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## Specific MicroRNAs Modulate Embryonic Stem Cell–Derived Neurogenesis

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**Key Words.** Embryonic stem cells • MicroRNA array • MicroRNA • Neurogenesis

### ABSTRACT

MicroRNAs (miRNAs) are recently discovered small non-coding transcripts with a broad spectrum of functions described mostly in invertebrates. As post-transcriptional regulators of gene expression, miRNAs trigger target mRNA degradation or translational repression. Although hundreds of miRNAs have been cloned from a variety of mammalian tissues and cells and multiple mRNA targets have been predicted, little is known about their functions. So far, a role of miRNA has only been described in hematopoietic, adipocytic, and muscle differentiation; regulation of insulin secretion; and potentially regulation of cancer growth. Here, we describe miRNA expression profiling in mouse embryonic stem (ES) cell–derived neurogenesis *in vitro* and show that a number of miRNAs are

simultaneously co-induced during differentiation of neural progenitor cells to neurons and astrocytes. There was a clear correlation between miRNA expression profiles in ES cell–derived neurogenesis *in vitro* and in embryonal neurogenesis *in vivo*. Using both gain-of-function and loss-of-function approaches, we demonstrate that brain-specific miR-124a and miR-9 molecules affect neural lineage differentiation in the ES cell–derived cultures. In addition, we provide evidence that signal transducer and activator of transcription (STAT) 3, a member of the STAT family pathway, is involved in the function of these miRNAs. We conclude that distinct miRNAs play a functional role in the determination of neural fates in ES cell differentiation. *STEM CELLS* 2006;24:857–864

### INTRODUCTION

The recent discovery of microRNAs (miRNAs) introduces a novel type of regulatory control over gene expression during plant and animal development [1, 2]. miRNAs are noncoding transcripts of 18–25 nucleotides (nt) derived from initially long primary transcripts, which are processed in the nucleus to ~70-nt precursor-miRNAs by the RNase III Droscha [3]. These precursors have a hairpin structure that undergoes cleavage by the enzyme Dicer [4, 5] to release the mature miRNA. The mature cytoplasmic miRNA is then incorporated into the RNA-induced silencing complex (RISC), an RNA-protein complex that mediates target mRNA cleavage or regulates its translation [2, 6]. In invertebrates, no genomic sequences have been identified with a perfect complementarity to known miRNAs. Several partially complementary mRNA targets have been validated and shown to control a broad spectrum of cell processes, including developmental timing, cell proliferation, cell death, and patterning of the nervous system [1]. It seems that miRNAs can

form extensive regulatory networks with a complexity comparable to that of transcription factors [7]. In mammals, approximately 250 miRNAs have been described, but very little is known about their mRNA targets and functions. One emerging function of miRNAs is control over lineage determination in hematopoiesis [8, 9]. These studies demonstrated that several miRNAs are dynamically regulated during early hematopoiesis and hematopoietic lineage commitment. One of the miRNAs was preferentially expressed in B-lymphoid cells, and its ectopic expression in hematopoietic stem cells led to an increase in the fraction of B-cells [8]. These results and the observation that expression of numerous miRNAs is cell- and tissue-specific [10–12] strongly suggest that miRNAs may modulate lineage differentiation of cells from different origins.

miRNAs have been shown to specify cell fates in the nervous system in worms [13, 14] and brain morphogenesis in fish [15], and their distinct expression patterns during mammalian brain development also suggest a role in neural differentiation in

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mammals [1, 16, 17]. However, a functional role for miRNAs in mammalian neurogenesis has not been described yet. Using oligonucleotide arrays designed to detect miRNA expression, we have demonstrated that specific miRNAs are precisely regulated during mammalian brain development [16]. To extend these studies, we now analyzed miRNA expression during mouse embryonic stem (ES) ES cell-derived neurogenesis *in vitro* and investigated a possible function of miRNAs in ES cell neural differentiation.

## MATERIALS AND METHODS

### ES Cell Differentiation

The mouse blastocyst-derived ES cell line D3 was propagated and maintained as previously described [18]. Differentiation of ES cells into neural progenitor cells, neurons, and astrocytes *in vitro* followed published protocols [19–22], with the modification of harvesting Nestin<sup>+</sup> precursors at stage 3 day 6 (3:6) for transfection assays with miRNAs.

### Analysis of miRNA Expression

RNA isolation from cultured cells and Northern blot analysis of miRNA were performed as previously described [16].

Oligonucleotide arrays of miRNA expression were performed and analyzed as described previously [16], but with probes specific for 135 miRNAs. Briefly, trimer DNA oligonucleotide probes (antisense to microRNAs) were spotted on Gene-Screen Plus membranes (PerkinElmer Life Sciences, Boston, <http://las.perkinelmer.com>) and permanently immobilized. For hybridizations, low molecular weight fractions of RNA enriched in molecules under 60 nt was obtained from total RNA and end-labeled by T4 polynucleotide kinase. Hybridizations and washes were carried out as described [16]. The experiments included at least three independent RNA samples for each stage of differentiation. To ensure accuracy of the hybridizations, each RNA sample was hybridized with three membranes. Hybridization signals for each spot of the array and background values at 15 empty spots were measured. Raw data was further processed automatically in Microsoft Excel. Hybridization signals that failed to exceed the average background value by more than three standard deviations were excluded from analysis. Upon normalization, the remaining data were averaged among triplicate arrays, and the resulting three data sets, each corresponding to an RNA sample, were considered independent measurements for the purposes of the two-tailed, two-sample *t* test when comparing different developmental stages. Also, the three data sets were averaged among three RNA samples to produce the set of expression levels at a given developmental stage. The expression ratio for each miRNA between given developmental stages was calculated as ratio between the corresponding expression levels.

