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Original Article

miR-206 is involved in the rat brain development by targeting the mRNA of lis-1

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Abstract: Increasing evidence indicates that microRNAs (miRNAs) play important roles in rat brain development. The present study aimed to characterize the functional role of neuronal microRNA, miR-206 in modulating cortical neuronal migration by targeting lis1. A miRNA microarray was profiled recently during the rat brain development. The result showed that the expression of miR-206 decreased in focal cortical dysplasia induced by liquid nitrogen in the immature rat. Few studies on miR-206's roles in the brain have been reported so far. The bioinformatic analysis indicated that one of miR-206's putative targets is Lis-1, a known gene related to cell proliferation and migration according to our previous study. The luciferase assay confirmed that Lis-1 mRNA was targeted by miR-206. Our data showed an inhibitory effect of miR-206 on the expression of Lis-1 in PC12 cells transfected by pre-miR-206 after 24 h. Whereas, the results showed a promoting effect in PC12 cells transfected by anti-miR-206 after 24 h. Cell migrate assays showed that miR-206 can significantly inhibit PC12 and HCN-2 cells migration treated with or without lis1 protein. The above results demonstrated that the reduction of miR-206 at least in part caused the upregulation of Lis-1 in the rat brain. Take these results together, we hypothesized miR-206 may have a role in the brain development by affecting the expression of Lis-1.

Keywords: Focal cortical dysplasia, miR-206, Lis-1, migration, transwell

Introduction

The maturation of brain undergoes three important stages (cell proliferation, neuronal migration, cortical organization) [1]. Cortical development performs an important role in the maturation of brain. Malformations of cortical development are increasingly recognized in association with severe epileptic syndromes, neuropsychological disorders and mental retardation. It is necessary to find the possible regulatory factors involved in these developmental events in the maturation of brain [2]. Lis-1, a brain-specific gene, encodes the non-catalytic subunit of platelet-activating factor acetyl hydrolase isoform 1B (PAFAH1B), this enzyme inactivates platelet activating factor (PAF). Lis-1, a known gene related to cell proliferation and migration, contributes to brain development in our previous study [19]. People with Miller-Dieker syndrome (MDS) or isolated lissencephaly sequence (ILS) have hemizygous deletion or mutation in the Lis1 gene [3]. ILS and MDS often result from haploinsufficiency at human chromosome 17p13.3; this chromosomal region includes the Lis-1 gene. Lis-1 is disrupted in both ILS and MDS patients and suggests that mutations within Lis-1 are responsible for defective neuronal migration [3].

MicroRNAs (miRNAs) are smallnon-coding RNAs that bind to the 3'UTR of target mRNAs to regulate its stability or the translational efficiency [4]. Over the last decade, research has indicated the important regulatory roles for miRNAs in development, differentiation, cell proliferation, apoptosis, metabolism and their involvement in the pathogenesis of diverse diseases [5]. Approximately 70% of known miRNAs are expressed in the mammalian brain, and level of many miRNAs change dramatically during the brain development. As a class of critical regulators of development and physiology, however, the roles for miRNAs in regulating the mammalian brain development are still poorly defined. Therefore, we profiled the expression of 391 miRNAs in

the rat brain between postnatal day 60 and 80 with a miRNA microarray in order to find whether there were some miRNAs implicated in the brain development in the rat. Actually, we have found that the expression of 84 miRNAs changed significantly. Herein, we studied a candidate in these 84 miRNAs, miR-206, which has not been reported in the studies on brain so far [6]. Several genes are predicted as miR-206 putative targets by multiple algorithms (TargetScan, miRanda and Pictar), These genes include Lis-1, Capn8, Gmfb, Mapk1, Dclk1, Vim, Mgst1, Gnb1, Klf15, and Cdkn1b. The results from the miRNA microarray showed that the expression of miR-206 decreased from day 20 to 80 during the rat brain development. What's more, Lis-1, a key gene related to cell proliferation and migration, is a predicted target for miR-206. It has been reported that Lis-1 can modulate cell proliferation and migration through its interaction with some proteins, such as Dynein [7].

