Original Paper

Mechanical Tension Promotes the Osteogenic Differentiation of Rat Tendon-derived Stem Cells Through the Wnt5a/Wnt5b/JNK Signaling Pathway

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Key Words

Rat tendon-derived stem cells (rTDSCs) • Osteogenic differentiation • Wnt5a • Wnt5b • JNK

Abstract

Background/Aims: Tendinopathy is a common sports injury that is manifested by the heterotopic ossification of tendon tissue. Tendon stem cells (TSCs) are prone to osteogenic differentiation under excessive tension. The underlying mechanisms remain poorly understood.

Methods: Uniaxial mechanical tension (UMT) served to stretch rat tendon-derived stem cells (rTDSCs) at 8% elongation (frequency: 1 Hz; 48, 60, or 72 hours). **Results:** The osteogenic differentiation of rTDSCs appeared after UMT along with increased mRNA expression of the osteogenic genes Runx2, Dlx5, Alpl, and Col1a1 and increased Runx2 protein expression. Wnt5a, Wnt5b and P-JNK protein levels were also upregulated after UMT stimulation. The inhibition of JNK expression by SP600125 and JNK1-shRNA decreased UMT-induced Runx2 protein expression, and the activation of JNK expression by anisomycin and JNK1-cDNA increased UMT-induced Runx2 protein expression. When shRNA knocked down Wnt5a and Wnt5b expression in rTDSCs, the induction of Runx2 and P-JNK expression by UMT was reduced. The inhibition of Runx2 expression could be rescued by the activation of JNK expression by anisomycin. **Conclusion:** UMT induced the osteogenic differentiation of rTDSCs via the Wnt5a/Wnt5b/JNK signaling pathway. Accordingly, this pathway may influence the heterotopic ossification of tendon tissue subjected to excessive tension.

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Introduction

Tendinopathy is one of the main injuries treated in orthopedics and sports medicine. The main pathological change in tendinopathy is heterotopic ossification and adipose tissue formation in tendon tissue [1]. The majority of scholars believe that tendon fiber injury caused by excessive tension loading is the leading cause of tendon disease. Bi et al. have demonstrated that tendon stem cells (TSCs) are the precursors of tendon cells and are capable of differentiating into multiple lineages and possess multiple differentiation capacity [2]. Gulotta et al. found that TSCs have a biological function similar to that of mesenchymal stem cells (MSCs) or bone marrow mesenchymal stem cells (BMSCs) and that they are directly involved in and play a critical role in the regeneration and repair of tendon micro-damage [3, 4]. Chen et al. found that platelet-rich plasma combined with TSCs is potentially effective for the treatment of tendinopathy [5]. Morphological changes in TSCs may influence the direction of cell differentiation. An *in vitro* study found that under 4% mechanical tension, TSCs mainly differentiated into tendon cells and were involved in the regeneration and repair of tendon tissue; under 8% tension, TSCs differentiated into non-tendon cells (adipose cells, chondrocytes and osteoblasts) [6]. However, the mechanism by which mechanical tension induces morphological and cytoskeletal changes in TSCs that lead to osteogenic differentiation is unclear. The c-Jun amino N-terminal protein kinases (JNKs) are involved in the phosphorylation of c-Jun at the N-terminal Ser-63 and Ser-73 sites in the transcriptional activation domain [7, 8]. Activated JNK exhibits mechanical sensitivity and is associated with osteogenic differentiation and bone formation [9-12], activation of the JNK signaling pathways upstream and downstream effectors upregulate the expression of the osteogenic gene Runx2 [13-16]. Tetsunaga et al. found that SW1353 chondrosarcoma cells increased Runx2 expression in response to mechanical tension via the JNK signaling pathway [17]. The Wnt/PCP (planar cell polarity) signaling pathway regulates the JNK signaling pathway. The activation of Wnt signaling pathway modulates the proliferation and differentiation of osteoblasts [18-20]. In vertebrates, the Wnt5a, Wnt5b, and Wnt11 ligands, as well as other ligands, activate Frizzled receptors and a co-receptor, Ror2, which then activate the G protein and Dvl within a trimetric complex that assembles into a Dvl/effector complex. The Dvl-Rac1 complex then activates JNK to regulate the cytoskeleton and cell adhesion [21-23]. Rac1 is a small molecule GTP enzyme that is involved in regulating cell differentiation and the osteogenic differentiation of MSCs [24, 25]. Ror2, a receptor tyrosine kinase (RTK)-related receptor, binds to Wnt5a and Wnt5b and is Dvl or Ca²⁺ dependent. Ror2 is associated with osteogenic differentiation and JNK activation [26-29]. A recent study by Park et al. found that the JNK signaling pathway promotes the osteogenic differentiation of mouse MC3T3-E1 cells [30]. Charoenpanich and others have recently shown that the Wnt5b signaling pathway is associated with the osteogenic differentiation of human mesenchymal stem cells (hMSC) induced by mechanical tension [31]. However, it is unclear whether the non-canonical Wnt-JNK signaling pathway regulates the mechanical stress-induced osteogenic differentiation of stem cells. We speculated that uniaxial mechanical tension (UMT) may induce the osteogenic differentiation of rat tendon-derived stem cells (rTDSCs) through the Wnt5a/b/Ror2/Rac1/JNK signaling pathway. This study aimed to explore whether UMT elicited the osteogenic differentiation of rTDSCs and to analyze the contribution of the Wnt5a/Wnt5b/JNK signaling pathway to the UMT-induced osteoblast differentiation of rTDSCs.

