MiR-206 Regulates Neural Cells Proliferation and Apoptosis via *Otx2*

Rui Wang^{1,2}, Yi Hu^{1,2}, Ge Song^{1,2}, Chan Juan Hao^{1,2}, Yi Cui^{1,2}, Hong-Fei Xia^{1,2} and Xu Ma^{1,2}

¹Reproductive and Genetic Center of National Research Institute for Family Planning, Beijing, ²Graduate School, Peking Union Medical College, Beijing

Key Words

miR-206 • Human Glioma Cells • Apoptosis • *Otx2*

and low expression of *miR-206* inhibits cell apoptosis. *Otx2* may play an important role in the process of *miR-206*-mediated cell apoptosis.

Copyright © 2012 S. Karger AG, Basel

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

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 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 and *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 luciferases gene in pGL3 control vector (Invitrogen, Carlsbad, CA, USA) and the constructs were verified by sequencing with the ABI 3730 DNA Analyzer platforms (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 *miR-206* mimics, pre-miR control, *miR-206* inhibitor or anti-miR control was transfected into cells by the lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction. The *miR-206* mimics, pre-miR control, *miR-206* inhibitor and anti-miR control were synthesized by GenePharma (GenePharma Co., Ltd, Shanghai, China).

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.

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 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.

Real time RT-PCR was used to detect the expression of *Otx2*. Total RNA (2 µg) was used as template for reverse transcription using the Iscript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The mRNA level of *Otx2* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were measured by real-time PCR using a FastStart DNA Master SYBR Green kit (Roche, Mannheim, Germany) and StepOne™ Real-Time PCR System (Applied Biosystems, Foster city, CA, USA) with specific primers for *Otx2* and *Gapdh* (Table 1). Each sample in each group was measured in triplicate and the experiment was repeated at least three times. The quantification was normalized to an endogenous control *Gapdh*.

Cell proliferation assay

Cell proliferation was estimated by the MTT assay. U343 and SK-N-SH were seeded in 96-well plates at low density (5×10³) in DMEM culture, and allowed to attach overnight. The cells were then transfected with *miR-206* 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 cells were incubated for further 4 h. The absorbance was recorded at A_{570nm} 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).

Flow cytometry analysis

Cells apoptosis was performed with flow cytometry analysis by Annexin V-FLUOS staining Kit (Roche, Mannheim,

Gene name	Primer sequence	Accession number	Size and location	Application
<i>Otx2</i> 3'-UTR (sense)	Forward/ <i>SpeI</i> : 5'-GGACTAGTACCTGTAGAACCCTCTT -TTTGTGGGT-3' Reverse/ <i>Pst</i> I: 5'-AACTGCAGGCAGATTGGTTTGTCC -ATTTCAT-3'	NM_021728	200bp (1171-1370)	PCR
<i>Otx2</i> 3'-UTR--mutant (deleting putative binding region)	The first part of sequence/ <i>SpeI</i> : 5'-GGACTAGTACCTGTAGAACCCTCTTT -TTGTGGGTGATTTTAAA-3' (This sequence was synthesized directly) The second part of sequence: Forward: 5'-GTTTTAGCCAGGCATTGGTT-3' Reverse/ <i>Pst</i> I: 5'-AACTGCAGGCAGATTGGTTTGTCC -ATTTCAT-3'	NM_021728	1021bp (1171-1206 plus 1227-1370)	PCR
<i>Otx2</i>	Forward: 5'-CAGCAACAACAGCAGAATGGAGGT-3' Reverse: 5'-TGGCCACTTGTTCCTCTCTGAA-3'	NM_021728	103bp (585-687)	qRT-PCR
<i>Gapdh</i>	Reverse: 5'-GTAGAGGCAGGGATGATGTTC-3' Forward: 5'-TGGTATCGTGAAGGACTCA-3'	NM_002046	129bp (606-734)	qRT-PCR

Table 1. Primer sequences

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).

Western Blot

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 40 µg samples were loaded into each lane of 8% polyacrylamide gels. The proteins were separated by electrophoresis, and transferred to PVDF membranes (Amersham Pharmacia Biotech, St Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-OTX2 polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. The specific protein-antibody complex was detected by using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The GAPDH signal was used as a loading control. The experiment has been repeated for three times. The bands were analyzed using Quantity one analyzing system (Bio-Rad, Hercules, CA, USA).

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 (Hhstage 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-

Fig. 1. Northern blot confirmation of differential expression of *miR-206* in SA-treated chick embryos. Chick embryos were treated with SA (100 nm) or saline as negative control for 72 h. The expression of *miR-206* in SA-treated chick embryos was detected by northern blot. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as *miR-206* intensity/*U6* intensity to normalize for gel loading and transfer. * indicates significant differences ($p < 0.05$).

