

RRM2 regulates osteogenesis of mouse embryo fibroblasts via the Wnt/ β -catenin signaling pathway

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Abstract. Osteoporosis is a widespread bone metabolic disease characterized by reduced bone mass and bone microstructure deterioration. Ribonucleotide reductase M2 (RRM2) is a key enzyme in DNA synthesis and repair. The present study investigated the effect of RRM2 on osteogenesis of mouse embryo fibroblasts (MEFs) and its molecular mechanism. Bioinformatics analysis revealed that RRM2 expression was increased during osteogenesis of MEFs triggered by bone morphogenetic protein 9. Subsequently, MEFs were used as a mesenchymal stem cell model and osteogenic inducing medium was used to induce osteogenic differentiation. RRM2 protein expression was measured by western blotting during osteogenic differentiation induction of MEFs. RRM2 levels in MEFs were upregulated and downregulated by RRM2-overexpressing recombinant adenovirus and small interfering RNA-RRM2, respectively. Bone formation markers (RUNX family transcription factor 2, osterix, distal-less homeobox 5, collagen type I α 1 chain, osteopontin and osteocalcin) were detected by reverse transcription-quantitative (RT-q) PCR and alkaline phosphatase (ALP) and Alizarin Red S staining were examined. The protein expression levels of β -catenin and the ratio of phosphorylated (p-) GSK-3 β to GSK-3 β were detected by western blotting and the RNA expression of downstream related target genes (β -catenin, axis inhibition protein 2 (AXIN2), transcription factor 7 like 2, lymphoid enhancer binding factor 1, c-MYC and Cyclin D1) in the Wnt/ β -catenin signaling pathway was measured by RT-qPCR. RRM2 protein expression increased as the osteogenic differentiation induction period was

extended. RRM2 overexpression increased osteogenic marker RNA expression, ALP activity, bone mineralization, the protein expression levels of β -catenin, the ratio of p-GSK-3 β to GSK-3 β and the RNA expression of downstream related target genes in the Wnt/ β -catenin signaling pathway, whereas RRM2 knockdown had the opposite effect. The findings of the present study revealed that RRM2 overexpression enhanced osteogenic differentiation, while RRM2 knockdown reduced osteogenic differentiation. RRM2 may regulate osteogenic differentiation of MEFs via the canonical Wnt/ β -catenin signaling pathway, providing a possible therapeutic target for osteoporosis.

Introduction

Osteoporosis is known as a widespread progressive bone metabolic disease characterized by reduced bone mass and bone microstructure deterioration, which leads to an increase in bone fragility and the risk of fracture (1,2). The imbalance of bone resorption and bone synthesis resulting from osteolysis over bone formation is a key factor in the progression of osteoporosis (3,4). The global prevalence of osteoporosis has been reported to be 18.3% (23.1% in women and 11.7% in men) (5). With the aging of the world's population, osteoporosis is becoming increasingly widespread and the personal and social expenses are rising year after year, making it a serious public health issue (2,5). As bone strength is compromised, osteoporotic fractures are a leading cause of bone-related disorders. The most serious consequences of osteoporosis are hip and vertebral fractures, in particular. Treatment for osteoporosis focuses on reducing bone loss, maintaining bone metabolism and lowering the risk of fracture (6). The majority of current treatments are lifestyle changes and pharmaceutical interventions (6,7). Bisphosphonates (BPs), calcitonin, denosumab, teriparatide and other pharmacological medications are commonly used to inhibit bone resorption or promote bone anabolism. The anti-sclerostin antibody romosozumab (8), stem cell-based treatments and gene therapies are all promising novel medicines.

In recent years, gene therapy and mesenchymal stem cell (MSC) therapy has been developed as novel regenerative medicine treatments (9,10). MSC therapy may have a bright future

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in the treatment of osteoporosis (11,12). MSCs appear to be multipotent stem cells, which may self-renew and specialize in multiple directions, and they serve a vital role in tissue regeneration and regenerative medicine (9). MSC transplantation is considered to enhance osteoblast differentiation, limit osteoclast activation and equilibrate bone formation and resorption, avoiding osteoporosis development (13). Mouse embryo fibroblasts (MEFs) are multipotent progenitor cells (14). MEFs have MSC-like properties *in vitro* and *in vivo* and are regarded as an important cell line for MSC-related research (15). Gene therapy uses gene technology to transfer genes that induce osteoblast differentiation from MSCs, enhancing their osteogenic differentiation capability, stimulating bone formation and helping to increase the therapeutic effect and efficacy of MSCs (16). Therefore, the present research focus is utilizing gene modifications to enhance osteogenesis of MSCs.

Ribonucleotide reductase M2 (RRM2) is considered to be a DNA synthesis enzyme that catalyzes the deoxy reduction of nucleosides to deoxyribonucleoside triphosphate. It is important for controlling cell proliferation and differentiation (17). RRM2 can enhance cardiac myocyte proliferation and act as a tumor biomarker, according to most studies (18,19). However, to the best of the authors' knowledge, its significance in osteogenic differentiation remains to be elucidated.

The present study used RRM2-overexpressing recombinant adenovirus (AdRRM2) and small interfering (si) RNA-RRM2 (siRRM2) to upregulate and downregulate RRM2 levels in MEFs and to investigate the influence and molecular mechanism of RRM2 on osteoblast differentiation for the first time. It is expected that this could provide a possible therapeutic target for the use of gene therapy based MSC therapy in osteoporosis.

Materials and methods

Cell culture and osteogenic induction. MEFs (Wt MEFs; cat. no. CRL-2991) were donated by American Type Culture Collection for the present study. Dulbecco modified Eagle's high glucose medium (DMEM; Chongqing Saimike Biotechnology Co., Ltd; www.saimikebio.com) supplemented with streptomycin (100 µg/ml), penicillin (100 U/ml) and 10% FBS (cat. no. s711-001s; Shanghai Shuangru Biotechnology Co., Ltd.) was used to cultivate the cells at 37°C with 5% CO₂ (20). Once cell confluence reached ~40% and following transfection with recombinant adenovirus or siRNA, DMEM was replaced by osteogenic inducing medium (OM). OM comprised DMEM, streptomycin (100 µg/ml), penicillin (100 U/ml), 10% FBS, dexamethasone (100 nM; Beyotime Institute of Biotechnology), L-ascorbic acid (50 µg/ml; MilliporeSigma) and β-glycerophosphate (10 mM; MilliporeSigma) (21). The medium was replaced every 2 days.

