

# Expression of PADI4 in patients with ankylosing spondylitis and its role in mediating the effects of TNF- $\alpha$ on the proliferation and osteogenic differentiation of human mesenchymal stem cells

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Received February 2, 2015; Accepted June 9, 2015

DOI: 10.3892/ijmm.2015.2248

**Abstract.** Peptidyl arginine deiminase, type IV (PADI4) plays an important role in inflammation and in the immune response, and it has been shown to be associated with rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (AS). However, little is known about the precise role of PADI4 in the pathogenic process *in vitro*. In this study, we aimed to investigate the expression of PADI4 in the synovial tissue of patients with AS and to determine the potential effects of PADI4 on human mesenchymal stem cell (hMSC) proliferation and osteogenic differentiation under normal and pathological conditions. Synovial tissues were collected from 18 patients with AS and 11 control subjects. The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis revealed that the expression of PADI4 was upregulated in the patients with AS. In the hMSCs, the protein expression of PADI4 was increased following treatment with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in a dose- and time-dependent manner. MTT assay revealed that TNF- $\alpha$  promoted hMSC proliferation. In addition, we found that TNF- $\alpha$  promoted the osteogenic differentiation of hMSCs, as demonstrated by an increase in alkaline phosphatase (ALP) activity, as well as an increase in the expression of bone morphogenetic protein 2 (BMP-2), runt-related transcription factor 2 (Runx2) and Osterix. The hMSCs were transfected with PADI4 siRNA to silence PADI4 expression. We found that, under normal conditions, the silencing of PADI4 did not have any effect on hMSC proliferation or osteogenic differentiation. However, in the presence of

TNF- $\alpha$ , hMSC proliferation and osteogenic differentiation were induced. These effects were attenuated by the silencing of PADI4. In conclusion, the findings of this study demonstrate that the expression of PADI4 differs between patients with AS and normal subjects. In addition, our data suggest that PADI4 plays a role in hMSC proliferation and differentiation, which are induced by TNF- $\alpha$ .

## Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disorder that primarily affects the spine and sacroiliac joint. It is a type of spondyloarthritis (SpA) and has a particularly strong genetic association with human leukocyte antigen (HLA)-B27 (1).

The simultaneous destruction and excessive formation of bone are the most distinctive features of AS (2-4), and these processes lead to the formation of syndesmophytes combined with systemic bone loss (5-8). There has been marked progress in the treatment of AS, due to the development of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) blocking agents, and anti-TNF- $\alpha$  therapy has become the standard of care for patients with AS over the past decade. However, to the best of our knowledge, the pathophysiological mechanisms responsible for the effects of TNF- $\alpha$  on new bone formation and osteoporosis in AS have not been fully elucidated.

Peptidyl arginine deiminase, type IV (PADI4) is an enzyme that catalyzes the conversion of arginine residues to citrulline residues. It is predominantly expressed in granulocytes and monocytes (9) and plays an important role in inflammation and the immune response (10-13). Emerging evidence has indicated that the expression of PADI4 is associated with the development of rheumatoid arthritis (RA), osteoarthritis (OA) and AS (11,14,15). However, little is known about the precise role of PADI4 in the pathogenic process *in vitro*.

In the present study, we examined the expression of PADI4 in the synovial tissue of patients with AS and in normal controls. We then carried out an *in vitro* experiment to investigate the potential effects of PADI4 on human mesenchymal stem cell (hMSC) proliferation and osteogenic differentiation under normal and pathological conditions. This study indicates that there is a novel mechanism underlying anti-TNF- $\alpha$  therapy for patients with AS.

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**Key words:** ankylosing spondylitis, peptidyl arginine deiminase type IV, tumor necrosis factor- $\alpha$ , osteogenic differentiation, proliferation

## Materials and methods

**Sample collection.** A total of 18 patients diagnosed with AS and 11 healthy control subjects with traumatic fractures were enrolled in the present study. The patients and controls provided informed written consent prior to enrollment in this study, and the study protocol was approved by the Ethics Committee of Xinyu Hospital of Nanchang University, Xinyu, China. Synovial tissue samples were collected during hip replacement surgery from the patients with AS and the healthy controls with traumatic fractures. The synovial tissue samples were immediately stored at  $-80^{\circ}\text{C}$  following dissection from the connective tissue.

