Research Article

Magnesium–Magnetic Field Synergy Enhances Mouse Bone Marrow Mesenchymal Stem Cell Differentiation into Osteoblasts Via the MAGT1 Channel

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Received 28 December 2021; Revised 12 February 2022; Accepted 14 February 2022; Published 3 March 2022

Academic Editor: Xiaoming Li

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Magnesium ion (Mg2+)–based materials are known to exert osteogenic effects that can be enhanced by the bioelectrical properties of magnetic fields. In this study, we examined the effect of a medium-strength static magnetic field (SMF), combined with a Mg2+–containing medium, on the proliferation and osteogenic differentiation of mouse bone marrow mesenchymal stem cells (BMSCs). Mouse BMSCs were divided into a control group, 7.5 mM Mg2+ group, 15 mT SMF group, and 7.5 mM Mg2+ plus 15 mT SMF group. Osteoblast proliferation was measured using a Cell Counting Kit-8 assay, whereas osteogenic differentiation was detected using alkaline phosphatase (ALP) staining and western blot analysis, respectively. The number and size of calcium nodules were determined using Alizarin Red staining. Compared with those in the control group, the ALP activity, calcium nodule formation, and osteogenic protein expression were promoted in other groups. In particular, Mg2+-SMF had a significant effect after 7 days of intervention and more effectively promoted BMSC differentiation and proliferation than either Mg2+ or the SMF alone, suggesting that Mg2+-SMF synergistically contributed to osteogenic differentiation and cell proliferation. To examine their roles in bone differentiation, the Magt1 and Creb1 genes were silenced in BMSCs, and the findings indicated that the synergistic intervention with Mg2+ and magnetic fields might exert osteogenic effects via the MAGT1 channel and CREB1 protein. This study provides an experimental basis for a potential Mg2+-SMF synergistic artificial bone material that could be clinically applied in the treatment of bone defects.

1. Introduction

Magnesium ion (Mg2+)–based materials are expected to be applied in clinical practice, as they exert osteogenic effects and their mechanical properties are similar to those of the natural bone. Unfortunately, the rate of degradation of pure Mg-based materials that are used in orthopedics is extremely high. In the bone tissue, wherein fluid circulation and metabolism are relatively slow, this degradation can cause excessive local Mg2+ and hydrogen accumulation, resulting in a highly alkaline environment, which adversely affects the blood supply to surrounding soft tissues as well as cell adhesion and the repair of bone defects.

Biodegradable orthopedic materials have been extensively studied in recent years [1, 2], and numerous studies have shown that Mg2+ can promote the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) [3–6]. In addition to promoting the differentiation of BMSCs into osteoblasts, Mg2+ can increase extracellular matrix (ECM) mineralization, resulting in excellent osteoinduction [7–9]. A previous study has confirmed that BMSCs display the best proliferation and
osteogenic differentiation when cultured with 7.5 mM Mg\(^{2+}\) [10]. In particular, Mg\(^{2+}\) can promote osteogenic differentiation by inducing cAMP responsive element binding protein 1 (CREB1) phosphorylation via the Mg\(^{2+}\) channel magnesium transporter 1 (MAGT1) [9]. Mg\(^{2+}\) can also induce BMSC differentiation into osteoblasts directly, via MAGT1, and promote the expression of intracellular osteogenic signaling molecules [alkaline phosphatase (ALP), OCN, COL1, and RUNX2]. Since osteogenic properties of Mg\(^{2+}\) are associated with its transmembrane transport, it is important to determine the mechanisms underlying the opening of the MAGT1 channel to induce Mg\(^{2+}\) influx. Therefore, an understanding of how to effectively open the MAGT1 channel in the cell membrane and cause an effective influx of Mg\(^{2+}\) is the key to achieving the full osteoinductive activity of Mg\(^{2+}\).