Further knowledge about the molecular mechanisms underpinning the cortical neuronal migration may provide insights for better therapeutic opportunities to treat malformations of cortical development patients. In this study, we undertook to examine the expressions profiles of miRNAs in the immature rat with focal cortical dysplasia induced by liquid nitrogen and aimed to identify miRNAs that might modulate cortical neuronal migration. Herein we identified and characterized a miRNA, miR-206, and found that the loss of miR-206 plays an important role in cortical neuronal migration through modulating Lis-1.

Materials and methods

miRNA microarray analysis

RNA labeling and hybridization on miRNA microarray chips were performed as previously described [8]. Brain tissues were pooled from Day 20 and Day 80 Sprague-Dawley rats, the total RNAs were extracted with Trizol reagent. Briefly, 50 μg total RNA was purified by using mirVANA miRNA isolation kit (Ambion, Austin, TX, USA) to enrich small RNA fraction. Purified RNA was labeled with Cy3 and hybridization was carried out on miRNA microarray chip (CapitalBio Corp, Beijing, China) containing 381 probes in triplicate.

qRT-PCR of miR-206

The steps of miRNA qRT-PCR were performed as described in the previous paper [6]. Briefly, 1 μg total RNA obtained from the rat brain was reverse transcribed with the stem-loop RT primer and then realtime PCR was performed. The primers used for miR-206 qRT-PCR were listed as follows: miR-206-forward: 5'-GAGTGCTGGAATGTAAGGAAG-3', miR-206-reverse: 5'-GCAGGGTCCGAGG-3'; U6-forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', U6-reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'.

Analysis of miR-206 predicted targets

The prediction of miR-206 targets was performed by the algorithms: miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), TargetScan (http://www.targetscan.org/vert_50/), miRanda (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/mirna.pl).

Cell culture and transfection

PC12 cells were maintained in DMEM high glucose medium, supplemented with 10% fetal bovine serum (FBS) and 5% horse serum. HCN-2 cells were maintained in DMEM high glucose medium, supplemented with 10% fetal bovine serum (FBS). All were cultured in a humidified incubator at 37°C with 5% CO_2. PC12 cells were transfected with 50 nM of either a non-targeting small RNA oligonucleotide as a negative control (control), or miR-206 mimics (stability enhanced miR-206 oligonucleotide) using Lipofectamine 2000 [9]. The transfected cells were harvested after 48 h, RNAs were extracted from the cells with Trizol reagent, proteins were extracted with the lysis buffer (20 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton-X-100, protease inhibitor [10]. The results of the following qRT-PCR or Western blot were obtained from three separate transfections.

qRT-PCR of Lis-1

The steps of mRNA qRT-PCR were performed as follows. Briefly, 1 μg total RNA obtained from the rat brain or PC12 cells was reverse transcribed with oligo (dT) 18 and then qPCR was performed. The primers used for Lis-1 qRT-PCR were listed as follows: Lis-1-forward: 5'-TGCCCAAGACTACTCAACCC-3', Lis-1-reverse: 5'-GCACCCTGTGACGAAAGC-3'; 18 s RNA-forward:

5'-AGCAACTGCGCCTGAAAC-3', 18 s RNA-reverse: 5'-CCCTGTCCCGCTCAACTA-3'.

Western blot for Lis-1

Cells were rinsed once with PBS and lysed in lysis buffer (20 mM Tris (pH 7.4), 5 mmol/l EDTA, 1% (V/V) TritonX-100, 0.15 mol/l NaCl) on ice for 10 min. Insoluble components were removed by centrifugation (12000 rpm, 5 min), and protein concentration was measured [11]. Cellular proteins (100 μg) were adjusted to a total concentration of 5 μg/μl. After boiling for 5 min in Loading Buffer, proteins were separated by 8% Tris-Glycine Gels for Lis-1 [6]. Western blotting was performed using Mouse anti-rat Lis-1 antibody (Abcam Biotech, USA) and goat anti-mouse secondary antibody. Immunocomplexes were visualized by Enhanced Chemiluminescence according to the manufacturer's protocol.

