MicroRNA-204-5p inhibits the osteogenic differentiation of ankylosing spondylitis fibroblasts by regulating the Notch2 signaling pathway

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Abstract. Ankylosing spondylitis (AS) is a chronic inflammatory systemic disease and is difficult to detect in the early stages. The present study aimed to investigate the role of microRNA *(miR)-204-5p* in osteogenic differentiation of AS fibroblasts. Bone morphogenetic protein 2 (BMP-2) was used to induce osteogenic differentiation. Cells were divided into the following groups: AS group, AS + BMP-2 group, AS + BMP-2 + miR-negative control group, AS + BMP-2 + *miR-204-5p* mimics group and AS + BMP-2 + *miR-204-5p* mimics + pcDNA-Notch2 group. The expression levels of *miR-204-5p*, Notch2, runt-related transcription factor 2 (RUNX2) and osteocalcin were detected via reverse transcription-quantitative PCR analysis. The binding site between Notch2 and *miR-204-5p* was predicted using TargetScan software and verified via the dual-luciferase reporter assay. Alkaline phosphatase (ALP) activity was assessed via the ALP assay, while the mineralized nodules area was determined via the Alizarin Red S staining assay. The results demonstrated that Notch2 is a target gene of *miR-204-5p*. Furthermore, treatment with BMP-2 significantly decreased *miR-204-5p* expression, and significantly increased ALP activity, the mineralized nodules area and the expression levels of Notch2, RUNX2 and osteocalcin in ligament fibroblasts (all P<0.05). Conversely, transfection with *miR-204-5p* mimics significantly increased *miR-204-5p* expression, and significantly decreased ALP activity, the mineralized nodules area and the expression levels of Notch2, RUNX2 and osteocalcin in ligament fibroblasts (all P<0.05). Notably, transfection with pcDNA-Notch2 significantly reversed the inhibitory effects induced by miR-204-5p mimics on the osteogenic differentiation of ligament fibroblasts (all P<0.05). Furthermore, *miR-204-5p* inhibited the osteogenic differentiation of ligament fibroblasts in patients with AS by targeting Notch2. Thus, *miR-204-5p* may negatively regulate Notch2 expression and may be a potential therapeutic target for AS. Collectively, the results of the present study provide a theoretical basis for the effective treatment of patients with AS.

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Introduction

Ankylosing spondylitis (AS) is a common chronic immune-mediated joint disease, which predominantly affects the spine and pelvis (1). Between May 2005 and May 2019, the total prevalence of AS in mainland China was 0.29% (2). AS is characterized by spinal pain, stiffness and new bone formation, which manifests ligament atrophy and joint stiffness (3). A previous study has demonstrated that there is no definite value in assessing the long-term prognosis and mortality of patients with AS (4). The number of patients with AS per 10,000 people is 23.8 in Europe, 16.7 in Asia, 31.9 in North America, 10.2 in Latin America and 7.4 in Africa (5). With the increasing incidence of AS, the therapeutic strategies of AS are also diversified, including the use of tumor necrosis factor blockers (6), radiotherapy (7), ultrasound combined exercise therapy (8) and surgical treatment (9). Furthermore, microRNAs (miRNAs/miRs) play a key role in regulating the immune function and autoimmunity (10). With the development of molecular targeting technology, research on miRNAs is of great interest for the treatment of AS.

miRNAs play a significant role in AS pathology by targeting the inflammation and bone remodeling genes (11). Notably, miR-204 regulates the transformation of mesenchymal stem cells into adipose and osteoblast cell lines (12). miR-204 is involved in the development of several diseases. For example, *miR-204-5p* plays a therapeutic role in aplastic anemic rats via the NF-kB signaling pathway (13), which is a target for AS treatment (14). Furthermore, the maintain bone morphogenetic protein (BMP)/SMAD (15), Wnt/β-catenin (16) and Notch (17) signaling pathways are involved in the process of AS. Specifically, the Notch2 signaling pathway is required to promote cell proliferation and maintain BMP signaling (18).

There is a positive regulatory association between the Notch and NF-κB signaling pathways (19). However, whether *miR-204-5p* is involved in the regulation of the Notch signaling pathway, and whether it has an impact on osteogenic differentiation of AS fibroblasts have not yet been fully investigated.

BMP-2 is a member of the transforming growth factor-β superfamily that is synthesized and secreted by osteoblasts (20). BMP-2 is considered a common osteogenic agent, which can induce undifferentiated mesenchymal cells into cartilage and bone tissues (21). A previous study demonstrated that BMP-2 facilitates the osteogenic differentiation of bone marrow-derived mesenchymal stem cells by inducing alkaline phosphatase (ALP) activity, promoting mineralization, enhancing adherence and mediating the expression and activation of osteogenic markers (22).

