*Original Research*

# MiR-21 functions oppositely in proliferation and differentiation of neural stem/precursor cells via regulating AKT and GSK-3β

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**Abstract:** MicroRNA involves in regulating behavior of neural stem/precursor cells (NSPCs), thus it offers the potential to treat central nervous system disease. However, the effect of miR-21 on NSPCs remains unknown. In this study, we demonstrated that miR-21 reduced proliferation and promoted neural differentiation in NSPCs via regulating the activation of AKT and GSK-3β signaling pathways in vitro. During differentiation of NSPCs, the expression of miR-21 was increased in a time-dependent manner by qRT-PCR. Synthesized pre-miR-21 or anti-miR-21 was transfected into NSPCs, thereby efficiently overexpressing or knocking down miR-21. Overexpression of miR-21 promoted the neural differentiation of NSPCs, as indicated by Tuj1 and PSA-NCAM staining. Interestingly, knocking down miR-21 had the opposite effect of neural differentiation in NSPCs. However, in proliferation area, overexpression of miR-21 decreased the cell viability by 3-((2-methyl-1,3-thiazol-4-yl) ethynyl)pyridine hydrochloride (MTT) assay, and inhibited the proliferation of NSPCs, as indicated by 5-Bromo-2-deoxyUridine (BrdU) staining. And likewise, knocking down miR-21 had the opposite effect of cell viability and proliferation. Western blot showed that overexpression of miR-21 enhanced the expression of Cyclin D1, however, knocking down miR-21 prevented its expression. Furthermore, we revealed that protein kinase B (AKT) and glycogen synthase kinase-3 beta (GSK-3β) signaling pathways were involved in the proliferation and neural differentiation of NSPCs. Overexpression of miR-21 activated AKT, and the p-GSK-3β was increased. Conversely, knocking down miR-21 blocked the activation of AKT, and decreased the phosphorylation level of GSK-3β. These results demonstrated that miR-21 promotes neural differentiation and reduces proliferation in NSPCs via regulating AKT and GSK-3β pathways. These findings may help to develop strategies for treatment of central nervous system diseases.

*Key words:* Neural stem/precursor cells, miR-21, proliferation, differentiation.

## Introduction

Neurogenesis in the brain of mammals will last a lifetime, however, the adult mammals neurogenesis is spatially restricted under normal conditions to two specific "neurogenic" brain regions, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus; and the subventricular zone (SVZ) of the lateral ventricles (1). There are many of neural stem/precursor cells (NSPCs) in the two areas above, and NSPCs are characterized by the capacity to continuously self-renew and generate a multitude of neuronal and glial lineages. In the neurogenic niche, these NSPCs can be activated in response to the physiological and pathophysiological stimuli, whereby they might participate in CNS repair and functional recovery (2). Neurogenesis, especially adult neurogenesis, is regulated by physiological and pathological activities at all levels, including the proliferation, differentiation and fate determination of progenitor cells, survival, maturation, and functional integration of newborn cells into the existing neuronal circuitry (3). A number of factors to regulate maintenance, activation, and fate choice of NSPCs, including extracellular signal molecules (like bFGF, EGF and glutamate); intracellular signal molecules (like Gli-1, AKT, notch1) and lots of microRNAs (miRNAs) (4-6).

One recent breakthrough is that miRNAs, a small non-coding RNA molecule (containing about 22 nucleotides), emerge as important regulatory mechanism of cell proliferation and differentiation. Among them, microRNA-21 (miR-21) is particularly important in terms of regulatory cell behaviors. In the process of differentiation of human adipose tissue-derived mesenchymal stem cells (hASCs), miR-21 will be transiently up-regulated and promotes differentiation by binding to target sequences in the untranslated region of TGFBR2 (7). Overexpression of miR-21 contributes to bone formation by promoting BMP9-induced osteogenic differentiation (8). More importantly, one of these neuronal repressor REST (RE1-silencing transcription factor; also called NRSF) regulatory miRNAs, including miR-21, specifically suppresses the self-renewal of mouse ES cells, corresponding to decreased the expression of Oct4, Nanog, Sox2 and c-Myc (9). However, the effect of miR-21 in NSPCs has remained unknown, let alone the mechanisms of miR-21 in regulation NSPCs proliferation and neural differentiation.

