REVIEW PAPER

MicroRNAs in Adult and Embryonic Neurogenesis

Changmei Liu · Xinyu Zhao

Abstract Neurogenesis is defined as a process that includes the proliferation of neural stem/progenitor cells (NPCs) and the differentiation of these cells into new neurons that integrate into the existing neuronal circuitry. MicroRNAs (miRNAs) are a recently discovered class of small non-protein coding RNA molecules implicated in a wide range of diverse gene regulatory mechanisms. More and more data demonstrate that numerous miRNAs are expressed in a spatially and temporally controlled manners in the nervous system, which suggests that miRNAs have important roles in the gene regulatory networks involved in both brain development and adult neural plasticity. This review summarizes the roles of miRNAs-mediated gene regulation in the nervous system with focus on neurogenesis in both embryonic and adult brains.

Keywords MicroRNA · Neurogenesis · Stem cells · Neural stem cells · Embryonic · Adult

Introduction

Neurogenesis is defined as a process that consists of the proliferation of neural stem/progenitor cells (NSCs) and the differentiation of these cells into new neurons that can integrate into the existing neuronal circuitry. After the completion of initial embryonic development, neurogenesis is largely restricted to the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus (Keller 2005; Zhao et al. 2008a). The

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presence of neurogenesis in these two regions persists through out adult life and has been demonstrated in many species including primates (Kornack and Rakic 2001; Pencea et al. 2001) and humans (Curtis et al. 2007; Eriksson et al. 1998).

MicroRNAs (miRNAs) are a family of non-coding small RNAs that can post-transcriptionally regulate gene expression by either inhibiting mRNA translation or leading to mRNA degradation. While post-transcriptional regulation of gene expression is a mechanism used in multiple aspects of neuronal development and functions of the central nervous system, the discovery of miRNAs further emphasizes the importance and complexity of post-transcriptional regulation. Structurally, mature miRNAs are single-stranded RNA molecules of about 21 nucleotides (nt) derived from a 70 to 100 nt hairpin-precursor. miRNAs are expressed at different levels and in a tissue-specific manner (Bartel 2004; Borchert et al. 2006). Bioinformatics tools predict hundreds of mRNA targets for each miRNA, suggesting that many genes are subject to miRNA-mediated regulation. More and more data demonstrate that miRNAs are involved in the control of stem cell self renewal, cell fate specification, neuronal dendritic development and synaptic plasticity, and neuronal homeostasis (Williams 2008). In this review, we discuss the recent findings in this exciting field with respect to embryonic and adult neurogenesis, and address emerging connections between the miRNA and miRNA-related pathways.

Neurogenesis and Neural Stem Cells

Stem Cells and Neural Stem Cells

Stem cells are undifferentiated cells and are defined by their ability for self-renewing division that results in at

least one daughter remaining as a stem cell and by their potential for generating multiple lineages of differentiated cell types. Both embryonic stem (ES) cells and adult stem cells fit this definition, however, their stem cell properties are largely different. ES cells can be isolated from an embryo at the blastocyst stage (Martin 1981). These cells are pluripotent, which means they are capable of differentiating into a large number of cell lineages of all three germ layer origins in both cell culture and in vivo (Keller et al. 1993; Kennedy and Keller 2003). Adult stem cells exist in many adult organs and have been shown to be involved in tissue repair and regeneration (Li and Zhao 2008). Among the best studied adult stem cells, hematopoietic stem cells (HSCs) could give rise to all of the cells in the blood, and neural stem cells (NSCs) (Kilpatrick and Bartlett 1993; Morshead et al. 1994), can differentiate to neurons and glial cells in the nervous system. Compared to ES cells, adult stem cells have much more restricted potentials. Although significant advances have been achieved towards the understanding of the biology of stem cells, how stem cells' basic properties are maintained remains to be answered. Recently, epigenetic regulations including DNA methylation, chromatin remodeling, noncoding RNAs, have come to the center stage of gene regulation during development and cell fate determination (Li and Zhao 2008). In this review we will focus on the functions of miRNAs.

Embryonic Neurogenesis

During development, neuroepithelial cells and radial glia lining the neural tubes are the NSCs that are responsive for generating the entire central nervous system (CNS). The three major cell types in the CNS arise from NSCs in a temporally defined sequence with neurons appear first, followed by astrocytes, and then oligodendrocytes (Temple 2001). Extensive effort has been put into understanding the molecular controls of this sequential cell genesis. Transcriptional factors (Gritti et al. 1999), DNA methylation (Shimozaki et al. 2005), Notch signaling (Sanosaka et al. 2009), etc. have been shown to be important factors in this process. Recently, the involvement of miRNAs has been discovered (see below). Since this embryonic neurogenic process is extremely vulnerable to prenatal exposure to toxins, pollutants, maternal physiological changes, and other environmental impact (Zhao et al. 2008a), understanding its regulatory mechanism is critical in preventing and treating neurodevelopmental disorders.

