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

Rajesh Ramachandran¹, Xiao-Feng Zhao¹ and Daniel Goldman¹,²

In zebrafish, retinal injury stimulates Müller glia (MG) reprogramming, allowing them to generate multipotent progenitors that replace damaged cells and restore vision. Recent studies suggest that transcriptional repression may underlie these events. To identify transcriptional repressors, we compared the transcriptomes of MG and MG-derived progenitors and identified insm1a, a 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 the suppression of the Wnt inhibitor dickkopf (dkk), 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, 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 scaring¹, zebrafish mount a robust regenerative response that culminates in the restoration of vision². 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 types³⁻⁹. Although mammalian MG can be coaxed to proliferate, their regenerative capacity is meagre and limited¹⁰⁻¹³. Therefore, understanding the mechanisms underlying successful retina regeneration in zebrafish may provide insights for developing strategies to stimulate MG reprogramming and retina regeneration in mammals.

Recent studies have identified a number of gene products and signalling cascades contributing to retina regeneration. These include an ascl1a–lin28–let-7 microRNA (miRNA) signalling pathway that supports the conversion of MG into progenitors⁴; an ascl1a–Dkk–Wnt signalling pathway that in conjunction with Pax6, Stat3 and Hspd1 regulates MG reprogramming and progenitor proliferation⁵,⁶,¹⁴,¹⁵; a heparin-binding epidermal-like growth factor (HB-EGF)-dependent signalling pathway that stimulates MG reprogramming and retina regeneration¹⁶; and Fgf, Mps1 and galectin-dependent signalling that contribute to photoreceptor regeneration¹⁴,¹⁷,¹⁸.

Although many of the signalling 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 formation⁵, and hb-egf gene repression helps modulate the size of the progenitor population¹⁶. Perhaps the most obvious role for repression is the curtailing of cell proliferation programs as progenitors begin to differentiate. Mechanisms underlying this last repression are of major interest because they prevent uncontrolled growth that may lead to tumour formation and glial scarring. Here we report the remarkable finding that a single transcriptional repressor, Insm1a, contributes to all of 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 fluorescence-activated cell sorting (FACS)-purified MG and MG-derived progenitors from gap43:gfp and 1016 tuba1a:gfp transgenic fish retinae, respectively³,¹⁹ (Supplementary Fig. S1a). 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 1,200 genes induced greater than two-fold and over 300 genes suppressed by at least 50%. Of these, we identified transcriptional repressors belonging to her, bel11, klf, eng and insm1 gene families (Supplementary Fig. S1b). PCR

¹Molecular and Behavioral Neuroscience Institute and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109, USA.
²Correspondence should be addressed to D.G. (e-mail: neuroman@umich.edu)

Received 13 June 2012; accepted 17 August 2012; published online 23 September 2012; DOI: 10.1038/ncb2586
with reverse transcription (RT–PCR) confirmed injury-dependent regulation of these genes and revealed an intriguing biphasic pattern of expression for insm1a (Supplementary Fig. S1c). insm1a messenger RNA was first detected around 6 h post injury (hpi), suppressed at 24 hpi and reappeared by 4 days post injury (dpi). This pattern of expression suggested that Ins1a may play multiple roles during retina regeneration.

Injury-dependent insm1a induction is necessary for retina regeneration

In situ hybridization and immunofluorescence microscopy at 4 dpi in 1016 tuba1a:gfp transgenic fish showed that insm1a was expressed in GFP$^+$ progenitors that also expressed the MG marker, glutamine synthetase (GS; Fig. 1a and Supplementary Fig. S2a). Furthermore, many,
but not all, of these insm1a+ cells incorporated 5-bromo-2′-deoxyuridine (BrdU; Fig. 1b,c). Importantly, Ins1a knockdown using two different lissamine-tagged morpholino-modified antisense oligonucleotides (MOs) electroporated into the retina at the time of injury showed that Ins1a was necessary for the generation of GFP+ and BrdU+ MG-derived progenitors (Fig. 1d,e and Supplementary Fig. S2b and Table S1). Ins1a-targeting morpholinos prevented insm1agfp reporter gene expression in zebrafish embryos (Supplementary Fig. S2c) and suppressed injury-dependent Ins1a 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 panretinal pattern of expression at 6 hpi that became restricted to the injury site by 2 dpi (Fig. 1f–h and Supplementary Fig. S3). This spatial and temporal expression pattern was reminiscent of that previously reported for ascl1a (ref. 5) and both of 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 that insm1a often associates with cells flanking BrdU+ progenitors (Fig. 1h,i). Quantification showed that ~30% of the progenitors expressed insm1a at 2 dpi, which increased to 40% by 4 dpi (Fig. 1j and Supplementary Table S2). These data, along with the observation that Ins1a knockdown blocks progenitor production by over 80% (Fig. 1e and Supplementary Table S1), suggested that Ins1a expression in dedifferentiated MG is necessary for them to produce progenitors for retinal repair.

