miR-150-5p inhibits osteogenic differentiation of fibroblasts in ankylosing spondylitis by targeting VDR

YUAN LI, WUFANG QI and YUQUAN SHI

Department of Rheumatology and Immunology, Tianjin First Central Hospital, Tianjin 300192, P.R. China

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Abstract. Dysregulated microRNAs (miRNAs or miRs) serve potential roles in inflammatory systemic disease, including ankylosing spondylitis (AS). The aim of the present study was to investigate the potential function of miR-150-5p in osteogenic differentiation of AS fibroblasts and its underlying mechanism. The expression of miR-150-5p and vitamin D receptor (VDR) in AS joint capsules and fibroblasts was detected by reverse transcription-quantitative (RT-q)PCR and western blotting. Following overexpression of miR-150-5p, the alteration in osteogenic gene expression was detected by RT-qPCR, western blotting and alkaline phosphatase activity assay, as well as alizarin red staining. The association between miR-150-5p and VDR was confirmed by luciferase assay and rescue experiments were performed. Patients with AS exhibited decreased expression of miR-150-5p in joint capsules. Treatment with bone morphogenic protein 2 (BMP-2) and transforming growth factor-β1 (TGF-β1) led to downregulation of miR-150-5p in AS fibroblasts. Enforced expression of miR-150-5p attenuated osteogenic differentiation of AS fibroblasts. These results demonstrated that miR-150-5p inhibited osteogenic differentiation of AS fibroblasts by targeting VDR. miR-150-5p overexpression decreased osteogenic transformation of fibroblasts by decreasing VDR expression in AS.

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory autoimmune disease involving the sacroiliac, axial and hip joints that is caused by genetic, environmental and immune disorders (1). The course of this disease progresses from the inflammatory stage of bone marrow edema to the chronic stage of ectopic ossification and joint fusion (2). The incidence of AS in the Chinese population is ~0.2% and tends to occur in young and middle-aged men, which brings a burden to the family and society (3,4). There are few cases of surgical treatment in clinical application due to high perioperative risk, cost, difficulty in operation and poor prognosis (5). Considering the adverse effects, such as increased risk of cardiovascular disease, non-steroidal anti-inflammatory drugs and anti-tumor necrosis factor-α therapy are not approved for long-term treatment of AS (6,7). Prevention of the ossification of ligament fibroblasts is one of the primary goals in treating AS (8,9). However, the underlying mechanism is not yet been fully understood.

MicroRNAs (miRNAs or miRs) are non-coding small RNAs comprising endogenous 21-23 nucleotides involved in regulation of eukaryotic gene expression at the post-transcriptional level by binding to the 3'-untranslated region (UTR) of target gene mRNA (10). Evidence has revealed abnormal expression of numerous miRNAs in AS, some of which may be used as molecular markers for diagnosis and prognosis of AS (11,12). Expression profile analysis of patients with AS showed 9 differentially expressed circulating miRNAs, among which miR-150-5p, located on chromosome 19q13.33, was significantly decreased in patients with AS compared with healthy controls (13). It was hypothesized that decreased miR-150-5p expression may be associated with the development of AS.

Materials and methods

Patients and tissue collection. The collection of clinical specimens involved was approved by the Ethics Committee of Tianjin First Central Hospital (approval no. TJIRB2015-198) and written informed consent was obtained from all subjects. Patients and controls were recruited from Tianjin First Central Hospital (Tianjin, China). A total of 20 patients with AS involving both hips who received joint replacement between November 2015 to December 2017, including 17 males and 3 females with an average age of 32.8 years, were included as the experimental group. The diagnostic criteria for patients with AS complied with the 1984 modified New York criteria (14). A total of 20 patients with femoral neck fracture who underwent open surgery or total hip arthroplasty without AS or rheumatoid arthritis history (15 males and 5 females; average age, 32.3 years) were selected as the control group between November 2015 and December 2017. The joint capsules were acquired during the surgery. Clinical characteristics of all subjects are listed in Table I.

