

Original article

Effect of lncRNA RMRP on proliferation, apoptosis, osteoblast differentiation and mineralization of bone marrow mesenchymal stem cells

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Abstract: *Background* — Long non-coding RNAs (lncRNAs) play a crucial role in bone growth and skeletal development. Ribonuclease (RNase) for mitochondrial RNA processing, commonly known as RMRP, is a small nucleolar ribonucleoprotein particle composed of RMRP lncRNA (lncRNA RMRP) and several protein subunits. Specifically, mutations in lncRNA RMRP are associated with cartilage-hair hypoplasia and skeletal developmental disorders in humans. For instance, mutations in lncRNA RMRP are observed in individuals with severe dwarfism, thus suggesting that RMRP functions in both chondrogenic and osteogenic processes. Results of a previous study have identified a potential role of RMRP in chondrogenic differentiation in mouse cell lines. However, the specific functions of RMRP in osteogenic processes remain unclear.

Methods — In the present study, RMRP expression was altered in murine bone marrow derived mesenchymal stem cells (BMSCs). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), cell counting, apoptosis and mineralization assays were used to investigate the effects of RMRP on BMSCs, osteoblasts and osteogenic differentiation.

Results — The results of the present study showed that RMRP expression was up-regulated in BMSCs and down-regulated during apoptosis. The RMRP knockdown resulted in decreased proliferation and viability of BMSCs and increased apoptosis. In addition, the RMRP knockdown increased osteoblast differentiation of BMSCs and affected the mineralization of osteoblastic cells.

Conclusion — The results of the present study indicated that RMRP may play a role in BMSCs, osteoblastic cells and osteogenic differentiation process. Therefore, the present study may provide a new theoretical basis for the role of RMRP in skeletal development.

Keywords: lncRNA, RMRP, bone marrow mesenchymal stem cells, osteoblast differentiation, mineralization.

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Introduction

Long non-coding RNAs (lncRNAs) are involved in various biological functions [1-3]. Results of previous studies demonstrated the regulatory roles of lncRNAs in numerous cellular physiological and pathological processes including bone growth and skeletal development [4-6]. Ribonuclease (RNase) for mitochondrial RNA processing, commonly known as RMRP, is a small nucleolar ribonucleoprotein particle composed of RMRP lncRNA (lncRNA RMRP) and several protein subunits. Notably, RMRP is required for the viability of eukaryotic cells and is present in osteocytes. RMRP is localized predominantly in the nucleolus and acts as an endoribonuclease cleaving several RNA substrates [7-10]. In humans, mutations in the RMRP gene lead to cartilage-hair hypoplasia [11, 12].

McKusick type metaphyseal chondrodysplasia, also known as cartilage-hair hypoplasia (CHH), is a rare autosomal recessive osteochondrodysplasia resulting from a mutation in the RMRP gene. CHH is associated with dwarfism and impaired skeletal development manifesting as short phalanges and metacarpals, lumbar lordosis, shortened and widened iliac wings, femoral bowing, increased ossification, and longer fibulae compared with

tibiae [13-16]. The skeletal features of CHH are indicative of chondrogenesis and osteogenesis processes. Few studies have focused on mechanism underlying how RMRP mutations can lead to cartilage dysplasia. A zebrafish model with a knockout mutation of RMRP suppressed the proliferation of Col2a1a cells and promoted apoptosis [3]. A previous study showed that downregulation of RMRP RNA expression in cells involved in chondrogenesis resulted in dysregulation of chondrogenic differentiation, thereby affecting hypertrophy. These results highlight the role of RMRP in chondrogenesis [5]. Thus, we hypothesize that lncRNA RMRP may also serve as an important regulator of osteogenesis through effects on cell proliferation, apoptosis, osteoblast differentiation, and mineralization; however, the specific roles in osteogenic progenitor cells and the osteogenesis process remain unclear.

Bone marrow mesenchymal stem cells (BMSCs) differentiate into several cell types, including osteogenic progenitor cells and osteoblasts [17]. Notably, only a few studies have focused on the effects of RMRP on BMSC proliferation, apoptosis, osteoblast differentiation, and mineralization. In the present study, murine BMSCs were used to evaluate the function of RMRP in stem cell proliferation, apoptosis, osteoblast differentiation, and

mineralization. In our study, RMRP expression was altered in murine BMSCs. We employed reverse transcription in BMSCs, followed by quantitative polymerase chain reaction (qPCR), cell counting, apoptosis, and mineralization assays to examine the expression and effects of RMRP on BMSCs, osteoblasts, and osteogenic differentiation. The design of our study is presented in [Figure 1](#).

Material and Methods

Animal experiments

C57BL/6J male mice (age: 6-8 weeks; SiLaiKe JingDa Experimental Animal Corporation) were used for the isolation of BMSCs. At the end of the experiments, the mice were euthanized using CO₂ at a volume displacement rate of 30% vol/min, followed by cervical dislocation, cardiac and respiratory arrest, and nebulization with 70% ethanol. All experiments were approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Research Center, Hunan Medical College (ethical approval No. 2021HY3108).

Preparation of cell culture medium

BMSC culture medium was prepared using α Minimum Essential Medium (α MEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. In addition, 200 μ L of 0.5 M ascorbic acid in PBS and 800 μ L of 1 M β -glycerol phosphate in PBS were added to 99 mL of BMSC culture medium to prepare osteoblast differentiation medium. All reagents were purchased from Dalian Meilun Biotech Co., Ltd.

BMSC culturing

BMSCs were collected, cultivated and purified from mice as previously described [18, 19]. Animals were euthanized using CO₂ followed by cervical dislocation, and treated with 70% ethanol. Humeri, femora, and tibiae were isolated and washed with PBS to remove blood cells and residual connective tissues, and the two

