CHAPTER FIVE

Diverse Functions of MicroRNAs in Nervous System Development

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Abstract

MicroRNAs (miRNAs) are integral parts of the gene regulatory networks that control most developmental processes. Through their regulatory action, miRNAs introduce an additional layer of genetic complexity that can translate into increased cellular diversity, something that is extremely relevant to nervous system structure. In addition, miRNAs sharpen the spatial and temporal boundaries between different cellular states during development. Here, we illustrate these roles with a number of specific miRNAs that act during distinct steps of neural development. We further discuss specific aspects of miRNA function that make these regulators particularly suited to provide the robustness and

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complexity that are essential for the dynamic nature of both the development and activity of the nervous system.

1. Introduction

The large diversity of neuronal and nonneuronal cells that form a nervous system arises from the combinatorial function of a large number of gene regulatory factors. Nowadays, it is evident that these regulators include not only transcription factors but also small regulatory RNAs, with micro-RNAs (miRNAs) being the most intensely studied ones. While our understanding of transcription factor involvement in neural development far exceeds that of miRNAs, here we review some of the more recent examples of the roles of miRNAs in nervous system development.

miRNAs are an abundant class of short (21–23-nt long) regulatory RNAs with versatile functions in biology (for review, see Bartel, 2009; Chekulaeva and Filipowicz, 2009). They are derived from longer primary transcripts (pri-miRNA) that form stem-loop structures, through the successive cleavages of two RNAse III-type enzymes. The first one, Drosha, excises the hairpin structure (pre-miRNA) from the longer pri-miRNA in the nucleus, while cleavage from the second one, Dicer, in the cytoplasm results in the production of the mature 21–23-nt long miRNA. Mature miRNAs associate with a protein complex with an Argonaute family member at its core, and it is in the context of this complex—also known as RNA-induced silencing complex (RISC)—that miRNAs carry out their regulatory roles. miRNAs are, in the vast majority of cases examined, negative regulators of their targets. In the context of the RISC, miRNAs can guide binding to partially complementary sequences in their target mRNAs and cause posttranscriptional repression of gene expression. The precise mechanism by which this repression is exerted is still under debate. Both translational repression—most likely at the level of translation initiation—and mRNA destabilization—likely triggered by mRNA deadenylation—have been shown to affect target protein levels. However, their relative contributions and how they are mechanistically related to one another are still poorly understood (for a recent review, see Djuranovic *et al*., 2011).

Regardless of our incomplete understanding of how miRNAs regulate their targets, their cellular and physiological effects are evident. miRNAs affect every step required for the proper development of a nervous system, from patterning of the nervous system to neuronal differentiation and plasticity. These functions have been described in nervous systems as simple as that of *Caenorhabditis elegans*, where the first miRNA with a role in neuronal differentiation was discovered (Johnston and Hobert, 2003), as

well as in the more complex vertebrate systems. Studies in this wide range of model organisms have been extremely fruitful as they have uncovered common themes in the roles of miRNAs in the nervous system and will likely help us understand their functions from an evolutionary perspective.

The first miRNAs shown to have a role in development of the correct architecture of the vertebrate nervous system were those of the mir-430 family in zebrafish (Giraldez *et al.*, 2005). Fish embryos lacking Dicer showed severe brain morphogenesis defects which could be rescued by injection of preprocessed mir-430 miRNAs. This pioneering study prompted numerous others that have identified other miRNAs and their targets that act at these early stages of neural development.

While a lot of the earlier evidence for involvement of miRNAs in all steps of vertebrate neural development was obtained from Dicer knockout experiments, results from Dicer knockouts should be interpreted carefully as Dicer is known to be involved in the processing of other essential noncoding RNAs (rRNAs, snoRNAs, tRNAs) (Cole *et al.*, 2009; Ender *et al.*, 2008; Liang and Crooke, 2011; Saraiya and Wang, 2008). Thus, we have chosen to focus here on some of the cases of nervous system development where specific miRNAs have been implicated.

2. Diverse Roles of miRNAs in Nervous System Development and Function

2.1. miRNAs act at all steps of neuronal development

2.1.1. Patterning

Patterning of the developing vertebrate neuroectoderm that gives rise to all different mature structures of the nervous system is a complex process that relies on multiple secreted signals emanating from specific regions. Combinations of these signals result in regional domains with particular patterns of transcription factor expression that will give rise to different neuronal and glial pools. A number of miRNAs have been implicated in refining the boundaries of these domains, perhaps fulfilling a role that is best suited for this kind of repressor. A few of these are presented below.

Patterning of the midbrain and anterior hindbrain has long been known to be controlled by an organizing center located at their boundary (the midbrain–hindbrain boundary or MHB), primarily through Wnt and Fgf signaling (Wurst and Bally-Cuif, 2001). Bally-Cuif and colleagues have shown that in zebrafish, mir-9 is involved in maintaining the MHB limits during development. mir-9 is expressed extensively within the neural tube except in the MHB (Leucht *et al.*, 2008). Ectopic expression of mir-9 causes a loss of this organizing center due to ectopic repression of genes required for MHB function. In contrast, loss of mir-9 causes an expansion of the

MHB along the antero-posterior axis. As mentioned, Fgf signaling from the MHB to the surrounding area is critical for proper patterning around this region, and it is in fact the target of mir-9 function. mir-9 seems to target directly at least three components of the Fgf pathway, *fgfr-1, fgfr-8,* and *canopy* (Leucht *et al.*, 2008). Interestingly, the patterning defect induced by ectopic mir-9 can be fully rescued by protecting *fgfr-1* from mir-9 targeting, arguing that most of the effect of mir-9 on the MHB is through this pathway. Importantly, Fgf signaling is part of a feedback loop that ensures MHB stability and mir-9 may be a part of such loop as well. Integration of miRNAs into known gene regulatory networks is a key aspect of their biological function and evolution and will be discussed in Section 4. In addition to its effect on patterning, mir-9 also affects neurogenesis at the boundary areas. This is a role for mir-9 that seems to be conserved in other areas of the developing nervous system as well as across organisms and will be discussed in Section 2.1.2.

Another well-characterized case of neural patterning during development is the spatial organization of different neuronal and glial types in the spinal cord. Two important mechanisms are used to define these diverse progenitor domains. For dorso-ventral patterning, sonic hedgehog (SHH) signaling from the ventral side of the neural tube and retinoic acid from the mesoderm define five domains of ventral neural progenitors with characteristic gene expression (for review, see Jessell, 2000). For antero-posterior regionalization of the spinal cord, distinct domains of *Hox* gene expression have been shown to be crucial (for review, see Dasen and Jessell, 2009). Two cases have been described where miRNAs are proposed to ensure the correct domains of expression in both mentioned aspects of spinal cord patterning.

In the first case, mir-17-3p restricts the expression of transcription factor OLIG2 to its known domain of expression (Chen *et al.*, 2011). While *Olig2* is normally expressed in the ventral progenitor pool that gives rise to motor neurons (pMN), it was also found to be transiently expressed in one of the neighboring ventral pools that produce V2 interneurons (p2). However, for p2 identity establishment, *Olig2* should be turned off and p2 cells should express IRX3, another transcription factor that forms a cross-repressive loop with OLIG2. mir-17-3p is expressed in the p2 domain (as well as in other Irx3-positive pools) but not in the pMN domain. mir-17-3p can directly repress *Olig2*, and thus, loss of this miRNA results in a significant loss of V2 interneurons due to ectopic expression of OLIG2 in the p2 pool.

