

MicroRNA-99a regulates early chondrogenic differentiation of rat mesenchymal stem cells by targeting the *BMPR2* gene

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Abstract Mesenchymal stem cells (MSCs) are candidates for the regeneration of articular cartilage as they possess the potential for chondrogenic differentiation. MSCs are easily obtained and expanded in vitro. Specific microRNAs (miRNAs) that regulate chondrogenesis have yet to be identified and the mechanisms involved remain to be defined. The miRNAs regulate biological processes by binding target mRNA to reduce protein synthesis. In this study, we show that expression of miR-99a and miR-125b-3p were increased during early chondrogenic differentiation of MSCs (rMSCs) derived from the Norwegian brown rat (*Rattus norvegicus*). MiR-99a knockdown promoted proteoglycan deposition and increased the expression of *ACAN* and *COL2A1* during early chondrogenic differentiation. MiR-99a knockdown promoted early chondrogenic differentiation of rMSCs. A dual-luciferase reporter gene assay showed that miR-99a targeted a putative binding site in the 3'-UTR of bone morphogenetic protein (BMP) receptor type 2 (BMPR2). Overexpression of miR-99a reduced the expression levels of BMPR2 protein. The expression of total p38 and p-p38 increased at 7 and 14 days during early chondrogenic differentiation of rMSCs. Reduction in levels of total p38 and p-p38 protein followed miR-99a overexpression during early chondrogenic differentiation of rMSCs. BMPR2 silencing reversed the effects of

miR-99a inhibition on proteoglycan deposition and protein expression of *ACAN*, *COL2A1*, total p38 and p-p38 during early chondrogenic differentiation of rMSCs. In conclusion, the findings of these in vitro studies in rat MSCs support a role for miR-99a as a negative regulator of early chondrogenic differentiation by directly targeting the *BMPR2* gene at an early stage.

Keywords Mesenchymal stem cells · Chondrogenic differentiation · MicroRNA-99a · BMPR2 · Bone morphogenetic protein receptor 2

Introduction

Osteoarthritis is a chronic, progressive degenerative form of arthritis and is a leading cause of chronic disability worldwide (Moyer and Hunter 2015). Degeneration of the articular cartilage is the main pathological change in osteoarthritis, leading to clinical symptoms (Luyten and Vanlauwe 2012). Repair of the damaged articular cartilage is a potentially beneficial future treatment option to ease joint pain and regain joint function and has the potential to bridge symptomatic conservative management before joint replacement (Jiang et al. 2015). A potential approach to cartilage repair is to use mesenchymal stem cells (MSCs), which can be stimulated to differentiate into a variety of cells, including cartilage (Barry et al. 2001; Caplan and Bruder 2001; Pittenger et al. 1999). MSCs are candidates for the regeneration of cartilage as they possess chondrogenic differentiation potential and are easily obtained and expanded in vitro (Csaki et al. 2008; Pittenger 2008).

Several transcription factors and growth factors that control chondrogenic differentiation of MSCs are regulated by microRNAs (miRNAs) that are short (21–24 nucleotides) non-protein-coding RNAs (Lewis et al. 2005). MiRNAs

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repress target mRNA expression through 5'-seed sequence interactions with miRNAs regulatory elements located in the 3'-untranslated region (UTR) of target mRNA (Lewis et al. 2005).

Previous studies have shown that miRNAs play critical roles in regulating cellular processes, such as cell proliferation, apoptosis and differentiation (Alvarez-Garcia and Miska 2005; Bartel 2004; Thompson and Cohen 2006). Specific miRNAs that regulate chondrogenesis have yet to be identified (Lee et al. 2014). There have been studies on miRNA expression patterns in chondrogenic differentiation in MSCs (Lin et al. 2009). Several miRNAs, including miR-127, miR-140, miR-99a, miR-145, miR-132 and miR-125b-3p, have been shown to have a changed expression profile at different stages during chondrogenic differentiation of mouse MSCs (mMSCs) (Yang et al. 2011b). In 2011, a study demonstrated that miR-145 regulates chondrogenic differentiation of MSCs by targeting Sox9 (Yang et al. 2011a). However, evidence for the role of other miRNAs, such as miR-127, miR-140, miR-99a, miR-132 and miR-125b-3p, in chondrogenic differentiation is still lacking.

The bone morphogenetic protein (BMP) receptor, BMPR2, is a member of the BMP receptor family of transmembrane serine/threonine kinases (Liu et al. 1995). The BMP ligands of this receptor are involved in endochondral bone formation and embryogenesis (Freyria et al. 2008). Recent studies have shown that total p38 and phosphorylated p38 (p-p38) increased during early chondrogenic differentiation and that miR-99a overexpression can decrease the expression of total p38 and p-p38; p38 regulated chondrogenesis and was a downstream protein of BMPs (Braem et al. 2012; Li et al. 2010). Recent studies have confirmed that *ACAN* and *COL2A1* are two important genes that define the chondrocyte phenotype; for this reason, we chose to include these genes in this study (Ito et al. 2015).

In view of these previous studies and the potential role for micro-RNAs in the regulation of chondrogenesis, we chose to study the expression of miRNAs, miR-127, miR-140, miR-99a, miR-145, miR-132 and miR-125b-3p in early chondrogenic differentiation in vitro, using MSCs (rMSCs) derived from the Norwegian brown rat, *Rattus norvegicus*.

Materials and Methods

Isolation and culture of rat mesenchymal stem cells (rMSCs)

All animal experiments were approved by the Local Ethics and Welfare Committee of the University of South China. Ten six-week-old male Norwegian brown rats weighing between 100 and 120 g were killed by controlled inhalation of CO₂. The hind legs, including the soft tissue, were removed and the separated bones (tibia, femur) were stored in PBS.

The rMSCs were collected and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) under the conditions of 5 % CO₂ at 37 °C. When the cells were 80–90 % confluent, they were cultured with the ratio of 1:2; the purified rMSCs were acquired after three generations. The purified third-generation rMSCs were removed from their culture plates with trypsin, which was added to 10 % FBS L-DMEM. The concentration of rMSCs obtained was 2×10^5 cells/mL.