### miRNA Duplexes and 2'-O-Methyl Oligonucleotides: Preparation and Delivery

RNA duplexes corresponding to miR-9/9\*, miR-124a, miR-22, and miR-125b were designed with 5' phosphate and two base overhangs on each strand, chemically synthesized by Dharmacon (Lafayette, CO, <http://www.dharmacon.com>) and annealed as recommended [23, 24]. 2'-O-Methyl oligonucleotides complementary

to miR-9, miR-124a, and miR-125b, chemically synthesized by Dharmacon, were composed entirely of 2'-O-methyl bases.

Transfections of miRNA duplexes to neural precursors (NPs) were carried out with NeuroPorter Reagent (GTS; Gene Therapy Systems, San Diego, CA, <http://genetherapysystems.com>) in 24-well plates according to the manufacturer's instructions. Briefly, 1.5  $\mu$ g of diluted miRNA (2 duplexes  $\times$  0.75  $\mu$ g or 4 duplexes  $\times$  0.375  $\mu$ g) per well were formulated with 3.75  $\mu$ l of NeuroPorter reagent in N2 serum-free medium. The transfection complex was added directly to the cells and replaced with a fresh medium after 24 h. This method resulted in uptake of the miRNA in more than 90% of the NPs as assessed by transfections with fluorescein RNA duplex. Two  $\mu$ g of 2'-O-methyl oligonucleotides per well were delivered similarly. Analyses of the effects of miRNAs on cell development were performed 4 to 16 days after transfections.

### Immunocytochemistry and Western Blots

Cells were analyzed by immunofluorescence staining as previously described [20–22] and examined using an LSM510 Meta confocal microscope equipped with ultraviolet, argon, and helium/neon lasers (Carl Zeiss, Thornwood, NY, <http://www.zeiss.com>). The following primary antibodies were used: rabbit antiglial fibrillary acidic protein, glial fibrillary acidic protein (GFAP) (DAKO, Carpinteria, CA, <http://www.dako.com>; 1:500), mouse anti- $\beta$ -III-tubulin (Covance, Richmond, CA, <http://www.covance.com>; 1:500) and rabbit anti- $\beta$ -III-tubulin (Covance; 1:2000). The secondary antibodies utilized were Alexa Fluor 488, 568, and 660 conjugated donkey immunoglobulin (Molecular Probes Inc., Eugene, OR, <http://probes.invitrogen.com>; 1:500).

For Western blots, the following antibodies were used: anti-signal transducer and activator of transcription (anti-STAT3) and anti-P-STAT3 (Cell Signaling Technology, Beverly, MA, <http://www.cellsignal.com>; 1:1000), anti-GFAP (BD Biosciences, San Diego, <http://www.bdbiosciences.com>; 1:500). Goat anti-rabbit and anti-mouse IgG-horseradish peroxidase secondary antibodies (1:10,000) were from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>).

### Flow Cytometry

The procedure for flow cytometry (FCM) was adapted from previously published protocols [25] with minor modifications. Briefly, cells at different stages of *in vitro* differentiation were mildly trypsinized (0.05% trypsin/EDTA; Invitrogen) and harvested in Ca<sup>+</sup>-free and Mg<sup>+</sup>-free phosphate-buffered saline (PBS), 2% fetal calf serum, 0.1% sodium azide. After washing, 10  $\mu$ g/ml propidium iodide (Fluka BioChemika, Buchs, Switzerland) was added and the cells washed again before fixation in 2% paraformaldehyde solution for 20 minutes on wet ice. In a second step, cells were incubated for 20 minutes on wet ice in PBS supplemented with 0.5% saponin (Invitrogen). The following staining steps with primary and secondary antibodies (see above) were performed in PBS/0.1% saponin. For FCM, cells were filtered through nylon mesh and analyzed in a BD FACS-Area cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>).

FCM analysis was set up in parallel to immunostainings and fluorescent terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) experiments, and it reflected tenden-

cies detected by those techniques through the time course of differentiation, such as reduction in Nestin<sup>+</sup>, increase in Tuj1<sup>+</sup>, and later increase in GFAP<sup>+</sup> cells, as well as relative amounts of apoptotic cells during the five stages of differentiation.

Apoptosis was assessed by staining of cultures with the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI, <http://www.promega.com>) and caspase activity was measured with CaspACE Assay System (Promega) according to the manufacturer's instructions.

### Statistical Analyses

For all statistical analysis including the values from the Western blot experiments, the Octave software (version 2.1.71; GNU, Madison, WI, <http://www.octave.org>) was used for performing analysis of variance. A *p* value below .05 was considered statistically significant.

