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Insm1a-mediated gene repression is essential for the formation and differentiation of Müller glia-derived progenitors in the injured retina

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Abstract

In zebrafish, retinal injury stimulates Müller glia (MG) reprograming; allowing them to generate multipotent progenitors that regenerate damaged cells and restore vision. Recent studies suggest transcriptional repression may underlie these events. To identify these repressors, we compared the transcriptomes of MG and MG-derived progenitors and identified *insm1a*, a transcriptional repressor exhibiting a biphasic pattern of expression that is essential for retina regeneration. Insm1a was found to suppress *ascl1a* and its own expression and link injury-dependent *ascl1a* induction with *dickkopf* (*dkk*) suppression, which is necessary for MG dedifferentiation. We also found that Insm1a was responsible for sculpting the zone of injury-responsive MG by suppressing *hb-egf*$_a$ expression. Finally, we provide evidence that Insm1a stimulates progenitor cell cycle exit by suppressing a genetic program driving progenitor proliferation. Our studies identify Insm1a as a key regulator of retina regeneration and provide a mechanistic understanding of how it contributes to multiple phases of this process.

In contrast to mammals where retinal injury results in glial scaring[1], zebrafish mount a robust regenerative response that culminates in the restoration of vision[2]. Müller glia (MG) are largely responsible for this remarkable phenomenon. They accomplish this by injury-induced cellular reprogramming that allows them to generate progenitors that are able to regenerate all retinal cell types[3–9]. Although mammalian MG can be coaxed to proliferate, their regenerative capacity is meager and limited[10–13]. Therefore, understanding the mechanisms underlying successful retina regeneration in zebrafish may provide insight for developing strategies to stimulate MG reprogramming and retina regeneration in mammals.

Recent studies have identified a number of gene products and signaling cascades contributing to retina regeneration. These include an Ascl1a/Lin28/*let-7* miRNA signaling

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Author Contributions

D.G. and R.R. conceived the study and designed experiments. R.R. and X-F.Z. performed the experiments. R.R., X-F.Z. and D.G. analyzed the data and wrote the manuscript.

Competing Financial Interests

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pathway that supports the conversion of MG into progenitors[4]; an Ascl1a/Dkk/Wnt signaling pathway that in conjunction with Pax6, Stat3 and Hspd1 regulates MG reprogramming and progenitor proliferation[5,6,14,15]; a heparin binding epidermal-like growth factor (HB-EGF)-dependent signaling pathway that stimulates MG reprogramming and retina regeneration[16]; and FGF, Mps1 and galectin-dependent signaling that contribute to photoreceptor regeneration[14,17,18].

Although many of the signaling molecules described above are positive effectors, it is becoming increasingly clear that gene repression is essential for retina regeneration. For example, *dickkopf* (*dkk*) gene suppression contributes to progenitor formation[5], and *hb-egf* gene repression helps modulate the size of the progenitor population[16]. Perhaps the most obvious role for repression is the curtailing of cell proliferation programs as progenitors begin to differentiate. Mechanisms underlying this latter repression are of major interest since they prevent uncontrolled growth that may lead to tumor formation and glial scarring. Here we report the remarkable finding that a single transcriptional repressor, Insm1a, contributes to all these repressive events, placing it among the key regulators of retina regeneration.

Results

We probed zebrafish whole genome microarrays (Agilent) to identify transcriptional repressors regulating retina regeneration (GEO accession:GSE36191). Probes were derived from FACS purified MG and MG-derived progenitors from *gfap:gfp* and *1016 tuba1a:gfp* transgenic fish retinas, respectively (Supplementary Fig. S1a)[3,19]. The advantage of using *1016 tuba1a:gfp* fish is that they specifically label MG-derived progenitors, free of contaminating MG and other cell types. This analysis identified over 1200 genes induced greater than 2-fold and over 300 genes suppressed by at least 50%. Of these, we identified transcriptional repressors belonging to *her*, *bcl11*, *klf*, *eng* and *insm1* gene families (Supplementary Fig. S1b). RT-PCR confirmed injury-dependent regulation of these genes and revealed an intriguing biphasic pattern of expression for *insm1a* (Supplementary Fig. S1c). *insm1a* mRNA was first detected around 6 hrs post injury (hpi), suppressed at 24 hpi and reappeared by 4 days post injury (dpi). This pattern of expression suggested that Insm1a may play multiple roles during retina regeneration.

Injury-dependent *insm1a* induction is necessary for retina regeneration

In situ hybridization and immunofluorescence at 4 dpi in *1016 tuba1a:gfp* transgenic fish[3] showed *insm1a* was expressed in GFP^+ progenitors that also expressed the MG marker, glutamine synthetase (GS) (Fig 1a, Supplementary Fig. S2a). Furthermore, many, but not all of these $insm1a^+$ cells incorporated BrdU (Fig. 1b, c). Importantly, Insm1a knockdown using 2 different lissamine-tagged morpholino-modified antisense oligonucleotides (MOs) electroporated into the retina at the time of injury showed Insm1a was necessary for the generation of GFP^+ and $BrdU^+$ MG-derived progenitors (Fig. 1d, e; Supplementary Fig. S2b; Supplementary Table S1). Insm1a-targeting morpholinos prevented *insm1a:gfp* reporter gene expression in zebrafish embryos (Supplementary Fig. S2c) and suppressed

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injury-dependent Insm1a protein induction at the injury site (Supplementary Fig. S2d, e), without affecting cell death (Supplementary Fig. S8).

Although *insm1a* was localized to MG-derived progenitors at 4 dpi, it exhibited a pan retinal pattern of expression at 6 hpi that became restricted to the injury site by 2 dpi (Fig. 1f–h; Supplementary Fig. S3). This spatial and temporal expression pattern was reminiscent of that previously reported for *ascl1a*[5] and both these RNAs localized to MG-derived progenitors at 2 dpi (Fig. 1g). Interestingly, close inspection of *insm1a* expression and BrdU incorporation at 2 dpi suggested *insm1a* often associates with cells flanking $BrdU^+$ progenitors (Fig. 1h, i). Quantification showed ~30% of the progenitors expressed *insm1a* at 2 dpi which increased to 40% by 4 dpi (Fig. 1j; Supplementary Table S2). These data, along with the observation that Insm1a knockdown blocks progenitor production by over 80% (Fig. 1e; Supplementary Table S1), suggested Insm1a expression in dedifferentiated MG is necessary for them to produce progenitors for retinal repair.

An Ascl1a/Insm1a regulatory loop

Because *ascl1a* and *insm1a* exhibited a similar pattern of expression in the injured retina, we wondered if there was a hierarchical relationship. An analysis of injury-dependent *ascl1a* and *insm1a* mRNA expression showed *insm1a* lagged behind that of *ascl1a* and that *insm1a* was transiently reduced at 24 hpi (Fig. 2a, b). Ascl1a knockdown suppressed *insm1a* expression (Fig. 2c–e; Supplementary Fig. S2e), while Insm1a knockdown caused a small increase in *insm1a* and *ascl1a* expression (Fig. 2e). Inspection of the *insm1a* promoter revealed 3 putative Ascl1a binding sites (Fig. 2f) and ChIP assays using myc-Ascl1a overexpressed in zebrafish embryos showed Ascl1a binds these sites (Fig. 2g). Coinjection of zebrafish embryos with an *insm1a:gfp-luciferase* reporter and increasing concentrations of *ascl1a* mRNA showed Ascl1a stimulates *insm1a* promoter activity (Fig. 2h). Although these data are consistent with Ascl1a directly regulating *insm1a* promoter activity, we were unable to confirm this direct regulation in adult retinas due to the lack of a suitable antibody for its detection.

Because Insm1a knockdown resulted in a small, but significant increase in *ascl1a* and *insm1a* expression (Fig. 2e), we suspected that Insm1a may feedback to regulate their promoters. Inspection of *ascl1a* and *insm1a* promoter sequences revealed putative Insm1a binding sites (Fig. 2i, k) and ChIP assays confirmed that endogenous Insm1a bound these sites in the injured retina (Fig. 2j, l). Furthermore, coinjection of zebrafish embryos with *ascl1a:gfp-luciferase* or *insm1a:gfp-luciferase* reporters harboring wild type or mutant promoters and increasing concentrations of *insm1a* mRNA showed that these Insm1a binding sites confer Insm1a-dependent regulation (Fig. 2m, n). This led us to speculate that Insm1a itself may be contributing to its transient suppression at 24 hpi (Fig. 2a, b), and Insm1a knockdown confirmed this suspicion (Fig. 2o). Together these experiments reveal an Ascl1a-Insm1a regulatory loop and an Insm1a autoregulatory loop that contributes to the dynamic expression pattern of *ascl1a* and *insm1a* during the course of regeneration.