Chondrogenic differentiation and cell sample preparation

The rMSCs were cultured in mesenchymal stem cell chondrogenic differentiation medium (MCDM) for 0, 7 and 14 days and harvested for quantitative real-time polymerase chain reaction analysis (qRT-PCR) for the detection of expression of miRNAs and runt-related transcription factor 2 (RUNX2).

To study the function analysis of miR-99a and miR-125b-3p, rMSCs were infected with lentivirus expressing miR-99a and miR-125b-3p inhibitor or negative control (NC). Infected rMSCs were cultured in MCDM for 14 days and cells were harvested for qRT-PCR, western blot, immunofluorescence and Alcian blue staining.

For the qRT-PCR, western blot and luciferase reporter assay to confirm miR-99a targeting, rMSCs were transfected with miR-99a mimics, negative control mimics, miR-99a mimics plus luciferase reporter vectors expressing wild BMPR2 3'UTR or mutant BMPR2 3'UTR, or NC mimics plus luciferase reporter vectors expressing wild-type BMPR2 3'UTR or mutant BMPR2 3'UTR for 48 h using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

To confirm whether miR-99a regulated chondrogenic differentiation through targeting BMPR2, rMSCs were infected with lentivirus expressing miR-99a inhibitor plus NC siRNA, or lentivirus expressing miR-99a inhibitor plus BMPR2 siRNA. Infected rMSCs were cultured in MCDM for 14 days. Cells were harvested for qRT-PCR, western blot, immunofluorescence and Alcian blue staining.

RNA extraction and quantitative real-time PCR analysis (qRT-PCR)

Total RNA was extracted from harvested cells using Trizol reagent (Invitrogen). To analyze miR-127, miR-140, miR-99a, miR-145, miR-132 and miR-125b-3p expression, reverse transcription PCR was performed using specific stem-loop RT primers (Table 1) in a Mir-X™ miRNA First Strand Synthesis Kit (Takara, Dalian, China). Quantitative real-time PCR was performed using a Mir-X™ miRNA qRT-PCR SYBR® Kit (Takara) on an Applied Biosystems 7500 system. U6 was used as an internal control.

Table 1 Primers for quantitative real-time RT-PCR

Primer name	Sequence (5'-3')
miR-127	TCGGATCCGTCTGAGCTTGGCT
miR-127 RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGCCAAGCT
miR-127 F	ACACTCCAGCTGGGTCTGGATCCGTCT
miR-140	CAGTGGTTTTACCCTATGGTAG
miR-140 RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCTACCATAG
miR-140 F	ACACTCCAGCTGGGCAGTGGTTTTACC
miR-99a	AACCCGTAGATCCGATCTTGTG
miR-99a RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCACAAGAT
miR-99a F	ACACTCCAGCTGGGAACCCGTAGAT
miR-132	TAACAGTCTACAGCCATGGTCG
miR-132 RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCGACCATG
miR-132 F	ACACTCCAGCTGGGTAAACAGTCTACAG
miR-125b-3p	ACGGGTTAGGCTCTTGGGAGCT
miR-125b-3p RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGCTCCCA
miR-125b-3p F	ACACTCCAGCTGGGACGGTTAGGCT
miR-145	GTCCAGTTTTCCCAGGAATCCCT
miR-145 RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGGGATTCCCT
miR-145 F	ACACTCCAGCTGGGGTCCAGTTTTCC
All R for miRNA	TGGTGTCTGGAGTCG
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
RUNX2 F	CCATCAGCGTCAACACCA
RUNX2 R	AGCCACCTTTACTTACACCC
GAPDH F	CCTCGTCTCATAGACAAGATGGT
GAPDH R	GGGTAGAGTCATACTGGAACATG
SOX9 F	ATCTGAAGAAGGAGAGCGAG
SOX9 R	CAAGCTCTGGAGACTGCTGA
COL2A1 F	ATCGCCACGGTCTACAATG
COL2A1 R	GGCCCTAATTTTCGGGCATC
ACAN F	GAAGTGGCGTCCAAACCAAC
ACAN R	AGCTGGTAATTGCAGGGGAC
BMPR2 F	CACTACGGCTGCTTCCCAGAAT
BMPR2 R	TCCCACAGACCATAACATGTGC

To quantify the levels of mRNA for *RUNX2*, *BMPR2*, *ACAN* and *COL2A1*, reverse transcription PCR was applied using a PrimeScript RT Reagent Kit with cDNA Eraser (Takara) and quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara). GAPDH was used as an internal control. The primer sequences used in qRT-PCR are shown in Table 1. Gene expression was measured in triplicate and quantified using the $2^{-\Delta\Delta CT}$ method normalized to control.

Lentivirus package and cell infection

A retroviral transduction system was established for the generation of miR-99a, miR-125b-3p and BMPR2 knockdown in rMSC. For miR-99a reduction, miR-99a inhibitor sequencing

CACAAGATCGGATCTACGGGTT was ligated into the shuttle vector pGLV3. For miR-125b-3p reduction, miR-125b-3p inhibitor sequencing AGTCCCAAGAG CCTAACCCGT was ligated into the shuttle vector pGLV3. For BMPR2 reduction, BMPR2 siRNA sequencing CGGGATGAAACTATAATCATT was ligated into the shuttle vector pGLV3.

The control expression vector contained a negative control inhibitor or siRNA sequence. pGLV3s encoding miR-99a inhibitor, miR-125b-3p inhibitor, BMPR2 siRNA or negative control sequence were generated by transient transfection of 293T cells. Vector production, concentration and titration were performed in accordance with standard procedure. Briefly, rMSCs were seeded at a low density in DMEM medium supplemented with 10 % FCS. After 24 h of proliferation, viral

transduction was performed on three consecutive days by adding fresh virus particles together with complete medium containing 6 µg/ml polybrene (Sigma) to the rMSC.