Materials and Methods

Isolation and culture of rTDSCs

rTDSCs were isolated as previously described [32]. Four 6-week-old male Sprague-Dawley rats weighing 150-200 g were used. The intact Achilles tendons were excised, the peritendinous tissues were removed, and the mid-substance tissues were minced and digested with type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 2.5 h at 37°C. To yield a single-cell suspension, the tissues were passed through a 70

µm cell strainer (Becton Dickinson, NJ, USA). The released rTDSCs were resuspended in growth medium (GM) containing Dulbecco's Modified Eagle Medium (DMEM), 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (all from HyClone, Logan City, Utah, USA). To enable colony formation, the isolated rTDSCs were cultured at 37°C in a 5% CO₂ atmosphere. Three days after initial plating, the rTDSCs were washed once with PBS to remove the non-adherent cells. At day 7, the rTDSCs were trypsinized as passage 0 (P₀).

Identification of rTDSCs

Immunofluorescence. The rTDSCs were fixed with 4% paraformaldehyde for 30 minutes, washed twice with PBS, and blocked in 2% goat serum for 1 hour. After washing with PBS, the rTDSCs were incubated with antibodies against nucleostemin, Oct-4 and Nanog (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 hours at room temperature. After washing with PBS, a Cy3-conjugated secondary antibody (Santa Cruz) was applied for 30 minutes. After counterstaining with DAPI (Sigma-Aldrich, St. Louis, MO, USA), the rTDSCs were visualized under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

Flow cytometry assay. rTDSCs (2×10^6 at P₂) were centrifuged at 12,000 g for 5 minutes; the supernatant was discarded, and the cells were re-suspended in 300 µl of PBS. A total of 3.0×10^5 events were counted for each sample. Antibody (1 µg) was added to each sample. The samples were then incubated for 30 minutes at room temperature, washed with PBS, and centrifuged at 12,000 g for 5 minutes; the supernatant was subsequently discarded. The cells in each sample were re-suspended in 300 µl of PBS and analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). The following antibodies were used: anti-CD31 (ab33858; Abcam, Cambridge, UK), anti-CD90 (ab33694; Abcam, Cambridge, UK), anti-CD44 (ab23396; Abcam, Cambridge, UK) and anti-CD34 (sc-7324, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Osteogenic differentiation assays

rTDSCs were cultured in GM. After 24 hours, the GM was carefully aspirated from each well, and 2 ml of induction medium was added (GM supplemented with 10 nM dexamethasone, 2 mM glutamine, 5 mM β-glycerophosphate, 50 mM ascorbate, 100 U/ml penicillin, and 10 µg/ml streptomycin; all from Cyagen Biosciences Inc, Santa Clara, CA, USA). The cells were fed with fresh induction medium every 3 days. After 21 days, the mRNA expression of Runx2, DIX5, wnt5a and wnt5b was determined. For Alizarin Red staining, the induction medium was removed, and the wells were rinsed twice with PBS. The cells were then fixed with 2 ml of 4% formaldehyde solution for 30 minutes, rinsed twice with PBS and stained with 1 ml of Alizarin Red working solution (Cyagen Biosciences Inc., Santa Clara, CA, USA) for 5 minutes. The wells were rinsed twice with PBS, and the cells were visualized under a light microscope (Olympus BX51, Tokyo, Japan).