Luciferase targeting assay and transwell experiment

We cloned 400 bp of Lis-1 3'UTR containing the 7 bp target site for miR-206 into the Spe I/Hind III sites of a luciferase gene in the pMIR-REPORT luciferase vector. PCRs were performed by rTag polymerase. PC12 cells (5×10^4) were co-transfected with 150 ng of pMIR-REPORT-Lis-1-3'-UTR plasmid and 25 nM of either a stability-enhanced non-targeting small RNA oligonucleotide (Gene Pharma) as a negative control (control), or miR-206 mimics (Gene Pharma) using Lipofectamine™ 2000 [12]. The transfected cells were harvested after 24 h and then assayed with Dual-Luciferase Reporter Assay System (Promega). The results were obtained from three separate experiments, and each one was done in triplicate.

Transwell migration assay was performed as described previously [13]. The cells migrated the membrane were fixed with methanol and stained with crystal violet. Photographs of randomly selected fields of the fixed cells were taken and cells were counted. Experiments were repeated independently three times.

Rat model of focal cortical dysplasia

Thirty-three days after birth, 30 Sprague-Dawley rats were obtained from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China). All animals were housed in a specific pathogen-free (SPF) facility and received human care according to the Chinese legal requirements. The experimental animal procedures were approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

The rat model of liquid nitrogen lesion-induced focal cortical dysplasia was established as previously described [14]. Twenty Sprague-Dawley rats with cortical dysplasia were randomly divided into two groups: the model group and the miR-206 administration group. In the model group, brain tissue was obtained on days 0, 20, 60 and 80, respectively. In the miR-206 administration group, miR-206 was injected into the rat brains using a rat brain locator at 5 mg/kg dose on days 40 and 50, and brain tissue was obtained on day 60. The control (healthy; n=10) group was administered the same volume of PBS, on day 60, brain tissue was rapidly removed and prepared for next experiment.

Statistical analysis

All experiments were performed in triplicate. Hierarchical Cluster analysis was carried out in Gene Cluster software. Comparisons between groups were made by Student's t-test. A *P*-value <0.05 was considered statistically significant [15].

Results

Cluster analysis for microarray data and qRT-PCR validation

We examined the expression profiles of miRNAs in immature rats with liquid nitrogen lesion-induced focal cortical dysplasia. The dendrogram generated by cluster analysis showed the separation of model from control samples on the basis of miRNA profiling (**Figure 1A**). The relative fold changes of miRNAs from qRT-PCR data were compared with microarray data (**Figure 1B**). These results indicated a downregulation of miR-206 expression during the rat brain development.

miR-206 targets the mRNA of Lis-1 by luciferase assay

We used multiple algorithms (TargetScan, miRanda and Pictar) to predict the putative targets of miR-206 and got several potential targets such as Lis-1, Capn8, Gmfb, Mapk1, Dclk1, Vim, Mgst1, Gnb1, Klf15 and Cdkn1b. The bio-

