

# Identification of many microRNAs that copurify with polyribosomes in mammalian neurons

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**Localized translation in mammalian dendrites may play a role in synaptic plasticity and contribute to the molecular basis for learning and memory. The regulatory mechanisms that control localized translation in neurons are not well understood. We propose a role for microRNAs (miRNAs), a class of noncoding RNAs, as mediators of neuronal translational regulation. We have identified 86 miRNAs expressed in mammalian neurons, of which 40 have not previously been reported. A subset of these miRNAs exhibits temporally regulated expression in cortical cultures. Moreover, all of the miRNAs that were tested cofractionate with polyribosomes, the sites of active translation. These findings indicate that a large, diverse population of miRNAs may function to regulate translation in mammalian neurons.**

translational control | noncoding RNA | messenger ribonucleoprotein complex (mRNP)

The role of microRNAs (miRNAs) in translational repression stems from studies of the first two miRNAs, *lin-4* and *let-7*, identified genetically in *Caenorhabditis elegans* (1–6). *lin-4* attenuates the translation, but not the mRNA abundance, of two target genes, *lin-14* and *lin-28*, by imperfect base-pairing to complementary sequences in the 3' UTR of the target mRNAs (2, 7). Similarly, *let-7* regulates *lin-41* and *hbl-1/lin-57* through partially complementary sequences in the target mRNA 3' UTR (3, 4, 8, 9). In *Drosophila melanogaster*, the *bantam* miRNA regulates the expression of its target, the proapoptotic *hid* gene (10). In these cases, the targets of miRNAs in animals are regulated at the posttranscriptional level through miRNA binding to imperfect antisense sites in the target mRNA 3' UTR.

Over 200 miRNAs have been identified from animals, plants, and yeast (11–25). miRNA cloning from a panel of mouse organs revealed that many were expressed in a tissue-specific manner and supports roles for miRNAs in development (14, 15). In addition, computational analyses have revealed in genomes many more miRNAs that have eluded cloning strategies, often because of their low abundance and/or their brief temporal window of expression, and have suggested that as much as 1% to 1.5% of a genome may be represented by miRNAs (23–26).

The function of both *lin-4* and *let-7* in the control of developmental timing in *C. elegans*, and the temporal and spatial control of *bantam* miRNA expression during fly development, may indicate that many of the miRNAs with unknown functions regulate the temporal sequence of gene expression during development or other regulatory pathways (5, 10). The aggregate function of the miRNAs also can be inferred from mutations in the genes encoding components of the general machinery required for all miRNAs. *lin-4* and *let-7* are detected as ≈70-nt precursors with predicted hairpin secondary structures, which are cleaved by the Dicer RNase (DCR-1) to release the 22-nt mature miRNAs (1, 3, 27–29). Mutations in Dicer and Argonaute proteins, two components required in processing miRNAs, cause profound developmental defects, including meristem patterning in plants and embryonic development in *C. elegans* (6, 30, 31).

Regulatory pathways rich in evidence for translational control are expected to use miRNAs. Translational control has been implicated in neural development as well as the maintenance and plasticity of neural connections (32, 33). Select mRNAs and translational machinery, including ribosomes and other noncoding RNAs, are localized to dendritic regions of neurons (32–34). In addition, axons have been demonstrated to be capable of localized translation (35–37). Some of these mRNAs encode proteins such as kinases and translational control factors that are attractive candidates to mediate synaptic changes (33, 34, 38). Elements in the 3' UTR of some mRNAs have been implicated in their localization (39–42). There is evidence that synaptic activity activates the translation of these localized mRNAs (32, 43). In addition, various neurotrophic signals regulate translation locally (37, 44–46). One attractive feature of localized translational control is that it can occur just in the region of synaptic activity, neatly solving the problem of how changes in gene expression in a neuron can modify only active synapses, when neurons can have thousands of synapses not destined for plastic changes (47). miRNAs would fit well into this model if their maturation, localization, or expression were modulated by synaptic activity.

We therefore sought to identify miRNAs from mammalian brain preparations and explored their regulation and possible function in translational control. We found many previously uncharacterized miRNAs and show that they are localized to polyribosomal fractions where translational control is likely to occur. We also found that the expression of particular brain miRNAs changes over time as neurons differentiate in culture. These data suggest that, as in the case of the *lin-4* and *let-7* miRNAs, some mammalian neural miRNAs may regulate the temporal sequence of developmental events by their control of translation of target mRNAs.