hrRNA and JNK1-cDNA). After fresh DMEM was added, the transfected rTD

Biochemical agents and shRNA and cDNA transfections

rTDSCs were exposed to 10 µM SP600125 (ab120065; Abcam, Cambridge, UK) or 50 µM anisomycin (CAS 22862-76-6; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 minutes before UMT stimulation. Recombinant lentiviral vectors expressing Wnt5b-shRNA and JNK1-shRNA were constructed by BioWit Technologies Co., Ltd (Shenzhen, China); the shRNAs were inserted into a lentiviral expression vector that contains the GFP (green fluorescent protein) gene as a reporter. The recombinant vectors were co-transfected into 293T cells; the titer of the recombinant Wnt5b-shRNA lentivirus was 8×10^8 infectious units per mL, and the titer of the recombinant JNK1-shRNA lentivirus was 1×10^8 infectious units per mL. The shRNA sequences targeting Wnt5b are as follows: forward: 5'-GGA CAA CTT GTC TGT CTT TGG TTC AAG AGA CCA AAG ACA GAC AAG TTG TCC TTT TTT CTC GAG G-3', reverse: 5'-GAT CCC TCG AGA AAA AAG GAC AAC TTG TCT GTC TTT GGT CTC TTG AAC CAA AGA CAG ACA AGT TGT C C-3'. The shRNA sequences targeting JNK1 are as follows: forward: 5'-CTG ACA AGC AGT TAG ATT CAA GAG ATC TAA CTG CTT GTC AGG GAT CTT TTT TCT CGA GG-3', reverse: 5'-GAT CCC TCG AGA AAA AAC TGA CAA GCA GTT AGA TCT CTT GAA TCT AAC TGC TTG TCA GGG ATC-3'. A recombinant lentiviral vector expressing Wnt5a-shRNA was constructed by GeneChem Co. Ltd (Shanghai, China). Wnt5a-shRNA was introduced into a lentiviral expression vector that expressed the GFP reporter gene. This recombinant vector was co-transfected into 293T cells, and the titer of the recombinant lentivirus was 3×10^8 infectious units per mL. The shRNA sequences targeting Wnt5a are as follows: forward: 5'-CCG GAA CCC TGT TCA GAT GTC AGA ACT CGA GTT CTG ACA TCT GAA CAG GGT TTT-3' and reverse: 5'-AAT TCA AAA ACC CTG TTC AGA TGT CAG AAC TCG AGT TCT GAC ATC TGA ACA GGG

TT-3'. A scrambled shRNA vector was constructed by BioWit Technologies Co., Ltd (Shenzhen, China); the scrambled shRNA sequence (TTC TCC GAA CGT GTC ACG T) was used as the universal negative control [33-35] (shRNA-control) throughout the study. Rat JNK1-cDNA was introduced into the lentiviral vector pLVX-rJNK1-mCMV-ZsGreen by BioWit Technologies Co., Ltd (Shenzhen, China) for overexpression. The pLVX-mCMV-ZsGreen vector was used as the scrambled control (cDNA-control). The titer of the recombinant lentivirus was 1×10^8 infectious units per mL. The cDNA sequences targeting JNK1 are as follows: forward: 5'-CAT CCA CGC TGT TTT GAC CTC CAT AGA AGA CAC CGA CTC TAC TAG AGG ATC TAT TTC CGG-3', reverse: 5'-TGA ATT CCT CGA GAC TAG TTC TAG AGC GGC CGC CAC CAT GAG CAG AAG TAA ACG TGA CAA-3'. rTDSCs were plated into 6-well plates at 1×10^5 cells per well and were maintained in DMEM overnight. When the cells reached 50% confluence, they were infected with lentiviral particles for 24 hours at a multiplicity of infection (MOI) of 40 (Wnt5a-shRNA, Wnt5b-shRNA, JNK1-shRNA and JNK1-cDNA). After fresh DMEM was added, the transfected rTDSCs cells were subcultured at an appropriate density. After an additional 3 days in culture, the infection efficiency was determined by counting GFP-expressing cells by fluorescence microscopy (Olympus BX51, Tokyo, Japan) and by real-time PCR (RT-PCR) and Western blot analysis.

Uniaxial mechanical tension

rTDSCs at P₂ were plated at a density of 2×10^4 cells/cm² in 2.5 ml of GM in 14 silicone culture dishes (B-Bridge International, Inc., Cupertino, CA, USA). The bottom of each dish was coated with type I collagen (Sigma-Aldrich, St. Louis, MO, USA). The cells were then incubated for 24 hours at 37°C in 5% CO₂ to facilitate cell adhesion and were allowed to reach 70-80% confluence. Seven silicone dishes (UMT group) were then stretched in an intelligent bionic uniaxial mechanical tension system (YAHU/XBQX-2000, Yahong Technologies Co., Ltd., Chongqing, China). The settings for the UMT were the following: 1 Hz frequency, 8% elongation, 4 hours at each tension, and a 4 hour interval. The other silicone dishes served as the non-loading control (NC) group.

