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Inhibition of miR-17-5p promotes mesenchymal stem cells to repair spinal cord injury

X.-H. YUE[1,2], L. GUO[3], Z.-Y. WANG[3], T.-H. JIA[1]

[1]Department of Orthopedics, Jinan Central Hospital Affiliated to Shandong University, Jinan, China
[2]Department of Orthopedics, Jinan Military General Hospital, Jinan, China
[3]Department of Spinal Surgery, Jinan Central Hospital Affiliated to Shandong University, Jinan, China

Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-17-5p (miR-17-5p) in the pathogenesis of spinal cord injury repair by mesenchymal stem cells (MSCs), and to investigate the possible underlying mechanism.

MATERIALS AND METHODS: MiR-17-5p mimics and negative controls were transfected into MSCs. Dual-luciferase reporter gene assay was used to verify the functional binding between miR-17-5p and its target mRNA. After overexpression or knockdown of miR-17-5p, the expression level of target genes in MSC cells was analyzed by Real-time quantitative polymerase chain reaction (qRT-PCR) and Western blot. The proliferation ability of cells was detected by cell counting kit-8 (CCK-8) assay. The effect of miR-17-5p and VEGF-A on angiogenesis was assessed by HUVEC assay. T8 spinal cord injury model was constructed in nude mice. All mice were divided into the negative control group, the SCI group, the miR-17-5p-NC group, the miR-17-5p-inhibitor group, and the miR-17-5p-inhibitor + sh-VEGF-A group. After injection of different treated MSCs at the lesion site, the proportion of intact tissue as well as reduced lumen volume was measured at 28 d. Meanwhile, the motor function of hind limbs was scored based on the Basso Beattie Bresnahan (BBB) standard scale at 7 d, 14 d, 21 d, and 28 d after transplantation, respectively.

RESULTS: A binding site of miR-17-5p was found on the mRNA of VEGF-A. The protein expression of VEGF-A was strikingly altered after overexpression or knockdown of miR-17-5p. Knocking down miR-17-5p expression significantly increased the protein level of VEGF-A and GDNF. Meanwhile, miR-17-5p down-regulation significantly enhanced the viability and the angiogenic ability of MSCs. However, simultaneous knockdown of miR-17-5p and VEGF-A showed the opposite results. After spinal cord injury, the proportion of intact spinal cord tissues in mice was significantly reduced, whereas reduced lumen volume was remarkably increased. After injection of MSCs alone, the proportion of intact tissues was significantly increased. After knocking down miR-17-5p, the proportion was further increased. However, no significant effect was found on the amount of intact tissues after knocking out VEGF-A. Moreover, the reduction in cavity volume appeared to present an opposite trend comparable to the proportion of intact tissues. The BBB scores were significantly decreased in the mice model, while remarkably increased after MSC transplantation. Furthermore, the BBB score was the highest in the miR-17-5p knockout group, while VEGF-A knockout had little effect on it. In addition, no significant difference was found in the mRNA expression GFP in the spinal cord of mice in different groups after MSCs treatment.

CONCLUSIONS: Inhibition of miR-17-5p up-regulates the expression of VEGF-A and GDNF in MSCs, and promotes the repair of spinal cord injury by MSCs.

Key Words
MicroRNA-17-5p (MiR-17-5p), Mesenchymal stem cells (MSCs), VEGF-A, Spinal cord injury repair.

Introduction

Spinal cord injury (SCI) is one of the most common spinal diseases. It is also a serious complication that can cause severe dysfunction of the limb below the injured segment. The incidence of SCI is increasing year by year. However, due to the lack of regenerative ability of neurons, the treatment of SCI has always been a difficult point in the medical field. This seriously threatens human health and brings a heavy burden to the society and families[1]. Currently, the main purpose of treatment plans for SCI is to reduce secondary injury and to maximize the retention of spinal cord residual function[2]. The main treatments can be divided into two categories, including spinal nerve protection and nerve regeneration therapy. Meanwhile, interventions include surgical decompression, spinal cord hypothermia, target-

