Embryonic ectoderm development protein is regulated by microRNAs in human neural tube defects

Pei-Pei Song, MS; Yi Hu, PhD; Chun-Mei Liu, MD; Mu-Ju Yan, MS; Ge Song, MS; Yi Cui, PhD; Hong-Fei Xia, PhD; Xu Ma, PhD

OBJECTIVE: The objective of the study was to investigate the expression and regulation of polycomb group (PcG) proteins in human neural tube defects (NTDs).

STUDY DESIGN: PcG proteins in human NTD fetuses and age-matched controls were detected by Western blot. The relation between PcG proteins and microribonucleic acids was predicted and confirmed by the bioinformatics method, real-time polymerase chain reaction (PCR), dual-luciferase activity assay, and Western blot. The trimethyl condition of histone H3 Lys27 (H3K27) was detected by immunohistochemical and immunofluorescence.

RESULTS: Embryonic ectoderm development protein (EED) was differentially detected in placenta, cerebral cortex, and spinal cord from NTDs and age-matched controls. MiR-30b can interact with 3′-untranslated region (UTR) of Eed and regulate endogenous EED expression in neural tissues. In addition, we found an inverse relationship between the miR-30b expression and the amount of trimethyl H3K27.

CONCLUSION: Differential expression of EED exists in the nerves system in human NTDs and that is regulated by miR-30b.

Key words: embryonic ectoderm development, microribonucleic acid, neural tube defects, polycomb proteins


Neural tube defects (NTDs) are congenital malformations of the brain and spinal cord caused by neural tube close failure. NTDs occur in approximately 1 of every 1000 pregnancies in the United States and in an estimated 300,000 fetuses worldwide. In the northern provinces of China, the incidence rate of NTDs is among the highest in the world, at about 6 per 1,000 births in rural areas. The etiology of NTDs is complicated, with both environmental and genetic contribution. Polycomb group (PcG) proteins are transcription regulatory proteins that control the expression of various genes from early embryogenesis to fetus. PcG proteins formed at least 2 classes of complexes named polycomb repressive complexes 1 (PRC1) and 2 (PRC2). PRC1 contains polycomb ring finger oncogene (BMI1), ring finger protein1, etc. PRC2 contains embryonic ectoderm development protein (EED), enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 homolog (SUZ12), etc.

Among many transcription factor genes that give rise to NTDs, PcG proteins have been found to be essential for neural tube closure. Mutations in genes that encoding most PRC1 proteins cause transformation of the axial skeleton as well as producing various neurological abnormalities by regulating Hox gene expression. Mutations in PRC2 coding genes, each of which eliminates detectable trimethyl histone H3 Lys27 (H3K27me3), cause early embryonic lethality soon after gastrulation. Although it is well known that PcG proteins participate in neurological abnormalities by regulating the downstream pathways, how the PcG proteins are regulated in occurrence of NTDs remains to be determined.

Microribonucleic acids (miRNAs), 21-24 nucleotide duplex RNAs, attenuate gene expression by pairing to 3′-untranslated region (UTR) of target transcripts inducing ribonucleic acid (RNA) cleavage or translational inhibition. The miRNAs can influence neural progenitor’s proliferation by mediating repression of target genes. The discovery that noncoding RNAs repress the transcription of many genes has raised the intriguing possibility that tissue-specific expression of PcG proteins could be regulated by noncoding RNAs. Up to now, the available knowledge in the literature about whether miRNAs anticipate in the regulatory of the PcG proteins is mainly from the report by Juan et al that miR-214 negatively feeds back on PcG by targeting the Ezh2 3′-UTR.

To explore the expression and regulation of PcG protein in abnormal develop-
opment of the neural tube, we detected the expression pattern of PcG from NTDs and age-matched controls and analyzed the relation of miRNAs and PcG proteins. In this study, we found that a significant change occurred in EED expression in human NTDs. The differential expression of EED was primarily regulated by miR-30b and then changed the H3K27me3 level in nervous tissues.

Materials and Methods

Sample collections

A population-based embryo development defects survey was performed in the Family Planning Technique Service Station in Qian’xi, Hebei Province, from December 2006 to December 2008.

