European Review for Medical and Pharmacological Sciences 2018; 22: 6212-6220

MicroRNA-615-3p promotes the osteoarthritis progression by inhibiting chondrogenic differentiation of bone marrow mesenchymal stem cells

J.-X. ZHOU, Z.-G. TIAN, L.-F. ZHU, W.-D. WU, S.-L. ZHOU, Y.-T. ZHAO, S. HUANG

Department of Orthopedics, The First People's Hospital of Wujiang District, Suzhou, China

Jianxin Zhou and *Zhigang Tian* contributed equally to this work

Abstract. – OBJECTIVE: To investigate whether microRNA-615-3p participates in the development and progression of osteoarthritis by regulating chondrogenic differentiation of bone marrow mesenchymal stem cells.

MATERIALS AND METHODS: Bone marrow mesenchymal stem cells (BMSCs) were isolated from rat bone marrow and identified by flow cytometry. After chondrogenic differentiation was induced in BMSCs, expression levels of chondrogenic-specific genes were then detected by quantitate Real-time polymerase chain reaction (qRT-PCR). Expression levels of inflammatory cytokines were detected by enzyme-linked immunosorbent assay (ELISA). Protein expression of SOX9 after overexpression or knockdown of microRNA-615-3p was detected by Western blot, respectively.

RESULTS: MicroRNA-615-3p was down-regulated in the process of chondrogenic differentiation of BMSCs. The mRNA expressions of chondrogenic-specific markers, COL2A1, COL10A1, ACAN and MATN3 were decreased after microRNA-615-3p overexpression in BMSCs. Overexpressed microRNA-615-3p down-regulated protein expression of SOX9. Expression levels of inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-α (IL-α) were increased after overexpression of microRNA-615-3p, while inhibition of microRNA-615-3p expression obtained the opposite result. In addition, overexpression of SOX9 rescued the effect induced by microRNA-615-3p on inflammatory cytokines.

CONCLUSIONS: MicroRNA-615-3p participates in the development and progression of osteoarthritis by increasing the expressions of inflammatory cytokines and inhibiting chondrogenic differentiation of BMSCs.

Key Words:

MicroRNA-615-3p, Bone marrow mesenchymal stem cells, SOX9, Inflammatory factors, Osteoarthritis.

Introduction

Osteoarthritis (OA) is a type of joint diseases resulting from breakdown of joint cartilage and underlying bone[1,2]. OA mainly occurs in joints with heavy load and frequent activity, such as the hip, knee, ankle and temporomandibular joint[3]. American Academy of Rheumatology proposed that the pathogenesis of OA is due to both systemic biological factors and local factors. Specifically, OA-induced the synthesis and degradation imbalance of articular cartilage and subchondral bone damages synovial tissue, subchondral bone and the surrounding ligaments, eventually leading to joint deformation and dysfunction in OA patients[4]. It is believed that age, hormones, obesity, joint damage and other factors are also involved in the development and progression of OA. Researches have indicated that a large number of biomolecules, such as inflammatory factors, signaling molecules and proteases, are capable of regulating the OA development.

MicroRNA (miRNA) is a non-coding RNA containing 19-25 nucleotides in length. It is commonly found in animals, plants and microorganisms, which is involved in regulating various physiological activities in the body. MiRNAs are highly conserved during their evolution. They can degrade the gene or inhibit the translation process by directly targeting the mRNAs, thereby interfering with the expression levels of target genes[5,6]. So far, miRNA function has been well recognized.

Pu et al[7] have shown that microRNA-615-3p is down-regulated in non-small cell lung can-

Corresponding Author: Sheng Huang, BM; e-mail: 180809972@qq.com

cer and inhibits proliferation and invasion of lung cancer. MicroRNA-615-3p is proved to negatively regulate osteogenic differentiation of the ligamentum flavum cells by inhibiting the osteogenic modulators GDF5 and FOXO1 at the post-transcriptional level[8]. However, the expression level of microRNA-615-3p in osteoarthritis has not been reported yet. Therefore, we studied the effect of microRNA-615-3p on the differentiation of MSCs *in vitro* to provide new ideas for the pathogenesis and treatment of osteoarthritis.

Materials and Methods

Isolation and Culture of BMSCs

Rats were sacrificed by dislocations of cervical vertebrae and then immersed in 75% ethanol for 5 min. Rat femurs were taken and placed in sterile culture dish. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM)/F-12) (1:1) medium (Thermo Fisher Scientific, Waltham, MA, USA) was used to wash the bone marrow cavity and the BMSCs were then collected. After the cell density was adjusted to 1×10^5/mL, isodose of L-DMEM medium containing 15% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was added. Cells were then inoculated into a 25 cm^2 plastic culture bottle and recorded as P0. The culture bottle was maintained in a 5% CO_2 incubator at 37°C. Half dose of the culture medium was replaced after cell adherence. Then, the culture medium was replaced every other day. The non-adherent cells were discarded until the culture medium was clear. This study was approved by the Animal Ethics Committee of The First People's Hospital of Wujiang District Animal Center.

