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Minireview

MicroRNAs Potentiate Neural Development

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MicroRNAs (miRNAs) are endogenously expressed noncoding RNAs that regulate mRNA expression. In vertebrates, more distinct miRNAs are expressed in the brain than in any other tissue, where they are hypothesized to function in neural development. Recent reports describing the effects of specific miRNAs during development, and studies employing miRNA depletion as neural commitment proceeds in the embryo, support a requisite role for miRNAs in cell-fate decisions and provide clues to their function in other aspects of nervous system development.

MicroRNAs are small noncoding RNAs that regulate gene expression in a variety of organisms by base pairing to mRNAs. miRNA expression is enriched in the nervous system (Krichevsky et al., 2003; Miska et al., 2004; Sempere et al., 2004; Wienholds et al., 2005), and distinct miRNA expression profiles are seen in germ cells, neural stem cells, and fetal brain (Kloosterman et al., 2006a; Wienholds et al., 2005), with levels changing dramatically upon neuronal differentiation (reviewed in Gangaraju and Lin, 2009). Recent studies have helped clarify the role of miRNAs in neural development. Together the data support the hypothesis that miRNAs influence cell differentiation and/or cell cycling in the developing CNS and may contribute to neurodevelopmental diseases. While the focus of this review is on miRNAs, it is important to note that other species of noncoding RNAs have functional importance in neural cells, including small nucleolar RNAs (snoRNAs) (Cavaille et al., 2000), small cytoplasmic RNAs (Tiedge et al., 1993), and endogenous small interfering RNAs (endo-siRNAs) (Carlile et al., 2009).

miRNA Biogenesis

miRNA transcription occurs via RNA polymerase II or RNA polymerase III, upstream of intergenic miRNAs or miRNAs residing in introns of coding or noncoding genes (reviewed in Liu et al., 2008) (Figure 1). For many miRNAs, the primary transcript (pri-miRNA) is processed in the nucleus by Drosha/DGCR8 to liberate an ~70 nt pre-miRNA product that is then exported into the cytoplasm by exportin V. Some miRNAs encoded in introns can bypass nuclear processing (mirtrons) prior to export (Berezikov et al., 2007; Ruby et al., 2007). The cytoplasmic RNase Dicer further processes the pre-miRNA into a 21–22 nt duplex, and one or both strands is then loaded into the Ago-protein-containing complex called the RNA induced silencing complex (RISC). Within RISC, the single-stranded mature miRNA forms partial complementary contacts on target mRNAs, which typically mediate mRNA degradation or translational inhibition.

miRNAs can be controlled posttranscriptionally by several pathways. For example, in embryonic stem cells, Lin28 inhibits let-7 maturation, but in neural stem cells, Lin28 is downregulated and let-7 processing proceeds (Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). In some cell types, inhibition of processing occurs through Lin28-mediated recruitment of an uridyl transferase (Heo et al., 2008). Following uridylation, pre-let-7 fails to proceed to Dicer processing and is degraded.

Alteration of the pri-miRNA sequence by the action of Adenosine Deaminase Acting on double stranded RNA (ADAR) enzymes can also affect miRNA processing (Kawahara et al., 2007a, 2008; Luciano et al., 2004; Yang et al., 2006) or alter the mRNAs they target (Kawahara et al., 2007b). The consequence of ADAR activity is the conversion of adenosine (A) to inosine (I). Because I is read as guanine for base pairing, consequences might include changes in stem-loop stability as well as changes in target mRNA selection. A to I editing of pri-miRNAs was demonstrated in human and mouse brain samples (Kawahara et al., 2007a). Given that the brain shows enriched ADAR activity compared to other tissues, editing-induced functional changes to miRNAs or creation of novel miRNA target sites in 3′ UTRs of mRNAs (Borchert et al., 2009) may facilitate expression of brain-specific genetic programs.

Some miRNAs have upstream RE1 silencing transcription factor (REST) elements that can repress their expression in non-neuronal cells and neural progenitor cells (reviewed in Ballas and Mandel, 2005). Interestingly, several of the miRNAs whose levels are controlled by REST target mRNAs that encode components of the REST repressor complexes, including REST, CoREST, MeCP2, and SCP1 (Conaco et al., 2006; Klein et al., 2007; Packer et al., 2008; Visvanathan et al., 2007). If REST inhibits the expression of miRNAs, what induces their expression? While not fully understood, Vo et al. showed that the transcription factor CREB occupies elements upstream of miR-132, suggesting that it may promote miRNA expression (Vo et al., 2005).

