Long noncoding RNA H19 accelerates tenogenic differentiation by modulating miR-140-5p/VEGFA signaling

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ABSTRACT

Rotator cuff tear (RCT) is a common tendon injury, but the mechanisms of tendon healing remain incompletely understood. Elucidating the molecular mechanisms of tenogenic differentiation is essential to develop novel therapeutic strategies in clinical treatment of RCT. The long non-coding RNA H19 plays a regulatory role in tenogenic differentiation and tendon healing, but its detailed mechanism of action remains unknown. To elucidate the role of H19 in tenogenic differentiation and tendon healing, tendon-derived stem cells were harvested from the Achilles tendons of Sprague Dawley rats and a rat model of cuff tear was established for the exploration of the function of H19 in promoting tenogenic differentiation. The results showed that H19 overexpression promoted, while H19 silencing suppressed, tenogenic differentiation of tendon-derived stem cells (TDSCs). Furthermore, bioinformatic analyses and a luciferase reporter gene assay showed that H19 directly targeted and inhibited miR-140-5p to promote tenogenic differentiation. Further, inhibiting miR-140-5p directly increased VEGFA expression, revealing a novel regulatory axis between H19, miR-140-5p, and VEGFA in modulating tenogenic differentiation. In rats with RTC, implantation of H19-overexpressing TDSCs at the lesion promoted tendon healing and functional recovery. In general, the data suggest that H19 promotes tenogenic differentiation and tendon-bone healing by targeting miR-140-5p and increasing VEGFA levels. Modulation of the H19/miR-140-5p/VEGFA axis in TDSCs is a new potential strategy for clinical treatment of tendon injury.

Key words: lncRNA; miRNA; tendon stem cell; rotator cuff tear repair

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Introduction

Rotator cuff tear (RCT) is a common injury that causes shoulder joint pain and muscle weakness.[1] Epidemiological studies have reported that 20% of people over 20 years of age and up to 25% of people over 50 years of age suffer from rotator cuff injury.[2,3] Rotator cuff injury is a primary cause of shoulder joint pain and dysfunction, and severely limits work efficiency and quality of life.[4] In Italy, 62.1 out of every 100,000 people have undergone rotator cuff repair surgery, and according to prediction models, the total amount spent on RCT in 2025 will reach one billion euros.[5] Previously, surgical treatment of RCT was thought to have satisfactory efficacy and outcomes, but recent studies have identified that the long-term efficacy of surgical treatment is uncertain,[6] and that conservative (non-surgical) treatment in fact reduced pain and improved function in patients with mild to moderate RCT.[7] About 11–36% of patients with RCT suffered from re-rupture due to unhealed tendon-bone injuries.[6] Thus, elucidating the detailed mechanisms of tendon injury and recovery to develop novel approaches to RCT treatment is a significant unmet clinical need.

Tendon-derived stem cells (TDSCs) were first identified in rat and human tendons in 2007.[8,9] As tenocyte precursor cells, TDSCs exhibit similar biological functions to mesenchymal stem cells (MSCs).[10] TDSCs exhibit strong multi-directional differentiation potential into tendon, fat, osteogenic, and cartilage tissues.[11] Promoting differentiation of TDSCs into tendon cells and inhibiting differentiation of TDSCs into non-tendon cells under pathological conditions have been identified as effective strategies for treatment of RCT.[12]

miRNAs are short non-coding RNA molecules 17-25 nucleotides in length, and control gene product levels by suppressing translation of target mRNAs, and in some cases by decreasing stability of target mRNAs. miRNAs are involved in a variety of physiological and pathological processes. An increasing body of evidence suggests that miRNAs are closely related to tendinopathy and have therapeutic potential in this context. For example, miRNA-499 is significantly upregulated in samples from injured tendons, and functions by targeting CELF2 and MYB.[13] In another set of miRNA screening experiments for degenerative RCT, expression levels of miR-29a and miR-29c were significantly downregulated in RCT patient serum and biopsy samples.[14] In human tendon stem/progenitor cells (TSPCs), miR-140-5p regulates Pin1 expression, which is decreased during TSPC aging.[15] However, the roles and mechanisms of miR-140-5p in TDSC development and tendon tear repair remain largely unknown.

Long non-coding RNA (lncRNA) plays an important role in transcriptional and post-transcriptional regulation of gene expression, and is a regulatory hub for transcriptional activity and mRNA expression of target genes. Several studies have suggested a relationship between lncRNA and tendon disease, but the regulatory mechanisms remain incompletely understood. Zheng et al. identified that eight dysregulated lncRNAs could be involved in fiber formation after tendon injury.[16] Inhibition of lncRNA KCNQ1OT1 suppressed the adipogenic and osteogenic differentiation of TDSCs via miR-138/PPARg/RUNX2 signaling,[17] and lncRNA H19 has been reported to accelerate TDSC differentiation, thereby promoting tendon development and repairing tendon injuries through the TGF pathway.[18] Growth factors such as VEGFA are major mediators of tendon-bone healing. They promote collagen formation, angiogenesis, and extracellular matrix formation, which are critical for tissue healing and regeneration.[19] VEGFA belongs the VEGF protein family (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor, PGF), and has great potential in tendon-bone healing.

In the present study, we used *in vitro* cultured TDSCs and an *in vivo* RCT rat model to examine the regulatory relationship between H19 and miR-140-5p in tenogenic differentiation during tendon injury recovery.

Materials and Methods

Animals

SD rats were supplied by the Institute of Laboratory Animal Science of Jinan University.