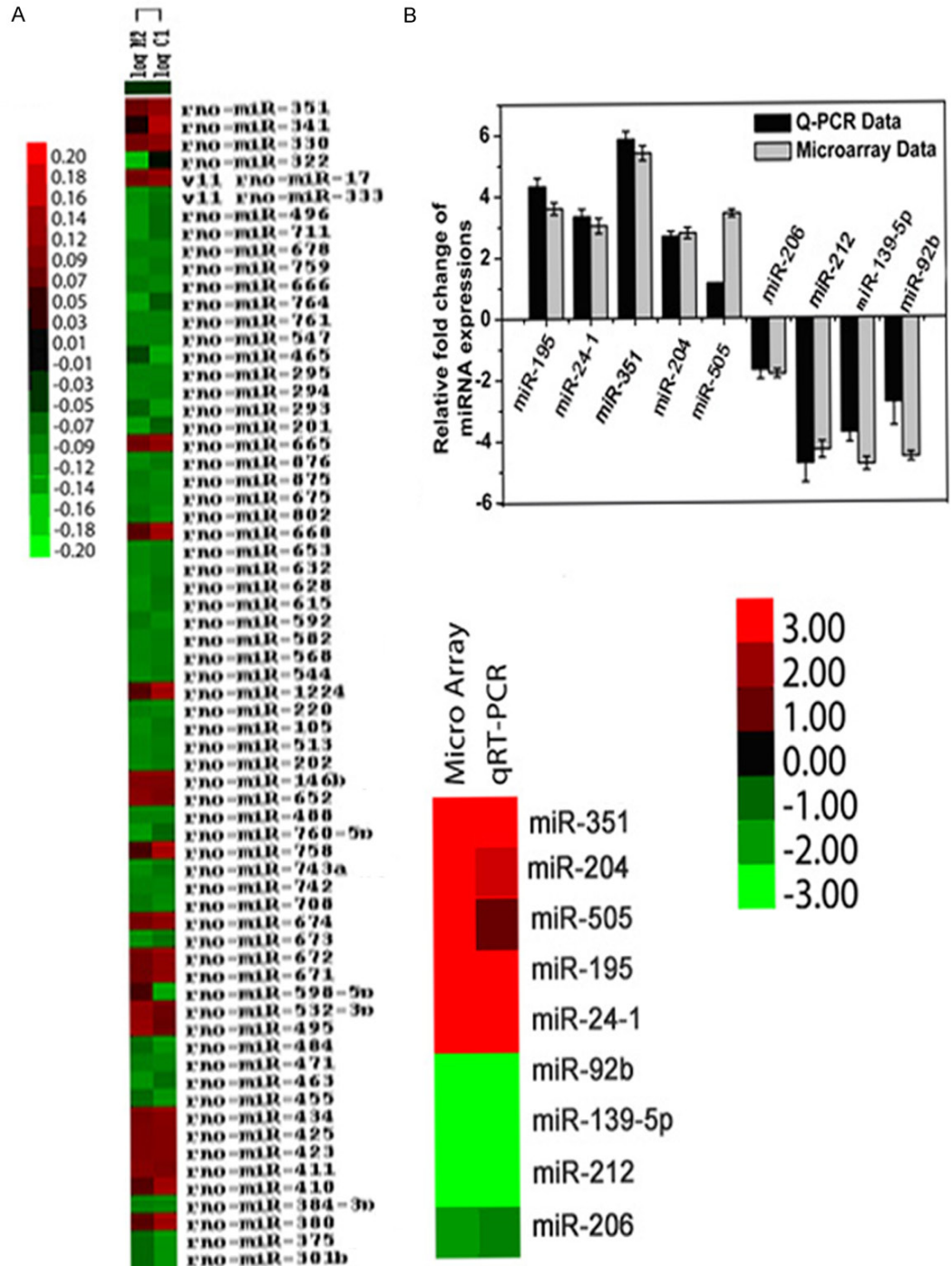

Figure 1. Cluster analysis of the aberrant microRNA (miRNA) expression in focal cortical dysplasia induced by liquid nitrogen in the immature rat in the microarray scan and qRT-PCR validation. A. Dendrogram generated by cluster analysis showed the separation of model from control samples on the basis of miRNA profiling. B. The relative fold changes of miRNAs from qRT-PCR data compared with microarray data.

