miR-148a-3p facilitates osteogenic differentiation of fibroblasts in ankylosing spondylitis by activating the Wnt pathway and targeting DKK1

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Abstract. Ankylosing spondylitis (AS) is a chronic inflammatory form of arthritis. MicroRNAs (miRNAs) have been identified to serve as therapeutic targets in various inflammatory diseases. The aim of the present study was to determine the functional mechanism of miR-148a-3p on AS. Specimens were collected from AS patients and non-AS patients. Fibroblasts were delivered with the aid of miR-148a-3p inhibitor. Cell staining was performed to observe the morphological changes, calcified nodules, and mineralization degree. The binding sites of miR-148a-3p and DKK1 were predicted on the Starbase website and subsequently verified by means of dual-luciferase reporter assay. AS fibroblasts with silenced miR-148a-3p were transfected with si-DKK1. Levels of RUNX2 and Osteocalcin, DKK1 and Wnt1 protein and phosphorylation level of β-catenin were detected by means of western blot analysis. Results of the present study denoted that AS upregulated miR-148a-3p in fibroblasts to exacerbate osteogenic differentiation, resulting in increased calcified nodules and mineralization degree. Silencing miR-148a-3p could reverse the upregulation of RUNX2 and Osteocalcin in AS fibroblasts and reduce the calcified nodules and mineralization degree. miR-148a-3p targeted DKK1. DKK1 knockdown averted the effect of silencing miR-148a-3p in AS fibroblasts. In addition, silencing miR-148a-3p reversed the upregulation of Wnt1 and β-catenin proteins in AS fibroblasts. To conclude, miR-148a-3p exacerbated the osteogenic differentiation of AS fibroblasts by inhibiting DKK1 expression and activating the Wnt pathway.

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Introduction

Ankylosing spondylitis (AS) is a progressive and debilitating form of arthritis with predominant onset before the age of 40, characterized by lower back pain and morning stiffness (1,2). Statistics indicate that one in 200 individuals may suffer from AS; however, a conclusive diagnosis is often made several years after the onset of symptoms (3). The average prevalence of AS is approximately 0.1-1.4% with slight male patient predominance (4). The pathogenesis of AS has been associated with several genetic factors and histocompatibility leukocyte antigen (HLA)-B27 (5). AS presents with numerous complications such as impaired spinal mobility, postural abnormalities, buttock pain, hip pain, peripheral arthritis, enthesitis and dactylitis (6). Under poor treatment, AS may progress to severe disability and impair the quality of life (7). The pathogenesis of AS is not fully determined yet and effective treatment methods should be investigated. Furthermore, the development of novel treatment approaches is crucial.

MicroRNAs (miRNAs) are small non-coding endogenously expressed RNAs that can serve as regulators of gene expression (8). Essentially, miRNAs can modulate cellular differentiation, inflammation and immune responses (9). miRNAs can also modulate the interaction between fibroblasts and osteoclasts in AS progression (10). An existing study determined the ability of miR-148a to serve as a potential disease-modifying agent in osteoarthritis (11). Moreover, an elevated expression of miR-148a was identified during the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (BMSCs) (12). Moreover, miR-148a-3p could regulate adipocyte and osteoblast differentiation (13). miR-148a-3p in extracellular vesicles derived from BMSCs alleviates osteonecrosis of the femoral head (14). Fibroblasts have been implicated as vital components in the ossification and ankylosis of ligament tissues (15). Accumulating research has established an association between the excessive proliferation of fibroblasts and heterotopic ossification with AS (16,17). However, the role of miR-148a-3p in osteogenic differentiation of human AS fibroblasts remains to be elucidated.

Dickkopf homologue 1 (DKK1) serves as a crucial component in the osteogenic differentiation of fibroblast in AS (18). DKK1 plays a fundamental role in the pathogenesis of

rheumatoid arthritis (19). Increasing evidence has elicited the regulation of DKK1 by different miRNAs in the osteogenic differentiation of fibroblasts in AS (20,21). The hypothesis is that miR-148a-3p participates in the osteogenic differentiation of fibroblasts in AS by regulating DKK1 expression. The aim of the present study was to determine miR-148a-3p expression in AS fibroblasts and its functional mechanism and to identify new therapeutic targets for osteogenic differentiation of AS fibroblasts.

Materials and methods

Ethic statement. The present study was performed in accordance with the Helsinki Declaration (22) and the experiment procedures were conducted with approval of the Ethics Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (Shanghai, China; approval no. 2017-IRBQYYS-057). All patients signed the informed consent.

Human samples. A total of 20 AS patients hospitalized in the Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine from May 2017 to May 2019 were chosen for sample collection. The 20 patients had undergone surgical intervention of total hip replacement due to severe and persistent pain worsening quality of life due to involvement of the hip joint on the basis of the 1984 modified New York criteria of American Rheumatism Association (23). The patients presented with notable findings such as inflammatory low backache, ossification of ankle joint, positive HLA-B27, and increased C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR). The samples used in the control group were provided by 20 non-AS patients who required hip replacement due to fracture of the femoral neck (excluding other types of osteoarthritis) caused by blunt trauma. The capsular ligament tissues were isolated during surgical intervention. Fibroblasts dissociated from ligament tissues were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd.).

Cell isolation and culture. Fibroblasts were isolated from the capsular ligament tissues of the enrolled patients. The ligament tissues were sectioned at 0.5 mm^3 and rinsed twice with phosphate-buffered saline (PBS). The ligament sections were placed in plates containing 5 ml of serum-free DMEM and 0.2 μg/ml Collagen I (Thermo Fisher Scientific, Inc.). The collagen fibers were removed using a 0.22-μm filter (MilliporeSigma) at 120 g. The precipitated cells were cultured in DMEM containing 20% serum and 1% streptomycin in 5% CO_2 for 72 h at 37˚C. The ligament sections were removed after observable growth of the fibroblasts from tissue fragments and adhered to the plate. The DMEM was replaced every 3 days. Fibroblasts were divided at the ratio of 1:3 and cultured to 80-90% confluence. The 3rd generation fibroblasts were used for subsequent experimentation. Briefly, the fibroblasts were cultured in normal medium supplemented containing a combination of 0.1 μl/l dexamethasone, 10 mmol/l β-glycerophosphate and 50 μl/l ascorbic acid to induce osteogenic differentiation (24).

