MiR-206 Regulates Neural Cells Proliferation and Apoptosis via Otx2

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Abstract
MiR-206 was involved in a series of cellular activities, such as the growth and development of skeletal muscle and the tumorigenesis. MiR-206 was characterized previously as a differentially expressed gene in sodium arsenite (SA)-induced neural tube defects (NTDs) in chick embryos via miRNA microarray analysis. However, the role of miR-206 in the pathological process of nerve cells remained elusive. In this study we found differential expression of miR-206 in SA-treated chick embryos by Northern blot analysis. Ectopic expression of miR-206 inhibited cell proliferation, and promoted cell apoptosis in U343 and SK-N-SH cell by using MTT, Edu Apollo assay and Flow cytometry analysis. Further investigation revealed that miR-206 can interact with 3'-untranslated region (UTR) of Otx2. MiR-206 mimics down-regulated the endogeneous Otx2 expression, whereas the miR-206 inhibitor obviously up-regulated the expression of Otx2. These findings indicate that overexpression of miR-206 promotes cell apoptosis and low expression of miR-206 inhibits cell apoptosis. Otx2 may play an important role in the process of miR-206-mediated cell apoptosis.

Introduction
MicroRNAs (miRNAs) are endogenous 19-25 nucleotide non-coding single-stranded RNAs that regulate gene expression by blocking translation or decreasing mRNAs stability [1]. Recently, it’s estimated that miRNAs account for approximately 1% of the human genome [1]. It has been shown that miRNAs play a critical role in several biological process, such as development, proliferation, differentiation, apoptosis and et al [2]. MiR-206 is one of the miRNAs family members. Our previous study found that miR-206 was up-regulated in sodium arsenite (SA)-induced neural tube defects (NTDs) in chick embryos [3]. Several studies indicated that miR-206 played an important role in the growth and development of skeletal muscle. Dey et al. reported that miR-206 induced the differentiation of myoblast and modulated the proliferation and differentiation of skeletal muscle.
muscle satellite cells by down regulation of pax7 [4]. However, it remains unclear whether miR-206 are associated with abnormal development of nerve cells.

In this study, we firstly explored the possible function of miR-206 in the pathological process of nerve cells, and found that overexpression of miR-206 significantly inhibited the proliferation and promoted apoptosis at the early stages in glioma cell U343 and neuroblastoma SK-N-SH. Down-regulation of miR-206 notably enhanced the proliferation capacity and suppressed cell apoptosis. Furthermore, we identified that Otx2 was one of the miR-206 target genes. The expression level of miR-206 was inversely correlated with the regulation of Otx2. Meanwhile, we also found that Otx2 was down-regulated in chick embryos with NTDs induced by SA. Altogether, miR-206 may modulate the proliferation and apoptosis of neural cells through regulating the expression of Otx2.

Materials and Methods

Plasmid Construction, cell culture and transfection

The Otx2 3'-UTR or Otx2 3'-UTR-mutant sequences were amplified by PCR from human genomic DNA using the primers in Table 1. After being double digested with Spe I and Pst I, the PCR product was cloned into the downstream of the stop codon of luciferase gene in pGL3 control vector (Invitrogen, Carlsbad, CA, USA) and the constructs were verified by sequencing with the ABI 3730 DNA Analyzer plates (Applied Biosystems, Foster City, CA, USA). The recombinant pGL3 was designated as Otx2-pGL3. The recombinant pGL3 mutation was designated as Otx2-pGL3-mutant.

Human glioma cell U343 and neuroblastoma cell SK-N-SH were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and cultured in DMEM (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum, 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO2. For transient transfections, the cells were incubated for further 4 h after transfection, and the absorbance was recorded at A570nm with a 96-well plate reader (Model 3550 microplate reader, Bio-Rad, Hercules, CA, USA). All experiments were repeated at least three times.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis of miRNA and mRNA

TaqMan microRNA assay was used to detect the expression of miR-206. Single-stranded cDNA was synthesized by using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster city, CA, USA) together with miRNA-specific TaqMan MGB probes: miR-206 or U6 (Applied Biosystems, Foster city, CA, USA). Each sample in each group was measured in triplicate and the experiment was repeated at least three times. The U6 snoRNA was used for normalization.

