Am J Stem Cell 2012;1(3):182-195
www.AJSC.us /ISSN:2160-4150/AJSC1207001

Review Article

Specification of neural cell fate and regulation of neural stem cell proliferation by microRNAs

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Received July 27, 2012; Accepted October 8, 2012; Epub November 30, 2012; Published December 10, 2012

Abstract: In the approximately 20 years since microRNAs (miRNAs) were first characterized, they have been shown to play important roles in diverse physiologic functions, particularly those requiring coordinated changes in networks of signaling pathways. The ability of miRNAs to silence expression of multiple gene targets hints at complex connections that research has only begun to elucidate. The nervous system, particularly the brain, and its progenitor cells offer opportunities to examine miRNA function due to the myriad different cell types, numerous functionally distinct regions, and fluidly dynamic connections between them. This review aims to summarize current understanding of miRNA regulation in neurodevelopment, beginning with miRNAs that establish a general neural fate in cells. Particular attention is given to miR-124, the most abundant brain-specific miRNA, along with its key regulators and targets as an example of the potentially far-reaching effects of miRNAs. These modulators and mediators enable miRNAs to subtly calibrate cellular proliferation and differentiation. To better understand their mechanisms of action, miRNA profiles in distinct populations and regions of cells have been examined as well as miRNAs that regulate proliferation of stem cells, a process marked by dramatic morphological shifts in response to temporally subtle and refined shifts in gene expression. To tease out the complex interactions of miRNAs and stem cells more accurately, future studies will require more sensitive methods of assessing miRNA expression and more rigorous models of miRNA pathways. Thorough characterization of similarities and differences in specific miRNAs' effects in different species is vital to developing better disease models and therapeutics using miRNAs.

Keywords: MicroRNA, stem cell, neural development, neural stem cell

Introduction

MicroRNAs (miRNAs) are one of four types of small, noncoding RNAs that also include small interfering RNAs (siRNAs), repeat associated small interfering RNAs (rasiRNAs), and Piwi-interacting RNAs (piRNAs). Initially synthesized as much longer hairpin loop RNA sequences, these primary miRNA transcripts (pri-miRNAs) are then enzymatically processed by Drosha and Dicer into mature miRNAs that are approximately 23 nucleotides in length. The mature miRNA can then be incorporated into an RNA-induced silencing complex (RISC) and guide it to the appropriate messenger RNA (mRNA) transcripts using the miRNA seed region, the 6-7 nucleotide sequence beginning at position 2 of the miRNA. This seed region allows the RISC complex to specifically target mRNAs with complementary sequences in the 3' untranslated region. In animals, miRNAs can silence target mRNAs by four distinct mechanisms: inhibition of translation initiation, inhibition of translation elongation, co-translational protein degradation, and premature termination of translation [1-3].

The small size of miRNAs belies their ubiquity and broad influence in numerous physiological processes, including developmental pathways and the rapid, dynamic changes in gene expression seen in stem cells. Different sets of miRNAs are expressed in pluripotent embryonic stem (ES) cells compared to differentiated cells; without mature miRNAs, ES cells will fail to differentiate and develop the three requisite germ layers. In many organisms, the absence of miRNA processing machinery will result in failure to maintain stem cell populations, resulting in embryonic lethality [4].

The ability of miRNAs to affect multiple mRNA targets also makes them effective in orchestrating changes in networks of pathways, similar to what occurs in neural development and neuropathologies. Aberrant expression of miRNAs leading to either downregulation or upregulation of downstream targets has been implicated in a number of neurodegenerative, neurodevelopmental, as well as psychiatric disorders. Several studies have detected decreased levels of the miR-29 family and increased expression of miR-146a in patients with Alzheimer's disease, while decreased expression of miR-133b and -9/9* has been shown in patients with Parkinson's disease and Huntington's disease, respectively [5-7]. Neurodevelopmental disorders resulting from miRNA dysfunction include Fragile X mental retardation (effects mediated through miR-125b and -132 in flies), Down syndrome (upregulation of let-7c, miR-99a, -125b-2, -155, and -802, all located on human chromosome 21, in patients), and Rett syndrome (increased miR-132 expression in mice) [8, 9]. Decreased levels of miR-132 and decreased levels of miR-134, -185, and -299 are associated with schizophrenia in humans and mice, respectively [10, 11].

Although growing evidence implicates the involvement of miRNAs in numerous aspects of human physiology and disease etiologies, the mechanisms by which miRNAs actually alter gene expression are only just coming to light. In particular, knowledge of miRNAs' role in neural development and dysfunction has exploded in the past several years. Not only have researchers found new correlations, they are beginning to make inroads into understanding functional interactions between miRNAs, their potential upstream regulators, and their downstream targets. This review will explore current thinking about miRNAs' roles in neural development, paying particular attention to regulatory mechanisms in mature neural cell types as well as neural stem cells.

Specification of neural development by miRNAs

Although miRNAs have been found to underlie the development and function of several organ systems, such as the heart, kidneys, and skeletal muscles [12], the nervous system and the brain in particular continue to tantalize researchers. With several different cell types, numerous functionally distinct regions, and fluidly dynamic connections between them, the brain presents a complex puzzle that must be approached from multiple angles.

Of the numerous miRNAs associated with the human brain, miR-124 is by far the most abundantly expressed [13]. It has been the subject of intense scrutiny since Lim *et al.* demonstrated that transfecting hsa-miR-124 into HeLa cells was sufficient to shift those cells' gene expression profile towards that of neurons [14]. Microarray analysis revealed downregulation of 174 genes consistent with those downregulated in the brain. Lim *et al*. were also able to downregulate 46 genes associated with heart and skeletal muscles, showing that miRNA transfection was sufficient to shift the gene expression profiles of HeLa cells toward that of specific differentiated tissues. To determine whether the gene downregulation was directly due to miR-124 binding with the target mRNA transcripts, two tools were used. Multiple Em for Motif Elicitation (MEME), a motif discovery tool, elucidated the six-nucleotide 3' UTR sequence of the downregulated mRNA transcripts, which proved to be complementary to the miRNA seed region in miR-124. Comparison of the frequencies of downregulated UTR hexamers versus all UTR hexamers confirmed the specific reduction of transcripts complementary to miR-124, indicating that the reduction in gene expression levels was due to specific targeting and not targeting of upstream regulatory genes [14].