Corresponding Author: Tanghong Jia, MD; e-mail: fzmi82@163.com

ing inflammation, and excitatory toxic drugs[3-6]. Nerve regeneration therapy mainly refers to the treatment of myelin-related inhibitors and adult cell transplantation. Researchers[7] have demonstrated that cell transplantation may be the most promising method to improve the recovery of neurological function after SCI. Stem cells are a class of cells with self-renewal and multi-directional differentiation potential. MSCs have not only the basic characteristics of stem cells, but also are easy to obtain, isolate, culture, proliferate and express foreign genes. They maintain the potential of multi-directional differentiation during long-term *in vitro* culture. Meanwhile, the genetic background of MSCs is quite stable. There are no ethical problems like neural stem cells and embryonic stem cells. MSCs also have the prerequisites for the source of nerve repair cells[8,9]. Investigations have found that MSCs can invade the injured site and phagocytize the necrotic tissue, thereby inhibiting scar formation that is not conducive to nerve regeneration[10]. In addition, MSCs can secrete a variety of nerve growth factors, which also promote the secretion of nerve growth factors in the central nervous system[11]. Scholars[12] have demonstrated that MSCs play a significant role in nerve regeneration, including promoting the regeneration of autologous nerve cells and directed differentiation of transplanted cells into neuron-like cells and glial-like cells. Meanwhile, BMSCs also facilitate local neovascularization and vascular remodeling, eventually improving neurotrophic situation[10]. MiRNAs are a class of highly conserved non-coding small RNA molecules that are widely found in living organisms. Mature miRNAs are involved in regulating gene expression by base-pairing with complementary sequences of targeted mRNAs. They also affect gene expression, cell cycle, and ontogenesis of eukaryotes[13]. Recent works have shown that miRNAs play a regulatory role in spinal cord development, spinal cord plasticity, and pathological changes after SCI. Some miRNAs may be effective targets for therapeutic intervention after SCI. Studies found that miR-124a, miR-20a, miR-486, and miR-133b are differentially expressed after SCI. It has been observed that miR-20a is significantly up-regulated, which is also related to motor neuron degeneration. Inhibition of miR-20a expression can effectively induce motor neuron survival and neurogenesis by regulating its target gene Ngn1[14]. Although miR-133b has a positive effect on motor function recovery and nerve regeneration after spinal cord transection in zebrafish, its effect is achieved by negative regulation of the protein expression of small GTPase RhoA[15]. Hong et al[16] have demonstrated that miR-17-5p is up-regulated in a SCI model, promoting astrocyte proliferation and glial scar formation. However, the role of miR-17-5p in the treatment of SCI by MSCs has not been reported yet.

Materials and Methods

Extraction of MSCs

4-week-old Sprague Dawley (SD) rats were sacrificed by cervical dislocation. The lower limb skin of the rat was cut and open, and the femur with muscle was removed and placed in 75% alcohol. The soft tissue covered by the femur of rats was peeled off and placed in phosphate-buffered saline (PBS). The rat femoral condyle was cut, and the medullary cavity was washed with Dulbecco's Modified Eagle's Medium (LG-DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). After repeated pipetting and rinsing, Ficoll-Paque was centrifuged. The cell suspension was reconstituted with LG-DMEM medium containing 10% FBS and counted under a light microscope using a hemocytometer. This study was approved by the Animal Center of Shandong University Ethics Committee.

Cell Culture

The density of MSCs was adjusted to 10^4-10^5cells/mL, and MSCs were inoculated into the culture flask. The cells were cultured in an incubator at 37°C, with 5% CO_2 and saturated humidity. Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO_2 incubator.

Lentiviral Transfection

Rat BMSCs were seeded into 24-well plates at a density of 2×10^6/mL, followed by culture for 48 h. Lentiviral supernatant containing miR-17-5p/sh-VEGF-A and the corresponding negative control were added to the culture medium. Subsequently, 5 mL/L polyamine were added to achieve a multiplicity of infection of 20-100. The cells were then cultured in an incubator for 24-72 h. The transfection condition was observed under an inverted fluorescence microscope.

SCI Model and MSC Treatment

Rats were anesthetized by intraperitoneal injection of chloral hydrate. After that, the rats were placed in the prone position, and the midline incision was taken. With the 8th thoracic vertebral body as the center, the paravertebral muscles were removed. Meanwhile, the T7-T9 stage spinous processes and lamina were also removed to completely expose the *dura mater*. The impact rod was used to hit the spinal cord from a height of 25 mm. MSCs were injected into the rat spine with a 25G needle at a rate of 10^6/500 μL, and reflux should be avoided during this process. Foam thrombin was subsequently injected. After surgery, 0.05 mg/kg buprenorphine was administered subcutaneously for analgesia. The dietary status and the skin around the wound were closely monitored.