The magnetic field environment can change the opening frequency of Mg\(^{2+}\) channel proteins on the cell membrane surface. Thus, the influx efficiency of Mg\(^{2+}\) can be improved to enhance its biological effect on osteogenesis. Numerous studies have shown that BMSC proliferation and osteoinduction can be accelerated by medium-strength magnetic fields (1 mT–1 T) [11–14], which include both pulsed electromagnetic fields (PEMFs) [15–17] and static magnetic fields (SMFs) [18–22]. Since magnetic fields are noninvasive and safe, they have the potential for broad clinical applications [23–27]. Although PEMFs are currently extensively studied for the induction of bone formation, SMFs have unique advantages for the preparation of artificial bone materials. For example, SMFs do not require powerful equipment for the biophysical stimulation of BMSCs, and it is easier to produce osteogenic effects when magnetic materials are added to artificial bone materials. SMFs can also exert important regulatory effects during bone metabolism and remodeling and have become an important biophysical tool for treating nonunions and promoting bone healing [28–30]. Studies have shown that SMFs can promote bone repair, bone deposition, and bone formation in vitro and in vivo [31–34]. In addition, SMFs can inhibit the reduction of the bone density caused by surgery or prostheses. Magnetic fields mainly exert osteogenic effects on BMSCs via electrical and mechanical receptors on the cell membrane that convert bioelectrical signals into biochemical signals, thereby activating intracellular signaling cascades. Indeed, osteogenesis-related ion channels, such as MAGT1, TWIK-related K\(^+\) channel 1, and ORAI calcium release-activated calcium modulator 1/2, have been shown to alter their opening frequencies under the action of magnetic fields, thereby promoting osteogenesis [35–37]. However, it remains unclear whether the bioelectrical effects of a magnetic field could be combined with Mg\(^{2+}\) to regulate the osteogenic properties of ion channels and thereby synergistically enhance osteogenic differentiation and ECM mineralization.

In this study, we aimed to combine two factors with recognized osteogenic effects, Mg\(^{2+}\) and SMFs, in order to explore whether they can synergistically enhance bone formation while eliminating the disadvantages of Mg\(^{2+}\) as a potential new biomaterial. The findings of this study provide an experimental basis for a putative new type of Mg\(^{2+}\)-SMF synergistic artificial bone material with excellent osteogenic properties.

2. Materials and Methods

2.1. Cell Culture. BMSCs were purchased from Cyagen, Inc. (Beijing, China) and were grown in a BMSC growth medium at 37°C with 5% CO\(_2\). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in T25 culture flasks for expansion. To induce osteogenic differentiation, BMSCs were cultured in DMEM with 50 mM ascorbic acid, 10 mM dexamethasone, and 10 mM β-glycerophosphate (Sigma–Aldrich, St. Louis, MO, USA) at 37°C with 5% CO\(_2\). The cells were divided into the following four groups: control (0 mM Mg\(^{2+}\) and 0 mT SMF), Mg\(^{2+}\) (7.5 mM Mg\(^{2+}\)), SMF (15 mT SMF), and Mg\(^{2+}\)-SMF (7.5 mM Mg\(^{2+}\) combined with 15 mT SMF) (Figure 1(a)), and their proliferation and osteogenic differentiation were observed. Mg\(^{2+}\) environment was added to the osteogenic induction medium in an appropriate proportion by anhydrous magnesium sulfate powder (aladdin@Shanghai, China), and sterilized by filtration with a 0.22 μm/28 mm filter (Beyotime, Shanghai, China).

2.2. Gene Silencing. Two genes (Magt1 and Creb1) were selected for silencing to verify their roles in the synergistic osteogenic effect of Mg\(^{2+}\) and the SMF. The same gene silencing method was used for both genes. BMSCs were cultured in 24-well plates for 36 h to reach 30–50% confluence. Transfection was performed with target gene-specific small interfering RNAs (siRNAs) using a siRNA transfection kit (RIBOBIO, Guangdong, China). The grouping included a nonspecific control (nc), siRNA (si), Mg\(^{2+}\)-SMF plus non-specific control (ms + nc), and Mg\(^{2+}\)-SMF plus siRNA (ms + si), as shown in Figure 1(b). After transfection, the cell plate was incubated at 37°C with 5% CO\(_2\) for 48 h. The efficiency of siRNA transfection was observed under a fluorescence microscope (Leica, Wetzlar, Germany); a 30–50% proportion of fluorescently labeled cells indicated good transfection and successful silencing of the target gene. The cells were then cultured in different intervention environments for different times, and the expression levels of the osteogenic genes were evaluated in each group.