**Cell culture and transfection.** Bone marrow-derived hMSCs (registration no. PCS-500-012; ATCC, Manassas, VA, USA) were cultured in mesenchymal stem cell basal medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 15 ng/ml insulin-like growth factor (IGF)-1, 125 pg/ml fibroblast growth factor-basic (FGF-b) and 2.4 mM L-Alanyl-L-Glutamine. The cells were grown at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in a humidified atmosphere. TNF- $\alpha$  was purchased from PeproTech (Rocky Hill, NJ, USA) and diluted in 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). Cells in the vehicle group were treated with 1% BSA only. Cell transfection with PADI4 siRNA or scrambled control siRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, 2  $\mu\text{g}$  siRNA were diluted with Opti-MEM I (Gibco-BRL, Grand Island, NY, USA) and incubated with Lipofectamine 2000 at  $37^{\circ}\text{C}$ . The lipid-DNA complexes were then added to each well, and the cells were incubated at  $37^{\circ}\text{C}$  for 4 h.

**Reverse transcription-quantitative (real-time) PCR (RT-qPCR).** Total RNA was extracted using TRIzol reagent (Invitrogen). A total of 6  $\mu\text{g}$  of each RNA sample was used for reverse transcription using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Quantitative PCR (qPCR) was performed on a Corbett Rotor-Gene 6000 system (Corbett Research, Westburg, Leusden, The Netherlands) using the PerfeCTa qPCR FastMix (Quanta Biosciences, Inc., Gaithersburg, MD, USA) following the manufacturer's instructions. The primer sequences were as follows: PADI4 forward, 5'-tttgggaacctggaagtgag-3' and reverse, 5'-ggcacaagctcaggaactc-3'; and  $\beta$ -actin forward, 5'-cattaaggagaagctgtgct-3' and reverse, 5'-gttgaaggtatgttctgga-3'.  $\beta$ -actin was used as a housekeeping gene. The Ct value was calculated using the  $\Delta\Delta\text{Ct}$  method.

**Protein extraction and western blot analysis.** Total protein was extracted using lysis buffer (150 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2% SDS and 1 mM PMSF). After 30 min on ice, the lysates were centrifuged for 1 min at  $4^{\circ}\text{C}$ , and the supernatant was then collected. The protein content was assessed using the bicinchoninic acid assay (BCA) method with reagents from Pierce Biotechnology, Inc. (Rockford, IL, USA). Protein (40  $\mu\text{g}$ ) was separated on a 12% SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk, the membranes were incubated with anti-PADI4 rabbit

polyclonal antibody (Cat. no. sc-98991, 1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-bone morphogenetic protein 2 (BMP-2) mouse monoclonal antibody (Cat. no. ab6285, 1:400 dilution), anti runt-related transcription factor 2 (Runx2) rabbit polyclonal antibody (Cat. no. ab102711, 1:400 dilution), anti-Osterix mouse monoclonal antibody (Cat. no. ab57335, 1:800 dilution) (all from Abcam, Cambridge, MA, USA) and anti  $\beta$ -actin mouse monoclonal antibody (Cat. no. BM0627, 1:1,000 dilution; Boster, Wuhan, China) at  $37^{\circ}\text{C}$  for 2 h. The membranes were washed 3 times with TBST and incubated with rabbit-anti mouse IgG (Cat. no. sc-358913, 1:2,000 dilution) or mouse-anti rabbit IgG (Cat. no. sc-2357, 1:2,000 dilution) horseradish peroxidase (HRP)-conjugated secondary antibody (both from Santa Cruz Biotechnology) at  $37^{\circ}\text{C}$  for 1 h. The signals were detected using an ECL detection kit (Pierce Biotechnology, Inc.). The intensity of the protein bands was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). The relative protein levels were normalized against  $\beta$ -actin.