In the current study mRNA expression was detected using reverse transcription-quantitative PCR (RT-qPCR). The binding site between Notch2 and *miR-204-5p* was predicted using TargetScan software and assessed via the dual-luciferase reporter assay. Moreover, ALP activity was assessed via the ALP assay, while the mineralized nodules area was determined via the Alizarin Red S staining assay. In addition, BMP-2 was used to induce osteogenic differentiation of AS fibroblasts, and the regulatory role of *miR-204-5p* on the osteogenic differentiation of AS fibroblasts, and the underlying molecular mechanism involving the Notch signaling pathway were assessed. Taken together, the results of the present study provide a theoretical basis for the treatment of patients with AS.

Materials and methods

Primary culture of ligament fibroblasts. A total of 20 patients with AS (20 men; age, 25-39 years; mean age, 30.2 years) who underwent surgical intervention at Shouguang People's Hospital between January 2016 and January 2018 were recruited in the present study. The bioptic tissues were collected from the 20 patients with AS. All patients were in the active stage, exhibiting inflammatory low back pain, notable ossification of the ankle joint, positive histocompatibility leukocyte antigen (HLA)-B27, and elevated levels of C-reactive protein and erythrocyte sedimentation rate (ESR). All patients met the New York Standard of the American College of Rheumatology revised in 1984 (23).

A total of 20 patients (20 men; age, 26-43 years; mean age, 31.5 years) who underwent hip arthroplasty for femoral neck fracture (excluding other types of osteoarthritis) between January 2017 and October 2017 were recruited as the control group in the present study. The hip ligament tissues were washed with physiological saline, immediately frozen in liquid nitrogen and stored at -80˚C until further experimentation. The present study was approved by the Ethics Committee of Shouguang People's Hospital (approval no. SGSRMXY-2020-09) and written informed consent was provided by all patients prior to the study start.

The hip ligament tissues of patients with AS were rinsed three times with PBS supplemented with 300 U/ml penicillin and 300 μg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.). The ligament tissues were subsequently cut into 1-mm^3-thick sections using ophthalmic scissors, and added into plates containing 5 ml serum-free DMEM medium and 0.2 μg/ml type I collagenase (all Invitrogen; Thermo Fisher Scientific, Inc.). The collagen fibers were removed by filtration at 1,000 r/min, through a 0.22 μm filter (EMD Millipore). The precipitated cells were cultured in DMEM medium supplemented with 20% serum and 1% streptomycin, at 37˚C in 5% CO_2 for 72 h.

Osteogenic differentiation of ligament fibroblasts. The osteogenic differentiation of ligament fibroblasts was induced by BMP-2 as previously described (24-26). Cells were divided into the following groups: AS group, AS + BMP-2 group, AS + BMP-2 + miR-negative control (NC) group, AS + BMP-2 + *miR-204-5p* mimics group and AS + BMP-2 + *miR-204-5p* mimics + pcDNA-Notch2 group. Cells were transfected with 50 nmol/l *miR-204-5p* mimics, miR-NC, pcDNA-Notch2 or pcDNA-NC (Shanghai GenePharma Co., Ltd.), using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.). The subsequent experiments were performed at 24 h post-transfection. Subsequently, cells were cultured in DMEM/H containing 10% fetal bovine serum, 0.05 mM vitamin C and 100 mM dexamethason (all Gibco; Thermo Fisher Scientific, Inc.). BMP-2 (200 ng/ml; Sigma-Aldrich; Merck KGaA) was added to all medium except the AS group. All cells were cultured in 5% CO_2 at 37˚C and induced for 14 days.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the hip ligament tissues and ligament fibroblasts using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Synthesis of cDNA using reverse transcriptase was performed with the PrimeScript RT Enzyme Mix I kit (Takara Bio, Inc.). The reaction mixtures were incubated at 37˚C for 60 min, 95˚C for 5 min and then held at 4˚C. A total of 5 μl diluted RNA (1:20) was used to determine the concentration and purity of total RNA. miScript SYBR Green PCR kit (Qiagen, Inc.) was used to conduct the qPCR analysis. RT-qPCR was performed on an ABI7500 quantitative PCR machine (Thermo Fisher Scientific, Inc.). U6 was used as the internal control for miRNAs, and GAPDH served as the internal control for other genes. The primer sequences (Guangzhou Ruibo Biotechnology Co., Ltd.) are listed in Table I. The reaction conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 34 sec. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (27).

ALP staining and calcium salt deposition staining. After 7 days of culturing, cells ($1x10^4$ cells/well) from each group were collected and fixed. ALP activity was assessed using the ALP activity assay kit (cat. no. A059-2-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. ALP activity was measured at a wavelength of 520 nm, using a microplate reader (Molecular Devices LLC).