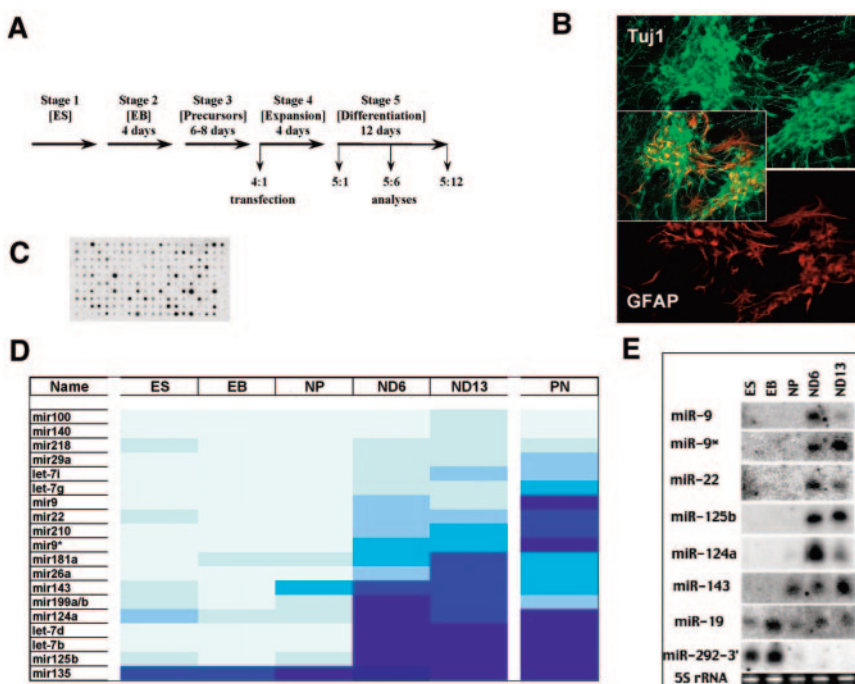
## RESULTS

### Expression Profiles of miRNA in ES Cell-Derived Neurogenesis

We used a stage-controlled ES cell differentiation protocol that includes embryoid body (EB) formation, selection and expansion of NPs and neural differentiation (ND) into neuronal and glial cell types strongly resembling neurogenesis and gliogenesis in vivo (Fig. 1A, 1B) [19–22]. Based on a previously described miRNA array technology for miRNA expression studies in the developing brain [16], we first determined miRNA expression profiles during the progressive stages of ES cell differentiation into neural cell populations. The following stages were analyzed: ES (stage 1), EB (stage 2), NP (stage 3), and ND (stage 5) (Fig. 1A). Primary cortical neurons were also analyzed to determine a pattern characteristic for neurons. For this study, the number of probes spotted on the membranes was expanded to display an expression profile of 135 vertebrate miRNAs (Fig. 1C).

The analysis of the miRNA array indicated that more than half of the miRNAs tested (70 of 135) changed at least twofold between any two stages. Among these, clusters of upregulated (Fig. 1D) and downregulated miRNAs were detected, as well as miRNAs with more complex expression patterns. More miRNAs were upregulated than downregulated, probably because the array consisted of miRNAs cloned from differentiated tissues and cells including brain and primary neurons [10, 11], rather than ES cell-expressed miRNAs [26] that had not been cloned at the time when the arrays were prepared.

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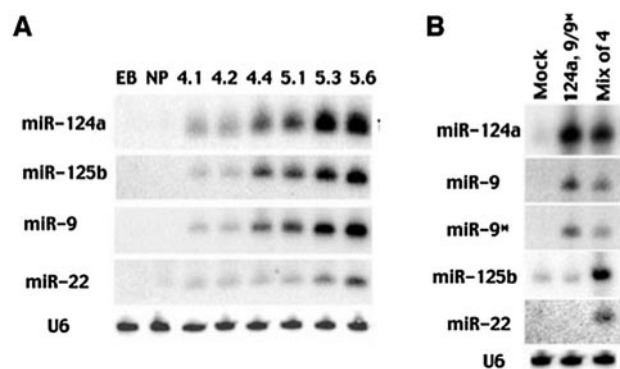


**Figure 1.** miRNA expression during ES cell-derived neurogenesis in vitro. **(A):** Schematic view of the five-stage ES cell differentiation protocol used in this study [19–22]. The different stages are indicated as follows: ES; EB; Precursors, selection of Nestin<sup>+</sup> cells; Expansion, expansion of Nestin<sup>+</sup> cells; Differentiation, neural differentiation into mature phenotypes. Times of transfection with miRNAs and analyses of their effects on cell development are indicated. **(B):** Immunocytochemistry demonstrating the development of the main components in the ES cell differentiation protocol: neurons (Tuj1, green) and astrocytes (GFAP, red). Inset shows composite image. Cells were stained at stage 5 day 12. **(C):** Representative view of an oligonucleotide array corresponding to stage 5 of ES cell differentiation. **(D):** Clusters of miRNAs induced during the course of differentiation. Three independent RNA samples corresponding to each stage of differentiation and to PNs were analyzed. After normalization, the three data sets were averaged to produce a set of expression levels at a given stage of differentiation. Colors from light to dark blue indicate relative intensities of miRNAs upregulated from stage 1 to stage 5 of differentiation (light blue corresponds to low intensity and dark blue to high intensity). **(E):** Validation of miRNAs induced from stage 3 (NP) to stage 5 (ND) by Northern blottings. Some of the miRNAs (miRNA-9\*, -22, and -125b) maintained their expression levels during the entire stage 5 (ND) of the differentiation protocol, whereas others (miR-9 and -124a) were only transiently elevated. MiR-19 and miR-292-3' demonstrate alternative expression patterns. 5S rRNA was detected by ethidium bromide staining of the gels prior to transfer to verify equal loading of total RNA. Abbreviations: EB, embryoid body; ES, embryonic stem cell; GFAP, glial fibrillary acid protein; ND, neural differentiation; NP, neural precursor; PN, primary neuron.

