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miR-21 promotes the differentiation of hair follicle-derived neural crest stem cells into Schwann cells

Yuxin Ni[1], Kaizhi Zhang[2], Xuejuan Liu[3], Tingting Yang[1], Baixiang Wang[1], Li Fu[1], Lan A[1], Yanmin Zhou[1]

1 Hospital of Stomatology, Jilin University, Changchun, Jilin Province, China
2 China-Japan Union Hospital, Jilin University, Changchun, Jilin Province, China
3 First Hospital of Jilin University, Changchun, Jilin Province, China

Corresponding author:
Yanmin Zhou, Ph.D., M.D., Hospital of Stomatology, Jilin University, Changchun 130021, Jilin Province, China, neurology@live.com.

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Abstract

Hair follicle-derived neural crest stem cells can be induced to differentiate into Schwann cells *in vivo* and *in vitro*. However, the underlying regulatory mechanism during cell differentiation remains poorly understood. This study isolated neural crest stem cells from human hair follicles and induced them to differentiate into Schwann cells. Quantitative RT-PCR showed that microRNA (miR)-21 expression was gradually increased during the differentiation of neural crest stem cells into Schwann cells. After transfection with the miR-21 agonist (agomir-21), the differentiation capacity of neural crest stem cells was enhanced. By contrast, after transfection with the miR-21 antagonist (antagomir-21), the differentiation capacity was attenuated. Further study results showed that SOX-2 was an effective target of miR-21. Without compromising SOX2 mRNA expression, miR-21 can down-regulate SOX protein expression by binding to the 3′-UTR of miR-21 mRNA. Knocking out the SOX2 gene from the neural crest stem cells significantly reversed the antagomir-21 inhibition of neural crest stem cells differentiating into Schwann cells. The results suggest that miR-21 expression was increased during the differentiation of neural crest stem cells into Schwann cells and miR-21 promoted the differentiation through down-regulating SOX protein expression by binding to the 3′-UTR of SOX2 mRNA.

Key Words: *nerve regeneration; microRNA; stem cells; Schwann cells; SOX2; hair follicle; neural crest stem cells; neurons; NSFC grant; neural regeneration*

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Introduction

The repair of injured peripheral nerve remains a great area of interest in neuroscience research. Recently, considerable attention has been paid to tissue engineering techniques, which are widely used in the repair of peripheral nerve injury (Gao et al., 2013; Marquardt and Sakiyama-Elbert, 2013; Pang et al., 2013). Schwann cells are part of the peripheral nervous system, and participate in nerve growth and regeneration, transmit nerve impulses, regulate the activity of motor nerves, and therefore are indispensable "seed cells" for peripheral nerve tissue engineering (Stefanescu et al., 2012; Nie et al., 2013). The majority of currently used Schwann cells are mainly the cells differentiated from autologous stem cells owing to the difficulties in harvesting autologous Schwann cells and the immunological rejection caused by allogeneic Schwann cells (Ren et al., 2012).

Neural crest stem cells, a kind of unique pluripotent stem cells in vertebrates, originate in the ectoderm at the margins of the neural tube in the embryonic stage and then develop into many tissues including the heart, smooth muscle and peripheral nerve (Hall, 2008; Sauka-Spengler and Bronner-Fraser, 2008; Huisman and Rivolta, 2012). Neural crest stem cells can be harvested from the heart, hair follicles, olfactory bulb and craniomaxillofacial tissue in adults (Achilleos and Trainor, 2012; Pelaez et al., 2013). Hair follicle-derived neural crest stem cells are mostly studied owing to the ease of harvesting and wide availability (Sieber-Blum and Grim, 2004). A previous study by our laboratory has shown that after induction by transforming growth factor-β1, hair follicle-derived neural crest stem cells can differentiate into smooth muscle cell-like cells expressing smooth muscle actin and calponin (Liu et al., 2013b). There is strong evidence that under the effects of different inducers, hair follicle-derived neural crest stem cells also possess the potential to differentiate into many different mature cell types including Schwann cells, melanocytes and neurons (El Seady et al., 2008; Sviderskaya et al., 2009; Lin et al., 2011; Dong et al., 2012). Accumulating evidence exists that hair follicle-derived neural crest stem cells, as an important source of Schwann cells, play a critical role in the repair of injured peripheral nerve by tissue engineering (Lin et al., 2009, 2011). However, the mechanism by which hair follicle-derived neural crest stem cells differentiate

into Schwann cells remains poorly understood and therefore brings difficulties for a better understanding of the regulation of Schwann cell differentiation (Bhatt et al., 2013). A recent study has shown that microRNAs (miRNAs), an important small molecule substance *in vivo*, play a crucial role in Schwann cell differentiation (Dugas and Notterpek, 2011).

miRNAs are a class of non-coding single-stranded small RNA molecules of 18–25 nucleotides that can bind to the 3′UTR of the mRNA molecules and regulate the protein expression of target gene (Amiel et al., 2012; Nana-Sinkam and Croce, 2013). Approximately 1,600 human miRNAs have been identified to participate in various pathophysiological processes, including stem cell differentiation, tumor formation and metastasis, cell apoptosis, inflammation and embryonic development (Garofalo et al., 2012; Kong et al., 2012; Rutnam and Yang, 2012; Koerner et al., 2013; Qi et al., 2013; Sethi et al., 2013). Studies using quantitative real time-PCR, microarray and northern blot technique have shown that miRNA expression alters during Schwann cell differentiation of precursor cells and subsequent myelination (Bremer et al., 2010; Verrier et al., 2010; Yun et al., 2010). After knocking out the *Dicer1* gene (a key enzyme for miRNA biogenesis), Schwann cells do not have the capacity to form myelin sheaths, while after silencing *Dicer1* gene expression, these cells greatly proliferated and cannot form myelin sheath with normal function (Dugas et al., 2010; Pereira et al., 2010). These findings suggest that miRNAs likely regulate Schwann cell differentiation, myelination and peripheral nerve growth and development. Nevertheless, the miRNAs included in the regulation and the mechanisms by which miRNAs regulate Schwann cell differentiation need to be further studied.