In this study, we attempt to disclose the contribution of miR-21 to the proliferation and differentiation in NSPCs and relate cell signaling pathways. Here, we used the sequence of anti-miR-21 and pre-miR-21, to observe the effects of the miR-21 on proliferation and differentiation. Our data demonstrated that miR-21 can promote neural differentiation and inhibit proliferation in NSPCs

via regulating the activation of AKT and GSK-3β.

## Materials and Methods

### Rat NSPCs culture

Pregnant 15.5 d SD rats were purchased from the Experimental Animal Center of Xi'an Jiaotong University Health Science Center (Certificate No. 22-9601018). All experimental protocols were approved by the Animal Care and Use Regulation of Xi'an Jiaotong University Health Science Center. All efforts were made to minimize animals' suffering and to keep the numbers of animals used to a minimum.

Rat NSPCs were prepared from E15.5 Sprague-Dawley rat embryos as previously described and with minor modification (10). Briefly, the cortex was carefully dissected in chilled sterile phosphate buffered saline (PBS) and incubated with TrypLE (Invitrogen) and 200 μM EDTA in PBS at 37°C for 10 min. Then the tissue was mechanically dissociated using a fire-polished Pasteur pipette and filtrated using a 40 μm Cell Strainer (BD Falcon, NJ). After centrifuged at 1000 g for 5 min at 4°C, cells were suspended and trypan blue-excluding cells were counted. Then the cells were seeded at a density of 500,000 cells /ml in non-adherent T75 flasks and incubated at 37°C and 5% $CO_2$ in a humidity incubator (Sanyo, Japan). The serum-free complete medium consisted of DMEM/F12 (1:1), 1% of N2, 2% of B27, 20 ng/ml of epidermal growth factor (EGF) and 10 ng/ml of basic fibroblast growth factor (bFGF). After five to seven days, cells propagated in primary neurospheres (P0 cells) with the diameter of approximately 80-200 μm, were dissociated into single cells. These cells were cultured in suspension at a density of 100,000 cells / ml and allowed to form the secondary neurospheres (P1 cells).

For single-cell adhesive culture, neurospheres were dissociated into single cells using TrypLE and plated in poly-D-lysine-coated 24 or 6 well plates in serum-free complete/differentiation medium overnight before using in experiments. For observing cell proliferation, single NSPCs were allowed to attach onto poly-D-lysine-coated coverslips with serum-free complete medium. For observing cell differentiation, single NSPCs were allowed to attach onto poly-D-lysine-coated coverslips with serum-free differentiation medium (DMEM/F12 supplemented with 1% N2 and 2% B27).

### RNA extraction and quantitative real-time PCR

The total RNA isolated from cultured NSPCs was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions and cDNA was synthesized according to the manufacturer's protocol (MBI Fermentas). Quantitative real-time PCR (qRT-PCR) was performed using a Maxima SYBR Green qPCR Master Mixes (MBI Fermentas), and PCR-specific amplification was conducted in the IQ5 Optical System real-time PCR machine (BioRad). The relative expression of genes was calculated with the $2^{-(\Delta\Delta Ct)}$ method. The sequences of primers specific to the published miR-21 sequences were used (7).

### Transfection of miRNA

Cells were plated in poly-D-lysine-coated 24-well or 6-well plates and transfection was performed shortly before or at 70% confluence. The microRNA precursors, inhibitors and negative controls of miR-21 were commercially purchased (Ambion, USA). cells were transfected with the negative control, pre-miR-21 or anti-miR-21 at 200 nM using Optifect Reagent (Invitrogen) following the manufacture's protocol. Cells were cultured for 24 hours before further treatments.

### MTT assay

Cell viability was evaluated by MTT assay. NSPCs were grown in poly-D-lysine-coated 24-well plates in complete medium and transfected microRNA precursors, inhibitors and negative controls of miR-21 for 24 h before experiments. At the end of each time point (2 h, 6 h, 12 h, 24 h and 48 h), the culture medium in each well were added MTT (0.5 mg/mL final concentration, Sigma) and incubated for 2 h at 37°C, then added 400 μl dimethyl sulfoxide (DMSO, Sigma) in the medium, then absorbed 200 μl medium into 96-well plate. The absorbance was measured at the wavelength of 490 nm using a multimicroplate spectrophotometer (Epoch, BioTek, VT, USA). Triplicate parallel wells were set in all of the experiments, and data were collected as the average of at least five independent experiments. At least three independent experiments were conducted for each assay.