Since melting temperatures of oligonucleotide probes to miRNA target hybrids vary significantly due to the small size and different G/C content of miRNAs, it is technically impossible to achieve absolutely specific hybridization for each miRNA in an array format [16, 17]. In particular, the arrays do not discriminate among close family members that differ by only one or two nucleotides. Therefore, we validated the miRNA arrays by Northern blot experiments and confirmed most of the changes in expression profiles seen by the array hybridizations (Fig. 1E). A particularly interesting transition was from NP to early ND stage, an interval with many validated changes in miRNA expression (Fig. 1D, 1E). During this transition, Nestin<sup>+</sup> neural precursors are expanded and acquire neuronal and glial cellular phenotypes [19–22]. Many of the miRNAs induced during this transition were identical to those up-regulated in brain development during the E13–E21 interval when neuronal differentiation is prominent [16, 17] and in the process of neuronal differentiation of P19 cells [12]. Among this set of miRNAs, some maintained a high expression level, whereas others were down regulated as cells underwent further differentiation in culture (Fig. 1E).

### Overexpression of miRNAs in Neural Precursors

To assign a function to those miRNAs induced in differentiating NPs, we chose five ND-induced miRNAs (corresponding to four pre-miRs) that were highly expressed in brain and primary neurons (Fig. 1D) [10, 16]. Among them, miR-124a and miR-9/9\* are strongly enriched in brain, whereas miR-125b and miR-22 are expressed in various tissues [12]. MiR-124a, -9/9\*, -125b, and -22 were undetectable in stage 3 of NP selection, were simultaneously co-induced in stage 4, and reached the maximal levels at stage 5 (ND) days 3 and 6 (5.3 and 5.6) (Fig. 2A). The onset of expression among this miRNA set correlated with the first appearance of differentiated neurons in culture and ranged from stage 4 day 1 (4.1) to stage 5 day 1 (5.1) (Fig. 2A demonstrates the earliest onset). The nearly synchronous induction of these miRNAs suggests a common role in differentiation.

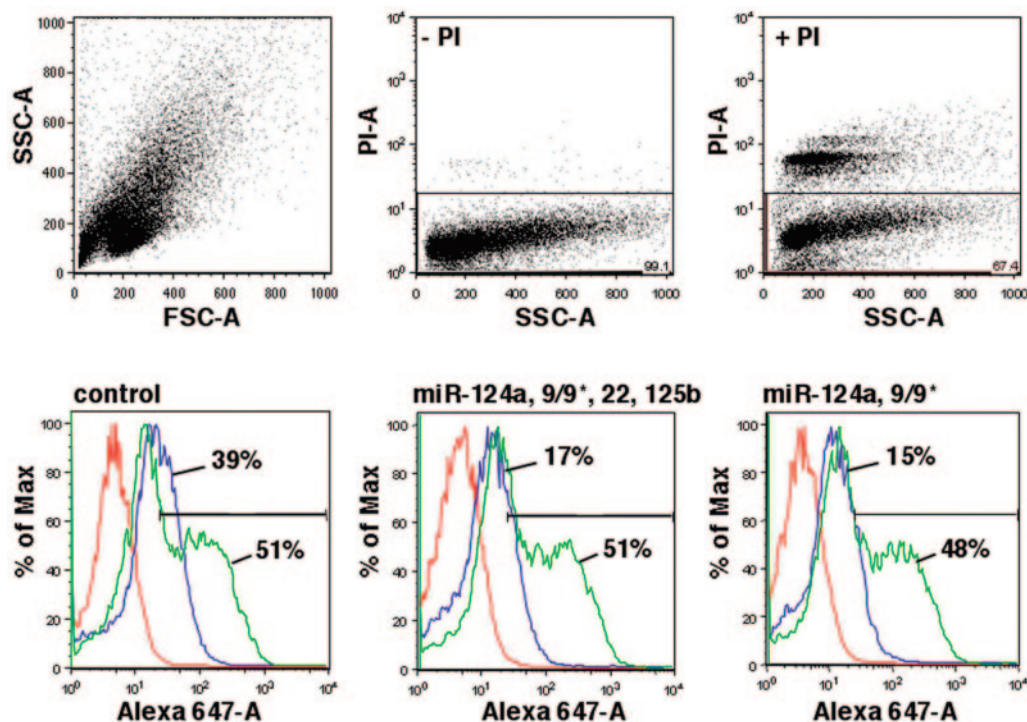


**Figure 2.** Overexpression of miRNAs during *in vitro* differentiation. (A): Kinetics of the induction of the endogenous miRNAs miR-9, -22, -124a, and -125b. Northern blot assays showing an induction of these miRNAs during expansion of NPs (stage 4) and at early differentiation (stage 5 days 1–6). (B): Overexpression of two duplexes (miR-9/9\* and miR-124a) and of four duplexes (miR-9/9\*, -22, -124a, and -125b) (Mix of 4) in NPs (stage 4 day 2). Northern blots demonstrating overexpression of the specific miRNAs 48 hours post-transfection. Abbreviations: EB, embryoid body; NP, neural precursor.