Flow cytometry analysis

Cells apoptosis was performed with flow cytometry analysis by Annexin V-FLUOS staining kit (Roche, Mannheim, Germany). The results were described as a ratio of transfected mimics, pre-miR control, miR-206 inhibitor and anti-miR control, respectively. There were 3 wells in each treatment group. Twenty microlitres MTT (5 mg/ml)(Sigma, St. Louis, MO, USA) were added to each well 48 h after transfection, and the absorbance was recorded at A570nm with a 96-well plate reader (Model 3550 microplate reader, Bio-Rad, Hercules, CA, USA). The experiment has been repeated for three times and the results were described as a ratio of transfected miR-206 mimics, or miR-206 inhibitor vs corresponding control.

Cell proliferation was also estimated using Cell-Light Edu Apollo DNA in vitro Kit (Guangzhou RiboBio Co., Ltd, Guangzhou, China). Proliferative cells were visualized and imaged using a Laser Scanning Confocal Microscopy (Carl ZEISS LSM 510 META, Jena, Germany). Proliferative cells were counted in different optical fields (magnification x100) selected in a random manner and analyzed by the software of AxioVision Rel. 4.8 (Carl Zeiss, Jena, Germany).

Northern blot analysis

Total RNA was isolated from cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 40 µg of total RNA per sample was subjected to electrophoresis on a 15% Urea-PAGE gel and transferred to a nylon membrane (Hybond N’, Amersham Pharmacia Biotech, St Albans, Herts, UK). After being UV-cross-linked and baked at 50°C for 30 min, the membrane was prehybridized at 42°C for 4 h and then hybridized with 32P-labeled miR-206 or U6 probe at 40°C overnight. Membranes were washed and exposed to PhosphorImager screens (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The bands were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). All experiments were repeated at least three times.
Germany), containing 2.5 µl annexin V-fluorescein isothiocyanate (FITC) stock and 5 µl 20 µg/ml PI to determine the phosphatidylserine (PS) exposure on the outer plasma membrane. After incubation for 15 min at room temperature in a light-protected area, the specimens were quantified by flow cytometry (BD Biosciences, San Jose, CA, USA), acquiring 8,000 events. Each treatment was repeated twice within an experiment. The experiment has been repeated for three times.

**Dual-Luciferase Activity Assay**

Luciferase activity assay was used to detect the interaction between 3'-UTR of Otx2 and miR-206. A 200-nt-long region of the 3'-UTR of Otx2 was amplified from human genomic DNA and cloned into the downstream of the stop site of luciferase coding gene in pGL3 (Otx2-pGL3). Deleting miR-206 target sites in Otx2 3'-UTR was used as control vector (Otx2-pGL3-mutant). Cells transfected with Otx2-pGL3 alone were also used as control and designated as untransfected cells. For the luciferase assay, U343 cells were seeded into 96-well plates. The cells were cotransfected with Otx2-pGL3, pRL-TK and miR-206 mimics, pre-miR control, miR-206 inhibitor or anti-miR control, respectively. In addition, the cells were also cotransfected with pGL3, Otx2-pGL3 or Otx2-pGL3-mutant, pRL-TK and miR-206 mimics. Two days later, cells were harvested and measured using the Dual-Luciferase Assay kit (Promega, Madison, WI, USA). pRL-TK containing renilla luciferase was co-transfected with firefly luciferase vector for data normalization. Each treatment was performed triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

**Embryo treatment and whole-mount immunofluorescence**

White Leghorn chick eggs (Bovan strain) were purchased from Merial Vital Laboratory Animal Technology Co., Ltd (Beijing, China). To begin embryonic development, chick eggs were placed in automatic tilting racks in an incubator (Grumpatch, Savannah, GA, USA) and incubated at 38°C and 60% humidity. Chick embryos were treated with SA (100 nM, Sigma, St. Louis, MO, USA) at Hamburger-Hamilton (HH) stages 6, 8 and 12 [5]. SA were directly injected into the center of the egg yolk via a...
small hole at the blunt end of the egg using an established protocol [6]. Embryos were harvested for analysis after incubation for 72 h (Hlstage 20).

