Development 137, 891-900 (2010) doi:10.1242/dev.042895

LIN28 alters cell fate succession and acts independently of the *let-7* microRNA during neurogliogenesis in vitro

Erica Balzer[1,2], Christian Heine[1,2], Qiang Jiang[3], Vivian M. Lee[3] and Eric G. Moss[1,2,*]

SUMMARY

LIN28 is an RNA-binding protein that is expressed in many developing tissues. It can block *let-7* (*Mirlet7*) microRNA processing and help promote pluripotency. We have observed LIN28 expression in the developing mouse neural tube, colocalizing with SOX2, suggesting a role in neural development. To better understand its normal developmental function, we investigated LIN28 activity during neurogliogenesis in vitro, where the succession of neuronal to glial cell fates occurs as it does in vivo. LIN28 expression was high in undifferentiated cells, and was downregulated rapidly upon differentiation. Constitutive LIN28 expression caused a complete block of gliogenesis and an increase in neurogenesis. LIN28 expression was compatible with neuronal differentiation and did not increase proliferation. LIN28 caused significant changes in gene expression prior to any effect on let-7, notably on *Igf2*. Furthermore, a mutant LIN28 that permitted let-7 accumulation was still able to completely block gliogenesis. Thus, at least two biological activities of LIN28 are genetically separable and might involve distinct mechanisms. LIN28 can differentially promote and inhibit specific fates and does not function exclusively by blocking *let-7* family microRNAs. Importantly, the role of LIN28 in cell fate succession in vertebrate cells is analogous to its activity as a developmental timing regulator in *C. elegans*.

KEY WORDS: MicroRNA, Neurogliogenesis, RNA-binding protein, *let-7* (*Mirlet7*), LIN28, LIN28B, Heterochronic gene, Mouse

INTRODUCTION

Tissue formation requires that distinct cell types descend from a common pool of stem cells. In most cases, generation of mature cell types occurs in a stereotypic sequence as different progenitors are born in succession from proliferating undifferentiated cells. For example, neural stem cells divide and differentiate to produce neurons first, then astrocytes and oligodendrocytes. This progression, which is necessary for nervous system formation, is evolutionarily conserved among diverse animals (Pearson and Doe, 2004). How neural stem cells coordinately stop making neurons and start making glial cells is not known.

The heterochronic genes of the nematode *Caenorhabditis elegans* are a model for such developmental sequence regulation (Moss, 2007). These mutants display either precocious or retarded phenotypes in which certain cell fates occur abnormally early or late, respectively (Ambros and Horvitz, 1984). One of these, *lin-28*, governs the succession of cell fates in the larva. It is highly expressed in the first larval stage and downregulated to allow the transition to later stages. Constitutive *lin-28* expression causes earlier fates to be repeated at the expense of normally later fates (Moss et al., 1997). Although homologs of heterochronic genes exist in mammals, whether they are part of a developmental timing regulatory mechanism is not known.

Mammals possess two *lin-28*-like genes, *Lin28* (or *Lin28a*) and *Lin28b* (Balzer and Moss, 2007; Guo et al., 2006; Moss and Tang, 2003). The LIN28 protein contains two RNA-binding domains: a cold-shock domain (CSD) and retroviral-type CCHC zinc knuckles. *Lin28* is expressed in embryonic stem (ES) cells and in diverse tissues within the embryo, including the developing nervous system, and in developing tissues of the adult (Richards et al., 2004; Yang and Moss, 2003; Yokoyama et al., 2008). As in *C. elegans*, levels of LIN28 protein are high in developing tissues and decrease as differentiation proceeds (Moss and Tang, 2003; Yang and Moss, 2003). *Lin28* is expressed in undifferentiated ES cells (Moss and Tang, 2003; Richards et al., 2004). Importantly, LIN28 can collaborate with the transcription factors OCT3/4 (POU5F1; also known as OCT4), SOX2 and NANOG to promote pluripotency (Yu et al., 2007).

LIN28 protein is predominantly cytoplasmic, although it may shuttle through the nucleus (Balzer and Moss, 2007; Moss and Tang, 2003). Initial reports proposed that LIN28 is associated with mRNAs and enhances translation of *Igf2* mRNA (Balzer and Moss, 2007; Polesskaya et al., 2007). However, LIN28 has been shown to specifically bind and block the processing of *let-7* (*Mirlet7*) microRNAs (miRNAs) (Hagan et al., 2009; Heo et al., 2008; Heo et al., 2009; Lehrbach et al., 2009; Newman et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008). Its recognition of *let-7* family pre-miRNAs is remarkably specific and appears to occur via the loop region of the miRNA precursor, rather than recognizing the mature miRNA sequence itself (Newman et al., 2008; Piskounova et al., 2008).

let-7 was the first heterochronic gene homolog and the first miRNA gene discovered outside of *C. elegans* (Pasquinelli et al., 2000). *let-7* is widely expressed in vertebrates and has been shown to be important in multiple cancers (Chan et al., 2008). Mammals have at least ten *let-7* family members with widespread and regulated expression, numerous predicted targets, and what are likely to be significant roles in development (Roush and Slack, 2008).

Because the role of LIN-28 in *C. elegans* is to control the succession of cell fates during tissue development, we wished to address whether vertebrate LIN28 has a similar function. We used

[1]Department of Molecular Biology, [2]Graduate School of Biomedical Sciences, The University of Medicine and Dentistry of New Jersey, Stratford, NJ 08084, USA. [3]Division of Developmental Biology, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI 53226, USA.

*Author for correspondence (mosseg@umdnj.edu)

Accepted 26 November 2009

in vitro differentiation of pluripotent mouse embryonal carcinoma (EC) cells to observe the effect of LIN28 on the succession of neural and glial cell fates from a common pool of undifferentiated cells. Like the stem cell factor OCT3/4, LIN28 is rapidly downregulated during neurogliogenesis in vitro. Prevention of this downregulation by constitutive expression of LIN28 caused a striking effect on the normal progression of fates: an increase in neurogenesis and prevention of gliogenesis. Importantly, the two effects were genetically separable and appeared to result from more than one molecular mechanism, at least one of which is independent of *let-7*. Our data suggest that the developmental role of LIN28 in vertebrates is analogous to its developmental timing role in *C. elegans*.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Embryonic day (E) 8.5-10.5 CD-1 mouse embryos were fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization as described (Wilkinson, 1992). The antisense *Lin28* probe was digoxigenin labeled and the color reaction was visualized by BM Purple (Roche).

Immunostaining on tissue sections

E8.5 and E9.5 CD-1 embryos were fixed in 4% paraformaldehyde overnight at 4°C and processed for cryosectioning. Sections (10 μm) were collected on SuperFrost Plus slides (VWR Scientific) and immunostained with primary antibodies to LIN28 (Moss and Tang, 2003) and SOX2 (Millipore). Fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch) were applied in the dark at room temperature. Fluorescence images were captured using a Zeiss Z1 microscope equipped with a monochrome MRm AxioCam camera.