In the second case, mir-196 has been implicated in the antero-posterior patterning of the spinal cord. Although further evidence should be sought, it seems likely that mir-196 delimits the domain of expression of *Hoxb8* in the caudal neural tube to ensure *Hoxb8* is absent in the prospective hindlimb field (Asli and Kessel, 2010). Ectopic HOXB8 expression in this lumbar area causes a decrease in the number of motor neurons. However, the precise

contribution of mir-196 to this restriction is difficult to assess. A consistent role for mir-196 in restricting *Hoxb8* expression had previously been reported during limb formation, where *Hoxb8* is expressed in the forelimb buds but not in the hindlimb buds (Hornstein *et al.*, 2005). Loss-of-function experiments knocking down mir-196 with an antagomir in developing chick embryos showed derepression of *Hoxb8* at more anterior somites consistent with a transformation of the last cervical vertebrae to a thoracic identity (McGlinn *et al.*, 2009). Interestingly, mir-196 has also been implicated in proper tail and spinal cord regeneration following amputation in axolotls (Sehm *et al.*, 2009). However, in this case, *Hox* gene expression does not seem to be the major target of mir-196.

Finally, the role of miRNAs in other patterning events, such as early blastoderm organization in *Drosophila* (Leaman *et al.*, 2005), neural tube closure (Maller Schulman *et al.*, 2008), and morphogenesis in the cortex and hippocampus (Davis *et al.*, 2008), have been proposed based on individual miRNA knockdown, target-gene analysis, and conditional loss of Dicer, respectively. These will not be discussed here, but further research in these areas should prove interesting.

2.1.2. Neurogenesis

Neurogenesis is a tightly controlled process by which neuroepithelial progenitors or other types of neural stem cells (NSCs), such as those involved in adult neurogenesis, become progressively committed until becoming postmitotic neurons (for reviews, see Conti and Cattaneo, 2010; Li and Jin, 2010). Along their path to become neurons, NSCs have the choice of proliferating to expand the progenitor pool or becoming more committed neural precursor cells (NPCs). In addition, NPCs can give rise to either glial cells (oligodendrocytes (OLs) and astrocytes) or neurons, and so during neurogenesis, alternative fates must be repressed. Both intrinsic and extrinsic factors are involved in these decisions. Among the intrinsic factors, the role of miRNAs is becoming increasingly evident, with two miRNAs, mir-9 and mir-124, being extensively implicated in neurogenesis.

In NSCs from adult mouse forebrains, gain of function of mir-9 causes a dose-dependent decrease in proliferation, and when these cells are induced to differentiate, mir-9 can cause a marked increase in the percentage of differentiated neurons and glia (Zhao *et al.*, 2009). In addition, introduction of mir-9 in NSCs in developing mouse embryos (at E13.5) reduced their proliferation and induced them to migrate to the cortical plate, as differentiated neurons do. These cells also lost expression of a progenitor marker and instead expressed a neuronal marker. A major target of mir-9 for these effects seems to be the transcription factor TLX/Nr2e1, known to be required for stem-cell renewal. Interestingly, TLX/Nr2e1 is a transcriptional repressor of mir-9-1 and mir-9-2 (there are three mir-9 encoding genes in the mouse genome), thereby establishing a cross-repressive feedback loop.

In another report using hESC-derived hNPCs, loss-of-function analysis of mir-9 revealed a somewhat different role. First, mir-9 was necessary for the proliferation of the hNPCs (Delaloy *et al.*, 2010). In addition, in the hESC-derived hNPCs, mir-9 inhibits neuronal migration by repressing Stathmin, a protein that increases microtubule instability. While the opposing effect of mir-9 in these two experimental setups could well be due to their inherent differences (perhaps the main difference is the origin of the studied progenitors), it is also possible that mir-9 gain- (in Zhao *et al.*, 2009) and loss-of-function (in Delaloy *et al.*, 2010) studies have uncovered different functions for this versatile miRNA, and that its effect on proliferation and differentiation results from the balance of its different targets at different developmental stages or in different neuronal or neuronal progenitor types. Further experiments with emphasis on comparing the function of miRNAs along different precursor or mature states of different types of neurons should clarify these discrepancies.

In vivo studies in vertebrate systems have been more consistent with a role for mir-9 as a promoter of neurogenesis, mostly by repressing inhibitors of neuronal differentiation. In mouse and zebrafish, mir-9 is found in proliferating progenitor cells as well as in mature neurons (Lagos-Quintana *et al.*, 2002; Leucht *et al.*, 2008; Wienholds *et al.*, 2005). As mentioned above, mir-9 is expressed in the NPCs just outside the zebrafish MHB where it represses the antineurogenic Hes bHLH transcription factors *her5* and *her9*, thereby promoting differentiation. This effect of mir-9 also explains why its ectopic expression causes loss of the MHB: not only does it repress MHB required genes, but it also causes premature differentiation and thus depletion of the progenitor pool in the MHB. In *Xenopus*, mir-9 has also been implicated in promoting neurogenesis through the inhibition of *hairy1*, another member of the Hes bHLH transcriptional repressors (Bonev *et al.*, 2011).

In the developing mouse brain, mir-9 function has been most extensively studied in the telencephalon. Using gain- and loss-of-function experiments by injection of mir-9 or a mir-9 antisense oligonucleotide, respectively, and more recently analysis of a mir-9-2/mir-9-3 double mutant mouse, Aizawa and colleagues have shown that mir-9 promotes differentiation of Cajal-Retzius cells in the medial pallium as well as other early-born neurons (E12.5–E13.5) (Shibata *et al.*, 2008, 2011). This early effect seems to be mediated by repression of *Foxg1* by mir-9. FOXG1 is a forkhead transcription factor with a known role in promoting proliferation of progenitor cells, and in the medial pallium, the expression patterns of mir-9 and *Foxg1* form reciprocal gradients. Interestingly, no effect on TLX/Nr2e1 was observed in these studies. In the absence of mir-9, increased proliferation of early-differentiating neural progenitors is observed in the medial pallium and also in the subpallium (in this case, mir-9 seems to target both *Foxg1* and *Gsh2*). Interestingly, in the mir-9-2/3 mutant at later stages (E16.5–E18.5),

there is a decrease of proliferation of progenitors in the pallium although it is unclear whether this is due to direct action of mir-9 on these progenitors or a consequence of its earlier effect. This decrease in later progenitor proliferation could be related to the decrease in proliferation of hNPCs described above. It is interesting to note that in the mouse and zebrafish, olfactory epithelia miRNAs of the mir-200 family also seem to regulate neurogenesis at least in part through their action on *Foxg1* (Choi *et al.*, 2008).

In addition to the targets mentioned so far, mir-9 and mir-9* (a miRNA partially complementary to mir-9 derived from the opposite strand of the same precursor hairpin) have been shown to repress the well-known, antineuronal transcriptional repressor, REST/NRSF (RE1 silencing transcription factor/neuron restrictive silencer factor) and its cofactor, CoREST (Conaco *et al.*, 2006; Packer *et al.*, 2008). While these studies were conducted in cell lines and their contribution *in vivo* should be further explored, this result is consistent with the proneural role of mir-9 and its coordinated function with mir-124 to promote neurogenesis as discussed below.

mir-9 has also been shown to have a role in a well-characterized model for neurogenesis, *Drosophila* sensory organ development (Li *et al.*, 2006). However, in this case, despite *mir-9* being highly conserved, its role seems to be different than that in vertebrate systems. First, *mir-9* expression in the nervous systems is not as prominent as in other organisms. *mir-9* is present in embryonic epithelial cells and in the larval wing disc, but not in the sensory organ precursors (SOPs) each of which will give rise to a neuron. In fact, ectopic *mir-9* expression causes a reduction in the number of SOPs mediated by repression of *Senseless*, a known proneural gene. As expected, loss of *mir-9* results in additional SOPs, suggesting that *mir-9* in *Drosophila* suppresses neuronal precursor specification in "nonneuronal" tissues. Another miRNA in *Drosophila*, *mir-7*, complements the function of *mir-9*. While *mir-9* is expressed in the non-SOP cells, *mir-7* is present in the SOP where it promotes expression of proneural genes such as *Atonal* and *Senseless* through its negative effect on *E(spl)* (Li *et al.*, 2009). Interestingly, *mir-7* is also part of a complex gene regulatory network—involving different players and a different target—that controls photoreceptor determination (Li and Carthew, 2005).

