European Review for Medical and Pharmacological Sciences 2018; 22: 8814-8821

MiR-143-3p regulates early cartilage differentiation of BMSCs and promotes cartilage damage repair through targeting BMPR2

J. TIAN[1,2], Y.-J. RUI[2], Y.-J. XU[2], S.-A. ZHANG[3]

[1]Soochow University, Suzhou, China
[2]Department of Orthopedics, Soochow University Affiliated Wuxi Ninth People's Hospital, Wuxi, China
[3]Orthopedics Institute, Soochow University, Suzhou, China

Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-143-3p (miR-143-3p) in cartilage injury, and to investigate the possible underlying mechanism.

MATERIALS AND METHODS: A chondrogenic differentiation cell model was established in bone marrow mesenchymal stem cells (BMSCs). The mRNA expression levels of runt-related transcription factor 2 (RUNX2), miR-143-3p and bone morphogenetic protein 2 (BMPR2) in BMSCs were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) after 0 d, 5 d and 10 d, respectively. Mesenchymal stem cells (MSCs) were transfected with miR-143-3p mimics and its control in accordance with the liposome method. Alcian blue colorimetric assay was used to evaluate proteoglycan deposition of MSCs. Meanwhile, qRT-PCR and Western blot were performed to analyze the expression levels of ACAN and COL2A1. Luciferase reporter gene assay was applied to verify the binding status of miR-143-3p and BMPR2 3'UTR. Also, proteoglycan deposition and the expression of ACAN and COL2A1 were detected after simultaneous transfection of miR-143-3p mimics and BMPR2 overexpression plasmid.

RESULTS: 0 d, 5 d and 10 d after inducing cartilage differentiation, the mRNA expression levels of RUNX2 and BMPR2 were markedly increased. However, the expression level of miR-143-3p was significantly decreased with the prolongation of induction period. After transfection with miR-143-3p mimics, the level of miR-143-3p in MSCs was remarkably increased. Alcian blue colorimetric assay and staining assay showed that the deposition of proteoglycans in the mimics group was significantly lower than that of the control group. Meanwhile, after overexpressing miR-143-3p, the levels of cartilage differentiation marker proteins including ACAN and COL2A1 were remarkably reduced. Luciferase report gene assay indicated that miR-143-3p could negatively regulate BMPR2 by binding to its 3'UTR. In addition, overexpression of BMPR2 could strikingly reverse the above effects of overexpressed miR-143-3p.

CONCLUSIONS: During chondrogenic differentiation, the level of miR-143-3p was decreased. Moreover, miR-143-3p could regulate the differentiation process by targeting BMPR2 in BMSCs.

Key Words:
Mesenchymal stem cells, Chondrocyte differentiation, MiR-143-3p, BMPR2.

Introduction

Age-related cartilage wear and degeneration, as well as cartilage damage caused by various traumas and sports injuries have increased gradually. It has also become a common orthopedic disease. Cartilage damage, mainly characterized by joint pain and dysfunction, can be resulted from a variety of reasons such as joint disease or trauma. Meanwhile, in severe cases, cartilage damage may lead to partial or complete loss of joint function[1]. Due to less blood supply and weak intrinsic repair ability, the repairing ability of adult articular cartilage is very limited. When the wound diameter is over than 4 mm[2], it can't be spontaneously repaired. The treatments of articular cartilage injury include subchondral bone plate drilling, micro-fracture, arthroplasty, periosteal perichondrium transplantation, autologous chondrocyte transplantation and others. Although some effects have been achieved, the above treatments cannot help to produce tissues with morphological, functional, and mechanical properties as normal cartilage[3].

Corresponding Author: Jian Tian, MD; e-mail: tianjian828@163.com

Mesenchymal stem cells (MSCs) belong to multi-tissue-derived adult stem cells derived from mesoderm. MSCs have the ability of self-proliferation and renewal, which can also differentiate into chondrocytes, osteoblasts, and adipocytes[4,5]. Certain specific biological factors, such as transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), and insulin-like growth factor (IGF) can induce MSCs to differentiate into chondrocytes, ultimately forming cartilage-like tissues[6]. The process of cartilage regeneration involves the regulation of multiple transcription factors, cell growth factors and related signaling pathways, including BMP, IGF, Wnt, Indian hedgehog (IHH), and parathyroid hormone-related peptides (PTHrP)[7]. Studies have shown that bone morphogenetic protein 2 (BMPR2) belongs to the transforming growth factor superfamily. BMPR2 plays an important role in a series of biological processes such as cell proliferation, differentiation and apoptosis. Meanwhile, it can induce osteogenetic and chondrogenic differentiation of stem cells *in vitro*[8]. Inducing chondrogenic differentiation of BMRP2 and inhibiting its osteogenic differentiation and endochondral bone formation are helpful methods for inducing chondrogenic differentiation of MSCs, promoting cartilage formation and maintaining the phenotype of chondrocytes.

