Ascl1a regulates Müller glia dedifferentiation and retina regeneration via a Lin-28-dependent, *let-7* miRNA signaling pathway

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Abstract

Unlike mammals, teleost fish mount a robust regenerative response to retinal injury that culminates in restoration of visual function[1, 2]. This regenerative response relies on Müller glia (MG) dedifferentiation into a cycling population of progenitor cells. However, the mechanism underlying this dedifferentiation is unknown. Here we report that genes encoding pluripotency factors are induced following retinal injury. Interestingly, the proneural transcription factor Ascl1a and the pluripotency factor Lin-28 are induced in MG within 6 hrs following retinal injury and are necessary for MG dedifferentiation. We demonstrate that Ascl1a is necessary for *lin-28* expression and that Lin-28 suppresses *let-7* miRNA expression. Furthermore we show that *let-7* represses expression of regeneration-associated genes like, *ascl1a*, *hspd1*, *lin-28*, *oct4*, *pax6b* and *c-myc*. Interestingly, *hspd1*, *oct4* and $c\text{-}myc_a$ exhibit basal expression in the uninjured retina and *let-7* may inhibit this expression to prevent premature MG dedifferentiation. The opposing actions of Lin-28 and *let-7* miRNAs on MG differentiation/dedifferentiation are similar to that of embryonic stem cells[3] and suggest novel targets for stimulating MG dedifferentiation and retina regeneration in mammals.

Despite structural and functional similarities between the teleost and mammalian retina, disease or injury of the mammalian retina leads to irreparable vision loss, while the injured teleost retina mounts a regenerative response that restores lost sight[1, 2]. Key to successful regeneration are MG, which dedifferentiate and assume progenitor properties following retinal injury[4–7]. In mammals, MG generally respond to injury by reactive gliosis that is accompanied by hypertrophy; rarely do these cells re-enter the cell cycle and regenerate new neurons[8–11]. These data suggest that a key difference between the regenerative responses of fish and mammals is the ability of MG to dedifferentiate in response to retinal injury. Because zebrafish mount a robust regenerative response following retinal injury, they

provide a useful model system for uncovering the underlying mechanisms of MG dedifferentiation. These mechanisms are likely to suggest novel strategies for improving MG dedifferentiation and retina regeneration in mammals.

We recently reported that the proneural basic-helix-loop-helix transcription factor Ascl1a is necessary for MG dedifferentiation and retina regeneration[12]. However, the mechanism by which Ascl1a mediates its effects remained unknown. We reasoned that MG dedifferentiation may share some aspects with reprogramming of somatic cells by pluripotency factors[13, 14]. Therefore we investigated if pluripotency factors were regulated during retina regeneration. For this analysis, total RNA was isolated from uninjured and injured retinas at 15 hrs post injury (hpi) when MG dedifferentiation is beginning; 2 days post injury (dpi) when MG begin proliferating, 4 dpi when MG proliferation is maximal, and 7 dpi when MG proliferation has stopped and cellular differentiation has begun[4, 12]. RT-PCR (Fig. 1a) and Real-time PCR (Fig. 1b) showed pluripotency factors *klf4, oct4* and *c-myc$_a$* were expressed in the uninjured retina and transiently increased around15 hpi. In contrast, pluripotency factors *lin-28, sox2, nanog* and *c-myc$_b$* were undetectable in the uninjured retina and induced either within 15 hpi (*lin-28, c-myc$_b$*) or by 2 dpi (*sox2, nanog*). We were particularly intrigued by the lack of detectable *lin-28* and *myc$_b$* expression in the uninjured retina and their rapid and high induction following retinal injury. Here we focused on the role *lin-28* plays in retina regeneration.

A more detailed RT-PCR analysis showed *lin-28* induction within 6 hrs post retinal injury, which paralleled that of *ascl1a* (Fig. 1c). *In situ* hybridization was used to visualize *lin-28* RNA expression, and immunofluorescence was used to identify glutamine synthetase (GS)-expressing MG at different time points following retinal injury in wild-type and *1016 tuba1a:gfp* transgenic fish. These latter fish express GFP and incorporate BrdU in MG-derived progenitors located at the injury site[4] (Supplementary Information, Fig. S1). Since BrdU is only metabolically available for the first 4 hrs following intraperitoneal injection[15], our observation that BrdU injection at 4 dpi identified an expanding population of cells at 5–7 dpi (Supplementary Information, Fig. S1) suggests that BrdU-labeled cells are proliferating. *In situ* hybridization showed *lin-28* expression is restricted to MG and MG-derived GFP$^+$ progenitors at 1and 4 dpi, respectively, that are located at the injury site (Fig. 1d; Supplementary Information, Fig. S2). Although *lin-28* and *ascl1a* gene expression was first detected at 6 hpi, this expression peaked around 3–4 dpi. At this latter time point, *ascl1a* and *lin-28* were co-expressed in proliferating MG-derived progenitors (Fig. 1e). Quantification showed that approximately 83% of the *ascl1a$^+$* cells also expressed *lin-28*, and 91% of the *lin-28$^+$* cells also expressed *ascl1a*. Of the BrdU$^+$ cell population, 80% expressed *lin-28*, and 82% expressed *ascl1a* (Supplementary Information, Fig. S3).

Lin-28 is an RNA binding protein whose expression is associated with embryonic stem cells and cancer[16]. In zebrafish, RT-PCR suggests that *lin-28* is a transiently expressed zygotic transcript whose expression peaks around 5 hours post fertilization (hpf) (Supplementary Information, Fig. S4). We were interested in determining if *lin-28* expression in the developing retina marked proliferating retinal progenitors similar to what we observed in the adult regenerating retina. Surprisingly, at 24 hpf *in situ* hybridization and BrdU labeling revealed *lin-28* expression in differentiated cells of the hindbrain and spinal cord with

undetectable expression in the retina (Supplemental Information, Fig. S4). These results, along with our analysis of pluripotency factor gene expression (Fig. 1a), suggests that MG-derived retinal progenitors are not identical to embryonic retinal progenitors, but rather share characteristics with embryonic stem cells and raise the intriguing possibility that MG-derived progenitors may not be restricted to regeneration of only the retina if placed in the appropriate environment.

Because Lin-28 is associated with embryonic stem cells and cancer, we were interested in determining if Lin-28 expression was necessary for injury-induced MG dedifferentiation. For this analysis we knocked down the expression of Lin-28 in the adult retina using electroporated morpholino-modified antisense oligonucleotides (MOs); a well characterized approach for suppressing mRNA translation in zebrafish[12, 17–19]. Because antibodies to zebrafish Lin-28 are not available, we confirmed the efficacy of the *lin-28*-targeting MOs by co-injecting a plasmid encoding a *lin-28-GFP* chimeric transcript into single cell zebrafish embryos with and without the *lin-28*-targeting MO and assaying GFP expression 24 h later (Supplementary Information, Fig. S5). We previously demonstrated that a non-specific control MO electroporated into the injured fish retina had no appreciable effect on the regenerative response, while 2 different MOs targeting *ascl1a* inhibit Müller glia proliferation following retinal injury[12]. We confirmed these results here and showed that MO-mediated knockdown of Lin-28 expression dramatically suppressed *1016 tuba1a:gfp* transgene expression and MG proliferation (Fig. 2). Quantification indicates that only about 6% of the *lin-28* MO #1 and 9% of the *lin-28* MO #2 positive cells were also BrdU$^+$ (Fig. 2b; Supplementary Information, Table S1). Hence, both *ascl1a* and *lin-28* are necessary for MG dedifferentiation and proliferation.

