

High expression of VEGFA in MSCs promotes tendon-bone healing of rotator cuff tear via microRNA-205-5p

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Abstract. – OBJECTIVE: To explore the role of vascular endothelial growth factor A (VEGFA) in tendon-bone healing of rotator cuff tear (RCT) and to investigate its possible mechanism.

MATERIALS AND METHODS: Mesenchymal stem cells (MSCs) were transfected with pcDNA-VEGFA. The viability of MSCs was detected by cell counting kit-8 (CCK-8) assay. Expression levels of type I and type II collagen in MSCs were detected by quantitative Real time-polymerase chain reaction (qRT-PCR). RCT was constructed in rats. Meanwhile, all rats were divided into MSCs group and MSCs-pcDNA-VEGFA group, respectively. Biomechanical test was performed to detect ultimate load of failure and stiffness in RCT rats. Dual-luciferase reporter gene assay was conducted to analyze the binding condition between microRNA-205-5p and VEGFA, which was further verified by Western blot and qRT-PCR.

RESULTS: VEGFA overexpression significantly promoted viability and proliferation of MSCs. Expression levels of type I and type II collagen were significantly upregulated after VEGFA overexpression in MSCs. Biomechanical test showed that VEGFA overexpression in RCT rats remarkably elevated ultimate load of failure and stiffness. Dual-luciferase reporter gene assay elucidated that VEGFA was the target gene of microRNA-205-5p. Furthermore, VEGFA negatively regulated microRNA-205-5p expression.

CONCLUSIONS: VEGFA promotes tendon-bone healing of RCT via inhibiting microRNA-205-5p expression.

Key Words:

RCT, VEGFA, MicroRNA-205-5p, MSCs.

Introduction

Rotator cuff tear (RCT) is a common disease in sports medicine, which is also a major cause of

shoulder pain and dysfunction^{1,2}. Degeneration of shoulder joint may occur if RCT is not repaired in time or the repair effect is poor^{3,4}. Even after surgical repair, it is difficult to heal RCT in complete level, accompanied by high non-healing rate of tendon-bone in RCT site. It is reported that 11-36% RCT patients may experience re-rupture due to non-healing of tendon-bone around RCT site⁵⁻⁷. Therefore, it is urgent to develop new repair strategies to improve the tendon-bone healing of RCT.

Mesenchymal stem cells (MSCs) are multi-potential stem cells mainly found in bone marrow tissue. MSCs exert potentials of self-renewal and differentiation. They can be differentiated into osteoblasts, fibrocartilage cells, muscle cells, muscle-bond cells, and fat cells. Meanwhile, they are important seed cells for tissue engineering. MSCs have a strong reproductive ability, which can still maintain good proliferation and differentiation ability after repeated passages^{8,9}. Easy collection and culture allow MSCs to participate in the renewal of various tissues. Therefore, they have shown important application prospects in various fields. Vascular endothelial growth factor (VEGF) is the most potent and specific pro-angiogenic factor. VEGF exerts an important role in tissue healing and regeneration. It has been found that the VEGF family includes five major members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF). VEGF-A is the most studied and recognized one. Meanwhile, VEGF165 is the most abundant and biologically strongest one. In recent years, VEGF has been proved to be significant in tendon-bone healing. Yoshikawa et al¹⁰ performed AcL reconstruction in 30 Japanese white rats. They have found that VEGF is highly expressed at the healing site of leg bone at 2 and

3 weeks after surgery. Boyer et al¹¹ performed RNA transfer technique in the reconstruction of cruciate ligament in dogs. Their results have indicated that mRNA level of VEGF reaches peak at 7 and 10 days after surgery. However, the application of exogenous VEGF in promoting the tendon-bone healing of RCT is rarely reported. MiRNAs are a class of endogenous, non-coding small RNAs with 21-24 nucleotides in length. They can control target gene expressions by binding to the 3'UTR¹²⁻¹⁴. MicroRNA-205-5p is located on lq32.2, which has been reported in various tumors. The function of microRNA-205-5p varies in different tumors. Current studies have pointed out that microRNA-250-5p is highly expressed in breast cancer, whereas lowly expressed in metastatic breast cancer¹⁵⁻¹⁷. So far, few studies have reported the exact role of microRNA-205-5p in tendon-bone healing of RCT. In the present study, we aimed to explore whether MSCs overexpressing VEGFA could improve tendon-bone healing of RCT. Moreover, we also detected whether microRNA-205-5p could affect VEGFA expression during the process of RCT recovery.