MicroRNAs (miRNAs) are a type of endogenous, short-chain non-coding RNAs with 18-25 nucleotides in length. Researchers have shown that miRNAs can promote the degradation of target mRNAs and inhibit relevant protein expression levels. Target genes may affect multiple biological processes such as cell proliferation, division, differentiation, metabolism, and apoptosis[9]. A large number of miRNAs can regulate the process of cartilage differentiation by regulating certain transcription factors such as Sox9, growth factors like TGF-β, BMP2, as well as fibroblast growth factors (FGF)[10,11]. Among differentially expressed miRNAs, the process of cartilage differentiation regulated by them is totally different. For example, miR-495 and miR-145 can down-regulate the expression of target gene Sox9, eventually leading to decreased mRNA levels of chondrocyte marker genes including Col2a1, Acan (proteoglycan), Col9a2, and Col11a1[12]. However, miR-335-5p promotes the differentiation of MSCs through targeting Daam1 and ROCK1. Both of the two molecules are negative regulators of Sox9[13]. Due to the complexity of miRNA function, the exact role of miRNAs in regulating chondrogenic differentiation still remain unclear. Moreover, the effect of miRNA-143-3p on chondrogenic differentiation of MSCs has not been reported yet.

Materials and Methods

Isolation and Culture of BMSCs

All animal experiments were approved by the local Ethics Committee. Totally 10 6-week-old male Norwegian brown rats weighing 100-120 g were sacrificed by CO_2. Subsequently, hind legs were collected, and bone tissue (tibia, femur) was separated. MSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator. When the cells grew to a density of 80-90%, cell passage was performed at a ratio of 1:2. Purified MSCs were collected after 3 subcultures. Trypsin was added to 10% FBS L-DMEM, meanwhile, third generation of purified MSCs were digested and collected. The concentration of MSCs was adjusted to 2 x 10^5 cells/mL. This study was approved by the Animal Ethics Committee of Soochow University Animal Center.

Induction of Chondrogenic Differentiation

MSCs were cultured in bone marrow mesenchymal stem cell chondrocyte differentiation medium (MCDM) for 0, 5 and 10 d, respectively. Then the cells were collected for subsequent experiments.

Cell Transfection

Before transfection, cells were seeded into 6-well plates. 10 μL NC/miR-143-3p mimics, 1 μg BMPR3 plasmid and 5 μL lipo2000 (Invitrogen, Carlsbad, CA, USA) were diluted to 250 μL serum-free culture medium, respectively. Then, the three mixtures were incubated at room temperature for 5 min. Subsequently, the former two solution were slowly mixed with the latter one, followed by incubation for 20 min. Finally, the mixture was added in cells. 6 h after, the cells were replaced with complete medium and cultured for 48 h.

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

RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform, and isopropanol.

Then, the concentration of extracted RNA was measured and stored at -80°C until use. Subsequently, extracted total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA). 1 μL cDNA sample was used, and SYBR Green method was applied for qRT-PCR detection. Primers used in this study were as follows: BMPR2 (F: 5'-CACTACGGCTGCTTCCCAGAAT-3', R 5'-TCCCACAGACCATAACATGTGC-3'); RUNX2 (F: 5'-CCATCAGCGTCAACACCA-3', R 5'-AGCCACCTTTACTTACACCC-3'); COL2A1 (F: 5'-ATCGCCACGGTCCTACAATG-3', R 5'-GGCCCTAATTTTCGGGCATC-3'); ACAN (F: 5'-GAAGTGGCGTCCAAACCAAC -3', R 5'-AGCTGGTAATTGCAGGGGAC -3'); miR-143-3p (F: 5'-GTGATAATGTTTAGGGATTTT-3', R: 5'-AAA TTAGGCTACATTATCAC-3').

Alcian Blue Colorimetry

After culture in inducing medium for 10 days, the content of proteoglycan in MSCs was determined by Alcian blue colorimetry. Culture medium in each group was discarded, and the cells were washed with phosphate-buffered saline (PBS) for 3 times. 100 μL cell lysate was added to each well, followed by centrifugation and supernatant collection. Subsequently, 0.5 mL 5 mg/L trypsin was added in the supernatant. After 24 h, 0.5 mL 5 mg/L papain was added to hydrolyze for 24 h. Then, 1 mL sample solution was added to the test tube, and 1.5 mL 1.4 g/L Alixin blue dye solution was added. After 10 min, the absorbance values of each group were measured at the wavelength of 620 nm.