Cell staining. Alizarin red staining was performed to detect the degree of calcification during heterotopic ossification. After a PBS rinse, the cells in 24-well plates were fixed with 95% ethanol for 30 min at 37˚C. Following fixation, the cells stained with Alizarin red solution (Sigma-Aldrich) were incubated for 30 min at 37˚C. Immunohistochemical staining (IHC) was performed on fibroblast with vimentin antibody (at a dilution ratio of 1:250; catalog no. ab92547; Abcam) using an IHC kit (Wuhan Boster Biological Technology Co., Ltd.) in strict accordance with the provided instructions. Hematoxylin and eosin (H&E) and BCIP/NBT staining were conducted based on the provided instructions of the corresponding kits (Nanjing Jiancheng Bioengineering Institute).

Cell transfection. Lipofectamine® 2000 (cat. no. 11668-019, Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the inhibitor negative control (NC), miR-148a-3p inhibitor, si-NC, and si-DKK1 (Shanghai GeneChem Co., Ltd.) (miRNA-inhibitor 50 nM, miRNA-mimic 30 nM, si-RNA 40-100 nM) into the experimental or 293T cells. The sequence of miR-148a-3p inhibitor, inhibitor NC, si-DKK1 and si-NC are presented in Table I. Briefly, miR-148a-3p inhibitor, si-DKK1, and corresponding controls were delivered into target cells using Lipofectamine RNAIMAX Transfection kit (Invitrogen; Thermo Fisher Scientific, Inc.) as per the protocol. Cells were seeded in 6-well plates ($1x10^6$ cells/well) one day prior to transfection. On the day of transfection, Lipofectamine RNAIMAX (1 μl) reagent was thoroughly mixed with 100 μl opti-MEM culture medium (Thermo Fisher Scientific) and the transfection complex at room temperature for 10 min and added in the 6-well plates. After 48-h transfection, the cells were detached with trypsin (Thermo Fisher Scientific) and washed once with PBS for subsequent experimentation. Cell grouping was as follows: the control group (fibroblasts of non-AS patients), the AS group (fibroblasts of AS patients), the AS + inhibitor-NC group (AS fibroblasts transfected with inhibitor NC), the AS + inhibitor-miR group (AS fibroblasts transfected with miR-148a-3p inhibitor), the AS + inhibitor-miR + si-NC group (AS fibroblasts transfected with miR-148a-3p and si-NC), and the AS + inhibitor-miR + si-DKK1 group (AS fibroblasts transfected with miR-148a-3p inhibitor and si-DKK1).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA content was extracted from the ligament tissues or cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using the cDNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Quantitative PCR was amplified using the SYBR® Premix Ex Taq™ kit (Takara Bio, Inc.). U6 or GAPDH served as the internal control. The reaction conditions were: Pre-denaturation at 95˚C for 120 sec, and 40 cycles of denaturation at 95˚C for 15 sec and extension at 60˚C for 60 sec. Relative expression was calculated based on the $2^{-\Delta\Delta Cq}$ method (25). The experiments on each sample were conducted three times independently. PCR primers are presented in Table II.

Western blot analysis. The total protein content was isolated from the ligament tissues or cells by radio immunoprecipitation assay lysis buffer and centrifuged at 4˚C, 12,000 x g for

Table I. Sequences of miR-148a-3p inhibitor, inhibitor NC, si-DKK-1 and si-NC.

Name of primer	Sequences (5'-3')
miR-148a-3p inhibitor	ACAAAGTTCTGTAGTGCACTGA
Inhibitor NC	TCTATGTGAAGTCACGAAGTCA
si-DKK1	AAAAUGACCGUCACUUUGCAA
si-NC	UGAACCGAAAUCAAUUCCAUG

miR, microRNA; DKK1, Dickkopf homologue-1.

Table II. RT-qPCR primer sequences.

Name of primer	Sequences
miR-148a-3p-F	TCAGTGCACTACAGAACTTTGT
miR-148a-3p-R	GAATACCTCGGACCCTGC
DKK1-F	GGTATTCCAGAAGAACCACCTTG
DKK1-R	CTTGGACCAGAAGTGTCTAGCAC
GAPDH-F	CATCACCATCTTCCAGGAGCG
GAPDH-R	TGACCTTGCCCACAGCCTTG
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT

F, forward; R, reverse; DKK1, Dickkopf homologue-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

10 min. Protein concentration was determined using the bicinchoninic acid kit. The protein sample (30 μg) was separated by 8% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. A membrane blockade was conducted using PBS containing 5% skimmed milk for 2 h at room temperature. Subsequently, the primary antibodies were added for incubation at 4˚C overnight. The membranes were co-incubated with the HRP-conjugated goat anti-rabbit IgG (at a dilution ratio of 1:2,000, ab97051, Abcam) secondary antibody for 2 h. Protein bands were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific) and estimated using the Image J software. Primary antibodies included in the present study were: runt-related gene 2 (RUNX2; at a dilution ratio of 1:5,000, ab76956, Abcam), Osteocalcin (at a dilution ratio of 1:1,000, ab133612, Abcam), DKK1 (at a dilution ratio of 1:1,000, ab109416, Abcam), Wnt1 (at a dilution ratio of 1:1,000, ab15251, Abcam), β-catenin (1:5,000, ab32572, Abcam), and p-β-catenin (at a dilution ratio of 1:5,000, ab75777, Abcam).

Dual-luciferase reporter assay. The binding sites of miR-148a-3p and DKK1 were predicted using the Starbase database (http://starbase.sysu.edu.cn/), RNAInter (http://www.rna-society.org/raid/search.html), Jefferson (https://cm.jefferson.edu/rna22/Precomputed/), and miRDB (http://mirdb.org/) websites. The binding and mutation sequences were cloned to the luciferase vector pGL3 (Promega Corporation) to construct the wild-type (DKK1-wt) and mutation-type (DKK1-mut) luciferase plasmids. The 293T cells (ATCC) were seeded in 6-well plates ($2x10^5$ cells/well) and subsequently incubated for 24 h. The constructed luciferase vectors and mimic NC or miR-148a-3p mimic (5'-UCAGUGCACUACAGAACUUUGU-3') (Shanghai GeneChem) (miRNA-mimic 30 nM) were co-transfected into the 293T cells using Lipofectamine 2000 in strict accordance with the provided instructions. Luciferase activity was detected using the dual-luciferase reporter assay kit (Beijing Solarbio Science & Technology Co., Ltd.) after 24 h of transfection. The cell experiments were conducted three times independently.