**Cell proliferation assay.** The cells were seeded onto 96-well plates and incubated with 10 ng/ml TNF- $\alpha$  for 12, 24, 48 and 72 h. Cell proliferation was examined by MTT assay. MTT reagent (20  $\mu\text{l}$ ; Beyotime Institute of Biotechnology, Shanghai, China) was added to the cell cultures and incubated at  $37^{\circ}\text{C}$  for 4 h. Subsequently, 150  $\mu\text{l}$  of dimethyl sulfoxide (DMSO) were added and mixed gently for 10 min. The absorbance at 570 nm was measured using a microplate reader (Ascent 354; Thermo Labsystems, Waltham, MA, USA).

**Measurement of alkaline phosphatase (ALP) activity.** ALP activity in the hMSCs was measured using an Alkaline Phosphatase Activity Colorimetric assay kit (BioVision Inc., Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, the cells were homogenized and centrifuged at 13,000  $\times$  g for 3 min. The samples and the p-nitrophenyl phosphate (pNPP) standard were added to the 96-well plates, and the ALP enzyme solution was then added to each well followed by incubation at room temperature for 60 min. The reactions were terminated by the addition of Stop Solution. The optical density at 405 nm was measured using a microplate reader (Ascent 354; Thermo Labsystems). The amount of purine nucleoside phosphorylase (pNP) generated by the ALP sample was calculated by applying sample readings to the standard curve.

**Statistical analyses.** Statistical analyses of the data were performed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). The student's t-test was used to assess the statistical differences between 2 groups. A P-value  $<0.05$  was considered to indicate a statistically significant difference.

## Results

**PADI4 expression is increased in the synovial tissues of patients with AS.** The expression levels of PADI4 in the synovial tissues from 18 patients with AS and 11 controls were measured by RT-qPCR and western blot analysis. As shown in Fig. 1, the relative mRNA expression of PADI4 was significantly higher in the patients with AS compared

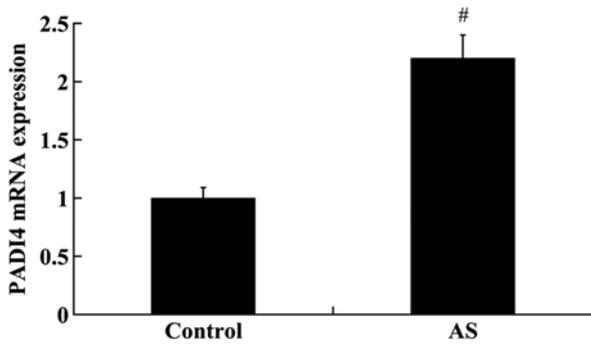


Figure 1. RT-qPCR analysis of peptidyl arginine deiminase, type IV (PADI4) mRNA expression in the synovial tissues from patients with ankylosing spondylitis (AS). <sup>#</sup>P<0.01 compared with the control.

