# Ankylosis progressive homolog upregulation inhibits cell viability and mineralization during fibroblast ossification by regulating the Wnt/β-catenin signaling pathway

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**Abstract.** Ankylosis progressive homolog (ANKH) is associated with fibroblast ossification in ankylosing spondylitis (AS). As the human ANKH gene is poorly characterized relative to its murine counterpart, the aim of the present study was to examine ANKH expression in ligament tissue isolated from patients with AS and the role played by this gene in AS-associated fibroblast ossification. Fibroblasts were isolated from ligament tissue collected from patients with AS and ligament tissue from individuals with spinal cord fractures, then cultured. Fibroblasts from patients with AS were subsequently transfected with an ANKH overexpression vector, while those collected from individuals with spinal cord fractures were transfected with small interfering RNA specific for ANKH. Cell viability, apoptosis and mineralization were analyzed using MTT assays, flow cytometry and Alizarin Red staining, respectively. Furthermore, ANKH mRNA and protein expression levels were analyzed using reverse transcription-quantitative PCR and western blotting analysis, respectively. The expression levels of osteogenesis markers, including alkaline phosphatase, osteocalcin, Runt-related transcription factor 2, c-Myc, as well as the β-catenin signaling protein, were also determined using western blotting. The results of the present study revealed that ANKH protein expression levels were downregulated in AS total ligament tissue extract, compared with spinal fracture ligament. Moreover, the fibroblasts derived from patients with AS exhibited an increased viability and reduced apoptosis rates, compared with the fibroblasts from patients with spinal fracture. Notably, ANKH overexpression inhibited viability, mineralization and ossification, increased the phosphorylation of β-catenin and downregulated β-catenin and c-Myc protein expression levels in fibroblasts from patients with AS. In addition, ANKH overexpression increased the ratio of p-β-catenin/β-catenin in fibroblasts from patients with AS. By contrast, ANKH silencing in fibroblasts from patients with spinal fracture resulted in the opposite effect. In conclusion, the findings of the present study suggested that ANKH may inhibit fibroblast viability, mineralization and ossification, possibly by regulating the Wnt/β-catenin signaling pathway.

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*Abbreviations:* ANKH, ankylosis progressive homolog; AS, ankylosing spondylitis; ALP, alkaline phosphatase; OCN, osteocalcin; Runx2, Runt-related transcription factor 2; RT-qPCR, reverse transcription-quantitative PCR

*Key words:* ankylosing spondylitis, ankylosis progressive homolog, fibroblast, ossification, Wnt/β-catenin signaling pathway

## Introduction

The chronic progression of ankylosing spondylitis (AS) is characterized by inflammatory bone erosion, intravertebral bone loss and abnormal bone overgrowth (1,2). Sacroiliac joints, the vertebral column and ligaments affected by inflammation have been discovered to gradually cause extensive bone generation, leading to stiffness and fusion of the spine and syndesmophyte formation (3). Although previous studies have examined genetic and environmental factors, sex, age and ethnicity as causative factors, to the best of our knowledge, the etiology of AS remains unclear (3-5). Genetic factors underlying AS pathogenesis have been widely studied (6-8), and several genes that have been identified as susceptibility factors of AS may support the diagnosis of AS, such as HLA-B27, HLA-B51 and ERAP1 (5,9,10). In addition, genetic associations with AS may also provide insight into the etiopathogenesis of this disease (11). Although the molecular functions of several genes have been studied in the context of AS, their potential pathogenic role and their possible therapeutic use have not been evaluated (12).

Ankylosis progressive homolog (ANKH), an amino acid of 492 base pairs in length, is a multichannel transmembrane protein that transports intracellular pyrophosphate (PPi) to the extracellular milieu (13). Extracellular PPi was discovered to be a regulator of pathological calcification and a potential inhibitor of calcium phosphate mineralization (14-16). ANKH is the human homolog of the murine ANK gene (15). It has been demonstrated that the loss of ANK transporter function in mice led to excessive mineralization due to reduced PPi transport (17), which was later confirmed by other studies (18,19).

Gurley *et al* (18) illustrated the role of ANK in PPi transport and suggested that ANKH mutations may cause skeletal diseases in humans. Ho *et al* (19) reported that ANK-deficient mice displayed increased PPi levels and presented with mineralization and skeletal ankylosis resembling AS. Thus, these previous studies indicated that ANK may regulate ectopic mineralization in animal models.

AS is characterized by pathological osteogenesis (20). Previous studies suggested that fibroblasts may be the target of heterotopic ossification because these cells belong to the same lineage as osteoblasts and were discovered to express osteogenesis-related genes, such as BMP2, osteocalcin and Runx2 (21,22). Fibroblasts are resident cells that preserve the extracellular matrix, both in healthy conditions and during inflammation (23). Thus, fibroblasts were suggested as likely contenders to be involved in osteogenesis (24). Fibroblasts and osteoblasts both originate from mesenchymal stem cell with overlapping phenotypes and through similar differentiation pathways, allowing fibroblasts to differentiate into osteoblasts and osteoblasts to differentiate into fibroblasts (25). In addition, the proliferative and osteogenic potential of fibroblasts was discovered to increase following the stimulation with fibronectin or BMP2 (26,27). Therefore, fibroblasts may serve as important mediators of osteogenesis.

The aim of the present study was to determine the function of ANKH in fibroblasts isolated from patients with AS. ANKH expression levels were analyzed in ligament tissue collected from patients with AS, and explored the effect of ANKH on the fibroblast cell viability, mineralization and ossification differentiation. From the results, it was hypothesized that the regulation of ANKH may affect fibroblast cell mineralization and ossification in AS.

