INVESTIGATING THE MECHANISM OF INTRACELLULAR SIGNALING OF MAGNESIUM IN HUMAN BONE MARROW STROMAL CELLS (hBMSCs): POTENTIAL ROLE IN OSTEOGENESIS

by

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Mostafa Salah Shehabeldin, B.D.S, M.S.

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Magnesium metal and its alloys have been investigated as promising biomaterials for internal bone fixation devices. Previous reports have shown that magnesium fixation devices enhance fracture healing in animal models while exhibiting biocompatibility and biodegradability. Moreover, mechanistic studies have indicated that mesenchymal stem cells (MSCs) display an osteogenic response to increased magnesium (Mg^{2+}) concentrations in culture medium. We sought to study the signaling pathways underlying Mg-mediated osteogenesis in human bone marrow stromal cells (hBMSCs). We hypothesized that Mg^{2+} could be inducing the osteogenic response of hBMSCs in a calcium sensing receptor (CaSR) dependent manner. In the present study, hBMSCs were stimulated with basal medium (BM) supplemented with 10 mM $MgSO_4$ (10-Mg), 4 mM $CaCl_2$ (4-Ca) or 10 mM $CaCl_2$ (10-Ca) at different time points. Western blot results showed a similar trend of CaSR phosphorylation in response to 10-Mg and 4-Ca media. Although this phosphorylation peaked at 15 minutes with both 10-Mg and 4-Ca media, it was not significantly higher than with BM alone. PKC-δ and Erk 1/2 are two downstream kinases of the CaSR. Both kinases have been reported to play a pivotal role in the differentiation of osteoprogenitors by regulating the expression of key osteogenic markers. We showed that hBMSCs exposure to 10-Mg medium resulted in significant phosphorylation of PKC-δ relative to BM. In contrast, Erk1/2 did not exhibit significant changes in its activity with 10-Mg medium. In addition, qPCR data showed upregulation of *Cx43*, *ALPL* and *Col10A1* following

hBMSCs treatment with 10-Mg medium. Similarly, a trend of upregulation of *Runx2* mRNA was seen with 10-Mg medium; however, this upregulation was not statistically significant. *VEGFA*, a key angiogenic marker, was downregulated at the mRNA level by 10-Mg medium at a late time point (3 weeks). Chemical blocking of CaSR by its selective antagonist NPS2143 only potentiated *ALPL* expression, but did not have any effect on *VEGFA* or *Col10A1*. In conclusion, we propose a potential mechanism by which PKC-δ and Cx43 could mediate the osteogenic response of hBMSCs to increased Mg^{2+} concentrations. The suggested functional activity of PKC-δ might be regulated by a CaSR upstream signaling mechanism.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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Finally, I would like to thank my Mom, my Dad and my brother who came all the way from Egypt to support me on that special day.

1.0 INTRODUCTION

Over the past decades, significant efforts have been made to develop bone fixation devices that support fracture healing while exhibiting biocompatibility and optimum mechanical properties. In this respect, titanium-based devices have been widely and reliably used in long bones and craniofacial fractures. This is due to the inherent biological inertness of titanium metal and the mechanical properties of its alloys that make them suitable for load bearing applications (Rack and Qazi, 2006). However, titanium implants have been associated with a number of long-term complications including pain, infection, screw migration and interference with pediatric bone growth (Berryhill et al., 1999). These complications often necessitate surgical intervention to remove the fixation device from the compromised site (O'Connell et al., 2009). This often results in significant tissue morbidity with greater risk of refracture upon device removal (Inan et al., 2016).

Due to the long-term problems associated with permanent implants (e.g. titanium- and stainless-steel-based), remarkable attention was directed toward degradable biomaterials as an alternative for orthopedic applications. Among those materials, magnesium (Mg) metal and its alloys appear to stand out as promising candidates due to their resorbability, biocompatibility and their mechanical properties that match those of natural bone (Castellani et al., 2011; Staiger et al., 2006). Several *in vivo* studies employing different animal models have been conducted to

assess the clinical feasibility of Mg devices. The results from these studies show a great potential for Mg-based devices (plates and screws or composite scaffolds) not only in supporting the normal bone healing process, but also in enhancing local bone formation (Brown et al., 2015; Chaya et al., 2015a; Chaya et al., 2015b; Kraus et al., 2012).

Since Mg metal and its alloys have been proposed as favorable bone biomaterials, a myriad of studies have been published on animal testing of Mg devices and optimization of their microstructural and degradation properties (Cheng et al., 2016; Iskandar et al., 2013; Johnson et al., 2013; Kim et al., 2014). Nevertheless, fewer studies have focused on examining the molecular mechanisms underlying Mg-mediated osteogenesis. Previous *in vitro* data showed that increased concentrations of magnesium sulfate ($MgSO_4$) or magnesium chloride ($MgCl_2$) in culture medium (up to 10 mM) enhanced the proliferation of human bone marrow stromal cells (hBMSCs) and endothelial cells (Maier et al., 2004; Yoshizawa et al., 2014b). Leem et al reported that the accelerated proliferation of hBMSCs in response to Mg was mediated by the cell surface receptor intergrin alpha-2, which was upregulated in these cells (Leem et al., 2014). In addition, a concentration of 10 mM $MgSO_4$ or 10 mM $MgCl_2$ in culture medium upregulated hBMSCs expression of certain genes involved in neovascularization and extracellular matrix mineralization. These genes included vascular endothelial growth factor (*VEGFA*), alkaline phosphatase (*ALPL*), and Collagen X (Col*10A1*) (Leem et al., 2014; Yoshizawa et al., 2014a; b). Yoshizawa *et al* found that the upregulated expression of *VEGFA* and Col*10A1* in response to Mg was accompanied by enhanced expression of their corresponding transcription factors (Yoshizawa et al., 2014b). These included hypoxia inducible factor-2α (HIF-2α) and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α.

To better simulate *in vivo* degradation of Mg implants, other *in vitro* studies employed pure Mg metal or a Mg alloy extract as a source of Mg ion in culture medium. These studies revealed intriguing results regarding the effects of Mg on mesenchymal stem cells (MSCs) gene expression and fate determination. For instance, Li et al. observed that extracts from pure Mg or Mg-Yttrium alloys enhanced the expression of genes that are crucial for human MSCs osteogenic differentiation such as TGFβ-1, BMP-2, SMAD-4 and FGF-2 (Li et al., 2014). Li et al also showed that these extracts downregulated the expression of genes that interfere with the osteogenic pathway such as TNF-α (a proinflammatory cytokine) and PPAR-γ (a marker for adipogenic differentiation). Another study by Luthringer and Romer showed that human embryonic perivascular cells (HUCPV) responded differently to a 5 mM Mg extract solution depending on the presence or absence of osteogenic factors in culture medium (Luthringer and Willumeit-Romer, 2016). In Luthringer and Romer study, the 5 mM magnesium extract exhibited a synergistic effect on the expression of alkaline phosphatase (ALPL) and osteocalcin (OCN) when the HUCPV are driven towards the osteoblastic phenotype. Interestingly, this synergistic effect was reversed in the absence of osteogenic factors.

It is evident from the aforementioned mechanistic studies that a clear understanding of the specific signaling pathways involved in the Mg-mediated osteogensis has not yet been reached. Therefore, we focused in the present study on further exploring the mechanism of intracellular signaling of Mg^{2+} in hBMSCs. The aim of this work was to examine the role of the calcium sensing receptor (CaSR) signaling pathway in Mg-mediated osteogenesis.

The CaSR is a G-protein-coupled receptor that is expressed by several cell types including parathyroid gland cells, kidney cells, preosteoblast and mature osteoblasts (House et al., 1997; Yamaguchi et al., 1998a; Yamaguchi et al., 1998b). Under normal physiologic conditions, the CaSR on parathyroid gland and kidney cells plays a critical role in monitoring changes in serum calcium, thereby helping in the maintenance of calcium homeostasis (Brown, 2013). The osteoblasts CaSR has been shown to act as an important mediator of bone development, mineralization and remodeling (Dvorak-Ewell et al., 2011). At an area of bone resorption, the CaSR expressed on the nearby osteoblasts senses the increases in local calcium levels. As a result, these osteoblasts are stimulated to proliferate and to migrate to the resorption site where they contribute to the formative phase of bone remodeling (Yamaguchi et al., 1998a).

