IL-6 Promotes Osteogenic Differentiation of Rat Tendon Stem Cells through the STAT3/Wnt5a Signalling Pathway

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Research

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Abstract

Background: Tendinopathy is currently the common clinical condition related to sports injury. The main pathological change in tendinopathy is ectopic ossification in tendon tissue, but the mechanisms have remained elusive. Studies have found that interleukin-6 (IL-6) is a major inflammatory mediator in chronic tendinopathy, and osteogenic differentiation of tendon stem cells (TSCs) is believed to be closely related to ectopic ossification of tendons.

Methods: Rat tendon-derived stem cell (rTDSC) culture model, Lentivirus transfection, Alkaline phosphatase staining, Real-time PCR and Western blotting were performed in this study.

Results: We showed that after IL-6 induction, the mRNA expression of Runx2, Alpl, Dlx5, and Wnt5a and the protein expression of phosphorylated STAT3, Runx2, and Wnt5a were increased in rTDSCs. Wnt5a shRNA and cDNA induced silencing and overexpression of Wnt5a inhibited and promoted osteogenic differentiation of rTDSCs, respectively. The addition of a STAT3 inhibitor inhibited osteogenic differentiation and Wnt5a mRNA and protein expression in rTDSCs, and this inhibition was reversed by cDNA induced Wnt5a overexpression.

Conclusion: We concluded that IL-6 promotes osteogenic differentiation of rTDSCs through the STAT3/Wnt5a signalling pathway.

Introduction

Tendinopathy refers to a condition of pain, swelling, and impaired performance of the tendon that is caused by overuse of the tendon [1-3]. Tendinopathy is not a disease unique to professional athletes, and it is also very common in the general population. The main pathological change in tendinopathy is ectopic ossification in tendon tissue [4,5]. TSCs are precursors of tendon cells and have multidirectional differentiation potential [6]. Additionally, TSCs tend to undergo osteogenic differentiation under the action of mechanical stress and inflammatory factors, suggesting that TSCs may be related to ectopic ossification of tendon tissue [7,8]. IL-6 expression is increased and IL-6 becomes a major inflammatory factor in chronic tendon disease and torn tendon tissue [9,10]. IL-6 expression is also increased in tendon tissue after mechanical stretching [11,12], suggesting that IL-6 may be related to pathological changes in tendons. The interleukin family can activate the JAK/STAT3 signalling pathway through ligand-specific receptors [13], and IL-6 can regulate the osteogenic differentiation of stem cells through STAT3 molecules [14-16]. Wnt5a belongs to the Wnt family of glycoproteins, which are released from activated cells [17,18]. Wnt5a can activate target genes located in the nucleus through canonical and noncanonical Wnt signalling pathways. Wnt5a works through canonical pathways to maintain the stemness and self-renewal of cells and controls cell polarity, cell adhesion, and movement through noncanonical pathways [19-22]. Additionally, Wnt5a can promote osteogenic differentiation of stem cells through noncanonical pathways [23,24]. IL-6 and cardiotrophin induce Wnt5a upregulation in rat myocardial cells through the STAT3 signalling pathway, but the mechanism is unknown [25]. IL-6 family cytokines, such as leptin, oncostatin M, ciliary neurotrophic factor, IL-6, and IL-11, bind to cell surface receptors and induce Wnt5a upregulation by IL6ST-JAK-STAT3. Then, Wnt5a binds to Frizzled receptors and coreceptors to activate the Wnt signalling pathway. IL-6 is significantly increased in the synovial fluid of patients with rheumatoid arthritis, and IL-6 can activate the IL6ST-JAK-STAT3 signalling pathway of synovial fibroblasts and is usually accompanied by elevated Wnt5a and FZD5 [26]. Recent studies have found that increased IL-6, IL-10, and COL1 in hepatic stellate cells can cause Wnt5a overexpression and are associated with liver fibrosis [27]. Additionally, IL-6 can induce overexpression of Wnt5a and promote melanoma cell movement [28]. However, the mechanism by which IL-6 regulates Wnt5a has not yet been thoroughly explored. Additionally, it is unclear whether IL-6 can induce osteogenic differentiation of stem cells by regulating Wnt5a.

Therefore, our study aimed to investigate whether IL-6 causes osteogenesis of rTDSCs through the STAT3/Wnt5a signalling pathway, which would probably provide new strategies for the treatment of tendinopathy.