## Materials and methods

*Patients and tissue samples*. Ligament tissue samples were collected from four patients with AS (male; age, 18-42) and four patients with spinal fracture (JK) (male; age, 22-44) who attended The People's Hospital of Xinchang Hospital in March 2019 for treatment, according to the modified New York criteria (28). The patients used nonsteroidal anti-inflammatory drugs or disease-modifying anti-rheumatic drugs, but not anti-tumor necrosis factor. Written informed consent forms were signed by all patients. The study was approved by The Ethics Committees of the People's Hospital of Xinchang (Xinchang, China; approval no. XC201902114).

*Cell culture*. The ligaments samples from patients with AS and JK were separated in 60-mm sterile Petri dishes, and washed three times with D-Hank's balanced salt solution (HBSS; cat. no. 14170161; Gibco; Thermo Fisher Scientific, Inc.). The tissues were then sectioned into 1-3-$mm^3$ blocks and washed with HBSS twice. The blocks were maintained in a 25-$cm^2$ culture flask, with 4 mm space in between tissue blocks. Next, 2 ml high-glucose DMEM (cat. no. 11965118; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. A3160901; Gibco; Thermo Fisher Scientific, Inc.) was added to the flask. The flask was placed vertically inside an incubator at 37˚C with 5% $CO_2$ for 4 h. The bottle was gently laid flat at the end of this incubation, and the medium was changed every three days thereafter. After 4-8 days, when the cells reached 80% confluence, they were washed with HBSS and added to 1 ml 0.25% trypsin solution (cat. no. R001100; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C. After 2 min, 3 ml DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS was immediately added to terminate cell dissociation. The cells were collected and centrifuged in a 15-ml centrifuge tube at 1,000 x g for 5 min at 4˚C. The cell suspension was then divided (1:2) for secondary culture at 37˚C with 5% $CO_2$. The medium was changed every two days, and the cells were further subcultured in an incubator at 37˚C with 5% $CO_2$ whenever confluence reached 80-90%. Cells in the third generation were used for experimentation following examination of cell morphology under an inverted phase-contrast microscope (magnification, x100; Chongqing UOP Photoelectric Technology Co., Ltd.).

*Immunocytochemistry*. Immunocytochemistry was performed to analyze the expression levels of vimentin in fibroblasts isolated from patients with AS and JK. Slides covered with fibroblast cells were washed with PBS three times for 5 min each time. After washing, 4% paraformaldehyde was added to each slide for 15 min at room temperature for fixed slides. Excess amounts of paraformaldehyde was removed, and the slices were then washed again with PBS three times for 5 min each time. Subsequently, 0.03% Triton X-100 was added to the slides for 25 min at room temperature and further incubated with 3% $H_2O_2$ for 10 min at room temperature. After blocking with 2% BSA (cat. no. 11021037; Gibco; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. After washing with PBS three times for 5 min each time, the slides were then incubated with 50 $\mu$l anti-vimentin antibody (1:200; cat. no. ab193555; Abcam) overnight at 4˚C. Following the primary antibody incubation, the slides were washed with PBS three times for 5 min each time, then incubated with 50 $\mu$l goat anti-rabbit IgG H&L (DyLight® 488) preabsorbed secondary antibody (1:100; cat. no. ab96899; Abcam) for 30 min at 37˚C and then washed again with PBS three times. Staining was then visualized using a DAB staining kit (cat. no. E670033; Sangon Biotech Co., Ltd.) for 3-5 min at room temperature, and the slides were examined under an inverted phase-contrast microscope (magnification, x400).

*Cell transfection*. The ANKH-pCMV6-XL5 overexpression plasmid (cat. no. SC120218) and empty vector negative control (NC; cat. no. PCMV6XL5) were purchased from OriGene Technologies, Inc. Fibroblasts from patients with AS were transfected with ANKH overexpression vector or NC. Fibroblasts from patients with JK were transfected with small interfering RNA (si) specific for ANKH (siANKH) or siNC, siANKH (5'-UCACUAUAAGCUAUCAGUGUG-3') and siNC (5'-UUCUCCGAACGUGUCACGU-3') were obtained from Guangzhou RiboBio Co., Ltd.

Fibroblasts were seeded into 24-well culture plates at a density of $2x10^4$ cells/well one day before transfection and cultured to 90% confluence in 0.5 ml medium with 10% FBS without antibiotics. On the day of transfection, 20 pmol siANKH/siNC, or 2 $\mu$g ANKH/NC were separately diluted

in 50 $\mu$l DMEM (cat. no. 12491015; Gibco; Thermo Fisher Scientific, Inc.). A volume of 1 $\mu$l Lipofectamine® 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) was separately diluted in 50 $\mu$l DMEM and incubated at room temperature for 5 min. The diluted siANKH, siNC, ANKH or NC was then mixed with diluted Lipofectamine® 2000 and placed at room temperature for 20 min, then added to the cells. Finally, the cells were incubated at 37˚C with 5% $CO_2$ for 48 h, then harvested for subsequent experimentation. Untransfected cells also served as an additional control group.