Adult Neurogenesis

In adult brains, neurogenesis largely ceases, but multipotent NSCs have been found to exist in many brain regions (Palmer et al. 1997). Experimental evidence suggests that adult NSCs originate from the embryonic neuroepithelial cells mostly radial glia located in the ventricular zone. These radial glia produce cortical neurons either directly or indirectly through intermediate progenitors cells at the end of neurogenesis the neurogenic radial glia become translocating cells that are astrocytes (Corbin et al. 2008; Noctor et al. 2004). A subset of astrocytes persist as stem cells in specialized niches in the adult brain and continuously generate large numbers of neurons that functionally integrate into restricted regions (Doetsch 2003). Neurogenesis in the SVZ has been shown to be important for olfactory function and olfactory learning (Alonso et al. 2006) and its age-dependent decline is likely a contributing factors of age-dependent olfactory impairment (Larson et al. 2005). Hippocampus is well established as the brain region involved in memory establishment and spatial learning (Dupret et al. 2008). The link between hippocampal neurogenesis and hippocampus-dependent learning has been suggested for years, although most evidence is correlational (Zhao et al. 2008a). Recently, more direct evidence is provided by inducing the death of new neurons in the hippocampus (Imayoshi et al. 2008) and by blocking the Wnt signaling pathway in the hippocampus using retrovirus (Jessberger et al. 2009). Both approaches resulted in certain learning deficits. Although neither paper can draw direct causal lines between adult hippocampal neurogenesis and learning, they indeed provide more convincing evidence. Many extrinsic stimuli and intrinsic mechanisms can affect adult neurogenesis, which include but is not limited to: physiological activities and enriched environment (Kempermann et al. 1997), hormones (Cameron et al. 1998), growth factors such as fibroblast growth factor (Fgf) and Vegf (Reynolds et al. 1992), transcription factors such as Tlx (Shi et al. 2004), Bmi-1 (Molofsky et al. 2003), and Mbd1 (Zhao et al. 2003), and Sox2 (Ferri et al. 2004; Graham et al. 2003) and diseases including epilepsy (Parent et al. 1997), stroke (Jin et al. 2006; Sun et al. 2005), and depression (Kempermann and Kronenberg 2003). The involvement of miRNAs in adult neurogenesis has only recently been revealed.

One fundamental question in neurogenesis research is why adult brains have more limited neurogenic potential when compared to embryonic brains. It is conceivable that adult neurogenesis is subjected to different regulation compared to embryonic neurogenesis. In adult brains, multipotent NSCs are in intimate contact with mature neurons, astrocytes, and oligodendrocytes and the activation of the NSCs have to be tightly controlled to prevent unwanted neoplastic transformation. Therefore, the fate of NSCs is largely affected by their microenvironment (so-called "stem niche") (Lie et al. 2002; Palmer et al. 1999; Shihabuddin et al. 2000; Weiss et al. 1996). In addition, adult NSCs also

process different intrinsic properties as compared to their embryonic counterparts, likely due to the differences in the genetic and epigenetic programs between adult and embryonic NSCs. For example, isolated embryonic NSCs can generate much more diverse types of neurons than adult NSCs upon transplantation into embryonic brains (Temple 2001). The self-renewal capacity of NSCs declines with age, which is partially due to increased expression of let-7b miRNA in NSCs of aged brains (Nishino et al. 2008). To further support this notion, mice lacking sonic hedgehog (Ahn and Joyner 2005), Tlx (Shi et al. 2004), Bmi-1 (Molofsky et al. 2003), and Mbd1 (Zhao et al. 2003) have profound deficits in postnatal neurogenesis but not in embryonic neural development. Consistent with this, we have demonstrated that Mbd1 mutant adult NSCs expressed abnormally higher levels of stem cell mitogen basic fibroblast growth factor (Fgf-2) and exhibited increased aneuploidy while NSCs isolated from Mbd1 mutant embryonic day 14 (E14) or neonate (P1) brains did not (Zhao et al. 2003). Understanding all aspects of molecular regulatory mechanism underlying neurogenesis is a fundamental step in mammalian biology and a critical progress towards therapeutic applications of stem cells.

miRNAs in Neurogenesis

In the past few years, many different classes of small non-coding RNAs have been identified in the brain and have been shown to exhibit diverse roles in normal brain development and functions. Among them, miRNAs are the best studied and are likely to be key post-transcriptional regulators in stem cells and neurogenesis.

Biogenesis of miRNAs

miRNAs are single-stranded RNAs of ∼22 nt in length that are originated from the sequences in the genome. The primary transcripts of miRNA (pri-miRNAs) are transcribed from the genomic DNA by RNA polymerase II. These pri-miRNAs are usually several kilo bases long and contain stem–loop structures. The cleavage at the stem of the hairpin generates stem–loop miRNA precursors of ∼70 nt, termed pre-miRNAs. This reaction takes place in the nucleus by the nuclear RNase III-type protein, Drosha, which forms a micro-processor complex with the double-stranded RNA-binding protein DGCR8. The pre-miRNAs are subsequently translocated to the cytoplasm by an exportin-5-dependent mechanism. Once in the cytosol, pre-miRNAs are further processed by a second RNA III enzyme, Dicer. The Dicer cleavage generates an imperfect, ∼22 nt siRNA-like duplex, which is then loaded onto Ago protein and forms the RNA-induced silencing complex

(RISC). Then the miRNA directs the RISC to its mRNA targets based on sequence homology (Bartel 2004; Carthew and Sontheimer 2009; Kim et al. 2009). The mechanism of miRNA-mediated gene regulation remains contentious. One of the main mechanisms as demonstrated by a large number of publications appears to be translational repression. Degradation of target mRNAs can also result from miRNA action (Kim et al. 2009). A few recent studies suggest that miRNAs might be able to activate protein (Vasudevan et al. 2007).

