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REVIEW

Lin28 and let-7: ancient milestones on the road from pluripotency to neurogenesis

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Abstract Beginning with their discovery in the context of stem cell fate choice in *Caenorhabditis elegans*, the microRNA (miRNA) let-7 and the RNA-binding protein Lin28 have been recognized as a regulatory pair with far-reaching impact on stem cell behavior in a wide range of organisms and tissues, including the mammalian brain. In this review, we describe molecular interactions between Lin28 and let-7 and the biological role that each plays in implementing stem cell programs that either maintain stem cell self-renewal and plasticity or drive lineage commitment and differentiation. For Lin28, considerable progress has been made in defining let-7-dependent and let-7-independent functions in the maintenance of pluripotency, somatic cell reprogramming, tissue regeneration, and neural stem cell plasticity. For the pro-differentiation activity of let-7, we focus on emerging roles in mammalian neurogenesis and neuronal function. Specific targets and pathways for let-7 have been identified in embryonic and adult neurogenesis, including corticogenesis, retinal specification, and adult neurogenic niches. Special emphasis is given to examples of feedback and feedforward regulation, in particular within the miRNA biogenesis pathway.

Keywords miRNAs · Stem cells · Post-transcriptional regulation · RNA-protein interactions · Neural differentiation

Introduction

During the development of the central nervous system (CNS), expression of the let-7 microRNA (miRNA) commences as rapidly proliferating, self-renewing neural stem cells (NSCs) exit the cell cycle and commit to the various neural lineages. In the adult, let-7 accumulates to high levels, particularly in post-mitotic neurons, making let-7 family members the most abundant miRNAs in the brain. In NSC niches, let-7 promotes differentiation and neuronal maturation by directing the post-transcriptional silencing of stem cell mRNAs. Targets for let-7 silencing include pluripotency factors, fetal oncogenes and suppressors of neural differentiation. In our view, an important function of let-7 is to feed back onto the miRNA pathway itself in order to set the stage for early overtly neurogenic miRNAs followed by miRNAs responsible for neuronal specification and outgrowth. The most prominent mechanism that excludes let-7 from stem cells involves the RNA-binding protein Lin28. Lin28 enforces stem-cell-specific gene expression patterns in part by directly interfering with the functional maturation of let-7. Our focus is on the relevance of this regulatory circuit for mammalian neurogenesis and neuronal function. We will therefore draw on, but not comprehensively cover, work on Lin28 and let-7 in other model organisms; for this, we suggest a number of excellent reviews (Büssing et al. 2008; Viswanathan and Daley 2010; Thornton and Gregory 2012). For more general treatments of miRNAs and their functions in neurogenesis, we recommend the contributions from Brüstle et al., Reh et al., and Abernathy et al. in this volume.

Because this review deals with the post-transcriptional regulation of let-7 expression and its function, it is important to begin with a general discussion of miRNA biogenesis and mode of action (summarized in Fig. 1). A more comprehensive treatment and citations of the original literature can be found in the reviews cited in the text (Bartel 2004; Fabian et al. 2010). miRNAs are short noncoding RNAs that are approximately 22 nucleotides (nt) in length and that act as antisense regulators by binding to and suppressing the expression of specific mRNAs (termed target mRNAs). Briefly, miRNAs are transcribed by RNA-polymerase II or, in rare

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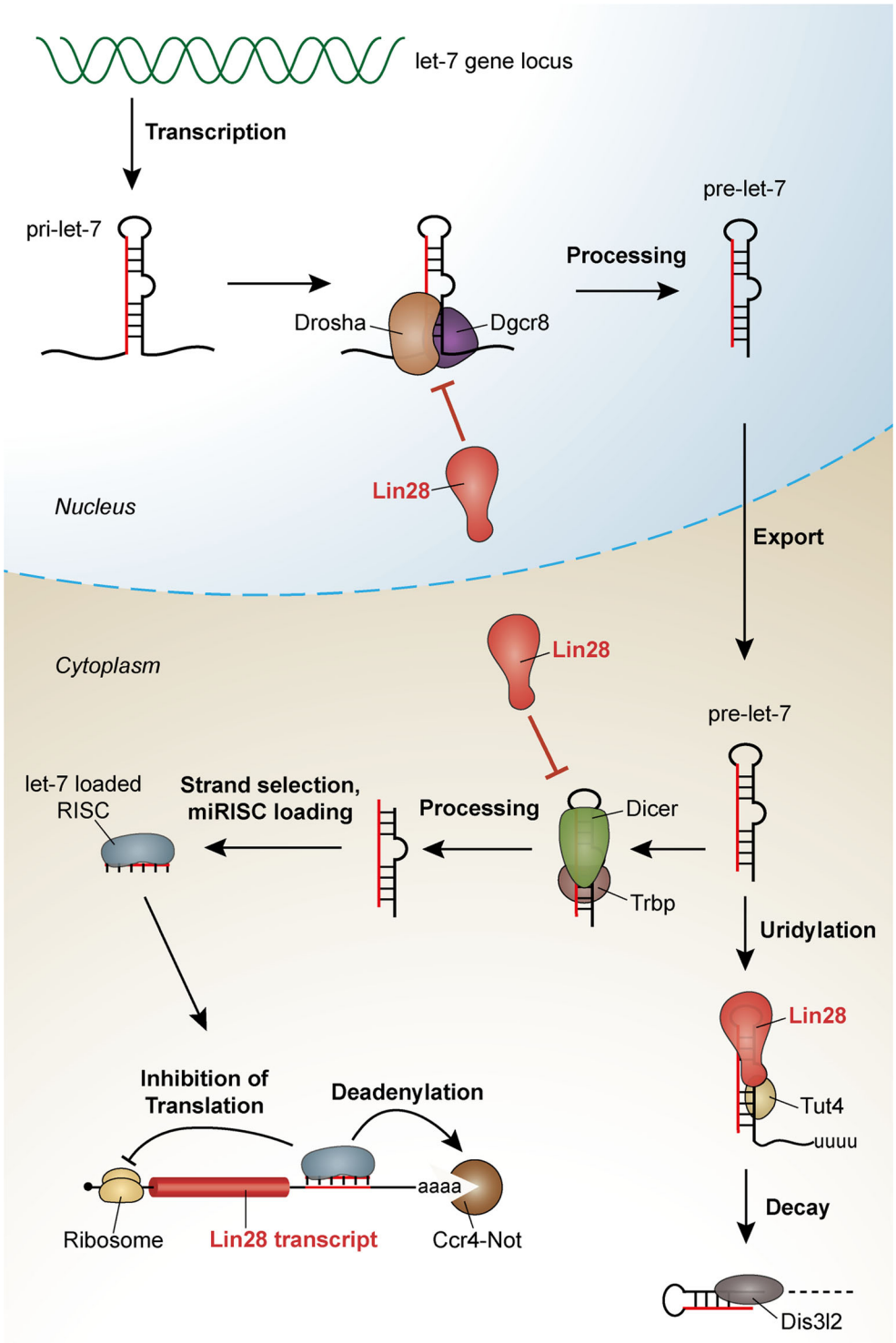