An Ascla1–Ins1a regulatory loop
As ascl1a and insm1a exhibited a similar pattern of expression in the injured retina, we investigated whether there was a hierarchical relationship. An analysis of injury-dependent ascl1a and insm1a mRNA expression showed that 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 and Supplementary Fig. S2e), whereas Ins1a knockdown caused a small increase in the insm1a and ascl1a expression levels (Fig. 2e). Inspection of the insm1a promoter revealed three putative Ascl1a-binding sites (Fig. 2f) and chromatin immunoprecipitation (ChIP) assays using myc–Ascl1a overexpressed in zebrafish embryos showed that Ascl1a binds these sites (Fig. 2g). Co-injection of zebrafish embryos with insm1agfp–luciferase reporter and increasing concentrations of ascl1a mRNA showed that 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 owing to the lack of a suitable antibody for its detection.

As Ins1a knockdown resulted in a small, but significant, increase in ascl1a and insm1a expression levels (Fig. 2e), we suspected that Ins1a may feedback to regulate their promoters. Inspection of ascl1a and insm1a promoter sequences revealed putative Ins1a binding sites (Fig. 2i,k) and ChIP assays confirmed that endogenous Ins1a bound these sites in the injured retina (Fig. 2j,l). Furthermore, co-injection of zebrafish embryos with ascl1agfp–luciferase or insm1agfp–luciferase reporters harbouring wild-type or mutant promoters and increasing concentrations of insm1a mRNA showed that these Ins1a-binding sites confer Ins1a-dependent regulation (Fig. 2m,n). This led us to speculate that Ins1a itself may be contributing to its transient suppression at 24 hpi (Fig. 2a,b), and Ins1a knockdown confirmed this suspicion (Fig. 2o). Together these experiments reveal an Ascl1a–Ins1a regulatory loop and an Ins1a autoregulatory loop that contributes to the dynamic expression pattern of ascl1a and insm1a during the course of regeneration.

Ins1a mediates dickkopf (dkk) gene repression in the injured retina
We previously demonstrated that the formation of MG-derived progenitors required dkk repression1. 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, whereas dkk1b exhibited the opposite pattern of expression (Fig. 3a,b). Importantly, Ins1a knockdown prevented injury-dependent dkk suppression (Fig. 3c). Inspection of the dkk1b promoter identified two putative Ins1a-binding sites that bound endogenous Ins1a in the injured retina (Fig. 3d,e). In addition, zebrafish embryos co-injected with a wild-type or mutant dkk1bgfp–luciferase reporter and increasing concentrations of insm1a mRNA showed that both Ins1a-binding sites contribute to dkk1b promoter repression by Ins1a (Fig. 3f). Finally, Ins1a knockdown in zebrafish embryos increased promoter activity from a co-injected dkk1bgfp–luciferase reporter and also prevented Ascl1a-dependent dkk1bgfp–luciferase reporter suppression (Fig. 3g). These data suggest that Ins1a represses dkk promoter activity in the adult retina and that this repressive mechanism can be recapitulated in zebrafish embryos by either Ins1a or Ascl1a overexpression. Taken together these data identify an Ascl1a–Ins1a–Dkk signalling cascade underlying MG reprogramming in the adult retina.

Ins1a 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 Ins1a 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 Ins1a 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 (Fig. 1d,e), Ins1a knockdown at 4 dpi greatly increased the number of progenitors (Fig. 4b–d and Supplementary Fig. S4b). This result was reminiscent of DAPT (N-[(3,5-difluorophenylacetyl)-l-alanyl]-S-phenylglycine t-butyl ester)-dependent Notch inhibition16 and suggested that Ins1a 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 response3, progenitor proliferation following Ins1a knockdown at 4 dpi was insensitive to Dkk overexpression (Fig. 4f and Supplementary Fig. S4c–f).

