

MMP-2 silencing reduces the osteogenic transformation of fibroblasts by inhibiting the activation of the BMP/Smad pathway in ankylosing spondylitis

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Abstract. Ankylosing spondylitis (AS) is a common type of rheumatoid disease, which has recently been demonstrated to be associated with the expression of matrix metalloproteinase (MMP)-2. The aim of the present study was to investigate whether MMP-2 interference reduced the osteogenic differentiation of fibroblasts and to explore the mechanism involved in the differentiation. Fibroblasts from patients with AS were divided into control, mock and small interfering (si)RNA-MMP-2 groups. Cell viability was assessed using the MTT assay. mRNA and protein expression levels of MMP-2, core-binding factor a1 (Cbfa-1) and bone morphogenetic proteins/Smad-signalling molecules (BMP/Smad) were measured using reverse transcription-quantitative polymerase chain reaction and western blotting. The results indicated that cell viability and fibroblast morphology did not differ significantly between healthy volunteers and patients with AS. However, MMP-2 expression levels in AS fibroblasts were substantially higher. *MMP-2* gene silencing markedly downregulated the expression of MMP-2 and Cbfa-1, and inhibited the activation of the BMP/Smad signalling pathway consequent to the reduction in levels of BMP-2, Smad1, Smad4 and Smad1/5/8. The results showed that MMP-2 gene silencing may reduce the osteogenesis of fibroblasts in AS by inhibiting the activation of the BMP/Smad signalling pathway.

Introduction

Ankylosing spondylitis (AS), a prototype of spondyloarthropathy, is a chronic autoimmune disease, which manifests in its early stages as inflammatory back pain, restricts the movements as it progresses and may eventually lead to complete disability (1-3). Ankylosis, resulting from ectopic ossification of tendons and ligaments, is generally accepted to be the pathological hallmark of AS. However, the underlying mechanism is still under investigation (4).

Matrix metalloproteinases (MMPs) are a family of proteins that play an important role in the development of inflammatory and immune diseases as well as in damaging cartilage and bone (5,6). MMPs are structurally and functionally related proteinases that share significant homology in their cytoplasmic domains (7). MMPs are responsible for the proteolytic degradation of the extracellular matrix (8). MMP-2 was up-regulated in numerous inflammatory processes and was involved in the degradation and remodelling of extracellular matrix (9,10). We speculated that elevated levels of MMP-2 protein expression were likely to be associated with the development of AS, which is also an inflammatory disease.

The principal aim of this study was to evaluate the effect of silencing *MMP-2* gene using siRNA technique, on the MMP-2 expression levels in fibroblasts from patients with AS, and also to investigate the effects of MMP-2 inhibition on the activation of downstream signalling pathways. Endogenous siRNA is a powerful tool that cells use to regulate developmental genes or modify DNA and chromatin (5,11). *In vitro* inhibition of MMP-2 gene can be achieved by gene silencing.

Materials and methods

Primary fibroblast isolation and culture. The research included 42 AS patients in Xinchang People's Hospital between October 2014 and December 2015 at the case group, which consisted of 31 males and 11 females. A total of 30 healthy volunteers who underwent routine physical examination in the hospital were included as the control

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group, including 20 males and 10 females. All the study subjects were aged from 20 to 43 years old, with the average of 31.18 ± 6.28 (Table I). The diagnostic criteria for AS patients complied with New York criteria (12). Basic clinical and pathological data of these patients were collected with their written informed consents. The present study was approved by the Ethics Committee of Xinchang People's Hospital (Xinchang, China).

