$MgCl_2$ promotes mouse mesenchymal stem cell osteogenic differentiation by activating the p38/Osx/Runx2 signaling pathway

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Abstract. Magnesium, an important inorganic mineral component in bones, enhances osteoblast adhesion and osteogenic gene expression. Mg^{2+}-containing hydroxyapatite promotes mouse mesenchymal stem cell (MMSC) osteogenic differentiation. In the present study, MMSCs were cultured in media containing different concentrations of $MgCl_2$ (0 and 20 mM) for different time periods. Western blotting and reverse transcription-quantitative PCR were performed to determine the expression levels of phosphorylated (p)-p38 mitogen-activated protein kinase (MAPK), the osteoblast-specific transcription factor Osterix (Osx), runt-related transcription factor 2 (Runx2), and p38 downstream genes, such as 27 kDa heat shock protein (hsp27), activating transcription factor 4 (Atf4), myocyte enhancer factor 2C (Mef2c) and CCAAT/enhancer-binding protein homologous protein (Ddit3). The facilitatory effect of $MgCl_2$ on MMSC osteogenic differentiation was assessed via Alizarin Red staining. The results suggested that $MgCl_2$ increased p38 phosphorylation compared with the control group. Downstream genes of the p38 signaling pathway, including Osx and Runx2, as well as several osteogenesis-associated downstream target genes, including Hsp27, Atf4, Ddit3 and Mef2c, were significantly upregulated in the Mg^{2+}-treated group compared with the control group. The increased osteogenic differentiation in the Mg^{2+}-treated group was significantly attenuated in MMSCs treated with SB203580, a specific inhibitor of the p38 signaling pathway. The results suggested that appropriate concentrations of $MgCl_2$ promoted MMSC osteogenic differentiation via regulation of the p38/Osx/Runx2 signaling pathway.

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Introduction

The adult body contains ~24 g Mg, of which ~60% is located in the skeleton, accounting for 0.26-0.55% of the mass percentage of the natural bone. Park *et al* (1,2) demonstrated that Mg^{2+} promoted osteoblast adhesion, stimulated alkaline phosphatase (ALP) and osteocalcin (OC) syntheses, and increased the expression levels of integrin, transcription factor distal-less homeobox 5 (Dlx5), osteogenesis marker gene ALP, bone sialoprotein (BSP), osteoblast-specific transcription factor Osterix (Osx) and OC. Crespi *et al* (3) reported that Mg^{2+} enhanced the expression of runt-related transcription factor 2 (Runx2), and initiated osteoblast synthesis of OC and bone matrix. Magnesium phosphate increases the expression of Runx2, ALP and osteopontin in osteoblasts (4). In our previous study, mouse mesenchymal stem cells (MMSCs) were cultured in Mg-containing hydroxyapatite (Mg-HA) solution for 72 h. The reverse transcription-quantitative PCR (RT-qPCR) results demonstrated that the mRNA expression levels of ALP, Osx and Runx2 in the Mg-HA group were significantly higher compared with MMSCs cultured without Mg-HA, suggesting that Mg-HA promoted MMSC osteogenic differentiation (5). To meet clinical requirements, several bone-related ions were added to synthetic HA to improve its effects, which indicated that Mg-HA was suitable for clinical application (6). Magnesium and hydroxyapatite composites can overcome the shortcomings of traditional bioceramics, including lack of bone formation inducing ability (7). In addition, extracts of biodegradable Mg alloys exhibited superior effects on human mesenchymal stem cell proliferation and differentiation compared with culture media (8).