Cell culture

Mouse P19 cells were obtained from the ATCC (Manassas, VA, USA). P19 cells were maintained in alpha-MEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 2.5% fetal bovine serum, 7.5% bovine serum and 50 units/ml penicillin/streptomycin. Cell lines were transfected using Fugene6 transfection reagent as described by the manufacturer (Roche, Indianapolis, IN, USA). Stably transfected cell lines were selected using 2 μg/ml puromycin (Sigma-Aldrich). To induce differentiation, 2×10^6 P19 cells were plated on bacterial-grade Petri dishes with 5×10^{-7} M all-trans retinoic acid (Sigma-Aldrich). After 5 days, aggregated cells were dissociated and 2×10^6 cells were plated on standard tissue culture dishes in DMEM supplemented with N2 supplement (Invitrogen, Carlsbad, CA, USA). Cells were fed and harvested every other day throughout the timecourse. For cell counts and proliferation assays, P19 cells were seeded in 10-cm^2 tissue-culture-grade dishes at 1×10^6 cells/ml. Cells were then harvested and again counted 24 and 48 hours later. For cell proliferation assays during differentiation, cells were aggregated as described above and 2×10^6 cells were replated after 5 days. Cells were then trypsinized and counted every other day.

Antibody production

A portion of LIN28B (residues 2-32 plus 173-271) was expressed in *E. coli* as a His-tagged fusion and purified. Polyclonal antisera were generated in rats by Covance (Denver, PA, USA). Anti-LIN28A (Moss and Tang, 2003) and anti-LIN28B antisera detect only their respective proteins and do not cross-react (data not shown).

SDS-PAGE and immunoblotting

Cell pellets were lysed in 1% NP40 lysis buffer (150 mM NaCl, 2.5 mM $MgCl_2$, 10 mM HEPES pH 7.5, 0.1% NP40, 1 mM DTT, 0.2 mM PMSF, 1× Roche Complete EDTA-free protease inhibitor cocktail). Equal amounts of protein were loaded and fractionated on 10% polyacrylamide Tris-HCl gels and transferred to PVDF membranes. Membranes were incubated with blocking solution for 1 hour and then probed with antibodies against: actin (1:1000; Sigma-Aldrich), LIN28A (1:2500), LIN28B (1:2500), GFAP (1:1000; Sigma-Aldrich), GFP (1:25,000; Covance), nestin (1:1000; Chemicon/Millipore, Bellerica, MA, USA), OCT3/4 (1:1000; Santa Cruz) and TuJ1 (1:1000; Covance). Bound mouse, rabbit or rat antibodies were detected using HRP-conjugated goat anti-rat (BioRad, Hercules, CA, USA), anti-rabbit (BioRad) or anti-mouse (Novus, Littleton, CO, USA) antibodies.

Immunofluorescence and microscopy

RA-induced cell aggregates were trypsinized and replated onto coverslips in DMEM supplemented with N2 (Invitrogen). At the indicated time points, cells were fixed with 4% formaldehyde. Fixed cells were washed with PBS and permeabilized in 0.1% Triton X-100 in PBS (PBST) for 5 minutes. Antibodies were diluted in PBST containing 2% BSA and incubated for 1 hour at room temperature: GFAP (1:1000; Sigma-Aldrich), LIN28A (1:500), nestin (1:1000; Chemicon/Millipore) and TuJ1 (1:1000; Covance). Goat anti-rat Alexa Fluor 488 and anti-mouse Alexa Fluor 594 (1:2000; Invitrogen) were used as secondary antibodies. Cells were subsequently stained with Hoechst 33342 (Invitrogen), washed and mounted onto slides and monitored by fluorescence microscopy. Quantitation was conducted on at least three independent differentiation experiments.

mRNA array

P19 and P19+Lin28A cell pellets were harvested on day 4 of differentiation. Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and gene expression profiling was performed using cDNA microarray technology at the CINJ Core Expression Array Facility (CINJ, New Brunswick, NJ, USA). The Affymetrix Mouse Genome 430A 2.0 Array (Affymetrix, Santa Clara, CA, USA) was used for analysis. A list of genes, the expression of which changed by at least 2-fold, was generated. GEO accession number: GSE19705.

Quantitative RT-PCR

Total RNA was isolated using the miRNA Cells-to-CT Kit (Applied Biosystems, Foster City, CA, USA) from ~2×10^4 cells. Mature miRNA levels were measured by quantitative (q) PCR using miRNA TaqMan probe kits (Applied Biosystems) and normalized using Sno202 RNA levels. *Igf2* mRNA levels were normalized to 18S levels. Relative fold changes were calculated using the Δ-Ct method (Thomson et al., 2006). Assays were performed on three biological replicates.

miRNA array

Global miRNA profiling was performed by Exiqon (Vedbaek, Denmark) using miRCURY LNA miRNA Arrays. The array represented all miRNAs as recorded in miRBase version 10.0 (www.mirbase.org), on RNA samples from P19 and P19+Lin28A cells differentiated to 5 days as described above. Total RNA was purified using the miRNA Isolation Kit (Ambion, Austin, TX, USA). GEO accession number: GSE19858.

Yeast three-hybrid assay

The three-hybrid assays were performed as described (Hook et al., 2005). Iron response element (IRE) and iron regulatory protein (IRP) plasmids, as well as vectors and strains, were provided by Marvin Wickens, University of Wisconsin. Plasmids containing human LIN28, LIN28 mutant variants and human YB-1 were fused to the activation domain in the vector pACTII, and RNA sequences fused to the MS2 stem loop in the vector pIIIA/MS2-2. Yeast strains were constructed by co-transformation into strain YBZ1. To visualize the interaction, X-Gal overlay was performed at 30°C for 6 hours and overnight. All RNA sequences that failed to interact with LIN28 were demonstrated to be expressed by RT-PCR (data not shown).

RESULTS

LIN28 is expressed in the developing mouse neural tube

LIN28 is abundant in a variety of developing tissues, including neuroepithelium, but its localization during neural development has not been precisely defined (Yang and Moss, 2003). To address this issue, we used in situ hybridization and immunostaining of wild-type mouse embryos (Fig. 1). By whole-mount in situ hybridization, *Lin28* mRNA was widespread in the neural tube at E8.5 (Fig. 1A). At E9.5, the signal in the neural tube was low at the forelimb level,

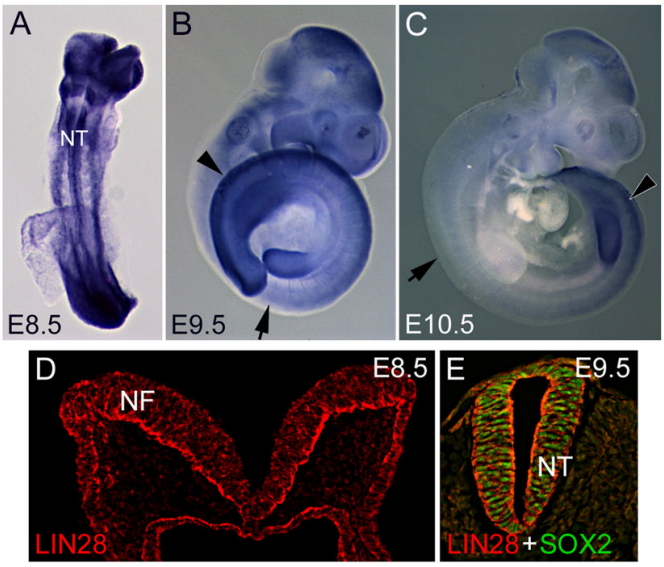

Fig. 1. Mouse LIN28 expression during normal neural development. (**A**) An E8.5 wild-type mouse embryo viewed from the dorsal side showing *Lin28* mRNA expression in the developing neural tube (NT). (**B**) An E9.5 embryo showing *Lin28* mRNA expression. The signal in the neural tube was low at the forelimb level (arrow), whereas it was still high at the hindlimb level (arrowhead). Expression was intense in the forelimb bud. (**C**) An E10.5 embryo showing that *Lin28* mRNA was almost undetectable in the neural tube at the forelimb level (arrow), but still present at the hindlimb level (arrowhead). Expression was detected in the hindlimb bud, but not in the forelimb bud. (**D**) Immunostaining of embryo sections. LIN28 protein (red) was expressed broadly in the neural fold early on in development. (**E**) In the caudal neural tube, where neural differentiation has not begun, LIN28 (red) was expressed throughout the neural tube and colocalized with SOX2 (green).

