miR-203a-3p.1 is involved in the regulation of osteogenic differentiation by directly targeting Smad9 in MM-MSCs

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Abstract. MicroRNAs (miRNAs) have emerged as important regulators of bone development and regeneration. The aim of the present study was to determine whether miR-203a-3p.1 is involved in osteogenic differentiation of multiple myeloma (MM)-mesenchymal stem cells (MSCs) and the potential underlying mechanism. MSCs were isolated from patients with MM and normal subjects and confirmed by flow cytometry using specific surface markers. The osteogenic differentiation capacity of MM-MSCs was identified by Alizarin Red S calcium deposition staining and reverse transcription-quantitative PCR (RT-qPCR) of typical osteoblast differentiation markers. The role of miR-203a-3p.1 in the osteoblast differentiation of MM-MSCs was determined by gain or loss of function experiments. The target of miR-203a-3p.1 was identified using bioinformatics (including the miRNA target prediction database TargetScan, miRDB, DIANA TOOLS and venny 2.1.0), luciferase reporter assay, RT-qPCR and western blotting. The expression levels of proteins involved in the Wnt3a/β-catenin signaling pathway were detected by western blot analysis. The results revealed that the osteogenic differentiation capacity of MM-MSCs was reduced when compared with normal (N)-MSCs, as demonstrated by a decrease in calcium deposition and mRNA expression of typical osteoblast differentiation markers, including ALP, OPN and OC. In addition, miR-203a-3p.1 was downregulated in N-MSCs following osteoblast induction, whereas no changes were observed in MM-MSCs. The downregulation of miR-203a-3p.1 resulted in increased osteogenic potential, as indicated by the increase in the mRNA expression levels of the typical osteoblast differentiation markers, including alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC). Bioinformatics and luciferase reporter assay analysis indicated that mothers against decapentaplegic homolog 9 (Smad9) may be a direct target of miR-203a-3p.1 in N-MSCs. The RT-qPCR and western blot assays revealed that overexpression of smad9 significantly enhanced the effect of miR-203a-3p.1 inhibitors on osteoblast markers, which indicated that miR-203a-3p.1 inhibitors may regulate the osteogenic differentiation of MM-MSCs by upregulating Smad9. In addition, the Wnt3a/β-catenin signaling pathway was activated following miR-203a-3p.1 inhibition. These results suggest that miR-203a-3p.1 may serve an important role in the osteogenic differentiation of MM-MSCs by regulating Smad9 expression.

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Introduction

Multiple myeloma (MM) is a clonal plasma cell malignancy that strongly depends on interactions with its microenvironment (1). A major complication of MM is the development of osteolytic lesions, which are caused by an imbalance between osteoclastic bone resorption and impaired osteoblastic bone formation and lead to severe bone pain, fractures, osteoporosis and hypercalcemia (2). Mesenchymal stem cells (MSCs) are involved in bone repair and regeneration as they can differentiate into osteoblasts and osteocytes (3). A previous study has indicated that the osteogenic differentiation of MSCs obtained from patients with MM (MM-MSCs) is impaired (4). Thus, an efficient strategy that induces osteogenic differentiation of MM-MSCs is required to improve the wellbeing of patients with MM.

MicroRNAs (miRNAs/miRs) are a large class of small, noncoding RNA molecules of 17-25 nucleotides that are involved in gene regulation at the post-transcriptional level by binding to the 3'-untranslated regions (UTRs) of target mRNAs and have been demonstrated to serve critical roles in a number of biological processes (5). Previous studies have suggested

that miRNAs contribute to bone development (6-8). Several miRNAs have been identified as regulators of osteogenesis; for instance, miR-133a-5p has been demonstrated to target runt-related transcription factor 2 (RUNX2) and inhibit the expression of osteoblast differentiation-associated markers (9). In addition, miR-20a promotes osteoblast differentiation and bone formation of human MSCs by co-regulating bone morphogenetic protein signaling (10). miR-203a is expressed in keratinocytes and affects their growth, differentiation and function (11). Additionally, miR-203a has been demonstrated to increase tumor growth in a number of types of cancer (12,13). A recent study reported that miR-203-3p inhibited osteogenesis in the jaws of diabetic rats and in rat bone marrow mesenchymal stem cells cultured in high-glucose medium by directly targeting mothers against decapentaplegic (Smad) homolog 1 (Smad1) (14). However, to the best of our knowledge, the role of miR-203a-3p.1 in the osteogenic differentiation of MM-MSCs has not been identified. Thus, the aim of the present study was to characterize the expression of miR-203a-3p.1 in MM-MSCs and to investigate its effects on osteoblast differentiation, as well as the potential molecular mechanisms.

Materials and methods

Patients and subjects. A total of five patients with newly diagnosed stage IIIA-IIIB of MM (age range, 40-63 years; 2 males, 3 females) and 5 normal healthy subjects (age range, 32-48 years; 3 males, 2 females) were recruited in the present study. The bone marrow samples were obtained at The General Hospital of Western Theater Command (Chengdu, China) from April 2017 to April 2018 according to the institutional guidelines. The present study was approved by the General Hospital of Western Theater Command (Chengdu, China). All volunteers provided written informed consent.