Mitogen-activated protein kinases (MAPKs) are a group of serine-threonine protein kinases that can be activated by different extracellular stimuli, such as cytokines, neurotransmitters, hormones, cell stress and cell adhesion (9,10). There are four independent MAPK signaling pathways composed of four signaling families: The MAPK/ERK family or classical pathway, the Big MAP kinase-1 family, c-Jun N-terminal kinase family and p38 family (9). MAPK signaling pathways are crucial for several physiological activities in the cell, including proliferation, differentiation and apoptosis (10-12). MAPK has been reported to be pivotal in regulating signaling pathways involved in osteogenic differentiation, specifically the p38/Osx

signaling pathway, where its activation promotes osteogenic differentiation (13). Yi *et al* (14) demonstrated that activation of the p38 signaling pathway facilitates osteogenic differentiation over fat cell differentiation during mesenchymal stem cell differentiation. *In vitro*, the inhibition of p38α and p38β can block early osteogenic differentiation (15,16). In addition, p38α favors the mineralization of long bones (17). The p38ignaling pathway can also phosphorylate the key transcriptional factor Runx2, and thus facilitate osteogenic differentiation (18). A combination of Runx2 and Osx can activate osteogenic lineage transcriptional patterns, such as increased mRNA expression levels of various bone-related genes, including ALP, BSP, osteocalcin, osteopontin and type I collagen (19). Independent of Runx2, the p38 signaling pathway can regulate the transcription factor Dlx5, which promotes osteogenic differentiation by activating BSP and Osx (20).

The 27 kDa heat shock protein (Hsp27) is expressed in a differentiation-related pattern and can regulate osteocalcin synthesis (21). Activating transcription factor 4 (Atf4) is an important transcription factor that serves a pivotal role in osteoblastic differentiation and bone formation (22,23). CCAAT/enhancer binding protein homologous protein (Ddit3) also increases osteoblastic differentiation potential (24,25). Myocyte enhancer factor 2C (Mef2c) significantly regulates osteogenic differentiation of various stem cells (26). Moreover, the functions of Hsp27, Atf4, Ddit3 and Mef2c are dependent on the p38 signal transduction pathway (27-29).

In the present study, the expression levels of phosphorylated (p)-p38, Osx, Runx2 and p38 downstream genes, including Hsp27, Atf4, Ddit3 and Mef2c, were assessed following Mg^{2+} treatment. Furthermore, the osteogenic differentiation potential of MMSCs in the Mg^{2+}-treated group was evaluated via ALP and Alizarin Red staining. The relationship between Mg^{2+} and the p38/Osx/Runx2 signaling pathway was explored to determine the mechanisms underlying the osteogenesis-boosting effect of $MgCl_2$.

Materials and methods

Cell line and cell culture. MMSCs (ScienCell Research Laboratories, Inc.) were cultured in mesenchymal stem cell culture medium (ScienCell Research Laboratories, Inc.; Mg concentration, 0.8112 mM) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and 100 μg/ml streptomycin and penicillin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37˚C.

ALP staining assay. MMSCs were seeded into 12-well plates ($1x10^5$ cells/well). After culture with mesenchymal stem cell culture medium for 24 h, different concentrations of $MgCl_2$ (0 and 20 mM) were added to the osteoblast differentiation medium (MODM; Mg concentration, 0.8112 mM; ScienCell Research Laboratories, Inc.) and the culture medium was changed every 2 days. MMSCs continuously cultured with mesenchymal stem cell culture medium, changed every 2 days, were considered as the undifferentiated negative control. Throughout osteogenic differentiation, the concentration of $MgCl_2$ remained constant. After 14 days of induction, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed twice with PBS. NBT/BCIP color substrate solution was freshly prepared by adding the Ready-to-Use tablet (Roche Diagnostics) into 10 ml double distilled water according to the manufacturer's protocol. Cells were stained with NBT/BCIP color substrate solution for 15 min at 25˚C, washed twice with distilled water and observed under an IX71-F22FL/DIC optical microscope (Olympus Corporation; magnification, x10).

ELISA. MMSCs were induced with $MgCl_2$ as aforementioned. After 14 days of induction, cells were harvested, rinsed with PBS, homogenized in 500 μl PBS and stored overnight at -20˚C. Subsequently, two freeze-thaw cycles were performed to break the cell membranes. The lysate was centrifuged for 5 min at 5,000 x g at 4˚C. The supernatant was collected and immediately assayed using a mouse osteocalcin ELISA kit (cat. no. CSB-E06917m; Cusabio Technology LLC) according to the manufacturer's protocol. The results were read using a microplate reader at a wavelength of 450 nm.