Expression and Regulation of miRNAs in ES Cells

The DGCR8 and Dicer gene knockout studies have confirmed the critical roles of miRNAs in ES cell proliferation and differentiation (Kanellopoulou et al. 2005; Wang et al. 2008b, 2007). Several miRNAs are highly expressed in embryonic stem cells and therefore may be essential in maintaining the pluripotent state and stemness of ES cells. By comparing undifferentiated with differentiated mouse ES cells, Houbaviy et al. (2003) has identified an ES cell-specific small RNA cluster, from which miR-293 is highly expressed. They also found many other miRNAs that were up-regulated following retinoic acid-induced ES cell differentiation and concluded that these miRNAs might be involved in cell lineage determination during early development (Houbaviy et al. 2005, Houbaviy et al. 2003). A similar cluster has also been identified in humans and was shown to be human ES cell-specific (Suh et al. 2004). In addition, the miR-290 cluster was found to be co-transcribed as polycistronic primary transcripts regulated by a common promoter. The functional importance of the miR-290 cluster in embryogenesis has been demonstrated by the embryonic lethality of homozygous mutant mouse embryos lacking this cluster (Houbaviy et al. 2003). Wang et al. has shown that multiple ES cell-specific miRNAs, such as miR-19, miR-20a, miR-20b, miR-294, and miR-295, etc. could rescue the cell proliferation defect of DGCR8 knockout ES cells. The rescued cells no longer accumulate in the G1 phase of the cell cycle, because these miRNAs function by suppressing several key regulators of the G1-S transition. More recently, two independent studies have provided strong evidence that miR-290 family of miRNAs may regulate ES cell maintenance and differentiation via epigenetic regulation of de novo DNA methylation (Wang et al. 2007). Interestingly, another recent study found that key ES cell transcription factors, including Oct4, Sox2, Nanog, and Tcf3, are associated with promoters of those miRNAs highly expressed in ES cells including the miR-290 cluster (Tay et al. 2008). These data reveal how key ES cell transcription factors promote the expression of ES cell miRNAs, therefore these miRNAs are integrative part of the regulatory circuitry controlling ES cell identity.

The let-7 miRNA family was among the first group of miRNAs that were suggested to regulate stem cell functions. This evolutionarily conserved family of miRNAs was first described in the worm *Caenorhabditis elegans* (Chalfie et al. 1981). In mammals, let-7 expression is similarly induced during embryonic development and has been suggested to negatively regulate stem cell self renewal in a variety of tissues. The functional role of let-7 miRNAs was further supported by a study of breast cancer stem cells (CSCs) (Yu et al. 2007). The CSCs represent a rare population of breast tumor cells that exhibit not only highly enriched tumor-initiating properties, but also key attributes that are unique to stem cells, including the abilities of self-renewal and differentiation. The expression of the let-7 family was significantly reduced in breast CSCs and the reduced let-7 level was required for self-renewal and maintenance of an undifferentiated state of the breast CSCs. Inhibition of let-7 activity with antisense oligonucleotides enhanced CSC self-renewal capability. Increased let-7 paralleled the reduced expression of both H-RAS and HMGA2, two known let-7 targets. HMGA2 is a known regulatory gene that promotes cell proliferation in human and mouse ES cells (Li et al. 2007, 2006). Silencing H-RAS in CSCs leads to reduced self-renewal, while silencing HMGA2 results in enhanced cell differentiation (Fig. 1). Interestingly, the let-7/HMGA2 pathway in CSCs described by Yu et al. is strikingly similar to that in ES cells. The finding that let-7 controls self-renewal and differentiation in CSCs through RAS and HMGA2 hint similar functional roles of let-7 miRNA in stem cells in general (Wang et al. 2009). For example, the let-7 expression level in ES cells is very low but is increased with cell differentiation and is inversely correlated with the expression levels of HMGA2 (Wang et al. 2009). This is further confirmed by the finding that let-7 and HMGA2 together regulate the expression of p16Ink4a. Increased let-7, decreased HMGA2, and increased p16Ink4a is partially responsible for age-dependent decline in self-renewal ability of brain NSCs (Nishino et al. 2008) (Fig. 1).