Materials and Methods

Isolation and Culture of MSCs

12-week-old Sprague-Dawley (SD) rats were executed with dislocation of cervical vertebra. Rat femur and tibia were collected under aseptic condition. The marrow cavity was washed with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA). After centrifugation at 1000 r/min for 5 min, MSCs were re-suspended in DMEM containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and maintained in a 5% CO₂ incubator at 37°C. When the confluence was up to 80-90%, cell passage was performed with 0.25% trypsin. This study was approved by the Animal Ethics Committee of Qingdao University Animal Center.

Lentivirus Infection

MSCs were seeded into 6-well plates at a density of 2.0×10^6 cells per well. After overnight culture, MSCs were infected with solution containing pcDNA-VEGFA. DMEM containing 10% horse serum and 5% fetal bovine serum (FBS) was replaced 24 h later. Subsequently, MSCs were cultured for another 24 h, followed by detection of GFP-positive cells using a fluorescence microscope.

Cell Transfection

MSCs were transfected with microRNA-205-5p mimics or microRNA-205-5p inhibitor according to the manufactures' instructions of siPORT NeoFX (Life Technologies, Carlsbad, CA, USA). 48 hours later, transfected MSCs were collected for the following experiments.

Construction of RCT in Rats

A 3-cm longitudinal incision was made on the anterolateral side of the shoulder. Tendons of supraspinatus muscle were exposed and separated sharply from the greater tuberosity. The remaining tendons and muscles on the greater tuberosity were completely cleaned up. A bone tunnel from the anterolateral medial to posterolateral of the greater tuberosity was created at the insertion site of supraspinatus tendon using a 4-0 suture. Rats were randomly assigned into control group, MSCs group and MSCs-pcDNA-VEGFA group, with 18 rats in each group. Rats in control group received implantation of 200 μ L fibrin glue (FG) in the tendon-bone interface. Meanwhile, 200 μ L FG and 2×10^6 MSCs or MSCs-pcDNA-VEGFA mixture was implanted in the tendon-bone interface in rats of MSCs group and MSCs-pcDNA-VEGFA group, respectively. Rats were sacrificed at 4th and 8th week for biomechanical test.

Biomechanical Testing

3 rats in each group were sacrificed at 4th and 8th week, respectively. Complete tendons of supraspinatus muscle and proximal humerus were harvested. Biomechanical test was performed using MTS 858 material testing system. Briefly, tensile load was first eliminated by 0-5 N pre-load for 10 times. Each sample was loaded with a crosshead speed of 14 mm/s, and the load-deformation curves were recorded. Finally, ultimate load of failure and stiffness was calculated using Sigma-Aldrich Plot 8.0 (St. Louis, MO, USA).

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

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). After amplification of cDNA, qRT-PCR was performed to detect the expressions of related genes in strict accordance with SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). Relative gene expression was detected using ABI Prism 7900HT system (Applied Biosystems, Foster City, CA, USA). Primer sequenc-

es used in this study were as follows: type I collagen, F: 5'-CCGTGAATGATAGTGAGGAACC-3', R: 5'-TGAACGATTTGCCACACACA-3'; Type II collagen, F: 5'-GTTGTCCTATAGAAGCACATG-3', R: 5'-ACATTTCCACAGCCCTGTGG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Counting Kit-8 (CCK-8)

