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Original Article

MiR-181a regulates the chondrogenic differentiation in pig peripheral blood mesenchymal stem cells

Daohong Zhao[1], Yanlin Li[2], Yan Li[3], Zhaowei Jiang[3], Duo Shen[4], Zhi Zhao[1], Fuke Wang[2]

[1]Department of Orthopedics, Second Affiliated Hospital of Kunming Medical University, Kunming, China; [2]Department of Sports Medicine, First Affiliated Hospital of Kunming Medical University, Kunming, China; [3]Department of Orthopedics, People Hospital of Dehong State, Yunnan Province, China; [4]Department of Orthopedics, People Hospital of Longchuan County, Yunnan Province, China

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Abstract: Articular cartilage injury and therapy are important clinical issues around the world. Mesenchymal stem cells (MSCs) have the ability to differentiate into chondrocytes, which makes MSCs good candidates for use in cartilage repairing. However the regulation and the mechanism of chondrogenesisin MSCs is still unclear. To clarify the factor and mechanism which contribute to the process of chondrogenic differentiation, we focus on miRNAs. Considering the role of miR-181a in chondrogenesis and osteoblast formation, we tested the expression of miR-181a in the induced chondrogenic differential pig PBMSCs by using qRT-PCR. And we identified miR-181a as an up-regulated miRNA in the TGF-β3-induced pig PBMSCs chondrogenic differentiation from the early stages and maintained elevated throughout the whole process. After inhibition of the endogenesis miR-181a expression by transfecting the miR-181a inhibitor, the western-blot results and immunofluorescence results indicated that the expression of differentiation-related protein COL2A1, BMP2 were decreased, together with the Alcian blue assay, proving the process of differentiation was inhibited significantly. Taken together, our results demonstrated that miR-181a might be necessary in chondrogenesis of MSCs. Even so, the mechanism of miR-181a on regulating the chondrogenesis still needed to be investigated in future work. And our data would provide an experimental evidence for the research of tissue engineering.

Keywords: Chondrogenesis, miR-181a, mesenchymal stem cells

Introduction

Mesenchymal stem cell (MSCs) were first identified and described by Friedenstein as a type of plastic-adherent, fibroblast-like cells and isolated from bone marrow [1]. Following the initial discovery, various studies have demonstrated that MSCs possess the potency of self-renewal [2] and multipotential differentiation, such as fat, tendon, cartilage, and bone [3-7]. Fu et al successfully isolated MSCs from mobilized peripheral blood (PB) of New Zealand White rabbits and found that PBMSCs share certain similar biological characteristics in vitro and chondrogenesis in vivo as BM MSCs, which makes PBMSCs a new source of seed cells used in articular cartilage repair [8].

Chondrogenesis is an essential process controlled by numerous environmental and endocrine factors in cartilage and bone development [9-14]. Although various signaling pathways, such as TGF-β, fibroblast growth factor, and Indian hedgehog, involved in chondrogenesis have already been defined, the other important factor and mechanisms promoting chondrogenesis process are worth to be elucidated, continuously.

microRNAs (miRNAs) are endogenous small noncoding ~22 nt RNAs and exert vital regulating functions in multiple organisms via negatively regulating the expression of target genes at the post-transcriptional level [15]. And they have been found to be involved in various fundamental physiological and pathological processes, such as cell proliferation [16, 17], apoptosis [18, 19], immunoresponses [20] and differentiation [21, 22]. The importance of miRNAs in skeletal development was initially demonstrated by studies deleting Dicer in skeletal cells in vivo [23]. In recent years, a mass of

microRNAs have been experimentally validated as key regulators in chondrogenesis. And there are an increasing number of studies have focused on the mechanisms of microRNAs regulation in chondrogenic differentiation of MSCs. miR-140 plays an important role in both cartilage development and homeostasis via regulating its downstream target genes, HDAC4 and Smad3 [24-27]. Large scale miRNA screening identifies that miR-574-3p up-regulated during chondrogenesis in MSCs. Furthermore, MiR-574-3p expression increases at early stage of chondrogenesis, and maintains at an elevated level throughout differentiation which exhibited a similar expression pattern to that of miR-140 [28]. Paik et al discovermiR-449 negatively regulates chondrocyte differentiation of MSCs [29]. Some miRNAs and their target genes may form a feedback loop, as miR-335 decreases-Rock1 and Daam1 to increase Sox9, which in turn increases Mest and miR-335 transcription by suppressing miR-29a and miR-29b [30]. There are some other microRNAs such as miR-24, miR-199b, miR-101, miR-124a, miR-199a, miR-18, miR-96 [31, 32], and miR-145 [33] were proved to regulate lineage determination during MSC differentiation. However, more evidences of the roles of miRNAs in regulating chondrogenic differentiation in PBMSCs are needed.