miRNAs in Central Nervous System

miRNAs are involved in almost every biological process, including developmental timing, cell differentiation, cell proliferation, cell death, and so on. The significance of miRNAs in the morphogenesis of the brain and neuronal system has also received much attention. Suppression of miRNA biogenesis by disrupting the zebrafish Dicer gene has provided the first evidence that miRNAs are necessary for the development of nervous system (Giraldez et al. 2005). Not surprisingly, a large number of unique miRNAs
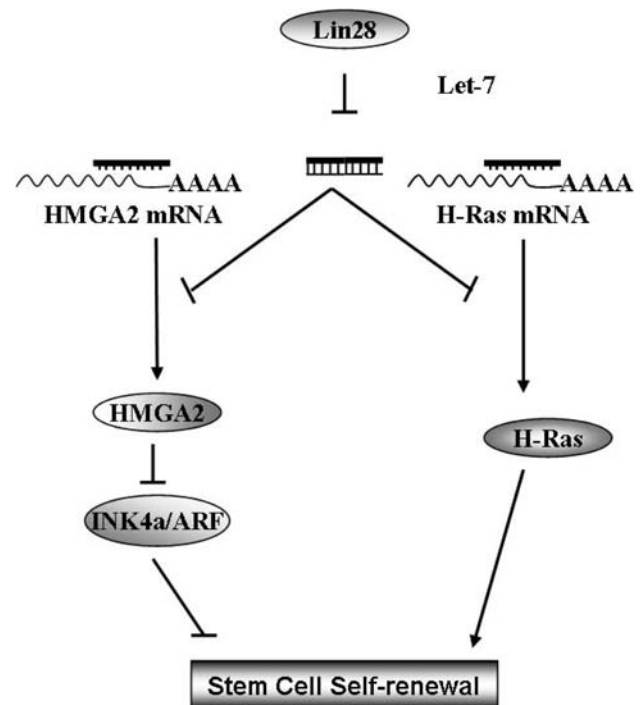

Fig. 1 Currently known functions of let-7 miRNA in stem cell self-renewal

have been isolated from the brain and neuronal cell lines (Lagos-Quintana et al. 2002). Using zebrafish as a model, researchers have found that miRNAs showed a wide diversity of expression profiles in neural cells, including expression in neural precursors and stem cells (e.g., miR-92b); expression associated with transition from proliferation to differentiation (e.g., miR-124); constitutive expression in mature neurons (e.g., miR-124 again); expression in both proliferative cells and their differentiated progenies (e.g., miR-9); regionally restricted expression (e.g., miR-222 in telencephalon); and cell-type specific expression (e.g., miR-218a predominantly in motor neurons; miR-26 and miR-29 predominantly in astrocytes) (Kapsimali et al. 2007). In mammals, a miRNA expression atlas has been generated by large scale small RNA cloning and sequencing (Landgraf et al. 2007). Both studies have confirmed the presence of CNS-specific miRNAs and identified new ones, such as miR-218, a miRNA that is expressed only in motor neurons in zebrafish, and miR-29a, a family of miRNAs that is absent in embryonic tissues but highly expressed in the adult cortex. These data provided an important resource for future functional studies of miRNAs in the CNS. Many of these miRNAs are associated with polyribosomes (Kim et al. 2004), suggesting that in the nervous system, like in other tissues, miRNAs are likely involved in the regulation of protein translation (Fiore et al. 2008).

The Potential Roles of miRNAs in Neurogenesis

As regulators of cell fate determination, miRNAs have first been identified in *Caenorhabditis elegans* (Lee et al. 1993). Extensive studies have explored the potential role of miRNAs in neuronal differentiation and fate determination using various model systems such as *C. elegans*, *Drosophila melanogaster* and zebrafish. More recently, the analysis has been extended to several mammalian animal and cellular models (Fiore et al. 2008). Many miRNAs are specifically expressed or enriched in mammalian brain tissues have been identified, such as let-7a, miR-218, and miR-125 (Sempere et al. 2004). The conserved miR-200 family of miRNAs, including miR-200a, miR-200b, miR-200c, miR-429, and miR-141, are enriched in olfactory tissue and have been shown to be important for olfactory neurogenesis (Choi et al. 2008).

miR-124a and miR-9 are two brain specific miRNAs and are among best studied brain miRNAs. In vitro, both miR-124 and miR-9 expression levels increase sharply during the transition from neuronal precursors to neurons in differentiating embryonic stem cells. Overexpression of both miRNAs promotes neuronal differentiation, while down regulation of them had the opposite effect (Conaco et al. 2006). Krichevsky et al. have demonstrated that a number of miRNAs are simultaneously co-induced during differentiation of neural progenitor cells to neurons and astrocytes. Using both gain-of-function and loss-of-function, they demonstrated that brain-specific miR-124a and miR-9 affect neuronal lineage differentiation through the signal pathway of signal transducer and activator of transcription (STAT) 3 pathway (Krichevsky et al. 2006). Interestingly, miR-9 could target NRSF/REST (neuronal restricted silencing factor/RE-1 Silencing Transcription factor) transcription factor, a global repressor of the transcription of neuronal genes in non-neuronal tissues while miR-9* could target co-REST, the co-factor of REST. The transcription factor REST/NRSF suppresses neuronal genes in non-neuronal cells by binding a conserved repressor element (RE1) in neuronal gene loci and recruiting the corepressor complex containing histone deacetylases and methyl CpG-binding protein MeCP2. Down regulation of REST during transition from progenitors to post-mitotic neurons allows neuronal gene expression. Downregulation miR-9 and miR-9* and subsequent increased levels of REST and coREST in neurons could be responsible for down regulation of neuronal genes in Huntington's disease (Packer et al. 2008). Recently, miR-9 was found to regulate the expression of Tlx, a gene that regulates the self renewal of NSCs (Shi et al. 2004). Interestingly, Tlx also regulates the expression of miR-9 and forms a regulatory loop that is critical for neurogenesis (Zhao et al. 2009). Among brain-enriched miRNAs, miR-124 might be the best characterized. The