In order to evaluate this role, and assuming that miRNAs target specific mRNAs, we ectopically overexpressed these miRNAs in NPs before the onset of their expression (Fig. 2A) with the goal to downregulate early expression of target proteins. We followed “rational siRNA/miRNA design” rules to design and synthesize small siRNA-like duplexes with a sense strand identical to a mature miRNA sequence and nearly complementary antisense strand (see Materials and Methods). It was previously demonstrated that transfection of such duplexes with relatively unstable 5' ends of the sense strand resulted in preferential incorporation of this strand into RISC and therefore ensured its stability and functionality [23, 24]. Synthetic sense and antisense RNA molecules were annealed to create four miRNA duplexes and transfected in different combinations to NPs. This design strategy in combination with a highly efficient cationic lipid transfection method (up to 90% uptake of the miRNAs as evaluated by transfections of fluorescent miRNA duplex marker) was successful for all miRNA duplexes tested and revealed high transiently elevated levels of all five miRNAs in the transfected cells (Fig. 2B).

To determine an effect of the transfected miRNAs on differentiating NPs, we evaluated multiple parameters including cell proliferation, appearance of specific cell markers, cell death, and timing of events through stage 5 of differentiation (ND). In the protocol used, differentiation of NPs requires formation of cell–cell contacts, which is achieved by growing the cells at high densities and leads to formation of multilayered cultures with mixed cell populations (Fig. 1B). Quantitative analysis of different cell types in such cultures by manual counting on slides after specific immunolabeling is technically problematic. We therefore used FCM to determine the proportion of neuronal and astroglial-like cell populations at different times post-transfection. Specific antibodies against the intracellular markers Tuj-1 and GFAP were used to discriminate neurons from astrocytes, respectively (Fig. 3). In addition, considering a high level of apoptosis concomitant with the *in vitro* differentiation conditions, we stained the cultures with propidium iodide (PI) to discriminate among live, apoptotic, and dead cells. Each of these populations was analyzed separately (Fig. 3).

This analysis revealed substantial alteration in the markers of neural lineage differentiation when the NPs were simultaneously transfected with a combination of four miRNA duplexes. We found that the proportion of GFAP<sup>+</sup> cells was significantly reduced when compared to cells, which were untransfected, mock-transfected or transfected to overexpress unrelated RNA duplexes (Fig. 4A). Overall, in three independent experiments, we observed 46%–51% reduction in the amount of GFAP<sup>+</sup> cells. Overexpression of this set of miRNAs caused a slight increase or no effect on the number of Tuj1<sup>+</sup> cells. The ratio between Tuj1<sup>+</sup> and GFAP<sup>+</sup> in the cultures, therefore, shifted significantly in favor of Tuj1<sup>+</sup> cells (Fig. 4B). Importantly, quantitative analysis of PI-positive cells, as well as assessment of caspase activity and TUNEL, indicated that the reduced number of GFAP<sup>+</sup> cells was not associated with the selective death of this cell population in culture (Fig. 4C and data not shown). Transfection of two brain-specific RNA duplexes, miR-124a and miR-9/9\*, was sufficient to cause a similar effect (47%–55% reduction in the amount of GFAP<sup>+</sup> cells) (Fig. 4). However, we could not detect a significant effect when either miR-124a or miR-9/9\* was delivered to NPs separately.



**Figure 3.** Representative example of a typical FCM analysis of cells harvested at stage 5 day 6 of culture. The upper row shows distribution of cells by FSC/SSC (left panel) and the determination of the fraction of PI-positive cells staining in the PI/SSC analysis (middle and right panels). The lower row demonstrates the fraction of Tuj1<sup>+</sup> (green line) and GFAP<sup>+</sup> (blue line) cells in mock-transfected cultures (left panel); in miR-124a and miR-9/9\* cotransfected cultures (right panel); and in miR-124a, miR-9/9\*, miR-22, and miR-125b cotransfected cultures (middle panel). Only the PI-negative cells (upper right panel) were included in this analysis. Abbreviations: PI, propidium iodide; FCM, flow cytometry; FSC, forward scatter; SSC, side scatter.