Cell transfection. Cells were seeded in a 6-well plate. Polybrene (4 µg/ml; Beyotime Institute of Biotechnology) was added, followed by RRM2-overexpressing recombinant adenovirus labeled with green fluorescence protein (AdRRM2; cat. no. GOSA0296619; Shanghai GeneChem Co., Ltd.) and negative control adenovirus labeled with green fluorescence protein (AdGFP; cat. no. ADCON267; Shanghai GeneChem Co., Ltd.) according to the manufacturer's standard protocol when cell confluence reached ~40%. After transfection at

37°C for 8-12 h, the medium was changed to OM. Green fluorescence was observed and images were captured 24 h after treatment using a fluorescence microscope (Olympus IX53; Olympus Corporation; magnification, x100). siRRM2 was purchased from Shanghai GeneBio Co., Ltd. The sequences of siRRM2 were: Forward, 5'-GAGUACCAUGAUUCUGG CAGAUGU-3' and reverse, 5'-ACAUCUGCCAGAUUAUCAU GGUACUC-3'. According to the manufacturer's recommended protocol, siRRM2 was configured as a working system with an ultimate concentration of 20 µM and cells were transfected using Lipo8000 Transfection Reagent (cat. no. C0533; Beyotime Institute of Biotechnology) when cell confluence reached ~40%. In the control group, only Lipo8000 was added without siRRM2. Subsequent experimentation was performed after transfection with recombinant adenovirus or siRNA for at least 2 days.

Bioinformatics analysis. Our previous RNA sequencing data of bone morphogenetic protein 9 (BMP9)-induced osteogenesis in MEFs (Table SI; MEFs respectively transfected with AdGFP or AdBMP9 were sequenced in Chongqing Genetic Biotech Inc. in 2019; <https://pan.baidu.com/s/11PhrQKn-p4Kw3oK52q5Dig?pwd=peyz>; lncRNA/expression_diff/GFP-VS-B9. GeneDiffExpFilter.xls) was imported into Rstudio software (RStudio 2021.09.1 Build 372 ©2009-2021 RStudio, PBC; <https://www.rstudio.com/>) and a Volcano plot [false discovery rate (FDR)=0.01; llog2 fold change (FC)=1.5] was generated using ggplot2 (ggplot2 version 3.3.5; <https://ggplot2.tidyverse.org>). FDR <0.01 and llog2FC >1.5 were first used to screen out the top 30 differentially expressed genes in Excel (Microsoft Excel suitable for Microsoft 365MSO; version 2205 Build 16.0.15225.20172), these were imported into Rstudio software and a heatmap was generated using Pheatmap (ComplexHeatmap version 2.10.0) (4,22).

Alkaline phosphatase (ALP) staining. MEFs were plated in 24-well cell culture plates and transfected using AdRRM2 or siRRM2 according to the experimental design. The cells were fixed for 10 min in a 4% paraformaldehyde solution at room temperature before being washed twice with PBS after 7 days. The cells were subsequently stained according to the manufacturer's instructions using NBT/BCIP kits (cat. no. C3206; Beyotime Institute of Biotechnology) (20).

Alizarin Red S (AR) staining. MEFs were plated on 24-well cell culture plates and transfected with the appropriate reagents, depending on the experimental requirements. After being stimulated for 21 days, the cells were fixed in a 4% paraformaldehyde solution at room temperature for 10 min before being washed twice with PBS. The cells were then stained with a 0.4% AR solution for 10 min at room temperature before being rinsed with distilled water (21). Finally, the plates were scanned and images were captured using a fluorescence microscope (Olympus IX53; Olympus Corporation; magnification, x100).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. MEFs were seeded in 6-well cell culture plates and transfected with appropriate reagents based on the experimental design. Yosi TRezol Reagent (cat. no. B1012; Wuhan Youshi

Table I. Primer sequences for quantitative PCR.

Gene	GenBank ID	Forward sequences (5'→3')	Reverse sequences (5'→3')
β-actin	NM_007393.5	CCACCATGTACCCAGGCATT	CGGACTCATCGTACTCCTGC
RRM2	NM_009104.2	TGGCTGACAAGGAGAACACG	AGGCGCTTTACTTTCCAGCTC
RUNX2	NM_001146038.2	GCCAATCCCTAAGTGTGGCT	AACAGAGAGCGAGGGGGTAT
OSX	NM_130458.3	GTCGGGGAAGAAGAAGCCAA	TAGGGGAACAGAGAGAGCCCC
DLX5	NM_010056.2	CTCAGCCACCACCCTCAT	TGGCAGGTGGGAATTGAT
COL1A1	NM_007742.3	CAGTCGCTTCACCTACAGCA	GGTGGAGGGAGTTTACACGA
OPN	NM_001204233.1	TGCACCCAGATCCTATAGCC	CTCCATCGTCATCATCATCG
OCN	NM_007541.2	AAGCAGGAGGGCAATAAGGT	TAGGGCAGCACAGGTCCTAA
β-catenin	NM_007614.3	GTGCAATTCCTGAGCTGACA	CTTAAAGATGGCCAGCAAGC
AXIN2	NM_015732.4	GAGGAGATCGAGGCAGAAGC	TGCATTCCGTTTTGGCAAGG
TCF7L2	NM_001142924.2	AACGAACACAGCGAATGTTTCC	CTCGGCATTTCTTAGGAGCG
LEF1	NM_010703.4	TCACTGTGAGGCGACACTTC	TGAGGCTTCACGTGCATTAG
c-Myc	NM_001177352.1	GCCCAGTGAGGATATCTGGA	ATCGCAGATGAAGCTCTGGT
Cyclin D1	NM_007631.3	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC

RRM2, ribonucleotide reductase M2; RUNX2, RUNX family transcription factor 2; OSX, osterix; DLX5, distal-less homeobox 5; COL1A1, collagen type I α1 chain; OPN, osteopontin; OCN, osteocalcin; AXIN2, axis inhibition protein 2; TCF7L2, transcription factor 7 like 2; LEF1, lymphoid enhancer binding factor 1.