Pathological Observation

On the 28th day after surgery, 6 rats in each group were deeply anesthetized with intraperitoneal injection of sodium pentobarbital. The left ventricle was perfused with normal saline until the effluent was clarified. After perfusion with 200 mL 4% paraformaldehyde, spinal cord tissue of the injured segment was taken for embedding. HE staining was performed along the longitudinal section of the spinal cord. Meanwhile, the area of each slice cavity was multiplied by the interval thickness (50 μm) to estimate the volume of the spinal cavity and averaged. The intact tissue was estimated by subtracting the volume of the cavity from the total volume.

Behavioral Observation

On the 4th, 14th, 21st, and 28th day after model transplantation, Basso Beattie Bresnahan (BBB) scores were evaluated by two experimental personnel. Both of them were familiar with the scoring criteria but blind to the specific group[17]. Grading processes were as follows: the rats were placed on a test bench, and the scores were obtained after 10 minutes of independent observation. After each scoring, the average of the two scores was taken as the final result and recorded for statistical analysis.

Collagen Gel Test

HUVEC endothelial cells were embedded in collagen gel and seeded into 24-well plates. The collagen was polymerized by placing it in an incubator for 15 minutes. Subsequently, the same amount of MSCs with different treatments was added to a chamber, followed by continuous culture in an incubator. The medium was changed daily. The culture images were photographed, and the angiogenesis of HUVEC was finally quantified.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was detected using CCK8 kit (Dojindo, Kumamoto, Japan). The suspension of MSCs was prepared, and 1×10^4 cells per well were seeded into 96-well plates. After 24 h of culture, the reaction was terminated and the medium was discarded. 10 μL CCK8 solution were added in each well and incubated for 4 h. The absorbance of each well at the wavelength of 450 nm was measured using a microplate reader.

Luciferase Reporter Gene Assay

Potential binding sites for VEGF-A and miR-17-5p were predicted by bioinformatics software (Starbase v2.0). The predicted binding sequence of miR-17-5p on VEGF-A was cloned by PCR and inserted into the luciferase reporter vector to establish the reporter vector VEGF-A-wt. After mutation of the binding site, relevant mutant reporter plasmid VEGF-A-Mut was established. Subsequently, VEGF-A-wt, VEGF-A-Mut (500 ng) and the miR-17-5p overexpression plasmid (100 nmol/L) were co-transfected into cells according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 h after transfection, the cells were collected. Finally, luciferase activity of each group was detected by Luciferase assay kit.

Total RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Collected cells were washed with PBS twice, then TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added. After extraction with chloroform, the aqueous phase was transferred to a new tube. The RNA in the aqueous phase was precipitated with isopropanol, washed with 75% ethanol and dried at room temperature. After dissolving with diethyl pyrocarbonate (DEPC) water, extracted RNAs were placed in a -20°C refrigerator for subsequent use. Extracted RNA was reverse transcribed into cDNA according to the instructions. QRT-PCR was performed using the SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). The specific reaction conditions were as follows: pre-denaturation at 95°C for 15 min, denaturation at 94°C for 15 s, 55°C for 30 s, extension at 72°C for 30 s, for a total of 40 cycles. Fluorescence was collected at 75-80°C and finally analyzed at 65-95°C for melting curve anal-

ysis. Primers used in this study were: GAPDH (F: 5'-TGAGATCAACGTGTTCCAGTG-3', R: 5'-ACCAGATGAAATGTGCCCC-3'), VEGF-A (F: 5'-CTGTACCTCCACCATGCCAA-3', 5'-GCTGCGCTGATAGACATCCA-3'), GDNF (F: 5'-AGAGGGGCAAAAATCGGGG-3', R: 5'-CCGCTGCAATATCGAAAGATCA-3'), GFP (F: 5'-AATCGGTTCTAAACGAGAG-3', R: 5'-ACAATCATAGCCTGCACGCTGC-3'), U6 (F: 5'-AACGCTTCACGAATTTGCGT-3', R: 5'-CCAAGCTTATGACAGCCATCATC-3') microRNA-17-5p (F: 5'-TCATAGGCAAATG-GATGAAAATGG-3', R: 5'-ATGTGTTCTGTG-CATCTAGGG-3').