Correspondence to: Dr Yuquan Shi, Department of Rheumatology and Immunology, Tianjin First Central Hospital, 24 Fukang Road, Nankai, Tianjin 300192, P.R. China
E-mail: shi_yuquan@126-web.net

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Cell isolation and culture. Fibroblasts were obtained from ligament tissue of joint capsules derived from patients with AS using tissue explant adherent method. Briefly, 1 mm^3 ligament pieces were washed three times with PBS and transferred into a 25-cm^2 tissue culture flask containing Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The medium was refreshed every 3 days. After 7 days culture in a humidified atmosphere containing 5% CO_2 at 37˚C, the adherent cells were digested with 0.25% trypsin at 37˚C for 2 min. Following centrifugation at 1,000 x g for 5 min at room temperature, the single cell suspension was sub-cultured in a ratio of 1:3 and fibroblasts at the third passage were used for subsequent experiments. For induction of osteogenic differentiation, cells were cultured at 37˚C in DMEM supplemented with 50 bone morphogenic protein 2 (BMP-2) and 10 ng/ml transforming growth factor-β1 (TGF-β1; both R&D Systems, Inc.) for 14 days (15).

Cell transfection. miRNA-150-5p mimics and negative controls (miR-NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. as follows: miR-150-5p mimics forward, 5'-UCUCCCAACCCUUGUACCAGUG-3' and reverse, 5'-CACUGGUACAAGGGUUGGGAGA-3' and miR-NC forward, 5'-UCACAACCUCCUAGAAAGAGUAGA-3' and reverse, 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'. Plasmid for vitamin D receptor (VDR) overexpression was constructed by inserting the amplified complementary DNA (cDNA) into pcDNA 3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). Fibroblasts were incubated at 37˚C overnight in antibiotics-free DMEM medium until 70% confluence, and transfection was performed with 50 nM oligonucleotides or 2 μg vectors using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions for 4-6 h at 37˚C before changing to DMEM medium. Following 48 h transfection, fibroblasts were stimulated at 37˚C with 50 BMP-2 and 10 ng/ml TGF-β1 for 14 days before further analysis.

Bioinformatics prediction. Bioinformatics algorithm from starBase v2.0 (starbase.sysu.edu.cn/starbase2/targetSite.php) was used to predict the target genes of miR-150-5p.

Luciferase reporter assay. Fibroblasts were co-transfected with either miR-150-5p mimics (forward, 5'-UCUCCCAACCCUUGUACCAGUG-3' and reverse, 5'-CACUGGUACAAGGGUUGGGAGA-3') or mimic NC (forward, 5'-UCACAACCUCCUAGAAAGAGUAGA-3' and reverse, 5'-UCUACUCUUUCUAGGAGGUUGUGA-3') and wild-type or mutant psiCHECK-2 dual luciferase vector (Promega Corporation) comprising 3'UTR of VDR. Following 48 h transfection using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C, luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from joint capsules and fibroblasts using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using Mix-X miRNA First-Strand Synthesis and PrimeScript™ RT reagent kits (all from Takara Bio, Inc.). Reverse transcription was performed at 37˚C for 15 min followed by 85˚C for 5 sec. The obtained cDNA was subjected to PCR amplification using the following thermocycling conditions: Denaturation at 92˚C for 10 sec, annealing at 55˚C for 20 sec and extension at 68˚C for 20 sec (40 cycles). The mRNA expression levels of miR-150-5p, VDR, collagen type I (Col I), osteopontin (OPN) and runt-related transcription factor 2 (Runx2) were quantified using SYBR Green PCR kit (Takara Bio, Inc.) and U6 or GAPDH was used as the internal reference. The primer sequences were as follows: miR-150-5p forward, 5'-ACACTCCAGCTGGGTCTCCCAACCCTTGTACCA-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; Col I forward, 5'-CCTGGATGCCATCAAAGTCT-3' and reverse, 5'-ACTGCAACTGGAATCCATCG-3'; OPN forward, 5'-GCTATCACCTCGGCCGTTGGGG-3' and reverse, 5'-CATTGCCTCCTCCCTCCCGGTG-3'; Runx2 forward, 5'-CTCCCTGAACTCTGCACCAA-3' and reverse, 5'-GTTCTGAAGCACCTGAAATGCG-3'; GAPDH forward, 5'-CCATGGAGAAGGCTGGGG-3' and reverse, 5'-CAAAGTTGTCATGGATGACC-3' and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The relative quantification of mRNA expression was calculated via the $2^{-\Delta\Delta Cq}$ method as previously described (16).

Table I. Clinical characteristics and parameters of patients with ankylosing spondylitis and controls.

Parameter	Patient (n=20)	Control (n=20)
Age, years	32.80±12.96	32.30±10.61
Male/female	17.00/3.00	15.00/5.00
BASFI	2.08±3.10	-
BASDAI	3.90±2.03	-
BASMI	2.80±1.05	-
ASDAS-CRP	2.85±1.11	-
Disease duration, years	14.30±8.50	-
WBC, $10^3/mm^3$	8.12±1.83	-
Hb, g/dl	12.93±2.68	-
HLA-B27, n (%)	18.00 (90.00)	-
CRP, nmol/l	101.12±110.78	14.08±17.14
ERS, mm/h	16.90±18.30	6.10±5.20

ASDAS, Ankylosing Spondylitis Disease Activity Score; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Mobility Index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; HLA, human leukocyte antigen; WBC, white blood cells.

