

## MicroRNA expression profiling during neural differentiation of mouse embryonic carcinoma P19 cells

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**MicroRNAs (or miRNAs) are small non-coding RNAs (21–25 nucleotides) that are involved in a wide range of activities related to the development and differentiation of cells. Comparison of the miRNA expression profiles of mouse P19 embryonic carcinoma cells with those of differentiated neural stem cells showed that the expression level of 65 miRNAs changed (2-fold) after differentiation. MiR-124a was dramatically up-regulated (more than 20-fold) while miRNAs of the miR-302 family and those in the miR-290–295 cluster were strongly down-regulated. Further analysis revealed that some important factors such as Oct4 and Sox2 appeared to be involved in the regulation of these miRNAs. These results may contribute to a better understanding of miRNA-regulated neural differentiation in early mouse embryos.**

**Keywords** microRNA; embryonic carcinoma P19 cell; neural stem cells; neural differentiation

Received: October 7, 2008 Accepted: November 28, 2008

### Introduction

The study of neuronal differentiation of embryonic stem (ES) cells has generated considerable interest in the last few years. These studies facilitated a better understanding of the fundamental aspects of neurogenesis. Since the first report on derivation of human ES cells, a number of studies have explored the possibility of directing the differentiation of ES cells towards neuronal development [1]. However, a number of challenges still need to be overcome, including gaining a better understanding of the mechanisms involved and developing

techniques that allow the generation of homogeneous neuronal and glial subtypes.

The mouse embryonic carcinoma (EC) P19 cell line is a teratocarcinoma cell line derived from transplanted epiblast mouse embryonic cells. Studies on P19 cells have provided a significant amount of information on the mechanisms of neural and skeletal muscle differentiation. Aggregated P19 EC cells can be induced to differentiate into neurons and glial cells in the presence of retinoic acid (RA) [2]. This method is commonly used for the molecular analysis of neural induction and differentiation [3]. However, only a small percentage of neural stem cells can be induced from P19 cells. Moreover, many different cell types exist in the induced cell population, and these non-neural cells may interfere with the detailed analysis of neural differentiation [4,5]. RA treatment also disturbs neural patterning and the neuronal identities of the ES cell aggregates [6]. Neural precursors produced by RA induction appear to be developmentally restricted, and can only generate a limited range of neural cell types [7]. In order to obtain a high percentage (about 95%) of neural stem cells from the P19 EC cells, we employed a method established by Xia *et al.* [8].

MicroRNAs (miRNAs) are a class of small, non-coding, regulatory RNA molecules. MiRNAs have been used as regulators of differentiation processes in different experimental models for neuronal development research [9]. Many miRNAs involved in the developmental processes exhibit a highly tissue-specific expression, e.g. some miRNAs are specifically expressed in the neural system [10–13]. Certain miRNAs appear to be correlated with the maintenance of pluripotency in cells during early mammalian development [14].

In this study, we aimed to understand the functions of miRNAs in neural development and neuron function rather than in the neuronal differentiation of ES cells. We used the microarray to monitor the expression profiles of mouse miRNAs during the course of P19 neuronal differentiation. The results indicated that there were significant differences between the miRNA expression in P19 EC cells and the resultant differentiated neural stem cells.

## Materials and Methods

### Cell culture and induction of differentiation

P19C6 cells, which are subclones of the mouse EC P19 cell line, were used in this study. The N2B27-induced neural differentiation of P19 cells was performed as described previously [8]. Briefly, the P19 cells from the stock culture were trypsinized into single cells, and they were allowed to aggregate in N2B27 serum-free medium for 4 days. The aggregates were then dissociated and re-plated onto poly-*L*-lysine-coated tissue culture dishes with N2B27 medium for up to 2 weeks. The cells were harvested on the first, second, third, and fourth days after N2B27 treatment. Untreated cells were used as controls.

### Construction of small RNA cDNA library

The total RNA was extracted with the RNArose reagent (Sangon, Shanghai, China). The cDNA libraries were constructed as described previously [15]. At least two independent reverse transcriptions/amplifications/hybridizations (technical replications) were performed for each sample.

### miRNA microarray

Each microarray contained 406 capture probes. The probes were perfectly matched for all miRNAs registered and annotated in the miRBase [16] at the Wellcome Trust Sanger Institute from human, mouse, and rat. There were 233 mouse miRNAs and 14 negative control probes (complementary to mRNAs, tRNAs, rRNAs, random sequences, etc.). All the microarray methods were performed as described previously [15], except that the hybridization temperature was adjusted to 45°C. The miRNA expression data have been submitted to the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>) under sample accession numbers GSM312842, GSM313002, GSM313003, GSM313005, and GSM313006 and series accession number GSE12600.