Induction and Differentiation of BMSCs

Osteogenic differentiation was induced by incubating cells in L-DMEM containing 0.25 mM vitamin C, 10 mM β-phosphoglycerate and 10 nM dexamethasone. Adipogenic differentiation was induced by incubating cells in DMEM containing 10 mM 3-isobutyl-1-methylxanthine, 10 mM indomethacin and 10 nM dexamethasone. Chondrogenic differentiation was induced by incubating cells in serum-free DMEM containing 1 ng/mL transforming growth factor-β1 (TGF-β1), 10 mol/L dexamethasone, 50 mg/L vitamin C, 6.25 mg/L insulin, 6.25 μg/mL transferrin and 1.25 mg/mL bovine serum albumin.

Detection of Surface Markers on BMSCs

The third-passage BMSCs in logarithmic growth phase were digested and washed to prepare for cell suspension. Cells were centrifuged at 1000 rpm/min for 5 min and resuspended in complete medium. CD29, CD90, CD45 monoclonal antibodies were added for incubation at room temperature for 20 min. The treated cells were centrifuged at 1000 rpm/min for 5 minutes. After centrifugation, the supernatant was discarded and cells were rinsed twice with phosphate-buffered saline (PBS). Cells were resuspended in PBS and transferred to a special test tube to detect the expressions of cell surface antigens by flow cytometry.

RNA Extraction and Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed with Superscript II reverse enzyme on a Light Cycler PCR (Roche, Basel, Switzerland) following the instructions of Light cycler-fast start DNA master SYBR green I. Primers used in this study were as follows: ALP (F: 5'-AAGGCTTCTTCTTGCTGGTG-3', R: 5'-GCCTTACCCTCATGATGTCC-3'), Runx2 (F: 5'-ACTTCCTGTGCTCCGTGCTG-3', R: 5'-TCGTTGAACCTGGCTACTTGG-3'), ADIPOQ (F: 5'-GGTGCTGAAGCCTACCAAC-3', R: 5'-AGGAAGAACAGACGGCAGAAC-3'), FABP4 (F: 5'-ACTGGGCCAGGAATTTGACG-3', R: 5'-CTCGTGGAAGTGACGCCTT-3'), COL2A1 (F: 5'-TGGACGATCAGGCGAAACC-3', R: 5'-GCTGCGGATGCTCTCAATCT-3'), COL10A1 (F: 5'-ATGCTGCCACAAATACCCTTT-3', R: 5'-GGTAGTGGGCCTTTTATGCCT-3'), ACAN (F: 5'-ACTCTGGGTTTTCGTGACTCT-3', R: 5'-ACACTCAGCGAGTTGTCATGG-3'), MATN3 (F: 5'-TCTCCCGGATAATCGACACTC-3', R: 5'-CAAGGGTGTGATTCGACCCA-3'), SOX9 (F: 5'-AGCGAACGCACATCAAGAC-3', R: 5'-CTGTAGGCGATCTGTTGGGG-3').

Enzyme-Linked Immunosorbent Assay (ELISA) Detection of Inflammatory Cytokines

Antibodies were diluted with coating buffer at a dose of 1-10 μg/mL and incubated at 4°C overnight. The solution was discarded the next day and washed with wash buffer three times for 3 min each. 0.1 mL of protein sample was added in the coated well and incubated at 37°C for 1 h. 0.1 mL of enzyme-labeled antibody was added

in each reaction well for 1-h incubation at 37°C. Subsequently, 0.1 mL of TMB substrate solution was added in each reaction well and samples were incubated at 37°C for 10-30 min. 0.05 mL of sulfuric acid was added to terminate the reaction. Optical density (OD) value at the wavelength of 450 nm was measured on the ELISA detector (R&D Systems, Minneapolis, MN, USA).

Cell Transfection

The fourth-passage BMSCs in logarithmic growth phase were digested, centrifuged and seeded in 12-well plates at a dose of 10×10^4/mL. Cell transfection was performed when the cell confluence was up to 90%. The miRNA-615 mimics, miRNA-615 inhibitor or negative control were transfected following the instruction of Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA), respectively. Transfected primers in this study were as follows: microRNA-615-3p, F: 5'-CTGCCTTTCACCTTGGAGAC-3', R: 5'-CGTTTCCTGGGGATGAGATA-3'; microRNA-615-3p inhibitor, F: 5'-AAGAGGGAGACCCAGGCUCGGA-3'; microRNA-615-3p mimics: F: 5'-UCCGAGCCUGGGUCUCCCUCUU-3', R: 5'- GAGGGAGACCCAGGCUCGGAUU -3'.

Western Blotting

Total proteins were extracted from cells by radioimmunoprecipitation assay (RIPA) method (Beyotime, Shanghai, China) and then quantified using bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA) based on the instructions. Proteins were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Statistical Analysis

Each experiment was repeated for three times. Statistical product and service solutions (SPSS 19.0, IBM, Armonk, NY, USA) statistical software were utilized for analyzing data. All data were expressed as mean ± standard deviation. Comparison of measurement data was conducted using *t*-test. $p < 0.05$ was considered statistically significant.