presence of miR-124 seems to define the identity of neural cells (Conaco et al. 2006) #12. For example, miR-124 has been shown to be necessary for the maintenance of neuronal identity in the chicken spinal cord (Conaco et al. 2006; Visvanathan et al. 2007). miR-124 seems to carry out such an important functions by suppressing at least three anti-neuronal differentiation pathways, as summarized in Fig. 2. First, miR-124, like miR-9 can also antagonize the function of REST, the repressor of neuronal gene expression. Small C-terminal domain phosphatase 1 (SCP1) is an anti-neural factor expressed in non-neural tissues, like REST, and is recruited to RE1-containing neural genes by REST. It has been shown that down regulation of REST during the transition from progenitors to post mitotic neurons leads to increased levels of miR-124. miR-124 expression then results in the degradation of non-neuronal transcripts, including the phosphatase SCP1. Therefore, miR-124 suppresses SCP1 expression and induces neurogenesis (Visvanathan et al. 2007). Second, miR-124 directly targets Polypyrimidine tract binding protein 1 (PTBP1, also called PTB/hnRNP I) mRNA, which encodes a global repressor of alternative pre-mRNA splicing in non-neuronal cells. One target of PTBP1 is a critical exon in the pre-mRNA of PTBP2 (nPTB/brPTB/PTBLP), a neural-enriched PTBP1 homolog. When this exon is skipped, PTBP2 mRNA is subject to nonsense-mediated decay (NMD). During neuronal differentiation, miR-124 reduces PTBP1 levels, leading to the accumulation of correctly spliced PTBP2 mRNA and a dramatic increase in PTBP2 protein (Makeyev et al. 2007). The authors also demonstrate that miR-124 plays a key role in the differentiation of progenitor cells to mature neurons. The connection between RNA silencing (miR-124) and splicing (PTB/nPTB) was confirmed by reciprocal expression analyses of these genes in several differentiated and precursor cell stages, shedding light on a new chapter of neuronal gene expression regulation (Fiore et al. 2008; Makeyev et al. 2007). The third target of miR-124 is Sox9, a SRY-box transcription factor. Chen et al. found that miR-124 is an important regulator of the temporal progression of adult neurogenesis in the SVZ of mice. Knockdown of endogenous miR-124 maintains isolated SVZ stem cells as dividing precursors, whereas ectopic miR-124 expression led to precocious and increased neuron formation. Furthermore, blocking miR-124 function during regeneration led to hyperplasia, followed by a delayed burst of neurogenesis. Over expressing Sox9 abolishes neuronal differentiation, whereas knocking down Sox9 led to increased neuron formation, similar to the effect of miR124 (Fig. 2) (Cheng et al. 2009). More recently, Yu et al. found that miR-124 could also regulate neurite outgrowth during neuronal differentiation. Over expressing miR-124 in differentiating mouse P19 cells promoted neurite outgrowth, whereas blocking miR-124 function delayed neurite
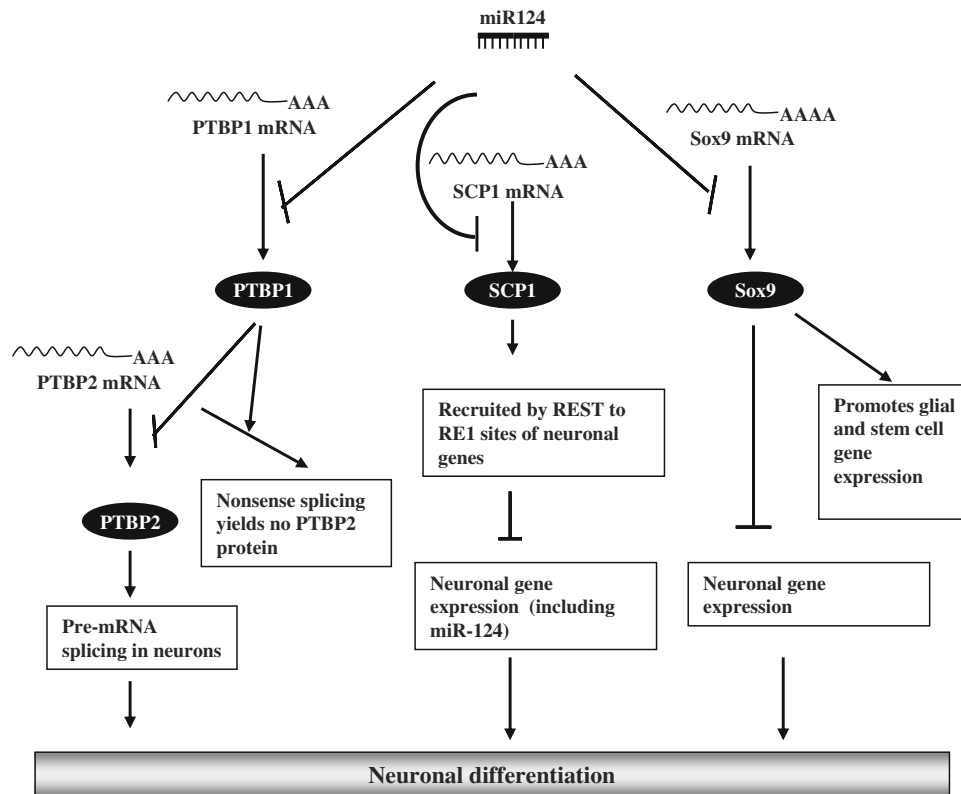
Fig. 2 Schematic summary showing how miR-124 may modulate multiple pathways involved in neuronal neurogenesis through translational regulation of its currently known mRNA targets. Polypyrimidine tract binding protein 1 (PTBP1) is a global repressor of alternative pre-mRNA splicing in non-neuronal cells. One target of PTBP1 is a critical exon in the pre-mRNA of PTBP2. When PTBP1 level is high, it inhibits neuronal PTBP2 protein level by alternative splicing. miR-124 down regulates PTBP1 therefore upregulates the level of PTBP2 in cells undergoing neuronal differentiation. Small