TDSC isolation and cell culture

TDSCs were harvested from the Achilles tendons of SD rats as previously described.[20] Briefly, rat Achilles tendons were digested with type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 2 h at 37°C. Dissociated cells were plated in 100 mm dishes at 200 cells/cm^2, and cultured in DMEM (Gibco, Thermo Scientific, Waltham, MA, USA) with 20% FBS (Gibco), 100 U/mL penicillin (Gibco) and 100 U/ml streptomycin (Gibco) for 10 days in a standard tissue culture incubator. TDSCs at passage 3 or 4 were used for subsequent experiments. TDSCs were confirmed as previously reported.[20]

Rat RCT model

Briefly, a longitudinal incision of approximately 1.5 cm was made on the anterolateral side of the shoulder. The supraspinatus tendon and muscle were exposed, and then half of the tendons were cut and sharply separated from the greater tuberosity. Subsequently, the supraspinatus tendons were sutured layer by layer using a 4-0 suture. Rats were randomly assigned into control, TDSC, or H19-overexpressing TDSC groups, with 10 rats in each group. In the control group, rats were injected with 200 µL fibrin glue in the tendon-bone interface. In the TDSC groups, fibrin glue with 2×10^6 TDSCs or H19-TDSCs was injected. Four or eight weeks later, tendons were subjected to biomechanical testing.

Biomechanical testing

Four or eight weeks after RCT, complete tendons of the rat supraspinatus muscle and proximal humerus were collected and subjected to biomechanical testing using an MTS 858 material testing system (MTS System, Minneapolis, MN, USA). A 0-5 N preload was applied. Samples were loaded with a crosshead speed of 14 mm/s. Ultimate force at failure (N) and stiffness (N/mm) were then measured and recorded using a Sigma-Aldrich Plot 8.0 (Sigma-Aldrich). Ultimate force was determined from the load-displacement curve. Stiffness was calculated by measuring the slope.

Generation of stable TDSCs

H19 overexpression plasmid and shRNA sequences were cloned into the lentiviral vector pLKO.1. Viruses were then transfected into HEK293 cells for packaging. Supernatants containing retrovirus particles were then collected and filtered using 0.45 µm Millex-HV filters (Millipore, Bedford, MA, USA) to remove cell debris. To construct stable cell lines, TDSCs were infected with vector control lentivirus, H19 overexpressing lentivirus, shRNA control lentivirus, or shH19 lentivirus; 48 h after infection, cells were selected by 2 µg/mL puromycin (Sigma-Aldrich), and the efficiency was determined by real-time quantitative PCR (RT-qPCR) assay. shRNA targeting sequences were as follows: shH19 5′-GTGCAGGTAGAGCGAGGTAAA-3′.[21]

Cell transfection

miR-140-5p mimic (5'-ACCAUAGGGUAAAACCACUGUU-

3'), miR-140-5p inhibitor (5'-CUACCAUAGGGU AAAACCACU-3'), and mimic and inhibitor negative controls (NCs) (5'-UUCUCCGAACGUGU CACGUTT-3') were purchased from GenePharma (Shanghai, China) and transfected with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol.

Sirius red staining

A Sirius red staining assay was applied to measure collagen formation. Briefly, TDSCs were cultured in a 12-well plate. For cell harvesting, medium was removed, cells were washed and fixed with 4% paraformaldehyde for 40 min, and subsequently incubated with 0.1% Sirius Red F3BA in a saturated picric acid solution for 45 min. The plate was measured by using a spectrophotometer (BioTek Instruments, Winooski, VT, USA) at 540 nm to quantify the red color.

Luciferase reporter assay

To generate luciferase reporter constructs for H19 and VEGFA, the pGL3 vector (Promega, Madison, WA, USA) was used. H19 or VEGFA (wild-type, WT or mutant, MUT) regions against the binding site of miR-140-5p were cloned into the pGL3 vector. Binding site mutants were generated using a Site-Directed Mutagenesis kit (Thermo Fisher Scientific). Plasmids with an internal reporter (Renilla) were then co-transfected into 293T cells using the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions. Fragments of miR-140-5p mimic, miR-140-5p inhibitor, or NC were co-transfected; 48 h later, cells were harvested, and luciferase activities were tested using a Luciferase Assay System (Promega) and normalized to the internal control signal.

RNA isolation and RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To reverse transcribe mRNA, 1 µg RNA was used to reverse-transcribe mRNA into cDNA with an EasyScript cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) was used. For miRNA cDNA synthesis, a TransScript miRNA First-Strand cDNA Synthesis SuperMix (TransGen) was used. RT-qPCR was performed using TransStart Top Green quantitative PCR SuperMix (TransGen). Primer sequences used are listed in Table 1. For quantification, genes were normalized to *Gapdh* and miRNA to U6.

Western blotting

Samples were lysed using RIPA buffer with protease inhibitors (Beyotime, China). Proteins were then separated by SDS-PAGE (10%) and transferred to PVDF membranes (Millipore). Membranes were blocked with milk and incubated with primary antibodies against VEGFA (#ab46154; Abcam, Cambridge, UK) and GAPDH (#ab8245, Abcam). After secondary antibody (Abclonal Biotechnology, Wuhan, China) incubation for 1 h at room temperature, blots were detected using enhanced chemiluminescence (ECL) (Beyotime). GAPDH was used as a loading control.

Statistical analyses

Statistical analyses were performed using SPSS (27.0). All data are presented as mean±SD. A two-tailed Student's *t*-test (for two groups) or analysis of variance test (ANOVA, for multiple groups, One-Way ANOVA with Tukey *post-hoc* test, or Two-Way ANOVA with Bonferroni *post-hoc* test) was used to compare unpaired samples; $p<0.05$ was considered statistically significant.