After 14 days of culturing, cells from each group were collected and stained with 2% Alizarin Red staining solution (pH 8.3; Nanjing KeyGen Biotech Co., Ltd.) at 37˚C for 10 min. The solution was discarded, cells were washed with PBS and subsequently observed under a phase contrast microscope

Table I. Primer sequences used for quantitative PCR.

Gene	Sequence (5'-3')
miR-204-5p (F)	TTCCCTTTGTCATCCTATGCCT
miR-204-5p (R)	TGGTGTCGTGGAGTCG
U6 (F)	GCTTCGGCAGCACATATACTAAAAT
U6 (R)	CGCTTCACGAATTTGCGTGTCAT
Notch2 (F)	CACAGGGTTCATAGCCATCTC
Notch2 (R)	GGAGGCGACCGAGAAGAT
RUNX2 (F)	AGCTTCTGTCTGTGCCTTCTGG
RUNX2 (R)	GGAGTAGAGAGGCAAGAGTTT
Osteocalcin (F)	CTTTGTGTCCAAGCAGGA
Osteocalcin (R)	CTGAAAGCCGATGTGGTCAE
GAPDH (F)	GAAGGTGAAGGTCGGAGTC
GAPDH (R)	GAAGATGGTGATGGGATTTC

F, forward; R, reverse; miR, microRNA; RUNX2, runt-related transcription factor 2.

(light microscope), and the mineralized nodules area was counted at five high-power fields (magnification, x100).

Western blotting. Ligament fibroblasts were lysed using RIPA lysate (Beyotime Institute of Biotechnology) at 4˚C for 30 min. The supernatants were collected via centrifugation at 7,200 x g at 4˚C for 10 min. Total protein was quantified using the bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and 60 μg protein/lane was separated via 10% separating gum and 5% concentrating gum. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked with 5% skim milk for 1 h at 37˚C. The membranes were incubated with primary antibodies against: Notch2 (cat. no. ab8926), runt-related transcription factor 2 (RUNX2; cat. no. ab23981), osteocalcin (cat. no. ab93876), GAPDH (cat. no. ab9485) and rabbit anti-human (all 1:5,000 and from Abcam) overnight at 4˚C. Following the primary incubation, membranes were incubated with horseradish peroxidase-labeled goat-anti-rabbit IgG secondary antibody (1:5,000; ca. no. ab6721; Abcam) for 1 h at 25˚C. The protein blots were visualized using an enhanced chemiluminescence kit (Invitrogen; Thermo Fisher Scientific, Inc.). Protein bands were assessed using a luminescent image analysis software (Quantity One 1-D Analysis software; version 4.6.9; Bio-Rad Laboratories, Inc.). GAPDH was used as the internal control.

Dual-luciferase reporter assay. TargetScan software v3.0 (http://starbase.sysu.edu.cn.) was used to predict the targeting relationship between *miR-204-5* and Notch2. A 3'-untranslated region (UTR) wild type (WT) plasmid of Notch2 (Notch2-3'-UTR-WT) was constructed according to the 3'-UTR sequence of Notch 2. Based on this plasmid, a binding site was mutated to construct a 3'-UTR mutant (MUT) plasmid (Notch2-3'-UTR-MUT). The construction and sequencing of the plasmids were performed by Sangon Biotech Co., Ltd. Subsequently, the constructed luciferase reporter plasmids, pmirGLO-Notch2-WT/pmirGLO-Notch2-MUT (Shanghai GenePharma Co., Ltd.) and *miR-204-5p* mimics/miR-NC were co-transfected into 293T cells (American Type Culture Collection) using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.). The luciferase activity was measured using the dual luciferase activity assay kit (Thermo Fisher Scientific, Inc.), 48 h post-transfection, and was normalized to *Renilla* luciferase activity.

Statistical analysis. Statistical analysis was performed using SPSS software (version 21.0; IBM Corp.) and data are presented as the mean ± standard deviation. All experiments were repeated three times. Unpaired Student's t-test was used to compare differences between two groups. One-way analysis of variance followed by Tukey's post hoc test was used to compare differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of miR-204-5p in hip capsules of patients with AS. *miR-204-5p* expression was significantly lower in the hip joint capsules of patients with AS than in patients with femoral neck fracture ($P<0.05$; Fig. 1A). Additionally, *miR-204-5p* expression was significantly decreased in BMP-2-induced AS cells compared with untreated-cells ($P<0.05$; Fig. 1B). Furthermore, transfection of *miR-204-5p* mimics significantly increased *miR-204-5p* expression in BMP-2-induced AS cells ($P<0.05$; Fig. 1B).

Upregulation of Notch2 expression in hip capsules of patients with AS. Notch2 mRNA expression was significantly higher in the hip joint capsules of patients with AS than that in patients with femoral neck fracture ($P<0.05$; Fig. 2A). Notch2 expression was significantly higher in the AS + BMP-2 group compared with the AS group, at both the mRNA and protein levels ($P<0.05$; Fig. 2B and C). Furthermore, the mRNA and protein levels of Notch2 were significantly decreased in the AS + BMP-2 + *miR-204-5p* mimics group compared with those in the AS + BMP-2 + miR-NC group ($P<0.05$; Fig. 2B and C).