## Methods

**Primary Cortical Cultures and Sucrose Density Gradients.** Procedures for culturing E18 rat cerebral cortices, cellular fractionation of cortical primary neurons, and linear sucrose gradients [15–45% (wt/wt)] have been described (48).

**miRNA Cloning and RNA Analysis.** The miRNA cloning procedure has been described (11). The starting material for the miRNA cloning combined primary cultures of dissociated E18 rat cortex that had been grown *in vitro* for 1.5, 7, and 14 days. Total RNA

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Abbreviations: miRNA, microRNAs; mRNP, messenger ribonucleoprotein complex.

Data deposition: The miRNAs reported in this paper have been deposited in the miRNA Registry database, [www.sanger.ac.uk/Software/Rfam/mirna](http://www.sanger.ac.uk/Software/Rfam/mirna) (miRNA nos. *mir-322-mir352*).

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R.n.	<i>mir-340</i>	TCCCTGTCCTCCAGGAGCTCATT--
R.n.	<i>mir-352</i>	TCCCTGAGGAGCCCTTTGAGCCGTG
C.e.	<i>lin-4</i>	TCCCTGAGACCTCAAGTGTGA----
M.m.	<i>mir-125a</i>	TCCCTGAGACCCCTTTAACCTGTG--
M.m./R.n.	<i>mir-125b</i>	TCCCTGAGACCCTAACTTGTGA---
H.s.	<i>mir-182a</i>	CAAAGTGCTGTTA <b>GTGCAG</b> GTTAG-
H.s.	<i>mir-182b</i>	CAAAGTGCTGTTAT <b>TGCAGG</b> ----
H.s./R.n.	<i>mir-93</i>	-AAAGTGCTGTT <b>CGTGCAG</b> GTTAG-
H.s.	<i>mir-94</i>	-AAAGTGCTGACAG <b>TGCAGAT</b> ---
H.s.	<i>mir-195</i>	CAAAGTGCTGACAG <b>TTCAGG</b> TAG-
H.s./R.n.	<i>mir-91</i>	CAAAGTGCTTACAG <b>TGCAGG</b> TAGT
H.s.	<i>mir-106</i>	AAAAGTGCTTACAG <b>TGCAGG</b> TAGC
H/M/R	<i>mir-20</i>	TAAAGTGCTTATAG <b>TGCAGG</b> TAG-
H/M/R	<i>mir-18</i>	TAAGTGCATCTAG <b>TGCAGATA</b> --

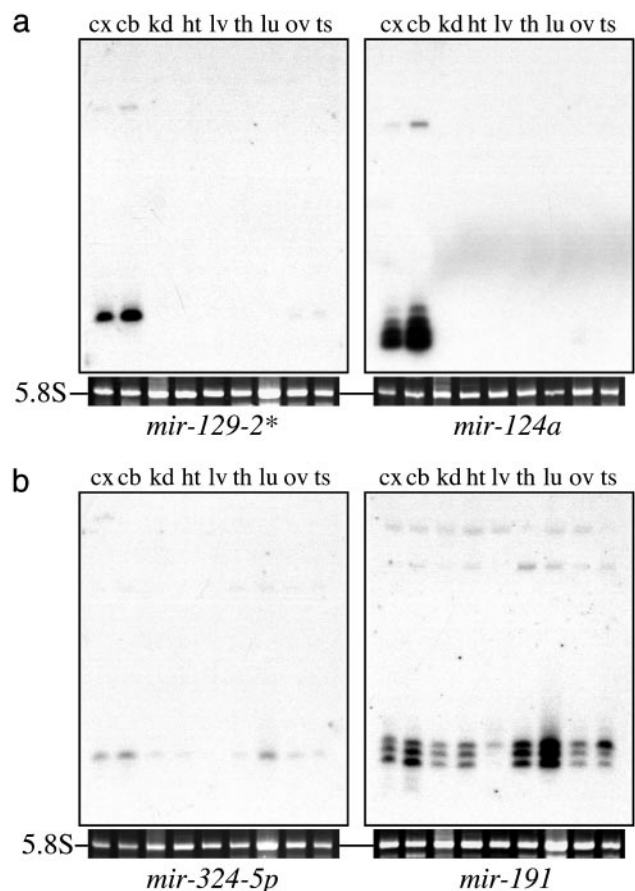
**Fig. 1.** miRNAs from rat cortical neurons cluster in miRNA "families." Previously identified miRNA sequences and miRNAs cloned in this study from combined E18 rat cortical neurons cultured for 1.5, 7, and 14 days were compiled and each sequence was aligned against all others by using a Smith-Waterman algorithm (EMBOSS 2.3.1; ref. 70). Complete hierarchical clustering was performed on the dissimilarity matrix generated from the scores of the pairwise sequence alignments. A dendrogram was generated from the clustering analysis (see Fig. 5) and was cut to yield a set of clusters, which were hand-adjusted to improve groupings of miRNAs that share common subsequences. Two examples of such clusters are presented here. Conserved sequences are highlighted in yellow. miRNAs identified in rat cortical neurons are labeled in red. R.n., *Rattus norvegicus*; M.m., *Mus musculus*; H.s., *Homo sapiens*; C.e., *Caenorhabditis elegans*. H/M/R designates clones found in human, mouse, and rat.