C-terminal domain phosphatase 1 (SCP1) is an anti-neural factor expressed in non-neural tissues. Neuronal restricted silencing factor/ RE-1 Silencing Transcription factor (REST) recruits SCP1 to RE1-containing neural genes (including miR-124) and repress their expression. miR-124 down regulates SCP1, which also ensure its own expression. Sox9 has been shown to promote expression of gilal genes and to maintain stem cell niche. Down regulation of Sox9 has been clearly demonstrated to promote neuronal differentiation (please see text for details and references)

outgrowth (Yu et al. 2008). These data demonstrate that miR-124 plays important roles in neuronal development.

In addition, Kim et al. found that a miRNA-guided RNA silencing pathway was linked to differentiation of dopaminergic neurons (DN). miR-133b, specifically is expressed in mammalian midbrain and is deficient in the context of Parkinson's disease patient samples. Overexpression of miR-133b in primary embryonic rat midbrain cultures represses dopaminergic differentiation, whereas its inhibition by a 2′-O-methyl-antisense oligonucleotide against miR-133b leads to an increase in DNs. miR-133b may also inhibit the maturation and function of midbrain DNs by repressing the translation of paired-like homeodomain transcription factor Pitx3. (Kim et al. 2007). miR-134, one of the first miRNAs found to be expressed in brain is localized in dendrites of mouse cultured hippocampal neurons. Its overexpression causes a significant decrease in dendritic spine size, and conversely its depletion leads to a small increase in spine volume. This indicates that miR-134 plays a role in spine morphogenesis. The target of

miR-134 is lim-domain containing protein kinase 1 (limk1). Limk1 mRNA and miR-134 colocalize within neuronal dendrites and miR-134 inhibits Limk1 expression locally. The unique feature of miR-134/Limk1 interaction is its reversibility: Brain-derived neurotrophic factor (BDNF), a neurotrophin released in response to synaptic stimulation, can release the inhibition on Limk1 translation imposed by miR-134. But the molecular mechanism underlying depression of limk1 mRNA translation is currently unknown (Fiore et al. 2008; Schratt et al. 2006).

Techniques for Studying miRNA Functions in Neurogenesis

Identification and Expression Analysis of miRNAs

The first hint that miRNAs might play a variety of roles within the nervous system came from cloning and expression analyses of miRNA, and more and more

miRNAs are being discovered. Three major approaches have been used to determine miRNAs with altered expression profiles in tissues or cells. The first approach uses miRNA microarray analysis, in which RNA samples containing endogenous miRNAs are hybridized to species-specific microarrays containing known miRNA in the species studied. This method can quickly and accurately identify miRNAs whose levels change in a specific phenotype compared to a control phenotype. This platform has been used widely (Choi et al. 2008) and miRNA microarrays are available from many vendors. However, this method is limited by its sensitivity due to the fact that many miRNAs are expressed at relatively low level. In addition the difficulty in resolving mature miRNAs because of their small size and the fact that many miRNAs differ from one another by as little as one nucleotide also hinder the utility of this method. In the second approach, real-time PCR can be used to distinguish the changed expression levels of miRNAs. With this method, mature miRNAs can be distinguished from primary miRNAs and precursor miRNAs using stem–loop reverse transcription primers. This method is more sensitive than microarray-based approaches and has been used widely (Chen et al. 2005; Mellios et al. 2008). Although the above methods are easier to operate and have been used widely, they can only assess known miRNAs. To study unknown miRNAs, next generation sequencing methods such as Illumina 1G, SOLiD, 454, have provided revolutionary options. Since these sequencing methods yield short sequences, they are ideal in identifying non-coding small RNAs. The number of reads obtained for each small RNA can serve as an indication of abundance therefore providing so called digital gene expression profile. The overview of this technology is beyond the scope of this review. Several recent reviews have given comprehensive summary on this technology (Morozova and Marra 2008; Pomraning et al. 2009). A few recent studies have demonstrated the concepts and feasibility of this method in small non-coding RNA analysis (Chi et al. 2009; Devor et al. 2009; Hafner et al. 2008; Xu et al. 2009) However, the high cost and bioinformatic demand for data analysis make these high throughput sequencing method prohibitory as a routine method for most laboratories. With the fast progress in sequencing technology, sequencing might.