There are antisense transcripts nearby and/or overlapping with miRNAs that can give rise to another miRNA that, in the case of *Drosophila*, serves to regulate genes within the same family as the sense-encoded miRNA (Stark et al., 2008a; Tyler et al., 2008). Transcripts antisense to miRNAs could possibly regulate the activity of the sense miRNA through competitive binding, similar to artificial "sponge" sequences sequestering

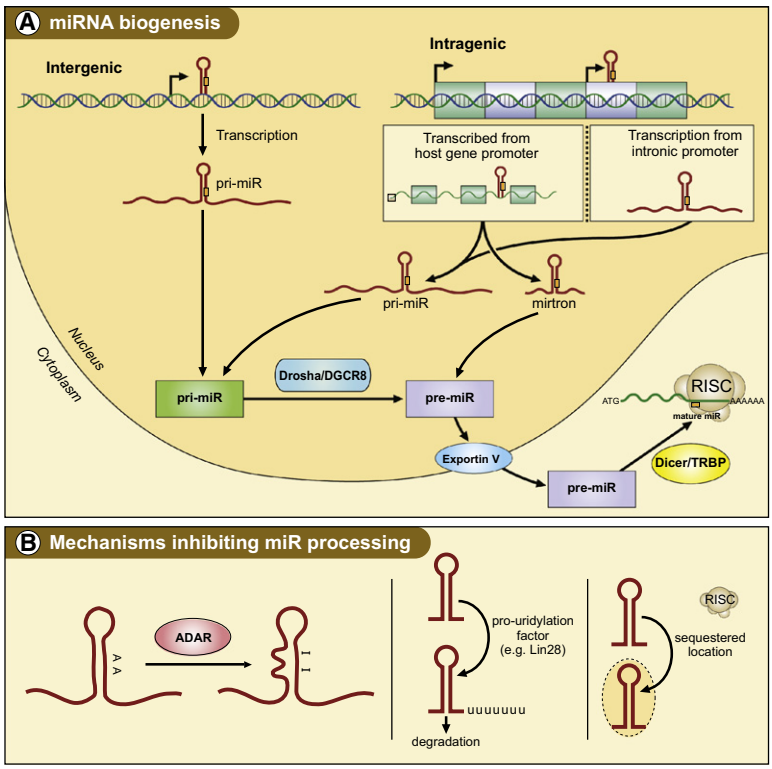

Figure 1. miRNA Biogenesis and Processing

(A) MiRNAs are expressed as pri-miRNAs or mirtrons as depicted. Pri-miRNAs are processed by Drosha/DGCR8 in the nucleus to pre-miRNAs prior to cytoplasmic export by exportin V. The pri-miR is shown encoding a single miRNA, but pri-miRNAs encoding several miRNAs are common. Intronic miRNA expression can be controlled from host gene promoters, or internal promoters (Ozsolak et al., 2008). Once in the cytoplasm, pre-miRNAs are further processed by Dicer/TRBP to form a 21–22 nt duplex. Association of the duplex with an argonaut protein complex forms the RISC, which can mediate inhibition of mRNA expression.

(B) miRNA levels can be controlled by transcription (e.g., promoter activity) or processing. Inhibition of processing can occur via blockage of Drosha processing in the nucleus (e.g., by ADAR activity [Yang et al., 2006]), export, or Dicer processing in the cytoplasm (e.g., by uridinylation [Rybak et al., 2008]). Inhibition of activity could also be controlled by sequestration of the miRNA, impeding miRNA:mRNA interaction.

endogenous miRNAs from their targets (Brennecke et al., 2003). Deep sequencing efforts to quantify the relative levels of these transcripts and the effects of loss of function of the antisense sequences will help define their role.