Alizarin Red staining assay. MMSCs were induced with $MgCl_2$ as aforementioned. After 21 days of induction, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and washed once with distilled water. Subsequently, cells were stained with Alizarin Red (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature, washed with distilled water and observed under an IX71-F22FL/DIC optical microscope (Olympus Corporation; magnification, x10). Alizarin Red staining analysis was conducted using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc).

Western blotting. MMSCs were seeded into 6-well plates ($3x10^5$ cells/well). After 24 h, $MgCl_2$ (20 mM) was added to the culture medium for 0, 5, 30 or 60 min to determine the immediate effect of p38 activation. In the other group, 20 mM $MgCl_2$ was applied to the culture medium for a longer period (1, 24 and 72 h) to detect the continuous activating effect. Subsequently, cells were washed with cold PBS at least three times and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) at 4˚C. Then laemmli sample buffer (2% SDS) was added. Total protein was quantified using a BCA protein quantification kit (Thermo Fisher Scientific, Inc.). Proteins (40 μg) were denatured in 1X SDS loading buffer, separated via 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The membranes were blocked for 1 h with 5% non-fat milk in TBS-Tween-20 (0.1% Tween-20) at room temperature with gentle agitation. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies targeted against: Runx2 (cat. no. ab76956; Abcam), anti-Osx (cat. no. ab22552; Abcam), p38 (cat. no. 8690S; Cell Signaling Technology, Inc.), p-p38 (cat. no. 9216S; Cell Signaling Technology, Inc.) and β-actin (cat. no. 3700S; Cell Signaling Technology, Inc.). All primary antibodies were used at a dilution of 1:1,000. Following primary incubation, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (cat. no. 7074S; Cell Signaling Technology, Inc.) and goat anti-rabbit (cat. no. 7076S; Cell Signaling Technology, Inc.) secondary antibodies for 2 h at room temperature. All secondary antibodies were used at a dilution of 1:2,000. Protein bands were visualized using SuperSignal™ West Femto Maximum Sensitivity substrate

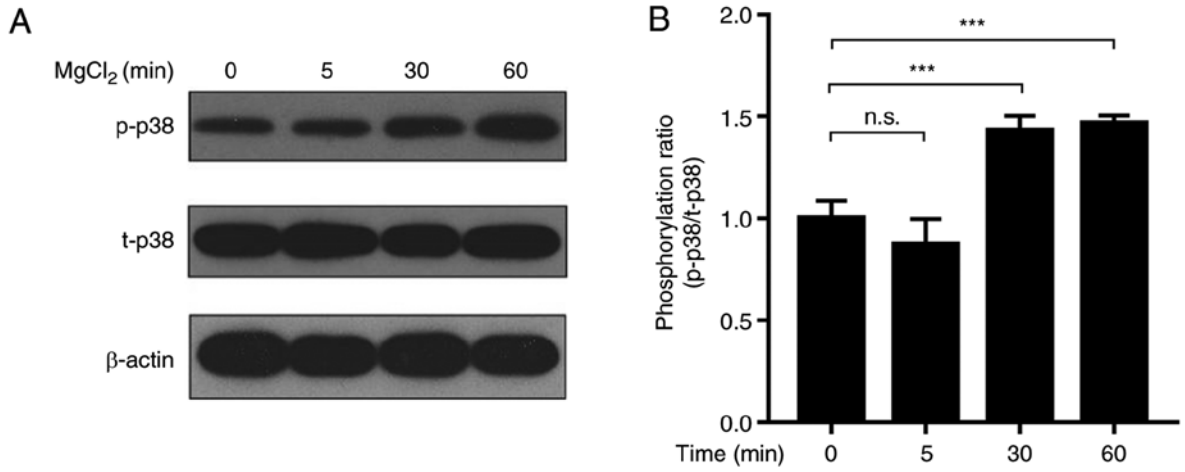

Figure 1. Effect of Mg^{2+} on the levels of p-p38 and t-p38 in mouse mesenchymal stem cells at different time points. Protein expression levels were (A) determined by western blotting and (B) semi-quantified. Data were analyzed using one-way ANOVA with Bonferroni's post hoc testing (n=3). ***P<0.001. p, phosphorylated; t, total; n.s., not significant.