Biotechnology Co., Ltd; www.yoshibio.com) was used to extract total RNA when the cell density was $\sim 2.5 \times 10^6$ /well. Nano Drop One equipment (Thermo Fisher Scientific, Inc.) was used to measure the concentration of the total RNA. Subsequently, 1 μ g total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (cat. no. RR037A; Takara Bio, Inc.) according to the manufacturer's procedure. Finally, the BIO-RAD CFX Connect Real-time system was utilized to perform RT-qPCR using 2X SYBR Green qPCR Master Mix (cat. no. B21202; Bimake Bio, Inc). The thermocycling conditions used for qPCR were: 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 20 sec, and extension at 70°C for 20 sec. The relative expression levels of mRNA were quantified using the $2^{-\Delta\Delta C_q}$ method and normalized to the internal reference gene β-actin (23-25). Tests were performed in at least three independent experiments. Primer sequences used for RT-qPCR are shown in Table I.

Western blotting (WB). MEFs were seeded in 6-well cell culture plates and treated based on the design of the experiment. The plates were washed with PBS and placed on ice and proteins were extracted with cold RIPA lysis solution (Beyotime Institute of Biotechnology). After centrifuging the samples at 12,000 \times g at 4°C for 15 min, the supernatant was absorbed. The Enhanced BCA Protein Assay Kit (cat. no. P0010; Beyotime Institute of Biotechnology) was performed to determinate the protein concentration. Following the addition of loading buffer and β-mercaptoethanol, the protein was denatured by boiling for 10-15 min. The proteins (30 μ g per lane) were separated by 10% SDS-PAGE and then electrotransferred onto PVDF membranes for 90 min at 210 mA. The membranes were blocked with TBS with 0.2% Tween-20 (TBST) buffer containing 5% BSA

(cat. no. 0123S; Chongqing Saimike Biotechnology Co., Ltd.) for ~ 1 h at room temperature. Subsequently, the membranes were incubated at 4°C with the primary antibodies of β-actin (1:10,000; cat. no. AC038; ABclonal Biotech Co., Ltd.), RRM2 (1:1,000; cat. no. sc-398294; Santa Cruz Biotechnology, Inc.), β-catenin (1:1,000; cat. no. sc-7963; Santa Cruz Biotechnology, Inc.), GSK-3β (1:1,000; cat. no. sc-377213; Santa Cruz Biotechnology, Inc.) and phosphorylated (p)-GSK-3β (1:1,000; cat. no. sc-373800; Santa Cruz Biotechnology, Inc.) overnight. After three washes with TBST, the membranes were incubated with HRP-labeled Goat Anti-Rabbit IgG(H+L) (1:3,000; cat. no. A0208; Beyotime Institute of Biotechnology) or HRP-labeled Goat Anti-Mouse IgG(H+L) (1:3,000; cat. no. A0216; Beyotime Institute of Biotechnology) for 1 h at room temperature. After three washes with TBST, Super ECL Detection Reagent (cat. no. 160072; Chongqing Saimike Biotechnology Co., Ltd.) was used to observe the protein bands on the membranes. ImageJ software (National Institutes of Health; version 1.51j8) was then used to measure the intensity of the bands (24,25).

Statistical analysis. The unpaired t test was used to compare two groups of data and one-way ANOVA followed by Bonferroni's post hoc test was performed to compare several groups of data using GraphPad Prism 8.0 statistical software (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RRM2 is upregulated during osteogenic differentiation. RRM2 was upregulated during the process of BMP9-induced osteogenic differentiation in MEFs, according to a bioinformatics analysis (Volcano plot and heat map) of prior data of

