



Magnesium ion stimulation of bone marrow stromal cells enhances osteogenic activity, simulating the effect of magnesium alloy degradation



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ABSTRACT

Magnesium alloys are being investigated for load-bearing bone fixation devices due to their initial mechanical strength, modulus similar to native bone, biocompatibility and ability to degrade in vivo. Previous studies have found Mg alloys to support bone regeneration in vivo, but the mechanisms have not been investigated in detail. In this study, we analyzed the effects of Mg^{2+} stimulation on intracellular signaling mechanisms of human bone marrow stromal cells (hBMSCs). hBMSCs were cultured in medium containing 0.8, 5, 10, 20 and 100 mM $MgSO_4$, either with or without osteogenic induction factors. After 3 weeks, mineralization of extracellular matrix (ECM) was analyzed by Alizarin red staining, and gene expression was analyzed by quantitative polymerase chain reaction array. Mineralization of ECM was enhanced at 5 and 10 mM $MgSO_4$, and collagen type X mRNA (*COL10A1*, an ECM protein deposited during bone healing) expression was increased at 10 mM $MgSO_4$ both with and without osteogenic factors. We also confirmed the increased production of collagen type X protein by Western blotting. Next, we investigated the mechanisms of intracellular signaling by analyzing the protein production of hypoxia-inducible factor (HIF)-1 α and 2 α (transcription factors of *COL10A1*), vascular endothelial growth factor (VEGF) (activated by HIF-2 α) and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 α (transcription coactivator of VEGF). We observed that 10 mM $MgSO_4$ stimulation enhanced *COL10A1* and VEGF expression, possibly via HIF-2 α in undifferentiated hBMSCs and via PGC-1 α in osteogenic cells. These data suggest possible ECM proteins and transcription factors affected by Mg^{2+} that are responsible for the enhanced bone regeneration observed around degradable Mg orthopedic/craniofacial devices.

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1. Introduction

Every year, more than 6.2 million cases of bone fracture are reported, with 56% of fractures in adults requiring internal fixation with biomedical devices such as plates and screws [1,2]. Bone fixation devices are most commonly made of non-degradable metallic alloys, such as titanium and stainless steel. Drawbacks to these traditional orthopedic alloys include stress shielding due to the mismatch in mechanical properties between the metal and the bone [3], and the need for secondary surgery to remove the fixation devices in some cases. Degradable polymers (e.g. poly

(lactic-co-glycolic acid) (PLGA), poly(L-lactic acid) and poly(ethylene glycol)) have been employed in order to avoid the secondary removal surgery; however, their compressive strengths are not ideal for load-bearing fracture repair cases [4], and foreign body reactions to the polymers have been reported [5–7]. In order to address these issues, magnesium alloys have been studied as a candidate material for bone fixation devices due to their bone-like mechanical properties, enhanced osteoconductivity compared to polymers and ability to safely degrade in vivo [3].

Mg alloys were first used for biomedical applications over 200 years ago; however, their development has accelerated in the last 10 years due to advances in alloy manufacturing and processing methods [8]. Numerous research groups have synthesized a wide range of magnesium alloys and characterized their microstructure, corrosion properties, mechanical properties, in vitro cytotoxicity

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and in vivo biocompatibility. In vivo Mg alloy studies have involved implantation of rods into rabbit tibiae [9], ulnae [10] and femora [11,12], rat femora [13] and guinea pig femora [14]. These in vivo studies found through microcomputed tomography, mechanical testing and histology analysis that the magnesium alloys safely degrade and allow osseointegration at the site of implantation. Additionally, comparisons of Mg alloy rods to polymer rods found that mineralization was increased surrounding the Mg samples [14]. Mg^{2+} concentrations were found to be increased in bone tissue immediately surrounding degrading Mg alloys in vivo [15]. This finding suggests that the mechanisms underlying enhanced bone regeneration observed in vivo can be recapitulated using Mg^{2+} salts in vitro.

Most in vitro studies of Mg alloys have focused on cell viability and proliferation to assess cytocompatibility. Previous studies used MTT and WST-1 assays to show that Mg alloys are cytocompatible with primary human mesenchymal stem cells [10], bone-derived cells [16], mouse fibroblasts [11,17], MG-63 human osteosarcoma cells [16], RAW264.7 macrophages [16] and MC3T3-E1 osteoblasts [17,18]. In addition, von Kossa and alkaline phosphatase stains were utilized to examine the effect of magnesium alloys on U2OS human osteosarcoma cell mineralization and osteogenic differentiation [19]. Furthermore, immunohistochemistry and flow cytometry were employed to study the mechanisms of cell adhesion on biomaterials when stimulated by Mg [20]. Overall, these in vivo studies have shown Mg-based devices to be promising for bone fracture fixation, and in vitro studies have shown enhancement of standard osteogenic markers in bone cells. However, to the best of our knowledge, this report is the first identification of specific intracellular signaling pathways through which Mg enhances bone regeneration.

We hypothesized that treating human bone marrow stromal cells (hBMSCs) with MgSO_4 , resulting in increased exposure of the cells to Mg^{2+} , would enhance osteogenic gene expression, matrix production and mineral deposition. We cultured hBMSCs with various concentrations of MgSO_4 , either with or without osteogenic factors. These treated cells were then analyzed for their matrix mineralization, gene expression and protein production in order to elucidate the intracellular signaling pathways involved in bone growth around Mg alloys. In this study, we found that increased MgSO_4 enhanced protein expression of collagen type X (COL10A1), vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1 α , HIF-2 α and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 α in hBMSCs. COL10A1 is abundant in fractured bone at early stages of healing and VEGF is a major angiogenic signaling protein. This work identified specific osteogenic pathways that are affected by Mg. The identification of these pathways and the optimal Mg concentrations to enhance their activity will lead to improved Mg bone fixation device design and other possible therapeutic uses for Mg.