As HB-EGF is released locally at the injury site and stimulates MG dedifferentiation18, its suppression by Ins1a would provide a convenient mechanism for restricting the zone of dedifferentiating MG. Indeed, insm1a and hb-egf, are co-expressed in BrdU+...
Figure 2 An Ascl1a–Insm1a regulatory loop. (a,b) RT–PCR (a) and qPCR (b) were used to assay injury-dependent insm1a, ascl1a and ribosomal protein l-24 gene expression; n = 6 biological replicates. (c) RT–PCR (left) and qPCR (right) show that Ascl1a knockdown inhibits insm1a induction. ∗P < 0.0001, n = 3 biological replicates. (d) ISH shows that Ascl1a knockdown inhibits insm1a induction. The asterisk flanks the injury site. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 10 μm. (e) RT–PCR (top) and qPCR (bottom) show Ascl1a knockdown inhibits insm1a induction, whereas Insml1a knockdown slightly increases ascl1a and insm1a expression levels. Relative to the control MO, *P < 0.0001 in left panel; *P < 0.01 in right panel, n = 6 biological replicates. (f,i,k) Diagram of insm1a (f,k) and ascl1a (i) promoters with putative Ascl1a– (f) and Insml1a– (i,k) binding sites. The solid line represents DNA sequences included in the insm1a:gfpluciferase vector used in m. The arrows indicate primers used in ChIP assays. The capital letters are conserved bases of the consensus site. (g) The embryo ChIP assay detects myc–Ascl1a bound to target sites in the insm1a promoter (ns indicates nonspecific site; 1 and 2 represent duplicates). Ab, antibody. (h) Ascl1a overexpression stimulates insm1a:gfpluciferase expression in embryos. Promoter activity is normalized light units. ∗P < 0.0002, n = 3 biological replicates. (i,l) ChIP assays using retinal extracts at 4 dpi show that endogenous Insml1a binds its target sites in the ascl1a (j) and insm1a (l) promoters (ns indicates nonspecific site; 1 and 2 represent duplicates). (m,n) Expression vectors harbouring wild-type (WT) and mutant (Mut) insm1a or ascl1a promoters (Pro) driving gfpluciferase expression were injected into embryos with increasing amounts of insm1a mRNA. Promoter activity is normalized light units. ∗P < 0.0004, n = 3 biological replicates in m and *P < 0.0001, n = 3 biological replicates in n. (e) RT–PCR (top) and qPCR (bottom) show that Insml1a knockdown at the time of injury increases the insm1a mRNA expression level at 1 dpi. ∗P < 0.0001, n = 3 biological replicates. Error bars are s.d.
Insma1 regulates dkk promoter activity. (a) ISH and immunofluorescence microscopy show that insma1 and dkk exhibit opposing expression patterns in the uninjured and injured retina. The arrows point to BrdU+ MG-derived progenitors with high levels of insma1 and low levels of dkk1b mRNAs. The asterisk flanks the injury site. Scale bar, 20 μm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (b) RT–PCR (top) and qPCR (bottom) using mRNA from FACS-purified non-MG and MG-derived progenitors (MG) from either 1016 tuba1a:gfp or gfap:gfp transgenic fish shows that insma1 is transiently induced in a panretinal fashion at 8 hpi and by 4 dpi it is specifically expressed in MG-derived progenitors. In contrast, dkk1b exhibits the opposite expression pattern. In the histogram, the first pair of bars at each time point represents insma1 expression. Relative to uninjured controls, *P < 0.001, n = 3 biological replicates. (c) RT–PCR (top) and qPCR (bottom) show that Insma1 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 the putative Insma1-binding sites. Promoter mutations in Insma1-binding elements are shown in red. The dotted line represents DNA sequences not included in the dkk1b promoter used in f. (e) ChIP assays using retinal extracts at 4 dpi show that endogenous Insma1 binds its target sites in the dkk1b promoter (ns indicates nonspecific site, 1 and 2 represent duplicates). Ab, antibody. (f) Insma1 overexpressed in zebrafish embryos regulates co-injected dkk1b:gfp-luciferase reporter activity through the Insma1-binding sites of the dkk1b promoter. Relative to 0 ng insma1, *P < 0.0001, n = 3 biological replicates. Insma1 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. Error bars are s.d.

Insma1 stimulates progenitor cell-cycle exit by suppressing a genetic program driving cell proliferation

Fish receiving an intraperitoneal (i.p.) injection of BrdU 3 h before euthanization showed that the fraction of proliferating progenitors expressing insma1 increased from ~40% at 4 dpi to ~80% at 6 dpi (Fig. 6a and Supplementary Fig. S5a and Table S2). As progenitor proliferation is decreasing during this time period, we reasoned that Insma1 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 Table S2).
Insm1a most likely mediates p57kip2 induction by suppressing an intervening repressor. One candidate was bcl11b (CTIP2) that inhibits p57kip2 gene expression in SK-N-MC cells. Interestingly, our microarray data suggested that bcl11b expression was reduced by ~80% in MG-derived progenitors (Supplementary Fig. S1b). Zebrafish harbour four different bcl11 genes and all four are coordinately repressed in a panretinal 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; ref. 5) and suggested a shared mechanism of regulation. Indeed, similarly to dkk (Fig. 3c; ref. 5), Insm1a or Ascl1a knockdown relieved injury-dependent bcl11a repression (Fig. 7c).