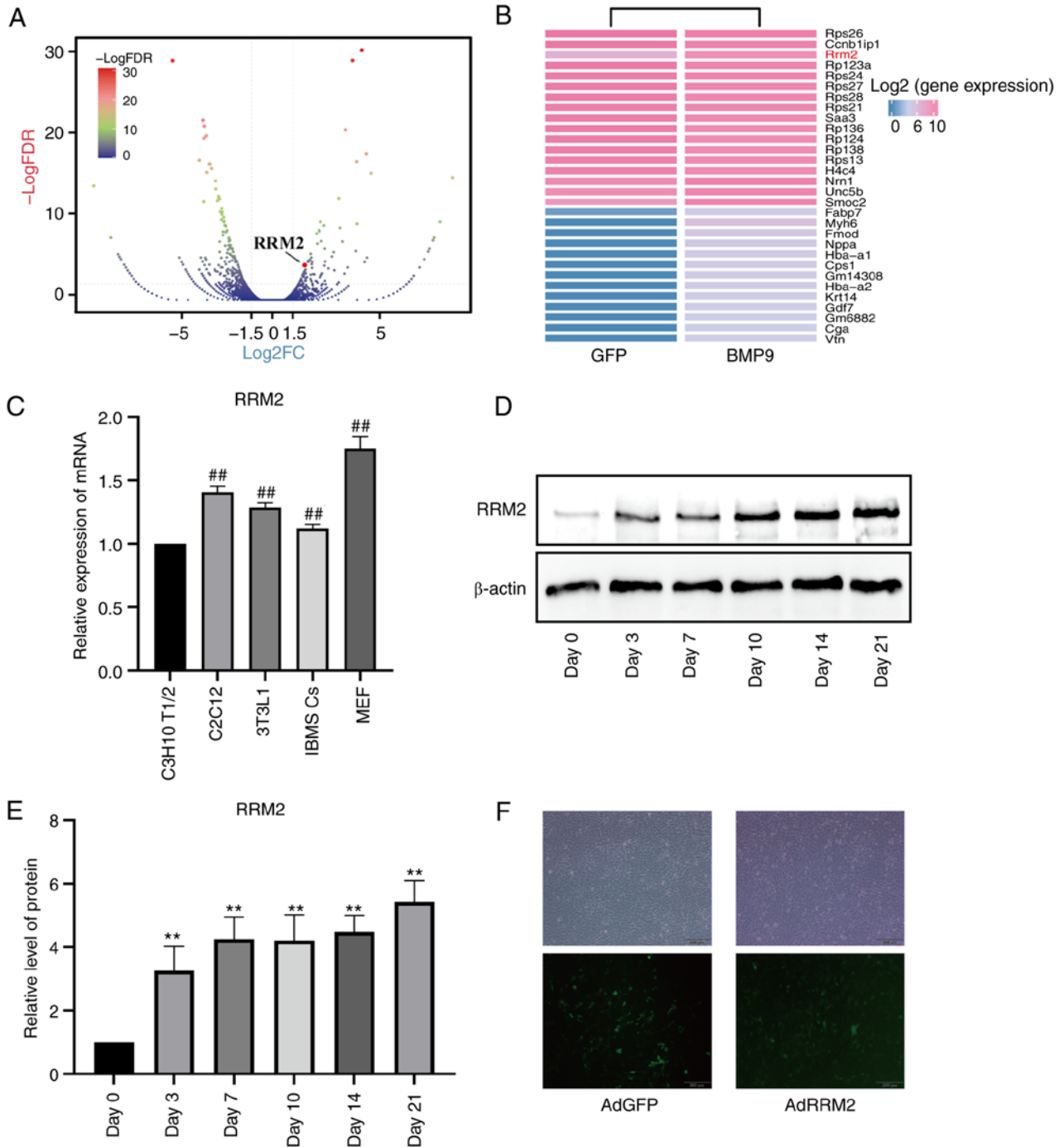


Figure 1. RRM2 is involved in the osteogenic differentiation of MEFs. (A) Volcano plot of differentially expressed genes. (B) The heat map of the top 30 differentially expressed genes. Red indicates upregulation and blue indicates downregulation. Differentially expressed genes (false discovery rate <0.01 ; log_2 fold change >1.5) were selected from bone morphogenetic protein 9-induced osteogenic differentiation RNA sequencing data (Table SI). (C) Reverse transcription-quantitative PCR analysis revealed endogenous RRM2 expression in some progenitor cells. (D and E) WB and semi-quantification of WB indicated RRM2 protein expression in MEFs cultured with osteogenic inducing medium from day 0 to 21. (F) Green fluorescence and cells were imaged 24 h after transfection with negative control adenovirus labeled with green fluorescence protein and RRM2-overexpressing recombinant adenovirus using a fluorescence microscope. Scale bar=200 μm (magnification, $\times 100$). ## $P<0.01$ vs. C3H10T1/2. ** $P<0.01$ vs. Day 0 group. RRM2, ribonucleotide reductase M2; MEFs, mouse embryo fibroblasts; WB, western blotting.

BMP9-induced osteogenic differentiation in MEFs (Table SI; Fig. 1A and B). These results implied that RRM2 might be intimately linked to osteogenic differentiation. The endogenous expression of RRM2 in progenitor cells was examined next and the RT-qPCR results revealed that RRM2 was detectable in all available stem cells (Fig. 1C). Subsequently, using MEFs as a MSC model, the present study investigated RRM2 expression

in MEFs during osteogenic induction and revealed that RRM2 expression increased as the osteogenic induction stage progressed (compared with day 0, the protein levels of RRM2 were increased 2.254, 3.248, 3.212, 3.484 and 4.433 times on day 3, 7, 10, 14 and 21, respectively; Fig. 1D and E). These results further support the notion that RRM2 may serve a role in favoring osteogenic differentiation of MSCs.