2. Materials and methods

2.1. Harvest, expansion and experimental culture of hBMSCs

hBMSCs were harvested from surgical waste in accordance with the US NIH regulations governing the use of human subjects under protocol 94-D-0188 or OHRS Assurance No. 4165 and established from colony-forming units as previously reported [21]. The osteogenic differentiation capabilities of these cells were confirmed by bone tissue formation following in vivo transplantation into immunocompromised mice (courtesy of Dr. Pamela Robey at National Institutes of Health). The cells were plated at 40,000 per cm^2 in Minimum Essential Medium Eagle Alpha Modifications (α -MEM; Life Technologies, Grand Island, NY) containing 20% fetal bovine

serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 1% penicillin and streptomycin (Life Technologies) and 1% L-glutamine (Life Technologies). We used this medium formulation as the “expansion medium”. Cells were cultured at 37 °C in an atmosphere of 5% CO_2 . Non-adherent cells were washed away 24 h later. For subculture, hBMSCs were detached with 0.05% trypsin–EDTA (Life Technologies) and expanded at a 1:3 ratio. Cells were passaged three times, harvested and then plated for experiments.

hBMSCs were cultured in either maintenance or osteogenic medium throughout the experiments. The “maintenance medium” consisted of α -MEM, 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine and a variable amount of MgSO_4 (5, 10 and 20 mM for Alizarin red staining assay, 10 and 100 mM for proliferation assay, and 10 mM for gene and protein expression analysis; Sigma Aldrich, St. Louis, MO). α -MEM, as purchased, contains 0.8 mM MgSO_4 (this concentration of MgSO_4 was considered the control group). Osteogenic differentiation of hBMSCs was induced by culturing in “osteogenic medium”, which contained α -MEM, 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 μM ascorbic acid, 100 nM dexamethasone and 10 mM β -glycerol phosphate (Sigma–Aldrich, St. Louis, MO). Finally, a “ SO_4^{2-} control medium” was formulated in the same manner as the maintenance medium, but with the MgSO_4 substituted by Na_2SO_4 (Fisher Scientific, Pittsburgh, PA). The contents of all medium used for cell culture are summarized in Table 1.

2.2. Cell proliferation assay

hBMSCs were plated at 1×10^5 per well in six-well plates in expansion medium. After 24 h, the medium was switched to maintenance or osteogenic medium containing 0.8, 10 or 100 mM MgSO_4 , with three biological replicates per group. Cells were detached with 0.05% trypsin–EDTA at 1, 3, 5 and 7 days, and the number of live cells was counted using a hemocytometer. The dead cells were excluded using the Trypan blue stain.

2.3. Alizarin red staining

hBMSCs were plated in six-well plates at a density of 1×10^5 cells per well in expansion medium. Twenty-four hours after plating, the medium was switched to 0.8, 5, 10 or 20 mM MgSO_4 osteogenic medium or Na_2SO_4 (SO_4^{2-} control medium), with three biological replicates per group, and cultured for 3 weeks. The cells were then fixed in 10% formalin for 1 h and washed with phosphate-buffered saline. The calcium nodules in the ECM were stained with a solution of 1% Alizarin red (Sigma Aldrich) in 2% ethanol for 5 min. Following incubation, the stain was removed and washed repeatedly with ddH_2O . Finally, the amount of Alizarin red bound to the calcium nodules was quantified by dissolving the stained ECM into 1% cetylpyridinium chloride (CPC) solution and reading the optical density at 540 nm using a plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA).

2.4. Assessment of gene expression

2.4.1. RNA extraction and purification

hBMSCs were plated in six-well plates at a density of 1×10^5 cells per well in maintenance or osteogenic medium (0.8 and 10 mM MgSO_4), with three biological replicates per group, and cultured for 3 weeks. Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Qiagen) to eliminate genomic DNA according to manufacturer's instructions. The quantity and quality of RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA samples were cleaned using RNA Clean & Concentrator™-5 (Zymo Research Corporation, Irvine, CA) until the ratio of absorbance readings at 230–260 nm was greater than

Table 1

Composition of the culture medium used in this study.

Medium	FBS (%)	Osteogenic factor	MgSO ₄ or Na ₂ SO ₄
Expansion	20	–	–
Maintenance	5	–	0.8, 5, 10, and 20 mM of MgSO ₄
Osteogenic	5	50 μ M ascorbic acid 100nM dexamethasone 10 mM β -glycerol phosphate	0.8, 5, 10, and 20 mM of MgSO ₄
SO ₄ ²⁻ control	5	–	0.8, 5, 10, and 20 mM of Na ₂ SO ₄

1.7 and at 260–280 nm was between 1.8 and 2.0, according to manufacturer's instructions for quantitative polymerase chain reaction (qPCR) arrays (SABiosciences, Frederick, MD).

2.4.2. Quantitative PCR array

From each sample, 500 ng of purified RNA was reverse transcribed to cDNA using an RT² First Strand Kit (SABiosciences) according to the manufacturer's instructions. qPCR array assays were performed using an RT² Profiler™ PCR Array: Osteogenesis (SABiosciences) and a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) according to manufacturers' instructions. Briefly, each experimental cDNA sample was mixed with RT² SYBR Green Master Mix and RNase-free water then plated into 96 wells of the 384-well qPCR array. The qPCR array is prefilled with four replicates of 84 different primer/probe sets of osteogenesis related genes, four different primer/probe sets of housekeeping genes, one genomic DNA control, three reverse transcription controls and three positive PCR controls. The thermal cycler was set to incubate once at 95 °C for 10 min to activation the HotStart DNA Taq polymerase. Amplification of DNA was performed for 40 cycles, consisting of 95 °C denaturing for 15 s and 60 °C annealing for 1 min. The fluorescence intensity for all wells was collected at the end of each cycle. The C_t values were calculated from the first cycle in which the fluorescence data for each well was greater than the fixed threshold. The C_t values were analyzed by the $\Delta\Delta C_t$ method, as previously described [22]. All C_t values greater than 35 were considered negative calls. The C_t values of each sample were normalized with the average C_t values of housekeeping genes (ΔC_t value). The difference in the ΔC_t value between the experimental and control wells was used as the $\Delta\Delta C_t$ value. The fold change between these two wells was calculated as $2^{(-\Delta\Delta C_t)}$.