Notch2 is a target gene of miR-204-5p. The binding site for Notch2 and *miR-204-5p* was predicted using TargetScan software (Fig. 3A). The luciferase activity of cells co-transfected with *miR-204-5p* mimics and pmirGLO-Notch2-WT was significantly lower than those co-transfected with *miR-204-5p* mimics and pmirGLO-Notch2-MUT ($P<0.05$; Fig. 3B).

miR-204-5p inhibits osteogenic differentiation of ligament fibroblasts by targeting Notch2. The ALP activity of the AS + BMP-2 group was higher than that in the AS group ($P<0.01$; Fig. 4A). Furthermore, the ALP activity in the AS + BMP-2 + miR-204-5p mimics group was significantly lower than that in the AS + BMP-2 + miR-NC group ($P<0.01$; Fig. 4A). Notably, transfection with pcDNA-Notch2 significantly reversed the inhibitory effect induced by miR-204-5p mimics on the ALP activity of ligament fibroblasts ($P<0.05$; Fig. 4A).

The mineralized nodules area in the AS + BMP-2 group was significantly increased compared with the AS group ($P<0.01$;

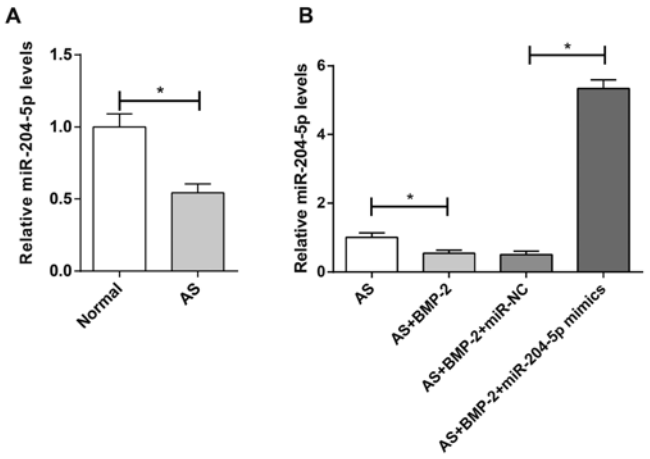

Figure 1. *miR-204-5p* expression in hip joint capsule tissues and ligament fibroblasts. (A) *miR-204-5p* expression in hip joint capsule tissues. (B) *miR-204-5p* expression in AS-derived ligament fibroblasts. U6 was used as the internal control. *P<0.05. miR, microRNA; AS, ankylosing spondylitis; BMP-2, bone morphogenetic protein 2; NC, negative control.

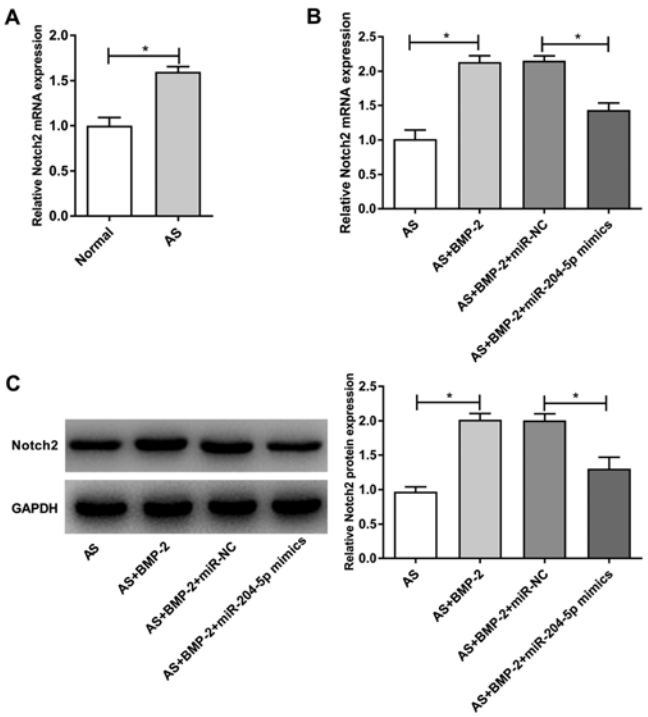

Figure 2. Notch2 expression in hip joint capsule tissues and ligament fibroblasts. (A) Notch2 expression in hip joint capsule tissues. (B) Notch2 mRNA expression in AS-derived ligament fibroblasts. (C) Notch2 protein expression in AS-derived ligament fibroblasts. GAPDH was used as the internal control. *P<0.05. AS, ankylosing spondylitis; BMP-2, bone morphogenetic protein 2; NC, negative control; miR, microRNA.