Lim *et al*. went on to generate mutant miR-124 with alterations at positions 5 and 6 (124mut5-6), i.e. within the seed region, and at positions 9 and 10 (124mut9-10), i.e. outside of the seed region. Although transfection of both mutant miR-124's into HeLa cells resulted in fewer numbers of downregulated transcripts, 124mut9-10 yielded far greater overlap with the expression pattern of wild-type miR-124 than 124mut5-6, whose expression pattern was quite distinct from either of the other miR-124 variants. These results indicated the importance and necessity of the intact miR-124 seed region in order to effectively downregulate genes to match the neuronal profile. Co-transfection of mutant 3' UTR segments from predicted targets linked to renilla luciferase reporter genes with miR-124 showed no

repressive effect compared to robust repression of wild-type 3' UTR segments [14]. Taken together, Lim *et al.* showed that miR-124 was able to specifically induce in HeLa cells a gene expression profile consistent with neurons via downregulation of particular transcripts.

Knowing that it was possible to assign neuronal fate to cells with good specificity using miR-124, the next step was to determine with greater temporal resolution whether miRNAs could be associated with different neurodevelopmental stages and specific cell types. Smith *et al*. used microarray analysis to quantify miRNA levels over a 28-day course of retinoic acid-driven differentiation in NTera2/D1 (NT2) human embryonal carcinoma cells, which are often used as a "surrogate" of pluripotent embryonic stem cells. In the course of their study, they found the miR-302 cluster was downregulated while the miR-181 family was upregulated, matching expression profiles in primary cultures of human neurons and astrocytes; other miRNA levels examined remained constant throughout retinoic acid treatment. Furthermore, paralogs of miR-92a (miR-17 and -106 clusters) stayed upregulated during neuronal differentiation while paralogs of miR-24 (miR-23a, -23b, and -29a clusters) stayed upregulated during astrocytic differentiation. The switch from a pluripotent state to committed cell fate typically occurred between 8-12 days of retinoic acid treatment, which was accompanied by marked upregulation of a number of miRNAs either known to be or thought to be controlled by the repressor called neuron-restrictive silencer element (NRSE, or RE1 silencing transcription factor (REST). This study demonstrated that not only are miRNAs capable of inducing neural development, different families are potentially associated with different types of cells, namely neurons versus astrocytes in this study, thus indicating specificity. Moreover, miRNA expression can also track different stages of development. Knowing that REST is typically downregulated in the first 8 days of retinoic acid treatment to allow neural miRNAs to be expressed, the authors posit that REST may provide the mechanism by which changes in miRNA expression leading to neural development are effected [15].

To better map the temporal change in miRNA expression during development of the brain, miRNA levels in brains from ICR mice at various ages were quantified using *Genopal*®-MICM DNA chips. Cerebrum, cerebellum, and hippocampus samples were taken at postnatal day 2 (P2), postnatal day 6 (P6), 1 month, 3 months, and 19 months postnatal; additional cerebrum and cerebellum samples were used at embryonic day 16.5 (E16.5). Stark differences in expression levels were noted between ages 1m or older versus younger ages, i.e. P2 and P6, indicating that the 1m mark is a critical point of change. This finding was corroborated by a rapid increase in brain weight from weeks 1-4 and the fact that miRNA expression profiles between cerebrum and cerebellum became markedly different at the 1m time point (miR-132, -138, -24, -29b, -137, -106a, -135a, and -19b). Over the course of development, several miRNAs were upregulated (miR-29a, -29b, -34a, -124a, -127, -129, -132, and let-7) while others were downregulated (miR-18, -19b, -20a, -106a, -130a, and -130b). In summary, this study corroborated and expanded Smith *et al*.'s findings with examination of additional miRNAs and identification of a critical 1-month window in which expression levels markedly change throughout the brain as well as between specific brain regions. The authors further noted that upregulation of let-7 and miR-124a with downregulation of the miR-17 and -92 clusters matched levels in *in vitro* neuronal differentiation of mouse embryonal carcinoma P19 and human teratocarcinoma NT2 cells. Downregulation of miR-17 and -92 clusters typically results in upregulation of *B-cell lymphoma 2*, *myocyte enhancer factor-2D*, and *zipper protein kinase* genes, indicating other promising pathways by which miRNA changes may occur [16].

A closer look at brain-specific miR-124: upstream regulators and downstream targets

Having established that miRNAs are important for different phases of neural development, much of the current research efforts have focused on elucidating upstream regulators and downstream effectors of miRNA function, particularly miR-124.

REST is a regulator that has been the focus of much attention due to its ability to potentially target numerous miRNAs. Enriched in non-neuronal tissue, REST will bind to RE1 sites to

actively repress neuronal genes. Corepressors associated with its repressor domains will recruit histone deacetylases and MeCP2, effectively silencing the REST target gene. In addition to the miR-124 family, miR-9 and miR-132 also lie near REST binding sites and thus may fall under REST control. In one study focusing on retinoic acid-driven neuronal differentiation in P19 cells, REST protein levels disappeared after 4 days of treatment. In the absence of REST, expression of mature miR-124a increased in tandem with the onset of terminal differentiation; in the presence of REST, miR-124a expression decreased as measured by luciferase reporter assays. Depletion of miR-124a using antisense 2'-O-methyl oligoribonucleotides (2'-OMe), which irreversibly binds to miRNAs and resists ribonuclease cleavage, resulted in increased expression of 10 of 17 nonneuronal transcripts identified by Lim *et al*. as being downregulated during neuronal differentiation. Overexpression of miR-124a lowered all 17 transcripts, corroborating Lim *et al.*'s findings and demonstrating REST's ability to inhibit miRNA expression. Derepression of miR-124a through lack of REST allows for neuronal differentiation [17].