Isolation and culture of both normal and AS fibroblasts were carried out as previously reported (4,13). Ligaments isolated from healthy volunteers and patients with AS, were placed into sterile flasks containing DMEM/F-12 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured for 2 h at 4°C. The ligaments were washed with phosphate-buffered saline (PBS; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) to remove any blood or attached tissue, and then cut into small blocks. After centrifuged at 1,000 rpm for 5 min, the precipitated cells were collected and cultured with Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 medium containing 1 mg/ml Type-I collagenase (Beijing Solarbio Technology Co., Ltd., Beijing, China) at 37°C for 4 h, incubated with 0.25% trypsin (Sinopharm Chemical Reagent Co., Ltd.) for 15 min and shaken at 20 min intervals. The ligament preparation was filtered with 200-mesh sieve (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China). The obtained filtrate was centrifuged at 800 rpm for 5 min. The predicated cells were re-suspended in DMEM/F-12 medium containing 20% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Sinopharm Chemical Reagent Co., Ltd.). The cells were seeded into plates at a density of 1×10^3 cells per well. After approximately 12-15 days of culture at 37°C, a monolayer of fibroblasts was obtained. The third-passage cells were used for the subsequent experiments. Pictures of fibroblast morphology were captured using a light microscope at a magnification of x20 (Thermo Fisher Scientific, Inc.).

Cell grouping and treatment. Fibroblasts isolated from patients with AS were randomly divided into control, mock (empty vector transfected controls) and siRNA-MMP-2 transfection group (vectors carrying siRNA against MMP-2). Recombinant plasmids were obtained from Nanjing Cobioer Biotechnology Co., Ltd. (Nanjing, China).

MTT assay. Fibroblasts in each group were seeded in 96-well plates at a density of 2×10^3 cells per well and allowed to attach overnight. Cells were cultured for 6 day(s), changing the culture medium every 24 h. The cell density was examined 6 times at one-day interval using MTT assay (Shanghai Haling Biotechnology Co., Ltd., Shanghai, China). In brief, the supernatant of each well was removed at specific time points and replaced with 120 µl of MTT solution (5 mg/ml in PBS) diluted 1:6 in medium, prior to use. Cells were incubated in 5% CO₂ incubator at 37°C for 3 h. Formazan, solubilized in 200 µl of DMSO (Sinopharm Chemical Reagent Co., Ltd.), was added to the cultures and incubated at 37°C for 5 min. Absorbance was read at 570 nm using a plate reader (Thermo Fisher Scientific, Inc.). All experiments were performed in triplicate. Cell growth rates were plotted.

RT-PCR. RNA was extracted by one-step TRIzol extraction as per manufacture's instruction. The expression of MMP-2, Cbfa-1, BMP-2, Smad1, Smad4 and p-Smad1/5/8 were determined by reverse transcription. PCR primer pairs were designed based on the sequences of different exons of the corresponding genes. All primers in the study were designed by Shanghai Sangon Biotech Co., Ltd., Shanghai, China. The specific primer sequences for each gene were listed as the follows: 5'TGTGTTGTCCAGAGGCAATG3' and 5'ATC ACTAGGCCAGCTGGTTG3' for MMP-2 (product: 107 bp); 5'TCGCCAGGCTTCATAGCAAA3' and 5'GGCCTTGGG TAAGGCAGATT3' for Cbfa-1 (product: 170 bp); 5'CGCTGT CTTCTAGCGTTGCT3' and 5'GGGGTGGGTCTCTGT TTCAG3' for BMP-2 (product: 191 bp); 5'ATTCTGTGAGTTC GCGGTTGA3' and 5'CACAGTTACTCGGTTGCCCT3' for Smad1 (product: 421 bp); 5'GCTGCAGAGCCCAGTTTA GA3' and 5'CCCCAAAGCAGAAGCTACGA3' for Smad4 (product: 147 bp); 5'GGCCGAGCTGCTAATAAAGTTG3' and 5'AAACAAGCTGGCCATTGACG3' for p-Smad1/5/8 (product: 429 bp) and 5'GTCATTCCAAATATGAGATGC GT3' and 5'GCTATCACCTCCCCTGTGTG3' for β-actin (product: 121 bp). The reactions were incubated at 95°C for 10 min and amplified using the following cycling parameters: 95°C for 10 sec, 58°C for 10 sec and 72°C for 30 sec. After totally 45 cycles, the primers were elongated at 60°C for 1 min. The relative gene expression levels of the target genes were analyzed through $2^{-\Delta\Delta C_q}$ method. β-actin was applied as the internal control to normalize the expression level of each gene.