Fig. 1 Overview of the micro RNA (miRNA) pathway and its microRNA Silbentrennung immer ohne Bindestrich intersection with Lin28. Lin28 binds primary and precursor let-7 forms and inhibits their biogenesis via three distinct activities. First, Lin28 prevents the nuclear cleavage of pri-let-7 by Drosha and Dgcr8. Second, cytosolic processing of pre-let-7 by Dicer and Trbp is inhibited by Lin28. Third, Lin28 recruits Tut4 to the let-7 precursor leading to the 3' uridylation and subsequent decay of pre-let-7. Mature let-7 in turn is able to repress Lin28 expression via conserved seed matches in the *Lin28* 3'UTR (*red*)

cases, by RNA-polymerase III as long primary transcripts (termed pri-miRNAs). pri-miRNAs have to undergo a sequence of two processing steps to generate the mature active miRNA. In an initial step, pri-miRNAs are cleaved by the nuclear microprocessor complex, which is composed minimally of the RNase III enzyme Drosha and the accessory

protein Dgcr8. This releases a hairpin-structured RNA referred to as the precursor-miRNA (pre-miRNA). pre-miRNAs are approximately 70 nt long and are substrates for active export to the cytoplasm, where they are processed in a second step by the RNase III enzyme Dicer to yield an RNA duplex of approximately 22 nt in length (for a review, see V.N. Kim et al. 2009). One strand of this duplex is selectively loaded onto one of the Argonaute (Ago) proteins, the main effector of the miRNA-induced silencing-complex (miRISC; Meister 2013). This complex is thought to scan mRNAs, primarily in the 3' untranslated region (UTR), for sites of partial sequence complementary to the Ago-bound miRNA (Bartel 2009). Complementarity to the 5' end of the miRNA (the so-called seed region) is particularly important for productive engagement of the miRISC. Once bound, the miRISC inhibits target mRNA utilization by inhibiting translation initiation, while enhancing deadenylation and mRNA decay (Huntzinger and Izaurralde 2011). The fact that a single miRNA can directly repress hundreds of mRNAs underlines the regulatory impact of this class of RNAs and highlights the necessity for the tight control of individual miRNA expression and activity.

The first miRNAs were identified in *Caenorhabditis elegans* as regulators of developmental timing (for reviews, see Pasquinelli and Ruvkun 2002; Resnick et al. 2010). Mutations in the lin-4 and let-7 genes perturb the transitions between larval stages. Therefore, they were included in the group of "heterochronic genes"—master regulators of the temporal progression of developmental stages (Ambros and Horvitz 1984). The expression of these miRNAs increases during development, which in turn leads to the repression of other protein-coding heterochronic genes by direct targeting. This negative regulation by miRNAs is necessary to switch between developmental stages.

One important action of the let-7 miRNA is to negatively regulate the heterochronic gene *Lin41* and to some extent *Lin28*. Worms mutant for let-7 fail to progress from the last larval to the adult stage, ultimately leading to vulval bursting and death of the animal (Reinhart et al. 2000). Despite its role in worm-specific developmental pathways, the let-7 miRNA is highly conserved and is present throughout the bilateral animal phyla, a seminal discovery for the miRNA field (Pasquinelli et al. 2000). Gene duplications during the course of evolution have resulted in about a dozen different let-7 precursors in mammalian genomes. Nevertheless, after processing, the mammalian let-7a isoform is identical in sequence to its *C. elegans* paralog (Pasquinelli et al. 2000). All other let-7 family members share high sequence similarity and 100 % identity in the important seed region. This illustrates the strong selective pressure on the sequence of the miRNA, indicative of conserved function. Members of the let-7 family are the most abundant miRNAs in a variety of adult mammalian tissues, including the brain (Pena et al. 2009). During embryonic development of the mouse brain, let-7 levels continuously rise between embryonic day 12 (E12) and postnatal day 0 (P0) (Wulczyn et al. 2007; see below), much as they do during *C. elegans* larval development (Van Wynsberghe et al. 2011).

The counterpart of let-7, the *Lin28* gene, encodes a highly conserved RNA-binding protein. Two mammalian paralogs of *C. elegans* Lin28 have been identified, named Lin28a (frequently referred to as Lin28) and Lin28b. In agreement with the structural conservation of let-7 and Lin28, their genetic interaction is also conserved. Mammalian *Lin28a* and *Lin28b* mRNAs each possess let-7 binding sites in their 3'UTRs and are subject to regulation by let-7 (Moss and Tang 2003; Guo et al. 2006; Rybak et al. 2008). The ability of let-7 to repress Lin28 expression (and vice versa, as we will discuss below) is a key feature in the reciprocal relationship between the two. Lin28 is expressed in self-renewing stem cells (Moss and Tang 2003), is frequently overexpressed in tumor cells (Iliopoulos et al. 2009) and has been associated with increased regenerative capability (Shyh-Chang et al. 2013b). In contrast, mature let-7 is absent in stem cells (Houbaviy et al. 2003; Wulczyn et al. 2007) and antagonizes proliferation. let-7 has been shown to be a bona fide tumor suppressor (Johnson et al. 2007) and to suppress the self-renewal capacity of NSCs (Nishino et al. 2008).

This evolutionarily ancient interaction of Lin28 and let-7 represents a powerful switch that seems to be important in developmental progression. Given the importance of Lin28 and let-7 as heterochronic regulators in *C. elegans*, this regulatory pair is also likely to play a key role in mammalian development. In the following review, we will take a closer look at the functions of Lin28 and let-7 and their co-regulation in the context of neural differentiation and will discuss current views on the contribution each can make to the determination of fundamentally opposing cell fates.

Lin28: from nematode to mouse development

The *Lin28* gene was first identified in the mid 1980s as a heterochronic regulator of *C. elegans* development (Ambros and Horvitz 1984). *Lin28* mutant worms show precocious patterns of stem cell division and commitment in which many events characteristic of the second larval stage are omitted and progress directly to the third larval stage (Ambros and Horvitz 1984; Euling and Ambros 1996). This phenotype led to the gene's name, viz., *Lin28* for "abnormal cell lineage 28". More than a decade after this genetic discovery, the sequence and protein architecture of Lin28 were resolved, revealing two conserved functional domains: an N-terminal cold-shock domain (CSD) coupled to two retroviral-type CCHC-zinc knuckles (CCHCx2). This combination of domains is uniquely found in Lin28 and is not shared by any other protein in bilateral animals (Moss and Tang 2003). Both domains are known to mediate RNA binding, which indicates a function for Lin28 in the post-transcriptional regulation of gene expression.

Analysis of the phenotype of *Lin28* showed that it is epistatic to another heterochronic gene termed lin-4. Cloning of lin-4 led to the realization that it encoded a small noncoding RNA referred to originally as a small temporal RNA. The recognition of potential complementarity between lin-4 and the 3'UTR of *Lin28* (and a second heterochronic gene, *Lin14*) suggested a mechanism for the direct antisense regulation of downstream targets such as *Lin28* by lin-4 (Moss et al. 1997). When the second small heterochronic RNA, let-7, was discovered, similar considerations led to the proposal that let-7 might directly regulate *Lin28* (Reinhart et al. 2000). Much attention then turned to the exploration of Lin28, let-7, and miR-125, the mammalian lin-4 paralog, in mice and humans.

Initial characterization suggested that *Lin28* genes also function in early organismal development in vertebrates (Moss and Tang 2003). During mouse development, Lin28 protein is widely expressed until around E12.5 (see below), although expression is maintained in cardiac and skeletal muscle and in some adult epithelia such as the bronchi and the intestinal crypts (Yang and Moss 2003). Importantly, mRNA was detected in mouse embryocarcinoma (EC) and embryonic stem (ES) cells but not in standard cell lines from somatic tissues. In EC cells, expression was downregulated in response to differentiation with retinoic acid. In addition, sites of potential complementarity to let-7 and miR-125 were identified in mouse and human sequences (Moss and Tang 2003). Together with early findings showing that let-7 and miR-125 are upregulated in differentiating cells (Pasquinelli et al. 2000; Houbaviy et al. 2003; Sempere et al. 2004), this was the first indication that the genes of the *C. elegans* pathway would prove important for mammalian stem cell biology.