Results

Phenotype and Multidirectional Differentiation Ability of BMSCs

BMSCs grew by static adherence after 4 days of inoculation. It was observed that cells grew into a short fusiform, appeared round and triangle shape with strong refraction (Figure 1A). Flow cytometry results indicated that the cell surface antigens of BMSCs were CD29-positive (99.89%), CD90-positive (96.12%) and CD45-negative (0.10%), which were compliant with the purity requirements of BMSCs (Figure 1B). The expressions of osteoblast-specific genes and adipocyte-specific genes were detected before and 14 days after differentiation induction. The expressions of ALP and Runx2 in osteogenesis-induced cells were increased on the 14th day (Figure 1C). Meanwhile, the expressions of ADIPOQ and FABP4 in adipogenesis-induced cells were also significantly increased (Figure 1D), indicating the multidirectional differentiation ability of cultured BMSCs. The expression level of microRNA-615-3p was detected on the 14th day as well, which was found to be significantly reduced during osteogenic and adipogenic differentiation (Figure 1E). Subsequently, the expression level of microRNA-615-3p before and on the 3rd, 5th, 7th day after chondrogenic induction was detected. Results showed that the expression level of microRNA-615-3p was significantly decreased in a time-dependent manner (Figure 1F). These data demonstrated that the expression level of microRNA-615-3p is gradually decreased alongside with the process of chondrogenic differentiation of BMSCs.

MicroRNA-615-3p Inhibited Chondrogenic Differentiation of BMSCs

Transfection of microRNA-615-3p mimics remarkably increased the expression level of microRNA-615-3p, while transfection of microRNA-615-3p inhibitor significantly inhibited its expression (Figure 2A). The mRNA expression of chondrogenic-specific genes COL2A1, COL10A1, ACAN and MATN3 were significantly decreased after microRNA-6153p knockdown in BMSCs induced with chondrogenic differentiation. Opposite results were obtained in BMSCs transfected with microRNA-615-3p mimic (Figure 2B, C, D and E). Researches have reported that SOX9 acts as a transcriptional activator and regulates the proliferation and differentiation of chondrocytes.