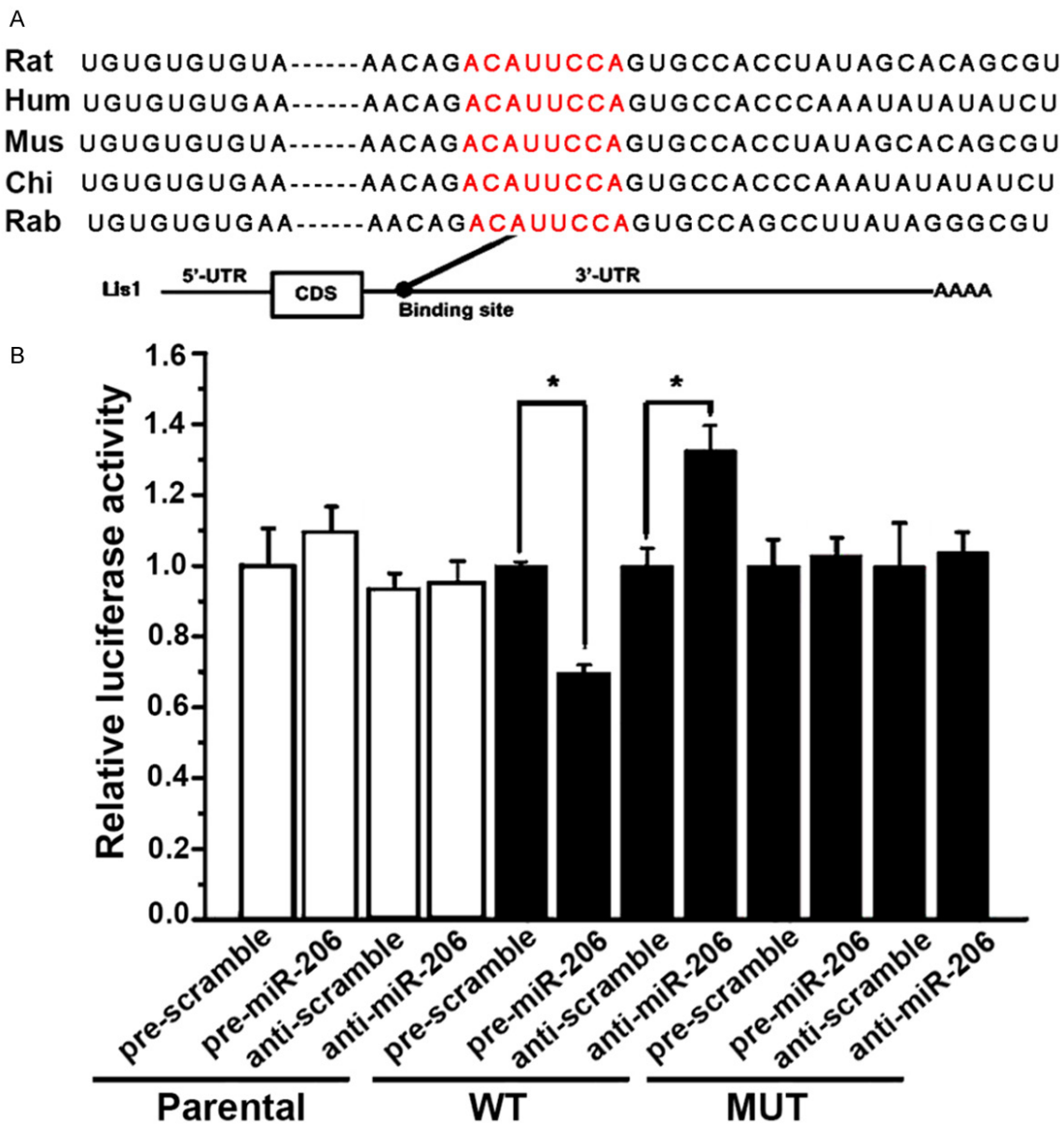

Figure 2. Predicted targets for miR-206. A. Schematic description of conserved binding sites for miR-206. The seed-recognizing site was demarked and all nucleotides in this region were completely conserved in several species. Hypothesized duplexes formed by interacting between the binding sites of Lis-1 3'UTR (top) and miR-206 (bottom) was illustrated, the predicted free energy of each hybrid was indicated. B. Analysis of luciferase activity in the PC12 cells transfected with pre-miR-206 or anti-miR-206. PC12 cells were co-transfected with a pre-miR-206 or anti-miR-206, luciferase reporter plasmid containing the wild-type (WT) or mutant (MUT) 3'UTR of Lis1 mRNA and β-gal as the control plasmid. Data are presented as the means ± SD (n=6). *, P<0.05.

informatic analysis was used to predict the target site of miR-206 in Lis-1 3' untranslated region (UTR) (**Figure 2A**). It could be seen from **Figure 2A** that mature miR-206 shared a same sequence in rat, mouse and human. There was one 7 bp target site in Lis-1 3'UTR which is conservative in mammals. In this paper, we focused on Lis-1 as it is a known gene related to cell proliferation and migration. Lis-1 contributed to brain development according to our previous study [19]. **Figure 2B** showed the wild-type or mutant Lis-1 3'UTR constructed in the luciferase reporter plasmid to perform the luciferase targeting assay. One contained the 7 bp target site for miR-206 and another contains the mutant 7 bp site. As shown in **Figure 2B**, the lucif-