In mice, mir-124 is expressed somewhat later than mir-9 during development, but it is also expressed in neuronal progenitors and mature neurons as it continues to be expressed into adulthood and is the most abundant miRNA in the adult mammalian brain (Deo *et al.*, 2006; Lagos-Quintana *et al.*, 2002). mir-124 also seems to both repress the expression of neural progenitor genes as well as induce the expression of neuronal genes to promote neuronal differentiation (Coolen and Bally-Cuif, 2009; Vo *et al.*, 2010). These roles of mir-124 are carried out through a number of targets.

In HeLa cells, transfection with mir-124 can cause a significant change in the transcriptome profile, making it more similar to that of the brain (Lim *et al.*, 2005). Changes in transcriptome specificity also arise from the effect of mir-124 on alternative splicing (Makeyev *et al.*, 2007). mir-124 targets a repressor of alternative splicing called PTBP1 in the nervous system; this, in turn, allows for alternative splicing of another splicing regulator, PTBP2, resulting in correctly spliced and functional PTBP2. Increased levels of PTBP2 in the nervous system correlate with neural specific alternative splicing patterns and are necessary for proper neuronal differentiation.

Other *in vitro* experiments using ESCs or other cell lines have shown that mir-124 induces neuronal like differentiation, although in most cases, additional proneurogenic factors were required to uncover the effect of mir-124 (reviewed in Maiorano and Mallamaci, 2010). More recently, the neurogenic effect of mir-124 has been explored *in vivo*. In mouse models, mir-124 has been shown to promote neurogenesis in the embryonic cerebral cortex (Maiorano and Mallamaci, 2009), as well as to control the timing of progression down the lineage during adult neurogenesis in the stem-cell niche of the subventricular zone (Cheng *et al.*, 2009). In the embryonic cortex, a dramatic upregulation in mir-124 expression in the precursors undergoing direct neurogenesis as well as gain-of-function experiments support the role of mir-124 as an inducer of neurogenesis (Maiorano and Mallamaci, 2009). Although no specific target of mir-124 was shown to be responsible for this effect, a more recent study also analyzing progenitors in the developing cortex identified the Ephrin *EfnB1* as a relevant target of mir-124 (Arvanitis *et al.*, 2010). Interestingly, mir-124 and *EfnB1* form a cross-repressive loop resulting in two states, one with high *EfnB1* and low mir-124 that correlates with maintenance of the progenitor pool, and one with high mir-124 and low *EfnB1* that seems to promote neuronal differentiation.

In the chick spinal cord, the role of mir-124 in neurogenesis has been somewhat controversial. In a first report, no effects of mir-124 overexpression or inhibition were observed (Cao *et al.*, 2007). However, a different study showed subtle yet noticeable increase in neuronal differentiation at the expense of proliferation of neural progenitors upon mir-124 overexpression (Visvanathan *et al.*, 2007), again suggesting that mir-124 plays a role in neurogenesis but may not be strictly sufficient. In the chick spinal cord, mir-124 is able to repress SCP1 (small C-terminal domain phosphatase 1) an antineural factor that is recruited by REST to its target genes. However, the effect of mir-124 in spinal cord neurogenesis is mediated only in part by repression of SCP1, as a mir-124-insensitive SCP1 transcript could only partially counteract the neurogenic function of mir-124. Subsequent work has shown that indeed, mir-124 acts on a number of additional targets to favor neurogenesis. Moreover, it is possible that mir-124 acts in conjunction

with mir-9/mir-9* to robustly reduce REST/SCP1-mediated repression of neural genes (Conaco *et al.*, 2006; Packer *et al.*, 2008).

This is not the only target that is shared by these miRNAs. In the mouse developing neural tube, mir-124 and mir-9* have also been shown to cooperate in promoting neurogenesis by repressing a progenitor-specific subunit of a Swi/Snf-like chromatin-remodeling complex termed BAF. Chromatin remodeling by BAF is important for the different steps of neural development and its subunit composition changes accordingly. By targeting a progenitor-specific subunit of BAF, BAF53a, mir-124, and mir-9* promote this subunit switch and among other effects, reduce the proliferation of neural progenitor cells (Yoo *et al.*, 2009).

The overall picture that emerges from these studies is that in vertebrates, mir-9 and mir-124 are integral to the successful transition between progenitors and differentiated neurons. They seem to orchestrate this transition through the regulation of multiple targets, all of which either promote progenitor proliferation or directly inhibit neuronal differentiation, and most of which are transcriptional regulators.

2.1.3. Neuron-class differentiation

In the previous section, we discussed the role of miRNAs in the first, more general aspects of neurogenesis of NSCs that become progressively committed to giving rise to neurons. However, miRNAs are also involved in the subsequent steps of defining what type of neurons those committed precursors will become and in controlling the progression of the differentiation program.

It is widely accepted that distinct cell types are the products of combinatorial "codes" of gene regulatory factors; these include, but are not limited to, transcription factors and miRNAs. This aspect of neural development has been widely studied in the nematode *C. elegans*, where specification of pan-neuronal features can be genetically separated from neuron-class specification and where several genes affecting the latter have been identified (Hobert, 2011). In *C. elegans,* mir-124 does not seem to be involved in neurogenesis as broadly as in other systems, but rather it is expressed in a subset of sensory neurons and it may play a role in shaping their cell-specific transcriptome (Clark *et al.*, 2010). One of the best-studied miRNAs involved in neuron-class specification, a miRNA called *lsy-6,* also comes from *C. elegans*. *lsy-6* is responsible for the subclass diversification of the ASE neurons, a pair of sensory neurons on either side of the head of the worm (Johnston and Hobert, 2003). Despite being bilaterally symmetric with respect to a number of criteria (position in the head, connectivity, morphology of its projections, and shared gene expression), each ASE neuron senses different environmental cues and responds to them in different ways. This is largely due to the presence of *lsy-6* only in the left ASE neuron (ASEL), where it represses the Nkx6-type transcription factor *cog-1*. *lsy-6*,

cog-1, and another zinc-finger transcription factor, *die-1*, form a bistable feedback loop that can exist in either one of two states: a high *lsy-6* and *die-1* state that results in the expression of an ASEL-specific gene battery and a high *cog-1* state that causes the ASE neuron to adopt an ASER fate (Johnston *et al.*, 2005).

Studies in *Drosophila* have also provided an interesting case: *mir-279* was identified in a screen because its loss resulted in ectopic CO_2-sensing neurons in the maxillary palps (MPs) in addition to the CO_2-sensing neurons normally present in the antenna of the fly (Cayirlioglu *et al.*, 2008). These ectopic sensory neurons in the MP were hybrids, with properties of both CO_2-sensing neurons and one of two specific subclasses of olfactory neurons. One of the targets of *mir-279* involved in this process is a transcription factor called Nerfin-1; however, ectopic expression of Nerfin-1 by itself is not sufficient to generate CO_2-sensing neurons, suggesting the involvement of additional targets. Interestingly, discovery of this role for *mir-279* may have uncovered an evolutionary path in which introduction of a miRNA was involved. In the fly, CO_2 causes an aversive response; however, in blood-feeding insects, CO_2 is attractive and it is sensed by neurons in the MP. It is interesting to speculate that the *mir-279*-mutant phenotype may have uncovered an intermediate hybrid state on which selective pressure could have acted to generate the diversity observed nowadays.