Methods

Isolation and culture of rTDSCs

All experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No.8023, revised 1978), all experimental protocols were approved by the ethics committee of Zunyi Medical University (Zunyi, China). Six male 6-week-old Sprague-Dawley rats weighing 200-250g were provided and fed by the animal laboratory. The bilateral Achilles tendon tissues of the rat were excised and minced, digested with type I collagenase (3 mg/ml Sigma-Aldrich, St Louis, MO, USA) at 37°C for 2.5 h, and passed through a 70 μm cell filter (Becton Dickinson, Franklin Lakes, NJ, USA) to generate an rTDSC single-cell suspension. Then, the suspension was resuspended in Dulbecco's Modified Eagle's Medium containing 100U/ml penicillin, 100mg/ml streptomycin, 10% foetal bovine serum, and 2mM glutamine (HyClone, Logan City, Utah, USA). The isolated rTDSCs were diluted to an appropriate density (50 cells/cm^2) and cultured at 37°C under 5% CO_2 to form clones. After 2 days, the cells were washed twice with Phosphate-buffered saline (PBS) to remove nonadherent cells. After 7 days, the cells were digested with trypsin and were considered P_0 cells; then, the P_0 cells were passaged to P_2, and P_2 passage cells were used for all experiments. The identification of stem cell characteristics of rTDSCs was performed as described previously [29]. rTDSCs were seeded in 6-well plates at a density of 6×10^4/well and induced with 0, 0.1, or 1.0 ng/ml IL-6 (REIL P-06011, Cyagen Biosciences Inc., Santa Clara, CA, USA) for 5 or 7 days. The STAT3 inhibitor Stattic was added to cells at a concentration of 50 μM to inhibit STAT3.

Lentivirus transfection

The wnt5a-shRNA recombinant lentiviral vector was provided by BioWit Technologies Co., Lt-d (Shenzhen, China). The shRNAs were embedded in a lentiviral vector containing green fluor-escent protein (GFP) and cotransfected into 293T cells, and the lentivirus titre was 3×10^9 infe-ctious units per mL. Wnt5a-cDNA (BioWit Technologies Co., Ltd, Shenzhen, China) was intr-oduced into the lentiviral vector pLVX-r wnt5a-mCMV-Zs Green as the overexpression vector, and the lentivirus titre was 1×10^9 infectious units per mL. The pLvx-mCMV-ZsGreen vector was used as a scrambled control (cDNA control). The shRNA sequences targeting Wnt5a wereas follows: forward: 5'-CTTTTTTCTCGAGGGATCCCAATTCTAGTTATTAATAGTA-3', reverse:5'-ATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAG-3'.The cDNA sequences targetin-g Wnt5a were as follows: forward: 5'-TAATAAAAGCTAATTCTTGGTGGTCCCTAAGTATGAATAA-3', reverse: 5'-CCCTGTTCAGATGTCAGAAGTATACATCATAGGAGCACAG-3'. rTDSCswere seeded in 6-well plates overnight at a density of 1×10^5 per well. When the cells reache-d 50% confluency, lentiviruses were added. After 48 h of transfection, the medium was replace-d with normal medium, and IL-6 was added 4 days later. The lentiviral transfection efficiencywas determined by real-time PCR (RT-PCR) and Western blot.

Alkaline phosphatase staining

rTDSCs were induced with IL-6 at concentrations of 0.1 ng/ml and 1 ng/ml for 7 days. After IL-6 induction, the rTDSCs were washed twice with PBS and then fixed with 4% paraformaldehyde and NBT/BCIP (Sigma-Aldrich, St. Louis, MO, USA) solution at room temperature for 30 minutes. rTDSCs were observed and photographed under a microscope (Olympus BX51, Tokyo, Japan).

Real-time PCR

rTDSC gene expression was determined by RT-PCR. Total RNA was extracted with TRIzol Re-agent (Takara, Dalian, China) and subjected to reverse transcription PCR. RT-PCR was perfor-med using the Bio-Rad iCycler IQ system (Bio-Rad, CA, USA). GAPDH was used as an inte–rnal reference, and the relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method.The primer sequences (Table 1) were used.

Table1 Sequence of the primers used for quantitative RT-PCR

Accession no	Gene name	Primer sequence
NM_017008.4	GAPDH	Forward: 5′TGACTTCAACAGCAACTC3′
		Reverse: 5′TGTAGCCATATTCATTG3′
NM_022631.1	Wnt5a	Forward:5′AATTCGTGGACGCACG3′
		Reverse:5′GCCAGCATGTCTTGAGG3′
NM_001278483.1	Runx2	Forward:5′GAACTCAGCACCAAGTCCTTT3′
		Reverse:5′CAGTGTCATCATCTGAAATACG3′
NM_ 013059.1	Alpl	Forward:5′GGCACCATGACTTCCCAGAA3′ Reverse:5′CACCGTCCACCACCTTGTAAA3′
		Forward:5′CTTATGCGGACTACGGCTACGC3′ Reverse:5′CCTGGGTTTACGAACTTTCTTTG3′
NM_ 012943.1	Dlx5	