2.3. SMF Exposure. An SMF exposure system was produced using a neodymium (Nd2Fe14B) disc magnet (2 mm thick, 35 mm in diameter; Xinhongchang Magnets, Guangdong, China) and a 6-well culture plate (Figure 2). Briefly, the magnetic disc was placed above a well to expose the culture to a north magnetic field, and its strength was altered by controlling the distance between the magnetic disc and the culture plate. To stimulate BMSC osteogenesis, we used the optimal magnetic field strength of 15 mT, as has been shown in a previous study [13]. A Gauss meter (TS200; Sanliang, Tokyo, Japan) was used to measure the SMF strength.

2.4. Cell Proliferation Assay. To measure cell proliferation, we used a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, BMSCs were seeded into 96-well plates
(2 × 10^3 cells/well) and cultured at 37°C with 5% CO₂ for 1, 3, 5, or 7 days, with or without SMF exposure, in a medium with or without 7.5 mM Mg²⁺. Cells that were cultured without SMF or Mg²⁺ exposure were used as a negative control. After incubation of plates with the CCK-8 reagents for 2 h, the absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.5. ALP Staining. BMSCs were seeded into a 6-well plate and cultured for 2 days to reach confluence. The cells were then cultured in an osteogenic medium containing DMEM supplemented with 10% FBS (Invitrogen, Waltham, MA, USA) and 50 μg/mL L-ascorbic acid (Sigma-Aldrich) at 37°C with 5% CO₂, for 7 days, with or without the SMF and/or Mg²⁺, and the osteogenic medium was changed every 2 days. ALP staining was performed using a 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium ALP color development kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions, and the cells were then observed under an optical microscope.

2.6. Alizarin Red Staining. Osteoblast differentiation was measured by quantifying the formation of mineralized bone nodules using an Alizarin Red staining assay. Briefly, BMSCs were seeded into a 6-well plate (4 × 10^4 cells/well) and cultured for 2 days to reach confluence. The cells were then cultured in the osteogenic medium at 37°C with 5% CO₂ for 30 days, with or without the SMF and/or Mg²⁺, then washed with phosphate-buffered saline, and stained with a 40 mM Alizarin Red solution for 10 min. After the cells were washed five times and decolorized with 10 mM sodium phosphate containing 10% cetylpyridinium chloride for 15 min at 26°C, Alizarin Red staining was quantified by Image J (National Institutes of Health, Bethesda, MD, USA).

2.7. Western Blot Analysis. ALP, RUNX2, OSX, and COL1 protein expression was measured using western blotting. Total protein was extracted from cells using radioimmuno-precipitation assay lysis buffer supplemented with 1% phenylmethanesulfonyl fluoride. Protein concentrations were measured using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were separated by sodium dodecyl
sulfate–polyacrylamide gel electrophoresis (10%) and transferred onto polyvinylidene fluoride membranes, which were then blocked with skim milk for 1 h and incubated with the following antibodies (Abcam, Shanghai, China) overnight at 4°C: anti-ALP (ab83259), anti-RUNX2 (ab23981), anti-Sp7/OSX (ab209484), and anti-collagen I (ab34710). The membranes were then washed three times with Tris-buffered saline containing 1‰ Tween 20 and incubated with IgG (heavy + light chains; ab205718) for 1 h. Protein bands were visualized using enhanced chemiluminescence detection (the protein bands were photographed after incubation with a chromogenic solution). Relative protein levels were determined by normalizing their expression to that of \( \beta \)-actin (ab119716).