Bone marrow (BM)-MSC isolation and propagation. MSCs were isolated from BM samples. Briefly, the BM fluid was mixed with an equal volume of Ficoll (Tianjin Haoyang Biological Products Technology Co., Ltd.), and mononuclear cells were obtained following centrifugation at 450 x g for 20 min at room temperature. The cells were seeded in a T25 cell culture bottle at 5,000 cells/cm^2 with Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The medium was replaced twice weekly until the cultures attained 80% confluence.

MSC identification by flow cytometry. In the primary cells, CD44-PE, CD90-FITC and CD105-PC5.5 were used to isolate MSCs. Briefly, MSCs were resuspended in 4 ml PBS following digestion and centrifugation. Subsequently, MSCs were incubated with 40 μl mouse anti-human CD90-FITC (1:100), CD44-PE (1:100) and CD105-PerCP-Cy™5.5 (1:100) for 30 min at 4˚C. MSCs were isolated using a FACScalibur flow cytometer (BD Biosciences). Following 4 culture passages, the MSCs with very low fluorescence value (Blank) were sorted, and single staining was used to identify MSCs. Following washing, trypsin digestion and centrifugation, the sorted MSCs were resuspended in 100 μl PBS (1x10^6 cells) and stained with 5 μl mouse anti-human CD90-FITC (1:20), CD44-PE (1:20), CD34-FITC (1:20), CD105-PerCP-Cy™5.5 (1:20) and CD45-PE antibodies (1:20) for 30 min at 4˚C. After rinsing twice with PBS and resuspending in 500 μl of PBS, the cells were analyzed using a FACScalibur flow cytometer (BD Biosciences) and Cell Quest 3.3 software (BD Biosciences), based on their characteristic immunophenotype of CD44$^+$, CD90$^+$, CD105$^+$, CD34$^-$ and CD45$^-$. The antibodies against CD45-PE (cat. no. 304008) and CD34-FITC (cat. no. 343503) were purchased from BioLegend, Inc. The BD Stemflow™ Human MSC Analysis kit (cat. no. 562245; BD Biosciences) included CD90-FITC, CD105- PerCP-Cy™5.5 and CD44-PE antibodies.

MSC differentiation. Following 3 passages, MSCs were seeded in 6-well plates at 1x10^5 cells/well with DMEM supplemented with 10% FBS and cultured until cell confluence reached 60-80%. Fresh DMEM containing 10% FBS, 1x10^{-8} mol/l dexamethasone, 10 mmol/l sodium β-glycerophosphate and 50 μg/ml vitamin C was used to incubate the MSCs for 2 weeks (15) and replaced twice a week to induce MSC differentiation into osteoblasts (16).

Alizarin Red S staining. Following osteogenesis differentiation, MSCs were washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. MSCs were stained with Alizarin Red S (1%, pH 4.2; Novon Scientific; Beijing Xinhua Luyuan Technology Co., Ltd.) for 5 min at room temperature. Images of the cells were captured using a light microscope.

Cell transfection. MSCs were seeded into a 6-well plate at a density of 1x10^5 cells/well the day prior to transfection. Transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol with 50 pmol/ml miR-203a-3p inhibitor or mimic (Guangzhou RiboBio Co., Ltd.) at 37˚C. Fresh medium was added at 6 h. Cells were harvested for analysis following transfection for 24 h.

For the transfection of Smad9, lentiviruses overexpressing Smad9 (lv-Smad9; Smad9 mRNA sequence, NM_001127217; vector name, GV492) and the corresponding control lentiviruses (lv-green fluorescent protein) were purchased from Shanghai GeneChem Co., Ltd. MSCs were seeded into a 6-well plate at a density of 1x10^5 cells/well. Lentivirus (1x10^8 TU/ml) infection was performed when the cells reached 20-40% confluence. The lentiviruses were transfected into MSCs with a multiplicity of infection of 30. After transfection for 8-12 h, fresh medium was added for further incubation. Cells were collected for RT-qPCR or western blotting analysis 72 h after transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells (1x10^5 cells/well) using TRIzol® reagent (Takara Biotechnology, Co., Ltd.). Following isolation, 4 μg RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent kit (Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocol at 37˚C for 15 min and 85˚C for 5 sec. qPCR was performed on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Premix Ex Taq II

(Takara Biotechnology, Co., Ltd.) in a 25 μl mixture containing 2 μl cDNA templates, 12.5 μl 2X SYBR Premix Ex Taq II, 1 μl each primer and 8.5 μl DNase/RNase-free H_2O. The thermocycling conditions were as follows: 3 min at 95˚C; 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec; followed by 72˚C for 30 sec. For the quantification of miRNA expression, RT was performed at 42˚C for 60 min and 70˚C for 10 min using Bulge-Loop™ miRNA RT-qPCR Primer and Bulge-Loop™ miRNA RT-qPCR Starter kit (cat. no. C10211-1; Guangzhou RiboBio Co., Ltd.). Gene expression levels were quantified at 95˚C for 10 min, followed by 40 cycles of 95˚C for 2 sec, 60˚C for 20 sec and 70˚C for 10 sec. The $2^{-\Delta\Delta Cq}$ value was used for comparative quantitation (17). β-actin and U6 small nuclear RNA genes were used as endogenous normalization controls. The primer sequences (Sangon Biotech Co., Ltd.) are listed in Table I. The catalogue number of miR-203a-3p primer was miRA0000264-1-200, and the catalogue number of U6 was miRAN0002-1-200.