2.4.3. Quantitative PCR

mRNA expression of *HIF1A*, *HIF2A*, *COL10A1* and 18S ribosomal RNA was analyzed by TaqMan ABI inventoried gene assays (Applied Biosystems) to confirm the mRNA expression data from qPCR arrays. *VEGFA* (NM_003376) was designed using Prime Express Software from ABI, Version 2.0 (forward 5'-CATGCAGATTATG CGGATCAA-3', reverse 5'-TTTGTGTGCTGTAGGAAGCTCAT-3', Taqman probe 5'-CCTCACAAGGCCAGCACATAGGAGA-3'). Real-time PCR reactions were conducted in a 7900HT Fast Real-Time PCR system (Applied Biosystems).

2.5. Western blotting

hBMSCs were plated into six-well plates at a density of 1×10^5 cells per well and cultured for 3 weeks in maintenance or osteogenic medium (0.8 and 10 mM MgSO₄), with three biological replicates per group. Proteins from cultured cells and ECM were extracted using M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The amount of proteins was quantified by colorimetric protein assay using Pierce® 660 nm Protein Assay Reagent (Thermo Fisher Scientific). The protein samples were reduced with sample buffer containing β -mercaptoethanol at 60 °C for 10 min, and sodium dodecyl sulfate–polyacrylamide gel

electrophoresis was performed with a 10% acrylamide gel. The proteins were transferred to a polyvinyl difluoride membrane (Bio-Rad, Hercules, CA) and Western blotting was performed using primary antibodies against COL10A1 (C7974), β -actin (A5441) (Sigma–Aldrich), VEGF (NB100-648), HIF-2 α (NB100-122), PGC-1 α (NB11-04676) (Novus Biologicals, Littleton, CO) and HIF-1 α (BD Transduction Laboratories, Franklin Lakes, NJ). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (R&D Systems, Minneapolis, MN) or anti-mouse IgM (Santa Cruz Biotechnology). The blots were developed with the Western Lightning® Plus-ECL (PerkinElmer, Inc., Waltham, MA). The intensity of the bands were measured by ImageJ (<http://imagej.nih.gov/ij/>), and normalized by β -actin.

2.6. Statistical analysis

The graphical presentations of data show the means \pm standard deviations. The proliferation assay and Alizarin red staining data were analyzed using a one-way analysis of variance (ANOVA) followed by post hoc *t*-tests. The *p*-values from the PCR array were calculated based on Student's *t*-tests of the replicate $2^{-\Delta C_t}$ values for each gene in the control group and treatment groups. Student's *t*-tests were also performed to calculate the differences in the optical intensities of specific Western blot bands obtained from cells treated with either 0.8 or 10 mM MgSO₄.

3. Results

3.1. Higher cell proliferation rate and extracellular mineralization induced by 5–10 mM MgSO₄

Cell proliferation rates of hBMSCs were significantly enhanced when grown in medium containing 10 mM MgSO₄ in both maintenance and osteogenic medium (Fig. 1), but were inhibited at 100 mM MgSO₄. Stronger Alizarin red staining was observed in the wells treated with 5 or 10 mM MgSO₄ (Fig. 2A and B). In comparison, Na₂SO₄ groups had fewer numbers of nodules, and lighter Alizarin red staining of the ECM. The quantified optical density of Alizarin red dissolved in CPC solution was significantly higher in the 5 and 10 mM MgSO₄ groups compared to the Na₂SO₄ control (Fig. 2C).

3.2. COL10A1 and IGF2 expression enhanced and ITGA3 expression decreased by 10 mM MgSO₄

The qPCR array assays yielded 14 out of 81 genes up- or down-regulated by greater than 2.0-fold when cultured in 10 mM MgSO₄ compared to 0.8 mM MgSO₄. The expression levels of all of the genes are listed in Supplemental Table 1. Among them, collagen type X (*COL10A1*), insulin-like growth factor 2 (*IGF2*) and integrin $\alpha 3$ (*ITGA3*) showed statistically significant difference of up-/down-regulation. hBMSCs treated with 10 mM MgSO₄ expressed significantly higher amounts of *COL10A1* compared to 0.8 mM MgSO₄ when cultured in both maintenance and osteogenic medium. The expression of *COL10A1* was increased by 2.5- and 4.0-fold,