Embryos were fixed with 4% paraformaldehyde in 0.1 mol/L PBS and stored at 4°C overnight, then immersed the embryos using 100% methanol and stored at -20°C until use. After washing in 100% methanol, embryos were rehydrated sequentially using 100%–25% methanol in 0.1 mol/L PBS and then incubated with rabbit anti-OTX2 polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA). After rinsing in PBS, the embryos were exposed to fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). At least three embryos were analyzed in each treatment group and the whole mount images were visualized under Zeiss lumar V12 fluorescence stereomicroscope under same parameters (Carl Zeiss, Jena, Germany). Fluorescence intensity was analyzed using Axiovision Rel.4.8 software (Carl Zeiss, Jena, Germany). We had the negative control which using FITC-conjugated IgG in the absence of primary antibodies in whole-mount immunofluorescence experiments. Quantification of fluorescence in each treatment group is performed after subtracting the background from the negative control values.

Statistical analysis
All statistical analyses were performed using the SPSS 16.0 statistical software package. Data are presented as mean±SEM from at least three independent experiments. Multiple group comparisons were performed using one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p<0.05$.

Results and Discussion

Differential expression of miR-206 in SA-treated chick embryos

MiR-206 was characterized previously as a differentially expressed gene in SA-induced NTDs in chick embryos via miRNA microarray analysis. In order to further approve the result, Northern blot was used to detect the expression of miR-206. We found that miR-206 expression was significantly increased in SA-treated chick embryos compared with controls ($P<0.05$; Fig. 1). The result suggests that miR-206 may play an important role in the development of nervous system.

Up-regulation of miR-206 inhibits the cell viability in U343 and SK-N-SH

To explore the possible function of miR-206 in the pathological process of nerve cells, the effect of miR-206 expression on the growth of nervous cells was detected using an in vitro cell lines model. Firstly, cells were respectively transfected with miR-206 mimics, pre-

miR control, miR-206 inhibitor or anti-miR control. qRT-PCR results showed that the amount of miR-206 was significantly enhanced in cells transfected with miR-206 mimics compared with pre-miR control, while miR-206 expression was obviously decreased in cells transfected with miR-206 inhibitor compared with anti-miR control (data not shown).

Cell proliferation and viability was measured by MTT assay. As shown in Fig. 2A, the relative proliferation rates in U343 and SK-N-SH cells transfected with miR-206 mimics was about 88.26% and 86.51%, respectively, as compared with the corresponding cells transfected with pre-miR control. The relative proliferation rates in U343 and SK-N-SH cells transfected with miR-206 inhibitor were increased about 14.95% and 18.39%, respectively, compared with the corresponding cells transfected with anti-miR control. These results showed that overexpression of miR-206 in human glioma cells markedly suppressed the cell viability, and conversely, knockdown of miR-206 clearly promoted the cell viability.
In order to further confirm the role of miR-206 in cell viability, the proliferation capacity of U343 cells was determined by Cell-Light Edu Apollo DNA in vitro kit (Fig. 2B). The results showed that the cell viability in U343 cells transfected with miR-206 mimics was decreased approximately 31.93% compared with pre-miR control. While the viability of U343 cells was increased about 28.83% after transfection with miR-206 inhibitor compared with the control.

All of these results indicated that overexpression of miR-206 markedly inhibited the neural cell viability and low expression of miR-206 visibly increased neural cell proliferation. Our findings are consistent with a recent observation that miR-206 is associated with cellular proliferative inhibition [7], and implying that abnormal expression of miR-206 may be involved in the growth of human glioma cells.

Overexpression of miR-206 promotes cell apoptosis
To further explore the role of miR-206 in controlling the growth of nervous cells, the apoptosis in U343 and SK-N-SH cells were determined by flow cytometry analysis.

As shown in Fig. 3, the early apoptotic cells (annexin V-FITC positive) in U343 cells transfected with pre-miR control or miR-206 mimics were about 10.2% or 14.2%, respectively. While the late apoptotic cells (annexin V-FITC/PI positive) in U343 were about 9.53% or 12.4%, respectively. The early apoptotic cells (annexin V-FITC positive) in SK-N-SH transfected with pre-miR control or miR-206 mimics were about 14.0% or 24.3%, respectively. While the late apoptotic cells (annexin V-FITC/PI positive) in SK-N-SH cells were about 6.59% or 7.17%, respectively. These results showed that
overexpression of miR-206 visibly promoted cell apoptosis in human glioma cells, especially during the early stage of apoptosis.