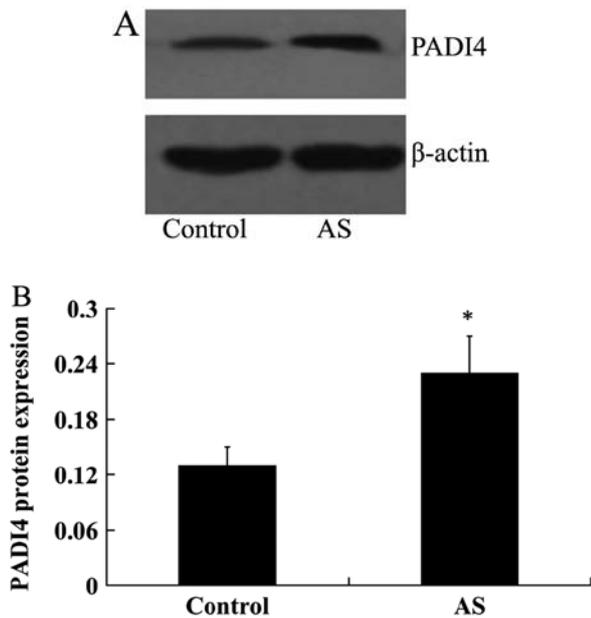


Figure 2. Western blot analysis of peptidyl arginine deiminase, type IV (PADI4) protein expression in the synovial tissues from patients with ankylosing spondylitis (AS). (A) Blots showing PADI4 and  $\beta$ -actin protein expression. (B) Relative protein expression of PADI4.  $\beta$ -actin was used as a loading control. <sup>\*</sup>P<0.05 compared with the control.

with the controls (fold change >2.0, P<0.01). The results from western blot analysis revealed that PADI4 protein expression was also increased in the patients with AS compared with the controls ( $0.23\pm 0.04$  vs.  $0.13\pm 0.02$ , P<0.05; Fig. 2).

**TNF- $\alpha$  promotes PADI4 expression in hMSCs.** In order to investigate the effect of TNF- $\alpha$  on PADI4 expression, the hMSCs were cultured with 10 ng/ml TNF- $\alpha$  for 6, 12, 24 and 48 h. Western blot analysis was then performed to measure the expression levels of PADI4 in the hMSCs. At 6 h, no significant difference in PADI4 expression was detected. However, the increased expression of PADI4 in the hMSCs was detected at 12, 24 and 48 h (Fig. 3), with the maximal response being observed at 48 h. The hMSCs were then cultured with 0.01, 0.1, 1 and 10 ng/ml TNF- $\alpha$  for 48 h. As shown in Fig. 4, PADI4 protein expression was increased in a dose-dependent manner by TNF- $\alpha$ , with the maximal response being observed following treatment with TNF- $\alpha$  at 10 ng/ml.

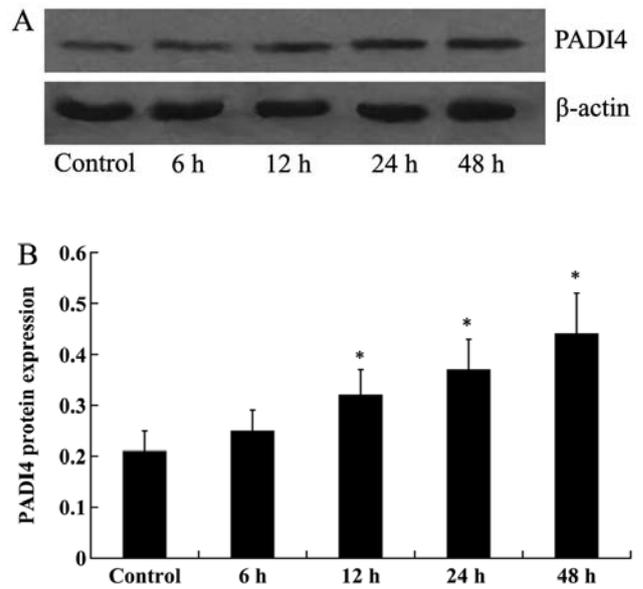


Figure 3. Western blot analysis of peptidyl arginine deiminase, type IV (PADI4) protein in human mesenchymal stem cells (hMSCs) following treatment with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) for 6, 12, 24 and 48 h. (A) Blots showing PADI4 and  $\beta$ -actin protein expression. (B) Relative protein expression of PADI4.  $\beta$ -actin was used as a loading control. <sup>\*</sup>P<0.05 compared with the control.