Western blot analysis. The MSCs from three normal healthy subjects were randomly selected and seeded into 6-well plates at $1x10^5$ cells/well after four culture passages. Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology, Ltd.). Protein concentration was quantified using a BCA Protein Assay kit (Wuhan Boster Biological Technology, Ltd.). The protein samples (20 μg) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore). Following blocking with 5% skimmed milk powder at 37˚C for 1 h, the membranes were incubated with primary antibodies against Smad9 (cat. no. ab115900; 1:500; Abcam), Wnt3a (cat. no. ab28472; 1:500; Abcam), β-catenin (cat. no. 8480; 1:1,000; Cell Signaling Technology, Inc.), glycogen synthase kinase (GSK)-3β (cat. no. 12456; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. 4970; 1:1,000; Cell Signaling Technology, Inc.) at 4˚C overnight, followed by a HRP-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. BA1054; 1:5,000; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h. The protein bands were visualized using a ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Inc.). Protein levels were calculated relative to β-actin.

Luciferase assay. miR-203a-3p.1 targets were predicted using bioinformatics software, including TargetScan (http://www.targetscan.org/), miRDB (http://mirdb.org), DIANA TOOLS (http://diana.imis.athena-innovation.gr) and venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). MSCs were plated in 24-well plates ($4x10^4$ cells/well). When the cultures attained 50% confluence, cells were co-transfected with the *Renilla* luciferase pRL-TK plasmid (100 ng/ml; Shanghai GenePharma Co., Ltd.) plus the recombinant Firefly luciferase pGL3 reporters containing the 3'-untranslated region (3'-UTR) of human Smad9 (2 μg/ml; Shanghai GenePharma Co., Ltd.) in combination with miR-203a-3p.1 mimic and miR-203a-3p.1 inhibitor using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Luciferase activity was detected at 24 h using a Dual-Luciferase Reporter Assay kit (cat. no. E1910; Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each tested well.

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')
ALP	F: GACCTCCTCGGAAGACACTCTG
	R: CGCCTGGTAGTTGTTGTGAGC
OPN	F: GCCGACCAAGGAAAACTCACT
	R: GGCACAGGTGATGCCTAGGA
OC	F: CCAGGCGCTACCTGTATCAATG
	R: ATGTGGTCAGCCAACTCGTCA
Smad9	F: GCAGCCTCAAGGTCTTCAACAAC
	R: CATGAAGATGAATCTCAATCCAGGA
β-actin	F: TGGCACCCAGCACAATGAA
	R: CTAAGTCATAGTCCGCCTAGAAGCA

ALP, alkaline phosphatase; OPN, osteopontin; OC, osteocalcin; Smad9, mothers against decapentaplegic homolog 9.

Statistical analysis. Statistical analysis was performed using SPSS 20.0 (IBM Corp.). The data are expressed as the mean ± standard deviation. Comparisons between two groups were analyzed by unpaired Student's t-test (for parametric data) or Mann-Whitney U test (for non-parametric data). Differences among multiple groups were compared by one-way analysis of variance (ANOVA) with Dunnett's post hoc test or two-way ANOVA with Bonferroni's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference, and $P<0.01$ was considered to indicate a highly significant difference.

Results

MSCs from patients with MM exhibit decreased osteogenic differentiation. Following 7 days of primary culture, the adherent cells exhibited colony growth and reached >40% confluence. The cells were fusiform and pleomorphic. Following 14 days of primary culture, the cells attained 60-70% confluence and had regular morphology and a long spindle shape (Fig. 1A). After 4 culture passages, the cell surface markers were detected by flow cytometry and the results revealed that MSCs were negative for CD34 and CD45, but positive for the CD44, CD90 and CD105 markers (Fig. 1B). These results suggested that the cultured cells were MSCs.

The osteogenic differentiation capacity of MSCs from patients with MM and normal subjects was investigated using Alizarin Red S staining, which revealed that the calcium deposition of MM-MSCs was lower compared with MSCs derived from normal healthy subjects (N-MSCs) (Fig. 1C). RT-qPCR results demonstrated an decrease in mRNA expression levels of typical osteoblast differentiation markers in MM-MSCs compared with N-MSCs, including alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC) (Fig. 1D). These results indicated that the osteogenic differentiation capacity of MM-MSCs may be reduced.