Previous studies show that miR-181a is involved in bone formation. miR-181a is upregulated upon osteoblast differentiation [34] and Bhushan et al provide evidence that miR-181 miRNAs (miR181a, b, c and d) promote osteoblast differentiation by downregulating TGF-β signaling [35]. It was also reported that chicken chondrocytes abundantly express miR-181a. MiR-181a can repress expression of Ccna2 (encoding for cyclin A2) and Acan, which may act as a negative feedback for cartilage homeostasis [36].

So in this study, to determine the role of miRNAs in chondrogenic differentiation of PBMSCs, we focused on miR-181a. Here, we showed that miR-181a had an important role in promoting chondrogenic differentiation of the pig peripheral blood mesenchymal stem cells. Importantly, suppression of miR-181a resulted in inhibiting chondrogenic differentiation. Accordingly, we identified for the first time that miR-181a acts as a key mediator to promote early chondrogenic differentiation.

Methods

Isolation of peripheral blood-derived mesenchymal stem cells (PBMSCs)

Peripheral blood (30 ml) was harvested from small-ear pigs (12-15 kg) which were provided by the center of laboratory animal science of Kunming University, China, collected in 5 ml vacuum collection tubes with sodium heparin, and diluted immediately with D-Hank's solution (Sigma) in a 1:1 proportion. The diluted blood was gently loaded onto Ficoll density gradient (GE Health care) in 10 ml tubes and centrifuged for 30 min at 1600 g at room temperature. Mononuclear cell fraction was collected and rinsed three times with D-Hank's solution, and then cultured in serum-free medium (Advcell) and incubated at 37°C with 5% CO_2 in a humidified incubator. The medium was replaced every three days. This study protocol was approved by the Animal Ethics Committee of Kunming University, China.

Flow cytometric analysis of the immunophenotyping of PBMSCs

The following antibodies conjugated to different fluorochrome were used to perform flow cytometric analysis on P3 PBMCs: PE-anti-CD44 (BD Biosciences), FITC-anti-CD90 (BD Biosciences), Biotin-anti-CD105 (BD Biosciences), APC-anti-CD45 (BD Biosciences), and PerCP-anti-CD34 (BD Biosciences). The harvested P3PBMSCs were washed with cold PBS, blocked with 1% BSA (Amresco), and then incubated with antibodies at 4°C for 30 min. After washing by PBS three times, all cells were analyzed on FACScan flow cytometer.

In vitro chondrogenic differentiation of PBMSCs

For chondrogenesis, P3 PB-MCSs were plated at 2×10^4 cells/cm^2 in 24-well plates and induced underosteogenic conditions (Advcell serum-free medium with 10^{-7} M dexamethasone, 50 μM L-ascorbic acid-2-phosphate, 10 ng/ml TGF-β3, 1% insulin-transferrin-selenium, 5 mM sodium pyruvate, 40 μg/ml L-proline, and 1% non-essential amino acid) for 14 days. CCK-8 kit was used to incubate with PB-MCSs according to the manufacturer's instructions. Cell viability was assessed by measurement