In addition to REST, retinal non-coding RNA3 (*Rncr3*) in the mouse retina, the dominant source of miR-124a, has also been shown to regulate miR-124a-1 expression. *Rncr3 -/-* animals exhibited CNS dysfunction characterized by retinal cone cell death as determined by immunostaining and TUNEL assays, small brain size, and aberrant axonal sprouting of dentate gyrus granule cells into the CA3 region as determined through Timm staining for hippocampal mossy fibers. Furthermore, *Lhx2* was identified as a target of miR-124a along with *Ptbp1* and *Ctdsp1* in humans and mice through luciferase reporter assays, which demonstrated the ability of miR-124a to reduce the activity of luciferase attached to wild-type or mutated seed sequences of the Lhx2 3'UTR. Loss of *Rncr3* resulted in reduction of miR-124a expression and subsequent elevation of LHX2 levels in concordance with the previously identified CNS abnormalities. Downregulation of *Lhx2* is thus necessary for retinal cone cell survival and proper development of hippocampal mossy fibers. Expression of *Lhx2* lies within the purview of miR-124a, which in turn is under *Rncr3* control, demonstrating an alternative mode of miR-124 regulation [18].

In addition to LHX2, some downstream effectors of miR-124 function include small C-terminal domain phosphatase 1 (SCP1), BAF53a, and PTBP1. SCP1 is an anti-neural factor expressed in nonneuronal tissues much like REST [19]. Electroporation of a vector containing the SCP1 ORF coupled to a chick β-actin promoter into the spinal cord of developing chick embryos caused SCP1 to be constitutively expressed in neuroepithelial cells. Forced SCP1 expression delayed the transition of progenitor cells from proliferation to terminal differentiation, prolonging the progenitor or nonneuronal state, whereas introduction of an inactive SCP1 mutant resulted in earlier neurogenesis. Luciferase reporter assays established that miR-124 specifically targeted SCP1 via conserved 3' UTR sequences. miR-124 inhibition of SCP1 ultimately promoted neuronal differentiation and neurogenesis while inhibition of miR-124 by 2'-OMe reduced neuronal differentiation as ascertained by immunostaining with post-mitotic marker $p27^{kip1}$ and neuronal marker NeuN. Co-transfection of SCP1 with miR-124 inhibition further reduced differentiation, suggesting that miR-124 may be sequestered by SCP1 [19].

In P19 cells that normally lack miR-124, transfection of miR-124 induced neurogenesis as determined by expression of two proneural basic helix-loop-helix (bHLH) factors, Ngn2 and NeuroD, and neuronal marker TuJ; SCP1 expression was reduced. Co-transfection of miR-124 with SCP1-ORF significantly attenuated neuronal differentiation, while co-transfection with a normal miR-124-responsive SCP1 3' UTR still allowed differentiation to occur. It is interesting to note that the constitutive expression of SCP1 failed to completely abolish miR-124's neurogenic effects, suggesting that there are other miR-124 targets that play a crucial role in inducing neuronal differentiation [19].

In switching from a proliferative state to a committed cell fate, progenitor cells must undergo a mitotic or cell cycle exit, i.e. loss of multipotency marked by a switch in ATP-dependent chromatin-remodeling mechanisms. The mammalian Swi2/Snf2-like ATPase chromatin remodeling complex BAF is composed of 11 subunits and is specific to neural progenitors. To switch from neural-progenitor-specific BAF (npBAF) to neuronal-specific BAF (nBAF), the BAF53a subunit must be exchanged for its

Table 1. Summary of major miR-124 targets implicated in neural development

MicroRNA	Target	Source
miR-9	BAF53a	Yoo *et al*., 2009
miR-124	Lhx2	Sanuki *et al*. 2011
	Ptbp1	Sanuki *et al*. 2011
	Ctdsp1	Sanuki *et al*. 2011
	SCP1	Visvanathan *et al*., 2007
	BAF53a	Yoo *et al*., 2009
	PTBP1	Makeyev *et al*., 2007
miR-128	??	Bruno *et al*., 2011

As the most abundant miRNA expressed in the brain, miR-124 concomitantly bears numerous targets that support normal neural development by controlling progenitors' cell cycle exit.

homolog, BAF53b. This switch is crucial for development and dendritic morphogenesis in post-mitotic neurons: BAF53a promotes progenitor proliferation while BAF53b is essential for the switch to differentiation [20].

Immunocytochemistry and EGFP reporter assays showed that miR-9* and miR-124 both target BAF53a. Mutation of BAF53a 3' UTR binding sites for these miRNAs resulted in persistent expression of BAF53a and, consequently, impaired activity-dependent dendritic growth. Interestingly, inhibition of BAF53a was only achieved if both miRNA binding sites were mutated; a single mutation was not sufficient to repress BAF53a expression. Subsequent overexpression of BAF53a *in vivo* repressed BAF53b expression, suggesting some form of antagonism between the two subunits [20]. *In vivo* overexpression of miR-9* and miR-124 in neural progenitors reduced proliferation, which was corrected with expression of miRNA-resistant BAF53a. Finally, transfection of REST, which normally inhibits miR-9* and miR-124, reactivated BAF53a expression in neurons. Co-transfection of REST along with miR-9* and miR-124 restored BAF53a expression to normal levels. In summary, REST inhibition of miR-9* and miR-124 results in de-repression of BAF53a, leading to inhibition of BAF53b. Since BAF53a promotes proliferation of neural progenitors while BAF53b mediates the mitotic exit into differentiation, the net effect of REST is to maintain neural progenitor proliferation via BAF53a [20].