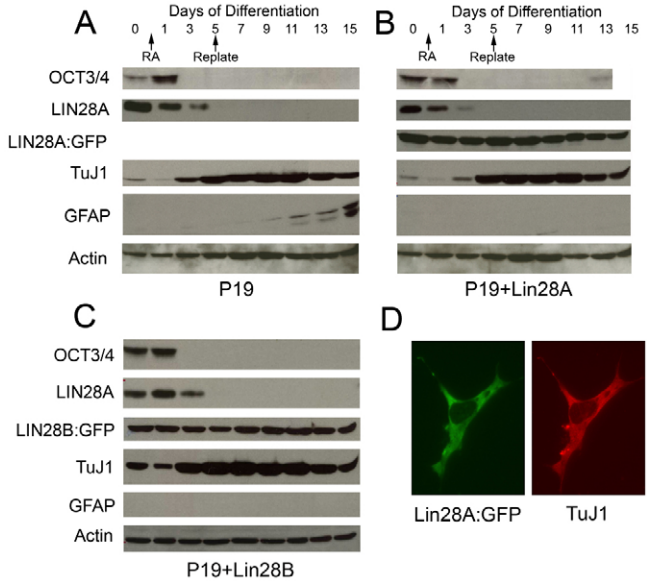

Fig. 2. Constitutive LIN28 specifically blocks the appearance of glial marker, but not of neuronal or pluripotency markers. (**A-C**) Immunoblots, using protein-specific antisera, of P19, P19+Lin28A and P19+Lin28B cells induced to differentiate with retinoic acid (RA), followed by plating without RA. Arrows indicate times of RA treatment and plating. (**D**) Immunofluorescence micrographs of a single P19+Lin28A cell after 11 days of differentiation, showing simultaneous expression of Lin28A::GFP (left) and the neuronal marker TuJ1 (right).

but still high at the more caudal, hindlimb level (Fig. 1B). At E10.5, *Lin28* mRNA was almost undetectable in the neural tube at the forelimb level, but still present at the hindlimb level (Fig. 1C).

By immunostaining, LIN28 protein was expressed broadly in the neural fold early on, at E8.5 (Fig. 1D). At E9.5 in the caudal neural tube, where neural differentiation has not begun, LIN28 was present throughout the neural tube (Fig. 1E). At this stage, LIN28 protein colocalized with SOX2, a marker of neural stem cells (Ellis et al., 2004). Later, LIN28 disappeared from much of the developing neural tube and was not detected in differentiated neural lineages (data not shown).

Constitutive LIN28 expression blocks glial differentiation of P19 cells

Undifferentiated mouse P19 EC cells have characteristics of stem cells and differentiate along a neuronal-glial lineage when grown as aggregates with retinoic acid (RA) (Bain et al., 1994; Rudnicki, 1987). After 4 days, induced cells differentiate for more than a week, first as neurons and then as glia. We monitored LIN28 and cell type-specific markers over this time by immunoblotting and immunofluorescence (Fig. 2). In P19 cells, LIN28 was rapidly downregulated by 3 days after the start of aggregation/RA treatment (Fig. 2A). OCT3/4, a canonical marker of pluripotent stem cells, was downregulated in the first 2 days. The neuron-specific differentiation marker TuJ1 (a class III β-tubulin; TUBB3) was upregulated by 3 days of aggregation and expressed continuously thereafter. TuJ1 was detected at a low level in untreated cells, possibly owing to spontaneous differentiation (Rudnicki, 1987). GFAP (glial fibrillary acidic protein), a marker of glial astrocytes, was not detectable until several days after replating and reached its peak by day 15.

To examine the effect of constitutive LIN28 expression on the succession of cell fates, we expressed the *Lin28* open reading frame fused to GFP from a CMV enhancer/chicken β-actin (CAG) promoter. P19 cells stably transfected with the constitutive LIN28A expression plasmid (P19+Lin28A cells) were maintained in the presence of puromycin. These cells behaved like P19 cells and did not show increased spontaneous differentiation in the absence of aggregation/RA treatment (data not shown). Immunoblotting confirmed that the LIN28A protein level was similar to that of endogenous LIN28A in undifferentiated cells and was maintained throughout the differentiation timecourse (Fig. 2B). We observed, by immunoblotting, that LIN28A did not significantly alter the protein expression profiles of OCT3/4, LIN28A and TuJ1 (Fig. 2B). However, constitutive LIN28A completely blocked the upregulation of GFAP (Fig. 2B). To test the possibility that the accumulation of GFAP was merely delayed, differentiation was carried out to 25 days, but no GFAP was detected (data not shown). In light of this observation, we confirmed that TuJ1-positive cells continued to express the exogenous LIN28A::GFP by fluorescence microscopy (Fig. 2D).

Indistinguishable results were seen when a LIN28B::GFP construct was constitutively expressed in P19 cells using the same constitutive promoter (P19+Lin28B cells; Fig. 2C). Similarly, continuous LIN28B expression prevented the appearance of GFAP but allowed for normal downregulation of OCT3/4. The timing of TuJ1 expression was apparently the same as in P19+Lin28A and P19

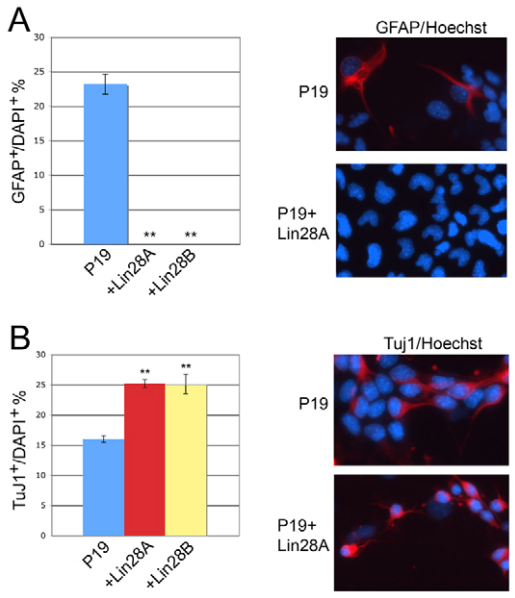

Fig. 3. Constitutive LIN28 expression blocks gliogenesis and promotes neurogenesis. (**A**,**B**) The bar charts show the percentage of P19, P19+Lin28A and P19+Lin28B cells expressing the glial marker GFAP at day 15 (A) or the neuronal marker TuJ1 at day 11 (B) after RA treatment. **, $P<0.01$ versus P19 cells. Error bars indicate s.e.m. for three independent experiments. Representative microscopy fields are shown to the right. Nuclei were stained with Hoechst.

cells. Thus, the continued expression of either LIN28A or LIN28B blocked the production of a glial cell type, but was compatible with the differentiation of neurons.