### Microarray data analysis

The analysis of microarray data was performed as described previously [9]. All hybridizations were

normalized by the total intensity and the Student's *t*-test was performed for the values. For each probe, we calculated the arithmetic mean of at least two replicates from two independent hybridizations at each time point. The probes with mean values less than 1000 at all time points were considered undetectable and were filtered out of the analysis. The probes that had *P* values <0.001 and changed more than 2-fold were selected for Northern blot validation.

### Northern blot analysis

Total RNA was extracted with the RNArose reagent. Total RNA (10 µg) was resolved on a 12% acrylamide/8 M urea gel, transferred onto nylon membranes, and cross-linked with UV light at 125 mJ/cm<sup>2</sup>. The DNA probes that were complementary to the miRNA sequences were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase (Fermentas, Glen Burnie, USA). The unincorporated [ $\gamma$ -<sup>32</sup>P]ATP was removed by filtration through a Sephadex G-25 column. Pre-hybridization and hybridization were carried out using PerfectHyb hybridization solution (Toyobo, Osaka, Japan) at 37°C. The blots were washed in 2 × saline-sodium citrate/0.1% sodium dodecyl sulfate according to the user manual. The membrane was measured by phosphorimager. We used 5S rRNA as the loading control for normalization. The DNA probes are listed in Supplementary data, Table S1.

### Analysis of promoters of the miRNA gene cluster and target miRNA

The preliminary information of the selected miRNAs was obtained from the website <http://microrna.sanger.ac.uk/> [16]. Gene sequences about 1 kb upstream of the gene clusters were extracted for promoter prediction. The BDGP Neural Network Promoter Prediction program (<http://www.fruitfly.org/>) was used for promoter prediction [17]. We then extracted sequences about 2 kb upstream of the predicted promoters and used TRANSFAC software [18] to predict the transcript factor-binding sites. The candidate target genes for the 15 miRNAs that showed significant changes were predicted using the miRanda [19,20] and TargetScan algorithms [21].

## Results

### miRNA expression changed significantly during neuronal differentiation of P19 cells

We used microarray analysis to examine the differential expression of miRNAs in the P19 EC cells and the

neural stem cells that were derived from the EC cells. An example of the scan images of non-induced p19 cells (labeled as p19) and neural stem cells (labeled as p19 N) are shown in Supplementary data, Fig. S1. The data indicated that there were significant changes in the miRNA expression during the differentiation of P19 cells. In comparison with the expression levels in the P19 cells, 34 miRNAs in the P19 N cells exhibited at least 2-fold up-regulation, while 31 miRNAs in the P19 N cells exhibited at least 2-fold down-regulation (**Table 1**).

We also observed that many miRNAs were expressed at low levels in the induced stem cells, a finding consistent with a previous study [22]. However, the number of up-regulated miRNAs was more than that of the down-regulated miRNAs. While 77 miRNAs exhibited at least 1.5-fold up-regulation, 49 miRNAs exhibited at least 1-fold down-regulation. The up-regulation of miRNAs appeared to be common during differentiation. However, while the ratios of up-regulation were not very high, those of down-regulation were much more significant. Although various kinds of miRNAs participated in this process, the most dramatic changes were observed in the miRNAs whose expression was reduced, which indicated that they were probably the key regulators in maintaining the multipotentiality of stem cells.