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Insm1a mediates *dickkopf* (*dkk*) gene repression in the injured retina

We previously demonstrated that the formation of MG-derived progenitors required *dkk* repression[5]. Interestingly, here we report an opposing pattern of *insm1a* and *dkk1b* gene expression. We found that *insm1a* was expressed throughout the retina at 6 hpi, but restricted to MG-derived progenitors at 4 dpi, while *dkk1b* exhibited the opposite pattern of expression (Fig. 3a, b)[5]. Importantly, Insm1a knockdown prevented injury-dependent *dkk* suppression (Fig. 3c). Inspection of the *dkk1b* promoter identified 2 putative Insm1a binding sites that bound endogenous Insm1a in the injured retina (Fig. 3d, e). In addition, zebrafish embryos coinjected with a wild type or mutant *dkk1b:gfp-luciferase* reporter and increasing concentrations of *insm1a* mRNA showed that both Insm1a binding sites contribute to *dkk1b* promoter repression by Insm1a (Fig. 3f). Finally, Insm1a knockdown in zebrafish embryos increased promoter activity from a co-injected *dkk1b:gfp-luciferase* reporter and also prevented Ascl1a-dependent *dkk1b:gfp-luciferase* reporter suppression (Fig. 3g). These data suggest Insm1a represses *dkk* promoter activity in the adult retina and that this repressive mechanism can be recapitulated in zebrafish embryos by either Insm1a or Ascl1a overexpression. Taken together these data identify an Ascl1a-Insm1a-Dkk signaling cascade underlying MG reprogramming in the adult retina.

Insm1a restricts the zone of MG-derived progenitors by suppressing *hb-egf* expression

We next investigated the significance of *insm1a* expression around 4–6 dpi when MG-derived progenitors are proliferating and differentiating. To bypass the block in regeneration that would result from Insm1a knockdown at the time of injury, we waited until 4 dpi to electroporate the control or *insm1a*-MOs (Fig. 4a) and confirmed their effectiveness in knocking down Insm1a at 6 dpi (Supplementary Fig. S4g). This delayed knockdown had no effect on cell death (Supplementary Fig. S8). In contrast to electroporation at the time of injury which blocks progenitor formation (Figs. 1d, e), Insm1a knockdown at 4 dpi greatly expanded the number of progenitors (Fig. 4b–d; Supplementary Fig. S4b). This result was reminiscent of DAPT-dependent Notch inhibition[16] and suggested that Insm1a suppression may underlie the expanded zone of progenitors in DAPT-treated retinas. Consistent with this idea, DAPT inhibited *insm1a* expression at 4 dpi (Fig. 4e). Interestingly, unlike the initial injury response[5], progenitor proliferation following Insm1a knockdown at 4 dpi was insensitive to Dkk overexpression (Fig. 4f; Supplementary Fig. S4c–f).

Since HB-EGF is released locally at the injury site and stimulates MG dedifferentiation[16], its suppression by Insm1a would provide a convenient mechanism for restricting the zone of dedifferentiating MG. Indeed, *insm1a* and *hb-egf*$_a$ are coexpressed in BrdU$^+$ dedifferentiated MG (Fig. 5a), and Insm1a knockdown at 4 dpi stimulated *hb-egf*$_a$ expression (Fig. 5b, c). Furthermore, we identified 2 putative Insm1a binding sites in the *hb-egf*$_a$ promoter and ChIP assays showed endogenous Insm1a in the injured retina bound these sites (Fig. 5d). Finally, HB-EGF$_a$ knockdown or inhibition of its receptor with PD158780 prevented progenitor expansion in Insm1a depleted retinas (Fig. 5e, f) without affecting cell death (Supplementary Fig. S8). These results suggest that a Notch-Insm1a-*hb-egf*$_a$ signaling cascade helps define the zone of reprogrammed MG flanking the injury site.

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Insm1a stimulates progenitor cell cycle exit by suppressing a genetic program driving cell proliferation

Fish receiving an IP injection of BrdU 3 hrs prior to sacrifice showed that the fraction of proliferating progenitors expressing *insm1a* increased from ~40% at 4 dpi to ~80% at 6 dpi (Fig. 6a; Supplementary Fig. S5a; Supplementary Table S2). Because progenitor proliferation is decreasing during this time period[3], we hypothesized that Insm1a may be associated with cell cycle exit. Our transcriptome analysis of MG and MG-derived progenitors identified 16 cell cycle-related genes that were induced in the injured retina (Supplementary Fig. S5b) and whose expression peaked around 4 dpi (Fig. 6b). We hypothesized that Insm1a may suppress these genes around 5–7 dpi and thereby promote cell cycle exit. Indeed, almost all of the genes examined showed higher expression at 6 dpi when retinas were electroporated with an *insm1a*-MO compared to a control MO (Fig. 6c, d).

Unique among the cell cycle genes is $p57^{kip2}$ (*cdkn1c*), a cyclin kinase inhibitor associated with cell cycle exit[20,21] whose expression was detected from ~6 hpi to 8 dpi (Fig. 6e). At 4 dpi, $p57^{kip2}$ was co-expressed with *insm1a* in MG-derived progenitors (Fig. 6f). While Insm1a and Ascl1a knockdown from 0–2 dpi had little effect on $p57^{kip2}$ expression (Fig. 6g), knockdown from 4–6 dpi dramatically suppressed its expression and increased progenitor proliferation (Fig. 6g, h). Thus, Insm1a stimulates progenitor cell cycle exit by inhibiting genes driving proliferation and stimulating expression of the cyclin-cdk inhibitor, $p57^{kip2}$.

Insm1a most likely mediates $p57^{kip2}$ induction by suppressing an intervening repressor. One candidate was *bcl11b* (CTIP2) that inhibit $p57^{kip2}$ gene expression in SK-N-MC cells[22]. Interestingly, our microarray data suggested *bcl11b* was reduced by ~80% in MG-derived progenitors (Supplementary Fig. S1b). Zebrafish harbor 4 different *bcl11* genes and all 4 are coordinately repressed in a pan retinal fashion shortly after injury (Fig. 7a, b). Using *bcl11a* as a representative member of this gene family, we found that its expression returned to non-MG, but remained repressed in MG-derived progenitors at 4 dpi (Fig. 7b). This expression profile was temporally and spatially similar to that of *dkk1b* (Fig. 3b)[5] and suggested a shared mechanism of regulation. Indeed, like *dkk* (Fig. 3c)[5], Insm1a or Ascl1a knockdown relieved injury-dependent *bcl11a* repression (Fig. 7c).

We next investigated if Insm1a directly regulated *bcl11a* gene expression. Inspection of the *bcl11a* promoter identified a single putative Insm1a binding site in its proximal region (Fig. 7d) and ChIP assays showed endogenous Insm1a bound this site in the injured retina (Fig. 7e). To test if this Insm1a binding site was functional we co-injected zebrafish embryos with *bcl11a:gfp-luciferase* reporters harboring wild type or mutant promoters and *insm1a* mRNA. This experiment showed that the Insm1a binding site was necessary for Insm1a-dependent promoter repression (Fig. 7f). Furthermore, Insm1a overexpression in zebrafish embryos inhibited *bcl11a* expression (Fig. 7g) and induced $p57^{kip2}$ expression (Fig. 7h); while Bcl11a overexpression suppressed endogenous $p57^{kip2}$ expression (Fig. 7i). These Insm1a-dependent gene expression changes observed in the embryo recapitulate that found in the

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adult injured retina and suggests Insm1a drives progenitor cell cycle exit, in part, by relieving Bcl11a-dependent repression of *p57^{kip2}* gene expression.

Insm1a knockdown transiently suppresses progenitor differentiation

One consequence of Insm1a driven cell cycle exit is progenitor differentiation. Therefore, we investigated if Insm1a knockdown at 4–6 dpi reduced the number of differentiating progenitors. HuC/D is often used as a marker of differentiating neurons. In the retina HuC/D also identifies amacrine and ganglion cells. We investigated if HuC/D could be used to identify differentiating cells in the regenerating retina and whether Insm1a was necessary for their formation. For these experiments fish received control or *insm1a*-targeting MO at the time of injury, an IP injection of BrdU at 4 dpi, MO electroporation 5 hrs post BrdU injection and then sacrificed at 6 dpi (Fig. 8a). Interestingly, double labeled BrdU$^+$ and HuC/D$^+$ cells migrating towards the outer nuclear layer (ONL) were readily identified in the control MO-treated retina, but not those with Insm1a knocked down (Fig. 8b, c) suggesting Insm1a drives differentiation in the injured retina.