In addition to post-transcriptional modification, miRNAs are also capable of regulating gene expression through other downstream mechanisms, namely alternative pre-mRNA splicing. For example, Makeyev *et al*. showed that miR-124 specifically targets polypyrimidine tract-binding protein 1 (PTBP1) and its homolog, PTBP2, both of whom act as repressors of alternative splicing. PTBP1 is highly expressed in nonneuronal cells and inhibits splicing by binding pyrimidine-rich sequences near its target genes. PTBP2 is enriched in the nervous system and prevents splicing of the SrcN1 exon and the glycine receptor α2 exon E3A. The switch from general to neuronal alternative splicing mechanisms, much like the BAF53b-mediated switch to neuronal chromatin-remodeling mechanisms, appears to be mediated by upregulation of PTBP2.

Exact regulatory mechanisms are not known for either protein, although both proteins have conserved miR-124 binding sites in their 3' UTRs. Based on this finding, it was shown that miR-124 downregulates PTBP1 more aggressively than PTBP2, suggesting that PTBP1 is a stronger miR-124 target than PTBP2. PTBP1 can in turn inhibit expression of PTBP2; PTBP1 binding of exon 10 in PTBP2 causes it to be skipped during splicing, resulting in a premature termination codon and nonsense-mediated decay of the PTBP2 mRNA. PTBP2 can also repress several PTBP1 exons, albeit weakly [21]. Ultimately, miR-124 reduces PTBP1 expression levels, preventing PTBP1-mediated repression of PTBP2 and raising levels of the latter such that the PTBP1/PTBP2 ratio is skewed in favor of neuronal differentiation. In this way, miR-124 is able to influence neuronal gene expression levels during differentiation via inhibition of PTBP1 and subsequent activation of PTBP2's alternative pre-mRNA splicing regulatory functions [21]. Looking beyond miR-124, miRNA regulation of inhibitors of Wnt pathway may mediate neural development [22]. miR-128 may regulate development by repressing NMD of various transcripts [23]. For a summary of miR-124's targets, see **Table 1**.

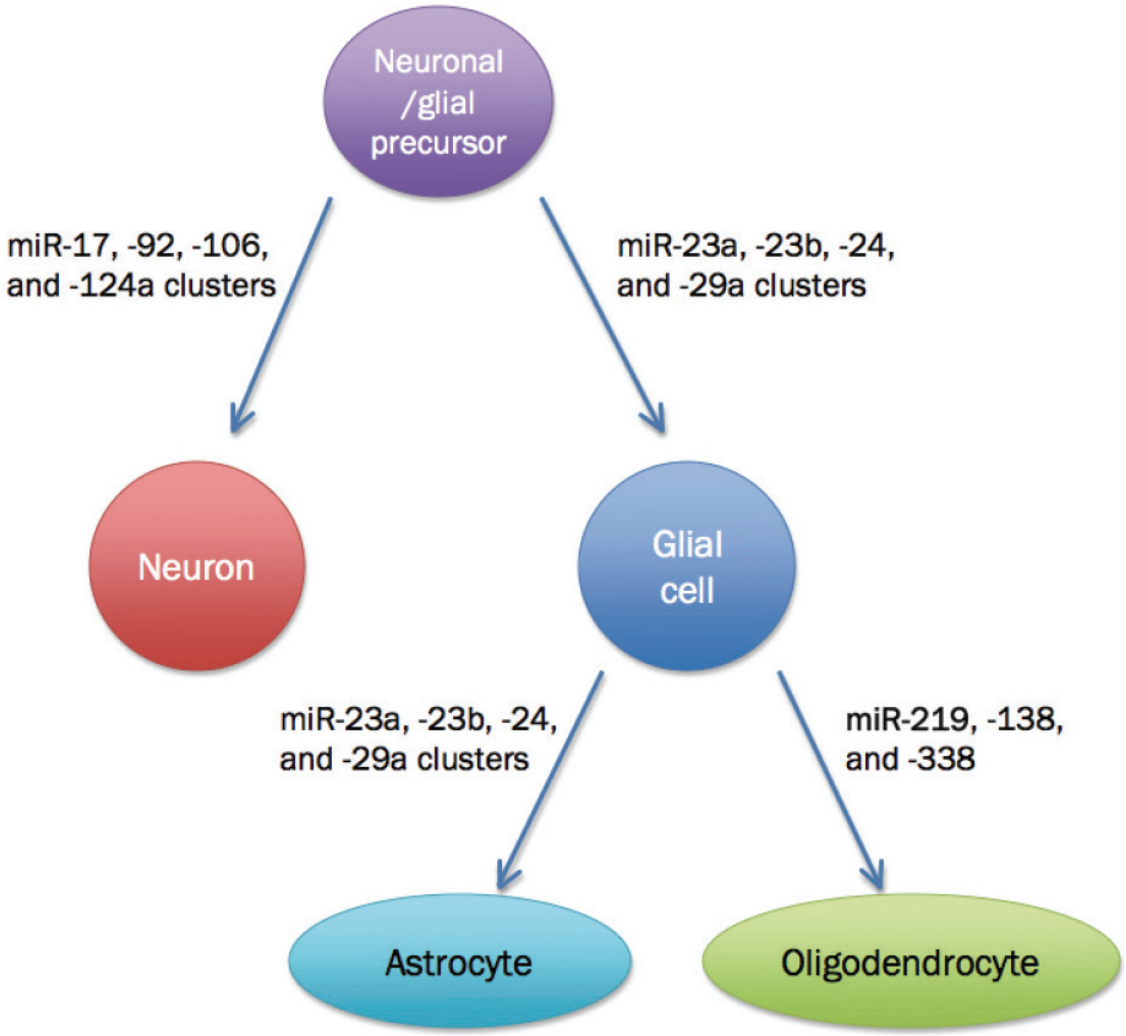

Figure 1. miRNA regulation of neuronal/glial progenitor differentiation. Neural cell fate is determined by a number of miRNAs. Although neurons and glial cells arise from a common progenitor, neuronal differentiation relies on miR-17, -92, -106, and -124a clusters while glial differentiation calls for miR-23a, -23b, -24, and -29a clusters. The addition of miR-219 plays a key role in determining the oligodendrocyte fate; miR-138 and -338 are also implicated in oligodendrocyte development.