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.

Fig. 2. The effects of *miR-206* on the proliferation of U343 and SK-N-SH cells. (A) U343 and SK-N-SH cells were transfected with the *miR-206* mimics, pre-miR control, *miR-206* inhibitor, or anti-miR control, respectively. After 48 h, cell proliferation was determined by MTT assay. All experiments were performed in triplicate and expressed as mean stimulation index (SI). SI was expressed as the ratio of the proliferation of stimulated cells (*miR-206* mimics or inhibitor) vs that of non-stimulated cells (pre-miR control or anti-miR control). (B) U343 cells were transfected with the *miR-206* mimics, pre-miR control, *miR-206* inhibitor, or anti-miR control, respectively. Two days later, U343 were treated with Cell-Light Edu and Hoechst stained nuclei. Red represents the proliferative cells. Blue indicates cell nuclei. The photographs were shown at x100 original magnification (left). Scale bar=100 μ m. The black histogram represents the ratio of percentage of cell viability and was expressed as ratio of Edu-positive cells vs total cells. * indicates significant differences ($p < 0.05$).

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

Fig. 3. Apoptosis in U343 and SK-N-SH were detected by flow cytometry. Cells were respectively transfected with the *miR-206* mimics, pre-miR control, *miR-206* inhibitor, or anti-miR control for 48 h. Then the single cell suspension was prepared and stained with annexin V/ phosphatidylinositol (PI) and subjected to flow cytometry analysis. Lower left quadrant, viable cells (annexin V-FITC and PI negative); lower right quadrant, early apoptotic cells (annexin V-FITC positive and PI negative); upper right quadrant, late apoptosis/necrosis cells (annexin V-FITC and PI positive). The percentage of annexin V-FITC and PI-positive cells was the average value of three repeated experiments.

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

Fig. 4. The prediction and confirmation of the *miR-206* target. (A) Schematic diagram for constructing the *miR-206* binding site into pGL3 control vector. (B) Confirmation of the target gene of *miR-206*. The 3' UTR segment of human *Otx2* predicted to interact with *miR-206* was amplified by PCR from human cDNA and inserted into the downstream of the luciferase reporter gene in the pGL3 control vector (*Otx2*-pGL3) for Dual-Luciferase assay. U343 cells were co-transfected with *Otx2*-pGL3, pRL-TK and pre-*miR* control, *miR-206* mimics (B1), anti-*miR* control or *miR-206* inhibitor (B2). Cells transfected with *Otx2*-pGL3 and pRL-TK were also used as control. Two days later, cell lysate was collected to perform Dual-Luciferase activities assay. (C) Mutation analysis of the *miR-206* binding site. U343 cells were co-transfected with *miR-206* mimics, pRL-TK and *Otx2*-pGL3, *Otx2*-pGL3-mutant or pGL3. When the binding site of *miR-206* in the 3'-UTR of *Otx2* was deleted, the luciferase activity was significantly increased in U343 cells. pRL-TK containing renilla luciferase was co-transfected with firefly luciferase vector for data normalization. * $p < 0.05$, or ** $p < 0.01$.

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

Fig. 5. *MiR-206* regulates *Otx2* expression. (A) OTX2 protein expression in *miR-206* mimics and inhibitor-treated U343 cells were detected by western blot. Total protein was separated by SDS-PAGE, then transferred to PVDF membranes. The membranes were probed with OTX2 or GAPDH antibody, then incubated with anti-rabbit IgG-HRP, and developed using the chemiluminescence (ECL) reagent. The bands were analysed using the Quantity One analyzing system (Bio-Rad Laboratory inc.). The expression of GAPDH served as an internal control. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as OTX2 intensity/GAPDH intensity to normalize for gel loading and transfer. (B) The expression of *Otx2* mRNA in *miR-206* mimics and inhibitor-treated U343 cells were detected by qRT-PCR. The expression of GAPDH served as an internal control. The products of qRT-PCR were subjected to electrophoresis on a 2% agarose gel. * indicates significant differences ($p < 0.05$).

Fig. 6. OTX2 is down-regulated in chick embryos with NTDs induced by SA. Chick embryos were treated with SA (100 nm) or saline as negative control for 72 h. (A) The expression of OTX2 was analyzed by whole-mount immunofluorescence. Fluorescence intensity was visualized with the Axiovision Rel. 4.8 software. Red arrow indicated positive signals. The histogram represents the mean optical densities (MOD) of positively signals of OTX2 in chick embryos that were determined by NIS-Elements BR Image processing and analysis system (NIKON, Japan). Scale bar=1000 μ m. (B) The expression of OTX2 was analyzed by western blot. The expression of GAPDH served as an internal control. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as OTX2 intensity/GAPDH intensity to normalized for gel loading and transfer. * $p < 0.05$, ** $p < 0.01$.

