

MiR-124 regulates osteoblast differentiation through GSK-3 β in ankylosing spondylitis

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Abstract. – **OBJECTIVE:** Ankylosing spondylitis (AS) is a spastic and spinal joint disease with the characteristic of pathological ossification. Bioinformatics analysis demonstrated that there is a complementary binding site between microRNA-124 (miR-124) and the 3'-UTR of glycogen synthase kinase-3 β (GSK-3 β) mRNA. We aimed to investigate the role of miR-124 in regulating GSK-3 β expression, Wnt/ β -catenin pathway activity, and osteoblast differentiation of spinal ligament fibroblasts.

PATIENTS AND METHODS: The ligament tissues of AS and the femoral neck fracture patients were collected. MiR-124 and GSK-3 β mRNA expressions were detected by using quantitative Real-time PCR (qRT-PCR). GSK-3 β and β -catenin protein expressions were detected by using Western blot. Ligament fibroblasts were isolated and induced to differentiate into osteoblasts. Alizarin red S staining (ARS) was used to identify osteoblast differentiation. Expressions of miR-124, GSK-3 β , β -catenin, Osterix, and runt-related transcription factor 2 (RUNX2) were detected during differentiation. The cells were divided into two groups as agomiR-normal control (NC) transfection group and agomir miR-124 transfection group. Alkaline phosphatase (ALP) activity and Alizarin Red S staining were detected.

RESULTS: MiR-124 and β -catenin expressions in the ligament of AS patients increased, while GSK-3 β level reduced compared with control. MiR-124, β -catenin, Osterix, and RUNX2 expressions gradually elevated, whereas GSK-3 β level gradually declined following increased osteoblasts differentiation. Antagomir miR-124 transfection significantly up-regulated the expression of GSK-3 β in osteoblast differentiation, significantly decreased the expression of β -catenin, Osterix, and RUNX2, and significantly inhibited osteoblast differentiation.

CONCLUSIONS: MiR-124 decreased and GSK-3 β elevated in AS ligament tissue. Down-regulation of miR-124 expression enhanced GSK-3 β expression, weakened Wnt/ β -catenin pathway activity, and inhibited the differentiation of ligament fibroblasts into osteoblasts.

Key Words:

Ankylosing spondylitis, miR-124, GSK-3 β , Wnt/ β -catenin pathway, Osteogenesis.

Introduction

Ankylosing spondylitis (AS) is a chronic progressive inflammatory disease with sacroiliac joints and spinal joints as the main lesions. It is characterized by pathological ossification, joints rigidity, spinal joint activity restriction, and function progressive loss^{1,2}. Fibroblasts are the main cells in the interstitial tissue around the joints that have the potential to differentiate into osteoblasts³. Excessive abnormal differentiation of fibroblasts into osteoblasts can lead to pathological ossification, which plays an important role in the pathogenesis of AS. It is an important source of cells for abnormal ossification of AS joints⁴⁻⁶.

Several studies showed^{7,8} that over-activation of the Wnt/ β -catenin pathway is closely related to the onset of AS. Glycogen synthase kinase-3 β (GSK-3 β) is an important negative regulator in Wnt/ β -catenin signaling pathway. GSK-3 β can reduce the expression of β -catenin protein, thus blocking the activation of Wnt/ β -catenin pathway and negatively regulating osteogenic differentiation. It plays a role in inhibiting pathological bone formation. It was found⁸ that as a negative regulator in the Wnt/ β -catenin pathway, the abnormally decreased expression of GSK-3 β is associated with AS. MicroRNA is a type of endogenous, non-coding, single-stranded, small-molecule RNA of about 22 to 25 nucleotides in length. It is an important epigenetic regulatory molecule that degrades mRNA or inhibits translation by targeting the 3'-untranslated region (3'-UTR). It is closely related to osteoblast differentiation, bone formation, and osteoclast activation. It was observed^{9,10} an abnormal increase of miR-124

expression in peripheral blood of AS patients, suggesting that miR-124 may be a promoting factor in the pathogenesis of AS. Bioinformatics analysis revealed that there is a complementary binding site between miR-124 and the 3'-UTR of GSK-3 β mRNA, indicating possible targeting relationship. We compared miR-124 and GSK-3 β expressions in the ligament tissue of AS patients and control, and investigated whether miR-124 and GSK-3 β abnormalities may participate in the pathogenesis of AS. In addition, cultured ligament fibroblasts were used to explore the impact of miR-124 in the regulation of GSK-3 β expression, Wnt/ β -catenin pathway activity, and osteoblast differentiation in spinal ligament fibroblasts.