We next investigated if there is any hierarchical regulation or interdependency between Ascl1a and Lin-28. Introduction of control MO into injured retinas of *1016 tuba1a:gfp* fish had little effect on the expression of GFP, *ascl1a* or *lin-28* (Fig. 3a,b), while *lin-28*-targeting MOs specifically suppressed GFP expression when assayed at 4 dpi (Fig. 3a). Interestingly, Ascl1a knockdown suppressed both GFP and *lin-28* mRNA expression (Fig. 3b). To further investigate *ascl1a* and *lin-28* interdependency, we examined their expression in MO-treated retinas at 2 dpi, a time correlating with MG dedifferentiation but prior to their reentry into the cell cycle[4]. Similar to the *in situ* hybridization studies (Fig. 3a,b), whole retina RT-PCR showed that *ascl1a*-targeting MOs suppress *lin-28* expression in a concentration-dependent manner, while *lin-28*-targeting MOs had little effect on *ascl1a* expression (Fig. 3c–f). Real-time PCR indicates that *ascl1a*-targeting MOs at 0.1mM and 0.25mM cause about a 40% and 99% reduction, respectively, in *lin-28* mRNA levels (Fig. 3f).

The above results suggest that Ascl1a is required for injury-dependent *lin-28* expression. To determine if Ascl1a can directly regulate *lin-28* expression, we cloned the zebrafish *lin-28* promoter and generated a *lin-28:luciferase* expression vector. Transfection of HEK293 cells with this vector and either a control or a *cmv:ascl1a* expression vector showed a concentration-dependent regulation of *lin-28* promoter activity by Ascl1a (Fig. 3g). Inspection of the *lin-28* promoter sequence identified 14 E-boxes (CANNTG) of which 6 appeared similar (CACCTG, CAGGTG, CAGCTG, CACGTG) to previously reported Ascl1 binding sites (CAGCTG and CAGGTG)[20, 21] (Fig. 3h). Two of these sites are clustered in

the distal promoter region (region 1 flanked by arrows in Fig. 3h) and 3 of these sites are clustered in a more proximal promoter region (region 2 flanked by arrows in Fig. 3h). To determine if Ascl1a can bind to regions 1 and 2, we performed a ChIP experiment where we injected mRNA encoding either Myc or Myc-Ascl1a into single cell zebrafish embryos and 30 hrs later immunoprecipitated Myc:DNA and Myc-Ascl1a:DNA complexes with an anti-Myc antibody. This ChIP experiment showed that Ascl1a can bind to *lin-28* promoter regions 1 and 2, consistent with the idea that Ascl1a directly regulates *lin-28* promoter activity (Fig. 3h).

Lin-28 inhibits *let-7* miRNA formation and stimulates its degradation22–24. Interestingly, Lin-28 is associated with cell proliferation, while *let-7* miRNAs are associated with cellular differentiation3, 16, 22. Therefore, it seemed reasonable to hypothesize that injury-dependent *lin-28* induction would lead to *let-7* miRNA suppression in dedifferentiated MG. Indeed, these RNAs were regulated in an opposing fashion in MG purified from uninjured and injured retinas (Fig. 4a), with *lin-28* mRNA increasing 170-fold and *let-7* miRNAs decreasing by 85% (Fig. 4b). Consistent with these data, *in situ* hybridization at 4dpi with an LNA-modified oligonucleotide probe targeting mature *let-7a* miRNA showed a dramatic injury-dependent reduction of *let-7a* expression in MG-derived progenitors that were identified by BrdU incorporation (Fig. 4c). Quantification of *let-7* miRNA-negative cells that co-labeled with BrdU showed that $81 \pm 1.52\%$ of the BrdU-positive cells were also *let-7*-negative (n=3). Quantification of injury-dependent *let-7* miRNA expression in uninjured and injured retinas showed a ~50% reduction at 15 hpi that increased to an ~80% at 4 dpi; by 7 dpi, *let-7* miRNAs begin to return to control levels (Fig. 4d). Consistent with the idea that Ascl1a regulates Lin-28 expression and that Lin-28 controls *let-7* miRNA levels22–24, Lin-28 or Ascl1a knockdown almost completely abrogated injury-dependent *let-7* miRNA suppression (Fig. 4e,f).

It is interesting that MG in the uninjured retina express genes associated with retinal progenitors25–28 and pluripotency (Fig. 1a,b). *Let-7* is associated with cellular differentiation and may contribute to maintaining the differentiated state of MG in the uninjured retina and promoting redifferentiation of MG-derived progenitors in the injured retina by suppressing expression of regeneration and pluripotency-associated genes. Indeed, *let-7* miRNAs are known to inhibit Myc and Lin-28 expression during cancer progression, embryonic stem cell development and neural commitment3, 22, 29. Interestingly, all 6 genes known to be necessary for retina regeneration (*ascl1a*, *lin-28*, *pax6$_a$*, *pax6$_b$*, *mps1*, and *hspd1*)12, 30, 31 harbor putative *let-7* binding sites32 (Supplemental Information, Table S2). Although *let-7* binding sites in the corresponding mouse genes are predicted; in general their sequences are not conserved between zebrafish and mouse. Of the genes necessary for regeneration, *ascl1a*, *lin-28* (Fig. 1) and *mps1*30 are undetectable in the uninjured retina, while *pax6a* expression4, 31, 33 is restricted to amacrine and ganglion cells. Basal *hspd1* expression has been reported in the uninjured retina30 but cell-type specificity remains uncharacterized; likewise *pax6$_b$* expression is not well characterized. We found that injury-dependent induction of *hspd1*, *pax6$_a$*, and *pax6$_b$* lagged behind that of *ascl1a*, *lin-28* and *c-myc$_b$*, with *hspd1* increasing ~3.5-fold, *pax6a* increasing ~ 4.5-fold and *pax6b* increased ~11-fold at 4 dpi (Fig. 5c and Supplementary Information, Fig. S6). Interestingly, of all

these regeneration-associated genes, only *hspd1* exhibits significant basal expression in GS$^+$ MG of the uninjured retina (Fig. 5a,b). Therefore, *hspd1* may represent a *let-7* target whose basal expression and injury-dependent induction are suppressed in a *let-7*-dependent manner.

To determine if *let-7* can regulate the expression of proteins that are essential for regeneration we appended a sequence encoding a Myc or Flag-tag to the 5' end of the *ascl1a*, *hspd1*, *lin-28* and *pax6$_b$* coding sequence and cloned them into the pCS2expression vector. Transfection of HEK 293 cells with these vectors, ± an expression vector harboring the zebrafish *let-7a,let-7f* pri-miRNA sequence (*ubC:let-7a,let-7f* ; Fig. 5e), showed a concentration- dependent suppression of Ascl1a, Hspd1, Lin-28 and Pax6$_b$ protein expression (Fig. 5d). We also investigated if *let-7* could regulate the expression of pluripotency factors Klf4, cMyc$_a$, cMyc$_b$ and Oct4, all of whose transcripts harbor putative *let-7* binding sites (Supplementary Information, Table S2) and, except for *c-myc$_b$*, exhibit basal expression in the uninjured retina (Fig. 1a). Interestingly, in addition to *let-7*-dependent suppression of pluripotency factor Lin-28, we also observed suppression of cMyc$_a$, cMyc$_b$ and Oct4 in transfected HEK 293 cells (Fig. 5d). Densitometric quantification of Western blots suggests robust suppression of Ascl1a, Hspd1, Lin-28, Pax6b, cMyc$_a$, and cMyc$_b$, while Oct4 is modestly suppressed and Klf4 appears unaffected (Supplementary Information, Fig. S7).