Western blotting

Total protein from rTDSCs was collected with RIPA buffer. The protein concentrations were determined using a BCA protein analysis kit (Thermo Fisher Scientific Inc.). Protein samples (30 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyethylene difluoride membrane. The membranes were incubated with 0.1% TBS-Tween containing 5% skimmed milk powder for 1 h at room temperature, and then the primary and secondary antibodies were added in sequence. Finally, the bands were visualized using a Li-Cor Odyssey Imager (LI-COR Biosciences, Lincoln, NE, USA). The following primary antibodies were used: anti-STAT3 (1:1000; ab68153, Abcam, Cambridge, UK), anti-STAT3 (phospho Y705; 1:1000; ab76315, Abcam, Cambridge, UK), anti-Wnt5a (1:500; ab110073, Abcam, Cambridge, UK), anti-Runx2 (1:1000; ab23981, Abcam, Cambridge, UK), GAPDH (1:1000; Pierce Biotechnology, USA), and anti-β-tubulin (1:1000; loading control; Pierce).

Statistical analysis

SPSS 17.0 statistics software was employed for statistical analysis (SPSS Inc., Chicago, IL, USA). All data are presented as the means ± SD (x±s), α=0.05. Student's t-test was applied when only two groups were compared, and P <0.05 was considered to indicate a statistically significant difference.

Results

IL-6 can induce osteogenic differentiation of rTDSCs

rTDSCs were induced with IL-6 at concentrations of 0 ng/ml, 0.1 ng/ml or 1 ng/ml for 5 or 7 days with 0 ng /ml IL-6 as the control. The qRT-PCR results showed that the expression levels of Runx2, Alpl, Dlx5 mRNA were higher in the treated group than in the control group (Fig. 1A-D, P<0.05). rTDSCs were induced with IL-6 at concentrations of 0 ng/ml or 1 ng/ml for 7 days with 0 ng /ml IL-6 as the control. The alkaline phosphatase staining results indicated that the alkaline phosphatase activity was higher in the treated group than in the control group (Fig. 1E). rTDSCs were induced with IL-6 at concentrations of 0 ng/ml, 0.1 ng/ml, or 1 ng/ml for 7 days, and the Western-blot results demonstrated that the protein expression level of Runx2 was higher in the treated group than in the control group (Fig. 1F). These data suggested that IL-6 can induce osteogenic differentiation of rTDSCs.

STAT3 and Wnt5a are activated by IL-6

rTDSCs were induced with IL-6 at concentrations of 0 ng/ml, 0.1 ng/ml or 1 ng/ml for 5 or 7 days with 0 ng/ml IL-6 as the control. qRT-PCR results showed that the expression of Wnt5a mRNA was higher in the treated group than in the control group (Fig. 2A and B, $P<0.05$). rTDSCs were induced with IL-6 at concentrations of 0.1 ng/ml or 1 ng/ml for 7 days. Western blot results demonstrated that the protein expression of Wnt5a (Fig. 2C) and P-STAT3 (Fig. 2D) was higher in the treated group than in the control group. These data demonstrated that STAT3 and Wnt5a were activated in rTDSCs after IL-6 induction.

Stattic inhibits IL-6-induced osteogenic differentiation and the expression of Wnt5a

After induction with IL-6 at a concentration of 1 ng/ml for 7 days, the mRNA expression of Runx2 (Fig. 3A) and Wnt5a (Fig. 3B) was decreased in the IL-6+Stattic group compared with that in the group treated with IL-6 alone ($P<0.05$). Compared with that in the control group, the expression of P-STAT3 was dramatically decreased in the IL-6+Stattic group (Fig. 3C). After rTDSCs were induced with IL-6 at a concentration of 1 ng/ml for 7 days, Western blot results demonstrated that the protein expression of Runx2 (Fig. 3D) and Wnt5a (Fig. 3E) was decreased in the IL-6+Stattic group compared with that in the group treated with IL-6 alone. These results demonstrated that STAT3 regulated the IL-6-induced osteogenic differentiation of rTDSCs and regulated the expression of Wnt5a.