Cytoskeletal staining

rTDSCs were plated in silicone dishes at a concentration of 2×10^4 cells/cm². When the rTDSCs had attached to the base film, UMT was applied to the silicone dishes at a frequency of 1 Hz with 4% or 8% elongation for 4 hours; each tension interval was 4 hours. After a total of 24 hours of UMT, rTDSCs were washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes and then washed twice with PBS. rTDSCs were subsequently treated with 0.3% Triton X-100 for 10 minutes, stained with 5 µg/ml rhodamine-phalloidin (p2141, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 hour and washed twice with PBS. rTDSCs were exposed to DAPI for 10 minutes to stain the nuclei and were washed twice with PBS. rTDSCs were observed using a confocal microscope (Carl Zeiss, Jena, Germany).

Alkaline phosphatase staining

rTDSCs that had been treated with 8% UMT for 72 hours and rTDSCs in the NC group were washed twice with PBS and fixed with 4% paraformaldehyde for 8 minutes. The rTDSCs were incubated with an NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at room temperature and were washed twice with PBS. rTDSCs were visualized using a light microscope (Olympus BX51, Tokyo, Japan).

Real-time PCR

Total RNA was isolated using TRIzol reagent (Takara, Dalian, China). After total RNA was reverse-transcribed, real-time PCR (RT-PCR) was performed using the Bio-Rad iCycler IQ system (Bio-Rad, CA, USA). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ formula; Gapdh was used as the internal control. The data were expressed as the fold change after 48, 60 and 72 hours of UMT compared with the NC group. At least three independent experiments were performed. The primer sequences are as follows: Gapdh (NM_017008.4) forward: 5'-TGA CTT CAA CAG CAA CTC-3', reverse: 5'-TGT AGC CAT ATT CAT TGT CA-3'; Wnt5a (NM_022631.1) forward: 5'-TTC CAA GTT CTT CCT AAT-3', reverse: 5'-TAT ACT TCT GAC ATC TG AA-3'; Wnt5b (NM_001100489.1) forward: 5'-GCA GGG TCA TGC AGA TAG G-3', reverse: 5'-CGG TAG CCA TAC TCC ACG ACG T-3'; Ror2 (NM_001107339.1) forward: 5'-CTT CCC ACT CTG AAA GGC TAC TT-3', reverse: 5'-TAC GAC AGG GGC ATC ATT CTT C-3'; Rac1 (NM_134366.1) forward: 5'-CTG CTC ATC AGT TAC ACG AC-3', reverse: 5'-GGA CGC AGT CTG TCA TAA TC-3'; Runx2 (NM_001278483.1) forward: 5'-GAA CTC AGC ACC AAG TCC TTT-3', reverse: 5'-CAG TGT CAT CAT CTG AAA TAC GC-3'; Dlx5 (NM_012943.1)

forward: 5'-CTT ATG CGG A CTA CGG CTA CG-3', reverse: 5'-CCT GGG TTT ACG AAC TTT CTT TG-3'; Col 1a1 (NM_053304.1) forward: 5'-TGG TGA GAC GTG GAA ACCT G-3', reverse: 5'-CTT GGG TCC CTC GAC TCC TA-3'; and Alpl (NM_013059.1) forward: 5'-GGC ACC ATG ACT TCC CAG AA-3', reverse: 5'-CAC CGT CCA CCA CCT TGT AA-3'.

Western blotting

The following primary antibodies were used: anti-Runx2 (1:1000; ab23981, Abcam, Cambridge, UK), anti-Wnt5a (1:400; ab110073, Abcam, Cambridge, UK), anti-Wnt5b (1:1000; GTX47175, GeneTex, Irvine, CA, USA), anti-JNK1/JNK2/JNK3 (1:1000; ab179461, Abcam, Cambridge, UK), anti-JNK1/JNK2/JNK3 (phospho-T183/T183/T221; 1:1000; ab124956, Abcam, Cambridge, UK), anti-JNK1 (1:1000; ab199380, Abcam, Cambridge, UK), anti-JNK1 (phospho-T183; 1:1000; ab47337, Abcam, Cambridge, UK), GAPDH (1:1000; Pierce BIC, USA), and β -tubulin (1:1000; loading control; Pierce). rTDSCs were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl, and 0.1% sodium dodecyl sulfate) with proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL, USA). Samples (30 μ g/lane) were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The results were visualized using a LiCor Odyssey Imager (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

The NC and UMT groups were compared using a Student's t-test. The data analysis was performed using SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). The statistical significance level was $P < 0.05$.