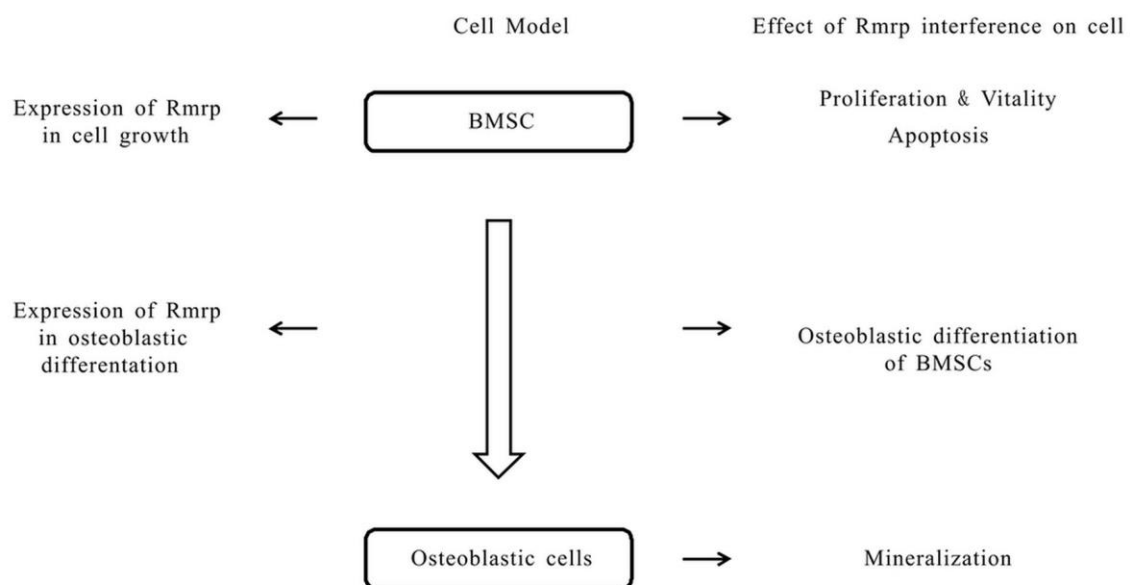
ends were cut just below the end point of the marrow cavity. The bone marrow was washed and suspended until the bones became pale. After removal of bone fragments, the suspended cells were cultured at 37 °C in a 5% CO₂ incubator. After 48 h, non-adherent hematopoietic stem cells were removed and adherent cells were detached with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd.). The cells were then centrifuged at 1000 G at room temperature for 5 min, resuspended, and plated or cultured for additional days. When 80-100% confluency was achieved, differentiation experiments were performed. The purity of cultured BMSCs is shown in [Figure S1](#).

Osteogenic differentiation

BMSCs were cultured in culture medium at 37 °C in an incubator or with 5% CO₂ until reaching 80-100% confluency. Then, the BMSC culture medium was removed and osteoblast differentiation medium was added. Osteoblast differentiation medium was replaced every other day. BMSCs were cultured in dishes at 37 °C in an incubator with 5% CO₂ for 21 days to complete the osteogenic differentiation process [20, 21].

Transfection

Ribo lncRNA Smart Silencer, consisting of three small interfering (si)RNA moieties and three antisense oligonucleotide (ASO) moieties, was synthesized by Guangzhou RiboBio Co., Ltd. Ribo m-RMRP Smart Silencer ([Table 1](#)) was used to knockdown lncRNA RMRP. Cells were transfected with Smart Silencer or negative control using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After the specified period, samples were collected for quantification as described previously [22]. The default concentration of Smart Silencer was optimized at 200 nM unless otherwise specified, and the default transfection duration was optimized at 24 h unless otherwise stated.



[Figure 1](#). Diagram of study design.

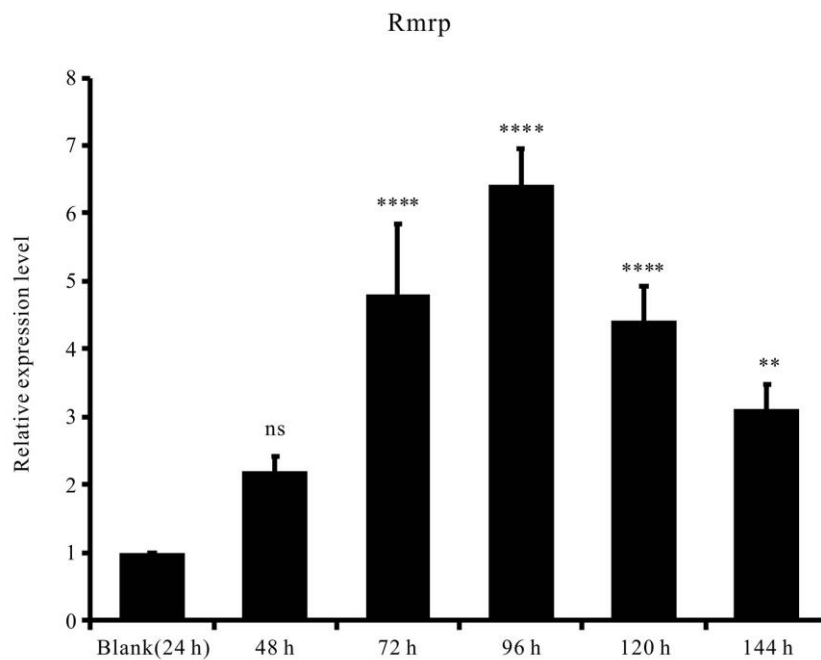


Figure 2. Regulation of RMRP expression in BMSCs. RT-qPCR analysis revealed RMRP expression levels in mouse-derived BMSCs at different time points (n=3 per group). The Blank group was collected 24 h after transfection with RMRP silencer. P(144 h)=0.0019. ns, not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. the Blank group. RMRP, RNase MRP; BMSC, bone marrow mesenchymal stem cells.

Table 1. Smart silencer sequences used in the present study

Name	Target sequence
Ribo™ m-Rmrp Smart Silencer	GTGCACACGCGCTAGACTT
	ACATACGAGGGACATGTTCC
	TCATCCGTCAGCTCACATAG
	ACTGTTAGCCCGCCAAGAA
	CAGCTCACATAGTGACGCA
	GGACATGTTCTTATCCTT

Table 2. The thermal cycling profile

Step	Temperature	Duration	Cycle(s)
Enzyme Activation	95°C	3 minutes	1
Denaturation	95°C	15second	
Annealing/Extension	60°C (default)	1 minute	
Melting Curve		95°C	

Table 3. Primer sequences used in the present study

Primer name	Forward	Reverse
β-actin	GAAGGCTATAGTCACCTCGGG	ATGGTAATAATGCGGCCGGT
Rmrp	CCGCAAGTCACTGTTAGCC	CACTGCCTGCGTCACTATGT
Cyclin B2	TGCCAAGCTTTCTCTGATGCT	GGGTTCTCCCTGTCCTCGTT
Viperin	TGCTATCTCTGCGACAGCTT	CCTTGACCACGGCCAATC
Runx2	GACGAGGCAAGAGTTTCACC	GGACCGTCCACTGTCACTTT
Alp	CCGATGGCACACCTGCTT	GGAGGCATACGCCATCACAT
Ocn	GCGGCCCTGAGTCTGACA	GCCGGAGTCTGTTCACTACCTT
Osterix	GTGGGAACAAGAGTGAGCTGG	GCCATAGTGAGCTTCTTCTGCGG

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using QuantStudio 5 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA was isolated from cells using TRIzol reagent and RNA extraction kit (Genstone Bio). RNA concentration was determined using a Nanodrop spectrophotometer, and RNA integrity was confirmed by gel electrophoresis. RNA samples were then reverse transcribed

into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). qPCR was conducted using BlasTaq™ 2X qPCR MasterMix (Applied Biological Materials, Inc.) according to the manufacturer's instructions (Table 2). β-actin served as a reference gene and was synthesized by Guangzhou RiboBio Co., Ltd (Table 3).