In the chick spinal cord, mir-9 has been implicated in the specification of different motor neuron subtypes (Otaegi *et al.*, 2011). mir-9 is transiently expressed in motor neurons of the lateral motor column (LMC), and its overexpression causes a change in identity of these neurons to that of the median motor column. This effect seems to be caused by the mir-9-mediated repression of *FoxP1*. Interestingly, mir-9 and *FoxP1* are coexpressed in LMC motor neurons where mir-9 has been proposed to tune FoxP1 levels. This is consistent with different *FoxP1* dose requirements to generate different motor neuron classes (Dasen *et al.*, 2008; Rousso *et al.*, 2008).

Another miRNA involved in neuronal differentiation is mir-133. In human and mouse midbrains, mir-133 seems to negatively regulate dopaminergic neuron differentiation through the repression of *Pitx3*, a *bicoid*-related transcription factor well known to promote dopaminergic neuron differentiation and survival. In turn, Pitx3 can transcriptionally activate mir-133 (Kim *et al.*, 2007). Why would mir-133 be expressed and repress a prodopaminergic factor in cells that have to become dopaminergic neurons? While the answer is still unclear, one clue may come from an analysis of *Pitx3* levels in different dopaminergic neuron populations in the brain. This study showed that *Pitx3* level is about six times higher in dopaminergic neurons from the ventral tegmental area than in neurons from the substantia nigra (Korotkova *et al.*, 2005). It will be interesting to know whether

mir-133 is equally present in these two areas and is perhaps involved in creating this difference, and how this difference may impinge on the function and susceptibility to degenerate of these two classes of dopaminergic neurons. Alternatively, mir-133 could be part of dynamic regulatory interactions required for the progression of differentiation from progenitors to mature dopaminergic neurons.

Such a role for a miRNA has been proposed for mir-96 in controlling the progression of differentiation in cochlear hair cells in mice. In this case, loss of mir-96 function (through a point mutation in its seed region) puts a brake in the differentiation program that leaves both inner and outer hair cells with an immature morphology, electrophysiology, and innervation pattern (Kuhn *et al.*, 2011). Therefore, mir-96 could be in charge of down-regulating genes required for initial specification but that could later impair the acquisition of the fully differentiated fate. Identifying the targets of mir-96 will be essential to pinning down its function.

2.1.4. Maturation

During neuronal maturation, the appropriate connections between neurons and their targets are established. In addition, in vertebrates, those neurons that fail to integrate into the corresponding circuits die through apoptosis, while those that form appropriate connections must inhibit apoptosis since they have to survive throughout the lifetime of the organism. miRNAs play roles in these processes as well.

A number of groups have reported roles for miRNAs in regulating dendritic spine development and plasticity. mir-132 is induced by synaptic activity and has been shown to increase dendritic length, branching, and spine density in *in vitro* models using primary cortical and hippocampal neurons from embryonic or newborn rats (Vo *et al.*, 2005). In addition, the expression of mir-132 in the developing rat hippocampus correlates with a period of active synaptogenesis (Impey *et al.*, 2010). The effect of mir-132 to promote spine formation seems to be mediated by p250GAP, a Rho-family GTPase that regulates actin dynamics (Vo *et al.*, 2005; Wayman *et al.*, 2008). More recently, the effect of mir-132 was confirmed *in vivo* in an adult neurogenesis model: knockout of mir-132 in newborn hippocampal neurons in adult mice decreased dendritic length and arborization (Magill *et al.*, 2010). mir-132 has also been implicated in regulating synaptic maturation during the neonatal period through its action on MeCP2 (Klein *et al.*, 2007). The interaction with MeCP2 results in a complex regulatory network that will be further discussed in Section 3.2.

The structure and function of synapses can also be regulated by mir-125b. Overexpression of this miRNA in cultured hippocampal neurons from rat embryos induced the formation of long and narrow spines with low mEPSC amplitude (Edbauer *et al.*, 2010). This morphology correlates with the timing of mir-125b expression; endogenous levels are high in younger

neurons that typically have filopodia-like projections. A target or targets of mir-125b regulating spine morphology are still unknown.

mir-134 is, like mir-124, a nervous system-specific miRNA. However, unlike mir-124 that is expressed earlier, mir-134 expression in the hippocampus peaks around postnatal day 13 correlating with the time of synaptic maturation (Schratt *et al.*, 2006). mir-134 localizes to dendrites, where it regulates spine morphology. First, in hippocampal neurons in culture, mir-134 overexpression resulted in decreased dendritic spine volume, without affecting the number of spines, through the repression of Lim-domain-containing protein kinase 1 (*Limk1*) (Schratt *et al.*, 2006). LIMK1 has been shown to regulate dendritic spine morphology by affecting dynamics of the actin cytoskeleton. However, upon neuronal activation—which is known to stimulate dendritic growth—the inhibition on *Limk1* is relieved. Surprisingly, in younger neurons, neuronal activity induces mir-134 expression and through downregulation of Pumilio 2, mir-134 promotes dendritic outgrowth (Fiore *et al.*, 2009; Khudayberdiev *et al.*, 2009). These seemingly opposite roles of mir-134 can be reconciled in two different ways. First, it is possible that mir-134 plays different roles at different time points, mediated through distinct targets. Alternatively, both effects could be part of homeostasis or plasticity mechanisms to coordinate global and local responses of a neuron to increased or decreased neuronal activity. For example, an increase in dendritic arborization has been shown to be accompanied by a decrease in the strength of individual synapses to maintain the overall excitability within a certain range (Peng *et al.*, 2009). In addition, localized effects of mir-134 in the synapse allow for differential downscaling or strengthening of individual spines. Whether all three, mir-132, mir-134, and mir-125b, act in the same cell to provide a coordinated, balanced response or whether they act at different times and/or different cell types need to be further explored to obtain a more comprehensive picture of their function during dendritic formation and plasticity.

In addition, three different miRNAs have been shown to affect synaptic strength and plasticity in three different organisms. In *Aplysia californica*, mir-124 is, as in *C. elegans*, restricted to sensory neurons. The sensory-motor synapse of *Aplysia* has been extensively studied, and it can be modulated by serotonin. Serotonin is known to promote long-term facilitation at this synapse through the activation of CREB. While mir-124 seems to target CREB in the sensory neuron, interestingly, serotonin was found to inhibit mir-124 biogenesis and thus relieve the repression this miRNA imposes on CREB (Rajasethupathy *et al.*, 2009). This arrangement forms a coherent feedforward loop (FFL), postulated to increase the specificity of the response.

The other two cases involve miRNAs regulating the strength of the neuromuscular junction (NMJ). The first report was from Kim and colleagues who showed that in *C. elegans*, *mir-1*, a conserved muscle-specific

miRNA, affects both pre- and postsynaptic function to couple changes in the activity of the muscle to that of the motor neuron (Simon *et al*., 2008). In the muscle, *mir-1* decreases sensitivity to acetylcholine (ACh) by targeting two subunits of the nicotinic ACh receptor, while in the motor neuron, it exerts a non-cell-autonomous effect mediated through a retrograde signal from the muscle that results in decreased ACh release. As opposed to promoting a transition, as proposed for mir-124 in the *Aplysia* sensory-motor synapse, *mir-1* is likely to be mediating a homeostatic response to refine the activity of the nematode NMJ. At the *Drosophila* larva NMJ, the *mir-310-313* cluster also plays a role in regulating synaptic strength, likely allowing for the establishment of synaptic homeostasis as well. The *mir-310* cluster negatively regulates synaptic strength at the NMJ during larval development when synaptic growth is fast, likely to promote homeostatic compensation (Tsurudome *et al*., 2010). In contrast to *mir-1* in the worm, the *mir-310* cluster exerts its function in the motor neuron, not in the muscle. In the neuron, it inhibits expression of a kinesin family member, *Khc-73*, which plays a role in recruiting specific proteins to the presynaptic active zones.