Materials and Methods

Main Reagents and Materials

Dulbecco's modified eagle medium (DMEM), osteogenic differentiation kit, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). RNA extraction reagent TRIzol and transfection reagent Lipo 2000 were purchased from Invitrogen (Carlsbad, CA, USA). HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Real-time PCR kit and SYBR were purchased from TaKaRa (Dalian, China). Rabbit anti-human GSK-3 β , β -catenin, Osterix, runt-related transcription factor 2 (RUNX2), and β -actin polyclonal antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody was purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Western and immunoprecipitation (IP) cell lysate (no inhibitor), and alkaline phosphatase (ALP) detection kit were purchased from Beyotime Biotech. (Shanghai, China). Luciferase activity detection kit was purchased from Beyotime Biotech. (Shanghai, China). Dual-Glo Luciferase Assay System was purchased from Promega (Madison, WI, USA). miR-NC, miR-124 mimic, antagomir-NC, antagomir miR-124, and pMIR-Report target gene plasmid vectors were purchased from RiboBio (Guangzhou, China). Collagenase was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alizarin red S (ARS) staining kit was purchased from Shanghai Jiemei Biotechnology (Shanghai, China).

Clinical Information

Thirty-two AS patients undergoing hip replacement surgery in Zhuzhou Central Hospital (Hunan China) were selected, including 25 males and 7 females with mean age of 28.7 ± 4.5 years. The hip ligament tissue was collected from the surgery. In addition, 22 cases of ligament tissue in patients with femoral neck fractures during the same period were included as control. Among them, there were 17 males and 5 females with average age of 26.6 ± 5.2 years. There was no significant difference in age and gender between the two groups. This study was approved by the Ethics Committee of Zhuzhou Central Hospital (Zhuzhou, China). All patients had signed informed consents and approved this study.

Ligament Fibroblasts Separation and Cultivation

The collected ligament tissue was placed in sterile phosphate-buffered saline (PBS) buffer to remove blood, fat, and muscle tissue. The ligament tissue was transferred to a 50 ml centrifuge tube and cut with scissors. After digested by 20 ml of 0.1% collagenase at 37°C for 90 min, the sample was centrifuged at 350 \times g for 10 min. Next, the cells were added with complete medium and inoculated into a 10 cm dish. After 12 to 15 days, the cells were overgrown and digested with 0.05% trypsin for passage. The cells in the third generation were used for experiments.

Osteogenic Differentiation

The third passage ligament fibroblasts were inoculated into 6-well plates. After the cells were grown to 80% confluence, the original DMEM medium was removed and osteogenic differentiation serum free medium (SFM) was added to induce differentiation. After 10 and 20 days, the cells were harvested to detect Osterix and RUNX2. The differentiation of osteoblasts was performed according to the ARS staining kit instructions.

ALP Activity Detection

A total of 100 μ l Western and IP cell lysates (without inhibitors) were added to 5×10^6 cells and lysed for 15 min. The lysate supernatant was transferred to a new Eppendorf (EP) tube. According to ALP test kit instructions, the cells were divided into blank control, standard, and sample groups, respectively. The blank well was added with 50 μ l buffer and 50 μ l chromogenic

substrates. The standard well was added with corresponding volume of standard working fluid. The sample well was added with 50 μ l chromogenic substrate, 20 μ l cell lysate supernatant, and 30 μ l buffer. The plate was incubated at 37°C for 10 min. At last, each well was added with 100 μ l of stop solution and the absorbance was measured at 405 nm (A).

Dual Luciferase Gene Reporter Recombinant Plasmid Construction

The RNA of HEK293 cells was used as a template to amplify the fragment containing the target binding site in the 3'-UTR region of GSK-3 β mRNA or its mutant fragment. After PCR amplification, the PCR product was double digested with Sac I and Hind III. The pMIR vector was digested under 37°C for 4 h. The purified product was recovered by 1.5% agarose gel electrophoresis and the purified PCR product was incubated with the vector overnight at 16°C for ligation. The ligated product was transformed into DH5 α competent cells and inoculated on a plate containing penicillin overnight at 37°C. A single positive clone was selected and shaken in an *E. coli* culture solution at 37°C overnight. The plasmid was extracted and the target sequence was identified by sequencing, named as pMIR-GSK-3 β -WT or pMIR-GSK-3 β -MUT.