Results

Involvement of H19 in tenogenic differentiation

TDSCs have reparative properties in tendon injury, exhibiting spontaneous tenogenic differentiation potentials *in vitro*.[22] H19 has previously been reported to promote tenogenic differentiation of human TDSCs.[18] To confirm this, rat TDSCs were isolated and cultured, and stable H19-overexpression (H19) and H19-shRNA (shH19) TDSCs were generated using a lentiviral system and confirmed by RT-qPCR. TDSCs cells were treated with TGF-β to induce tenogenic differentiation as previously reported.[23,24] As shown in Figure 1A, RT-qPCR demonstrated that the expression of H19 was markedly increased. In the H19 overexpression group, collagen formation significantly increased, as revealed by Sirius red staining (Figure 1B and Supplementary Figure 1A for enlarged details). mRNA levels of tenogenic markers and extracellular matrix (ECM) markers, including *Scx, Mkx*, *Cola1*, *Fmod*, *Tnmd*, and *Dcn*, were significantly upregulated in the H19 overexpression group (Figure 1C). Contrastingly, H19 expression was decreased in the shH19 group (Figure 1D and Supplementary Figure 1B for enlarged details). Genetic silencing of H19 resulted in decreased collagen formation (Figure 1E), and decreased levels of tenogenic and ECM

Table 1. Primer sequences for real-time qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')
H19	TAAAGCAGCTGGGGTGGTGAG	TGACTGGCAGGCACATCCAC
Scx	AGCCCAAACAGATCTGCACCTT	CTTCCACCTTCACTAGTGGCATCA
Mkx	CTCAAGGACAACCTCAGCCTGAG	CGTTGCCCTGAACATACTTGTTGTAC
Tnmd	ATGGGTGGTCCCACAAGTGAA	CTCTCATCCAGCATGGGATCAA
Col1a1	GTCCGAGGTCCTAATGGAGATGC	GGTCCAGGGAATCCGATGT
Col2a1	CCAGGTCCTGCTGGAAAA	CCTCTTTCTCCGGCCTTT
Fmod	CAAGGCAACAGGATCAATGAG	CTGCAGCTTGGAGAAGTTCA
Dcn	GACTCCACGACAATGAGATCACC	GTTGCCATCCAGATGCAGTTC
Vegfa	GCACATAGGAGAGATGAGCTTCC	CACGCCTTGGCTTGTCACAT
Gapdh	TGATTCTACCCACGGCAAGTT	TGATGGGTTTCCCATTGATGA

markers (Figure 1F). These data suggest that H19 is closely related to tenogenic differentiation, which is consistent with previous reports.

H19 targets miR-140-5p to promote tenogenic differentiation

Next, we sought to determine the detailed mechanism of H19 regulation of tenogenic differentiation. Using bioinformatic tools Starbase-ENCORI,[25] we screened candidate miRNAs and found a putative H19 binding site on miR-140-5p (Figure 2A). Overexpression of a miR-140-5p mimic significantly decreased, while a miR-140-5p inhibitor increased, expression of H19 in TDSCs (Fig. 2B). To further assess the potential direct interaction between H19 and miR-140-5p, a dual-luciferase reporter gene assay was used. miR-140-5p significantly reduced the luciferase activity of H19 WT but not mutant H19 (H19 MUT) (Figure 2C). Furthermore, miR-140-5p mimics were transfected into TDSCs with or without H19 overexpressing plasmids. Overexpression of miR-140-5p significantly suppressed tenogenic differentiation with TGF-βtreatment, as demonstrated by Sirius red staining (Figure 2D and Supplementary Figure 1C for enlarged details). However, additional overexpression of H19 markedly abolished the suppressive effect of miR-140-5p (Figure 3D). Taken together, these findings demonstrate that miR-140-5p directly interacts with H19 during tenogenic differentiation.

miR-140-5p targets VEGFA

Next, we sought to determine the potential downstream target genes of miR-140-5p. We screened candidate targets by bioinformatics prediction *via* TargetScan[26] and ENCORI[25] and identified VEGFA as a gene of interest, as its 3'-UTR region contained a miR-140-5p target sequence (Figure 3A). To assess the direct relationship between VEGFA and miR-140-5p, a dual-luciferase reporter assay was performed. miR-140-5p mimic transfection significantly decreased the wild-type VEGFA (VEGFA WT) reporter but not the mutant VEGFA reporter (VEGFA MUT) (Figure 4B). Furthermore, mRNA (Figure 3C) and protein (Figure 3D) levels of VEGFA were measured in the presence and absence of miR-140-5p transfection. miR-140-5p mimics significantly suppressed, while miR-140-5p inhibitors promoted, expression of VEGFA (Figure 3 C,D). These data indicate that miR-140-5p directly targets VEGFA.