We suspected that the excessive proliferation following Insm1a knockdown at 4 dpi may result in MO dilution so that progenitors may ultimately restore Insm1a levels and return to a normal program of regeneration. To investigate this possibility, we followed a similar experimental paradigm as described above except we gave fish daily IP injections of BrdU from 4–7 dpi before sacrificing them at 30 dpi (Fig. 8d). Differential interference contrast (DIC) microscopy showed complete recovery of retinal lamination at the injury site (Fig. 8e). Immunofluorescence detection of BrdU and retinal cell type-specific proteins showed that progenitors migrated to all 3 retinal layers regenerating zpr1$^+$ photoreceptors, PKC$^+$ bipolar cells, GS$^+$ MG, HuC/D$^+$ amacrine and putative ganglion cells, and Zn5$^+$ differentiating ganglion cells (Fig. 8e, f; Supplementary Fig. S6). Interestingly, Insm1a knockdown had little effect on the distribution of differentiated cell types. In contrast, progenitors originally residing in the expanded zone flanking the injury site were generally confined to a single lamina of the INL and predominantly regenerated GS$^+$ MG (Fig. 8e, f; Supplementary Fig. S6; Supplementary Table S3). These data suggested that MG-derived progenitors were eventually able to overcome the cell cycle trap imposed by Insm1a knockdown. However, unlike in previous studies where we found that progenitors residing outside the injury zone could regenerate all cell types at 18 dpi[5,16], this study suggests that at later times (30 dpi) MG selectively survive (Fig. 8f). We suspect these are the original reprogrammed MG that reacquired mature MG characteristics. Although we did not investigate the reason why newly regenerated non-MG cells flanking the injury site eventually died off, it may be due to their inability to integrate into functional circuits like those at the injury site.

Discussion

Insm1 is a transcriptional repressor associated with neuroendocrine tumors[23], small cell lung cancer[24], and the terminal cell divisions associated with neurogenesis[25–28]. Although Insm1 plays an important role during nervous system development, surprisingly little is known about Insm1's action in the adult nervous system. Furthermore, the mechanisms controlling

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insm1 gene induction and underlying Insm1's action on progenitor differentiation remain poorly understood.

Zebrafish harbor 2 *insm1* genes referred to as *insm1a* and *insm1b*[29]. In the adult retina, *insm1a* is normally expressed in the neurogenic ciliary marginal zone, but can also be found in the central retina of fish with photoreceptor degeneration[30]. Our studies suggest that Insm1a is a multifaceted transcriptional repressor that plays an essential role in the formation, expansion and differentiation of MG-derived progenitors during retina regeneration. These studies highlight how a single transcription factor can assume diverse roles at different stages of regeneration and point to the cellular environment as a critical factor in determining Insm1a function. Furthermore, our studies revealed signaling mechanisms underlying injury-dependent *insm1a* gene induction and also identified mechanisms by which Insm1a acts to control MG reprograming, along with the proliferation and differentiation of MG-derived progenitors. These results may have important implications for stimulating retina regeneration in mammals and for preventing uncontrolled MG proliferation in diseased and damaged human retinas. Furthermore our studies suggest mechanisms underlying *Insm1* gene function and action in mammals.

Although Insm1 is best known as a transcriptional repressor that is associated with terminal cell division and neuronal differentiation, we uncovered a number of novel roles for Insm1a during retina regeneration. First, Insm1a links *ascl1a* gene induction with *dkk* gene repression, which we previously showed was necessary for MG dedifferentiation and retina regeneration[5]. Second, using a novel protocol of delayed MO electroporation and gene knockdown, we found that Insm1a regulates the zone of injury-responsive MG flanking the injury site. This later function of Insm1a was only observed if MG were allowed to initially dedifferentiate and generate progenitors, suggesting that knockdown of Insm1a in these progenitors stimulated reprogramming of neighboring MG so they could generate additional progenitors. A possible mechanism underlying this effect was suggested by our finding that Insm1a controls *hb-egf*$_a$ gene expression, whose product was recently shown to stimulate MG reprogramming and progenitor formation in the uninjured retina[16].

Investigation of the mechanisms initiating injury-dependent Insm1a induction identified Ascl1a, a gene product that is a nodal point for a number of signaling cascades during retina regeneration[4,5,31,32]. We previously showed that *ascl1a* gene expression is regulated by HB-EGF and is among the earliest gene inductions following retinal injury[16]. Here we show Ascl1a is necessary for injury-dependent *insm1a* induction. Thus, together these studies identify an HB-EGF/Ascl1a/Insm1a/*dkk* signaling cascade as a critical signaling mechanism underlying the formation of injury-induced MG-derived progenitors. Interestingly, Ascl1 and Insm1 induction are also associated with *Dkk* repression in certain human lung cancers[24,33] and pancreatic endocrine tumors[34,35], perhaps suggesting a conserved signaling pathway.

In addition to regulating *dkk* gene expression, Insm1a also feeds back to inhibit *hb-egf*$_a$, *ascl1a* and *insm1a* promoter activity. This kind of feedback is often associated with oscillations[36,37] and appears to underlie the biphasic pattern of *insm1a* gene expression during retina regeneration and also helps restrict injury-responsive MG to the injury site.

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Insm1a knockdown at 4 dpi not only expanded the zone of MG reprogrammed to produce progenitors, but also dramatically increased progenitor proliferation at the expense of differentiation. This action is consistent with Insm1's role in mammals where it is associated with cell cycle exit. Insm1 appears to stimulate cell cycle exit by sequestering cyclin D1 with its proline-rich cyclin D1 binding domain[38]. However, this domain is missing in zebrafish Insm1a (Supplementary Fig. S7), suggesting another mechanism of action. Remarkably, we found that Insm1a not only suppressed a gene expression program that drives cell proliferation, but also relieved repression of $p57^{kip2}$, a cyclin kinase inhibitor that along with $p27^{kip1}$ drives cell cycle exit during mouse retina development[20,39]. In the mouse retina, reduced expression of $p27^{kip1}$ is associated with MG proliferation and reactive gliosis[40]. Interestingly, our transcriptome analysis of MG and MG-derived progenitors revealed that $p27^{kip1}$ is constitutively expressed in these two populations. Whether this constitutive expression in the injured zebrafish retina helps prevent a gliotic response and promotes a regenerative one is not known.

In summary, our data suggest Insm1a plays at least 3 important roles during retina regeneration (Fig. 8g, h). First, it contributes to MG reprogramming and the generation of progenitors by inhibiting Dkk expression and releasing the Wnt/β-Catenin pathway from inhibition. Second, it helps sculpt the zone of injury-responsive MG by regulating *hb-egf*$_a$ gene expression. Third, it contributes to the cessation of retina regeneration by stimulating cell differentiation via the suppression of genetic programs driving cell proliferation. Finally, our studies revealed mechanisms by which Insm1a mediates these effects and the signaling pathways underlying *insm1a* gene regulation in the injured retina. These studies place Insm1a among the key factors underlying retina regeneration and provide novel insight into signaling pathways that may help shift the response of MG in the injured mammalian retina from reactive gliosis towards retinal repair.

Methods

Animals, heat shock, drugs and retinal injury

Zebrafish were kept at 26–28 °C on a 14h/10h light/dark cycle. *1016 tuba1a:gfp, gfap:gfp,* and *hsp70:dkk1b-gfp* transgenic fish have been previously described[3,19,41]. Embryos were obtained by natural matings. Heat shock was performed by transferring *hsp70:dkk1b-gfp* fish to at 36.5 °C water bath for 1 h every 12 h beginning at 3 dpi and ending at 6 dpi. The EGFR inhibitor, PD158780 (Sigma-Aldrich) was used at 10 μM. Drugs were delivered intravitreally through the front of the eye using a Hamilton syringe equipped with a 30 gauge needle. Retinal lesions were performed as previously described[3]. Briefly, fish were anesthetized in tricaine methane sulfonate and the right eye was gently rotated from its socket and the retina stabbed 4–8 times (once or twice in each quadrant) through the sclera with a 30-gauge needle inserted the length of the bevel. All experiments were repeated a minimum of 3 times.