Results

Identification and osteogenic differentiation of rTDSCs

The shape of the rTDSCs from P_0 to P_3 was similar to elongated fibroblasts (Fig. 1A). The flow cytometry analysis showed that rTDSCs exhibited positive staining for the MSC marker CD90 but negative staining for CD44, the hematopoietic cell marker CD34 and the endothelial cell marker CD31 (Fig. 1B). The immunofluorescence analysis demonstrated that the stem cell markers Oct-4, Nanog and nucleostemin were expressed in rTDSCs (Fig. 1C). rTDSCs have the capacity to differentiate into osteoblasts. After 21 days of osteogenic induction, the RT-PCR analysis revealed that the mRNA expression of Runx2 and Dlx5 was higher in the osteogenic media (Osteo-M) group than in the GM group (Fig. 1D). Mineralized calcium deposits were observed by Alizarin Red staining after 21 days of osteogenic induction; however, no mineralized calcium deposits were found in the GM group (Fig. 1E). Compared to the GM group, the mRNA expression of Wnt5a and Wnt5b was higher in the Osteo-M group (Fig. 1D), indicating that Wnt5a/Wnt5b may be associated with the osteogenic differentiation of rTDSCs.

UMT induced the osteogenic differentiation of rTDSCs

UMT for 48, 60, or 72 hours at 8% elongation and a frequency of 1 HZ increased the mRNA expression of the osteogenic genes Runx2, Dlx5, Alpl, and Col1a1 in rTDSCs compared with the NC group (Fig. 2A-C). Similarly, Runx2 protein expression in rTDSCs was higher after 48, 60, or 72 hours of UMT compared with the NC group (Fig. 2D). The alkaline phosphatase staining level in the 72-hour UMT group was higher than that in the NC group (Fig. 2E).

Upregulation of Wnt5a, Wnt5b, Ror2, and Rac1 in rTDSCs after UMT

UMT for 48, 60, or 72 hours increased the mRNA expression of non-canonical Wnt5a/Wnt5b in rTDSCs as well as that of Ror2 and Rac1 compared with the NC group (Fig. 3A-C). The Wnt5a and Wnt5b protein levels were higher in rTDSCs exposed to 48 and 60 hours of UMT compared with the NC group (Fig. 3D-E).

Fig. 1. Identification and osteogenic differentiation of rTDSCs. (A) Shape of rTDSCs from P₀-P₃. Scale: 100 μ M. (B) rTDSCs were stained for MSC markers (CD90 and CD44), an endothelial cell marker (CD31), and a hematopoietic stem cell marker (CD34) by flow cytometry; the positive population was enclosed by the line. (C) Expression of the stem cell markers Nanog, Oct-4 and nucleostemin (red dot). Bar: 10 μ m. (D) Osteogenic differentiation of rTDSCs. After rTDSCs were cultured in growth medium (GM) and osteogenic medium (Osteo-M) for 21 days, the mRNA expression levels of Runx2, DIX5, Wnt5a and Wnt5b were determined (n = 3; **P < 0.01). (E) Mineralized calcium deposits. rTDSCs were cultured in GM and Osteo-M for 21 days and stained with Alizarin Red (Scale: 50 μ m).

Regulation of Runx2 expression by Wnt5a/Wnt5b in rTDSCs after UMT

Wnt5a/Wnt5b mRNA (Fig. 4A-B) and protein (Fig. 4C-D) expression was significantly decreased in rTDSCs after transfection with Wnt5a-shRNA and Wnt5b-shRNA for 3 days compared with the shRNA-control group. After 60 hours of UMT, Runx2 protein expression was lower in the 8%+Wnt5a-shRNA and 8%+Wnt5b-shRNA groups compared with the 8%+shRNA-control group (Fig. 4E).

UMT induced cytoskeletal changes and regulated Runx2 expression in rTDSCs through JNK

In rTDSCs exposed to 8% elongation, actin was depolymerized and clearly broken, and the mean fluorescence intensity was decreased compared with the NC group and the 4% elongation group, suggesting that irreversible cytoskeletal changes had occurred (Fig. 5A). After 48 and 60 hours of UMT, P-JNK1/JNK2/JNK3 levels were higher in the UMT groups than in the NC group (Fig. 5B). To verify whether JNK regulated UMT-induced Runx2 expression, rTDSCs were treated with the JNK inhibitor SP600125 and the JNK activator anisomycin for 30 minutes before UMT induction. SP600125 decreased Runx2 mRNA expression, and anisomycin increased Runx2 mRNA expression in rTDSCs after UMT induction for 48 and 60 hours compared with the 8%+no drug group (8%+Veh) (Fig. 5D). After UMT for 60 hours, Runx2 protein expression was higher in the 8%+Veh group than in the NC group, and Runx2 protein expression was higher in the anisomycin group than in the 8%+Veh group; however, SP600125 decreased Runx2 protein levels compared to the 8%+Veh group (Fig. 5C).