In the *C. elegans* heterochronic pathways, *Lin28* is more strongly associated with lin-4 than let-7, although *Lin28* mutants can partially compensate for the loss of let-7 (Reinhart et al. 2000). The potential for functional interplay between let-7 and Lin28 was suggested by their reciprocal expression patterns during the differentiation of mammalian stem cells (Wu and Belasco 2005). Evidence for a direct functional link arose from studies of let-7 regulation. Although the mature 22-nt forms of let-7 are absent in undifferentiated EC and ES cells (Houbaviy et al. 2003), let-7 genes are transcribed, and both primary and precursor forms of let-7 are present (Wulczyn et al. 2007). Extracts from undifferentiated cells have less activity in an in vitro processing assay using the let-7 precursor as substrate and contain let-7 precursor-specific binding activity visible by electrophoretic mobility shift assays. This complex disappears upon the differentiation of the cells and is absent in somatic cell lines (Wulczyn et al. 2007). In 2008, the groups of Richard Gregory and Scott Hammond reported that Lin28 interfered with let-7 maturation by inhibiting Drosha processing in the nucleus (Viswanathan et al. 2008; Newman et al. 2008), whereas our group and the group of Narry Kim presented evidence for Lin28 action at the level of cytoplasmic Dicer processing (Rybak et al. 2008; Heo et al. 2008; see Fig. 1).

How does Lin28 repress let-7 maturation?

An ES-cell-specific pre-let-7-binding complex can be eliminated by short interfering RNA (siRNA) treatment against Lin28 and reconstituted with purified Lin28 (Rybak et al. 2008). The recovery of Lin28 after affinity purification of EC and cancer cell lysates with immobilized pre-let-7 RNA provided additional evidence for a direct interaction (Viswanathan et al. 2008; Newman et al. 2008; Heo et al. 2008). Mutational analysis and competition assays have revealed that Lin28 binds pre-let-7 at a region named the precursor element (preE), which contains the loop of the precursor hairpin structure (Newman et al. 2008; Heo et al. 2008; Nam et al. 2011). The preE shows substantially higher sequence variability compared with the precursor part harboring the mature miRNA. Nevertheless, a consensus motif, highly enriched in let-7 family members, has been identified. This GGAG motif is always located 3' to the terminal loop and is present in a majority of vertebrate let-7 precursors but is absent in invertebrates. Mutational analysis has shown that the GGAG motif is essential for the repressive effect of Lin28 on pre-let-7 maturation (Heo et al. 2009). Artificial insertion of a GGAG motif into the precursor of miR-16, an miRNA that is normally not affected by Lin28, is sufficient to introduce binding and regulation by Lin28 (Heo et al. 2009). Nuclear magnetic resonance (NMR) spectroscopy and X-ray crystal structure analysis of Lin28 in complex with let-7 isoforms have given further insights into the way that binding and regulation is accomplished. Lin28 interacts with the let-7 precursor in a bipartite manner. Both RNA-binding domains contact the let-7 preE, but in a different manner and at different locations. The CSD recognizes structural informations and inserts itself into the terminal loop. The CCHCx2 module specifically interacts with a GGAG sequence motif more distal to the loop (Nam et al. 2011; Mayr et al. 2012). As determined by NMR spectroscopy, the protein region in between the two domains seems to be highly flexible to allow for the optimal arrangement and binding of both domains to preE-let-7 (Nam et al. 2011). This flexibility might explain the ability of Lin28 to bind let-7 family members that share the GGAG motif but whose precursor sequence and size differ considerably.

The structural analysis has also revealed that Lin28 binding partially denatures the stem-loop structure, thereby extending the loop at the expense of the terminal end of the stem (Nam et al. 2011; Mayr et al. 2012). This region of the precursor carries the internal Dicer cleavage site. Since Dicer needs double-stranded RNA substrates for activity (Hutvagner et al. 2001; Zhang et al. 2002), the denaturation of the pre-let-7 stem by Lin28 is probably responsible for the loss of Dicer cleavage observed in in vitro pre-let-7 processing assays with recombinant Dicer and Lin28 (Rybak et al. 2008; Heo et al. 2008). However, this inhibition is enforced by a second

mechanism: the recruitment of the terminal uridylyl transferase Tut4, which catalyzes the addition of multiple uridines to the 3' end of the precursor (Heo et al. 2009; Hagan et al. 2009; Yeom et al. 2011). The presence of such oligouridine tails make pre-let-7 molecules refractory to Dicer processing and mark them for rapid degradation (Heo et al. 2009), most likely by the 3' to 5' exonuclease Dis3l2 (Chang et al. 2013). siRNA treatment against Dis3l2 increases the pool of oligouridylated pre-let-7, whereas levels of mature let-7 remain constant. Unlike Tut4 and Lin28, Dis3l2 is apparently not necessary for the inhibition of Dicer (see Fig. 1). Interestingly, Dis3l2 loss-of-function leads to Perlman syndrome (Astuti et al. 2012), a severe developmental disturbance with similarities to Lin28 gain-of-function phenotypes of overgrowth and tumorigenesis detailed below.

Compared with the interference of cytoplasmic processing by Dicer, considerably less is known about the way that Lin28 inhibits the initial nuclear cleavage event by Drosha and the Microprocessor (Viswanathan et al. 2008; Newman et al. 2008; see Fig. 1). Unlike mammalian ES cells in which the cytoplasmic mechanism might predominate, nuclear inhibition has been found to be the major effect of Lin28 in *C. elegans* (Van Wynsberghe et al. 2011). Lin28b has been proposed to act preferentially in the nucleus compared with Lin28a (Piskounova et al. 2011); given the similarity in the mode of let-7 binding between the two proteins, isoform-specific associated factors might be responsible for the difference in activity. Nuclear roles for Lin28 are likely to gain in importance, commensurate with the growing interest in miRNA functions in the nucleus.

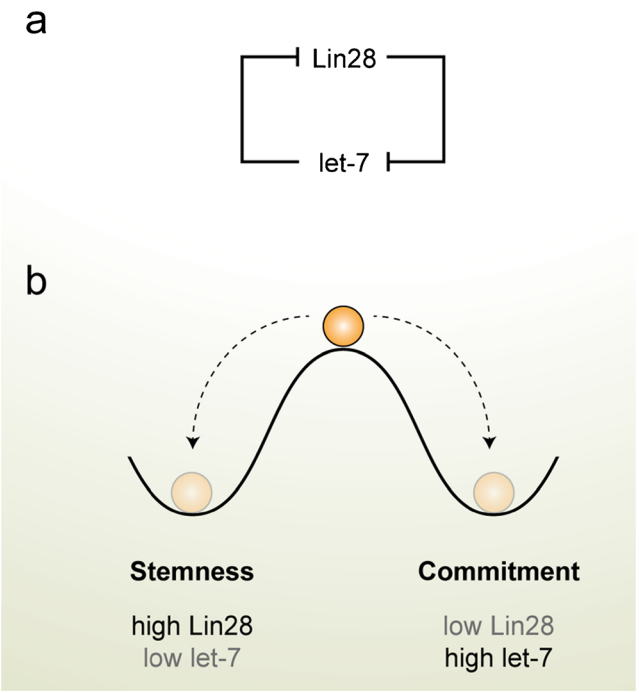

Fig. 2 Representation of regulatory interactions between Lin28 and let-7. **a** Lin28 and let-7 form a double-negative feedback loop by virtue of mutual inhibition. **b** This regulatory circuit builds a bi-stable switch with two mutually exclusive states: high Lin28 and low let-7 levels in stem cells or low Lin28 and high let-7 levels in committed cells. Factors that initially shift the equilibrium beyond the transition state during differentiation remain to be identified

Lin28 and let-7 form a regulatory circuit

An important additional feature of the Lin28 pathway is that the mRNAs for both proteins contain let-7 binding sites, making the expression of both proteins sensitive to the level of let-7 in the cell (Rybak et al. 2008; see Fig. 1). Because any reduction of Lin28 will in turn lead to less repression of let-7 processing, and therefore more let-7 and less Lin28, the system is self-amplifying. Of course, the reverse is also true: any increase in Lin28 will lead to a reduction in let-7 processing and therefore less let-7 and more Lin28. The self-reinforcing nature of this feedback mechanism represents a bi-stable switch with two mutually exclusive outcomes: Lin28*on*—let-7*off* or Lin28*off*—let-7*on* (see Fig. 2). Since this regulatory concept is conserved in evolution and bears an extensive developmental impact in *C. elegans*, there is considerable interest in determining the role of this pathway in mammalian development.