Western blotting. Total proteins were extracted with RIPA lysis buffer containing 1 mM PMSF (Beijing Solarbio Technology Co., Ltd.). The protein samples were boiled for 10 min in loading buffer and subjected to SDS-PAGE/immunoblotting analysis. Separated protein bands were transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.) at 25 V for 30 min. The membranes were blocked with 5% skimmed milk powder in PBS. The membrane was washed thrice with Tris buffered saline containing Tween-20 (TBST) (Sinopharm Chemical Reagent Co., Ltd.), and incubated in secondary antibodies diluted 1:1,000, at 37°C for 1 h. After incubation, it was washed thrice, for 5 min each time, with TBST. Protein bands were visualized using LumiPico® ECL Reagent.

Statistical analysis. All data were expressed as means ± standard deviation (SD). Student's two-tailed t-test, and one-way ANOVA followed by a Tukey's multiple comparison test were performed using IBM SPSS version 20 for statistical comparisons. Statistical significance was defined as $P < 0.05$ and $P < 0.01$.

Results

No obvious difference in the morphology of normal and AS fibroblasts. Ligament fibroblasts in the control and AS groups were isolated from tissues of healthy volunteers and patients with AS, respectively. No significant difference in cell morphology was observed between normal and AS fibroblasts. Gender difference was not manifested in morphology of healthy or AS fibroblasts, but the incidence rate of men was apparently

higher than women (Table I). Cells in both control and AS groups adhered to the culture flasks and were spindle-shaped, as observed under a light microscope (Fig. 1A).

No significant difference in cell viability between normal and AS fibroblasts. Cell proliferation profiles of normal and AS fibroblasts were investigated using the MTT assay. The results showed that both normal and AS fibroblasts had an initial lag phase, followed by a steady increase in cell proliferation from day 2 to day 6 (Fig. 1B). There was no significant difference in cell viability between the control and AS groups at any of the time points tested ($P>0.05$).

MMP-2 mRNA expression level in AS group was much higher than that in the control group. The expression levels of MMP-2 mRNA in both normal and AS fibroblasts were measured using RT-PCR. The MMP-2 mRNA expression level in AS fibroblasts at 0.093 ± 0.012 , was approximately four times higher than that in normal cells (0.025 ± 0.0023) ($P<0.01$) (Fig. 1C).

MMP-2 expression was substantially inhibited in the siRNA-MMP-2 group. The expression levels of MMP-2 mRNA and protein were examined in control, mock and siRNA-MMP-2 groups, using RT-PCR and western blotting, respectively. There was no significant quantitative differences in the protein levels of MMP-2 between the control and mock group ($P>0.05$). However, MMP-2 gene expression was dramatically inhibited in cells transfected with siRNA-MMP-2, compared to that in normal cells, from 0.72 ± 0.05 in normal cells to 0.15 ± 0.012 in siRNA-MMP-2 transfected cells ($P<0.01$) (Fig. 2A and B).

MMP-2 gene silencing down-regulated the expression of Cbfa-1 in fibroblasts. The effect of MMP-2 gene silencing on the expression of Cbfa-1 was analysed by RT-PCR and western blotting. Control and mock groups did not differ significantly in the expression levels of Cbfa-1 mRNA and protein ($P>0.05$). Results of both RT-PCR and western blotting indicated that the expression of Cbfa-1 was markedly down-regulated by silencing the MMP-2 gene in siRNA-MMP-2 group, which suggested the positive correlation between the expressions of MMP-2 and Cbfa-1 ($P<0.01$) (Fig. 2C and D).

MMP-2 gene silencing had an inhibitory effect on the BMP/Smad signalling pathway in AS fibroblasts. Quantitative analysis of the expression of BMP/Smad pathway components was carried out using RT-PCR and western blotting. The results of RT-PCR showed that the mRNA expression levels of BMP-2, Smad1, Smad4, and Smad1/5/8 were all dramatically reduced in the siRNA-MMP-2 group, compared to those in the control as well as mock groups ($P<0.01$). Western blot analysis showed the inhibitory effect of MMP-2 gene silencing on the expression levels of BMP-2, Smad1, Smad4 and Smad1/5/8 proteins, as reflected in the significant differences observed between siRNA-MMP-2 and control groups ($P<0.01$) (Fig. 3).