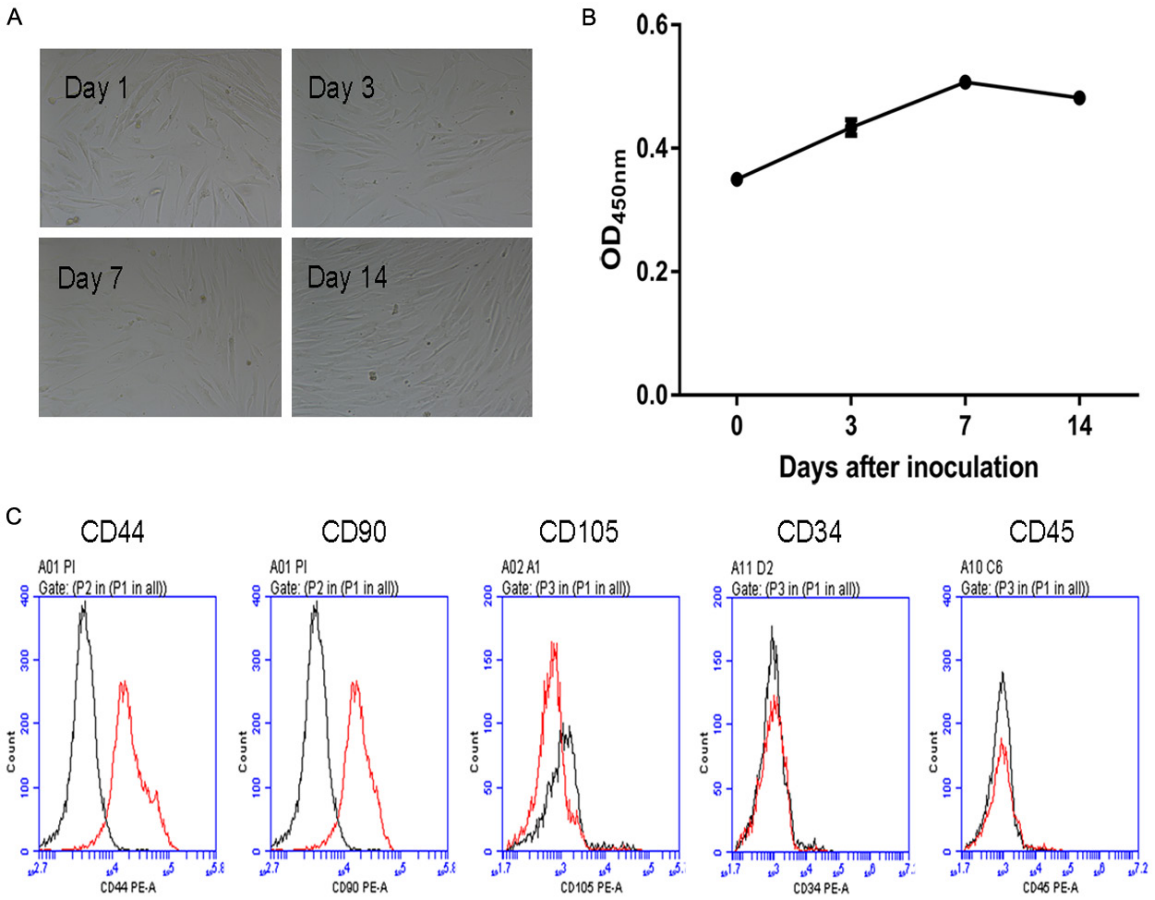

Figure 1. The characterization of cultured PBMCs. A. The chondrocyte of cultured PBMCs was observed by inverted phase contrast microscope at day 1, day 3, day 7, and day 14. B. The proliferation assay of cultured PBMCs. C. Cultured PBMSCs were immunostained with antibodies for CD44, CD90, CD105, CD34, and CD45. The stained cells were analyzed using flow cytometry to detect the surface markers specific for PBMSCs.

of absorbance at 450 nm using a microplate reader.

Transfection assay

To demonstrate the function relevance ofmiR-181a, miR-181a inhibitor or its negative control (GenePharm, Shang) was transfected, respectively, into induced-differentiation PBMSCs with Lipofectamine 2000 transfection agent following the manufacturer's instruction.

Alcian blue stain

To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures were collected at indicated time points (day 3, day 7 and day 14) of induction and sulfated cartilage glycosaminoglycans (GACs) were measured by alcian blue staining. The pellets for alcian blue staining were routinely fixed by 4% paraformaldehyde, dehydrated and paraffin imbedded. 5 μm sections were stained by 0.5% alcian blue for 20 min. The stained pellet sections were mounted and evaluated microscopically.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, subsequently washed twice with PBS, blocked with 5% BSA and 0.1% Triton X-100 in PBS and proceeded to immunocytochemistry with primary antibodies against BMP2 (Abcam) or COL2A1 (Abcam). Alexa-647-conjugated secondary antibodies (RICKY) were used. Nuclei were counter-stained with DAPI (Thermo Fisher Scientific) and visualized using the confocal microscope (OLYMPUS).