Transgenic mouse models for Lin28 have begun to address these questions. Mice constitutively overexpressing Lin28 are viable but manifest a tissue overgrowth phenotype and show a delayed onset of puberty (Zhu et al. 2010). Gain- and loss-of-function mutations of several let-7 targets (*Imp1/Igf2bp1*, *Hmga2*, *c-Myc*, see Table 1) display developmental phenotypes consistent with Lin28 action via let-7 in growth control (Trumpp et al. 2001; Hansen et al. 2004; Weedon et al. 2007). *Lin28a* knockout models show opposite, yet more severe, phenotypes with over 90 % of newborn animals dying within 1 day after birth. Body mass in the few surviving animals is reduced by 30–50 %. Similar observations have been made in the *Lin28b* knockout mouse, although the phenotype is milder and more restricted to postnatal development. Deletion of both isoforms leads to embryonic lethality between E9.5 and E12.5 (Shinoda et al. 2013).

The model of Lin28 and let-7 as bi-stable switch is appealing in its robustness and simplicity (see Fig. 2). Nevertheless, as is becoming increasingly clear, this interaction is only the core of a more complex regulatory circuit. Transcriptional regulation is likely to affect the balance between *Lin28* and let-7. In tumor cells, c-Myc and NF-κB have been shown to promote transformation and oncogenesis by transactivating Lin28b expression and, as a consequence, reducing mature let-7 levels (Chang et al. 2009; Iliopoulos et al. 2009). The finding that *c-Myc* and its homolog *n-Myc* are, in turn, both targets of the let-7 miRNA (H.H. Kim et al. 2009; Melton et al. 2010; Molenaar et al. 2012) suggests that this extension of the circuit is also subject to feedback regulation (for

Table 1 Targets of let-7 and their functions in neural development (*SVZ* subventricular zone, *iPS cells* induced pluripotent stem cells, *miRNA* micro RNA, *mTOR* mammalian target of rapamycin)

let-7 target	Function	Reference
Ascl1	• Dedifferentiation of Müller glia cells into retinal progenitors	Ramachandran et al. 2010
	• Maintenance of neural progenitor proliferation and neurogenic potential	Cimadamore et al. 2013
Hmga2	• Adult stem cell plasticity in the SVZ	Nishino et al. 2008
	• Fetal vs adult stem cell identity	Yuan et al. 2012; Copley et al. 2013;
	• Oncogenesis	Mayr et al. 2007; Lee and Dutta 2007; F. Yu et al. 2007
Hspd1	• Dedifferentiation of Müller glia cells into retinal progenitors	Ramachandran et al. 2010
Igf1r, Insr, Irs2	• Regulation of the mTOR pathway	Frost and Olson 2011; Shinoda et al. 2013
Imp1	• Expansion of neural stem cells during cortical development	Nishino et al. 2013
Lin28a	• Regulation of proliferation and neurogenesis in neural precursors	Cimadamore et al. 2013
	• Dedifferentiation of Müller glia cells into retinal progenitors	Ramachandran et al. 2010
	• Maintenance of stemness	Rybak et al. 2008
	• Reprogramming into iPS cells	Melton et al. 2010
Lin28b	• Maintenance of early progenitors in retinal neurogenesis	La Torre et al. 2013
Lin41	• Maintenance of stemness, inhibition of miRNA activity	Slack et al. 2000; Rybak et al. 2009
	• Axonal regeneration in larval vs adult neurons	Zou et al. 2013
c-Myc	• Dedifferentiation of Müller glia cells into retinal progenitors	Ramachandran et al. 2010
	• Pluripotency gene networks and oncogenesis	Koscianska et al. 2007; Kumar et al. 2007
n-Myc	• Development of neuroblastomas	Molenaar et al. 2012
	• Maintenance of stemness	Melton et al. 2010
Pax6	• Dedifferentiation of Müller glia cells into retinal progenitors	Ramachandran et al. 2010
Protogenin	• Maintenance of early progenitors in retinal neurogenesis	La Torre et al. 2013
Sal4	• Maintenance of stemness	Melton et al. 2010
Tlx	• Cell cycle progression of neural stem cells	Zhao et al. 2009

a review, see Büssing et al. 2008). However, an answer to the important question of how the balance between Lin28 and let-7 levels is controlled during development remains elusive. Apart from transcriptional control, additional miRNAs might also be involved. Both vertebrate *Lin28* mRNAs possess a number of additional miRNA-binding sites in their 3'UTRs, of which miR-9, miR-30, and miR-125 are known to be functional (Rybak et al. 2008; Zhong et al. 2010).

Lin28 functions beyond let-7

So far, the inhibitory effect on let-7 miRNA biogenesis is the best-studied regulatory function of Lin28. Although let-7 is a key target of Lin28, several results indicate important additional let-7 independent functions. For example, muscle-specific loss of Lin28 in mice results in insulin insensitivity and impaired glucose uptake, although let-7 levels remain constant (Zhu et al. 2011). The influence of Lin28 on metabolic pathways has been attributed to the direct regulation of mRNA translation by Lin28 (Shinoda et al. 2013). The first evidence that Lin28 can directly bind mRNAs and stimulate their translation was reported for the insulin-like growth factor two (*Igf2*) mRNA (Polesskaya et al. 2007) and then extended to additional mRNAs (Lei et al. 2012). Two recent studies have made use of RNA-protein crosslinking and deep-sequencing (the CLIP-Seq method) to determine the genome-wide RNA-binding repertoire of Lin28. These experiments have not only verified the known association of Lin28 with pre-let-7 miRNAs, but also revealed an abundance of mRNAs bound to Lin28 (Wilbert et al. 2012; Cho et al. 2012). Bound RNAs are enriched for 3'UTR sequences that contain the same GGAG sequence motif responsible for the recognition of the let-7 precursor. Apart from this agreement, the two studies differ in their results and show primarily either enhancement (Wilbert et al. 2012) or inhibition (Cho et al. 2012) of mRNA translation upon Lin28 binding. Apparently, the outcome of Lin28 binding varies depending on the sequence and context of the substrate mRNA. For example, the expression of multiple splicing factors has been shown to be positively regulated by Lin28 (Wilbert et al. 2012), but the translation of secretory and membrane proteins seems to be inhibited by Lin28 (Cho et al. 2012).

The Lin28-let-7 axis in pluripotency

As previously noted, Lin28 is highly expressed in early embryonic tissues and in pluripotent ES and EC cells (Moss and

Tang 2003; Yang and Moss 2003). Stem cell specificity of Lin28 has been attributed to direct transcriptional regulation by the core ES cell factors Oct4, Sox2 and Nanog, based on ChIP-Seq assays (Marson et al. 2008). There is also evidence that the pluripotency factor c-Myc directly transactivates Lin28b transcription (Chang et al. 2009). Additionally, Sox2 physically interacts with Lin28a in a nuclear protein-protein complex (Cox et al. 2010), suggesting that Lin28 plays a direct role within the pluripotency network. Indeed, a cocktail of Lin28 together with Oct4, Nanog and Sox2 is sufficient to reprogram human fibroblasts into induced pluripotent stem (iPS) cells (J. Yu et al. 2007). Compared with the more widely used Yamanaka cocktail of pluripotency factors (OSKM: Oct4, Sox2, Klf4 and c-Myc; Takahashi and Yamanaka 2006), the activity of Lin28 is thought to match c-Myc most closely. In their original characterization, Thomson and co-workers found that Lin28 enhanced but was dispensable for iPS cell generation (J. Yu et al. 2007). However, subsequent examination at the level of single cells identified Lin28 expression as an early and predictive event in what is described as the stochastic phase of reprogramming (Buganim et al. 2012). The stochastic phase is characterized by the heterogeneous activation of a small group of early genes that include *Lin28* (namely *Lin28*, *Esrrb*, *Utf1* and *Dppa2*). This early phase lays the groundwork for an hierarchical deterministic phase under the control of Sox2 in which full pluripotency is achieved. The importance of *Lin28* and the other stochastic phase genes is emphasized by the finding that they can be used instead of the Yamanaka factors to drive somatic cells to pluripotency (Buganim et al. 2012). In these assays, Lin28 is reported to increase the rate of cell proliferation (Hanna et al. 2009). However, the relative activities of reprogramming factors such as Lin28 might be dependent on the assay conditions, as Yamanaka's group found that Lin28 is less important for initiation is but critical for the maturation of fully reprogrammed iPS cell colonies that can be stably expanded (Tanabe et al. 2013).