*Cell viability assay*. Fibroblasts from patients with AS or JK were seeded into 96-well culture plates at 200 $\mu$l per well, then incubated for 1-6 days. Following the incubation, an MTT assay was used to detect cell viability. Briefly, 20 $\mu$l MTT (5 mg/ml; cat. no. M6494; Invitrogen; Thermo Fisher Scientific, Inc.) was added to the cells for 4 h at 37˚C. After discarding the supernatant, 100 $\mu$l DMSO (cat. no. D12345; Invitrogen; Thermo Fisher Scientific, Inc.) was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm at the initial timepoint, then every day over a time course of 6 days using a microplate reader. In a separate experiment, absorbance was read at 24, 48 and 72 h following transfection; the rest of the experimental details were unchanged.

*Flow cytometric analysis of apoptosis*. Apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) staining kit (cat. no. V13242; Invitrogen; Thermo Fisher Scientific, Inc.). Fibroblasts ($2x10^5$ cells/well) were cultured in 6-well plates for 24 h at 37˚C, then washed with cold PBS and centrifuged at 1,000 x g (5 min, 4˚C). The cells were subsequently resuspended in 1X Annexin binding buffer, 5 $\mu$l Annexin V-FITC and 5 $\mu$l PI was added to the cells. The cells were incubated at room temperature for 15 min, then added to 400 $\mu$l 1X Annexin binding buffer. Cell apoptosis was assessed by flow cytometry using a FACScan instrument (BD Biosciences) equipped with CellQuest software (version 5.1; BD Biosciences). The apoptosis rate was calculated as the sum of early-apoptotic and late-apoptotic ell frequencies. Unstained cells, as well as Annexin V and PI single-stained cells were used as controls, and the gates for positive staining were set according to these controls.

*Reverse transcription-quantitative PCR (RT-qPCR)*. Relative ANKH mRNA expression levels were analyzed using RT-qPCR. Briefly, total RNA was extracted from fibroblasts using TRIzol® reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration was measured using a NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Inc.). In addition, RNA integrity was confirmed by visualization of the fragments in 1.5% agarose gel electrophoresis (Invitrogen; Thermo Fisher Scientific, Inc.) followed by ethidium bromide and visualized under ultraviolet light.

RT into cDNA was performed using a PrimeScript RT Reagent kit with gDNA Eraser (cat. no. RR047A; Clontech Laboratories, Inc.). Briefly, 7 $\mu$l RNA, 1 $\mu$l gDNA Eraser and 2 $\mu$l 5X gDNA Eraser buffer were added into a reaction tube and incubated at 42˚C for 2 min. The 10 $\mu$l sample was then mixed with 1 $\mu$l PrimeScript RT enzyme mix I, 1 $\mu$l RT primer mix, 4 $\mu$l 5X PrimeScript buffer II and 4 $\mu$l RNase-free distilled water. All samples were incubated at 37˚C for 15 min and at 85˚C for 5 sec, then allowed to cool down on ice. The resulting cDNA concentration and OD values were measured on a NanoDrop 8000 spectrophotometer. RT-qPCR was subsequently performed using a 7500 Real-Time PCR system (cat. no. 4351105; Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR® Green PCR Master Mix (4312704, Thermo Fisher Scientific, Inc.). The thermocycling conditions consisted of an initial denaturation at 95˚C for 10 min followed by 40 cycles at 94˚C for 5 sec and 60˚C for 1 min. The experiment was carried out three times. The relative expression levels of the mRNA was analyzed using the $2^{-\Delta\Delta Cq}$ method (29), and ANKH expression was normalized to GAPDH. All primers used in in the present study were purchased from Sangon Biotech Co., Ltd. and the primer sequences are presented in Table I.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

| Gene | Primer sequence (5'→3') |
|---|---|
| Ankylosis progressive homolog | F: GTGGGCCTGGTGTTTGTGAA |
| | R: CCTTCTCGTCTTGCTCCCC |
| GAPDH | F: TGGATTTGGACGCATTGGTC |
| | R: TTTGCACTGGTACGTGTTGAT |

F, forward; R, reverse.

*Alizarin Red staining*. A 0.1% Alizarin Red dye liquor (pH 8.3) was prepared by dissolving 0.1 g Alizarin Red powder (cat. no. 130-22-3; Guidechem) in 100 ml Tris-HCl buffer (0.1 mol/l; cat. no. AP-9005-125; Thermo Fisher Scientific, Inc.). Fibroblasts isolated from the ligament tissue of patients with AS or JK ($2x10^4$ cells/well) were seeded into six-well plates. A cover glass was added to the 6-well plate before the cells were inoculated. When the cells reached ~50% confluence, the samples were collected, washed in PBS, then fixed in 4% methanol for 15 min at room temperature. The cells were then washed again with PBS three times for 3 min each time. Each cover glass was incubated with 0.1% Alizarin Red dye liquor for 30 min at 37˚C, then washed using double steaming water three times for 3 min each time. The slides were examined under an optical microscope (magnification, x400), and Alizarin Red staining absorbance was read at 510 nm for quantification.