(cat. no. 34095; Thermo Fisher Scientific, Inc.). Protein expression was semi-quantified using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.) with β-actin as the loading control.

RT-qPCR. MMSCs were seeded into 6-well plates ($3x10^5$ cells/well). After 24 h, $MgCl_2$ (20 mM) and 10 μM SB203580 (cat. no. S1076; Selleck Chemicals), a specific inhibitor of the p38 signaling pathway, were added to the culture medium. After 48 h, total RNA was extracted from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (cat. no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's instruction. Briefly, 1 ng total RNA was reverse transcribed with 3 steps: 10 min incubation at 25˚C, 120 min incubation at 37˚C and 5 min incubation at 85˚C. Subsequently, qPCR was performed using SYBR Select Master Mix (cat. no. 4368577; Applied Biosystems; Thermo Fisher Scientific, Inc.) and a ViiA 7 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 95˚C for 30 sec; followed by 40 cycles of 95˚C for 5 sec, 58˚C for 15 sec and 72˚C for 30 sec. The sequences of the primers used for qPCR were: Hsp27 forward, 5'-GTCCCTGGATGTCAACCACT-3' and reverse, 5'-GACTGGGATGGTGATCTCGT-3'; Atf4 forward, 5'-CCTGAACAGCGAAGTGTTGG-3' and reverse, 5'-TGGAGAACCCATGAGGTTTCAA-3'; Ddit3 forward, 5'-AAGCCTGGTATGAGGATCTGC-3' and reverse, 5'-TTCCTGGGGATGAGATATAGGTG-3'; Mef2c forward, 5'-ATCCCGATGCAGACGATTCAG-3' and reverse, 5'-AACAGCACACAATCTTTGCCT-3'; and GAPDH forward, 5'-AGCTTCGGCACATATTTCATCTG-3' and reverse, 5'-CGTTCACTCCCATGACAAACA-3'. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (30) and normalized to the internal reference gene GAPDH.

Cell apoptosis. MMSCs were seeded into 6-well plates at $3x10^5$ cells per well overnight with mesenchymal stem cell culture medium. Subsequently, the medium was changed to MODM with different concentration of $MgCl_2$ (0, 2, 20, 50 and 100 mM). Cells were harvest to analyze with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to manufacturer's protocols. In brief, the MMSCs were washed twice with cold PBS and then harvest with trypsin digestion. Resuspended in 100 μl binding buffer, cells were then incubated with 5 μl FITC Annexin V and 5 μl propidium iodide (PI) for 15 min at room temperature avoiding light. Then, 400 μl extra binding buffer was added to each sample before they were analyzed with a flow cytometer (FACSCanto II; BD Biosciences). The results were analyzed with FlowJo v10 software (FlowJo LLC).

Cell proliferation. MMSCs were seeded into 96-well plates ($5x10^3$ cells/well) overnight with mesenchymal stem cell culture medium in triplicate. MODM with different concentrations of magnesium (0, 2, 20 and 100 mM) were changed every other day. Cell proliferation assay was performed using CellTiter 96™ Aqueous One Solution Cell Proliferation (MTS) assay (Promega Corporation) every day for 5 days according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism software (version 7; GraphPad Software, Inc.) and SPSS software (version 19.0; IBM, Corp.). Experiments were repeated at least three times and quantified data are presented as the mean ± standard deviation. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. Comparisons between two groups were analyzed using unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of Mg^{2+} on the levels of p-p38 and total (t)-p38 in MMSCs. Previously, it was reported that Mg^{2+} promoted mouse bone marrow-derived MMSC proliferation and osteogenic differentiation (7). Moreover, different concentrations of Mg^{2+} displayed contrasting effects on osteoblast differentiation, suggesting there would be a peak in its enhancing effect with the