In addition to calcium, other divalent (Mg^{2+} and Sr^{2+}) and trivalent (La^{3+} and Gd^{3+}) ions, as well as some calciomimetic compounds, have been recognized as allosteric activators of the CaSR (Riccardi and Brown, 2010; Saidak et al., 2009). McCormick et al. showed that moderate stimulation of HEK cells overexpressing CaSR with 2.5 mM $CaCl_2$ in culture medium resulted in significant phosphorylation of the CaSR at its intracellular domain residue Thr-888 ($CaSR^{T888}$) (McCormick et al., 2010). Davies et al. observed that the phosphorylation of the $CaSR^{T888}$ domain in response to calcium and other calciomimetics is mediated by protein kinase C (PKC), which acts in a feedback mechanism to control the receptor activity (Davies et al., 2007). Davies et al. also showed that this PKC-dependent feedback phosphorylation of the $CaSR^{T888}$ domain declines at higher calcium concentrations due to an opposing $CaSR^{T888}$ phosphatase activity. Ward and Riccardi suggested that these successive cycles of $CaSR^{T888}$ phosphorylation

and dephosphorylation are responsible for the oscillations in intracellular Ca^{2+} levels following CaSR activation extracellularly (Ward and Riccardi, 2012).

Previous studies have described the chain of cellular events that follow CaSR activation (Chakravarti et al., 2012; Hofer and Brown, 2003). In brief, stimulation of the CaSR by an agonist causes the receptor to activate the membrane bound phospholipase C (PLC) through receptor coupling with the heterodimeric G-protein subunits $G_{q\alpha}$ and $G_{11\alpha}$. Activated PLC then hydrolyses Phosphatidyl Inositol Bisphosphate (PIP2) into Diacylglycerol (DAG) and Inositol Triphosphate (IP3). The latter binds to its receptor on endoplasmic reticulum (ER) causing mobilization of Ca^{2+} from intracellular stores. The elevated intracellular Ca^{2+} and the DAG released from the PIP2 hydrolysis activate several intracellular protein kinases such as the protein kinase C (PKC) isoforms. PKC isoforms have been classified into either conventional or novel isoforms. The conventional isoforms include PKC α, β and γ, which need both DAG and elevated intracellular Ca^{2+} to be activated. On other hand, the novel PKC isoforms (PKC δ, θ and ε) do not require elevated intracellular Ca^{2+} and can only be activated by DAG (Newton, 2010) (Figure 1) .

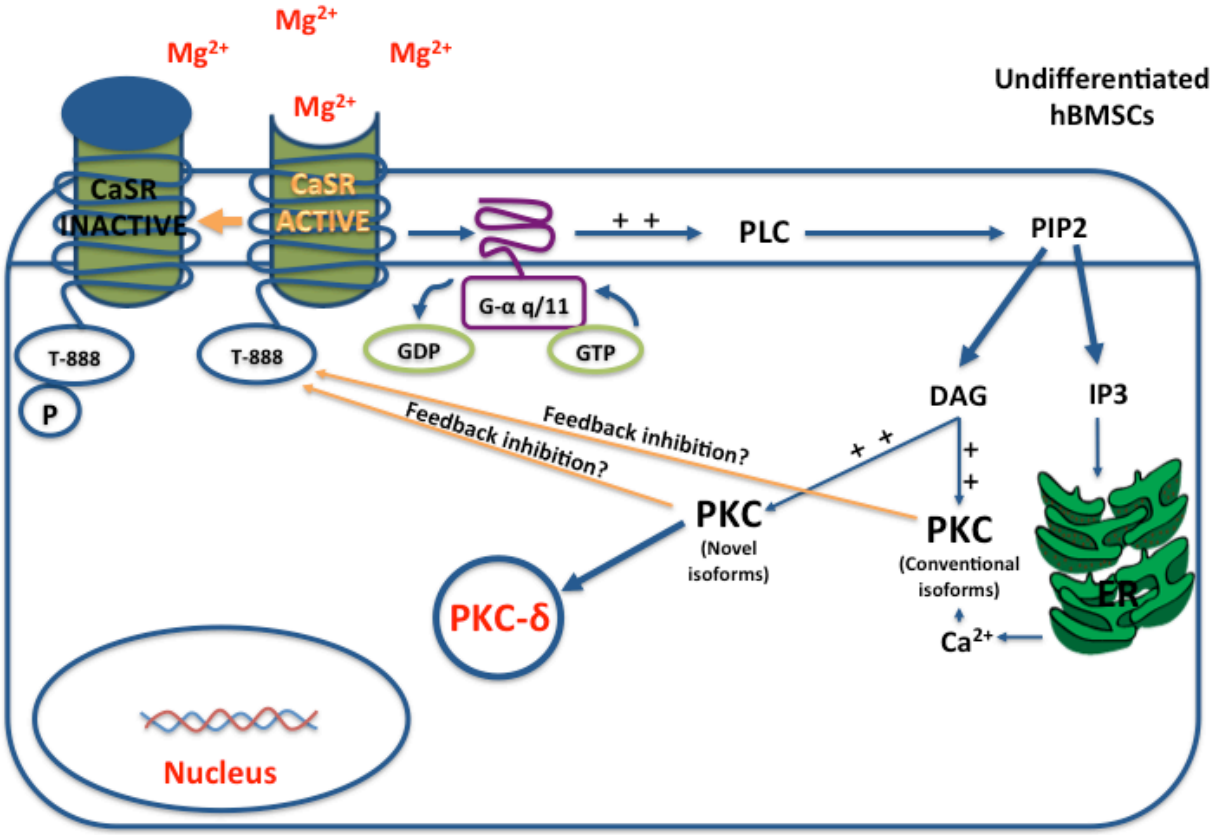


Figure 1. A diagram showing the CaSR-PKC signaling pathway.

Among all the PKC isoforms (conventional and novel), PKC-δ has been reported to have the strongest implication in the osteogenic pathway. For instance, Lee et al. showed that the upregulation of the osteogenic markers ALPL and OCN during the osteogenic differentiation of hBMSCs was associated with enhanced expression and phosphorylation of PKC-δ (Lee et al., 2014). Lee et al. also observed that the silencing of PKC-δ with shRNA resulted in inhibition of ALP activity and matrix mineralization. Similarly, Lima et al. reported that PKC-δ abrogation by siRNA or its specific blocker Rottlerin resulted in inhibition of the transcriptional activity of

OCN in FGF-2 induced MC3T3 cells (Lima et al., 2009). Moreover, a study by Liu et al. showed that pharmacological blocking of PKC-δ inhibited ALPL expression and dexamethasone-induced osteogenic differentiation of hMSCs (Liu et al., 2010). In addition, many studies have pointed to a role for PKC-δ in mediating Runx2 expression by MC3T3 cells exposed to FGF-2 (Lima et al., 2009; Niger et al., 2013). These studies showed that this effect of PKC-δ was potentiated by the gap junction protein connexin43 (Cx43). Cx43 was previously reported to play a critical role in osteoblasts and osteocytes survival and function (Gramsch et al., 2001; Li et al., 2006).

The extracellular-signal-regulated kinases (Erk1/2) are another group of intracellular kinases involved in the CaSR signal transduction (Holstein et al., 2004; Ward, 2004). Increasing the concentration of extracellular Ca^{2+} or addition of other CaSR polycationic agonists have been reported to induce the phosphorylation of the Erk1/2 kinases in MC3T3 cells (Yamaguchi et al., 2000). The Erk1/2 kinases are in the form of two-associated serine/threonine kinases that have been implicated in a wide range of cellular processes including cell proliferation, migration and differentiation (Roskoski, 2012). In bone, the Erk1/2 kinases have been shown to play an important role in the growth, differentiation, integrin expression and function of human osteoblasts (Lai et al., 2001). During skeletal development, the Erk1/2 pathway has been proposed to act as a key mediator of osteoblasts differentiation by enhancing the phosphorylation and activity of Runx2 (Ge et al., 2007).