Fifty mothers containing harbored NTDs fetuses and age-matched controls were surveyed by a questionnaire that contained the general condition (Table). The serum folic acid level in these pregnant women was analyzed by electrochemiluminescence (Supplemental Table). There was no significant difference between the NTD group and the normal group. Twenty-five fetuses with NTDs (anencephaly, n = 11; spina bifida, n = 14) were taken as subjects and age-matched normal fetuses as controls.

The control groups were obtained from allowable therapeutic abortions. In the control group, therapeutic abortions were performed when there was a serious threat to the mother’s health or for a lethal threat, for example, the accident or serious pregnancy reaction to pregnant mother. In the case group, induced abortions were performed when the fetus was found to have spina bifida or anencephaly by B-mode ultrasound. The placenta, cerebral cortex, and spinal cord were excised and frozen in liquid nitrogen for RNA and protein analysis.

This study was approved by the Ethics Committee of the National Research Institute for Family Planning. The collection of fetal tissues followed the procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration.

RNA and protein extraction

To detect the expression of PcG proteins and miRNAs in the placenta, spinal cord, and cerebral cortex, we extracted protein and total RNA from these tissues. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s standard protocol and total protein using Cellytic mammalian tissue lysis/extraction reagent (Sigma, St Louis, MO).

Western blot analysis

Western blot analysis was used to detect the expression of PcG proteins. Seventy-five microgram proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene fluoride membranes (Amersham, St Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-EZH2, BMI1, EED, or SUZ12 polyclonal antibody or mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The specific protein-antibody complexes were detected by using horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Detection by the chemiluminescence reaction was carried using the enhanced luminol-based chemiluminescent kit (Millipore, Billerica, MA). The β-actin signal was used as a loading control. The experiment has been repeated at least 3 times. The bands were analyzed using Quantity One analyzing system (Bio-Rad Laboratories, Hercules, CA).

Cell cultures and transfection

Cell cultures and transfection were used to detect the relations of EED and miRNAs and the effect of miRNAs on endogenous EED expression and H3K27me3 level. U343 cells were cultured in Dulbecco’s modified Eagle’s medium/nutrient F-12 Ham’s (DMEM/F-12) culture medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 10 mg/ml streptomycin.

The miRNA mimics, miRNA inhibitor, pre-miR control, or anti-miR control purchased from GenePharma Co (Shanghai, China) was transfected into the U343 cells by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Forty-eight hours later, the cells were collected to extract protein and RNA.

Real-time reverse transcript polymerase chain reaction (RT-PCR)

Real time RT-PCR was used to detect the expression of EED. Total RNA (2 μg) was used as template for reverse transcription using Superscript III (Invitro-

---

**TABLE**

Characteristics of test case and control group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>Age, y</td>
<td>28.43 ± 6.77</td>
<td>29.41 ± 5.23</td>
<td>.50</td>
</tr>
<tr>
<td>Husband’s age, y</td>
<td>30.50 ± 6.64</td>
<td>31.48 ± 5.18</td>
<td>.52</td>
</tr>
<tr>
<td>Nationality (Han)</td>
<td>24</td>
<td>24</td>
<td>1.00</td>
</tr>
<tr>
<td>Menarche age, y</td>
<td>14.31 ± 1.03</td>
<td>14.53 ± 1.04</td>
<td>.48</td>
</tr>
<tr>
<td>Blood type (A/B/AB/O)</td>
<td>8/7/1/9</td>
<td>6/7/3/9</td>
<td>.76</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>61.92 ± 8.75</td>
<td>65.50 ± 10.15</td>
<td>.13</td>
</tr>
<tr>
<td>Gestational week</td>
<td>23.17 ± 8.28</td>
<td>25.29 ± 7.56</td>
<td>.43</td>
</tr>
<tr>
<td>The number of abortions</td>
<td>1.27 ± 0.65</td>
<td>1.25 ± 0.46</td>
<td>.88</td>
</tr>
<tr>
<td>The number of spontaneous abortions</td>
<td>0.21 ± 0.52</td>
<td>0.032 ± 0.18</td>
<td>.093</td>
</tr>
<tr>
<td>Fetal sex (female/male)</td>
<td>19/6</td>
<td>20/5</td>
<td>.88</td>
</tr>
<tr>
<td>Member with regulatory menstrual cycle</td>
<td>21</td>
<td>24</td>
<td>.22</td>
</tr>
</tbody>
</table>