Luciferase Reporter Gene Assay

1 \times 10⁵ HEK-293T cells were inoculated in a 24-well plate and incubated for 24 h. 100 ng of pMIR-GSK-3 β -WT (or pMIR-GSK-3 β -MUT) and 50 nmol miR-124 mimic (or miR) were co-transfected into HEK293T cells by Lipo2000 and continued to culture for 48 h. Dual-Glo Luciferase Assay System Kit was adopted to detect dual luciferase activity. The plate was added with 100 μ l passive lysis buffer cell lysate and shaken slowly for 15 min at room temperature. Next, the 20 μ l cell lysate were added with 100 μ l luciferase assay reagent II (LARII) to test the firefly luciferase activity on a fluorescence luminometer, and then added with 100 μ l renilla fluorescein reagent to detect Renilla luciferase activity, calculating the relative activity value of firefly luciferase activity to Renilla luciferase activity.

Quantitative Real-time PCR (qRT-PCR)

PrimeScript™ RT reagent Kit was used to reverse transcribe RNA to complementary DNA (cDNA) for q-PCR reaction. The reverse tran-

scription reaction system contained 0.5 μ l OligodT Primer (50 μ M), 0.5 μ l Random 6 mers (100 μ M), 0.5 μ l PrimeScript RT Enzyme Mix, 1.0 μ g RNA, 2 μ l 5 \times PrimeScript Buffer, and RNase Free H₂O. The reaction conditions were 37°C for 15 min and 85°C for 5 s. qPCR reaction system contained 10.0 μ l SYBR Fast qPCR Mix, 0.8 μ l Forward Primer (10 μ M), 0.8 μ l Reverse Primer (10 μ M), 2.0 μ l cDNA, and 6.4 μ l RNase Free dH₂O. q-PCR reaction conditions were pre-denatured at 95°C for 10 min, denatured at 95°C for 10 s, followed by 40 cycles of annealing at 60°C for 20 s and extension at 72°C for 15 s, on the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

Western Blot

Western- and IP cell lysates were used to lyse the transfected cells. The supernatant was used to determine the protein concentration. A total of 40 μ g proteins were separated by 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and 5% concentrated gel. Then, the protein was transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 90 min. Next, the membrane was blocked with 5% skim milk at room temperature for 60 min and incubated in primary antibody (GSK-3 β , β -catenin, Osterix, RUNX2, and β -actin at 1:1000, 1:1000, 1:1000, 1:1000, 1:5000, respectively) at 4°C overnight. After that, the membrane was incubated with HRP-labeled secondary antibody (1: 10000) at room temperature for 60 min and finally detected by enhanced chemiluminescence (ECL) assay.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean \pm standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among groups; miR-124 and GSK-3 β mRNA expressions in the ligament were compared by the Mann-Whitney U test. The correlation between the expression of miR-124 and GSK-3 β in the ligament of AS patients was adopted via Spearman method. *p* < 0.05 was considered as statistically significant.

Results

The Targeted Regulatory Relationship Between miR-124 and GSK-3 β

MicroRNA.org website online prediction showed that there is a targeted complementary binding site between miR-124 and the 3'-UTR of GSK-3 β mRNA (Figure 1A). Dual luciferase gene reporter assay revealed that transfection of miR-124 mimic significantly reduced the relative luciferase activity of pMIR-GSK-3 β -WT transfected HEK293T cells. Transfection of miR-124 inhibitor significantly increased the relative luciferase activity of pMIR-GSK-3 β -WT transfected HEK293T cells. However, miR-124 mimic or miR-124 inhibitor exhibited no marked effect on relative luciferase activity in pMIR-GSK-3 β -MUT transfected HEK293T cells (Figure 1B), suggesting that there is a targeted regulatory relationship between miR-124 and GSK-3 β mRNA.