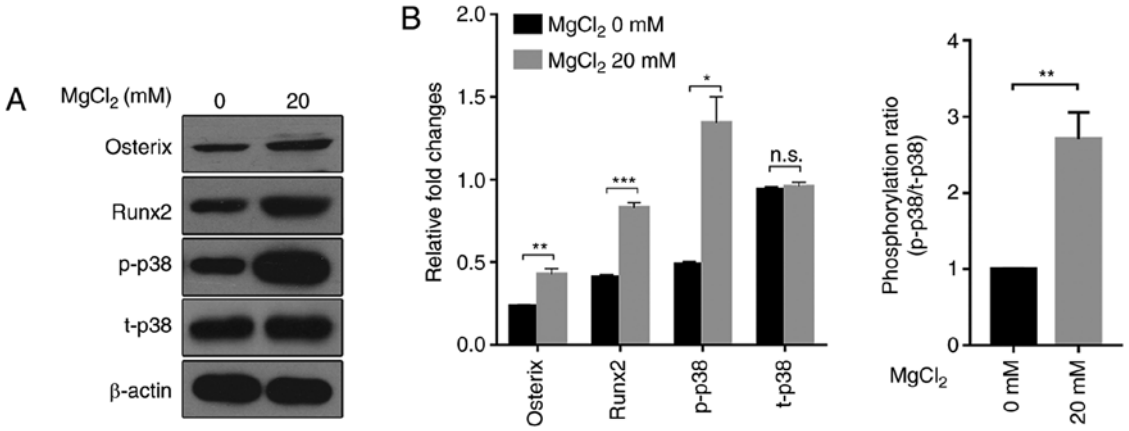

Figure 2. Effect of Mg^{2+} on the expression levels of Osterix and Runx2 in MMSCs. Protein expression levels were (A) determined by western blotting and (B) semi-quantified. Right panel shows the ratio of p-p38/t-p38. Data were presented as the mean ± SD and analyzed using Student's t-test. (n=3). *P<0.05, **P<0.01 and ***P<0.001. Runx2, runt-related transcription factor 2; MMSC, mouse mesenchymal stem cell; p, phosphorylated; t, total; n.s., not significant.