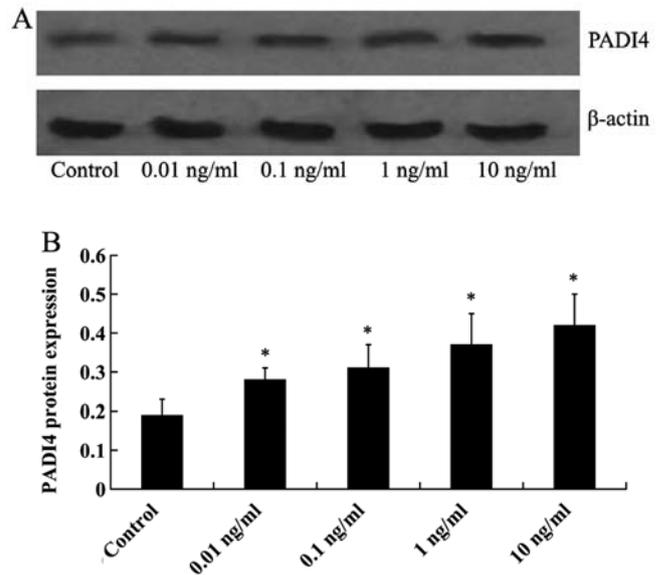


Figure 4. Western blot analysis of peptidyl arginine deiminase, type IV (PADI4) protein in human mesenchymal stem cells (hMSCs) following treatment with 0.01, 0.1, 1 and 10 ng/ml tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). (A) Blots showing PADI4 and  $\beta$ -actin protein expression. (B) Relative protein expression of PADI4.  $\beta$ -actin was used as a loading control. <sup>\*</sup>P<0.05 compared with the control.

**Silencing of PADI4 attenuates the TNF- $\alpha$ -induced proliferation of hMSCs.** In order to silence PADI4 expression in the hMSCs, the cells were transfected with siRNA against PADI4. As shown by the results of western blot analysis, PADI4 protein expression was significantly decreased in the hMSCs transfected with PADI4 siRNA in the presence or absence of TNF- $\alpha$  (P<0.01; Fig. 5).

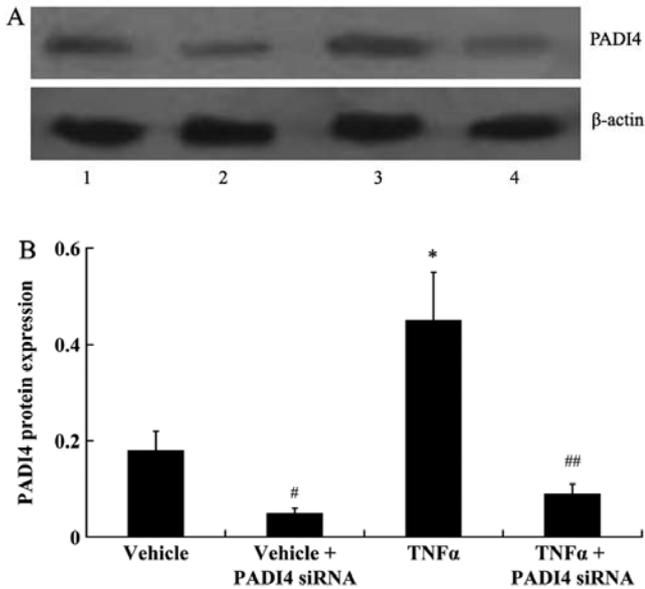


Figure 5. Western blot analysis of peptidyl arginine deiminase, type IV (PADI4) protein expression in human mesenchymal stem cells (hMSCs) following transfection with PADI4 siRNA in the presence or absence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). (A) Blots showing PADI4 and  $\beta$ -actin protein expression. Lane 1, vehicle-treated group; lane 2, vehicle + PADI4 siRNA-treated group; lane 3, TNF- $\alpha$ -treated group; lane 4, TNF- $\alpha$  + PADI4 siRNA-treated group. (B) Relative protein expression of PADI4.  $\beta$ -actin was used as a loading control. \* $P$ <0.01 and # $P$ <0.05 compared with the vehicle-treated group. ## $P$ <0.01 compared with the TNF- $\alpha$ -treated group.