miRNA Function in Early Neurogenesis

While an absolute requirement for miRNAs in the initiation of neurulation has not been reported, they are plausible players. Dicer null zebrafish undergo nervous system differentiation (Giraldez et al., 2005), but they have prominent morphological nervous system defects. When Dicer is deleted in the developing mammalian nervous system, gross histological aberrations, and in some cases embryonic lethality, occur (Choi et al., 2008; Cuellar et al., 2008; Damiani et al., 2008; Davis et al., 2008b; De Pietri Tonelli et al., 2008; Kim et al., 2007; Stark et al., 2008b). While these studies support a strict requirement for Dicer in neural development, these phenotypes may not be due solely to miRNA depletion; Dicer is required for the maturation of some snoRNAs (Taft et al., 2009) and endo-siRNAs (Golden et al., 2008). And while a reduction of abundant brain-enriched miRNAs (e.g., miR-9 and miR-124) has been reported in Dicer null embryos, it is doubtful that all mature miRNAs are affected. Indeed, one challenge of studies utilizing conditional, cell-type-specific dicer ablation is determining exactly when and which miRNAs are lost. miRNA half life in vivo could be highly variable, and functional effects could persist days after Dicer inactivation (Davis et al., 2008b). Finally, Dicer depletion could elevate pri-miRNAs and pre-miRNAs, impacting cell division and differentiation in a manner independent from loss of mature miRNA(s) (Stark et al., 2008b).

To refine our understanding of the role of miRNAs in development, investigators have assessed how specific miRNAs impact protein complexes with defined functions. One early example of miRNAs' importance in cell-fate decisions is the elegant description of left-right asymmetry decisions in *C. elegans* sensory neurons (Chang et al., 2004; Johnston and Hobert, 2003). More recently, Slack and colleagues found that loss of the let-7 target gene, *Mlin41*, causes neural tube defects, indicating a role for miRNAs in neural tube closure in mouse (Maller Schulman et al., 2008). Other target proteins required for neural tube closure may be under miRNA control. For example, miR-9* and miR-124 inhibit the neural progenitor cell specific BAF53a and BAF45a, reducing proliferation and inducing differentiation (Yoo et al., 2009). One could envision that other miRNAs might also inhibit expression of the prodifferentiation homologs of these proteins, BAF53b and BAF45b. An imbalance of miRNAs controlling these opposing complexes could shift developmental transitions between progenitor cells and their differentiated phenotypes.

The interplay between miRNAs and development extends beyond direct miRNA targets. For example, miR-124 also impacts neuronal gene expression broadly by reducing the expression of the splicing regulator, PTBP1 (Makeyev et al., 2007). Because PTBP1 induces nonsense-mediated decay of PTBP2, an important neuronal cell splicing regulator, the loss of PTBP1 stabilizes PTBP2, thus increasing its proneuronal splicing activity.

miRNAs in Cortical Development

miRNAs play a role in cortical neogenesis (Tables 1 and 2). Two recent studies assessed the consequences of Dicer depletion in olfactory progenitor cells at approximately embryonic day 9.5 (E9.5), a point which closely coincides with olfactory neogenesis. Choi et al. noted a reduced number of neuron-committed progenitor cells and mature neurons (Choi et al., 2008), while Makeyev et al. observed disorganization throughout the cortex, ectopic expression of PTBP1, and loss of the postmitotic post-migratory marker MAP2 (Makeyev et al., 2007). Apoptosis was increased, but the studies did not include a detailed analysis of progenitor cell pools or their proliferation. miRNAs also play a role in progenitor cell maintenance and differentiation and neocortex formation (De Pietri Tonelli et al., 2008). Deletion of Dicer in the developing cerebral cortex by E9.5 led to the