Discussion

AS, a prototype of the spondyloarthritis group of diseases, is a chronic systemic inflammatory and autoimmune

Table I. Clinical information of study subjects.

Clinical items	Case group (n=42)
Age of treatment (median, years)	26
Age of onset (median, years)	21
Course of disease (median, years)	5
Male no. (%)	31 (73.81)
Female no. (%)	11 (26.19)
HLA-B27 positive rate no. (%)	36 (85.71)
Hip joint involvement no. (%)	12 (28.57)
Peripheral joints involvement no. (%)	20 (47.62)
Enthesitis no. (%)	8 (19.05)
Extra-articular manifestations (iritis or urethritis) no. (%)	2 (4.76)
Family history of spondyloarthritis no. (%)	12 (28.57)
History of axial joints and peripheral joints trauma no. (%)	7 (16.67)
Bilateral inflammation <II no. (%)	5 (11.90)
Bilateral inflammation in SIJ II no. (%)	17 (40.48)
Bilateral or unilateral inflammation in SIJ III no. (%)	23 (54.76)
Bilateral I or unilateral inflammation in SIJ IV no. (%)	8 (19.05)
SIJ, sacroiliac joint.	

rheumatic disease with a high disability rate (14). The disease process is characterized by ectopic ossification of spine and peripheral joints (15). In late stages of AS, which is regarded as the beginning of irreversible disability, cartilage is progressively replaced by bone, eventually leading to joint ankyloses. Excessive MMP-2 production is associated with collagen degradation in the joints, which is considered as one of the major causes of AS (16). Fibroblasts from patients with AS were isolated for the investigations described in this report. First, we showed that the MMP-2 expression level obviously differed between normal and AS fibroblasts, with the levels in AS fibroblasts nearly four-fold higher compared to those in normal cells. Silencing MMP-2 gene in AS fibroblasts resulted in a remarkable reduction in the expressions of Cbfa-1 and components of BMP/Smad signalling pathways.

Fibroblasts, one of the major cell types in connective tissue, produce collagen-rich extracellular matrix and play a key role in trauma repair as well as in pathologic ectopic ossification (17,18). Fibroblasts can differentiate into osteoblasts under specific conditions, although they are from the same lineage. A variety of bone growth factors participate in the regulation of cell proliferation, differentiation, and bone metabolism (19). New bone formation involves the recruitment of osteoprogenitor cells. The rate of mature bone formation depends on the half-life of osteoblasts, cell proliferation rate and their functional differentiation (20). Osteoblast differentiation is regulated by signalling factors. According to recent reports, osteoblasts express two osteogenic marker genes, Cbfa-1 and