IL-6 regulates the osteogenic differentiation of rTDSCs through the STAT3/Wnt5a signalling pathway

Wnt5a protein (Fig. 4A) expression was significantly decreased in rTDSCs after transfection with Wnt5a shRNA for 4 days compared with that in rTDSCs transfected with the shRNA control, and Wnt5a protein expression was significantly increased in rTDSCs after transfection with Wnt5a cDNA for 4 days compared with that in rTDSCs transfected with the cDNA control (Fig. 4B). After transfection with Wnt5a shRNA and Wnt5a cDNA for 4 days, P-STAT3 expression was not significantly changed compared with the control groups (Fig. 4C). It was demonstrated that Wnt5a had no effect on P-STAT3 expression. To determine whether Wnt5a regulated IL-6-induced Runx2 expression, after rTDSCs were transfected with Wnt5a shRNA or Wnt5a cDNA for 4 days, 1 ng/ml IL-6 was added to the Wnt5a shRNA, Wnt5a cDNA and nonlentiviral transfection groups for 7 days, and 0 ng/ml IL-6 was added as a control group; the IL-6-induced upregulation of Runx2 protein (Fig. 4D) and Runx2 mRNA (Fig. 4F) expression was decreased in the Wnt5a shRNA-transfected group and increased in the Wnt5a cDNA-transfected group compared to those in the 1 ng/ml IL-6-transfected group. These data demonstrated that Wnt5a regulated the IL-6-induced osteogenic differentiation of rTDSCs. The IL-6-induced upregulation of Runx2 mRNA and protein expression was suppressed in rTDSCs treated with 50 μM Stattic compared to that in the 1 ng/ml IL-6-treated group, and Runx2 mRNA expression (Fig. 4G) and Runx2 protein expression (Fig. 4E) were restored by transfection with Wnt5a cDNA. These results demonstrated that IL-6 promoted the osteogenic differentiation of rTDSCs through the STAT3/Wnt5a signalling pathway.

Discussion

The main pathological change in tendinopathy is ectopic ossification in tendon tissue. Inflammatory factors are considered to be important factors that cause pain in tendinopathy and pathological changes in tendon tissue [30, 31]. IL-6 is the main inflammatory mediator in injured tendon tissue [32] and regulates the osteogenic differentiation of stem cells through the STAT3 signalling pathway [33], and studies have found that the STAT3/Wnt5a signalling pathway regulates stem cell self-renewal [34]. Whether STAT3 regulates Wnt5a and causes stem cell osteogenic differentiation is still unclear.

In our study, IL-6 induced rTDSCs to differentiate into osteogenic cells. After IL-6 induction, Wnt5a mRNA and protein expression and P-STAT3 levels were increased. Wnt5a regulated the differentiation of rTDSCs to osteogenesis but had no effect on STAT3. Stattic inhibited IL-6-induced osteogenic differentiation and Wnt5a mRNA and protein expression, and this inhibition was reversed by cDNA-induced Wnt5a overexpression. This indicates that IL-6 promotes the osteogenic differentiation of rTDSCs through the STAT3/Wnt5a signalling pathway.

Most studies consider osteogenic differentiation and adipogenic differentiation to be mutually inhibitory [35]. Wnt molecules can promote osteogenic differentiation by inhibiting the adipogenic differentiation of stem cells through noncanonical signalling pathways, but some studies have suggested that Wnt5a can promote adipogenic differentiation of stem cells [36]. Whether IL-6 also promotes adipogenic differentiation of stem cells through the STAT3/Wnt5a signalling pathway remains unclear.

Conclusions

Our study found that IL-6 can induce osteoblast differentiation of rTDSCs through the STAT3/Wnt5a signalling pathway. Additionally, STAT3 is the upstream regulator of the STAT3/Wnt5a signalling pathway. This study provides new strategies for the prevention and treatment of tendinopathy.

Abbreviations

IL-6: interleukin-6; TSCs: tendon stem cells; rTDSC: Rat tendon-derived stem cells; PBS: Phosphate-buffered Saline; GFP: green fluorescent protein; ALP: Alkaline phosphatase; P-STAT3: phosphorylated STAT3; RT-PCR: Real-time PCR

Declarations

Acknowledgements

Not applicable.

Authors' contributions

XL analyzed and interpreted the data, prepared all figures, drafted the manuscript, revised it, collected and analyzed the data and drafted the manuscript; YJ, JP, and XL collected the data and provided technical assistance; JP polished the language of the article. All authors contributed to the data interpretation and manuscript preparation. All authors approved the final submitted submission.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Ethics approval

All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the ethics committee of Zunyi Medical University (Zunyi, China).