MSCs were seeded into 96-well plates and DMEM was discarded until 80% of adherence. 6 replicates were set in each group. Briefly, 20 μ L of CCK-8 solution (Dojindo, Kumamoto, Japan) were added in each well, followed by incubation in dark for 2 h. Optical density (OD) value at the wavelength of 490 nm was determined using a microplate reader.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by the bicinchoninic acid (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Protein sample was separated by gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibodies, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Dual-Luciferase Reporter Gene Assay

The binding sites of microRNA-205-5p and VEGFA were predicted, and wild-type and mutant-type VEGFA were constructed. MSCs were first seeded into 12-well plates. Subsequently, they were co-transfected with 50 pmol/L microRNA-205-5p mimics or inhibitor and 80 ng wild-

type or mutant-type VEGFA for 48 h, respectively. After washing with PBS, MSCs were then incubated with 1 $\times$ PLB for complete lysis. Luciferase activity was finally detected according to relative commercial kit instructions (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Quantitative data were represented as mean $\pm$ standard deviation ($\bar{x} \pm s$). *t*-test was used to compare the differences between two groups. $p < 0.05$ was considered statistically significant.

Results

VEGFA Overexpression Promoted MSCs Proliferation

To explore the role of VEGFA in tendon-bone healing of RCT, we constructed pcDNA-VEGFA and negative control. After 72 h of transfection with lentivirus, the protein expression of VEGFA in MSCs was remarkably upregulated (Figure 1A). Subsequently, CCK-8 results indicated VEGFA overexpression significantly increased the viability of MSCs (Figure 1B). The above results elucidated that VEGFA overexpression promoted MSCs proliferation.

VEGFA Overexpression Promoted the Protective Role of MSCs in Tendon-Bone Healing of RCT

Rats underwent RCT were injected with MSCs or MSCs overexpressing VEGFA, respectively.

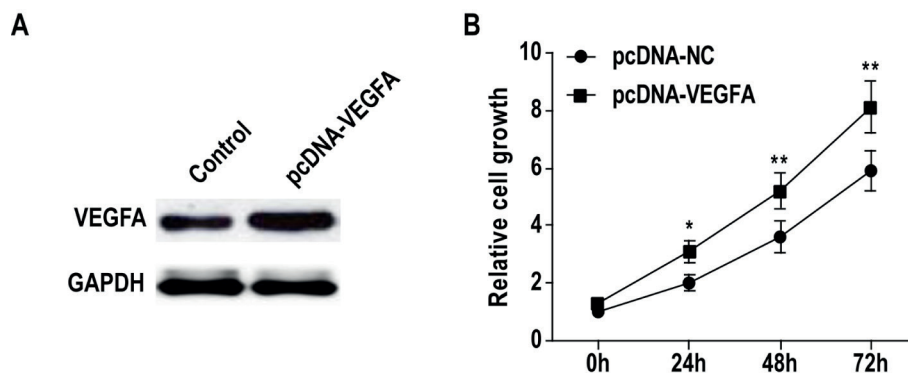


Figure 1. VEGFA overexpression promoted MSCs proliferation. **A**, After 72 h of transfection with lentivirus, the protein expression of VEGFA in MSCs was remarkably upregulated (Figure 1A). **B**, CCK-8 results indicated significantly higher viability in MSCs after VEGFA overexpression.