MiR-124 Elevated, While GSK-3 β Reduced in the Ligament Tissue of AS Patients

qRT-PCR demonstrated that GSK-3 β mRNA level in the ligament of AS patients was apparently decreased (Figure 2A), while miR-124 was significantly enhanced compared with control

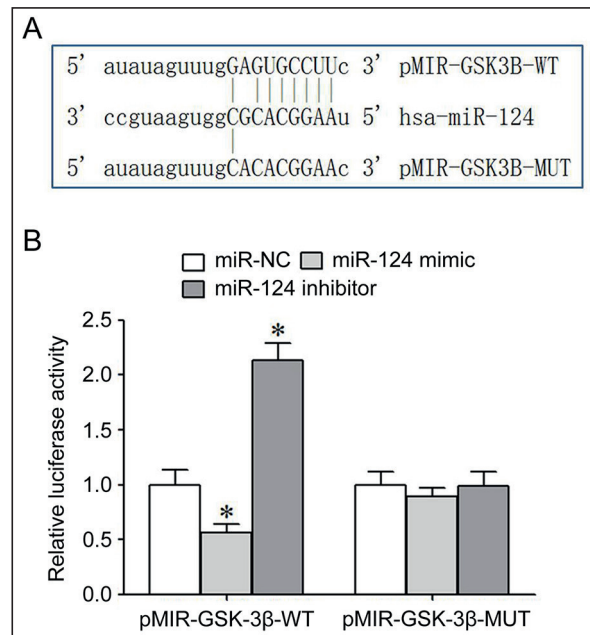


Figure 1. The targeted regulatory relationship between miR-124 and GSK-3 β . (A) The complementary binding site between miR-124 and the 3'-UTR of GSK-3 β mRNA. (B) Dual luciferase reporter assay. * $p < 0.05$, compared with miR-NC.

(Figure 2B). Spearman rank correlation analysis exhibited that there was a significant negative correlation between miR-124 and GSK-3 β mRNA

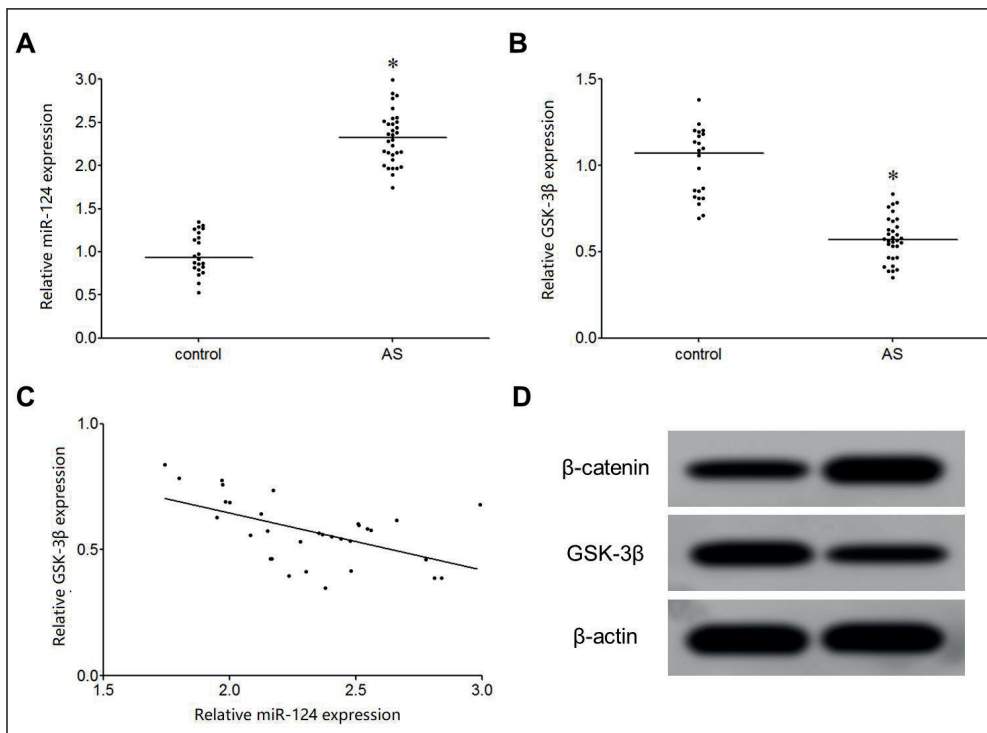


Figure 2. MiR-124 elevated, while GSK-3 β reduced in the ligament tissue of AS patients. (A) GSK-3 β mRNA expression detected by qRT-PCR. (B) miR-124 expression detected by qRT-PCR. (C) Correlation analysis between miR-124 and GSK-3 β mRNA expression. (D) Protein expression detected by Western blot. * $p < 0.05$, compared with control.

expressions in the ligament of AS patients ($r = -0.51, p < 0.01$) (Figure 2C). Western blot showed that GSK-3 β protein expression in the ligament of AS patients was significantly decreased compared with control (Figure 2D).