Western Blot

Total protein of each group was lysed using RIPA lysate (Beyotime, Shanghai, China). Subsequently, the protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After electrophoresis in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with primary antibodies (1:1 000 dilution) at 4°C overnight. The next day, the membranes were washed with Tris-buffered saline and Tween 20 (TBST), and incubated with the corresponding secondary antibody (1:3 000 dilution) at room temperature for 2 h. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) were used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. *t*-test was performed to analyze the difference between groups. $p \leq 0.05$ was considered statistically different.

Results

MiR-17-5p Could Bind to VEGF-A

Previous investigations[16] have demonstrated that miR-17-5p is up-regulated in the SCI model and may promote the development of SCI. We then used bioinformatics to predict the target gene of miR-17-5p, and found that there was a miR-17-5p binding site on VEGF-A mRNA (Figure 1A). This suggested that miR-17-5p might regulate VEGF-A expression. Luciferase reporter gene assay results showed that the luciferase activity was significantly reduced in the VEGFA-WT 3' UTR group. However, there was no significant difference in VEGFA-MUT 3'UTR luciferase activity (Figure 1B). To further verify the regulatory effect of miR-17-5p on VEGF-A expression, we detected the mRNA and protein expression levels of VEGF-A after overexpression of miR-17-5p in cells. Results indicated that there was no significant change in mRNA level (Figure 1C), while the protein expression of VEGF-A was remarkably down-regulated. Meanwhile, the protein level of VEGF-A was significantly up-regulated after knockdown of miR-17-5p (Figure 1D). These results indicated that miR-17-5p could target to VEGF-A and inhibit VEGF-A expression.

Knockdown of miR-17-5p Promoted VEGF-A Expression and Enhanced Angiogenesis

Results found that the mRNA expression of VEGF-A was not significantly altered after knockdown of miR-17-5p in cells. In addition to VEGF-A, we also examined the effect of miR-17-5p on GDNF expression. Results demonstrated that knockdown of miR-17-5p and VEGF-A had little effect on the mRNA expression of GDNF (Figure 2A). Meanwhile, we inhibited miR-17-5p expression in cells, and detected the protein expression of VEGF-A and GDNF. Western blot demonstrated that VEGF-A and GDNF were both remarkably increased when compared with the control group. Knockdown of VEGF-A in cells reversed the increased level of VEGF-A and GDNF induced by miR-17-5p down-regulation (Figure 2B). Multiple studies have found that VEGF-A plays an important role in cell proliferation. In the present study, we found that the viability of MSCs was significantly increased after miR-17-5p knockdown, which could be offset by inhibiting VEGF-A expression (Figure 2C). Additionally, collagen gel assay was performed to test the angiogenesis ability of MSCs in HUVEC cells. Results indicated that the angiogenesis ability of MSCs in the miR-17-5p-inhibited group was significantly up-regulated. Meanwhile, low expression of VEGF-A also counteracted the promoting effect (Figure 2D). The above results suggested that knockdown of miR-17-5p promoted the expression of VEGF-A and the angiogenesis ability of MSCs.