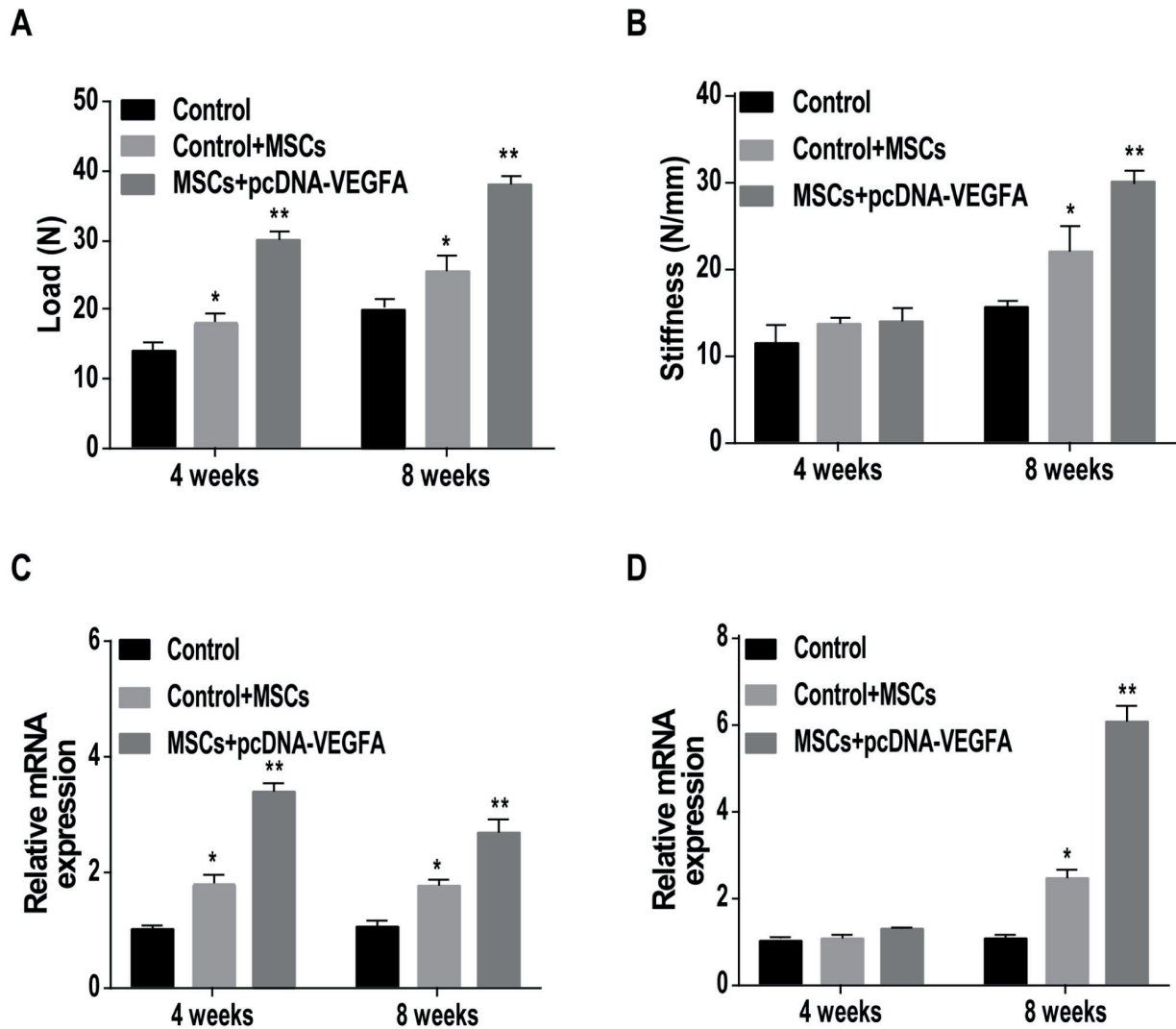


Figure 2. VEGFA overexpression promoted the protective role of MSCs in tendon-bone healing of RCT. **A**, Ultimate load to failure in MSCs-pcDNA-VEGFA group was significantly higher than control group and MSCs group detected at 4th and 8th week. **B**, Stiffness remarkably increased in MSCs-pcDNA-VEGFA group at 8th week. **C-D**, The mRNA levels of type I and type II collagen in control group, MSCs group and MSCs-pcDNA-VEGFA group at 4th and 8th week.