Effects of miR-203a-3p.1 on osteogenic differentiation in MM-MSCs. To determine whether miR-203a-3p.1 is associated with osteogenesis, the expression levels of miR-203a-3p.1

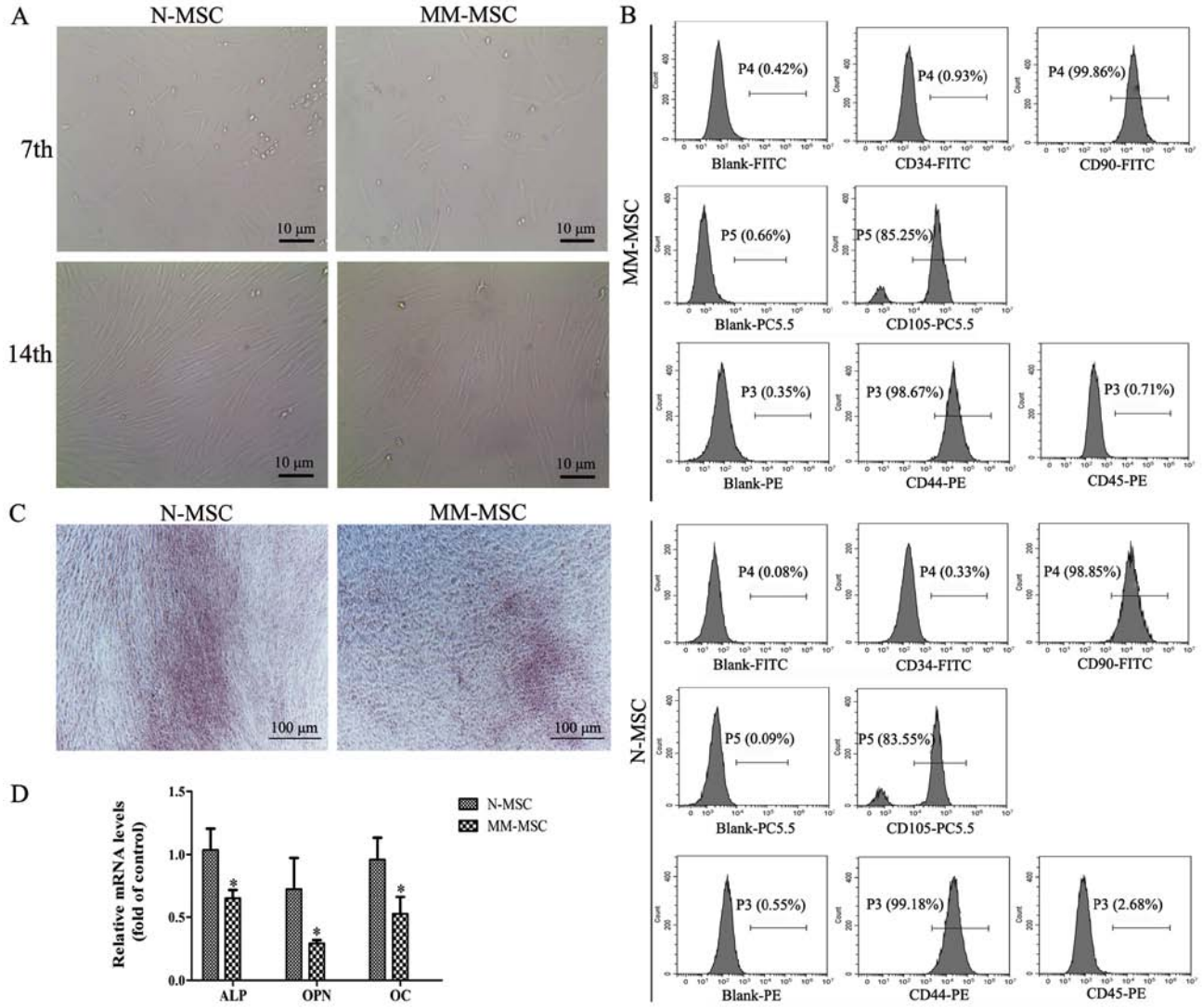

Figure 1. MM-MSCs exhibit a reduced osteogenic differentiation capacity. (A) Images of MSCs in primary culture on the 7th and 14th day captured using an inverted microscope (magnification, x400). (B) The surface markers of the third generation MM-MSCs and N-MSCs were identified by flow cytometry Blank group, isolated MSCs from the primary cells at the fourth generation with no fluorescence detected. (C) Following 21 days of osteogenic induction, calcium deposition was evaluated using Alizarin Red S staining (magnification, x40). (D) Reverse transcription-quantitative PCR was performed to detect the mRNA levels of ALP, OPN and OC following osteogenic induction in N-MSCs and MM-MSCs. *P<0.05 vs. N-MSC. MM, multiple myeloma; N, normal; MSCs, mesenchymal stem cells; ALP, alkaline phosphatase; OPN, osteopontin; OC, osteocalcin; CD, cluster of differentiation.

in MSCs from patients with MM and normal subjects were analyzed by RT-qPCR. The results revealed that the expression of miR-203a-3p.1 in MM-MSCs was significantly lower compared with that in N-MSCs. In addition, the expression of miR-203a-3p.1 in N-MSCs was significantly decreased following osteoblast induction, whereas no evident change was observed in MM-MSCs; therefore, it was hypothesized that the decrease in the expression of miR-203a-3p.1 contributes to osteogenic differentiation in N-MSCs, whereas in MM-MSCs, the decreased expression of miR-203a-3p.1 was inhibited, which resulted in the reduced osteogenic differentiation capacity of MM-MSCs. To study the role of miR-203a-3p.1 on osteogenic differentiation, MM-MSCs cells were transfected with an miR-203a-3p.1 mimic and inhibitor. RT-qPCR results revealed that the miR-203a-3p.1 mRNA expression levels were significantly increased in the mimic group and decreased in the inhibitor group compared with the corresponding negative control groups (Fig. 2B). In N-MSCs transfected with the miR-203a-3p.1 inhibitor, mRNA expression levels of ALP, OPN and OC increased; however, the overexpression of miR-203a-3p.1 had no significant effects on osteogenic differentiation marker expression (Fig. 2C). These results indicate that miR-203a-3p.1 inhibition may increase osteogenic differentiation.