Cell Counting Kit-8 (CCK-8) assay

Cells were seeded in 24-well plates at a density of 1×10⁴ cells/well. After 24 h of culture at 37 °C, cells were transfected with RMRP siRNAs to promote cell adhesion. After 48 h, 72 h, and 96 h, 0.25% trypsin was added to each well for 2 min to provide detachment, and cells were sampled for counting. Samples were analyzed using a cell counter (DeNovix CellDrop).

BMSCs were seeded in 48-well plates at a density of 0.5×10⁴ cells/well. After 24 h of culture at 37 °C, cells were transfected with RMRP siRNAs to promote cell adhesion. After 6 h, 24, 36 h and 48 h, 10 μL of CCK-8 reagent (Dalian Meilun Biotech Co., Ltd.) was added to each well, and the cells were incubated in a humidified incubator at 37 °C with 5% CO₂ for additional 4 h. The absorbance of each well was measured at 450 nm.

Cell apoptosis

Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Dalian Meilun Biotech Co., Ltd.) was used to examine cell apoptosis. The cells were seeded in 12-well plates at a density of 1×10⁵ cells/well. After 24 h of culture, the cells were transfected with RMRP siRNA to ensure cell adhesion. BMSCs were then digested and suspended in 0.25% EDTA-free trypsin for 2 min at room temperature. Cells were collected, resuspended in binding buffer, and normalized to a concentration of 1×10⁶ cells/mL. Samples were stained at room temperature with 5 μL annexin V-FITC and 5 μL PI in the dark for 15 min and then analyzed by

fluorescence microscopy and flow cytometry. The percentage of apoptotic cells was determined using a FACS Caliber system (Beckman Coulter, Inc.).

AKP/alkaline phosphatase (AKP/ALP) activity assays

Cell lysates were prepared using cell lysis buffer (Beyotime Institute of Biotechnology) as described previously [22]. After homogenization and centrifugation at 5,180 G for 10 min at 4°C, the supernatant or culture medium was collected. AKP/ALP activity was assessed using ALP enzymatic colorimetric kit (Beijing Solarbio Science & Technology Co., Ltd.). P-nitrophenol yield was measured at 510 nm using a UV2600 UV/VIS spectrophotometer (Shimadzu). The lysate solution was normalized to total cellular protein using Nanodrop 2000.

Mineralization assay

After reaching 80–100% confluency, BMSCs were cultured at 37 °C in osteogenesis-inducing medium for 21 days to trigger osteoblast mineralization. The cells were then stained with 2% Alizarin Red-S (pH 4.2; Beijing Solarbio Science & Technology Co., Ltd.) to assess mineralization. Visualization was performed using a camera system (Nikon Corporation) and a Diaphot inverted microscope. Qualitative assessment of mineralization was

performed based on the concentration of Alizarin Red-S observed using nuclear imaging.

Statistical analysis

Statistical analysis was performed using SPSS (version 13.1; SPSS, Inc.) and GraphPad prism 8 (GraphPad Software). All data are presented as mean ± standard deviation. Two-tailed Student's t-test was used for comparisons between two groups using SPSS. One-way ANOVA with Dunnett's test was used for comparisons between multiple groups using GraphPad Prism 8. Experiments were performed in triplicate, and representative experiments were presented. $P < 0.05$ was considered statistically significant.

Results

Regulation of RMRP expression in BMSCs

To investigate the expression of RMRP in BMSCs, cells were isolated from murine bone marrow. The results of the present study showed that RMRP expression was significantly elevated during the growth stages of BMSCs. RMRP expression reached the highest point when BMSCs were in the logarithmic growth phase and subsequently decreased as proliferation ceased (Figure 2). These results may highlight the regulatory role of RMRP in BMSC growth.