Statistical analysis. Statistical data were processed using the SPSS 21.0 statistical software (IBM Corp.). Data were all measurement data. The experimental data are presented as mean ± standard deviation (SD). GraphPad Prism 8.0 (GraphPad Software Inc.) was utilized for graphing. Normal distribution of data was assessed using the Shapiro-Wilk test. Data comparisons between two groups were analyzed using an unpaired t-test, and data comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. The P-value was obtained through two-sided test. In all statistical references, a value of $P<0.05$ was considered to indicate statistical significance.

Results

miR-148a-3p was highly expressed in fibroblasts of AS patients. Initially, the miR-148a-3p expression pattern in the harvested ligament tissues was detected by RT-qPCR, with results revealing that the miR-148a-3p expression pattern in the ligament tissues of AS patients had increased relative to the non-AS patients (Fig. 1A; $P<0.001$). Fibroblasts were identified by H&E staining and IHC staining (Fig. 1B). Fusiform fibroblasts with regular ellipsical nucleus in the control group were identified while long fusiform or flat star-shaped fibroblasts were observed in the AS group ($P<0.001$). IHC staining showed a positive expression pattern of the specific fibroblast marker vimentin (18) in normal fibroblasts and a decreased expression pattern of fibroblast marker vimentin (in dark brown) in AS group compared to the control group ($P<0.001$). Following induction of osteogenic differentiation, the results of BCIP/NBT and Alizarin red staining showed an increase in calcified nodules with mineralization degree in fibroblasts of the AS group relative to the control group (Fig. 1C; $P<0.001$). These results indicated that miR-148a-3p may participate in the osteogenic differentiation of fibroblasts.

Silencing miR-148a-3p inhibited the osteogenic differentiation of AS fibroblasts. To further investigate the function of miR-148a-3p, the miR-148a-3p inhibitor was transfected into the AS fibroblasts (Fig. 2A). BCIP/NBT and Alizarin red staining showed that silencing miR-148a-3p reversed the degree of increased calcified nodules and mineralization (Fig. 2B). Protein levels of osteogenic proteins RUNX2 (26) and Osteocalcin (27) were detected by western blot analysis (Fig. 2C). The result showed that levels of RUNX2 and Osteocalcin were significantly increased in AS fibroblasts ($P<0.001$), but were reduced in AS fibroblasts transfected with the miR-148a-3p inhibitor ($P<0.001$). These results