Consent for publication

Applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

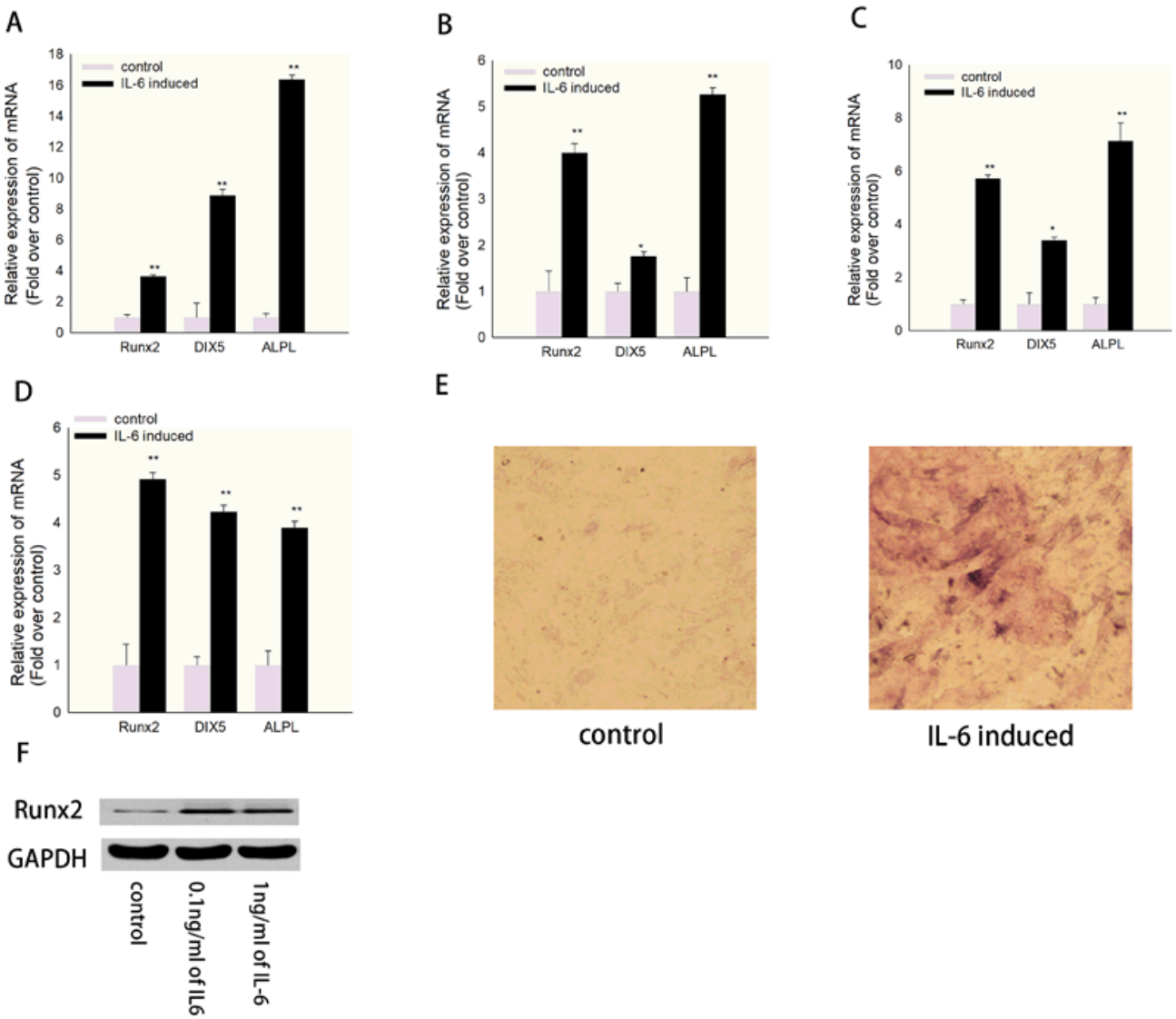

Figure 1

IL-6 induced osteogenic differentiation of rTDSCs. The mRNA expression of osteogenic genes (Runx2, Dlx5, and Alpl) in rTDSCs after induction with 0.1 ng/ml IL-6 for 5 days (A) or 7 days (B) and after induction with 1 ng/ml IL-6 for 5 days (C) or 7 days (D) compared to the expression in the control group (n = 3; *P < 0.05; **P < 0.01). ALP staining of rTDSCs after 7 days of induction with 1 ng/ml IL-6 compared to that of control rTDSCs (E Scale bar: 50 μm). Runx2 protein levels in rTDSCs after treatment with 0.1 ng/ml or 1 ng/ml IL-6 for 7 days relative to the control rTDSCs (F). GAPDH was used as the loading control.