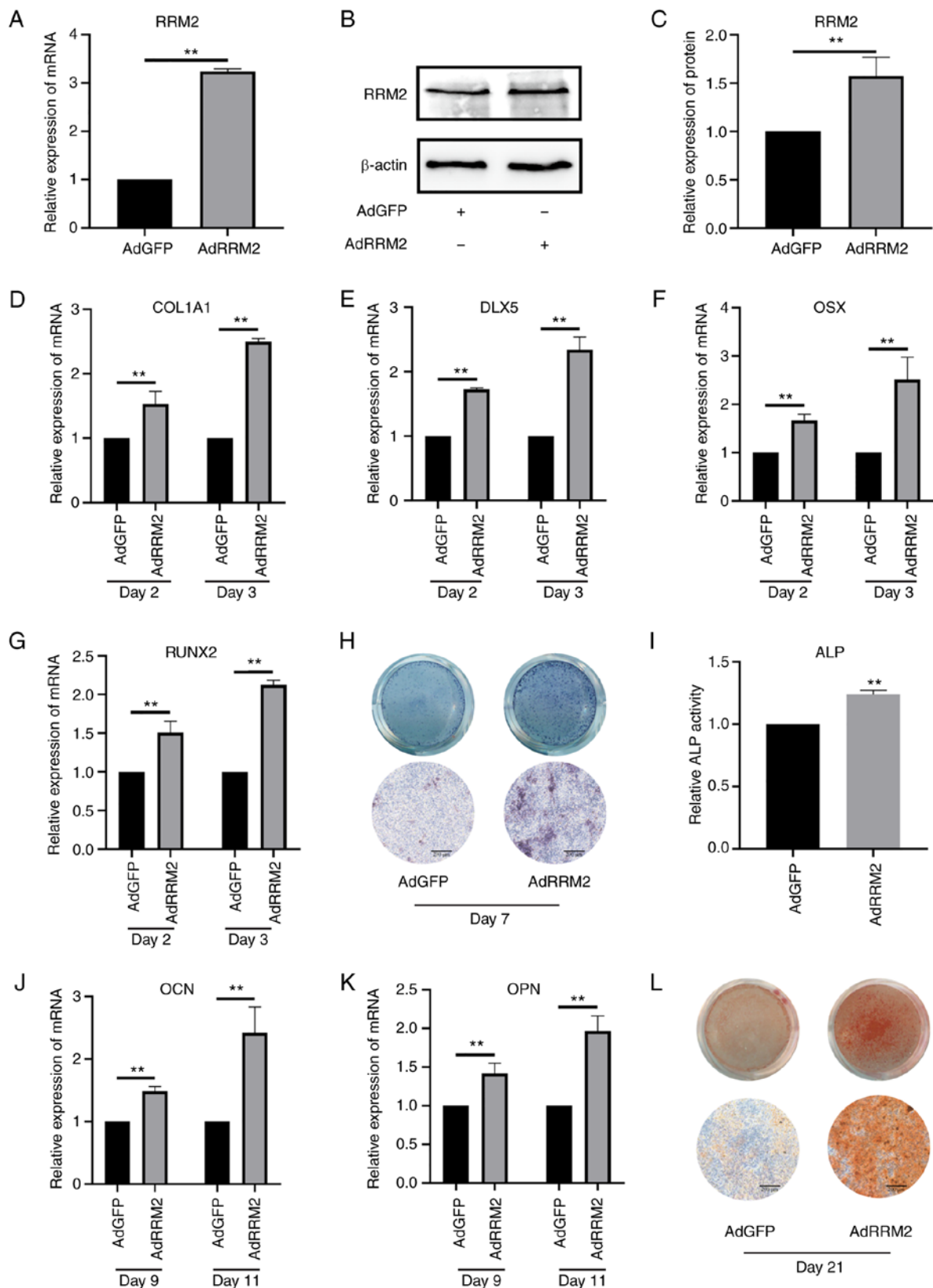


Figure 2. RRM2 promotes osteogenic differentiation in MEFs. (A) RT-qPCR analysis revealed the expression levels of RRM2 in MEFs after infection by AdRRM2. (B) WB and (C) semi-quantification of WB revealed RRM2 protein expression in MEFs following infection by AdRRM2. RT-qPCR analysis indicated the expression levels of early osteogenic markers (D) COL1A1, (E) DLX5, (F) OSX and (G) RUNX2 in MEFs after infection by AdRRM2 for 2 or 3 days. (H) ALP staining and (I) quantification of ALP staining revealed the effect of RRM2 on ALP activity in MEFs after infection by AdRRM2 for 7 days. RT-qPCR analysis indicated the expression levels of late osteogenic markers (J) OPN and (K) OCN of MEFs after infection by AdRRM2 for 9 or 11 days. (L) AR staining indicated the effect of RRM2 on late bone mineralization in MEFs after infection by AdRRM2 for 21 days. **P<0.01 vs. AdGFP group. RRM2, ribonucleotide reductase M2; MEFs, mouse embryo fibroblasts; AdRRM2, RRM2-overexpressing recombinant adenovirus; WB, western blotting; RT-qPCR, reverse transcription-quantitative PCR; COL1A1, collagen type I α 1 chain; DLX5, distal-less homeobox 5; OSX, osterix; RUNX2, RUNX family transcription factor 2; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; AR, Alizarin Red S; AdGFP, negative control adenovirus labeled with green fluorescence protein.

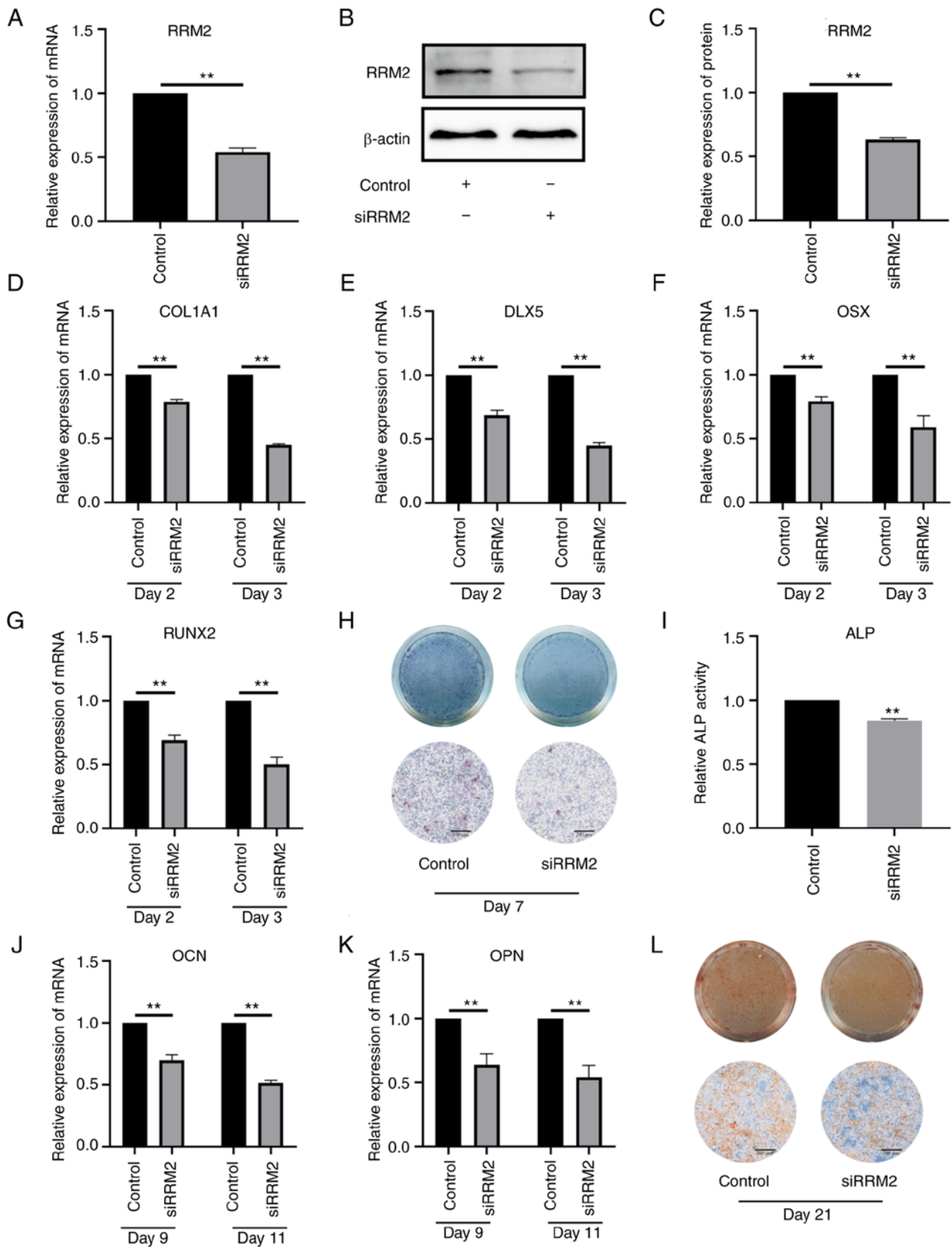


Figure 3. RRM2 knockdown reduces osteogenic development in MEFs. (A) RT-qPCR analysis indicated RRM2 expression in MEFs after transfection with siRRM2. (B) WB and (C) semi-quantification of WB revealed the protein expression levels of RRM2 in MEFs after transfection with siRRM2. RT-qPCR analysis indicated the expression levels of early osteogenic makers (D) COL1A1, (E) DLX5, (F) OSX and (G) RUNX2 in MEFs after RRM2 was knocked down using siRRM2. (H) ALP staining and (I) quantification of ALP staining of MEFs transfected with siRRM2 after culture for 7 days. RT-qPCR analysis revealed the expression levels of late osteogenic makers (J) OPN and (K) OCN of MEFs after RRM2 was knocked down using siRRM2. (L) AR staining of MEFs treated with siRRM2 after culture for 21 days. ** $P < 0.01$ vs. control group. RRM2, ribonucleotide reductase M2; MEFs, mouse embryo fibroblasts; RT-qPCR, reverse transcription-quantitative PCR; siRRM2, small interfering RNA-RRM2; WB, western blotting; COL1A1, collagen type I $\alpha 1$ chain; DLX5, distal-less homeobox 5; OSX, osterix; RUNX2, RUNX family transcription factor 2; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; AR, Alizarin Red S.