### Cell culture BrdU incorporating assay

For the cell proliferation assay, 10 μM BrdU solution was added to NSPCs plated on coverslips for 2 h. The cells were then fixed with 4% PFA for 20 min at room temperature. The BrdU labeled cells were visualized using immunostaining and normalized using propidium iodide (PI, Sigma-Aldrich) to stain the cells.

### Immunostaining

Cultured NSPCs were fixed in 4% PFA for 20 min and 3 samples for each group were used for immunocytochemistry. To expose the BrdU, the cell samples were pretreated with 2 N hydrochloric acid (HCl) for 30 min at 37°C, then the samples were neutralized with 0.1 M borate buffer (pH8.5) for 10 min, followed by 0.01 M PBS wash (all of the washes were performed three times, 5 min/rinse). All of the coverslips were permeabilized using 0.3% Triton X-100 (Sigma-Aldrich) for 20 min, then they were rinsed, and blocked for 1 h with 10% normal goat serum. All of the primary antibodies were diluted in 0.01 M PBS plus 2% bovine serum albumin (BSA, St Louis, Sigma-Aldrich). The following primary antibodies were used: mouse anti-Tuj1 monoclonal (1:200, Millipore), mouse anti-PSA-NCAM monoclonal (1:200, Millipore) and sheep anti-BrdU polyclonal (1: 200, Abcam, UK). The samples were incubated with primary antibodies overnight at 4°C, after which they were incubated in secondary antibodies. For a negative control, the samples were incubated in PBS instead of primary antibodies. Then, the samples were incubated with appropriate fluorescein conjugated IgG for 2 h. The secondary antibodies were as follows: Alexa Fluor 488 donkey anti-mouse IgG (1:600; Invitrogen, USA), Alexa Fluor 488 donkey anti-sheep IgG (1:800, Invitrogen, USA). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI,

1μg/mL, Sigma-Aldrich) or PI (10 μg/mL)for 10 min at 37°C, they were washed and then mounted with an anti-fade mounting medium (Invitrogen). Immunostaining positive cells were observed using a BX51 fluorescent microscope equipped with a DP70 digital camera (both from Olympus, Japan).

## Western blot analysis

NSPCs were cultured on a PDL-coated 60 mm dish. After treatment, the cells were rinsed three times in PBS and incubated in lysis buffer (50 mMTris-HCl, pH 7.6, 150 mMNaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5% so-dium Deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 20 mM $Na_4P_2O_7$, 10% glycerol, Pierce, Rockford, IL, USA) supple-mented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA) for 15 min on ice, after which they were sonicated (VCX500, Sonics, CT). Cell lysates were cleared by centrifugation at 12000 rpm for 15 min at 4°C. Protein concentrations of samples were estima-ted using the BCA assay (Pierce, Rockford, IL, USA). The samples were mixed with loading buffer and boiled for 5 min. Proteins (20 μg for each sample) were re-solved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly-vinylidene fluo-ride (PVDF) membranes (Bio-Rad, Hercules, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and subsequently probed with specific primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit anti-Cy-clin D1 polyclonal (1:1000, Cell Signaling Technology, USA), rabbit anti-phospho-AKT monoclonal (1:1000, Cell Signaling), rabbit anti-AKT polyclonal (1:2000, Cell Signaling), rabbit anti-phospho-GSK3β polyclonal (1:1000, Cell Signaling), rabbit anti-GSK3β polyclonal (1:2000, Cell Signaling), mouse anti-β-Actin monoclo-nal (1:10000, Sigma-Aldrich). The membranes were rinsed and further incubated with horseradish peroxi-dase-conjugated anti rabbit or anti mouse IgG (both 1:100000, Sigma-Aldrich) for 1 h at room temperature. Following by the secondary antibody incubation, the membranes were rinsed and the immunoreactive bands were visualized by enhanced chemiluminescent subs-trate according to the manufacturer's protocol (Pierce) and exposed to X-ray film (Fuji, Japan). The results were collected using a G: Box gel imaging system (Syngene, Cambridge, UK) and quantified using an NIH ImageJ 3.5 software. The relative levels of the target proteins were calculated and normalized by β-Actin, which was used as an internal control. All of the Western blot data are presented in samples from at least 3 independent experiments.