As mentioned, the regulation of apoptosis is essential during the development and maturation of the nervous system. It is well known that during their maturation, neurons that establish successful connections become decreasingly sensitive to apoptosis induced by a number of insults. miRNA profiling of developing sympathetic neurons revealed that mir-29 levels are significantly increased in mature neurons compared to developing ones (Kole *et al*., 2011). This increase is not limited to sympathetic neurons; it is also observed in cerebellar and cortical neurons during their maturation. In neuronal cultures, mir-29 has been shown to repress the apoptotic pathway by targeting at least four different BH3-only inducers of apoptosis. In the developing retina of *Xenopus*, mir-24a plays a similar role in restricting apoptosis through repression of the proapoptotic factors *caspase 9* and *apaf1* (Walker and Harland, 2009).

A summary of the different stages of neuronal development and the discussed miRNAs acting at each stage is presented in Fig. 5.1.

2.2. miRNAs in glia development

Glial cells account for at least half of the cells in the human brain and their functions range from providing structural support, regulating the balance of water, ions, and nutrients in the brain and maintaining the blood–brain barrier, to providing the myelin sheaths that are so essential for neuronal conduction and even modulating neuronal synaptic transmission (for review, see the Nature Insight on Glia issue, editorial by Chouard and Gray, 2010). Glial cells derive from the same NPCs that give rise to neurons, through a similar series of stages. Perhaps not surprisingly at this

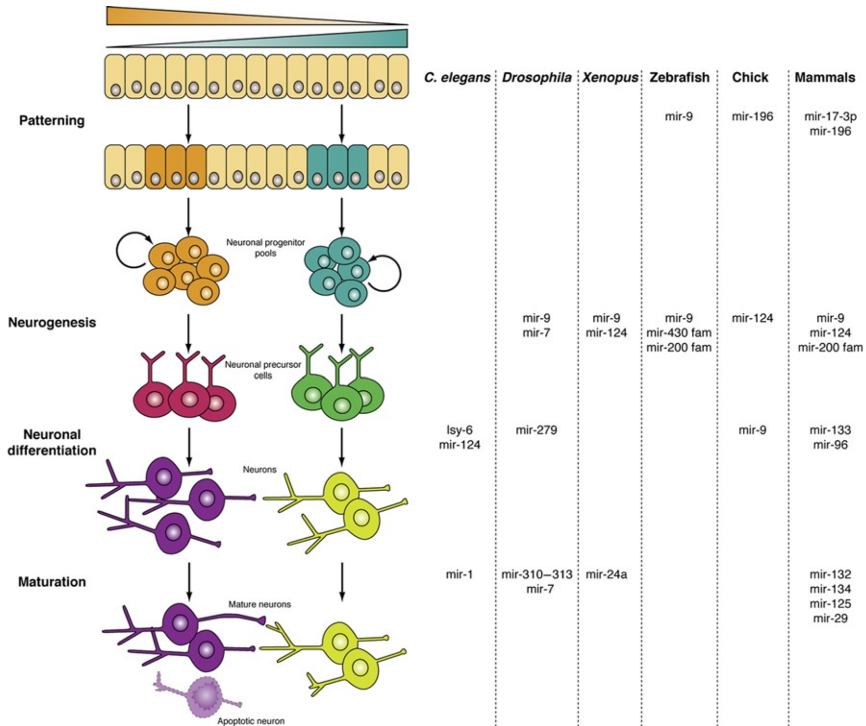

Figure 5.1 **Summary of the miRNAs acting during different stages of neuronal development. Only those miRNAs discussed in the text have been included.**

point, miRNAs have been implicated in practically all steps of glia development, particularly in the two glial types that produce myelin, OLs (in the CNS), and Schwann cells (in the PNS). Here, we will illustrate the state of our knowledge by discussing the roles of miRNAs in OL development.

The differentiation of oligodendrocyte progenitor cells (OPCs) into OLs occurs in at least two steps. First, a series of intrinsic and external signals promote differentiation into a premyelinating OL that is able to extend processes to contact axons and initiate sheath formation. Subsequently, myelin sheaths start being produced, and at this stage, new processes cannot be extended to contact new axons. Thus, the timing of these events is important for proper myelination. At least five different miRNAs are involved in OL differentiation. mir-19, from the mir-17-92 cluster, first promotes the expansion of the OPC pool, inducing proliferation through the inhibition of its target *PTEN* (Budde *et al.*, 2010). OPCs are then induced to differentiate by a number of factors including mir-219 and mir-338. Together, these two miRNAs target a number of OPC-expressed

genes that impair differentiation (mostly transcription factors and signaling pathways) (Dugas *et al.*, 2010; Zhao *et al.*, 2010). mir-23 also represses an OPC-expressed gene, Lamin B1 (*Lmnb1*), that otherwise inhibits the morphological differentiation of OLs (Lin and Fu, 2009). Finally, mir-138 specifically promotes the first stage of OL differentiation, acquisition of the premyelinating OL state, while preventing progression to the later stage, thus extending the intermediate stage that is necessary to establish the number of sheaths that a cell will form (Dugas *et al.*, 2010).

In *C. elegans*, a single miRNA, *mir-228*, has been identified, to date, to be expressed in sheath and/or socket cells that are glia-like support cells for the ciliated sensory neurons in the worm (Pierce *et al.*, 2008). While these cells do not produce myelin, it will be interesting to see whether *mir-228* plays a role in some step during their differentiation.

2.3. miRNAs have highly context-dependent functions

In addition to the commonalities of miRNA functions in diverse systems, it has also become evident that even the most conserved miRNAs can play substantially different roles in different organisms. Even in the same organism, the same miRNA can have distinct roles in different cell types or at different time points in development. This observation that miRNAs can have distinct context-dependent functions has been explored by Gao (2010), and we will expand it here with more recent examples from the literature.

First, even highly conserved miRNAs can have diverse roles in different organisms. For example, as we have seen above, mir-9 promotes neurogenesis in multiple vertebrate systems (Bonev *et al.*, 2011; Conaco *et al.*, 2006; Lagos-Quintana *et al.*, 2002; Leucht *et al.*, 2008; Packer *et al.*, 2008; Wienholds *et al.*, 2005; Zhao *et al.*, 2009). However, in *Drosophila*, it acts in nonneural cells to repress neuronal differentiation (Li *et al.*, 2006). Another case of seemingly distinct functions in different organisms is provided by mir-124. While in vertebrate systems it seems to promote neurogenesis in multiple types of neuronal precursors, in *Aplysia* and in *C. elegans*, mir-124 is only expressed in sensory neurons, and at least in *Aplysia*, while it is not yet known whether it plays a role in neurogenesis, it has been shown to be involved in synaptic plasticity (Rajasethupathy *et al.*, 2009). In *C. elegans*, its precise function is still unknown, but in its absence, the neurons where it is expressed are still present, arguing against a strong role in neurogenesis (Clark *et al.*, 2010).

The second level at which the same miRNA can display segregation of function is at the tissue level within the same organism. A number of miRNAs fit this category, with distinct targets and thus different effects in different tissues. mir-138 was presented above as an important player in OL differentiation; however, it has also been implicated in spine morphogenesis

(Siegel *et al.*, 2009) and cardiac patterning (Morton *et al.*, 2008). As mentioned, mir-338 is involved in OL differentiation but plays a role in axonal function as well (Aschrafi *et al.*, 2008), while mir-132 in addition to its role in dendritic outgrowth also modulates circadian rhythms and has a function in innate immunity (Cheng *et al.*, 2007; Lagos *et al.*, 2010).