MiR-124 Increased, while GSK-3 β Declined During the Osteogenic Differentiation Process of Ligament Fibroblast

ARS staining revealed that ligament fibroblast staining was negative before the induction of differentiation. ARS staining was positive after 10 days and 20 days of induction (Figure 3A). qRT-PCR showed that the expression of miR-124, β -catenin, Osterix, and RUNX2 mRNAs gradually increased, whereas the expression of GSK-3 β mRNA gradually reduced during the differentiation of ligament fibroblasts into osteoblasts (Figure 3B). Western blot analysis demonstrated that

the expression of β -catenin, Osterix, and RUNX2 proteins gradually enhanced, whereas the expression of GSK-3 β protein gradually decreased with the prolongation of ligament fibroblast differentiation (Figure 3C).

Down-Regulation of miR-124 Inhibited the Osteogenic Differentiation of Ligament Fibroblasts

qRT-PCR showed that compared with antagomir-NC group, the expression of miR-124 was apparently reduced, while the expression of GSK-3 β mRNA was obviously increased in the differentiation of ligament fibroblasts to osteoblasts in antagomir miR-124 transfection group (Figure 4A). qRT-PCR found that compared with the antagomir-NC group, the expression of β -catenin, Osterix, and RUNX2 mRNA was significantly declined during the differentiation of ligament fibroblasts into osteoblasts after transfection of