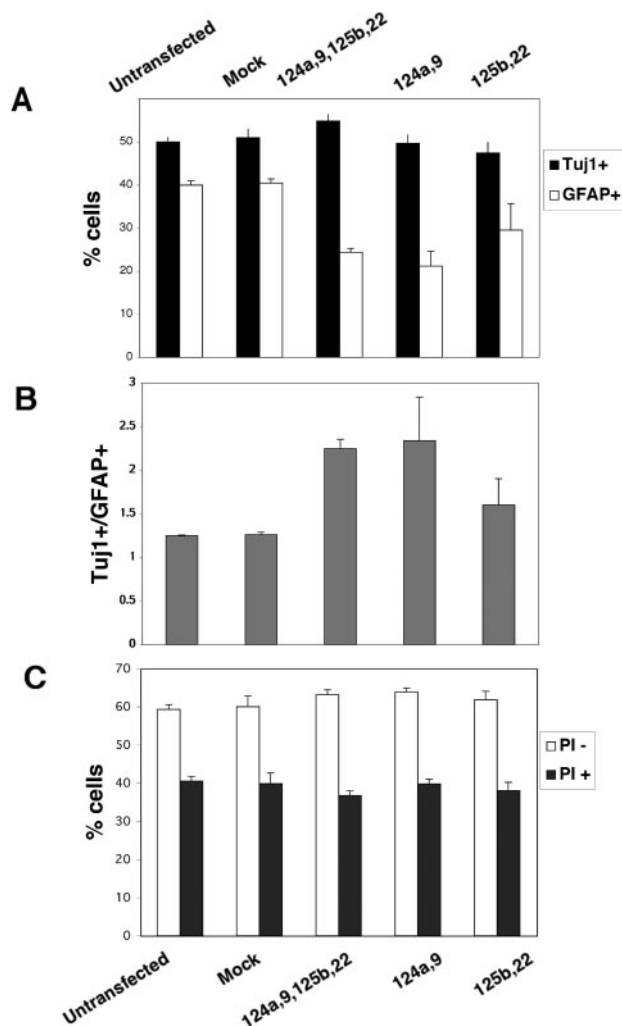
### Inhibition of miRNAs

Recently, 2'-*O*-methyl oligonucleotides were characterized as sequence-specific inhibitors of miRNA function and miRNA-directed RISC activity [17, 27, 28]. These molecules stoichiometrically bind and irreversibly inactivate miRNA, providing a valuable tool to disrupt the function of a single miRNA in vitro and in vivo. We used 2'-*O*-methyl oligonucleotides complementary to miR-124a, miR-9, or miR-125b to test the effect of loss-of-function of the miRNAs on the markers of neural differentiation. These oligonucleotides were transfected into NPs during their expansion in culture at the time of onset of the miRNA expression, and the cells were analyzed by Northern blots 2–4 days later. These analyses showed that the target miRNAs became markedly reduced (Fig. 5A), probably due to the formation of highly stable complexes with the blocking oligonucleotide that prevented miRNA detection even in the strong denaturing conditions used for the Northern blots. The effect was sequence-specific: each miRNA was blocked by the corresponding antisense oligonucleotide but not by scrambled or unrelated oligonucleotides. FCM analyses performed as described above revealed a significant drop in the ratio between Tuj1<sup>+</sup> and GFAP<sup>+</sup> cells when NPs were treated with the miR-9-blocking 2'-*O*-methyl oligonucleotide (Fig. 5B). This change was mainly caused by a reduced number of Tuj1<sup>+</sup> neurons (29%–31% reduction), whereas the proportion of GFAP<sup>+</sup> cells slightly increased. The level of PI staining and caspase activity in Tuj1<sup>+</sup> and GFAP<sup>+</sup> cells was not altered, indicating that the observed effect was not associated with increased apoptosis of

neurons. In contrast, neither miR-125b nor miR-124a knock-down caused significant effects on the differentiating NPs.

### miR-124a and miR-9 Affect Phosphorylation of STAT3

Several signaling pathways can be potentially implicated in neuronal and glial differentiation of ES cells [29, 30]; for example, activation of the signal transducer and activator of transcription 3 (STAT3) pathway plays an important role in neuronal development, particularly in inhibiting neuronal terminal differentiation [31, 32], and selectively enhances differentiation of neural precursors along a glial lineage [33, 34]. In addition, multiple studies demonstrated that STAT3 phosphorylation at Tyr705 leads to its activation and nuclear translocation with subsequent binding to target mRNAs [35, 36]. We therefore analyzed the levels of STAT3 Tyr705 phosphorylation and found that specific inhibition of miR-9 by its antisense 2'-*O*-methyl oligonucleotide led to significantly increased levels of phosphorylated STAT3, whereas the total level of STAT3 protein was stable (Fig. 6). On the other hand, in cells overexpressing miR-124a and miR-9, the level of STAT3 phosphorylation was reduced. This suggests that the effect of the miR-9 inhibitors on reduced neuronal differentiation (Fig. 5B) may be mediated by STAT3 activation, whereas reduction of STAT3 signaling in cells overexpressing miR-124a and miR-9 might lead to the reduced astrocytic lineage differentiation observed in our experiments (Fig. 4).