The early apoptotic cells (annexin V-FITC positive) in U343 cells transfected with anti-miR control or miR-206 inhibitor were about 16.3% or 7.29%, respectively. While the late apoptotic cells (annexin V-FITC/PI positive) in U343 were about 10.1% or 9.82%, respectively. The early apoptotic cells (annexin V-FITC positive) in SK-N-SH cells transfected with anti-miR control or miR-206 inhibitor were about 18.4% or 9.23%, respectively. While the late apoptotic cells (annexin V-FITC/PI positive) in SK-N-SH cells were about 9.14% or 6.68%, respectively. These results showed that knockdown of miR-206 observably inhibited cell apoptosis in human glioma cells, especially during the early stage of apoptosis.

In previous study, miR-206 levels were found to increase during late stages of human foetal muscle development [8], and up-regulation of miR-206 could enhance early cell apoptosis. Therefore, we speculated that miR-206 might inhibit the differentiation of muscle cells by inducing cell apoptosis, and then regulate muscle formation. Similarly, we speculate that overexpression of miR-206 may inhibit the differentiation of nerve cells by...
promoting cell apoptosis, and then lead to the abnormal development of nerve cells.

**Otx2 is a direct target of miR-206**

To figure out the molecular mechanisms in which the miR-125b are involved we looked for its target gene. MiR-206 targets were predicted using target prediction programs, TargetScan, PicTar and miRanda algorithms, and found that Otx2 was a potential target of miR-206. Otx2 is the earliest homeobox-containing gene to be expressed in the neuroectoderm, which has also been isolated from other species, including sea urchin [9], Xenopus [10], zebrafish [11], chick [12] and et al. Otx2 is expressed in the entire ectoderm and visceral endoderm before gastrulation at E5.5-6.0 in mouse [13, 14]. Otx2 was required for proper segregation of early regional identities anterior and posterior to the mid-hindbrain boundary (MHB) and for conferring competence to the anterior neuroectoderm in responding to forebrain-midbrain- and rostral hindbrain-inducing activities [15-17]. In the absence of Otx2, expression of important regulatory genes, such as Hesx1/Rpx, Six3, Pax2, Wnt1, failed to be initiated or maintained in the neural plate [15]. Accordingly, Otx2 expression is a caudal limit marker of the midbrain/hindbrain boundary separating the mesencephalic and isthmo/cerebellar regions, indicating that Otx2 may play an important role in neurodevelopment during embryogenesis.

To validate whether Otx2 is indeed the target gene of miR-206, a human Otx2 3'-UTR fragment containing...
a complementary site for the seed region of miR-206 was cloned into the downstream of the stop codon of firefly luciferase gene (Fig. 4A). MiR-206 mimics or inhibitor were co-transfected with Otx2-pGL3. A luciferase reporter assay system was used to analyze the binding capacity between miR-206 and 3′-UTR of Otx2. Compared with pre-miR control, the luciferase activity was mildly suppressed by the miR-206 mimics, about 9% (Fig. 4B1). There was no significant difference in luciferase activity between the untransfected cell and
pre-miR control. The luciferase activity was visibly up-regulated by the miR-206 inhibitor compared with anti-miR control, about 14% (Fig. 4B2). There was no significant in luciferase activity difference between the untransfected cell and anti-miR control. These results indicated that miR-206 affected the binding capacity between pre-miR 3'-UTR of Otx2.

Base mutation was performed to further confirm the binding site for miR-206 in 3'-UTR of Otx2 (Fig. 4C). U343 cells were co-transfected with miR-206 mimics, pRL-TK and Otx2-pGL3, Otx2-pGL3-mutant or pGL3. No significant difference in luciferase activity was observed between Otx2-pGL3-mutant and pGL3. Compared with Otx2-pGL3-mutant, the enzyme activity was reduced about 92% in U343 cells transfected with Otx2-pGL3 (p<0.01). These data indicated that miR-206 may suppress gene expression through binding with seed sequence at the 3'-UTR of Otx2, and Otx2 may be a direct target of miR-206.