Table 1. Genetic Depletion of miRNA Production in Mouse Brain

	[a]Foxg1-Cre; Dicer$^{loxP/loxP}$	[b]Emx-1-Cre X Dicer$^{loxP/loxP}$	[c]Microdeletion Syntenic to Human 22q11.2	[d]Rag1AG-Cre (α-CamKII Promoter);Dicer$^{loxP/loxP}$	[e]Chx10-Cre;Dicer$^{loxP/loxP}$
Location/time of enzyme loss	E9.5 concurrent with olfactory neurogenesis	From E9.5 in the dorsal telencephalon	Presuming all tissues, timing unknown. Genetic deletion of DGCR8-containing region; (deletes other genes in the region) creates model haploinsufficient for DGCR8.	From E15.5 in cortex and hippocampus	Mosaic expression in developing retina at P16
Confirmed time of miR loss	E13.5 miR200a is reduced from olfactory epithelium	E10.5	Pri-miRs rise, but 19% of mature miRs expressed in prefrontal cortex and 10% of miRs expressed in hippocampus are reduced	For miR132 14%–20% loss at P15, 60% by P21	No change at ~P30, but strong decreases in several miRNAs at 3 and 7 months
Survival	Death in utero	To weaning	Normal in hemizygous deletion	Homozygotes die early postnatal (40% by P2, 100% by P20)	Normal
Patterning	Defined regions of olfactory structure are present	Normal		Normal	Normal
Brain size	Small forebrain and eyes	Small forebrain	Normal with DGCR8 haploinsufficiency	Small forebrain and reduced brain mass at P21	
Cell loss	Loss of olfactory markers by E10.5, profound by E16.5	In emerging lamina by E12.5, later in proliferative regions		Not directly measured, but enlarged lateral ventricles and smaller cortex	Extensive retinal degeneration
Apoptosis	Present by E10.5, persistent at E12.5	Present by E12.5, widespread by E14.5		Apoptotic cells near VZ at P0, limited apoptotic signal at P15	Extensive cell loss from 3 months on
Histopath	At E10.5, no gross change in olfactory pits, but 18% neuron loss. At E13.5, marked thinning with loss of olfactory neuron markers.	Decreased proliferation affects SVZ > VZ, present by E14.5. Reduced cortical thickness and disorganization.	In hippocampus, decrease in dendrite complexity, spine number and size	Decreased dendritic branching and increased apical dendritic spine length in hippocampus at P21	Rosette formation at P16, then progressive remodeling of retinal lamina between P16 and P45
Cortical layers		Distinct cortical layers fail to form		Normal	
Behavior			Hyperactive, but more fearful of novelty. Reduced performance on fear-context task and spatial memory task.	At P14, tremors, ataxia, unison hindlimb movement, clasping	ERG amplitudes decreased in homozygous and heterozygous animals

[a] Choi et al., 2008.
[b] De Pietri Tonelli et al., 2008.
[c] Stark et al., 2008b.
[d] Davis et al., 2008b.
[e] Damiani et al., 2008.

Table 2. MiRNAs and Neural Development

miRNA	Function	Targets	Regulation	Reference
let-7	Neural tube closure	Mlin41		Maller Schulman et al., 2008
miR-9	Promotes NPC differentiation in mouse cortex, maintains organizer activity at midbrain-hindbrain boundary in zebrafish	TLX, REST, Fgf8, FGFR1	RE1 sites in promoters block nonneural transcription	Conaco et al., 2006; Packer et al., 2008; Shibata et al., 2008; Zhao et al., 2009
miR-9*		BAF53a, BAF45a, CoREST	As miR-9	Yoo et al., 2009; Packer et al., 2008
miR-10	Control of zebrafish branchial nerve migration	HoxB1a, HoxB3a		Woltering and Durston, 2008
miR-17	Blocks neural differentiation		Repressed by retinoic acid	Beveridge et al., 2009
miR-34		Cell cycle genes, predicted to target Notch pathway, Wnt pathway, Math1	p53, CREB	
miR-124	Promotes neuronal transcriptome	BAF53a, BAF45a, SCP1, PTBP1, Sox9	RE1 sites in promoters block nonneural transcription	Yoo et al., 2009; Conaco et al., 2006; Visvanathan et al., 2007; Makeyev et al., 2007; Cheng et al., 2009
miR-125	May promote cerebellar granule cell differentiation	Smo, Gli		Ferretti et al., 2008
miR-132		MeCP2	RE1 sites in promoters block nonneural transcription; CREB induces transcription	Conaco et al., 2006; Klein et al., 2007; Vo et al., 2005
miR-324-5p	May promote cerebellar granule cell differentiation	Smo, Gli		Ferretti et al., 2008
miR-326	May promote cerebellar granule cell differentiation	Smo, Gli		Ferretti et al., 2008

reduction of some specific miRNAs by E10.5. By E12.5, the cerebral cortex was thinned, and there was increased apoptosis in newborn neurons. Animals were microcephalic and failed to thrive past P24–P25. Interestingly, assessment at E13.5 revealed no impact of miRNA depletion on apical progenitors in the ventricular zone and basal progenitors in the subventricular zone. By E14.5, however, there was depletion of mitotic apical and basal progenitor cells. Thus, loss of miRNAs in neural progenitor cells early in corticogenesis profoundly impacts production of neurons and subsequently decreases progenitor cell renewal. The extent to which loss of progenitor cells is due to altered asymmetric divisions, precocious neuronal differentiation, or apoptosis is unclear. Also unclear is whether these defects reflect loss of many miRNAs or a select few.