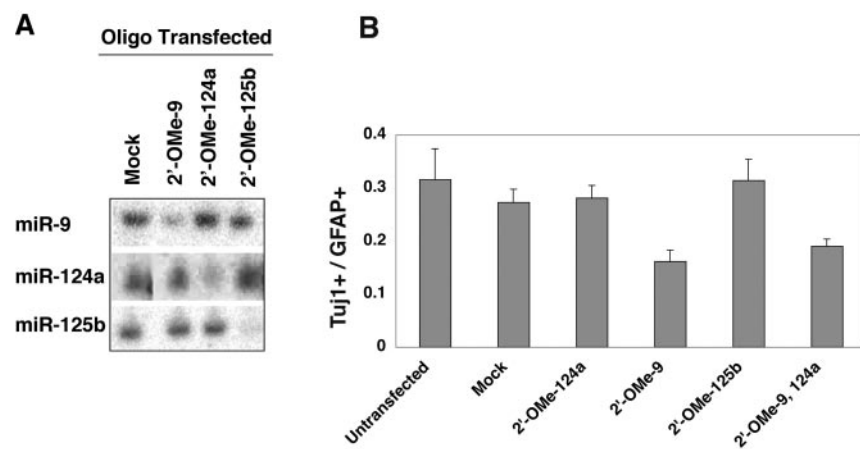
**Figure 4.** Effects of overexpression of miRNAs on neuronal and astroglial cell differentiation and cell death. **(A):** Percentages of Tuj1<sup>+</sup> (black) and GFAP<sup>+</sup> (white) cells among the differentiating neural progenitor cells in the different miRNA transfection conditions: untransfected, mock-transfected, mix-transfected (miR-9/9\*, -22, -124a, and -125b), miR-124a+miR-9/9\*, and miR-125b+miR-22. The average of three culture replicates for each combination is shown, with error bars indicating the standard deviation (using analysis of variance,  $p < .03$  for the comparisons of GFAP<sup>+</sup> cells in mock- and mix-transfected cells, and for mock- and miR-124a+miR-9/9\*-transfected cells). In independent repetitions of these experiments, similar decreases in the relative number of GFAP<sup>+</sup> cells were observed in cultures overexpressing miR-9/9\* and miR-124a when compared to the mock-transfected cultures. However, between individual experiments, the absolute percentages of Tuj1<sup>+</sup> and GFAP<sup>+</sup> cells differed, which was attributed to the heterogeneity of the ES cells. **(B):** To compensate for variations between individual experiments, the ratio of Tuj1<sup>+</sup> to GFAP<sup>+</sup> cells in cultures overexpressing the different combinations of specific miRNAs was determined. In all experiments, there was a significant increase of the Tuj1<sup>+</sup>/GFAP<sup>+</sup> ratios after transfections of four miRNA duplexes or miR-124a+miR-9/9\* combined transfections. **(C):** Percentages of PI-positive (black) and PI-negative (white) cells at stage 5 day 6 of culture in the different miRNA transfection conditions: untransfected, mock-transfected, mix-transfected (miR-9/9\*, -22, -124a, and -125b), miR-124a+miR-9/9\*, and miR-125b+miR-22. The average of three culture replicates for each combination is shown, with error bars indicating the standard deviation. The treatment of the cultures with the miRNAs did not affect the percentages of PI-positive or PI-negative cells. Abbreviation: PI, propidium iodide.

## DISCUSSION

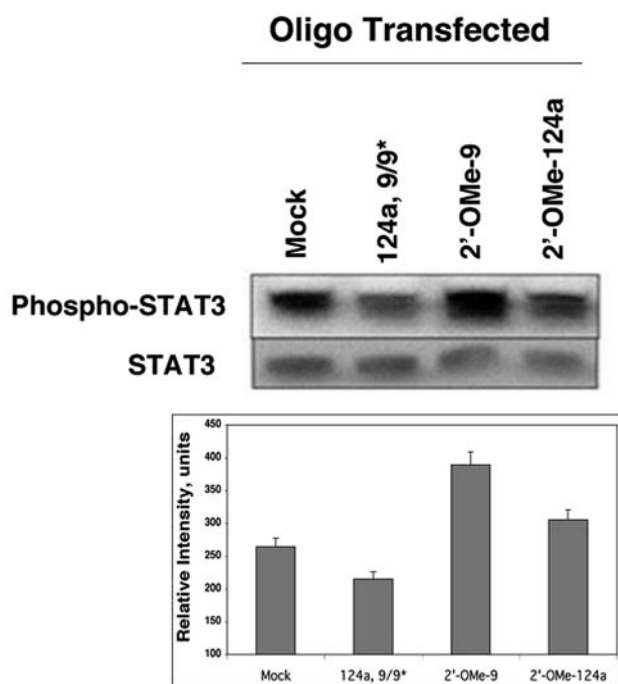
miRNAs have been shown to specify cell fates in the nervous system in worms [13, 14] and brain morphogenesis in zebrafish [15], and these factors, as well as their distinct expression patterns during mammalian brain development, suggest a role in neural differentiation in mammals [1, 16, 17]. However, a functional role of miRNAs in mammalian neurogenesis has not been described yet. Here, we demonstrate that in vitro transient overexpression or inhibition of brain-specific miRNAs in ES cell-derived neural precursor cells significantly reduced differentiation along either glial or neuronal cell lineages and could alter the balance between neurogenesis and gliogenesis.