The objective of this study was to investigate the signaling events underlying the osteogenic response of hBMSCs to increased Mg concentrations. To achieve this, we started

with comparing the effects of increased Mg^{2+} and Ca^{2+} concentrations in culture medium on the activity of hBMSCs CaSR. Next, we evaluated the activity of two CaSR downstream molecules PKC-δ and Erk1/2 in response to hBMSCs stimulation with high levels of Mg^{2+} in culture medium. Lastly, we assessed hBMSCs expression of selected genes that are linked to the CaSR downstream pathway and to the osteogenic pathway under different culturing conditions and at different time points. The findings presented in this work could serve as a guide for future studies aiming at further elucidating the cellular mechanisms involved in Mg-mediated osteogenesis.

2.0 HYPOTHESIS

Our hypothesis is that undifferentiated hBMSCs exhibit their osteogenic response to Mg^{2+} through the calcium sensing receptor (**CaSR**) and its downstream kinases **PKC-δ** and **Erk1/2.**

3.0 MATERIALS AND METHODS

3.1 Human bone marrow stromal cells (HBMSCs) harvesting, expansion and culture

hBMSCs at passage 1 were obtained from Dr. Pamela Robey at the National Institute of Health. The cells were plated at 40,000 per cm^2 in Minimum Essential Medium Eagle (MEM) Alpha Modifications (α-MEM) (Life Technologies, Grand Island, NY) containing 16.5% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 1% penicillin and streptomycin (Life Technologies) and 1% L-glutamine (Life Technologies) at 37°C in an atmosphere of 5% CO_2. After 24 hours, non-adherent cells were washed away then adherent hBMSCs were detached with 0.05% of trypsin-EDTA (Life Technologies) and subcultured in T175 flasks in expansion medium. At 80-90% confluency, the cells were passaged and this was repeated for up to three times. For experiments, cells between passage 3 and 4 were used.

3.2 Western blotting

3.2.1 CaSR^{T888} phosphorylation assay

hBMSCs were plated into 6-well plates at a density of $3x10^5$ cells per well. The cells were serum starved for 24 hours, then stimulated with 5% FBS MEM-alpha medium supplemented with either 10 mM $MgSO_4$ (10-Mg) or 4 mM $CaCl_2$ (4-Ca) (table 1) for 5, 10, 15 and 20 minutes (three replicates for each treatment group). The plain 5% FBS alpha-MEM medium was used for the control wells and was referred to as basal medium (BM). At the end of each time point, total protein from cultured cells was extracted using RIPA buffer that contained 150 mM sodium chloride, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), 5mM sodium fluoride and proteinase inhibitor cocktail (Roche). Extracted protein samples were quantified by colorimetric protein assay using Pierce® 660 nm Protein Assay Reagent (Thermo Fisher Scientific). Next, the samples were denatured using loading buffer containing β-mercaptoethanol at 95˚C for 5 minutes and SDS-page was performed with 10% acrylamide gel then the samples were transferred to polyvinyl difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Western blotting was done using primary antibodies against phosphor-CaSR^{T888} (ab182619) (Abcam) at a dilution of 1:750 and against β-actin (A5441) (Sigma-Aldrich). The blots were developed with the Western Lightning® Plus-ECL (PerkinElmer, Inc., Waltham, MA). The intensity of the bands was quantified by ImageJ, and normalized by β-actin. The relative

expression in the control samples was considered as baseline (fold change = 1) and was used to calculate the fold change in the treated groups.

3.2.2 PKC-δ phosphorylation assay

hBMSCs were plated into 60 mm cell culture dishes at a density of $0.8x10^6$ cells per dish. The day of the experiment, the cells were serum starved for 2 hours then stimulated with either plain 5% FBS alpha-MEM medium (BM) or BM supplemented with 10 mM $MgSO_4$ (10-Mg) (table 1) for 10 and 15 minutes. Total protein was extracted, quantified and samples were denatured as described previously. Next, the samples were run into 10% polyacrylamide gel then the protein was transferred to PVDF membrane. For western blotting, antibodies against phospho-PKCδ (Ser643) **(**9376P) at a dilution of 1:1000 (Cell Signaling Technology) and against β-actin (A5441) (Sigma-Aldrich) were used. The blots were developed and quantified as described previously.

3.2.3 Erk1/2 phosphorylation assay

hBMSCs were plated into 6-well plates at a density of $3x10^5$ cells per well. The cells were then stimulated with either BM alone or BM containing 10 mM $MgSO_4$ (10-Mg), 10 mM $CaCl_2$ (10-Ca) (table 1) (3 replicates per treatment group) for 6, 12 and 24 hours. Total protein was extracted and quantified, SDS- page and gel transfer were performed as described previously. Antibodies against Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (9101S) and p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (9102S) (Cell signaling Technology) at a dilution of

1:1000 were used for western blotting. The non-phosphorylated total Erk1/2 bands were used for normalization during band intensity quantification with ImageJ software.

3.3 Analysis of gene expression

3.3.1 RNA extraction

hBMSCs were plated into 6-well plates at a density of $3x10^5$ cells per well for 3 independent experiments. The first experiment involved treatment of hBMSCs with basal medium (BM) alone or BM containing 10 mM $MgSO_4$ (10-Mg), 4 mM $CaCl_2$ (4-Ca) (table 1) for 1, 3 and 7 days. In the second experiment, hBMSCs were treated with either the 10-Mg medium or BM for 1, 2 and 3 weeks. For the third experiment, hBMSCs were cultured for 1, 3 and 7 days with either BM alone or BM containing one of the following: 10 mM $MgSO_4$ (10-Mg), 10 mM $MgSO_4$+ 3μm NPS 2143 (Selective blocker for CaSR) (Sigma) (Mg-10 + NPS), 3μm NPS 2143 alone (NPS) or DMSO alone (DMSO) (3 replicates per treatment group). At the end of each time point, total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Qiagen) to eliminate DNA contamination according to manufacturer's instructions. The quantity and quality of RNA was measured using a nanophotometer.

3.3.2 Quantitative Polymerase Chain Reaction (q-PCR)

qPCR was performed with TaqMan ABI inventoried gene assays (Applied Biosystems) to analyze the mRNA expression of *RUNX2*, *GJA1* (Cx43*), ALPL*, *COL10A1* and *VEGFA*. The assays were conducted in QuantStudio(TM) 6 Flex System instrument (Applied Biosystems). All Ct values greater than 35 were considered as negative readings and the fold change for each of the samples was calculated using the $\Delta\Delta C_t$ method. First, the ΔC_t value was calculated by normalizing the C_t value of each sample to that of the housekeeping gene. Then, the difference in the ΔC_t values between the experimental and control wells of each time point was calculated as the ($\Delta\Delta C_t$). The fold change for each sample was calculated as $2^{(-\Delta\Delta Ct)}$.

3.4 Statistical analysis

The data are presented as means $\pm$ standard deviations. Statistical comparison of the quantified western blot bands was done using either Student's *t-test* or one-way ANOVA with *post-hoc Tukey* correction. q-PCR data were analyzed by comparing the fold change results from each treatment group using Student's *t-test* or one-way ANOVA with *post-hoc Tukey* correction. The results of all statistical tests were considered significant at $p<0.05$.

Table 1. Composition of media used in this study*

Medium	Abbreviation	FBS	$MgSO_4$	$CaCl_2$
Expansion medium	N/A	16.5%	0.8 mM	1.8 mM
Basal medium	BM	5%	0.8 mM	1.8 mM
Magnesium medium	10-Mg	5%	10 mM	1.8 mM
Calcium media	4-Ca	5%	0.8 mM	4 mM
	10-Ca	5%	0.8 mM	10 mM

* It should be noted that the magnesium and calcium media used for hBMSCs stimulation were prepared by adding $MgSO_4$ or $CaCl_2$ salts (Sigma) to the BM already containing an initial concentration of 0.8 mM $MgSO_4$ and 1.8 mM $CaCl_2$.

4.0 RESULTS

4.1 Magnesium modulates the activity of the calcium sensing receptor in a manner similar to calcium by changing the phosphorylation state of its Thr-888 domain.

