

## Original Article

# MiR-495 targeting dvl-2 represses the inflammatory response of ankylosing spondylitis

Wenxi Du<sup>1\*</sup>, Liming Yin<sup>2\*</sup>, Peijian Tong<sup>1</sup>, Junjie Chen<sup>1</sup>, Ying Zhong<sup>1</sup>, Jiefeng Huang<sup>1</sup>, Shufang Duan<sup>3</sup>

<sup>1</sup>Department of Orthopedics, <sup>2</sup>Institute of Hematology, The First Affiliated Hospital of Zhejiang Chinese Medical University, P. R. China; <sup>3</sup>Department of Endocrinology, The Second Affiliated Hospital of Zhejiang Chinese Medical University, P. R. China. \*Equal contributors.

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**Abstract:** Ankylosing spondylitis (AS) is a type of rheumatic inflammatory disease. miRNAs participate in the process of regulating inflammatory response and bone differentiation. Herein, we aimed to test the effect of miR-495 on AS. The serum and tissues were obtained from traumatic fracture (health) and AS patients. The human fibroblast-like synovial (HFLS) cells were extracted from AS tissues. The contents of inflammatory factors and dishevelled 2 (DVL-2) were examined using enzyme-linked immunosorbent assay (ELISA). The ossification factors were detected by immunohistochemistry assay. Osteoclast was assessed by tartaric acid acid phosphatase (TRAP) assay. The cell viability and luciferase activity were measured using cell counting kit-8 (CCK-8) and dual-luciferase reporter system. The levels of factors were evaluated using quantitative real-time PCR (qRT-PCR) and western blotting. DVL-2 was a target gene for miR-495, according to the MicroRNA.org website and luciferase activity assay. The expressions of miR-495 and DVL-2 were negative correlated in AS. miR-495 and si-DVL-2 did not affect the cell viability. miR-495 and si-DVL-2 obviously inhibited inflammatory response by down-regulating tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 levels, and facilitated bone differentiation by up-regulating osteoprotegerin (OPG) and receptor activator for nuclear factor- $\kappa$ B ligand (RANKL) levels in HFLS cells. Besides, miR-495 and si-DVL-2 increased the expression of wnt3a, runt-related transcription factor 2 (RUNX-2) and  $\beta$ -catenin and reduced the phosphorylation of  $\beta$ -catenin. Collectively, miR-495 depressed inflammatory response and promoted bone differentiation of HFLS cells, and this was accompanied by mediating wnt/ $\beta$ -catenin/Runx-2 pathway by targeting DVL-2.

**Keywords:** MiR-495, ankylosing spondylitis, dishevelled 2, inflammatory response

## Introduction

Ankylosing spondylitis (AS), a chronic and progressive inflammatory disease of bone and soft tissue, mainly causes damage to the spine, multiple joints of the whole body, the adjacent muscle and ligaments. About half of AS patients develop peripheral joint disease, and the incidence of hip involvement is high [1]. In the early stage of AS joint onset, there is an inflammatory change based on synovial disease, followed by bone erosion then new bone formation and osteophyte that could be observed, ultimately leading to whole joint ossification and ankylosis [2, 3]. Therefore, to fully understand the factors affecting AS inflammation and ossification is of great guiding significance in the diagnosis, prognosis and treatment of patients.

miRNAs are a class of non-coding single strand RNA of endogenous chromosomes with a short

sequence of 21~25 nt in length, which was found in various eukaryotic cells and viruses in recent years. They have a high conservatism in evolution [4, 5]. miRNAs can cause target mRNA degradation or inhibit its translation by complementing the specific base of target mRNA, thus regulating the transcriptional expression of genes [6]. Abnormal regulation of miRNAs is linked to all types of diseases, such as cancer, cardiovascular disease, metabolic disease [7-10]. Furthermore, miRNAs participate in the process of regulating inflammatory response and bone differentiation [11, 12]. It has been found that miR-495 suppresses new bone regeneration in mice [13]. However, the effect of miR-495 on inflammatory response and bone differentiation of AS is still not clear.

The classic wnt/ $\beta$ -catenin signaling pathway is the binding of wnt ligand to its specific receptor

(Frizzled (FZD) and LDL receptor-related protein 5/6 (LRP5/6)), which transmits the signal to the cytoplasm. Subsequently, dishevelled (DVL) reduces the phosphorylation of  $\beta$ -catenin by inhibiting the function of the protein complex GSK-3 $\beta$ /Axin/APC, thereby the continuous increase of  $\beta$ -catenin in the cytoplasm is transferred to the nucleus, and  $\beta$ -catenin combines with T cytokines/lymphokine enhancing factor (TCL/LEF) to form the transcriptional agonist. Finally, the transcription of downstream target genes of wnt/ $\beta$ -catenin signaling pathway is initiated [14-16]. The wnt/ $\beta$ -catenin signaling pathway plays an important role in regulating the differentiation of stem cells and adjusting bone formation [17]. The activation of wnt signaling pathway can promote the formation of bone sclerosis and osteophyte. Therefore, wnt signaling pathway is considered to be one of the key ways to regulate the ectopic osteogenesis of AS [18, 19].

In the present study, the expression of miR-495 in traumatic fracture (health) and AS patients was measured. The target gene of miR-495 was identified according to the microRNA.org website. The effects and mechanism of miR-495 on the inflammatory response and bone differentiation of AS were explored.

### Materials and methods

#### *The source of blood and tissue*

From Oct.2015 to Apr.2017, a total of 34 patients, including 16 cases of traumatic fracture (health) and 18 cases of AS, were recruited from The First Affiliated Hospital of Zhejiang Chinese Medical University. The serum and tissues were obtained from 34 patients. All subjects in this study voluntarily participated in the project, and signed informed consent. The experiment was approved by the Ethics Committee of the hospital.

#### *Cell extraction and culture*

Human fibroblast-like synovial (HFLS) cells were extracted from AS tissues. In brief, tissues were washed using PBS for 3 times, and shredded by aseptic scissors. Tissues were digested by 2.0 g/L collagenase II (Gibco, USA) at 37°C for 1 hour (h). After digesting, tissues were centrifuged at 1000 g for 20 min. The supernatant was removed. High-glucose Dulbe-

cco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA) containing 50% fetal bovine serum (FBS; Gibco) was added into the precipitation, which was repeatedly blown through a straw. Next, the suspension was placed in a 50 mL culture bottle and incubated at 37°C with 5% CO<sub>2</sub> in an incubator (BPN-40RHP; Yiheng, Shanghai, China). After 24 hours of hatching, cells gradually formed from tissue blocks, and were then slowly added into DMEM containing 10% FBS to continue culture. The cells were passed to the third generation to carry out the later experiment.