Smad9 is a target of miR-203a-3p.1. The miRNA target prediction databases TargetScan, miRDB, DIANA TOOLS and venny 2.1.0 were used to identify the target genes of miR-203a-3p.1 in osteogenesis. A conserved putative target site for miR-203a-3p.1 was identified in the 3'-UTR of the Smad9 gene (Fig. 3A). To assess whether Smad9 may be regulated by miR-203a-3p.1, the N-MSCs were transfected with miR-203a-3p.1 mimic and inhibitor, respectively. The luciferase assay revealed that the miR-203a-3p.1 mimic significantly repressed the luciferase activity and the miR-203a-3p.1 inhibitor increased luciferase activity (Fig. 3B). RT-qPCR and western blotting results demonstrated that the mRNA and

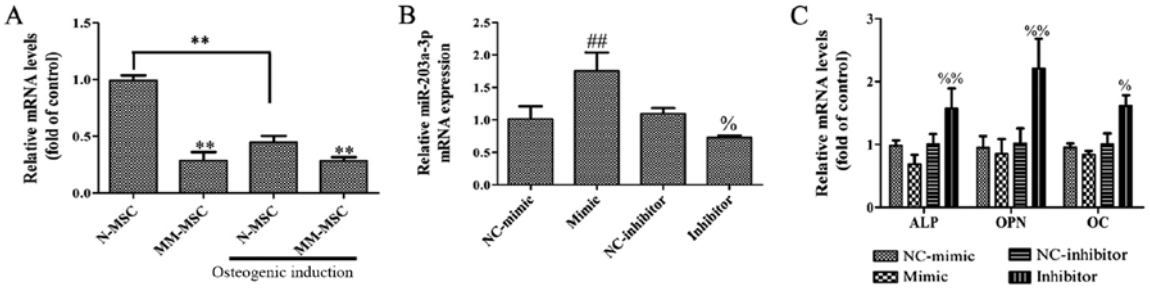

Figure 2. Effects of miR-203a-3p.1 inhibition on the osteogenic differentiation of N-MSCs. (A) The miR-203a-3p.1 mRNA expression levels in N-MSCs and MM-MSCs were detected by RT-qPCR prior to and following osteoblast induction. (B) The miR-203a-3p.1 mRNA expression levels in N-MSCs were detected by RT-qPCR following transfection with miR-203a-3p.1 mimic or inhibitor. (C) The mRNA expression levels of ALP, OPN and OC in N-MSCs were detected by RT-qPCR following transfection with miR-203a-3p.1 mimic or inhibitor. **P<0.01 vs. N-MSC; $^{\#\#}$P<0.01 vs. NC-mimic; $^{\%}$P<0.05 and $^{\%\%}$P<0.01 vs. NC-inhibitor. miR, microRNA; MM, multiple myeloma; N, normal; MSCs, mesenchymal stem cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ALP, alkaline phosphatase; OPN, osteopontin; OC, osteocalcin; NC, negative control; mimic, miR-203a-3p.1-mimic; inhibitor, miR-203a-3p.1-inhibitor.