Isolation of RNA and quantitative RT-PCR

Total RNA was isolated using Trizol Agent (Invitrogen), and miRNAs were reverse transcribed using MirX™ microRNA First-Strand Synthesis kit (Clontech). cDNA were synthe-

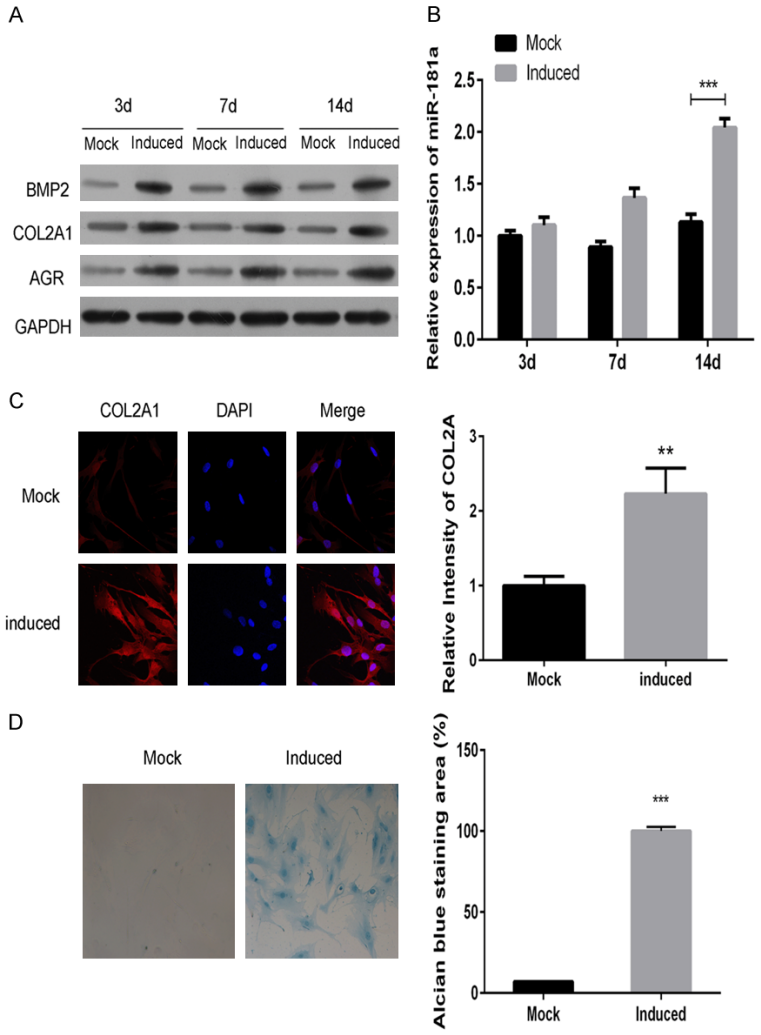

Figure 2. MiR-181a is up-regulated during TGF-β3-induced pig PBMSCs chondrogenic differentiation. Pig PBMSCs were treated with TGF-β3. A. After 3 days, 7 days and 14 days of treatment, the expression of chondrogenic differentiation markers, such as BMP2, COL2A1, and AGR were measured via western-blot. B. After 3 days, 7 days and 14 days of treatment, the expression ofMiR-181a was measured via qRT-PCR. C. After 14 days of treatment, the expression of chondrogenic differentiation markers COL2A1 was measured via immunofluorescence. D. After 14 days of the treatment, the differentiated cells were measured by alcian blue staining.

sized from miRNAs was quantified using SYBR Green qPCR master Mix (Bestar). The primer sequences were 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTGAGTT-3' and 5'-ACACTCCAGCTGGGAACATTCAACGCTGTCGG-3'. The relative abundance of miR-181a was normalized to the expression of a U6 and calculated using the ΔΔCt method.

Western blot analysis

Cells lysates were prepared using RIPA buffer (Beyotime Biotechnology) for 30 minutes on ice, and the protein concentration was quantified using BCA protein assay kit (Thermo). The samples (30-50 μg protein) were separated by 10% polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, USA). The membranes were blocked with 5% BSA (Amresco, USA), and incubated with specific antibodies followed by incubation with HRP-conjugated secondary immunoglobulin antibodies (BOSTER). The primary antibodies used in the studies are as follows: GAPDH (Abcam), BMP2 (Abcam), COL2A1 (Abcam), AGR (Abcam). ECL chromogenic substrate (Millipore) was used and signals were recorded on X-ray film. GAPDH antibody was taken as loading control.