#### *Cell transfection*

miR-495 mimics, miR-495 inhibitor, miRNA negative control, DVL-2 siRNA and siRNA negative control (20 nM) were obtained from the United States Dharmacon Company. The vectors were transfected into HFLS cells reached 50-60% confluence by Lipofectamine 2000 (Sxbio, Shanghai, China) for 24 h.

The DVL-2 3'UTR and DVL-2 3'UTR mutant were inserted to the firefly luciferase pMIR-REPORT™ vector (Hibio, Hangzhou, China). HEK-293T cells were transfected with the firefly luciferase plasmid (500 ng) and miR-495 mimics/control mimics by Lipofectamine 2000 for 24 h. Besides, 50 ng pRL-TK (encoding Renilla luciferase) (Promega, Madison, WI, USA) was co-transfected into the HEK293T cells for 48 h.

#### *Luciferase activity assay*

Luciferase activity assay was performed as previously described [20]. Briefly, after HEK293T cells transfection, the dual-luciferase reporter system (Promega) was used to evaluate the luciferase activity.

#### *Enzyme linked immunosorbent assay (ELISA)*

The content of the corresponding inflammatory factors was detected using ELISA kit (Mibio, Shanghai, China). Briefly, the tested samples and standard products were added to the enzyme labeled plate at 37°C for 1 h. The solution was removed, and the enzyme labeled plate was washed by PBS for 3 times. Chromogenic antibody was dripped into each well and incubated at 37°C for 1 h. The plate was washed using washing liquid for 5 times. Enzyme labeled solution was added into the

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**Table 1.** The sequences of primers

Primer name	Sequence (5'-3')	Product size (bp)
miR-495-Forward	AAACAACATGGTGCA	
miR-495-Reverse	GAGCAGGCTGGAGAA	218
DVL-2-Forward	CATCCAGCCAATTGACCCTG	
DVL-2-Reverse	AGGGATGGTGATCTTGAGCC	241
OPG-Forward	AGTGTCTTTGGTCTCCTGCT	
OPG-Reverse	TCTGCGTTTACTTTGGTGCC	206
RANKL-Forward	ACTACACCAAGTACCTGCGT	
RANKL-Reverse	TAGGATCCATCTGCGCTCTG	210
Wnt3a-Forward	ATCGAGTTTGGTGGGATGGT	
Wnt3a-Reverse	CGCTGTCGTACTTGTCTTGG	238
Runx-2-Forward	TCCTCCCAAGTAGCTACCT	
Runx-2-Reverse	AGGACTTGGTGCAGAGTTCA	211
$\beta$ -actin-Forward	GGGAAATCGTGCCTGACATT	
$\beta$ -actin-Reverse	AGGTAGTTTCGTGGATGCCA	219

each well at 37°C for 15 min. The reaction was terminated using terminating solution. The OD value at 450 nm was read by the SMR16.1 multimode reader (Uscn Kit Inc. Wuhan, Hubei, China).

### Immunohistochemistry assay

The tissue slices were washed by distilled water for 3 times. The slices were soaked in the citrate buffer (pH=6.0) at room temperature for 30 min. The hydrogen peroxide reagent is dripped to the slices at room temperature for 10 min. The slices were sealed using goat serum at room temperature for 20 min. The corresponding primary antibodies (anti- $\beta$ -catenin, cat.no.ab6302, dilution: 1:800; anti-osteoprotegerin (OPG), cat.no.ab73400, dilution:1:1000; anti-receptor activator for nuclear factor- $\kappa$ B ligand (RANKL), cat.no.ab9957, dilution:1:600; Abcam, Shanghai, China) were added to the slices at 4°C for 24 h. Next day, the secondary antibodies (Goat anti-mouse IgG H&L, cat.no.ab6708, dilution:1:6000; Rabbit anti-mouse IgG H&L, cat.no.ab6728, dilution:1:7000; Abcam) were dripped onto the slices at room temperature for 20 min. After reaction, the slices were stained by DAB staining solution (Leica, Shanghai, China) at room temperature for 15 min. Next, the slices were cleaning by distilled water for 3 times. The slices were dyed using hematoxylin (Solarbio, Beijing, China). The slices were observed under a fluorescence microscope (MF43; Mshot, Guangzhou, Guangdong, China).

### Tartaric acid phosphatase (TRAP) assay

TRAP staining (Sigma-aldrich) was used to assess the osteoclast in AS tissues. The tissue slices were fixed by 4% polyoxymethylene at 37°C for 25 min, and washed by PBS for 3 times. After washing, the slices were stained with the staining solution that contained fast garnet GBC base solution, sodium Nitrite solution, naphthol AS-BI phosphate solution, acetate and tartrate in the darkness at 37°C for 1 h. The slices were cleaned by purified water for 3 times. The slices were observed under a fluorescence microscope.

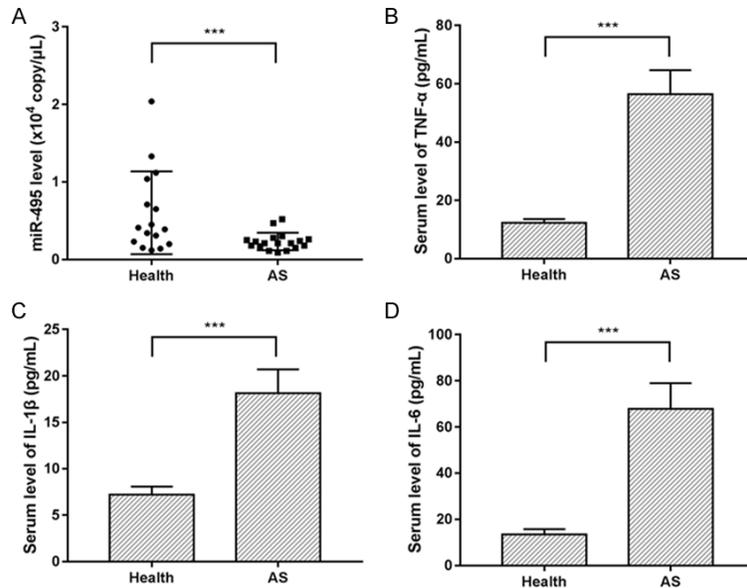
### Cell counting kit-8 (CCK-8) assay

CCK-8 (MSK, Wuhan, China) was used to determine the viability of HFLS cells. In brief, the cells were seeded in 96-well plates ( $2.5 \times 10^3$  cells/well) for 24 h. Some cells were treated with PBS (control), miRNA negative control (NC), miR-495 mimics (mimics) and miR-495 inhibitor (inhibitor), while the others were subjected to PBS (control), siRNA negative control (NC) and DVL-2 siRNA (si-DVL-2) for 12, 24 and 48 h, respectively. Next, the cells were treated with CCK-8 reagent and transferred onto the incubator for 4 h. The OD value at 450 nm was assessed by the SMR16.1 multimode reader.