Florescence-activated cell sorting (FACS) and microarray

RNA was obtained from FACS purified MG and MG-derived progenitors at 4 dpi as previously described[5]. Briefly uninjured and injured retinas were isolated from *gfap:gfp* and

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1016 tuba1a:gfp transgenic fish. GFP$^+$ MG from *gfap:gfp* uninjured retinas and GFP$^+$ MG-derived progenitors from *1016 tuba1a:gfp* retinas at 4 dpi were isolated by treating retinas with hyaluronidase and trypsin and then sorted on a BC Biosciences FACSViDa 3 laser high speed cell sorter. Four uninjured retinas from *gfap:gfp* fish yielded 235,000 GFP$^+$ cells, while 30 injured retinas from *1016 tuba1a:gfp* fish yielded 140,000 GFP$^+$ cells. Total RNA was isolated using TRIzol (Invitrogen) and underwent one round of amplification to generate probes for screening a Zebrafish 44K microarray (Agilent, #G2519F). Duplicate samples were analyzed. Microarray data have been submitted to GEO (GSE36191).

Primers and Plasmid construction

All primers are listed in Supplementary Table S4. *ascl1a*, *insm1a*, *bcl11a* promoters were amplified from zebrafish genomic DNA using primer pairs Xho*Ascl*1aPro-F and Bam*Ascl*1aPro-R (~6kb), Xho-*insm1a*-Pro-F & Bam-*insm1a* Pro-R (~3kb) and Xho-*bcl11a* Pro-F & EcoR1-*bcl11a* Pro-R (~2kb), respectively. The PCR amplicons were digested and cloned into a pEL luciferase expression vector to create *ascl1a:gfp-luciferase*, *insm1a:gfp-luciferase* and *bcl11a:gfp-luciferase* constructs. The *dkk1b:gfp-luciferase* construct was described previously[5]. Site-directed mutagenesis was done as previously described[4].

insm1a and *bcl11*a cDNA were amplified from zebrafish retina RNA at 4 dpi using primer pairs Bam-*insm1a*-F and Xho-*insm1a*-R (1.1kb) and Cla-*bcl11a*-F and Cla-*bcl11a*-R (2.4kb). The PCR amplicons were cloned into their respective enzyme sites in pCS2$^+$-MT (*insm1a*) and pCS2$^+$ (*bcl11a*) plasmid to obtain *cmv:myc-insm1a* and *cmv:bcl11a*. The *cmv:myc-ascl1a* construct was described previously[4]. All primers used in this study are listed in Supplementary Table S1.

RNA isolation, RT-PCR and qPCR

Total RNA was isolated from control and injured retinas using TRIzol (Invitrogen). Random hexamers and Superscript II reverse transcriptase (Invitrogen) were used to generate cDNA. PCR reactions used Taq polymerase and gene-specific primers (Supplementary Table S4) with previously described cycling conditions[4]. qPCR was carried out in triplicate with Absolute SYBR Green Fluorescein Master Mix (Thermo Scientific) on an iCycler real-time PCR detection system (BioRad). The ΔΔCt method was used to determine relative expression of mRNAs in control and injured retinas and normalized to ribosomal protein *L-24* mRNA levels.

mRNA synthesis, embryo microinjection and ChIP assay

pCS2$^+$ and pCS2$^+$-MT plasmids harboring cDNA inserts were linearized and capped mRNAs were synthesized using the mMESSAGE mMACHINE (Ambion). Single cell zebrafish embryos were injected with ~200 pl of solution generally containing 0.02 pg of Renilla luciferase mRNA (normalization), 2 pg of *promoter:gfp-luciferase* vector and 0–4 pg of *ascl1a*, *insm1a* or *bcl11a* mRNA. To ensure reproducibility a master mix was made for daily injections and ~200 embryos were injected at the 1–2 cell stage. 24 hrs later, embryos were divided into 3 groups (~65 embryos/group) and lysed for dual luciferase reporter assays (Promega).

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ChIP assays to analyze endogenous Insm1a binding to various promoters in the adult injured retina was performed using ~100 adult retinae collected at 4 dpi after dark adaptation. Chromatin was isolated and sonicated as described previously[42]. The sonicated chromatin was distributed into 3 equal aliquots; 2 were probed with an anti-zebrafish Insm1a antibody (AnaSpec, Fremont, CA. Cat No # 55795-2, 1:250 dilution) and the third served as a control. For ChIP assays in embryos with myc-tagged protein overexpression, embryos were injected with 4 pg of myc-tagged mRNA and ChIP assays performed as previously described[42]. Primers used for ChIP assays are described in Supplementary Table S4.

Morpholino (MO) electroporation

Lissamine-tagged MOs (Gene Tools, LLC) (~0.5 μl of 0.02–0.5 mM) were introduced at the time of injury using a Hamilton syringe. MO delivery to cells was accomplished by electroporation as previously described[31]. The control, *ascl1a*- and *hb-egf*$_a$-targeting MOs have been previously described[16,31]. The *insm1a*-targeting MOs are: *insm1a* MO1 5′-ATGCCCCCGGCAAATCCGCATCTCA-3′ and *insm1a* MO2 5′-GCTTGACTAAAAATCCTCTGGGCAT-3′. Because antibodies detecting zebrafish Insm1a are unavailable, we evaluated the efficacy of these MOs *in vivo* in zebrafish embryos using an indirect assay. For this purpose, we prepared cDNA from injured retinas and used primers MO-Hind-*insm1a*-F and MO-Bam-*insm1a*-R (Supplementary Table S4) to amplify a 121 bp 5′ *insm1a* cDNA fragment that harbored both of the *insm1a*-MO target sites. This product was appended to the coding N-terminus of GFP in the *cmv:gfp* expression vector and injected into zebrafish embryos with either lissamine-tagged control (0.5mM) or lissamine-tagged *insm1a*-targeting MOs (0.25mM) in separate experiments. After 24 hrs, embryos were assayed under fluorescent microscopy for GFP expression (Supplementary Fig. S2c).

BrdU labeling, tissue preparation, *in situ* hybridization (ISH) and immunofluorescence (IF)

BrdU labeling was accomplished by injecting 20 μl of BrdU (20 mM) IP 3 h prior to sacrifice unless otherwise indicated. Some animals received multiple injections of BrdU over multiple days. Fish were overdosed with tricaine methane sulfonate and eyes were dissected, enucleated, fixed and sectioned as previously described[3,43]. ISH was performed on retinal sections with digoxigenin-labeled cRNA probes (DIG RNA labeling kit, Roche Diagnostics)[44]. Fluorescent ISH was performed according to the manufacturer's instructions (Perkin-Elmer). Sense control probes consistently gave no signal above background. IF protocols and antibodies were previously described[3–5,43]. Insm1a IF was performed using anti-zebrafish Insm1a antibody (Anaspec, Fermont, CA. Cat No # 55795-2) at 1:100 dilution. For BrdU IF, sections were treated with 2N HCl at 37 °C for 20 min, rinsed in 0.1 sodium borate (pH 8.5) for 10 min and then processed using standard procedures[43]. For lineage tracing experiments retinal sections from a single eye were distributed across 6 slides. Each slide was first processed for immunofluorescent detection of cell type-specific markers (one marker per slide) and then a 2N HCl epitope retrieval protocol was performed to identify BrdU$^+$ cells[3,8]. Each slide was used to react with a different cell type-specific marker, while BrdU$^+$ cells were detected on all slides. The total number of BrdU$^+$ cells and the number of co-labeled BrdU$^+$ cells that also stained for a particular cell type marker were quantified on each slide.