MiR-206 regulates endogenous Otx2 expression

Although Otx2 was identified as a target gene for miR-206, it was unknown whether miR-206 could regulate endogenous Otx2 expression. U343 cells were transfected with miR-206 mimics and inhibitor to analyze whether the change in the expression of miR-206 affected endogenous Otx2 level (Fig. 5). Compared with pre-miR control, the level of OTX2 protein detected by western blot was significantly down-regulated by miR-206 mimics (Fig. 5A). The level of Otx2 mRNA detected by qRT-PCR was also significantly decreased by miR-206 mimics (Fig. 5B). Additionally, OTX2 protein level in U343 cells was up-regulated by miR-206 inhibitor compared with anti-miR control (Fig. 5A). The level of Otx2 mRNA was also significantly increased by miR-206 inhibitor (Fig. 5B). These results showed that the expression of endogenous Otx2 mRNA and protein were regulated by miR-206.

In this study, we found that there was a massive difference in fluorescence between mutant and wild-type constructs, while the difference between miR-206 mimics and pre-miR control is minimal (9%). This discrepancy may be caused by the following reasons: firstly, the experimental purposes of both are different. Base mutation was performed to analyze the binding site for miR-206. When the binding site of miR-206 in the 3'-UTR of Otx2 was deleted, the luciferase activity was significantly higher than that in 3'-UTR of Otx2 containing the binding site of miR-206 under the condition of overexpression miR-206. These results confirm that the mutated bases in the 3'-UTR of Otx2 are indeed the bind site of miR-206. However, overexpression or knockdown of miR-206 was performed to analyze the binding ability of miR-206 with 3'-UTR of Otx2 without considering the effects of other miRNAs and 3'-UTR of mRNA. According to our experimental results, we can say that miR-206 and 3'-UTR of Otx2 have mildly binding capacity under complex intracellular environment. Secondly, the research objects of both are different. Base mutation was performed to analyze the luciferase activity in cells transfected by 3'-UTR of Otx2 with or without the binding site of miR-206 in the case of the same miRNAs expression. However, overexpression or knockdown of miR-206 was performed to analyze the effect of miR-206 expression changes on luciferase activity in the case of the same Otx2-pGL3, which endogenous miR-206 could interact with Otx2-pGL3 to partially counteract the effect of overexpression of miR-206 on the luciferase activity.

The studies from Torero Ibad et al. showed that Otx2 could promote the survival of damaged adult retinal ganglion cells (RGCs) [18]. Knockdown of Otx2 expression by siRNA inhibited medulloblastoma cell growth in vitro [19]. Otx2 mutant in neurectoderm cells of forebrain could result in apoptosis [20]. Therefore, miR-206 might affect cell viability and apoptosis, mainly through regulating the expression of Otx2 in the abnormal development of nerve cells.

Confirmation of target gene of miR-206 in vivo

To further confirm the target gene of miR-206 in vivo, the whole-mount immunofluorescence assay was performed to detect the expressive change of OTX2 in SA-induced NTDs in chick embryos. As shown in Fig. 6A, compared with the control, the fluorescence signals of OTX2 was remarkably decreased after treatment with SA, especially in the forebrain and midbrain (P<0.05). Meanwhile, in order to further validate the result, western blot analysis was used to detect the effect of SA on the protein level of OTX2. As shown in Fig. 6B, we found that OTX2 was down-regulated in chick embryos treated by SA, compared with the control (P<0.01). The expression pattern was inversed with miR-206, indicating that miR-206 might regulate OTX2 expression in vivo. In conclusion, it’s the first time to elucidate the possible role of miR-206 in abnormal development of nerve cells. All these facts indicated that miR-206 induced abnormal development of neural cells by inhibiting cells viability and promoting cell apoptosis at early stage, at least in part by blocking Otx2 mRNA transcript and translation. We
believe that this study will lead to a better understanding of the important role of miR-206 in the progress of neurodevelopment and the molecular mechanisms involved in miR-206 mediated abnormal development of nervous cells.

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