H19/miR-140-5p regulates VEGFA expression to promote tenogenic differentiation

After determining the relationships between H19/miR-140-5p

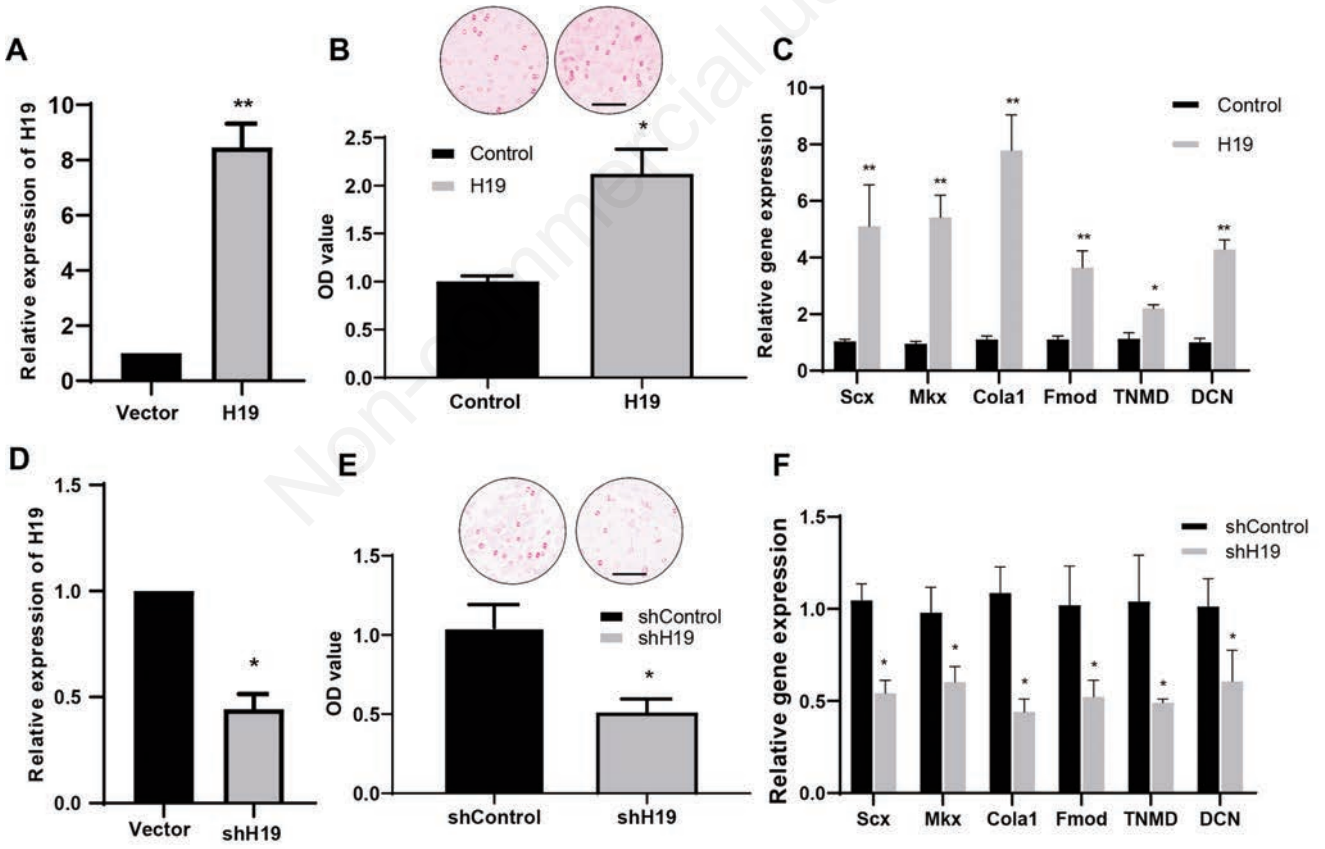

Figure 1. Figure 1. H19 regulates tenogenic differentiation of TDSCs. A) H19 was upregulated in TDSCs by H19-encoding plasmids. B) Sirius red staining of Control and H19-overexpressing TDSCs; scale bar: 20 μm. C) RT-qPCR detection of gene expression of tenogenic markers, including *Scx, Mkx, Cola1, Fmod, Tnm,* and *Dcn,* during tenogenic differentiation of TDSCs in the specified groups. D) H19 expression was decreased in TDSCs by plasmids containing H19 shRNA. E) Sirius red staining of shControl and shH19 groups; scale bar: 20 μm. F) mRNA expression level of tenogenic markers in shControl and shH19 groups; *p<0.05; **p<0.01, relative to Vector or shControl group.

and miR-140-5p/VEGFA, we determined if H19/miR-140-5p functioned via VEGFA to regulate tenogenic differentiation. A dual-luciferase VEGFA reporter assay was conducted with or without miR-140-5p mimic or H19 overexpression plasmids (Figure 4A). miR-140-5p significantly reduced the level of VEGFA reporter, which was reversed by H19 overexpression (Figure 4A). Moreover, mRNA (Figure 4B) and protein (Figure 4C) levels of VEGFA were measured in these treatment groups. Both the mRNA and protein levels of VEGF were decreased by miR-140-5p mimic transfection, which was reversed by H19 co-transfection (Figure 4C). Furthermore, miR-140-5p significantly inhibited tenogenic differentiation, and this effect was rescued by H19 overexpression (Figure 4D and Supplementary Figure 1D for enlarged details). Taken together, these data indicate that the H19/miR-140-5p axis regulates VEGFA during tenogenic differentiation.

H19 overexpression promotes rotator cuff tear repair

To further evaluate the role of H19-promoted tenogenic differentiation, a rat RCT model was used. Rats with surgically induced RCT were injected with vehicle, normal TDSCs, or H19-overexpressing TDSCs (H19-TDSCs) at the lesion site. Biomechanical testing was then performed 4 or 8 weeks later. Ultimate load to failure was significantly improved in the H19-TDSC group relative to the normal TDSC group at both 4 and 8 weeks post-RCT (Figure 5A). However, stiffness was significantly increased by H19-TDSCs at week 8 but not week 4 post-RCT (Figure 5B). Tendon samples of the RCT site were collected and subjected to RT-qPCR. mRNA levels of *Col1A1* were significantly increased in both the TDSC and H19-TDSC groups relative to control on week 4 and week 8, and were further increased in the H19-TDSC group relative to the TDSC group at both time points (Figure 5C). mRNA levels of *Col2a1* followed the same trend (Figure 5D). These data indicate that H19 overexpression enhances