We then focused on the *hspd1* mRNA to determine if the predicted *let-7* binding site was functional. For this analysis single cell zebrafish embryos were injected with increasing amounts of *ubC:let-7a,let-7f* and either *insm1a:luciferase-hspd3'UTRwt* or *insm1a:luciferase-hspd3'UTRmut* that harbor a wild type or a mutant (4 base change in putative *let-7* binding site seed sequence) zebrafish *hspd1* 3' UTR behind the *luciferase* coding sequence and under control of the zebrafish *insm1a* promoter (Fig. 5e). This analysis showed that the *hspd1* 3' UTR conferred *let-7*-dependent regulation on reporter expression and that this regulation was mediated by the *let-7* binding sites (Fig. 5f). These data suggest that injury-dependent *let-7* regulation can have a dramatic impact on gene expression during retina regeneration.

In conclusion, our studies have uncovered a mechanism by which Ascl1a contributes to retina regeneration and suggests that Ascl1a-dependent regulation of the pluripotency gene *lin-28* is crucial for stimulating MG dedifferentiation into a cycling population of progenitor cells. We propose that Ascl1a-dependent induction of Lin-28 contributes to MG dedifferentiation, in part, by decreasing *let-7* miRNA levels, thus relieving repression of regeneration-associated mRNAs that are essential for MG dedifferentiation (Supplementary Information, Fig. S8). Because some pluripotency factors and regeneration-associated genes exhibit basal expression in MG of the uninjured retina and are inhibited in a *let-7*-dependent manner, it appears *let-7* may also help maintain MG in a differentiated state in the absence of retinal injury. Because Lin-28 is an RNA binding protein that can shuttle between the nucleus and cytoplasm and may regulate other genes, including those that control the cell cycle16, 34, we suspect a much wider role for Lin-28 in retina regeneration. Likewise, Ascl1a is likely to have many additional targets in addition to *lin-28* and identification of these targets should further our understanding of mechanisms underlying MG

dedifferentiation and retina regeneration. A distinguishing feature of dedifferentiated MG is the expression of *lin-28*, which is not shared with other adult stem cell populations or with retinal progenitors generated during development and suggests MG-derived progenitors may share properties with embryonic stem cells. The opposing relationship between Lin-28 protein and *let-7* miRNAs is similar to that reported in embryonic stem cells[3] and cancer[29], and suggests mammalian MG dedifferentiation may be enhanced by manipulating this signaling pathway.

Methods

Animals

Zebrafish were kept at 26–28 °C on a 14hr light, 10 dark cycle. Transgenic *gfap:GFP* zebrafish were kindly provided by Dr. David Hyde (University of Notre Dame)[35] and *1016 tuba1a:gfp* fish (previously *1016 a1TI:pEGFP*) fish were previously described[4, 12]. Embryos for injections were obtained by natural mating of wild type adults.

Plasmid construction

cDNAs and genomic DNAs were amplified by RT-PCR using zebrafish adult retinal mRNA and embryonic genomic DNA, respectively. The *ascl1a* cDNA was amplified using ascl1a-F and ascl1a-R primers that harbor an *Eco*RI and *Xho*I site, respectively at their 5' ends (Supplementary Information, Table S3) and cloned into *pCS2+MT* vector (provided by David Turner, University of Michigan) to generate *cmv:myc-ascl1a*. A 3.1 kb *lin-28* promoter, whose 3' end was just upstream of the translation start site, was amplified using lin-28-pro-F and lin-28-pro-R primers harboring an *Xho*I and *Apa*I restriction site, respectively, at their 5' ends (Supplementary Information, Table S3) and cloned into a luciferase expression vector to create *lin-28:luciferase*. A 3 kb *insm1a* promoter, whose 3' end was just upstream of the translation start site, was amplified using insm1a-pro-F and insm1a-pro-R primers harboring an *Xho*I and *Bam*HI site, respectively, at their 5' ends (Supplementary Information, Table S3) and cloned in the the *pEL* vector (provided by Michael Uhler, University of Michigan) that harbors a GFP-luciferase fusion to generate *insm1a:luciferase*. The 3'UTR of *hspd1* was amplified using hspd1-UTR-F and hspd1-R primers that harbor an *Eco*RI and *Nhe*I site, respectively, at their 5' ends (Supplementary Information, Table S3) and inserted at the 3' end of the *luciferase* cDNA in expression vector *insm1a:luciferase* to generate *insm1a:luciferase-hspd3'UTR*. The *pre-let7a* and *pre-let-7f* miRNAs are encoded on a contiguous 1.5 kb genomic DNA fragment, which was amplified using primers Pre-let-7af-F and Pre-let-7af-R harboring an *Eco*RI and *Xba*I site, respectively, at their 5' ends (Supplementary Information, Table S3) and cloned into the second intron of the *UI4-GFP-SIBR* vector[36] (provided by David Turner).

Site-directed mutagenesis

We mutated 5 nucleotides in the putative *let-7* binding site in the *hspd1* 3' UTR using site directed mutagenesis. A typical PCR amplification contained approximately 50 ng of dsDNA (either *insm1a:luciferase-hspd13'UTR*), 20 pM of each of the two oligonucleotide primers (mut-hspd1-F and mut-hspd1-R; Supplementary Information, Table S3), 2 μl of 10 mM dNTP mix, 0.2 μl (1 unit) of proofreading thermo polymerase (PCR extender system, 5

Prime) and 2.5 μl of 10X reaction buffer in a reaction volume of 25 μl. Amplification conditions were: 94 °C for 5 min without enzyme, followed by 2 min on ice; Polymerase was then added and 30 cycles of denaturation at 93 °C for 30 sec, annealing at 58 °C for 1 minute and extension at 68 °C for 10 min was performed. Following PCR, the product was treated with *Dpn*I to and mutagenized DNA purified and electroporated into XL1-Blue cells. Transformed cells were selected on LB-kanamycin plates and colonies harboring mutant sequences were identified by PCR and confirmed by DNA sequencing.

RNA isolation, RT-PCR, Real-time PCR and TaqMan PCR

Total RNA was isolated from control and injured retinas using Trizol (Invitrogen). cDNA synthesis was performed using random hexamers and Superscript-II reverse transcriptase (Invitrogen). PCR reactions used *Taq* and gene specific primers (Supplementary Information, Table S3) under the following conditions: denaturation at 93 °C for 15 sec, annealing at 60 °C for 30 sec and extension at 68 °C for 60 sec. Accession numbers for mRNAs assayed by PCR are in Supplementary Information, Table S2. Real-time PCR reactions were carried out in triplicate with ABsolute SYBR Green Fluorescein Master Mix (ThermoScientific) on an iCycler real-time PCR detection system (BioRad). The   Ct method was used to determine relative expression of mRNAs in control and injured retinas and normalized to *L-24* or *β-actin* mRNA expression levels.

miRNA quantification was performed using TaqMan microRNA probes (Applied Biosystems) according to the manufacturer's instructions. Briefly, total RNA was reverse transcribed using stem-loop RT primers (Applied Biosystems) and Real-time PCR performed with a TaqMan PCR kit on an Applied Biosystems 7300 sequence detection system.