Most of the data are means ± SD. Measuring data were analyzed by using Student t test and counting material data by χ² examination. P < .05 was considered statistically significant.

gen). The mRNA levels of Eed and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were measured by real-time PCR using a FastStart universal SYBR Green master (Roche, Mannheim, Germany) and ABI prism7000 sequence detection system (Applied Biosystems, Foster City, CA). The sequences of primers were as follows: For Eed, forward, 5'-AGAAAGCTGACGTGACGAGAACA-3’ and reverse 5'-AGGTGC-ATTGGCGTGTTTGATAGG-3’; and for Gapdh, forward 5'-TGATGTGTGTCACAACCAGGGAT-3’ and reverse 5’-GGTGAGAGGGAAGAGCTGAAGT-3’. Each sample in each group was measured in triplicate and the experiment was repeated at least 3 times. The quantification was normalized to an endogenous control Gapdh.

**TaqMan miRNA assay**

TaqMan miRNA assay was used to detect the expression of miRNAs that regulated EED. Single-stranded cDNA was synthesized by using TaqMan miRNA reverse transcription kit (Applied Biosystems) and then amplified by using TaqMan universal PCR master mix (Applied Biosystems) together with miRNA-specific TaqMan MGB probes: miR-30b, miR-30c, or miR-181b (Applied Biosystems). Each sample in each group was measured in triplicate and the experiment was repeated at least 3 times. The U6 small nuclear RNA was used for normalization.

**Luciferase (LUC) activity assay**

Luciferase activity assay was used to detect the interaction between 3’-UTR of Eed and miRNAs. A 166 nt long region of the 3’-UTR of Eed was amplified from human genomic DNA and cloned into the downstream of the stop site of luciferase coding gene in pGL3. Eed 3’-UTR, deleted miR-30b, miR-30c, and miR-181b target sites, was used as control vector (Supplemental Figure S1). For the luciferase assay, U343 cells were seeded into 96 well plates. The cells were cotransfected with Eed 3’-UTR inserted vector or control vector, renilla luciferase expression vector, and synthesized miRNA mimics, inhibitors, and controls. Two days later, cells were harvested and assayed using the dual-luciferase assay kit (Promega, Madison, WI). Each treatment was performed triplicate in 3 independent experiments. The results were expressed as relative luciferase activity (firefly LUC/Renilla LUC).

**Immunohistochemistry**

Immunohistochemistry was used to analyze the H3K27me3 level in tissues. Paraffin-embedded microarray slides (5 μm) including placenta, cerebral cortex, and spinal cord were deparaffinized and rehydrated and then incubated with anti-H3K27 polyclonal antibody (Millipore) and corresponding fluorescein isothiocyanate-conjugated secondary antibody (Jackson Immunoresearch Laboratories). Positive cells were counted in different optical fields (magnification ×400) selected in a random manner and counted at least 500 cells for each sample.
random manner and counted at least 500 cells for each sample.

Statistical analysis
Western blot results of NTDs and normal controls were estimated using the rank sum test to analyze the association between the PcG expression and the NTDs’ occurrence. \( P < .05 \) was considered significant.

The results of Western blot, real-time PCR, immunohistochemistry, and immunofluorescence were analyzed by 1 way analysis of variance. All values are reported as the mean \( \pm \) SE. When significant effects of treatment were indicated, Duncan’s multiple-range test was used for group comparisons. All statistical analyses were performed using SPSS version 14.0 (SPSS Inc, Chicago, IL). A value of \( P < .05 \) was considered statistically significant.