### Quantitative real-time PCR (qRT-PCR)

Total RNA was obtained by TRIzol Reagent (Takara, Dalian, China). 1  $\mu$ g of RNA was used to synthesize cDNA using cDNA synthesis kit (Promega). Reaction reagents of reverse transcription were as follows: RNA 1  $\mu$ g, primer Oligod T 1  $\mu$ L, Random primers 1  $\mu$ L, nuclease-free water to a final volume of 5  $\mu$ L. The reaction was set at 70°C for 4 min. cDNA was amplified by SYBR Premix Taq<sup>TM</sup> II kit (Takara). Reaction reagents of qPCR contained 25  $\mu$ L SYBR Green Mix, 19  $\mu$ L distilled water, 4  $\mu$ L cDNA and 1  $\mu$ L forward/reverse primer. The sequences of primers were listed in **Table 1**. The reaction was set as follows: predegeneration: at 92°C for 5 min, (denaturation: at 92°C for 20 s; annealing: at 59°C for 40 s) for 30 cycles, extending: at 68°C for 30 s.  $\beta$ -actin was expressed as internal control. The formula  $2^{-\Delta\Delta CT}$  was performed to compare gene expression.

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**Figure 1.** The expression levels of miR-495 and inflammatory factors in traumatic fracture (health) patients and Ankylosing Spondylitis (AS) patients. The tissues and serum were obtained from 34 patients, including 16 cases of traumatic fracture (health) and 18 cases of AS. A. The mRNA level of miR-495 was detected by RT-qPCR in the tissues of health patients and AS patients. miR-495 was low expressed in AS patients. B-D. The contents of inflammatory factors (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) were measured using enzyme linked immunosorbent assay (ELISA). The inflammatory response appeared in AS patients \*\*\* $P < 0.001$ , versus health.

### Western blotting

Total proteins were obtained by high RIPA lysis buffer (Leagene, Beijing, China). Proteins were separated from sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and bound to a PVDF membrane (Kaihong; Shenzhen, Guangdong China). TBST buffer containing 5% skimmed milk was used to seal membrane at 37°C for 1 h, and the membrane was incubated with the corresponding primary antibodies (anti-DVL-2, cat.no.ab22616, dilution:1:1000; anti-OPG, cat.no.ab73400, dilution:1:1000; anti-RANKL, cat.no.ab9957, dilution:1:600; anti-Wnt3a, cat.no.ab28472, dilution:1:600; anti-runt-related transcription factor 2 (RUNX-2), cat.no.ab76956, dilution:1:1000; anti- $\beta$ -catenin, cat.no.ab6302, dilution:1:800; anti-phosphorylated- $\beta$ -catenin (p- $\beta$ -catenin), cat.no.ab27798, dilution:1:1000; anti- $\beta$ -actin, cat.no.ab8226, dilution:1:2000; Abcam) at 4°C for 24 h. After being incubated with the primary antibodies, the membrane was incubated with the secondary antibodies (Goat anti-mouse IgG H&L, cat.no.ab6708, dilution:1:6000; Rabbit

anti-mouse IgG H&L, cat.no.ab6728, dilution:1:7000; Abcam) at 37°C for 60 min. The blots were detected using ECL chromogenic solution (Biodragon, Beijing, China).

### Statistical analysis

SPSS.20 software was performed to analyze the data. The correlation coefficient was calculated to measure the strength of the association between the miR-495 and DVL-2 data using a linear fit. The experimental data were presented as mean  $\pm$  SD. One-way ANOVA and the post hoc test (LSD) were carried out to analyze the differences between groups. The experiment was performed for 3 times for each sample. The value of  $P < 0.05$  was presented as a significant difference.

## Results

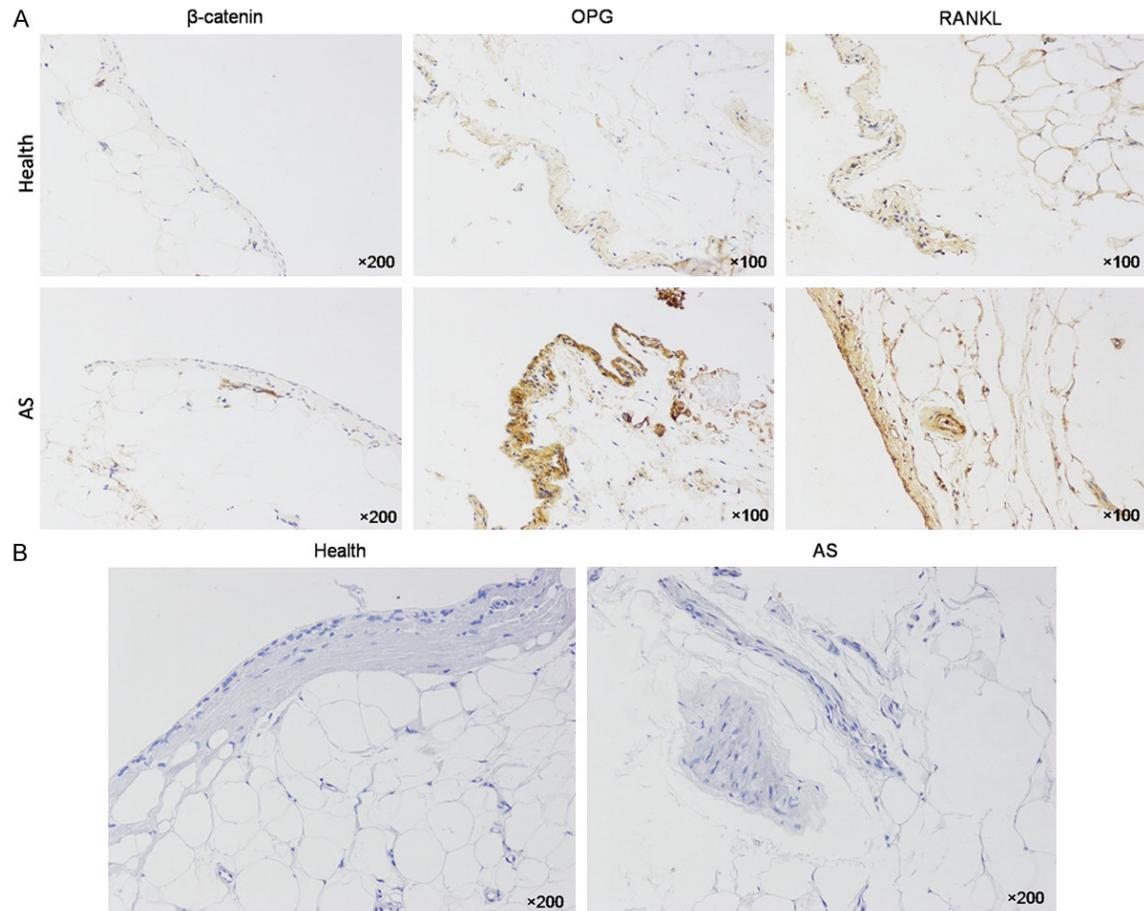
### miR-495 was low expressed and inflammatory response appeared in AS patients