LIN28 promotes neurogenesis

To quantify the effects of LIN28 on differentiation, cultures of P19 or P19+Lin28A cells were induced to differentiate and examined for the number of neurons and glial cells with marker-specific antisera (Fig. 3). Cells were examined when neural differentiation and astrocyte accumulation were high, at days 11 and 15, respectively (Fig. 3A). Whereas greater than 20% of P19 cells were positive for GFAP at day 15, fewer than 0.001% of P19+Lin28A cells or P19+Lin28B cells expressed this glial marker (Fig. 3A). Surprisingly, the number of cells expressing TuJ1 at day 11 increased ~1.6-fold when either LIN28A or LIN28B was constitutively expressed in P19 cells (Fig. 3B).

To determine whether the increase in TuJ1-positive cells at day 11 was due to a shift in the peak of neurogenesis or an increase in the number of differentiating cells, control and LIN28-expressing cells were examined by immunofluorescence at 2-day intervals for TuJ1 and nestin. TuJ1 is a marker of early neurons and nestin is a marker of neural progenitors (Lendahl et al., 1990; Menezes and Luskin, 1994). Both TuJ1 and nestin were detected during the aggregation phase of induction, but did not show significant differences between the cell lines as determined by immunoblotting (Fig. 2 and data not shown). Although the days of peak expression were similar in the two cell lines, the number of TuJ1-positive cells was significantly higher at all timepoints in the P19+Lin28A cells (Fig. 4A). This observation indicates that the number of differentiating neurons was greater in P19+Lin28A cells than in P19 cells for most of the timecourse. The proportion of cells expressing nestin was also statistically higher at 9 and 11 days in P19+Lin28A cells; however, by day 13, the numbers of nestin-positive cells were equal in the two lines (Fig. 4B). These observations suggest that LIN28 increases the total number of cells differentiating as neurons, and does not simply shift the peak of neuronal differentiation.

Constitutive LIN28 expression does not increase overall cell number

The increase in cells undergoing neurogenesis as a result of constitutive LIN28 expression raised the possibility that LIN28 increases proliferation. To test this, the growth of undifferentiated P19 and P19+Lin28A cells was measured at 24 and 48 hours after the plating of RA-treated aggregates (Fig. 4C). No difference in total cell numbers between the cell lines was observed, suggesting that LIN28 does not have the ability to alter proliferation of the undifferentiated cells. To address whether LIN28 stimulated

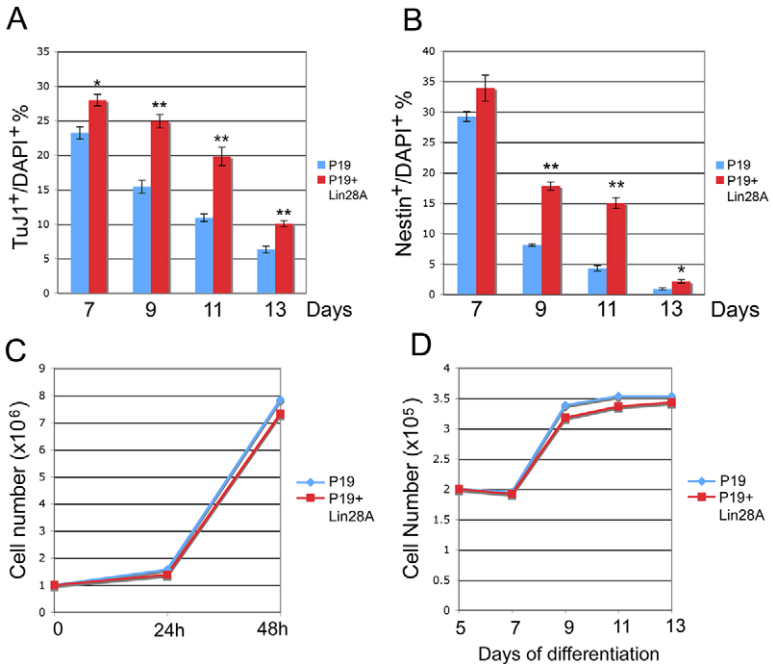

Fig. 4. Constitutive LIN28 expression increases differentiating neurons without increasing overall cell number. (**A**,**B**) Percentage of P19, P19+Lin28A and P19+Lin28B cells expressing the neuronal marker TuJ1 (A) or the neuronal progenitor marker nestin (B) during the differentiation timecourse at the indicated days after RA treatment. *, $P<0.05$; **, $P<0.01$ versus P19. Error bars indicate s.e.m. for three independent experiments. (**C**) Numbers of P19 and P19+Lin28A cells after 24 and 48 hours of growth with no RA treatment. The two sets of data were not significantly different ($P=0.94$). (**D**) Numbers of P19 and P19+Lin28A cells during a differentiation timecourse. After RA treatment and replating at day 5, counts were made every other day for 8 days. Differences between cell lines: day 2, $P=0.85$; day 4, $P=0.45$; day 6, $P=0.54$; day 8, $P=0.74$.

proliferation during differentiation, cell counts were made at 2-day intervals following aggregation/RA treatment (Fig. 4D). Based on the increase in TuJ1-positive cells, an increase of at least 10% would be expected if LIN28 induced proliferation of cells prior to neuronal differentiation. However, no significant difference in total cell numbers was observed between the cell lines. LIN28 expression does not therefore appear to stimulate proliferation, although we have not ruled out the possibility that it simultaneously promotes both proliferation and cell death equally. Nevertheless, the increase in neurogenesis cannot be explained by an increase in overall cell number, suggesting that LIN28 governs cell fate choice.

The conserved domains are differentially required for the effect of LIN28 on cell fates

To test the requirement for the two conserved RNA-binding domains of LIN28, mutant forms of the protein were constitutively expressed in P19 cells. The mutant CSD contained alanines in place of three conserved surface aromatic residues, and the mutant CCHC contained alanines in place of two conserved histidines, as described previously (Balzer and Moss, 2007). Each mutant domain was in the context of a full-length LIN28A protein fused to GFP. Each mutant protein was expressed at levels comparable to the wild-type proteins and localized normally to the cytoplasm (data not shown) (Balzer and Moss, 2007). Cells expressing the mutants were differentiated as before and the expression of markers assessed by immunoblotting (Fig. 5). As with the wild-type protein, constitutive expression of the mutants had no effect on OCT3/4 downregulation or TuJ1 upregulation. Likewise, the mutant CSD cell line (P19+mutCSD) behaved like LIN28-expressing cells in preventing the accumulation of GFAP-positive cells (Fig. 4B). By contrast, the mutant CCHC line (P19+mutCCHC) differentiated normally, indicating that this protein lacked biological activity despite retaining the ability to interact specifically with RNA, as discussed below (Fig. 5C).