Western Blot

After culture in MCDM medium for 10 d, MSCs of each group were first washed with PBS. Then, cell lysates were transferred to EP tubes after lysis. The cells were pulverized using ultrasonication, and the supernatant was collected after centrifugation. A total of 2 μL protein sample was used for concentration measurement. After mixing with 5×sodium dodecyl sulphate (SDS) and boiling, protein samples were separated by polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk at room temperature for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. After washing with Tris-Buffered Saline with Tween 20 (TBST) (Beyotime, Shanghai, China) for three times, the membranes were incubated with secondary antibody at 37°C for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Alcian Blue Dyeing

The culture medium was discarded, and the cells were fixed with 4% paraformaldehyde for 20 min. After washing twice with PBS, the cells were co-incubated with Alcian blue dye solution for about 30 min. Then, the cells were washed with distilled water for 5-10 min. Subsequently, the staining results of each treatment group were observed and captured. Finally, the differences between groups were compared.

Luciferase Reporter Gene Assay

The full-length sequence of wild-type 3' untranslated region (UTR) of BMPR2 and the mutant was amplified. Both the sequences were verified by DNA sequencing. Briefly, 100 ng vector and 200 nmol/l miR-143-3p mimics or NC were co-transfected into cells. 48 h after transfection, the cells were harvested. Finally, the activity of luciferase and Renilla were measured by dual-luciferase reporter gene assay (Promega, Madison, WI, USA). The experiment was repeated three times independently.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation (SD). The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-143-3p Was Lowly Expressed During Cartilage Differentiation of MSCs

MSCs were induced by bone marrow MSC chondrocyte differentiation medium (MCDM) for 0d, 5d, and 10d, respectively. Then the level of RUNX2 was detected by qRT-PCR. Results showed that BMSCs differentiated into early chondrocytes, and the mRNA level of RUNX2 gradually increased on 0, 5, and 10 d of chondrocyte differentiation (Figure 1A). Meanwhile, miR-143-3p was gradually down-regulated at these time points (Figure 1B). Previous studies have

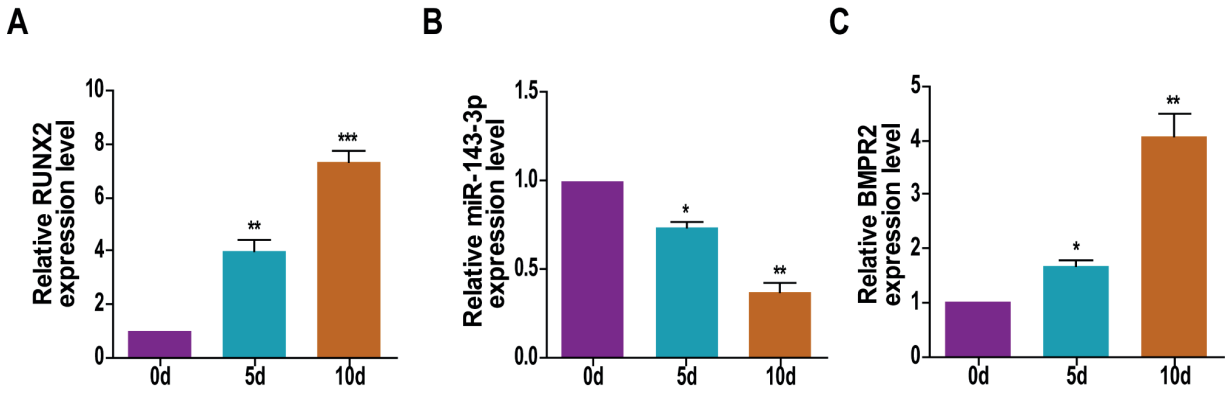

Figure 1. Expression of miR-143-3p in MSCs during cartilage differentiation. ***A,*** During the process of cartilage differentiation, the mRNA expression level of RUNX2 in MSCs was significantly increased with the prolongation of induction days. ***B,*** The expression level of miR-143-3p was markedly decreased with the prolongation of induction days. ***C,*** The mRNA expression level of BMPR2 was remarkably increased with the prolongation of induction days.

shown that BMPR2, a BMP ligand, is involved in endochondral bone formation and embryonic development. Hence, we examined the expression level of BMPR. Results found that BMPR expression increased with the prolongation of induction time, which was opposite to the level of miR-143-3p (Figure 1C).