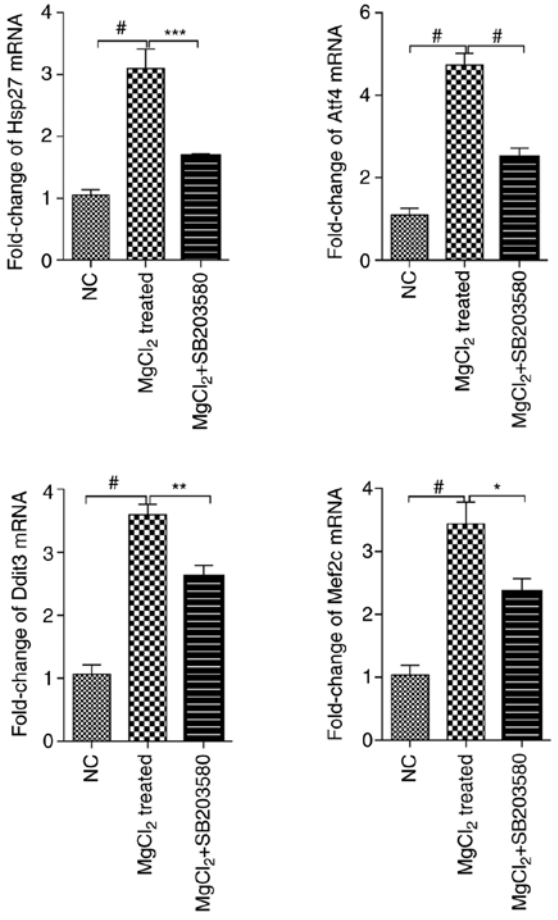

Figure 3. Effect of Mg^{2+} on the expression levels of Hsp27, Atf4, Ddit3 and Mef2c is abrogated by SB203580-mediated p38 inhibition. MMSCs were cultured with media containing 20 mM $MgCl_2$ for 48 h, with or without 10 μM SB203580. Data were analyzed using one-way ANOVA with Bonferroni's post hoc testing. *P<0.05, **P<0.01, ***P<0.001; $^{\#}$P<0.0001. Hsp27, 27 kDa heat shock protein; Atf4, activating transcription factor 4; Ddit3, CCAAT/enhancer-binding protein homologous protein; Mef2c, myocyte enhancer factor 2C; MMSC, mouse mesenchymal stem cell; NC, negative control.

appropriate dose (31). Based on the preliminary findings of the present study, the optimal concentration of Mg^{2+} for osteogenic differentiation was 20 mM (Fig. S1), as it notably facilitated osteogenesis and partially decreased apoptosis induced by MODM (0 $MgCl_2$ group). To verify our observations, cell proliferation was analyzed with MTS at the early differentiating stage (Fig. S1C). The 0, 2, and 20 mM $MgCl_2$ groups exhibited better proliferation than 100 mM group although no significant difference was observed. Thus, 20 mM Mg^{2+} was selected for subsequent experiments. The p38 signaling pathway has been hypothesized to be closely associated with osteogenic differentiation (18); therefore, the effect of Mg^{2+} on the p38 signaling pathway was further examined. The results indicated that 20 mM Mg^{2+} increased the ratio of p-p38/t-p38 in MMSCs in a time-dependent manner (Figs. 1 and S2).

Effect of Mg^{2+} on the expression levels of Osx and Runx2 in MMSCs. Downstream genes in the p38 signaling pathway, such as Osx and Runx2, are key genes involved in osteogenic differentiation (12). The western blotting results indicated that Mg^{2+} treatment significantly increased the expression levels of Osx and Runx2 compared with the control group, suggesting that Mg^{2+} enhanced the activity of the p38 signaling pathway (Fig. 2). Furthermore, compared with the control group, Mg^{2+} also increased the ratio of p-p38/t-p38, an indicator of activation of the signaling pathway, highlighting a potential mechanism underlying Mg^{2+}-mediated promotion of MMSC osteogenic differentiation.

Effect of Mg^{2+} on the expression levels of p38 downstream genes. To further investigate the hypothesis that the p38 signaling pathway mediated the effects of Mg^{2+} treatment, the expression of other crucial p38 downstream genes, specifically Hsp27, Atf4, Ddit3 and Mef2c, was evaluated. The mRNA expression levels of Hsp27, Atf4, Ddit3 and Mef2c in the $MgCl_2$ group were significantly increased compared with the control group (Fig. 3). The results suggested that Mg^{2+} increased the expression levels of p38 downstream target genes via activation of the p38 signaling pathway.

Moreover, blocking of the p38 signaling pathway using SB203580 resulted in downregulation of Hsp27, Atf4, Ddit3 and Mef2c expression levels in $MgCl_2$-treated cells (Fig. 3), supporting the hypothesis that Mg^{2+} increased the