To quantify these effects, cell lines constitutively expressing the domain mutants were induced to differentiate and the number of TuJ1- and GFAP-positive cells determined at days 11 and 15, respectively. As with wild-type LIN28, constitutive expression of the mutant CSD protein completely blocked the appearance of GFAP-positive cells, whereas the mutant CCHC protein had no effect (Fig. 5D). Interestingly, both the CSD and CCHC mutants failed to increase the numbers of differentiating neurons in the cultures, and in fact had slightly negative effects on the production of neurons as compared with the wild-type protein (Fig. 5E). These observations indicate that the CCHC domain is necessary for the inhibition of gliogenesis, whereas the CSD domain is not, and that both domains are required for the increase in neurogenesis caused by constitutive LIN28.

LIN28 blocks the accumulation of *let-7* family miRNAs during differentiation

let-7 miRNAs have been shown to accumulate markedly after aggregation/RA treatment of P19 cells, an effect attributed primarily to post-transcriptional regulation (Thomson et al., 2006). LIN28 has been found to specifically bind to, and block, the processing of let-7 precursors (Heo et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008). Therefore, it was expected that constitutive expression of LIN28 would inhibit the accumulation of let-7 during the differentiation of P19 cells. To test this, cells expressing LIN28 were differentiated as described above and the expression of *let-7g* was monitored by real-time qRT-PCR. Whereas mature let-7g began to accumulate in P19 cells at day 5, as expected, let-7g levels remained consistently low in cells expressing LIN28 throughout the differentiation timecourse (Fig. 6A).

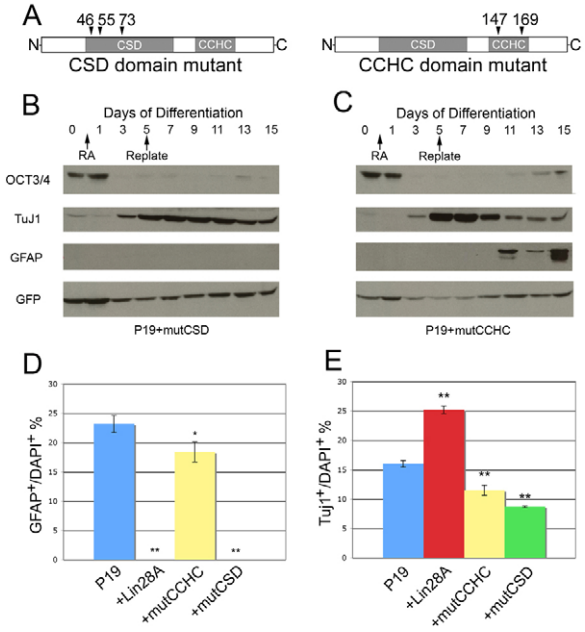

Fig. 5. Domain requirements for LIN28 activity in neurogliogenesis. (**A**) Locations of CSD and CCHC mutations in the LIN28A protein; numbers indicate amino acid residues from the N-terminus. (**B**,**C**) Immunoblots, using protein-specific antisera, of P19+mutCSD (B) and P19+mutCCHC (C) cells induced to differentiate with RA, followed by plating without RA. Arrows indicate times of RA treatment and plating. GFP expression from the constitutive promoter served as a gel loading control. (**D**,**E**) Percentage of P19, P19+Lin28A, P19+mutCSD and P19+mutCCHC cells expressing the neuronal marker TuJ1 at day 11 (E) or the glial marker GFAP at day 15 (D) after RA treatment. *, $P<0.05$; **, $P<0.01$ versus P19 cells. Error bars indicate s.e.m. for three independent experiments.

To determine whether other miRNAs are up- or downregulated by LIN28 expression, miRNA profiles were compared between LIN28-expressing and wild-type P19 cells. RNA from cells at day 5 of differentiation was examined on microarrays containing probes for all mouse miRNAs (miRBase version 10.0). Of 580 mouse miRNAs assayed, seven accumulated to significantly different levels in the two lines: let-7a, let-7d, let-7f, let-7g, miR-152, miR-302d and miR-483 (Fig. 6B). In all cases, the miRNA level was lower in the LIN28-expressing cells than in the wild-type P19 cells (reduced by at least half), with members of the *let-7* family showing the greatest reduction (reduced by at least two-thirds).

To identify when let-7 accumulation was first inhibited by constitutive LIN28 expression, real-time qRT-PCR was performed at days 3, 4 and 5 of differentiation. In differentiating P19 cells, no significant increase in let-7a or let-7g expression was observed until day 5 of the differentiation timecourse (Fig. 6C), as observed previously (Thomson et al., 2006). let-7 levels were consistently low in the presence of constitutive LIN28. Therefore, the effect of constitutive LIN28 on let-7 accumulation began at day 5 of differentiation.

LIN28 can influence gene expression prior to its inhibition of let-7 accumulation

To determine whether gene expression is altered prior to *let-7* upregulation, mRNA expression profiling was conducted on P19 and P19+Lin28A cells at day 4 of differentiation. The mRNA levels of 14 genes were repeatedly altered at least 2.5-fold by expression

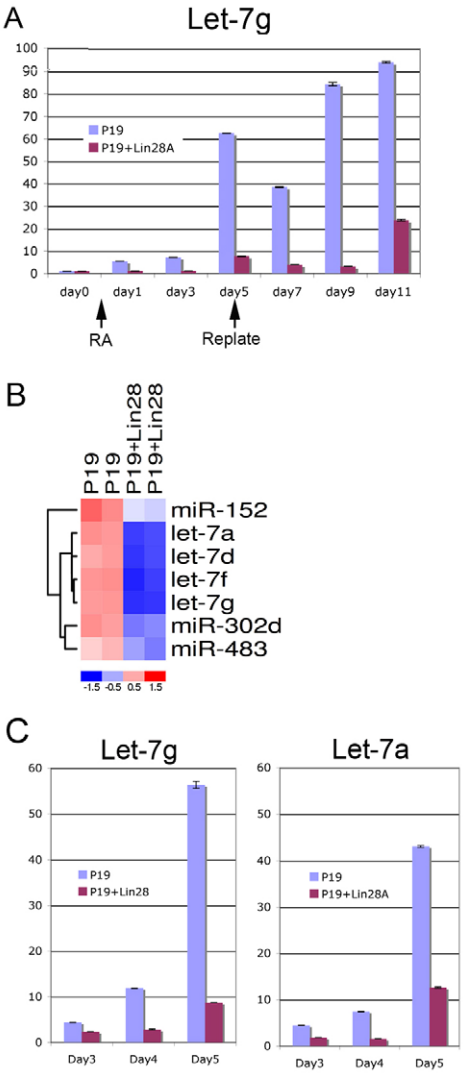

Fig. 6. LIN28 blocks let-7 accumulation during neurogliogenesis in vitro. (**A**) Levels of mature let-7g miRNA expression during RA-induced differentiation of P19 or P19+Lin28A cells as measured by qRT-PCR. (**B**) Heat map showing two-way hierarchical clustering of miRNA levels compared between P19 and P19+Lin28A cells at day 5 of differentiation, as measured by miRNA microarray. The miRNA clustering tree is shown on the left, and the sample clustering tree is at the top. The color scale shown at the bottom illustrates the relative expression level of an miRNA across all samples: red represents an expression level above the mean, blue expression below the mean. (**C**) Levels of mature let-7g and let-7a at days 3, 4 and 5 of differentiation in P19 or P19+Lin28A cells as measured by qRT-PCR. Error bars indicate s.e.m. for three independent experiments.