Immunoblotting. Total protein was extracted from cultured fibroblasts using RIPA lysis buffer (Pierce; Thermo Fisher Scientific, Inc.). The protein quantification was performed using bicinchoninic acid method (Thermo Fisher Scientific, Inc.). Equal amounts of protein (40 μg) were electrophoresed via 10% SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (MilliporeSigma). After blocking in 5% skimmed

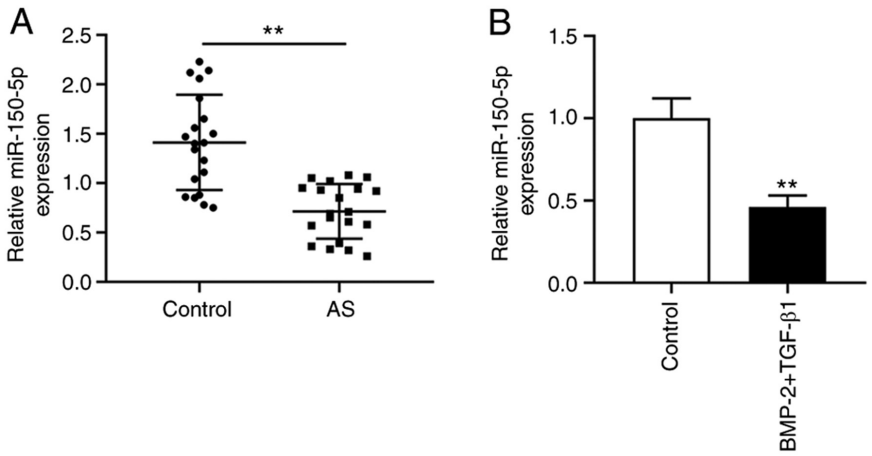

Figure 1. Downregulation of miR-150-5p in AS joint capsules and fibroblasts. (A) miR-150-5p expression in joint capsule tissues obtained from AS patients and non-AS controls (n=20/group) was determined by reverse transcription-quantitative PCR. (B) miR-150-5p expression in BMP-2 and TGF-β1-treated AS fibroblasts and untreated cells. Data are presented as the mean ± SD. **P<0.01 vs. control. miR, microRNA; AS, ankylosing spondylitis; BMP, bone morphogenetic protein; TGF, transforming growth factor.