At the molecular level, Lin28 might act at multiple levels to enforce pluripotency. In addition to the physical interaction with Sox2 mentioned above, Lin28 has been proposed to act as a specific positive regulator of the Oct4 mRNA (Qiu et al. 2010; Peng et al. 2011). However, we will focus our attention on the role of Lin28 in the regulation of the let-7 miRNA. As discussed in the previous section, Lin28 acts as a specific inhibitor of let-7 maturation to prevent the initiation of the prodifferentiation program mediated by let-7 (see Fig. 1). The Blelloch group has used ES cells depleted for miRNAs by the deletion of the miRNA processing co-factor Dgcr8 as a tool that enables the functional analysis of individual miRNAs. They were able to show that let-7 and ES-cell-specific miRNAs (e.g., the miR-290 cluster) have opposing effects on pluripotency. Introduction of stem-cell-specific miRNAs such as miR-294 or miR-130b into miRNA-deficient *Dgcr8*$^{-/-}$ ES cells enhances the expression of pluripotency markers including Lin28, Sal4 and Myc. Most likely, these miRNAs act indirectly by targeting an unidentified common repressor (or set of repressors) of pluripotency. let-7 acts more directly and has been shown to silence mRNAs for *Lin28*, *Sal4* and *n-Myc*. Moreover, in the absence of the normal population of stem cell miRNAs in *Dgcr8*$^{-/-}$ ES cells (but not in wild-type ES cells), the overexpression of let-7 is sufficient to suppress self-renewal. Accordingly, antagonizing let-7 activity improves the efficiency of the dedifferentiation of somatic cells to iPS cells, thus mimicking the activity of Lin28 (Melton et al. 2010; Worringer et al. 2014).

Taken together, these results define the role of the bi-stable double-negative feedback loop between Lin28 and let-7. High Lin28 levels suppress let-7 and promote self-renewal and pluripotency, and conversely, high let-7 levels inhibit Lin28 and promote differentiation (see Fig. 2). A remarkable example of this regulatory loop has been described in the hematopoietic system. An inverse expression pattern of Lin28 and let-7 has been found to be a key molecular feature distinguishing fetal from adult hematopoietic stem cells (HSCs) in mice and humans. As a master regulator of fetal HSC identity, ectopic Lin28b alone is capable of reprogramming adult HSCs into a fetal-like state by inhibiting let-7 maturation and derepressing the let-7 target gene *Hmga2* (Yuan et al. 2012; Copley et al. 2013).

The Lin28-let-7 axis in tissue regeneration and repair

The ability of Lin28 to induce self-renewal in differentiated cells might also be useful during tissue regeneration, which involves (among other processes) dedifferentiation to replace lost or damaged cells. In mammals, regeneration capacity is highest in juvenile animals and declines with age. Genome-wide association studies (GWAS) for age at menarche discovered a variant within Lin28b as the first genetic marker associated with the timing of many aspects of puberty in humans (Ong et al. 2009). To study the role of Lin28 in puberty, Daley and co-workers developed an inducible Lin28a transgenic mouse model (iLin28 Tg). Consistent with the human GWAS findings for Lin28b, Lin28a overexpression delayed the onset of puberty and promoted growth. Analysis of metabolic and endocrine mechanisms of overgrowth in these transgenic mice revealed increased glucose metabolism and insulin sensitivity. This metabolic shift was then shown to protect against diabetes induced by a high fat diet during aging (Zhu et al. 2010, 2011). By contrast, conditional deletion of *Lin28a* and *Lin28b* or overexpression of let-7 caused dwarfism and glucose intolerance. let-7-mediated repression of multiple components of the insulin-phosphatidyl-inositol-3-kinase-mTOR pathway, including *Igf1r*, *Insr*, and *Irs2*, was demonstrated to be partly responsible for these metabolic effects (Frost and Olson 2011;

Shinoda et al. 2013). Follow-up studies with the iLin28 Tg mouse line revealed that the re-expression of Lin28 was sufficient to reprogram the developmental age of tissues and enhance their postnatal regenerative capacity (Shyh-Chang et al. 2013b). Activation of Lin28a promoted hair regrowth, improved digit repair after amputation and accelerated ear wound healing. Lin28 acted at least in part by repressing let-7, leading to increased mesenchymal cell proliferation and enhanced repair. However, Lin28 also acted independently of let-7 by directly regulating the translation of mRNAs including multiple metabolic enzymes such as phosphofructokinase and pyruvate dehydrogenase. The net effect of Lin28 is to increase glycolysis and oxidative phosphorylation, which together are able to increase cell migration and proliferation, at least in vitro. Support for the relevance of this effect in vivo has been obtained by blocking the beneficial effects of Lin28a on tissue regeneration by using inhibitors of oxidative phosphorylation in the iLin28 Tg model (Shyh-Chang et al. 2013b).

Recent advances in metabolomics indicate that modulation of the bioenergetic pathways used by cells influences not only regenerative capacity, but also cell fate and lineage specification (Vander Heiden 2009; Folmes et al. 2011; Folmes et al. 2012; Shyh-Chang et al. 2013a). For instance, ES and iPS cells show reduced mitochondrial oxidative phosphorylation and elevated aerobic glycolysis (Kondoh et al. 2007; Prigione et al. 2010; Folmes et al. 2011). Despite the lower efficiency of ATP production in comparison with oxidative phosphorylation, glycolysis provides a source of biosynthetic substrates that are essential for cell growth. Conversely, the activation of mitochondrial oxidative metabolism to maximize ATP generation can prime stem cells to differentiate (Chung et al. 2007). Unlike proliferative ES cells, most adult stem cells, including NSCs, are largely quiescent. NSCs ensure life-long tissue renewal capacity by suppressing oxidative phosphorylation to avoid cellular damage from reactive oxygen species (Renault et al. 2009). In contrast, proliferative neural progenitors show upregulated glycolysis (Gershon et al. 2013), lipogenesis (Knobloch et al. 2013) and oxidative phosphorylation (Renault et al. 2009). Metabolic regulation is increasingly thought to reinforce, or even prime, neural differentiation programs in stem cell niches (for a review, see Shyh-Chang 2013a). Additional work will be required to assess the relevance of metabolic regulation for the activity of Lin28 and let-7 in neural differentiation.

Although the work of the Daley group and their collaborators has highlighted the ability of Lin28 and let-7 to influence metabolic pathways directly, abundant evidence has been presented for additional regulatory roles. In the context of growth regulation, Lin28 has been shown to bind mRNAs of diverse cyclins and cyclin-dependent kinases to accelerate cell cycle progression (Xu et al. 2009; Li et al. 2012; Hafner et al. 2013). In addition, Lin28-associated mRNAs are enriched for RNA-binding proteins, including ribosomal proteins. Increased ribosome synthesis might also contribute to the ability of Lin28 to promote cellular growth (Peng et al. 2011). Finally, the direct effects of Lin28 on translational control occur in the context of the derepression of let-7 targets. Together, the regulatory pair of Lin28 and let-7 integrates the biosynthetic and metabolic demands of rapid stem cell proliferation. Disregulation of this circuit has not only been implicated in tumors, including common malignancies of the CNS, but might also present the opportunity to improve the regenerative capacity and metabolic resilience of cells and tissues of the nervous system as organisms age.