Biomechanical test was performed at 4th and 8th week. Results showed that ultimate load to failure in MSCs-pcDNA-VEGFA group was significantly higher than control group and MSCs group at 4th and 8th week (Figure 2A). However, no significant difference in stiffness was found among the three groups at 4th week. However, stiffness in MSCs-pcDNA-VEGFA group remarkably increased at 8th week (Figure 2B). The mRNA levels of type I and type II collagen in tendon-bone of RCT site were detected by qRT-PCR. Both MSCs group and MSCs-pcDNA-VEGFA group showed significantly upregulated type I collagen expression at 4th and 8th week, which was more pro-

nounced in MSCs-pcDNA-VEGFA group (Figure 2C). No significant difference in the mRNA level of type II collagen was found among the three groups at 4th week. Meanwhile, type II collagen expression gradually increased at 8th week in both MSCs and MSCs-pcDNA-VEGFA groups. In addition, MSCs-pcDNA-VEGFA group showed the highest level of type II collagen at 8th week (Figure 2D).

VEGFA was the Target Gene of microRNA-205-5p

TargetScan predicted the presence of microRNA-205-5p binding sites in the 3'UTR sequence

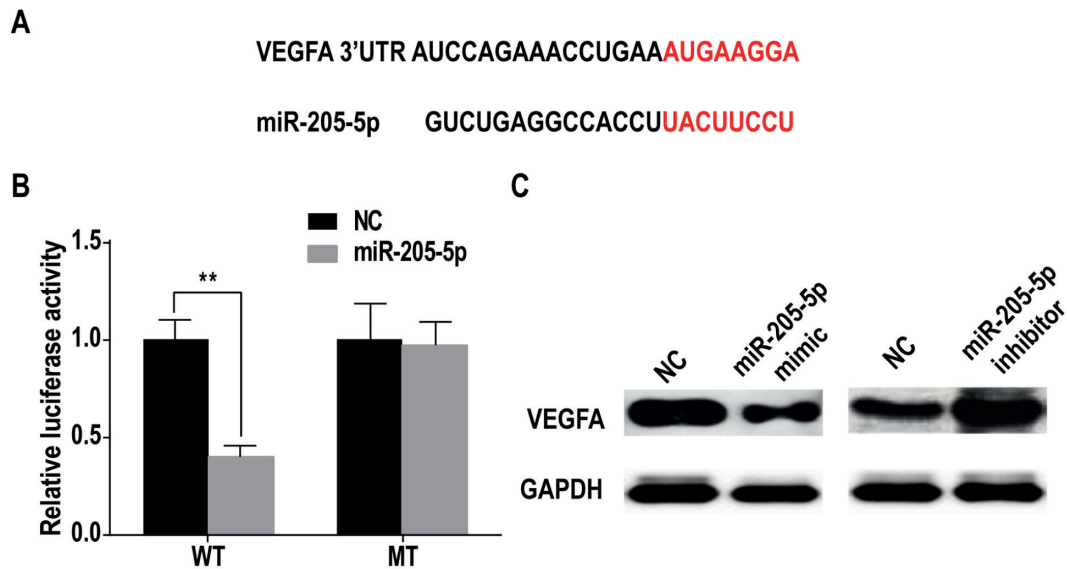


Figure 3. VEGFA was the target gene of microRNA-205-5p. **A**, Binding site for microRNA-205-5p and VEGFA. **B**, Luciferase activity of MSCs transfected with wild-type VEGFA and microRNA-205-5p mimics was significantly lower than those transfected with mutant-type VEGFA and microRNA-205-5p mimics. **C**, Western blot elucidated that microRNA-205-5p overexpression downregulated protein expression of VEGFA in MSCs.

of VEGFA (Figure 3A). Dual-luciferase reporter gene assay was further performed to verify the binding condition. Results showed that luciferase activity of MSCs transfected with wild-type VEGFA and microRNA-205-5p mimics was significantly lower than those transfected with mutant-type VEGFA and microRNA-205-5p mimics (Figure 3B). Western blot elucidated that microRNA-205-5p overexpression significantly downregulated VEGFA expression in MSCs (Figure 3C). On the contrary, microRNA-205-5p knockdown remarkably upregulated VEGFA expression in MSCs.