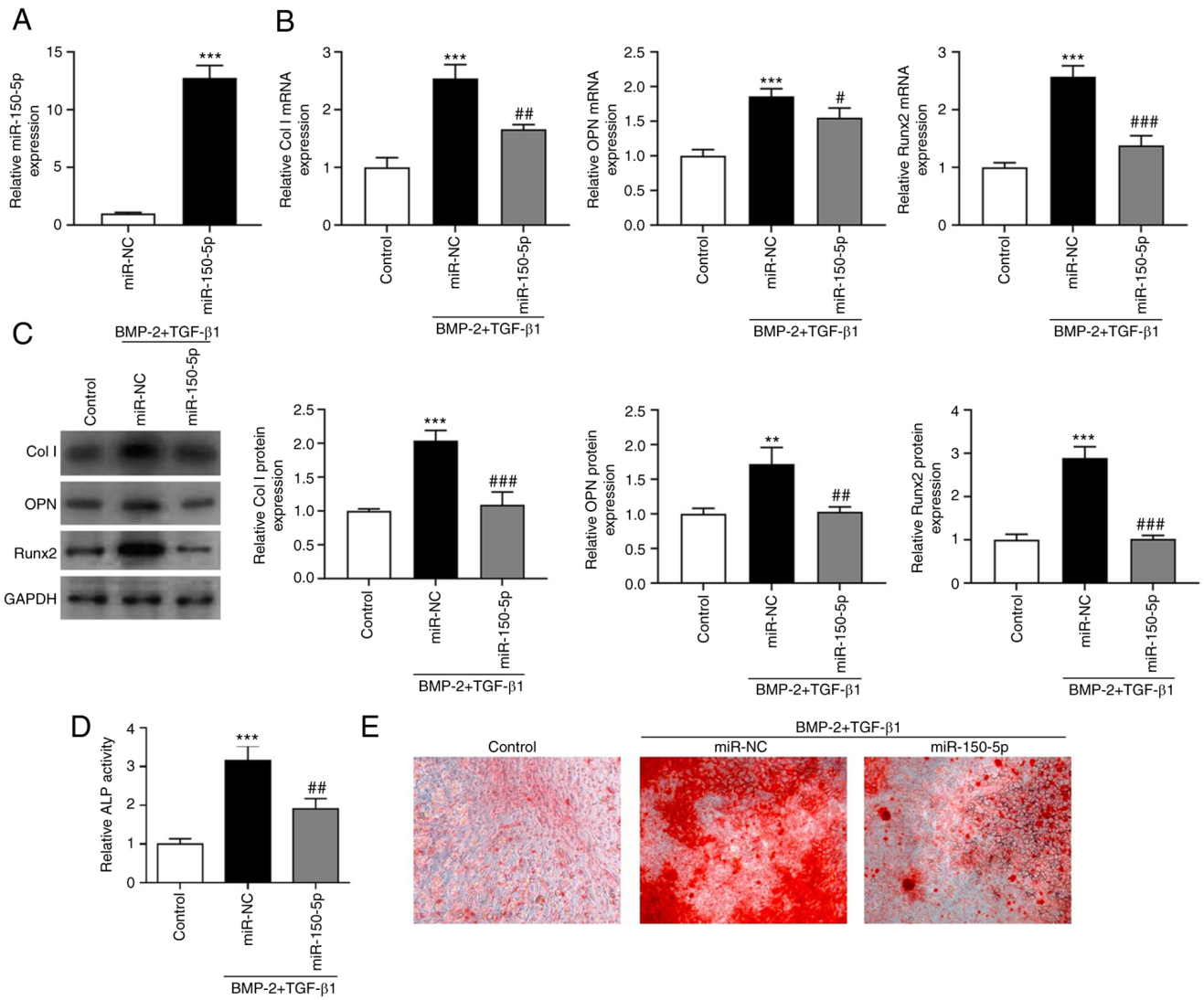

Figure 2. Overexpression of miR-150-5p suppresses osteogenic differentiation of AS fibroblasts. (A) Transfection efficiency. (B) mRNA expression of Col I, OPN and Runx2 determined by reverse transcription-quantitative PCR. (C) Protein levels of Col I, OPN and Runx2 determined by western blotting. (D) ALP activity. (E) Alizarin red staining for mineralization in BMP-2 and TGF-β1-induced AS fibroblasts transfected with miR-150-5p mimics or miR-NC or untreated cells (magnification, x100). Data are presented as the mean ± SD. **P<0.01, ***P<0.001 vs. control; #P<0.05, ##P<0.01, ###P<0.001 vs. miR-NC. miR, microRNA; AS, ankylosing spondylitis; BMP, bone morphogenetic protein; TGF, transforming growth factor; Col I, collagen type I; OPN, osteopontin; ALP, alkaline phosphatase; NC, negative control.