Western blot

The cell lysates were extracted with lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1 % TritonX-100, 1 % deoxycholate, 0.1 % SDS, 1 mM PMSF and 0.2 % aprotinin (Sigma). Total protein concentrations were determined with the BCA Protein Assay kit (Beyotime). Equal amounts of protein were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocking in 10 % non-fat dried milk in TBST for 2 h, the blots were incubated with anti-BMP2 (Abcam, diluted 1:2000) anti-p38 (Abcam, diluted 1:1000), anti-p-p38 (Abcam, 1:2000), anti-ACAN (Abcam, diluted 1:2000), anti-COL2A1 (Abcam, diluted 1:2000), or anti-GAPDH antibody (Abcam, diluted 1:5000) at 4 °C overnight. GAPDH was used as an internal control. After washing with TBST, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, diluted 1:2000) at room temperature for 1 h. The blots were visualized by an ECL Plus Western Blotting Substrate (Pierce) following the manufacturer's instructions. Western blotting was carried out in triplicate.

Immunofluorescence microscopy

Cells from each group of rMSCs were grown on coverslips. At the end of the intervention, the cells were washed with PBS and fixed for 20 min with 4 % paraformaldehyde at room temperature. The cells were permeabilized for 5 min in 0.2 % Triton X-100 and washed with PBS. To block non-specific background staining, the samples were incubated in a solution containing 10 % normal goat serum for 30 min. After washing with PBS, the cells were incubated for 1 h at room temperature with primary anti-ACAN or anti-COL2A1 antibody (1:100) diluted in PBS supplemented with 1 % BSA.

The cells were then rinsed with PBS and incubated with Alexa Fluor594-conjugated secondary antibodies for 60 min at room temperature. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) by incubation for 5 min

at room temperature. After washing with PBS, the stained cells were mounted in medium suitable for fluorescence microscopy and examined under a confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). Immunofluorescence microscopy was carried out in triplicate.

Alcian blue staining for cell chondrogenic differentiation

After the cells were cultured for 7 or 14 days, the culture medium was removed, the cells were rinsed gently with PBS twice and then fixed with −20 °C methanol for 30 min. After rinsing with PBS twice, the wells were stained with Alcian blue in 0.1N HCl for 30 min. The wells were then rinsed with distilled water 3 times, and finally images were captured of the stained wells. To quantify proteoglycan, the matrix-associated dye was extracted with 6 M guanidine-HCl (Sigma-Aldrich, Oakville, ON, Canada) at 200 µl/well and measured at 620 nm. Alcian blue staining and proteoglycan quantification was carried out in triplicate.

Reporter vector construction and luciferase reporter assay

The full-length wild-type 3' untranslated region (UTR) of *BMP2* and mutant 3' UTR of *BMP2* were amplified and cloned into the psi-CHECK-2 vector (Promega, USA). The primer sequences used in reporter vector construction are shown in Table 2.

All plasmids were confirmed by DNA sequencing. A total of 293 cells were plated on 24-well plates and were co-transfected with 100 ng plasmid and 200 nmol/L of miR-99a mimic or NC. Cell lysates were harvested 48 h after transfection and then firefly and *Renilla* luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Three independent experiments were performed.

Statistical analysis

All results were presented as mean ± standard deviation (SD) and analyzed using SPSS19.0 software. The Kruskal–Wallis test was used to determine the statistical significance. A *P* value < 0.05 was considered statistically significant.

Table 2 Primers for luciferase reporter construction

Primer name	Sequence (5'–3')
BMP2- XhoI F	CCGctcgagACTAATGACTAAAAACGCAAT
BMP2- NotI R	ATAAGAATgcccgcCCTAAGAACCACCTTCAAAC
BMP2- mut F	CAGAACCCTCCTTCTTGTACGATCCATCTTTCGTTGGTGTGTTTTG
BMP2- mut R	CAAAACACACCAACGAAAGATGGATCGTAAACAAGAAGGAGGGTTCTG

Results

The expression profile of six miRNAs during early chondrogenic differentiation in rat mesenchymal stem cells (rMSCs)

Purified rat mesenchymal stem cells (rMSCs) were induced by mesenchymal stem cell chondrogenic differentiation medium (MCDM) and following isolation showed early chondrogenic differentiation by detection of *RUNX2* expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

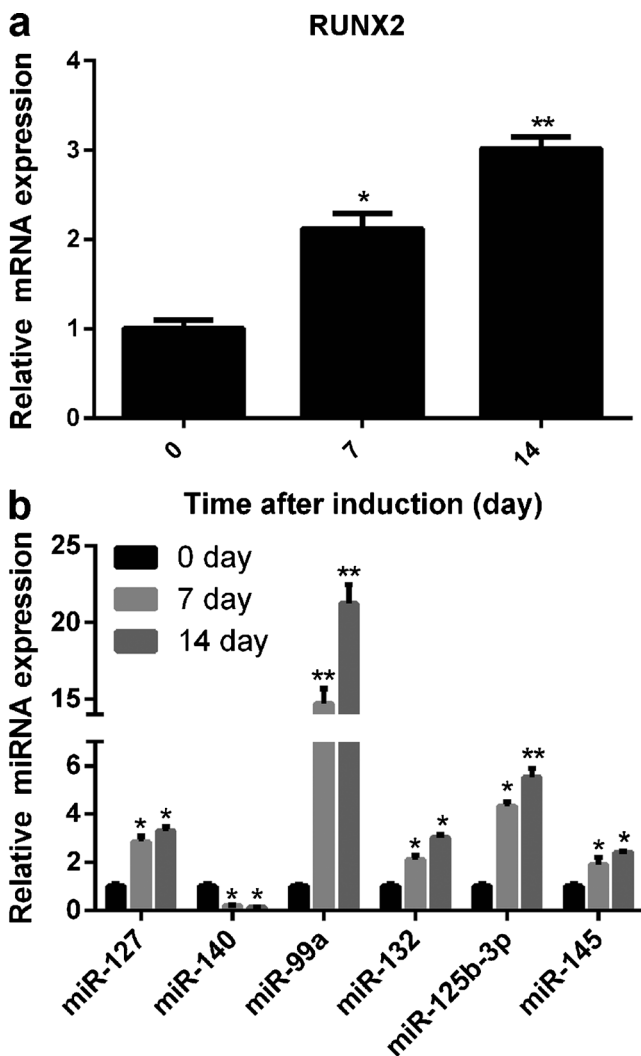


Fig. 1 The expression of miR-127, miR-140, miR-99a, miR-145, miR-132, and miR-125b-3p during early chondrogenic differentiation of rat mesenchymal stem cells detected by quantitative reverse transcription polymerase chain reaction. **a** *RUNX2* mRNA expression level at 0, 7, and 14 days during chondrogenic differentiation. **b** The expression of miR-127, miR-140, miR-99a, miR-145, miR-132, and miR-125b-3p at 0, 7, and 14 days during chondrogenic differentiation. Experiments were carried out in triplicate. Data are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, when compared with day 0