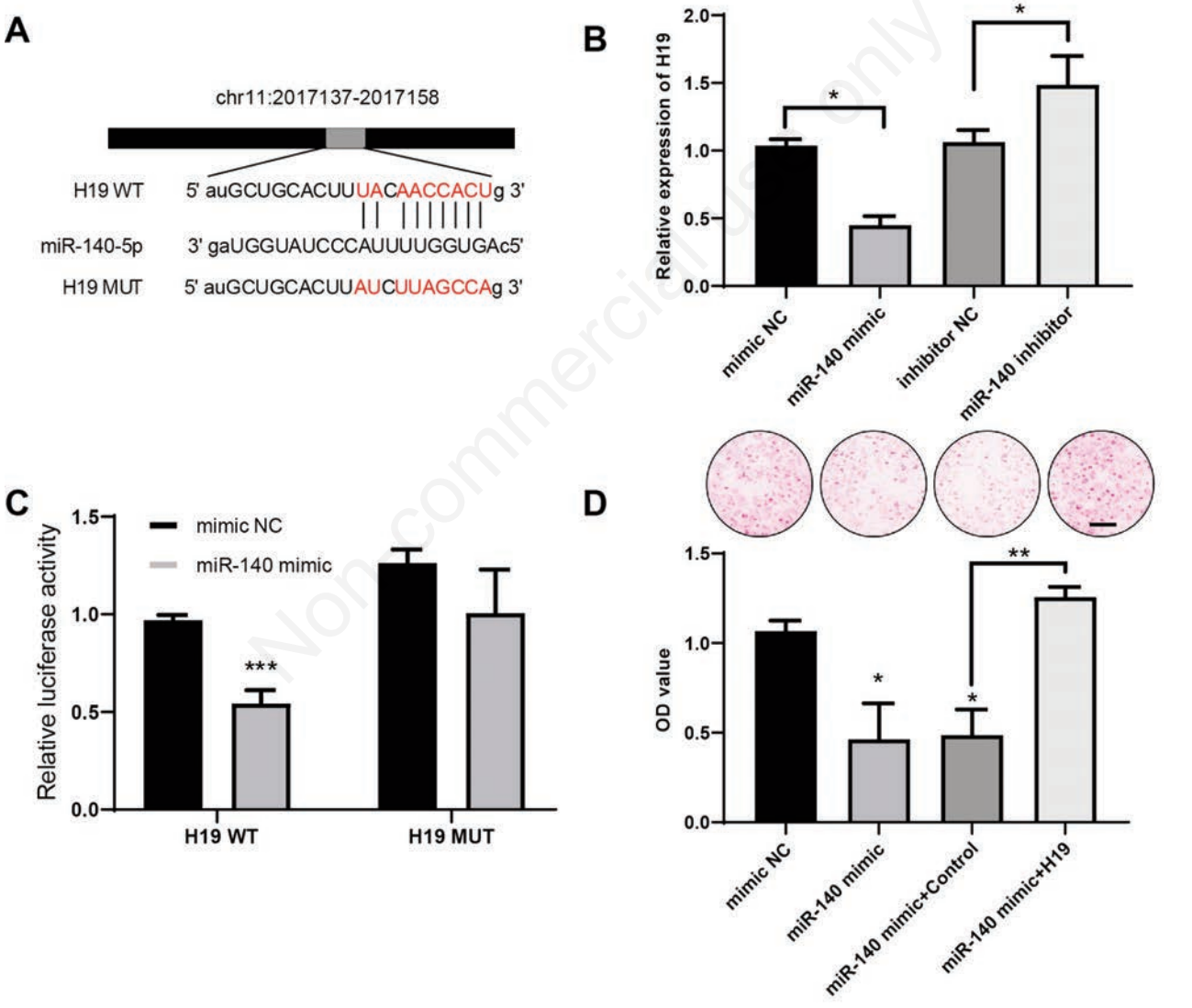

Figure 2. H19 directly targets miR-140-5p. A) Bioinformatic prediction of target sites in H19 against miR-140-5p. B) The RNA level of H19 in TDSCs was detected by RT-qPCR upon transfection of miR-140-5p mimic/inhibitor or negative control (NC). C) TDSCs were transfected with luciferase reporter plasmids containing H19 wild-type (WT) or mutant (MUT) gene, with or without miR-140-5p mimic or NC; luciferase activity was then detected. D) miR-140-5p mimic or NC were transfected into TDSCs; Sirius red staining was performed to assess tenogenic differentiation; scale bar: 20 μm; *p<0.05; **p<0.01.

the protective effect of TDSCs during tendon-bone healing of rat RCT.

Discussion

In the present study, we demonstrated that H19 is involved in tenogenic differentiation. H19-overexpressing TDSCs promoted functional recovery of rat RCT. Bioinformatic analyses and luciferase reporter assays demonstrated that H19 targeted miR-140-5p, and miR-140-5p directly targeted VEGFA. Furthermore, H19/miR-140-5p regulated VEGFA expression during tenogenic differentiation of TDSCs. Taken together, these results point to a novel role of the H19/miR-140-5p/VEGFA axis in tenogenic differentiation of TDSCs in RCT repair.