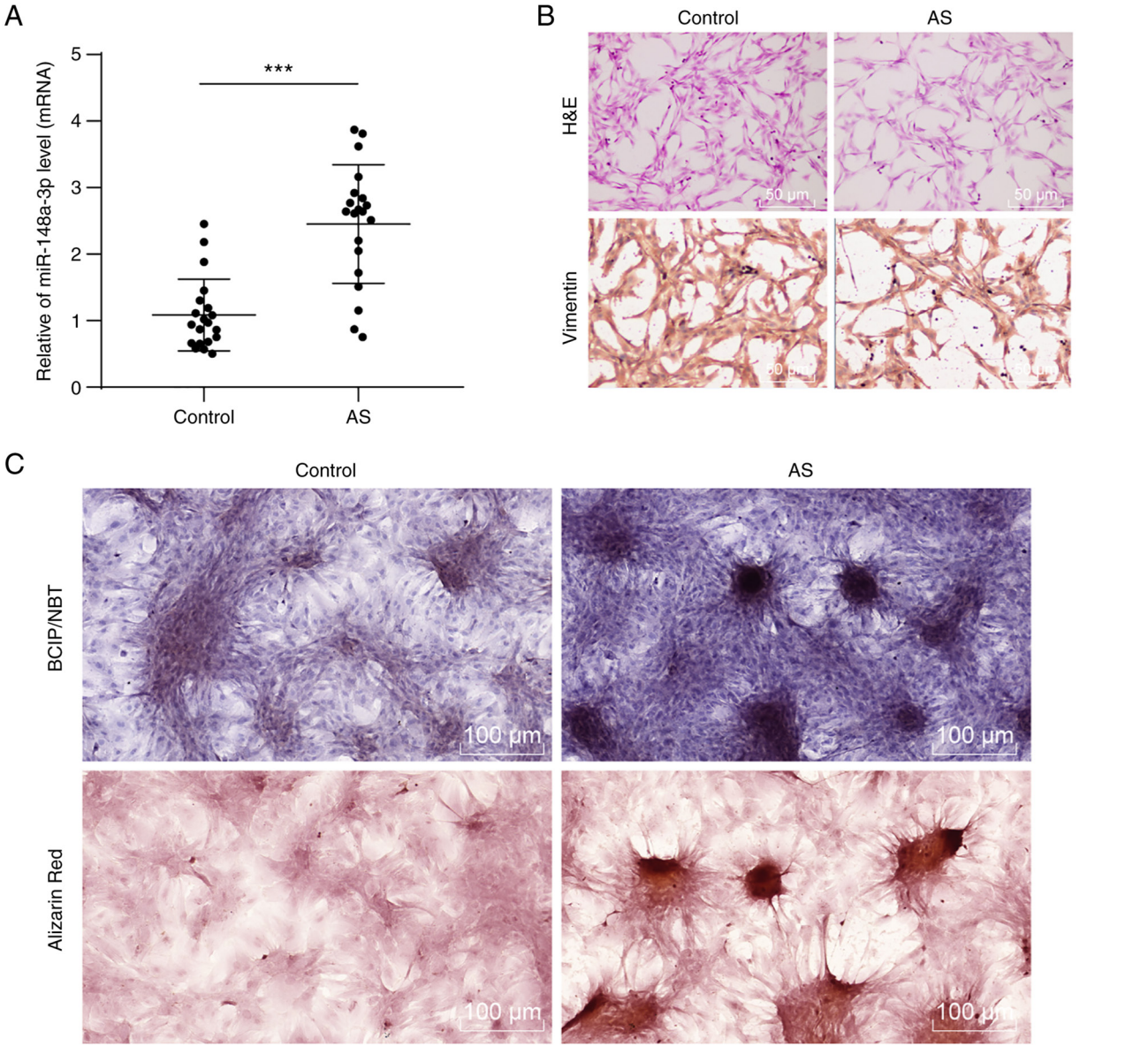

Figure 1. miR-148a-3p was highly expressed in fibroblasts of AS patients. Ligament tissues were collected from AS patients and non-AS patients. Morphological changes of AS fibroblasts were observed. (A) miR-148a-3p expression was detected by RT-qPCR, N=20, ***P<0.001; (B) H&E and IHC staining were performed; (C) BCIP/NBT and Alizarin red staining were performed.

demonstrated that silencing miR-148-3p could inhibit the osteogenic differentiation of AS fibroblasts.

miR-148a-3p targeted DKK1. To examine the functional mechanism of miR-148a-3p in AS fibroblasts, the downstream target genes of miR-148a-3p were predicted through a combination of the Starbase database (http://starbase.sysu.edu.cn/), RNAInter (http://www.rna-society.org/raid/search.html), Jefferson (https://cm.jefferson.edu/rna22/Precomputed/) and miRDB (http://mirdb.org/) websites, and intersections were determined (Fig. 3A). DKK1 was identified among the intersections; the significance of DKK1 in the osteogenic differentiation of AS fibroblasts was previously indicated (18). Thus, miR-148a-3p affected AS fibroblasts via DKK1. To verify our hypothesis, the binding sites of miR-148a-3p and DKK1 were initially predicted through the Starbase website (Fig. 3B), and their binding relation was verified by a dual-luciferase reporter assay in the 293T cells (Fig. 3C). Subsequently, the DKK1 expression pattern was detected in fibroblasts by RT-qPCR (Fig. 3D). The result showed that the DKK1 expression pattern was downregulated in AS fibroblasts (P<0.001), while silencing miR-148a-3p inverted the downregulation of DKK1 (P<0.001). These results suggested that miR-148a-3p targeted DKK1.

DKK1 knockdown reversed the inhibitory effect of miR-148a-3p knockdown on the osteogenic differentiation of AS fibroblasts. To verify the preceding results, si-DKK1 was transfected into the AS fibroblasts treated with silencing miR-148a-3p to observe the effect on AS fibroblasts. First of all, the transfection efficiency of si-DKK1 was verified by means of RT-qPCR (Fig. 4A). Subsequently, BCIP/NBT and Alizarin red staining showed that DKK1 knockdown in AS fibroblasts with silencing miR-148a-3p could increase calcified nodules

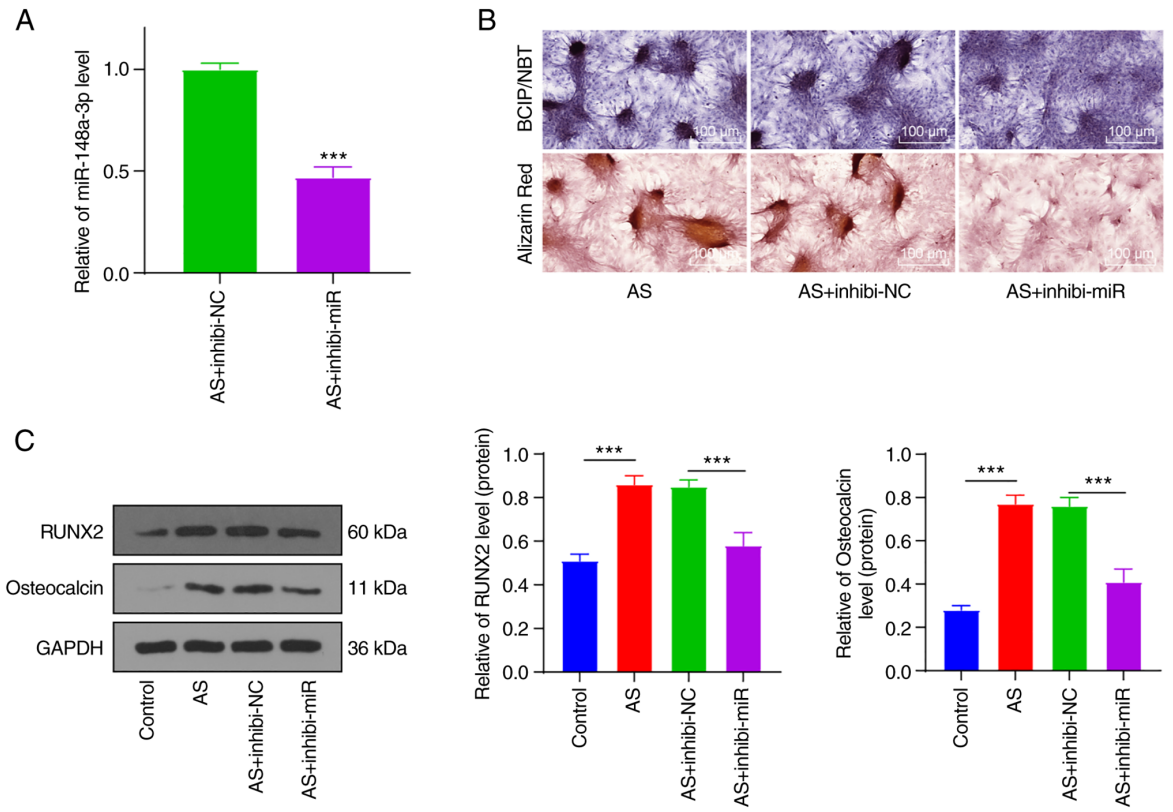

Figure 2. Silencing miR-148a-3p inhibited the osteogenic differentiation of AS fibroblasts. miR-148a-3p inhibitor was transfected into AS fibroblasts to downregulate miR-148a-3p and observe the effect on osteogenic differentiation. (A) miR-148a-3p expression was detected by RT-qPCR; (B) BCIP/NBT and Alizarin red staining were performed; (C) protein levels of RUNX2 and Osteocalcin were detected by western blot analysis. Cell experiment was repeated three times independently. Data were all measurement data. Data in panel A were analyzed using unpaired t-test and data in panel C were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. ***P<0.001.