Overexpression of MiR-143-3p Inhibited Proteoglycan Deposition As Well As the Expression of ACAN and COL2A1 in MSCs

To determine whether miR-143-3p mediated early chondrogenic differentiation, we transfected miR-143-3p mimics into MSCs. QRT-PCR results demonstrated that the expression of miR-143-3p was significantly increased (Figure 2A). Then, Alcian blue staining was performed to evaluate proteoglycans in MSCs in early cartilage differentiation. Results indicated that, compared with the control group, polysaccharide deposition of the miR-143-3p overexpression group was markedly reduced (Figure 2B). Cells stained with Alcian blue also confirmed this result (Figure 2C). Subsequently, qRT-PCR and Western blot were performed to assess the effect of miR-143-3p overexpression on the expression level of ACAN and COL2A1. Of note, we found that both the mRNA and protein expression levels of ACAN and COL2A1 were significantly reduced in the miR-143-3p overexpression group (Figure 2D, 2E).

BMPR2 Was Confirmed As a Target Gene of MiR-143-3p

To further investigate the possible mechanism of miR-143-3p inhibiting early cartilage differentiation of MSCs, we explored the target genes of miR-143-3p. Bioinformatics analysis found that BMPR2 was the target gene of miR-143-3p. Then, a dual-luciferase reporter vector containing wild-type/mutant BMPR2 3'-UTR (Figure 3A) and miR-143 -3p mimics/negative control (NC) were co-transfected into MSCs. The luciferase activity was detected 48 h after transfection. Results demonstrated that, when miR-143-3p was present, the luciferase activity of MSCs transfected with wild-type BMPR2 3'-UTR reporter gene remarkably reduced. When transfected with the vector containing mutant BMPR2 3'-UTR, no significant decrease in luciferase activity was observed even if miR-143-3p was present (Figure 3B). In addition, up-regulated miR-143-3p was found to down-regulate the expression of BMPR2 both on mRNA and protein level (Figure 3C, 3D), suggesting that miR-143-3p could bind to BMPR2 and inhibit its expression.

Overexpression of BMPR2 Could Reverse the Increase of Proteoglycan Deposition as Well as the Level of ACAN and COL2A1 Induced by MiR-143-3p Overexpression

After 10 d of culture in chondrogenic differentiation medium, the mRNA levels of ACAN and COL2A1 were remarkably elevated in the miR-143-3p&BMPR2 group when compared with the miR-143-3p overexpression group alone (Figure 4A). To determine whether miR-143-3p affected proteoglycan deposition of MSCs *via* BMPR2, we co-transfected miR-143-3p mimics and BMPR2 overexpression plasmids into MSCs. Staining results suggested that the deposition of proteoglycans in the miR-143-3p&BMPR2 group was