Fig. 4B). Furthermore, the mineralized nodules area in the AS + BMP-2 + *miR-204-5p* mimics group was significantly decreased compared with the AS + BMP-2 + miR-NC group (P<0.01; Fig. 4B). Notably, transfection with pcDNA-Notch2 significantly reversed the inhibitory effect induced by miR-204-5p mimics on the mineralized nodules area of ligament fibroblasts (P<0.05; Fig. 4B).

miR-204-5p inhibits the expression of RUNX2 and osteocalcin by targeting Notch2. Transfection with pcDNA-Notch2 significantly increased Notch2 protein expression in ligament fibroblasts (P<0.01; Fig. 5A). The expression of RUNX2 and osteocalcin in the AS + BMP-2 group were significantly increased compared with the AS group, at both the mRNA and protein levels (P<0.01; Fig. 5B and C). Furthermore, the expression of RUNX2 and osteocalcin in the AS + BMP-2 + *miR-204-5p* mimics group were significantly decreased compared with the AS + BMP-2 + miR-NC group, at both the mRNA and protein levels (P<0.01; Fig. 5B and C). Notably, transfection with pcDNA-Notch2 significantly reversed the inhibitory effect induced by miR-204-5p mimics on the expression of RUNX2 and osteocalcin in ligament fibroblasts (P<0.05; Fig. 5B and C).

Discussion

AS is an autoimmune disease characterized by fibroblast ossification (28). Notably, inhibition of the ossification of AS fibroblasts is a common treatment for patients with AS (28). The present study aimed to determine whether *miR-204-5p* regulates the Notch signaling pathway, and subsequently affects the osteogenic differentiation of AS fibroblasts. The results demonstrated that *miR-204-5p* expression decreased in the hip capsule tissues of patients with AS, and Notch2 was identified as the target gene of *miR-204-5p*. Furthermore, *miR-204-5p* inhibited the osteogenic differentiation of AS fibroblasts by downregulating the expression of Notch2, RUNX2 and osteocalcin. Heterotopic ossification is one of the most prominent features of AS (29), and osteogenic differentiation of fibroblasts plays a key role in the heterotopic ossification of AS (30). miRNAs play important roles in regulating cell-cell interactions between osteoclasts and fibroblasts (31). For example, *miR-204-5p* is involved in the adjustability of adipogenesis and osteogenic differentiation of bone marrow stem cells (32). Zhang *et al* (33) reported that downregulating *miR-204-5p* expression increases RUNX2 expression and promotes osteoblast proliferation. Consistent with previous findings, the results of the present study demonstrated the overexpression of *miR-204-5p* inhibited RUNX2 expression, thereby inhibiting osteogenic differentiation of fibroblasts. In addition, overexpression of miR-204 has been reported to promote adipocyte differentiation and inhibit osteogenic differentiation, while miR-204 knockdown exerts the opposite effects (34). Taken together, these results suggest that *miR-204-5p* inhibits osteogenic differentiation, and thus can be used to treat patients with AS.

The results of the present study demonstrated that *miR-204-5p* inhibited the osteogenic differentiation of fibroblasts by targeting Notch2. Lee *et al* (35) and Cai *et al* (36) have reported that Notch2 is a target gene of *miR-204-5p*. In addition, Notch family members and their ligands are involved in the formation of articular cartilage at different locations, and the coordination of the ossification and extension of growth plates (37). Notably, the Notch signaling pathway significantly enhances BMP-2-induced osteogenesis of embryonic fibroblasts (38). BMP-2 is a well-known bone formation stimulating factor (39). However, downregulation of miR-204 expression by BMP-2 increases RUNX2 expression and enhances osteogenic differentiation (40). *miR-204-5p* also