Results
The expression pattern of PcG proteins in placenta, cerebral cortex, and spinal cord
The expression pattern of different PcG proteins in the different tissues from NTDs and normal controls was diversity (Figure 1). In the placenta, the expression level of EZH2 and BMI1 was not recognizably different between NTDs and the control group. SUZ12 protein expression was more in NTDs than that in the controls (\( P < .05 \)). The protein level of the EED was higher in NTDs than in normal fetuses (\( P < .05 \)). These results showed that EED was detected differentially in all tissues, particularly in nervous tissues.

Prediction of miRNAs that regulate EED expression
To explore whether miRNAs anticipate in the regulation of EED expression, an online search of miRNA by miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), and TargetScan databases (http://www.targetscan.org/mta.shtml) were used to analyze the miRNAs that regulated EED expression and found that EED expression may be modulated by \( \text{miR-30b}, \text{miR-30c}, \text{and miR-181b} \) (Figure 2, Aa). As shown in Figure 2, Aa, both \( \text{miR-30b} \) and \( \text{miR-30c} \) bind to the same 2 miRNA responsive elements (MREs), and \( \text{miR-181b} \) binds to a single MRE in the 3’-UTR of Eed.

The binding sites that are predicted to anneal with Eed mRNA residues are highly conserved among different species. Thermodynamic analysis by RNAhybrid indicates that binding ability is strong between the sequence complementarities of \( \text{miR-30b}, \text{miR-30c}, \text{or miR-181b} \) with Eed 3’-UTR (Figure 2, Ab-d).
sion of miR-30b was markedly up-regulated \((P < .05)\), and that of miR-30c or miR-181b was not significantly different in cerebral cortex in the NTD group compared with control the group (Figure 2, Bb).

The miR-30b and miR-181b expression was remarkably down-regulated \((P < .05)\), and miR-30c had no obvious change in spinal cord in the NTD group compared with the control group (Figure 2, Bc). The expression trend of miR-30b or miR-181b in nervous tissues and the expression trend of miR-30c in the placenta were reversed with that of EED protein, suggesting that miR-30b, miR-30c, or miR-181b might regulate EED expression.

Confirmation of the relation of EED and miR-30b, miR-30c, or miR-181b

To further identify the relationship between the predicted miRNAs and Eed, we set up a luciferase reporter assay. The miRNA expression after transfection of miR mimics and inhibitor was detected by TaqMan miRNA assay and dual-luciferase activity assay (Figure 3, B, D, and F and Supplemental Figure S2). The luciferase activity was significantly decreased by the miR-30b or miR-30c mimics compared with the pre-miR control. There was no visible change between miR-181b mimics and pre-miR control. Furthermore, the luciferase activity was visibly up-regulated by the miR-30c inhibitor compared with the anti-miR control. There was no visible difference between the miR-181b inhibitor and anti-miR control.

Base deletion was also performed to further confirm the binding site for miRNAs. The histogram in Figure 3, C, E, and G, shows that enzyme activity was separately reduced 66.2%, 53.5%, or 6.9% in those cotransfected with miR-30b mimics, miR-30c mimics, or miR-181b mimics and EED 3’-UTR sense-inserted vector compared with Eed 3’-UTR control vector.

The effect of miR-30b, miR-30c, and miR-181b on endogenous EED expression

Although miR-30b and miR-30c were identified to bind to Eed 3’-UTR, it was unknown whether these miRNAs could regulate endogenous EED expression. U343 cells were transfected with miR-30b and miR-30c mimics to detect whether these miRNAs could affect endogenous EED expression (Figure 4). Compared with precontrol, the EED protein level was significantly down-regulated by miR-30b or miR-30c mimics \((P < .05)\), and the level of Eed mRNA was not significantly changed. Compared
with anti-miR control, EED protein level in U343 cells was remarkably up-regulated by the miR-30b or miR-30c inhibitor, and the level of Eed mRNA was also not significantly different. The EED protein and mRNA level in U343 cells were not obviously affected by the miR-181b mimics or inhibitor. These results further confirm that EED expression was regulated by miR-30b and miR-30c.