*Western blotting*. Proteins were extracted from ligament tissue and fibroblast by using RIPA (cat. no. 89900; Thermo Fisher Scientific, Inc.). Protein concentration in the supernatant was measured using a Pierce™ BCA Protein assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). A total of 20-40 $\mu$g protein lysate was separated by SDS-PAGE on 12% gels (cat. no. P0012A; Beyotime Institute of Biotechnology), then transferred onto PVDF membranes (cat. no. FFP28; Beyotime Institute of

Biotechnology). The membranes were subsequently blocked using 5% non-fat milk in TBS containing 0.1% Tween 20 (cat. no. TA-999-TT; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The membranes were then incubated overnight at 4˚C with the following primary antibodies: Anti-GADPH (cat. no. ab8245; 1:1,000; 36 kDa; Abcam), anti-ANKH (cat. no. ab90104; 1:1,000, 54 kDa; Abcam), anti-alkaline phosphatase (ALP; cat. no. ab83259; 1:1,000, 39 kDa; Abcam), anti-osteocalcin (OCN; cat. no. ab93876; 1:500; 11 kDa; Abcam), anti-Runt-related transcription factor 2 (Runx2; cat. no. ab23981; 1:1,000; 60 kDa; Abcam), anti-phosphorylated (p)-β-catenin (cat. no. 9561; 1:1,000, 92 kDa; Cell Signaling Technology, Inc.), anti-β-catenin (cat. no. 9562; 1:1,000, 92 kDa; Cell Signaling Technology, Inc.) and anti-c-Myc (cat. no. ab32072; 1:1,000; 57 kDa; Abcam). Following the primary antibody incubation, the membranes were incubated with a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (cat. no. ab205718; 1:5,000, 42 kDa; Abcam) at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence reagent kit (cat. no. 345818; EMD Millipore). Protein expression levels were analyzed and normalized to GAPDH using ImageJ software (version 1.5, National Institutes of Health).

*Statistical analysis*. Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc.) and data are presented as the mean ± SEM of three experiments. An unpaired Student's t-test was used to analyze statistical differences between two groups. Multi-group comparisons were performed using one-way ANOVA followed by a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*ANKH expression levels in ligament tissue from patients with AS*. The expression levels of ANKH were investigated in ligament tissues collected from patients with AS and JK controls (n=4/group) using western blot analysis. The results revealed that ANKH expression levels were significantly downregulated in the AS group compared with the JK group (P<0.001; Fig. 1A and B).

*Fibroblast isolation and culture*. Fibroblasts from the AS and JK groups were isolated from the ligament and cultured. The majority of the cells were shuttle-shaped and flat, with 2-3 protuberance, displaying the typical characteristics of fibroblasts (Fig. 1C). Notably, there were no observable differences in the morphology between the two groups. The intermediate filament protein vimentin is an important protein in mammalian fibroblasts (30). Fibroblasts from both groups were used for immunocytochemical staining of vimentin; the expression levels of vimentin in the AS and JK groups were positive (Fig. 1D).

*Cell viability and apoptosis of fibroblasts*. Cell viability and apoptosis were investigated in the fibroblasts from patients with AS and JK controls. The fibroblast viability in the AS group was significantly increased compared with the JK group (P<0.05 at days 1-3; P<0.001 at days 4-6; Fig. 1E). Moreover, the apoptotic rate, representing both early and late apoptosis, was significantly reduced in fibroblasts from patients with AS compared with the JK group (P<0.001; Fig. 1F).

*ANKH overexpression inhibits AS fibroblast viability, mineralization and ossification*. The effect of ANKH on cell viability, mineralization and ossification differentiation in AS was evaluated by transfecting ANKH overexpression vectors into fibroblasts from patients with AS and siANKH into fibroblasts from patients with JK. In the AS group, the relative ANKH mRNA expression levels were significantly upregulated following the transfection with the ANKH overexpression vector compared with the control and NC groups (P<0.001; Fig. 2A). By contrast, the transfection with siANKH in JK-derived fibroblasts significantly downregulated ANKH expression levels compared with the control and siNC group (P<0.001; Fig. 2B).

An MTT assay was performed to determine cell viability in both groups at 24, 48 and 72 h following transfection. Following transfection with the ANKH overexpression vector, the viability of fibroblasts in the AS group was significantly reduced compared with the control and siNC groups at each time point (P<0.05; Fig. 2D). However, the cell viability in the JK group was significantly increased, particularly 48 and 72 h after transfection, compared with the control group and the siNC group at each time point (P<0.05; Fig. 2D).

Moreover, Alizarin Red absorbance in ANKH-overexpressing fibroblasts from the AS group was significantly reduced compared with the control and NC groups (P<0.001; Fig. 2E and F). In comparison, Alizarin Red absorbance in the ANKH-silenced JK group was significantly increased compared with the siNC and control groups (P<0.001; Fig. 2G and H).

The expression levels of ossification markers were analyzed following transfection using western blotting analysis. ALP, OCN and Runx2 expression levels were significantly downregulated following ANKH overexpression in AS fibroblasts compared with the control and NC groups (P<0.001; Fig. 3A and B). By contrast, the expression levels of these ossification markers were significantly upregulated following siANKH silencing in JK fibroblasts compared with the control and siNC groups (P<0.001; Fig. 3C and D).

*ANKH overexpression affects the Wnt/β-catenin signaling pathway in fibroblasts*. The Wnt/β-catenin signaling pathway governs the differentiation of ossification and plays an important role in heterotopic ossification (25,31). The overexpression of ANKH significantly promoted β-catenin phosphorylation, whilst significantly downregulating the expression levels of β-catenin and c-Myc in AS fibroblasts compared with the control and NC groups (P<0.001; Fig. 3E and F). However, siANKH significantly upregulated the expression levels of β-catenin (P<0.05) and c-Myc (P<0.001), while inhibiting β-catenin phosphorylation (P<0.001) compared with the control and siNC groups in JK fibroblasts (Fig. 3G and H). In addition, the p-β-catenin/β-catenin ratios were significantly increased in the ANKH group compared with the untransfected control and NC groups in the AS-derived fibroblasts; however, the p-β-catenin/β-catenin ratio was significantly reduced in the siANKH group compared with the untransfected control and siNC groups in JK fibroblasts (P<0.001; Fig. 3I and J).