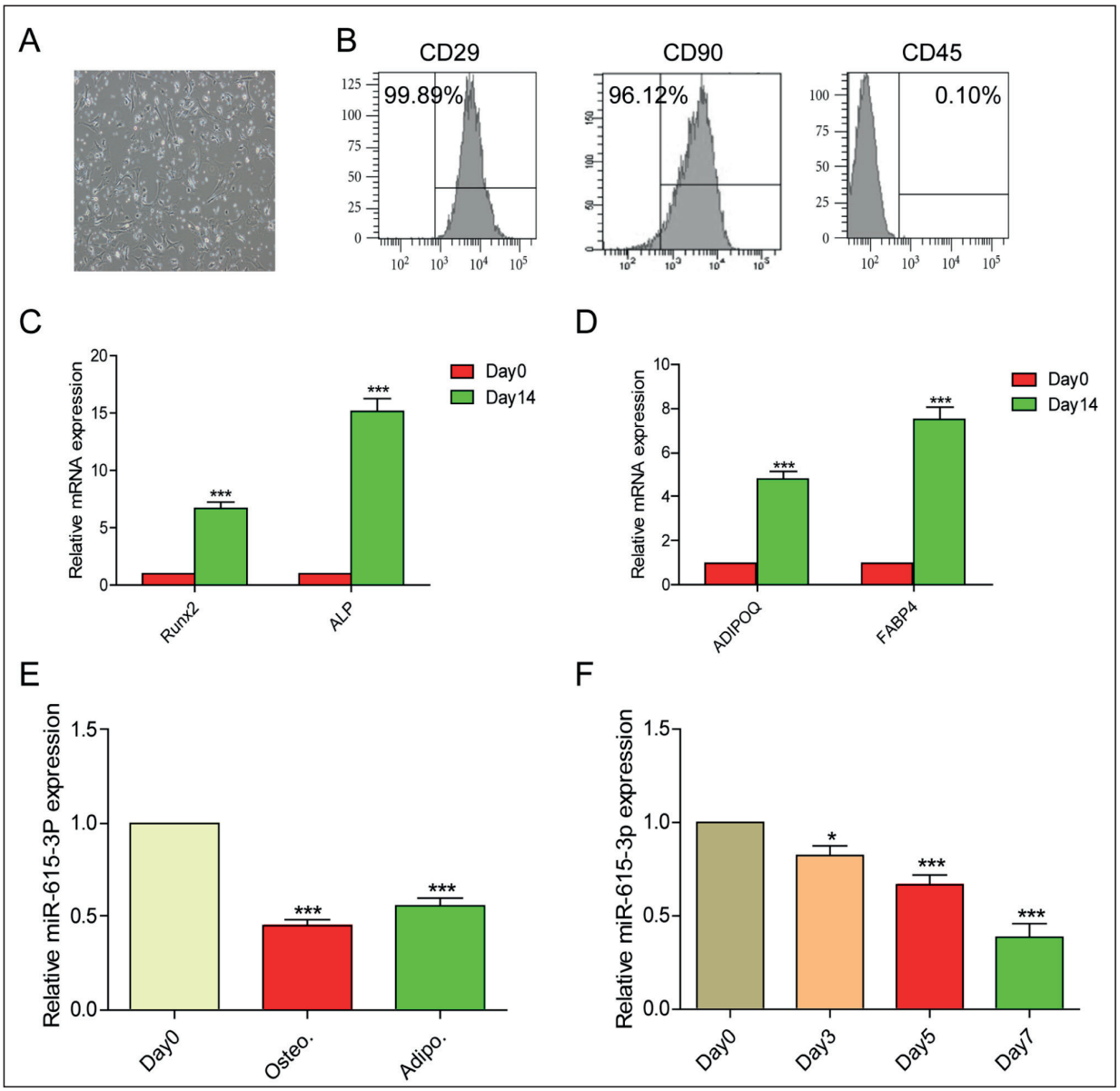

Figure 1. Phenotype and multidirectional differentiation ability of BMSCs. BMSCs grew into a short fusiform on the 4th day of inoculation. Flow cytometry indicated the cell surface antigens of BMSCs were CD29-positive (99.89%), CD90-positive (96.12%) and CD45-negative (0.10%). The mRNA expressions of osteoblast specific genes, ALP and Runx2 in BMSCs after osteoblast induction. The mRNA expressions of adipocyte-specific genes, ADIPOQ and FABP4 after adipogenic induction. The expression of microRNA-615-3p in BMSCs 14 days after osteoblast and adipogenic induction. The expression level of microRNA-615-3p on the 3rd, 5th and 7th day after chondrocyte induction.

In this study, the protein expression of SOX9 was detected after BMSCs were transfected with microRNA-615-3p mimics or microRNA-615-3p inhibitor and experienced 7-day chondrogenic induction. After overexpression of microRNA-615-3p, the expression of SOX9 was significantly decreased, while knockdown of microRNA-615-3p obtained the opposite result (Figure 2F). These data indicated that microRNA-615-3p inhibits the expression of chondrogenic-specific genes and may inhibit chondrogenic differentiation.

MicroRNA-615-3p Promoted the Expressions of Inflammatory Cytokines

Inflammatory cytokines participate in multiple physiological and pathological processes, including the regulation of immune function, hema-