In the cortical hem (a narrow strip of tissue in the developing forebrain that lies between the emerging choroid plexus and the more rostral neural tissue), miR-9 promotes the differentiation of NPCs and impacts progenitor cell pools (Shibata et al., 2008). Overexpression of miR-9 at E11.5 but not E14.5 caused ectopic production and location of Cajal-Retzius neurons, implying that miR-9-overexpressing progenitor cells, which consequently possess reduced levels of the miR-9 target FoxG1, differentiate prematurely and cause disorganization. Similar results are seen with another miR-9 target, TLX, a nuclear receptor essential for neural stem cell self-renewal (Zhao et al., 2009). Overexpression of miR-9 at E13.5 reduces TLX levels, leading to inappropriate migration and premature differentiation.

Depleting miRNA pools later in cortical development is also detrimental. Mice with Dicer loss at ~E15.5 in postmitotic excitatory forebrain neurons have microcephaly, alterations in hippocampal neuron spine density and branching, and ataxia by P15 (Davis et al., 2008b). Fifty percent of the mice die by postnatal day 10, and none survive beyond weaning. In contrast to Dicer depletion at E9, there is no effect on cortical lamination, despite a progressive decline in miR-124 and miR-132.

miRNA depletion also affects the adult neurogenesis niche. Work from the Doetsch lab found that miR-124 modulates the transitory progression of adult neurogenesis within the subventricular zone (SVZ) by influencing expression of the transcription factor Sox 9 (Cheng et al., 2009). Increased levels of Sox 9 maintained purified SVZ stem cells as dividing precursors, while ectopic expression of miR-124 led to precocious and increased neuron formation.

Hindbrain Development Requires miRNAs

The rhombomeres of the hindbrain play a crucial role in cerebellar differentiation and brainstem patterning. The nested expression of the Hox gene cluster in rhombomeres is well established, but recent work has revealed an additional level of regulation. This cluster also harbors miRNAs that regulate the Hox genes and noncoding RNAs that overlap and probably help restrict Hox gene expression patterns (reviewed in Lund, 2009). For example, miR-10 regulates *HoxB1a* and *HoxB3a* in zebrafish, and loss of that control impairs migration of branchial nerves (Woltering and Durston, 2008). Another critical factor for appropriate

hindbrain development is Fgf8 (reviewed in Sato et al., 2004). Fgf8 and its downstream signals are prime candidates for miRNA-mediated regulation because the strength of the Fgf8 signal determines differentiation into the mesencephalonic or the more caudal rhombomere 1. Interestingly, Leucht and colleagues found that miR-9 targets Fgf8 and its receptor, Fgfr1, in the zebrafish midbrain-hindbrain boundary (Leucht et al., 2008).

In the developing cerebellum, granule cell differentiation is induced by production of sonic hedgehog (Shh) by Purkinje cells. Several interactions between miRNAs and the hedgehog pathway have been shown in fly (Friggi-Grelin et al., 2008). Also, recent work has demonstrated increased levels of miRs-125, -324-5p, and −326 during cerebellar development in humans (Ferretti et al., 2008). These miRNAs repress the Shh pathway members Smoothened and Gli in human medulloblastoma cell lines, indicating roles in prodifferentiation signaling pathways during cerebellar development. The developing cerebellum also requires secretion of BMP, Wnt1, and retinoic acid (RA) by the roof plate of the fourth ventricle. Interestingly, the miR-34 family of miRNAs is predicted to target Wnt1 and Math1, a bHLH transcription factor expressed in the rhombic lip. BMP signals through Smads, which can control miRNA levels by direct induction of transcription or by promoting pri-miRNA processing (Davis et al., 2008a). As in the Shh pathway, miRNAs regulate BMP/SMAD signaling to repress proliferation and tissue growth. A link between RA and miRNAs has also been shown, as the miR-17 cluster is repressed by RA in human neuroblasts, causing derepression of genes important for differentiation (Beveridge et al., 2009).