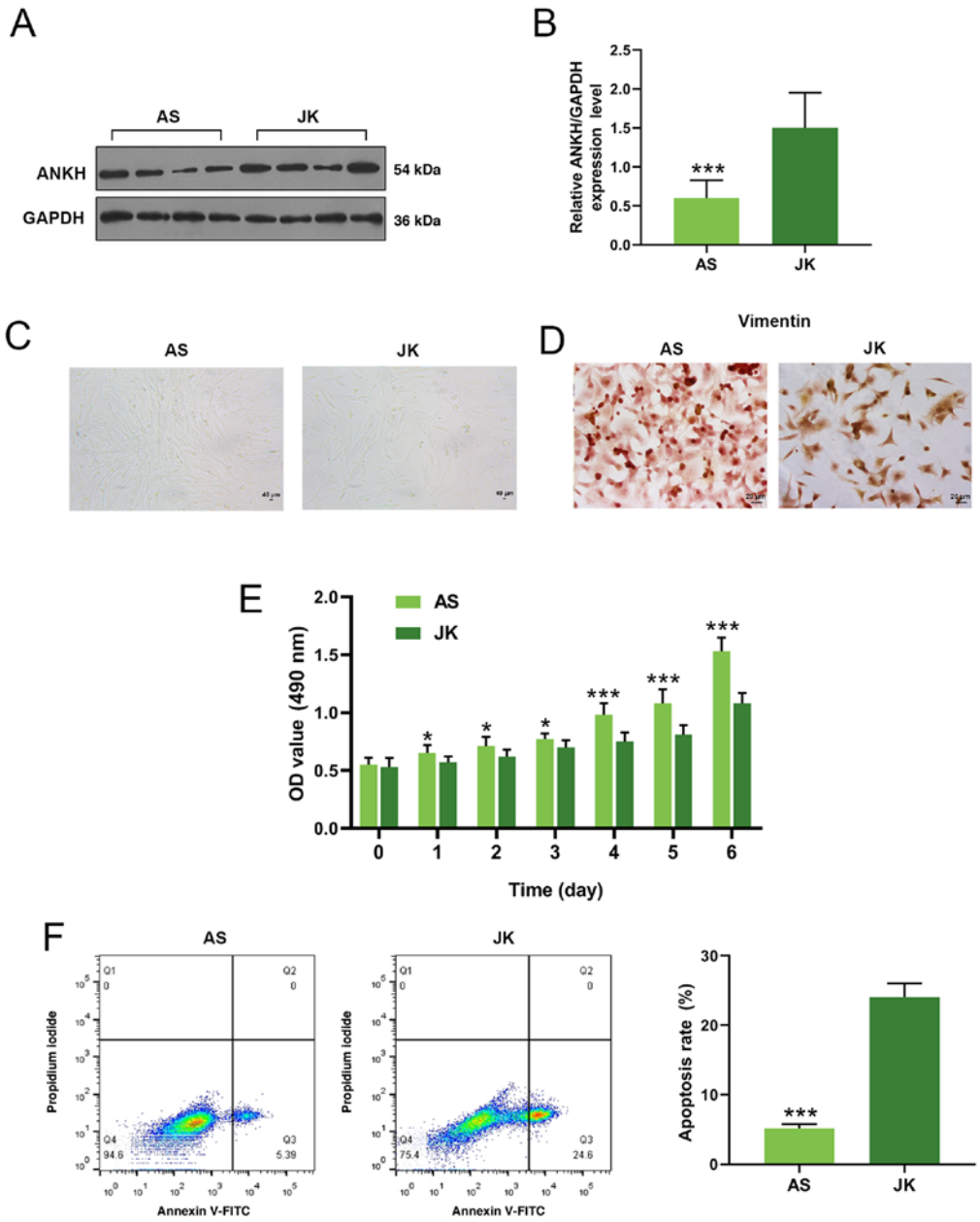


Figure 1. ANKH protein expression levels, cell viability and apoptosis in fibroblasts from patients with AS and JK controls. (A) ANKH protein expression levels in ligament tissue collected from patients with AS and JK controls were analyzed using western blotting. (B) Densitometry data for ANKH expression levels from part (A). Data are presented as the mean ± SEM. (C) Fibroblasts morphology was examined under a light microscope. Magnification, x100. (D) Immunocytochemistry was performed to analyze vimentin expression levels in AS and JK fibroblasts. Magnification, x400. (E) MTT assays were performed to measure cell viability over a time course of six days. (F) Apoptotic rates of AS or JK fibroblasts were analyzed using flow cytometry. Each dot plot represents necrotic cells (Q1), late apoptotic cells (Q2), early apoptotic cells (Q3) and viable cells (Q4). The apoptosis rate (late and early apoptosis) is summarized as a graph on the right-hand side. $^{*}$P<0.05, $^{***}$P<0.001 vs. JK. ANKH, ankylosis progressive homolog; AS, ankylosing spondylitis, JK, spinal fracture; OD, optical density; Q, quadrant.

## Discussion

The aim of the present study was to evaluate the role of ANKH in the ossification and mineralization of fibroblasts in patients with AS. The overexpression of the ANKH protein inhibited the viability and mineralization of fibroblasts in patients with AS. However, ANKH silencing led to the opposite effect, indicating a potential anti-osteogenic role for ANKH in AS.