In order to assess the expression of miR-495 and the contents of inflammatory factors in AS patients, qRT-PCR and ELISA analyses were performed in the study. As qRT-PCR shown, the level of miR-495 in AS patients was lower than that in health patients (**Figure 1A**,  $P < 0.001$ ). The ELISA results showed that the contents of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 in AS patients were higher than those in health patients (**Figure 1B-E**,  $P < 0.001$ ).

### Bone differentiation in AS patients

To analyze the ossification of AS patients, osteoblast-related factors and osteoclasts were respectively detected using immunohistochemistry and TRAP assays. The immunohistochemistry revealed that the AS group had an obvious brown staining, compared to health group. The results indicated strong positive expressions of  $\beta$ -catenin, OPG and RANKL in AS group (**Figure 2A**). The TRAP staining observed

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**Figure 2.** Bone differentiation in Ankylosing Spondylitis (AS) patients. A. The expression levels of  $\beta$ -catenin ( $\times 200$ ), osteoprotegerin (OPG) ( $\times 100$ ) and receptor activator for nuclear factor- $\kappa$ B ligand (RANKL) ( $\times 100$ ) were determined in the tissues of traumatic fracture (health) patients and AS patients using immunohistochemistry assay. B. The osteoclasts were detected in the tissues of health patients and AS patients using tartaric acid acid phosphatase (TRAP) assays ( $\times 200$ ).

a small cell dyed red in the AS group (**Figure 2B**), meaning that the expressions of positive cells (osteoclasts) in AS group was higher than those in health group.

*MiR-495 had no effect on the viability of HFLS cells and inhibited inflammatory response*

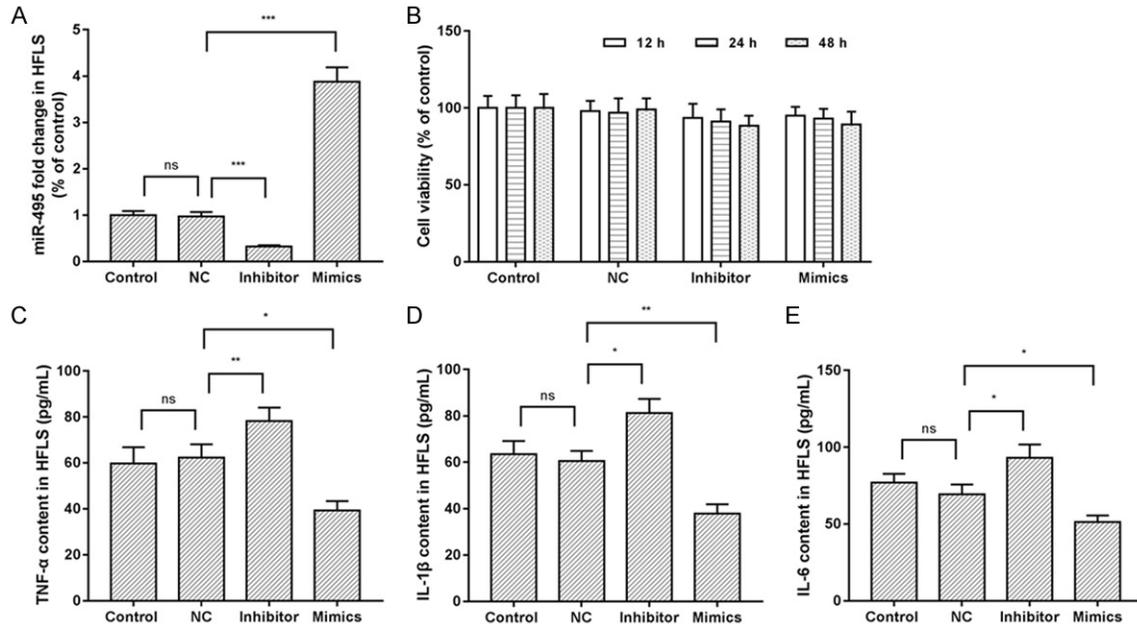
In order to explore the transfection efficiency and effect of miR-495 on HFLS cells, qRT-PCR, CCK-8 and ELISA were performed. As qRT-PCR observed, the mRNA levels of miR-495 was high in mimics group but low in inhibitor group, compared to NC group (**Figure 3A**,  $P < 0.001$ ). The cell viability remained stable when the cells were transfected with miR-495 mimics and miR-495 inhibitor (**Figure 3B**). The ELISA data found that the contents of TNF- $\alpha$ , IL-1 $\beta$  and IL-6

were significantly reduced in mimics group but markedly increased in inhibitor group (**Figure 3C-E**,  $P < 0.05$ ).