Fig. 2. UMT-induced osteogenic differentiation of rTDSCs. (A-C) The mRNA expression of osteogenic genes (Runx2, Dlx5, Alpl and Col 1a1) in rTDSCs after 8% UMT for 48, 60, and 72 hours compared to the expression in the NC group (n = 3; *P < 0.05; **P < 0.01). (D) Runx2 protein levels in rTDSCs after UMT for 48, 60, or 72 hours relative to the NC group. Runx2 protein expression was quantitated by densitometric analysis, and GAPDH was used as the loading control. (E) ALP staining of rTDSCs after 72 hours of 8% UMT compared to the NC group (Scale: 50 μ m). NC group: non-loading control group.

JNK regulated UMT-induced Runx2 expression in rTDSCs as confirmed by transfection with JNK1-cDNA and JNK1-shRNA

P-JNK1 expression was significantly decreased in rTDSCs after transfection with JNK1-shRNA for 3 days compared with shRNA-control (Fig. 6A). P-JNK1 expression was significantly increased in rTDSCs after transfection with JNK1-cDNA for 3 days compared with cDNA-control (Fig. 6B). After 60 hours of UMT, Runx2 protein expression was higher in rTDSCs transfected with JNK1-cDNA compared with the 8%+cDNA-control group, and Runx2 protein expression was lower in rTDSCs transfected with JNK1-shRNA compared with the 8%+shRNA-control group (Fig. 6C).

Wnt5a/Wnt5b regulated UMT-induced Runx2 expression in rTDSCs through JNK

To determine whether Wnt5a and Wnt5b regulated UMT-induced Runx2 expression through the JNK pathway, rTDSCs were transfected with Wnt5a-shRNA and Wnt5b-shRNA for 3 days; 30 minutes before UMT, anisomycin was added to the Wnt5a-shRNA and Wnt5b-shRNA groups. The UMT-induced upregulation of Runx2 mRNA expression was suppressed in rTDSCs transfected with shRNA-Wnt5a and shRNA-Wnt5b compared to the 8%+shRNA-

Fig. 3. UMT-induced Wnt5a, Wnt5b, Ror2 and Rac1 expression. The mRNA expression of Wnt5a, Wnt5b, Ror2 and Rac1 in rTDSCs after UMT for 48 hours (A), 60 hours (B) and 72 hours (C) relative to the NC group (n = 3; *P < 0.05; **P < 0.01). Protein expression of Wnt5a (D) and Wnt5b (E) in rTDSCs after UMT for 48 and 60 hours relative to the NC groups. β -tubulin was used as the loading control. NC group: non-loading control group.

Fig. 4. Wnt5a and Wnt5b regulated the UMT-induced osteogenic differentiation of rTDSCs. The mRNA expression levels of Wnt5a (A) and Wnt5b (B) in rTDSCs after 3 days of shRNA treatment relative to scrambled shRNA control (shRNA-control) (n = 3; *P < 0.05). The Wnt5a (C) and Wnt5b (D) protein levels were determined by densitometric analysis relative to the shRNA-control groups. β -tubulin was used as the loading control. (E) 3 days after transfection with Wnt5a-shRNA, Wnt5b-shRNA and Wnt5a-shRNA+Wnt5b-shRNA, rTDSCs were treated with 8% UMT for 60 hours. The Runx2 protein levels are shown relative to the 8%+shRNA-control group. GAPDH was used as the loading control. Sh-con: shRNA-control; ShWnt5a: Wnt5a-shRNA; ShWnt5b: Wnt5b-shRNA.

control group. Runx2 mRNA expression was restored by the addition of anisomycin (Fig. 7A). The P-JNK1 level were lower in the 8%+shRNA-Wnt5a and 8%+shRNA-Wnt5b groups than in the 8%+shRNA-control group after UMT for 60 hours. The UMT-induced upregulation of P-JNK1 level was suppressed in rTDSCs transfected with Wnt5a-shRNA and Wnt5b-shRNA;