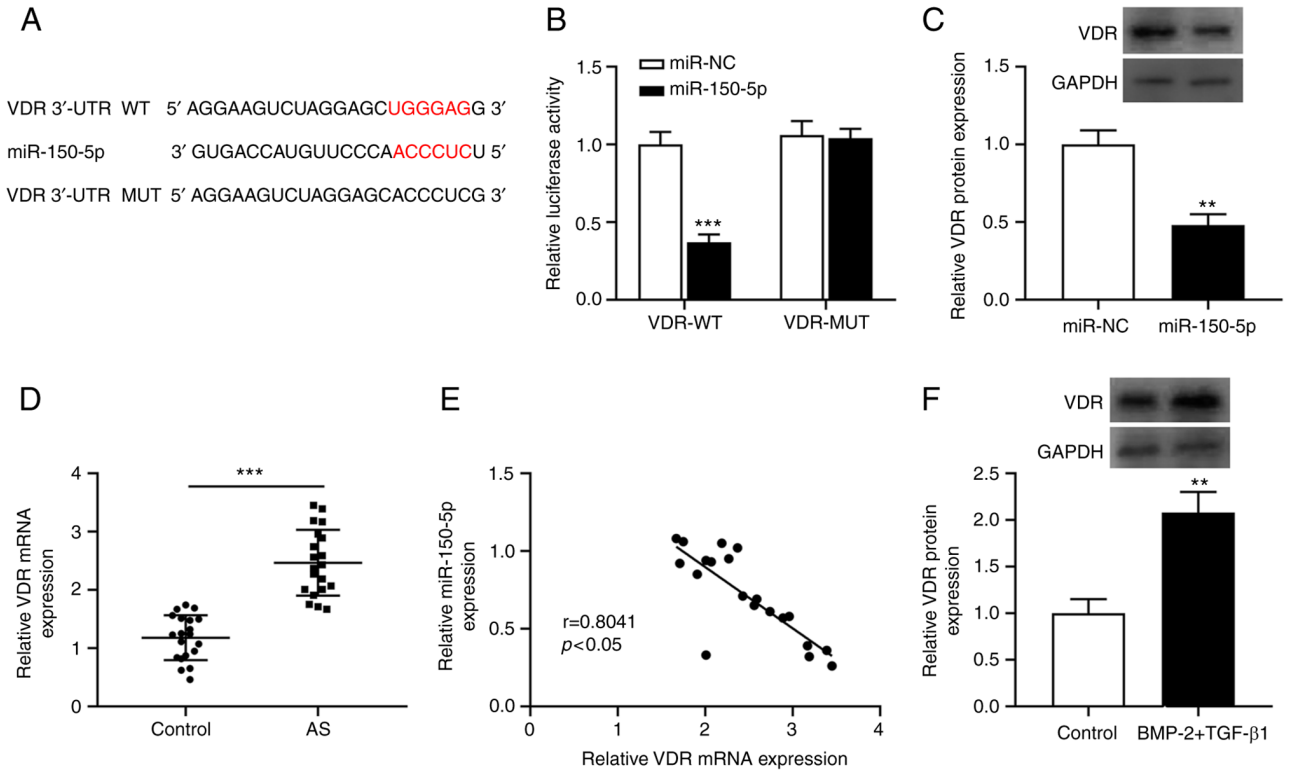

Figure 3. miR-150-5p decreases VDR expression by targeting VDR 3'-UTR. (A) Schematic diagram of the predicted miR-150-5p binding sites to VDR. (B) Luciferase assay in AS fibroblasts transfected with pmirGLO-VDR-WT or -MUT reporters and miR-150-5p mimics or miR-NC. (C) Protein levels of VDR in AS fibroblasts transfected with miR-150-5p mimics or miR-NC. (D) mRNA expression of VDR in joint capsule tissues obtained from patients with AS and non-AS controls (n=20/group) determined by reverse transcription-quantitative PCR. (E) Spearman's correlation analysis of miR-150-5p and VDR mRNA expression in AS joint capsules. (F) VDR protein levels in BMP-2 and TGF-β1-treated AS fibroblasts or untreated cells. Data are presented as the mean ± SD. **P<0.01, ***P<0.001 vs. miR-NC or control. miR, microRNA; AS, ankylosing spondylitis; BMP, bone morphogenetic protein; TGF, transforming growth factor; VDR, vitamin D receptor; UTR, untranslated region; WT, wild-type; MUT, mutant; NC, negative control.

milk for 1 h at room temperature, the membranes were incubated with primary antibody (1:1,000) overnight at 4˚C and horseradish peroxidase-conjugated IgG secondary antibody (1:5,000) for 1 h at room temperature. The primary antibodies against Col I (cat. no. ab6308), OPN (cat. no. ab8448), Runx2 (cat. no. ab76956), VDR (cat. no. ab134826) and GAPDH (cat. no. ab8245) and secondary antibody (cat. no. ab8245) were all purchased from Abcam. Densitometric analysis was processed using enhanced chemiluminescence reagent (Beckman Coulter, Inc.). Protein bands were semi-quantified using Image J software (version 1.8.0; National Institutes of Health).

Determination of alkaline phosphatase (ALP) activity. ALP activity was assessed using ALP assay kit (cat. no. A059-2-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. Fibroblasts in each group were collected and seeded in 6-well plates at a density of $1x10^4$ cells/well. A total of 50 μl p-nitrophenyl phosphate (Sigma-Aldrich; Merck KGaA) was added to each well. The reaction was stopped after 15 min at room temperature. The absorbance at 520 nm was determined by a microplate reader.

Alizarin red staining. Fibroblasts in each group were fixed in 70% ethanol for 1 h at 4˚C. After washing with distilled water three times, 40 mM alizarin red solution was added for 10 min at 37˚C. Cells were rinsed with distilled water and washed with PBS. The stained cells were observed under an inverted microscope (light microscope; magnification, x100). Photomicrographs were obtained using a charge-coupled device camera. Alizarin red was eluted with 10% cetylpyridinium chloride and the optical density value was measured at 510 nm to measure ossification.

