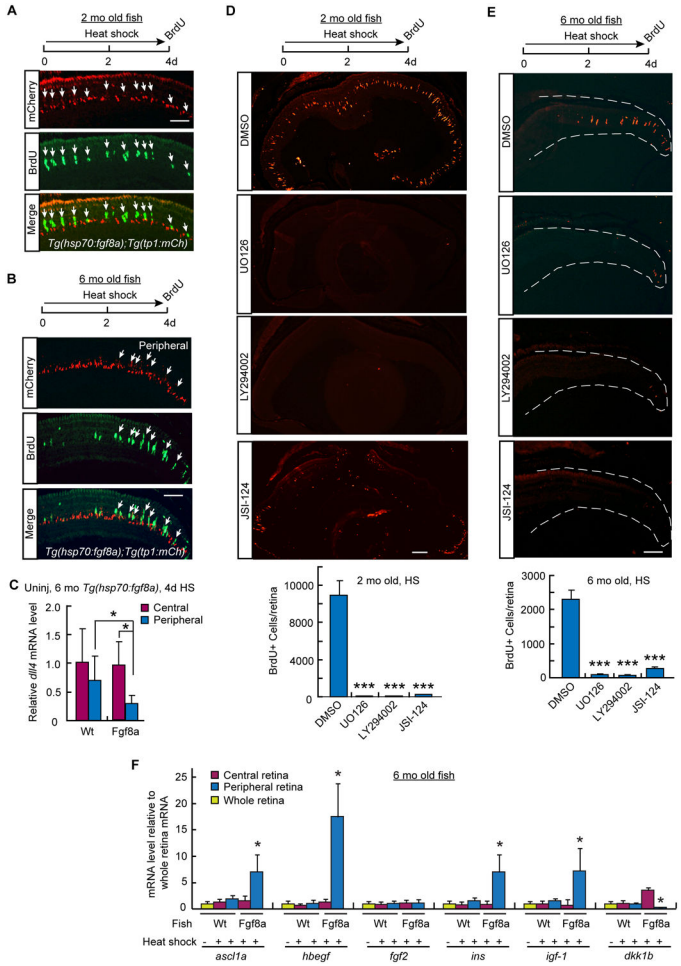

Figure 6. Signaling pathways contributing to Fgf8a-dependent MG proliferation in the uninjured retina

(**A, B**) mCherry and BrdU immunofluorescence in uninjured retinas from 2 mo (**A**) and 6 mo (**B**) old *hsp:70:fgf8a;tp1:mCherry* fish that received HS for 4 days. Arrows point to BrdU+/mCherry- cells in the inner nuclear layer of the central retina (**A**) and retinal periphery (**B**); scale bar is 100 μm. (**C**) qPCR analysis of *dll4* mRNA expression in central retina (2/3) and remaining retinal periphery in Wt and *hsp70:fgf8a* (Fgf8a) fish that were heat shocked for 4 days; n=3 individual experiments, error bars are s. d. **P*<0.05. (**D, E**) BrdU immunofluorescence in uninjured retinas from 2 mo old (**D**) and 6 mo old (**E**) *hsp70:fgf8a* fish treated with DMSO, MAPK inhibitor (UO126), PI3K inhibitor (LY294002) or Jak/Stat3 inhibitor (JSI-124). Shown is the whole retina for the 2 mo old fish and the peripheral retina for the 6 mo old fish; scale bar is 150 μm in (**D**) and 100 μm in (**E**). Quantification of BrdU+ cells/retina is shown below the images; n=3 individual experiments, error bars are s. d. ****P*<0.001. (**F**) qPCR analysis of indicated RNAs isolated from whole retina, central retina (2/3) and remaining peripheral retina of a 6 mo uninjured Wt or *hsp70:fgf8a* (Fgf8a) fish +/-HS for 4 days before sacrifice; n=3 individual experiments, error bars are s. d. **P*<0.05. See also Figure S6.

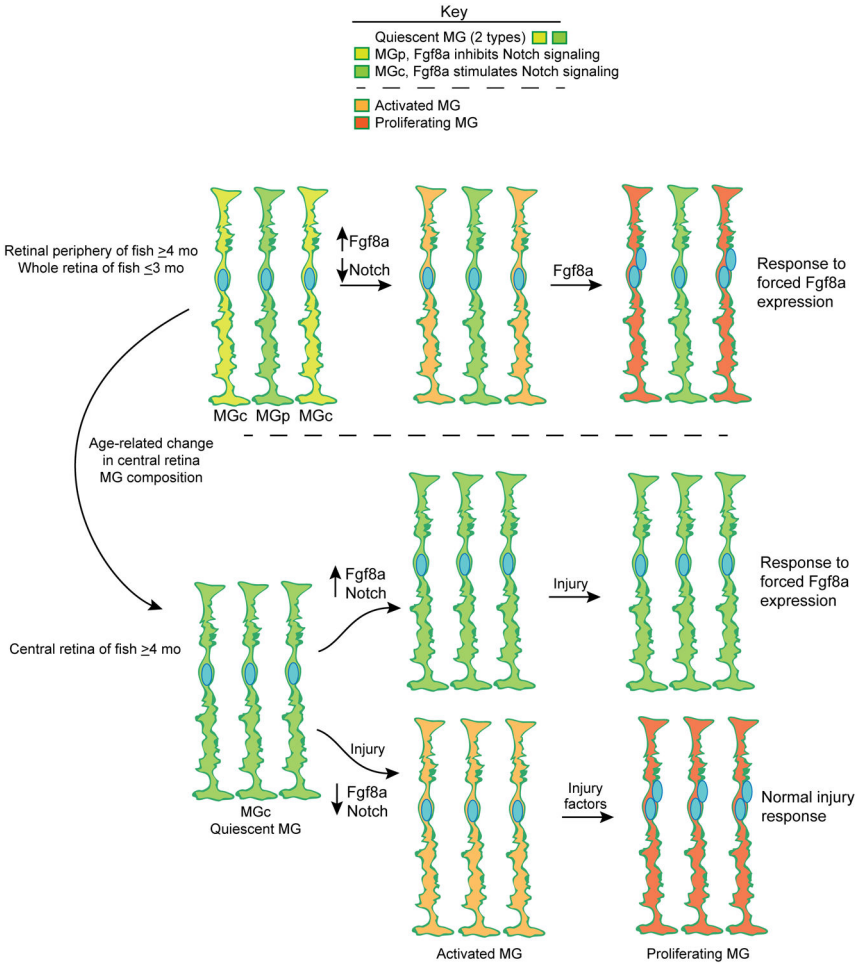

Figure 7. Model summarizing Fgf8a effects on Notch signaling and MG proliferation in uninjured and injured retina young and old fish retina

This model proposes that there are at least 2 different MG populations (light and dark green) in the uninjured retina that are distinguished by their response to Fgf8a signaling. Both populations contribute to the central and peripheral regions of the young retina; however, one of these MG types (dark green) predominate in the central region of the adult retina. In young retina, one MG population (light green) responds to increased Fgf8a by suppressing Notch signaling and transitioning to an active state (orange) that facilitates their proliferation (red). This population exhibits reduced Notch signaling and proliferation in response to retinal injury (not shown). The other MG population (dark green) that that predominates in the central region of the older retina, transitions to an activated state (low Notch signaling) in response to retinal injury. This MG population is also distributed in young retina and responds to injury in a similar fashion as in the older retina. These MG respond to forced Fgf8a expression with increased Notch signaling, which prevents their transition to an activated state. Although the action of Fgf8a on Notch signaling differs in these 2 different MG populations; in both populations, MG activation is associated with reduced Notch signaling.