Statistical analysis

The statistical analysis for the results was carried out using the Student's t test, and the data were expressed as the mean ± standard deviation. Values of $P<0.05$ or 0.01 were considered statistically significant.

Results

Characterization of cultured PBMSCs

The freshly cultured pig PB-MCSs appeared spindle shape after the initial 3 days. After the initial 3 days, the PBMSCs changed to typical polymorphic fibroblast-like morphology. After being subcultured every 3 days, the cells appeared to be a relatively homogeneous morphology (**Figure 1A**). To confirm whether PBMSCs cultured up to passages 3 have characteristics of general MSCs, the proliferation of PBMSCs was analyzed using CCK-8 assay. And it showed that PBMSCs at passage 3 grew quickly during the initial 7 days, after the initial

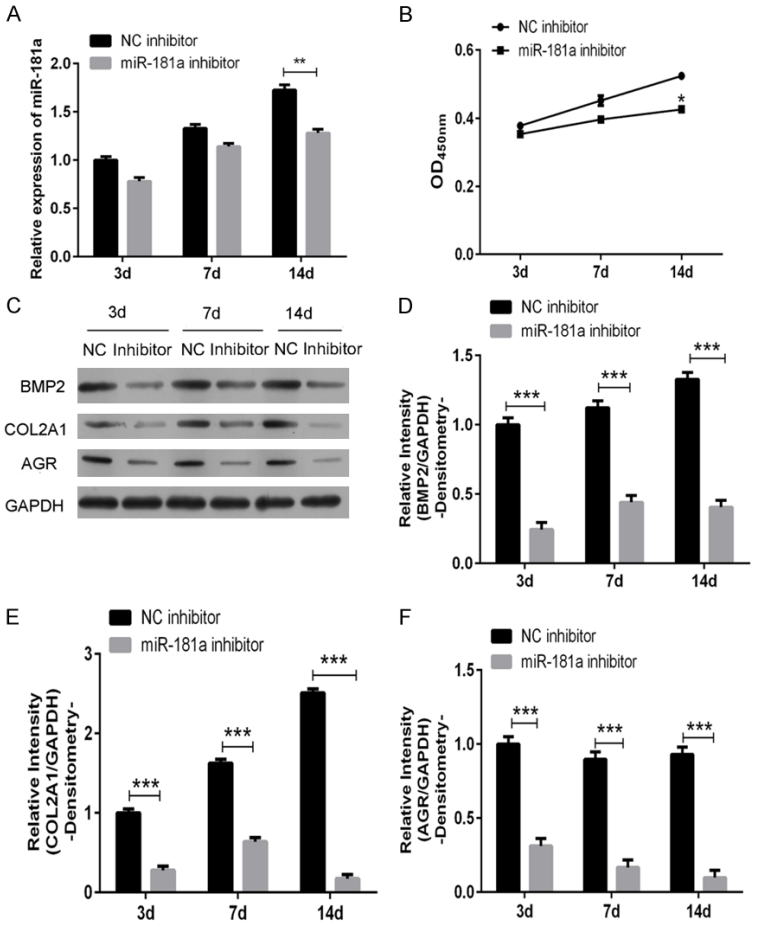

Figure 3. MiR-181a increases chondrogenic differentiation in PBMSCs. Pig PBMSCs were treated with TGF-β3 together with transfected with miR-181a inhibitor or its NC control. A. After 3 days, 7 days and 14 days of treatment, the expression of miR-181a was measured via qRT-PCR. B. The proliferation assay of treated PBMCs within 72 hours. C-F. After 3 days, 7 days and 14 days of treatment, the expression of chondrogenic differentiation markers, such as BMP2, COL2A1, and AGR were measured via western-blot.