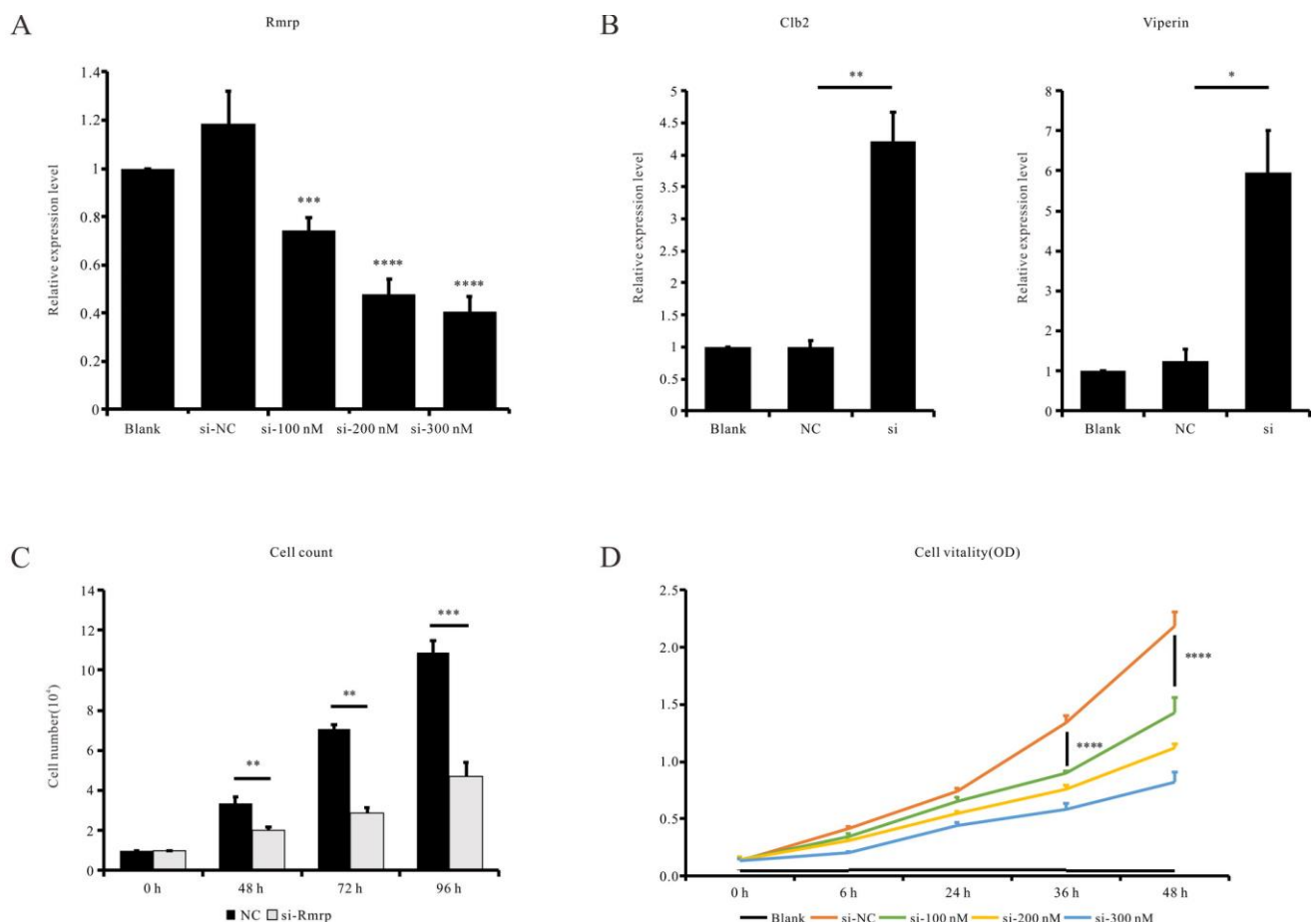


Figure 3. Downregulation of RMRP knockdown reduces BMSC proliferation and viability. (A) RT-qPCR confirmed the transfection effectiveness of RMRP silencer at different concentrations in BMSCs. (B) mRNA expression levels of RMRP substrate RNAs; viz., Clb2 and viperin, in BMSCs after RMRP knockdown; $P(\text{Clb2})=0.0052$. $P(\text{viperin})=0.0137$. (C) BMSC numbers at different time points after RMRP knockdown; $P(48\text{ h})=0.0359$. $P(72\text{ h})=0.0024$. (D) Cell Counting Kit-8 assays were used to examine BMSCs at different time points after RMRP knockdown. Each experiment included triplicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. RMRP, RNase MRP; BMSC, bone marrow mesenchymal stem cells.

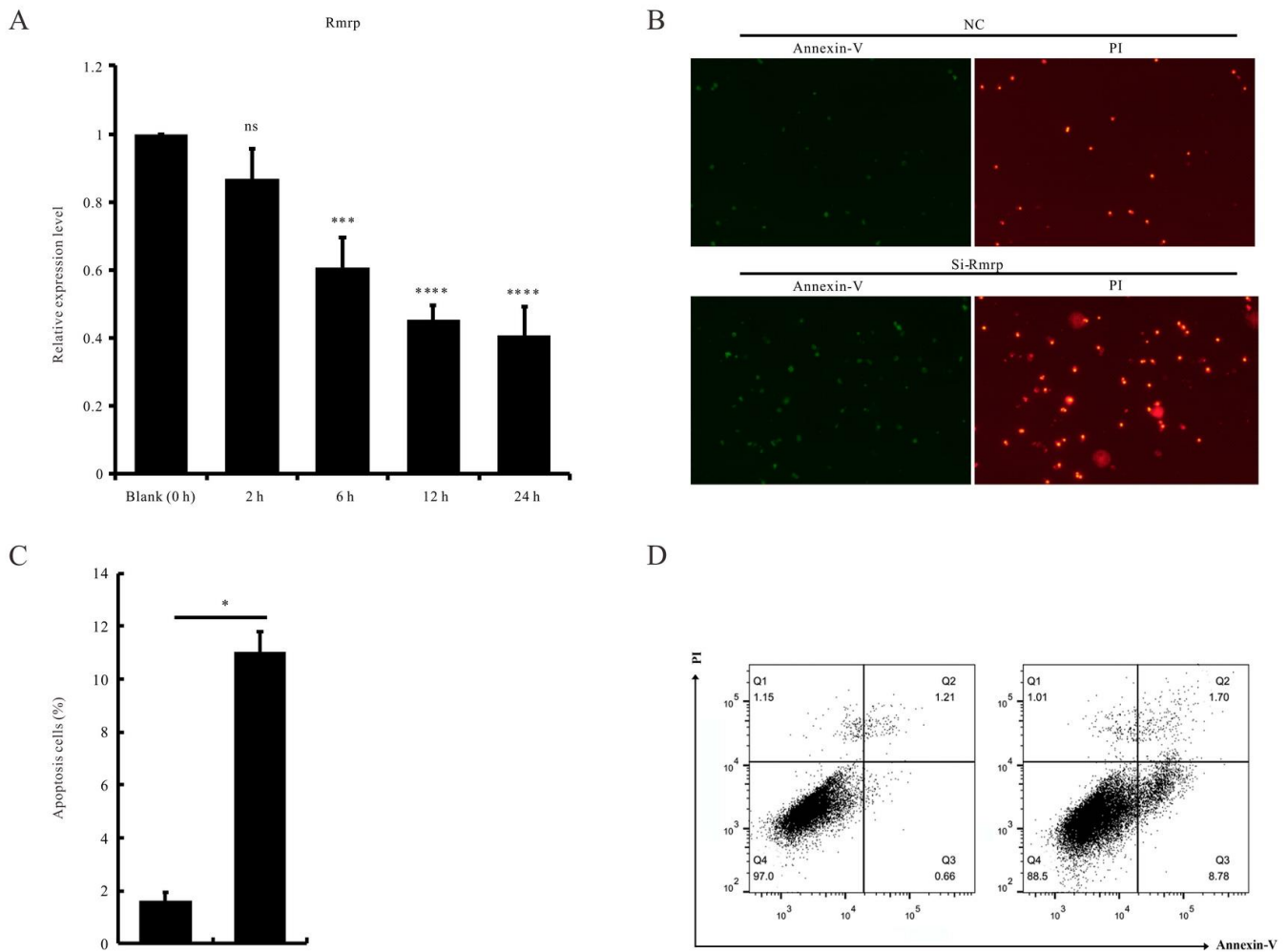


Figure 4. RMRP knockdown increased BMSC apoptosis. (A) RT-qPCR analysis of RMRP expression in BMSCs induced to undergo apoptosis (n=3 per group). (B) Annexin V/PI staining of BMSCs after RMRP knockdown (green, annexin V; red, PI). (C) Statistical summary of flow cytometry; P=0.0260. (D) Flow cytometry analysis showed that transfection with RMRP silencer promoted BMSC apoptosis. Q1 (annexin V-FITC/PI+), necrosis group; Q2 (annexin V-FITC/PI+), late apoptosis group; Q3 (annexin V-FITC/PI-), early apoptosis group; and Q4 (annexin V-FITC/PI-), functioning cells. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. RMRP, RNase MRP; BMSC, bone marrow mesenchymal stem cells; PI, propidium iodide.