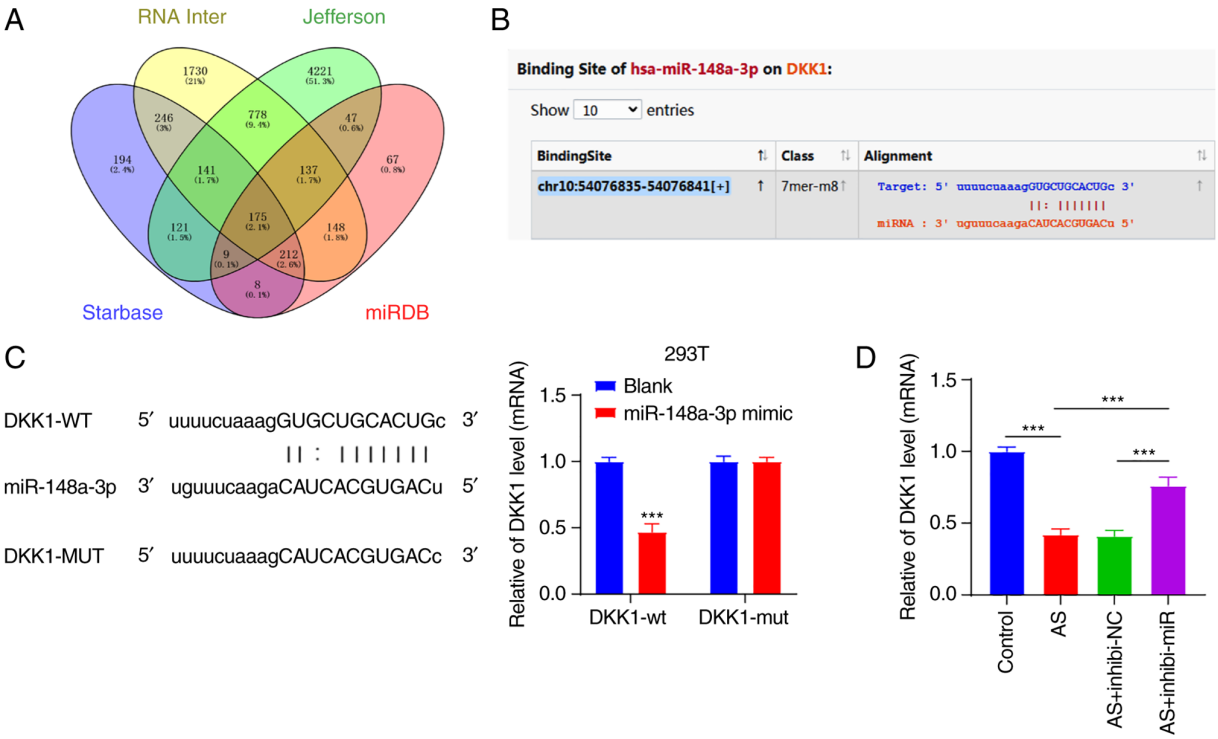

Figure 3. miR-148a-3p targeted DKK1. (A) Intersections of target genes were obtained on the Starbase, miRDB, RNAInter and Jefferson websites; (B) binding sites of miR-148a-3p and DKK1 were predicted through the Starbase website; (C) binding association of miR-148a-3p and DKK1 was verified by the dual-luciferase reporter assay; (D) DKK1 expression was detected by RT-qPCR. Cell experiment was repeated three times independently. Data were all measurement data. Data were expressed as mean ± SD. Data in panel C were analyzed using unpaired t-test and data in panel D were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. ***P<0.001.