miRNAs associated with specific neural regions and cell types

In recent years, more associations between miRNA clusters and specific neural cell types have been found. As previously shown, the miR-124 family has been linked to general neural differentiation. Deo *et al.* detected miR-124a throughout the mouse brain using *in situ* hybridization studies, albeit mostly in gray matter regions and not white matter [24]. Subsequent *in vivo* studies in mouse hippocampal neurons have shown miR-124a to play a key role in axogenesis [18], corroborating the neuronal specificity of miR-124a. Meanwhile, Northern blotting and RNase protection assays showed that miR-134 regulates dendritic spine development in rat hippocampal neurons [25]. *In situ* hybridization studies in mice also found particular miRNAs associated with the choroid plexus (miR-449, -204, and -224) [24, 26] as well as the ventricles (miR-9 and -92) [24].

With regard to glia, miR-24 paralogs such as the miR-23a, -23b, and -29a clusters were linked to astrocytes [15]. MiRNAs implicated in oligodendrocyte development included miR-219, -138, and 338 with miR-219 exhibiting the greatest induction during oligodendrocyte differentiation [27]. When Dicer1, an enzyme cru-

cial for cleaving pre-miRNA to yield mature miRNA, was selectively deleted from oligodendrocyte precursor cells (OPCs) and oligodendrocytes (OLs) in mice, a shivering phenotype surfaced that usually indicates CNS demyelination. The deficits associated with the shivering phenotype were partially rescued by miR-219, which proved to be necessary and sufficient for promoting oligodendrocyte differentiation. The positive regulation of oligodendrocyte differentiation by miR-219 was effected through repression of platelet-derived growth factor receptor alpha (PDGFRa), SRY-box containing gene 6 (Sox6), forkhead box J3 (FoxJ3), and zinc finger protein 238 (ZFP238). These targets normally encourage the proliferation of OPCs and repress OL differentiation, so that miR-219 inhibition of these factors would shift the equilibrium toward OL differentiation as determined by microarray analysis and immunohistochemistry in rodent neural cell cultures [27].

Another putative target was Elongation of very long chain fatty acids protein 7 (ELOVL7), which is highly expressed in mature OLs and help in the elongation of very long fatty acid chains. The inhibitory effect of miR-219 on ELOVL7, as determined by Western blot analysis and luciferase reporter assays upon miR-219 expression and deletion, would prevent excessive accumulation of lipids, whereas a lack of miR-219 would cause increased lipid accumulation, as detected by Oil Red-O staining. The buildup of lipids could ultimately result in demyelination, oxidative damage, and decreased peroxisomal function, triggering inflammation, neural degeneration, and growth retardation that shorten the animal's lifespan [28]. Altogether, miR-219 appears to play a key role in facilitating OPC differentiation and lipid homeostasis. For a summary of these key miRNAs governing differentiation of neural precursors into mature neural cell types, see **Figure 1**.

miRNAs in synaptic function and morphology

Having discussed individual components of the brain, one can begin to put the pieces together to better understand the role of miRNAs in the functional unit of the nervous system: the synapse. miRNAs are important for neural function as well as morphology. In examining pyramidal neurons in the prefrontal cortex of *Dgcr9* +/- mice, miRNA expression was decreased to a significant degree at P25, most notably with miR-134 and -491. Voltage and current clamp recordings on L5 pyramidal neurons in the medial prefrontal cortex were taken in order to determine the functional, electrophysiological consequences of this reduction in miRNA expression. Increased input resistance and decreased whole-cell capacitance without other changes indicated alteration of whole-cell electrical properties without changes in the membrane or leak conductances. Decreased frequency of spontaneous excitatory postsynaptic currents (EPSCs) without changes in amplitude or spontaneous inhibitory postsynaptic currents (IPSCs) were observed only beginning in the P25-30 time frame. Typically, the inverse relationship is seen between EPSCs and IPSCs: a rise in EPSCs and drop in IPSCs usually occurs roughly 3-4 weeks postnatal. These deficits persisted despite raising the experimental temperature to physiologic conditions [29].

To ascertain what morphological changes might account for the electrophysiological deficits, Golgi staining and three-dimensional reconstruction of L5 pyramidal neurons were performed. While the soma and apical dendrites remained unaffected, the basal dendrites exhibited fewer branch points and reduced total length, resulting in decreased basal dendrite complexity. It is interesting to note that basal dendrite elaboration coincides with the determination of intrinsic electrical properties in pyramidal neurons, indicating a connection between morphology and physiologic function [29]. The results of this study show that miRNA deficit, particularly in miR-134 and -491, can upset the balance of excitation/inhibition beginning roughly P25-30, a critical time point also identified by Eda *et al.* Thus, miRNAs are important for the development of proper morphology as well as functionality.