miR-21 is a miRNA closely related to stem cell differentiation and neural regeneration (Cioffi et al., 2010; Strickland et al., 2011). Yu et al. (2012a, b) reported that miR-21 expression was significantly increased in injured peripheral nerve tissue. However, a study using microarray technique has shown that miR-21 expression was gradually increased in myelinating Schwann cells of mouse sciatic nerve (Gokey et al., 2012). These findings indicate that miR-21 is associated with Schwann cell differentiation and remyelination. However, to the best of our knowledge, the role of miR-21 in Schwann cell differentiation of stem cells and the underlying mechanisms are poorly understood. This study isolated neural crest stem cells from human hair follicle, induced them to differentiate into Schwann cells and detected miR-21 expression change using quantitative real time (RT)-PCR. In addition, intracellular miR-21 expression was interfered to investigate the effect of miR-21 expression on Schwann cell differentiation. This study further sought to find the possible gene target of miR-21 responsible for regulating Schwann cell differentiation.

Materials and Methods

Materials

Human skin tissue containing hair follicles was provided by healthy adults (irrespective of sex and age) who were physically examined in the Department of Dermatology, Norman Bethune First Hospital, Jilin University, China. Informed consent was obtained from participants. Skin harvesting and use of samples was approved by the Ethics Committee, Jilin University, China. HEK-293 cells were purchased from Baoman Biotechnology Co., Ltd., Shanghai, China. Human Sox-2 shRNA lentiviral plasmids (sc-108080) and copGFP control lentiviral plasmids (sc-108084) were purchased from Santa Cruz Biotechnology, Dallas, TX, USA.

Isolation of hair follicle-derived neural crest stem cells

Fresh human skin tissue was harvested and then stored in ice-cold PBS. Neural crest stem cells were isolated using a previously published method (Krejci and Grim, 2010; Yu et al., 2010). Briefly, after several PBS washes and careful scraping of attached adipose tissue, skin tissue was cut into small blocks and then treated with Dulbecco's Modified Eagle's Medium (DMEM) containing 4.8 mg/mL dispase (Invitrogen, Grand Island, NY, USA) at 4°C overnight. Under a light microscope (Olympus, Shanghai, China), hair follicles were isolated from deciduous epidermis, collected and then washed with PBS. The hair follicles were treated twice with 0.25% trypsin/ethylenediamine tetraacetic acid (Invitrogen) for 30 minutes each. The resultant cell suspension was filtered with a 40-μm cell strainer (BD Falcon, Bedford, MA, USA). After counting, cells were seeded in a Petri dish, in which human embryonic stem cell medium (Gibco, Shanghai, China) containing 4 ng/mL basic fibroblast growth factor (Sino Biological, Beijing, China) was added. Cells were then incubated in a 5% CO_2 incubator at 37°C. Culture medium was renewed once every 3 days.

Screening of neural crest stem cells by flow cytometric cell sorting

Cells were collected, washed and then prepared into single cell suspension. Neural crest stem cells were screened using flow-cytometric cell sorting as described previously (Jiang et al., 2009; Yang and Xu, 2013). Briefly, cells were diluted with PBS to a final concentration of 10–20 × 10^6 cells/mL. They were treated with a mixture of human natural killer-1 and p75 mouse monoclonal antibody (1:100; BD Bioscience Pharmingen Inc., San Jose, CA, USA) in the tube at 4°C for 40 minutes. After washes with ice-cold PBS, cells were centrifuged at 4°C and 800 × *g* for 5 minutes. After re-suspension with PBS, cells were sorted by flow cytometry (BD Bioscience Pharmingen Inc.), and human natural killer-1/p75 double-positive cells (neural crest stem cells) were collected.

Induced differentiation of hair follicle-derived neural crest stem cells into Schwann cells

Neural crest stem cells were induced to differentiate into Schwann cells as published previously (Nie et al., 2013). Briefly, neural crest stem cells were collected and treated with MesenPRO medium (Invitrogen) containing 20 ng/mL neuregulin-1 (Sino Biological) in a 5% CO_2 incubator at 37°C for 28 days. The culture medium was replaced once every 2 days.

Oligonucleotide transfection

To investigate the effect of miR-21 on differentiation, 200 nmol/L of agomir-21, agomir-NC, antagomir-21 or antagomir-NC

(Riobo, Guangzhou, China) were added into 1640 medium (Gibco) before induction. Agomir and antagomir were chemically modified with cholesterol-conjugated RNA molecules that could be easily transfected into cells without Lipofectamine 2000. Agomir and antagomir activated and inhibited endogenous miRNA, respectively.

Knock out of SOX2 gene from neural crest stem cells

Methods used for gene knock out were reported in our previous study (Liu et al., 2013b). Briefly, neural crest stem cells were seeded in a 24-well plate. When cells reached over 50% confluence, polybrebe (Santa Cruz Biotechnology) was added until a final concentration of 5 μg/mL and then cells were transfected with human Sox-2 shRNA lentiviral plasmid (multiplicity of infection = 15). The plate was shaken once every 15 minutes to enhance the transfection efficacy. Culture medium was then discarded the next day and cells were incubated for 1–2 days with 500 μL polybrene-free complete culture medium. Thereafter, the culture medium containing 8 μg/mL puromycin was replaced once every 3 days to kill non-transfected cells. Thus, the surviving cells were SOX2-KD-neural crest stem cells.