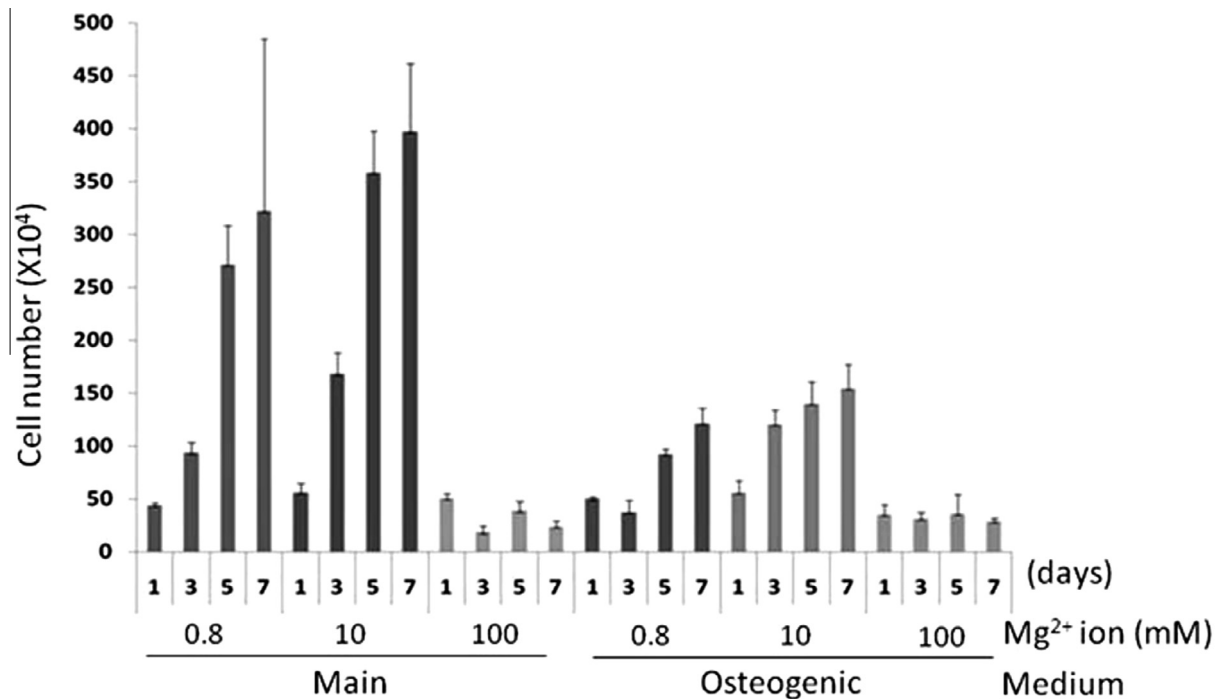


Fig. 1. 10 mM MgSO₄ increased proliferation rate of hBMSCs. The result of the cell count by the Trypan blue assay shows the proliferation rate of hBMSCs after stimulation with medium containing 0.8 mM (the original concentration in the culture medium), 10 mM and 100 mM MgSO₄. Statistical significance was observed between all three different concentrations of MgSO₄ medium at days 3, 5 and 7 (3-way ANOVA; medium type, $p < 0.001$; MgSO₄ concentration, $p < 0.001$; time point, $p < 0.001$).

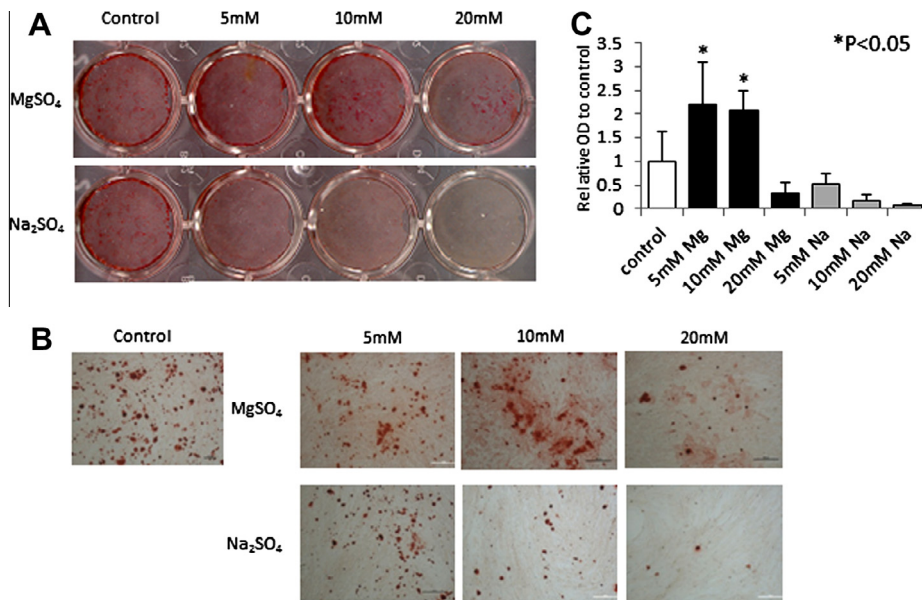


Fig. 2. 5 and 10 mM MgSO₄ induced higher deposition of mineral into ECM. (A) Alizarin red staining of ECM of hBMSCs after 3 weeks of culture in osteogenic medium containing 0.8, 5, 10 and 20 mM MgSO₄ or Na₂SO₄. (B) 10× images of ECM stained with Alizarin red. The scale bar is 200 μm. (C) The amount of Alizarin red was quantified by dissolving in 10% CPC solution. The relative OD at 562 nm to control is shown.

respectively, in maintenance and osteogenic medium (Fig. 3A and B). In addition, the expression of *IGF2* was increased by 2.4-fold when cultured in 10 mM MgSO₄ maintenance medium compared to 0.8 mM MgSO₄ maintenance medium. Furthermore, the expression of *ITGA3* in the osteogenic medium with 10 mM MgSO₄ was decreased by 0.42-fold compared to cells cultured in 0.8 mM MgSO₄ (Fig. 3A and B). The other genes up-regulated in 10 mM MgSO₄ were collagen type III (*COL3A1*), matrix metalloproteinase (MMP)-8, growth differentiation factor-1 (*GDF1*), cartilage oligomeric matrix protein (*COMP*), collagen type XI (*COL11A1*), MMP2,

cathepsin K (*CTSK*) and Twist-related protein 1 (*Twist1*) (>2.0-fold). The genes down-regulated were alkaline phosphatase (*ALPL*), vascular cell-adhesion molecule-1 (*VCAM1*) and collagen type XII (*COL12A1*) (<0.5-fold) (Fig. 3A and B).