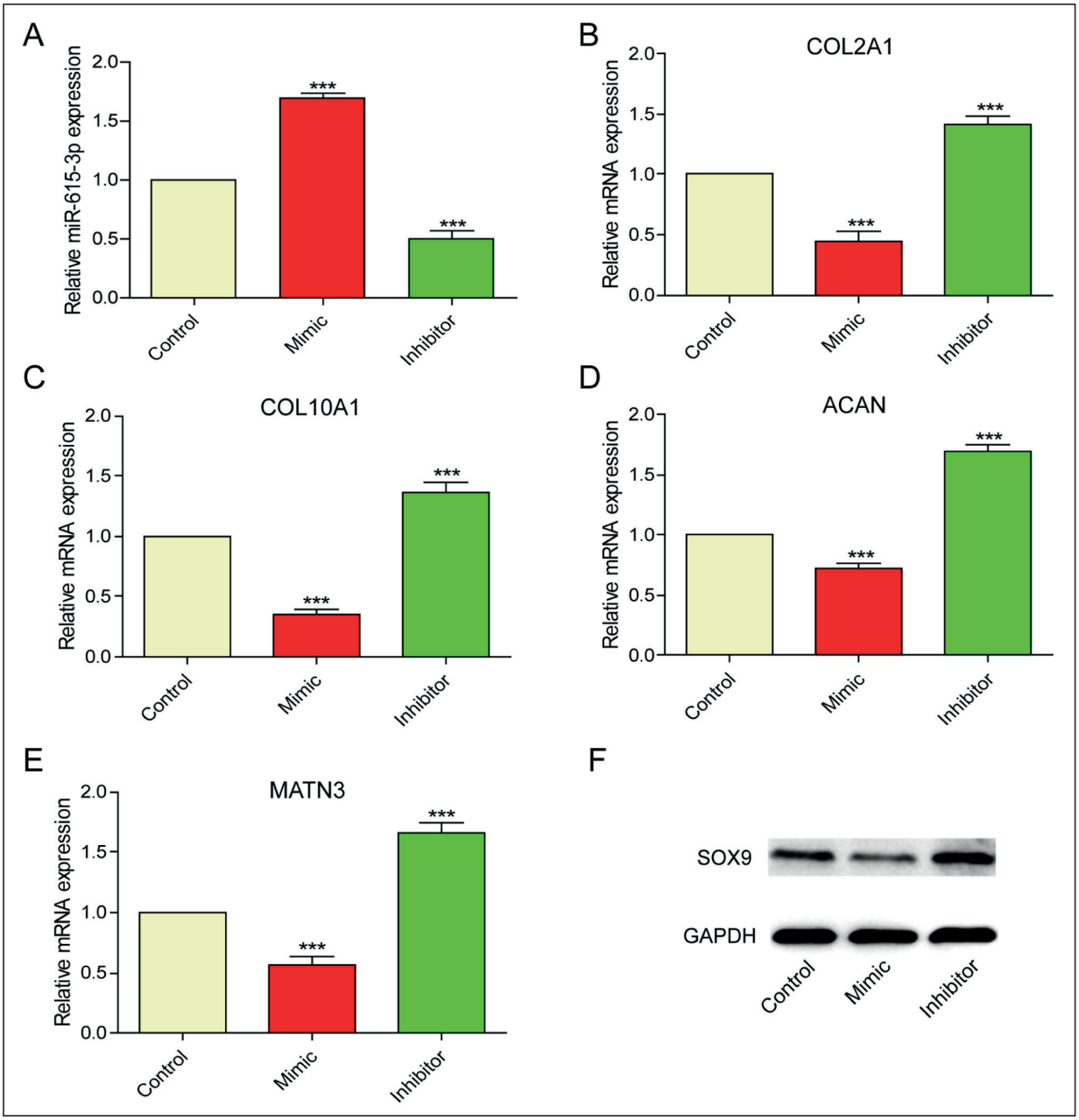

Figure 2. MicroRNA-615-3p inhibited chondrogenic differentiation of BMSCs. ***A,*** The expression level of microRNA-615-3p in BMSCs after overexpression or knockdown of microRNA-615-3p, respectively. ***B-E***, The expressions of chondrogenic specific genes COL2A1 ***(B)***, COL10A1 ***(C)***, ACAN ***(D)*** and MATN3 ***(E)*** after miR-6153p knockdown in BMSCs induced with chondrogenic differentiation. F. The expression of SOX9 in BMSCs after overexpression or knockdown of microRNA-615-3p, respectively.

topoiesis, inflammatory reaction and tumor formation. It is also well recognized that they play important roles in the progression and pathogenesis of OA. After induction of chondrogenic differentiation for 7 days in BMSCs transfected with microRNA-615-3p mimics or microRNA-615-3p inhibitor, the expressions of IL-1, IL-6, IL-8 and TNF-α were detected. Results revealed that the expressions of IL-1, IL-6, IL-8 and TNF-α were significantly increased after overexpression of microRNA-615-3p (red columns in Figure 3A, B, C and D), whereas they were significantly reduced after microRNA-615-3p knockdown (green columns in Figure 3A, B, C and D). Our