Group management

Prior to (0 week) and 1, 2, 3 and 4 weeks after induced differentiation, neural crest stem cells were collected and intracellular miR-21 expression was detected using quantitative real time (RT)-PCR. To investigate the effect of miR-21 expression on cell differentiation, prior to and 20 days after induction, neural crest stem cells were divided into five groups: namely agomir-21, agomir-NC, antagomir-21, antagomir-NC and control. Prior to and 20 days after induction, cells in the above four groups were treated with agomir-21, agomir-NC, antagomir-21 and antagomir-NC, respectively at a final concentration of 200 μmol/L and cells in the last group were not treated. At 40 days of induction, cell differentiation in each group was observed using immunofluorescence staining and quantitative RT-PCR. To investigate the effect of miR-21 on SOX2 expression in neural crest stem cells, neural crest stem cells were seeded in a 12-well plate and then divided into five groups: namely control, miR-21 mimic, mimic-NC, miR-21 inhibitor and inhibitor-NC. When cells reached over 50% confluence, miR-21 mimic, mimic-NC, miR-21 inhibitor and inhibitor-NC at a final concentration of 200 μmol/L were added to the four groups, respectively. After incubation for 48 hours, SOX2 protein and mRNA expression was detected. To investigate whether SOX2 participates in miR-21 promotion of stem cell differentiation, neural crest stem cells were divided into antagomir-21 and antagomir-NC groups and cells in these two groups were treated with antagomir-21 and antagomir-NC, respectively, at a final concentration of 200 μmol/L. SOX2-KD-neural crest stem cells were divided into antagomir-21 + SOX2 KD and antagomir-NC + SOX2 KD groups and treated with antagomir-21 and antagomir-NC, respectively, at a final concentration of 200 μmol/L. After induction for 40 days, the differentiation of neural crest stem cells was detected by immunofluorescence staining and quantitative RT-PCR.

Detection of cell differentiation by flow cytometry

To investigate cell differentiation, neural crest stem cells were washed three times with PBS for 3 minutes at room temperature. Then cells were treated with 0.1% Triton for 10 minutes. After three PBS washes for 3 minutes each, cells were blocked with 10% serum for 1 hour, treated with mouse anti-human S100 monoclonal antibody labeled with FITC (BD Bioscience Pharmingen Inc.) and mouse anti-human glial fibrillary acidic protein monoclonal antibody labeled with Cy3 (BD Bioscience Pharmingen Inc.) at a ratio of 1:100 at 4°C. Thirty minutes later, cells were washed three times with PBS for 3 minutes each. Subsequently, cells were suspended with 0.3 mL PBS and analyzed with flow cytometry (BD Bioscience Pharmingen Inc.).

Detection of miR-21 and SOX2 mRNA expression by quantitative RT-PCR

miR-21 and SOX2 mRNA expression was detected by PCR according to a previously described method (Liu et al., 2013b). Briefly, total RNA was extracted using the Trizol method (Sigma-Aldrich, St. Louis, MO, USA) and cDNA synthesis was performed using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). cDNA (2.5 μL), 1 μL miR-21 specific primer (RiboBio, Guangzhou, Guangdong Province, China) or SOX2 mRNA primer, S100 and glial fibrillary acidic protein mRNA primer were mixed with TransStart™ SYBR Green qPCR Supermix (TransGen Biotech) and quantitative RT-PCR was performed to detect miR-21 and SOX2 mRNA expression. The green fluorescence intensity of PCR product SYBR was detected using a PCR instrument (ABI, Grand Island, NY, USA). U6 was used as an internal control of miR-21 and GAPDH mRNA as an internal control of SOX2, S100 and glial fibrillary acidic protein mRNA.

Detection of SOX2 expression by western blot assay

Neural crest stem cells were collected, centrifuged and lysed for 30 minutes. Protein concentration was determined by the bicinchoninic acid method (Krejci and Grim, 2010). Each protein sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoretically transferred onto a polyvinylidene fluoride, and blocked with 5% defatted milk for 2 hours. Each membrane was treated with rabbit anti-SOX2, GAPDH polyclonal antibody (Santa Cruz Biotechnology) at 1:2,000 dilution for 2 hours at room temperature, and rinsed with Tris-buffered saline with Tween, six times for 10 minutes each. After washing, membranes were treated with horseradish peroxidase-labeled goat anti-rabbit IgM (Santa Cruz Biotechnology) at 1:2,000 dilution for 2 hours at room temperature, and developed by enhanced chemiluminescence. GAPDH (Santa Cruz Biotechnology) was used as an internal control.

Detection of miR-21 effects on fluorescence expression using Luciferase assay

To investigate the mechanism by which miR-21 promotes the differentiation of neural crest stem cells, we retrieved information for the miR-21 target from Pictar (http://pictar.

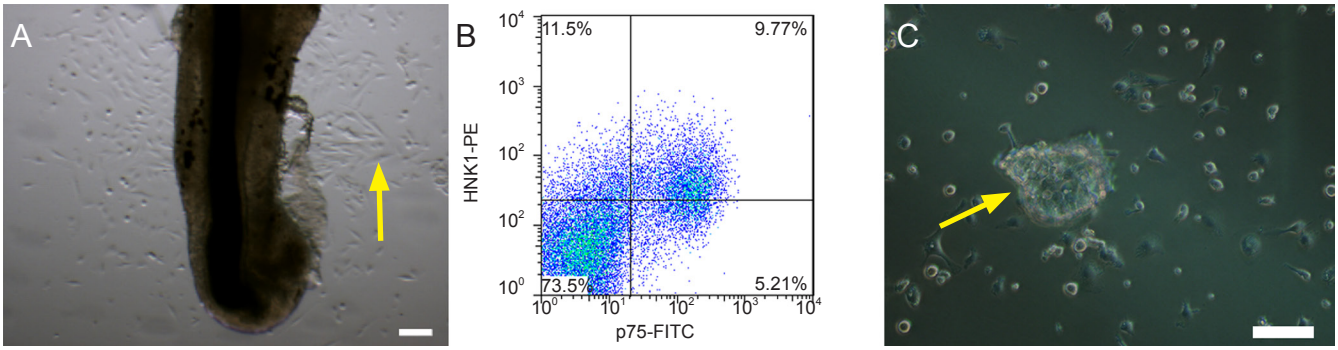

Figure 1 Isolation of hair follicle-derived neural crest stem cells.
(A) A small number of shuttle-shaped cells (arrow) appear around the hair follicles at 48 hours after culture in medium. (B) The HNK1/p75 double-positive cells are sorted out by flow cytometric cell sorting. (C) Small cell clumps (arrow) of neural crest stem cells. Bars: 50 μm. HNK1: Human natural killer-1.