Western blotting was used to examine the phosphorylation of the $CaSR^{T888}$ domain in response to increased Mg^{2+} and Ca^{2+} levels. The BM containing 10 mM $MgSO_4$ (10-Mg) slightly enhanced the phosphorylation of the $CaSR^{T888}$ in hBMSCs at 5 minutes (1.37 fold). This effect was not observed with either the 4 mM $CaCl_2$ medium (4-Ca) or the basal medium (BM) (Figure 2). In addition, tracking of the $CaSR^{T888}$ phosphorylation over time showed that it peaks at 15 minutes with the 10-Mg and the 4-Ca media but not with BM (Figure 3a, b and c). It should be noted that none of the changes seen with the $CaSR^{T888}$ phosphorylation were statistically significant.

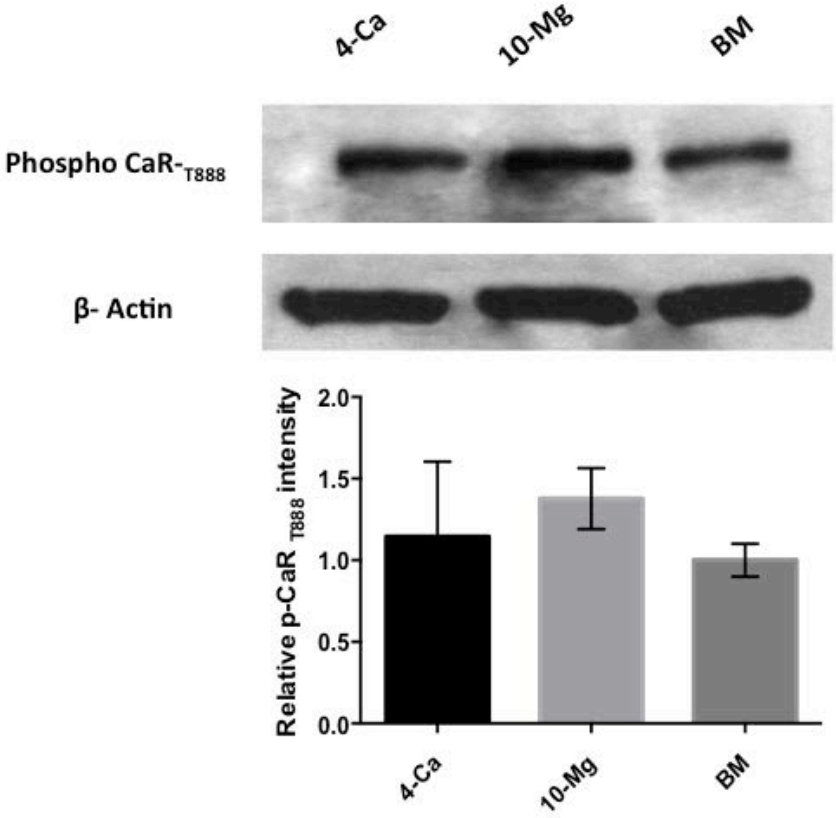


Figure 2. 10 mM $MgSO_4$ in basal medium BM (10-Mg) slightly enhanced the phosphorylation of the $CaSR^{T888}$. No significant changes in $CaSR^{T888}$ phosphorylation were seen with the medium supplemented with 4 mM $CaCl_2$ (4-Ca).

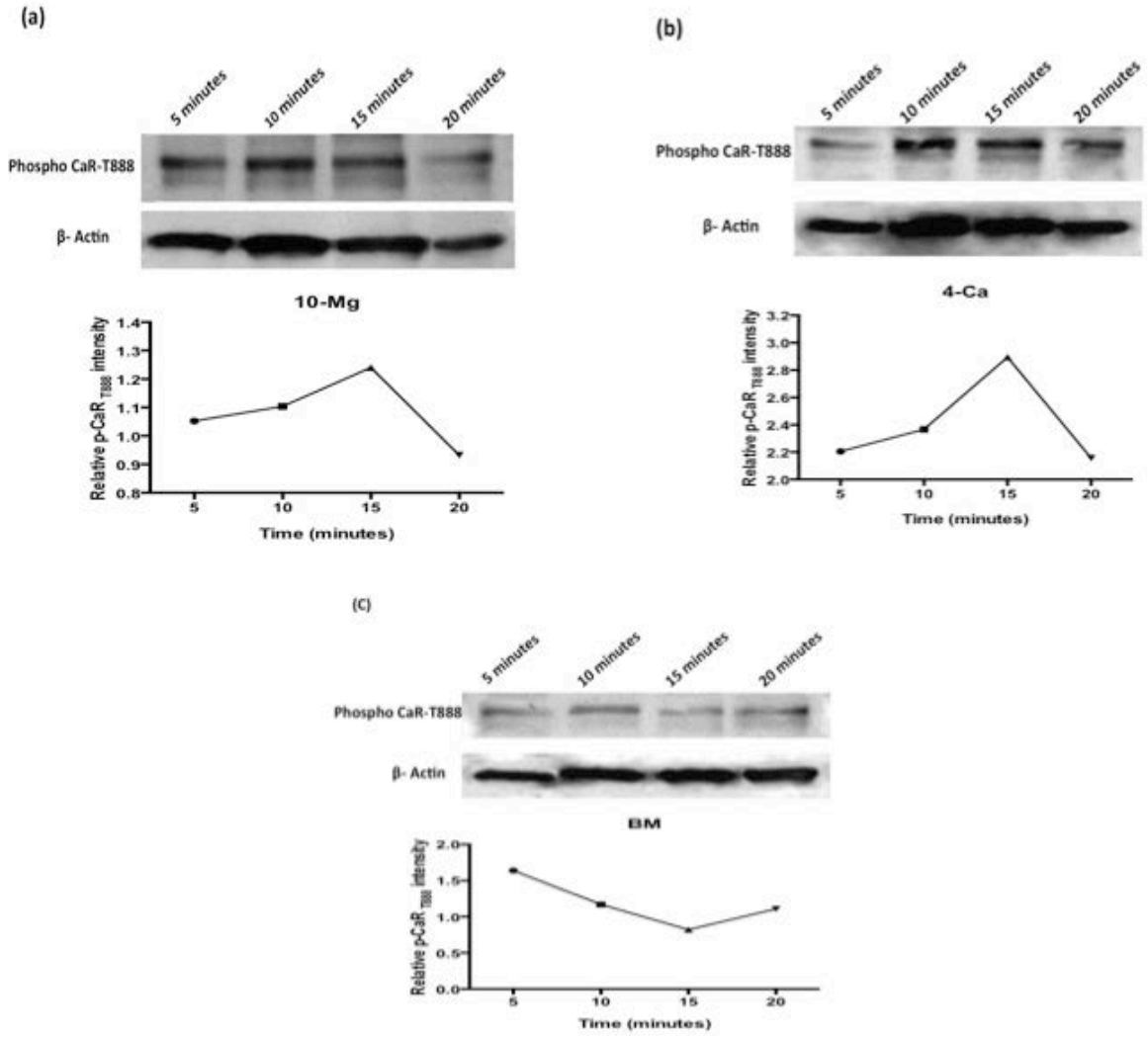


Figure 3. The expression of phospho-CaSR^{T888} in response to hBMSCs treatment with BM alone (c) or BM containing 10 mM $MgSO_4$ (10-Mg) (a) or 4 mM $CaCl_2$ (4-Ca) (b) was followed over time (5, 10, 15 and 20 minutes). The phosphorylation peaked with 10-Mg (a) and 4-Ca media, but not with BM.

4.2 10 mM $MgSO_4$ in basal medium (10-Mg) increased phospho-PKC-δ expression.

In contrast to basal medium (BM), the 10-Mg medium enhanced the phosphorylation of PKC-δ in hBMSCs at 10 (Figure 4a) and 15 minutes (Figure 4b). This effect of increased magnesium concentration on PKC-δ activity was more robust at 15 minutes (3.34-fold) than at 10 minutes (1.62-fold); however, it was statistically significant only at 10 minutes.