7 days the PBMSCs stop growing (**Figure 1B**). The PBMSCs analyzed using flow cytometry. It is well known that MSCs express CD44, CD90 and CD105, whereas do not express CD34 and CD45, hematopoietic stem cell marker. Immunophenotypic analyses by flow cytometry indicated that the cells at P3 were strongly positive for CD44, CD90 and CD105, while negative for CD34 and CD45 (**Figure 1C**).

miR-181a is up-regulated during TGF-β3-induced pig PBMSCs chondrogenic differentiation

Chondrogenesis of the PBMSCs acquired by the previously mentioned methods. After induction of chondrogenic differentiation for 14 days, western-blot analysis showed a significant increase in the protein expression levels of chondrogenesis markers including BMP2, COL2A1 and AGR after induction for 3 days, 7 days and 14 days (**Figure 2A**). And we confirmed that miR-181a increased in the third day of chondrogenesis and up-regulation maintained for 2 weeks during differentiation by using qRT-PCR (**Figure 2B**). Additionally, immunofluorescence and confocal imaging showed the same result as western-blot analysis (**Figure 2C**). As shown in **Figure 2D**, the differentiation cells were positively stained for alcian blue staining.

MiR-181a was reported as a bone formation-relevant miRNA. These results showed that increased expression of miR-181a was associated with the differentiation of MSCs towards chondrocytes.

miR-181a promotes chondrogenic differentiation in PBMSCs

Ultimately, to identify the effects of miR-181a on chondrogenic differentiation of PBMSCs, we examined the expression of BMP2, COL2A1 and AGR protein in NC inhibitor, and miR-181a inhibitor-transfected PBMSCs. MiR-181a inhibitor suppressed the expression of miR-181a in PBMSCs throughout the transfected process (**Figure 3A**). And the down-regulated miR-181a inhibited the proliferation of PBMSCs in 3 days (72 hours) (**Figure 3B**). Meanwhile miR-181a inhibitor decreased the protein expression of BMP2, COL2A1 and AGR compared with the NC inhibitor-transfected PBMSCs, which showed that miR-181a may increase protein expression of BMP2, COL2A1 and AGR during chondrogenic differentiation of PBMSCs by testing with weatern-blot and immunofluorescence (**Figures 3C-F** and **4A**, **4B**). We further examined the chondrogenic differentiation potential of miR-181a inhibitor-

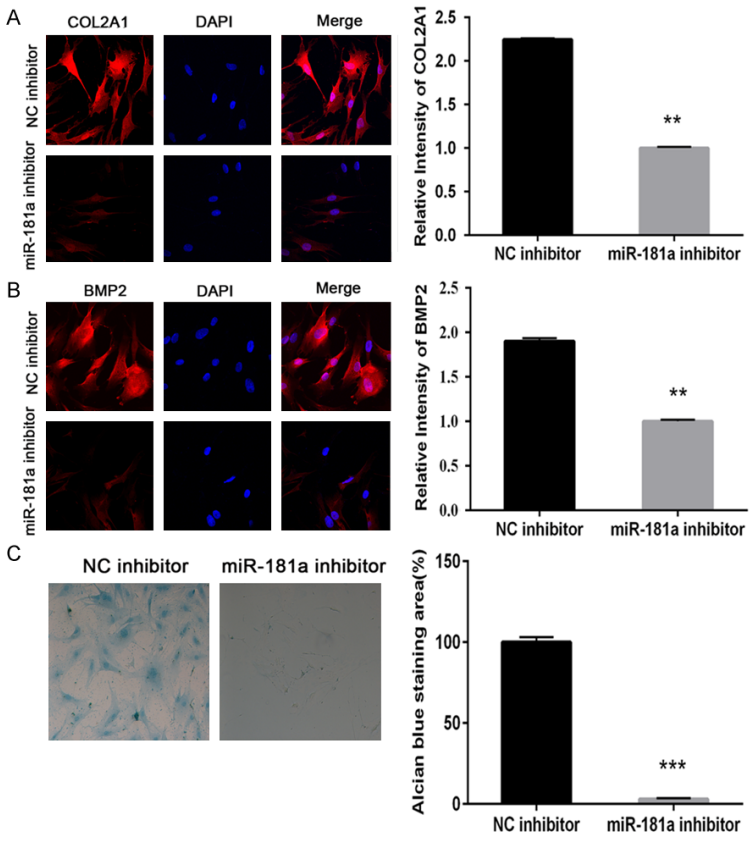

Figure 4. MiR-181a increases chondrogenic differentiation in PBMSCs. Pig PBMSCs were treated with TGF-β3 together with transfected with miR-181a inhibitor or its NC control. A, B. After 14 days of treatment, the expression of chondrogenic differentiation markers COL2A1 and BMP2 was measured via immunofluorescence. C. After 14 days of the transfection of anti-miR-181a or its negative NC control, the differentiated cells were measured by alcian blue staining.

transfected PBMSCs by alcian blue staining. Inhibition of endogenous miR-181a expression in PBMSCs by transfection of miR-181a inhibitor, under the same induction conditions as above, resulted in suppressing chondrogenic differentiation as shown by a significant decrease in alcian blue staining intensity (**Figure 4C**). Collectively, our data demonstrated that miR-181a act as a key positive regulator of chondrogenic differentiation.