## Statistical analysis

All of the data are reported as mean ± SD from at least three independent in vitro experiments. Statistical comparisons of MTT, qRT-PCR, immunostaining and western blot data between different groups were made by Tukey's test after one-way ANOVA. And compa-risons between two means were performed using the Student's t-test for paired data. Both of them used SPSS statistical software (version 12.0). P < 0.05 was consi-dered statistically significant.

## Results

### Changes in miR-21 levels during differentiation of NSPCs

To observe the express of miR-21 during differen-tiation of NSPCs by qRT-PCR, NSPCs were plated in poly-D-lysine-coated 24-well plates in serum-free dif-ferentiation medium or serum-free complete medium, then extracted total RNA by the method of TRIzol at different time points (from 1 d to 7 d). The result showed that compared to cells in the serum-free complete me-dium, the expression of miR-21 in the serum-free diffe-rentiation medium was significantly increased in a time-dependent manner (Fig. 1). However, in the serum-free complete medium, there was little difference in the expression of miR-21 at different time points. These fin-dings may indicate that miR-21 seems to be involved in the proliferation and differentiation of NSPCs.

### MiR-21 promotes the neural differentiation of NS-PCs

To further verify the role of miR-21 on NSPCs differentiation, we detected the positive rate of PSA-NCAM and Tuj1 by immunostaining. Single NSPCs were plated onto poly-D-lysine-coated glass coverslips in serum-free differentiation medium. One day later, synthesized pre-miR-21 or anti-miR-21 was transfected into NSPCs, thereby efficiently overexpressing or knoc-king down miR-21. After three days of culture, NSPCs were fixed in 4% PFA and detected the number of PSA-NACM and Tuj1 positive cells by immunostaining. The result showed that compared to the miR-control group, overexpression of miR-21 significantly increased the positive rate of PSA-NACM (Fig. 2A, C) and Tuj1 (Fig. 2B, D). Interestingly, knock down miR-21 had the op-posite effect of neural differentiation in NSPCs. These phenomenon demonstrated that miR-21 could promote the neural differentiation of NSPCs.

### MiR-21 inhibits the proliferation of NSPCs

To determine the effect of miR-21 on proliferation of NSPCs. First of all, we detected cell viability by MTT assay. Single NSPCs were plated onto poly-D-lysine-coated glass coverslips, 24-well plate in serum-free
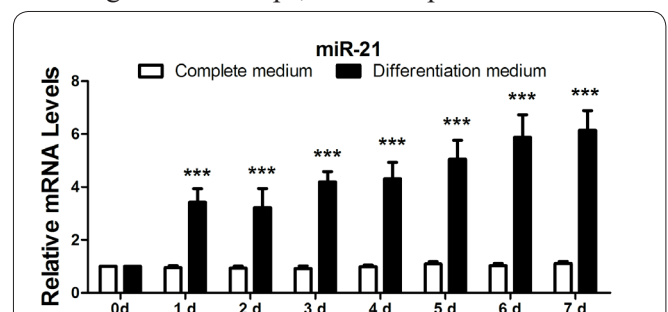