The ANKH gene encodes a multi-channel transmembrane protein, which has a previously characterized role in genetic susceptibility to AS (32,33). A previous study suggested that ANKH did not significantly affect the susceptibility to or clinical manifestations of AS (32), which contradicted the results of another study (33), in which ANKH was associated with genetic susceptibility to AS in a sex-specific manner (33).

Previous studies have also evaluated the role of ANKH in heterotopic ossification (34,35). For instance, Gurley *et al* (34) demonstrated that the deletion of the ANK gene caused progressive mineralization and joint disease, leading to stiffness of the spine, a symptom similar to AS in humans. Moreover, loss of ANKH function resulted in pathological

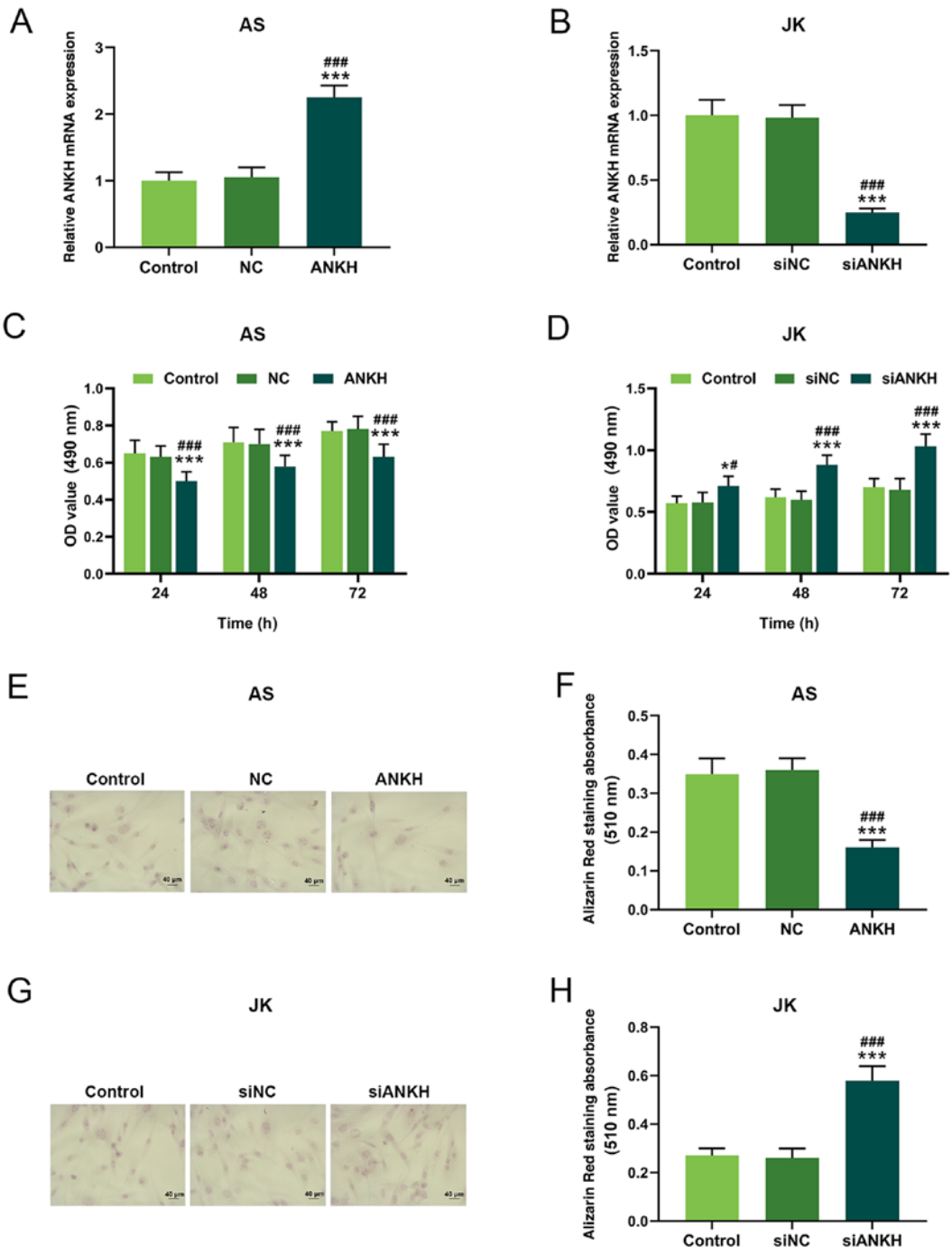


Figure 2. ANKH expression levels affect cell viability and mineralization. ANKH mRNA expression levels in fibroblasts from (A) patients with AS transfected with an ANKH overexpression vector or NC or (B) patients with JK transfected with siANKH or siNC. Cell viability following (C) ANKH overexpression in the AS group and (D) ANKH silencing in the JK group. (E) Mineralization levels were evaluated using Alizarin Red staining in ANKH-overexpressing AS fibroblasts. Magnification, x400. (F) Mineralization levels were semi-quantified in fibroblasts from patients with AS following ANKH overexpression. (G) Mineralization levels were evaluated using Alizarin Red staining following ANKH silencing in JK fibroblasts. Magnification, x400. (H) Mineralization levels were semi-quantified in fibroblasts from patients with JK following siANKH transfection. $^{*}$P<0.05, $^{***}$P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. NC or siNC. ANKH, ankylosis progressive homolog; AS, ankylosing spondylitis, JK, spinal fracture; OD, optical density; si, small interfering RNA; NC, negative control.

hydroxyapatite formation (35). Consistent with these previous studies, the present findings demonstrated that the expression levels of ANKH in ligaments from patients with AS were downregulated compared with the control subjects.