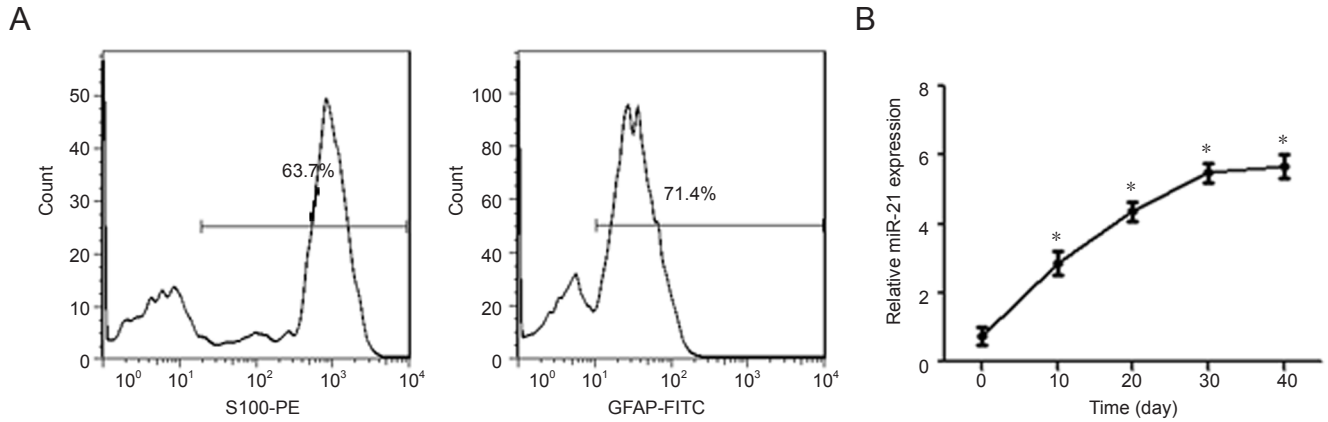

Figure 2 miR-21 expression during the differentiation of neural crest stem cells (NCSCs) into Schwann cells.
(A) Flow cytometry for differentiated NCSCs. (B) Quantitative real time (RT)-PCR tests showed that miR-21 expression was gradually increased with prolonged time (*i.e.*, 10, 20, 30 and 40 days of induction by neuregulin-1). All measurement data are expressed as mean ± SD. The experiment was repeated in triplicate. One-way analysis of variance and Student-Newman-Keuls test were used for comparison between groups. $*P < 0.05$, *vs.* 0 day.

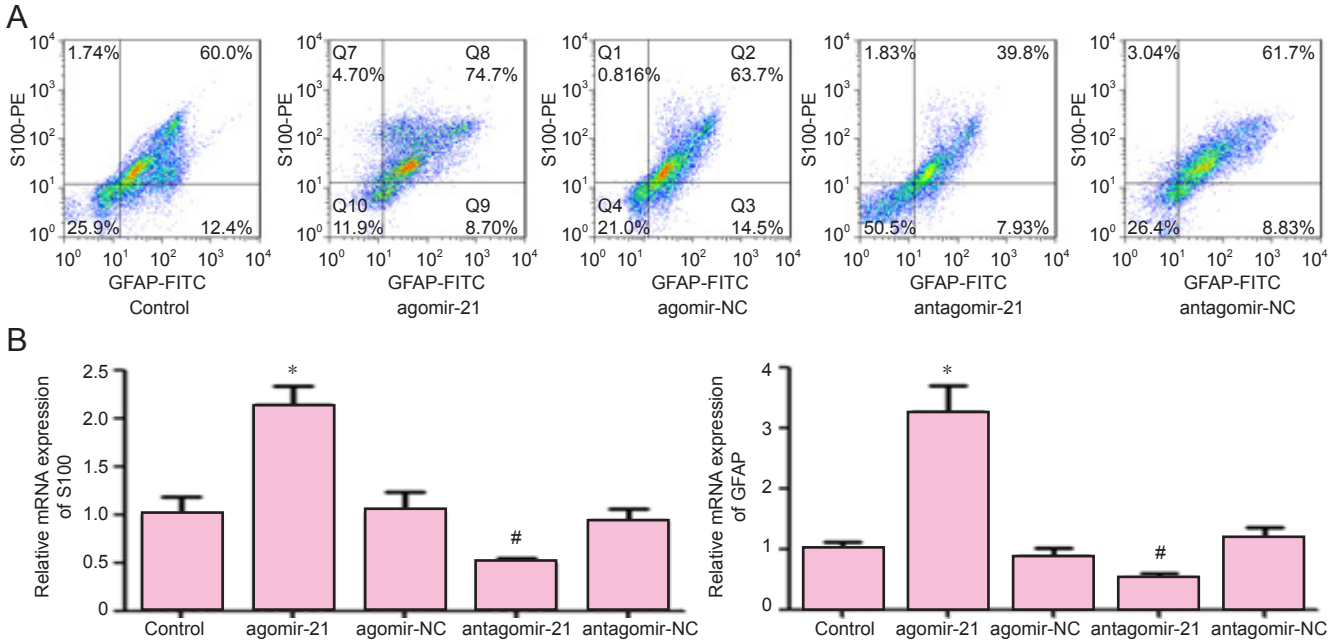

Figure 3 Regulatory role of miR-21 in the differentiation of neural crest stem cells.
(A) Flow cytometry showed S100- and GFAP-positive cells in the agomir-21, agomir-NC, antagomir-21 and antagomir-NC groups after 30-day induction by neuregulin-1. (B) Quantitative RT-PCR detected S100 and GFAP mRNA expression in the agomir-21, agomir-NC, antagomir-21 and antagomir-NC groups after 30-day induction by neuregulin-1. It took S100 and GFAP mRNA expression in the control group as 1. All measurement data are expressed as mean ± SD. The experiment was performed in triplicate. One-way analysis of variance and Student-Newman-Keuls test were used for comparison between groups. $*P < 0.05$, *vs.* agomir-NC group; $\#P < 0.05$, *vs.* antagomir-NC group. agomir-21: miR-21 agonist; antagomir-21: miR-21 inhibitor; GFAP: glial fibrillary acidic protein.

mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi), Targetscan (http://www.targetscan.org/) and miRBase (www.mirbase.org) databases and found that a 7-mer-long sequence at the 3′UTR region of the SOX2 gene was matched to miR-21. To determine if this sequence could down-regulate SOX2 mRNA translation after binding to miR-21, the SOX2 mRNA 3′UTR containing this site was included in the luciferase reporter system (SOX2-3′UTR-wt) and simultaneously the mutational SOX2 mRNA 3′UTR was also inserted into this reporter system (SOX2-3′UTR-mut). miR-21 was transfected into HEK-293 cells together with luciferase empty vector, SOX2-3′UTR-wt or SOX2-3′UTR-mut. Subsequently, HEK-293 cells were cultured in RPMI 1640 culture medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a 5% CO_2 incubator at 37°C. Full length rat SOX2-3′UTR containing miR-21 binding site was amplified by PCR, and then cloned into the region between *Hin*d III and *Sac* I sites in the downstream region and named SOX2-3′UTR-wt. After being subjected to point mutation in the Easy Mutagenesis System kit (TransGen Biotech), SOX2-3′UTR-wt was named as SOX2-3′UTR-mut. HEK293T cells were seeded in a 24-well plate and then divided into three groups: empty vector (400 ng empty vector was added), SOX2-3′UTR-wt (400 ng SOX2-3′UTR-wt plasmid was added) and SOX2-3′UTR-mut (400 ng SOX2-3′UTR-mut plasmid was added). In addition to lip2000 (Invitrogen), miR-21 and 20 ng pRL-TK (Ribobio, Guangzhou, China) were added as internal controls in each group. Two parallel wells were designated for each group, in which mimic-NC (Ribobio) rather than miR-21 mimic was added as negative control (miR-con). After transfection for 36 hours, cells were collected for fluorescence intensity detection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

All data were statistically processed using SPSS 18.0 software (SPSS, Chicago, IL, USA). Measurement data were expressed as mean ± SD. One-way analysis of variance and Student-Newman-Keuls test were used for comparison between groups. A level of $P = 0.05$ was considered statistically significant.

Results

Isolation and culture of hair follicle-derived neural crest stem cells

Human hair follicle bulges were inoculated in culture medium. Forty-eight hours later, a small number of shuttle-shaped cells appeared at the edge of hair follicle bulges, as shown in Figure 1A. When there were a large number of shuttle-shaped cells, they were digested with trypsin and stained with human natural killer-1 and p75 antibodies. Human natural killer-1/p75 double-positive cells were sorted by flow-cytometric cell sorting and accounted for 10% of all cells (Figure 1B). After neural crest stem cells were cultured in culture medium for 5 days, small typical clumps of stem cells formed (Figure 1C).

miR-21 expression during neural crest stem cell differentiation into Schwann cells

Flow-cytometric cell sorting showed that at day 30 of induction, 63.8 ± 3.7% of cells expressed S100 and 72.6 ± 4.9% of cells expressed glial fibrillary acidic protein. These results indicate that after induction by neuregulin-1, neural crest stem cells differentiated into Schwann cell-like cells (Figure 2A). Quantitative RT-PCR tests showed that miR-21 expression was detected at different time periods of induction. miR-21 expression was about two times higher at day 10 of induction compared with the level prior to induction and increased gradually thereafter ($P < 0.05$). This result demonstrates that miR-21 plays a regulatory role during neural crest stem cell differentiation into Schwann cells (Figure 2B).

miR-21 promoted neural crest stem cell differentiation into Schwann cells

After transfection with miR-21 agonist (agomir-21) or miR-21 antagonist (antagomir-21), neural crest stem cells were induced with 20 ng/mL neuregulin-1 to investigate whether miR-21 interference regulates Schwann cell differentiation. Results showed that after neuregulin-1 induction, 75% of agomir-21-transfected neural crest stem cells expressed both S100 and glial fibrillary acidic protein simultaneously, which was significantly higher than that in the corresponding negative control (agomir-NC) group (64%, $P < 0.05$). After neuregulin-1 induction, 41% of antagomir-21-transfected neural crest stem cells expressed both S100 and glial fibrillary acidic protein simultaneously, which was significantly lower than that in the corresponding negative control (antagomir-NC) group (62%, $P < 0.05$; Figure 3).

We also detected S100 and glial fibrillary acidic protein mRNA expression in each group using quantitative RT-PCR. Results showed that after neuregulin-1 induction, S100 mRNA expression in the agomir-21 group and antagomir-21 group was significantly higher than that in the agomir-NC group ($P < 0.05$), but lower than that in the antagomir-NC group ($P < 0.05$). After neuregulin-1 induction, glial fibrillary acidic protein mRNA expressions in the agomir-21 group and antagomir-21 group were significantly higher than that in the agomir-NC group ($P < 0.05$), but lower than that in the antagomir-NC group ($P < 0.05$; Figure 3B). These findings demonstrate that miR-21 can promote the differentiation of neural crest stem cells into Schwann cells.

miR-21 down-regulated SOX2 protein expression by binding to the 3′-UTR of SOX2 mRNA