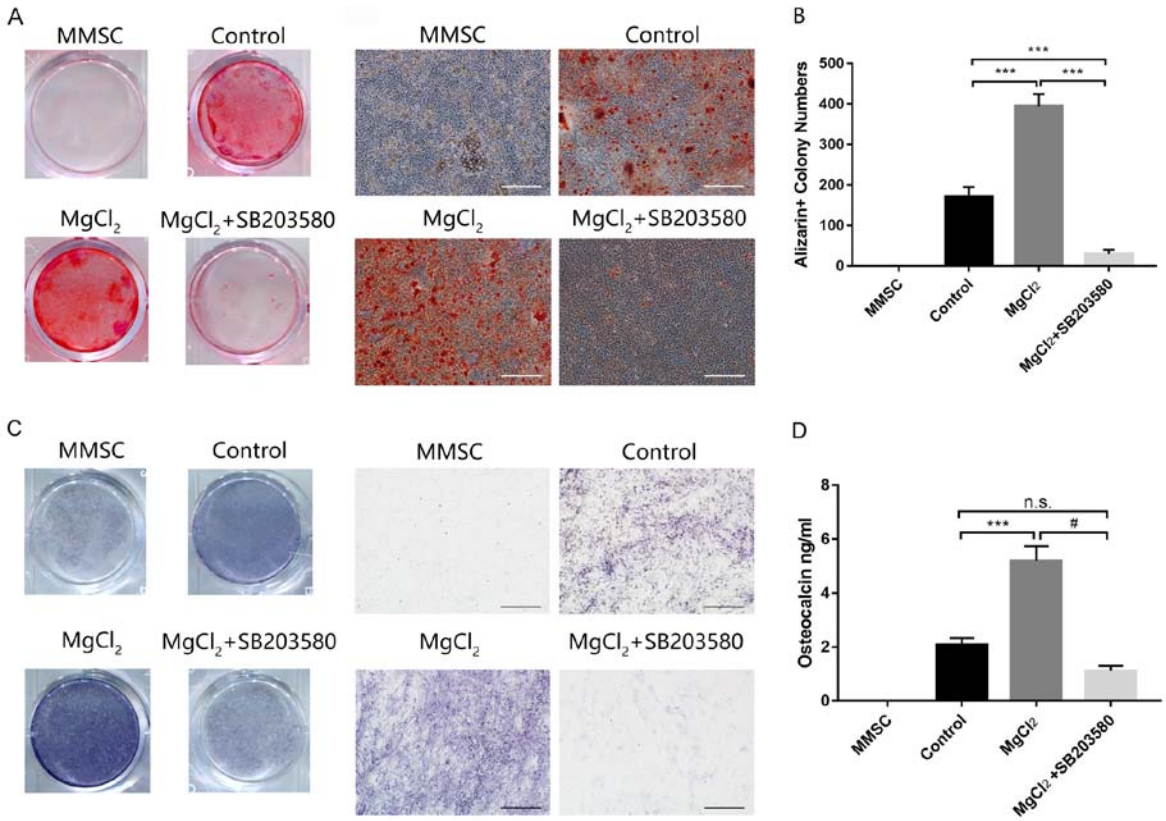

Figure 4. Mg^{2+} increases MMSC osteogenic differentiation. Effects of Mg^{2+} on extracellular calcium deposition were (A) determined by Alizarin Red S staining and (B) quantified. (C) Effects of Mg^{2+} on ALP activity were determined. Scale bar, 100 μm (D) Osteocalcin protein expression levels were assessed by performing ELISA. ***P<0.001 and $^{\#}$P<0.0001. MMSC, mouse mesenchymal stem cell; ALP, alkaline phosphatase; n.s., not significant.

expression of osteogenesis-associated genes via activation of the p38 signaling pathway.

Effect of Mg^{2+} on MMSC osteogenic differentiation. MMSCs were induced with MODM containing glutamine, ascorbic acid, β-glycerol phosphate disodium and dexamethasone to determine whether Mg^{2+} promoted osteogenic differentiation. MMSCs were treated with $MgCl_2$ 20 mM with or without SB203580. Control cells were incubated with MMSC media. After 21 days, fully differentiated MMSCs were fixed, and calcium node formation was analyzed by performing Alizarin Red staining (Fig. 4A). Compared with the control group, $MgCl_2$ significantly increased the number and size of calcium nodes. SB203580 significantly reduced Mg^{2+}-induced osteogenic differentiation (Fig. 4B). The ALP staining results and quantification of osteocalcin protein levels via ELISA were consistent with the Alizarin Red staining results (Fig. 4C and D). Collectively, the results suggested that Mg^{2+} promoted MMSC osteogenic differentiation, which was dependent on activation of the p38 signaling pathway.