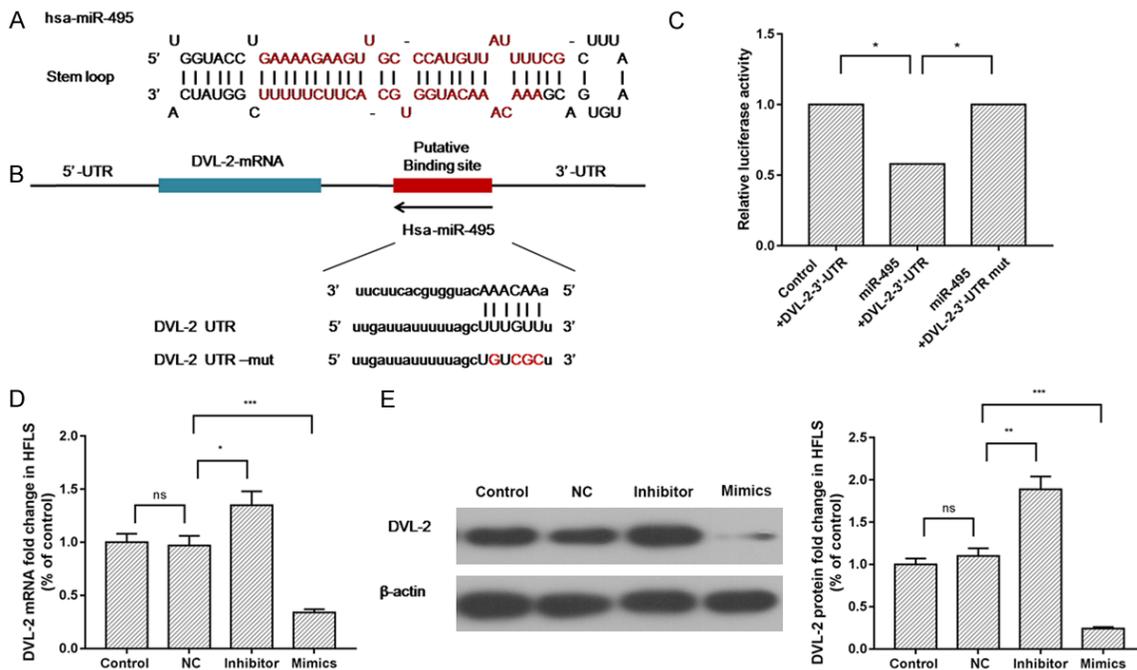
*DVL-2 was a target gene of miR-495 and miR-495 repressed DVL-2 expression*

microRNA.org website was used to predict miR-495 target genes. The data showed that the binding site of DVL-2 and miR-495 was obtained (**Figure 4A, 4B**). The luciferase reporter results showed that the luciferase activity was obviously repressed in miR-495+DVL-2-3'UTR, while no change in miR-495+DVL-2-3'UTR mut (**Figure 4C**,  $P < 0.05$ ) was identified. As qRT-PCR and western blotting shown, miR-495 mimics remarkably decreased DVL-2 expression, however, miR-495 inhibitor increased DVL-2 expres-

## The roles of miR-495 in AS

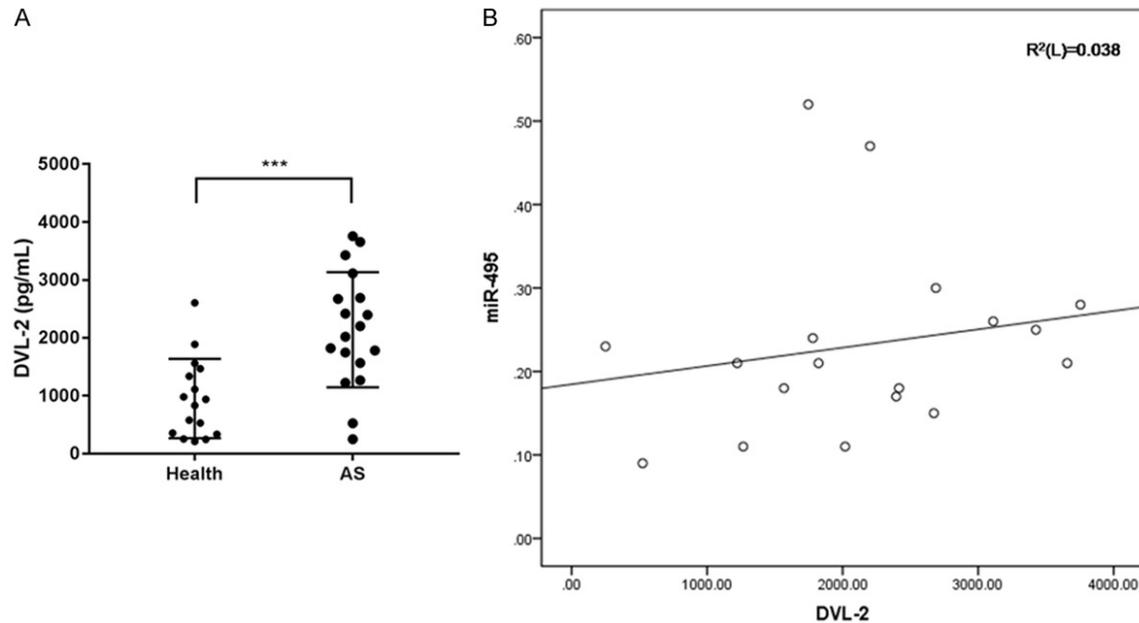


**Figure 3.** Effect of miR-495 on the cell viability and inflammatory response. Human fibroblast like synovial (HFLS) cells were subjected to PBS (control), miRNA negative control (NC), miR-495 mimics (mimics) and miR-495 inhibitor (inhibitor). A. QRT-PCR was performed to assess the mRNA level of miR-495. miR-495 mimics significantly increased the miR-495 expression, while miR-495 inhibitors had an opposite effect. B. The cell viability was measured using CCK-8. miR-495 had no effect on the viability of HFLS cells. C-E. The contents of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were detected by enzyme linked immunosorbent assay (ELISA) and miR-495 mimic reduced the TNF- $\alpha$ , IL-1 $\beta$  and IL-6 contents in HFLS cells. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, versus NC.