2.8. Statistical Analysis. All experiments were conducted at least three times. Data were processed using the GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA) and expressed as the mean ± standard deviation. Differences between two groups were determined using the Student’s t-test, and one-way analysis of variance with Tukey’s multiple comparison test was used for multiple-group comparison. \( P \)-values of <0.05 were considered statistically significant.

3. Results

3.1. Effects of \( \text{Mg}^{2+} \) and the SMF on Differentiation and Proliferation of BMSCs. ALP staining showed (Figure 3(a)) that osteogenic differentiation was significantly enhanced in BMSCs treated with both \( \text{Mg}^{2+} \) and the SMF compared with that in BMSCs treated with either \( \text{Mg}^{2+} \) or the SMF alone and in the control group. Thus, the combination of \( \text{Mg}^{2+} \) and the SMF showed a synergistic effect and strongly promoted cell osteogenesis.

To determine the effect of the combination of \( \text{Mg}^{2+} \) with the SMF on ECM mineralization, BMSCs were stained with Alizarin Red after 28 days in culture. Cells treated with \( \text{Mg}^{2+} \) and the SMF in combination had the highest number of calcium nodules, while there was little difference in the number of nodules between the other groups.
of calcium nodules between BMSCs treated with Mg²⁺ or the SMF (Figure 3(b)). Thus, the combination of Mg²⁺ and the SMF showed the best performance in promoting ECM mineralization.

To confirm that Mg²⁺ and the SMF increased osteogenesis, protein expression of various osteogenic markers was measured. As shown in Figures 3(c) and 3(d), the combination of Mg²⁺ and the SMF increased the expression levels of the COL1, RUNX2, Sp7, and ALP proteins to a greater extent than either treatment alone. Thus, Mg²⁺ and the SMF synergistically increased the expression of representative osteogenic proteins in BMSCs.

To determine the effects of Mg²⁺ and the SMF on BMSC proliferation, cells were grown under different conditions, and their proliferation was measured on days 1, 3, 5, and 7. Cell proliferation did not significantly differ among the groups on day 1 (Figure 3(e)). However, the proliferation of BMSCs treated with Mg²⁺ or the SMF and, particularly, with both 7.5 mM Mg²⁺ and 15 mT SMF was higher than that in the control group on days 3 and 5. Moreover, on
day 7, the proliferation was higher for BMSCs treated with both Mg\(^{2+}\) and the SMF than for those treated with Mg\(^{2+}\) and the SMF separately, and cell proliferation was higher in all three treatment groups than in the control group. Thus, the combination of Mg\(^{2+}\) and the SMF had the strongest proliferative effect on BMSCs. Taken together, these results showed that Mg\(^{2+}\) and the SMF synergistically enhanced the proliferation ability of BMSCs and their differentiation into osteoblasts compared with those in the groups treated with Mg\(^{2+}\) or SMF alone.

3.2. Effects of Magt1 and Creb1 Silencing on Osteogenesis. To investigate the roles of the MAGT1 channel and CREB1 protein in the synergistic osteogenic effects of Mg\(^{2+}\) and the SMF, both genes were silenced by transfecting BMSCs with the corresponding siRNAs. For Magt1 silencing, the siRNA transfection efficiency was 42% (Figure 4(a)). ALP staining (Figure 4(b)), Alizarin Red staining (Figure 4(c)), and western blot analysis (Figures 4(d)–4(e)) revealed that the levels of osteogenic markers were lower in the siRNA group than in the nonspecific control siRNA group and in the Mg\(^{2+}\)-
SMF-treated siRNA group than in the Mg\(^{2+}\)-SMF-treated nonspecific control siRNA group. Taken together, these findings suggested that Mg\(^{2+}\) and the SMF induced osteogenesis in BMSCs via the MAGT1 channel. The siRNA transfection efficiency was 38% (Figure 5(a)) in Creb1 silencing. Similar findings were obtained in ALP staining (Figure 5(b)), Alizarin Red staining (Figure 5(c)), and western blot analysis (Figures 5(d)–5(e)) when Creb1 was silenced, indicating that the CREB1 protein also played a key role in the synergistic osteogenic mechanism of Mg\(^{2+}\)-SMF. These data indicated that the MAGT1 channel and CREB1 protein played important roles in the synergistic osteogenic effects of Mg\(^{2+}\) and the SMF.