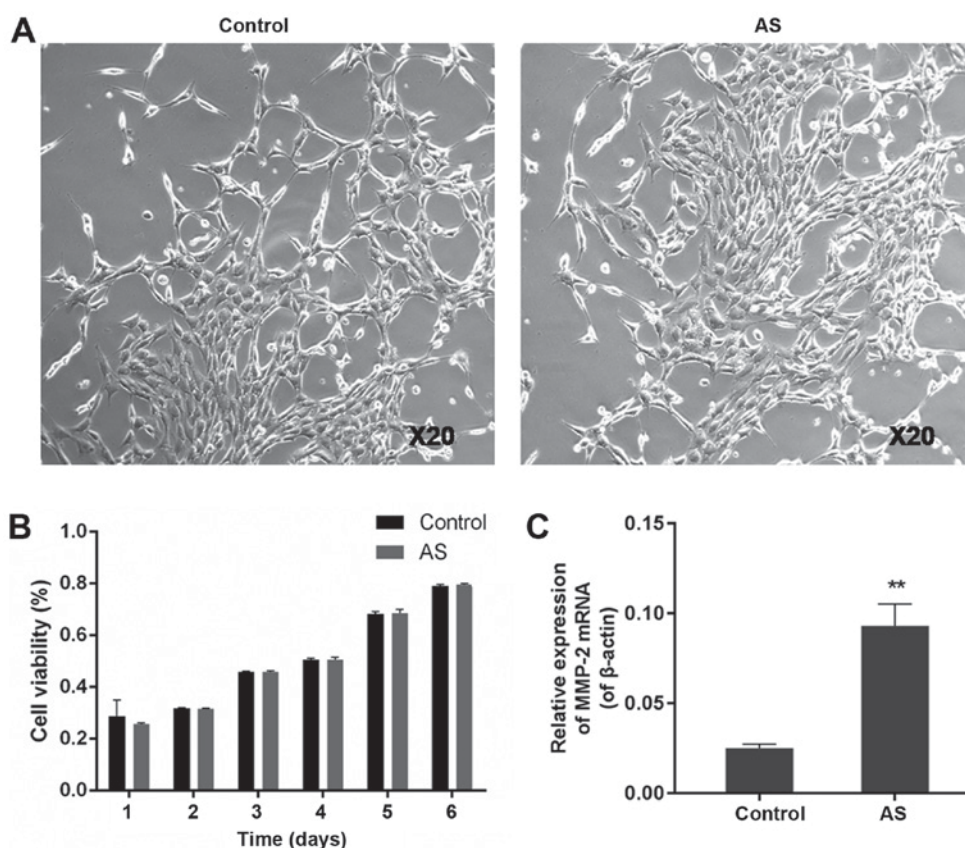


Figure 1. Morphology, viability and MMP-2 expression in fibroblasts from healthy volunteers and AS patients. (A) No significant difference was observed in morphology of fibroblasts from healthy volunteers (left) and AS patients (right) under microscope (magnification, x20). (B) No significant difference was detected in cell viability of fibroblasts from healthy volunteers and AS patients. (C) MMP-2 mRNA was highly expressed in AS fibroblasts. Data were presented as mean \pm standard deviation, n=3. **P<0.01 vs. control group (fibroblasts from healthy volunteers).