Cell culture, transfection and Western blots

HEK293 cells were seeded in a 24 well plate at ~40% confluence and grown in DMEM, 10% fetal bovine serum, antibiotics and antimycotics at 37 °C incubator with 5% CO_2. Cells were transfected 24 hrs post plating. For assaying *lin-28* promoter activity, cells were transfected with *lin-28:luciferase* and varying amounts of *cmv:ascl1a* along with an *SV40:Renilla luciferase* normalization vector. Forty-eight hrs later cells were harvested for luciferase assays. For examining *let-7*-dependent regulation of gene expression, cells were transfected with 50 ng of pCS2 vector harboring flag-tagged (*hspd1*, *klf4*, *c-myc_a*, *c-myc_b*) or myc-tagged (*ascl1a*, *lin-28*, *oct4*, *pax6_b*) cDNA, along with 0, 50, 200 or 500 ng of the *ubC:let-7a/let-7f* and 50 ng of the *β-actin2:mCherry* normalization vector. Forty-eight hrs post transfection cells were harvested and protein expression assayed on Western blots. Western blots were quantified by densitometry using NIH Image J software (Version 1.43). Normalization to mCherry expression or endogenous β-actin expression gave similar results.

mRNA synthesis and microinjections

Capped mRNAs encoding Myc and Myc-Ascl1a were synthesized *in vitro* from linearized *cmv:myc-ascl1a* using the mMESSAGE mMACHINE kit (Ambion) according to manufacturer's directions. Approximately 50 pg of mRNA (1 nl) was injected into single cell embryos. Embryos were harvested for ChIP experiments at 30 hpf. ChIP was performed

as previously described37. For examining if the *hspd1* 3' UTR confers *let-7*-dependent regulation in zebrafish, we injected single cell zebrafish embryos with approximately 200 pl of a stock solution containing 0.1 ng/μl of *Renilla luciferase* mRNA (for normalization), 20 ng/μl of *insm1a:luciferase-hspd13'UTRwt* or *insm1a:luciferase-hspd13'UTRmut* and 0, 1, 5 or 20 ng/μl of *ubC:let-7a/let-7f*. Embryos were harvested at 24 hpf for dual luciferase reporter assays (Promega). Graph shows average of experiment performed in triplicate. Error bars are standard error of the mean.

Retinal injury, BrdU labeling, FACS and MO-mediated gene knockdown

Eye lesions have been previously described4, 12, 38. Briefly, fish were anesthetized and under microscopic visualization, the right eye was pulled from its socket and stabbed 4–8 times through the sclera with a 30g needle inserted to the length of the bevel. Lissamine-tagged MOs (Gene Tools, LLC) (~0.5 μl of a 0.1–0.5 mM) were delivered at the time of injury using a Hamilton syringe attached to the needle. MO delivery to cells was facilitated by electroporation as previously described12. The control and 2 different *ascl1a*-targeting MOs were previously described12. *lin-28*-targeting MOs were: MO #1 TGAGATGCGGATTTGCCGGGGGCAT and MO #2 ACTAGGCCATACAATTAACTGCTTT. Because antibodies that recognize zebrafish Lin-28 are not available, we used an indirect method to confirm the efficacy of the *lin-28*-targeting MO. For this analysis we appended the MO target sequence to the amino terminus of EGFP in the *cmv:egfp* expression vector and injected zebrafish embryos with this expression vector along with either lissamine-tagged control or *lin-28*-targeting MOs. EGFP expression, 24 h later, was used to assay MO efficacy (Supplementary Information, Fig. S6). For BrdU labeling, adult fish received a 20 μl injection of BrdU (20 mM) 3 hrs prior to sacrifice; embryos were immersed in 20 mM BrdU, 4% DMSO for 3 hrs prior to harvesting. FACS sorting of GFP$^+$ MG was performed by dissecting uninjured retinas from 2 *gfap:gfp* transgenic fish or 4 days post injured retinas (10 needle pokes/retina) from 10 *1016 tuba1a:gfp* transgenic fish. Retinas were collected in 0.5 ml Leibovitz's L15 media, treated for 15 min with 1mg/ml hyaluronidase at room temp and then dissociated in 0.01% trypsin for an additional 10 min with frequent trituration. A single cell suspension was confirmed by microscopy and cells were washed in L15 medium before sorting on a BC Biosciences FACSViDa 3 laser high-speed cell sorter. A total of approximately 90,000 GFP$^+$ cells were obtained from 12 injured retinas and 235,000 GFP$^+$ cells were obtained from 2 uninjured retinas.

Tissue preparation, immunohistochemistry and *in situ* hybridization

Adult fish were overdosed with tricaine methane sulfonate and eyes were enucleated, followed by removal of the lens and then immersion in fresh 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for ~16 hrs. After fixation, samples were cryoprotected in phosphate-buffered 20% sucrose before embedding with Tissue-Tek O.C.T. compound (Sakura, Finetek). Embedded samples were kept frozen at −80 °C until sectioned to 8 microns on a CM3050S cryostat (Leica). Sections were collected on Superfrost/Plus slides (Fisher Scientific), dried and stored at −80 °C. Immunohistochemistry was performed as previously described4, 38 using the following primary antibodies: rat anti-BrdU (dividing cell marker, 1:400; Abcam); rabbit anti-GFP (1:1000; Invitrogen); mouse anti-glutamine

synthetase (GS) (Müller glia marker, 1:500; Chemicon/Millipore). Secondary antibodies were conjugated to Alexa Fluor 488 and used at the following dilutions: 1:500 for anti-mouse, 1:250 for anti-rat and 1:1000 for anti-rabbit. For BrdU staining, sections were pretreated with 2N HCl for 20 min at 37 °C and then soaked in 100 mM sodium borate for 10 min. Following immunhistochemical staining, slides were rinsed with water and allowed to dry in the dark prior to cover-slipping with 2.5% PVA (PVA-polyvinyl alcohol)/DABCO (1,4 diazabicyclo [2.2.2]octane). Slides were examined in a Zeiss Axiophot fluorescence microscope equipped with a digital camera or an Olympus FluoView FV1000 confocal imaging system.

Combined *in situ* hybridization and antibody staining were performed on retinal sections as described previously39. Double *in situ* hybridizations were done according to manufacturer's instructions (Perkin Elmer). Sense control probes were generated and showed no signal above background (data not shown). *In situ* hybridization for *let-7a* miRNA expression was performed using a zebrafish *let-7a* LNA probe (Exiqon). The probe was diluted to 1 μM in prehybridization buffer and hybridization and wash conditions were as previously described40.

Statistical Analyses

Data were analyzed by analysis of variance followed by a post-hoc Fisher's PLSD t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Sherpa T, et al. Ganglion cell regeneration following whole-retina destruction in zebrafish. Dev Neurobiol. 2008; 68:166–181. [PubMed: 18000816]

2. Mensinger AF, Powers MK. Visual function in regenerating teleost retina following cytotoxic lesioning. Vis Neurosci. 1999; 16:241–251. [PubMed: 10367959]

3. Melton C, Judson RL, Blelloch R. Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. Nature. 2010; 463:621–626. [PubMed: 20054295]

4. Fausett BV, Goldman D. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci. 2006; 26:6303–6313. [PubMed: 16763038]

5. Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci. 2007; 27:7028–7040. [PubMed: 17596452]

6. Fimbel SM, Montgomery JE, Burket CT, Hyde DR. Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. J Neurosci. 2007; 27:1712–1724. [PubMed: 17301179]

7. Thummel R, Kassen SC, Montgomery JE, Enright JM, Hyde DR. Inhibition of Muller glial cell division blocks regeneration of the light-damaged zebrafish retina. Dev Neurobiol. 2008; 68:392–408. [PubMed: 18161852]

8. Ooto S. Potential for neural regeneration in the adult mammalian retina. Nippon Ganka Gakkai Zasshi. 2006; 110:864–871. [PubMed: 17134034]