A few studies have uncovered that the same miRNA can have different effects in different time points along the development of the same tissue. mir-124 seems to have two different effects during eye development in *Xenopus*. During the optic vesicle stage, mir-124 is necessary and sufficient to repress neurogenesis and promote proliferation of retinal progenitors (Liu *et al.*, 2010). This effect is at least in part through the repression if *NeuroD1*, a known inducer of neuronal differentiation. Interestingly, if after injection with mir-124 for overexpression animals are examined later, at the optic cup stage, a reduction in cell proliferation is observed, more consistent with the role of mir-124 in promoting neurogenesis presented above (Qiu *et al.*, 2009). While further work is necessary to explore whether this later effect of mir-124 is due to a direct effect on proliferation or a consequence of its earlier effect on the progenitor pool, it is interesting to note that careful temporal analysis of miRNA function could address some of the discrepancies mentioned above (e.g., Section 2.1.2).

Another interesting case came from a study in *Xenopus,* where loss of mir-9 caused different effects on neurogenesis in neural progenitors from the forebrain as compared to those in the hindbrain (Bonev *et al.*, 2011). In the hindbrain, knockdown of mir-9 caused an increase in proliferation of neural progenitors, consistent with its role in limiting proliferation and promoting differentiation in other organisms. In contrast, in the forebrain, knockdown of mir-9 reduced the number of progenitors due to increased apoptosis, but when apoptosis was blocked, a similar increase in progenitor proliferation was observed. Interestingly, in both cases, mir-9 seems to exert its function through the repression of *hairy1,* and protection of *hairy1* from mir-9 repression phenocopies both the increased proliferation in the posterior progenitors and the increased apoptosis in the anterior progenitors. This suggests that the specificity of function is downstream of the miRNA target and that context-dependent functions can arise through a variety of mechanisms.

Finally, a given miRNA can cause different effects even within the same cell. This is of particular relevance to neurons, where localization in different cellular compartments can lead to distinct functions. One example presented above is that of mir-134 which localizes to puncta in the dendritic terminals of neurons where it can have a local effect on the strength of individual spines by targeting Limk1 but also seems to be able to cause more global effects on dendritic outgrowth by targeting a more general regulator, Pumilio2 (Khudayberdiev *et al.*, 2009).

The fact that miRNAs have functions that can be so dependent on the cellular context is consistent with a number of observations that suggest that

miRNAs evolve in a highly dynamic manner with a high birth rate and presumably also a high rate of incorporation into preexisting gene regulatory networks (Grimson *et al.*, 2008; Heimberg *et al.*, 2008; Hobert, 2008; Liu *et al.*, 2008; Lu *et al.*, 2008). The rapid evolvability of miRNAs could result in the acquisition of diverse targets in different organisms or different tissues. Interestingly, in those cases where the miRNAs are 100% conserved and still have distinct targets, some other kind of constraint, either structural or that imposed by one shared target, must also be in place.

In addition to evolutionary implications, the fact that cellular context can have such an impact on miRNA function has a very practical consequence: it is definitely an important point to keep in mind when analyzing potential targets of a miRNA, especially since so many approaches to validate miRNA targets rely on artificial expression of both miRNA and target in heterologous systems.

3. Neuronal-Specific Aspects of miRNA Function

3.1. Subcellular localization and spatial regulation

As most posttranscriptional regulators, miRNAs carry out their function in the cytosol. In neurons, the cytosol is divided in distinct subcellular domains, the soma, the dendrites, and the axon, and miRNAs have been detected in all these compartments. Translation regulation of synaptic-specific mRNAs in the neurites has been shown to provide a fast response, at the site where it is required, to allow for synaptic plasticity, and miRNAs are able to participate in these localized regulatory responses. Importantly, RISC is also present at synaptic terminals, and some of its components are regulated by neuronal activity, something we will discuss in Section 3.2.

While a few miRNAs have been observed to be present or even enriched in neuronal processes by *in situ* hybridization, four studies undertook an unbiased approach to identify dendrite- and axon-enriched miRNAs. Using different methods, all four managed to isolate miRNAs from either the soma or the projections and identified a number of miRNAs that are enriched in the different compartments.

First, Kosik and colleagues isolated miRNAs from the soma or the dendrites from hippocampal neurons in culture and identified mir-26 and mir-292-5p as being highly enriched in dendrites (Kye *et al.*, 2007). Smalheiser and colleagues fractionated adult mouse brains and isolated miRNAs from different synaptic fractions (synaptoneurosomes, enriched in dendritic spines and synaptosomes, enriched in synaptic membranes); this led to the identification of subsets of miRNAs enriched in these synaptic fractions as compared to whole brain homogenates (Lugli *et al.*, 2008). Further analysis of these miRNAs will reveal whether they have specific functions at the

synapse. Work from Schratt and colleagues identified a number of miRNAs enriched in synaptosomes prepared from hippocampal neurons in culture. Among these, mir-218 and mir-138 were the most significant (Siegel *et al.*, 2009). The authors further showed that mir-138 is a negative regulator of dendritic spine size, acting through the local repression of the synthesis of the depalmitoylation enzyme Lypla1/APT1 which, in turn, affects the membrane localization of a G protein involved in Rho signaling and actomyosin contraction. Finally, Kaplan and colleagues characterized the pool of miRNAs present in distal axons and compared them to those in the soma of sympathetic neurons in culture (Natera-Naranjo *et al.*, 2010). Kaplan and colleagues had previously shown that mir-338, which was not detected as being significantly enriched in axons in their later work, is present in axons as shown by *in situ* hybridization and is able to regulate cytochrome *c* oxidase IV, ATP levels, and thus the rate of neurotransmitter uptake in axons (Aschrafi *et al.*, 2008). Therefore, even those miRNAs which are not significantly enriched in these compartments can potentially have localized roles, for example, if their targets are spatially restricted.

While there is little overlap between the miRNAs identified in all these different studies, which could be due to the differences in samples and methodologies, these will likely prove to be useful resources for further studies. In addition, some conclusions are shared by different studies. For example, mir-124, one of the most abundant neuronal miRNAs, has been shown by two of these studies to be enriched in the soma (this is in contrast to mir-124 in *Aplysia* where it has been seen in the projections of sensory neurons), suggesting some kind of exclusion mechanism from the processes.

Having miRNA-mediated regulation in the synaptic terminals in addition to the regulation in the soma has two obvious advantages. First, a miRNA present both in the processes and in the soma can cause two types of effects as illustrated by mir-134 and mir-132, a rapid, localized one at the former (typically by targeting synaptic proteins), and perhaps a slower, more sustained one at the latter (typically by targeting transcriptional or posttranscriptional regulators). And second, it can allow for uncoupled global and local responses carried out by the same miRNA, as discussed for mir-134 in Section 2.1.4. Further characterization of how miRNAs are transported through the different compartments and how this transport is regulated will be critical to our understanding of how miRNAs contribute to neuronal development and function.

3.2. Activity-dependent regulation of miRNA biogenesis and activity

Neuronal activity is an important player during the maturation phase of neuronal development, as it modulates the establishment and refinement of neuronal connections, mainly through its effects on dendrite morphology

and synaptic plasticity. Upon neuronal activation with brain-derived neurotrophic factor (BDNF) or KCl, a number of signaling events, most notably activation of the CaMKII (calcium/calmodulin-dependent kinase II) phosphorylation cascade, result in local synaptic changes as well as in the activation of transcription factors in the nucleus. Two of these transcription factors, CREB and MeF2, have been shown to activate the transcription of mir-132 and the cluster containing mir-134, respectively (Fiore *et al.*, 2009; Wayman *et al.*, 2008).