The Lin28-let-7 axis in neurogenesis

Having discussed Lin28 and let-7 broadly, we will now focus on the specific roles of each in neurogenesis, beginning with let-7. In addition to the temporal regulation of let-7 discussed above, another significant feature of let-7 and miRNAs in general is their lineage specificity (Lagos-Quintana et al. 2002). For example, miRNA populations in ES cells are dominated by a limited set of mature miRNAs such as the miR-200, miR-291-4 and miR-302 clusters (Houbaviy et al. 2003; Suh et al. 2004). During neural differentiation and embryonic brain development, miRNA expression is upregulated in terms of both diversity and abundance (Miska et al. 2004; Sempere et al. 2004; Smirnova et al. 2005). Similar expression dynamics have been found in comparisons of let-7 family members with several highly expressed, brain-enriched miRNAs (e.g., miR-9, miR-124, miR-125, and miR-128; Smirnova et al. 2005; Wulczyn et al. 2007; Landgraf et al. 2007). These early studies are in general agreement with later experiments involving deep sequencing in order to catalog miRNA expression exhaustively during brain morphogenesis. In one comprehensive study of the adult human and macaque brain, four of the five most highly expressed miRNAs were let-7 family members, and all eight let-7 family members ranked in the top 25, together accounting for close to 15 % of the total population of mature miRNAs (Shao et al. 2010). Although the quantification of deep sequencing results might be subject to systematic errors, the let-7 miRNA family clearly exerts a powerful influence on gene expression in the CNS.

Many studies have addressed the global importance of miRNAs for brain development and function by targeting components of the miRNA biogenesis pathway (i.e., Drosha, Dgcr8, Dicer or Argonautes). This field is too extensive to review here, but recent reviews are available (McNeill and Van Vactor 2012; Sun et al. 2013; Bian et al. 2013). To date, difficulties have been experienced in attributing phenotypes observed in miRNA biogenesis knockouts to the specific loss

of let-7. The presence of twelve let-7 genes in the mouse genome has precluded targeted deletion thus far, although lines that allow reduced expression by targeting individual let-7 gene clusters or inducible overexpression are increasingly becoming available (Zhu et al. 2010; Frost and Olson 2011; Park et al. 2012). Therefore, even in the more tractable *C. elegans* and *D. melanogaster* models, in which deletion alleles for let-7 family members are available, specific roles for let-7 are just beginning to be studied (Abbott et al. 2005; Sokol et al. 2008). Nevertheless, a number of strategies have successfully addressed the question of specific let-7 functions in the nervous system and, in particular, neurogenesis. One such strategy is to characterize mRNAs targeted by let-7 as it is upregulated during neurogenesis. This can be performed experimentally or by mining bioinformatic target site predictions and has succeeded in identifying a number of important targets including *Lin41*, *c-Myc*, *Hmga2*, *Tlx*, *Lin28a* and *Lin28b*. Several of these interactions have been mentioned in the context of stem cell maintenance, but specific roles in neurogenesis have also been described (see Table 1).

Neurodevelopmental genes targeted by let-7

Lin41 is the original let-7 target gene, first described in a screen for genes downstream of let-7 in the *C. elegans* heterochronic pathway (Reinhart et al. 2000; Slack et al. 2000). Loss-of-function mutants in Lin41 cause the precocious terminal differentiation of seam cells, a distinct group of stem cells underlying the larval cuticle (Slack et al. 2000). This is the opposite of the reiterating seam cell divisions caused by loss of let-7. The mouse *Lin41* gene (also referred to by the gene symbol *Trim71* in mouse and humans) has been disrupted by gene-trap mutation, and the resultant mice display embryonic lethality between E9.5 and E13.5 (depending on the strain) and failure of neural tube closure (Maller Schulman et al. 2008). When analyzed at E9.5, neuroepithelial cells throughout the neural tube in *Lin41*$^{-/-}$ mice proliferate less and differentiate prematurely (Chen et al. 2012), consistent with the phenotype in *C. elegans*. Targeting of the *Lin41* 3'UTR by let-7 was first described in *C. elegans* (Slack et al. 2000; Bagga et al. 2005) and is evolutionarily conserved in zebrafish, *Xenopus* and mammals (Lin et al. 2007; O'Farrell et al. 2008; Rybak et al. 2009). In mouse, Lin41 and let-7 show reciprocal expression patterns in stem cell niches of the developing embryo and in adult tissues (Schulman et al. 2005; Rybak et al. 2009). Furthermore, Lin41 has been shown to suppress miRNA activity and to cooperate with Lin28 in suppressing let-7 activity in stem cells (Rybak et al. 2009). Like *Lin28*, *Lin41* has recently been demonstrated to be an iPS cell gene whose reactivation is essential for the suppression of prodifferentiation genes (Worringer et al. 2014).

In its role as a tumor suppressor, let-7 has been shown to target various cyclins and cyclin-dependent kinases to slow down cell cycle progression (Johnson et al. 2007; Dong et al. 2010; for an excellent review, see Büssing et al. 2008). Another target, c-Myc, is relevant for pluripotency gene networks and cancer. The introduction of let-7 into cancer cells represses c-Myc translation (Koscianska et al. 2007; Kumar et al. 2007). During neurogenesis, this repression is mediated by increasing levels of let-7. In the differentiating embryonic neuroepithelium, downregulation of c-Myc is reinforced by Trim32 (an ortholog of Lin41/Trim71). Upon asymmetric progenitor cell division, the Trim32 protein and mRNA are preferentially distributed to the daughter cell destined to become a neuron (Schwamborn et al. 2009; Kusek et al. 2012). Trim32 has two activities: it directly interacts with and ubiquitinates c-Myc to stimulate its proteolytic degradation and also interacts with and enhances let-7 activity by an unknown mechanism (Schwamborn et al. 2009).

Another let-7 target is Tlx, a forebrain-restricted transcription factor that is expressed in early embryonic development (until E13.5), testis and adult stem cell niches (Monaghan et al. 1995). It maintains stem cell plasticity and regulates the timing of neurogenesis in the cortex (Roy et al. 2004). Tlx promotes cell cycle progression of neural progenitors by recruiting histone deacetylases to the promoters of cell cycle inhibitors such as p21 and Pten, thereby repressing their transcription (Sun et al. 2007). Another target for Tlx-mediated transcriptional repression is the neurogenic miRNA miR-9 (Zhao et al. 2009). During the differentiation of adult NSCs, Tlx is downregulated by several members of the let-7 family. Ectopic overexpression of let-7 acts via Tlx inhibition to reduce the proliferative capacity of adult NSCs and promote neural and glial differentiation, as determined by an increase of cells positive for Tuj1 and GFAP (glial fibrillary acidic protein) (Zhao et al. 2010, 2013). Once released from Tlx-mediated inhibition, miR-9 might assist neural differentiation by the feedback inhibition of Tlx and by targeting REST and its co-factor Co-REST, a master inhibitor of neuronal gene transcription highly expressed in uncommitted stem cells (Packer et al. 2008; Zhao et al. 2009).

The oncofetal mRNA-binding protein Imp1 represents another let-7 target with direct involvement in mammalian neurogenesis. Mice lacking Imp1 show significantly smaller cerebral cortices compared to wild-type animals. This reduction of cortical thickness is attributable to a decreased self-renewal capacity of *Imp1*$^{-/-}$ NSCs and a precocious neuronal maturation of Pax6^{+} neural precursors. The let-7-induced downregulation of Imp1 is necessary for the developmental transition from highly proliferative fetal NSCs to the more quiescent stem cells found in adult animals. This let-7-mediated cell fate switch during mammalian corticogenesis resembles the ancient role of let-7 in the *C. elegans* heterochronic pathway (Nishino et al. 2013; see Table 1 for a summary of all let-7 targets).

