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Research paper

# MicroRNA-506-3p regulates neural stem cell proliferation and differentiation through targeting TCF3

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ABSTRACT

Neural stem cells (NSCs) are self-renewing, multipotent and undifferentiated precursors that retain the capacity for differentiation into both glial and neuronal lineages. MicroRNAs (miRNAs) are small noncoding RNAs that play important roles in cell development, differentiation and apoptosis. Recent studies have shown that TCF3 affects neural stem cell proliferation and differentiation. In this study, we predicted that microRNA-506-3p would target TCF3 and demonstrated that miR-506-3p negatively regulates TCF3 expression. The expression level of miR-506-3p was significantly increased during NSC differentiation. In addition, we found that miR-506-3p overexpression increased NSC differentiation and reduced NSC proliferation, indicating an important role of miR-506-3p in NSC. Moreover, the downstream of TCF3, Wnt signaling was significantly decreased with miR-506-3p transfection. These findings suggest that miR-506-3p played an important role in regulating NSC proliferation and differentiation via targeting TCF3, and provide a promising avenue for future in-depth research into the functions of miR-506-3p and TCF3 in nervous system development.



## 1. Introduction

Neural stem cells (NSCs) are a type of undifferentiated, self-renewing precursor cell that retain the ability to differentiate to both glial and neuronal lineages (Liu et al., 2014). NSCs are found in the central nervous system of mammals and can serve as a source of cell replacement therapy for neurological disorders (Aranha et al., 2011). Although increasing evidence has indicated the importance of NSCs in clinical interventions, much more needs to be learned about neural stem cells before their use in clinical applications (Lopez-Ramirez and Nicoli, 2014). Thus, it is urgent to understand the molecular mechanisms of NSC proliferation and differentiation.

MicroRNAs (miRNA) are naturally existing small noncoding RNAs 20–24 nucleotides in length. Distinguished from small interfering RNA (siRNA), miRNAs regulate gene expression in a translational and post-transcriptional manner by binding to specific sites in target mRNA (Kobayashi et al., 2012). It has been reported that miRNAs play important roles in numerous cellular processes, including cell proliferation, differentiation and apoptosis, and regulate gene translation in mammalian neurons (Kim et al., 2004). Dysregulation of miRNA expression has been reported in a number of cancers, including laryngeal cancer, gastric cancer and breast cancer (Dong et al., 2014). Studies have also shown that miRNAs are involved in the regulation of neurological diseases, such as glioma and temporal lobe epilepsy (Aronica et al., 2010). Recent studies have suggested that miRNAs play an important role in stem cell self-renewal and differentiation by controlling the expression of stem cell regulators (Morgado et al., 2015). However, the precise role of miRNAs in regulating NSCs remains poorly understood.

TCF3, also named Tcf7-like 1 or Tcf7l1, is a member of the Lef/Tcf family, which is a group of transcription factors with important roles in development, stem cell homeostasis and malignancy. It has been reported that TCF3 is a vital regulator of embryonic stem cell (ESC) function (Cole et al., 2008). Moreover, TCF3 plays a key role in patterning and cell fate specification during early embryonic development (Yi et al., 2011). Studies have also demonstrated that TCF3 is involved in the maintenance and specification of progenitor cells in the central nervous system (Kim et al., 2011). The canonical Wnt/β-catenin signaling pathway has a variety of roles in neural stem cell regulation throughout adult life, including elevating the differentiation of NSCs, and TCF3 has been suggested to be a negative mediator of Wnt/β-catenin signaling in NSCs (Kuwahara et al., 2014). Therefore, TCF3 is likely to be a key regulator of NSCs. Targeting TCF3 to manipulate NSC may provide new insights into developing NSC-based cell therapies for neurodegenerative disorders.

In the present study, using bioinformatics software programs, we predicted miR-506-3p to be the target miRNA for TCF3, and further investigated the potential role of miR-506-3p in the proliferation and

*Abbreviations:* NSCs, neural stem cells; miRNAs, microRNAs; siRNA, interfering RNA; ESC, embryonic stem cell; HEK, human embryonic kidney; GFAP, glial fibrillary acidic protein; Tuj1, Neuronal Class III β-Tubulin.

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differentiation of neural stem cells. qRT-PCR and western blot analysis showed that the expression of miR-506-3p was significantly increased, whereas TCF3 was decreased during differentiation of NSCs. By performing overexpression and knockdown of miR-506-3p, we found that miR-506-3p plays an important role in the proliferation and differentiation of NSCs. Furthermore, we confirmed the target reaction between miR-506-3p and TCF3, thereby demonstrating that overexpression of miR-506-3p is capable of downregulating TCF3 expression and regulating downstream gene expression. Taken together, our results suggest that miR-506-3p plays a vital role in regulating NSC proliferation and differentiation, at least partly through targeting and regulating TCF3.

## 2. Materials and methods

### 2.1. Cell culture

All neural stem cells used in the present study were isolated from the forebrains of adult male C57BL6 mouse (provided by Laboratory Animal Center of Xi'an Jiaotong University), using the Percoll gradient method as described previously (Palmer et al., 1999). All animal experiments were performed in accordance with the protocol approved by the Animal Care and Use Committee of Xi'an Jiaotong University. The cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) with 1% N2 supplement (Gibco, Rockville, MD, USA), 1 mM L-glutamine (Sigma, St. Louis, MO, USA), 20 ng/ml basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), 20 ng/mL epidermal growth factor (PeproTech), 1% penicillin–streptomycin (Invitrogen) and 50 ng/mL heparin (Sigma). Cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37 °C. The cultures were passaged at 2-week intervals with 1:4 splitting ratio, and we used the third generation of passages cells in the following experiments.