9. Wan J, et al. Preferential regeneration of photoreceptor from Muller glia after retinal degeneration in adult rat. Vision Res. 2008; 48:223–234. [PubMed: 18155124]

10. Karl MO, et al. Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci U S A. 2008; 105:19508–19513. [PubMed: 19033471]

11. Takeda M, et al. alpha-Aminoadipate induces progenitor cell properties of Muller glia in adult mice. Invest Ophthalmol Vis Sci. 2008; 49:1142–1150. [PubMed: 18326742]

12. Fausett BV, Gumerson JD, Goldman D. The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci. 2008; 28:1109–1117. [PubMed: 18234889]

13. Yu J, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007; 318:1917–1920. [PubMed: 18029452]

14. Hochedlinger K, Plath K. Epigenetic reprogramming and induced pluripotency. Development. 2009; 136:509–523. [PubMed: 19168672]

15. Zupanc GK, Horschke I. Proliferation zones in the brain of adult gymnotiform fish: a quantitative mapping study. J Comp Neurol. 1995; 353:213–233. [PubMed: 7745132]

16. Nimmo RA, Slack FJ. An elegant miRror: microRNAs in stem cells, developmental timing and cancer. Chromosoma. 2009; 118:405–418. [PubMed: 19340450]

17. Eisen JS, Smith JC. Controlling morpholino experiments: don't stop making antisense. Development. 2008; 135:1735–1743. [PubMed: 18403413]

18. Thummel R, et al. Inhibition of zebrafish fin regeneration using in vivo electroporation of morpholinos against fgfr1 and msxb. Dev Dyn. 2006; 235:336–346. [PubMed: 16273523]

19. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. Zebrafish. 2009; 6:69–77. [PubMed: 19374550]

20. Li J, et al. Identification and analysis of the mouse basic/Helix-Loop-Helix transcription factor family. Biochem Biophys Res Commun. 2006; 350:648–656. [PubMed: 17027923]

21. Bertrand N, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. Nat Rev Neurosci. 2002; 3:517–530. [PubMed: 12094208]

22. Rybak A, et al. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat Cell Biol. 2008; 10:987–993. [PubMed: 18604195]

23. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008; 320:97–100. [PubMed: 18292307]

24. Heo I, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell. 2009; 138:696–708. [PubMed: 19703396]

25. Blackshaw S, et al. Genomic analysis of mouse retinal development. PLoS Biol. 2004; 2:E247. [PubMed: 15226823]

26. Jadhav AP, Roesch K, Cepko CL. Development and neurogenic potential of Muller glial cells in the vertebrate retina. Prog Retin Eye Res. 2009; 28:249–262. [PubMed: 19465144]

27. Roesch K, et al. The transcriptome of retinal Muller glial cells. J Comp Neurol. 2008; 509:225–238. [PubMed: 18465787]

28. Trimarchi JM, Stadler MB, Cepko CL. Individual retinal progenitor cells display extensive heterogeneity of gene expression. PLoS One. 2008; 3:e1588. [PubMed: 18270576]

29. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007; 39:673–677. [PubMed: 17401365]

30. Qin Z, Barthel LK, Raymond PA. Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish. Proc Natl Acad Sci U S A. 2009; 106:9310–9315. [PubMed: 19474300]

31. Thummel R, et al. Pax6a and Pax6b are required at different points in neuronal progenitor cell proliferation during zebrafish photoreceptor regeneration. Exp Eye Res. 2010; 90:572–582. [PubMed: 20152834]

32. Miranda KC, et al. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell. 2006; 126:1203–1217. [PubMed: 16990141]

33. Thummel R, et al. Characterization of Muller glia and neuronal progenitors during adult zebrafish retinal regeneration. Exp Eye Res. 2008; 87:433–444. [PubMed: 18718467]

34. Xu B, Zhang K, Huang Y. Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells. RNA. 2009; 15:357–361. [PubMed: 19147696]

35. Kassen SC, et al. Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish. Dev Neurobiol. 2007; 67:1009–1031. [PubMed: 17565703]

36. Chung KH, et al. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. Nucleic Acids Res. 2006; 34:e53. [PubMed: 16614444]

37. Lindeman LC, Vogt-Kielland LT, Alestrom P, Collas P. Fish'n ChIPs: chromatin immunoprecipitation in the zebrafish embryo. Methods Mol Biol. 2009; 567:75–86. [PubMed: 19588086]

38. Senut MC, Gulati-Leekha A, Goldman D. An element in the alpha1-tubulin promoter is necessary for retinal expression during optic nerve regeneration but not after eye injury in the adult zebrafish. J Neurosci. 2004; 24:7663–7673. [PubMed: 15342733]