Discussion

In the present study, the facilitatory effects of $MgCl_2$ on MMSC osteogenic differentiation, including the effects on p38 signaling pathway activation, were assessed. Magnesium, the second most abundant intracellular cation in the human body, serves a key role in a number of essential cellular processes, including DNA replication and repair, and cell proliferation (32,33). As a result, magnesium sulfate is the necessary component in most of the commercialized culture media used for adhesive cells (34). In the present study, the culture media used for MMSCs and differentiation contained 0.8112 mM $MgSO_4$ as background magnesium. Due to the low background concentration, it was assumed that the magnesium present in the media would not affect the results of the present study. High concentrations of $MgCl_2$ (50 or 100 mM) resulted in a dose-dependent increase in apoptosis compared with the 0 mM $MgCl_2$ group. Conversely, 2 and 20 mM $MgCl_2$ reduced apoptosis rate compared with both the control group and the high concentration groups. Furthermore, 20 mM $MgCl_2$ displayed an increased capacity to facilitate osteogenic differentiation among the different concentrations of $MgCl_2$ used in the present study. The signaling pathways underlying Mg^{2+}-mediated increases in osteogenesis were evaluated, and the results indicated that $MgCl_2$ increased activation of the p38 signaling pathway compared with the control group. Additionally, the results suggested that the expression levels of key p38 downstream osteogenic genes were significantly increased by $MgCl_2$ treatment compared with the control group. Collectively, the results indicated a potential key role for intracellular Mg^{2+} in MMSC osteogenesis.

It has been reported that four downstream genes are closely associated with MMSC osteogenic differentiation, and the characteristics and formation of osteoblasts (35-38). Activation of the p38 signaling pathway phosphorylates Hsp27, thereby stimulating osteoblast mineralization (35). Atf4 is an essential transcription factor that participates in the synthesis of

osteoblast proteins (36). Pereira *et al* (24) reported that Ddit3 induced osteogenic differentiation, and it has also been demonstrated that Mef2c serves an important role in regulating gene expression in osteoblasts (37).

Apart from those four genes, Runx2, another downstream gene of p38 pathway, is also considered as a key effector and marker of osteoblast differentiation (3,4). Its transcription also reported to be influenced by other pathways like TRPM7/PI3K signaling pathway (38,39). Transient receptor potential M-type 7 (TRPM7), a non-selective cationic channel with constitutive activity, is an important regulator of entry of several extracellular metal ions, and serves an important regulatory role in bone sclerosis and remodeling (38). It has been reported that Mg^{2+} can upregulate the expression of Runx2 via a TRPM7/PI3K signaling pathway, which results in increased expression of the osteogenic marker ALP (39). Therefore, TRPM7 serves an important role in Mg^{2+}-mediated BMSC osteoblast differentiation.

Moreover, magnesium accelerates osteogenesis by affecting the secretion of the growth factor (40). Fibroblast growth factor (FGF) regulates the proliferation of osteoblast precursor cells via the p38 signaling pathway, and regulates Runx2 function, thereby promoting osteoblast differentiation and maturation (40). Mg^{2+} can increase the expression of FGF-2, and promote BMSC proliferation and osteogenic differentiation (8). Therefore, Mg^{2+} may promote BMSC proliferation and osteogenic differentiation by activating the p38 signaling pathway via FGF-2.

In the present study, Mg^{2+} was derived from $MgCl_2$. The means by which extracellular Mg^{2+} ions from biomaterials are transported into cells affects the expression levels of transcription factors and requires further investigation.

In conclusion, Mg^{2+} promoted MMSC osteogenic differentiation by activating the p38 signaling pathway, and blocking the p38 signaling pathway abrogated the effects of Mg^{2+} on differentiation.

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Availability of data and materials

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

Authors' contributions

XYN designed the study and contributed to writing the manuscript. XBX and SN performed the experiments. SN contributed to the critical revision of the manuscript and provided important intellectual feedback. All authors read and approved the final manuscript.

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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