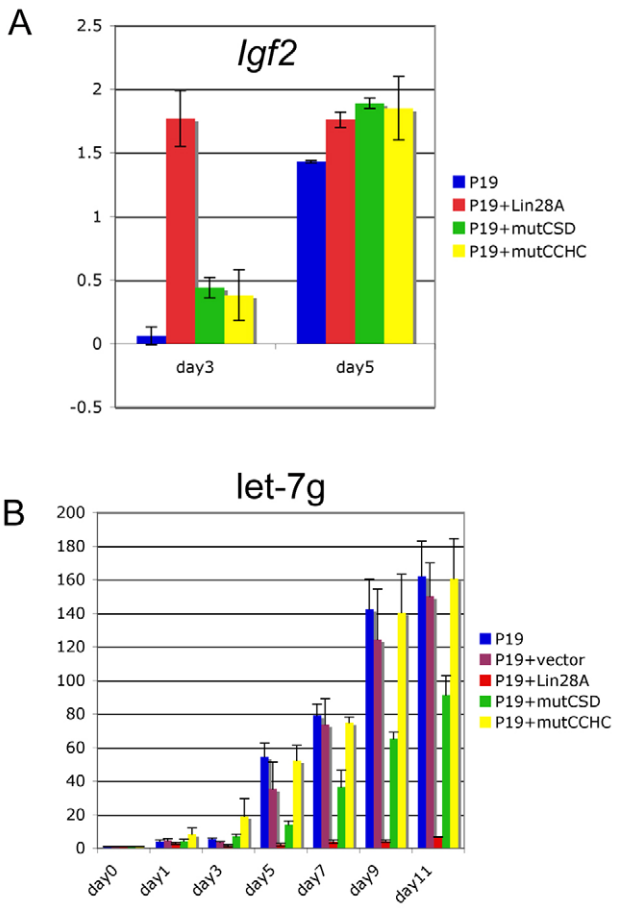

Fig. 7. The conserved RNA-binding domains of LIN28 are differentially required for its effect on gene expression. (**A**) Levels of *Igf2* mRNA in P19 cells expressing LIN28A, LIN28AmutCSD and LIN28AmutCCHC at days 3 and 5 of differentiation as measured by qRT-PCR. (**B**) Levels of mature *let-7g* miRNA expression during RA-induced differentiation of P19, P19+vector, P19+Lin28A, P19+mutCSD and P19+mutCCHC cells as measured by qRT-PCR. Error bars indicate s.e.m. for three independent experiments.

of LIN28 on day 4 (see GEO accession GSE19705). Among the most upregulated were *Peg3* (5.4-fold) and *Sall4* (4.1-fold), which both encode zinc-finger transcription factors that are involved in early embryonic cell fate decisions (Relaix et al., 1996; Zhang et al., 2006). *Igf2*, which was previously shown to be positively regulated by LIN28 during muscle differentiation (Polesskaya et al., 2007), also showed significantly higher expression at day 4 of neurogliogenesis in vitro. Several genes were also observed to be significantly downregulated (see GEO accession GSE19705). Therefore, although LIN28 dramatically inhibits *let-7* miRNA accumulation at day 5, it upregulates, at least 1 day earlier, the expression of several genes with known roles in differentiation.

The conserved domains are differentially required for the effects of LIN28 on gene expression

Because wild-type LIN28 and the two LIN28 mutants had different effects on cell fates, we determined whether they also produced different effects on gene expression. First, cell lines expressing wild-type LIN28, the CSD mutant and the CCHC mutant, as well as wild-type P19 cells, were differentiated and assayed for *Igf2* mRNA at 3 and 5 days of differentiation (Fig. 7A). Whereas wild-type LIN8 showed elevated *Igf2* mRNA, as expected based on the microarray results, neither the CSD nor the CCHC mutant showed any difference from P19 cells. This observation indicates that both domains are required for the effect of LIN28 on *Igf2* expression.

To examine the inhibition of let-7 accumulation, differentiation of each cell line was conducted and mature let-7g was assayed by qRT-PCR every 2 days from day 1 to day 11 (Fig. 7B). Cells expressing LIN28 blocked let-7g accumulation and control cells

Table 1. Interaction of LIN28 protein with pre-miRNA sequences

No.	Sequence	LIN28A	CSD mutant	CCHC mutant	CSD CCHC mutant	LIN28B	YB-1	IRP
1	pre-let-7a	++	++	++	–	++	–	–
2	pre-let-7g	++	++	++	–	++	–	–
3	pre-let-7f	++	++	++	–	++	–	–
4	pre-miR-15a	–	nd	nd	nd	–	–	–
5	pre-miR-16-1	–	nd	nd	nd	–	–	–
6	pre-miR-17	–	nd	nd	nd	–	+/–	–
7	pre-let-7g loop	++	++	++	–	++	–	–
8	pre-let-7g loop C-to-A*	++	++	++	–	++	–	–
9	pre-miR-152	+/–	+/–	–	–	–	+/–	–
10	pre-miR-302d	+	–	+	–	+	–	–
11	pre-miR-483	+/–	–	+/–	–	+/–	–	–
12	*Igf2* leader 3	–	nd	nd	nd	–	–	–
13	Ce pre-let-7	++	–	++	–	++	–	–
14	Vector	–	–	–	–	–	–	–
15	IRE	–	–	–	–	–	–	+

++, strong induction of β-galactosidase detectable in 6 hours; +, strong induction detectable in 24 hours; +/–, intermediate induction in 24 hours; –, no β-galactosidase activity detectable in 24 hours.
nd, not determined. Ce, *C. elegans*. CSD, cold-shock domain; IRE, iron response element.
*C-to-A mutant of Piskounova et al. (Piskounova et al., 2008).

showed the expected increase in let-7g. Interestingly, cells expressing the CCHC mutant, which accumulated and localized normally but did not inhibit gliogenesis, also showed the normal rise in let-7g levels. This observation suggests that the CCHC domain is required for LIN28 to block let-7 accumulation. By contrast, cells expressing the CSD mutant, which blocked gliogenesis to the same extent as the wild-type protein, showed substantial accumulation of let-7g, although not as much as in the controls. This observation suggests that the CSD domain is required for LIN28 to completely inhibit let-7g accumulation, although it is not required for blocking differentiation of GFAP-positive glial cells.

Thus, the two RNA-binding domains of LIN28 showed differential effects on gene expression: the CCHC domain was required both to inhibit let-7 accumulation and to positively regulate *Igf2* mRNA, whereas the CSD domain was required to positively regulate *Igf2*.

Each of the two conserved domains of LIN28 interact with pre-let-7 sequences

Because the two domains of LIN28 differentially affected both cell fate and gene expression, we wished to determine whether one or both domains recognize specific RNA sequences. Several groups have determined that one or both of these domains is required to bind and/or block let-7 processing (Heo et al., 2008; Newman et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008). We tested our mutants using a recent modification of the yeast three-hybrid system (Hook et al., 2005). This assay is similar to the yeast two-hybrid system, except that RNA tethers the DNA-binding protein to the transcriptional activation domain: the DNA-binding LexA protein is fused to the RNA-binding MS2 coat protein, the MS2 RNA stem-loop is expressed contiguously with the RNA sequence of interest, and the RNA-binding protein of interest is linked to the activation domain. Interaction between the RNA and protein of interest is detected by the expression of a β-galactosidase reporter.