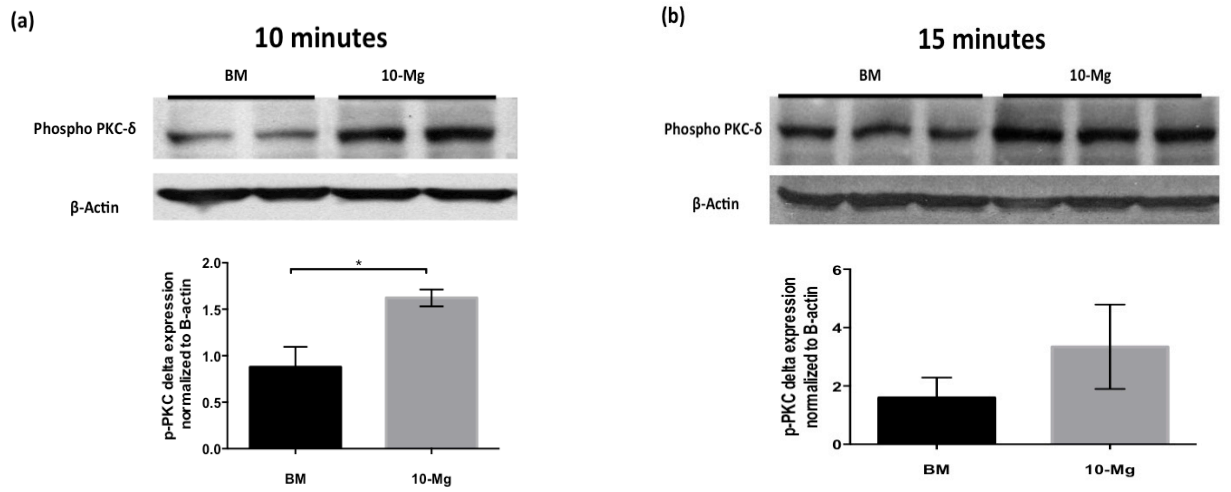


Figure 4. 10 mM $MgSO_4$ in BM (10-Mg) enhanced the expression of phospho-PKC-δ relative to BM alone. The phosphorylation was increased by 1.62-fold at 10 minutes (a) and by 3.34-fold at 15 minutes (b). However, statistical significance between BM and 10-Mg medium was only present at 10 minutes (student's t-test, $P<0.05$).

4.3 Components of the Erk1/2 signaling pathway exhibited a trend of upregulation with the BM containing 10 mM $CaCl_2$ (10-Ca). However, the 10 mM $MgSO_4$ medium (10-Mg) had no effect.

A trend of upregulation of the Erk1/2 subunits was seen after 6 and 24 hours stimulation of hBMSCs with the 10-Ca medium. However, neither the 10-Mg medium nor the BM had an effect on the activity of the Erk1/2 pathway (Figure 5).

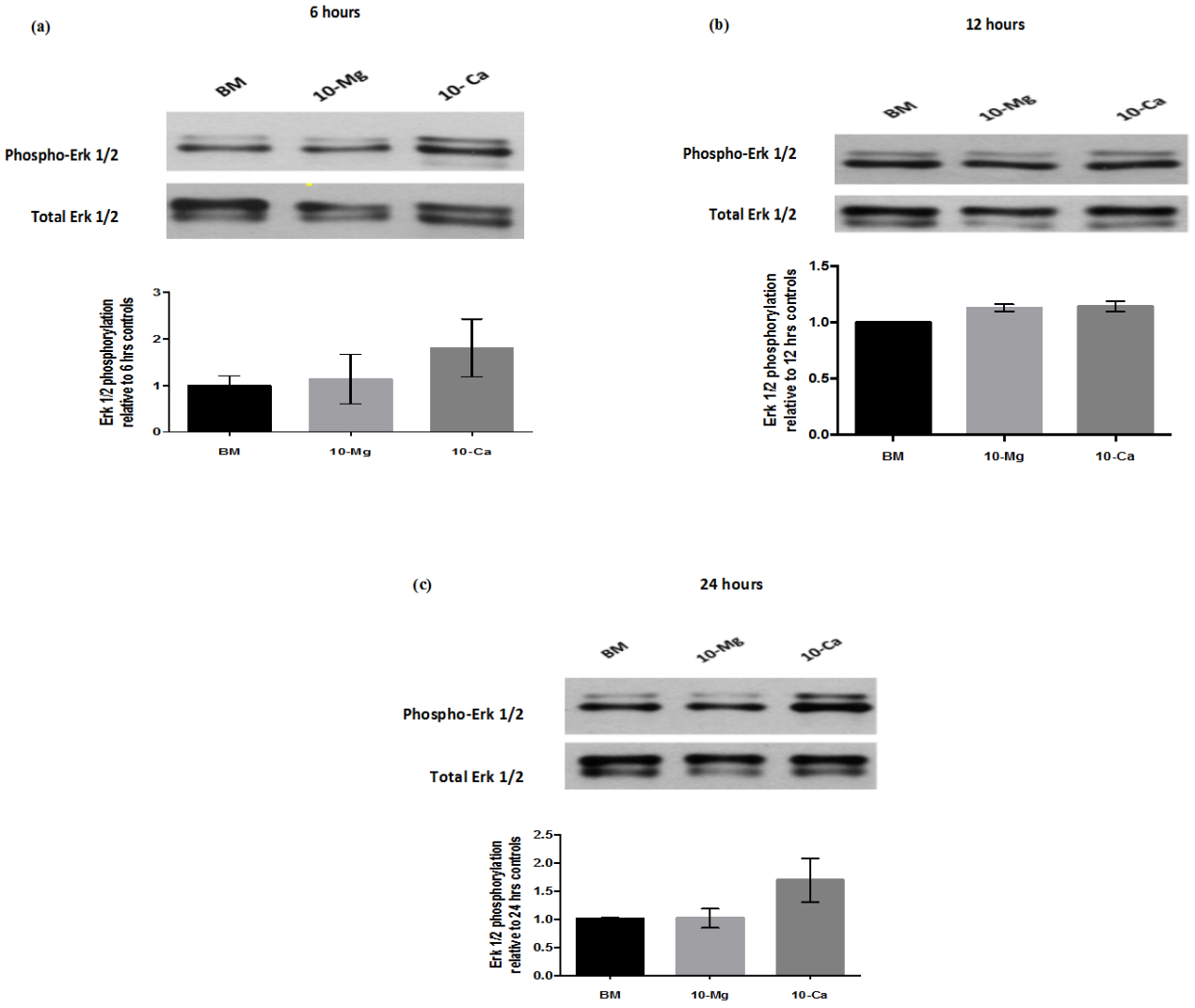


Figure 5. hBMSCs were treated with 10 mM $MgSO_4$ (10-Mg) or 10 mM $CaCl_2$ (10-Ca) in BM or basal medium (BM) alone for 6 hours (a), 12 hours (b) or 24 hours (c). Erk1/2 subunits showed a trend of upregulation with the 10-Ca medium treatment at 6 and 24 hours, whereas the 10-Mg medium and BM had no effect.

4.4 A trend of upregulation of *Runx2* expression was seen with 10 mM $MgSO_4$ in BM (10-Mg). On the other hand, 4 mM $CaCl_2$ in BM (4-Ca) did not exhibit this effect.

In contrast to the 4 mM $CaCl_2$ medium (4-Ca) or basal medium (BM), the 10 mM $MgSO_4$ medium (10-Mg) caused a gradual increase in *Runx2* mRNA expression over the course of hBMSCs stimulation for 1, 3 and 7 days (Figure 6). The highest expression of *Runx2* was seen at 7 days with 2.33-fold increase in the samples treated with the 10-Mg medium. However, this enhanced expression was not statistically significant at any of the time points.