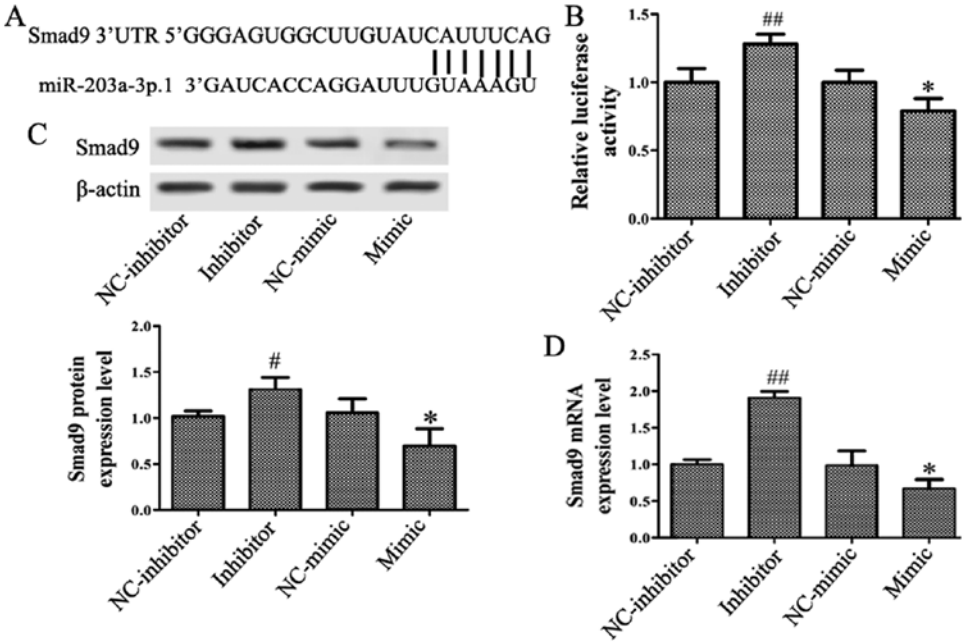

Figure 3. miR-203a-3p.1 targets Smad9 in N-MSCs. (A) Putative miR-203a-3p.1-binding sequence of Smad9. (B) N-MSCs were co-transfected with miR-203a-3p.1 mimic or inhibitor and the reporter plasmid containing the 3'-UTR of Smad9. miR-203a-3p.1 inhibitor enhanced luciferase activity, while the miR-203a-3p.1 mimic reduced luciferase activity. (C and D) Smad9 protein and mRNA expression levels were detected by western blot and RT-qPCR following transfection with miR-203a-3p.1 inhibitor or mimic, respectively. miR-203a-3p.1 inhibitor increased the expression levels of Smad9 protein and mRNA, while miR-203a-3p.1 mimic decreased the expression levels of Smad9 protein and mRNA. *P<0.05 vs. NC-mimic; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. NC-inhibitor. miR, microRNA; MM, multiple myeloma; MSCs, mesenchymal stem cells; UTR, untranslated region; Smad9, mothers against decapentaplegic homolog 9; NC, negative control; mimic, miR-203a-3p.1-mimic; inhibitor, miR-203a-3p.1-inhibitor.

protein levels of Smad9 were reduced when miR-203a-3p.1 was overexpressed; by contrast, the expression of Smad9 increased following treatment with miR-203a-3p.1 inhibitor (Fig. 3C and D). These results indicated that miR-203a-3p.1 may target Smad9 and negatively regulate Smad9 expression.

Inhibition of miR-203a-3p.1 mediates osteogenic differentiation through Smad9. To investigate the association between miR-203a-3p.1 and Smad9 during osteogenic differentiation, a lentiviral vector overexpressing Smad9 was used. Western blotting results demonstrated that Smad9 protein expression levels were significantly increased in N-MSCs following lentiviral vector transfection (Fig. 4A). Furthermore, co-transfection of miR-203a-3p.1 inhibitor with lv-Smad9 increased calcium deposition and mRNA expression levels of ALP, OPN and OC in MM-MSCs (Fig. 4B and C).

Thus, overexpression of Smad9 appeared to enhance the miR-203a-3p.1-knockdown-mediated promotion of osteogenic differentiation. These results indicated that the inhibition of miR-203a-3p.1 increased osteogenic differentiation, in part via the upregulation of Smad9 expression.

miR-203a-3p.1 may inhibit the osteogenic differentiation of N-MSCs by inhibiting the Wnt3a/β-catenin signaling pathway. To assess whether the impairment of osteogenic differentiation in N-MSCs was due to abnormalities in the Wnt3a/β-catenin