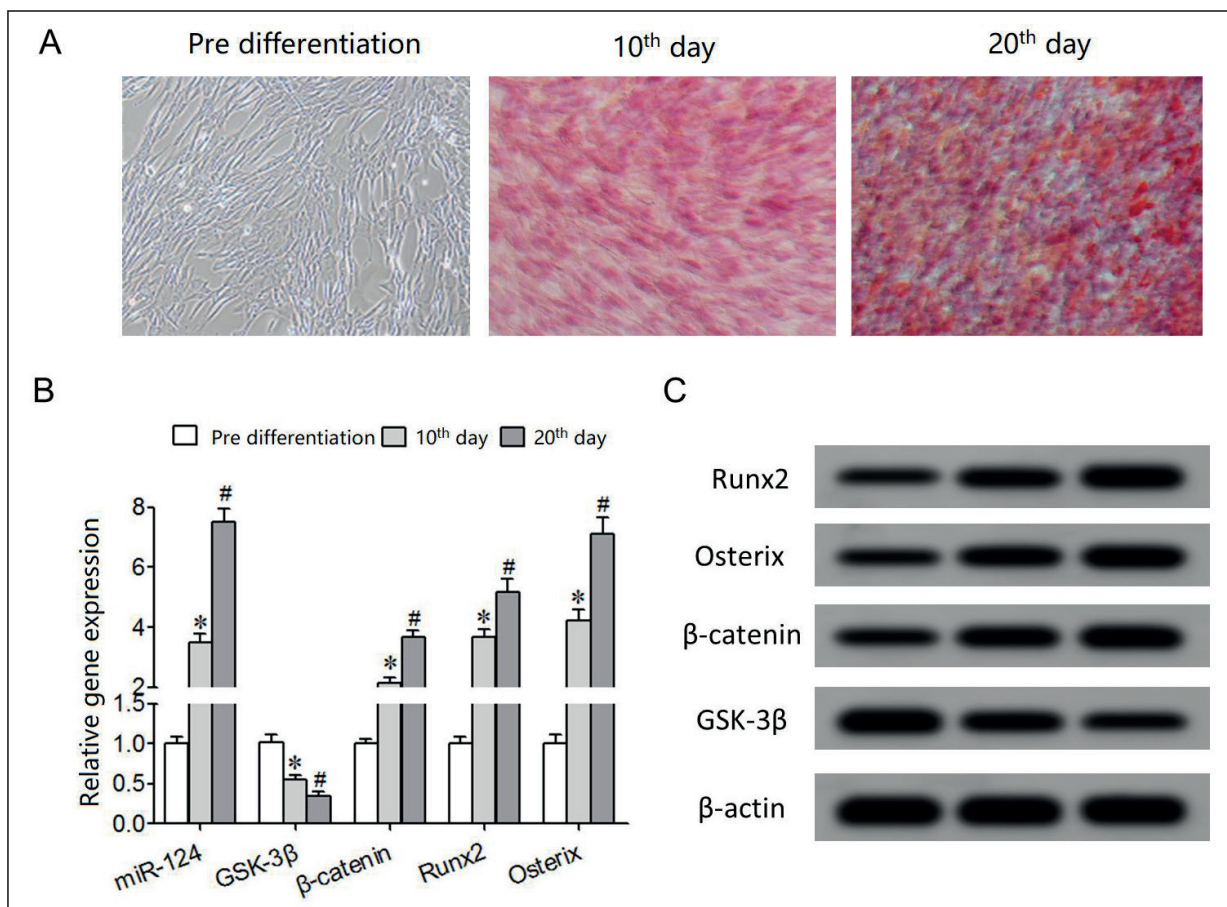


Figure 3. MiR-124 increased, while GSK-3 β declined during the osteogenic differentiation process of ligament fibroblast. (A) Osteogenic differentiation detected by ARS staining (200 \times). (B) Gene expression detected by qRT-PCR. (C) Protein expression detected by Western blot. * $p < 0.05$, compared with pre-differentiation, # $p < 0.05$, compared with the 10th day.