VEGF is highly upregulated at the surgical sites of leg bones in rats[27] and during the reconstruction of the cruciate ligament in dogs, *Vegf* mRNA levels peak at about 10 days after surgery.[28] VEGF and BEGF are highly expressed in the bone-tendon junction during acute injury healing.[19] Furthermore, VEGF-overexpressing MSCs promote tendon-bone healing of RCT by modulating miR-205-5p.[29] Engineered nanoparticles carrying the VEGFA gene significantly promote tendon-bone healing in flexor tendon repair models.[30,31] Collectively, these findings demonstrate an important role for VEGFA in regulating tendon-bone healing. In the present study, we found that VEGFA was upregulated in RCT, and that modulating H19/miR-140-5p directly affected VEGFA expression in TDSCs. However, VEGFA is only one of many downstream growth

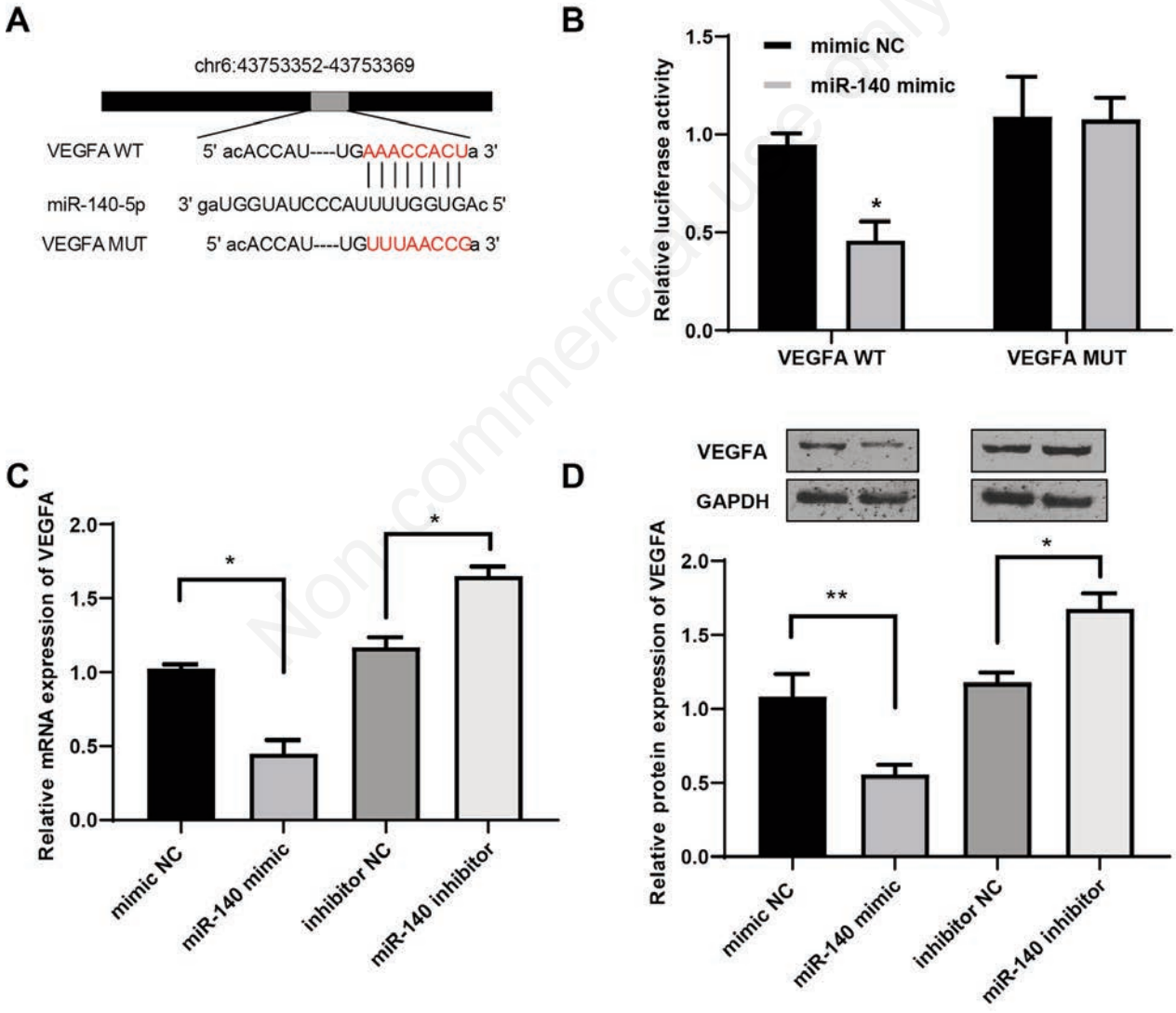

Figure 3. VEGFA is a direct target of miR-140-5p. A) Bioinformatics analysis of target sites for miR-140-5p in the VEGFA sequence. B) Luciferase analysis assay to assess the relationship between miR-140-5p and VEGFA. mRNA (C) and protein (D) levels of VEGFA were detected when miR-140-5p mimic/inhibitor or NC fragments were transfected into TDSCs. *p<0.05, **p<0.01. relative to mimic/inhibitor NC group.

effectors, and we postulate that the upstream switch of H19 may play an important role in regulating tenogenic differentiation and tendon-bone healing of RCT by regulating multiple downstream targets.

Traumatic injuries and degeneration of the rotator cuff are major causes of shoulder pain and dysfunction, causing chronic pain and restricted mobility.[32] As the aging population increases, shoulder pain in the elderly is projected to become an increasing socioeconomic burden. Both traumatic and degenerative RCT are age-related,[2] and are linked to intrinsic and extrinsic patient-specific factors. miRNAs have been identified as potential biomarkers and diagnostic tools for RCT.[33] miRNAs regulate tenocyte function and tendon-related gene expression during tendon development and injury repair.[34] For example, during the inflammatory response of RCT, miR-25 is downregulated in chronic rotator cuff tendinopathy. miR-25 plays a critical role during inflammatory responses via modulating the downstream target HMGB1, which is upregulated in RCT.[35] NF-kB is also an important mediator of chronic and acute tendon injuries.[36,37] miR-140-3p is significantly suppressed in both chronic tendinopathy and degenerative RCT, and miR-140-3p also negatively regulates NF-kB inflammatory signaling by inhibiting target gene expression of NCOA1 and NRIP1.[38] Thus, miR-140 could promote inflammation during tendon injuries. In the present study, we demonstrated that miR-140-5p was downregulated in RCT samples and TDSCs, which was consistent with the former observation of miR-140-3p. Furthermore, suppressing miR-140-5p upregulated expression of the downstream target VEGFA to promote the tenogenic differentiation of TDSCs. The role of miR-140-5p in regulating the inflammatory response in RTC will be investigated in future studies.