Moreover, the cellular morphology of fibroblasts from patients with AS and control subjects was also examined. Typical fibroblast cellular characteristics and positive vimentin expression confirmed that cultured cells isolated from the ligament retained a fibroblast phenotype. However, fibroblasts from patients with AS displayed increased viability and reduced apoptotic rates compared with the controls, indicating that AS pathogenesis may be related to fibroblast growth. This observation is consistent with a previous study, in which fibroblasts modulated osteoblast metabolism and

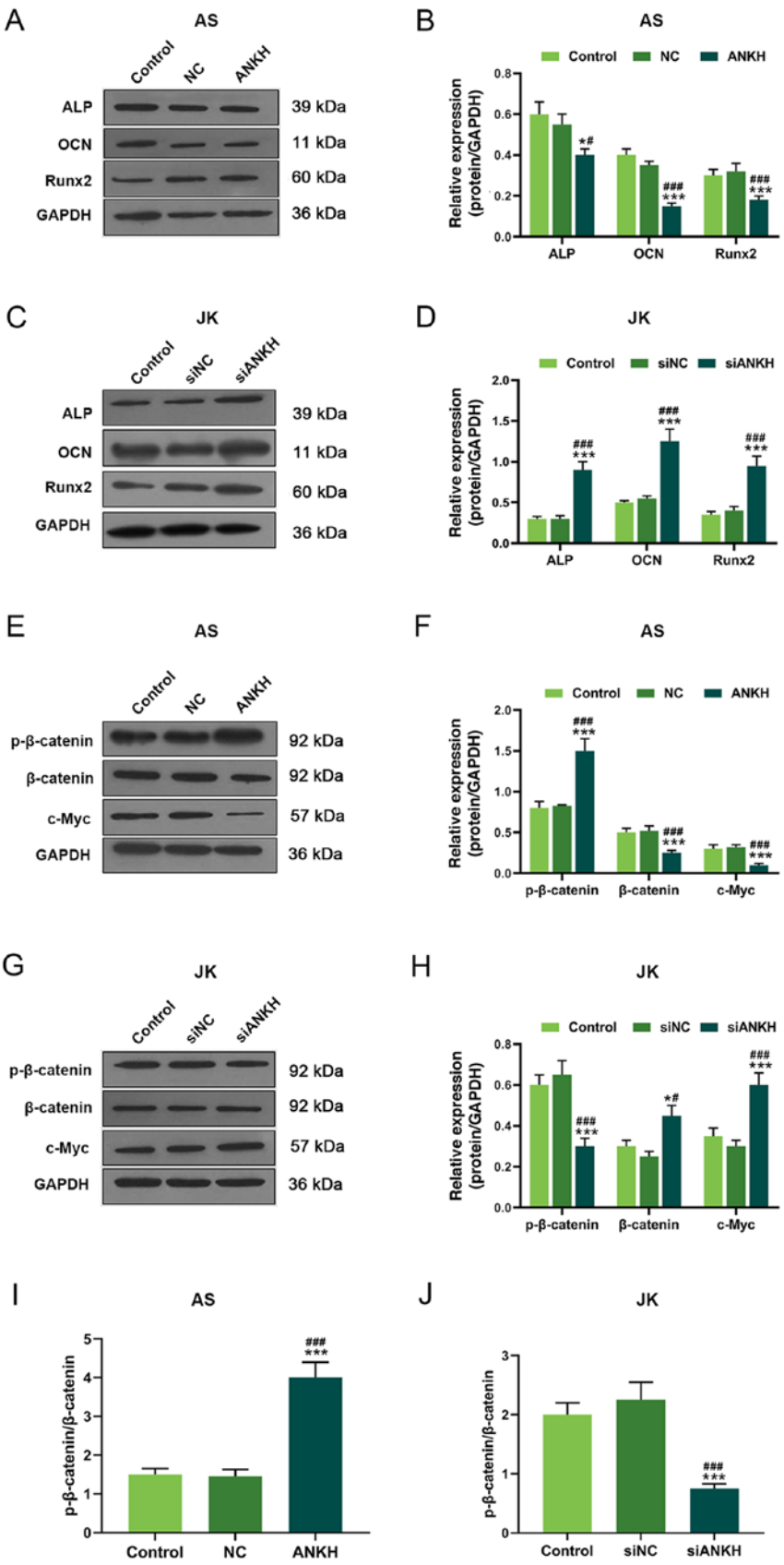


Figure 3. ANKH regulates the expression levels of ossification markers and Wnt signaling-related proteins. (A) Expression levels of ALP, OCN and Runx2 in AS fibroblasts overexpressing ANKH were analyzed using western blotting. (B) Semi-quantification of the expression levels presented in part (A). (C) Expression levels of ALP, OCN and Runx2 in JK fibroblasts transfected with siANKH were analyzed using western blotting. (D) Semi-quantification of the expression levels presented in part (C). Expression levels of p-β-catenin, β-catenin and c-Myc in AS fibroblasts overexpressing ANKH were analyzed using western blotting. (F) Semi-quantification of the expression levels presented in part (E). (G) Expression levels of p-β-catenin, β-catenin and c-Myc in JK fibroblasts transfected with siANKH. (H) Semi-quantification of the expression levels presented in part (G). p-β-catenin: Total β-catenin expression ratios following (I) ANKH overexpression in AS fibroblasts or (J) ANKH silencing in JK fibroblasts. $^{*}$P<0.05, $^{***}$P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. NC or siNC. ANKH, ankylosis progressive homolog; AS, ankylosing spondylitis, JK, spinal fracture; si, small interfering RNA; NC, negative control; p-, phosphorylated; ALP, alkaline phosphatase; OCN, osteocalcin; Runx2, Runt-related transcription factor 2.

osteogenesis (24). Therefore, it was hypothesized that inhibiting fibroblast viability or promoting apoptosis may prevent pathological osteogenesis in patients with AS.