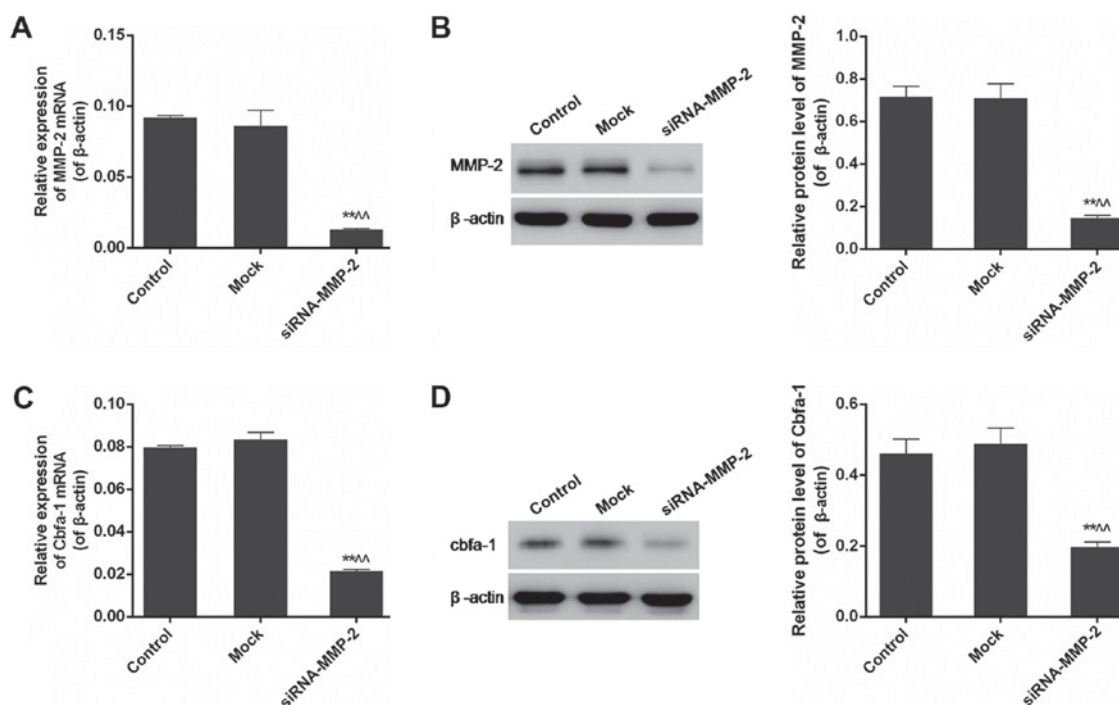


Figure 2. Influences of MMP-2 gene silencing on the expression of MMP-2 and Cbfa-1 in AS fibroblasts. (A) *MMP-2* gene silencing substantially inhibited the expression of *MMP-2* mRNA in AS fibroblasts. (B) A remarkable reduction of *MMP-2* protein level was detected in siRNA-MMP-2 group compared to control group. (C) *MMP-2* gene silencing obviously down-regulated the expression of *Cbfa-1* mRNA in AS fibroblasts. (D) A dramatic decrease of *Cbfa-1* protein level was tested in siRNA-MMP-2 group compared to control group. Data were presented as mean \pm standard deviation, n=3. ***P<0.01 vs. control group (AS fibroblasts), **P<0.01 vs. Mock group.