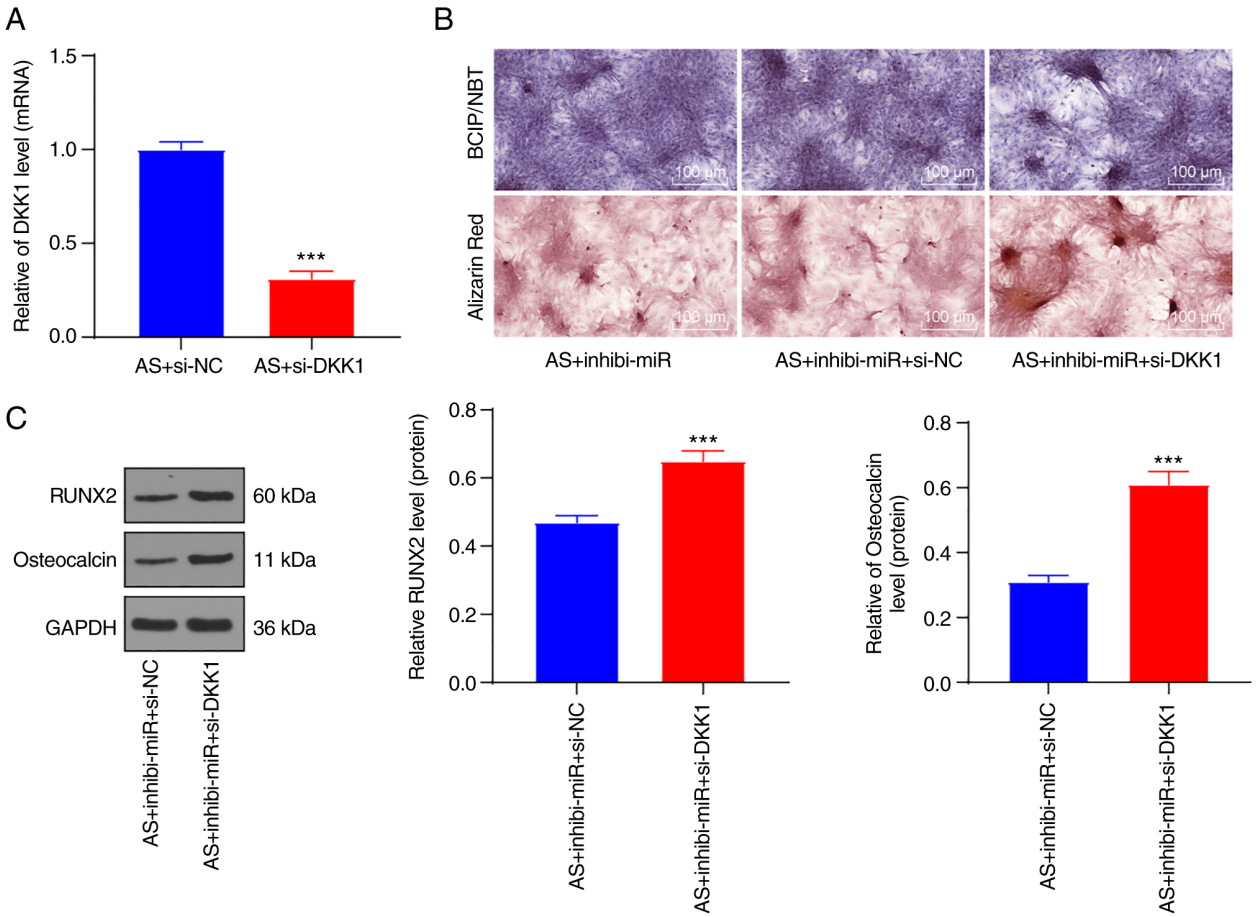

Figure 4. DKK1 knockdown reversed the inhibitory effect of miR-148a-3p knockdown on the osteogenic differentiation of AS fibroblasts. si-DKK1 was transfected into AS fibroblasts with silencing miR-148a-3p and the effect was observed. (A) DKK1 expression was detected by RT-qPCR; (B) BCIP/NBT and Alizarin red staining were performed; (C) protein levels of RUNX2 and Osteocalcin were detected by western blot analysis. Cell experiments were repeated three times independently. Data were all measurement data. Data were expressed as mean ± SD. Data were analyzed using unpaired t-test. ***P<0.001.

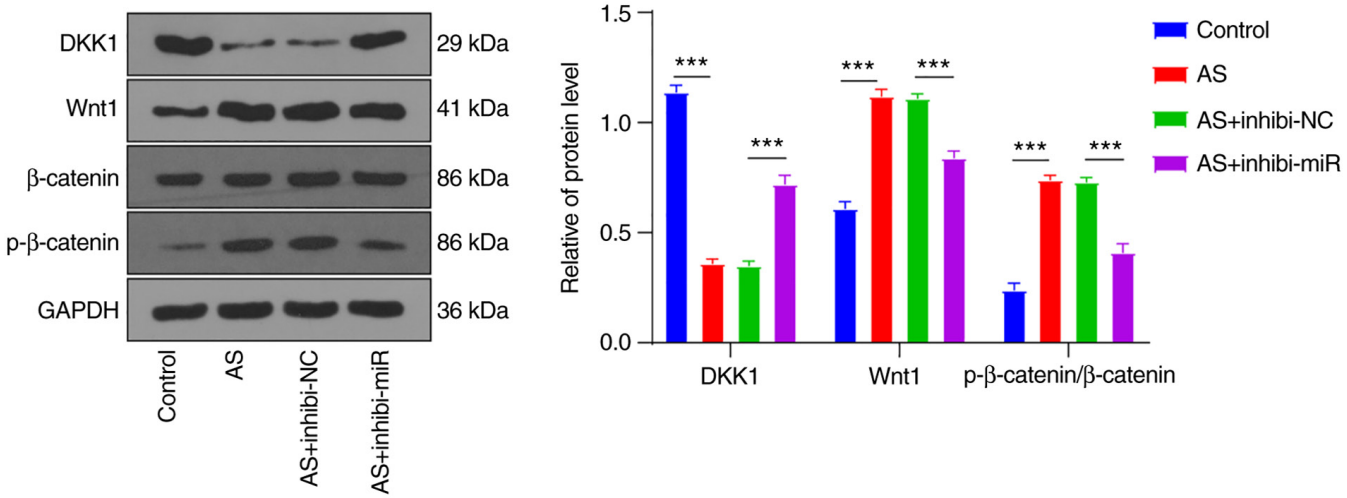

Figure 5. miR-148a-3p affected the osteogenic differentiation of AS fibroblasts by modulating the Wnt/β-catenin pathway via DKK1 regulation. The effect of miR-148a-3p on the Wnt/β-catenin pathway was observed by detecting the levels of DKK1 and Wnt pathway proteins in AS fibroblasts with silencing miR-148a-3p with AS fibroblasts with DKK1 overexpression as controls. Cell experiments were repeated three times independently. Data were all measurement data. Data were expressed as mean ± SD. Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. ***P<0.001.

and the mineralization degree (Fig. 4B). Western blot analysis showed that the downregulation of RUNX2 and Osteocalcin induced by miR-148a-3p inhibitor was reversed after DKK1 knockdown (Fig. 4C; P<0.001). Briefly, DKK1 knockdown could invert the inhibition of silencing miR-148a-3p on the osteogenic differentiation of AS fibroblasts.