39. Jowett T. Double in situ hybridization techniques in zebrafish. Methods. 2001; 23:345–358. [PubMed: 11316436]

40. Wang WX, et al. The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. J Neurosci. 2008; 28:1213–1223. [PubMed: 18234899]
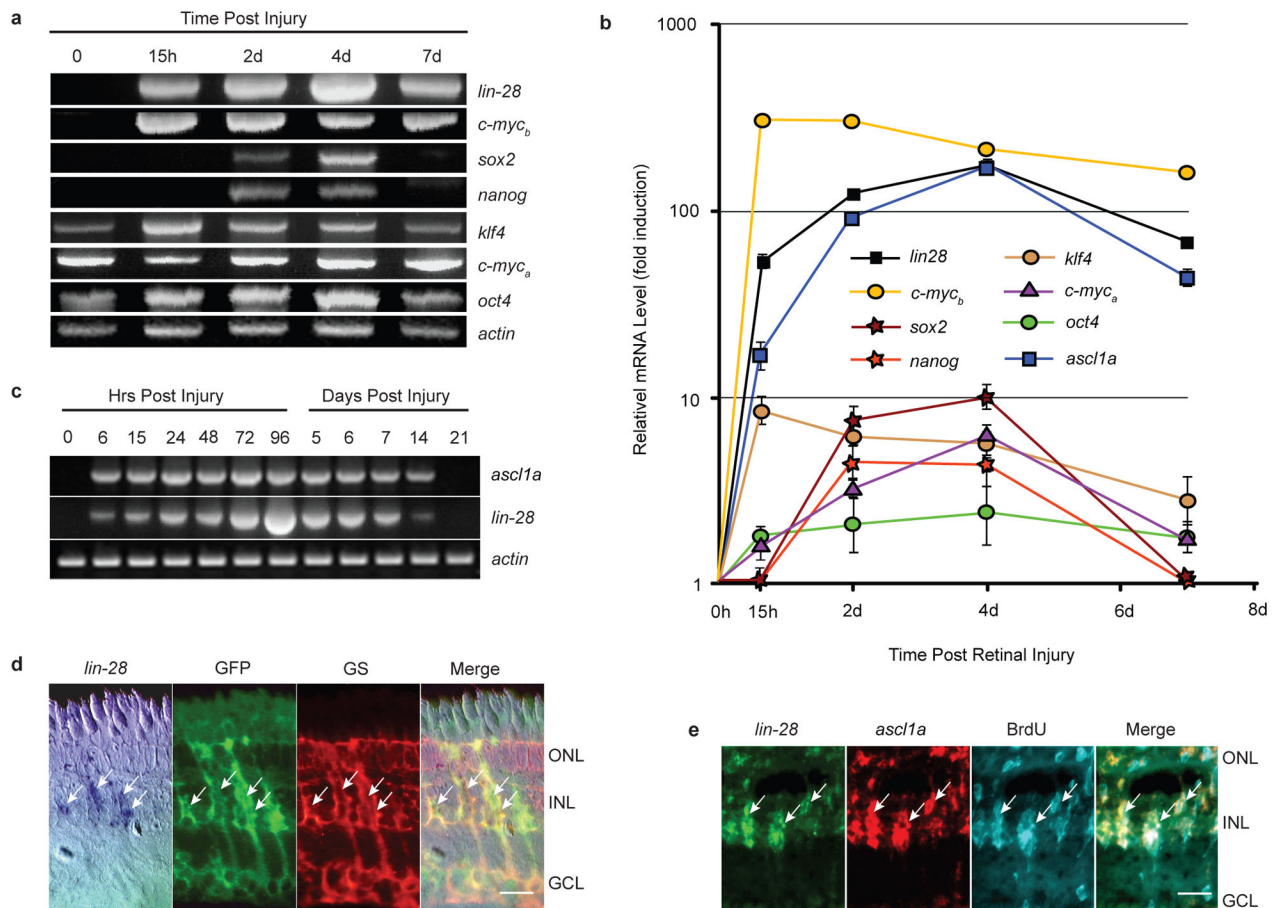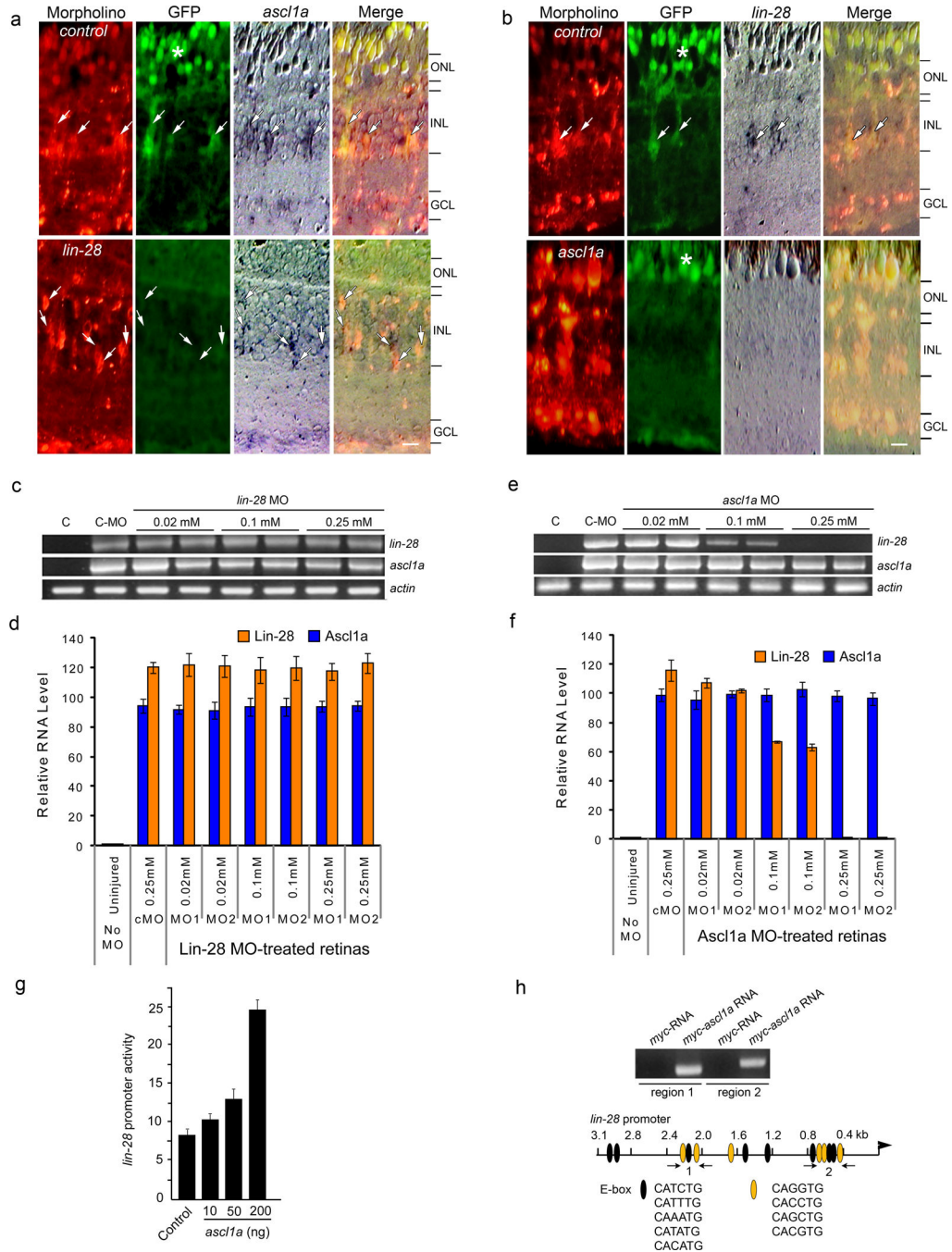
Figure 1.