Regulation of Lin28 and let-7 in neurogenesis

Targeted disruption of miRNA biogenesis has demonstrated that global loss of miRNAs prevents the execution of neurogenic programs. At the same time, more specific roles for individual miRNAs, and let-7 in particular, have begun to be revealed. Among the most interesting insights provided by studies on let-7 relate to the question: "Who regulates the regulators?" In contrast to their downstream targets and effects, considerably less is known about the mechanisms that control the expression and activity of neurogenic miRNAs such as let-7 during development. The neurogenic miRNAs miR-124 and miR-9 are the best-studied examples of the integration of miRNAs into transcriptional control circuits involved in mammalian NSC specification (for an excellent review, see Cochella and Hobert 2012). Overlapping the feedforward circuit just described between Tlx, miR-9 and REST, the neurogenic miRNA miR-124 is also subject to regulation by REST. In NSCs, the miR-124 gene locus is occupied and repressed by the REST-complex (Conaco et al. 2006). Components of the REST-complex, in turn, are post-transcriptionally inhibited by miR-124 as its levels rise during the course of neurogenesis (Visvanathan et al. 2007). As detailed in the previous sections, Lin28 and let-7 form a similar double-negative feedback loop, but one that operates primarily at the post-transcriptional level (see Fig. 2). Several recent studies have explored the roles of Lin28a and Lin28b in the control of neurogenesis via their ability to suppress the pro-differentiation activity of let-7.

Expression of the Lin28a protein is not specific for the nervous system but is widespread early in development, becoming more restricted to diverse epithelial stem cell niches as development progresses (Yang and Moss 2003). In the neuroepithelium, Lin28a is present throughout the neural tube until E11.5, after which expression drops (see Fig. 3a). The loss of the protein roughly coincides with the transition to radial glia progenitor populations, which do not express Lin28a (Yang and Moss 2003; Cimadamore et al. 2013). This expression pattern suggests that the Lin28 to let-7 switch is an early event triggered at the onset of neurogenesis (see Fig. 3a). One caveat is that considerably less is known about the expression pattern of the Lin28b homolog. Data from genome-wide expression profiling during neural development suggest that Lin28b expression is also downregulated during the course of neurogenesis, but that the reduction is somewhat delayed compared with Lin28a (Hartl et al. 2008).

One transcription factor that has been placed upstream of Lin28 and let-7 in NSCs is Sox2 (Cimadamore et al. 2013; see Fig. 3b). Lin28 expression has been demonstrated in several distinct $Sox2^{+}$ populations including cultured human ES-cell-derived NSCs, mouse E11 neuroepithelium and adult mouse NSCs present in the subgranular zone of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles.

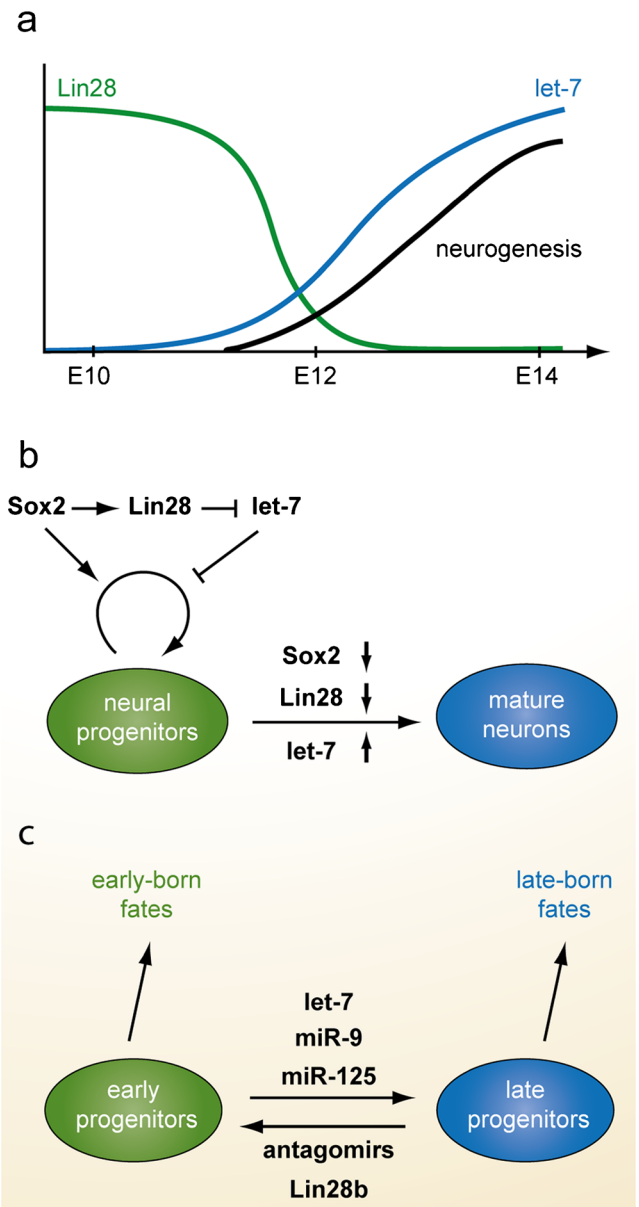

Fig. 3 The Lin28-let-7 axis in neurogenesis. **a** During telencephalic neurogenesis Lin28 mRNA and protein levels rapidly decline after embryonic day 12.5 (E12.5). As a consequence, mature let-7 levels increase. The targeting of inhibitors of neurogenesis by let-7, in turn, allows neurogenic differentiation. **b** Sox2 transcriptionally activates Lin28 expression in embryonic and adult neural progenitors. Lin28 in turn represses let-7, thereby ensuring self-renewal and cell cycle progression of neural progenitors. Upon in vitro differentiaton, Sox2 and Lin28 levels drop. Consequently, most let-7 species are upregulated (Cimadamore et al. 2013). **c** In mouse retina, early-born neuronal cell fates are adopted by the progeny of early progenitors and late-born neuronal and glial cell fates by the progeny of late progenitors. Lin28b is required for early progenitor identity, and the induction of neurogenic microRNAs (let-7, miR-9, miR-125) at E16.5 drives the transition from early to late progenitor identity (La Torre et al. 2013)

Conditional deletion of Sox2 in vivo or short-hairpin RNA-mediated depletion in vitro results in decreased levels of Lin28 expression. Apparently, Sox2 is bound to the Lin28

promoter and directly induces Lin28 expression through the recruitment of histone deacetylase complexes (Cimadamore et al. 2013). Sox2 is a well-known essential regulator of NSC proliferation and neurogenesis (Ferri et al. 2004). Depletion of Sox2 in the adult neurogenic areas results in a complete loss of neurogenesis (Favaro et al. 2009). Interestingly, part of the neurogenic function of Sox2 seems to be mediated by Lin28. Overexpression of Lin28 partially compensates for the adverse effects of Sox2 depletion on cultured NSC proliferation and apoptosis. Lin28 probably acts, at least in part, by inhibiting let-7, because ectopic let-7 expressed from lentiviral vectors also reduces the proliferation of cycling NSCs and strongly increases apoptosis during neural differentiation (see Fig. 3b). In addition, let-7 has been shown to downregulate two neurogenic basic-helix-loop-helix (bHLH) transcription factors in this model: Ascl1/Mash1 and Neurogenin. In the case of Ascl1, this regulation appears to be mediated in part by direct let-7 targeting of the *Ascl1* 3'UTR. This suggests that at least part of the requirement for Sox2 in neurogenesis is to suppress let-7 by maintaining Lin28 expression (Cimadamore et al. 2013) implying that premature let-7 expression is detrimental to early stages of neurogenesis. One test of this notion would be to determine to what degree Lin28 can compensate for the defects in the neurogenic program observed in conditional *Sox2* mutant mice. More information on the regulation of the Sox family and the interaction between miRNAs and proneural transcription factors is provided in the contributions from Reiprich and Wegner and from Reh et al., respectively, in this volume.