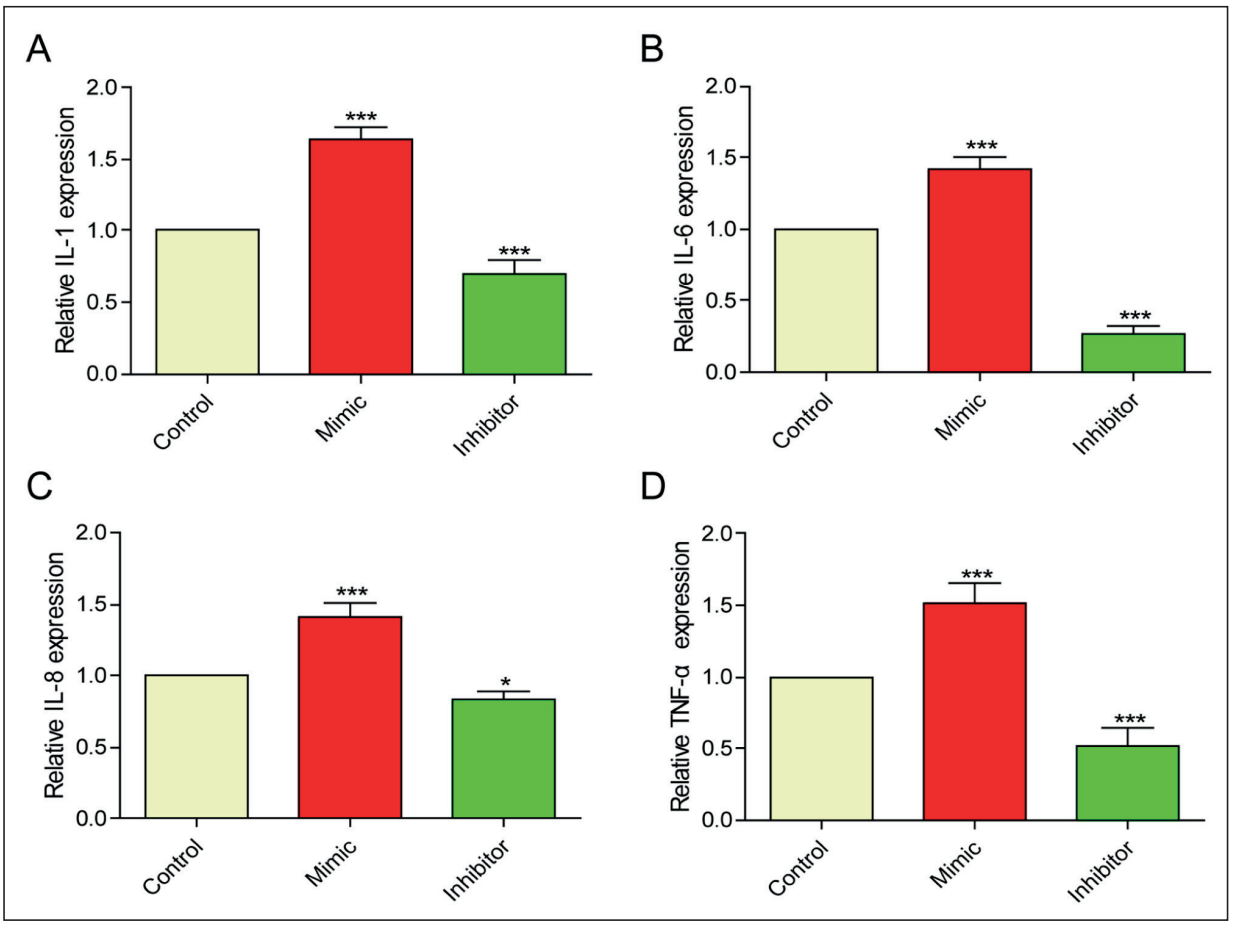

Figure 3. MicroRNA-615-3p promoted the expressions of inflammatory cytokines. The expressions of IL-1 ***(A)***, IL-6 ***(B)***, IL-8 (***C)*** and TNF-α ***(D)*** in BMSCs after overexpression or knockdown of microRNA-615-3p, respectively.

results indicated that microRNA-615-3p may participate in the OA pathogenesis by promoting the expressions of inflammatory cytokines.

MicroRNA-615-3p Promoted the Expressions of Inflammatory Cytokines by Inhibiting the Chondrogenic Differentiation of BMSCs

Since SOX9 could regulate chondrogenic differentiation and its expression was significantly reduced after overexpression of microRNA-615-3p, we hypothesized that microRNA-615-3p contributes to the OA development by promoting SOX9-mediated chondrogenic differentiation of BMSCs. First, the expression level of SOX9 was significantly elevated after transfection with SOX9 overexpression plasmid (Figure 4A). Subsequently, BMSCs were assigned into three groups, namely control group, microRNA-615-3p overexpression group, and SOX9 and microRNA-615-3p overexpression group. The expressions of IL-1, IL-6, IL-8 and TNF-α in BMSCs after 7 days of chondrogenic differentiation were significantly increased in microRNA-615-3p overexpression group (red columns in Figure 4B, C, D and E), which were reversed to the normal levels after overexpression of SOX9 (green columns in Figure 4B, C, D and E). These results indicated that microRNA-615-3p promotes the expressions of inflammatory cytokines by inhibiting the chondrogenic differentiation of BMSCs.

Discussion

Osteoarthritis (OA) is one of the joint diseases that seriously affects human health and life. It frequently occurs in people over 54 years old severely endangering their physical health. The main pathological features of OA include reduced