In order to investigate the role of ANKH in fibroblast viability and differentiation, the effects of ANKH overexpression and silencing on fibroblasts were examined. The present findings demonstrated an association between ANKH expression levels and fibroblast viability and mineralization. ANKH overexpression in fibroblasts from patients with AS reduced cell viability and mineralization. Indeed, Alizarin Red staining indicated that the number of mineralized nodules was reduced following ANKH overexpression, suggesting a negative association between ANKH expression levels and fibroblast cell mineralization. Skubutyte *et al* (36) demonstrated that ANK prevented pathological mineralization. Similarly, Ho *et al* (19) revealed that the ANK gene prevented mineralization, whereas ANK deficiency accelerated mineralization in joints. Thus, it may be possible to reduce fibroblast mineralization through ANKH overexpression in order to prevent pathological bone formation.

The expression levels of osteogenic markers, such as ALP, OCN and Runx2, were analyzed to determine the levels of osteogenic differentiation and mineralization in fibroblasts from patients with AS. ALP is a phenotypic marker for early-stage osteoblast differentiation that serves a role in bone mineralization (37). In the present study, ANKH overexpression downregulated ALP expression levels in fibroblasts from patients with AS. This result was consistent with a previous study, which demonstrated that ANK was negatively associated with ALP expression levels in bone marrow stromal cells (38). OCN is a marker unique to osteoblasts and a late marker of osteoblast differentiation (38,39). In the present study, the expression levels of OCN and Runx2 were downregulated in ANKH-overexpressing cells. Runx2 can stimulate the transcription of osteoblast-related genes, such as those encoding OPN and OCN (40). Hill *et al* (41) also demonstrated that ANKH overexpression suppressed the mineralization and ossification of fibroblasts.

Wnt/β-catenin signaling induces mesenchymal stem cell differentiation, a precursor of osteoblastic activity (42). Day *et al* (43) suggested that Runx2 expression levels were possibly regulated by β-catenin upregulation. Conversely, the inhibition of Wnt signaling was illustrated to impair osteogenic differentiation in another previous study (44). β-catenin serves an important role in osteocyte viability, differentiation, proliferation and new bone formation (45,46). In fact, during the early and late stages of fracture repair, β-catenin activation is essential for osteoblast differentiation (45,46), and the loss of β-catenin resulted in increased bone resorption (47). The present findings were consistent with these aforementioned previous studies. Canonical Wnt/β-catenin signaling was reported to stimulate the proliferation and differentiation from fibroblasts to myofibroblast cell (48). In the present study, ANKH silencing reduced p-β-catenin expression levels, thereby offering a possible mechanism through which the cell viability and mineralization of normal fibroblasts were stimulated. However, ANKH overexpression in fibroblasts from patients with AS led to the opposite result. In addition, c-Myc is an important downstream target protein of the Wnt signaling pathway that can regulate proliferation, differentiation and apoptosis (49). Loveridge *et al* (50) reported that downregulated c-Myc protein levels prevented chondrocyte mineralization. In the present study, a negative association was identified between ANKH and c-Myc expression levels. Indeed, the overexpression of ANKH in fibroblasts from patients with AS downregulated the expression levels of c-Myc, resulting in reduced mineralization, whereas ANKH silencing in normal fibroblasts led to c-Myc upregulation. Thus, ANKH overexpression may reduce mineralization and ossification in AS through c-Myc downregulation and increased β-catenin phosphorylation and p-β-catenin/β-catenin, thereby inhibiting pathological bone formation.

However, there were some limitations to the present study. The immunofluorescence identification of JK fibroblasts by vimentin is also required. In addition, the positive effects of ANKH on AS fibroblasts was only determined *in vitro*; thus, the present findings should be further validated *in vivo*. Moreover, the potential use of the ANKH gene in clinical treatment also requires validation. Finally, although this preliminary study suggested that ANKH may regulate the Wnt/β-catenin pathway to inhibit the viability, mineralization and ossification of fibroblasts, the regulatory mechanism underlying this pathway requires further investigation.

In conclusion, the findings of the present study revealed the role of ANKH in fibroblasts isolated from the ligaments of patients with AS. The results discovered that ANKH expression levels were downregulated in ligaments of patients with AS. Moreover, fibroblasts from patients with AS displayed increased cell viability and reduced levels of apoptosis. ANKH overexpression was discovered to inhibit cell mineralization and ossification, which was likely mediated through its effect on ossification markers and the Wnt/β-catenin signaling pathway. Therefore, these results suggested that ANKH overexpression may prevent or delay new bone formation in 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**

XH designed the study. XH and YD acquired, analyzed and interpreted the data, drafted the manuscript and critically revised it for important intellectual content. XH and YD authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity 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 Committees of The People's Hospital of Xinchang (approval no. XC201902114). All procedures involving human participants were in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was provided by all patients.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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