**Figure 4.** DVL-2 was a target gene of miR-495 and miR-495 repressed DVL-2 expression Human fibroblast like synovial (HFLS). A, B. The target gene of miR-495 was predicted according to the microRNA.org website. C. The luciferase activity was tested by dual-luciferase reporter system and the luciferase activity was obviously repressed in miR-495+DVL-2-3'UTR. D, E. The levels of DVL-2 were examined by qRT-PCR and western blotting. miR-495 repressed the mRNA and protein levels of DVL-2 in HFLS.  $\beta$ -actin was expressed as internal control. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, versus NC.

## The roles of miR-495 in AS



**Figure 5.** The expression level of miR-495 in traumatic fracture (health) patients and Ankylosing Spondylitis (AS) patients and the correlation between miR-495 and DVL-2. A. The content of DVL-2 in AS patient was examined by enzyme linked immunosorbent assay (ELISA). DVL-2 expression in AS group was higher than that of health group. B. The correlation between the miR-495 and DVL-2 level was analyzed. The expression level of miR-495 in AS patients was negatively correlated with DVL-2.

sion (Figure 4D, 4E,  $P < 0.05$ ). Additionally, as ELISA data shown, DVL-2 expression in AS group was higher than that in health group. The expression level of miR-495 in AS patients was negatively correlated with DVL-2 (Figure 5).

*Silence of DVL-2 had no effect on the viability of HFLS cells and inhibited inflammatory response*

qRT-PCR, CCK-8 and ELISA were carried out in order to explore the transfection efficiency and the effect of DVL-2 on HFLS cells. As qRT-PCR and western blotting revealed, the mRNA and protein levels of DVL-2 were reduced in cells transfected with DVL-2 siRNA, compared to NC group (Figure 6A, 6B,  $P < 0.001$ ). The cell viability remained stable when cells were transfected with DVL-2 siRNA (Figure 6C). The ELISA data showed that the contents of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly reduced in si-DVL-2 group (Figure 6D-F,  $P < 0.05$ ).

*MiR-495 and DVL-2 silencing enhanced the expression of OPG and RANKL*

To analyze the role of miR-495 mimics and si-DVL-2 in the ossification of HFLS cells, the osteoblast related factor (OPG) and osteoclast

related factor (RANKL) were detected using qRT-PCR and western blotting. The qRT-PCR data observed that in comparison with mimics NC and si NC, the mRNA levels of OPG and RANKL were up-regulated in mimics and si-DVL-2 groups (Figure 7A, 7B,  $P < 0.05$ ). The expression trend of protein was the same as that of mRNA (Figure 7C,  $P < 0.05$ ).

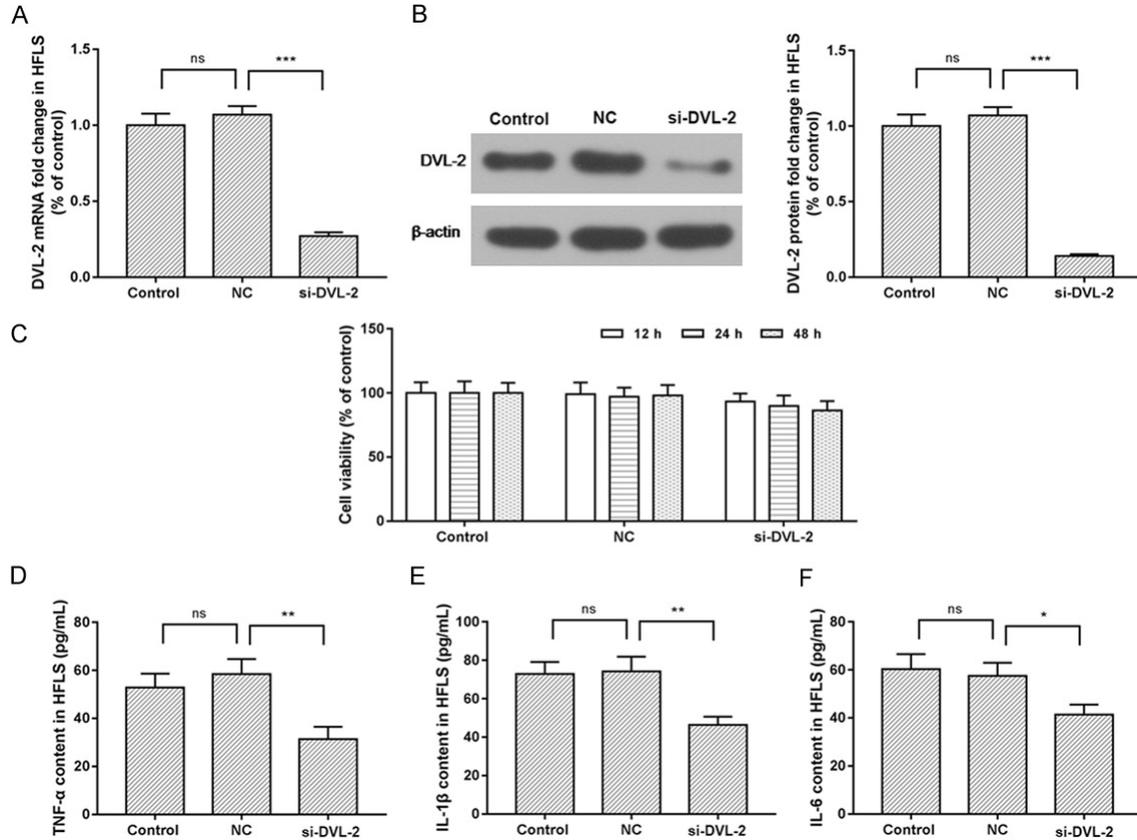
*MiR-495 and DVL-2 silencing regulated the wnt/ $\beta$ -catenin/Runx-2 pathway*

To study the effect of miR-495 mimics and si-DVL-2 on wnt/ $\beta$ -catenin/Runx-2 pathway, related factors were detected using qRT-PCR and western blotting. The qRT-PCR data revealed that miR-495 and si-DVL-2 markedly up-regulated the mRNA expression of wnt3a and Runx-2 (Figure 8A, 8B,  $P < 0.05$ ). Meanwhile, though miR-495 and si-DVL-2 up-regulated the proteins expressions of wnt3a, Runx-2 and  $\beta$ -catenin, they down-regulated the phosphorylation of  $\beta$ -catenin (Figure 8C,  $P < 0.05$ ).