MicroRNA-205-5p Promoted Tendon-Bone Healing of RCT in Rats

Knockdown of microRNA-205-5p by lentivirus transfection remarkably decreased its expression level in MSCs (Figure 4A). However, the protein expression of VEGFA was significantly increased (Figure 4B). Biomechanical test found that VEGFA knockdown elevated ultimate load to failure and stiffness in RCT rats (Figure 4C and 4D).

Discussion

Due to the relatively high failure rate of RCT repair, the methods to improve the mechanical properties after surgery have been well con-

cerned. Current treatments focus on improving the ecological environment around RCT sites, regenerating natural binding sites and preventing the formation of scar tissue. In this study, we demonstrated that VEGFA overexpression promoted tendon-bone healing of RCT. Meanwhile, microRNA-205-5p was a regulator of VEGFA in the healing process of RCT. Studies have shown that MSCs can be utilized for repairing RCT. Wang et al¹⁸ have indicated that transfection of BMP12 into MSCs of rhesus promotes cell differentiation into tendon cells. Koch et al¹⁹ have demonstrated that growth/differentiation factor-5 (GDF-5) contributes to tendon tissue engineering therapy with MSCs. At present, many methods have emerged to promote tendon-bone healing. However, they are all still in the experimental stage. Fibrocartilage band between leg bones is a typical characteristic structure in normal tibia. Once impaired, it is difficult to regenerate. Recent studies have provided promising applications for repairing fibrocartilage band using MSCs. For example, Lim et al²⁰ have used bio-protein gel combined with MSCs to promote tibia healing after cruciate ligament reconstruction in rabbits. Histological observation reveals that the cartilage region with mature tibial interface is inserted into the tendon graft after 8 weeks. Fibrocartilage cells and type II collagen are well arranged and perpendicular to the tibial interface. These