The functions of miRNAs in tendinopathy are a topic of intense investigation, but the role of lncRNAs remains largely obscure.[39,40] Mandy and colleagues used deep sequencing of transcriptome analysis to identify that several lncRNAs are changed in uninjured

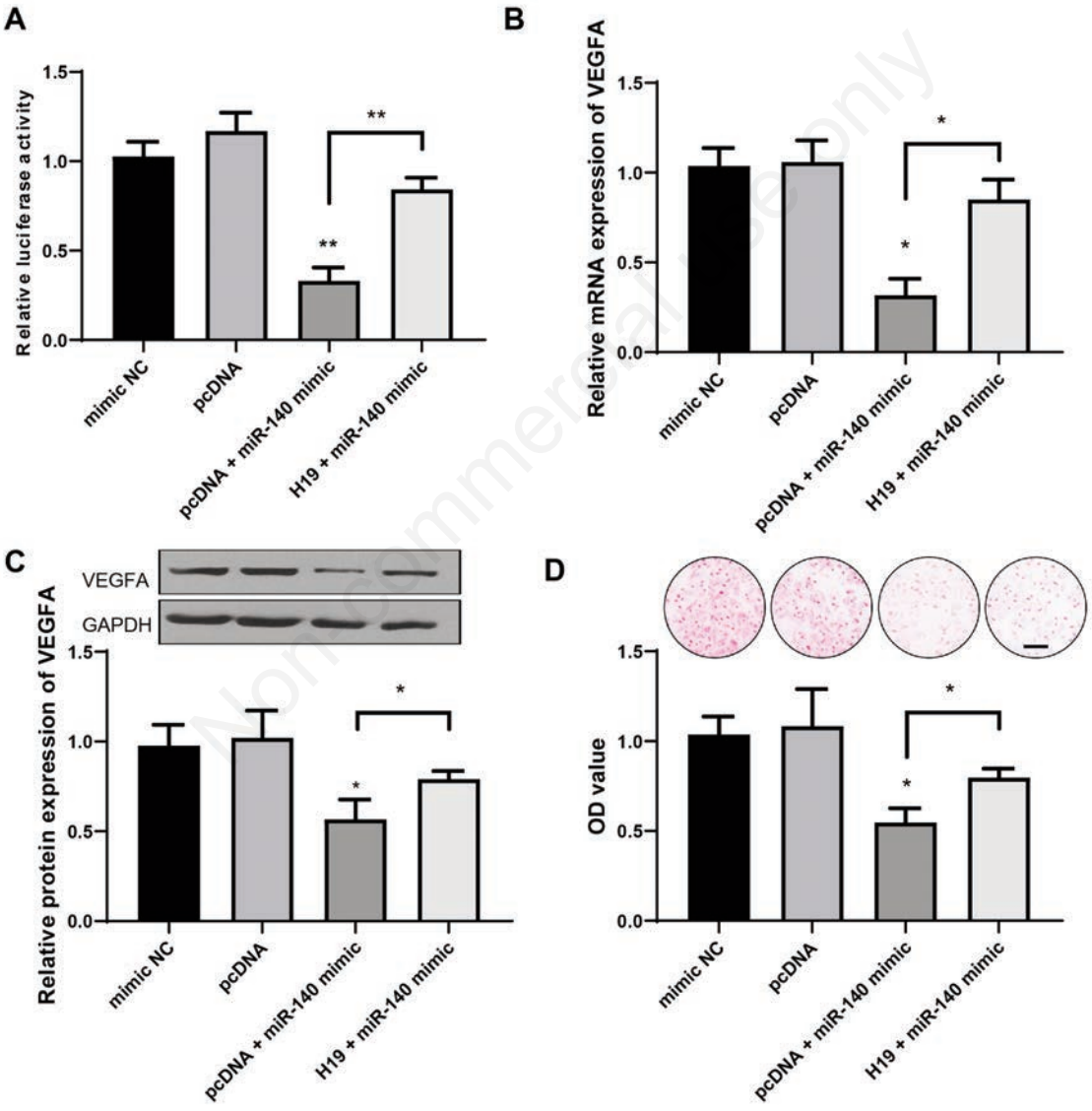

Figure 4. The H19/miR-140-5p/VEGFA axis regulates tenogenic differentiation. A) A VEGFA luciferase assay was performed when miR-140-5p mimic or NC fragments were transfected with or without H19 overexpression plasmids. mRNA (B) and protein (C) levels of VEGFA with or without miR-140-5p mimics or H19 overexpression plasmids were determined by RT-qPCR and western blotting, respectively. D) Sirius red staining was performed to assess the tenogenic differentiation effect in TDSCs treated as in (A); scale bar: 20 μm. *p<0.05, **p<0.01 relative to mimic NC or the indicated group.