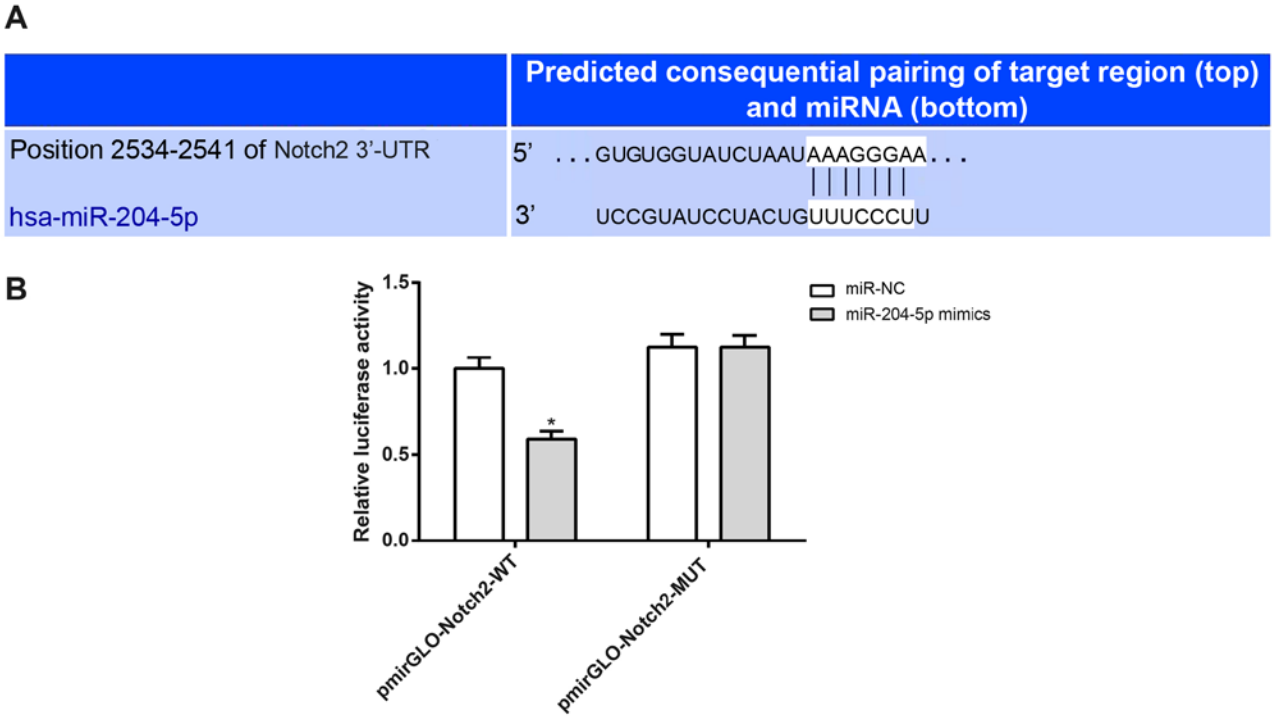

Figure 3. Notch2 is the target gene of *miR-204-5p*. (A) The target site for Notch2 and *miR-204-5p* was predicted using TargetScan software. (B) Detection of dual-luciferase reporter activity. *P<0.05 vs. the miR-NC group. miR, microRNA; NC, negative control; UTR, untranslated region; WT, wild-type; MUT, mutant.