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Microscopy, cell counts and Statistical Analysis

Slides were examined with a Zeiss Axiophot microscope equipped with Fluorescence optics or an Olympus FluoView FV1000 confocal imaging system. Cell counts were determined by counting fluorescently labeled cells ($BrdU^+$, retinal cell type-specific$^+$ or *insm1a*$^+$) in retinal sections visualized using fluorescent microscopy. All sections of the retina were examined and at least 3 individuals were used. Data were analyzed for statistical significance using Stat View software (SAS Institute). Statistical comparisons were conducted using a two-tailed unpaired Student's *t* test to analyze data from single parameter experiments. For all other experiments an analysis of variance was performed followed by a Bonferroni/Dunn post hoc *t* test. Error bars represent standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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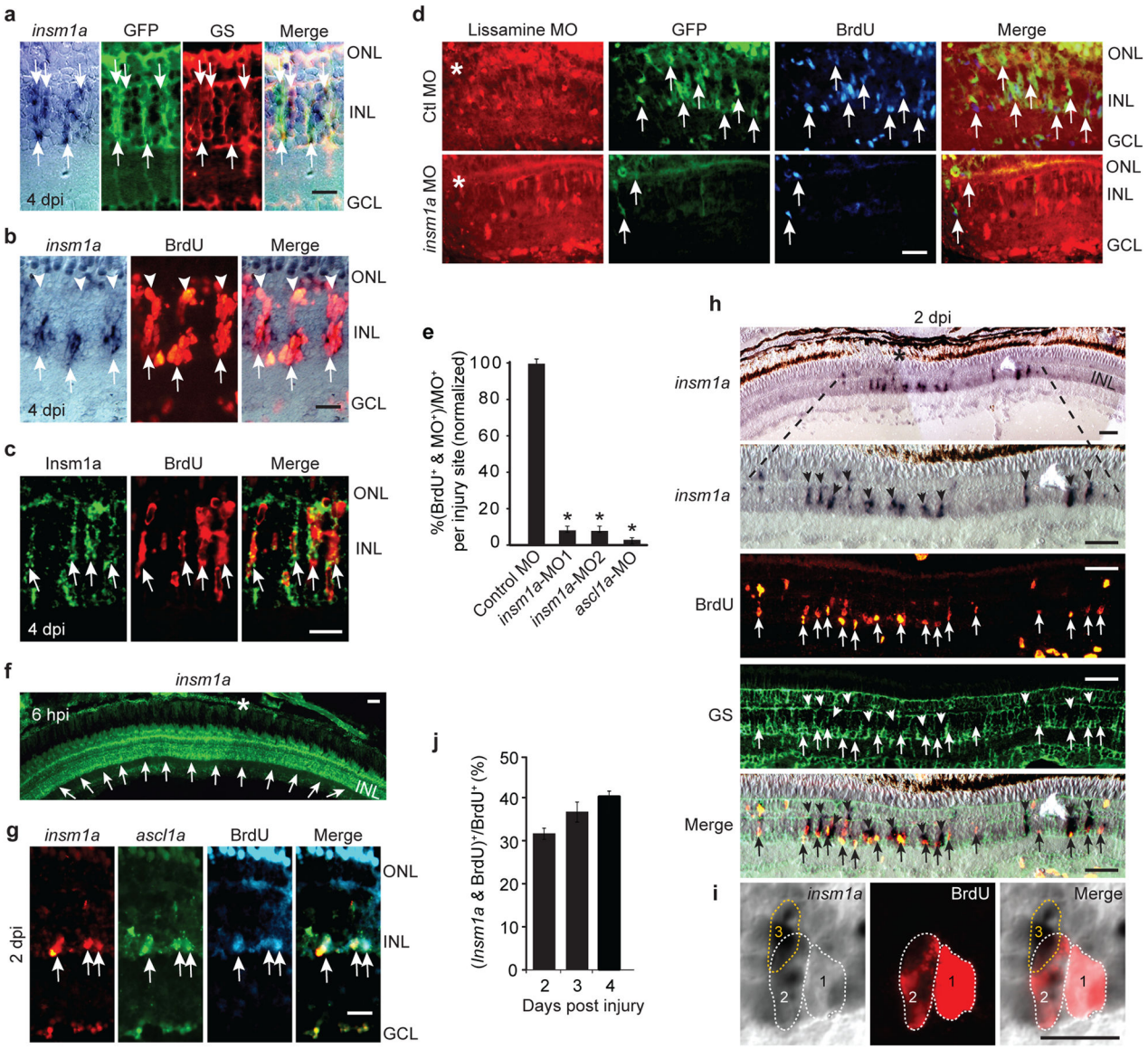

Figure 1.

Insm1a is necessary for MG dedifferentiation in the injured retina. **(a, b)** ISH and IF shows *insm1a* RNA is expressed in GFP$^+$, GS$^+$ and BrdU$^+$ MG-derived progenitors of *1016 tuba1a:gfp* transgenic fish at 4 dpi. Arrows in **(a, b)** identify co-labeled cells, while arrowheads in **(b)** identify *insm1a*$^-$/BrdU$^+$ cells localized to the injury site. **(c)** A single 0.5 micron thick confocal Z-section shows Insm1a protein in BrdU$^+$ MG-derived progenitors at 4dpi, arrows point to co-labeled cells localized to the injury site. **(d)** Control (Ctl) or *insm1a*-targeting lissamine-labeled MOs were electroporated into the retina of *1016 tuba1a:gfp* transgenic fish at the time of retinal injury and 3 hrs prior to sacrifice, at 4 dpi, fish received an IP injection of BrdU. Arrows point to GFP$^+$ and BrdU$^+$ co-labeled progenitors. Asterisk marks the injury site. **(e)** Quantification of the number of MO$^+$ cells at the injury site, which are also BrdU$^+$. Data is normalized to the control MO which is set to 100%. **P*<0.0001, n=3 biological replicates. **(f)** ISH shows *insm1a* RNA is pan retinal at 6 hpi. Asterisk flanks the

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injury site. **(g)** ISH shows *insm1a* RNA is restricted to the injury site at 2 dpi. **(h)** ISH shows *insm1a* RNA is restricted to the injury site at 2 dpi. Dotted lines indicate region shown in remaining panels where ISH was used to detect *insm1a* RNA and IF was used to detect BrdU or GS. Note *insm1a* RNA (arrowheads) flanking $BrdU^+$ cells (arrows). Asterisk marks the injury site. **(i)** A single 0.5 micron thick confocal Z-section shows *insm1a* RNA expression in $BrdU^+$ cell (labeled 2) and a flanking $BrdU^-$ cell (labeled 3) at 2dpi. A $BrdU^+$ cell lacking *insm1a* expression is also shown (labeled 1). **(j)** Quantification of the number of $BrdU^+$ cells that are also *insm1a*$^+$ at 2–4 dpi, n=3 biological replicates. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 10 microns in **(a, b, f, i)**; 20 microns in **(c, d, h, g)**.

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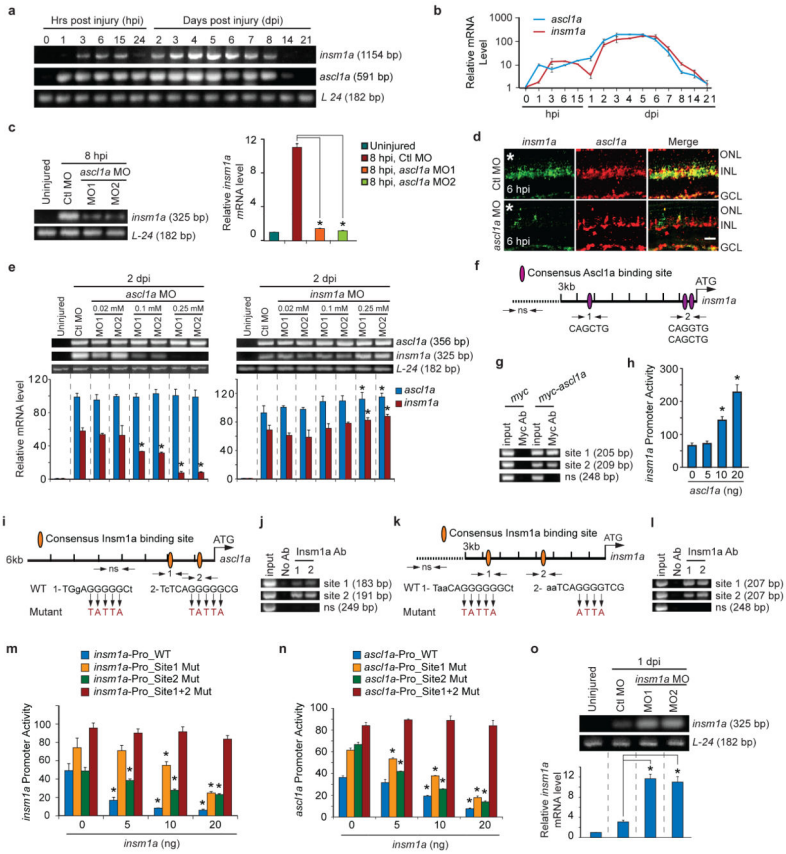

Figure 2.