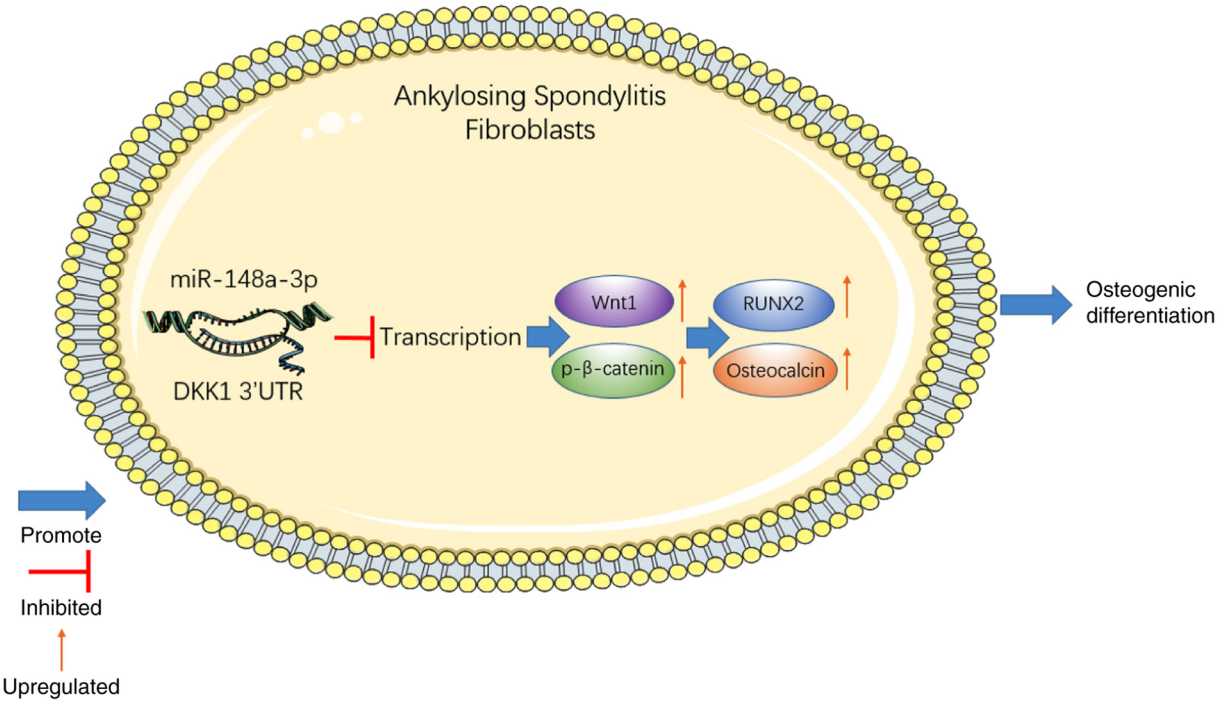

Figure 6. The mechanism of miR-148a-3p in osteogenic differentiation of AS fibroblasts. miR-148a-3p was highly expressed in AS fibroblasts. miR-148a-3p targeted DKK1, activated the Wnt/β-catenin pathway (increased the expressions of Wnt1 and p-β-catenin) and thus facilitated expressions of RUNX2 and Osteocalcin and ultimately promoted osteogenic differentiation of fibroblasts.

miR-148a-3p affected the osteogenic differentiation of AS fibroblasts by modulating Wnt/β-catenin pathway via DKK1 regulation. The participation of Wnt proteins has been identified in the osteogenic differentiation of AS fibroblasts. Therefore, the levels of DKK1, Wnt protein Wnt1, and p-β-catenin/β-catenin in AS fibroblasts with silenced miR-148a-3p were determined by western blot analysis with controls in AS fibroblasts revealing overexpression of DKK1 (Fig. 5). The result showed that the protein changes of DKK1 were consistent with the RT-qPCR result. Protein levels of Wnt1 and p-β-catenin/β-catenin were elevated in AS fibroblasts (all $P<0.001$), while silencing miR-148a-3p reversed the upregulation of Wnt1 and p-β-catenin/β-catenin proteins (all $P<0.001$), which was consistent with the result of overexpressing DKK1 in AS fibroblasts. The aforementioned results revealed that miR-148a-5p suppressed the DKK1 expression pattern and thus activated the Wnt/β-catenin pathway for participation in the osteogenic differentiation of AS fibroblasts (Fig. 6).

Discussion

AS may manifest as a multifocal disorder with numerous symptoms, affecting skeletal and extra skeletal organs and can radically increase the risk of multiple diseases (6). Several miRNAs, endogenous, non-coding small RNAs which can regulate mRNA gene expression, have been suggested to serve as definitive markers or therapeutic targets of AS (8). Numerous miRNAs have been implicated in the fundamental functionality of AS physiological and pathological processes (10). In the present study, the effect of miR-148a-3p on AS was evaluated, and the results highlighted that miR-148a-3p stimulated the osteogenic differentiation of AS fibroblasts by inhibiting the DKK1 expression and activating the Wnt pathway. Heo *et al* observed a weakly positive osteogenic differentiation of fibroblasts during identification of the differentiation capability of bone marrow mesenchymal stem cells with fibroblasts as negative controls (28). According to Ding *et al*, osteogenic differentiation of fibroblasts occurs in AS patients (24). The present findings identified the property of osteogenic differentiation of fibroblasts in AS patients, and therefore the molecular mechanism of osteogenic differentiation with fibroblasts in AS patients was examined.

As a common and genetically heterozygous inflammatory rheumatic disease, AS is characterized by progressive ankylosis and inflammation of hip, sacroiliac joints and spine, and new bone formation; miRNAs also show different expression patterns with the development of AS (8). miR-148a was upregulated in rheumatoid arthritis (10). Moreover, previous findings determined the ability of miR-148a-3p to regulate adipocyte or osteoblast differentiation by targeting lysine-specific demethylase 6b (13). Newly initiated ossification of ligaments is characteristic of AS with progression of pathological bone formation leading to loss of joint function and disability (29). Additionally, miR-148a-3p is a potential contributor to heterotopic ossification in AS in light of preceding literature (30). In the present study, an elevated miR-148a-3p expression was identified in the AS ligament tissues. Osteogenic differentiation of fibroblasts is the primary cause of osteophyte formation and ankylosis in AS (31). Previous results validated the functionality of vimentin fragments as potential markers of rheumatoid synovial fibroblasts (32). Findings of the present study denoted a reduced expression of vimentin in AS fibroblasts, while the concentration of calcified nodules and mineralization degree were increased. Moreover, the

miR-148a-3p from BMSCs-derived extracellular vesicles can facilitate the osteogenic differentiation in osteonecrosis of the femoral head (14). The current results validated the participation of miR-148a-3p in the osteogenic differentiation of fibroblasts in AS. To further verify the effect of miR-148a-3p on AS fibroblasts, the miR-148a-3p inhibitor was transfected into AS fibroblasts, the result of which suggested that the calcified nodules and mineralization degree were increased. Research has implicated RUNX2 and Osteocalcin as vital factors in osteoblast differentiation (33,34). The results of this study demonstrated markedly elevated expressions of RUNX2 and Osteocalcin in AS fibroblasts. However, the upregulation was reversed by silencing miR-148a-3p in AS fibroblasts. Similarly, miR-148a-3p downregulation could impede the differentiation of rabbit preadipocytes (35). The aforementioned results suggested that miR-148a-3p knockdown radically inhibited the osteogenic differentiation of AS fibroblasts.