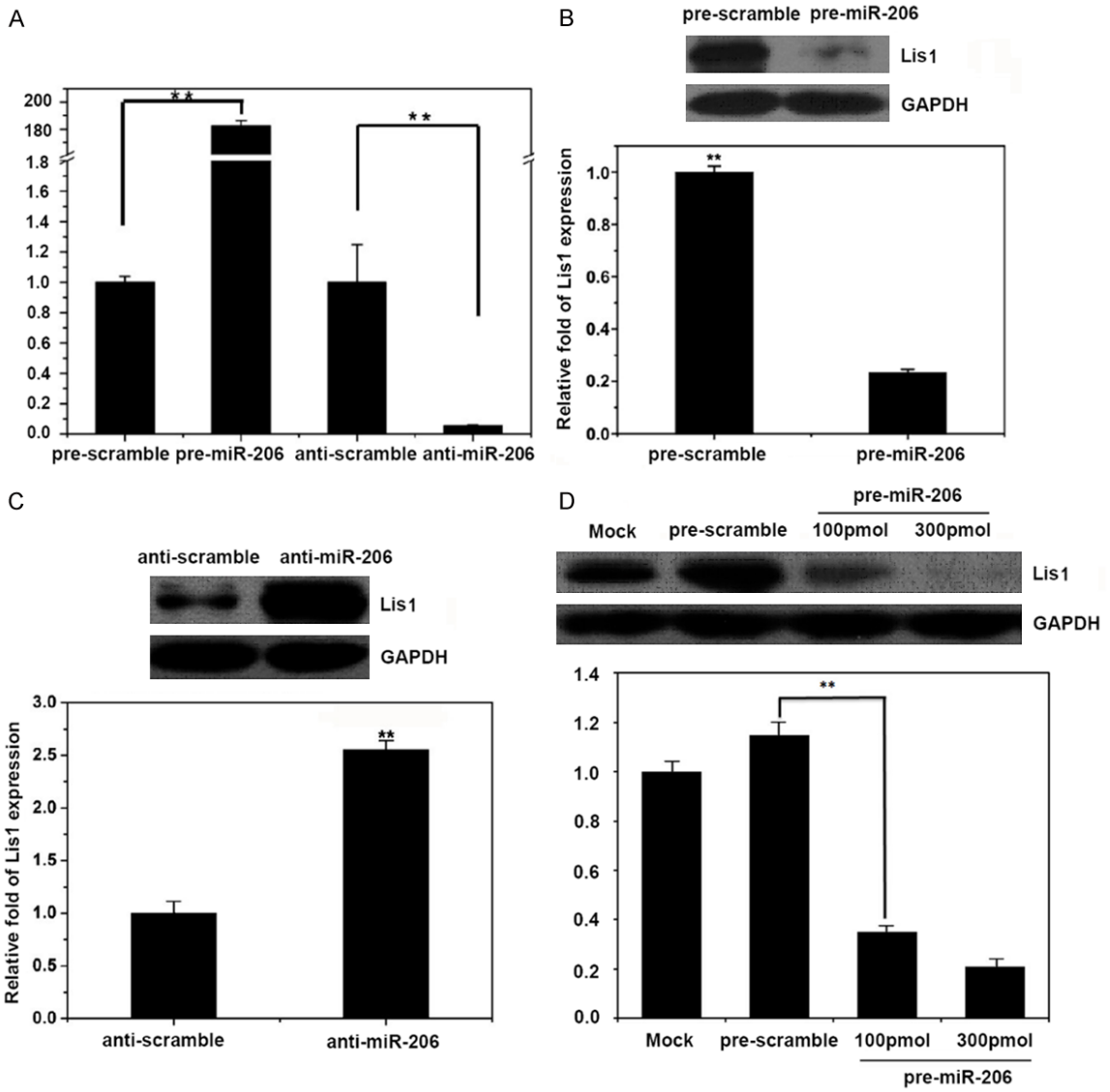

Figure 3. Protein levels of Lis-1 measured by Western Blotting after over expression or knock down of miR-206 in PC12 cell line. A. The relative miR-206 level after the transfection of pre-miR-206 or anti-miR-206. B. The relative Lis-1 protein expression after the transfection of pre-miR-206. C. The relative Lis-1 protein expression after the transfection of anti-miR-206. D. The relative Lis-1 protein expression after the transfection of pre-miR-206 at different doses. Data are presented as the means ± SD (n=6). **, P<0.01; *, P<0.05.

erase activity of the wild-type plasmid was suppressed by about 42% in PC12 cells transfected with miR-206 mimics after 24 h, while the activity of the mutant plasmid was not repressed. This result confirmed that Lis-1 mRNA was directly targeted by miR-206.