To investigate the mechanism by which miR-21 regulates the differentiation of neural crest stem cells into Schwann cells, we retrieved databases to find the effective target of miR-21, and found that a 7-mer-long sequence at the 3′-UTR of SOX2 mRNA was matched to miR-21 (Figure 4A). To investigate whether this sequence can down-regulate SOX2 mRNA translation by binding to miR-21, the SOX2 mRNA 3′UTR containing this site was included in the luciferase reporter system (SOX2-3′UTR-wt) and simultaneously the mutational SOX2 mRNA 3′UTR (SOX2-3′UTR-mut) was also inserted into this reporter system. Then miR-21 was

co-transfected into HEK-293 cells together with luciferase empty vector, SOX2-3′UTR-wt plasmid or SOX2-3′UTR-mut plasmid. Results showed that the fluorescence intensity in the SOX2-3′UTR-wt group was significantly lower than that in the negative control (miR-con) group. However, there was no significant difference in inflorescence intensity between the SOX2-3′UTR-mut group or empty vector group and miR-con group (Figure 4B). These findings suggest that miR-21 regulates SOX2 mRNA translation by binding to the 3′-UTR of SOX2 mRNA.

We also transfected neural crest stem cells with agomir-21 and its negative control (agomir-NC), with antagomir-21 and its negative control (antagomir-NC), and then detected SOX2 expression by western blot assay and SOX2 mRNA expression by quantitative RT-PCR. Results showed that agomir-21 can significantly decrease intracellular SOX2 expression ($P < 0.05$), while antagomir-21 can significantly increase intracellular SOX2 expression ($P < 0.05$; Figure 4C). SOX2 mRNA expression was not influenced by agomir-21 or antagomir-21 (Figure 4D). These findings suggest that miR-21 can regulate SOX2 protein expression at the post-transcriptional level by binding to the 3′-UTR of SOX2 mRNA.

SOX2 over-expression blocked the regulatory effect of miR-21 on Schwann cell differentiation

We silenced SOX2 expression in neural crest stem cells using the lentiviral shRNA approach and compared these SOX2-knocked out neural crest stem cells (SOX2-KD-neural crest stem cells) with those non-interfered neural crest stem cells (control) to investigate the role of SOX2 in miR-21 regulation of neural crest stem cell differentiation. Flow cytometry and quantitative RT-PCR results showed that antagomir-21-transfected neural crest stem cells exhibited a significantly decreased differentiation capacity than antagomir-NC-transfected cells ($P < 0.05$). However, there was no significant difference in the differentiation capacity between antagomir-21-transfected SOX2-KD-neural crest stem cells and antagomiR-NC-transfected SOX2-KD-neural crest stem cells (Figure 5). These results demonstrate that SOX2 knockout can block the effect of antagomir-21 against Schwann cell differentiation, indicating that SOX2 is one downstream pathway by which miR-21 regulates the differentiation of neural crest stem cells.

Discussion

Hair follicle-derived neural crest stem cells are widely accepted as the ideal source of "seed cells" in regenerative medicine owing to their multipotent differentiation capacity, their easy harvesting and wide availability. However, a key problem has been encountered regarding how to acquire highly purified neural crest stem cells (Sieber-Blum and Grim, 2004; Lin et al., 2011). Hair follicle-derived neural crest stem cells are often isolated by culturing hair follicles according to the characteristics of hair follicle-derived neural crest stem cell migration and chemotaxis (Yu et al., 2010). This method is easy to perform and economical, but is time consuming. One issue should be noted that hair follicles contain many stem cell types, including mesenchymal stem cells, which exhibit the characteristics of migration. Neural crest stem cells harvested by culturing hair follicles are often not satisfactory. Flow-cytometric cell sorting employed in this study can help isolate cells expressing only p75 and human natural killer-1, the specific antigens of neural crest stem cells. After culture, the isolated cells form typical lumps of neural crest stem cells.

Under the stimulation of proper inducers, neural crest stem cells can differentiate into Schwann cells successfully. A previous study using immunofluorescence staining showed that approximately 78 and 85% of cells express the specific markers of Schwann cells after culture with the medium containing 20 ng/mL neuregulin-1 for 40 days. A long induction period will influence the regulatory effect of miR-21 on the differentiation of neural crest stem cells, so a 4-week induction was designated in this study. Through immunofluorescence staining, approximately 65% and 73% of cells expressed S100 and glial fibrillary acidic protein, respectively, after a 4-week induction. Results from this study also showed that miR-21 expression was obviously increased at day 10 of induction, approximately 2-fold over that prior to induction, further increased thereafter and tended to be stable at day 30. Together with a previous finding that miR-21 expression is up-regulated after myelin sheath injury (Yu et al., 2012a, b), we preliminarily conclude that miR-21 plays a regulatory role in the process of neural crest stem cell differentiation into Schwann cells.

miR-21, one of the early discovered miRNAs, widely exists in animals and plants and is highly conserved across species. It is located at the fragile site FRA17B on chromosome 17q23.2 and contains independent transcriptional units. The transcription of miR-21 is regulated by various factors including STAT3, AP-1 and NFI (Kumarswamy et al., 2011). STAT3 is an important factor that regulates miR-21 transcription and STAT3 activation in some tumors leads to a significant increase in miR-21 expression (Iliopoulos et al., 2010; Bornachea et al., 2012). A study showed that after binding to cell membrane surface receptor, neuregulin-1 can strengthen STAT3 phosphorylation in the downstream pathway (Liu and Kern, 2002). It is presumed that the increase in miR-21 during induced differentiation of neural crest stem cells into Schwann cells is likely attributable to STAT3 phosphorylation induced by the effect of neuregulin-1 in the downstream pathway.