sequenced. Eighty-six distinct miRNAs were identified (Tables 1 and 2); 40 were distinct from previously reported mammalian miRNAs, and the remaining 46 matched those previously identified (13–16, 21, 23, 51). Of the 40 new miRNAs, 32 miRNAs (*mir-322* to *mir-352*) were entirely new and, in the case of *mir-324*, a mature miRNA was identified from both the 5' and 3' arms of the precursor hairpin (annotated as *mir-324-5p* and *mir-324-3p*, respectively). Five other new miRNAs were cloned also from the opposite strand of known precursors and have been tagged with an \* to indicate the origins of these miRNAs (e.g., the miRNA cloned from the opposite strand of *mir-20* was annotated as *mir-20\**; the others are *mir-7-1\**, *mir-129-2\**, *mir-140\**, and *mir-151\**; see Tables 1 and 2). Finally, three new miRNAs were close enough to existing miRNAs, but derived from distinct loci, to receive the same *mir* number followed by a new letter suffix (*mir-135b*, *mir-148b*, and *mir-101b*).

The number of times a miRNA was cloned varied greatly, ranging from 71 clones for *mir-125* to a single clone for 32 of the 86 miRNAs; in all, 29 of the 86 miRNAs were isolated four or more times (Tables 1 and 2). The corresponding rat and/or mouse genomic sequences and their locations were identified for each miRNA. The 5' or 3' flanking genomic sequences then were tested for the ability to fold into canonical  $\approx 70$ -nt miRNA precursor hairpin structures by using the MFOLD program ([www.bioinfo.rpi.edu/~zukerm/rna](http://www.bioinfo.rpi.edu/~zukerm/rna); refs. 50 and 52). Predicted precursors for 32 of the 40 newly identified rat miRNAs were found in both rat genome traces and the assembled mouse genome sequence ([ftp://ftp.ncbi.nih.gov/genomes/M\\_musculus](http://ftp.ncbi.nih.gov/genomes/M_musculus) and [ftp://ftp.ncbi.nih.gov/pub/TraceDB/rattus\\_norvegicus](http://ftp.ncbi.nih.gov/pub/TraceDB/rattus_norvegicus)), and, in nearly all of those cases, sequence conservation included the predicted precursor (Table 2). The six new rat miRNAs that were not identified in the mouse genome may be rat-specific, and two rat miRNAs identified in the mouse genome but not in rat genome traces may reflect incompleteness of the rat genome assembly (see Tables 1 and 2).

All 40 new miRNAs were examined by Northern blotting to total RNA from adult rat cortex, and 17 of the miRNAs were

detected (Table 1). The remaining 23 either may be expressed at levels below the detection threshold for Northern blotting or may exhibit temporally restricted expression patterns. In general, the number of times a particular miRNA was cloned correlated with the intensity of the signal by Northern blotting. In fact, 22 of the 23 new miRNAs that escaped detection by Northern blotting were cloned only once or twice (Table 1). Two recent studies demonstrate that many bona fide miRNAs evade detection by Northern blotting; it is only when an amplified library of small RNAs is queried by a second PCR-based amplification method with primers complementary to the miRNA under study that it can be detected (24, 26). Furthermore, although the miRNAs were cloned from primary E18 cortical cultures, which are enriched for neurons, testing expression by Northern blotting for all of the cloned miRNAs necessitated a large quantity of total RNA, which we procured from the abundant RNA source of adult rat cortex. Therefore, the developmental difference in the cortical material used for miRNA cloning and Northern blotting also may explain the lack of detection of 23 of the 40 new miRNAs identified in this study.