Three miRNAs have been shown to be important to synaptic function: miR-134, miR-138, and miR-132. miR-134 has been shown to localize to the dendrites of mature hippocampal neurons and negatively regulates dendritic spine morphology. A key target is LIM domain kinase 1 (LIMK1), which is part of the Rac-LIMK1 cascade. LIMK1 phosphorylates actin depolymerizing factor (ADF) cofilin, which normally instigates actin polymerization and growth of dendritic spines. These processes

Table 2. Summary of major miRNAs implicated in synaptic development and function

MicroRNA	Effect	Known Targets	Source
miR-132	Increased dendritic spine growth (canonical); decreased spine density?	p250RhoGTPase	Kosik, 2009; Schratt, 2009; Siegel *et al.*, 2011
miR-134	Electrical balance of EPSC/IPSC Decreased growth and maturation of dendritic spines	? LIMK1	Schofield *et al*., 2010; Kosik, 2009; Schratt, 2009; Siegel *et al.*, 2011
miR-138	Decreased dendritic spine growth, no change in abundance of synapses	LYPLA1, APT1	Kosik, 2009; Schratt, 2009; Siegel *et al.*, 2011
miR-491	Electrical balance of EPSC/IPSC	?	Schofield *et al*., 2010

Three miRNAs that control the morphology and subsequently the function of synapses are miR-132, -134, and -138. Growth effects are chiefly mediated through signaling cascades: miR-134 acts on the Rac-LIMK1 cascade while miR-132 and -138 target the RhoA-ROCK cascade.

are associated with synaptic growth in long-term potentiation (LTP), a key feature of synaptic plasticity. By inhibiting LIMK1, miR-134 effectively decreases the growth and size of dendritic spines, thus impairing LTP. However, this inhibitory effect can be rescued with application of brain-derived neurotrophic factor (BDNF), a protein released during heightened neuronal activity, suggesting that miR-134 expression can be modified by neuronal activity. It is thought that miR-134's effects on synaptic plasticity may be mediated by cAMP response element binding protein (CREB), a cellular transcription factor, and/or Pumilio homolog 2 (PUM2), an RNA-binding protein that regulates spatial and temporal miRNA expression. In short, miR-134's net effect is to repress dendritic spine growth and maturation through inhibition of LIMK1, thus playing a potential role in LTP-induced synaptic remodeling.

The following two miRNAs, miR-138 and -132, regulate dendritic spine morphology primarily through the RhoA-ROCK pathway, albeit with differing net effects. Activation of the small GTPase protein RhoA (Ras homolog gene family, member A) by Gα13 initiates the RhoA-ROCK cascade, ultimately resulting in actomyosin contraction and shrinking of dendritic spine size.

miR-138 negatively regulates dendritic spine morphology by targeting a depalmitoylating enzyme called lysophospholipase 1 (LYPLA1) or Acyl-Protein-Thioesterase 1 (APT1). APT1 catalyzes the depalmitoylation or inactivation of Gα13, thereby preventing activation of RhoA so that dendritic spine growth can occur. Since APT1 ultimately promotes spine growth, miR-138 repression of APT1 will lead to a reduction in spine growth without change to the number of synapses.

miR-132 was initially thought to positively regulate dendritic spine growth and target p250RhoGTPase, which functions similarly to Gα13 by activating the RhoA-ROCK pathway. By inhibiting p250, miR-132 prevents activation of the RhoA-ROCK pathway such that the net effect is of dendritic spine growth. miR-132 has also been shown to target methyl CpG binding protein 2 (MeCP2), a gene important for silencing other genes and whose dysfunction has been implicated in Rett syndrome. In addition to targeting p250 and MeCP2, miR-132 is activated by Ca2+-sensitive CREB and BDNF. Some recent studies showed overexpression of miR-132 did not increase but rather decreased spine density while increasing average dendritic spine width, but the regulatory mechanisms still require further investigation [30]. To date, the function(s) of miR-132 remain controversial although positive effects on dendritic spine growth have been demonstrated [13, 30, 31].

Proper development of synaptic morphology and function depend on a number of miRNAs. In particular, miR-134, -138, and -132 are important regulators of dendritic spine growth, which is important for neuroplasticity. For a summary of their known targets and effects, see **Table 2**. These miRNAs can modulate gene expression via numerous signal transduction pathways. Without proper miRNA expression, electrophysiological and morphological abnormalities may occur, impeding neuronal function.

miRNA regulation of neural stem cell proliferation and differentiation

At this point in time, there is enough information to link some miRNAs to specific populations and regions of cells thanks to numerous microarray studies. The accumulation of knowl-

edge has spurred the investigation of upstream and downstream components of potential signaling pathways. However, elucidating the connection between these islands of information in order to understand mechanisms of cellular differentiation still poses a challenge. One aspect of this effort is chronicling the function of different miRNAs implicated in initiating differentiation.

Previous work in our lab has demonstrated that members of the miR-17 family play important roles in embryonic development and regulate differentiation by targeting the mRNA of key transcription factors, such as Signal Transducer and Activator of Transcription 3 (STAT3). As shown by *in situ* hybridization using locked nucleic acid miRNA specific probes, miR-17 family members (miR-17-5p, -20a, -93, and -106a) were all differentially expressed in E4.0 blastocysts. In particular, miR-93 localized to differentiating primitive endoderm and trophectoderm tissues of the blastocysts; expression of miR-93 and -17-5p was also seen in the mesoderm of gastrulating embryos. The expression of STAT3, predicted to be a key target of the miR-17 family, was specific to the inner cell mass and was minimally present or absent in regions of high miR-93 expression, suggesting that miR-93 might be responsible for STAT3 downregulation [32].

To further investigate the role of miR-17 family miRNAs in differentiation, two models were used: blastocyst cell types and embryonic stem (ES) cells. Analysis by qRT-PCR revealed consistent upregulation of miR-93 upon initiation of differentiation, specifically in the putative primitive endoderm and trophectoderm of the blastocyst. Transfection of miR-93 inhibitors into ES cells disrupted endodermal differentiation and delayed expression of Brachyury, a marker of mesodermal differentiation. Addition of a miR-93 mimic proved more effective than a miR-20 mimic at increasing Brachyury expression. Although miR-20 and miR-93 both have different effects on different germ layers, miR-93 more potently induced differentiation than miR-20 [32].