We first determined whether this assay reflected the interactions reported by others. Consistent with previous observations, we detected LIN28 interaction with three *let-7* family pre-miRNAs, let-7a, let-7f and let-7g, but not with three unrelated pre-miRNA sequences, miR-15a, miR-16 and miR-17 (Table 1, lines 1-6). LIN28 also did not interact with the MS2 RNA sequence without an insert, nor with the iron regulatory element (IRE) control insert (Table 1, lines 14 and 15). Neither YB-1 (YBX1) nor the iron regulatory protein (IRP; aconitase 1) recognized let-7 precursors (Table 1, lines 1-6). YB-1 is an evolutionarily distinct protein that also contains a CSD domain. The IRP is a standard yeast three-hybrid control protein, which only interacted with the IRE (Table 1, line 15). Piskounova et al. demonstrated that LIN28 could bind the pre-let-7 'loop' sequence, which lacks the mature *let-7* miRNA sequence; in addition, they found that a C-to-A point mutation in the pre-let-7g loop sequence significantly reduced binding of LIN28 in an in vitro binding assay (Piskounova et al., 2008). We observed LIN28 interaction with both the unmodified loop sequence and C-to-A version in the yeast three-hybrid assay, indicating that this assay is more sensitive and less discriminatory than the in vitro binding assay performed by Piskounova et al.

We then assessed the specificities of the two LIN28 mutants used in the differentiation experiments. Interestingly, both the CSD mutant and CCHC mutant forms of LIN28 showed strong interactions with all three *let-7* family member precursors, whereas a mutant altered in both domains did not (Table 1, lines 1-3). This observation suggests that each domain has specificity for pre-let-7 that is independent of the other, and that no other domain of LIN28 is sufficient to recognize pre-let-7. Because three non-*let-7* miRNAs were also inhibited by LIN28 in the miRNA array analysis, we tested whether LIN28 could bind the precursor sequences of these miRNAs (Table 1, lines 9-11). LIN28 did not show a strong interaction with precursor forms of miR-152 and miR-483, but did interact with pre-miR-302d. The interaction with pre-miR-302d required the CSD, suggesting that the two conserved domains of LIN28 have somewhat different sequence preferences (Table 1, line 10). Based on previous reporter experiments, it is possible that LIN28 interacts directly with the 5′ UTR (leader 3) of the *Igf2* mRNA (Polesskaya et al., 2007). However, the yeast three-hybrid assay did not detect such an interaction, possibly because the RNA was not properly presented or additional factors are required (Table 1, line 12). Interestingly, human LIN28 recognized pre-let-7 of *C. elegans* only through its CSD domain (Table 1, line 13). Worm and mammalian pre-let-7 sequences differ substantially, suggesting that a simple sequence motif does not define the specificity of LIN28. LIN28B displayed the same interactions as LIN28A, suggesting that there is very little, if any, difference in their specificities (Table 1).

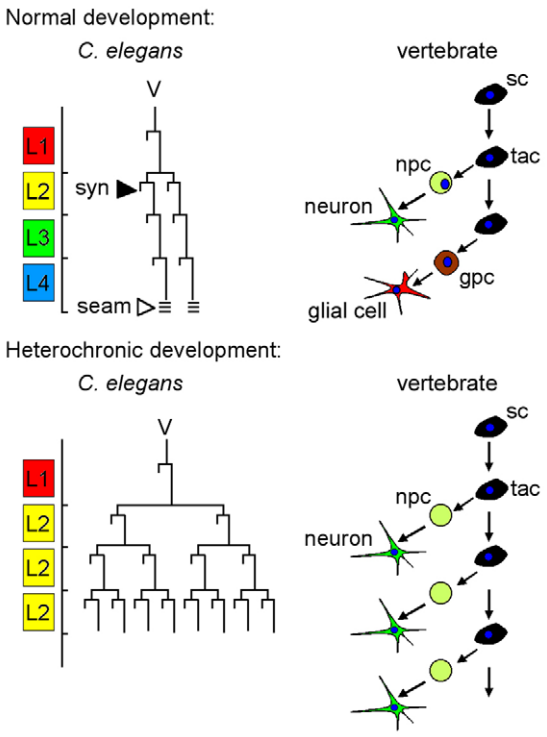

Fig. 8. The conserved role of LIN28 in developmental timing. (Top) In normal *C. elegans* larval development (left) and vertebrate neurogenesis in vitro (right), differentiated cells derive from the successive production of distinct cell types from a common pool of dividing cells. In both cases, LIN28 is expressed early on and is downregulated to permit the normal succession of cell fates. (Bottom) When LIN28 activity is continuous, early fates continue at the expense of later fates. In *C. elegans* (left), the L2-specific cell lineage pattern is repeated indefinitely. In vertebrate neurogliogenesis in vitro (right), LIN28 may block a transition within undifferentiated cells that produces glial cell fates while permitting neural fates to continue. V, lateral epidermal blast cell of the *C. elegans* larva; syn, epidermal syncytial nucleus; seam, differentiated epidermal seam cell; sc, stem cell; tac, transit amplifying cell; npc, neural progenitor cell; gpc, glial progenitor cell.

DISCUSSION

When we prevent the downregulation of LIN28 that normally occurs during differentiation, opposite effects on two cell fates are observed. Constitutive LIN28 completely blocks the accumulation of glial cells, which normally occurs late in the differentiation process, but increases the number of neurons by ~50%, which begin to accumulate soon after the start of RA treatment. Proneural transcription factors, such as Mash1 (Ascl1) and neurogenin, similarly promote neurogenesis while blocking gliogenesis (Nieto et al., 2001; Sun et al., 2001). However, LIN28 expression does not cause increased differentiation on its own, and instead alters the choice of cell fates once differentiation has commenced, favoring neuronal over glial differentiation.

The effect of LIN28 on cell fates during neurogenesis in vitro resembles that during *C. elegans* larval development (Fig. 8). In both cases, a succession of differentiated cells derives from a common pool of dividing cells (Fig. 8, top). In *C. elegans*, LIN-28 is expressed in the first larval stage in epidermal cells and is downregulated by the end of the second stage (Morita and Han, 2006; Moss et al., 1997; Seggerson et al., 2002). Constitutive expression results in the reiteration of second-stage cell fates, and, consequently, the number of cells that join an epidermal syncytium (sy) increases at the expense of those cells that form mature lateral seam (se) cells (Moss et al., 1997). In neurogliogenesis in vitro, constitutive expression of LIN28A or LIN28B results in an increase in neurons, an early fate, at the expense of glial cells, a later fate. This fate alteration can be interpreted as a retarded heterochronic phenotype (Fig. 8, bottom right). Because LIN28 is expressed in a variety of tissues in mammals, it might be involved in similar fate choices throughout development (Yang and Moss, 2003).