An Ascl1a/Insm1a regulatory loop. **(a)** RT-PCR and **(b)** qPCR were used to assay injury-dependent *insm1a*, *ascl1a* and ribosomal protein *l-24* gene expression, n=6 biological replicates. **(c)** RT-PCR and qPCR shows Ascl1a knockdown inhibits *insm1a* induction. **P*<0.0001, n=3 biological replicates. **(d)** ISH shows Ascl1a knockdown inhibits *insm1a* induction. Asterisk flanks the injury site. Scale bar: 10 μm. **(e)** Ascl1a knockdown inhibits *insm1a* induction, while Insm1a knockdown slightly increases *ascl1a* and *insm1a* expression. Relative to control MO, **P*<0.0001 in left-hand panel; **P*<0.01 in right-hand panel, n=6 biological replicates. **(f, i, k)** Diagram of *insm1a* **(f, k)** and *ascl1a* **(i)** promoters with putative Ascl1a **(f)** and Insm1a **(i, k)** binding sites. Solid line represents DNA sequences included in *insm1a:gfp-luciferase* vector used in panel (**m**). Arrows indicate primers used in ChIP assays. Capital letters are conserved bases of consensus site. **(g)** Embryo ChIP assay detects myc-Ascl1a bound to target sites in the *insm1a* promoter (ns indicates non-specific site, 1 and 2 represent duplicates). **(h)** Ascl1a overexpression stimulates *insm1a:gfp-luciferase* expression in embryos. Promoter activity is normalized light units. **P*<0.0002, n=3 biological replicates. **(j, l)** ChIP assays using retinal extracts at 4 dpi show endogenous Insm1a binds its target sites in the *ascl1a* **(j)** and *insm1a* **(l)** promoters (ns indicates non-specific site, 1 and 2 represent duplicates). **(m, n)** Expression vectors harboring wild type (WT) and mutant (Mut) *insm1a* or *ascl1a* promoters (Pro) driving *gfp-luciferase* expression were injected into embryos with increasing amounts of *insm1a* mRNA. Promoter mutations are shown in **(i, k)**. Promoter activity is reported as normalized

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light units. Relative to 0 ng *insm1a* **P*<0.004, n=3 biological replicates in **(m)** and **P*<0.0001, n=3 biological replicates in **(n). (o)** RT-PCR and qPCR shows Insm1a knockdown at the time of injury increases *insm1a* mRNA expression at 1 dpi. **P*<0.0001, n=3 biological replicates.

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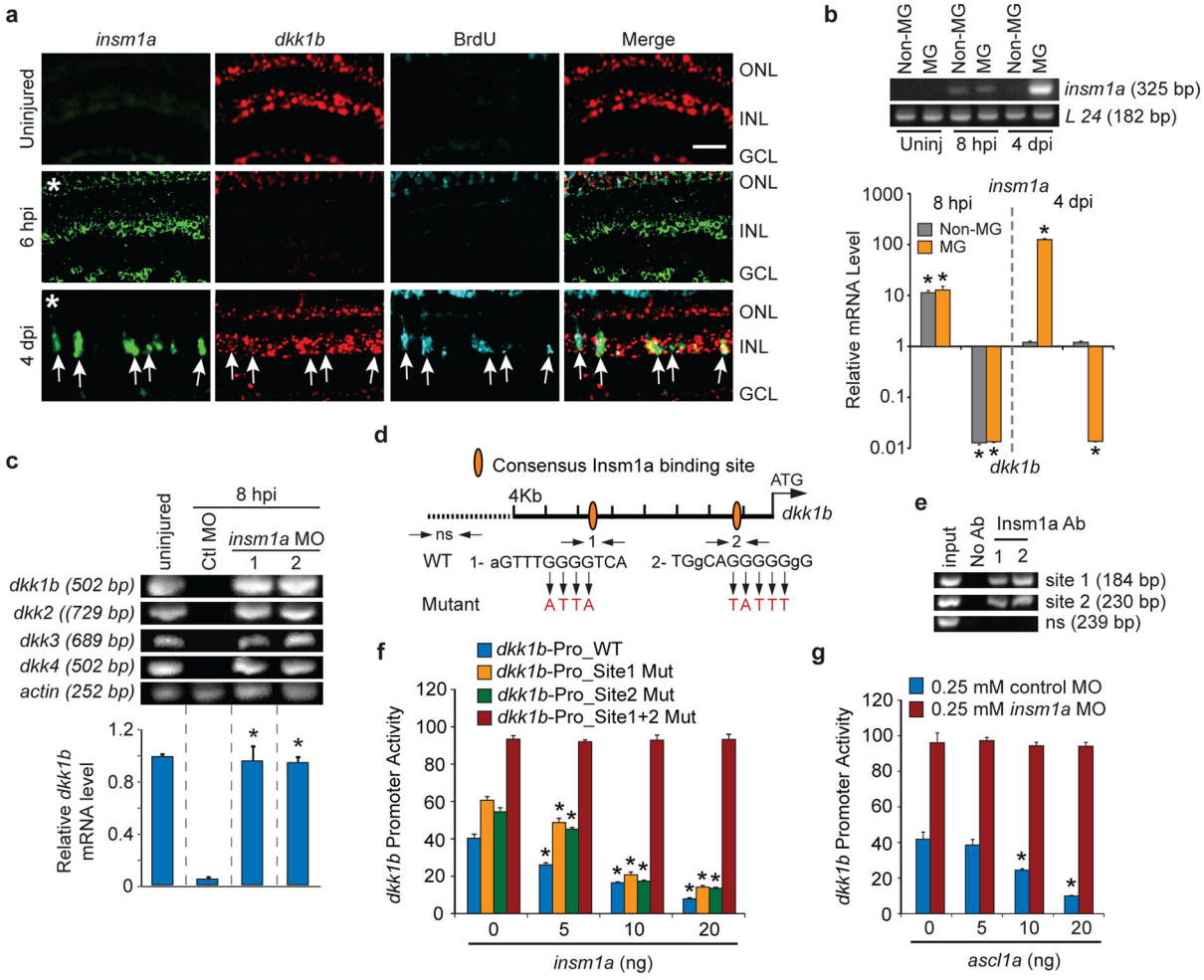

Figure 3.

Insm1a regulates *dkk* promoter activity. **(a)** ISH and IF show *insm1a* and *dkk* exhibit opposing expression patterns in the uninjured and injured retina. Arrows point to BrdU^{+} MG-derived progenitors with high levels of *insm1a* and low levels of *dkk1b* mRNAs. Asterisk flanks the injury site. Scale bar: 20 microns. **(b)** RT-PCR and qPCR using mRNA from FACS purified non-MG and MG-derived progenitors (MG) from either *1016 tuba1a:gfp* or *gfap:gfp* transgenic fish, shows *insm1a* is transiently induced in a pan retinal fashion at 8 hpi and by 4 dpi it is specifically expressed in MG-derived progenitors (MG). In contrast, *dkk1b* exhibits the opposite expression pattern. In the graph, the first pair of bars at each time point represents *insm1a* expression. Relative to uninjured controls, **P*<0.001, n=3 biological replicates. **(c)** RT-PCR and qPCR show that Insm1a knockdown at the time of injury restores *dkk* expression in the injured retina. Relative to control MO, **P*<0.0001. **(d)** Diagram of the *dkk1b* promoter and putative Insm1a binding sites. Promoter mutations in Insm1a binding elements are shown in red. Dashed line represents DNA sequences not included in *dkk1b* promoter used in panel (**f**). **(e)** ChIP assays using retinal extracts at 4 dpi show endogenous Insm1a binds its target sites in the *dkk1b* promoter (ns indicates non-specific site, 1 and 2 represent duplicates). **(f)** Insm1a overexpressed in zebrafish embryos regulates co-injected *dkk1b:gfp-luciferase* reporter activity via the *dkk1b* promoter's Insm1a

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binding sites. Relative to 0 ng *insm1a*, **P*<0.0001, n=3 biological replicates. **(g)** Insm1a knockdown in zebrafish embryos induces *dkk1b* promoter activity and blocks Ascl1a-dependent suppression of *dkk1b* promoter activity. Relative to 0 ng *ascl1a*, **P*<0.0001, n=3 biological replicates. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

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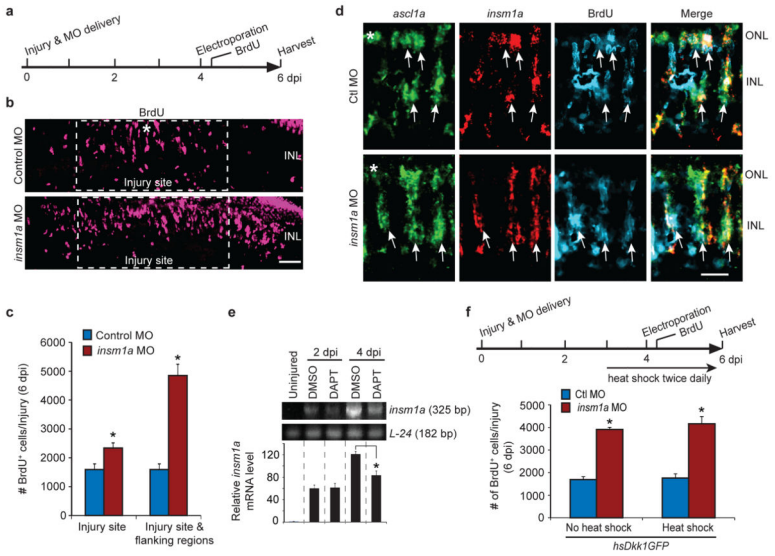

Figure 4.