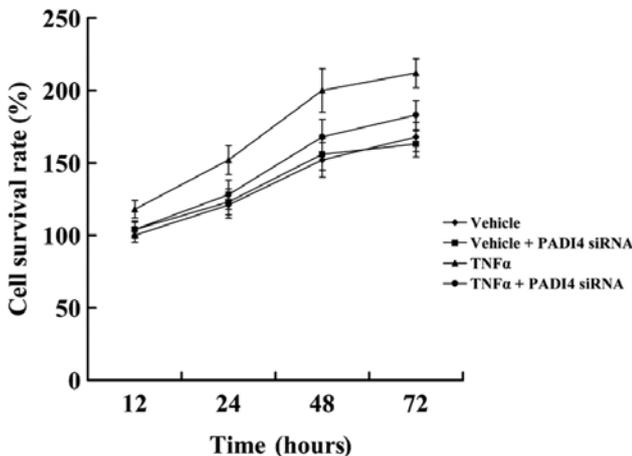


Figure 6. MTT assay of human mesenchymal stem cell (hMSC) proliferative ability following transfection with peptidyl arginine deiminase, type IV (PADI4) siRNA in the presence or absence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).

Subsequently, MTT assay was used to examine the proliferation of hMSCs in which PADI4 was silenced by siRNA. As shown in Fig. 6, treatment with 10 ng/ml TNF- $\alpha$  resulted in a significant induction of hMSC proliferation in comparison to the untreated cells. In the absence of TNF- $\alpha$ , cell viability did not differ significantly between the control and PADI4-silenced hMSCs. However, in the presence of TNF- $\alpha$ , the PADI4-silenced hMSCs proliferated at a lower rate than the control cells.

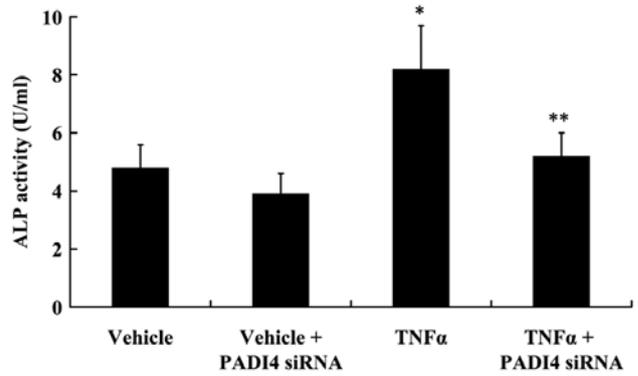


Figure 7. Alkaline phosphatase (ALP) activity in human mesenchymal stem cells (hMSCs) following transfection with peptidyl arginine deiminase, type IV (PADI4) siRNA in the presence or absence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). \* $P$ <0.05 compared with the vehicle-treated group. \*\* $P$ <0.05 compared with the TNF- $\alpha$ -treated group.

*Silencing of PADI4 attenuates the TNF- $\alpha$ -induced osteogenic differentiation of hMSCs.* To investigate the osteogenic differentiation of hMSCs, ALP activity and the expression of BMP-2, Runx2 and Osterix was examined. TNF- $\alpha$  (10 ng/ml) was used to treat the hMSCs for 48 h. As shown in Fig. 7, ALP activity increased significantly following treatment with TNF- $\alpha$  compared with the control (not treated with TNF- $\alpha$ ;  $P$ <0.05). The silencing of PADI4 did not alter ALP activity in the untreated cells. However, the silencing of PADI4 resulted in a significant decrease in ALP activity in the TNF- $\alpha$ -treated hMSCs ( $P$ <0.05; Fig. 7). The results from western blot analysis indicated that TNF- $\alpha$  significantly increased the expression of BMP-2, Runx2 and Osterix in the hMSCs ( $P$ <0.05). In the absence of TNF- $\alpha$ , the expression of BMP-2, Runx2 and Osterix did not change significantly following transfection of the hMSCs with PADI4 siRNA. However, in the presence of TNF- $\alpha$ , the expression of BMP-2, Runx2 and Osterix decreased significantly following transfection with PADI4 siRNA ( $P$ <0.05; Fig. 8).