The relation between the expression of miR-206 and Lis-1 in PC12 cell line

According to the result of the expression of miR-206, we confirmed that miR-206 was transfected into PC12 cells (**Figure 3A**). Our data showed an inhibitory effect of miR-206 on the expression of Lis-1 in PC12 cells transfected by pre-miR-206 after 24 h (**Figure 3B**). Whereas, the results showed the promoting effect of miR-206 on the expression of Lis-1 in PC12 cells transfected with anti-miR-206 after 24 h (**Figure 3C**). The results indicated that the transfection of miR-206 inhibited the expression of Lis-1 protein by about 60% and 90% by using 100 pmol and 300 pmol pre-miR-206 (**Figure 3D**), respectively. These results at least confirmed that miR-206 can be involved in the

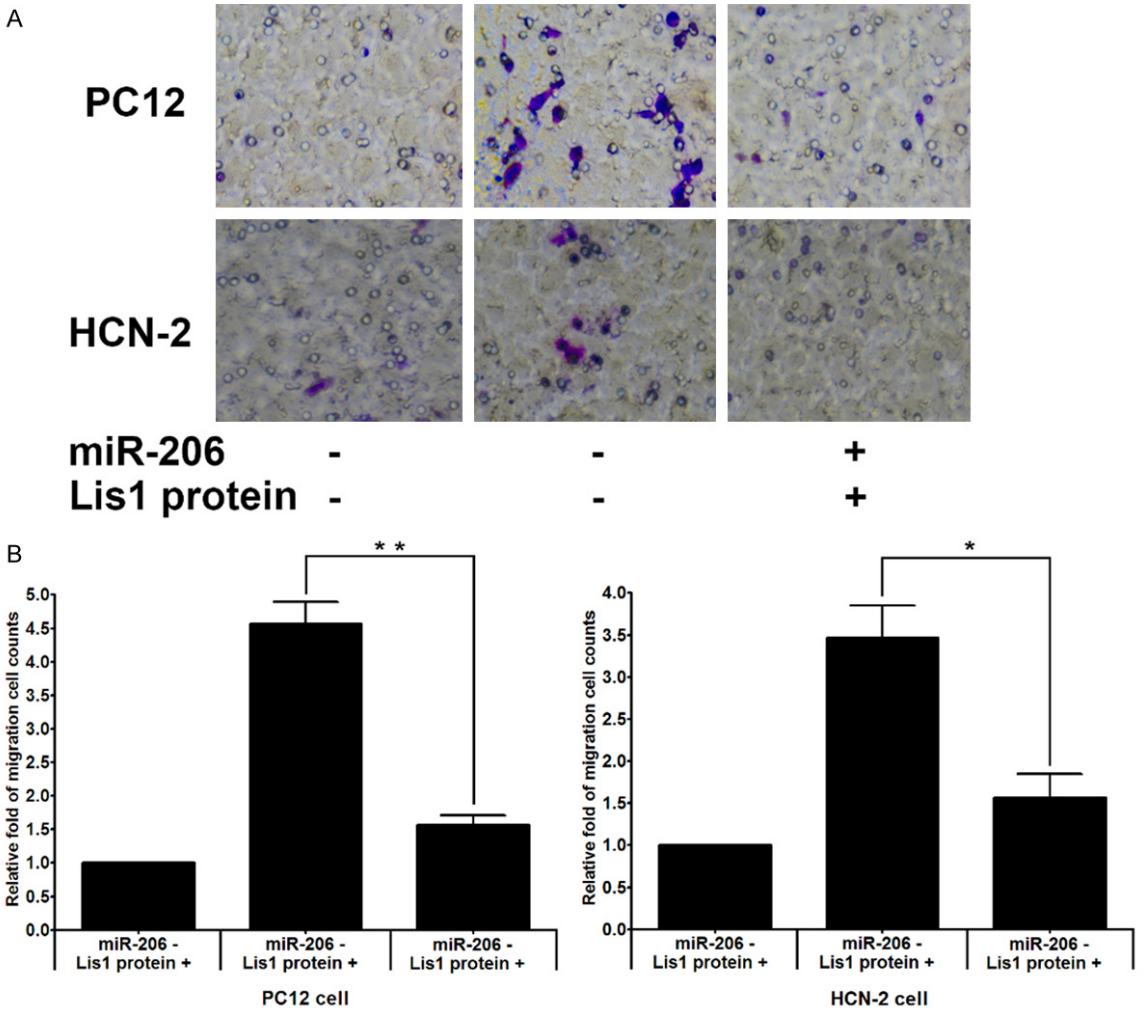

Figure 4. Cell migration experiments in PC12 and HCN-2 cells. A. Cell migration identified by Transwell experiments in PC12 and HCN-2 cells treated with or without Lis1 protein. Originalmagnification, ×200. B. Relative fold of cell count of cell migration experiments in PC12 and HCN-2 cells. Data are presented as the means ± SD (n=3). **, P<0.01; *, P<0.05.

brain development by affecting the expression of Lis-1.

miR-206 inhibits cell migration

Transwell assays revealed that miR-206 significantly inhibited the migration of PC12 and HCN-2 cells treated with Lis1 protein (**Figure 4**). The migration ability of the PC12 and HCN-2 cells was enhanced only upon the addition of Lis1 protein; this enhanced migration was attenuated by transfection with miR-206.

Discussion

The rat brain experiences a series of important events during its postnatal development. It is reasonable to consider that the development of rat brain is a highly regulated event and a series of regulatory factors might be involved in the brain development [12]. As known, miRNA is implicated in various developmental, differentiation, cell proliferation, migration and apoptosis pathways of diverse organisms [16]. So we believe that some miRNAs may play important roles in the brain development. In fact, just as expected, some miRNAs were found to be associated with the rat brain development [17]. However, we found that miR-206 might be also related to the rat brain development. It was found that the expression of miR-206 decreased from day 20 to 80 in focal cortical dysplasia induced by liquid nitrogen in the immature rat. Due to the significant change of miR-206 expression during the rat brain devel-