Insm1a regulates the zone of injury-responsive MG. **(a)** Experimental time line. Control or *insm1a*-targeting MO was injected into the retina at the time of injury and 4 days later delivered to cells by electroporation; 4 hrs later, fish received an IP injection of BrdU and were then sacrificed at 6 dpi. **(b)** BrdU IF on retinal sections shows expansion of the zone of proliferating progenitors following Insm1a knockdown. Asterisk marks the injury site. Boxed area represents the injury site quantified in **(c)**. Scale bar: 20 microns. **(c)** Quantification of $BrdU^+$ cells shown in **(b)**. Relative to control MO **P*<0.016 at the injury site and **P*<0.0001 for injury site and flanking regions, n=3 biological replicates. **(d)** ISH and IF shows colocalization of *ascl1a* and *insm1a* expressing cells with $BrdU^+$ progenitors in control and Insm1a knockdown retinas at 6 dpi. Asterisk flanks the injury site. Scale bar: 10 microns. **(e)** Notch inhibition with DAPT from 0–4 dpi inhibits *insm1a* induction. **P*<0.0001. **(f)** Experimental time line with *hsp70:dkk1b-gfp* transgenic fish that received heat shock twice daily from 3–6 dpi. Graph shows quantification of $BrdU^+$ cells/injury site under the indicated conditions. **P*<0.001, n=3 biological replicates. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

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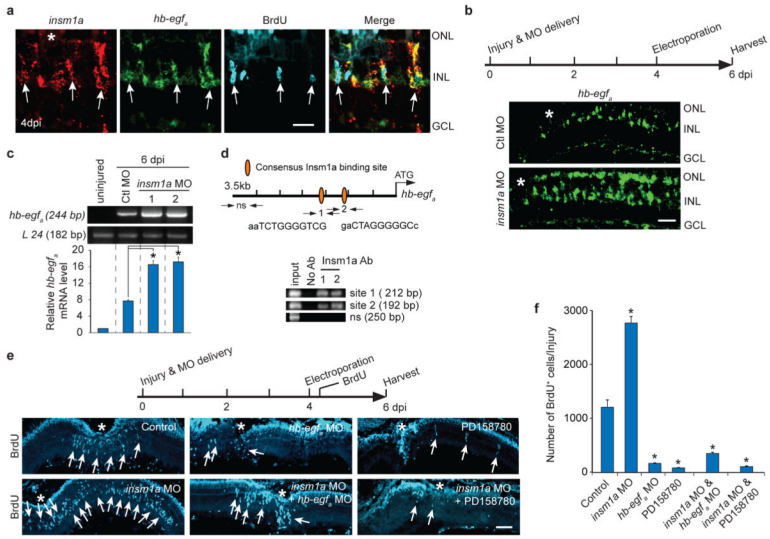

Figure 5.

Insm1a restricts the zone of dedifferentiating MG by suppressing *hb-egf$_a$* gene expression. **(a)** Fluorescent ISH and IF shows *insm1a* and *hb-egf$_a$* mRNAs are co-expressed in BrdU$^+$ MG-derived progenitors at 4 dpi. Asterisk flanks the injury site. Scale bar: 10 microns. **(b)** Top is experimental time line. Bottom shows *hb-egf$_a$* ISH on control and Insm1a knockdown retinal sections at 6 dpi. Asterisk marks the injury site. Scale bar: 20 microns. **(c)** RT-PCR and qPCR show Insm1a knockdown from 4–6 dpi stimulates *hb-egf$_a$* mRNA expression. **P*<0.0001, n=3 biological replicates. **(d)** Diagram of *hb-egf$_a$* promoter and putative Insm1a binding sites. Arrows indicate primers used for ChIP assay shown below the promoter diagram. ChIP assays using retinal extracts at 4 dpi show endogenous Insm1a binds its target sites in the *hb-egfa$_a$* promoter (ns indicates non-specific site, 1 and 2 represent duplicates). **(e)** Expansion of the zone of dedifferentiating MG by Insm1a knockdown at 4 dpi requires *hb-egf$_a$* expression and EGFR activation. Top shows experimental time line. Below the diagram of the experimental time line is shown representative BrdU IF photomicrographs of retinal sections at 6 dpi. MOs were delivered to cells at 4 dpi by electroporation. EGFR activity was inhibited with PD158780. Asterisk marks the injury site. Scale bar: 20 microns. **(f)** Quantification of the results shown in **(e)**. Relative to control, **P*<0.0001, n=3 biological replicates. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

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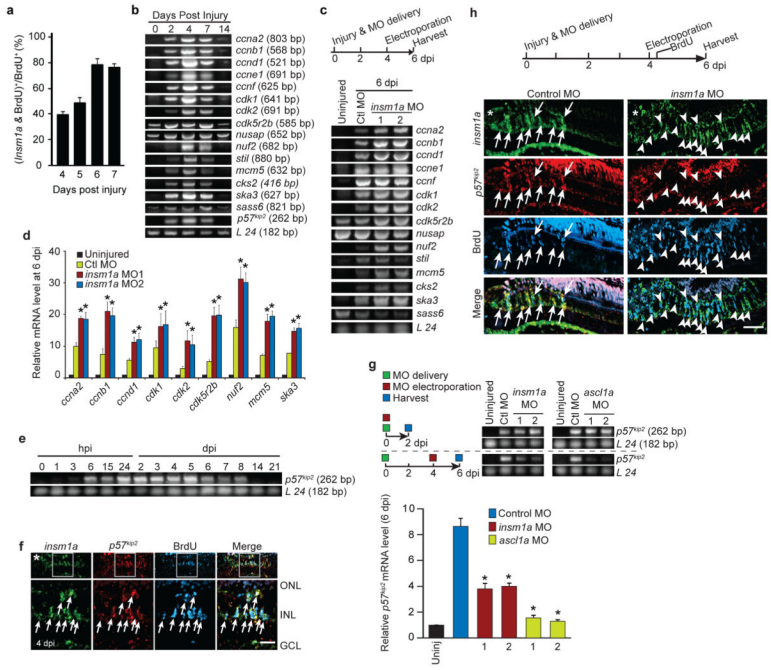

Figure 6.

At 4–6 dpi Insm1a inhibits a genetic program driving cell proliferation and stimulates a genetic program driving cell cycle exit. **(a)** Quantification shows that the number of BrdU$^+$ cells that are also *insm1a*$^+$ increases from 4–6 dpi. **(b)** RT-PCR shows the injury-dependent regulation of genes encoding cell cycle regulators. **(c, d)** RT-PCR and qPCR shows that the normal suppression of cell cycle genes at 6 dpi is generally prevented by Insm1a knockdown. Relative to control MO, **P*<0.003, except for *cdk1* data with a **P*<0.02, n=4 biological replicates. **(e)** RT-PCR shows the time course of injury-dependent *p57^{kip2}* expression. **(f)** ISH and IF shows that *insm1a* and *p57^{kip2}* mRNAs are coexpressed in BrdU$^+$ progenitors. Asterisk marks the injury site. Scale bar: 10 microns. **(g)** Insm1a or Ascl1a knockdown from 0–2 dpi with 2 different MOs had no effect on injury-dependent *p57^{kip2}* induction, while knockdown from 4–6 dpi suppressed this induction. Left-hand panel is Experimental time line; middle panel is RT-PCR analysis at 2 dpi (top gels) or 6 dpi (bottom gels); and right-hand panel is qPCR analysis at 6 dpi. Relative to control MO, **P*<0.0001, n=4 biological replicates. **(h)** ISH and IF show Insm1a knockdown suppresses *p57^{kip2}* expression and expands the zone of *insm1a*$^+$/BrdU$^+$ MG-derived progenitors. Asterisk marks the injury site. Scale bar: 20 microns. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

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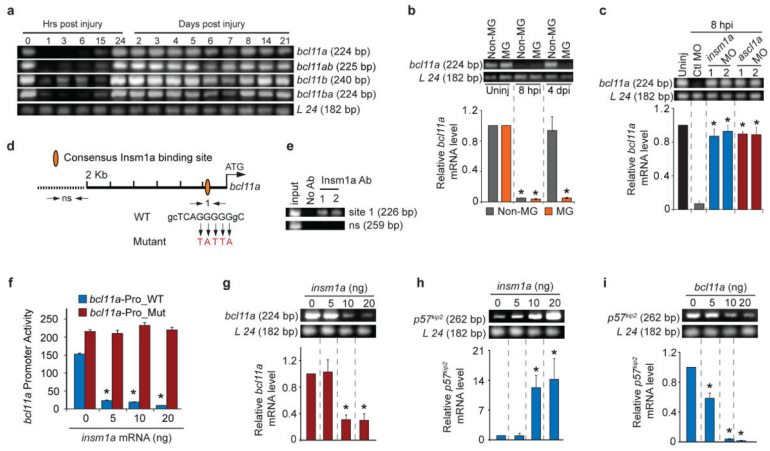

Figure 7.