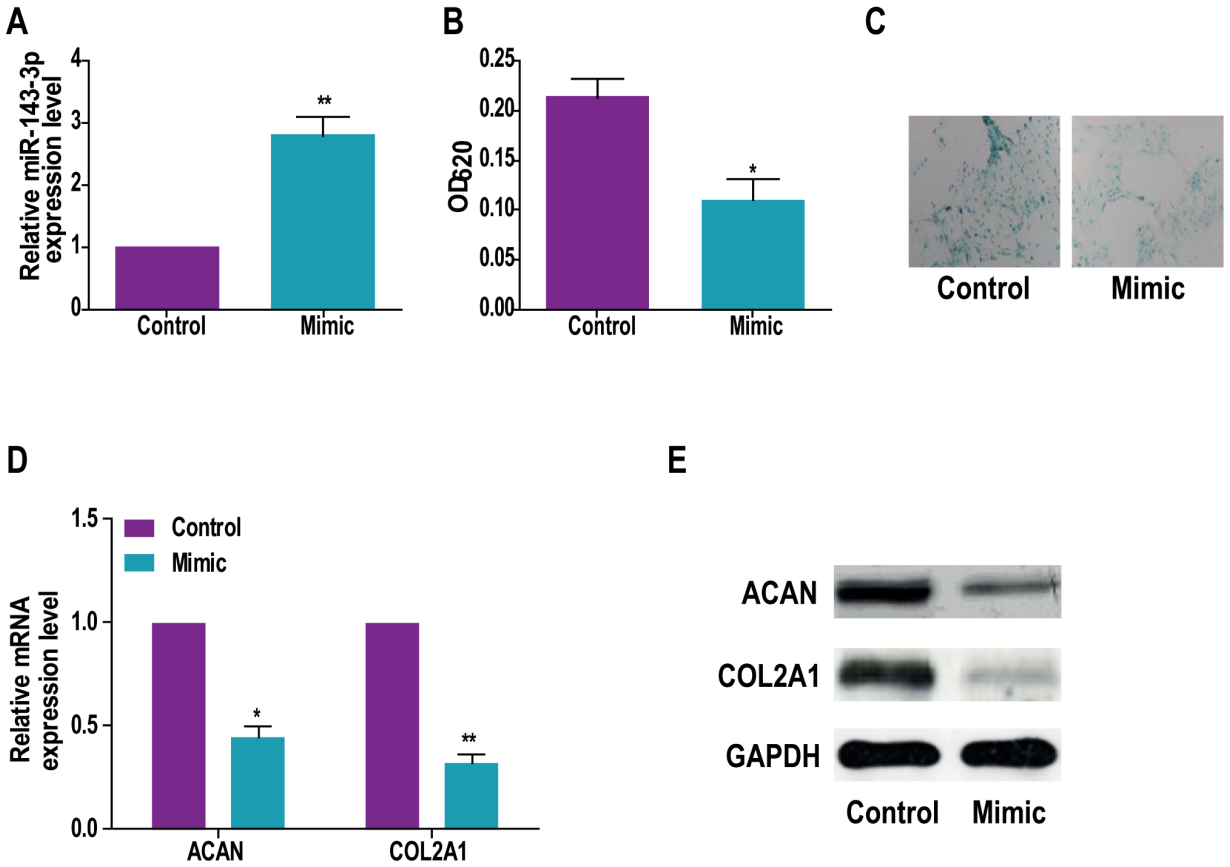

Figure 2. MiR-143-3p inhibited chondrogenic differentiation of MSCs. ***A,*** After overexpression of miR-143-3p, the expression level of miR-143-3p was significantly increased. MSCs were cultured in mesenchymal stem cell chondrogenic differentiation medium for 10 d. ***B,*** The absorbance value of dissolved Alcian blue was significantly decreased at 620 nm; ***C,*** Alcian blue staining in MSCs was remarkably reduced after 10 d of culture in differentiation medium; ***D,*** The mRNA levels of ACAN and COL2A1 were significantly decreased. ***E,*** The protein levels of ACAN and COL2A1 were obviously reduced.

more than that of the miR-143-3p overexpression group (Figure 4B). These results demonstrated that the up-regulation of BMPR2 could reverse the inhibitory effect of overexpressed miR-143-3p on proteoglycan deposition as well as ACAN and COL2A1 expression.

Discussion

MSCs can differentiate into different tissues, including bone, cartilage, and adipose tissue. Currently, MSCs are commonly used as seed cells for bone and cartilage tissue engineering, which have also exerted broad application prospects in regenerative medicine[14]. With the advantages of simple and convenient acquisition and culture, bone marrow-derived MSCs are most widely used and studied[15]. Previous studies have shown that chondrogenic differentiation of bone marrow MSCs is mainly regulated by the transcription factor Sox9, which induces the expression of COL2A and chondroitin sulfate. Meanwhile, chondrogenic differentiation of MSCs is a complex biological process involving numerous transcription factors, cytokines and signaling pathways[16].

MiRNAs regulate transcription factors and signaling pathways involved in cartilage differentiation at the post-transcriptional level, thereby regulating stem cell chondrogenic differentiation[17]. Differences in target genes regulated by miRNAs may lead to totally different roles in cartilage differentiation. Among differentially expressed miRNAs, miR-335-5p, miR-140 and miR-23b can positively regulate the chondrogenic differentiation of MSCs by regulating the expression of Sox9 and PKA genes[18,19]. In addition, a recent study[20] have indicated that miR-410 can