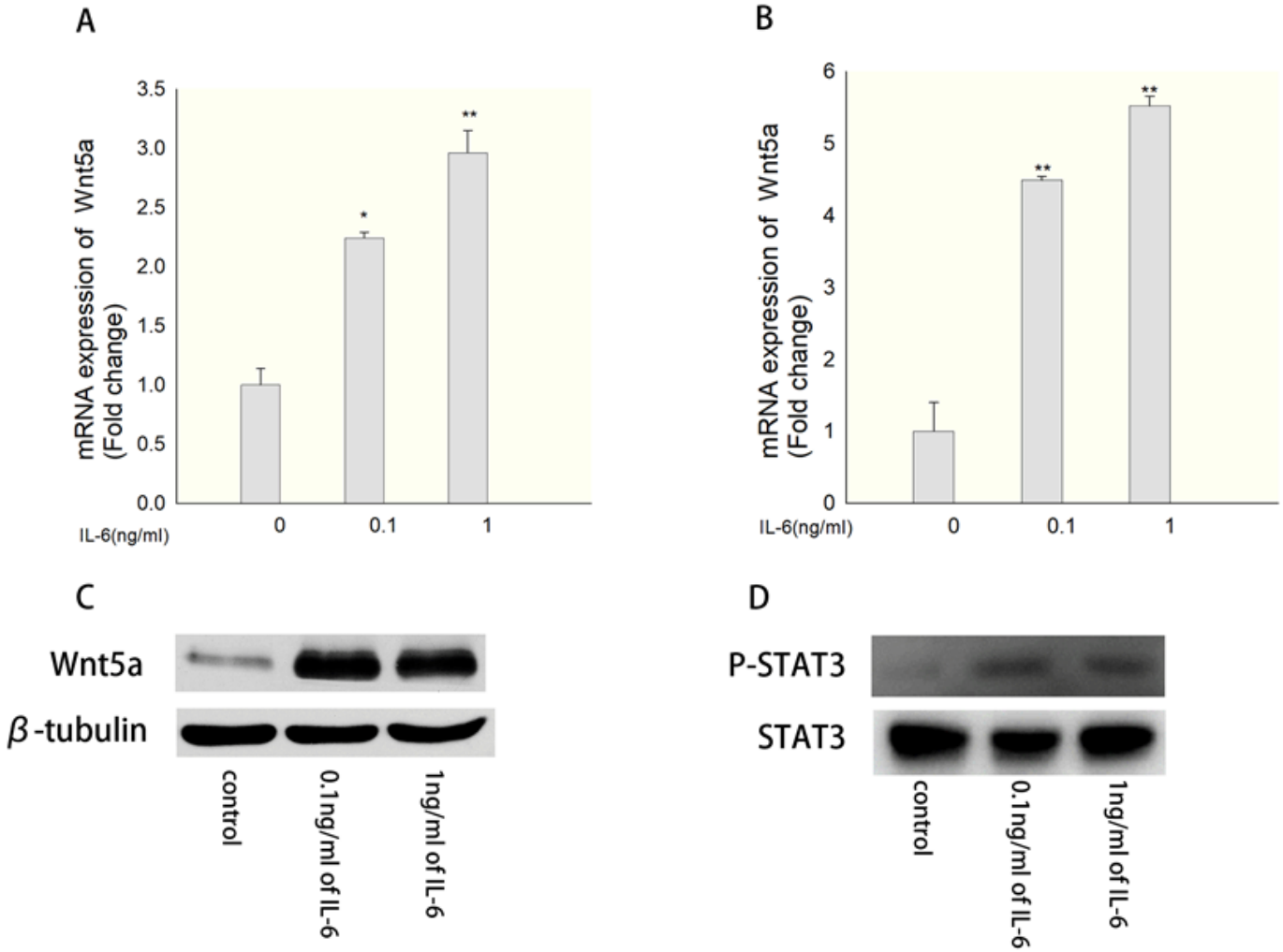

Figure 2

STAT3 and Wnt5a are activated by IL-6. The mRNA expression of Wnt5a in rTDSCs after treatment with 0.1 ng/ml or 1 ng/ml IL-6 for 5 days (A) or 7 days (B) compared to the expression in the control groups (n = 3; *P < 0.05; **P < 0.01). Wnt5a protein (C) and P-STAT3 (D) levels in rTDSCs after treatment with 0.1 ng/ml or 1 ng/ml IL-6 for 7 days relative to that in the control groups. β-Tubulin and STAT3 were used as the loading controls.