Effects of RMRP knockdown on BMSC proliferation and viability

To determine the effect of RMRP on BMSC proliferation and viability, siRNA-RMRP and ASO-RMRP (RMRP-Smart Silencer) were transfected into BMSCs and differentiating osteoblasts to suppress RMRP expression. ALP served as a marker of osteoblast differentiation.

The results of our study revealed that the negative control siRNA did not significantly affect the expression of RMRP. However, siRNA-RMRP statistically significantly reduced the expression of RMRP by >50% at an optimized concentration (Figure 3A). We also confirmed that the mRNA expression levels of RNase MRP substrates (viz., Clb2 and viperin mRNA) were reduced, implying a functional decrease in RMRP activity (Figure 3B).

After transfection of siRNA-RMRP into BMSCs for 24 h, 48 h and 72 h, the cells were harvested and analyzed by bright field microscopy. We observed a marked decrease in the number of

BMSCs in the RMRP silencer transfected group, and this decrease was most noticeable at 48 h and 72 h (Figure 3C). Furthermore, the CCK-8 assay results demonstrated a significant decrease in BMSC viability in the RMRP silencer transfected group, and this decrease was most noticeable at 36 h. However, the observed inhibitory effects were dependent on the silencer concentration (Figure 3D).

In contrast, after induction of osteoblast differentiation for ≥3 days, no significant differences in cell numbers were observed between RMRP silencer-transfected cells and the control group. These findings suggest that RMRP knockdown can inhibit BMSC growth and proliferation while having no detectable effect on the viability of differentiating osteoblasts.

Furthermore, according to our preliminary experiment which results suggest that lncRNA-RMRP expression was not increased following using RMRP overexpression, we did not investigate the effects of RMRP overexpression on the proliferation and viability of BMSCs.

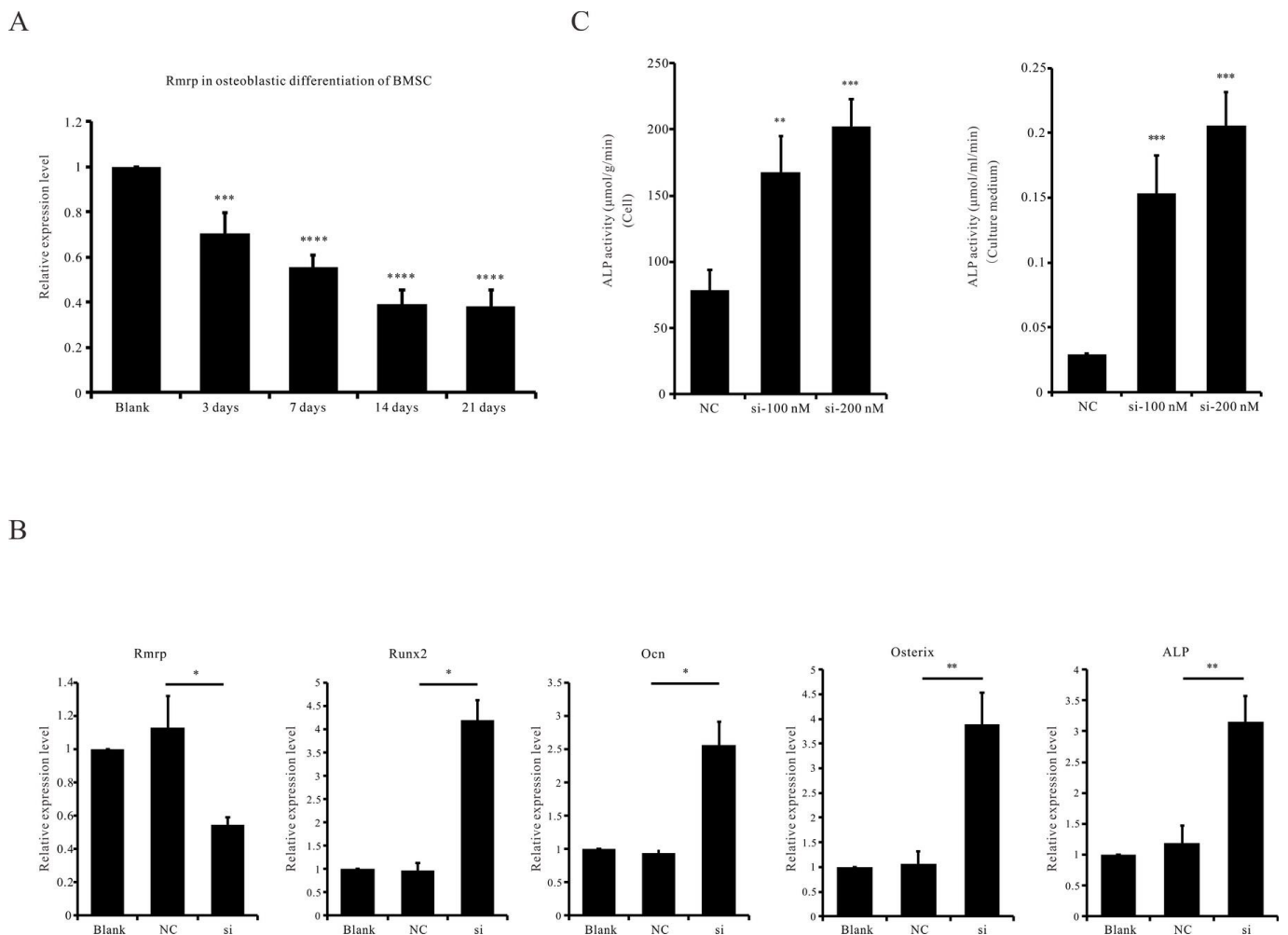


Figure 5. RMRP knockdown enhances BMSC osteoblast differentiation. (A) RT-qPCR analysis of RMRP expression in BMSC osteoblast differentiation. Blank panels were harvested at the beginning of induction. (B) RT-qPCR analysis of osteoblast marker genes in differentiating BMSCs. siRNA panels were transfected with 200 nM RMRP silencer; P(Rmrp)=0.0263, P(Runx2)=0.0103, P(Ocn)=0.0193, P(Osterix)=0.0076, P(ALP)=0.0029. (C) AKP/ALP activity in cells and culture medium. Each experiment included three replicates; P(si-100nM, Cell)=0.0044. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

RMRP knockdown increased BMSC apoptosis

We performed RT-qPCR to determine the changes in RMRP expression levels during apoptosis. The results of our study showed that RMRP expression levels were significantly reduced in BMSCs induced by apoptosis compared with the control group (Figure 4A).