Increased mir-132 levels upon CREB phosphorylation have two described consequences. As mentioned above, mir-132 promotes dendritic growth and branching, through its effect on the actin cytoskeleton. In addition, mir-132 downregulates MeCP2 (methyl CpG-binding protein 2), a broad transcriptional regulator with a strong implication in the neurodevelopmental disorder, Rett syndrome (Klein *et al.*, 2007). Among MeCP2's targets is BDNF itself; thus downregulation of MeCP2 by mir-132 results in a decrease in BDNF transcription, suggesting that mir-132 plays a role in neuronal homeostasis. Interestingly, CREB-mediated activation of mir-132 also occurs in the suprachiasmatic nucleus, where it plays a role in modulation of the circadian clock by light (Cheng *et al.*, 2007), again illustrating how network modules involving miRNAs can adopt different functions in different contexts. Notably, the miRNA *bantam* in *Drosophila* also plays a role in circadian rhythm modulation (Kadener *et al.*, 2009). The molecular oscillations that underlie the circadian rhythms are sustained by interconnected feedforward and feedback loops; miRNAs were likely an advantageous addition to these networks to enhance not only their robustness but also their flexibility (O'Neill and Hastings, 2007).

Just as miRNA, biogenesis can be stimulated by neuronal activity, so can miRNA catabolism (Krol *et al.*, 2010). Filipowicz and colleagues found that many miRNAs decay with much faster rates in neurons than in nonneuronal cells and that miRNA turnover in neurons is regulated by neuronal activity. For example, blocking glutamate receptors in hippocampal neurons slowed the decay of mir-124, -128, -134, and -138, while adding glutamate made it faster (Krol *et al.*, 2010). A rapid turnover of miRNAs (given by fast rates of degradation but also fast rates of biogenesis) likely allows neurons to adjust their repertoire of miRISC to the changing environment, in order to respond accordingly by changing its morphology or adjusting the strength of its synapses.

Finally, not only are the levels of miRNAs themselves affected by neuronal activity but so is the composition of the RISC. Work in both *Drosophila* olfactory interneurons (Ashraf *et al.*, 2006) and in rat hippocampal neurons (Banerjee *et al.*, 2009) has shown that upon neuronal activation, the DExD-box protein Armitage/MOV10, which is found at the synapses, is degraded via the ubiquitin–proteasome pathway. Reduction in the level of this key component of the silencing complex results in the release of a number of

synaptic mRNAs from the repressed state and into the polysome fraction. In both systems, CaMKII protein synthesis was increased, but interestingly, so were Limk1 and Lypla1/APT1 in the rat neurons. These two mRNAs had previously been shown to be targets of mir-134 (Schratt *et al.*, 2006) and mir-138 (Siegel *et al.*, 2009), respectively, and their repression had been shown to be relieved at the synapse by neuronal activity.

Overall, miRNA-mediated translational repression seems to be deeply integrated in the dynamic responses that neurons elicit during activity and to maintain homeostasis. Their subcellular localization, the rapid kinetics, and reversibility of their function and their target diversity make them very suitable to fulfill this role.

3.3. A role for miRNAs in generating neuronal diversity

The cellular diversity in the nervous system is unparalleled by that of any other organ system. Even an organism with a simple nervous system, such as *C. elegans*, has at least 118 different classes of neurons. In general, the properties of each cell class are a consequence of the genes each class expresses, and this is, in turn, defined by the gene regulatory factors present in each cell type. Transcription factors (TFs) and miRNAs are the two most abundant and diverse classes of gene expression regulators, and it has been proposed that combinatorial “codes” of TFs and miRNAs can define all different cell types (Hobert, 2004).

The magnitude of the contribution of miRNAs to generating this diversity is beginning to be grasped. While a number of examples have been provided so far, many more are likely to be uncovered. Given their numbers, high evolvability, their diverse spatial and temporal expression patterns (Kapsimali *et al.*, 2007), and their ability to modify preexisting genetic networks to produce stable, heritable phenotypes, miRNAs are very good candidates to introduce an additional level of complexity.

We have already discussed the role of *lsy-6* in diversifying two sensory neurons in *C. elegans* that would otherwise be practically identical. In this case, it seems likely that the incorporation of a single regulatory factor (*lsy*-6) into preexisting regulatory networks during evolution could be responsible for this diversification. *lsy-6* is a nematode-specific miRNA; however, it is not present in all nematodes. While species such as *Caenorhabditis briggsae*, *Caenorhabditis remanei*, and *Caenorhabditis brenneri* have homologs of *lsy-6* and its target *cog-1, Pristionchus pacificus,* a more distant relative, does not seem to have a *lsy-6* homolog, and interestingly, while the *cog-1* ORF is conserved with that of *C. elegans,* their 3′UTRs are not. Further analysis of the properties of the ASE sensory neurons in *Pristionchus* will likely provide new insight into the incorporation of miRNAs into gene regulatory networks during evolution.

Other miRNAs seem to distinguish different neuronal—and glial—types during development, as discussed throughout this chapter. For example, mir-9 distinguishes cells outside the zebrafish MHB from the progenitors in the actual MHB; mir-17-3p is important to robustly diversify two pools of progenitors in the mouse spinal cord, and the presence of *mir-279* allows for the generation of distinct classes of sensory neurons in *Drosophila*.

Notably, miRNAs may also play a role in maintaining the identity of distinct neuronal classes postdevelopmentally. mir-16 seems to be present at higher levels in noradrenergic than in serotoninergic neurons, and it is able to repress serotonin-metabolic enzymes in noradrenergic neurons (Baudry *et al*., 2010). Both *in vivo* and *in vitro*—in a bipotent neuroectodermal cell line that can differentiate into either serotoninergic or noradrenergic neurons—mir-16 inhibits the expression of the serotonin transporter (directly) as well as of tryptophan hydroxylase (likely indirectly). Loss of mir-16 in noradrenergic neurons gives them the ability to synthesize, store, and degrade serotonin without affecting the noradrenaline metabolism. These findings suggest that miRNAs are likely involved in maintaining well-defined neuronal classes.

Overall, it seems likely that miRNAs with their versatile repressive abilities have been able, during evolution, to segregate functions contained in common precursors into distinct neuronal subpopulations. Further studies analyzing this role of miRNAs as diversifiers, to generate the vast neuronal complexity, are necessary and will undoubtedly prove extremely interesting.

4. Integration of miRNA Function into Gene Regulatory Networks

4.1. Switches or modulators with a few or "hundreds" of targets?

To fully understand the contribution of miRNA regulation to a biological phenomenon, it is important to answer questions such as does a given miRNA act by regulating one or a few major targets, or is it really able to target dozens or hundreds of them, as prediction algorithms and genome-wide transcriptome and proteome analyses propose? And, do miRNAs act as molecular switches, turning off the expression of their targets, or do they modulate their expression to lower but still detectable levels? These questions have been raised in multiple occasions since the birth of the field, and while here we discuss them briefly, for further reading we suggest, among others, Flynt and Lai (2008).

The proposal that a miRNA can target numerous mRNAs is based on bioinformatic studies that use combinations of RNA sequence, secondary structure, and conservation information. These predict that each miRNA

could have dozens, or even "hundreds," of target mRNAs. In addition, the finding that many miRNAs cause not only translation repression but also a decrease in the levels of their target mRNA has led to the use of microarray analysis to identify potential miRNA targets. In these studies, as well as in proteomic analyses, typically the level of numerous mRNAs and proteins change, in general quite subtly, when a given miRNA is knocked out or overexpressed, and the subsequent statistical analyses in general show enrichment among these for potential targets of the miRNAs under study (based on the same prediction algorithms). This has been taken as proof that each miRNA indeed targets a large number of mRNAs. Unfortunately, it seems to be disregarded that our prediction algorithms still suffer from very high false-positive rates and that in these genome-wide analyses the majority of the changes observed could be indirectly caused by miRNA misexpression, even those that have a putative miRNA binding site. In addition, methods to validate miRNA targets usually rely on overexpression of the miRNA and a sensor in a heterologous system, which also likely produces false positives. Moreover, as mentioned above, a miRNA can have distinct targets in different cell types or even in the same cell type at distinct time points. It is therefore crucial to conduct appropriate experiments to interpret the target-prediction data correctly. Therefore, while it is a valid possibility that a miRNA causes its effects by targeting dozens of mRNAs, it has not been satisfactorily proven yet, and it is in fact very challenging to do so, as it requires systematic testing of all predicted targets in the proper experimental setup with rigorous quantitative approaches.