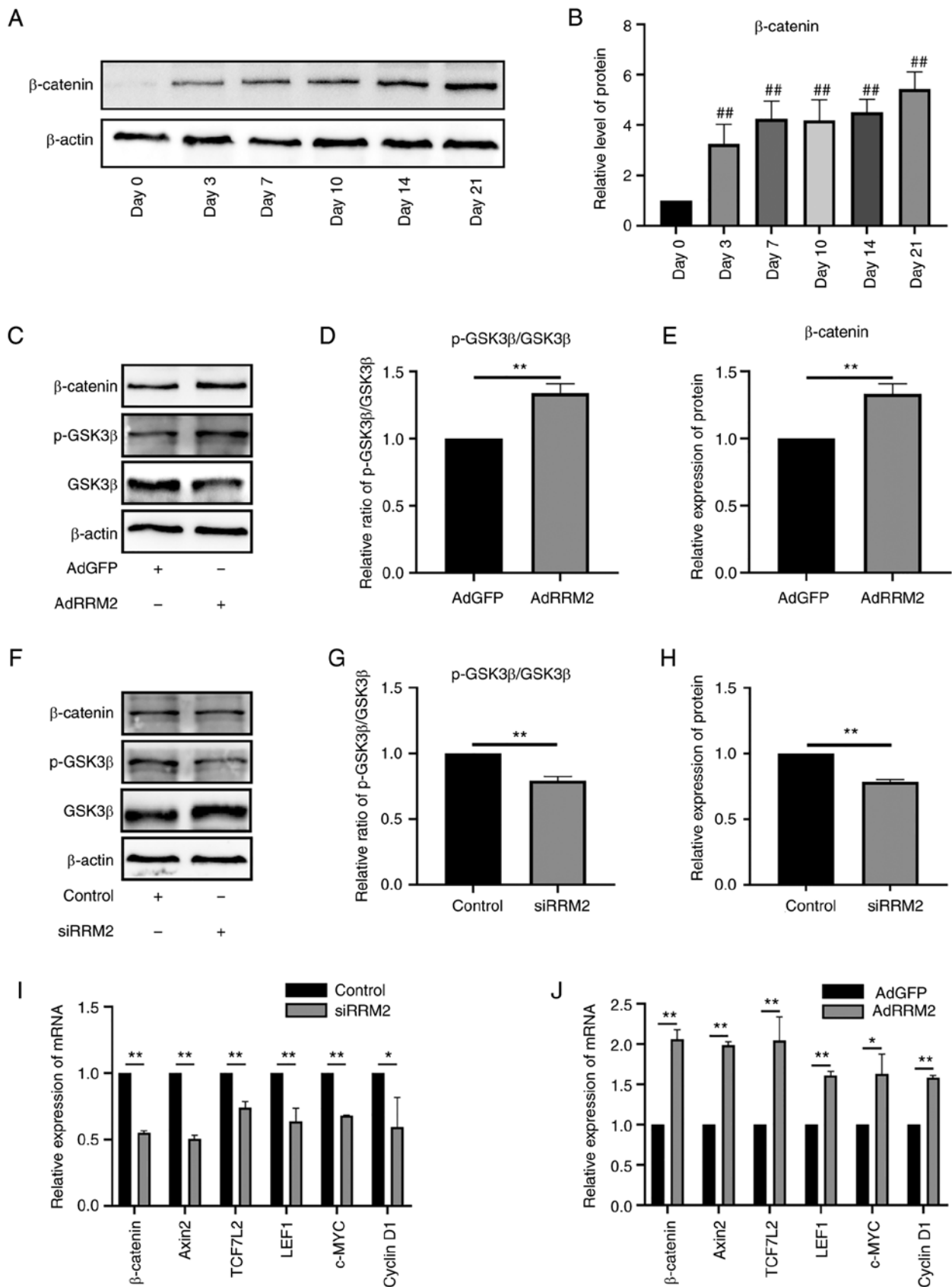


Figure 4. Effects of RRM2 on the Wnt/β-catenin signaling pathway. (A) WB and (B) semi-quantification of WB revealed the protein expression levels of β-catenin in MEFs cultured with osteogenic inducing medium from day 0 to 21. (C) WB and (D and E) semi-quantification of WB revealed the protein expression levels of β-catenin and the ratio of p-GSK-3β to GSK-3β in MEFs after infection by AdRRM2. (F-H) WB and semi-quantification of WB indicated the protein expression levels of β-catenin and the ratio of p-GSK-3β to GSK-3β in MEFs after RRM2 was knocked down using siRRM2. (I and J) Reverse transcription-quantitative PCR analysis revealed the expression levels of the downstream related target genes of the Wnt/β-catenin signaling pathway (β-catenin, Axin2, TCF7L2, LEF1, c-MYC and Cyclin D1) in MEFs following infection by AdRRM2 or siRRM2. ##P<0.01 vs. Day 0 group. *P<0.05; **P<0.01. RRM2, ribonucleotide reductase M2; WB, western blotting; MEFs, mouse embryo fibroblasts; p-, phosphorylated; AdRRM2, RRM2-overexpressing recombinant adenovirus; siRRM2, small interfering RNA-RRM2; TCF7L2, transcription factor 7 like 2; LEF1, lymphoid enhancer binding factor 1.