The function of miR-21 is complex and is an area of interest in diverse fields including embryonic development, tumorigenesis, fibrosis and immune reaction (Chen and Wang, 2013; Smigielska-Czepiel et al., 2013; Zhang et al., 2013). During embryonic development, miR-21 expression is gradually increased and regulates branching morphogenesis *via* target genes RECK and PDCD4 (Hayashi et al., 2011). In addition, miR-21 expression is up-regulated in many tumors including glioma, lung cancer and liver cancer and miR-21 promotes tumor growth by binding different target genes (Darido et al., 2011; Hermansen et al., 2013; Liu et al., 2013c). Results from this study showed that after transfection with agomir-21, neural crest stem cells had an increased capacity to differentiate into Schwann cells, while after transfection with antagomir-21, the differentiation capacity was

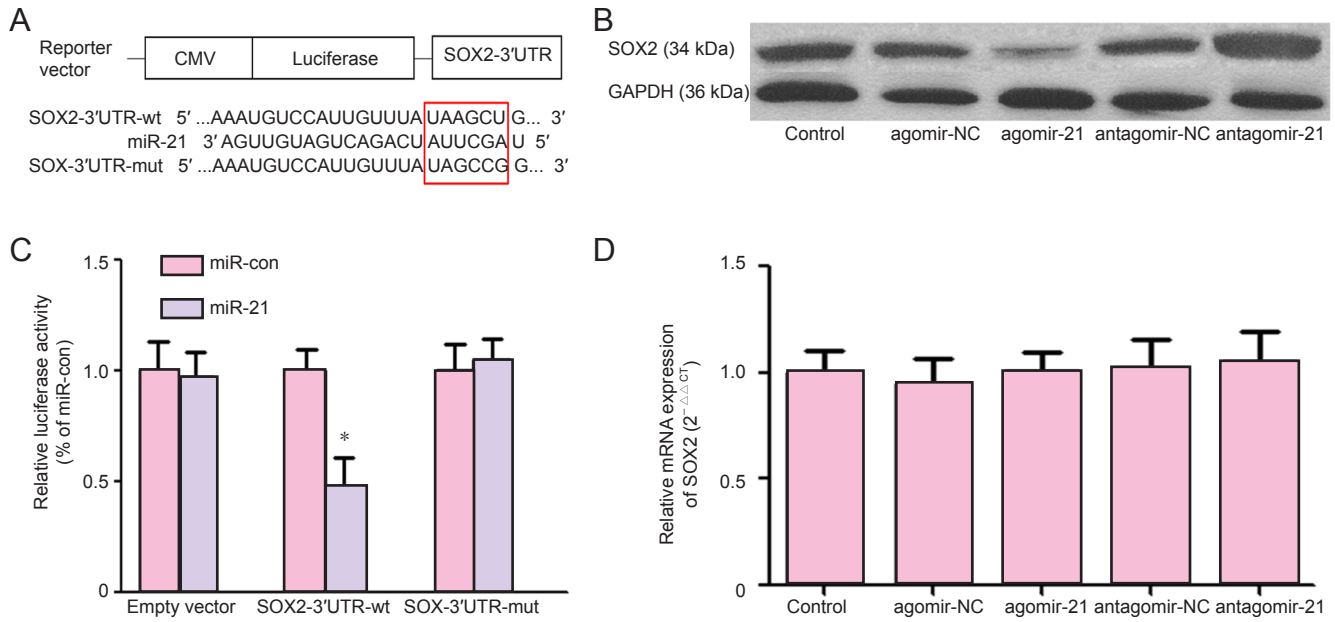

Figure 4 miR-21 down-regulated SOX2 expression by binding to the 3′-UTR of SOX2 mRNA.
(A) The combined (SOX2-3′UTR-wt) and mutational sequences of miR-21 (SOX2-3′UTR-mut) were cloned into reporter vectors. (B) Luciferase assay results showed that after miR-21 transfection, the fluorescence intensity in the SOX2-3′UTR-wt group was significantly decreased compared with the negative control (miR-con) group; however, there was no significant difference in the fluorescence intensity between SOX2-3′UTR-mut group or empty vector group and miR-con group. (C) Western blot assay for SOX2 expression in cells. (D) Quantitative real time (RT)-PCR showed that miR-21 could not influence mRNA level of SOX2. All measurement data are expressed as mean ± SD. The experiment was performed in triplicate. One-way analysis of variance and Student-Newman-Keuls test were used for comparison between groups. *$P < 0.05$, *vs.* miR-con group. agomir-21: miR-21 agonist; antagomir-21: miR-21 inhibitor.