Moreover, annexin V/PI staining and fluorescence flow cytometry revealed a fourfold increase in the percentage of apoptotic BMSCs after RMRP knockdown vs. the control group (Figure 4B-D). Notably, there was no significant difference in the percentage of apoptotic osteoblasts and the control group after RMRP knockdown. These findings suggest that RMRP expression may be regulated during apoptosis and RMRP knockdown may boost BMSC apoptosis.

RMRP knockdown enhances the BMSC osteoblast differentiation

Osteoblast differentiation was triggered in BMSCs using osteogenesis induction medium. The results of RT-qPCR analysis showed that the expression levels of RMRP were significantly

reduced during osteogenic differentiation of murine BMSCs (Figure 5A). In addition, the mRNA expression levels of key markers of osteoblast differentiation (viz., ALP, Runx2, osteocalcin and Osterix) were statistically significantly increased after RMRP knockdown (Figure 5B). Our study demonstrated that RMRP knockdown increased AKP/ALP activity by more than threefold in both the cytoplasm and the culture medium (Figure 5C). Thus, RMRP knockdown could enhance osteoblast differentiation of BMSCs.

Effect of RMRP knockdown on the mineralization of osteoblasts

In our study, cells were stained with Alizarin Red to identify whether RMRP knockdown-mediated dysregulation of osteogenic differentiation was accompanied by functional changes at different time points. To maintain RMRP knockdown during osteogenic differentiation, RMRP silencer duplexes were transfected at 2, 9 and 16 days after the onset of differentiation, and the effectiveness of RNA knockdown was confirmed by qPCR (Figure 6A). Mineralization levels were quantified using Alizarin

Red staining, and our results showed that they were elevated on day 14 (Figure 6B). These findings suggest that RMRP knockdown may promote mineralization of osteoblasts in vitro.

Discussion

Most mammalian genomes contain non-coding genes (ncRNAs) that are transcribed [23, 24]. Previous studies demonstrated that a high percentage of ncRNAs are functional

[25, 26]. In particular, functional ncRNAs include regulatory RNAs such as microRNAs and lncRNAs [27]. MicroRNAs regulate gene expression by binding to the 3'-untranslated regions in target transcripts [28]. lncRNAs are transcribed by RNA polymerase II and III and are >200 nucleotides in length. Previous studies confirmed that lncRNAs play key roles in the post-transcriptional, translational, and epigenetic coordination of gene expression in body development and formation of disorders [29].

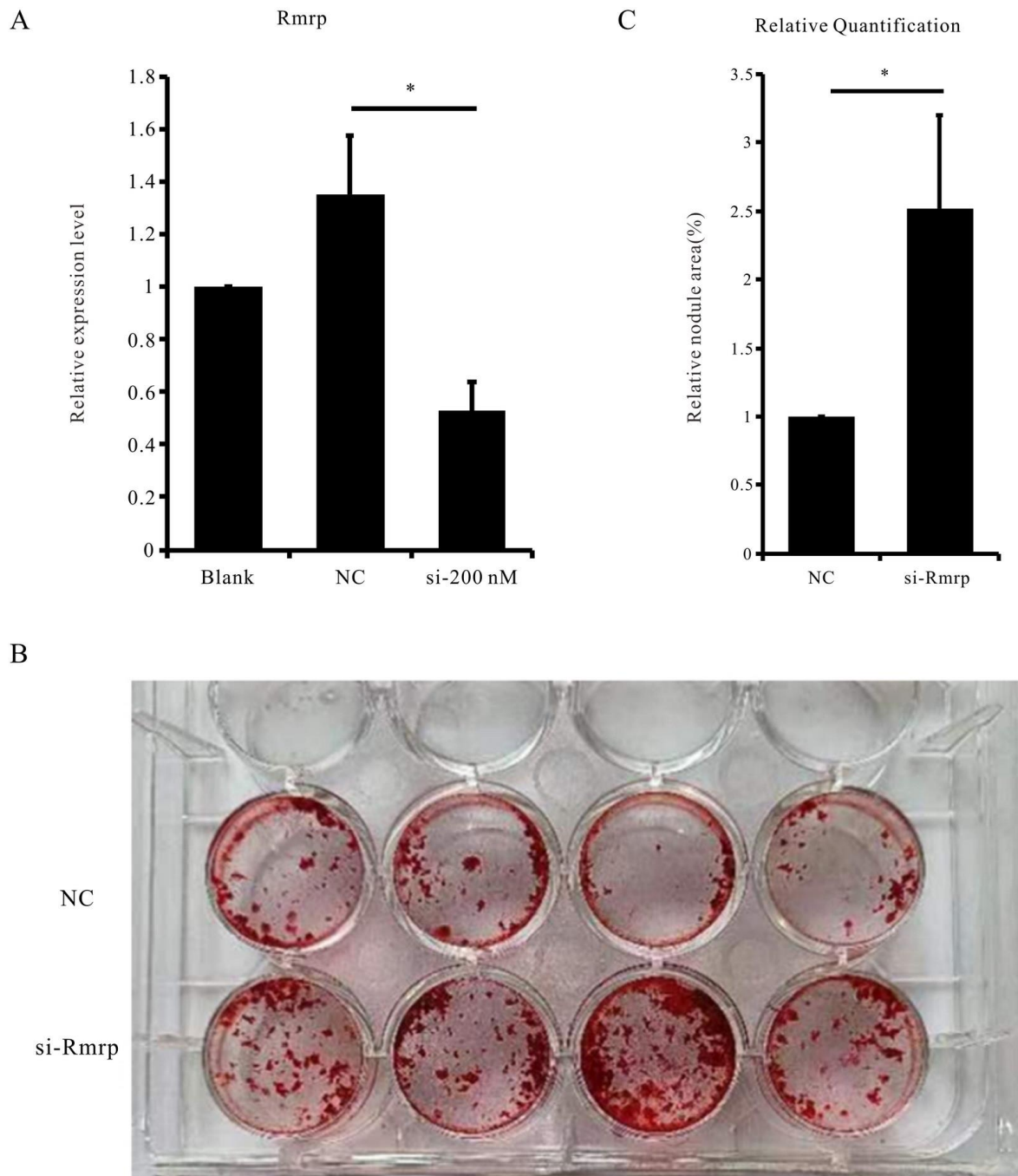


Figure 6. RMRP knockdown affects mineralization of osteoblasts. (A) RT-qPCR analysis of RMRP expression in BMSC osteoblast differentiation (n=3 per group); P=0.0323. (B) Mineralization was increased after RMRP knockdown at day 14 of osteogenic differentiation. (C) Mineralization of cells transfected with negative control or si-RMRP was quantified using ImageJ; P=0.0210. *P<0.05, **P<0.01. RMRP, RNase MRP; siRNA, small interfering RNA.