**Fig. 2.** Tissue-specific expression patterns of miRNAs cloned from rat cortex. Northern blots of total RNA isolated from adult rat tissues were assayed with probes against 12 miRNAs. Eight miRNAs were expressed predominantly in the cortex and cerebellum. Two miRNAs are shown in *a*: *mir-129-2\** and *mir-124a*, previously cloned from mouse (14); the others (not shown) with brain-restricted expression patterns include the rat homologue of *mir-103* cloned from the Gemin3–Gemin4–eIF2C2 complex in human HeLa cells (16), *mir-128* from mouse (14), and four previously uncharacterized miRNAs (*mir-323*, *-326*, *-329*, and *-344*). Four miRNAs exhibit a broader expression pattern. Two miRNAs are shown in *b*: *mir-191* and *mir-324-5p*; the others with broad expression patterns were *mir-335* and *mir-151\**. The tissues analyzed were cortex (cx), cerebellum (cb), kidney (kd), heart (ht), liver (lv), thymus (th), lung (lu), ovary (ov), and testis (ts).



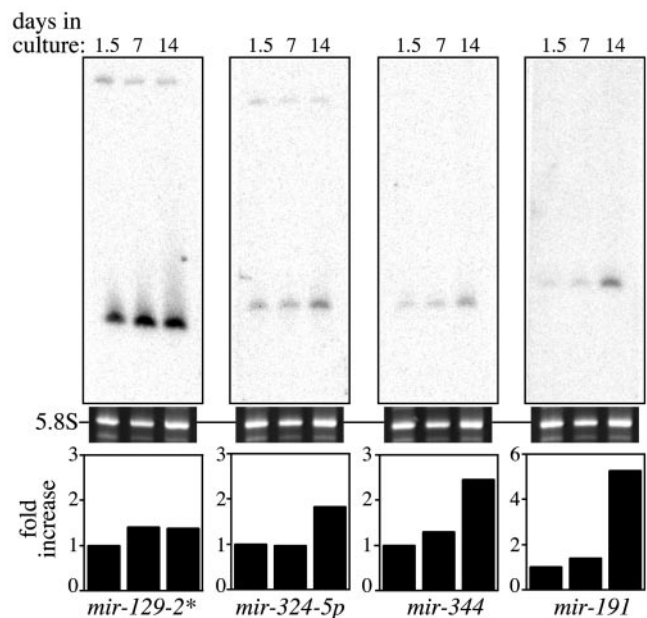
(*mir-128*, *-326*, *-344*, and *-129-2\**) in the mRNP and polyribosome fractions (Fig. 3c), suggesting that if miRNAs regulate such translational transitions, they occur without the release of these miRNAs from active translational machinery. However, to conclude that all miRNAs behave in the same manner as the four miRNAs examined here will require a survey of many more miRNAs and the analysis of neurons under a greater variety of stimulatory and quiescent conditions.

The localization of miRNAs to polyribosomes is suggestive that all of the miRNAs tested, and perhaps all miRNAs in mammalian neurons, mediate the translational control of target mRNAs on polyribosomes. These findings are consistent with the observation in *C. elegans*: *lin-4* and its target *lin-14* mRNA both associate with polyribosomes, even after *lin-4* down-regulates the translation of the *lin-14* mRNA and indicates that repression occurs after the initiation of translation (57).

Recent studies demonstrate that translational control in dendrites and axons is essential for developmental processes such as axon growth, guidance, and collapse, as well as dendritic maturation (36). We examined whether the expression of miRNAs identified in neurons might be temporally regulated and correspond to the stages of neuronal differentiation. Primary neurons in culture follow a well established set of morphological events that lead to mature, differentiated neurons (58–61). Cultures of rat E18 cortical neurons were plated and harvested at three stages of growth: 1.5 days *in vitro* (DIV) cultures, when cortical neurons adhere to the plates, begin growing minor neurites and extend one of these minor neurites as the incipient axon; 7 DIV, when axonal projections have emerged, dendritic elaboration is well underway, and the number of synapses increases dramatically; and 14 DIV, when dendritic processes have formed and additional synapses are established and stabilized. We examined the expression of 12 miRNAs by Northern blot analysis and discovered that 7 (*mir-128*, *-191*, *-323*, *-324-5p*, *-326*, *-329*, and *-344*) increased in expression  $\approx$ 2- to 5-fold from day 1.5 to day 14 in cortical cultures (Fig. 4). The expression level of the other miRNAs tested remained relatively constant over the time course. The temporal regulation of a subset of miRNAs correlated with these neural developmental events, and the precedence for miRNA function in regulation of timing during animal development suggests a potential role for miRNA-mediated gene regulation during neuronal differentiation and development.