Fig. 5. UMT induced cytoskeletal changes and regulated Runx2 expression in rTDSCs through JNK. (A) rTDSCs were treated with 4% and 8% UMT for 24 hours, and the cytoskeleton was stained with rhodamine-phalloidin. Scale: 20 μm. (B) Western blot analysis of phosphorylated JNK (P-JNK1/JNK2/JNK3) in rTDSCs treated with UMT for 48 and 60 hours. Total-JNK1/JNK2/JNK3 was used as the loading control. (C) Before UMT, rTDSCs were treated with 10 μM SP600125 or 50 μM anisomycin for 30 minutes. Runx2 mRNA expression was detected after UMT treatment for 48 and 60 hours. The data are presented as the fold change relative to the NC group (n = 3; **P < 0.01). (D) Runx2 protein expression in the 8%+Veh, 8%+SP600125 and 8%+anisomycin groups after UMT for 60 hours relative to the NC group. GAPDH was used as the loading control. NC group: non-loading control group; 8%+Veh: 8% UMT without drug treatment.

this inhibition could be reversed by the JNK activator, anisomycin (Fig. 7B). The UMT-induced upregulation of Runx2 protein expression was suppressed in rTDSCs transfected with shRNA-Wnt5a and shRNA-Wnt5b compared to the 8%+shRNA-control group. Runx2 protein expression was restored by the addition of anisomycin (Fig. 7C).

Discussion

Tendon tissue calcification is one of the main manifestations of tendinopathy. The majority of scholars believe that tendinopathy is caused by chronic fatigue and excessive tension loading, leading to tendon microfiber injuries. These microfiber injuries are considered an initial risk factor for tendon injuries [6, 36]. In 2007, Bi et al. identified stem cells in tendon tissue [2], named TDSCs. TDSCs have the capacity for multipotent differentiation, and studies have shown that TDSCs undergo osteogenic differentiation in response to mechanical stress [37]. Lui et al. proposed that TDSCs differentiate into non-tenocytes in response to mechanical stress, which may be the cause of the heterotopic ossification of tendon tissue [38]; however, the mechanisms for this remain unclear. This study aimed to investigate the mechanism of TDSC osteogenic differentiation in response to

Fig. 6. JNK1 regulated the UMT-induced Runx2 expression in rTDSCs as confirmed by transfection with JNK1-cDNA and JNK1-shRNA. (A) Phosphorylated JNK1 (P-JNK1) levels in rTDSCs after transfection with JNK1-cDNA for 3 days compared with cDNA-control. (B) P-JNK1 levels in rTDSCs after transfection with JNK1-shRNA for 3 days compared with shRNA-control. Total-JNK1 was used as the loading control. (C) After 60 hours of UMT induction, Runx2 protein expression in rTDSCs transfected with JNK1-cDNA was compared with that in the 8%+cDNA-control group, and Runx2 protein expression in rTDSCs transfected with JNK1-shRNA was compared with that in the 8%+shRNA-control group. GAPDH was used as the loading control.

Fig. 7. Wnt5a and Wnt5b regulated UMT-induced Runx2 expression in rTDSCs through JNK. (A) rTDSCs were transfected with Wnt5a-shRNA and Wnt5b-shRNA for 3 days; 30 minutes before UMT, anisomycin was added to the Wnt5a-shRNA and Wnt5b-shRNA groups. After UMT for 60 hours, an RT-PCR analysis of Runx2 mRNA expression in rTDSCs was performed. The data are presented as the relative change compared to the 8%+shRNA-control group ($n = 3$; $*P < 0.05$). AM: anisomycin. (B) rTDSCs were transfected with Wnt5a-shRNA and Wnt5b-shRNA for 3 days; 30 minutes before UMT, anisomycin was added to the Wnt5a-shRNA and Wnt5b-shRNA groups. Western blot of P-JNK1 after 60 hours of UMT was performed with total JNK1 as the loading control. (C) rTDSCs were transfected with Wnt5a-shRNA and Wnt5b-shRNA for 3 days; 30 minutes before UMT, anisomycin was added to the Wnt5a-shRNA and Wnt5b-shRNA groups. Western blot of RUNX2 after 60 hours of UMT was performed with GAPDH as the loading control.

mechanical stress. Studies have shown that applying intermittent mechanical tension to MSCs (every few hours, several times a day, for several days) promotes osteogenic differentiation, whereas applying continuous mechanical tension to MSCs inhibits osteogenic differentiation