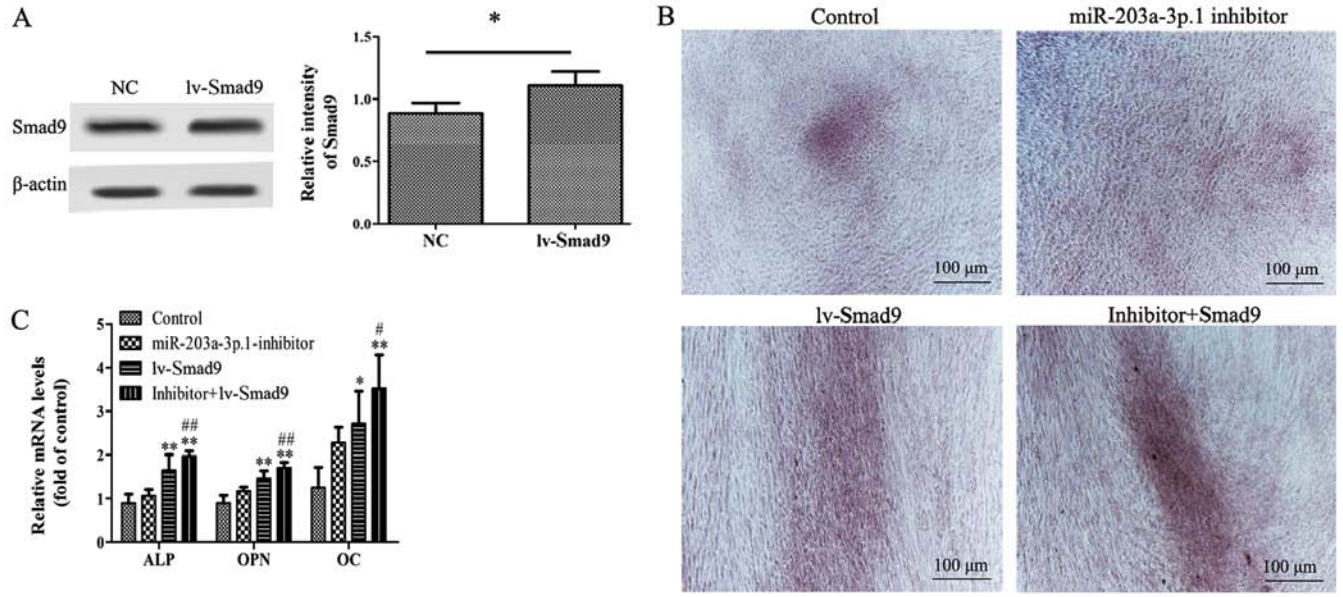

Figure 4. Effects of Smad9 overexpression on miR-203a-3p.1 inhibitor-mediated osteogenic differentiation in N-MSCs. (A) Smad9 protein expression levels were detected by western blot analysis following transfection with lentivirus. (B) N-MSCs transfected with miR-203a-3p.1 inhibitors were infected with lv-Smad9. Images of Alizarin Red S staining were captured using an inverted microscope (magnification, x40). Co-transfection of miR-203a-3p.1 inhibitor with lv-Smad9 increased the calcium deposition. (C) The ALP, OPN and OC mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction following transfection with miR-203a-3p.1 inhibitor or lentiviral vector. *P<0.05 and **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. miR-203a-3p.1 inhibitor group. miR, microRNA; MM, multiple myeloma; MSCs, mesenchymal stem cells; ALP, alkaline phosphatase; OPN, osteopontin; OC, osteocalcin; Smad9, mothers against decapentaplegic homolog 9; lv-, lentivirus; inhibitor: miR-203a-3p.1-inhibitor.

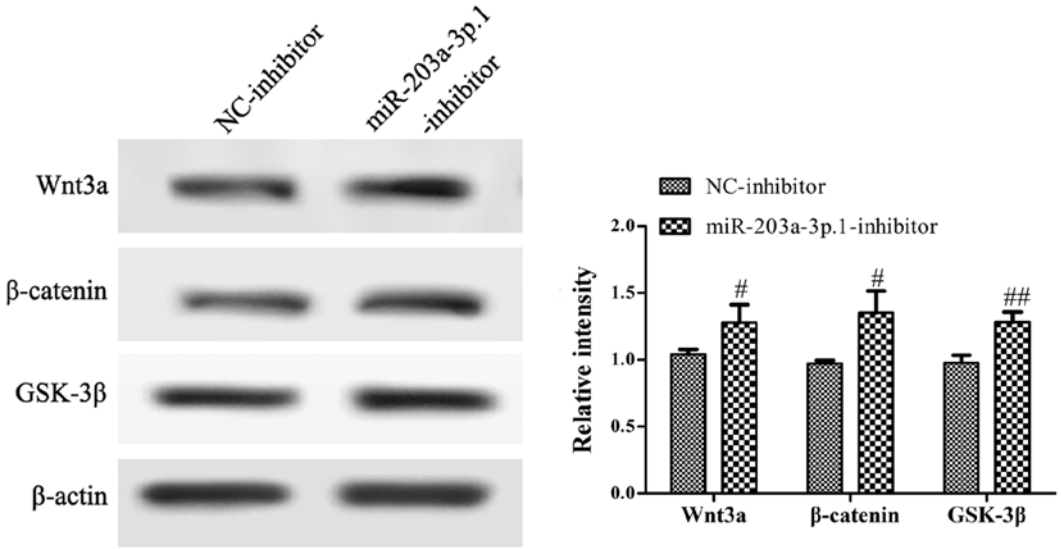

Figure 5. Inhibition of miR-203a-3p.1 activates the Wnt3a/β-catenin signaling pathway. The key proteins of the Wnt3a/β-catenin signaling pathway were detected using western blotting following N-MSC transfection with miR-203a-3p.1-inhibitor. $^{\#}$P<0.05 and $^{\#\#}$P<0.01. miR, microRNA; MM, multiple myeloma; MSC, mesenchymal stem cells; NC, negative control; inhibitor, miR-203a-3p.1-inhibitor; GSK-3β, glycogen synthase kinase 3β.

signaling pathway, the protein expression levels of genes involved in the Wnt3a/β-catenin signaling pathway were analyzed by western blotting. The results revealed that the miR-203a-3p.1 inhibitor significantly increased the expression of Wnt3a, β-catenin and GSK-3β (Fig. 5).