We next investigated whether Insm1a directly regulated bcl11a gene expression. Inspection of the bcl11a promoter identified a
Ins1a knockdown transiently suppresses progenitor differentiation

One consequence of Ins1a-driven cell-cycle exit is progenitor differentiation. Therefore, we investigated whether Ins1a 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 whether HuC/D could be used to identify differentiating cells in the regenerating retina and whether Ins1a was necessary for their formation. For these experiments, fish received control or ins1a-targeting MO at the time of injury, an i.p. injection of BrdU at 4 dpi, MO electroporation 5 h post BrdU injection and were then euthanized at 6 dpi (Fig. 8a). Interestingly, double labelled 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 Ins1a knocked

single putative Ins1a-binding site in its proximal region (Fig. 7d) and ChIP assays showed that endogenous Ins1a bound this site in the injured retina (Fig. 7e). To determine whether this Ins1a-binding site was functional we co-injected zebrafish embryos with bcl11a:egfp–luciferase reporters harbouring wild-type or mutant promoters and ins1a mRNA. This experiment showed that the Ins1a-binding site was functional as it co-injected zebrafish embryos with a single putative Ins1a-binding site in its proximal region (Fig. 7d) and ChIP assays showed that endogenous Ins1a bound this site in the injured retina (Fig. 7e). To determine whether this Ins1a-binding site was functional we co-injected zebrafish embryos with bcl11a:egfp–luciferase reporters harbouring wild-type or mutant promoters and ins1a mRNA. This experiment showed that the Ins1a-binding site was functional as it

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for the ChIP assay below. Bottom, ChIP assays using retinal extracts at 4 dpi show that endogenous Ins1a binds its target sites in the hb-egfa promoter (ns indicates nonspecific site, 1 and 2 represent duplicates). Ab, antibody. (e) Expansion of the zone of dedifferentiating MG by Ins1a knockdown at 6 dpi requires hb-egfa expression and EGFR activation. Top, experimental time line. Bottom, representative BrdU immunofluorescence micrographs of retinal sections at 6 dpi. MOs were delivered to cells at 4 dpi by electroporation. EGFR activity was inhibited with PD158780. The asterisk marks the injury site. (f) Quantification of the results shown in e. Relative to control, *P < 0.0001, n = 3 biological replicates. Scale bars, 10 μm (a) and 20 μm (b,e). Error bars are s.d.

Figure 5 Ins1a restricts the zone of dedifferentiating MG by suppressing hb-egfa gene expression. (a) ISH and immunofluorescence microscopy shows that ins1a and hb-egfa mRNAs are co-expressed in BrdU+ MG-derived progenitors at 4 dpi. The asterisk marks the injury site. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (b) Top, experimental time line. Bottom, hb-egfaISH on control and Ins1a-knockdown retinal sections at 6 dpi. The asterisk marks the injury site. (c) RT–PCR (top) and qPCR (bottom) show that Ins1a knockdown transiently suppresses progenitor differentiation. Top, experimental time line. Bottom, representative BrdU activation. Top, experimental time line. Bottom, representative BrdU

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Insm1a knockdown suppresses p57kip2 expression and expands the zone of Insm1a−BrdU+ MG-derived progenitors. The arrows identify triple-labelled cells; the arrowheads identify Insm1a−BrdU+ double-labelled cells. The asterisk marks the injury site. Scale bars, 10 μm (f) and 20 μm (h). Error bars are s.d.

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 that we gave fish daily i.p. injections of BrdU from 4 to 7 dpi before euthanizing 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 three 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 and Supplementary Fig. S6). Interestingly, Insm1a knockdown had little effect on the distribution of differentiated cell types at the injury site. In contrast, progenitors originally residing in the expanded zone flanking the injury site were generally confined to a single lamina of the inner nuclear layer (INL) and predominantly regenerated GS+ MG (Fig. 8e,f and Supplementary Fig. S6 and 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 (refs 5,16), this study suggests that at later times (30 dpi) MG selectively survive (Fig. 8f). We suspect that 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 as do newly regenerated cells at the injury site.

**DISCUSSION**

Ins1 is a transcriptional repressor associated with neuroendocrine tumours, small-cell lung cancer and the terminal cell divisions associated with neurogenesis. Although Ins1 plays an important role during nervous system development, surprisingly little is known about the action of Ins1 in the adult nervous system. Furthermore, the mechanisms controlling ins1 gene induction and the underlying action of Ins1 on progenitor differentiation remain poorly understood.