Finally, regulation of STAT3 expression by miR-17 family members was explored using CMV-promoter luciferase gene constructs containing various binding sites for the miRNAs. Since STAT3 possesses two predicted binding sites for the miR-17 family within its 3'UTR, constructs were made with mutations at either the first (pMIR-S3st1mt), second (pMIR-S3st2mt), or both binding sites (pMIR-S32xmt) in addition to the wild-type 3'UTR (pMIR-S3). The positive control (pMIR-miR20) contained two synthesized binding sites specific for miR-20, enabling it to be readily silenced by any endogenous miR-17 family miRNAs. The negative control (pMIR-Hoxa11) contained the 3'UTR from the Hoxa11 gene, which is predicted to be a miR-181 target and not a miR-17 family target so that it should be resistant to silencing. Endogenous miR-17 family miRNAs predictably reduced luciferase activity in pMIR-S3. A modest decrease was seen in pMIR-S3st2mt (mutation in the second binding site) while no decrease was seen in pMIR-S3st1mt and pMIR-S32xmt, indicating that the first binding site was more important than the second for miRNA binding. The miR-93 mimic was more effective at silencing pMIR-S3 than the miR-20 mimic, confirming miR-93's potency. When applying the inhibitors, which should result in greater if not maximum luciferase activity, the miR-20 inhibitor yielded attenuated reporter function while the miR-93 inhibitor resulted in complete disinhibition of luciferase function matching that seen with pMIR-S32xmt. These data indicate that miR-93 binds to and has a profound silencing effect on STAT3, thereby regulating differentiation in ES cells and also likely in embryonic development [32].

Recent findings from our lab provide further evidence for post-transcriptional regulation of differentiation. Ribosomal S6 kinase (S6K) is necessary for normal development of ES cells and human induced pluripotent stem cells (iPSCs) into cardiomyocytes. In addition to being upregulated in beating cardiomyocytes compared to surrounding, non-beating differentiated cells in ES cell culture, S6K appears to be an intermediary kinase responsible for enhancing translation of a class of genes whose mRNA contains 5' terminal oligopyrimidine (5'TOP) sequences. This group of genes includes connexin 43 (*Cx43*), desmoplakin (*Dsp*), and phosphatase and tensin homolog (*PTEN*), all of which are important for cardiac development. Repression of S6K in murine ES cells using short interfering RNA (siRNA) resulted in downregulation of these 5'TOP mRNAs without any change in non-5'TOP mRNAs, such as NkX2.5, cardiac troponin, Gata4, actin, and β-tubulin. Cardiomyoge-

nesis was attenuated with a marked decrease in average and total number of beating areas in both ES cells and human iPSCs. As early as 7 days post-siRNA transfection, the cells displayed an unexpected shift toward the neuronal lineage. In fact, increased expression level of neurofilament M confirmed differentiation toward a neural fate. This differential effect of S6K was confirmed *in vivo* with E9.5 embryos that showed high expression of S6K in cardiomyocytes in the nascent heart tube and low expression of S6K in the neural tube [33]. In total, there is strong evidence for post-transcriptional control of neural development through predictable as well as unexpected, pleiotropic mechanisms.

Deepening the investigation, a number of miRNAs have been implicated in promoting proliferation of neural stem cells (NSCs) into mature neural cell types. Ichi *et al*. noted increased methylation of histone H3 at lysine residue 27 (H3K27) in homozygous *Splotch* ($Sp^{-/-}$) mice that present with neural tube defects secondary to impeded thymidylate synthesis. Increased H3K27 methylation was correlated with decreased production of the demethylase, KDM6B, which is a known target of miR-138, -148a, -185, and -339-5p. The net increase in histone methylation rendered the histone inactive, thereby skewing NSC towards proliferation rather than differentiation. This condition was rescued with administration of folate, which rescued KDM6B levels and decreased H3K27 methylation so that gene transcription could occur, leading to differentiation [34].

In other studies, miR-31 and -29 were shown to promote proliferation of NSCs in the rat spinal cord [35]. Interestingly, expression in adult neural stem/progenitor cells of miR-25 alone was shown to promote proliferation *in vitro*, but expression of the entire miR-106b~25 cluster further led to increased ability to produce neurons, suggesting more subtle roles in balancing proliferation and differentiation [36]. miR-29, -31, and the -222-221 cluster were shown to promote proliferation of NSCs in embryonic rat spinal cords, with astrocytes also expressing miR-29 [35]. Lastly, miR-124 is typically associated with neuronal differentiation, but Weng *et al*., found that it is required for neuroblast proliferation in *Drosophila melanogaster*, suggesting that miR-124 may have differential effects on different organisms due to evolutionary divergence in target sites [37]. Wei *et al*. also found no difference in miR-124 expression in NSCs versus motor neurons in embryonic rat spinal cords [35].

With regard to NSC differentiation, miR-196 was shown to closely regulate Hoxb8 patterning in the developing neural tube both *in vitro* and *in vivo* [38]. LIN28, which can promote pluripotency by inhibiting *let-7* miRNA processing, was found to be highly expressed in undifferentiated murine cells and downregulated following differentiation *in vitro*. Interestingly, overexpression skewed differentiation towards neurogenesis rather than gliogenesis even with a mutated form of LIN28 that allowed let-7 accumulation, suggesting that LIN28 is able to operate independently of let-7 to promote gliogenesis [39]. More detailed research has shown that both let-7 and miR-137 affect neural stem cell development via the nuclear receptor TLX, which normally promotes proliferation and suppresses differentiation of neural stem cells. The presence of let-7 or miR-137 will result in downregulation of TLX, thereby promoting neuronal differentiation [40, 41]. Chen *et al*. found miR-17-3p to be important for *in vivo* murine spinal cord patterning via Olig2/Irx3 cross-repression [42], while miR-23 and -124 were both found to act on Sox9 expression in neuroepithelial cells of the rat spinal cord, promoting neuronal rather than glial development [43].

Other miRNAs that promoted neural differentiation include let-7 [40, 41, 44], miR-9 [45-48], -17-3p [42], -23 [43], -34a [49], -124 [35, 48, 50], -126 [35], -134 [51], -135 [52], -137 [40, 41], -181 [53], -183 in mice [54], -196 [38], and -371-3 for human ES cells and induced pluripotent stem cells [55]. Mukhopadhyay *et al*. also found evidence for miR-106b~25, -19a and -19b, and -17~92 amongst many others playing a pro-differentiation role. For a summary of miRNAs that have been shown to promote neural stem cell differentiation, see **Table 3**.