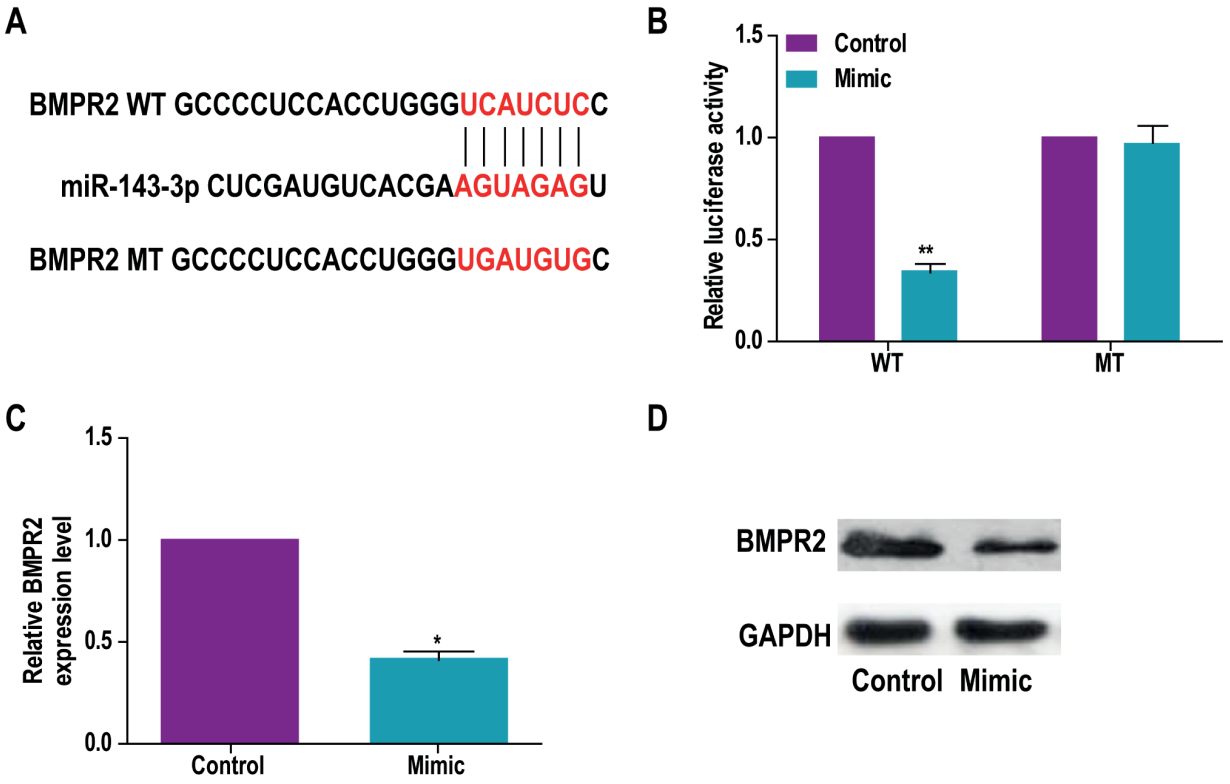

Figure 3. BMPR2 was confirmed as the target gene of miR-143-3p. ***A,*** Target gene prediction website predicted the presence of binding sites for BMPR2 and miR-143-3p. ***B,*** Luciferase reporter gene assay found that wild-type BMPR2 could quench fluorescence. ***C,*** After overexpression of miR-143-3p, the mRNA level of BMPR2 was significantly decreased. ***D,*** After overexpression of miR-143-3p, the protein level of BMPR2 was significantly decreased.

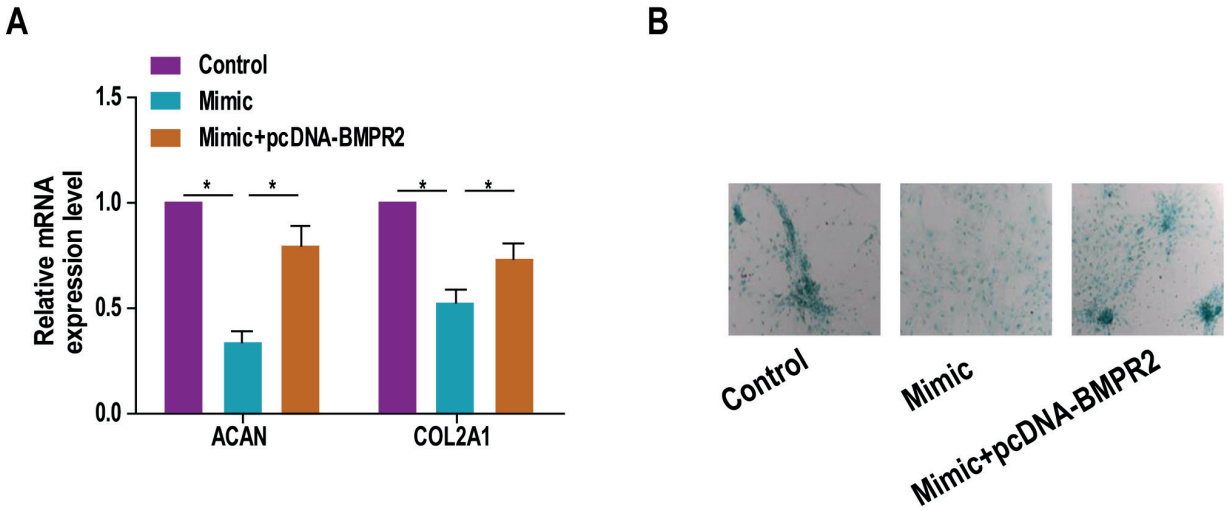

Figure 4. Overexpression of BMPR2 could reverse the inhibitory effect caused by miR-143-3p up-regulation. ***A,*** Overexpression of BMPR2 could reverse the decrease of ACAN and COL2A1 expression levels caused by miR-143-3p up-regulation. ***B,*** Overexpression of BMPR2 could reverse the decrease of Alcian blue staining caused by miR-143-3p overexpression.