**Figure 1.** The expression of miR-21 was increased during diffe-rentiation of NSPCs. The expression of miR-21 was detected by qRT-PCR. NSPCs were plated in poly-D-lysine-coated 24-well plates in serum-free differentiation medium or serum-free com-plete medium, then extracted total RNA by the method of TRIzol at different time points (from 1 d to 7 d). Each value represents the mean ± SD of three independent experiments (n = 5). \*\*\*P < 0.001 versus the same time point of complete medium group.
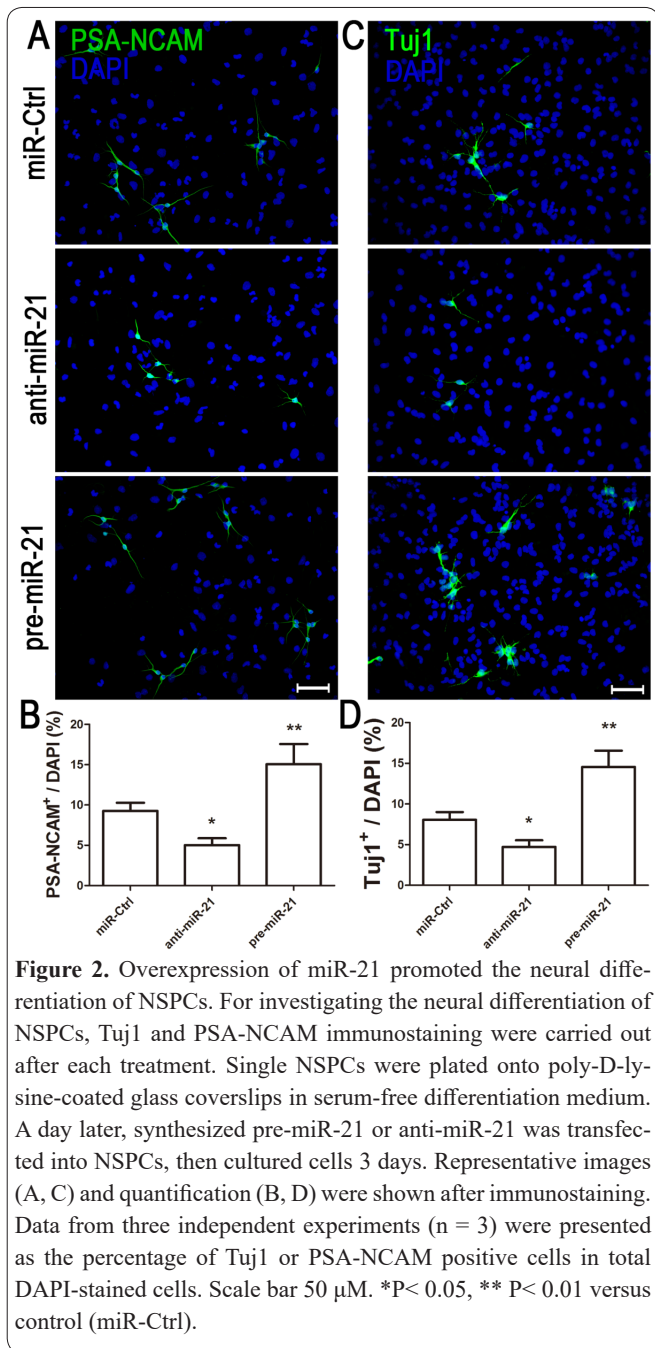
**Figure 2.** Overexpression of miR-21 promoted the neural differentiation of NSPCs. For investigating the neural differentiation of NSPCs, Tuj1 and PSA-NCAM immunostaining were carried out after each treatment. Single NSPCs were plated onto poly-D-lysine-coated glass coverslips in serum-free differentiation medium. A day later, synthesized pre-miR-21 or anti-miR-21 was transfected into NSPCs, then cultured cells 3 days. Representative images (A, C) and quantification (B, D) were shown after immunostaining. Data from three independent experiments (n = 3) were presented as the percentage of Tuj1 or PSA-NCAM positive cells in total DAPI-stained cells. Scale bar 50 µM. *P< 0.05, ** P< 0.01 versus control (miR-Ctrl).