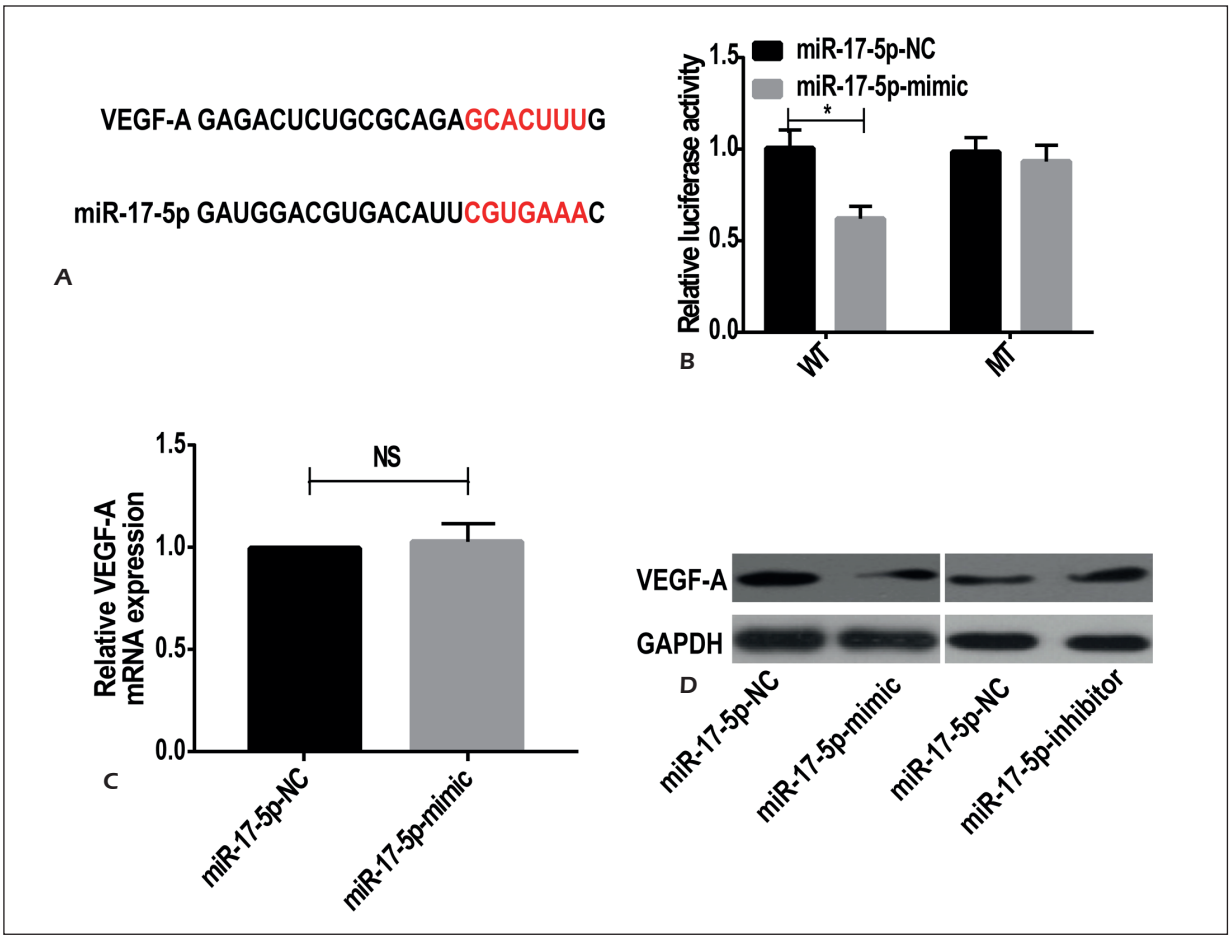

Figure 1. MiR-17-5p could target VEGF-A. **A**, The binding site of miR-17-5p on VEGF-A mRNA predicted by bioinformatics website. **B**, After co-transfection with wild-type VEGF-A and miR-17-5p mimics in MSCs, fluorescence was quenched. **C**, Overexpression of miR-17-5p did not change the mRNA expression of VEGF-A in MSCs. **D**, The protein expression of VEGF-A in MSCs was decreased after overexpression of miR-17-5p, while was increased after knockdown of miR-17-5p.

Knockdown of miR-17-5p Had No Effect on the Treatment of SCI by MSCs

We further explored whether miR-17-5p and VEGF-A had therapeutic effects in SCI. We established a spinal cord continuation model in nude mice and injected different groups of MSCs for treatment. Samples were collected 28 days later, and histological evaluation was performed. Results showed that the proportion of intact tissues was significantly increased after injection of MSCs alone. Meanwhile, the proportion of intact spinal cord tissue in the miR-17-5p inhibitor group was further increased when compared with the miR-17-5p NC group. However, knockdown of VEGF-A had no significant effect on the proportion of intact spinal cord tissue (Figure 3A). In contrast, the volume of spinal cord cavity in the miR-17-5p inhibitor group was significantly lower than that of the miR-17-5p NC group. Knocking down VEGF-A had no significant effect on cavity volume (Figure 3B). In addition, significant dyskinesia occurred in both lower extremities of rats after modeling. The results demonstrated that the scores of rats injected with miR-17-5p inhibitor were remarkably higher than those injected with MSCs alone. Knockdown of VEGF-A had no significant effect on the motor function of lower limbs in rats (Figure 3C). These results suggested that knockdown of miR-17-5p had no significant effect on the treatment of SCI mediated by VEGF-A up-regulation.

Survival and Spread of Transplanted MSCs In Vivo

To understand the survival and spread of transplanted MSCs, we extracted the mRNA of spinal cord in rats at different time points after MSCs injection, and detected the expression level of GFP.