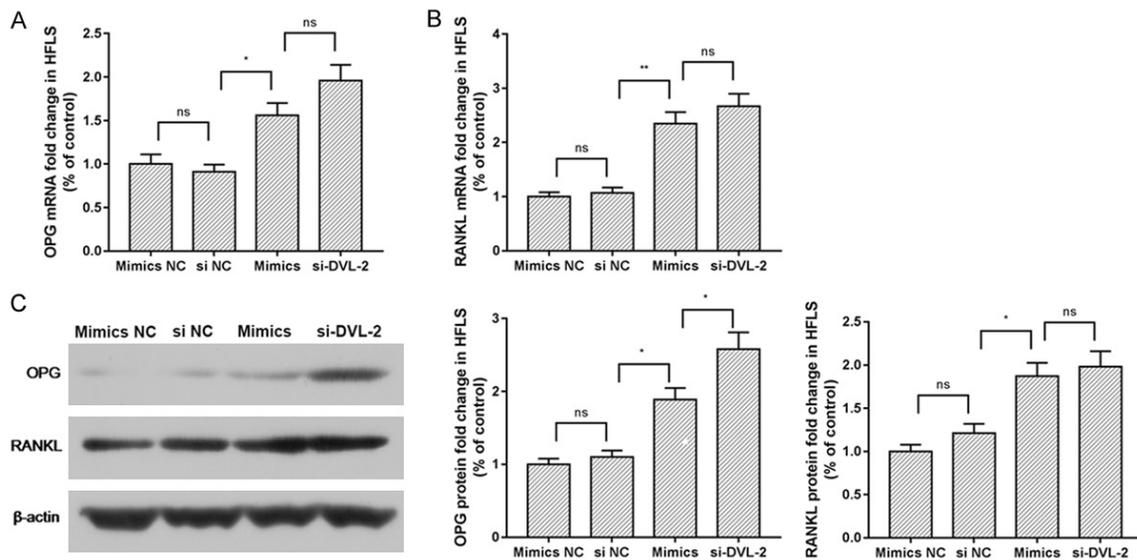
### Discussion

In this study, we found that miR-495 was highly expressed in AS. The phenomena of inflammation and bone differentiation were clearly pre-

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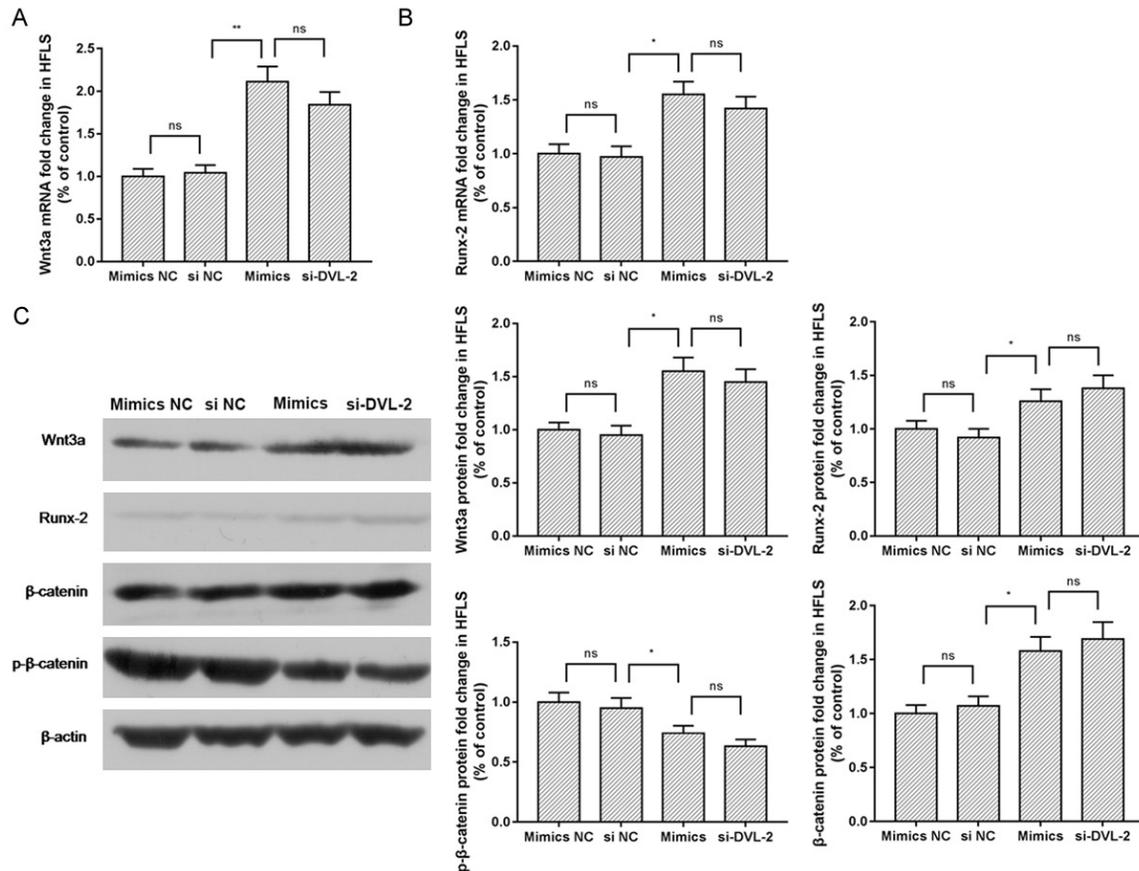


**Figure 6.** Effect of DVL-2 on the cell viability and inflammatory response. Human fibroblast like synovial (HFLS) cells were treated with PBS (control), siRNA negative control (NC) and DVL-2 siRNA (si-DVL-2). The mRNA and protein levels of DVL-2 were assessed using qRT-PCR (A) and western blotting (B). The mRNA and protein levels of DVL-2 were reduced in cells transfected with DVL-2 siRNA. (C) CCK-8 was carried out to test the cell viability. DVL-2 silencing had no effect on the viability of HFLS cells. (D-F) The contents of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were determined by enzyme linked immunosorbent assay (ELISA). DVL-2 silencing inhibited inflammatory response. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , versus NC.