Functional Studies of miRNAs

To understand how miRNAs regulate stem cell function, it is necessary to manipulate their expression in the cells or animals. Several methods have been developed by ours and other laboratories to either overexpress or down regulate miRNAs.

First, individual double-stranded small RNA duplex (miRNA mimics) can be used to augment the function of endogenous miRNA. Conversely, miRNA inhibitors, such as 2-O′-methyl oligo that can hybridize to specific endogenous miRNA thus blocking their incorporation into RISC, can be used to suppress the function of endogenous miRNAs. This method is particularly useful for performing functional screening of currently known miRNAs, if the phenotypic assay is amenable to high throughput screening. On the other hand, miRNAs mimics and inhibitors can also be used to identify and validate miRNA targets.

Second, for overexpression of miRNAs, researchers can use small hairpin RNA expression cassettes or use endogenous pri-miRNA gene to express miRNAs. Since miRNAs are expressed as hairpin pre-miRNA from a vector, the expression can last much longer than transfected miRNA mimics. The most commonly used promoters for expressing miRNAs are U6 and H1 RNA polymerase III promoters as well as some RNA Polymerase II promoters. For example, Zhao et al. used pcDNA-CMV promoter to express miR-221/222 in breast cancer cells (Zhao et al. 2008b). Mott et al. (2007) used Pri-miRNA to express miR-29 in H69 cell line. Since NSCs are difficult cells for DNA transfection, viral vector-based miRNA expression methods are extremely useful for miRNA over expression in NSCs (Barkho et al. 2008; Li et al. 2008) (Fig. 3). For example, Mellios et al. (2008) used self-inactivating lentiviral vector to express miR-30a in isolated neuron cells. We have used lentiviral vectors to express miR-137 in primary adult NSCs (Zhao unpublished data).

Expressing miRNAs in animals are much more difficult. Due to the small size of miRNAs, creating transgenic and knockout mice is more challenging for miRNAs than for regular protein coding genes. In 2008, Lu et al. cloned an 1.1 kb miR17-92 cluster DNA from C57BL/6 mouse genomic DNA and used mouse surfactant protein C (Sftpc) gene to overexpress this cluster in embryonic lung epithelium of two transgenic mouse lines (Lu et al. 2007). Recently, Mayoral et al. (2009) also created transgenic mice expressing miR-221-222 in ES cells using bacterial artificial chromosome clones and found that these transgenic ES cells exhibited dramatically reduced cell proliferation. Gene knockout technology has also been used for deleting miRNAs. Thai et al. (2007) used a β-galactosidase (LacZ) promoter to replace a major portion of the second exon, including miR-155,in the bic gene and then generated a loss-of function mutant mice named bic/miR-155$^{-/-}$. To explore the function of miR-126, Wang et al. replaced the region of intron 7 of the Egfl7 gene containing miR-126 with a neomycin-resistance cassette flanked by loxP sites. After breeding with mice expressing Cre recombinase under control of the CAG promoter, the resulting miR-126$^{-/-}$ mice do not express either the precursor or the mature
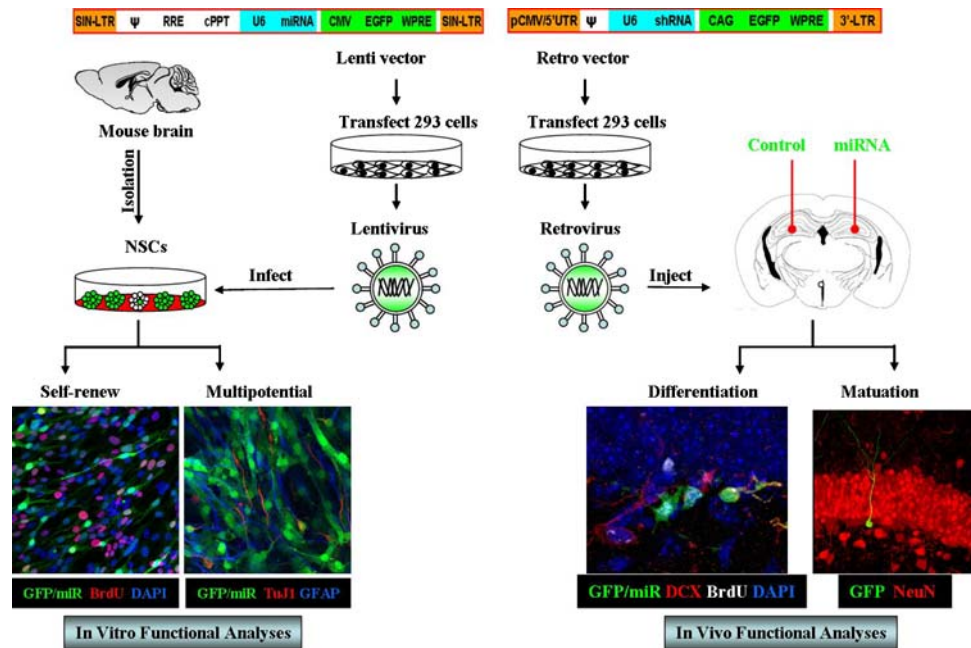
Fig. 3 Studying functions of mRNAs in neurogenesis using recombinant viruses. Both lentivirus and retrovirus can be engineered to express both miRNAs and GFP (*top panel*). Production of these viruses can be achieved by transfecting into 293T cells. Lentivirus can infect cultured primary NSCs at high efficiency and infected cells expressing miRNA (also GFP+) can be analyzed for their proliferation and differentiation potential (*left panel*). Retrovirus infect only diving cells therefore can be used to label dividing neuroprogenitors in adult brains. The labeled cells can then be tracked for their differentiation (1 week post injection) and neuronal maturation (4 weeks post injection) capabilities (*right panel*). BrdU, bromodeoxyuridine used to label dividing cells. Cell lineage markers used for flurescent imaging: TuJI, a marker for immature neurons. GFAP, a marker for astrocytes; NeuN, a marker for mature neurons; DCX, a marker for immature neurons; DAPI, a nuclear dye