Discussion

MM is a common malignancy characterized by the abnormal proliferation of clonal plasma cells in BM (18). One of the characteristics of MM is bone lesions due to a severe imbalance in bone remodeling (19). MSCs serve as a basic cellular unit of embryonic bone formation, which has a key role in fracture repair and bone regeneration (20). However, the osteogenic differentiation of MSCs obtained from patients with MM is impaired, which leads to decreased osteogenesis, increased adipogenesis and osteonecrosis (4,21,22). Maintaining a balance between the levels of osteoclasts and osteoblasts regulates bone homeostasis (23). Osteoblasts promote bone formation by secreting ALP and the bone

matrix protein that induces bone matrix mineralization (24). In the present study, the osteogenic differentiation capacity of MM-MSCs was significantly lower compared with that of N-MSCs, as indicated by the decrease in calcium deposition and the mRNA expression levels of the typical osteoblast differentiation markers, including ALP, OPN and OC. These results demonstrated that the osteogenic differentiation of MSCs was inhibited in patients with MM. Thus, understanding the mechanisms leading to the decreased osteogenic differentiation capability of MM-MSCs may explain the osteogenesis defects in patients with MM. In further studies, more bone marrow samples will be used to verify this result and further study the mechanism underlying decreased osteogenic differentiation of MSCs in patients with MM.

miRNAs have emerged as essential regulatory molecules of gene expression that participate in the regulation of bone homeostasis through transcriptional inhibition or mRNA cleavage (25). The majority of miRNAs have been described as signaling network nodes that serve a vital role in osteoblastic differentiation processes; for instance, miR-99a serves as a novel regulator of lysine demethylase 6B to regulate the osteogenic differentiation of BM stromal cells (26). In addition, miR-590-5p promotes osteoblast differentiation by indirectly protecting and stabilizing the Runx2 protein by targeting Smad7 gene expression in MSCs (27). Tang *et al* (14) demonstrated that miR-203-3p participates in the suppression of diabetes-associated osteogenesis in the jaw bone through targeting smad1. The results of the present study revealed decreased expression levels of miR-203a-3p.1 in MM-MSCs. Following osteoblast induction, the miR-203a-3p.1 mRNA expression level in N-MSCs was significantly decreased, whereas no change was observed in MM-MSCs. These results indicated that the downregulation of miR-203a-3p.1 may contribute to the osteogenic differentiation of normal MSCs. In agreement with these results, the typical osteoblast differentiation markers ALP, OPN and OC were upregulated in MM-MSCs following treatment with the miR-203a-3p.1 inhibitor. No changes were observed in ALP, OPN and OC mRNA levels in MM-MSCs overexpressing miR-203a-3p.1. These results suggested that the inhibition of miR-203a-3p.1 may increase osteoblast differentiation of MM-MSCs.

Transforming growth factor (TGF)-β signaling is an important pathway in osteoblastic differentiation. Through putative target prediction, the present study identified that miR-203a-3p.1 may target Smad9, which is an important component of the TGF-β signaling pathway. Previous studies have reported that several SMADs, including Smad3, Smad7 and Smad5, are involved in bone formation, remodeling and maintenance (27-29). Smad9 is upregulated during chondrocyte differentiation (30), whereas its expression and role in osteoclast differentiation has not been studied. In the present study, a conserved putative target site for miR-203a-3p.1 was identified in the 3'-UTR of Smad9. Furthermore, the RT-qPCR and western blotting assays revealed a negative association between Smad9 and miR-203a-3p.1. Luciferase reporter analysis indicated that Smad9 may be a direct target of miR-203a-3p.1 in MM-MSCs. Rescue experiments demonstrated that overexpression of Smad9 significantly enhanced the effect of the miR-203a-3p.1 inhibitor on osteoblast marker expression, which indicated that inhibition of miR-203a-3p.1 mediated promotion of osteogenic differentiation partially by upregulating Smad9. In further studies, Smad9 gene silencing will be used to validate the effects of Smad9 overexpression on osteoblast marker expression.

Wnt proteins are a large family of highly conserved secreted signaling molecules that mediate essential biological processes such as embryogenesis, organogenesis and tumorigenesis (31). In addition, Wnt/β-catenin is an important signaling pathway that regulates osteoblast differentiation and bone formation. Chen *et al* (32) reported that the knockdown of Sirtuin-7 increased the osteogenic differentiation of human BM-MSCs by activating the Wnt/β-catenin signaling pathway. In addition, baicalein promoted the osteogenic differentiation of human periodontal ligament cells by activating the Wnt/β-catenin signaling pathway (33). In the present study, the partial inhibition of miR-203a-3p.1 increased the protein expression levels of Wnt3a, β-catenin and GSK-3β in N-MSCs. These results indicated that the miR-203a-3p.1 inhibitor enhanced the osteoblast differentiation of MM-MSCs potentially by activating the Wnt/β-catenin signaling pathway.

In conclusion, the present study demonstrated that miR-203a-3p.1 was downregulated in MM-MSCs and may participate in osteogenic differentiation. Furthermore, the inhibition of miR-203a-3p.1 increased osteoblast differentiation by directly targeting Smad9. The potential mechanism may be associated with the activation of the Wnt3a/β-catenin signaling pathway. The present study revealed a potential function of miR-203a-3p.1 in the osteogenic differentiation of MM-MSCs, which may be targeted to develop a promising therapeutic against myeloma bone disease in the future.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FYF, RD, YS and XZ conceived and designed the experiments. FYF, RD, LQ, QW, YZ and LG performed the experiments. CZ, PK, JZ, NZ and ZL analyzed the data and assisted with the experiments. FYF, RD, YS and XZ wrote the manuscript. YS and XZ revised the manuscript and supervised the study. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of General Hospital of Western Theater Command (Chengdu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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