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 difference in luciferase activity between the untransfected cell and anti-miR control. These results indicated that *miR-206* affected the binding capacity between *miR-206* and 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 neuroectoderm 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.

Acknowledgements

This work was supported by the National Basic Research Program of China (No. 2007CB511905) and the Natural Science Foundation of China (No. 30800396).

References

- Bartel DP: MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215-233.
- Zhao Y, Srivastava D: A developmental view of microRNA function. *Trends Biochem Sci* 2007;32:189-197.
- Cui Y, Han Z, Yi H, Song G, Hao C, Xia H, Ma X: MicroRNA-181b and microRNA-9 mediate arsenic-induced angiogenesis via NRP1. *J Cell Physiol* 2012;227:772-783.
- Dey BK, Gagan J, Dutta A: miR-206 and -486 induce myoblast differentiation by downregulating Pax7. *Mol Cell Biol* 2011;31:203-214.
- Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. *Dev Dyn* 1992;195:231-272.
- Rosenquist TH, Ratashak SA, Selhub J: Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. *Proc Nat Acad Sci USA* 1996;93:15227-15232.
- Chen X, Yan Q, Li S, Zhou L, Yang H, Yang Y, Liu X, Wan X: Expression of the tumor suppressor miR-206 is associated with cellular proliferative inhibition and impairs invasion in ERalpha-positive endometrioid adenocarcinoma. *Cancer Lett* 2012;314:41-53.
- Koutsoulidou A, Mastroiannopoulos NP, Furling D, Uney JB, Phylactou LA: Expression of miR-1, miR-133a, miR-133b and miR-206 increases during development of human skeletal muscle. *BMC Dev Biol* 2011;11:34.
- Gan L, Mao CA, Wikramanayake A, Angerer LM, Angerer RC, Klein WH: An orthodenticle-related protein from *Strongylocentrotus purpuratus*. *Dev Biol* 1995;167:517-528.
- Pannese M, Polo C, Andreazzoli M, Vignali R, Kablar B, Barsacchi G, Boncinelli E: The *Xenopus* homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* 1995;121:707-720.
- Mercier P, Simeone A, Cotelli F, Boncinelli E: Expression pattern of two otx genes suggests a role in specifying anterior body structures in zebrafish. *Int J Dev Biol* 1995;39:559-573.
- Bally-Cuif L, Gulisano M, Broccoli V, Boncinelli E: c-otx2 is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryo. *Mech Dev* 1995;49:49-63.
- Ang SL, Conlon RA, Jin O, Rossant J: Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. *Development* 1994;120:2979-2989.
- Simeone A, Acampora D, Mallamaci A, Stornaiuolo A, D'Apice MR, Nigro V, Boncinelli E: A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J* 1993;12:2735-2747.
- Rhinn M, Dierich A, Shawlot W, Behringer RR, Le Meur M, Ang SL: Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* 1998;125:845-856.
- Li JY, Joyner AL: Otx2 and Gbx2 are required for refinement and not induction of mid-hindbrain gene expression. *Development* 2001;128:4979-4991.
- Martinez-Barbera JP, Signore M, Boyl PP, Puelles E, Acampora D, Gogoi R, Schubert F, Lumsden A, Simeone A: Regionalisation of anterior neuroectoderm and its competence in responding to forebrain and midbrain inducing activities depend on mutual antagonism between OTX2 and GBX2. *Development* 2001;128:4789-4800.
- Torero Ibad R, Rhee J, Mrejen S, Forster V, Picaud S, Prochiantz A, Moya KL: Otx2 promotes the survival of damaged adult retinal ganglion cells and protects against excitotoxic loss of visual acuity in vivo. *J Neurosci* 2011;31:5495-5503.
- Di C, Liao S, Adamson DC, Parrett TJ, Broderick DK, Shi Q, Lengauer C, Cummins JM, Velculescu VE, Fuhs DW, McLendon RE, Bigner DD, Yan H: Identification of OTX2 as a medulloblastoma oncogene whose product can be targeted by all-trans retinoic acid. *Cancer Res* 2005;65:919-924.
- Rhinn M, Dierich A, Le Meur M, Ang S: Cell autonomous and non-cell autonomous functions of Otx2 in patterning the rostral brain. *Development* 1999;126:4295-4304.