The RNA component of mitochondrial RMRP is a widely expressed transcript present in a number of eukaryotes, including humans and mice [30]. RMRP plays a regulatory role in RNA processing in the mitochondrial and ribosomal compartments [31], and this transcript is distributed in the mitochondria and nucleus [32, 33]. Previous studies revealed that RMRP expression is reduced in a patient with RMRP mutation, indicating that RMRP mutation may destabilize the structure of RMRP, leading to its degeneration [47]. Mutations in the RMRP gene have been associated with CHH in humans, an autosomal recessive inherited multisystem disorder characterized by skeletal dysplasia, dwarfism, thin and sparse hair, hypotrichosis, and varying degrees of immune dysfunction [34]. Patients with CHH exhibit changes in the metaphyses and epiphyses. In particular, changes in the metaphyses may include irregularities and cystic lesions that affect all bones of the hands and tubular bones. In addition, the epiphyses appear enlarged and rounded, and the hips, knees, and ankles of patients have a globular appearance. Notably, conoid epiphyses have also been described previously. Patients may also have short phalanges and metacarpals, impaired elbow pronation and supination, lumbar hyperlordosis, shortened and widened iliac wings, femoral bowing and increased ossification, and longer fibulae compared to tibiae [35, 36]. The observed changes in skeletal features imply that both chondrogenesis and osteogenesis are impaired in CHH, and these changes may be mediated by RMRP mutations. Notably, the lncRNA RMRP plays a key role in the initial stages of development in mice, and insertion of DNA elements upstream of RMRP results in early embryonic lethality [33]. RMRP downregulation was used to establish a zebra fish model of CHH, and the results confirmed impaired bone chondrogenesis and ossification accompanied by increased activation of Wnt/ β catenin signaling.

The results of a previous study showed that RMRP plays a key role in cell proliferation and apoptosis. Other studies discovered that RMRP downregulation can affect cell proliferation and modulate cell cycle stages [37–39]. RMRP was downregulated in hepatocellular carcinoma cells, while RMRP overexpression increased apoptosis in cells [40]. Lu et al. (41) demonstrated that RMRP knockdown promoted proliferation and suppressed apoptosis in osteoarthritic chondrocytes [41]. Moreover, combined single-cell and transcriptome analyses were performed in fibroblasts collected from healthy individuals and CHH patients. The results showed that multiple pathways influencing the regulation of apoptosis, bone and cartilage formation, and PI3K-Akt signaling were affected. Furthermore, cell cycle studies showed that CHH cells were arrested from the G₂ phase to mitosis [42].

Results of a previous study suggested that increased expression of RMRP RNA in hypertrophic chondrocytes may lead to reduced levels of Clb2 in healthy growth plates of mice resulting in altered CDK1 activity and total mitotic arrest. However, the specific role of RMRP RNA expression in the proliferation and apoptosis of murine chondrogenic cells is yet to be fully explained.

Although the effects of RMRP on cell proliferation, viability and apoptosis have been previously explored, the functional role of RMRP in the proliferation and apoptosis of BMSCs and osteoblasts is still unclear. Notably, these processes are crucial in osteoblast formation and bone repair. In our study, mouse BMSCs were used to investigate the impact of RMRP on stem cell proliferation, apoptosis, and osteoblast differentiation and mineralization. Our

results revealed that RMRP expression was regulated in BMSC proliferation and apoptosis, while RMRP knockdown suppressed the viability and proliferation of BMSCs. Moreover, RMRP knockdown had no significant effect on the viability or apoptosis of differentiating osteoblasts, which ceased to proliferate actively. As a complex that is involved in RNA processing, RNase MRP cleaves cyclin B2 mRNA at its 5'UTR at the end of mitosis to remove the 5' cap [48]. In our present study, cyclin B2 mRNA accumulated in BMSCs after RMRP silencing, thus indicating that RMRP may affect cell proliferation via the cyclin B2 pathway. Collectively, these results suggest that the effects of RMRP on cell growth and apoptosis may be related to the cell growth stages. RMRP is known to play a regulatory role in RNA processing in mitochondrial compartments. As for the relationship between mitochondrial and cellular apoptosis, we suspect that RMRP downregulation increases BMSC apoptosis through mitochondrial functional deficiency.

Previous studies have reported cases of osteochondrodysplasia [43–45]. The patient (12 years old) suffered from osteoporosis and global demineralization despite pamidronate infusions every 3 months from 8 to 10 the years of age [13]. These findings are consistent with the zebrafish CHH model used to further verify that RMRP knockdown was associated with chondrodysplasia, abnormal bone ossification and impaired mineralization. The results of a previous study showed that RMRP knockdown during the chondrogenic differentiation of ATDC5 cells derived from the AT805 teratocarcinoma cell line suppressed overall hypertrophy. Furthermore, delayed ossification and rare hypertrophic chondrocytes were observed in the growth plate of a CHH patient, highlighting that RMRP may play a functional role in chondrogenic differentiation. A previous study generated a homozygous mouse model with a G270T point mutation in RMRP, and its results showed that this mutation had no effect on skeletal development [46]. These findings demonstrate the need for further research.

However, previous *in vitro* studies have mainly focused on elucidating the underlying mechanisms of chondrogenesis impairment mediated by defects in RMRP. Currently, the specific role of RMRP in osteoblast differentiation and mineralization remains unknown. Our results demonstrate a significant reduction in RMRP expression during osteoblast differentiation of murine BMSCs, and RMRP knockdown promotes the mRNA expression of ALP, Runx2, Osterix and osteocalcin. Previous studies have shown that RMRP can act as a classical molecular scaffold, bind to several proteins and RNA, and play a role as a promoter of target gene [46]. Since RMRP silencing alter genes of osteoblasts in BMSCs, it was of great interest to figure out whether RMRP affects these genes through the same mechanism, and chromatin isolation by RNA purification (ChIRP) should be performed in the future to test this hypothesis. Because AKP/ALP activity is an indicator of the osteogenic activity intensity, we examined the former after RMRP knockdown in our study. The results demonstrated that RMRP knockdown enhanced AKP/ALP activity in the cytoplasm and culture medium. In particular, in the chondrocyte lineage, the mRNA expression levels of ATDC5, ALP, and AKP/ALP activity were significantly reduced in the cytoplasm after RMRP knockdown. Thus, RMRP, as a ubiquitously expressed gene, may affect the behavior of osteocytes and chondrocytes differently.