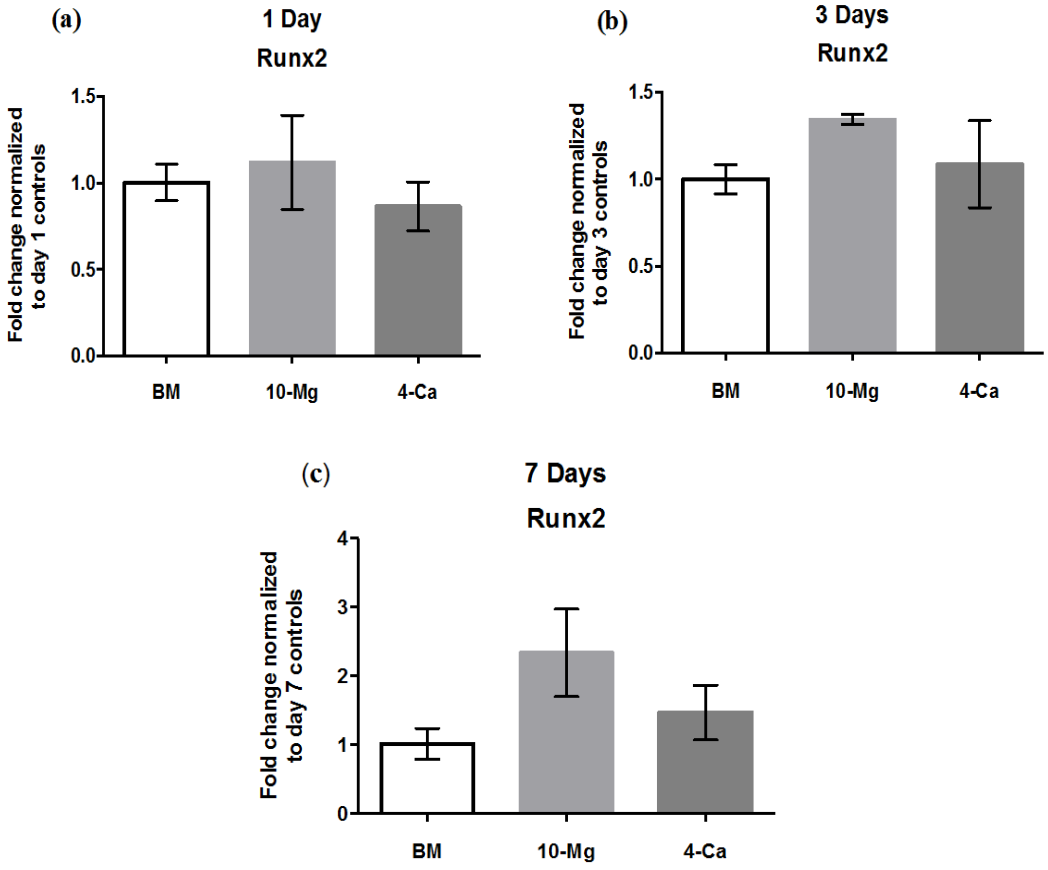


Figure 6. *Runx2* mRNA expression in response to hBMSCs treatment with BM, or BM with 10 mM $MgSO_4$ (10-Mg) or 4 mM $CaCl_2$ (4-Ca) for 1 day (a), 3 days (b) and 7 days (c).

4.5 Both 4 mM $CaCl_2$ (4-Ca) and 10 mM $MgSO_4$ (10-Mg) in basal medium slightly enhanced the mRNA expression of *Cx43* (*GJA1*).

The 4 mM $CaCl_2$ medium (4-Ca) slightly upregulated the mRNA expression of *Cx43* at 3 and 7 days with 1.53 and 1.7 fold change respectively (Figure 7b and c). On the other hand, the 10 mM $MgSO_4$ medium (10-Mg) resulted in 1.47 fold increase of *Cx43* mRNA expression only at 7 days (Figure 7 C).

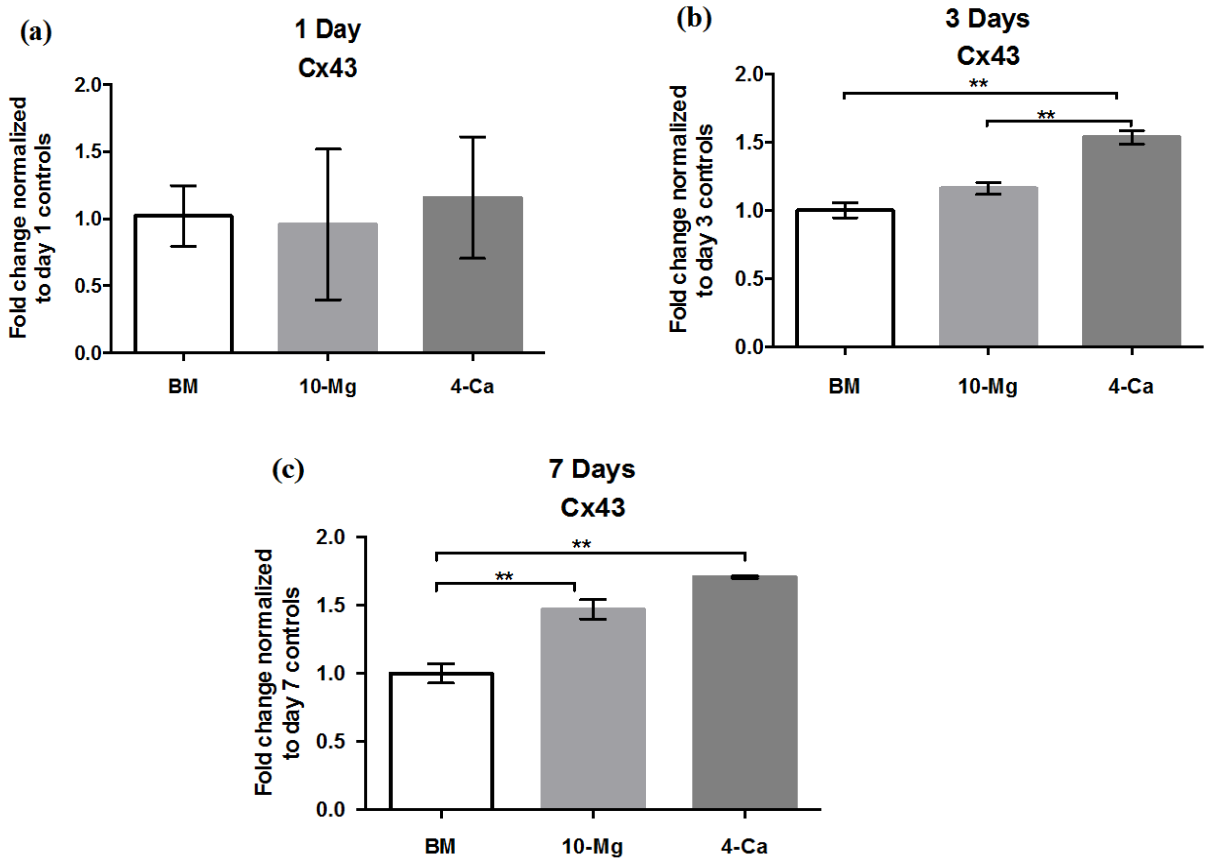


Figure 7. *Cx43* mRNA expression in hBMSCs cultured in BM alone or BM supplemented with 10 mM $MgSO_4$ (10-Mg) or 4 mM $CaCl_2$ (4-Ca) for 1 (a), 3 (b) and 7 days (c) (One-way ANOVA with post-hoc Tukey correction, $p<0.05$).

4.6 10 mM $MgSO_4$ in basal medium (10-Mg) enhanced the expression of *ALPL* and *Col10A1*.

hBMSCs cultured in BM with 10 mM $MgSO_4$ (10-Mg) upregulated the mRNA expression of *ALPL* (2.09-fold) and *Col10A1* (5.06-fold) at 2 weeks (Figure 8d and f). At 3 weeks, no significant changes were seen in either genes. *VEGFA* did not exhibit significant changes with the 10-Mg medium at 1 and 2 weeks; however, its expression was downregulated at 3 weeks (0.59-fold) (Figure 8h).