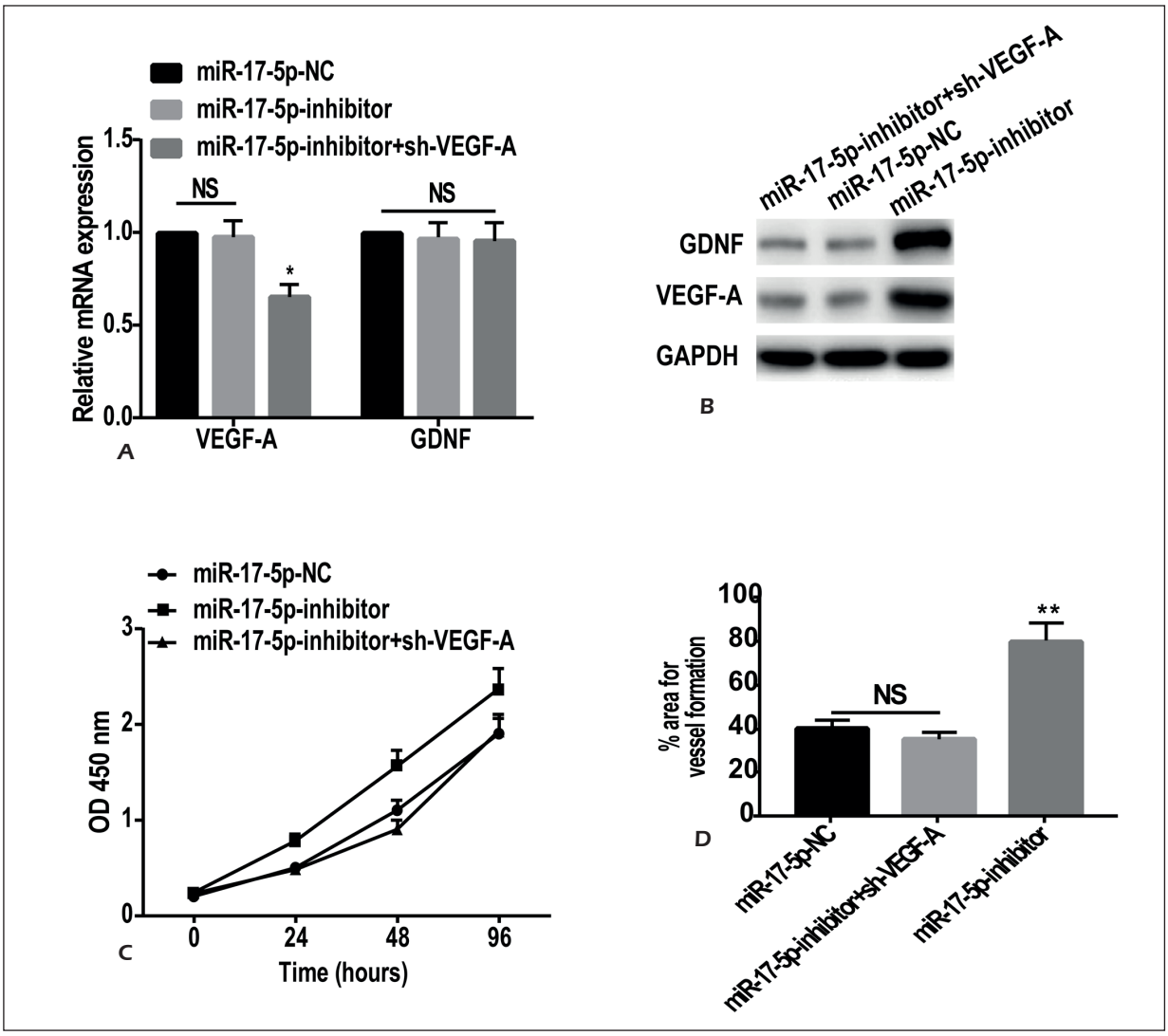

Figure 2. Knockdown of miR-17-5p increased VEGF-A expression and promoted angiogenesis in MSCs. **A**, Knockdown of miR-17-5p did not affect the mRNA expression of VEGF-A, but decreased the mRNA expression of VEGF-A. Various treatments had little effect on the mRNA expression of GDNF. **B**, After knockdown of miR-17-5p, the protein expression of VEGF-A and GDNF were significantly increased in MSCs cells. **C**, CCK-8 assay showed that the viability of MSCs was increased after knocking down miR-17-5p. **D**, HUVEC collagen gel assay found that the angiogenic ability of MSCs was increased after knockdown of miR-17-5p. Meanwhile, this ability was attenuated after knocking down VEGF-A at the same time.