ascl1a and *lin-28* mRNAs are rapidly induced in dedifferentiating MG following retinal injury. (**a**) RT-PCR shows induction of pluripotency factor mRNAs following retinal injury. (**b**) Real-time PCR quantification of pluripotency factor mRNA levels during retina regeneration. Data represent means ± s.d. (n=3 individual fish; compared to uninjured retina, P=0.0001 or less for *lin28*, *c-myc$_b$*, and *ascl1a* expression at all time points post retinal injury; P=0.0001 for *klf4* at 15 hpi, and 2 and 4 dpi; P=0.02 for *klf4* at 7 dpi; P=0.0001 or less for *sox2*, *c-myc$_a$* and *nanog* at 2 and 4 dpi; P= 0.0425 for *c-myc$_a$* at 7 dpi; P=0.0178 and 0.0069 for oct4 at 2 and 4 dpi, respectively). Note Y-axis is fold induction in log scale and normalized to 0 hr time point that is assigned a value of 1. (**c**) RT-PCR shows *ascl1a* and *lin-28* are coordinately induced following retinal injury. (**d**) *In situ* hybridization and immunofluorescence shows *lin-28* RNA co-localizes with *1016 tuba1a:gfp* transgene expression in glutamine synthetase (GS)$^+$ MG at 4 dpi. Scale bar is 10 microns. (**e**) *lin-28* and *ascl1a* double fluorescence *in situ* hybridization and BrdU immunofluorescence show *lin-28* and *ascl1a* are co-expressed in proliferating MG-derived progenitors at 4 dpi. Three hrs prior to sacrifice, adult fish, whose retina was injured 4 days earlier, received an intraperitoneal injection of BrdU. Scale bar is 10 microns. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; BrdU, bromodeoxyuridine; GS, glutamine synthetase; GFP, green fluorescent protein.
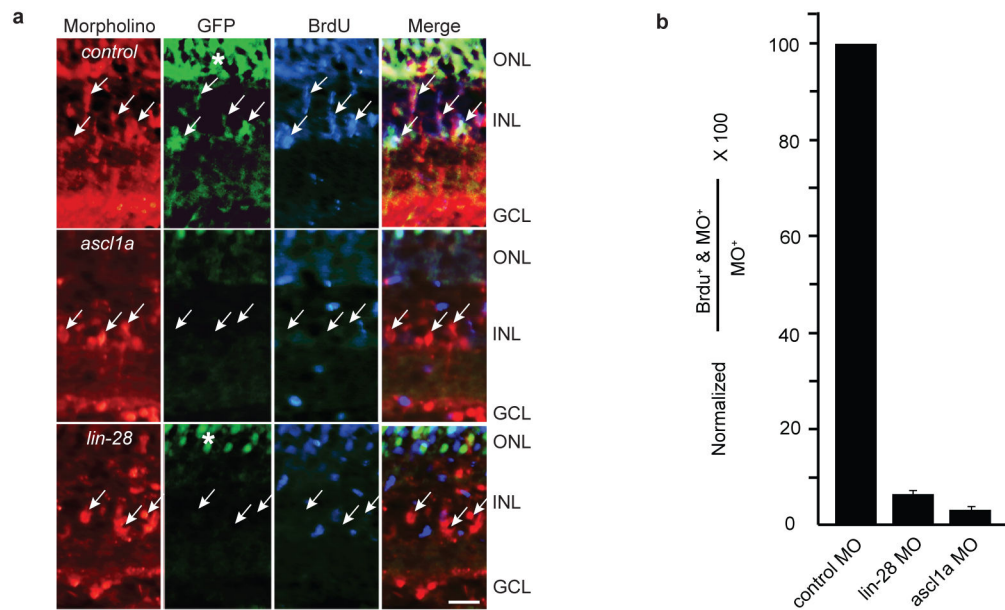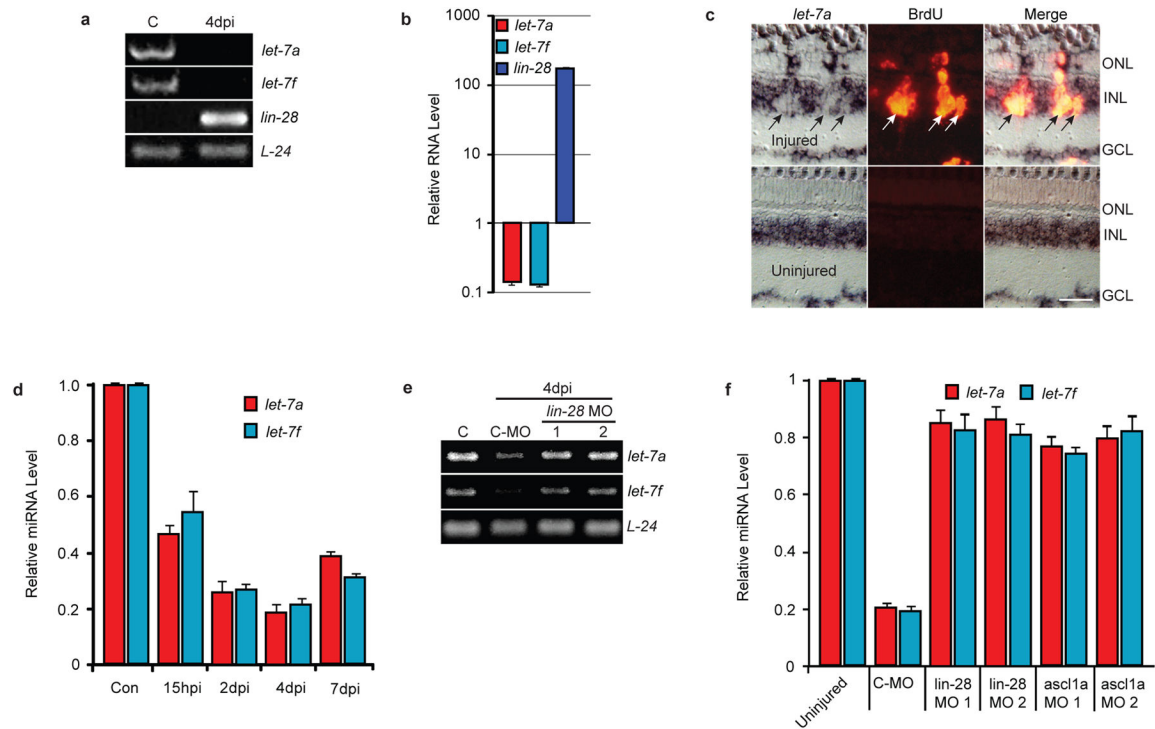
Figure 2.
Ascl1a and Lin-28 knockdown inhibit *1016 tuba1a:gfp* transgene expression and MG-derived progenitor proliferation. (**a**) *Control*, *ascl1a* or *lin-28* lissamine-tagged MOs were electroporated into the retina of *1016 tuba1a:gfp* transgenic fish at the time of retinal injury and 3 hrs prior to sacrifice, at 4 dpi, fish received an intraperitoneal injection of BrdU. Arrows point to cells harboring lissamine-tagged MO. GFP and BrdU immunofluorescence show Ascl1a and Lin-28 knockdown suppress *1016 tuba1a:gfp* expression and proliferation of MG-derived progenitors. (**b**) Quantification of the total number of MO^+ cells that are in S phase as indicated by BrdU uptake. Data represent means ± s.d. (n=3 individual fish; compared to control MO, *lin-28* MO and *ascl1a* MO *P*=0.000178 or less). (*) identifies autofluorescence in ONL. Scale bar is 10 microns and applies to all photomicrographs.
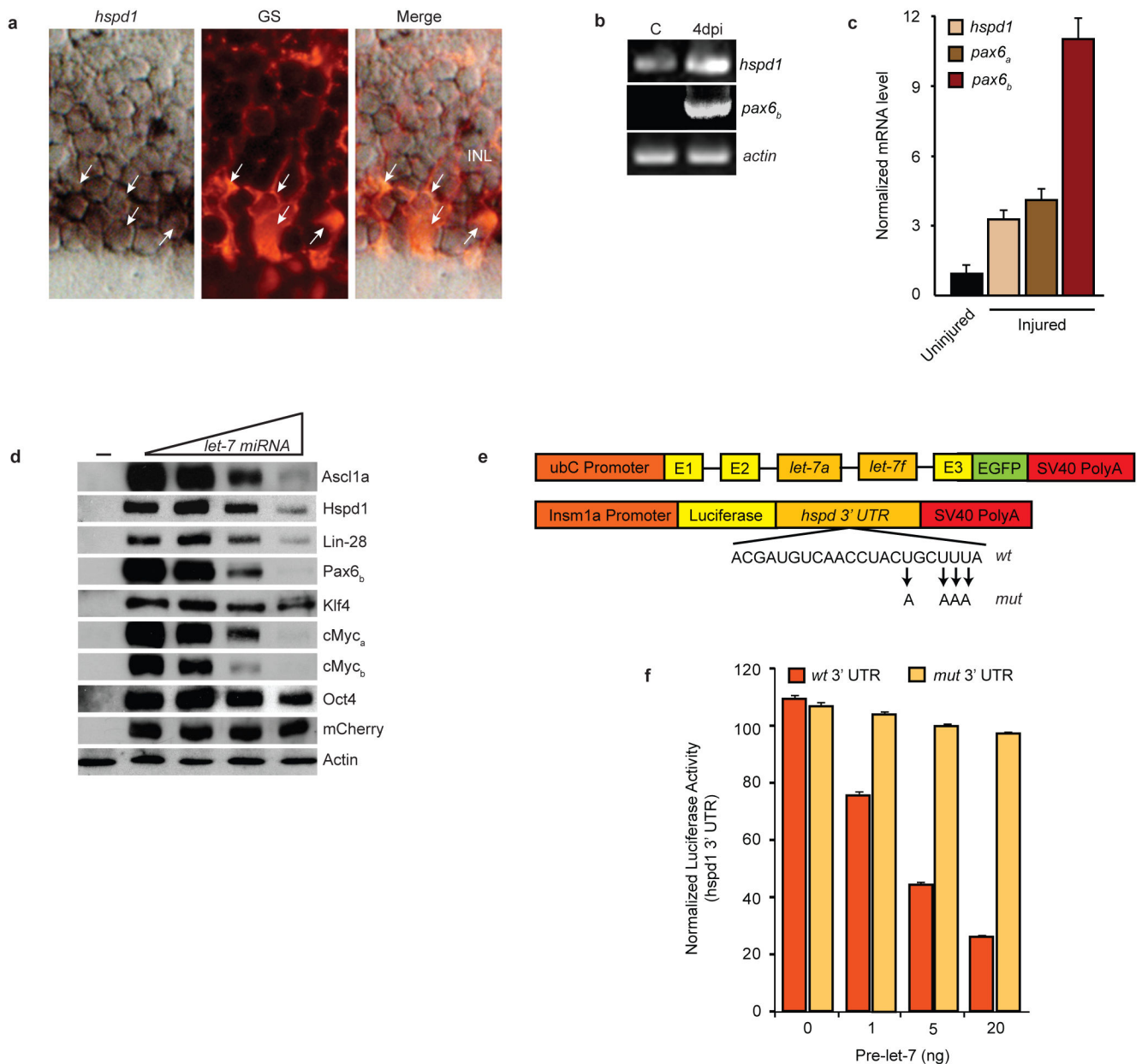
Figure 3.
Ascl1a regulates *lin-28* expression. (**a, b**) Lissamine-labeled *control*, *ascl1a* or *lin-28*-targeting MOs were electroporated into injured retinas of *1016 tuba1a:gfp* transgenic zebrafish. At 4 dpi GFP, and *ascl1a* and *lin-28* mRNA expression was detected by immunofluorescence and *in situ* hybridization, respectively. *Control* MO-treated retinas retain GFP, *ascl1a* and *lin-28* expression (arrows), while Lin-28 knockdown suppresses GFP expression and knockdown of Ascl1a suppresses both GFP and *lin-28* expression. Scale bar is 10 microns. (**c,d**) RT-PCR shows Lin-28 knockdown has no effect on injury-dependent