3.3. qPCR validation of *COL10A1* and *VEGF* and expression levels of *HIF2A*

The mRNA expression of *COL10A1* and *VEGF* was confirmed by qPCR showing significantly increased mRNA expression levels in

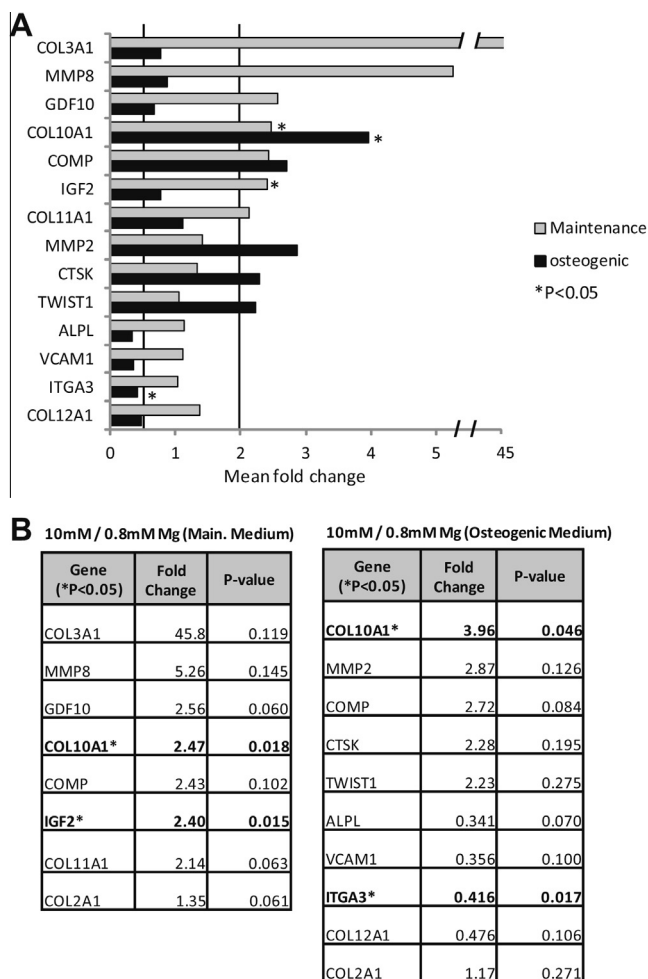


Fig. 3. 10 mM Mg ion enhanced COL10A1 and IGF2 expression and decreased ITGA3 expression. hBMSCs were cultured in maintenance or osteogenic medium with 0.8 mM (control) or 10 mM MgSO₄ for 3 weeks. The osteogenic mRNA expression was analyzed by quantitative PCR arrays. The genes listed changed by >2-fold or <0.5-fold, and the *C_t* value was <30. COL2A1 data is shown to prove that hBMSCs did not differentiate into chondrogenic cells, *n* = 3. (A) Mean fold change of 10 mM Mg ion groups compared to 0.8 mM Mg ion (control) groups; (B) mean fold change and *p*-value.

the 10 mM MgSO₄ groups in both maintenance (1.3-fold) and osteogenic (2.6-fold) medium compared to the 0.8 mM MgSO₄ group (Fig. 4). However, there was no significant change in VEGF expression with 10 mM MgSO₄ (Fig. 4). In addition, we examined the *HIF1A* and *HIF2A* mRNA expression, since they have been reported to be the most potent transcriptional activators of *COL10A1* expression [23]. However, we did not observe any change in mRNA expression of *HIF1A* and *HIF2A* in 10 mM MgSO₄. As discussed below, examination of the literature [24] showed that *HIF1A* and *HIF2A* are modulated at the protein level. Thus, we performed Western blot analysis to determine protein expression levels following the treatment with MgSO₄.

3.4. COL10A1, VEGF, HIF-1 α , HIF-2 α and PGC-1 α protein expression enhanced by 10 mM MgSO₄

The protein expression of hBMSCs cultured in MgSO₄ for 3 weeks was analyzed by Western blotting. Although a statistically significant change was observed only in maintenance medium group, expression of COL10A1 was enhanced 1.5- to 1.9-fold

relative to control in cells cultured with 10 mM MgSO₄ in maintenance medium (Fig. 5A and B), confirming that Mg enhances osteogenic differentiation. VEGF (an important osteogenic factor) expression was also increased by 10 mM MgSO₄ under both culture conditions. Since both COL10A1 and VEGF are transcriptionally activated by HIF-1 α and HIF-2 α , we examined their protein expression and found that only HIF-2 α levels were increased by 10 mM MgSO₄ in maintenance medium; however, the increase was not statistically significant (Fig. 5A and B). In contrast, protein levels of PGC-1 α , another important transcriptional activator of VEGF, increase in osteogenic medium with 10 mM MgSO₄ (Fig. 5A and B).

4. Discussion

The data show that hBMSCs proliferate faster, and their ECM mineralizes more in vitro, when 10 mM MgSO₄ is added. Using the time points and Mg concentrations of 10 mM, which showed the highest Alizarin red staining, we performed quantitative PCR arrays to analyze osteogenic gene expression and determined that *COL10A1* (an ECM component of healing bone) gene expression was increased in both undifferentiated (2.5-fold) and osteogenic-differentiated (4.0-fold) hBMSCs. The data obtained in these studies support the hypothesis that increasing Mg²⁺ enhances hBMSCs, osteogenic gene expression, matrix production and mineral deposition.