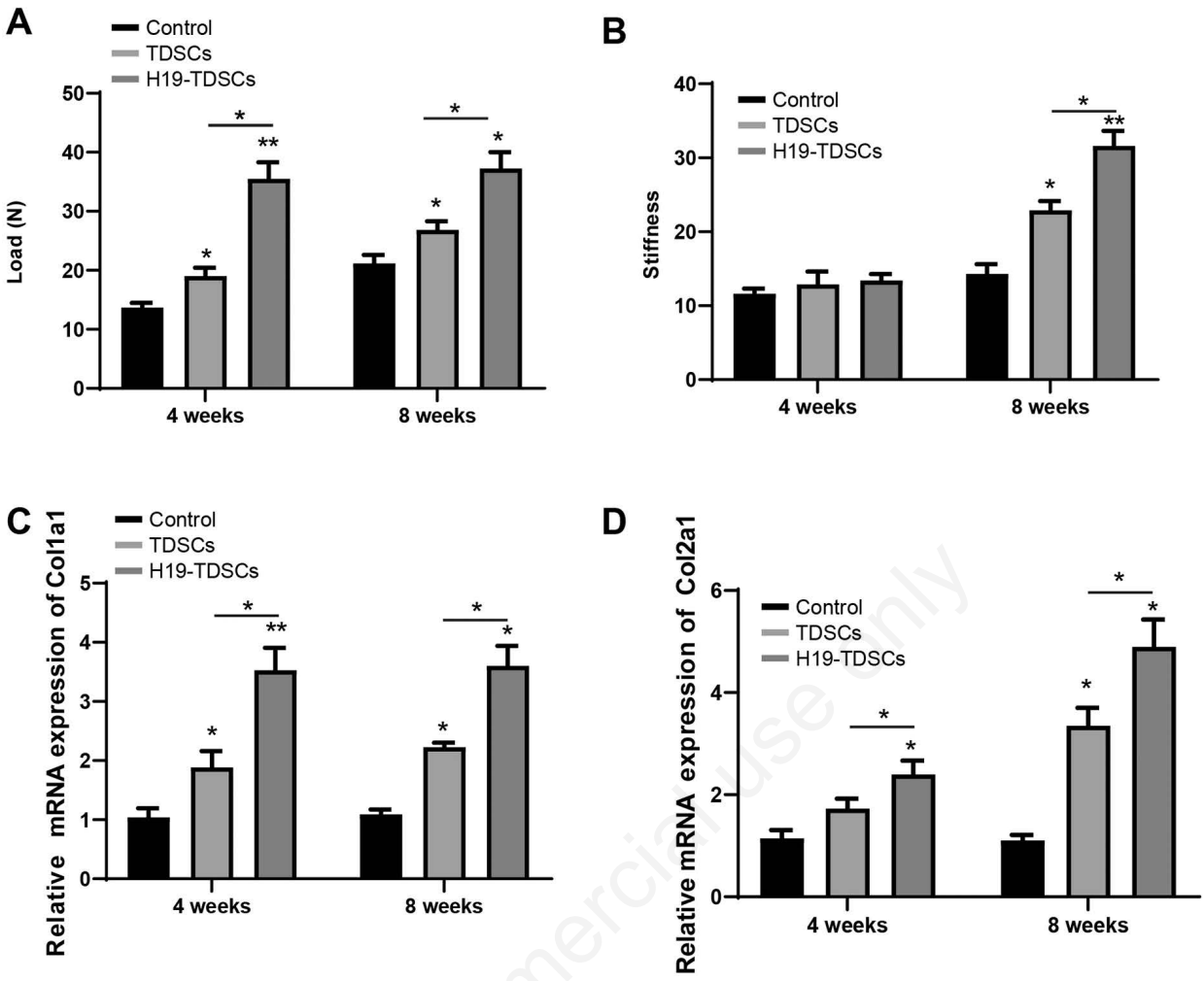

Figure 5. H19 overexpression increases the protective effect of TDSCs in tendon-bone healing of RCT. Lentiviral H19 overexpression vectors were introduced to TDSCs to generate a stable cell line. Cells were then injected into the lesions of RCT rats. Four and eight weeks later, tendons were subjected to mechanical testing. A) Ultimate load to failure in the H19-TDSC group was significantly increased relative to the control group at the 4- and 8-week points. B) Stiffness markedly increased in the H19-TDSC group at the 8-week point. C,D) mRNA expression levels of type I and II collagen were detected by RT-qPCR. *p<0.05, **p<0.01, relative to control or indicated TDSC group.

human Achilles tendon , leaving their functions to be further explored.[41] Also, several lncRNAs have been identified from analysis of transcriptomic data from tissue-engineered tendons.[42] LncRNA KCNQ1OT1 regulates adipogenic and osteogenic differentiation of TDSCs by suppressing miR-138-regulated expression of PPARg and RUNX2.[17] LncRNA TUG1 regulates osteogenic differentiation of TSPCs by promoting bFGF ubiquitination.[43] Furthermore, lncRNA H19 accelerates tenogenic differentiation of TDSCs and promotes tendon healing *via* modulation of miR-29b-3p-regulated TGF-b1 expression.[18] Further, prior studies suggest that H19 has potential roles in regulating mammalian muscle growth and development.[44] In the present study, we found that H19 was upregulated in RCT samples, and that H19 overexpression in TDSCs promoted tendon healing in RCT by modulating the miR-140-5p/VEGFA axis. In general, H19 is an important regulator for the functional recovery of tendon injuries. However, a broad spectrum of H19-regulated downstream effectors should be further evaluated by RNA-seq or other methods, which will be the topic of future studies.

In summary, we identified a regulatory axis in which H19 targets miR-140-5p to modulate VEGFA expression during tenogenic differentiation of TDSCs in RCT healing. Future studies of the H19/miR-140-5p/VEGFA axis could open a new direction for the development of novel therapies for the clinical treatment of RCT.

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