As shown in Fig. 1a, *RUNX2* mRNA gradually increased at 0, 7 and 14 days during chondrogenic differentiation. These results not only supported the presence of chondrogenic differentiation but also indicated that cells were suitable for the detection of the expression profile of miRNAs, miR-127, miR-140, miR-99a, miR-145, miR-132 and miR-125b-3p at these time points.

The qRT-PCR results showed that the expression of miR-127, miR-99a, miR-145, miR-132 and miR-125b-3p increased during early chondrogenic differentiation (Fig. 1b). The expression of miR-140 decreased during early chondrogenic differentiation (Fig. 1b). Among the increased miRNAs, the expression levels of miR-99a and miR-125b-3p showed the greatest expression levels, thus these two miRNAs were chosen for further investigation of early chondrogenic differentiation.

MiR-99a knockdown promoted proteoglycan deposition in rMSCs

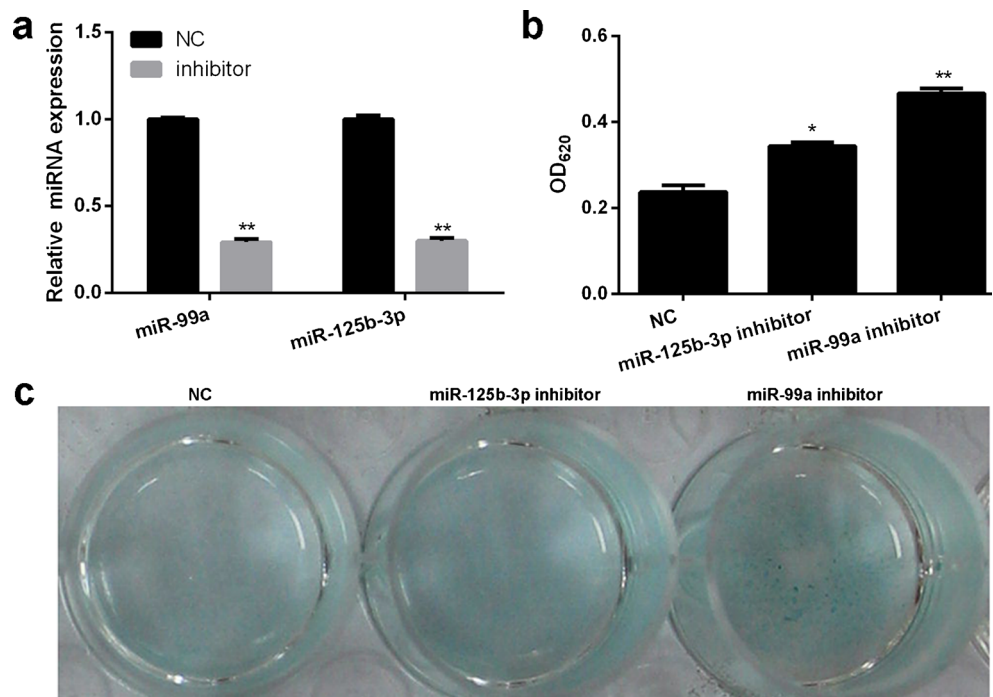
To determine whether miR-99a and miR-125b-3p mediated early chondrogenic differentiation, miRNA inhibitor construct was packaged into lentiviral particles and infected into rMSCs. The qRT-PCR analysis showed miR-99a and miR-125b-3p expression levels decreased in the miR-99a and miR-125b-3p lentivirus expression inhibitor group when compared with the negative control group (Fig. 2a).

Following Alcian blue staining to detect the proteoglycans in rMSCs in early chondrogenesis, the effect of miR-99a or miR-125b-3p silencing was demonstrated. Alcian blue was extracted from the stained cell cultures (Fig. 2b); more proteoglycan deposition was found in the miR-99a- or miR-125b-3p-silenced group compared with the control group. Images of the Alcian blue-stained cell culture wells also confirmed this result (Fig. 2c). In addition, miR-99a knockdown resulted in a stronger effect on the production of cell proteoglycan deposition when compared with miR-125b-3p knockdown.

MiR-99a knockdown increased the expression of *ACAN* and *COL2A1* during early chondrogenic differentiation

The effect of miR-99a or miR-125b-3p knockdown on the expression of the genes *ACAN* and *COL2A1* was assessed using qRT-PCR, western blot and immunofluorescence. After the cells had been cultured for 14 days in MCDM, *ACAN* and *COL2A1* mRNA expression of the miR-99a inhibitor group increased when compared with the normal control group (Fig. 3a). No increase in *ACAN* and *COL2A1* mRNA expression was observed in the miR-125b inhibitor group when compared with the

Fig. 2 MiR-99a knockdown promoted proteoglycan deposition of rat mesenchymal stem cells (rMSCs). **a** rMSCs were infected with lentivirus expressing miR-99a inhibitor, miR-125b-3p, or normal control (NC) and the expression of endogenous miR-99a and miR-125b-3p were detected by quantitative reverse transcription polymerase chain reaction. **b** The absorbance value of the solubilized Alcian blue at 620 nm after rMSCs were cultured in mesenchymal stem cell chondrogenic differentiation medium for 0, 7 and 14 days. **c** Photomicrograph of Alcian blue staining of proteoglycan in the extracellular matrix. Experiments were carried out in triplicate. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01, when compared with NC



normal control group (Fig. 3a). Western blots showed that ACAN and COL2A1 protein expression of the

miR-99a inhibitor group also increased when compared with the normal control group (Fig. 3b-h).