Zebrafish harbour two ins1 genes referred to as ins1a and ins1b (ref. 29). In the adult retina, ins1a is normally expressed in the neurogenic ciliary marginal zone, but can also be found in the central retina of fish with photoreceptor degeneration. Our studies suggest that Ins1a 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 the function of Ins1a. Furthermore, our studies revealed signalling mechanisms underlying injury-dependent ins1a gene induction and also identified mechanisms by which Ins1a acts to control MG reprogramming, 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 Ins1 inactivation and action in mammals.

Although Ins1a is best known as a transcriptional repressor that is associated with terminal cell division and neuronal differentiation, we uncovered a number of roles for Ins1a during retina regeneration. First, Ins1a links ascl1a gene induction with dkk gene repression, which we previously showed was necessary for MG dedifferentiation and retina regeneration. Second, using a protocol of delayed MO electroporation and gene knockdown, we found that Ins1a regulates expressions of endogenous p57<sup>kip2</sup> and p57<sup>kip2</sup>. This last function of Ins1a was observed only if MG were allowed to initially dedifferentiate and generate progenitors, suggesting that knockdown of Ins1a in these progenitors stimulated reprogramming of neighbouring MG so they could generate additional progenitors. A possible mechanism underlying this effect was suggested by our finding that Ins1a controls bcl11ab gene expression, whose product...
was recently shown to stimulate MG reprogramming and progenitor formation in the uninjured retina. Investigation of the mechanisms initiating injury-dependent Ins1a induction identified Ascl1a, a gene product that is a nodal point for a number of signalling cascades during retinal regeneration. We previously showed that ascl1a gene expression is regulated by HB-EGF and is among the earliest gene induced following retinal injury. Here we show that Ascl1a is necessary for the expression of injury-induced MG-derived progenitors. Interestingly, Ascl1 and Ins1a induction are also associated with DKK repression in certain human lung cancers and pancreatic endocrine tumours, perhaps suggesting a conserved signalling pathway.

In addition to regulating dkk gene expression, Ins1a also feeds back to inhibit VEGF, ascl1a and ins1a promoter activity. This kind of feedback is often associated with oscillations and seems to underlie the biphasic pattern of ins1a gene expression during retina regeneration and also helps restrict injury-responsive MG to the injury site. Ins1a knockdown at 4 dpi not only expanded the zone of MG reprogrammed to produce progenitors, but also markedly increased progenitor proliferation at the expense of differentiation. In its role as a regulator of MG proliferation, Ins1a seems to stimulate cell-cycle exit by sequestering cyclin D1 with its proline-rich cyclin-D1-binding domain. However, this domain is missing in zebrafish Ins1a, suggesting another mechanism of action. Remarkably, we found that Ins1a not only suppressed a gene expression program that drives cell proliferation, but also relieved repression of p57kip2, a cyclin kinase inhibitor that along with p27kip1 drives cell-cycle exit during mouse retina development. In the mouse retina, reduced expression of p27kip1 is associated with MG proliferation and reactive gliosis. Interestingly, our transcriptome analysis of MG and MG-derived progenitors revealed that p27kip1 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.

Figure 8. Ins1a knockdown transiently suppresses progenitor differentiation. (a) Experimental time course showing BrdU labelling 4 h before MO electroperoration at 4 dpi and analysis at 6 dpi. (b) HuC/D and BrdU immunofluorescence microscopy shows that in control MO-treated retinae BrdU* cells migrate towards the ONL, whereas in ins1a MO-treated retinae HuC/D migrating cells are rarely detected. The arrows identify HuC/D*/BrdU*. INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer. (c) Quantification of the data shown in b. *P < 0.0001, n = 3 biological replicates. (d) Experimental time course showing daily injections of BrdU from 4–7 dpi and analysis on 30 dpi. (e) BrdU immunofluorescence microscopy shows that BrdU localized to all three retinal layers at the site of injury (dashed outline) in control and ins1a MO-treated retinas; however, in the regions flanking the injury site in ins1a MO-treated retinae, BrdU is confined to predominantly a single lamina in the INL (bracketed region). DIC microscopy suggests that both control and ins1a MO-treated retina were repaired by 30 dpi. (f) Retinal-cell-type-specific antibodies and BrdU immunofluorescence microscopy were used to quantify the cell types regenerated with and without Ins1a knockdown at 30 dpi; n = 3 biological replicates. (g) Signalling pathways and genes that are regulated by Ins1a. (h) Summary of the action of Ins1a at different times and stages of retina regeneration. Scale bars, 10 μm (b) and 20 μm (e). Error bars are s.d.
Our data suggest that Insm1a plays at least three important roles during retinal 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 hbe-gf gene expression. Third, it contributes to the cessation of retinal regeneration by stimulating cell differentiation through the suppression of genetic programs driving cell proliferation. Finally, our studies revealed mechanisms by which Insm1a mediates these effects and the signalling pathways underlying insm1a gene regulation in the injured retina. These studies place Insm1a among the key factors underlying retinal regeneration and provide insight into signalling pathways that may help shift the response of MG in the injured mammalian retina from reactive gliosis towards retinal repair.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