Conclusion

It is clear that a number of pro-proliferative and pro-differentiating miRNAs (along with their targets) are involved in appropriate neural development. Research has shown that neural development is specifically tied to certain miRNAs, such as miR-124, and that miRNAs are

Table 3. Targets of miRNAs that promote differentiation of neural stem cells

MicroRNA	Targets	Source
Let-7	TLX	Sun *et al*., 2011; Zhao *et al*., 2010
	Mlin41/Trim71	Maller Schulman *et al*., 2008
miR-9	Hairy1	Bonev *et al*., 2011
	Stathmin	Delaloy and Gao, 2010
	Notch-1	Jing *et al*., 2011
	STAT3	Mukhopadhyay *et al*., 2011
miR-17-3p	Olig2/Irx3	Chen *et al*., 2011
miR-17~92, -106a~363, -106b~25 (paralogous clusters)	Various components of MAPK cascade	Mukhopadhyay *et al*., 2011
miR-19a and -19b	Lrp2 (Megalin), RhoB, IGF, Wnt, and MAPK pathways	Mukhopadhyay *et al*., 2011
miR-23	Sox9	Farrell *et al.,* 2011
miR-34a	SIRT1, p53	Aranha *et al.*, 2011
miR-124	Sox9	Farrell *et al.,* 2011
	Integrins (e.g. Itgβ1, Itga7, Itgα3, Itgα11), STAT3	Mukhopadhyay *et al*., 2011
	SNAI2	Xia *et al*., 2012
miR-125	Mlin41/Trim71	Maller Schulman *et al*., 2008
miR-126	HOXA9, HOXA3?	Wei *et al.,* 2010
miR-134	Chrdl-1, Dcx	Gaughwin *et al.*, 2011
miR-135	?	Arnold *et al*., 2011
miR-137	TLX	Sun *et al*., 2011; Zhao *et al*., 2010
miR-181	LIN28	X. Li *et al*., 2012
miR-183	mSEL-1L	Cardano *et al*., 2011
miR-196	Hoxb8	Asli *et al*., 2010
miR-371-3	BMP inhibitors (e.g. BAMBI, CHRD, CRIM1)?	Kim *et al.*, 2011

Until they receive signals to undergo differentiation, NSCs will remain in a proliferative state. miRNAs that promote differentiation will often target transcription factors such as TLX and Sox9. NSCs exiting the proliferative state will become neuronal and/or glial precursors and may express different miRNA profiles before arriving at a particular cell fate. Note: LIN28 is a RNA-binding protein, not a miRNA.

associated with particular cell types as well as synaptic morphology and function. Regulation of neural stem cell proliferation and differentiation also appears to fall under the influence of miRNAs.

The dynamic roles that miRNAs play are gradually becoming more apparent, particularly in developmental and stem cell biology where rapid, subtle changes in miRNA expression can efficiently effect the necessary dramatic changes in gene transcription. The ubiquity of both stem cells and miRNAs suggest that they hold the key to modeling or mimicking physiologic systems, which can prove especially helpful in complex neurological conditions such as Alzheimer's disease or addiction. Stem cells are excellent tools for studying the far-reaching effects of miRNAs due to their pluripotency and therapeutic potential.

One challenge lies in determining whether miRNAs are a cause or effect of changes in transcriptional regulation. Although studies have provided evidence that certain miRNAs are necessary and sufficient for post-transcriptional modifications to occur, the exact mechanisms connecting miRNAs to correlated effects remains elusive. In order to tease out whether miRNAs are a cause, effect, or a combination of both in any physiologic mechanism, more sensitive methods of detecting miRNA expression must be developed. Since many of the effects of miRNA repression can be subtle and transient, experiments with finely controlled temporal parameters would help shed more light on mechanisms of miRNA function. In addition, there are a number of published studies that draw contradictory conclusions about a particular miRNA, indicating greater complexity behind their function. Designing experiments for ES

cells and iPSCs may help elucidate the multifactorial effects of miRNAs more thoroughly. Their pluripotency would also allow researchers to observe effects without the influence of other factors, or else addition of the influencing factor could be tightly regulated.

Lastly, translating purported miRNA pathways from *in vitro* to *in vivo* models will require further investigation. Although miRNAs are generally highly conserved, differences do exist between humans and other mammals. Recent studies have begun to explore how well conserved miRNAs are between humans and mammals commonly used for research, such as mice, rats, pigs, and non-human primates. For example, despite the fact that human miR-1271 and mouse miR-96 have similar *in vitro* regulatory activities and both cause hearing loss in their respective species, they differ slightly in sequence. This difference results in different *in vivo* functions, identifying them as paralogs with distinct, non-conserved functions [56]. Thorough study of similarities and differences between miRNA paralogs will allow us to collect more accurate data and extrapolate more meaningful conclusions. Stem cells and iPSCs can contribute to the effort by providing a tabula rasa upon which miRNAs can act. The resultant effects can be measured without the interference of other cell types. Furthermore, researchers can use stem cells and iPSCs to examine antagonistic and/or synergistic miRNAs to tease out more subtle interactions between them.

The intersection of miRNAs and stem cells represents a singular opportunity to investigate novel developmental pathways. Although the process of neural development has been well established, the addition of miRNAs brings fresh perspective to understanding and elucidating regulatory mechanisms. Future research stands to uncover new connections between miRNAs and stem cells, deepening a knowledge base already undergoing rapid expansion.

Acknowledgements

Sincere thanks must be extended to Shannon M. Larabee for her scientific expertise, help in assembling figures, and unwavering support.

Declarations

The authors declare that they have no conflicts of interest, financial or otherwise.

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