Statistical analysis. Data are presented as the mean ± SD of three independent experimental repeats. Comparisons between groups were assessed by unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test using SPSS 19.0 (IBM Corp.). Spearman's correlation analysis was used to determine the association between miR-150-5p and VDR expression in AS joint capsules. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-150-5p in AS joint capsules and fibroblasts. To determine whether miR-150-5p serves a key role in the pathogenesis of AS, joint capsules from patients with AS and non-AS controls with femoral neck fracture were used to analyze expression levels of miR-150-5p via RT-qPCR. mRNA expression of miR-150-5p was significantly lower in patients with AS compared with controls (Fig. 1A). miR-150-5p expression in AS patient-derived fibroblasts following co-treatment with BMP-2 and TGF-β1 was detected. miR-150-5p expression was significantly decreased in BMP-2

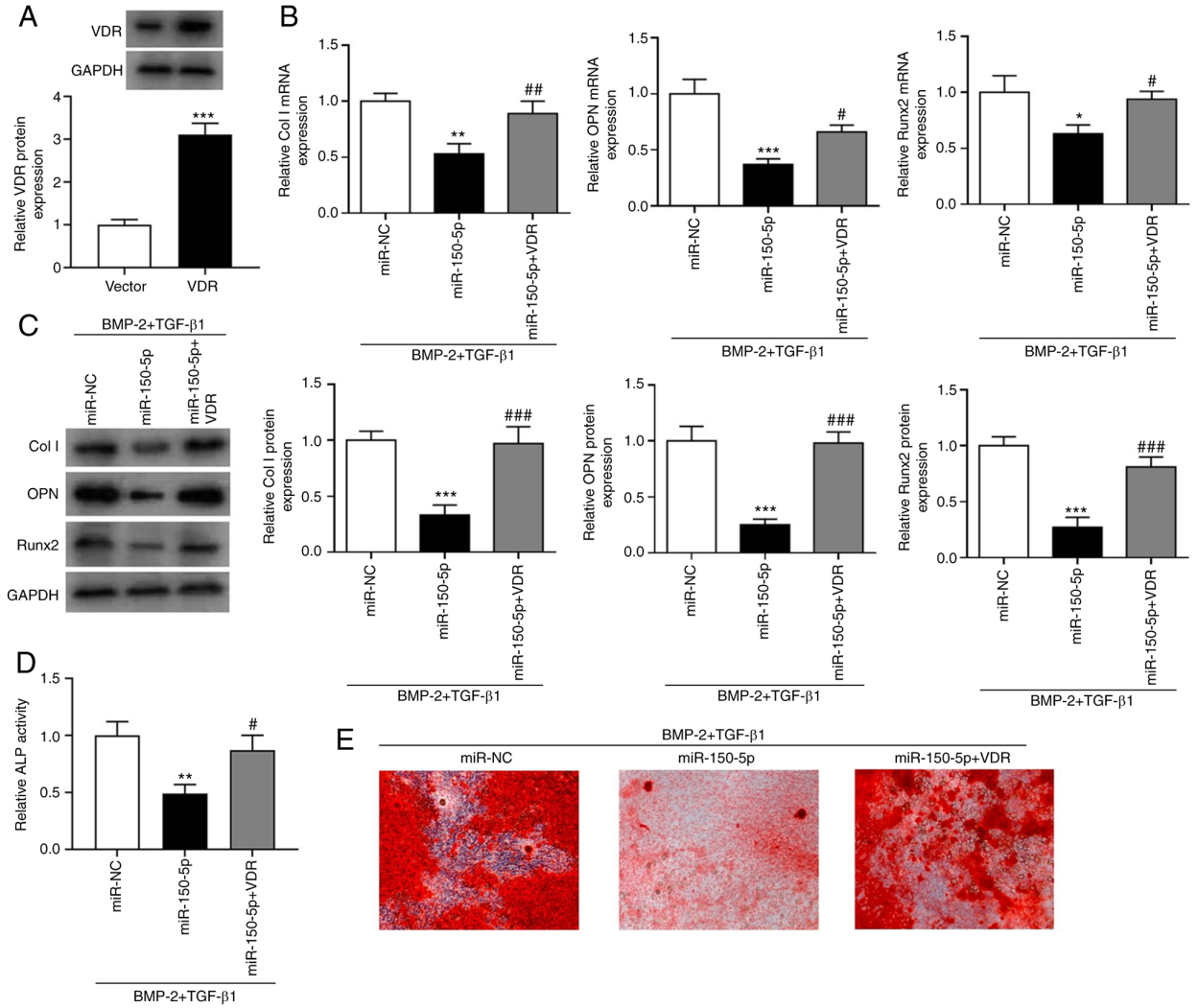

Figure 4. miR-150-5p regulates osteogenic differentiation in AS fibroblasts by downregulating VDR. (A) Transfection efficiency. (B) mRNA expression of Col I, OPN and Runx2. (C) Protein levels of Col I, OPN and Runx2. (D) ALP activity. (E) Alizarin red staining for mineralization in BMP-2 and TGF-β1-treated AS fibroblasts transfected with miR-NC or miR-150-5p mimics and with VDR (magnification, x100). Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. Vector or miR-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. miR-150-5p. miR, microRNA; AS, ankylosing spondylitis; BMP, bone morphogenetic protein; TGF, transforming growth factor; VDR, vitamin D receptor; UTR, untranslated region; Col I, collagen type I; OPN, osteopontin; ALP, alkaline phosphatase; NC, negative control.

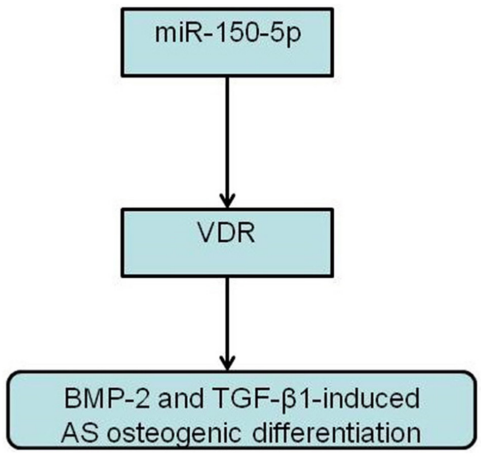

Figure 5. Schematic diagram of the potential role of miR-150-5p/VDR pathway in BMP-2 + TGF-β1-induced AS osteogenic differentiation. Arrows indicate downregulation. miR, microRNA; AS, ankylosing spondylitis; BMP, bone morphogenetic protein; TGF, transforming growth factor; VDR, vitamin D receptor.

and TGF-β1-treated AS fibroblasts compared with untreated cells (Fig. 1B).