4. Discussion

Mg\(^{2+}\)-based materials exert good osteogenic effects, and their mechanical properties are similar to those of the natural bone; however, these materials have not been applied clinically because of their high rate of degradation in vivo. Mg\(^{2+}\) can promote osteogenic differentiation by inducing CREB1 phosphorylation via the MAGT1 channel (Figure 6). According to this osteogenic property of Mg\(^{2+}\), we need to find a means to promote the influx to improve the utilization efficiency of Mg\(^{2+}\), so that only less Mg\(^{2+}\) is required to achieve a higher osteogenic effect. We found SMFs because it can change the opening frequency of Mg\(^{2+}\) channels to promote magnesium influx. Although SMFs can exert osteogenic effects via electrical and mechanical receptors on the cell membrane, it remains unclear whether the bioelectrical effects of a magnetic field could synergistically enhance osteogenic differentiation in combination with Mg\(^{2+}\). Herein, we combined Mg\(^{2+}\) and an SMF to explore whether they can synergistically enhance bone formation while eliminating the disadvantages of Mg\(^{2+}\)-based biomaterials. Notably, we found that proliferation and osteogenic differentiation were significantly higher in BMSCs cotreated with Mg\(^{2+}\) and the SMF than in those treated with either intervention alone. Further gene silencing experiments suggested that these effects might be related to Magt1 and Creb1. Collectively, our data demonstrated that Mg\(^{2+}\) and the SMF synergistically promoted the proliferation and osteogenic differentiation of BMSCs via the MAGT1 channel and CREB1 protein.

Several related studies have clearly demonstrated that certain Mg\(^{2+}\) concentrations and certain SMF intensities can promote BMSC proliferation and osteogenesis. Similarly, we observed that a synergistic intervention with Mg\(^{2+}\)-SMF significantly promoted the proliferation and induced osteogenic differentiation of BMSCs, which may be due to the effect of the SMF on MAGT1 channels on the cell membrane [38]. A previous study has shown that the opening frequency of ion channels on the cell membrane can change under the action of an SMF [39]. Therefore, SMFs can change the balance of the ion flow and membrane potential to promote bone formation and can also increase the opening frequency of MAGT1 channels to enhance the influx of Mg\(^{2+}\) to synergistically improve the osteogenic effect (Figure 7). Magnetic fields can modulate cellular functions, including cell morphology, cell cycle distribution, differentiation, proliferation, and gene expression [40]. This modulation may be due to electrodynamic interactions (Hall effect), magnetomechanical interactions, and radical pair effects [36]. Several studies have reported that different magnetic field environments have different effects on the cell differentiation ability. In particular, the effect of the magnetic field strength on cells has been a focus of research. As reported, S. Yamaguchi-Sekino, T. Kira, M. Sekino et al. found that the differentiation ability of cells was inhibited under the high-intensity magnetic field environment of 7 T [41]. They consider that SMFs may interfere with the opening of ion channels and hinder BMSC osteogenesis. However, we found that a medium-strength (15 mT) SMF promoted cell proliferation and osteogenesis. These differences are likely to be caused by two-way differences in the magnetic field strength in a cellular environment. At present, it is believed that the high-intensity magnetic field environment has an inhibitory effect on the differentiation ability of cells, whereas a medium-intensity magnetic field has a more
beneficial biological effect. Our results confirmed that the medium-strength magnetic field was consistent with other articles to promote the osteogenic differentiation of BMSCs. In our experiments, compared with the control group, the SMF group showed stronger osteogenic properties in the results of ALP staining, Alizarin Red Staining and Western Blot.