## Discussion

Using whole genome SNP scanning, PADI4 has been identified as a risk factor for RA (16-18), and the elevated expression of PADI4 has been detected in the synovial membrane and synovial fluid of patients with RA (11,14). In the present study, we examined the expression of PADI4 in patients with AS. Similar to the results of the above-mentioned studies obtained from patients with RA, in our study, the expression of PADI4 was found to be significantly increased in the synovial tissues of patients with AS both at the mRNA and protein level.

AS is characterized by massive bone loss and ectopic bone formation (2-4). Bone remodeling is affected by multiple factors, including cytokines, hormones and signaling molecules (19-21). A number of cell types, such as osteoblasts (OBs), osteoclasts (OCs) and osteocytes (OYs) have been shown to be involved in this process (22,23). In general, the activation of OCs is linked with bone loss and the development of erosion. By contrast, the activation of OBs and the inhibition of OCs are associated with new bone formation and ossification (24).

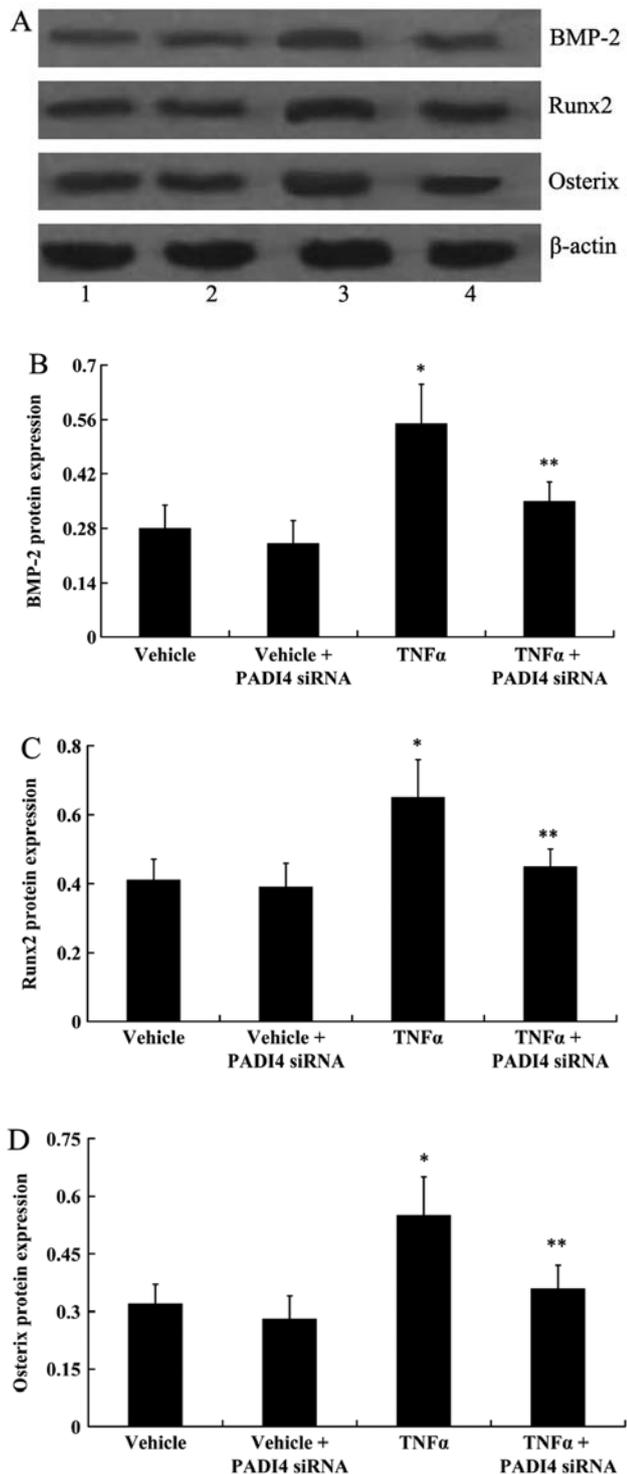


Figure 8. Western blot analysis of bone morphogenetic protein 2 (BMP-2), runt-related transcription factor 2 (Runx) and Osterix protein expression in human mesenchymal stem cells (hMSCs) following transfection with peptidyl arginine deiminase, type IV (PADI4) siRNA in the presence or absence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). (A) Blots showing BMP-2, Runx2, Osterix and  $\beta$ -actin protein expression. Lane 1, vehicle-treated group; lane 2, vehicle + PADI4 siRNA-treated group; lane 3, TNF- $\alpha$ -treated group; lane 4, TNF- $\alpha$  + PADI4 siRNA-treated group. (B) Relative protein expression of BMP-2. (C) Relative protein expression of Runx2. (D) Relative protein expression of Osterix.  $\beta$ -actin was used as a loading control. \* $P < 0.05$  compared with the vehicle-treated group. \*\* $P < 0.05$  compared with the TNF- $\alpha$ -treated group.

TNF- $\alpha$  is a highly potent pro-inflammatory molecule in the immune system. It is well known that TNF- $\alpha$  plays an

important role in the regulation of bone homeostasis and is involved in the pathogenesis of chronic immune and inflammatory joint diseases (25,26). It has been established that TNF- $\alpha$  acts as a stimulator of osteoclastogenesis (27-31) and an inhibitor of osteoblastogenesis (32-36). These actions are dependent on the TNF- $\alpha$  concentration, exposure time, and on the cell type (26,37,38). In