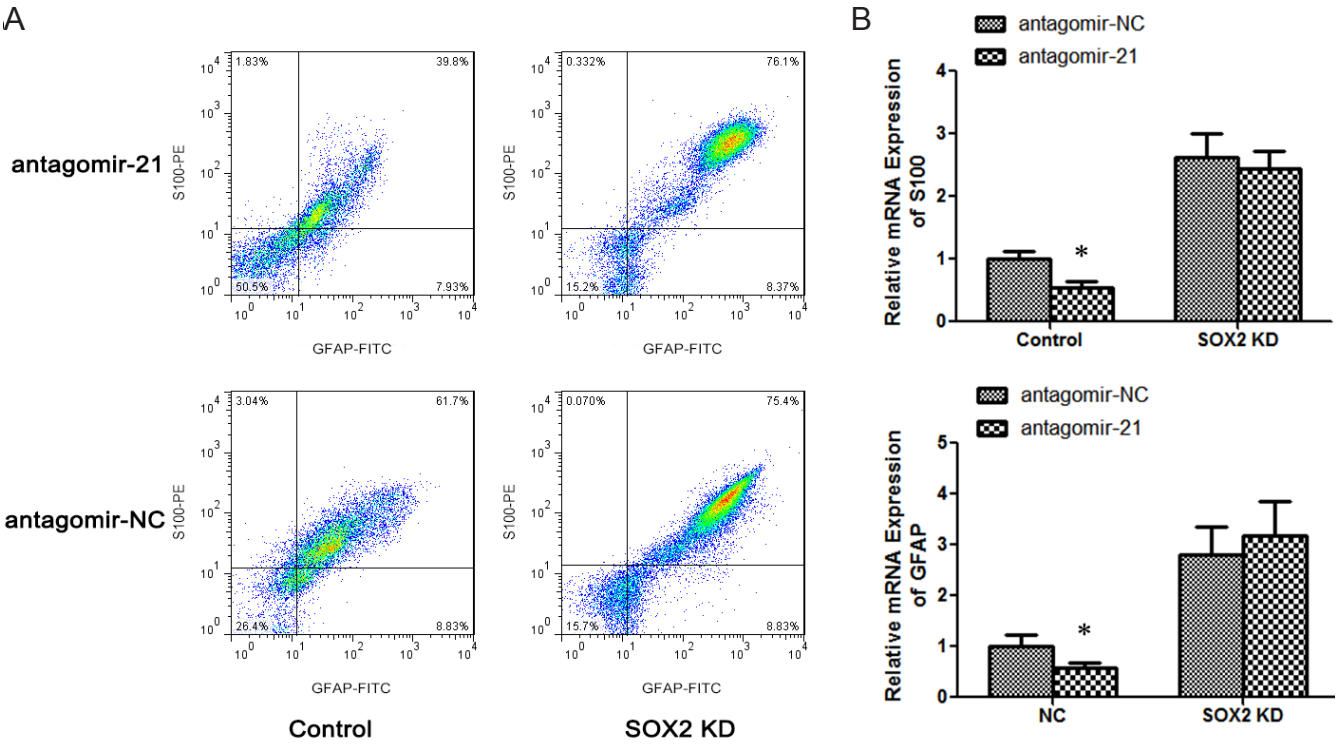

Figure 5 SOX2 knockout blocked the effect of antagomir-21 against Schwann cell differentiation.
(A) Through flow cytometry, the number of S100/glial fibrillary acidic protein (GFAP) double-positive cells was significantly lower after transfecting neural crest stem cells (NCSCs) with antagomiR-21 than with antagomiR-NC; however, there was no significant difference in the number of S100/GFAP double-positive cells after transfecting SOX2 knocked out NCSCs (SOX2-KD-NCSCs) with antagomir-21 versus antagomiR-NC. (B) Quantitative RT-PCR results showed that S100 and GFAP mRNA expression was significantly lower in antagomir-21-transfected NCSCs than in antagomir-NC-transfected NCSCs; however, there was no significant difference in S100 and GFAP mRNA expression between antagomir-21-transfected SOX2-KD-NCSCs and antagomir-NC-transfected SOX2-KD-NCSCs. S100 and GFAP mRNA expression was considered 1 in the control group. All measurement data are expressed as mean ± SD. The experiment was performed in triplicate. One-way analysis of variance and Student-Newman-Keuls test were used for comparison between groups. *$P < 0.05$, *vs.* antagomir-NC group.

attenuated. This suggests that in addition to aforementioned functions, miR-21 can also promote the differentiation of neural crest stem cells into Schwann cells. Together with the fact that neuregulin-1 promotes miR-21 transcription, it is considered that regulation of miR-21 expression can be used as an important means for neuregulin-1 promotion of stem cell differentiation.

To investigate the mechanism by which miR-21 regulates the differentiation of neural crest stem cells, we sought to find miR-21 targets *via* databases. Results showed that a 7-mer-long sequence at the 3′UTR region of SOX2 mRNA was matched to miR-21. This suggests that SOX2 is likely to be a downstream target gene *via* which miR-21 functions. SOX2 gene encodes a 317 amino acid protein and it exerts the regulatory function of transcriptional factors by binding to target genes through an HMG domain (Gracz and Magness, 2011; Sarkar and Hochedlinger, 2013). SOX2 participates in the embryonic development of vertebrates and maintains the undifferentiated state, multipotent differentiation and self-renewing capabilities of neural stem cells (Liu et al., 2013a; Maucksch et al., 2013). Evidence documents that SOX2 inhibits neural stem cells to differentiate into Schwann cells and subsequent myelination (Le et al., 2005; Adameyko et al., 2012). Therefore, this study selected SOX2 as a potential target of miR-21 and investigated whether miR-21 regulates the differentiation of neural crest stem cells by influencing SOX2 expression. Luciferase assay results showed that after transfection with agomiR-21, the fluorescence intensity of fluorescence reporter plasmid containing SOX2-3′UTR-wt was significantly decreased, while the fluorescence intensity of that containing SOX2-3′UTR-mt was not altered. Western blot assay results showed that when miR-21 was over-expressed, SOX2 protein expression in neural crest stem cells was decreased, while when miR-21 expression was silenced with antagomir-21, SOX2 protein expression was increased. This indicates that miR-21 can inhibit SOX protein expression by binding to the 3′-UTR of miR-21 mRNA.

Although SOX2 is a downstream target of miR-21, whether it participates in miR-21 promotion of neural crest stem cell differentiation needs to be further investigated. For this reason, we transfected antagomiR-21 into SOX2-KD-neural crest stem cells to investigate the relationship of SOX2 and miR-21 in the differentiation of neural crest stem cells. Results showed that antagomiR-21-transfected SOX2-KD-neural crest stem cells exhibited stronger differentiation capacity than non-transfected SOX2-KD-neural crest stem cells and non-interfered neural crest stem cells. These results demonstrate that SOX2 knockout can block the inhibitory effect of antagomiR-21 on the differentiation of neural crest stem cells and directly indicate that SOX2 is an important downstream target of miR-21 in the promotion of neural crest stem cell differentiation.

Taken together, after neuregulin-1 transfection, miR-21 expression was increased and miR-21 promoted the differentiation of neural crest stem cells into Schwann cells through down-regulating SOX protein expression by binding to the 3′-UTR of SOX2 mRNA. This study adds to the findings surrounding miRNAs and Schwann cell differentiation and provides a new insight into the repair of injured peripheral nerve by tissue engineering.

Author contributions: *Ni YX and Zhou YM designed and evaluated this study. Graphs were created by Liu XJ. All authors participated in experiment performance and data analysis, and approved the final version of this paper.*
Conflicts of interest: *None declared.*

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