opment, we are keen on wondering whether miR-206 is involved in the development of rat brain by modulating the expression of the certain targets. Several genes are predicted as miR-206 putative targets by multiple algorithms (TargetScan, miRanda and Pictar) [9]. These genes include Lis-1, Capn8, Gmfb, Mapk1, Dclk1, Vim, Mgst1, Gnb1, Klf15, and Cdkn1b. Some previous studies have reported their roles in organisms [18].

In our previous study, through the analysis of proteomics of freeze-lesion model of focal cortical dysplasia, we found the Up-regulated expression of Lis-1 gene (data not show) [19]. As it is known, Lis-1 is a key gene related to cell proliferation and migration, which contribute to brain development [3]. We are interested in this gene because our previous study focused on Lis-1 [19].

Notably, we can see from **Figure 4** that the expression of Lis-1 rose dramatically from day 20 and maintained a high level between day 20 to 80. It makes sense that such a high expression of Lis-1 between day 20 and 80 is very important since Lis-1's roles in promoting cell proliferation and migration are urgently needed in this period during the rat brain development. Cell migrate assays showed that miR-206 can significantly inhibit PC12 and HCN-2 cell migration treated with or without Lis-1 protein. Since we validated that miR-206 targeted the mRNA of Lis-1 and inhibited its expression, it leads to a conclusion that the upregulation of Lis-1 is at least partially caused by the reduction of miR-206 in the rat brain. As the upregulation of Lis-1 can advance the occurrence of some developmental events in the rat brain, miR-206 can be involved in the rat brain development through its effect on Lis-1 expression. Of course, miR-206 may also contribute to the rat brain development by affecting the expression of several other putative targets. The further study on the validation for the other predicted targets and their involvement in the brain development will be undertaken.

In conclusion, we examined the expressions profiles of miRNAs in the immature rat with focal cortical dysplasia induced by liquid nitrogen and identified miRNAs that might modulate cortical neuronal migration. We identified and characterized a miRNA, miR-206, and found that the reduction of miR-206 at least in part caused the upregulation of Lis-1 in the rat brain. The above results demonstrated that miR-206 may have a role in the brain development by affecting the expression of Lis-1. Based on the results of the present study, functional validation should be performed in the future by confirming the differentially expressed miRNAs.

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Disclosure of conflict of interest

None.

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