To investigate whether RMRP knockdown alters osteogenic differentiation at different differentiation stages, Alizarin Red staining was performed on Day 14 after the onset of

differentiation. The results of our study showed that RMRP knockdown enhances the mineralization of osteoblasts during osteogenic differentiation.

Taken together, the results of the present study indicate that RMRP expression is regulated in murine BMSCs, and RMRP may play a key role in proliferation and apoptosis. RMRP knockdown suppresses the viability and proliferation of BMSCs while enhancing apoptosis. Furthermore, RMRP knockdown promotes osteoblast differentiation of BMSCs and enhances osteoblast mineralization.

However, our study has limitations. For example, we did not perform RMRP overexpression, which could have additionally confirmed the effect of RMRP on BMSCs. Preliminary results showed that the expression of lncRNA RMRP was not increased after RMRP overexpression. Notably, RMRP acts as a housekeeping gene that is abundantly expressed in the nucleus and mitochondria of eukaryotes. Therefore, RMRP overexpression may exhibit low levels of efficacy and stability in the nucleus and mitochondria, and the abundant expression of RMRP in the cytoplasm may not mimic the behavior of RMRP in cells. Thus, further experiments using lentivirus transfection are needed. In addition, BMSCs were the only cell type used in the present study. To overcome this limitation, further verification should be performed on NIH/3T3 (mice) and human BMSCs (primary BMSCs and cell line). We plan to construct a lentivirus cell line to stabilize the overexpression level of RMRP. A recent study showed the effect of RMRP in ATDC5 cells; however, there are controversial opinions that ATDC5 cell line is not derived from hyaline cartilage, so it may be interesting to test the effects of RMRP in primary cartilaginous chondrocytes.

Since CHH is a rare autosomal recessive disease, we do not have enough CHH patient samples to conduct clinical analysis and validation. The lack of clinical analysis in humans and survival prognosis analysis in the disease is a limitation of this study as well.

In addition, regarding the effect of RMRP on osteoblast differentiation and mineralization, it is necessary to test the effect of RMRP in vitro in a mouse fracture model. In addition, the current mouse model of RMRP only presents an immune phenotype without gross skeletal defect. It is believed that the mutation site is closely related to the specific phenotype. Another animal model is the zebrafish RMRP^{-/-} mutation model, which presents a cartilaginous and ossifying phenotype. Regarding the symptoms of CHH in patients with RMRP mutation, the most interesting model may be the mouse model with RMRP 70 A>G mutation (this is the most common mutation in CHH patients), which can cause relapse of skeletal dysfunction.

Conclusion

In conclusion, our study has revealed that RMRP may function in chondrogenic cells, BMSCs and osteoblasts, and may mediate the abnormalities of ossification in patients with CHH. Hence, our results may provide a novel theoretical basis for the role of RMRP in skeletal dysplasia phenotypes.

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Availability of data and materials

The data presented in this study are available on request from the corresponding author.

Author contributions

JG: conceptualization, paper writing. YM: methodology, software. SX: formal analysis, draft manuscript preparation. XZ: data curation, investigation. GT: visualization, investigation, validation. JT: funding acquisition, study supervision, review.

Ethical approval and consent to participate

The study animals and protocols were approved by the Ethics Committees of the First Affiliated Hospital of HUNAN University of Medicine. All animals were bred in SPF animal center and executed after anesthesia to minimize animal suffering.

Conflict of interest

None declared by the authors.

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Supplementary

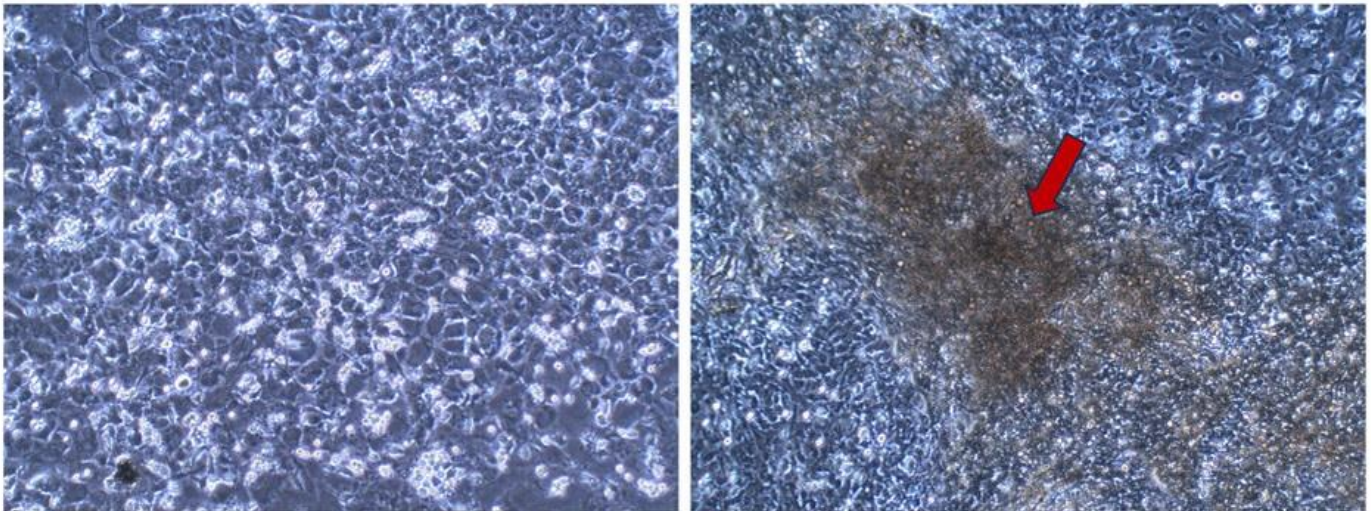


Figure S1. Purity determination of cultured BMSCs. Left, cultured BMSCs; right, osteoblasts during mineralization (red arrow, calcium nodules). BMSCs, bone marrow-derived mesenchymal stem cells.