**Figure 3.** Overexpression of miR-21 inhibited the proliferation of NSPCs. Single NSPCs were plated onto poly-D-lysine-coated glass coverslips, 24 or 6 well plate in serum-free complete medium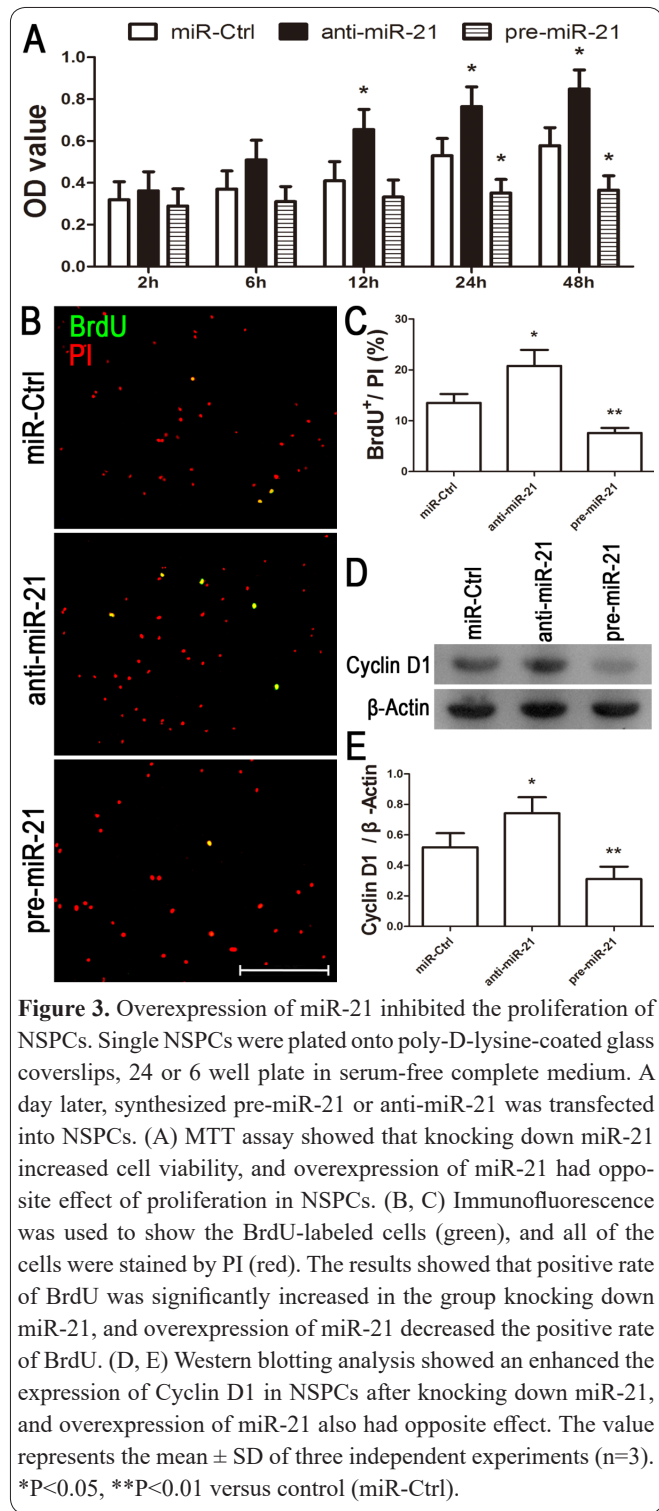. A day later, synthesized pre-miR-21 or anti-miR-21 was transfected into NSPCs. (A) MTT assay showed that knocking down miR-21 increased cell viability, and overexpression of miR-21 had opposite effect of proliferation in NSPCs. (B, C) Immunofluorescence was used to show the BrdU-labeled cells (green), and all of the cells were stained by PI (red). The results showed that positive rate of BrdU was significantly increased in the group knocking down miR-21, and overexpression of miR-21 decreased the positive rate of BrdU. (D, E) Western blotting analysis showed an enhanced the expression of Cyclin D1 in NSPCs after knocking down miR-21, and overexpression of miR-21 also had opposite effect. The value represents the mean ± SD of three independent experiments (n=3). *P<0.05, **P<0.01 versus control (miR-Ctrl).

complete medium. A day later, synthesized pre-miR-21 or anti-miR-21 was transfected into NSPCs, and then detected cell viability at different time points by MTT assay. The result showed that knocking down miR-21 could enhance the cell viability of NSPCs. Obvious proliferation was observed in 12 h, and over time, the effect would be more evident, reaching a peak in the 24 h. In contrast, pre-miR-21 attenuated the cell viability of NSPCs. Cells were overexpressed miR-21 for a further 24 h or 48 h, and the cell viability was attenuated (Fig. 3A). To further verify the role of miR-21 on NSPCs proliferation, proliferating cells were distinguished from viable cells by BrdU staining and Western blotting to detect the changes in the expression of cell cycle protein Cyclin D1. Compared to the miR-control group, the results showed that the positive rate of BrdU was significantly increased from 13.51% ± 1.75% to 20.82% ± 3.15% in the group of knocking down miR-21, and the positive rate of BrdU was 7.58% + 1.04% in the group of overexpression of miR-21, which is significantly lower than knocking down miR-21 (Fig. 3B, C). The

changes in the cell cycle protein Cyclin D1 expression were similar to the BrdU staining results, which showed that knocking down miR-21 can significantly promote the expression of Cyclin D1, whereas overexpression of miR-21 worked in the opposite direction (Fig. 3D, E). These data suggested that miR-21 inhibits the proliferation of NSPCs.