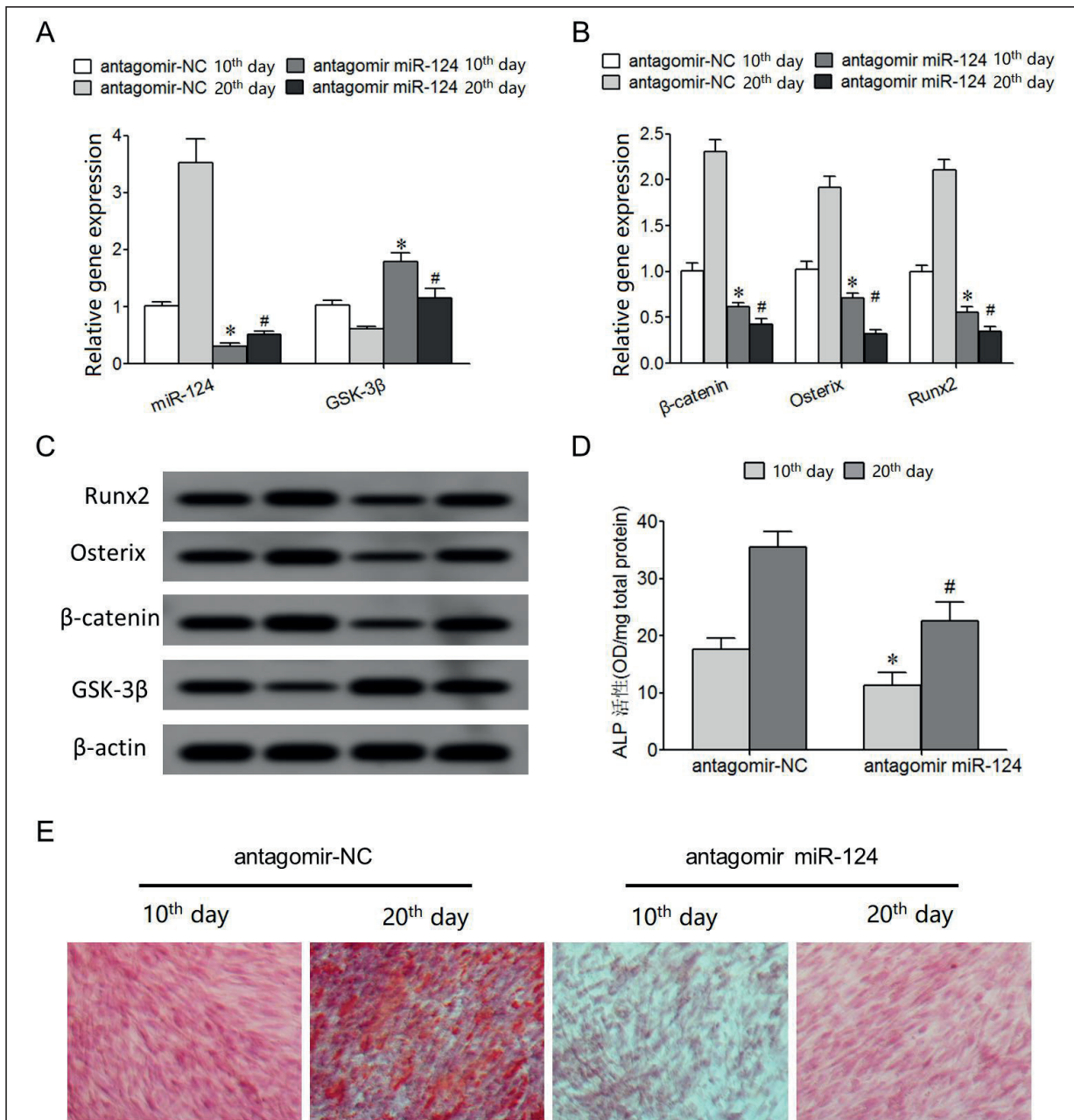


Figure 4. Down-regulation of miR-124 inhibited the osteogenic differentiation of ligament fibroblasts. **(A)** miR-124 and GSK-3 β mRNA expression detected by qRT-PCR. **(B)** β -catenin, Osterix, and RUNX2 mRNA expressions detected by qRT-PCR. **(C)** Protein expression detected by Western blot. **(D)** ALP activity detection. **(E)** Osteogenic differentiation detected by ARS staining (200 \times). ** $p < 0.01$, compared with antagomir-NC on the 10th day, # $p < 0.05$, compared with antagomir-NC on the 20th day.

antagomir miR-124 (Figure 4B). Western blot observed that transfection of antagomir miR-124 significantly up-regulated the expression of GSK-3 β protein and significantly decreased the expression of β -catenin, Osterix, and RUNX2 proteins during osteoblast differentiation (Figure 4C). ALP staining exhibited that the activity

of ALP on the 10th and 20th day of osteoblast differentiation in antagomir miR-124 transfected group was apparently lower than that in antagomir-NC group (Figure 4D). ARS staining showed that transfection of antagomir miR-124 significantly suppressed the osteoblastic differentiation of ligament fibroblasts (Figure 4E).

Discussion

AS mainly involves the sacroiliac joints and axial joints of the spine. Following the disease progresses, multiple joints, such as the lumbar spine, thoracic spine, and cervical spine may be ascendingly invaded, leading to ossification and rigidity, pathological osteogenesis, and fibrosis of the lesion joints^{11,12}. AS frequently occurs in young people aged 16-30 years, especially in young men. It is often accompanied by varying degrees of lesion in eye, lung, and muscle, resulting in the different degrees of limitation of patient's bending and walking activities, which is a serious impact on occupational capacity and quality of life^{13,14}. The pathogenesis of AS is complex and its etiology is not clear. Current studies suggest that genetic^{15,16} and immune dysfunction^{17,18} are involved in the pathogenesis of AS. At present, there is no effective treatment for AS to stop the progress. Therefore, searching for target molecules that inhibit the osteogenic differentiation of fibroblasts can provide a theoretical basis and reference value for the treatment of AS. Wnt/ β -catenin signaling pathway is a highly conserved pathway in species evolution, closely related to tissue and embryonic development, individual growth, immune response, and tumor formation¹⁹. When the Wnt/ β -catenin pathway is activated, Wnt can bind with frizzled protein (Frz) receptors and low-density lipoprotein receptor related proteins 5 and 6, LRP5/6, phosphorylate disheveled (Dvl) to send signal to GSK-3 β , thus inhibiting β -catenin phosphorylation by GSK-3 β and degrading APC-Axin-GSK-3 β complex. It further increases β -catenin stability and accumulation in the cytoplasm, which enters the nucleus to combine with T-cell factor/lymphoid enhancing factor (TCF/LEF) to promote transcription and expression of the target gene^{20,21}. Wnt/ β -catenin signaling pathway is closely related to bone formation. Wnt/ β -catenin signaling pathway plays an important role in promoting the differentiation of mesenchymal fibroblasts into osteoblasts and inhibiting their differentiation into adipocytes and chondrocytes^{5,22}. Wnt/ β -catenin signaling pathway can directly facilitate the differentiation of osteoblasts by upregulating the expression of osteoblast-specific genes, such as RUNX2²³, Osterix²⁴, and ALP, and suppress the differentiation of mesenchymal fibroblasts into adipocytes to indirectly promote their differentiation into osteoblasts by downregulating