In contrast, it has been easier to validate those cases where a miRNA has one or a few major targets. In these cases, the strongest evidence has come from studying genetic interactions. For example, reduction in the dose of a major target can fully (or almost fully) suppress the loss-of-function phenotype of the miRNA. Or alternatively, protection of the predicted target from miRNA repression fully (or almost fully) suppresses the miRNA overexpression phenotype. We have discussed some of these examples along the previous sections, but to name a couple, the function of *lsy-6* can be fully accounted for by its effect on *cog-1*; mir-9 has a handful of targets, but specific targets seem to mediate distinct functions in different cell types or at different times. Other examples include *mir-8* and its target *atrophin* in the *Drosophila* nervous system (Karres *et al.*, 2007) and mir-150 and its target c-Myb in the mouse B-cell lineage (Xiao *et al.*, 2007).

Regarding the mode of action of miRNAs, experimental evidence suggests two main types of miRNAs: those that are coexpressed with their targets and thus likely modulate the target concentration and those that are mutually exclusive and are therefore considered to have a switch-like behavior. Whether a miRNA will act as a modulator or as a switch depends on a number of variables. An important one to consider is the cellular concentration of the miRNA, given that the number of turnovers a given

miRNA-RISC can perform seems to be rather low. According to a number of studies, miRNAs are present at a range of 10–10,000 copies per cell (Chen *et al.*, 2005; Kye *et al.*, 2007). It seems unlikely that a miRNA that is present at very low abundance will have a switch-like behavior, while those that are present at higher concentrations have a higher probability of acting that way. In addition, in neurons, miRNAs are distributed across the soma and the processes as we previously discussed. So, for example, a miRNA that is present even at 1000 copies per cell might end up at one or less copies per dendrite, and this will have a direct impact on the type of regulatory role that miRNA can execute.

The mode of action will also of course be dependent on the target mRNA concentration, and so it will be important as well how many additional targets that miRNA has. At the same cellular concentration, a miRNA with several targets is less likely to be able to switch all of them off, while a miRNA with one or a few targets has a higher chance of fully repressing its target.

Importantly, the observed effect of a miRNA on its target may not be reflective of the direct consequence of miRNA-mediated repression, but rather it may be the result of additional interactions. In fact, this is probably one of the main contributors to the mode of action of a miRNA given the fact that most miRNAs form complex gene regulatory networks with their targets. Thus, even a modest effect of a miRNA on its target can be amplified by feedback loops to end up in mutually exclusive expression of the two. In such a case, the miRNA could be acting as a genetic switch even without being a strict molecular switch. These types of interactions will be discussed in the next section.

4.2. Network motifs involving miRNAs

It is evident from a number of the examples we have explored so far that many miRNAs regulate the expression of specific transcription factors and that, in turn, these TFs feedback to regulate expression of the miRNAs themselves. More directed analyses have shown that a few network motifs that integrate transcriptional and miRNA-mediated posttranscriptional regulation are overrepresented in regulatory networks in organisms ranging from *C. elegans* to humans (Li *et al.*, 2009; Martinez and Walhout, 2009; Martinez *et al.*, 2008; Osella *et al.*, 2011; Tsang *et al.*, 2007). Such network motifs have been implicated not only in providing robustness and stability to genetic programs, maintaining stable steady-state levels of the regulatory factors, but also in providing precise transitions (temporal and spatial) in response to external stimuli or intrinsic signals (Alon, 2007).

The two most recurring motifs are feedback and feedforward loops. In a typical double-negative feedback loop, a miRNA represses a target, a TF, which, in turn, represses expression of the miRNA itself (Fig. 5.2A).

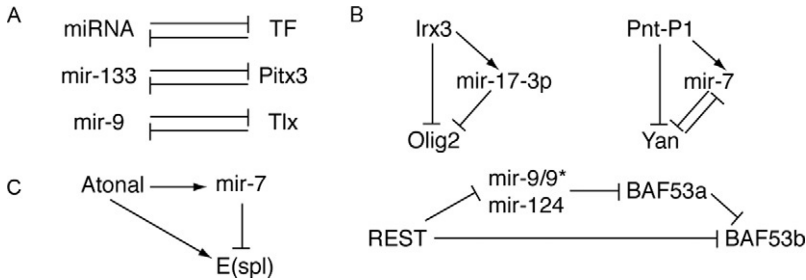

Figure 5.2 Examples of regulatory network motifs including miRNAs. (A) Examples of simple double-negative feedback loops involving one miRNA and one transcription factor. These small motifs are typically embedded in more complex networks. (B) Examples of coherent feedforward loops involving a few of the miRNAs mentioned in the text. (C) An incoherent feedforward loop involving a miRNA that has been implicated in providing robustness to a broader gene regulatory network.

This type of configuration has two possible outcomes. First, if the miRNA is able to fully turn off the TF, and the TF is able to shut down expression of the miRNA, this will result in a bistable switch where only one of the two components can be active at a given time. These relatively simple motifs are typically embedded in more complex networks. Which one of the two remains active will depend on additional input biasing the loop to one or the other side, or alternatively, initial stochastic fluctuations can be amplified to result in one or the other state. In cases where the TF is under an auto-regulatory positive feedback, a negative feedback loop with a miRNA can rather act as a noise filter and provide stability against fluctuations in the level of the TF that could trigger an unwanted response, increasing the specificity of a response.

Feedforward motifs also provide a number of advantageous properties to gene regulatory networks. These can belong to two main classes, coherent and incoherent FFLs. Coherent FFLs are those in which an upstream regulator affects a target through two different paths, both of which affect the target levels in the same direction (i.e., both activate or both repress the target). In contrast, in incoherent FFLs, the two regulatory paths cause opposite effects on the target level (i.e., one activates and one represses the target). Coherent FFLs can provide robustness to a biological response and could reinforce a switch-like effect, as is illustrated by the relationship of *Irx3*, *Olig2*, and mir-17-3p in the mammalian spinal cord (Fig. 5.2B). In addition, coherent FFLs will also likely affect the dynamics of activation and repression of the target(s).

One of the functions of incoherent FFLs is to buffer noise in gene expression, defining and maintaining the steady-state level of a network component. This results in more stable states, preventing random switching to the alternative state due to stochastic fluctuations (Fig. 5.2C). Examples

of the robustness conferred by both coherent and incoherent FFLs are given by the role of *mir-7* in photoreceptor and SOP differentiation in *Drosophila*. *mir-7* participates in complex interlocking loops and its function is essential to stabilize the expression of different network components in face of environmental fluctuations (Li *et al.*, 2009).

To sum up, addition of a miRNA to an existing gene regulatory network will have distinct consequences depending on the position of its target in the network. In some cases, it will provide a switch-like effect to remove expression of its target and generate a new cellular state, while in others, it will dampen fluctuations and thus provide robustness to a preexisting cellular state.

5. Concluding Remarks

miRNAs are a class of gene regulatory factors with versatile functions and as such they have been adopted during the course of evolution to serve a variety of purposes. During development, miRNAs are a source of robustness and reproducibility; they control spatial and temporal gene expression to allow for proper patterning and specification of different structures. At the same time, they provide heritable variability, by diversifying genetic programs and thus increasing the complexity of any system. Their modes of action and their molecular properties make them particularly suited to play regulatory roles in the dynamic cellular environment of the nervous system. And while their roles are already evidently widespread, we expect to find them involved in many more.

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