induction of *ascl1a* mRNA at 2 dpi (**c**), while Ascl1a knockdown blocks injury-dependent induction of *lin-28* mRNA at 2 dpi (**d**). Lane C is control uninjured retina. (**e,f**) Real-time PCR quantification of the effects of Lin-28 (**e**) or Ascl1a (**f**) knockdown on *lin-28* and *ascl1a* mRNA levels at 2 dpi. Data are normalized to uninjured retinas and represent means ± s.d. from 3 replicas of a single experiment. (**g**) Ascl1a regulates *lin-28* promoter activity. HEK293 cells were transfected with *lin-28:luciferase* and the indicated amounts of *cmv:ascl1a* along with *SV40:Renilla luciferase* for normalization. Normalized promoter activity is reported as means ± s.d. (n=3; compared to control, *P*=0.0123 for 50 ng Ascl1a expression vector and *P*=0.0001 for 200 ng of Ascl1a expression vector). (**h**) Ascl1a binds to regions of the *lin-28* promoter that harbor multiple E-boxes. ChIP analysis of zebrafish embryos (single cell stage) injected with either *myc*-RNA or *myc-ascl1a* mRNA. Immunoprecipitated chromatin was assayed by PCR using primers (arrows) flanking putative Ascl1a binding sites 1 and 2 (ethidium bromide stained gel shown). The predicted fragments sizes of 287 bp and 323 bp were amplified. The 3.1 kb *lin-28* promoter diagramed below the gel shows consensus E-box binding sites (ovals). Orange ovals are putative Ascl1 binding sites.

Figure 4.
Lin-28 regulates *let-7* miRNA levels in MG-derived progenitors. (**a**) RT-PCR shows *let-7* miRNA expression is high and *lin-28* expression is low in differentiated MG (lane C), while *let-7* miRNA expression is suppressed and *lin-28* expression is highly induced in MG-derived progenitors at 4 dpi. (**b**) Real-time PCR quantification of *lin-28* mRNA and TaqMan PCR quantification of *let-7a* and *let-7f* miRNA levels in purified MG and MG-derived progenitors at 4 dpi. Data are normalized to uninjured retina and represent means ± s.d. A single sample, consisting of MG purified from 2 uninjured fish and MG-derived progenitors purified from 10 injured fish, was assayed in triplicate. (**c**) *let-7a in situ* hybridization (LNA probe) and BrdU immunofluorescence shows a dearth of *let-7a* miRNA in BrdU+ MG-derived progenitors of the injured retina at 4dpi. Scale bar is 20 microns. (**d**) TaqMan PCR quantification of *let-7* miRNA levels in uninjured and injured retinas at different times after injury. Data represent means ± s.d. (n=3 fish; compared to control uninjured retina, *P*=0.0001 or less for *let-7a and let-7f* at all time points). (**e**) RT-PCR shows Lin-28 knockdown with 2 different MOs relieves injury-dependent *let-7a* and *let-7f* miRNA suppression. Lane C is uninjured retina. (**f**) Lin-28 or Ascl1a knockdown relieves injury-dependent *let-7* miRNA suppression. TaqMan PCR was used to quantify *let-7a* and *let-7f* miRNA levels. Data represent means ± s.d. from 3 replicas of a single experiment.

Figure 5.

let-7 miRNAs suppress expression of regeneration and pluripotency-associated genes. (**a***) hspd1 in situ* hybridization and glutamine synthetase (GS) immunofluorescence shows *hspd1* RNA co-localizes (arrows) with GS-expressing MG in the uninjured retina. (**b**) RT-PCR shows *hspd1* mRNA expression in uninjured retina that is increased following retinal injury, while *pax6b* expression is undetectable in the uninjured retina and dramatically induced following retinal injury. (**c**) Real-time PCR quantification of *hspd1*, *pax6a* and *pax6b* mRNA levels in uninjured and 4 day post injured retina (injured). Data represent means ± s.d. (n=3 fish; compared to uninjured retina, *P*=0.0001 or less for *hspd1, pax6a and pax6b,* respectively, at 4 dpi. (**d**) *let-7*-dependent suppression of zebrafish proteins that are necessary for retina regeneration or associated with pluripotency. HEK 293 cells were

transfected with flag or myc-tagged regeneration or pluripotency-associated gene expression vectors (50 ng) with increasing concentrations (0, 50, 200, 500 ng) *ubC:let-7a,let-7f* along with *pCS2:mCherry* for normalization. (−) lane indicates untransfected cells. Forty-eight hrs later cells were harvested and proteins resolved by denaturing SDS-PAGE. Western blots were probed with anti-mCherry, anti-Flag or anti-Myc antibodies. *let-7*-dependent regulation of protein expression was quantified by densitometry (Supplementary Information, Fig. S7). Experiments were repeated 3 times with similar results. See Fig. S9 for full scans. (**e**) Constructs used to overexpress *let-7* and investigate the function of putative *let-7* miRNA binding sites in the *hspd1* 3' UTR. Mutations in the *let-7* binding site seed sequence are indicated. (**f**) The *hspd1* 3' UTR *let-7* binding site at position 1970 confers *let-7*-dependent regulation on luciferase expression. Luciferase activity from 24 hpf zebrafish embryos that were co-injected at the single cell stage with the luciferase reporter and the *let-7* miRNA expression vector described in (**e**) along with capped *Renilla luciferase* mRNA for normalization. Data represent means ± s.d. (n=3 fish; compared to *wt* 3'UTR construct in absence of *pri-let-7* miRNA or mut 3' UTR construct with *pri-let-7* miRNA, *P*=0.0001 or less for luciferase-wt 3' UTR activity treated with 1, 5 and 20 ng of *ubC:let-7a,let- 7f* construct.