GFP is a transgene existing in transplanted MSCs, which does present in host cells. Compared with D0 (sampled immediately after transplantation), no significant decrease or increase was found in GFP expression in D2, D7, D14, and D28 spinal cord tissues of the three groups injected with MSCs. These indicated that MSCs survived well *in vivo* and underwent limited proliferation during the experiment (Figure 4). The above findings all demonstrated that up-regulation of VEGF-A after miR-17-5p inhibition did not impair the therapeutic potential of MSCs in the treatment of SCI in rats.

Discussion

SCI is currently recognized as one of the refractory diseases. With the characteristics of high incidence, disability, cost and low mortality, SCI seriously threatens human health. Therefore, the treatment of SCI has become a world issue[17,18]. As one kind of stem cells in early mesodermal development, MSCs have the ability to differentiate into various cells originating from the mesoderm, such as osteoblasts, chondroblasts, myocytes and adipocytes[19]. Under the action of

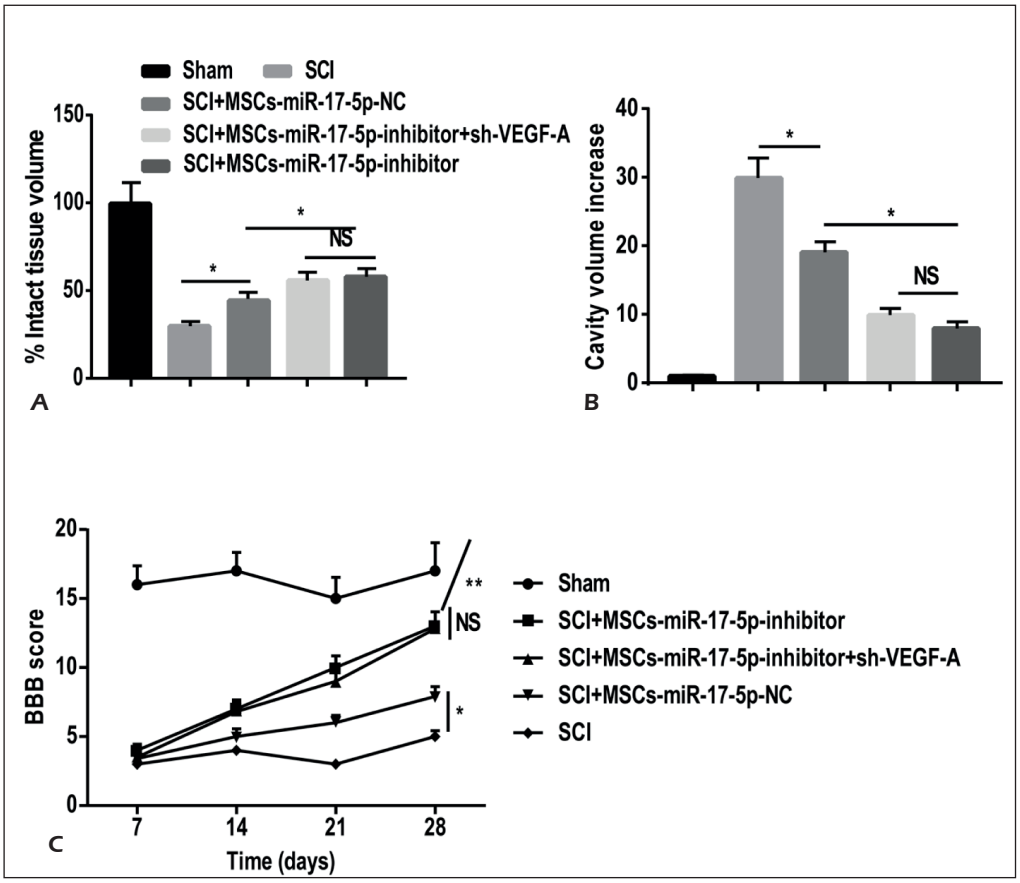

Figure 3. Knockdown of miR-17-5p did not affect MSCs treatment of SCI. **A**, The proportion of intact tissue and reduced lumen volume were detected in rats of different model groups. **B**, After injection of MSCs alone, the proportion of intact tissues was increased. The number of completed tissues in the MSCs treated group after knockdown of miR-17-5p was significantly higher than that of the MSCs alone group. The reduced cavity volume had a reverse trend with the proportion of intact tissue. **C**, The BBB score results were consistent with the results of the proportion of the full organization.