Lin28 and let-7 and the timing of retinal neurogenesis

Together with the work on *Imp1* discussed above, perhaps the most clear-cut parallels to the traditional view of Lin28 and let-7 as heterochronic regulators of stem cell fate have been obtained in studies of retinal neurogenesis (La Torre et al. 2013, see Fig. 3c). Retinal progenitors progress through distinct maturation stages during the generation of the stratified retinal neuroepithelium. Deletion of Dicer leads to a heterochronic phenotype in that early cell fates (outer ganglion and horizontal cells) are reiterated at the expense of late cell types (bipolar cells and Müller glia; Georgi and Reh 2010; Davis et al. 2011; Iida et al. 2011). In more recent work, the onset of let-7 expression, together with miR-125 and miR-9, has been shown to correlate with the transition phase between E12 and P0, when progenitors should shift production from early to late cell types (La Torre et al. 2013). Blocking these three late progenitor-specific miRNAs with a cocktail of specific antagomirs partially replicates the temporal shift seen in the *Dicer* knockout by inappropriately reiterating the production of early cell fates. The reverse experiment has also been successful: in *Dicer* knockout retinas lacking all miRNAs, electroporation of synthetic analogs of the three miRNAs is sufficient to rescue the loss of late cell fates. By examining mRNAs misregulated in *Dicer* knockout retinas followed by filtering for miRNA-binding sites, two downstream targets common to all three miRNAs were identified: *Lin28b* and *Protogenin*. Ectopic expression of Lin28b and Protogenin is sufficient to induce the reiteration of early progenitor cell fate (i.e., heterochronic ganglion cell production) in E16 retina. The classic heterochronic let-7 target, *Lin41*, is also upregulated in *Dicer* knockouts, but this was not studied further. This study by Reh and co-workers represents perhaps the clearest demonstration of an intrinsic miRNA-mediated "clock" in mammalian NSCs (La Torre et al. 2013).

Roles for let-7 and Lin28 in neural regeneration and aging

Another role for let-7 has been described in the changing properties of NSCs and neurons during aging. In aging NSCs, let-7b and the let-7 target gene *Hmga2* show reciprocal expression patterns (Nishino et al. 2008). *Hmga2*, which carries eight let-7-binding sites in its 3'UTR, has previously been shown to be a major target of let-7 in cancer cells (Mayr et al. 2007; Lee and Dutta 2007; F. Yu et al. 2007). Screening for miRNA expression changes associated with aging in the subventricular stem cell niche identified let-7b as significantly upregulated. Increased levels of let-7b correlate with decreased expression of Hmga2 and reduced self-renewal capacity, providing a mechanism for declining stem cell potency in the neurogenic niche during aging (Nishino et al. 2008). How let-7b and its targeting of Hmga2 are differentially regulated during the aging process has yet to be determined.

Another aspect of aging in which let-7 has been implicated is the age-related decline in neuronal regenerative capacity. Using *C. elegans* as a model, Chang and coworkers studied axon regrowth after laser dissection of a specific neuronal subtype termed AVM. Mutants for the *C. elegans* Argonaute gene *Alg-1* do not display the decline in regeneration normally seen in adult neurons (Zou et al. 2013). Expression and mutational analysis and rescue experiments have identified let-7 as being uniquely responsible for this decline in regenerative capacity. The authors subsequently identified *Lin41* as the let-7 target gene responsible for the differential regenerative capacity of larval and adult AVM neurons. In the mouse, Lin41 expression is extinguished in CNS neurons from E13 into adulthood, and so these results might not be directly transferable to mammalian axon injury. However, they do open the exciting possibility that embryonic regenerative capacity might be reactivated by manipulating the activity of let-7 or its gene targets.

Experimental support for just such a prospect comes from studies of Lin28 and let-7 in the zebrafish retina, with interesting parallels to the work on mammalian retinogenesis

discussed above. Unlike mammals, zebrafish are able to restore injured retina by promoting the dedifferentiation of Müller glia cells into a cycling population of retinal progenitors. Upon injury, the dedifferentiation transcription factor Ascl1/Mash1 transactivates *Lin28* and *c-Myc*. Introduced briefly in a previous section, Ascl1 is a well-characterized proneural bHLH transcription factor that is highly expressed in neural progenitors and is essential for neurogenesis in the retina and other regions of the telencephalon (Tomita et al. 1996; Cai et al. 2000; Brzezinski et al. 2011). In response to Lin28 induction, let-7 levels decrease, thus relieving repression of regeneration-associated mRNAs essential for glial dedifferentiation, including *Ascl1*, *Hspd1*, *c-Myc*, *Pax6* and *Lin28* itself (Ramachandran et al. 2010; Pollak et al. 2013; see Table 1). This direct link between Ascl1 and Lin28, together with the inhibition of Ascl1 by let-7 mentioned above, underscores the importance of the Lin28-let7 pathway in retinal neurogenesis and has the potential for use in regenerative approaches beyond retina injury.

Concluding remarks and outlook

Work on Lin28 and let-7 has been, and most likely will continue to be, a driving force in revealing the molecular novelty of miRNA action in development. Early models for Lin28 and let-7 function in neurogenesis suggested that Lin28 is required in embryonic progenitor cells to suppress the pro-differentiation activity of let-7 (Rybak et al. 2008; Schwamborn et al. 2009). This view is consistent with the role of Lin28 in retinal neurogenesis (La Torre et al. 2013) but has not been rigorously tested in other neurodevelopmental contexts in vivo. As in the retina, let-7 on its own is most likely not sufficient to orchestrate the complete process of neurogenesis. More probably, let-7 represents an essential early factor that is permissive for the neural differentiation program but is dependent on other neurogenic miRNAs to complete neurogenesis and guide neuronal fate specification and functional integration. The increasing use of genetically engineered mouse models should allow more directed and comprehensive investigations in the near future. For example, conditional knockouts should soon provide answers to the question of functional redundancy versus specific roles for the two *Lin28* genes and even the many let-7 isoforms. A greater challenge will be to distinguish between the direct effects of Lin28 on mRNA translation and the indirect effects achieved by let-7 inhibition. Specific mutagenesis of *Lin28* might allow the two functions to be separated (Balzer et al. 2010).

Another issue that looms large is that of context dependence. Various studies have placed more emphasis on the identification of a limited number of key downstream genes (for example, Lin28b and Protogenin in retinal neurogenesis) as opposed to more global roles for let-7, such as the regulation of cellular metabolism or the cell cycle. This is also a technical issue: one proteomic study has found that ectopic let-7 affects the abundance of over one hundred proteins in HeLa cells, with the degree of regulation in general being less than four-fold (Selbach et al. 2008). Are there situations in which the sum of the regulatory output is greater than the parts? If so, reliance on rescue experiments as the gold standard for defining critical targets carries the risk of oversimplifying our view of regulatory pathways. As is already clear, the regulatory repertoire of let-7 differs depending on the biological system under investigation, with only partial overlap between targets identified during ES cell differentiation, retinal neurogenesis, tissue repair or even stem cell differentiation in intestinal crypts (Melton et al. 2010; La Torre et al. 2013; Madison et al. 2013; Shyh-Chang et al. 2013b). One key to let-7 specificity might be combinatorial interactions, as shown in the retina for the early neurogenic trio of let-7, miR-9 and miR-125 (La Torre et al. 2013). Unsurprisingly, the regulatory power of the Lin28/let-7 circuit can also play important roles after neurogenesis is completed. Although outside the scope of this review, we should mention that Lin28 and let-7 have been shown to regulate the timing of axonal outgrowth for certain *C. elegans* neuronal subtypes (Olsson-Carter and Slack 2010) and to modulate synaptic plasticity by influencing the translational response to neurotrophins (Huang et al. 2012).

Finally, progress in understanding the way that Lin28 and let-7 affect neurogenesis both in early CNS development and in the adult neurogenic niches of the SVZ and dentate gyrus might prove relevant for future efforts directed at improving repair and regeneration in the CNS. Just as let-7 suppression and Lin28 activation accompany iPS cell generation, manipulation of the circuit in vivo might conceivably be used to encourage regenerative neuron replacement or to enhance axonal and dendritic regrowth in damaged or aging tissue.

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