Insm1a stimulates *p57^{kip2}* expression by suppressing *bcl11* gene expression. **(a)** RT-PCR shows the temporal pattern of *bcl11* family members following retinal injury. **(b)** RT-PCR and qPCR using mRNA from FACS purified non-MG and MG show *bcl11a* mRNA is transiently suppressed in a pan retinal fashion at 8 hpi and returns to non-MG by 4 dpi, but remains repressed in MG-derived progenitors (MG) at this time. Relative to uninjured control, **P*<0.0001, n=3 biological replicates. **(c)** RT-PCR and qPCR shows Insm1a or Ascl1a knockdown prevents injury-dependent *bcl11a* mRNA suppression. Relative to control MO, **P*<0.0001, n=3 biological replicates. **(d)** Diagram of *bcl11a* promoter and putative Insm1a binding site. Dashed line represents DNA sequences not included in *bcl11a* promoter used in panel (**f**). Arrows represent primers used in ChIP assay. Insm1a binding site mutations are in red. **(e)** ChIP assays using retinal extracts at 4 dpi show endogenous Insm1a binds to the *bcl11a* promoter's Insm1a binding site (ns indicates non-specific site, 1 and 2 represent duplicates). **(f)** Insm1a suppresses *bcl11a* promoter activity in zebrafish embryos injected with *insm1a* mRNA and a wild type (WT) or mutant (Mut) *bcl11a:gfp-luciferase* reporter. Promoter mutations shown in **(d)**. Relative to 0 ng *insm1a*, **P*<0.0001, n=3 biological replicates. **(g, h)** RT-PCR and qPCR show Insm1a overexpression in zebrafish embryos suppresses endogenous *bcl11a* mRNA, but increases endogenous *p57^{kip2}* mRNA levels. **P*<0.0001 for **(g)** and **P*<0.003 for **(h),** n=3 biological replicates. **(i)** Bcl11a overexpression in zebrafish embryos inhibits expression of endogenous *p57^{kip2}*. **P*<0.0001, n=3 biological replicates.

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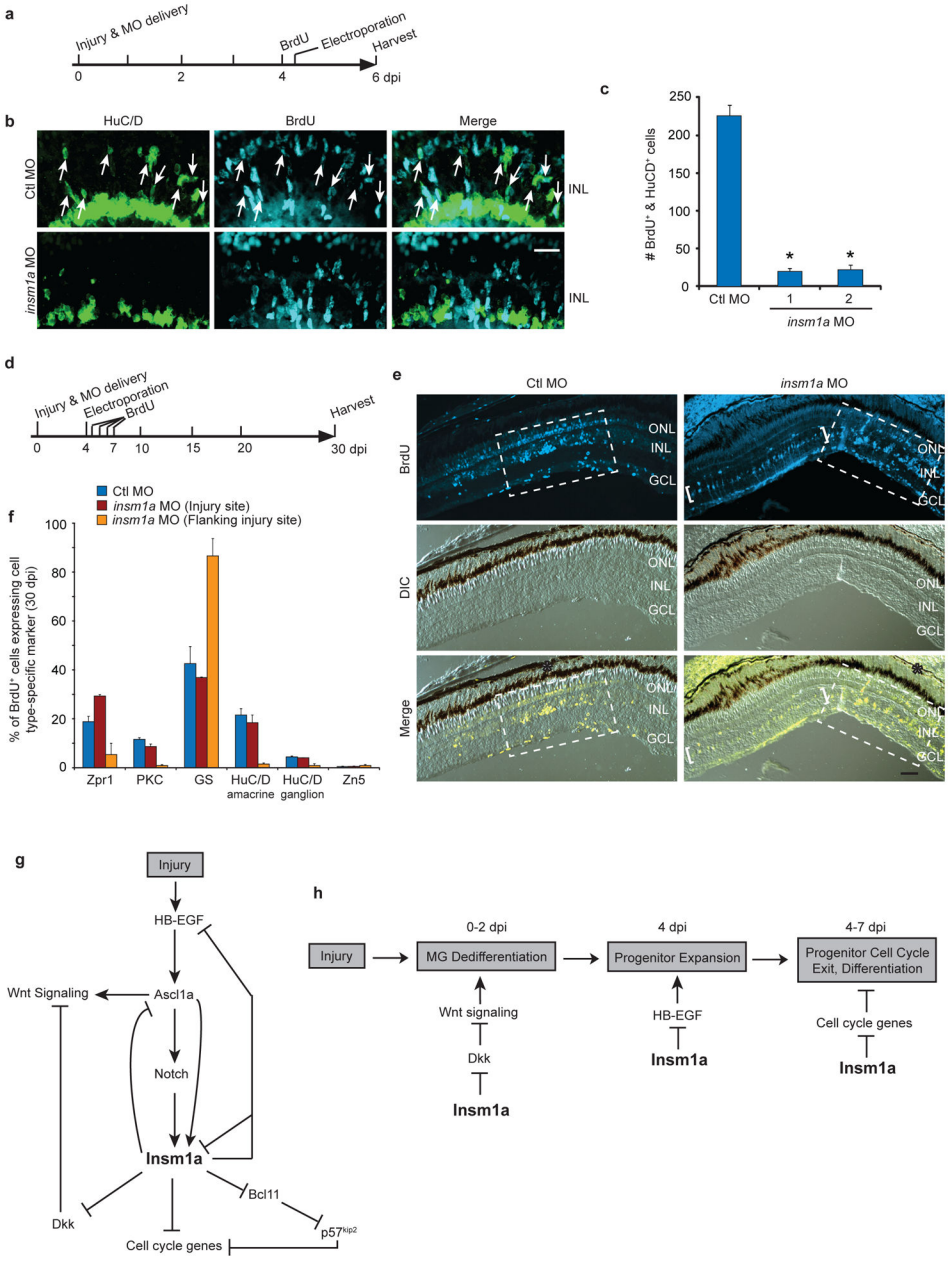

Figure 8.

Insm1a knockdown transiently suppresses progenitor differentiation. **(a)** Experimental time course showing BrdU labeling 4 hrs prior to MO electroporation at 4 dpi and analysis at 6 dpi. **(b)** HuC/D and BrdU IF shows that in control MO-treated retinas $BrdU^+$ cells migrate towards the ONL, while in *insm1a* MO-treated retinas HuC/D migrating cells are rarely detected. Scale bar: 10 microns. **(c)** Quantification of data shown in **(b)**. **P*<0.0001, n=3 biological replicates. **(d)** Experimental time course showing daily IP injections of BrdU from 4–7 dpi and analysis on 30 dpi. **(e)** BrdU IF shows BrdU localized to all 3 retinal layers at the site of injury (dashed box) in control and *insm1a* MO-treated retinas; however, in in regions flanking the injury site in *insm1a* MO-treated retinas, BrdU is confined to

predominantly a single lamina in the INL (bracketed region). DIC microscopy suggests both control and *insm1a* MO-treated retinas were repaired by 30 dpi. Scale bar: 20 microns. **(f)** Retinal cell type specific antibodies and BrdU IF were used to quantify the cell types regenerated with and without Insm1a knockdown at 30 dpi, n=3 biological replicates. **(g)** Signaling pathways and genes that are regulated by Insm1a. **(h)** Summary of Insm1a's action at different times and stages of retina regeneration. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

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