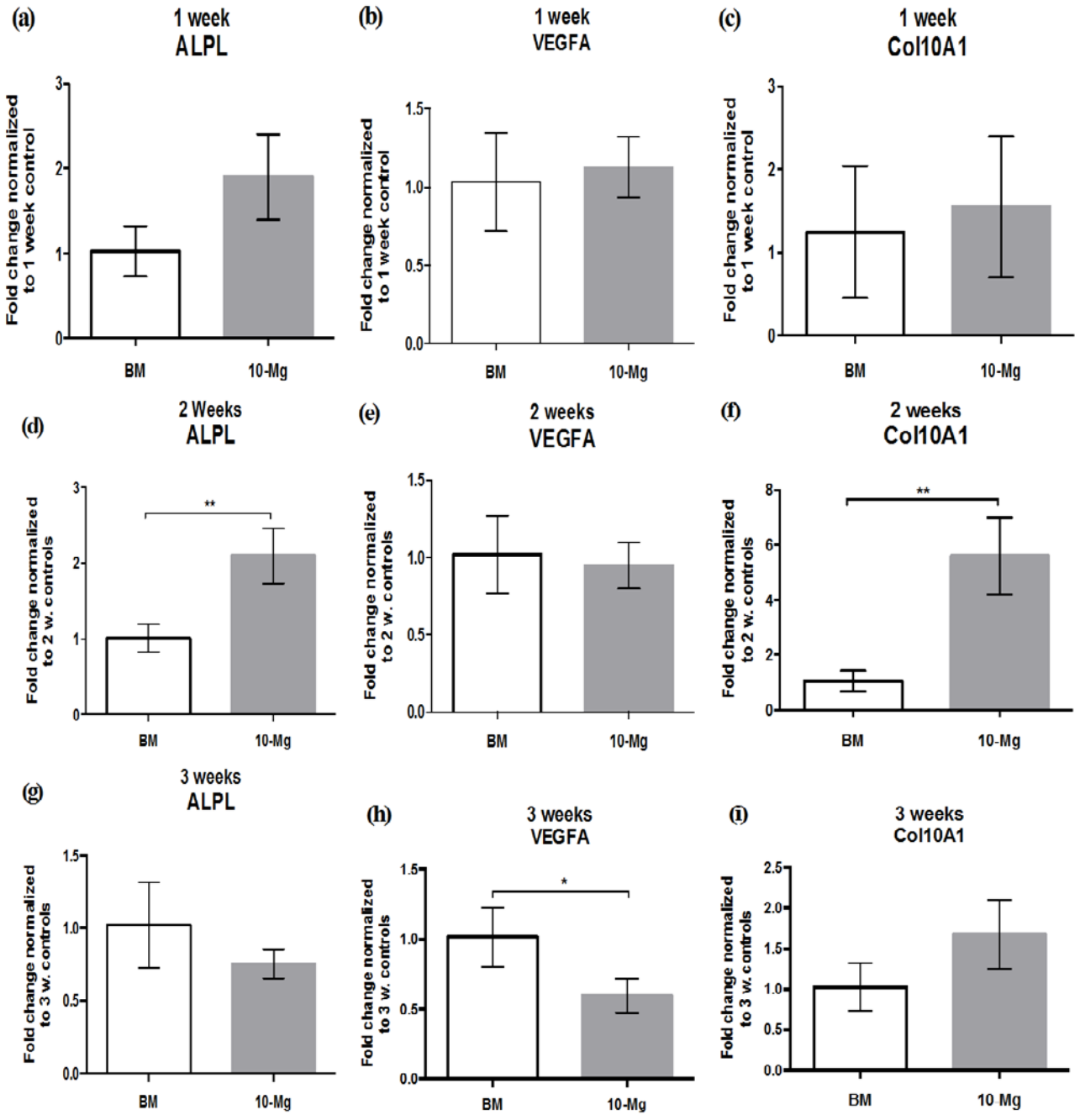
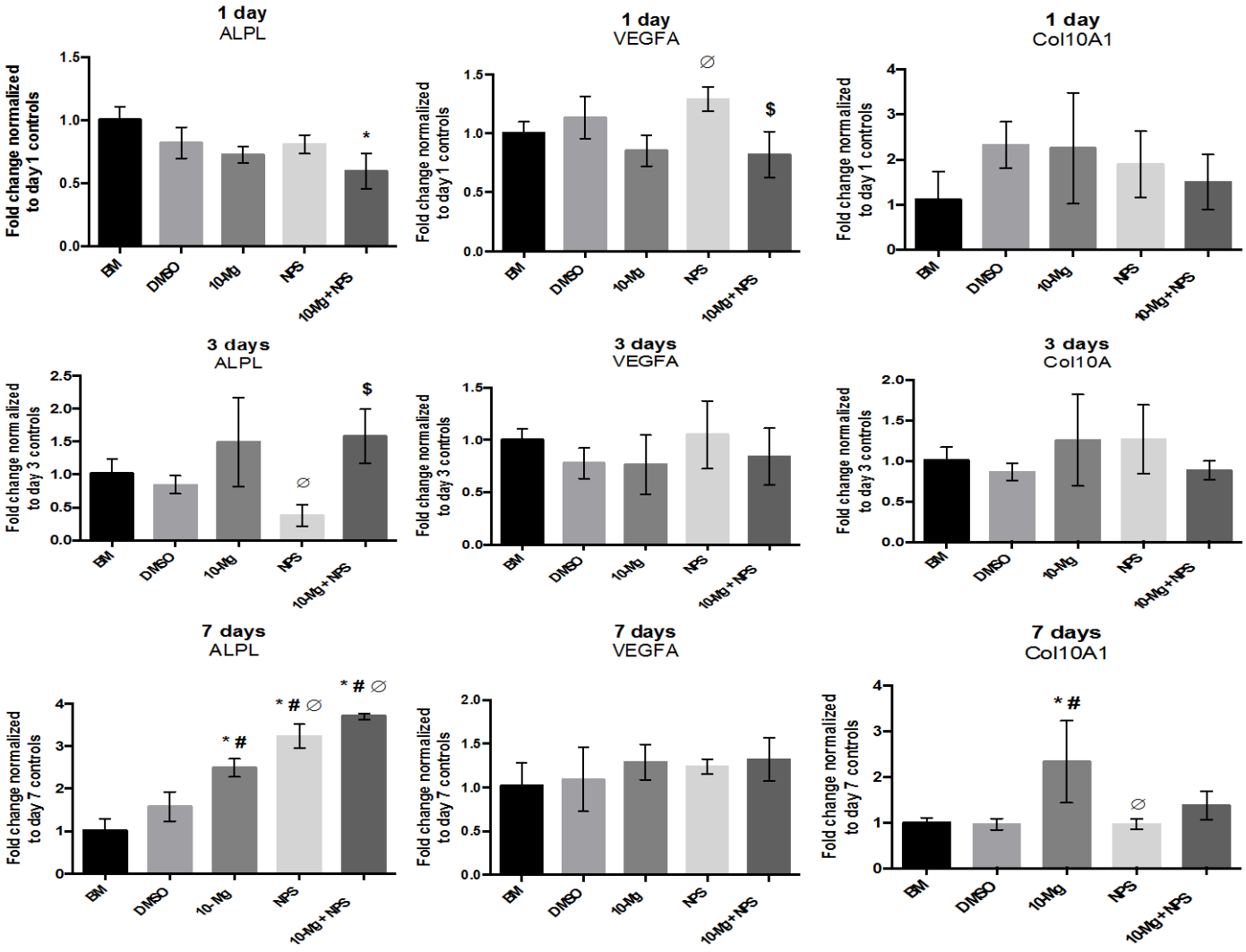


Figure 8. mRNA expression of *ALP*L, *VEGFA* and *Col10A1* following hBMSCs treatment with BM containing 10 mM $MgSO_4$ (10-Mg) or BM alone for 1, 2 and 3 weeks (Student's t-test, $p<0.05$).

4.7 Selective blocking of CaSR potentiated the effect of Mg on *ALPL* mRNA expression, but did not have a significant effect on *VEGFA* and *Col10A1* expression.

At 7 days, the selective blocker for the CaSR NPS2143 upregulated *ALPL* mRNA expression when added to BM either alone (NPS) (3.23-fold) or in combination with 10 mM $MgSO_4$ (10-Mg) (3.7-fold). However, blocking the CaSR did not cause any significant changes on the expression of either *VEGFA* or *Col10A1* (Figure 9).



***** = Significantly different from **BM** group. #= Significantly different from **DMSO** group.

∅ = Significantly different from **10-Mg** group. $ = Significantly different from **NPS** group.