Our finding that all miRNAs tested are localized to polyribosomes indicates that neuronal miRNAs, as a class, may act as translational regulators of neuronal gene expression. miRNAs are attractive candidates for this translational control. Those that are not expressed in early cortical cultures but are expressed later may regulate only their target mRNAs at later stages of neural development, for example, to mediate a temporal switch in neural type or pathfinding. Temporal sequences in neural type have been noted in the development of the vertebrate retina (62). In *Drosophila*, neuroblasts of the central nervous system produce specific lineages of neurons and glia according to the position and timing of division (63–65). This temporal sequence of neuroblast divisions is controlled by a temporal progression of transcription factor expression (*hunchback*  $\rightarrow$  *Kruppel*  $\rightarrow$  *pdm*  $\rightarrow$  *castor*) in neuroblasts (66, 67). Given the precedent for plant miRNAs to target transcription factors, miRNAs are good candidates to mediate the temporal regulation of these transcription factors in *D. melanogaster*. The mammalian miRNAs that we have shown to be temporally regulated during cortical development may act on analogous or even homologous target mRNAs.

Given that the miRNA localization on polysomes did not change in KCl-activated neural preparations (Fig. 3c), it is unlikely that the activity of these miRNAs is regulated by their localization to polyribosomes. In addition, we did not see



**Fig. 4.** Temporal expression of miRNAs in primary rat cortical cultures. Dissociated neurons from E18 rat forebrain were plated and harvested after 1.5, 7, and 14 days in culture. Northern blots of equal amounts of total RNA isolated from these primary cultures were assayed with probes against 12 miRNAs. Three of seven miRNAs (*mir-191*, *-324-5p*, and *-344*) that increase in expression level from day 1.5 to day 14 cultures are shown (the remaining miRNAs, *mir-128*, *-323*, *-326*, and *-329*, are not shown). *mir-129-2\** (shown) is among five miRNAs tested (*mir-103*, *-124a*, *-335*, *-129-2\**, and *-151\**) that display a relatively constant level of expression. The quantitation of the miRNA expression levels was normalized to day 1.5 cultures.

changes in the precursor to mature miRNA product ratio in these models of synaptic activity (data not shown), suggesting that it also is unlikely that the activity of these miRNAs is regulated at the level of processing. However, because we have not yet assigned any targets or functions to these miRNAs, it is possible that the particular miRNAs tested are regulated by neural events other than KCl exposure. Alternatively, only a subset of miRNAs may be regulated through localization to the polyribosome or by conversion of the precursor miRNA to the mature form.

The identification of translational machinery within dendritic spines suggests that the polyribosome-associated miRNAs also may be localized to dendrites to regulate local translation. Although *in situ* hybridization of the 21- to 25-nt miRNAs to subcellular locations remains a challenging technical problem, a recent study in *D. melanogaster* indicates that the fragile X mental retardation protein (FMRP in mammals; dFMR1 in fly) colocalizes with ribosomal translational apparatus, as well as with miRNAs, *in vivo* (68). FMRP also localizes to dendritic sites and interacts with polyribosomes in mammalian neurons (69). Given the requirement of FMRP for normal human cortical function, miRNAs also may be present in translationally active zones in dendrites, functioning in a complex with FMRP.

Despite the identification of >200 miRNAs from plants and animals, very few miRNA targets have been experimentally confirmed. A major challenge in elucidating the function of miRNAs will be to identify the specific target genes that they regulate. Unlike the near-perfect complementarity between plant miRNAs and the targets, computational approaches for target discovery have been complicated by the imperfect complementarity between an miRNA and its target transcript in animals. However, as dendritically localized mRNAs are iso-

lated, it is possible that this more limited sequence space of potential complementarity to the neuronally expressed miRNAs will allow the computational mapping of miRNAs to target mRNAs. Assigning mRNA targets to these miRNAs will prove an important step in the delineation of their roles in the control of the temporal sequence of neural development and in the regulation of synaptic remodeling.

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