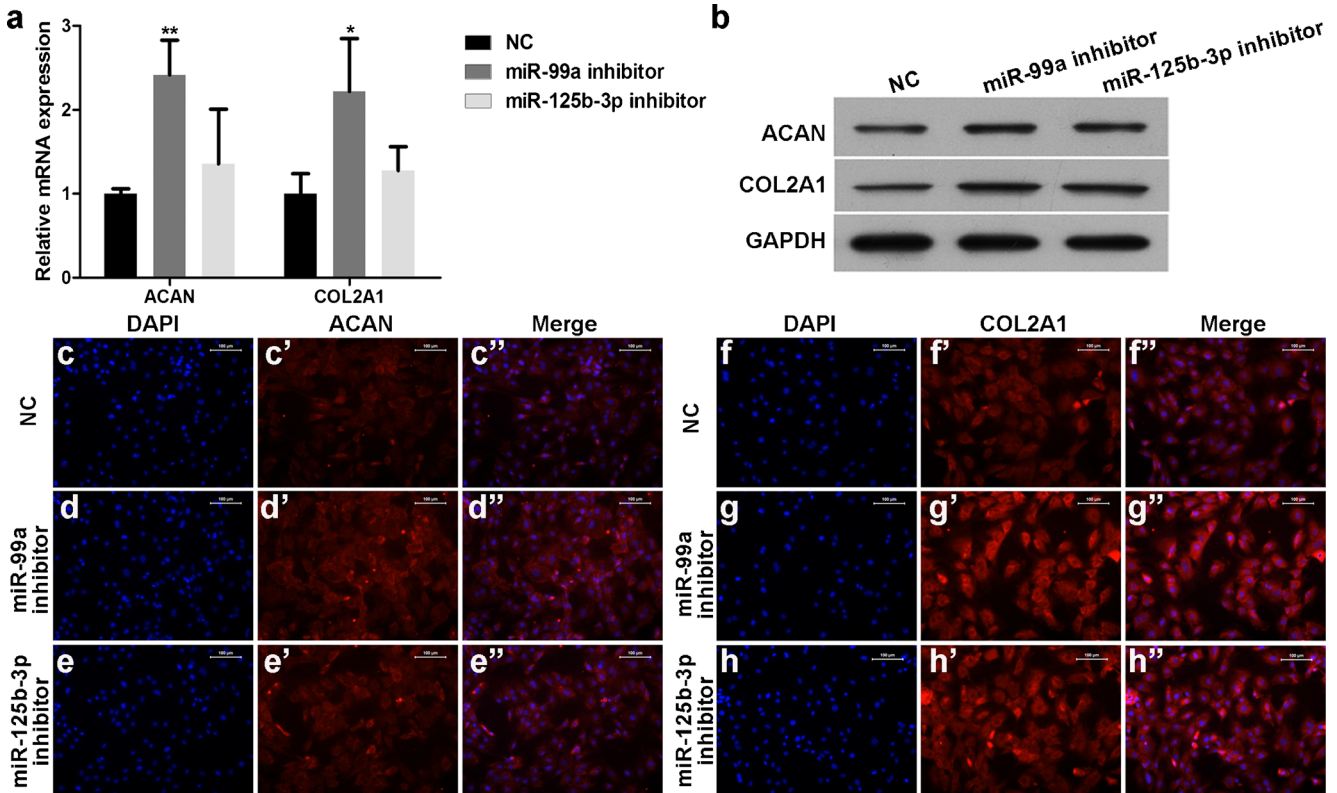


Fig. 3 The expression of ACAN and COL2A1 after miR-99a or miR-125b-3p knockdown during chondrogenic differentiation. miR-99a- or miR-125b-3p-silenced rat mesenchymal stem cells (rMSCs) or negative control. **a** rMSCs were cultured in mesenchymal stem cell chondrogenic

differentiation medium for 14 days and harvested for qRT-PCR. **b** Western blot. **c–h** Immunofluorescence analysis. Scale bar 100 μm. Experiments were carried out in triplicate. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01, when compared with normal control

BMPR2 as a possible target of miR-99a

To further study the underlying mechanism by which miR-99a knockdown promoted early chondrogenic differentiation of rMSCs, we explored miR-99a targets using the bioinformatics algorithm available at: microRA.org.

Analysis of the data from this study confirmed that BMPR2 was a potential target of miR-99a based on putative conserved target sequences at 3'-UTR of BMPR2. To confirm the relationship between miR-99a and BMPR2, we first examined the protein levels of BMPR2 at different stages during chondrogenic differentiation of rMSCs, including induction at 0, 7 and 14 days. The results showed that increased expression levels of BMPR2 protein were detected at 7 and 14 days during chondrogenic differentiation when compared with day 0 (Fig. 4a).

In addition, the expression of total p38 and p-p38 was also detected during early chondrogenic differentiation of rMSCs. The expression of total p38 and p-p38 increased at 7 and 14 days during chondrogenic differentiation of rMSCs (Fig. 4a).

To further examine whether miR-99a directly targets BMPR2, luciferase reporter vectors containing wild-type or mutant versions of the predicted miR-99a binding sequences in the BMPR2 3'-UTR (Fig. 4b) were co-transfected with miR-99a mimic or normal controls into rMSCs. Luciferase assays were performed 48 h following transfection. A decrease in luciferase activity of the reporter in the wild-type BMPR2 3'-UTR containing vector was observed in the presence of miR-99a (Fig. 4c) when compared with NC. This decrease in reporter activity was not seen when the reporter was in the vector containing the mutant BMPR2 3'-UTR (Fig. 4c), despite the presence of miR-99a, indicating that sequences in the 10480–10485 bp region of the BMPR2 3'-UTR interact with miR-99a, inhibiting expression of BMPR2.

On examination of the effect of miR-99a overexpression on BMPR2 messenger RNA (mRNA) and protein levels, miR-99a overexpression did not cause degradation of BMPR2 mRNA (Fig. 4d). However, a reduction in the level of endogenous BMPR2 protein was observed (Fig. 4e). A reduction in the level of total p38 and p-p38 protein was also observed (Fig. 4e).