For in vitro neurogenesis, we used a previously published five-step mouse ES cell differentiation protocol, which resembles several steps of embryogenesis, such as EB formation and differentiation of NPs into neurons and astroglia [19–22]. To determine miRNA expression profiles during ES cell differentiation, we performed miRNA array analysis and observed temporal expression of groups of miRNAs at specific stages of cell development. Since multiple miRNAs are co-induced during the NP to ND transition, they likely have multiple targets and pleurotropic combinatorial effects on differentiation. In addition, several miRNAs may cooperatively target the same mRNA, modulating expression of the encoded protein in a combinatorial way. These scenarios imply unlimited numbers of different regulatory combinations created by a network of co-expressed miRNAs that could contribute to a highly complex cell response. In this study, we have focused on five highly abundant miRNAs from those simultaneously induced in the transition from NP to ND cell stage. Among these miRNAs, miR-124a, and miR-9, which are expressed almost exclusively in the brain [12], also show prominent effects on ES cell-derived neurogenesis. Early overexpression of these miRNAs in neural precursors reduced the number of GFAP<sup>+</sup> cells (astrocytes) differentiated in culture. Inhibition of miR-9 expression alone or in combination with miR-124a caused a reduction of Tuj1<sup>+</sup> cells (neurons). Since miR-124a is expressed preferentially in embryonic neurons, whereas miR-9 is expressed in both neurons and glia [37] and our unpublished data), our results suggest that early overexpression of miR-124a in NPs prevents gliogenesis, whereas miR-9 expression contributes to neurogenesis. It should be noted that overexpression or inhibition of these miRNAs could have also affected other cells or factors in the differentiation cultures. Although flow cytometry with specific antibodies against Tuj1 and GFAP can be used for quantitative studies to discriminate neurons from astrocytes [25], GFAP-positive early neuronal (granule cell) and astrocytic neural precursors from the subventricular zone have been described [38–40]. Therefore, the observed effects of the miRNAs in our studies might, in part, be on a common neuronal and glial precursor, which has not been committed to either lineage. In addition, it is interesting to note that another class of small RNA molecules, dsRNA, can specify fates of adult neural stem cells by acting at the level of transcription [41]. It seems, therefore, that determination of the neural fate can be influenced by different types of small noncoding RNA modulators, which are involved in both transcriptional and post-transcriptional regulation.

The brain-specific miRNAs miR-9 and miR-124a, which we identified as mediators of neurogenesis, are conserved in mammals



**Figure 5.** Effects of miRNA inhibition on neural lineage differentiation. **(A):** Northern blot experiments showing selective inhibition of the endogenously induced miRNAs miR-9, miR-124a, or miR-125b in NPs with sequence-specific 2'-O-methyloligonucleotides 48 hours after transfection (stage 4 day 2). **(B):** The effect of miRNA inhibition on neural differentiation was analyzed by FCM as described in Figure 3, and the ratio of Tuj1<sup>+</sup> to GFAP<sup>+</sup> cells was determined. There was a significant reduction of the Tuj1<sup>+</sup>/GFAP<sup>+</sup> cell ratio when miR-9 was inhibited alone (2'-OMe-9) or together with miR-124a (using analysis of variance,  $p < .05$ ). Shown is the average of three culture replicates for each condition, with error bars indicating standard deviations. Abbreviations: miRNA, microRNA; FCM, flow cytometry.



**Figure 6.** Effect of miRNA overexpression and inhibition on STAT3 Tyr705 phosphorylation. Western blot analyses with anti-STAT3 and anti-phospho-STAT3 antibodies showed a reduction of phosphorylated STAT3 after transfection with miR-124a and miR-9/9\*. Conversely, inhibition of miR-9 by specific 2'-O-methyloligonucleotide (2'-OMe) led to a significant increase of phosphorylated STAT3 levels when compared to mock-transfected controls ( $p < .05$ ). The lower panel demonstrates relative levels of phospho-STAT3 normalized for total STAT3.

and associated with polysomes in the brain [10, 16], suggesting that they act, at least partially, at the level of translation. Hundreds of mRNA targets have been predicted computationally for these two miRNAs [42–44]. Some of them seem to be related to neurogenesis and/or to gliogenesis; in particular, our results imply that miR-9 and miR-124a act in the STAT3 signaling pathway, targeting some STAT3 upstream factor or factors but not the STAT3 mRNA itself. Though activation of STAT3 promotes differentiation of astrocytes

in the developing central nervous system [33, 34] and suppresses neurogenesis of neural stem cells [32], there is no clear understanding of STAT3 signaling in ES cell neuro- and gliogenesis. Multiple upstream factors, including Janus kinases, cytokines (LIF and CNTF) and their receptors (GP130, LIFR, CNTFR) and functional partners, could affect STAT3 phosphorylation. To our knowledge, none of these molecules have been consistently predicted as a very potent target for miR-9 and/or miR-124a. Therefore, further experiments in combination with bioinformatics approaches will be required to determine the molecular mechanism of miR-9 and miR-124a function in ES cell-derived neural cell differentiation.

## SUMMARY

Taken together, we demonstrate that distinct miRNAs can play a functional role in modulating neural differentiation of ES cells. Moreover, a clear correlation between miRNA expression profiles in ES cell-derived neurogenesis in vitro and in embryonal neurogenesis in vivo [16, 17] not only validates the stem cell model for studying miRNA regulation in neural development but also suggests that miRNAs can function as mediators of neural fate determination in mammalian brain development. Our results are in line with previously reported functions of miRNAs in lineage determination of hematopoietic cells [8] and in adipocyte development [45] and thus indicate important roles of miRNAs as molecules involved in regulating cell fates in mammals. Our data demonstrate an ability of miRNA to mediate neural differentiation of ES cells and suggest new mechanisms involved in the complex regulatory schemes that lead to neuronal and glial maturation.

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## DISCLOSURES

The authors indicate no potential conflicts of interest.

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