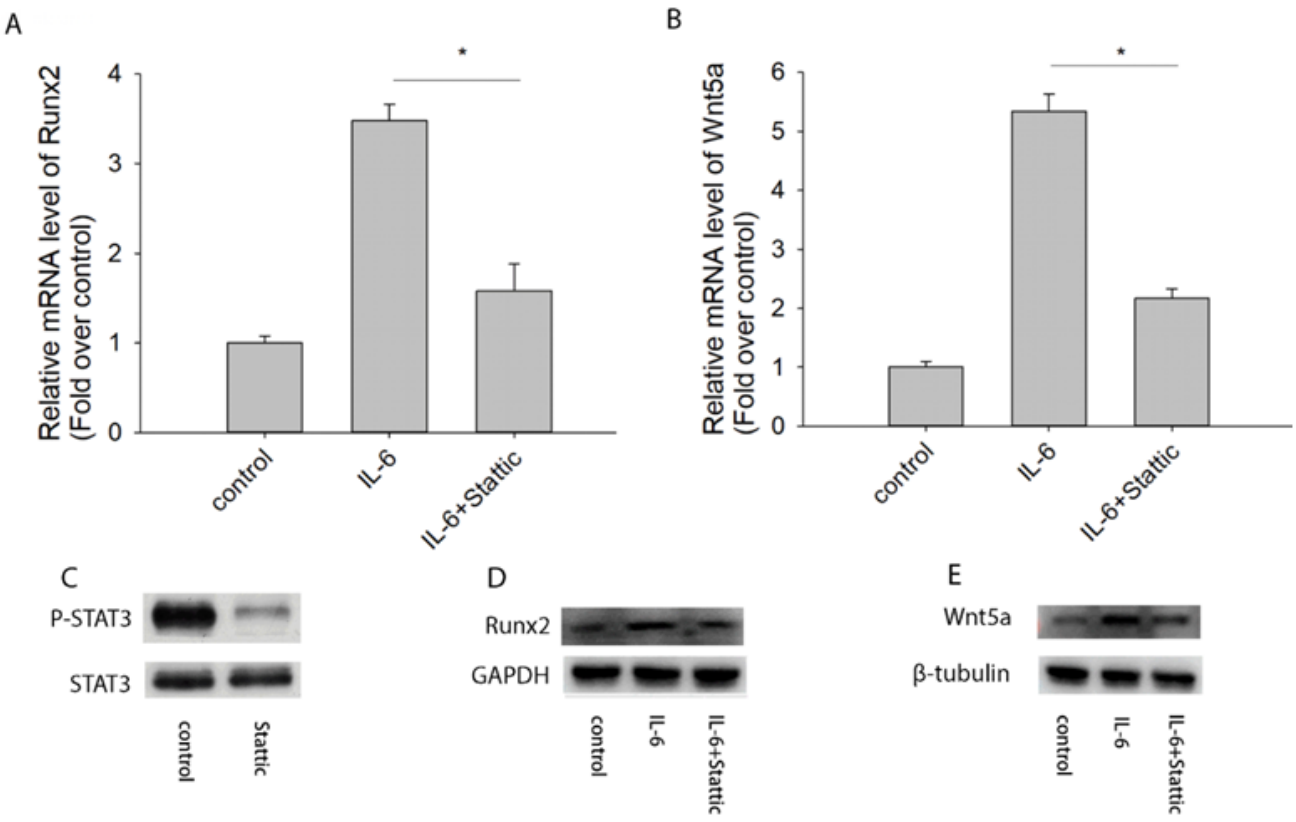

Figure 3

STAT3 regulated IL-6-induced osteogenic differentiation of rTDSCs and regulated the expression of Wnt5a. After 0 ng/ml IL-6 (control), 1 ng/ml IL-6, or 1 ng/ml IL-6 plus 50 μM Stattic were added for 7 days, the mRNA expression of Runx2 (A) and Wnt5a (B) in the treated rTDSCs was compared to that in the control group (n = 3; *P < 0.05). After rTDSCs were induced with Stattic at concentrations of 0 (control) or 50 μM for 7 days, the P-STAT3 level in the treated group was compared to that in the control group (C). STAT3 was used as the loading control. After rTDSCs were induced for 7 days with 0 ng/ml IL-6, 1 ng/ml IL-6, or 1 ng/ml IL-6 plus 50 μM Stattic, the Runx2 (D) and Wnt5a protein (E) levels in the treated groups were compared to those in the control groups. β-Tubulin and GAPDH were used as the loading controls.