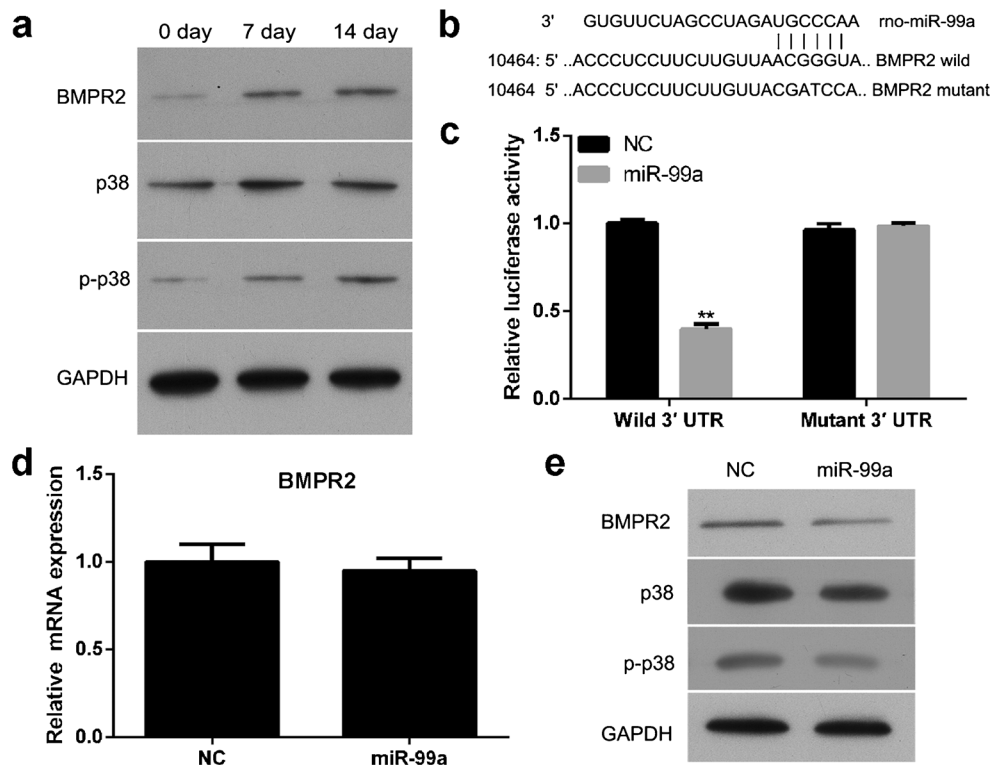


Fig. 4 Bone morphogenetic protein (*BMP*) receptor, *BMPR* is a direct target of miR-99a. **a** Western blot showing *BMPR*, p38 and p-p38 protein expression levels at 0, 7 and 14 days during chondrogenic differentiation. *GAPDH* protein was used as an internal loading control. **b** Predicted duplex formation between the wild-type or mutant *BMPR* 3' UTR and miR-99a. **c** Luciferase activity of wild-type (3'UTR-wild) or mutant (3'UTR-mutant) *BMPR* 3'UTR containing reporters, in rat mesenchymal stem cells (rMSCs) transfected with a miR-99a mimic or

normal control (NC). **d** Quantitative reverse transcription polymerase chain reaction of *BMPR* mRNA in rMSCs transfected with a miR-99a mimic or NC. Data were normalized to *GAPDH* mRNA. **e** Western blot of *BMPR*, p38 and p-p38 in rMSCs transfected with a miR-99a mimic or NC. *GAPDH* protein was used as an internal loading control. Experiments were carried out in triplicate. Data are expressed as mean \pm SD, * P < 0.05, when compared with NC

BMPR2 silencing reverses the effect of miR-99a inhibitor on proteoglycan deposition of rMSCs

To examine whether miR-99a affected proteoglycan deposition of rMSCs through *BMPR2*, rMSCs were infected with both miR-99a inhibitor plus *BMPR2* siRNA-expressing lentivirus. As shown in Fig. 5a, b, *BMPR2* mRNA and protein levels decreased following infection with both miR-99a inhibitor plus *BMPR2* siRNA expressing lentivirus, compared with both miR-99a inhibitor plus NC siRNA expressing lentivirus. In addition, miR-99a plus *BMPR2* silencing decreased the expression of total p38 and p-p38 (Fig. 5b).

Following Alcian blue staining that was carried out to analyze the effect of miR-99a plus *BMPR2* silencing on proteoglycan deposition of rMSCs, when Alcian blue was extracted from the stained cell cultures (Fig. 5c), less proteoglycan deposition was found in the miR-99a plus silencing group compared with the miR-99a silencing group. The images of the stained rMSC culture wells gave a similar result (Fig. 5d).

BMPR2 silencing reverses the effect of miR-99a inhibitor on the expression of ACAN and COL2A1 during early chondrogenic differentiation

The effect of miR-99a and *BMPR2* knockdown on the expression of *ACAN* and *COL2A1* was assessed using qRT-PCR and protein expression levels were determined using western blot and immunofluorescence.

After cells were cultured for 14 days in chondrogenic differentiation medium, *ACAN* and *COL2A1* mRNA expression in the miR-99a plus *BMPR2* knockdown group decreased when compared with the miR-99a knockdown group (Fig. 6a). Similarly, *ACAN* and *COL2A1* protein expression of the miR-99a and *BMPR2* knockdown group decreased when compared with the miR-99a knockdown group (Fig. 6b-f).

Discussion

This in vitro study of chondrogenic differentiation in rat mesenchymal stem cells (rMSCs) adds to the recently published studies on the role of microRNAs (miRNAs) in the regulation of chondrogenic differentiation in human, mouse and rat MSCs, including miR-495, miR-199a, miR-145, miR-140 and miR-193b (Buechli et al. 2013; Hou et al. 2015; Lee et al. 2014; Lin et al. 2009; Yang et al. 2011a). In this study, the expression of miR-127, miR-99a and miR-125b-3p in early chondrogenic differentiation were up-regulated, which is consistent with recently published findings (Yang et al. 2011b). The ligands of the bone morphogenetic protein (BMP) receptor type 2 (*BMPR2*) as a potential target for miRNAs in chondrogenesis, are supported by the findings of this present study.