In this study, hBMSCs proliferated 1.2 times more in 10 mM MgSO₄ medium relative to medium with 0.8 mM MgSO₄. This finding is consistent with a previous report that the proliferation rates of human articular chondrocytes [25] and microvascular endothelial cells [26] were enhanced with 5–10 mM MgSO₄. Our data shows that cell proliferation was inhibited at higher concentration (>20 mM) of MgSO₄. We speculate that this is due to the cytotoxicity of Mg ion at higher concentration, this cytotoxicity also being reported for other metal ions (Na, Cr, Mo, Al, Ta, Co, Ni, Fe, Cu, Mn and V) in osteoblasts [27]. As shown in the Alizarin red staining data, 10 mM MgSO₄ stimulation resulted in the largest increase in ECM mineralization compared to control medium. Previous reports have shown the addition of 5–10 mM MgSO₄ to tissue culture medium to enhance glycosaminoglycan production and redifferentiation (up-regulation of collagen type I and melanoma inhibitory activity) of human articular chondrocytes [25]. These findings are consistent with our data showing that 5 and 10 mM MgSO₄ are the most effective concentrations for stimulating ECM mineralization. In addition, Lu et al. [28] showed increased alkaline phosphatase activity in MG63 when Mg-doped hydroxyapatite cement was added. Furthermore, an Mg²⁺-containing fluoridated hydroxyapatite coating also enhanced osteocalcin expression in MG63 cells [29]. These results support our findings of osteogenic marker enhancement by MgSO₄ stimulation.

Summarizing our qPCR array data, chondrogenesis of hBMSCs by MgSO₄ stimulation was suggested because the up-regulated genes included chondrocyte markers, such as *COL3A1*, *COL10A1*, *COL11A1* [30] and *COMP* [31]. However, since the mRNA expression of collagen type II (*COL2A1*) was not significantly increased (data not shown) and mineral nodule formation was observed (Fig. 2), we speculate that hBMSCs might not have differentiated to chondrocyte at this time point. Moreover, it is reported that the *COL2A1* expression is up-regulated in human mesenchymal stem cells 10–21 days after being cultured in chondrogenic conditions [32], which indicates that hBMSCs in MgSO₄ containing medium did not differentiate into chondrocytes due to lack of *COL2A1* up-regulation. Moreover, up-regulation of *MMP2* suggests the enhancement of cell migration through the ECM [33]. Other genes up-regulated by MgSO₄ stimulation are *IGF2* (insulin-like growth