Cell transfection with AdRRM2 and effect of RRM2 overexpression on osteogenic differentiation in MEFs. AdRRM2 was transfected into MEFs using polybrene when cell confluence reached ~40%. The cell and fluorescence images were captured under a fluorescence microscope 24 h later (Fig. 1F). WB and RT-qPCR analysis 2 days later revealed that RRM2 protein and mRNA were successfully overexpressed in MEFs (RRM2 mRNA and RRM2 protein expression levels in the AdRRM2 group were 3.239 and 1.573 times of those in the AdGFP group; Fig. 2A-C), suggesting that AdRRM2 was effectively transfected. The effect of RRM2 overexpression on osteogenic differentiation was then investigated. According to the RT-qPCR results, early osteogenic markers were upregulated in the AdRRM2 group [in the AdRRM2 group, the levels of early osteogenic markers RUNX family transcription factor 2 (RUNX2), osterix (OSX), distal-less homeobox 5 (DLX5) and collagen type I $\alpha 1$ chain (COL1A1) were 1.507, 1.665, 1.724 and 1.531 times of those in the AdGFP group on day 2 and 2.123, 2.510, 2.338 and 2.496 times of those in the AdGFP group on day 3 respectively; Fig. 2D-G] and late osteogenic markers were similarly upregulated [the levels of late osteogenesis-related genes osteopontin (OPN) and osteocalcin (OCN) in the AdRRM2 group were 1.416 and 1.485 times of those in the AdGFP group on day 9 and 1.965 and 2.419 times of those in the AdGFP group on day 11; Fig. 2J and K]. RRM2 overexpression was found to increase ALP activity and calcium salt accumulation in MEFs (Fig. 2H, I and L), as indicated by ALP and AR staining. According to these findings, overexpression of RRM2 could promote osteogenic differentiation.

siRRM2 transfection and effects of RRM2 knockdown on osteogenic differentiation in MEFs. Lipofectamine[®] 8000 was used to transfect siRRM2 into MEFs according to the manufacturer's standard technique. According to WB and RT-qPCR, RRM2 protein and mRNA were knocked down in MEFs in 2 days (RRM2 mRNA and protein levels in the siRRM2 group were 54.0 and 63.3% of those in the control group; Fig. 3A-C), suggesting that siRRM2 was effectively transfected. The effect of RRM2 knockdown on osteogenic differentiation was then investigated. The expression levels of early and late bone formation markers were considerably reduced when RRM2 was knocked down (the levels of early osteogenesis-related genes RUNX2, OSX, DLX5 and COL1A1 in the siRRM2 group were 69.1, 79.1, 68.6 and 78.7% of those in the control group on day 2 and 50.1, 58.9, 45.0 and 45.1% of those in the control group on day 3 respectively; the levels of late osteogenic markers OPN and OCN in the siRRM2 group were 63.8 and 69.6% of those in the control group on day 9 and 54.1 and 51.4% of those in the control group on day 11 respectively; Fig. 3D-G, J and K). ALP and AR staining were also performed (Fig. 3H, I and L) and it was revealed that knockdown of RRM2 reduced ALP activity and calcium salt accumulation in MEFs. According to these results, knockdown of RRM2 decreased the osteogenic differentiation capability of MEFs.

RRM2 influences osteogenesis of MEFs via the Wnt/ β -catenin signaling pathway. β -catenin protein expression in osteogenic differentiated MEFs increased gradually compared with that in undifferentiated MEFs, similar to the change of RRM2 during osteogenic differentiation (compared with that on day

0, β -catenin protein expression was increased 2.252, 3.251, 3.192, 3.501 and 4.432 times on day 3, 7, 10, 14 and 21, respectively; Fig. 4A and B), indicating that the osteogenic effect of RRM2 was associated with β -catenin expression. WB demonstrated that β -catenin protein expression in the AdRRM2 group was 1.330 times higher than that in the AdGFP group and the ratio of p-GSK-3 β to GSK-3 β was 1.337 times higher than that in the AdGFP group. β -catenin protein expression in the siRRM2 group was 78.2% of that in the control group and the ratio of p-GSK-3 β to GSK-3 β was 79.2% of that in the control group (Fig. 4C-H). The present study further evaluated the effects of RRM2 on the downstream related target genes [β -catenin, Axin2, transcription factor 7 like 2 (TCF7L2), lymphoid enhancer binding factor 1 (LEF1), c-MYC and Cyclin D1] of the Wnt/ β -catenin signaling pathway in MEFs (Fig. 4I and J). RT-qPCR results demonstrated that the mRNA expression levels of β -catenin, Axin2, TCF7L2, LEF1, c-MYC and Cyclin D1 in the AdRRM2 group were 2.056, 1986, 2.043, 1.608, 1.630 and 1.579 times respectively of those in AdGFP group and the mRNA expression levels of β -catenin, Axin2, TCF7L2, LEF1, c-MYC and Cyclin D1 in the siRRM2 group were 54.9, 50.4, 73.7, 63.5, 68.0 and 59.4% respectively of those in the control group. When RRM2 was overexpressed, β -catenin protein expression, the ratio of p-GSK-3 β to GSK-3 β and the mRNA expression levels of the downstream related target genes of the Wnt/ β -catenin signaling pathway were increased, while the results were the opposite when RRM2 was knocked down. The Wnt/ β -catenin signaling pathway may be activated by RRM2.

Taken together, RRM2 overexpression promoted osteogenic differentiation of MEFs, whereas RRM2 knockdown reduced osteogenic differentiation of MEFs. RRM2 may influence osteogenesis of MEFs via the canonical Wnt/ β -catenin signaling pathway.

Discussion

Osteoporosis is known as a widespread progressive bone metabolic disease characterized by reduced bone mass and bone microstructure deterioration, which leads to an increase in bone fragility and the risk of fracture (1). BPs, calcitonin, denosumab (Prolia) and anabolic agents such as parathyroid hormone and teriparatide are pharmacological options since an imbalance between bone absorption and bone formation in the body causes osteoporosis (6,7). Gene therapy and MSC therapy have been developed as potential treatments for osteoporosis as novel strategies in bone regeneration medicine (11,16). Gene-based modifications to improve osteogenesis of MSCs and thereby enhance bone regeneration are a focus of investigation.

RRM2 is known as a crucial enzyme involved in DNA synthesis and repair, which contributes to regulating cell proliferation and differentiation (26). By activating the hippo-yes-associated protein signaling pathway, RRM2 can enhance myocardial cell proliferation after myocardial ischemia reperfusion injury (19). RRM2 has the potential to be exploited as a tumor marker for lung and liver cancer, as well as a therapeutic target (18,27). To the best of the authors' knowledge, there have not been any studies on the role of RRM2 in osteogenic differentiation.