Proteoglycans are an important component of cartilage that can be quantified using Alcian blue histochemical staining, as in the present study for cultured rMSCs (Gomoll and Minas 2014). In this study, Alcian blue staining for proteoglycan

Fig. 5 Bone morphogenetic protein receptor 2 (*BMPR2*) silencing reverses the effect of miR-99a inhibitor on proteoglycan deposition of rat mesenchymal stem cells (rMSCs). **a**, **b** rMSCs were infected with lentivirus expressing miR-99a inhibitor plus *BMPR2* siRNA, or lentivirus expressing miR-99a inhibitor plus NC and the expression of endogenous *BMPR2* was detected by quantitative reverse transcription polymerase chain reaction. **c** The absorbance value of the solubilized Alcian blue at 620 nm after infected rMSCs were cultured in mesenchymal stem cell chondrogenic differentiation medium for 14 days. **d** Photomicrograph of Alcian blue staining of proteoglycan in the extracellular matrix. Experiments were carried out in triplicate. Data are expressed as mean \pm SD. $**P < 0.01$

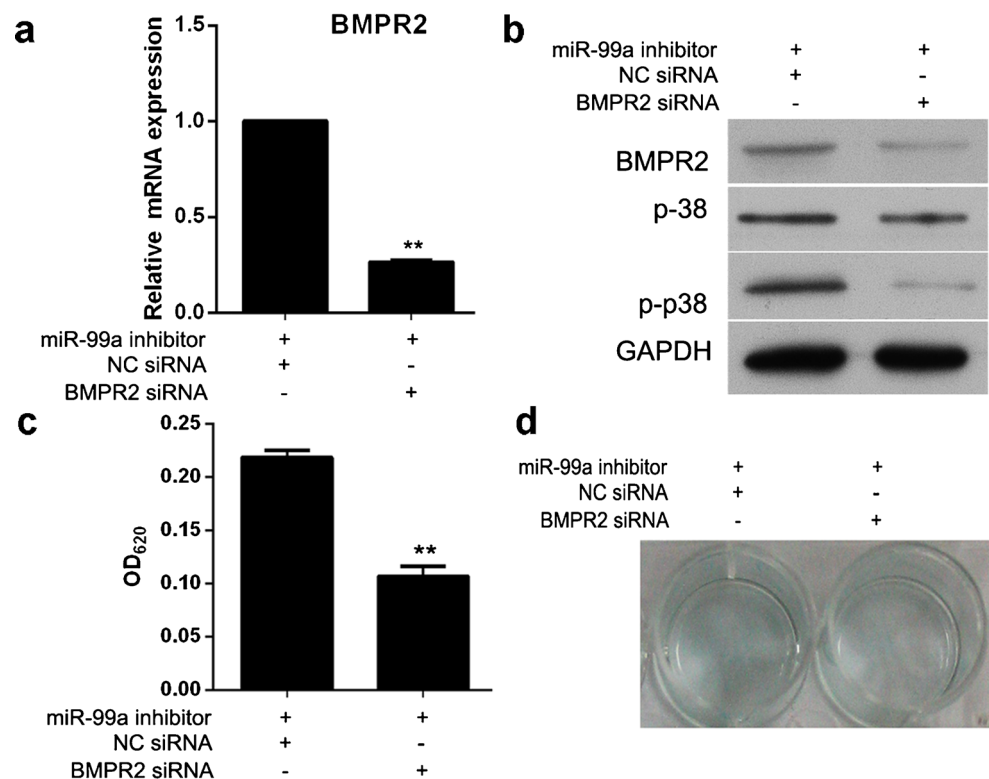
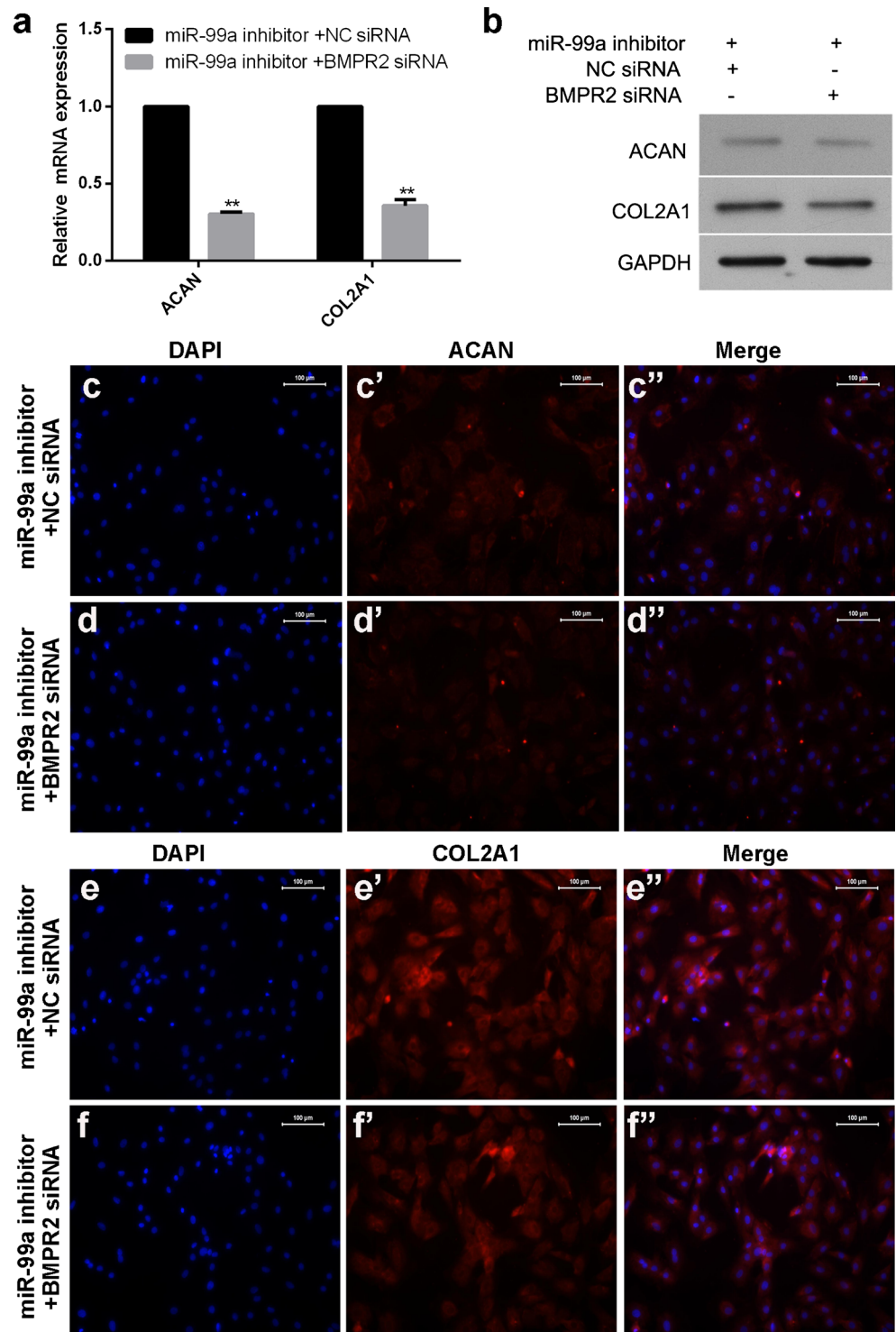