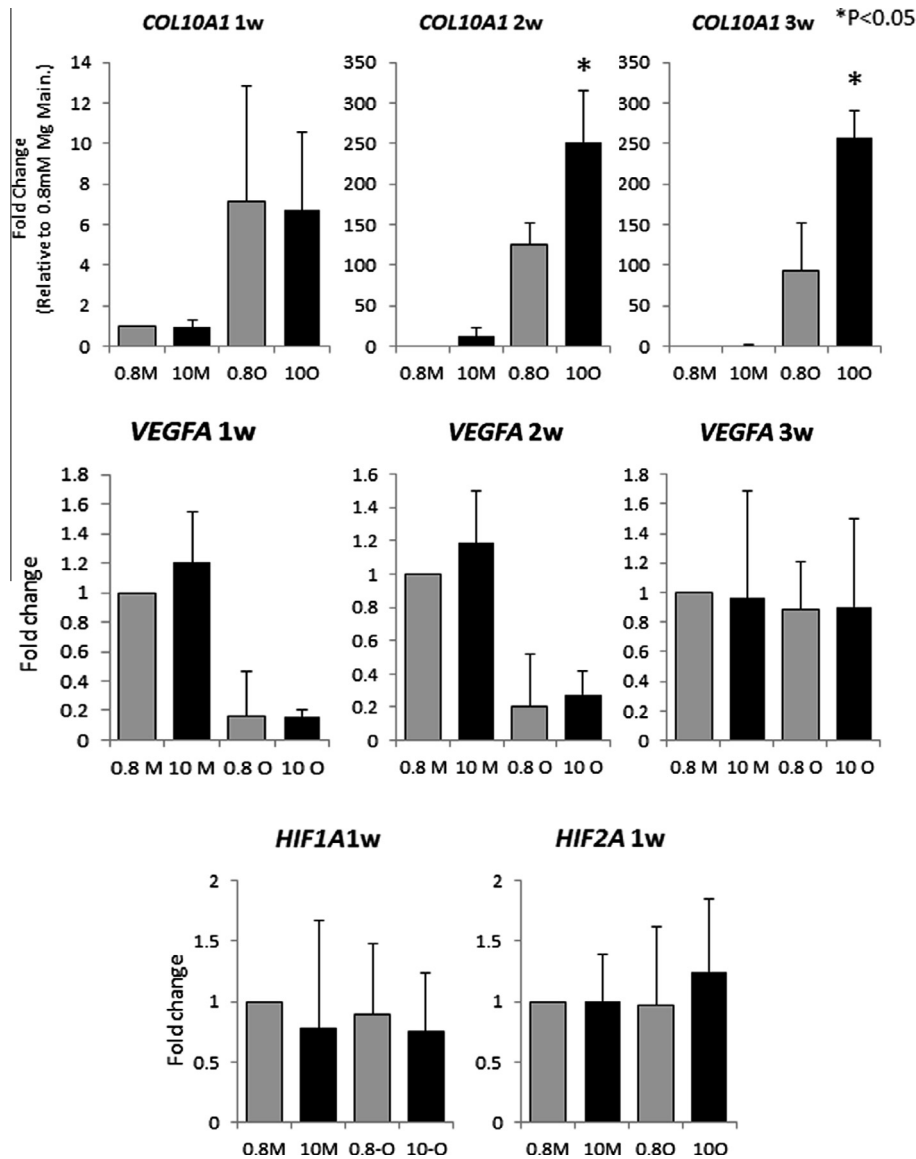


Fig. 4. 10 mM MgSO₄ changed *COL10A1* but not *VEGFA*, *HIF1A* and *HIF2A* mRNA expression. hBMSCs were cultured in maintenance or osteogenic medium with 0.8 mM (control) or 10 mM Mg ion for 1, 2 and 3 weeks. The mRNA expression of *COL10A1*, *VEGFA*, *HIF1A* and *HIF2A* was analyzed by the quantitative PCR. 0.8 M: maintenance medium containing 0.8 mM MgSO₄; 10 M: maintenance medium containing 10 mM MgSO₄; 0.8 O: osteogenic medium containing 0.8 mM MgSO₄; 10 O: osteogenic medium containing 10 mM MgSO₄, *n* = 3. **p* < 0.05 with Student's *t*-test between two samples indicated with a bar.

factor 2), *GDF10* (growth differentiation factor 10), *CTSK* and *Twist1*. *IGF2* plays an important role in long bone growth [34], and its up-regulation by Mg ion is indicative of the effect of Mg on bone growth. *GDF10* (also known as BMP-3b) is reported to be associated with the osteogenic differentiation of primary osteogenic cells [35] and is also suggested to increase the osteogenic inducing activity of BMP-2 [36]. Cathepsin K is known as an osteoclast enzyme and is reported to accelerate trabecular bone turnover [37]. *Twist1* is essential for osteoblast differentiation, but overexpression may inhibit osteogenesis [38]. These changes may explain the enhanced osteogenesis of hBMSCs when stimulated with MgSO₄. *VEGFA* expression did not change significantly in qPCR arrays (Fig. 3) or confirmatory qPCR, including at earlier time points (Fig. 4).

On the other hand, some genes were down-regulated (0.34- to 0.48-fold) when assessed by qPCR. Integrin alpha 3 (*ITGA3*) expression in hBMSCs was significantly decreased when 10 mM Mg in osteogenic medium was used. *ITGA3* has been reported to be an

important receptor for osteoblast to bind to the protein kinase C-binding protein NELL1 (osteoinductive protein) in ECM [39]. Decreased expression of *ITGA3* may indicate the enhanced migration of cells rather than adhesion, which could explain the enhanced bone regeneration in the tissue surrounding Mg alloys in a previous report [14]. *VCAM1*, related to osteoclast activity, is up-regulated by NFκB [40], but its expression was suppressed in the present study. This may indicate the deactivation of osteoclast activity.

Since *COL10A1* gene expression was significantly increased in both maintenance and osteogenic medium, the intracellular signaling pathway related to *COL10A1* up-regulation was further investigated in this study. The Western blotting results (Fig. 3) indicate the different pathways of *COL10A1* and *VEGF* expression under MgSO₄ stimulation depending on the differentiation status of hBMSCs. In the maintenance medium, 10 mM MgSO may have increased *COL10A1* and *VEGF* levels by increasing the stability of HIF-2α protein. On the other hand, the production of HIF-2α was

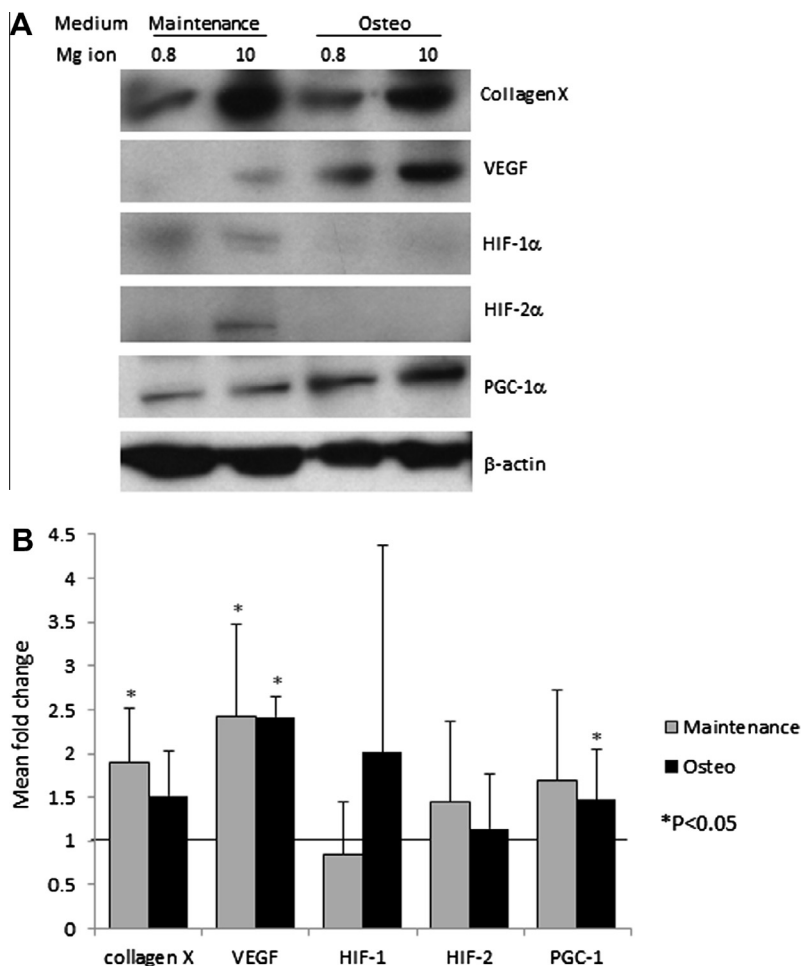


Fig. 5. 10 mM Mg ion enhanced collagen type X, VEGF, PGC-1 α , HIF-1 α and HIF-2 α protein expression. hBMSCs were cultured in maintenance or osteogenic medium with 0.8 mM (control) or 10 mM MgSO₄ for 3 weeks. The protein expression level was analyzed by Western blotting (A) and quantified by ImageJ. (B) Bar graph representing the ratio of expression level of 10 mM MgSO₄ samples normalized to 0.8 mM MgSO₄ samples in either maintenance or osteogenic medium, $n = 3$. The horizontal line represents the expression level without the addition of MgSO₄. * $p < 0.05$ with Student's t -test compared to 0.8 mM MgSO₄ samples in the same kind of medium (maintenance or osteogenic).

very low in the osteogenic medium; thus, the increase in PGC-1 α expression in osteogenic medium may have contributed to the increased transcription of *COL10A1* and *VEGF*.