## MiR-21 influence on the activation of AKT and GSK-3β signaling pathway

To explore possible involvement of miR-21 signaling pathway in the regulation the behavior of NSPCs, we investigated the activation of AKT and GSK-3β by western blot analysis. Single NSPCs were plated onto poly-D-lysine-coated 6-well plates in serum-free differentiation medium. A day later, synthesized pre-miR-21
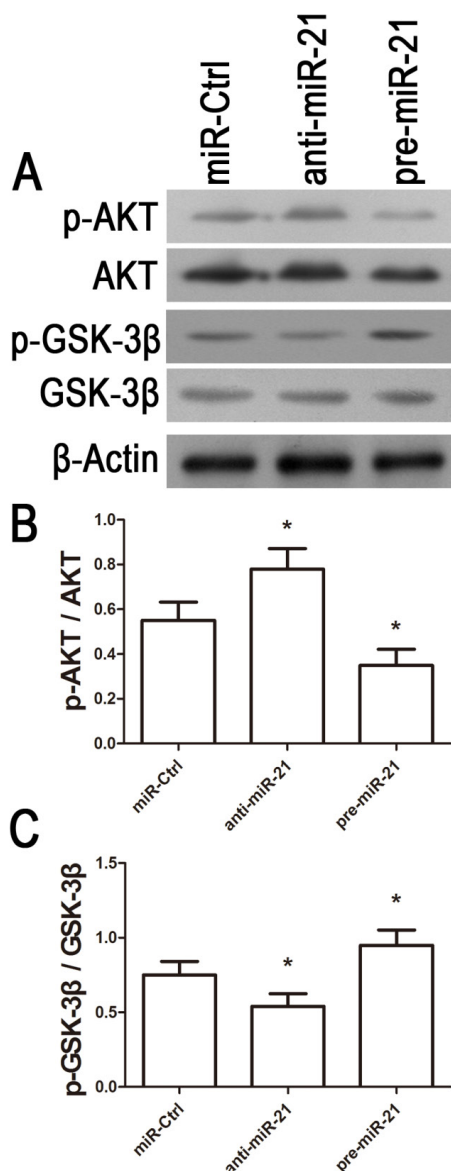
X. Gao *et al.* 2016 | Volume 62 | Issue 12

MiR-21 influence on the proliferation and differentiation of NSPCs.



**Figure 4.** miR-21 had impact on the activation of AKT and GSK-3β signaling pathway. Single NSPCs were plated onto poly-D-lysine-coated 6-well plates in serum-free differentiation medium. A day later, synthesized pre-miR-21 or anti-miR-21 was transfected into NSPCs, then cultured cells 3 days. (A) Western blot analysis showed knocking down miR-21 promoted the activation of AKT, however, the phosphorylation levels of GSK-3β is decreased, and overexpression of miR-21 was counterproductive. Western blotting band quantification for the ratio of p-AKT/AKT (B) and p- GSK-3β/ GSK-3β (C) was presented. The value represents the mean ± SD of three independent experiments (n=3). *P<0.05 versus control (miR-Ctrl).

or anti-miR-21 was transfected into NSPCs, and cultured cells within three days. Then, we detected the changes in the AKT and GSK-3β phosphorylation levels by Western blotting (Fig. 4A-C). The results showed that compared to the miR-control group, knocking down miR-21 promoted the activation of AKT, however, the phosphorylation levels of GSK-3β is decreased. And likewise, overexpression of miR-21 was counterproductive. These phenomenon may indicate that MiR-21 can affect the activation of AKT and GSK-3β signaling pathway.

## Discussion

In the present study, we reported that miR-21 reduces proliferation and promotes neural differentiation in NSPCs. Furthermore, the effect of miR-21 on cellular behavior in NSPCs might be due to influence on the activation of AKT and GSK-3β in cultured rat NSPCs.