[39]. In this study, osteogenic differentiation of rTDSCs was observed after intermittent UMT, as was increased mRNA expression of osteogenic genes (Runx2, Dlx5, Alpl, and Col 1a1), increased Runx2 protein expression and positive ALP staining, indicating that UMT induced the osteogenesis differentiation of rTDSCs. The current research showed that, in addition to the UMT-induced osteogenic differentiation of rTDSCs, the mRNA and protein expression of the non-canonical Wnt molecules Wnt5a/Wnt5b and the mRNA expression of Ror2 and its downstream small molecule GTP enzyme Rac1 were also elevated. Previous studies have shown that Wnt5a/Wnt5b mRNA is enhanced in stem cells under mechanical stress and that this effect is associated with osteogenic differentiation [29-31]. In a study by Shi et al., Wnt5a mRNA expression increased in rTDSCs after UMT, although Wnt5b mRNA expression was not increased [40]; these results differed from those of our study, and this discrepancy may be due to differences in the mechanical tension system or in the mechanical elongation, frequency, periodicity or duration. When shRNAs were used to knock down the expression of Wnt5a and Wnt5b, the UMT-induced osteogenic differentiation of rTDSCs was inhibited, indicating that the UMT-induced osteogenic differentiation of rTDSCs was regulated by Wnt5a and Wnt5b. Cytoskeletal changes were observed in rTDSCs after UMT. JNK is a cytoskeleton regulator, and the phosphorylation of JNK1/JNK2/JNK3 increased after UMT. The JNK activator anisomycin promoted the UMT-induced mRNA and protein expression of the osteogenic gene Runx2; the converse was observed with the JNK inhibitor SP600125. These results indicated that JNK may modulate the UMT-induced osteogenic differentiation of rTDSCs. However, anisomycin and SP600125 are not JNK-specific. Anisomycin can induce p38-MAPK, and SP600125 can inhibit a number of kinases in addition to JNK [41]. p38-MAPK also regulates stress-stimulated osteogenic differentiation [42]. The JNK gene has three isoforms, JNK1, JNK2, and JNK3; among them, JNK1 is mainly associated with osteogenic differentiation [43-45]. Therefore, we used JNK1-cDNA and JNK1-shRNA to confirm the effects of JNK signaling. JNK1-cDNA promoted UMT-induced Runx2 protein expression, whereas JNK1-shRNA inhibited Runx2 protein expression. The mechanism for this may involve force transmission, in which activated JNK and ERK1/2 convert mechanical stress to intracellular molecular events that lead to gene expression, protein synthesis, cell proliferation and cell differentiation [46, 47]. When rTDSCs were exposed to UMT after shRNA-mediated knockdown of Wnt5a/Wnt5b, JNK activity decreased, and osteogenic differentiation was inhibited. The inhibition of osteogenesis was rescued by the JNK activator, anisomycin, or by JNK1-cDNA, indicating that Wnt5a/Wnt5b is located upstream of JNK and regulates the function of JNK. These data suggested that the effect of Wnt5a/Wnt5b on UMT-induced osteogenesis was mediated by JNK. The non-canonical Wnt pathways include Wnt/PCP, Wnt/JNK, Wnt/calcium and Wnt/Rho [11]. The Rho and JNK signaling pathways both regulate the cytoskeleton and are associated with osteogenic differentiation, but the relationship between them is unclear. Studies have shown that JNK is independent of Rho but is regulated by Rac GTPase [48, 49]. Some scholars believe that in the process of eye development, JNK is another downstream effector of Dvl and is activated by RhoA, thereby regulating the PCP signaling pathway [50, 51]. Studies by Wang et al. have shown that Wnt5a/JNK regulates cell movement independent of or dependent on the RhoA pathway [52]. Whether the RhoA and JNK pathways regulate each other during the UMT-induced osteogenic differentiation of TDSCs remains to be studied.

Both the current study and Wang et al. observed increased mRNA expression of adipogenic-related genes, such as PPARgamma2, during the UMT-induced osteogenic differentiation of rTDSCs [6]. However, many studies have shown that osteogenesis and adipogenesis are mutually inhibitory [53, 54]. Whether the non-canonical Wnt signaling pathway regulates both adipogenic and osteogenic differentiation under mechanical stress awaits further study.

In conclusion, our study showed that the UMT-induced osteogenic differentiation of rTDSCs was regulated by the Wnt5a/Wnt5b/JNK signaling pathway. This work provides a potential molecular mechanism for the calcification of tendon tissue resulting from mechanical tension.

Abbreviations

TSCs (tendon stem cells); UMT (uniaxial mechanical tension); rTDSCs (rat tendon-derived stem cells); MSCs (mesenchymal stem cells); GM (growth medium); DMEM (Dulbecco's Modified Eagle Medium); Osteo-M (osteogenic media); NC (non-loading control); AM (anisomycin).

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Disclosure Statement

None declared by any author.

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