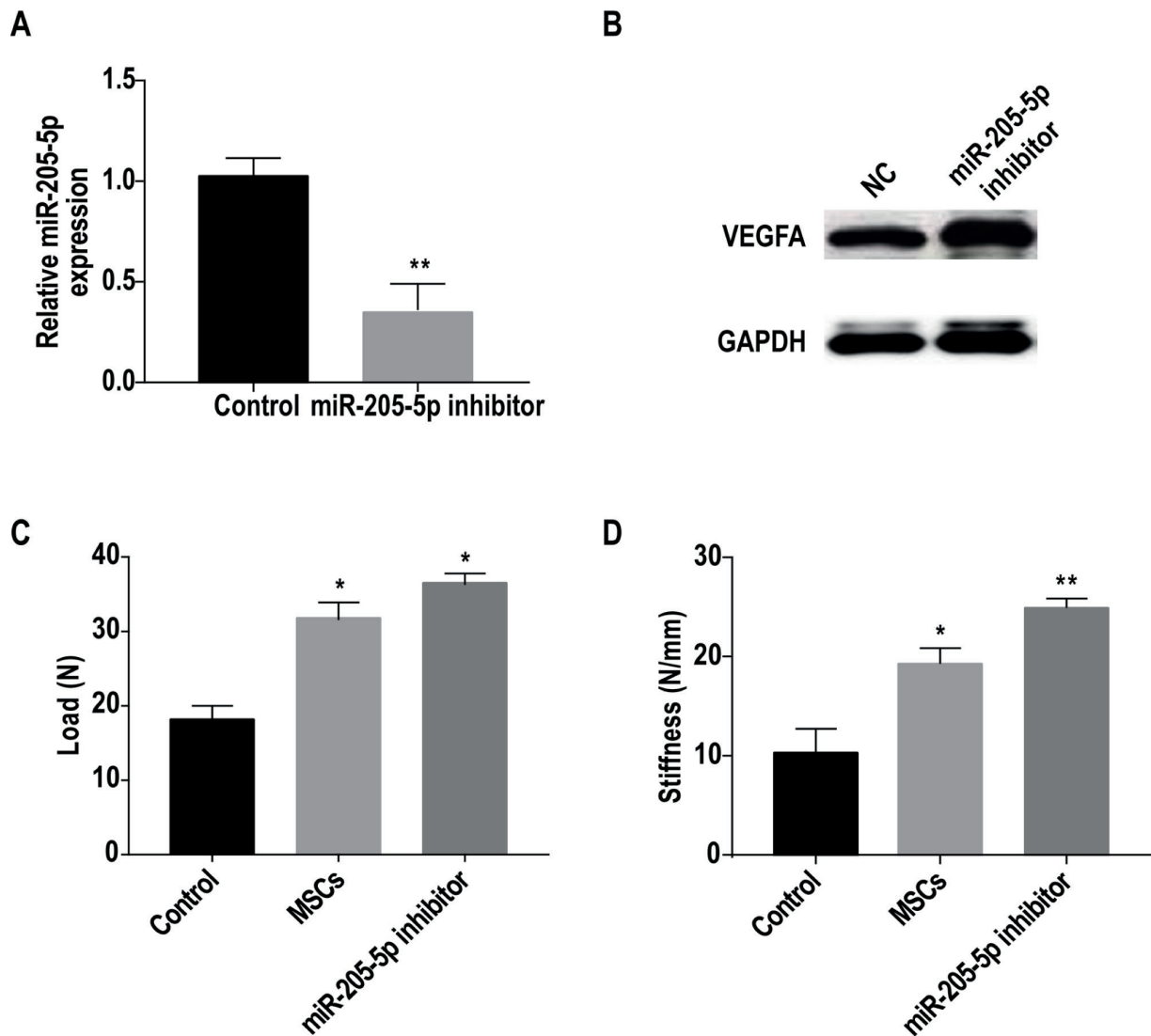


Figure 4. MicroRNA-205-5p promoted tendon-bone healing of RCT in rats. *A-B*, Knockdown of microRNA-205-5p by lentivirus transfection remarkably decreased its expression level in MSCs, whereas the protein expression of VEGFA increased. *C-D*, VEGFA knockdown significantly elevated ultimate load to failure and stiffness in rats.

findings may provide some improved potential methods to promote tendon-bone healing. VEGF exerts an important function in vascularization and reconstruction after ligament reconstruction. Boyer et al²¹ have found that the mRNA level of VEGF achieves peak at 7th and 10th day after anterior cruciate ligament reconstruction in dogs. However, it is reversed to baseline at 14th day. Nagashima et al²² have found VEGF is abundantly expressed in synovial lining cells and fibroblasts near the micro-vessels in the synovial tissues of normal and osteoarthritic knees. They believe that VEGF is derived from synovial tissue of the knee joint. At present, exogenous application of

VEGF has little effect on tendon-bone healing. In this study, we found that high expression of VEGFA could promote the proliferation of MSCs and improve tendon-bone healing of RCT. Overexpression of VEGFA significantly increased ultimate load to failure at 4th and 8th week. Our findings indicated that VEGFA up-regulation also elevated stiffness at 8th week. Compared with those of control group, VEGFA overexpression upregulated the expressions of type I and type II collagen in RCT site. MicroRNA Dicer is absent in osteoblasts, chondrocytes and osteoclasts, suggesting the vital role of miRNA in the normal development of bone formation and

metastasis^{23,24}. We found that microRNA-205-5p knockdown in MSCs remarkably promoted tendon-bone healing of RCT in rats. Dual-luciferase reporter gene assay verified that VEGFA was the target gene of microRNA-205-5p. Knock down of microRNA-205-5p significantly upregulated VEGFA expression, thereby promoting tendon-bone healing of RCT.

Conclusions

We observed that VEGFA promotes tendon-bone healing of RCT *via* inhibiting microRNA-205-5p expression.

Conflict of interest

The authors declare no conflicts of interest.

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