**H3K27me3 detection**

Because EED can bind to repressive methyl-lysine marks and then stimulate PRC2 methyltransferase activity to maintain the H3K27me3 station, H3K27me3 was detected in placenta, cerebral cortex, and spinal cord in human NTDs (Figure 5, A). The H3K27me3 level was significantly increased in spinal cord ($P < .05$) and decreased in the cerebral cortex ($P < .05$). The expression trend of H3K27me3 was opposite with that of miR-30b/miR-30c and the same with that of EED. To further confirm the result, the effect of miR-30b/miR-30c on H3K27me3 level was detected in U343 cells (Figure 5, B). The H3K27me3 level was decreased with the transfection of the miR-30b/miR30c mimics ($P < .05$) and increased with the transfection of the miR-30b/miR30c inhibitor ($P < .05$).

**Comment**

In this study, we collected the samples of NTDs and controls from Qianxi, Hebei Province, in which the incidence rate of NTDs was higher than the annual prevalence rate of NTDs in China. Qianxi is a rural area in north China. Many reports showed that the occurrence of NTDs was higher in the rural than in the urban areas and higher in the north than south, which was related with low socioeconomic status, atrocious weather, and environmental pollution. In addition, although the serum folic acid level was not significantly different between the NTD group and the age-matched controls, it was remarkably different between NTDs in high-incidence areas and that in low-incidence areas. We therefore speculated that there are other factors to be involved in the occurrence of NTDs in high-incidence areas of NTDs.

The PcG family had been found to be essential for the development of the nervous system. To investigate the relation of the PcG family and neural tube closure, we detected the distribution of 4 core polycomb family members (EZH2, EED, SUZ12, and BMI1) in the placenta and neural tissues from normal fetuses and fetuses with NTDs. We found that EED was differentially detected in placenta and nervous tissues between the NTD and control groups. The EZH2 in the placenta, SUZ12 and BMI1 in the spinal cord were weakly expressed. These results were similar with the report by Gunster et al that PcG protein expression was variable, depending on the tissue. We also found that only EED expressed differentially in all tissues in human NTDs.

EED expression was significantly increased in the placenta and spinal cord and decreased in the cerebral cortex. There were reports to show that the carboxy-terminal domain of EED specifically binds to histone tails carrying trimethyl-lysine residues associated with repressive chromatin marks and that this leads to the allosteric activation of methyltransferase activity of PRC2 and inhibit gene expression. Mutations in the EED that prevent it from recognizing repressive trimethyl-lysine marks
reduce global methylation and disrupt development.26

The placenta is a temporary organ formed during the pregnancy, which is essential for the normal development of the embryo. The increase of EED in the placenta may repress the expression of many genes that participate in placenta development and then result in the dysfunction of placenta and affect embryo development. EED knockdown prolongs the neurogenic phase of neural precursor cells (NPCs) and delayed the onset of astrogenic phase by repressing the promoter of the proneural gene neurogenin1.27

The decrease of EED in cerebral cortex may limit the neurogenic competence of NPCs and then lead to the abnormal development of the embryo brain. The augmentation of EED in the spinal cord may promote the overdifferentiation of NPCs and then cause the asynchrony between the spinal cord development and its outer organization and may contribute the occurrence of spina bifida.

All these findings support the content that EED may play an important role in the process of neural tube closure. Because the etiology of NTDs is complex,28 it may hardly determine that these findings are secondary to the NTD or the cause of the NTD.

The expression of protein can be affected by several factors, and we tried to explore whether there were relationships between miRNAs and the EED in the process of NTD occurrence because our previous studies showed that miRNAs played an important role in development of the nervous system.29,30 In this study, dual-luciferase activity assay showed that miR-30b and miR-30c can be able to bind to the Eed 3′-UTR. Western blot analysis showed that EED protein level in U343 cells was remarkably down-regulated by miR-30b or miR-30c mimics and up-regulated by the miR-30b or miR-30c inhibitor, and the level of Eed mRNA was not significantly changed.

These results confirmed that EED was the target gene of miR-30b and miR-30c, but miR-30b or miR-30c regulated only the EED protein level, not the mRNA level. In addition, we found that the EED protein level was inverted with that of miR-30c in placenta and with that of miR-30c in cerebral cortex.