#### Confirmation of miRNA microarray data

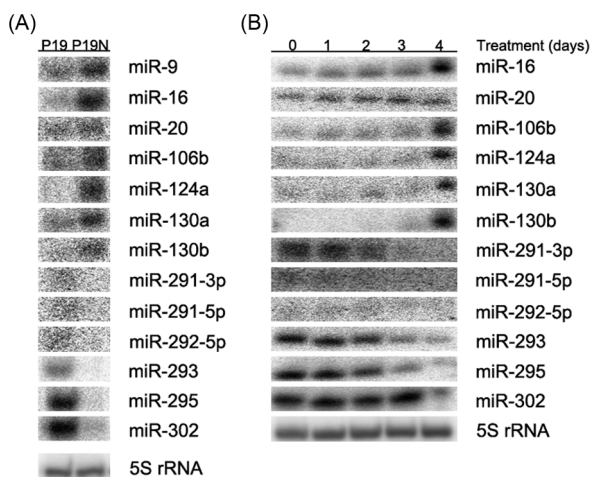
In order to confirm the results from the miRNA microarray experiments, we chose 13 miRNAs for northern blot analysis. The results are shown in **Fig. 1**. Seven of the 13 miRNAs, i.e. miR-9, miR-16, miR-20a, miR-106b, miR-124a, miR-130a, and miR-130b, were up-regulated in P19 N [**Fig. 1(A)**]. Interestingly, the expression of miR-124a, a brain-specific miRNA [23], increased after the differentiation [**Fig. 1(A)**]. These results were consistent with those of a previous study [23]. In contrast, miR-302, an miRNA specifically expressed in ES cells [24], was detected at high levels in the undifferentiated P19 cells; however, it was hardly detected in the differentiated P19 cells [**Fig. 1(A)**]. The miRNAs of the miR-290–295 cluster, which is composed of miR-290, miR-291a, miR-292, miR-291b, miR-293, miR-294, and miR-295, are also known as the ES-specific miRNAs [14]. The expression levels of miR-291, miR-292, miR-293, and miR-295 decreased gradually during the differentiation of P19 cells [**Fig. 1(A)**].

As the neural stem cells began to appear on the third day and account for 95% of the cell population on the fourth day of the induction period, the number of pluripotent cells (Oct4<sup>+</sup>Sox<sup>+</sup>) reduced gradually [8]. To

**Table 1** List of miRNAs showing differential expression in the non-induced P19 cells compared with the induced P19 neural stem cells

Up-regulated		Down-regulated	
miRNA name	P19 N/ P19	miRNA name	P19 N/ P19
mmu-miR-103	2.14	mmu-miR-182	0.2255
mmu-miR-106b	2.062	mmu-miR-183	0.2114
mmu-miR-124	20.31	mmu-miR-200b	0.2151
mmu-miR-130a	2.623	mmu-miR-22	0.2402
mmu-miR-130b	2.037	mmu-miR-23a	0.306
mmu-miR-134	2.03	mmu-miR-23b	0.1901
mmu-miR-135a	3.019	mmu-miR-24	0.2163
mmu-miR-135b	2.544	mmu-miR-27a	0.2576
mmu-miR-153	7.773	mmu-miR-27b	0.2496
mmu-miR-154	2.382	mmu-miR-29a	0.2827
mmu-miR-16	2.377	mmu-miR-29b	0.4937
mmu-miR-181a	3.066	mmu-miR-302a	0.2939
mmu-miR-181b	3.058	mmu-miR-302b	0.2189
mmu-miR-181c	2.98	mmu-miR-302c	0.2662
mmu-miR-191	5.966	mmu-miR-302d	0.2721
mmu-miR-20a	2.107	mmu-miR-31	0.2876
mmu-miR-210	4.212	mmu-miR-499	0.3616
mmu-miR-219	8.036	mmu-miR-7	0.3457
mmu-miR-221	2.312	mmu-miR-96	0.1544
mmu-miR-26a	2.231	mmu-miR-10b	0.4221
mmu-miR-26b	2.243	mmu-miR-290-5p	0.0951
mmu-miR-335-5p	8.425	mmu-miR-291a-3p	0.0626
mmu-miR-33	2.407	mmu-miR-291a-5p,	0.1476
		mmu-miR-291b-5p	
mmu-miR-369-3p	2.891	mmu-miR-292-3p	0.1082
mmu-miR-381	2.827	mmu-miR-292-5p	0.0653
mmu-miR-382	2.433	mmu-miR-293	0.0561
mmu-miR-410	2.462	mmu-miR-294	0.0775
mmu-miR-151-3p	2.362	mmu-miR-295	0.0705
mmu-miR-153	7.497	mmu-miR-298	0.2364
mmu-miR-300	2.013	mmu-miR-346	0.4983
mmu-miR-341	2.293	mmu-miR-96	0.1882
mmu-miR-376a	3.663		
mmu-miR-410	2.094		
mmu-miR-9	2.136		

study the elaborate changes in miRNAs during this process, the P19 cells were collected, the total RNA was isolated, and Northern blot analysis was carried out to analyze the miRNA expression patterns on the first, second, third, and fourth day after N2B27 induction.



**Fig. 1 Validation of the results from miRNA microarray studies by northern blot analysis** (A) miRNA expression in non-induced P19 cells (P19) and induced P19 neural stem cells (P19 N). (B) miRNA expression during 4 days of P19 induction. The names of the miRNAs are mentioned at the right side of each panel. 5S rRNA served as a loading control.