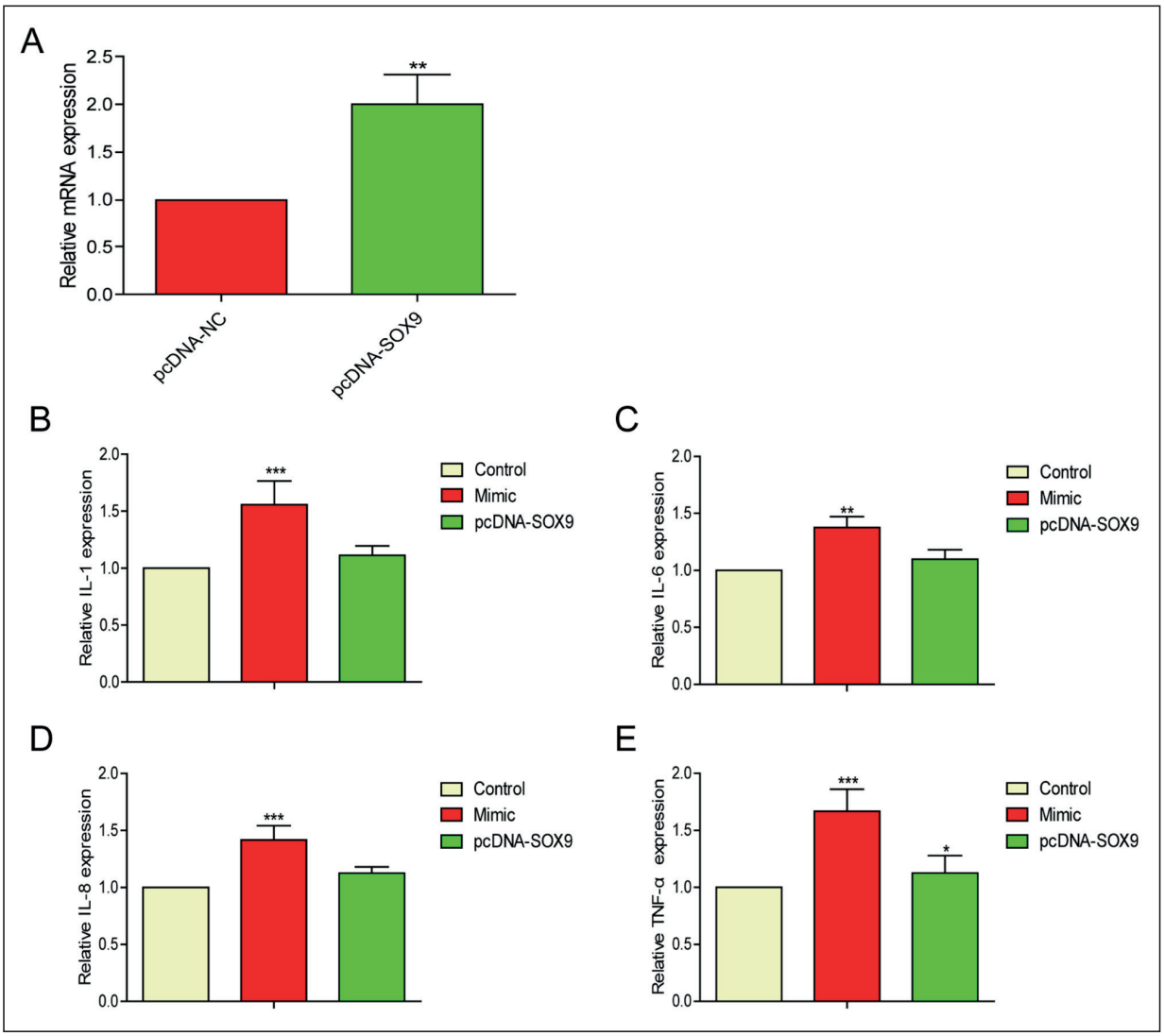

Figure 4. MicroRNA-615-3p promoted the expressions of inflammatory cytokines by inhibiting the chondrogenic differentiation of BMSCs. The expression level of SOX9 in BMSCs after overexpressing SOX9. ***B-E,*** The expressions of IL-1 ***(B)***, IL-6 ***(C)***, IL-8 ***(D)*** and TNF-α ***(E)*** in BMSCs after 7 days of chondrogenic differentiation.

number of chondrocytes, metabolic disorders of extracellular matrix (ECM), inflammatory response of synovial membrane and subchondral bone remodeling. These pathological changes will eventually lead to joint deformities and dysfunction in OA patients. Severe OA would lead to long-term disability, with a fatality rate of up to 53%[10]. Furthermore, OA development results in the aggravation of ECM catabolism, increased secretion of matrix metalloproteinase family and proteoglycan. These pathological lesions further progress to the irreversible damage of ECM[11] and chondrocyte apoptosis in the surface and deep layer of cartilage.

Chondrocytes, as the only cells in articular cartilage tissues, are associated with abnormal changes in the cellular physiology and development of OA[12]. Although chondrocytes have a relatively low density in cartilage tissue of about 14,000 cells/mm^3, they are important components involved in the balance of chondrogenic metabolism. Chondrocytes exert a crucial role in maintaining their physicochemical properties, as well as the structural and functional integrity of cartilage ECM[13]. In this study, the effects of BMSCs on chondrogenic differentiation and OA development were studied. MiRNAs have multiple biological functions in the body and

are involved in regulating many physiological activities including cell division, differentiation, apoptosis and organ formation[14]. Studies have shown that miRNAs exert an essential role in regulating the development and progression of OA. Microarray and *in situ* hybridization results showed that there were 9 upregulated miRNAs in the knee joint of OA patients, including miR-483, miR-22, miR-377, etc. Meanwhile, there were 7 down-regulated miRNAs in the knee joint of OA patients, including miR-140, miR-29a, miR-25, etc.[15]. At present, researchers have confirmed some certain OA-related miRNAs can regulate the chondrocytes apoptosis, metabolic balance of ECM and other life processes by inhibiting target gene expression. The present study found that the expression level of microRNA-615-3p was significantly decreased during the induction of chondrogenic differentiation. In addition, overexpressed microRNA-615-3p inhibited chondrogenic differentiation of BMSCs.