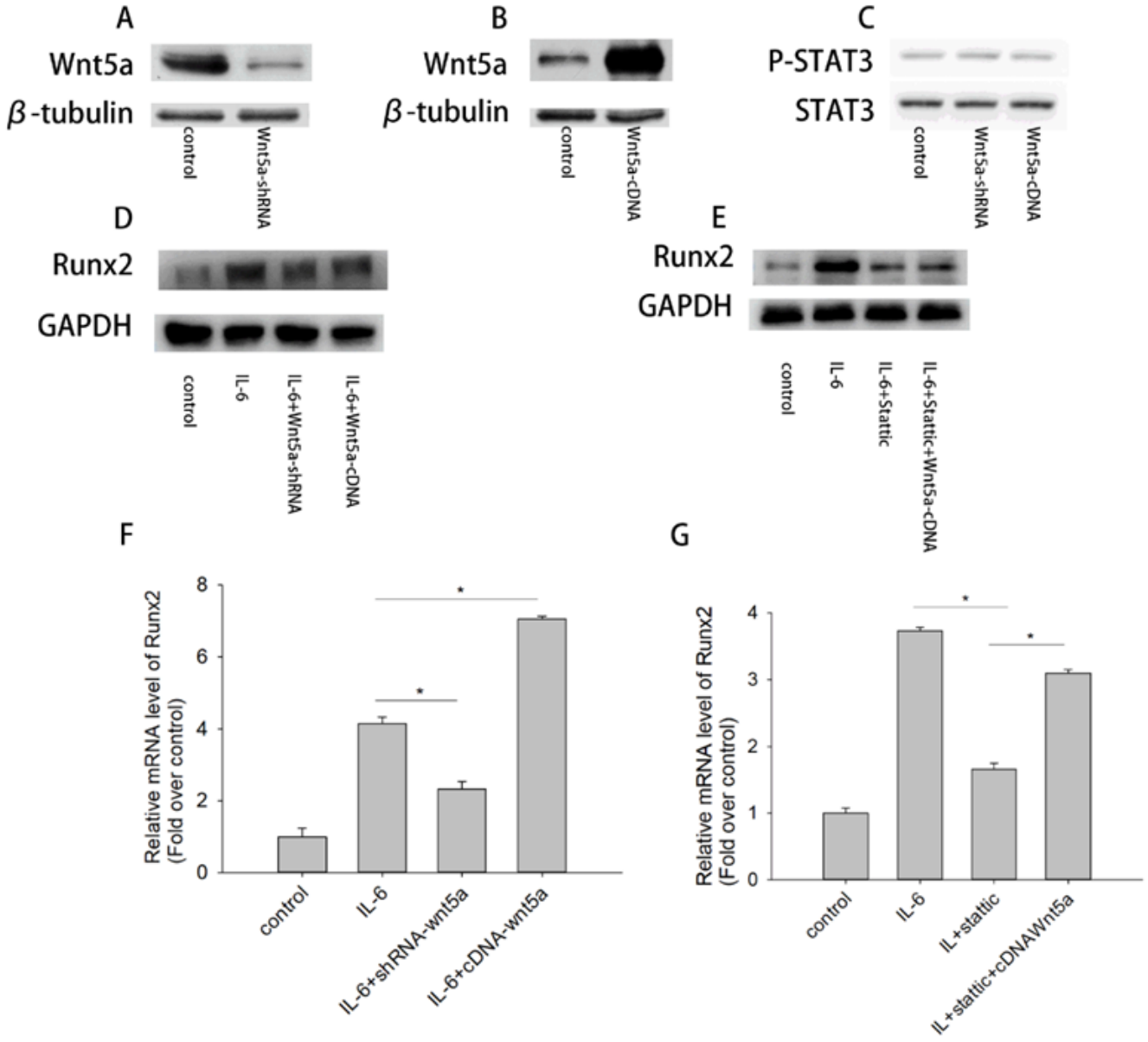

Figure 4

IL-6 regulated the osteogenic differentiation of rTDSCs through the STAT3/Wnt5a signalling pathway. Wnt5a protein levels in rTDSCs after transfection with Wnt5a shRNA (A) and Wnt5a cDNA (B) for 4 days compared with those in the controls. P-STAT3 levels in rTDSCs after transfection with Wnt5a shRNA or Wnt5a cDNA for 4 days compared with those in the controls (C). After 7 days of induction with 1 ng/ml IL-6, Runx2 protein expression in rTDSCs transfected with Wnt5a shRNA or Wnt5a cDNA was compared with that in the IL-6 control group (D). After 7 days of induction with 1 ng/ml IL-6, Runx2 mRNA expression in rTDSCs transfected with Wnt5a shRNA or Wnt5a cDNA was compared with that in the IL-6 control group (F) (n = 3; *P < 0.05). Runx2 protein expression (E) and mRNA expression (G) in the IL-6, IL-6+Stattic and IL-6+Stattic+Wnt5a cDNA groups after 7 days of induction with 1 ng/ml IL-6 relative to those in the control group (n = 3; *P < 0.05).