FIGURE 5
Detection of the expression of H3K27me3

Aa, The H3K27me3 level was detected in spinal cord, Ab, cerebral cortex, and Ac, placenta by immunohistochemistry using anti-H3K27me3 antibody. Black arrows indicate positive immunoreactivity. Ba, The H3K27me3 level was detected in U343 cells transfected by miR-30b mimics, Bb, pre-miR control, Bc, miR-30b inhibitor, and Bd, anti-miR control by immunofluorescence. Green represents H3K27me3 staining, blue indicates nuclear staining, and cyan-blue represents an overlap of green and blue. The histogram represents the mean optical densities (MOD) of positive signals of H3K27me3. These values are expressed as percentage of positive cells (mean ± SD). Asterisk indicates P < .05.

NTD, neural tube defects.

miR-30b in the cerebral cortex and spinal cord in human NTDs.

These results indicated that miR-30b may play an important role in neural tissues and miR-30c may execute functions in placenta. In addition, the seed sequence of miR-30b was the same as that of miR-30c that bound to MREs in the 3′-UTR of the EED. It was presumed that miR-30b and miR-30c may competitively combine with 3′-UTR of Eed in different tissues, and miR-30b preferential bound with 3′-UTR of Eed and interfered miR-30c to bind with 3′-UTR of Eed in neural tissues and vice versa in placenta.

It is thought that the gene-silencing activity of the PRC2 depends on its ability to trimethylate H3K27. The carboxy-terminal domain of EED specifically binds to histone tails carrying trimethyllysine residues and propagates the gene-silencing activity. In this study, we found that the trimethyl state of H3K27 was strong in spinal cord and weak in cerebral cortex, which was parallel with the expression tendency of EED and inverse with miR-30b. The H3K27me3 level was decreased with miR-30b/miR30c overexpression and increased with miR-30b/miR30c low expression in U343 cells. These results imply that the change of H3K27me3 level may be affected by miR-30b/miR30c via regulating EED expression.

In summary, this was the first study to report that the EED was differentially detected in the placenta and nervous tissues from NTDs. Although the EED was the target gene of miR-30b and miR-30c, the expression of the EED was primarily regulated by miR-30b in neural tissues in the abnormal development of neural tube, and miR-30b affected the H3K27me3 level via regulating EED expression in the occurrence of NTDs.

REFERENCES


SUPPLEMENTAL TABLE
Maternal serum folate levels between normal fetal and NTDs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal</th>
<th>NTDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample number</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Mean</td>
<td>7.1184</td>
<td>7.6752</td>
</tr>
<tr>
<td>SE</td>
<td>0.56282196</td>
<td>0.622613877</td>
</tr>
<tr>
<td>SD</td>
<td>2.814109806</td>
<td>3.113069386</td>
</tr>
<tr>
<td>Median</td>
<td>6.76</td>
<td>7.34</td>
</tr>
<tr>
<td>Maximum</td>
<td>2.14</td>
<td>2.16</td>
</tr>
<tr>
<td>Minimum</td>
<td>12.72</td>
<td>14.6</td>
</tr>
</tbody>
</table>

Units are nanograms per milliliter. Reference range is female, 3.5–9.0 (P = 0.4464). NTD, neural tube defects.


SUPPLEMENTAL FIGURE S1
Prediction of miRNAs that regulate EED expression

A. Predicted binding region of Eed 3'-UTR by using target scan. B. Letters in red are the nucleotides deleted.

EED, embryonic ectoderm development protein; miRNA, microribonucleic acids; UTR, untranslated region.


SUPPLEMENTAL FIGURE S2
TaqMan miRNA real-time RT-PCR was used to detect these miR expression after transfection

TaqMan miRNA real-time RT-PCR was used to detect these miR expressions of miR-30b, miR-30c, and miR-181b after transfected with miR-30b, miR-30c, or miR-181b mimics or inhibitor. The expression of miR-30b, miR-30c, and miR-181b expression was normalized to U6 small nuclear RNA. Asterisk indicates P < .05.

miRNA, microribonucleic acids; RT-PCR, reverse transcript polymerase chain reaction.