Under normal physiological conditions, the apoptosis and proliferation of articular chondrocytes, as well as the synthesis and degradation of cartilage ECM are strictly regulated to maintain the structure and function stability of articular cartilage. A large number of cytokines, especially inflammatory factors, maintain the dynamic balance of articular cartilage. Inflammatory factors can stimulate the secretion of cytokines by chondrocytes, thus triggering OA. Some studies have reported that the expression levels of IL-1[16], IL-6[17], TNF-α[18] in joint fluid of OA patients were significantly increased. Moreover, higher expressions of these cytokines predicted worse articular cartilage injury. In our study, the expressions of these inflammatory factors were significantly increased after overexpression of microRNA-615-3p.

SOX9 gene is an important gene related to the early embryonic development, including sex determination and chondrogenesis. SOX9 possesses the HMG domain located at the hydroxyl end and the transcription activation domain located at the amino terminal. Gordon et al[19] suggested that SOX9 can affect abnormal cartilage formation, thereby resulting in short bones and other skeletal dysplasia. Our study found that overexpression of microRNA-615-3p significantly reduced the expression level of SOX9 but increased the expressions of inflammatory cytokines. Moreover, the increased expressions of inflammatory cytokines were recovered to normal levels after overexpression of SOX9.

Conclusions

We found that, microRNA-615-3p was overexpressed in chondrogenic differentiation of BMSCs, which could inhibit chondrogenic differentiation of BMSCs and promote the expressions of inflammatory cytokines through SOX9. We provided theoretical basis and new direction for exploring the mechanism of the progression and development of osteoarthritis.

Funding Acknowledgements

This work was supported by Science and Technology Plan Project Fund of Suzhou City (No. SYSD2014047).

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Pelletier JP, Martel-Pelletier J, Mehraban F, Malemud CJ. Immunological analysis of proteoglycan structural changes in the early stage of experimental osteoarthritic canine cartilage lesions. J Orthop Res 1992; 10: 511-523.
2) Adams ME. Cartilage hypertrophy following canine anterior cruciate ligament transection differs among different areas of the joint. J Rheumatol 1989; 16: 818-824.
3) Abramson SB, Attur M. Developments in the scientific understanding of osteoarthritis. Arthritis Res Ther 2009; 11: 227.
4) Hart DJ, Spector TD. The classification and assessment of osteoarthritis. Baillieres Clin Rheumatol 1995; 9: 407-432.
5) Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001; 294: 853-858.
6) Ge DW, Wang WW, Chen HT, Yang L, Cao XJ. Functions of microRNAs in osteoporosis. Eur Rev Med Pharmacol Sci 2017; 21: 4784-4789.
7) Pu HY, Xu R, Zhang MY, Yuan LJ, Hu JY, Huang GL, Wang HY. Identification of microRNA-615-3p as a novel tumor suppressor in non-small cell lung cancer. Oncol Lett 2017; 13: 2403-2410.
8) Yin J, Zhuang G, Zhu Y, Hu X, Zhao H, Zhang R, Guo H, Fan X, Cao Y. MiR-615-3p inhibits the osteogenic differentiation of human lumbar ligamentum flavum cells via suppression of osteogenic regulators GDF5 and FOXO1. Cell Biol Int 2017; 41: 779-786.
9) Fitzgerald GK, Piva SR, Irrgang JJ. Reports of joint instability in knee osteoarthritis: its prevalence and relationship to physical function. Arthritis Rheum 2004; 51: 941-946.

10) Goldring MB, Goldring SR. Osteoarthritis. J Cell Physiol 2007; 213: 626-634.

11) Bertrand J, Cromme C, Umlauf D, Frank S, Pap T. Molecular mechanisms of cartilage remodelling in osteoarthritis. Int J Biochem Cell Biol 2010; 42: 1594-1601.

12) Carames B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M. Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. Arthritis Rheum 2010; 62: 791-801.

13) Kim HA, Blanco FJ. Cell death and apoptosis in osteoarthritic cartilage. Curr Drug Targets 2007; 8: 333-345.

14) Ambros V. The functions of animal microRNAs. Nature 2004; 431: 350-355.

15) Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. PLoS One 2008; 3: e3740.

16) Marks PH, Donaldson ML. Inflammatory cytokine profiles associated with chondral damage in the anterior cruciate ligament-deficient knee. Arthroscopy 2005; 21: 1342-1347.

17) Stannus OP, Jones G, Blizzard L, Cicuttini FM, Ding C. Associations between serum levels of inflammatory markers and change in knee pain over 5 years in older adults: a prospective cohort study. Ann Rheum Dis 2013; 72: 535-540.

18) Webb GR, Westacott CI, Elson CJ. Chondrocyte tumor necrosis factor receptors and focal loss of cartilage in osteoarthritis. Osteoarthritis Cartilage 1997; 5: 427-437.

19) Gordon CT, Tan TY, Benko S, Fitzpatrick D, Lyonnet S, Farlie PG. Long-range regulation at the SOX9 locus in development and disease. J Med Genet 2009; 46: 649-656.