promote cartilage differentiation of human BMSCs by down-regulating Wnt3a. MiRNAs, such as miR-29a, miR-29b, miR-495, and miR-193b, can ultimately inhibit stem cell differentiation in early stage by regulating target genes including Foxo3A, Sox9, and TGF-β2. These findings un-

doubtedly reveal that miRNAs also play a vital role in cellular events of cartilage formation.

BMPR2, the receptor of BMP, is a member of the transmembrane serine/threonine kinase BMP receptor family. These ligands of BMPII receptor BMPR2 can be served as targets of miRNAs in cartilage formation. It exerts a cartilage effect by activating Sox9 (cartilage key factor) *via* the Smad1/5/8 and P38 signaling pathways[21-23]. It has been reported that miR-99a can regulate the chondrogenic differentiation of rat MSCs in early stage through binding to BMPR2[24]. Therefore, we detected the expression of BMPR2 during the chondrogenic differentiation of MSCs. Results showed that the expression of BMPR2 was opposite to that of miR-143-3p. MiRNAs can induce the degradation of target genes through base pairing with the 3'-UTR of target mRNAs, thereby inhibiting mRNA translation. Furthermore, luciferase reporter gene assay showed that miR-143-3p could target to BMPR2 and inhibit its expression. Proteoglycan is an important component of cartilage. Alcian blue staining indicated that the up-regulation of miR-143-3p suppressed proteoglycan deposition of MSCs, which could be reversed by BMPR2 overexpression. Moreover, COL2A1 and ACAN are specific marker proteins of hyaline cartilage. Both of the two molecules were found remarkably decreased after miR-143-3p overexpression. However, simultaneous overexpression of BMPR2 could reverse the above changes.

Conclusions

We showed that miR-143-3p was down-regulated during early cartilage differentiation of BMSCs. Meanwhile, it could inhibit the deposition of proteoglycans as well as the expression of ACAN and COL2A1 in MSCs by targeting BMPR2, thus regulating early chondrogenic differentiation of stem cells.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Khan IM, Francis L, Theobald PS, Perni S, Young RD, Prokopovich P, Conlan RS, Archer CW. In vitro growth factor-induced bio engineering of mature articular cartilage. Biomaterials 2013; 34: 1478-1487.
2) Nettles DL, Elder SH, Gilbert JA. Potential use of chitosan as a cell scaffold material for cartilage tissue engineering. Tissue Eng 2002; 8: 1009-1016.
3) Madeira C, Santhagunam A, Salgueiro JB, Cabral JM. Advanced cell therapies for articular cartilage regeneration. Trends Biotechnol 2015; 33: 35-42.
4) Gao XL, Cao MG, Ai GG, Hu YB. Mir-98 reduces the expression of HMGA2 and promotes osteogenic differentiation of mesenchymal stem cells. Eur Rev Med Pharmacol Sci 2018; 22: 3311-3317.
5) Jiang T, Kai D, Liu S, Huang X, Heng S, Zhao J, Yu CB, Loh XJ, Zhu Y, Mao C, Zheng L. Mechanically cartilage-mimicking poly(PCL-PTHF urethane)/collagen nanofibers induce chondrogenesis by blocking NF-kappa B signaling pathway. Biomaterials 2018; 178: 281-292.
6) Blunk T, Sieminski AL, Gooch KJ, Courter DL, Hollander AP, Nahir AM, Langer R, Vunjak-Novakovic G, Freed LE. Differential effects of growth factors on tissue-engineered cartilage. Tissue Eng 2002; 8: 73-84.
7) Pathi S, Rutenberg JB, Johnson RL, Vortkamp A. Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. Dev Biol 1999; 209: 239-253.
8) Urata M, Kokabu S, Matsubara T, Sugiyama G, Nakatomi C, Takeuchi H, Hirata-Tsuchiya S, Aoki K, Tamura Y, Moriyama Y, Ayukawa Y, Matsuda M, Zhang M, Koyano K, Kitamura C, Jimi E. A peptide that blocks the interaction of NF-kappaB p65 subunit with Smad4 enhances BMP2-induced osteogenesis. J Cell Physiol 2018; 233: 7356-7366.
9) Vicente R, Noel D, Pers YM, Apparailly F, Jorgensen C. Deregulation and therapeutic potential of microRNAs in arthritic diseases. Nat Rev Rheumatol 2016; 12: 496.
10) Chang T, Xie J, Li H, Li D, Liu P, Hu Y. MicroRNA-30a promotes extracellular matrix degradation in articular cartilage via downregulation of Sox9. Cell Prolif 2016; 49: 207-218.
11) Chen J, Cui L, Yuan J, Zhang Y, Sang H. Circular RNA WDR77 target FGF-2 to regulate vascular smooth muscle cells proliferation and migration by sponging miR-124. Biochem Biophys Res Commun 2017; 494: 126-132.
12) Martinez-Sanchez A, Dudek KA, Murphy CL. Regulation of human chondrocyte function through direct inhibition of cartilage master regulator SOX9 by microRNA-145 (miRNA-145). J Biol Chem 2012; 287: 916-924.
13) Wang Y, Yang T, Zhang Z, Lu M, Zhao W, Zeng X, Zhang W. Long non-coding RNA TUG1 promotes migration and invasion by acting as a ceRNA of miR-335-5p in osteosarcoma cells. Cancer Sci 2017; 108: 859-867.
14) Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, Meeusen S, Althage A, Cho CY, Wu X, Schultz PG. A stem cell-based approach to cartilage repair. Science 2012; 336: 717-721.
15) Barry F, Murphy M. Mesenchymal stem cells in joint disease and repair. Nat Rev Rheumatol 2013; 9: 584-594.