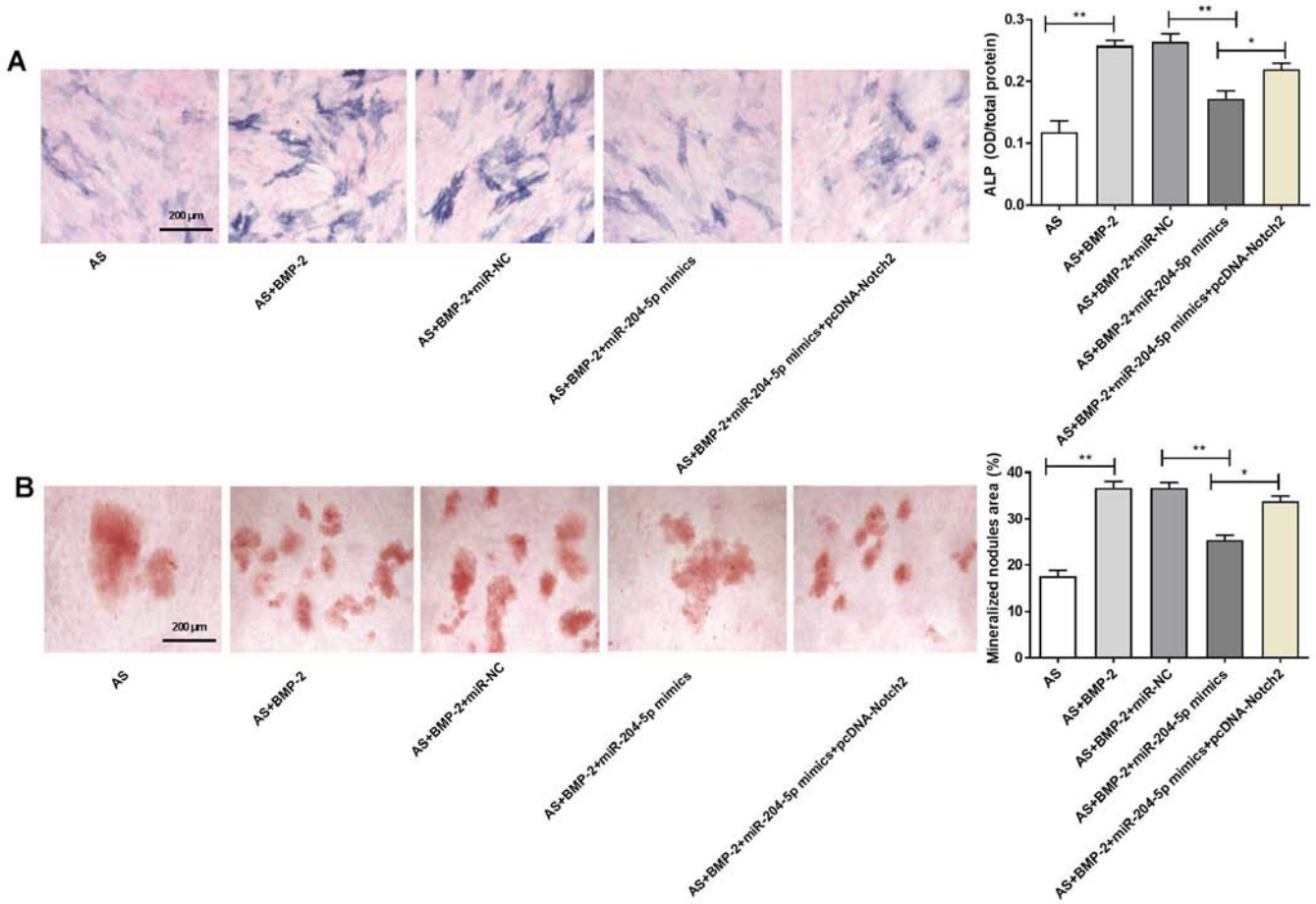

Figure 4. *miR-204-5p* inhibits the osteogenic differentiation of ligament fibroblasts by targeting Notch2. (A) ALP staining and (B) Alizarin Red S staining. *P<0.05 vs. the AS + BMP-2 + miR-204-5p mimics group; **P<0.01 vs. the AS and AS + BMP-2 + miR-NC groups, respectively. miR, microRNA; ALP, alkaline phosphatase; AS, ankylosing spondylitis; BMP-2, bone morphogenetic protein 2; NC, negative control; OD, optical density.