Overexpression of miR-150-5p suppresses osteogenic differentiation of AS fibroblasts. The association between miR-150-5p and osteoblast differentiation was assessed by overexpressing miR-150-5p in AS fibroblasts via transfection of miR-150-5p mimics. The overexpression transfection efficiency was confirmed by RT-qPCR (Fig. 2A). The mRNA and protein expression levels of osteogenic genes, including Col I, OPN and Runx2 were significantly elevated in BMP-2 and TGF-β1-induced AS fibroblasts and miR-150-5p upregulation significantly reversed these changes (Fig. 2B and C). The osteogenic phenotype in AS fibroblasts treated with BMP-2 and TGF-β1 was confirmed by increased ALP activity, whereas miR-150-5p mimics resulted in decreased ALP activity (Fig. 2D). Additionally, mineralization visualized by alizarin red staining showed more mineral deposition in AS patient-derived fibroblasts following co-treatment with BMP-2 and TGF-β1 compared with control, whereas

miR-150-5p overexpression suppressed the mineralization of fibroblasts (Fig. 2E).

miR-150-5p decreases VDR expression by targeting VDR 3'-UTR. Bioinformatics analysis (starBase) predicted VDR as a candidate target gene of miR-150-5p (Fig. 3A). To verify whether VDR was a direct target of miR-150-5p, luciferase activity assay was performed. The relative luciferase activity of AS fibroblasts co-transfected with miR-150-5p mimics in the presence of wild-type VDR 3'-UTR was significantly decrease, while miR-150-5p overexpression did not affect the relative luciferase activity of mutant 3'-UTR-VDR (Fig. 3B). VDR protein expression was significantly decreased in AS fibroblasts transfected with miR-150-5p mimics (Fig. 3C). Increased expression of VDR mRNA was observed in joint capsules of patients with AS (Fig. 3D), which was negatively correlated with miR-150-5p mRNA expression (Fig. 3E). VDR protein expression in BMP-2 and TGF-β1-treated AS fibroblasts was significantly higher compared with untreated cells (Fig. 3F).

miR-150-5p regulates osteogenic differentiation in AS fibroblasts by downregulating VDR. To verify whether the role of miR-150-5p in osteogenic differentiation was mediated by downregulating VDR expression, rescue assay was performed using AS fibroblasts. High transfection efficiency was confirmed by western blotting following 48 h transfection with VDR overexpression plasmid (Fig. 4A). Ectopic expression of VDR antagonized inhibition of osteogenic differentiation potential induced by miR-150-5p overexpression in AS fibroblasts treated with BMP-2 and TGF-β1, as evidenced by the upregulation of Col I, OPN and Runx2 (Fig. 4B and C) and ALP activity (Fig. 4D). Additionally, restoration of VDR expression abrogated the inhibitory effects induced by miR-150-5p overexpression in AS fibroblasts following co-treatment with BMP-2 and TGF-β1 (Fig. 4E).