With a decrease in the percentage of pluripotent cells and an increase in the number of neural stem cells, the miRNAs miR-16, miR-106b, miR-130a, miR-130b, and miR-124a were observed to be up-regulated and dramatic changes occurred on the fourth day after induction. However, the expression levels of miR-291, miR-292, miR-293, and miR-295 decreased steadily [Fig. 1(B)]. In addition, the expression of miR-302 increased gradually at first and then decreased on the fourth day. These results indicated that the down-regulated gene clusters may participate in the maintenance of stem-cell multipotency and the regulation of early embryonic development.

## Discussion

### Similar expression tendency of the miRNA cluster

The tendency of miRNA genes to occur in clusters [25] prompted us to analyze the chromosomal distribution of the differentially expressing miRNAs. We found that except for some miRNAs that were expressed alone,

**Table 2 Similar expression tendency of the miRNA cluster in the distal 12 regions of chromosome 12**

ID	Chromosome	Start	End	P19 N/P19
mmu-mir-337	12	110823999	110824095	1.251
mmu-mir-431	12	110828657	110828747	1.822
mmu-mir-433	12	110829925	110830048	1.865
mmu-mir-127	12	110831056	110831125	1.842
mmu-mir-434	12	110832716	110832809	1.649
mmu-mir-136	12	110833537	110833598	1.422
mmu-mir-341	12	110849710	110849805	2.293
mmu-mir-370	12	110856468	110856546	2.241
mmu-mir-379	12	110947270	110947335	1.854
mmu-mir-323	12	110950718	110950803	1.773
mmu-mir-494	12	110953528	110953612	1.301
mmu-mir-495	12	110956964	110957026	1.606
mmu-mir-376b	12	110961668	110961749	2.796
mmu-mir-376a	12	110961991	110962058	3.663
mmu-mir-300	12	110962523	110962601	2.013
mmu-mir-381	12	110965032	110965106	2.827
mmu-mir-382	12	110971981	110972056	2.433
mmu-mir-134	12	110972349	110972419	2.03
mmu-mir-154	12	110976643	110976708	2.382
mmu-mir-409	12	110981368	110981446	1.439
mmu-mir-369	12	110981628	110981706	2.891
mmu-mir-410	12	110981925	110982005	2.462

most of these miRNAs showed cluster distribution and they were distributed either in the intron of the protein gene or in the gene-spacer region. On the basis of the microarray data, nine of the 10 miRNA clusters were found to exhibit increased expression levels after their differentiation (Supplementary data, Table S2), while seven other clusters exhibited decreased expression level (Supplementary data, Table S3). It appeared that miRNAs in the same gene cluster usually changed their expression in the same way (i.e. either as an increase or as a decrease in expression).

Previous studies have shown that there are 50 pre-miRNA sequences located in the distal region of chromosome 12 in mice and these were organized as a large cluster that was conserved in the human 14q32 domain [25]. Our microarray included 33 miRNA genes of this cluster and we detected that 22 miRNAs exhibited significantly increased expression levels (**Table 2**). Most of these intensive-distribution miRNAs exhibited similar

expression tendencies, which may imply that the distal region of chromosome 12 in mice becomes active during differentiation.

### Potential binding sites for transcript factors and target genes that may participate in regulation

It was reported that miRNAs usually formed a reciprocal negative feedback loop in the neural system [26]. Using a computer-assisted approach, many potential binding sites for transcript factors were predicted (data not shown). MiRNAs suppress the mRNA translation or affect its stability by pairing with the 3'-untranslated region of the target mRNA. Further, we predicted a total of 248 potential target genes for the 15 significantly changed miRNAs using the miRanda [19,20] and TargetScan algorithms [21]. Most of these target genes were signal transducers, transporters, kinases, transcription regulators, and enzyme regulators (**Table 3**). Interestingly, most of the predicted target genes encoded

**Table 3 Predicted target genes regulated by the dramatically changed miRNAs with dramatically changed expression levels**