Discussion

Expounding the mechanism of chondrogenesis is distinctly important resulting from the growing importance of articular cartilage injury and repair. In this study, we investigated the function of miRNA-181a in the process of chondrogenic differentiation in pig PBMSCs.

PBMSCs were reported to have the potency of multipotential differentiation and self-renew. Manipulation the generation of desired cell types differentiated from PBMSCs was noticed in the field of cell-based therapies of articular cartilage injury or tissue engineering. There are some classical signaling pathway involved in the process of PBMSCs chondrogenic differentiation, including fibroblast growth factor (FGF) signaling pathway [37, 38], TGF-β/BMP signaling pathway [39-41] and Wnt/β-catenin signaling pathway [42-46]. Recently, a large number of novel factors including miRNAs have been verified in regulating bone- and cartilage-formation. Bhushan et al provided evidence that miR-181a was upregulated in C2-C12 cells, MC3T3-E1 cells, and primary calvarial osteoblasts upon osteoblast differentiation [35]. Meanwhile, miR-181a is abundantly expressed in chicken chondrocytes [36]. Considering that miRNAs have an important role in manipulating MSCs to expand, we focused on the role of miR-181a in the process of chondrogenic differentiation in MSCs.

The previous study indicated that miRNAs would induce the process of chondrogenic differentiation. In our study, we found that miR-181a was up-regulated during TGF-β3-induced pig PBMSCs chondrogenic differentiation from the early stages and maintained elevated throughout the whole process, while the western-blot results and immunofluorescence results indicated that the expression of differentiation-related protein COL2A1, BMP2 and AGR were decreased, together with the Alcian blue assay proving the process of differentiation was inhibited significantly after inhibiting the endogenesis miR-181a. All these results demonstrated that miR-181a act as a key positive regulator of chondrogenic differentiation in vitro.

Although we identified a novel miRNA on regulating the chondrogenic differentiation of MSCs, the mechanism of miR-181a on modulating the process of chondrogenic differentiation was still unclear. As reported, three major target genes including C/EBPb, Sox9 and Adam9 have been implicated in mediating the effects of miRNAs in regulating chondrogenesis [47].

Considering defect of articular cartilage is an unique challenge on clinic, more and more researchers focus on the tissue engineering field as the therapeutic strategy of articular cartilage injury. PBMSCs are appropriate cells for cartilage tissue engineering with the advantage of amount and the ability to differentiate into functional cartilage and maintain a chondrocyte phenotype long-term. Our results proved that miR-181a plays an important role in the process of chondrogenic differentiation from PBMSCs, suggesting that miR-181a has the potential to be the novel target to induce the generation of cartilage artificially.

In summary, we present evidences for the important role of miR-181a on the regulation of MSCs chondrogenesis, also suggest that the up-regulation of miR-181a during MSC differentiation might be required for chondrocyte lineage maintenance. And the up-regulated miR-181a might influence the expression of some differentiation process protein via post-transcriptional regulation, resulting in the promotion of chondrogenic differentiation. Such hypothesis will be investigated in the future work.

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Disclosure of conflict of interest

None.

Abbreviations

PBMSCs, Peripheral blood Mesenchymal stem cells; COL2A1, collagen type II alpha 1 chain; BMP2, bone morphogenetic protein 2; qRT-PCR, quantitative real-time polymerase chain reaction; TGF-β3, transforming growth factor beta 3; AGR, aggrecan; HDAC4, (histone deacetylase 4); Smad3, (SMAD family member 3); Sox9, SRY-box9; Adam9, ADAM metallopeptidase domain 9.

Address correspondence to: Yanlin Li, Department of Sports Medicine, First Affiliated Hospital of Kunming Medical University, 295 Xichang Road, Kunming 650032, China. E-mail: yanlinliedu@yeah.net; 852387873@qq.com

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