Discussion

The present study aimed to investigate expression of miR-150-5p and its molecular mechanism in progression of AS. miR-150-5p expression was decreased in hip capsule tissue of patients with AS and decreased the osteogenic differentiation capability of AS fibroblasts. VDR was confirmed as a direct target gene of miR-150-5p. miR-150-5p targeted VDR to exert anti-osteogenic effects in AS fibroblasts (Fig. 5).

In recent years, researchers have proposed aberrant expression of miRNAs in AS serves role in regulating osteoblast differentiation (17,18). For example, miR-124 increases osteoblast differentiation of AS fibroblasts via glycogen synthase kinase-3β-mediated Wnt/β-catenin pathway signaling (19). Moreover, miR-17-5p improves osteogenic differentiation and heterotopic ossification in AS via downregulation of ANKH (20). Overexpression of miR-96 enhances osteoblast growth and differentiation as well as bone formation in AS via activation of Wnt signaling pathway by targeting sclerostin (21). The present study demonstrated that miR-150-5p was downregulated in AS patients-derived hip joint tissue and ligament fibroblasts. The involvement of miR-150-5p has been reported in several types of disease, especially immune disease. For example, Chen *et al* (18) showed that exosomal miR-150-5p serves a therapeutic role in rheumatoid arthritis (RA) by decreasing migration and invasion of fibroblast-like synoviocytes, synoviocyte hyperplasia and angiogenesis. Qiu *et al* (19) reported that miR-150-5p inhibits apoptosis of RA synovial fibroblasts and promotes proliferation by downregulating suppressor of cytokine signaling 1. During osteoarthritis (OA) progression, overexpression of miR-150-5p suppresses proliferation and induces apoptosis and extracellular matrix degradation of OA chondrocytes (22,23). To the best of our knowledge, the present study is the first to demonstrate decreased miR-150-5p expression in AS fibroblasts treated with BMP-2 and TGF-β1. BMP-2 is a widely studied growth factor that enhances bone tissue regeneration (24,25). TGF-β1 serves a key role in regulating cell proliferation and differentiation and promoting osteogenesis (26,27). Therefore, the combination of BMP-2 with TGF-β1 is hypothesized to promote bone induction and regeneration and to exhibit a synergistic effect on induction of osteogenic differentiation (28-30). *In vitro* study of BMP-2 and TGF-β1-treated AS fibroblasts confirmed that miR-150-5p overexpression suppressed osteogenic potential, as shown by decreased expression of osteogenic markers.

VDR is a nuclear transcription factor that regulates bone metabolism and the inflammatory process by binding with specific ligands (31,32), such as steroid hormone 1alpha,25(OH)2-vitamin D3 (1,25(OH)2-D3) (33,34). VDR gene polymorphsim confers increased risk of AS in Chinese Han population (35,36). Zhang *et al* (33) recently demonstrated that long non-coding RNA H19 initiates IL-17A/IL-23 inflammatory pathway in AS pathogenesis by upregulating VDR by targeting miR-675-5p and miR-22-5p. The inhibitory effect of miR-351 in targeting VDR in osteogenic differentiation of bone marrow mesenchymal stem cells has been demonstrated (37). Here, VDR was highly expressed in AS fibroblasts and miR-150-5p inhibited expression of VDR by binding to its 3'-UTR. VDR overexpression abolished the anti-osteogenic role of miR-150-5p in AS fibroblasts. However, certain limitations should be considered. Other mechanisms underlying miR-150-5p-associated pathways that contribute to AS pathogenesis in different types of cell need further validation. Furthermore, additional animal models are required to confirm the role of miR-150-5p in AS. Lack of controls treated with BMP-2- and TGF-β1-alone is as a limitation of the present study and should be investigated in future.

In summary, the present study demonstrated that miR-150-5p directly targeted VDR and prevented osteogenic differentiation of AS fibroblasts, indicating a potential novel therapeutic option for 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

YL performed experiments and wrote the manuscript. WFQ performed experiments and analyzed the data. YQS designed the study and revised the manuscript. All authors have read and approved the manuscript. YL and YQS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Tianjin First Central Hospital (approval no. TJIRB2015-198). All subjects provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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