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**Figure 7.** Effect of miR-495 and DVL-2 silencing on the expressions of OPG and RANKL. Human fibroblast like synovial (HFLS) cells were administrated with miRNA negative control (mimics NC), miR-495 mimics (mimics), siRNA negative control (si NC) and DVL-2 siRNA (si-DVL-2). The mRNA levels of OPG (A) and RANKL (B) were measured using qRT-PCR. The mRNA levels of OPG and RANKL were up-regulated in miR-495 mimics and si-DVL-2 groups. (C) Protein expressions of OPG and RANKL were examined by western blotting. The protein levels of OPG and RANKL were up-regulated in mimics and si-DVL-2 groups. \* $P < 0.05$ , \*\* $P < 0.01$ , versus mimics NC and si NC.



**Figure 8.** Effect of miR-495 mimics and si-DVL-2 on wnt/ $\beta$ -catenin/Runx-2 pathway. A, B. The mRNA levels of wnt3a and Runx-2 were detected using qRT-PCR. miR-495 and si-DVL-2 markedly up-regulated the mRNA expression of wnt3a and Runx-2. C. Western blotting was carried out to test the protein levels of wnt3a, Runx-2,  $\beta$ -catenin and p- $\beta$ -catenin. miR-495 and si-DVL-2 up-regulated the proteins expression of wnt3a, Runx-2, and  $\beta$ -catenin, whereas down-regulated the phosphorylation of  $\beta$ -catenin. \* $P < 0.05$ , \*\* $P < 0.01$ , versus mimics NC and si NC.

sented in AS. DVL-2 was identified as the target gene for miR-495. The levels of miR-495 and DVL-2 were a negative correlation in AS. Though miR-495 and si-DVL-2 did not affect the viability of HFLS cells, they inhibited the inflammatory response, increase bone differentiation in HFLS cells and regulated the expressions of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, OPG and RANKL. Moreover, miR-495 and si-DVL-2 enhanced the levels of wnt3a, Runx-2 and  $\beta$ -catenin and reduced the phosphorylation of  $\beta$ -catenin.

AS is a type of rheumatic inflammatory disease, and AS patients usually present inflammatory

reaction, which causes bone differentiation [18, 21, 22]. In this study, we detected the inflammatory factors (TNF- $\alpha$ , IL-1 $\beta$  and IL-6), bone differentiation factors (OPG and RANKL) and osteoclast in traumatic fracture (health) and AS patients. Consistent with previous theories, our results showed obvious inflammatory reaction and bone differentiation in AS patients. Researchers have found that fibroblasts were the generation cells of AS ligament ossification, and that fibroblasts in AS joint capsule were also in an excessive proliferation state [23-25]. In the study of AS sacral coxa, it has been illustrated that the destruction of bone and carti-

lage was closely related to the proliferation of fibroblasts [26]. With the change of fibroblast proliferation degree, the local inflammation gradually shifted from invasion and destruction to new bone formation [27]. Hence, we extracted fibroblast like synovial cells from AS tissue as the research object in the later stage.

It has been proved that miRNAs were involved in AS development [28]. Mounting evidence has proved that some miRNAs, for example, miR-126-3p, miR-29a, miR-130, were low expression the AS patients [28-30]. In this study, our data showed that miR-495 was markedly and lowly expressed in AS patients. Furthermore, Wang Y had confirmed that miR-199a-5p depresses the inflammatory response of AS by down-regulating TNF- $\alpha$ , IL-17 and IL-23 [31]. Li C have indicated that miR-29a inhibited the expression of TNF- $\alpha$  [32]. We speculated that miR-495 might repress the inflammatory response of AS by regulating inflammatory factors. As expected, we observed that miR-495 decreased the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in HFLS cells, while miR-495 inhibitor increased the expression of the inflammatory factors. This result suggested that miR-495 suppressed the inflammatory response of AS by downregulating TNF- $\alpha$ , IL-1 $\beta$  and IL-6.

Subsequently, DVL-2 was identified as the target of miR-495 and highly expressed in AS. Our results found that the expression of miR-495 was negative correlated with DVL-2 in AS. Several groups have reported that miRNAs took part in the regulation of bone differentiation, metabolism and formation [33-36]. Huang X had validated that miR-29c-3p promotes the osteoblasts differentiation through targeting DVL-2 in BMSCs [37]. Thus, we speculated that miR-495 and DVL-2 played a certain role in the ossification of AS. In the process of AS peripheral joint ossification, the coupling imbalance between osteoblasts and osteoclasts is a condition that cannot be neglected in ossification [38]. RANKL/RANK/OPG system, as an important signal transduction pathway for regulating osteoblast and osteoclast differentiation, is undoubtedly a link between bone formation and bone absorption [39, 40]. In the study, our data showed that miR-495 and si-DVL-2 increased the expressions of OPG and RANKL in HFLS cells. This phenomenon explained that miR-495 and si-DVL-2 expedited the bone dif-

ferentiation of AS by up-regulating OPG and RANKL levels.

DVL-2 is a key regulatory factor in the wnt/ $\beta$ -catenin pathway [41, 42]. miRNAs are involved in the regulation of wnt/ $\beta$ -catenin pathway in a variety of diseases [43-45]. Hence, we speculated that miR-495 and DVL-2 might regulate the wnt/ $\beta$ -catenin pathway in AS. As expected, miR-495 and si-DVL-2 up-regulated the expressions of wnt3a, Runx-2 and  $\beta$ -catenin, and down-regulated p- $\beta$ -catenin level in HFLS cells. The result confirmed that miR-495 and DVL-2 silencing mediated the wnt/ $\beta$ -catenin/Runx-2 pathway in AS.

In our study, there were some limitations, for instance, lacking of more histology and IHC assays, or validation experiments of wnt/ $\beta$ -catenin/Runx-2 pathway. Thus, an in-depth study is worth performing on the pathological mechanism of miR-495/DVL-2 related to AS.

In conclusion, DVL-2 was identified as the target gene for miR-495. The expressions of miR-495 and DVL-2 were negatively correlated in AS. miR-495 and the knockdown of DVL-2 repressed inflammatory response by down-regulating TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels and accelerated bone differentiation of HFLS cells by up-regulating OPG and RANKL levels and mediating the wnt/ $\beta$ -catenin/Runx-2 pathway.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Shufang Duan, Department of Endocrinology, The Second Affiliated Hospital of Zhejiang Chinese Medical University, No. 318 Chaowang Road, Hangzhou 310005, Zhejiang, P. R. China. Tel: 86-571-85267000; E-mail: shufangd\_duan@163.com

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