Name	P19 N/ P19	Predicted target
miR-153	7.773	<i>Fgf13, Gata1, Sox18, Map4k5, Dpp4, Mapk7, Lrp8, Mapk10, Smad7, Grk4, Fst, Shc3p1, Six6os1, Rab20, Src, Ctb2, Bmp5, Fgfr3, Grb2, Wnt16, Neurod6</i>
miR-191	5.966	<i>Pou2f1, Plcd1, Hoxa2, Cdc5l, Pou3f2, Hdac1, Hdac5, Rexo2, Hoxc8, Hoxd10, Chrdl2, Hes1, Otx2, Numbl, Dkk4</i>
miR-219	8.036	<i>Hes5, Wnt16, Rab35, T(Brachyury), Rab17, Rab13, Lrp11, Plcb4, Cer1, Gata4, Gas2, Hoxd13, Gsc, Plcg2, Cdc7, Shh, Dvl2</i>
miR-301a	4.915	<i>Hat1, Rab34, Fstl5, Hdac2, Myc, Nfatc3, Wnt1, Gsc, Bmp5, Rab26, Smad2, Pou4f3, Pou3f2, Cerk, Zic3, Neurog1, Fgf18, Fgf7, Map3k9, Map2k1, Hoxa5, Pax9, Bmpr1b, Ncstn, Sos2</i>
miR-335-5p	8.425	<i>Hoxd8, Rab34, Pou4f3, Nfatc3, Cdc42, Six2, Hoxd13, T(Brachyury), Hoxd12, Qsox2, Dkk1, Pou6f1, Hes1, Dvl1, Shc2, Wnt10b, Hes2, Sos1</i>
miR-290-5p	0.0951	<i>Sox2, Hdac2, Rexo1, Nfatc1, Map3k1, Fgfr11, Nf2, Rexo1, Sos2, Map3k4, Fgf23, Plcb1, Dicer1</i>
miR-291a-3p	0.0626	<i>Hesx1, Vim, Dpp3, Lrp3, Pou2f1, Notch4, Zic1, Smad4, Lrp4, Fgfbp1, Map3k1, Smad6, Pax6</i>
miR-291a-5p	0.1476	<i>Hoxa9, Hoxb1, Nfatc3, Dpp3, Map3k5, Pou1f1, Bmp1, Nes, Mapk7, Map3k8, Rab21, Rab28, Psen1, Raf1, Pou3f2, Dpp8, Hoxd8</i>
miR-292-3p	0.1082	<i>Cdc40, Rab13, Mapk12, Nf2, Hoxd10, Numbl, Rab32, Plcb3, Fgf6, Neurog1, Gata6, Gas2</i>
miR-292-5p	0.0653	<i>Hdac2, Nanog, Map3k4, Map3k1, Hoxc4, Hdac4, Hoxb1, Bmpr1b, Hoxb4, Pou6f2, Cdc37, Hdac11, Fgf23, Sos2, Rexo1, Fstl3, Dicer1, Paxip1, Hoxc5, Hoxd1, Neurog1, Numb, Fgfr11, Fgf17, Wnt6, Sox2</i>
miR-293	0.0561	<i>Fgf8, Plce1, Lrp1, Shc3, Wnt7a, Rab13, Junm2, Gata3, Plcd3, Rab24, Cdc7, Dicer1, Plcg1, Dkk1</i>
miR-294	0.0775	<i>Vim, Notch4, Smad6, Zic3, Map2k3, Lrp4, Lrp3, Pou2f1, Pax5, Fgfbp1, Wnt7b, Nanos1, Pou6f1, Map3k1, Fgf16</i>
miR-295	0.0705	<i>Vim, Map3k11, Zic3, Wnt16, Neurog3, Lrp3, Dpp3, Rab28, Pax6, Hdac8, Rab15, Map3k1, Gas2</i>
miR-302	0.2189	<i>Hesx1, Fgf13, Pou2f1, Rab15, Vim, Smad2, Lrp4, Zic3, Procr, Lrp2, Lrp11, Dpp3, Neurog3, Rab38, Pou6f1, Hoxa13, Fgf10</i>
miR-302*	0.3213	<i>Pax3, Map3k1, Fstl3, Hoxa5, Stat4, Hoxa10, Hoxb1, Nfat5, Fgf18, Map4k4, Hdac2, Sox2, Hdac8</i>

for the predicted transcript factors. The mechanisms behind the regulation of miRNAs by these factors in neural development processes still need to be elucidated. In conclusion, these miRNAs, with significantly changed expression levels, could play important roles in the maintenance of stem-cell multipotency and neuronal differentiation in early embryonic development.

## Supplementary Data

Supplementary data is available at *ABBS* online.

## Funding

This work was supported by the grants from the National Key Basic Research and Development Program (2005CB724602), the National Natural Science Foundation of China (No. 30430210), and the Chinese Academy of Sciences (KSCX2-YW-R-096 and KSCX1-YW-R-64).

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