Significantly, we found that the promotion of neurogenesis and block of gliogenesis by LIN28 are genetically separable. Mutating the CSD domain causes LIN28 to lose the ability to promote neurogenesis while retaining its ability to block gliogenesis, whereas the CCHC domain appears to be required for all biological activity of LIN28. This finding suggests that the switch in cell fates from neuron to glia is not simply a choice between two alternatives, but rather a result of two separate regulatory processes.

The first reports on the molecular mechanism of LIN28 focused on its role as an mRNA-binding protein (Balzer and Moss, 2007; Polesskaya et al., 2007). In one instance, the positive regulation of *Igf2* translation was connected with the role of LIN28 in myoblast differentiation and muscle regeneration (Polesskaya et al., 2007). Subsequently, LIN28 was discovered to bind *let-7* family precursors and then shown to block biogenesis of the mature miRNA with high specificity (Heo et al., 2008; Newman et al., 2008; Piskounova et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). It is not clear under what circumstances and to what relative extent the two mechanisms of LIN28 operate to control cell fate.

Hammond and colleagues first noted the rapid rise in let-7 levels during ES and EC cell differentiation that results from post-transcriptional regulation (Thomson et al., 2006). An inhibition of let-7 processing by LIN28 explains the block in let-7 accumulation in these cells (Piskounova et al., 2008; Viswanathan et al., 2008). Indeed, we show in this report that continuous LIN28 expression blocks let-7 accumulation at the same time that it alters cell fates, suggesting that let-7 levels might govern the cell fate choice. However, the rapid increase in let-7 levels occurs with some delay after the downregulation of LIN28: we observe a significant drop in LIN28 levels by day 3 of differentiation, whereas the jump in mature let-7 occurs by day 5. The mechanism by which LIN28 inhibits let-7 precursor processing in vivo has been described in two ways: (1) interfering with the initial, Drosha (Rnasen)-mediated step in the nucleus and (2) the subsequent, Dicer-mediated step in the cytoplasm (Heo et al., 2008; Heo et al., 2009; Viswanathan et al., 2008). It is conceivable that it takes 24 hours or more for the downregulation of LIN28 to result in the great increase in mature let-7 levels observed during differentiation.

Prior to the rise in let-7 levels, LIN28 causes significant changes in the expression of other regulatory factors, notably *Igf2* mRNA, and thus acts independently of let-7 to promote their expression. Mouse *Igf2* mRNA does not contain any predicted let-7 sites (based on PicTar, TargetScan and Miranda algorithms), and therefore seems unlikely to be repressed by let-7. The post-transcriptional regulation of *Igf2* by LIN28 may increase the *Igf2* mRNA level in differentiating EC cells because the promotion of translation can increase mRNA stability (Balagopal and Parker, 2009; Polesskaya et al., 2007). Of the other genes upregulated by LIN28, mouse *Sall4* is predicted to contain one potential let-7 site in its 3′ UTR, whereas *Peg3* contains none. Although the post-transcriptional regulation of *Igf2* by LIN28 has been shown to occur through the 5′ UTR of the

gene, we did not observe a direct interaction between LIN28 and the *Igf2* 5′ UTR in our yeast three-hybrid assay. Therefore, the mechanism by which LIN28 regulates these genes remains obscure.

Interestingly, the differential effects on cell fates caused by LIN28 correlate with the differential effects seen on gene expression. The early increase in *Igf2* mRNA parallels the increase in neurons: both occur prior to the time at which let-7 levels normally rise, and both require the two conserved RNA-binding domains of LIN28. *Igf2* has a documented role in the formation of neural tumors and promotes neurogenesis (Corcoran et al., 2008; Mori et al., 2001; Sim et al., 2006). Thus, the importance of *Igf2* in neurogenesis is consistent with it being involved in the increase in neurons observed with constitutive LIN28.

The later phenotypic effect, the inhibition of gliogenesis, correlates with the complete block of let-7 accumulation: both occur later in differentiation and both require the CCHC domain. This correlation suggests that the inhibition of gliogenesis is due to a severe reduction in let-7 levels. Our attempts to prove in this system that the level of let-7 indeed promotes gliogenesis, by reducing or increasing let-7 expression directly, have been negative, possibly owing to the number of let-7 variants involved. Both redundancy and non-equivalence have been observed in let-7 variants in *C. elegans* (Abbott et al., 2005). Admittedly, the reduction in let-7 caused by the CSD mutant is ambiguous: either a high let-7 level is required for gliogenesis, or let-7 is irrelevant to glial differentiation.

It is striking that extremely low let-7 levels seen with constitutive LIN28 expression are compatible with neuron formation, especially as *let-7* expression has been linked to neural differentiation (Rybak et al., 2008; Wulczyn et al., 2007). Surprisingly, we observe that neurons increase with continued LIN28 expression and reduced let-7 levels. During development, high let-7 levels are generally associated with differentiation and low levels with less differentiation (Roush and Slack, 2008). Our data suggest that, at least under certain circumstances, low let-7 levels are indeed compatible with differentiation in some lineages.

The relative roles of LIN28A and LIN28B also remain unclear at present. Diverse vertebrates contain two or more LIN28 paralogs that are highly similar in sequence. Many developmental regulatory genes have duplicated since the origin of vertebrates (Zhang, 2003). In some cases, duplicate paralogs have overlapping, but partially distinct, functions. We have observed that *Lin28a* and *Lin28b* display generally similar, but not identical, expression patterns in the mouse embryo and slightly different expression profiles during neurogliogenesis in vitro, with LIN28A levels high and LIN28B levels low in undifferentiated cells (Fig. 2; data not shown). Although they may have identical targets and function redundantly, differences in LIN28A and LIN28B expression could define any distinct roles they might have in development.

Our major finding is that the effect of LIN28 on gliogenesis in this system is distinct from its effect on neurogenesis. LIN28 is one of a small set of proteins, and the only one that is not a transcription factor, whose activities when combined efficiently generate induced pluripotent stem (iPS) cells from differentiated cells (Yu et al., 2007). Because little is known about how LIN28 normally functions in development, one could infer that it is a ‘pluripotency factor’ that drives cells towards and/or maintains the undifferentiated state. Because LIN28 is expressed in a broad range of developing tissues and can promote the differentiation of neurons, we believe that LIN28 is not strictly a pluripotency factor. Here, we present evidence that the role of LIN28 in vertebrate cells is like that of its *C. elegans* homolog in promoting early-occurring fates over later-occurring fates in a regulated succession. The heterochronic mutants of *C. elegans* are unique among developmental mutants in that they alter the succession of diverse types of developmental events in a variety of cell lineages (Ambros and Horvitz, 1984). The conserved role of LIN28 might be to control the order of succession of a sequence of cell fates in a variety of tissues. The question that remains is: under what circumstances does LIN28 promote pluripotency versus differentiation? The usefulness of LIN28 in generating stem cells of clinical value depends on knowing how it functions in a developmental context.

Acknowledgements

We thank Kevin Kemper for plasmid construction and expert technical assistance; Marvin Wickens for the yeast three-hybrid system strains and plasmids; and Kevin Kemper, Bhaskar Vadla and Michael Henry for helpful comments on the manuscript. This work was supported by the Graduate School for Biomedical Sciences and University of Medicine and Dentistry of New Jersey.

Competing interests statement

The authors declare no competing financial interests.

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