addition, TNF- $\alpha$  possesses osteogenic differentiation capabilities. Mesenchymal stem cells are multipotent stromal cells and can differentiate into various cell types, including those of connective tissue and bone (39). In the present study, we found that TNF- $\alpha$  induced the proliferation of hMSCs, which is consistent with the results of previous studies (26,40). BMP-2 and ALP are well-known osteogenetic proteins, and RUNX2 and Osterix are two critical regulators of osteogenic differentiation. In this study, we examined the expression of proteins associated with osteogenic differentiation. We found that TNF- $\alpha$  promoted the osteogenic differentiation of hMSCs, as demonstrated by an increase in ALP activity, and an increase in the expression of BMP-2, Runx2 and Osterix.

It has been previously reported that TNF- $\alpha$  induces PADI4 translocation to the nucleus and that this is followed by the regulation of the expression of various genes (41). In the present study, we investigated to the best of our knowledge, this is for the first time that the effect of TNF- $\alpha$  on PADI4 expression has been studied. In this study, the time- and dose-dependent induction of PADI4 protein expression by TNF- $\alpha$  was observed in the hMSCs. To further elucidate the effects of PADI4 on hMSC proliferation and osteogenic differentiation under normal and pathological conditions, the cells were transfected with PADI4 siRNA in order to silence PADI4 expression. We observed that, under normal conditions, the silencing of PADI4 did not have any effect on hMSC proliferation or osteogenic differentiation. However, in the presence of TNF- $\alpha$ , hMSC proliferation and osteogenic differentiation were induced; these effects were attenuated by the silencing of PADI4. These results indicate that both PADI4 and TNF- $\alpha$  are involved in the induction of hMSC proliferation and osteogenic differentiation by TNF- $\alpha$ .

In conclusion, in this study, we demonstrated that the expression of PADI4 differs between patients with AS and normal subjects. TNF- $\alpha$  increased the protein expression of PADI4 in hMSCs in a dose- and time-dependent manner. Our data indicate that PADI4 plays a role in hMSC proliferation and differentiation, which is induced by TNF- $\alpha$ . This study identified a novel pathophysiological mechanism responsible for new bone formation and osteoporosis in patients with AS; thus, PADI4 may emerge as a novel therapeutic target in AS.

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