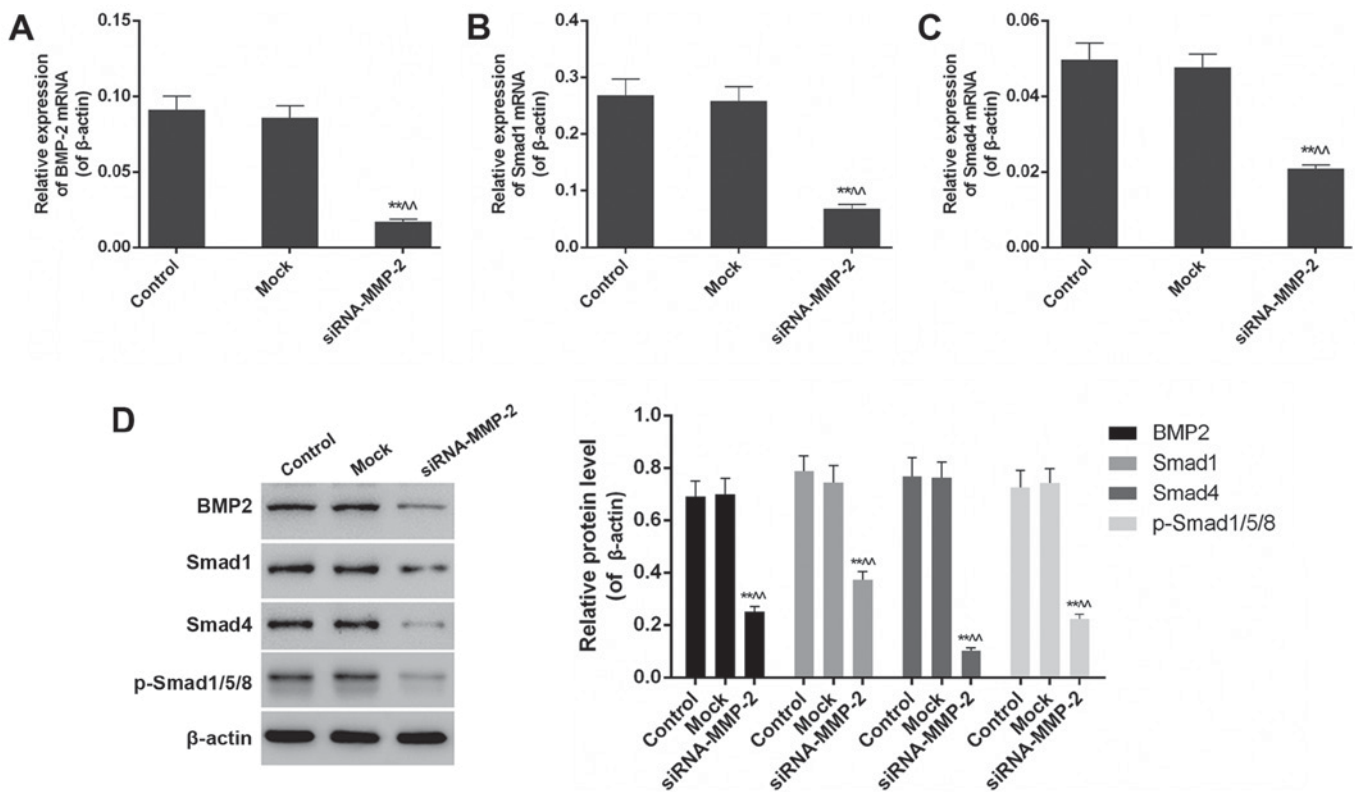


Figure 3. The effects of *MMP-2* gene silencing on the activation of BMP/Smad signaling pathway in AS fibroblasts. (A) *MMP-2* gene silencing down-regulated the expression of BMP-2 mRNA in AS fibroblasts. (B) *MMP-2* gene silencing down-regulated the expression of Smad1 mRNA in AS fibroblasts. (C) *MMP-2* gene silencing down-regulated the expression of Smad4 mRNA in AS fibroblasts. (D) The protein levels of components in BMP/Smad pathway including BMP-2, Smad1, Smad4 and p-Smad1/5/8 were substantially decreased in siRNA-MMP-2 group compared to control one. Data were presented as mean \pm standard deviation, n=3. **P<0.01 vs. control group (AS fibroblasts), ***P<0.01 vs. Mock group.

osteocalcin (21,22). As a Runt-related osteoblast-specific transcription factor, Cbfa-1 gene exerts its effects at earlier stages of the disease, as opposed to osteocalcin, which is involved in osteoblast differentiation (17,23,24). The expression of Cbfa-1 is regulated by its upstream signalling molecules (18).

BMPs are members of the transforming growth factor β superfamily. Several lines of studies showed that BMPs could activate the downstream signalling molecules in the Smad protein family, stimulate mesenchymal cell differentiation, and irreversibly induce bone and cartilage formation by regulating the expression of Cbfa-1 (25-27). BMP-2 can modulate osteoblastic differentiation through the canonical BMP/Smad pathway. This signalling pathway is initiated by type II BMP receptors, which after activation, propagate the BMP signals by phosphorylating BMP-specific Smad1, Smad5 and Smad8. p-Smad1/5/8 then binds to Smad4 to form a complex which gets translocated to the nucleus and activates or represses the transcription of osteogenic genes. The activation of BMP/Smads signalling pathway is an important mechanism of the osteogenic differentiation of AS fibroblasts and endochondral bone formation in ankylosing enthesitis. Untimely activation of the signalling cascades may promote AS processes (28-30).

We showed in this study that the expression of MMP-2, which is responsible for the degradation of non-fibrillar and denatured collagens, was obviously elevated at both transcriptional and translational levels in fibroblasts from patients with AS. Cbfa-1, one of the essential transcription

factors, which regulates osteoblast differentiation and bone formation, was observed to be highly affected by the silencing of MMP-2 gene, as demonstrated by its weak expression in the siRNA-MMP-2 group (31). The expression of Cbfa-1 was positively correlated with that of MMP-2. It was also found that MMP-2 inhibition by siRNA technique resulted in decreased expression of BMP-2, which in turn reduced the levels of Smad signalling protein levels remarkably. BMP molecules induce ligand-dependent type I and type II receptor heterodimerization, which activates p-Smad1/5/8 that bind Smad4 (32). The inhibition of BMP/Smad signalling by siRNA-MMP-2 was also associated with the inhibition of Cbfa-1 expression. Thus, inhibition of BMP/Smad signalling has the potential to effectively prevent osteoblastic differentiation in pathologic ectopic ossification (13). Our results showed that the level of expression of MMP-2 in AS fibroblasts can be markedly inhibited by siRNA-MMP-2. Therefore, MMP-2 silencing can be considered a promising therapeutic strategy for AS treatment.

This study investigated *in vitro*, the signalling mechanism of MMP-2 and the effect of its silencing in fibroblasts isolated from patients with AS. Our findings demonstrated that MMP-2 gene can be effectively down-regulated by transfecting the AS fibroblasts with siRNA-MMP-2. MMP-2 down-regulation also led to decreased expression of Cbfa-1 and BMP/Smad signalling proteins. The MMP-2 silencing, therefore, could serve as a novel therapeutic approach for AS treatment.

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