To examine the downstream mechanism of miR-148a-3p in AS fibroblasts, the downstream target genes of miR-148a-3p were predicted. A negative correlation was identified between the DKK1 level and AS severity (36). Therefore, miR-148a-3p could impact AS fibroblasts via DKK1. The binding sites of miR-148a-3p and DKK1 were predicted using the Starbase website. The target association of miR-148a-3p and DKK1 was verified by the dual-luciferase reporter assay. The results of the present study identified the downregulation of DKK1 in AS fibroblasts; however; the downregulation could be abolished after silencing miR-148a-3p. Briefly, miR-148a-3p could target DKK1. The effects of the downregulation of DKK1 were determined in a previous study, where this downregulation exacerbated fibroblast proliferation and enhanced the osteogenesis of fibroblasts in AS (37). In the present study, the concentration of calcified nodules and mineralization degree were increased after DKK1 knockdown. The results showed that the downregulation of RUNX2 and Osteocalcin mediated by silencing miR-148a-3p was inverted after DKK1 knockdown. Pathologically, the downregulation of DKK1 can facilitate the osteogenic differentiation of human adipose-derived MSCs in the progression of bone repair and reverse the suppression of primary human osteoblast differentiation (38,39). Conjointly, findings of this study denoted that DKK1 knockdown reversed the inhibition of silencing miR-148a-3p in the osteogenic differentiation of AS fibroblasts.

DKK1 is a natural inhibitor of the Wnt pathway (40,41). Wnt proteins are essential for normal bone homeostasis, especially in osteoblastic new bone formation (42-44). Wnt1 is a protein that can activate the Wnt pathway with β-catenin serving as a mediator (45). In the current study, miR-148a-3p in the fibroblasts of AS patients regulated DKK1 transcription, evidenced by deviations in the expressions of Wnt1 and p-β-catenin proteins while silencing miR-148a-3p reversed the downregulation. Thus, a fundamental role of DKK1 in AS fibroblasts as an endogenous inhibitor of the Wnt pathway was indicated. Wnt1 in the canonical Wnt signaling pathway is predicted as the target gene of hsa-miR-148a-3p with significant regulation in osteoporosis (46). The ability of human recombinant DKK1 to facilitate the differentiation of adipose-derived stem cells via the Wnt signaling pathway was determined (47). Moreover, miR-148a can modulate adipocyte differentiation of MSCs via the Wnt signaling (48). DKK1 has been reported to serve as an endogenous inhibitor of the Wnt pathway *in vivo* (49). The aforementioned results suggest that miR-148a-3p in fibroblasts of AS patients regulated DKK1 transcription, accompanied by expression alterations in Wnt1 and p-β-catenin proteins. Thus, that DKK1 plays an essential role in AS fibroblasts as an endogenous inhibitor of the Wnt pathway. Collectively, results of the present study identified the participation of miR-148a-3p in osteogenic differentiation by inhibiting DKK1 expression and activating the Wnt/β-catenin pathway.

To conclude, the current study demonstrated that the high expression of miR-148a-3p promoted the osteogenic differentiation of AS fibroblasts by downregulating DKK1 and activating the Wnt/β-catenin pathway. Novel insight has been provided for the management of osteogenic differentiation of AS fibroblasts. Nevertheless, the participation of miR-148a-3p in the osteogenic differentiation of AS fibroblasts was not verified in an animal experiment, and therefore requires subsequent validation. Moreover, the osteogenic differentiation of AS fibroblasts may be modulated by multiple pathways including the Wnt/β-catenin pathway. Whether miR-148a-3p affects the osteogenic differentiation of AS fibroblasts by modulating other pathways requires further investigation. Lastly, the effect of AS on patients comes mainly from the ankylosis of spine, which is often caused by heterotopic ossification. The molecular mechanism in AS from the perspective of heterotopic ossification was also examined. Fibroblasts were isolated from the ligaments of AS and non-AS patients, cultured with the same medium, and osteogenically induced with the same method, followed by observation of the effect of AS on osteogenic differentiation of fibroblasts. However, apoptosis-related changes were not detected in this study. ANKH, a multichannel transmembrane protein, has been reported to affect the metabolism of AS fibroblasts and inhibit fibroblast viability, ossification and mineralization (50). Recent findings have elucidated that ANKH plays a regulatory role in fibroblast viability, ossification and mineralization (50). In the current study, the Starbase database predicted ANKH as a downstream target gene of miR-148a-3p. miR-148a-3p might modulate fibroblasts via ANKH. We predicted that ANKH is the downstream target gene of miR-148a-3p on Starbase database, and further exploration should be conducted. Future studies should focus on exploring whether miR-148a-3p can serve as the new target for the clinical treatment for osteogenic differentiation of AS fibroblasts, whether miR-148-3p can regulate the osteogenic differentiation of AS fibroblasts through any other mechanisms and the molecular mechanism of miR-148a-3p in fibroblasts via targeting ANKH.

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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

WS, SL and HJ substantially contributed to the conception and the design of the study. WS and HY were responsible for the acquisition, analysis and interpretation of the data. HJ, SL and HY contributed to manuscript drafting or critical revisions of the intellectual content. WS and HJ approved the final manuscript to be published, and SL agreed to be accountable for all aspects of the work, so that any questions relating to research integrity or scientific accuracy in any part of the study are appropriately investigated and resolved. WS and HJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the Helsinki Declaration and the experiment procedures were conducted with approval of the Ethics Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (Shanghai, China; approval no. 2017-IRBQYYS-057). All the patients signed the informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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