MicroRNAs are small non-coding RNA molecules that occur naturally and downregulate protein expression by translational blockade of the target mRNA or by promoting mRNA decay. In this study, one fascinating finding was that in the serum-free complete medium, the expression of miR-21 maintained relatively constant within seven days. However, the expression of miR-21 was increased in the serum-free differentiation medium (without bFGF and EGF). This phenomenon may suggest that stable expression of miR-21 is the key factor to maintain neural stem/precursor cells properties, and overexpression of miR-21 promotes the neural differentiation of NSPCs.

Most previous research on miR-21 has identified that miR-21 plays a significant role in cancer biology and diagnostics, overexpression of miR-21 can inhibit proliferation. For example, compared to the matched normal breast tissues, miR-21 was highly overexpressed in breast tumors, and anti-miR-21 suppressed both cell growth in vitro and breast tumor growth in the xenograft mouse model (11). One of reason of miR-21 influence on cell behaviors is that miR-21 suppresses the expression of phosphatase and tensin homolog (PTEN). This phenomenon had been verified in several cancers and cell types including cholangiocytes (12), breast cancer (13), hepatocellular carcinoma (14) and vascular smooth muscle cells (15). As we know, PTEN is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI-3-K) signaling pathway and thus prevents Akt activation (16), and activation of PI-3-K pathway makes contribution to proliferation and pro-survival (17, 18). Therefore, miR-21 functions in many cell types as an anti-apoptotic, pro-survival and proliferation factor may through promoting the activation of AKT. However, in this study, our result showed that overexpression of miR-21 can inhibit proliferation and promoting neural differentiation may through preventing the activation of AKT and its downstream of GSK-3β. Previous research showed that overexpression of miR-21 enhanced adipogenic differentiation during the process of differentiation of hASCs (7), and miR-21 also can suppresses the self-renewal of mouse ES cells (9). These phenomenon may suggest that in terms of proliferation, miR-21 has oppositely effected of cancer cell and stem/precursor cell. And one possibility is that miR-21 has diverse impacted on the activation of AKT in different types of cells.

The glycogen synthase kinase-3 beta (GSK-3β), one of the downstream effector proteins of AKT, is taken part in a wide range of cellular processes. Previous research showed that GSK-3β was implicated in many processes in the nervous system, including differentiation, specification, and polarity, plasticity and axon growth (19-21). GSK-3β phosphorylation and inactivation of GSK-3β lead to enhanced neurite outgrowth. The major target of GSK-3β in controlling cytoskeleton dynamics is collapsing response mediator protein-2 (CRMP-2). The

binding activity of CRMP-2 to tubulin is decreased by the phosphorylation by GSK-3β. Nonphosphorylated CRMP-2 binds to tubulin heterodimers to promote microtubule assembly, thereby enhancing elongation of neuronal processes (22). Even through AKT can influence the phosphorylation level of GSK-3β, wnt/β-catenin pathway also be able to regulate the activation of GSK-3β. Secreted Wnt glycoproteins interact with the frizzled family of receptors and through disheveled mediated signaling inhibit GSK-3β (23). In our research, the activation of AKT did not lead to increase the phosphorylation level of GSK-3β. Conversely, the phosphorylation level of GSK-3β was decreased when AKT was activated. It may suggest that AKT had little influence on GSK-3β, others intracellular signal molecules (like Wnt, PKA, PKC) were played a vital role in regulating the activation of GSK-3β (24).

The G1 phase is a vital phase in the mammalian cell cycle because it determines cell fate and responds to extracellular environment (25). D-type cycles (D1, D2, D3) influence the cell cycle in different ways (26). Cyclin D1 plays an important role in the proliferation and differentiation of NSPCs (27). Recent research has demonstrated that Cyclin D1 knockdown significantly blocked embryonic neural stem cell proliferation. In our study, we found that knocking down miR-21 could enhance the expression of Cyclin D1, and promote cell entry into the mitotic phase, whereas overexpression of miR-21 had the opposite impact on NSPCs. These results showed that one of the reasons why miR-21 inhibits the proliferation of NSPCs was that miR-21 would reduce the expression of Cyclin D1 and decrease the cell numbers in the mitotic phase.

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