miRNA Levels Vary in Neurodevelopmental Disorders

It is not surprising that this class of regulators has been linked to developmental disorders. Indeed, miRNAs are dysregulated in the prefrontal cortex of schizophrenic and schizoaffective patients (Perkins et al., 2007). Mature miR-181b is elevated in samples from the auditory cortex, thought to be the anatomical substrate of auditory hallucinations in schizophrenia, in schizophrenic patients relative to controls (Beveridge et al., 2008). Two predicted targets of miR-181b are visinin-like 1, a calcium sensor gene, and the glutamate receptor subunit GRIA2, both of which were decreased in patient brains. Other work found that mice with a chromosomal deletion syntenic to microdeletions found in individuals at high risk for schizophrenia had haploinsuffiency in DCGR8 (Stark et al., 2008b) (Table 1). Reduced DCGR8, a component of the microprocessor complex important for miRNA maturation, would appear incongruous with the elevated miRNA levels found in patient brain samples by Beveridge and colleagues. However, miRNAs embedded within short introns can bypass nuclear processing (Berezikov et al., 2007; Ruby et al., 2007).

miRNA dysfunction may contribute to autism spectrum disorder (ASD), as microdeletions or duplications at 15q13.2q13.3, a region encoding at least one miRNA, can cause phenotypes with ASD features (Miller et al., 2008). Additional studies on patient tissues confirm that miRNAs are not globally dysregulated, although several were expressed at different levels in autistic samples (Abu-Elneel et al., 2008). Interestingly, neurexin and SHANK3, genes with known genetic links to autism, are among the predicted targets of the putatively dysregulated miRNAs.

Rett syndrome is a neurodevelopmental disorder resulting from loss of function of MeCP2, which becomes apparent in toddlers and progresses with spastic movements, epilepsy, and loss of motor and communicative skills. MeCP2 expression increases transcription of the cortical trophic factor BDNF, which promotes transcription of miR-132. However, MeCP2 is also a target of miR-132. In differentiated neural tissue, MeCP2 transcripts harbor long 3′ UTRs with active miR-132 sites, in contrast to MeCP2 transcripts in other tissues, which have short 3′ UTRs that lack miR-132 binding sites (Klein et al., 2007). Thus, miR-132 acts to decrease its own production, which may "protect" the cell against fluctuating MeCP2 levels.

Tourette's syndrome is a genetically influenced developmental neuropsychiatric disorder which may be linked to the gene encoding Slit and Trk-like 1 (SLITRK1). In patient samples, Abelson et al. found a frameshift mutation in SLITRK1 and a variant in the binding site for miRNA hsa-miR-189 in the SLITRK1 3′ UTR (Abelson et al., 2005). The consequence of the mutation in the 3′ UTR is enhanced miR-189 repression, suggesting a rare genetic basis for Tourette's syndrome that may be driven by a miRNA.

Fragile X syndrome is a common cause of mental retardation and results from loss of function of *FMRP1*. The FMRP1 protein has been shown to interact with the RISC components ARGONAUTE (AGO) and DICER in *Drosophila* and mammalian cell lines (Jin et al., 2004), suggesting a link with the miRNA pathway. Further, FMRP1 is located in P bodies and cytoplasmic granules, which are thought to be the location of miRNA-mediated transcript repression. While FMRP1 is associated with the miRNA machinery and can directly bind and repress mRNA targets, it is not known whether small RNAs play a role (reviewed in Li et al., 2008).

miRNAs may also participate in environmentally induced neurodevelopmental disorders. For example, Sathyan et al. found that ethanol exposure induced changes in expression levels of miRNAs postulated to modulate cell damage responses in neural progenitor cells (Sathyan et al., 2007). It is almost certain that the number of disorders in which miRNAs participate will increase as our abilities to query miRNA function and miRNA-mRNA interactions become more sophisticated.

Conclusions

Until recently, the hypotheses we formed about miRNAs in neural development were based on very focused and difficult to generalize models and by analogies to other organ systems. However, the rapid development of the miRNA field has clarified their functions at key steps. One clear role that has emerged for miRNAs is to facilitate the coordinated transitions in the proteome that temper stem cell renewal and promote neuronal differentiation. Future investigations are likely to reinforce their importance in development and disease in neural and nonneural systems.

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