This research was supported by NEI grant ROI EY 018132 from the NIH. We thank D. Hyde (University of Notre Dame, USA) for gfp:gfp transgenic fish; R. Moon (University of Washington, USA) for latep32dklk1b-gfp transgenic fish; the University of Michigan Flow Cytometry Core for cell sorting; A. Dombrowski (Wayne State, USA) for microarray screen; R. Thompson (University of Michigan, USA) for assistance with microarray data organization; M. Uhler and D. Turner (University of Michigan, USA) for providing the pE3 and pCS2 vectors, respectively; J. Beals (University of Michigan, USA) for help with confocal microscopy; the Goldman laboratory for helpful comments and suggestions during the course of this research; and R. Karr for fish care.

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. analysed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/dvod finder/doi:10.1038/nrc2586

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METHODS

Animals, heat shock, drugs and retinal injury. Zebrafish were kept at 26–28 °C on a 14 h:10 h light/dark cycle. 1016 tuba1a:gfp, glap:gfp and hsp70:dkl1b:gfp transgenic fish have been previously described. Embryos were obtained by natural matings. Heat shock was performed by transferring hsp70:dkl1b:gfp fish to a 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. Briefly, fish were anaesthetized in tricaine methane sulphonate 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 three times.

FACS and microarray. RNA was obtained from FACS-purified MG and MG-derived progenitors at 4 dpi as previously described. Briefly, unjured and injured retinas were isolated from glap:gfp and 1016 tuba1a:gfp transgenic fish. GFP+ MG from glap:gfp unjured retinas and GFP+ MG-derived progenitors from 1016 tuba1a:gfp retinas at 4 dpi were isolated by treating retinae with hyaluronidase and trypsin and then sorted on a BD Biosciences FACSVia 3 laser high-speed cell sorter. Four unjured retinas from glap:gfp fish yielded 235,000 GFP+ cells, whereas 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, G#S219F). Duplicate samples were analysed. Microarray data have been submitted to GEO (GSE36191).

Primers and plasmid construction. All primers are listed in Supplementary Table S4. asc1a, insm1a and bcl1a1a promoters were amplified from Zebrafish genomic DNA using primer pairs Xho-asc1a-Pro-F and Bam-asc1a-Pro-R (≈6 kb), Xho-insm1a-Pro-F and Bam-insm1a-Pro-R (≈3 kb), and Bam-insm1a-Pro-R and EcoR1-bcl1a1a-Pro-R (≈2 kb), respectively. The PCR amplicons were digested and cloned into a pET luciferase expression vector to create ascl1a:gfp–luciferase, insm1a:gfp–luciferase and bcl1a1a:gfp–luciferase constructs. The dkk1b:gfp–luciferase construct was described previously. Site-directed mutagenesis was done as previously described. insm1a and bcl1a1a are a complementary DNA were amplified from zebrafish retina RNA at 4 dpi using primer pairs Bam-insm1a-F and Xho-insm1a-R (1.1 kb), and Cla-bcl1a1a-F and Cla-bcl1a1a-R (2.4 kb). The PCR amplicons were cloned into their respective enzyme sites in pCS2+-MT (insm1a) and pCS2+-bcl1a1a plasmid to obtain cmvmyc–insm1a and cmvcycl1a. The cmvmyc–asc1a construct was described previously. 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 retinae 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. Quantitative PCR (qPCR) was carried out in triplicate with Absolute SYBR Green Fluorescent Master Mix (Thermo Scientific) on an Icycler real-time PCR detection system (BioRad). The ΔΔCt method was used to determine the relative expression of mRNAs in control and injured retina and normalized to ribosomal protein l-24 mRNA levels.

mRNA synthesis, embryo micro-injection and ChIP assay. pCS2+ and pCS2+-MT plasmids harbouring cDNA inserts were linearized and capped mRNAs were synthesized using the Message Machine Kit. Single-cell zebrafish embryos were injected with ~200 pl of solution containing 0.2 pg of Renilla luciferase mRNA and 2 pg of promoter-gfp–luciferase vector and 0–4 pg of asc1a, insm1a or bcl1a1a mRNA. To ensure reproducibility, a master mix was made for daily injections and ~200 embryos were injected at the 1–2 cell stage. At 24 h later, embryos were divided into 3 groups (~65 embryos/group) and lysed for dual luciferase reporter assays (Promega).