16) Luchetti F, Canonico B, Bartolini D, Arcangeletti M, Ciffolilli S, Murdolo G, Piroddi M, Papa S, Reiter RJ, Galli F. Melatonin regulates mesenchymal stem cell differentiation: a review. J Pineal Res 2014; 56: 382-397.

17) Zhao W, Zhang S, Wang B, Huang J, Lu WW, Chen D. Runx2 and microRNA regulation in bone and cartilage diseases. Ann N Y Acad Sci 2016; 1383: 80-87.

18) Lin X, Wu L, Zhang Z, Yang R, Guan Q, Hou X, Wu Q. MiR-335-5p promotes chondrogenesis in mouse mesenchymal stem cells and is regulated through two positive feedback loops. J Bone Miner Res 2014; 29: 1575-1585.

19) Karlsen TA, Jakobsen RB, Mikkelsen TS, Brinchmann JE. microRNA-140 targets RALA and regulates chondrogenic differentiation of human mesenchymal stem cells by translational enhancement of SOX9 and ACAN. Stem Cells Dev 2014; 23: 290-304.

20) Zhang Y, Huang X, Yuan Y. MicroRNA-410 promotes chondrogenic differentiation of human bone marrow mesenchymal stem cells through down-regulating Wnt3a. Am J Transl Res 2017; 9: 136-145.

21) Broege A, Pham L, Jensen ED, Emery A, Huang TH, Stemig M, Beppu H, Petryk A, O'Connor M, Mansky K, Gopalakrishnan R. Bone morphogenetic proteins signal via SMAD and mitogen-activated protein (MAP) kinase pathways at distinct times during osteoclastogenesis. J Biol Chem 2013; 288: 37230-37240.

22) Harper RL, Reynolds AM, Bonder CS, Reynolds PN. BMPR2 gene therapy for PAH acts via Smad and non-Smad signalling. Respirology 2016; 21: 727-733.

23) Broege A, Pham L, Jensen ED, Emery A, Huang TH, Stemig M, Beppu H, Petryk A, O'Connor M, Mansky K, Gopalakrishnan R. Bone morphogenetic proteins signal via SMAD and mitogen-activated protein (MAP) kinase pathways at distinct times during osteoclastogenesis. J Biol Chem 2013; 288: 37230-37240.

24) Zhou X, Wang J, Sun H, Qi Y, Xu W, Luo D, Jin X, Li C, Chen W, Lin Z, Li F, Zhang R, Li G. MicroRNA-99a regulates early chondrogenic differentiation of rat mesenchymal stem cells by targeting the BMPR2 gene. Cell Tissue Res 2016; 366: 143-153.

25) Zhou X, Wang J, Sun H, Qi Y, Xu W, Luo D, Jin X, Li C, Chen W, Lin Z, Li F, Zhang R, Li G. MicroRNA-99a regulates early chondrogenic differentiation of rat mesenchymal stem cells by targeting the BMPR2 gene. Cell Tissue Res 2016; 366: 143-153.