the expression of CEBP α and PPAR γ ^{24,25}. GSK-3 β , a negative regulator, is a key component of the Wnt/ β -catenin signaling pathway. GSK-3 β phosphorylates serine/threonine residues at the amino terminus of β -catenin protein, and forms complexes with axin and adenomatous polyposis coli (APC) to degrade β -catenin protein, thereby reducing its level in the cytoplasm and restraining its function. It was showed⁸ that, as a negative regulator in the Wnt/ β -catenin pathway, the abnormally decreased expression of GSK-3 β is associated with AS. It was found^{9,10} abnormal increase of miR-124 expression in peripheral blood of AS patients, suggesting that miR-124 may be a promoting factor in the pathogenesis of AS. This work was to investigate the role of miR-124 in regulating GSK-3 β expression, Wnt/ β -catenin pathway activity, and osteoblast differentiation of spinal ligament fibroblasts. Dual luciferase gene reporter assay demonstrated that transfection of miR-124 mimic significantly reduced the relative luciferase activity of pMIR-GSK-3 β -WT transfected HEK293T cells, whereas transfection of miR-124 inhibitor significantly enhanced the relative luciferase activity in HEK293T cells transfected with GSK-3 β -WT, indicating that there is a targeted regulatory relationship between miR-124 and GSK-3 β mRNA. Spearman rank correlation analysis exhibited that there was a significant negative correlation between the expression of miR-124 and GSK-3 β mRNA in the ligament of AS patients. Our results revealed that compared with the control group, the expression of miR-124 was significantly elevated, while the expression of GSK-3 β was markedly decreased in the ligament of AS patients, suggesting that miR-124 may play a role in down-regulating GSK-3 β to participate in the pathogenesis of AS. Xia et al¹⁰ showed that the expression of miR-124 was significantly increased in peripheral blood of AS patients compared with the control group, revealing that elevated miR-124 may be an AS promoting factor. Huang et al⁽⁸⁾ observed that compared with the control group, GSK-3 β was apparently decreased in peripheral blood cells of AS patients, suggesting that the decreased expression of GSK-3 β may be involved in the pathogenesis of AS. Since the abnormal differentiation of ligament fibroblasts to osteoblasts plays a role in AS, this work also observed the expression changes of miR-124 and GSK-3 β in ligamentous tissue of AS patients. Therefore, we further investigated the role of miR-124 and

GSK-3 β in regulating the differentiation of ligament fibroblasts into osteoblasts. Our findings showed that miR-124, β -catenin, Osterix, and RUNX2 levels gradually increased, while GSK-3 β expression gradually decreased during the osteoblast differentiation of ligament fibroblasts, indicating that elevated miR-124 expression is involved in down-regulating GSK-3 β expression, enhancing Wnt/ β -catenin pathway activity, and promoting osteoblast differentiation in ligament fibroblasts. Further exploration showed that after transfection of antagomir miR-124 in ligament fibroblasts, the expression of GSK-3 β was significantly increased, and the expressions of β -catenin, Osterix, and RUNX2 were markedly declined. Moreover, it was showed that miR-124 down-regulation can elevate GSK-3 β expression, weaken the Wnt/ β -catenin pathway activity, and inhibit the differentiation of ligament fibroblasts into osteoblasts. Zou et al⁵ found that compared with the control group, fibroblasts derived from AS patients exhibited stronger ability to differentiate into osteoblasts, which was attributed by the reduction of DKK-1, another important negative regulation factor in the Wnt/ β -catenin pathway. Over-expression of DKK-1 in fibroblasts significantly enhances the Wnt/ β -catenin pathway and its differentiation ability to osteoblasts, indicating that the reduction of the negative regulatory mechanism in the Wnt/ β -catenin pathway can lead to excessive osteogenic differentiation of fibroblasts, while increasing the expression of negative regulators in the Wnt/ β -catenin pathway can suppress fibroblasts to osteoblasts differentiation. However, in contrast, this study connected miR-124 and GSK-3 β , and revealed that miR-124 inhibits GSK-3 β expression and regulates Wnt/ β -catenin signaling during osteoblast differentiation. The specific role of miR-124 in the regulation of GSK-3 β expression and osteoblast differentiation in AS remains to be further validated by animal studies in the future, which is also a deficiency of this study.

Conclusions

We showed that miR-124 decreased and GSK-3 β elevated in AS ligament tissue. Down-regulation of miR-124 expression enhanced GSK-3 β expression, weakened Wnt/ β -catenin pathway activity, and inhibited the differentiation of ligament fibroblasts into osteoblasts.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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