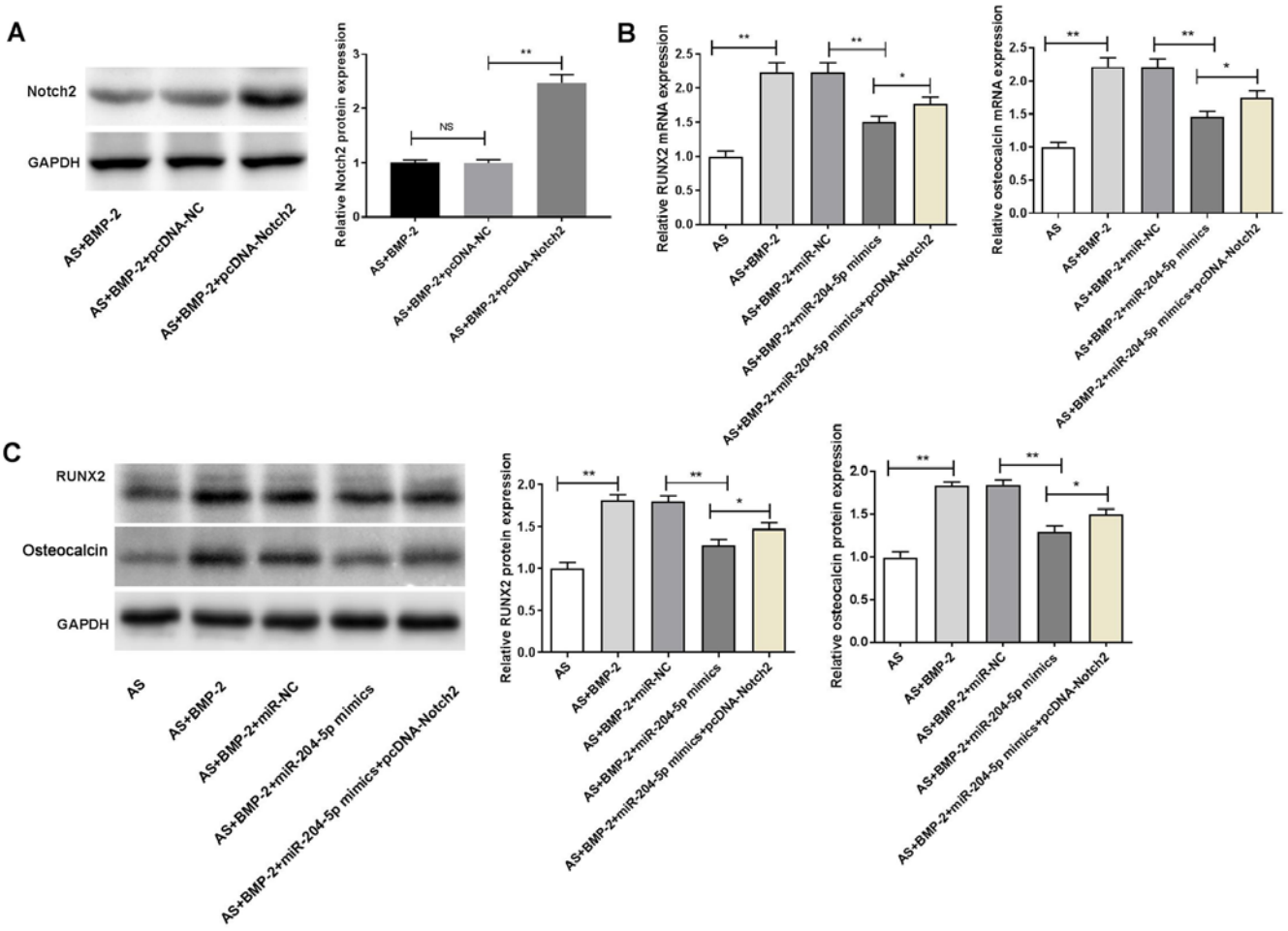

Figure 5. *miR-204-5p* inhibits the expression of RUNX2 and osteocalcin. (A) Notch2 protein expression following transfection with pcDNA-Notch2. (B) mRNA expression of RUNX2 and osteocalcin. (C) Protein expression of RUNX2 and osteocalcin. GAPDH was used as the internal control. *P<0.05 vs. the AS + BMP-2 + miR-204-5p mimics group; **P<0.01 vs. the AS and AS + BMP-2 + miR-NC groups, respectively. miR, microRNA; RUNX2, runt-related transcription factor 2; AS, ankylosing spondylitis; BMP-2, bone morphogenetic protein 2; NC, negative control.

functions in inhibiting the osteogenic differentiation of AS fibroblasts by targeting RUNX2 (41).

RUNX2 and osteocalcin are key factors involved in the bone-repair process (42). The level of RUNX2 mRNA is higher in patients with AS than that in healthy controls (43). RUNX2 controls the differentiation and formation of osteoblasts by upregulating the transcription of the BMP-2 gene to differentiate osteoblast precursors into osteocytes (44). Furthermore, suppressing RUNX2 can initiate osteogenic differentiation, which participates in the anti-osteogenic differentiation of AS fibroblasts (45). The results of the present study indicated that *miR-204-5p* inhibited the osteogenic differentiation of fibroblasts by inhibiting RUNX2 expression. Yu *et al* (41) demonstrated that *miR-204-5p* positively regulates RUNX2 expression to promote osteogenic differentiation of calcific aortic valve disease. Conversely, Wang *et al* (46) reported that *miR-204* inhibits RUNX2 expression and plays a negative role in regulating osteogenic differentiation. These previous findings suggest that the inhibition of RUNX2 expression contributes to the inhibitory effect induced by *miR-204-5p* on osteogenic differentiation.

Osteocalcin is the principle non-collagen component of the bone, which is considered a specific indicator of bone formation (47). Osteocalcin expression is notably higher in patients with AS than that in the control group (48). Furthermore, osteocalcin expression is significantly higher in patients with ankle stiffness and hip involvement than that in healthy controls (49). In the current study, osteocalcin expression was decreased in AS. *miR-204-5p* controls the osteogenic differentiation of fibroblasts by inhibiting osteocalcin expression (31). Thus, when *miR-204-5p* is inhibited, osteocalcin expression increases (31). Additionally, the expression of osteocalcin is downregulated by inhibiting RUNX2 expression and disrupting the activation of RUNX2 (50). Taken together, these results suggest that *miR-204-5p* is an important target to inhibit osteogenic differentiation through inhibiting the expression of RUNX2 and osteocalcin.

The current study had some limitations. Firstly, a relatively small number of studies have come from China, which limited the ability to identify the relationships between the *miR-204-5p* and AS. Moreover, the mechanism of *miR-204-5p* regulation on AS was only based on the experiments *in vitro*, and thus requires further investigation *in vivo*. In addition, the detailed mechanisms of action of *miR-204-5p* on AS are yet to be elucidated.

The present study investigated the osteogenic differentiation of ligament fibroblasts from patients with AS. The results demonstrated that *miR-204-5p* inhibited the expression of RUNX2 and osteocalcin in AS ligament fibroblasts by targeting Notch2, which provides a theoretical basis for the effective treatment of AS.

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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 on reasonable request.

Authors' contributions

JZ: Substantial contributions to the conception and design of the work. YZ: Substantial contributions to acquisition of data. BL: Substantial contributions to interpretation of data. JZ and YZ: Performed the experiments. BL: Performed the data analysis. JZ and YZ: Drafting the manuscript and revising it critically for important intellectual content. BL: Revised the manuscript for critically important intellectual content. JZ, YZ and BL: Final approval of the version to be published. JZ, YZ and BL: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shouguang People's Hospital (Shouguang, China; approval no. SGSRMXY-2020-09) and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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