Like the strength of the magnetic field, different types of magnetic fields have different effects on cell differentiation. A constant SMF with moderate intensity can induce the differentiation of BMSCs into osteoblasts by promoting the expression of related proteins. The sinusoidal electromagnetic field has a certain induction effect on the osteogenic differentiation of BMSCs, and the sinusoidal electromagnetic field of 1.0-2.0 mT, 10-50 Hz has the greatest effect on the differentiation of bone marrow mesenchymal stem cells [42]. So we fixed the most suitable magnetic field strength environment for experiments.

Magnetic field position and orientation also have different effects on cell differentiation. As reported by Lin, S. Y, Li, J et al., upon intervention of BMSCs in parallel and perpendicular magnetic field directions, the cells produced different differentiation states [43]. This may be due to the different angles of the magnetic poles and magnetic field lines to the cell. So we fixed the magnetic poles (using north magnetic poles for all interventions) to eliminate experimental errors.

To the best of our knowledge, this study is the first to demonstrate a synergistic effect between Mg2+ and the SMF in promoting BMSC proliferation and osteogenesis; however, the study has a few limitations. First, all experiments were performed using mouse BMSCs; therefore, further studies should examine the effects of Mg2+ and the SMF on BMSCs from other species, such as rabbits and humans. Similarly, since we only studied BMSCs cultured for 7 days, future experiments should extend the culture period and observe changes in osteogenic markers at different times. Finally, our research was based on the observations at the cellular level and thus may not reflect the process of bone formation in an organism. Subsequent studies are required to address these issues.

5. Conclusions

This study demonstrated that a medium-strength SMF intervention, combined with an appropriate Mg2+ concentration, could increase the expression of the COL1, Sp7, RUNX2, and ALP proteins in BMSCs via MAGT1 and CREB1 and significantly enhance the proliferation and osteoblast differentiation of BMSCs. Thus, Mg2+ and the SMF could be combined to develop new artificial bone materials with improved osteogenic properties to enhance bone cell proliferation and differentiation and promote the healing of bone defects. Future studies should identify the specific pathways through which Mg2+ and the SMF affect bone formation.

Abbreviations

MAGT1: Magnesium transporter 1
SMF: Static magnetic field
BMSCs: Bone mesenchymal stem cells
CCK-8 kit: Cell counting Kit-8
ALP: Alkaline phosphatase
CREB1: cAMP responsive element binding protein 1
PEMF: Pulsed electromagnetic fields
TREK1: TWIK-related K+ channel 1
ORAI1/2: ORAI calcium release-activated calcium Modulator1/2.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.
Authors’ Contributions

Youwen Deng and Cijun Shuai designed this work. Yifan Wang and Xin Wu integrated and analyzed the data. Yifan Wang, Xin Wu and Pei Feng wrote this manuscript. Pei Feng and Wei Tan edited and revised the manuscript. All authors approved this manuscript.

Funding

This work was supported by The Natural Science Foundation of China (81472058, 52105352); Hunan Science and Technology Innovation Program (2018SK2105, 422000008); Science and Technology Program of Huizhou (No.2020Y253); Medical Science and Technology Research Fund Project of Guangdong Province (B2021166); Postgraduate Research and Innovation Project of Central South University (1053320210754).

Acknowledgments

The authors thank the members in Hunan Engineering Laboratory for Orthopedic Biomaterials and the department of spine surgery of the Third Xiangya Hospital of the Central South University.

Supplementary Materials

Supplementary 1. rawdata-ALP: Alkaline phosphatase (ALP) staining by 5-bromo-4-chloro-3-indolyl-phosphate/Nitro-Blue-Tetrazolium ALP color development kit.

Supplementary 2. rawdata-ck8: Cell proliferation assay by cell counting kit (cck8).

Supplementary 3. rawdata-red: Alizarin red staining, osteoblast differentiation was measured by quantifying the formation of mineralized bone nodules using an alizarin red staining assay.

Supplementary 4. rawdata-siRNA: Silence genes in BMSCs by transfecting the cells with corresponding siRNAs.

Supplementary 5. rawdata-wb: Western blot analysis. ALP, Runx2, Osx, and Col-I protein expression were measured using western blotting.

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