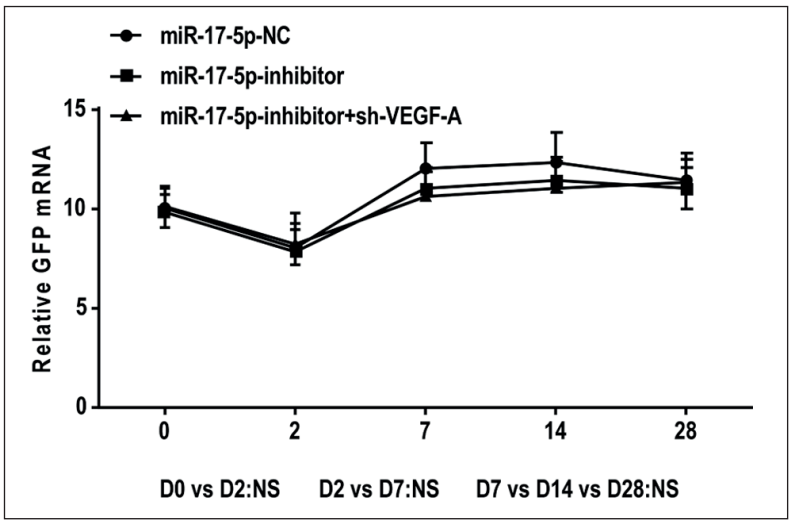

Figure 4. Survival and spread of transplanted MSCs *in vivo*. The mRNA was extracted from the spinal cord of mice at different times after injection with MSCs. The expression of GFP was detected. GFP was present in transplanted MSCs cells but was not present in host cells. There was no significant difference in GFP expression between different days.

appropriate inducing factors, MSCs can differentiate into neural cells and other tissue cells, which also express specific markers. MSCs have now become stem cells possessing broad application prospects[20]. Currently, phase I clinical trials of MSCs for the treatment of SCI have been successfully carried out. In addition, previous clinical trials have demonstrated that it is safe to implant autologous MSCs in an orderly manner[21]. At present, researches[22, 23] have illustrated that a variety of miRNAs are involved in the regulation of SCI development, including the pathophysiological process and the recovery process. For example, miR-219 plays an important role in the regulation of astrocyte proliferation, oxidative stress, and inflammatory response. Meanwhile, miR-219 can cause lipid accumulation in the white matter of the brain through activating its downstream gene ELOVL7. This suggests that up-regulation of miR-219 may attenuate SCI[24]. Meanwhile, up-regulation of miR-126 can reduce the axonal apoptosis rate and enhance the recovery of motor function through targeting PIK3R2 and the growth factor-related regulatory gene SPRED1[25, 26]. Previous works[16] have shown that miR-17-5p is up-regulated in a SCI model. Vascular endothelial growth factor can stimulate the functional recovery of rats after traumatic SCI[27]. Numerous investigations[28, 29] have demonstrated that VEGF-A is beneficial for the recovery after SCI, in which angiogenesis plays a vital role. GDNF is considered as an important survival factor for motor neurons, thereby serving its important clinical value for the treatment of SCI[30]. We found that miR-17-5p could target VEGF-A and regulate the protein expressions of VEGF-A and GDNF. Inhibition of miR-17-5p could strikingly increase the viability and angiogenic ability of MSCs through regulating VEGF-A. We also demonstrated that the increased level of VEGF-A mediated by lowly-expressed miR-17-5p had no inhibitory effect on the therapeutic potential of MSCs for SCI. Moreover, inhibiting VEGF-A expression showed no evident influence on the repair of SCI caused by lowly-expressed miR-17-5p.

Conclusions

We found that the inhibition of miR-17-5p can enhance the ability of MSCs to promote angiogenesis by regulating VEGF-A expression. Moreover, this has no connection with the repair of SCI promoted by VEGF-A.

Conflict of Interests

The authors declare that they have no conflict of interest.

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