ChIP assays to analyse endogenous Insn1a binding to various promoters in the adult injured retina were performed using ~100 adult retinae collected at 4 dpi after dark adaptation. Chromatin was isolated and sonicated as described previously. The sonicated chromatin was distributed into three equal aliquots; two were probed with an anti-zebrafish Insn1a antibody (AnaSpec, catalogue number 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 were performed as previously described. Primers used for ChIP assays are described in Supplementary Table S4.

Morpholino electroporation. Lissamine-tagged MOs (Gene Tools, LLC; ~0.5 pl 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. The control, ascl1a- and hh-ceg4-targeting MOs have been previously described. Sensm1a-targeting MOs are: sensm1a MO1 5′-ATGCCGCCGGCAAAATGCGCATTTCA-3′ and sensm1a MO2 5′-GCTGATGAAATCTCTCGGACAT-3′. As antibodies detecting zebrafish Insn1a 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 retinae and used primers MO-Hind-insm1a-F and MO-Bam-insm1a-R (Supplementary Table S4) to amplify a 121-base pair 5′ insm1a cDNA fragment that harboured both of the insm1a-MO target sites. This product was appened to the coding amino terminus of GFP in the cmvcycl1a expression vector and injected into zebrafish embryos with either lissamine-tagged control (0.5 mM) or lissamine-tagged insm1a-targeting MOs (0.25 mM) in separate experiments. After 24 h, embryos were assayed under fluorescent microscopy for GFP expression (Supplementary Fig. S3c).

BrdU labelling, tissue preparation, in situ hybridization and immunofluorescence microscopy. BrdU labelling was accomplished by i.p. injection of 20 μl of BrdU (20 mM) 3 h before euthanization unless otherwise indicated. Some animals received multiple injections of BrdU over multiple days. Fish were overdosed with tricaine methane sulphonate and eyes were dissected,enucleated, fixed and sectioned as previously described. In situ hybridization (ISH) was performed on retinal sections with digoxigenin-labelled complementary RNA probes (DIG RNA labelling kit, Roche Diagnostics)39. Fluorescent ISH was performed according to the manufacturer’s instructions (Perkin-Elmer). Sense control probes consistently gave no signal above background. Immunofluorescence microscopy protocols and antibodies were previously described. Insn1a immunofluorescence microscopy was performed using anti-zebrafish Insn1a antibody (Anaspec, catalogue number 55795–2) at 1:100 dilution. For BrdU immunofluorescence microscopy, sections were treated with 2 N HCl at 37 °C for 20 min, rinsed in 0.1 sodium borate (pH 8.5) for 10 min and then processed using standard procedures41. For lineage tracing experiments retinal sections from a single eye were distributed across six slides. Each slide was first processed for immunofluorescent detection of cell-type-specific markers (one marker per slide) and then a 2 N HCl epitope retrieval protocol was performed to identify BrdU+ cells44. Each slide was used to react with a different cell-type-specific marker, and BrdU+ cells were detected on all slides. The total number of BrdU+ cells and the number of co-labelled BrdU+ cells that also stained for a particular cell-type marker were quantified on each slide.

Microscopy, cell counts and statistical analysis. Slides were examined with a Zeiss Axioshot microscope equipped with fluorescence optics or an Olympus Fluoview FV1000 confocal imaging system. Cell counts were determined by counting fluorescently labelled cells (BrdU+ or retinal-cell-type-specific insm1a−) in retinal sections visualized using fluorescent microscopy. All sections of the retina were examined and at least three experiments were used. Data were analysed for statistical significance using Stat View software (SAS Institute). Statistical comparisons were conducted using a two-tailed unpaired Student’s t-test to analyse 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 s.d.

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Figure S1 Injury-dependent regulation of transcriptional repressors. 
(a) Representative scatter plots used to FACS purify GFP+ cells from 
gfap:gfp and 1016 tuba:gfp transgenic fish. Cells from wild type (Wt) 
non-transgenic fish do not fall into the R3 window, whereas GFP+ MG 
and MG-derived progenitors from transgenic fish sort into this window.