### 2.2. Cell differentiation

The third generation of passages cells was treated with separate differentiation medium the day after passage. For astrocyte differentiation, the cells were cultured in DMEM/F12 medium containing 50 ng/mL basic fibroblast growth factor (bFGF) and 5 mM forskolin (Sigma), 1% N2 supplement and 0.5% fetal bovine serum (FBS; Gibco) for 3 days (Jang et al., 2010). For neural differentiation, cells were treated with 0.5% FBS and 1 mM retinoic acid (Sigma) for 3 days in N2 supplemented medium (Park et al., 2016).

### 2.3. qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For miRNA detection, reverse transcription was performed using the one-step Primescript miRNA cDNA synthesis kit (Takara, Dalian, China). For mRNA detection, cDNA was generated by using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). To analyze gene expression, an RT-qPCR mixture system containing cDNA templates, primers and SYBR Green qPCR Master Mix were subjected to qRT-PCR quantification according to standard methods. Primers for miR-506-3p: forward: 5′-TAA GGC ACC CTT CTG AGT AGA-3′, reverse: 5′-GCG AGC ACA GAA TTA AT ACG AC-3′; U6: forward, 5′-AGA GCC TGT GGT GTC CG-3′, reverse: 5′-CAT CTT CAA AGC ACT TCC CT-3′; TCF3: forward: 5′-CGA GCT GGC CCT CAA CAG CC-3′, Reverse: 5′-CCG GAC CTT CTT GGG CTG CG-3′; β-actin: forward: 5′-CTG AAT CCC AAA GCC AAC AG-3′, reverse: 5′-ATA CCR CAA GAC TCC ATA CC-3′. β-Actin and U6 SnRNA were used as the internal control for mRNA and miRNA, respectively. Relative gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method. All PCR reactions were performed on a StepOne Plus RT-PCR instrument (Applied Biosystems, Shanghai, China).

### 2.4. Western blot analysis

Cells were lysed with lysis buffer and mini protease inhibitor. Then centrifuged at 12,000 rpm for 20 min, the concentrations of supernatant proteins were quantified using a BCA Protein Assay Kit (Thermo scientific, IL, USA). A total of 25 μg proteins extracted from NSCs were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were then blocked in 2.5% nonfat milk for 1 h at 37 °C. After washing with Tris-buffered saline containing Tween, the membranes were incubated with primary antibodies including rabbit anti-TCF3 antibody (1:800), rabbit anti-Wnt3A antibody (1:800), rabbit anti-active β-catenin antibody (1:800), anti-β-actin antibody (1:800) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. Then, a peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, Hubei, China) diluted 1:1000 was added and incubated for 1 h at room temperature. The immunoreactive protein bands were visualized using an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK).

### 2.5. Cell transfection

MiRNA mimics and inhibitors specific for miR-506-3p (Sangon, Shanghai, China) were used to increase or silence the expression of miR-506-3p in NSCs, respectively. NSCs were seeded in 6-well plates at a density of $1 \times 10^5$/well and cultured overnight. Then, cells were transfected with miR-506-3p mimics, miR-506-3p mimic control, miR-506-3p inhibitor, or miR-506-3p inhibitor control for a final concentration of 50 nM using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions for 48 h. The sequence of the miR-506 mimic: 5′-UAA GGC ACC CUU CUG AGU AGA-3′; miR-506 inhibitor: 5′-UCU ACU CAG AAG GGU GCC UUA-3′. The transfection efficiency was assessed by qRT-PCR. For TCF3 overexpression, pcDNA3.1-TCF3 vectors harboring no 3′-UTR were co-transfected with miR-506-3p mimics into NSCs using Lipofectamine2000 and incubated for 48 h.

### 2.6. Cell proliferation assay

Cell proliferation was detected using a commercial BrdU assay (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. NSCs were first seeded in 96-well plates at a density of $5 \times 10^3$ cells/well and transfected with 50 nM of miR-506-3p mimics or inhibitor for 48 h. Then, 10 μL of BrdU solution was added and further incubated for 2 h. Next, the medium was discarded and 100 μL of the Fixing/Denaturing solution was added. After incubation for 15 min at room temperature, the solution was discarded and 100 μL of primary antibody solution was added. After incubation for 1 h at room temperature, cells were washed with wash buffer and incubated with 100 μL of secondary antibody solution for 30 min. After washes with wash buffer, 100 μL of substrate solution was added and allowed for reaction at room temperature for 30 min. Finally, the optical density (OD) value at 450 nM of the reaction solution was read with a micro-plate reader (Bio-Rad).

### 2.7. Dual-luciferase reporter assay

A 600 bp sequence from the 3′-UTR of TCF3 containing a putative miR-506-3p binding site was amplified by PCR using the cDNA of human genomic DNA as the template. The sequence for the mutation within the miR-506-3p binding site was amplified by the point mutation method using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The sequences were then subcloned into the pmirGLO Luciferase reporter vector (Promega, Madison, WI, USA) with *Xba*I and *Not*I, and the recombinant plasmids were assessed by DNA sequencing. For the detection of luciferase activity, 10 ng of the recombinant plasmids were co-transfected with 50 nM of miR-506-3p mimics into human embryonic kidney