Bioinformatics analysis of RNA sequencing data of BMP9-induced osteogenesis of MEFs was conducted and revealed that RRM2 was upregulated, thus it was hypothesized that RRM2 might be intimately related to osteogenic differentiation. RRM2 expression during the osteogenic induction of MEFs was then detected and it was revealed that RRM2 expression increased with the extension of osteogenic induction time. AdRRM2 and siRRM2 were used to upregulate and downregulate the levels of RRM2 in MEFs to investigate the effect of RRM2 on osteogenic differentiation. RRM2 overexpression could increase the expression levels of bone formation markers RUNX2, OSX, DLX5, COL1A1, OPN and OCN and increase ALP activity and calcium nodule formation, whereas RRM2 knockdown had the opposite effect. RUNX2 is an important transcription factor in bone formation and its activation stimulates the expression of downstream osteogenic genes, such as OSX, DLX5, COL1A1, OPN and OCN (28). ALP is an early osteogenic marker that can enhance mineralization (29). RRM2 increases the activity of ALP in MEFs and induces the formation of calcium nodules. These results suggested that RRM2 could promote osteogenesis in MEFs.

The Wnt signaling pathway is important in osteoporosis (30). Bone remodeling is balanced by Wnt signaling cascades, which promote bone formation and inhibit bone resorption (31). Wnt signals are divided into canonical Wnt signals and noncanonical Wnt signals (32). The central components in the canonical Wnt/ β -catenin signaling pathway are GSK-3 β and β -catenin. The function of the Wnt/ β -catenin signaling pathway is dependent on the amount of β -catenin in the cytoplasm and GSK-3 β is critical in regulating the amount of β -catenin. GSK-3 β enzymatic activity is adversely controlled by N-terminal phosphorylation of the enzyme's serine residues (Ser 9), while phosphorylation of β -catenin by GSK-3 β promotes its degradation (33). Canonical Wnt signals are transmitted to the β -catenin signaling cascade via frizzled (Fzd) family receptors and the low-density receptor related protein (LRP)5/LRP6 coreceptor. In the absence of Wnt ligand, β -catenin levels in the cytosol are low due to the negative control exerted by the β -catenin 'destruction complex', which includes GSK-3 β , Axin, casein kinase 1 and adenomatous polyposis coli (32,33). When members of the Fzd family of proteins and LRP5/6 recognize Wnt protein at the cell surface, the activated complex phosphorylates and stabilizes the disheveled protein, inhibiting the degradation activity of the β -catenin degradation complex and promoting the stabilization and accumulation of β -catenin in the cytoplasm (34). β -catenin is then translocated to the nucleus, where it binds to the T-cytokine/lymphoid enhancer complex (35) and stimulates transcription of downstream target genes. Wnt signaling pathways can be activated by upregulation or downregulation of genes. By knocking down Foxf1 or leukocyte cell derived chemotaxin 2, the Wnt/ β -catenin signaling pathway is activated, promoting bone marrow stromal cell (BMSC) osteogenesis (30,36). Krüppel-like factor 2 overexpression promotes osteoblast differentiation by boosting RUNX2 expression and interacting with RUNX2 (37). Overexpressed FOXQ1 enhances Wnt/ β -catenin signals in BMSCs by binding to annexin A2, resulting in osteogenic differentiation (38).

The present study revealed that β -catenin protein expression was increased during the osteogenic differentiation of

MEFs and the osteogenic effect of RRM2 may be related to β -catenin. When RRM2 was overexpressed, β -catenin protein expression and the ratio of p-GSK-3 β to GSK-3 β were increased. The mRNA expression levels of downstream related target genes (β -catenin, Axin2, TCF7L2, LEF1, c-MYC and Cyclin D1) of the Wnt/ β -catenin signaling pathway were increased when RRM2 was overexpressed but decreased when RRM2 was knocked down. These results suggested that RRM2 promoted the expression of p-GSK-3 β , β -catenin and downstream related target genes in the Wnt/ β -catenin signaling pathway and RRM2 may influence osteogenesis of MEFs by stimulating the canonical Wnt/ β -catenin signaling pathway.

The present study has some flaws. It is not clear how RRM2 affects the Wnt signaling pathway or a specific link in the pathway. Whether RRM2 affects osteogenesis through other signaling pathways will be further studied. In the present study, *in vitro* cell experiments were carried out and *in vivo* experiments in animals are required to prove its osteogenic effect. To the best of the authors' knowledge, it is a novel development that gene combination therapy can promote osteogenic differentiation. BMP9, a member of the TGF- β class, is important in the generation of bone progenitor cells from MSCs (39,40). All-trans retinoic acid and BMP9 have been revealed to work together to counter the effects of cyclooxygenase 2 inhibitors on bone formation by stimulating the Wnt/ β -catenin signaling pathway (20). The influence and mechanism of the RRM2 single gene in osteogenesis were investigated in the present study, the combined application of RRM2 and BMP9 will also become our research direction. Exosomes, also called extracellular vesicles, are nanoparticles that are both biocompatible and bioactive (41). Exosomal metastasis associated lung adenocarcinoma transcript 1 derived from BMSCs has been demonstrated to promote osteogenic activity and alleviate osteoporosis symptoms in a mouse model by acting as a microRNA-34c sponge to increase SATB homeobox 2 expression (42). Whether RRM2-delivering exosomes can promote osteogenic differentiation more effectively is also our research direction. The research and application of novel biomaterials in bone repair is becoming increasingly attractive. The strawberry-like Ag-decorated pBT (Ag-pBT) nanoparticles PVDF (PVDF/4Ag-pBT) scaffold with good mechanical properties, enhanced antibacterial activity and piezoelectric properties might have great potential in orthopedic application (43). The porous PLLA-PGA/Ag loaded PMBG composite scaffold prepared by introducing Ag-loaded mesoporous bioactive glass modified with polydopamine into polymer matrices (poly-L-lactic acid and poly-glycolic acid blends, with mass ratio of 1:1) could be a promising therapy option for infected bone defects (44). With the rapid changes in technology, the biomedical use of exosomes or innovative biomaterials will provide a novel technique for the clinical care of osteoporosis.

Previous studies (17-19,26,27) on RRM2 have mainly focused on cancer markers and cell proliferation. To the best of the authors' knowledge, the present study was the first to demonstrate that RRM2 enhanced osteogenic differentiation of MEFs, potentially by activating the canonical Wnt/ β -catenin signaling pathway, providing a possible target for osteoporosis treatment.

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Availability of data and materials

The datasets used during the current study are available upon reasonable request from the corresponding author.

Authors' contributions

HJ, HC, HG, YD, SL and WH conceptualized, designed and coordinated the study. HC and HG performed RT-qPCR and WB. YD and JJ conducted data analysis and statistical analysis. The manuscript was written by HC. HC and HG confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agreed to be held accountable for the current study to ensure that any doubts about the integrity of any portion of the work are properly addressed and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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