(b) Microarray transcriptome analysis of MG and MG-derived progenitors 
at 4 dpi identifies candidate transcriptional repressors whose expression 
is regulated by retinal injury. (c) RT-PCR using total retinal RNA shows 
temporal expression pattern of transcriptional repressors at different times 
post injury.
Figure S2  *insm1a* and *ascl1a* mRNAs co-localize in MG-derived progenitors and Ins1a knockdown prevents MG dedifferentiation. (a) *insm1a* ISH along with GFP and GS IF show *insm1a* is expressed in dedifferentiated MG of the injured retina. Asterisk marks the injury site. (b) Control (Ctl) or *insm1a*-targeting MOs delivered at the time of injury inhibit MG-dedifferentiation as determined by GFP IF in *1016 tuba1a:gfp* transgenic zebrafish. Asterisk marks the injury site. (c) Zebrafish embryos were injected with *sCMV:gfp* expression vectors that had the *insm1a* MO target sequence appended to the 5' end of the *gfp* sequence, along with either a control MO or one of two different *insm1a*-targeting MOs. *Ins1a* MOs targeted either the *insm1a* 5' UTR or sequences flanking the initiator codon. The number of GFP+ embryos was quantified 24 hrs post injection. (d) IF shows Ins1a protein induction at the injury site at 4 dpi. Asterisk flanks the injury site. (e) Ins1a (green) and BrdU (blue) IF shows that when retinas are electroporated with MOs at the time of injury, *insm1a* and *ascl1a* targeting MOs, but not the control MO, knocks down injury-induced Ins1a expression and inhibits cell proliferation when assayed at 4 dpi. Asterisk marks the injury site. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 10 micron in (a, b, d & e); 100 micron in (c).
Figure S3 Injury-dependent *insm1a* mRNA expression at 6 hpi (a) and 2 dpi (b). Asterisk in (a) marks injury site. ISH (fluorescent detection) was used to assay *insm1a* at 6 hpi and ISH (peroxidase detection) was used to assay *insm1a* at 2 dpi. Scale bar: 150 micron.
Figure S4 Insm1a regulates the zone of dedifferentiating MG via an hb-egf-dependent mechanism. (a) Experimental time line. (b) insm1a ISH shows an expanded zone of insm1a-expressing dedifferentiated MG in the injured and Insm1a knockdown retina compared to the control injured retina. Low and high magnification images are shown. Asterisk marks the injury site. (c) Experimental time line. (d) IF for Dkk-GFP fusion expression after heat shock shows induction in all 3 retinal layers. Lissamine fluorescence shows MO was delivered throughout the retina. Asterisk flanks the injury site. (e, f) BrdU IF shows expansion of the zone of proliferating progenitors following Insm1a knockdown in hsp70:dkk1b-gfp fish is not prevented by Dkk1b overexpression. Asterisk marks the injury site. (g) Top diagram shows experimental time. Insm1a (green) and BrdU (blue) IF shows that delayed electroporation of insm1a targeting MO at 4 dpi, knocks down injury-induced Insm1a expression and stimulates cell proliferation when assayed at 6 dpi. Asterisk marks the injury site. Scale bar: 50 micron in (b, e, f) left panels; 20 micron in (b, e, f) right panels; 20 micron in (d); 20 micron in (g) upper panels and 10 micron in (g) lower panels.
**Figure S5** *insm1a* expression correlates with cell cycle exit. (a) *insm1a* ISH and BrdU IF show that from 4-7 dpi an increasing percentage of BrdU+ cells co-express *insm1a*. Arrows point to *insm1a*+/BrdU+ cells and arrowheads point to *insm1a*-/BrdU+ cells. Asterisk flanks the injury site. (b) Candidate cell cycle genes identified in a microarray-based transcriptome analysis of MG and MG-derived progenitors that may be regulated by *insm1a*. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 10 micron.
Figure S6  Progenitor differentiation in Insm1a knockdown retinas. Top diagram is the experimental time line. Below are representative photomicrographs of lineage tracing of progenitors (BrdU+) differentiated at 30 dpi at and located at the injury site or located in a region flanking the injury site. Double IF was performed using retinal cell type specific antibodies and anti-BrdU antibodies. Antibodies were as follows: Zpr1, photoreceptor; PKC, bipolar cell; GS, MG; HuC/D, in INL was used to detect amacr ine cells and in the GCL was used to detect putative ganglion cells; Zn5, differentiating ganglion cells. The predominant cell type detected in flanking regions of Insm1a knockdown retinas was MG. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; PKC, protein kinase C. Scale bar: 10 micron.
**Figure S7** Alignment of zebrafish (ZF) Insm1a and human (HS) Insm1 sequence. Yellow highlighted region is the proline-rich cyclin D1 binding domain found in human Insm1. Zinc fingers are highlighted in green. Asterisks mark conserved residues contributing to zinc finger.
Figure S8 TUNEL staining for apoptotic cells at different times post retinal injury and following various treatments. (a) Ouabain was used as a positive control for cell death and shows extensive cell death in all 3 retinal layers (arrows). (b-c) Minor cell death in the control and Insm1a MO electroporated retina at 2dpi (b) and 4dpi (c). Asterisk marks the injury site. (d) Cell death at 6 dpi following various treatments. Asterisk marks the injury site. Scale bar: 20 microns. (e) Quantification of TUNEL+ cells at various stages of retina regeneration and under various experimental conditions.