Our data showing the mechanisms involved in Mg²⁺ signaling are schematically represented in Fig. 6. HIF-1 α and HIF-2 α are transcription factors that are known to be stabilized in the cytosol under hypoxia [41] and, in response to various metals [42,43], activate a number of genes, including VEGF and glycolytic genes. They are important transcription factors for bone development and regeneration [44,45], as well as for inducing tissue remodeling (VEGF) and metabolic genes (e.g. glycolytic). Potier et al. [46] reported that hypoxia induced osteogenic differentiation and angiogenic factor expression in human mesenchymal stem cells, and Grayson et al. [47] reported that hypoxia induced proliferation and ECM production in mesenchymal stem cells. Nickel and cobalt are known to enhance HIF expression [42]; however, to the best of our knowledge, this is the first report of up-regulation of HIFs by Mg²⁺. Since Mg deficiency causes loss of response to hypoxia in paraganglion cells [48], it may be related to the regulation of reactive oxygen species response by Mg²⁺ intake via Ca²⁺ channels. PGC-1 α is a HIF-independent transcriptional coactivator of VEGF [49] and is also known to regulate chondrogenesis in human mesenchymal stem cells [50]. However, the activation of PGC-1 α by Mg²⁺ has not been reported. PGC-1 α expression is increased

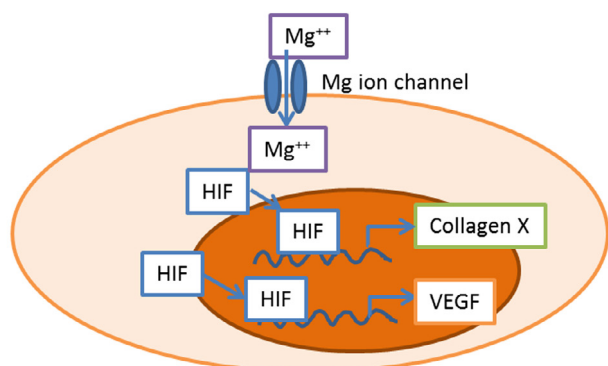
in response to Ca²⁺ activation of a calcineurin/calmodulin signaling complex [51]. Mg²⁺ also binds to calcineurin [52], and thus may act through a similar activation cascade to induce PGC-1 α in hBMSC in osteogenic medium via activation of specific transcription factor (Fig. 6).

To the best of our knowledge, this is the first report to show a possible Mg²⁺-stimulated intracellular signaling pathway in hBMSCs that may lead to the enhanced ECM mineralization observed in vitro and the enhanced bone regeneration observed in vivo [14]. Our findings, supported by those in the literature, suggest that an adequate concentration of Mg²⁺ should be maintained in healing bone tissue by adjusting the corrosion rate of Mg-based bone fixation devices. Also, the excessive deposition of COL10A1 by Mg²⁺ could be applied to treating defective bone diseases such as Schmid-type metaphyseal chondrodysplasia, osteogenesis imperfecta and osteoarthritis.

5. Conclusions

We have shown that concentrations of 10 mM Mg²⁺ in tissue culture medium resulted in the up-regulation of COL10A1 and VEGF in hBMSCs. The induction pathways of these proteins may differ, depending on the differentiation status of hBMSCs. It

Undifferentiated hBMSCs



Osteoblastic hBMSCs

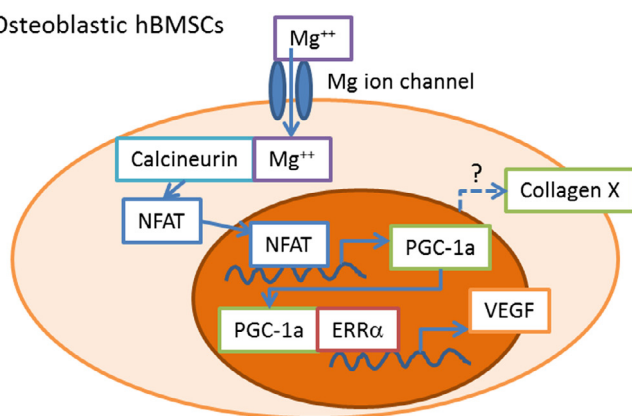


Fig. 6. Schematic of hypothesized intracellular signaling cascades by Mg ion stimulation of hBMSCs. We hypothesized that the addition of MgSO_4 will cause an increase in concentration of intracellular Mg ion in undifferentiated BMSCs. HIFs are then translocated into the cell nucleus and induce production of COL10A1 and VEGF. On the other hand, in the differentiated BMSCs, the Mg ion activates PGC-1 α production (via an unknown transcription factor), which induces the production of VEGF.

appears that undifferentiated cells are stimulated via HIF-2 α , whereas osteogenic cells that are stimulated via PGC-1 α . These findings are critical to our understanding of how Mg-based resorbable metals affect bone healing and regeneration.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2 and 6, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2014.01.027>.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2014.02.002>.

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