miR-126 (Wang et al. 2008a). In addition to the classic knockout mice, lentivirus-based knockout technology was also used in antagonizing endogenous miRNAs. Gentner et al. used lentiviral vectors to specifically knock down miRNAs by overexpressing miRNA target sequences (so called "miRNA sponge") from a RNA polymerase ΙΙ promoter. These vectors effectively inhibited expression of both reporter constructs and the natural targets of these miRNAs. Using this method, the authors showed that hematopoietic stem cells stably overexpressing miR-223 sponge sequence exhibited the similar phenotypes as miR-223 genetic knockout mice, indicating that the miRNA sponge method could lead to robust in vivo interference of endogenous miRNA function (Gentner et al. 2009). However, overexpressing short RNAs may have of-target effects unrelated to the specific miRNA, therefore such method needs to be used with caution.

To study in vivo neurogenesis, several groups have used retrovirus to deliver siRNAs in adult brains (Duan et al. 2007). Such method (Fig. 3) could be easily adapted to miRNA. Since retrovirus only infects dividing cells, they are ideal tools for overexpressing either miRNAs or siRNAs against miRNAs in dividing neuroprogenitors in vivo. The fate of these individually infected cells can then be tracked by co-expressed GFP and analyzed using

immunohistology and electrophysiology. The limitation of this method is the relatively low infection efficiency of virus and invasive nature of viral injection. Expressing miRNAs using promoters that are both cell type specific (e.g., Nestin for progenitor cells and doublecortin for young neurons) and inducible (e.g., by Tamoxifen, Tetrocyclin) would be a better method. Several lines of Tamoxifen inducible Nestin promoter driven Cre transgenic mice have been used to study temporal and spatial gene regulation in neurogenesis (Balordi and Fishell 2007; Burns et al. 2007; Imayoshi et al. 2008; Lagace et al. 2007), demonstrating the feasibility of this method for adaption to studying miRNA functions in neurogenesis.

miRNA Target Prediction

To fully understand the regulatory functions of miRNAs in a biological process, it is essential to identify the downstream targets of miRNAs. The computational identification of miRNA targets and the validation of miRNA–target interaction are critical steps in revealing the contribution of miRNAs to cellular functions. To date, the software programs for predicting targets of miRNAs, such as Targetscan, Pictar, Miradna, etc.; are largely based on the complementarities of the miRNA "seed sequence" to the 3′ untranslated

regions (UTR) of candidate mRNAs. However, recent studies indicate that miRNAs could also target the coding region as well as 5′ UTRs (Lytle et al. 2007; Orom et al. 2008). In addition, the sequences surrounding the seed sequences also seem to contribute to target specificity. Therefore, more research and updated software are needed.

Conclusions and Perspectives

Tens of thousands of small RNAs, especially miRNAs have been discovered, and more miRNAs are expected to be discovered owing to rapidly expanding DNA sequencing power. It has become more and more evident that miRNAs are important players in the regulation of different aspects of neuronal cell biology and more functions will undoubtedly be revealed in the near future. These relatively recent findings undoubtedly add another layer of complexity to established gene regulatory networks. Although miRNAs have been shown to regulate neurogenesis, the extend of such regulation remains unclear. Is one miRNA sufficient for regulating multiple targets in one biological process, or are multiple miRNAs required to effectively regulate one target gene? With the fast development of new technologies, such as more efficient sequencing methods, more sensitive proteomics, and advance in system biology, we will begin to understand how the signaling pathways orchestrated by miRNAs impact on neurogenesis and related pathological conditions. This knowledge will provide critical clues for miRNA-based therapeutic strategies for many developmental and adult brain diseases that currently have no known etiology or cures.

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