Fig. 6 Bone morphogenetic protein receptor 2 (*BMPR2*) silencing reverses the effect of miR-99a inhibitor on the expression of *ACAN* and *COL2A1* during early chondrogenic differentiation. Rat mesenchymal stem cells (rMSCs) were infected with lentivirus expressing miR-99a inhibitor plus *BMPR2* siRNA, or lentivirus expressing miR-99a inhibitor plus normal control (NC) and cultured in mesenchymal stem cell chondrogenic differentiation medium for 14 days and harvested for **a** Quantitative reverse transcription polymerase chain reaction. **b** Western blot. **c–f** Immunofluorescence analysis. Scale bar 100 μ m. Experiments were carried out in triplicate. Data are expressed as mean \pm SD. $**P < 0.01$



deposition for miR-99a or miR-125b-3p silencing showed that both miR-99a and miR-125b-3p silencing could promote proteoglycan deposition of rMSCs; miR-99a knockdown had a greater effect on promoting proteoglycan deposition when compared with miR-125b-3p knockdown. These results were supported by the findings that an increase of ACAN and COL2A1 protein expression was observed in both miR-99a

and miR-125b inhibitor groups compared with the normal control group, confirming the importance of these genes in chondrocyte differentiation (Ito et al. 2015). From these in vitro findings, we concluded that both miR-99a and miR-125b may play a role in regulating early chondrogenic differentiation of rMSCs, with miR-99a being particularly important.

The miRNAs exert their phenotypic effect by either inducing mRNA degradation of target genes or inhibiting mRNA translation through imperfect base-pairing with the 3'-untranslated region (3'UTR) of target mRNAs (Alvarez-Garcia and Miska 2005; Bartel 2004; Thompson and Cohen 2006). Among the target genes of miR-99a, BMPR2 is a putative target gene; this is because the ligands of BMPR2 are BMPs, which are involved in endochondral bone formation and embryogenesis (Freyria et al. 2008; Liu et al. 1995). In this study, BMPR2 was identified as a direct target of miR-99a in early chondrogenic differentiation of rMSCs. This conclusion is supported by several findings of this study. First, the complementary sequence of miR-99a was identified in the 3'UTR of BMPR2 mRNA suggesting this 3'UTR interacts with miR-99a. Second, the over-expression of miR-99a led to a significant reduction in BMPR2 protein expression. Third, over-expression of miR-99a suppressed luciferase reporter activity of the BMPR2 3'UTR-containing vector. Fourth, this effect was abolished by mutation of the miR-99b binding site in the BMPR2 3'UTR. Finally, this study has shown that BMPR2 silencing could reverse the effect of miR-99a on early chondrogenic differentiation of rMSCs.

Furthermore, the results of this study indicated that miR-99a may influence the expression of total p38 and p-p38 by targeting BMPR2 during early chondrogenic differentiation of rMSCs. This conclusion is supported by the following study findings. First, the expression of total p38 and p-p38 increased at 7 and 14 days during chondrogenic differentiation of rMSCs. Second, a clear reduction in the level of total p38 and p-p38 protein was also observed following miR-99a over-expression during early chondrogenic differentiation of rMSCs. And third, BMPR2 silencing reversed the effect of miR-99a inhibitor on total p38 and p-p38 protein of rMSCs during early chondrogenic differentiation of rMSCs.

This *in vitro* study was preliminary in nature and had some limitations. The culture of cell populations in laboratory conditions cannot replicate the cell environment in normal and diseased tissues. Care should be taken in extrapolating *in vitro* animal studies of bone marrow-derived cells to peripheral tissue-based disease states and human cell and tissue studies.

Previous studies have shown that p38 is activated during chondrogenic differentiation and that the BMP family of genes and proteins could enhance chondrogenesis by activating p38 mitogen-activated protein (MAP) kinase (Andres-Bergos et al. 2012; Jin et al. 2006; Nakamura et al. 1999). Although these previous studies support our findings and conclusions, the relationship between miR-99a, BMPR2 and p38 remains unclear and requires further studies on their roles and interactions in chondrogenesis.

In conclusion, this study demonstrated that miR-99a is increased during early chondrogenic differentiation of rMSCs. The knockdown of miR-99a expression up-regulated the expression of the direct target gene BMPR2 and resulted in the

promotion of early chondrogenic differentiation of rMSCs. These findings support a role for miR-99a in early chondrogenesis and may provide a novel mechanism in the miRNA-mediated regulation of early chondrogenic differentiation of MSCs.

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