Figure 9. mRNA expression of *ALPL*, *VEGFA* and *Col10A1* following hBMSCs treatment with plain basal medium(BM), or BM supplemented with 10 mM $MgSO_4$ (10-Mg), 10 mM $MgSO_4$ + 3um NPS2143 (10-Mg + NPS), NPS2143 alone (NPS) and dimethyl sulfoxide (DMSO) for 1, 3 and 7 days (One-way ANOVA with post-hoc Tukey correction, $p<0.05$).

5.0 DISCUSSION

In this study, we investigated the molecular mechanisms involved in Mg-mediated osteogenesis. We showed that Mg^{2+} modulates the activity of hBMSCs CaSR in a manner similar to Ca^{2+}; however, neither 10 mM $MgSO_4$ nor 4 mM $CaCl_2$ in culture medium significantly enhanced the phosphorylation of the $CaSR^{T888}$ active-domain. Additionally, our data indicated that the 10 mM $MgSO_4$ medium enhanced the activity of the CaSR downstream kinase PKC-δ, whereas no significant changes were seen with the Erk1/2. Using time points ranging from days to weeks, we found that the 10 mM $MgSO_4$ medium increased hBMSCs mRNA expression of *Cx43* (1.47-fold), *ALPL* (2.09-fold) and *Col10A1* (5.06-fold) and reduced the expression of *VEGFA* (0.59-fold). Blocking the CaSR with its selective blocker NPS2143 upregulated the mRNA expression of *ALPL*, whereas no significant changes were observed with *Col10A1* and *VEGFA*. The data presented in this work could provide a guideline for further studies aimed at exploring the biological effects of Mg-based devices on the bone healing process.

The CaSR has been recognized by its unique ability to interact with several divalent and trivalent cations other than Ca^{2+}. Mg^{2+} has been proposed as one of those cations; however, it requires two- to threefold the concentration of Ca^{2+} in order to influence the CaSR activity

(Hofer and Brown, 2003). Along those lines, we sought to examine the activity of hBMSCs CaSR in response to Mg^{2+} and Ca^{2+}. We used concentrations of 10 mM $MgSO_4$ and 4 mM $CaCl_2$ in culture medium to examine the phosphorylation of the $CaSR^{T888}$ domain in hBMSCs. We found that neither the 10 mM $MgSO_4$ nor the 4 mM $CaCl_2$ media were able to significantly enhance the phosphorylation of the $CaSR^{T888}$. Nonetheless, a similar trend of $CaSR^{T888}$ phosphorylation peaking at 15 minutes was seen with both the Mg^{2+} and the Ca^{2+} media. A possible explanation for the lack of robust $CaSR^{T888}$ phosphorylation is that the selected Mg^{2+} and Ca^{2+} concentrations were too high to sustain their effects on the CaSR activity at the designated time points. McCormick et al. previously showed that moderate stimulation of HEK-293 cells overexpressing CaSR with Ca^{2+} concentrations ranging between 2-2.5 mM resulted in a more sustained $CaSR^{T888}$ phosphorylation (up to 20 minutes) (McCormick et al., 2010). This effect was not evident at higher Ca^{2+} concentrations as reported by McCormick. Therefore, we propose that future experiments should include lower concentrations of $MgSO_4$ (7.5 to 8 mM) and $CaCl_2$ (2-2.5 mM) than the ones used in this study. This would allow for a more reliable assessment of Mg^{2+} and Ca^{2+} effects on hBMSCs CaSR activity.

To analyze the effects of Mg on the CaSR-dependent signaling pathways, we evaluated the activity of two CaSR downstream kinases PKC-δ and Erk1/2 in Mg-induced hBMSCs. First, we examined the phosphorylation of PKC-δ in hBMSCs stimulated with 10 mM $MgSO_4$ in BM (10-Mg) or BM alone. Our results showed enhanced phosphorylation of PKC-δ in response to the 10-Mg medium. PKC-δ has been reported to play a pivotal role in the osteogenic differentiation of hBMSCs by upregulating osteogenic markers such as Runx2 and ALPL (Lee et al., 2014). Therefore, we examined the expression of those markers by q-PCR in hBMSCs cultured with 10

mM $MgSO_4$. Our data indicated that the 10 mM $MgSO_4$ medium enhanced the expression of *ALPL* at 1 and 2 weeks, whereas no significant change was seen with *Runx2*, even though a trend of upregulation was observed at all time points. A previous report showed that the gap junction protein Cx43 potentiates the positive effect of PKC-δ on Runx2 expression by FGF-2 induced osteoblasts (Lima et al., 2009). Hence, we decided to evaluate the contribution of Cx43 in Mg-mediated osteogenesis by assessing its mRNA expression in hBMSCs stimulated with Mg. Our qPCR data showed that 10mM $MgSO_4$ and 4 mM $CaCl_2$ upregulated Cx43 mRNA expression. Although the upregulation of Cx43 by Mg was minimal (1.47-fold), we speculate that the gap junctional activity of Cx43 could amplify the osteogenic response of hBMSCs to Mg by potentiating PKC-δ activity.

The Erk1/2 is the second CaSR downstream molecule that we investigated. We observed the phosphorylation of the Erk1/2 complex in hBMSCs cultured with 10 mM $MgSO_4$, 10 mM $CaCl_2$ or basal medium (BM) at 6, 12 and 24 hours. A trend of upregulation of Erk1/2 was seen with the $CaCl_2$ medium at 6 and 24 hours; however, this upregulation was not statistically significant. On the other hand, the 10 mM $MgSO_4$ did not cause any changes in the Erk1/2 activity. A previous report showed that the Erk1/2 pathway is activated upon the adhesion of integrins to ECM molecules such as Collagen I (Popov et al., 2011). The most significant of those integrins is integrin alpha-2 (ITG-α2), which was shown to be upregulated along with Erk1/2 in human osteoblasts cultured on β-TCP (Lu and Zreiqat, 2010). Interestingly, ITG-α2 expression was also upregulated in hBMSCs stimulated with Mg, where it could mediate the interaction of those cells with the ECM components (Leem et al., 2014). Accordingly, there is a possibility that Mg^{2+} activates the Erk1/2 pathway in either an ITG-α2 or a CaSR dependent

manner. However, this effect might not be detectable with the Mg concentration or at the time points used in the current study.

A number of specific genes have been implicated in the osteogenic response of hBMSCs to Mg. These included: *ALPL*, *VEGFA* and *Col10A1*. Those genes have been reportedly upregulated in response to 10 mM $MgSO_4$ stimulation of hBMSCs (Leem et al., 2014; Yoshizawa et al., 2014a; b). We sought to investigate whether the hBMSCs CaSR would have a role in the enhanced expression of those genes in response to Mg. To achieve that, a selective antagonist for the CaSR called NPS2143 was added to hBMSCs cultured in the presence or absence of 10 mM $MgSO_4$. This antagonist acts by promoting the inactive conformation of the CaSR, thus rendering it less sensitive to agonist stimulation (Huang and Breitwieser, 2007). Our qPCR data indicated that CaSR blocking with NPS2143 did not have detectable effects on the mRNA expression of *VEGFA* and *Col10A1*. However, NPS2143 potentiated *ALPL* mRNA expression by hBMSCs when added to the culture medium either alone or in combination with 10 mM $MgSO_4$. This is in contrast to a previous study that showed inhibition of ALP activity in parathormone (PTH) stimulated bone marrow stromal cells exposed to NPS2143 (Gowen et al., 2000). However, the NPS2143 concentration used in this study was higher (10 uM) than the one used in our experiments (3 uM). Thus, there is a possibility that NPS2143 exhibits a positive effect on ALPL expression when used at a lower concentration, potentially by indirectly activating other parallel osteogenic pathways. Another approach for blocking the CaSR is to use a CaSR neutralizing antibody (Anti-CaSR). The Anti-CaSR has been reported to inhibit *Collagen I* and *ALPL* expression by rat BMSCs exposed to 10 mM $CaCl_2$ (Gonzalez-Vazquez et

al., 2014). Therefore, we suggest that the AntiCaSR should be used in future studies aiming to evaluate the functional role of CaSR in Mg-mediated osteogenesis.

In the present study, no osteogenic factors were added to the hBMSCs stimulation media in all experiments. Previous reports suggested that the pathways activated by Mg in hBMSCs vary depending on the presence or absence of osteogenic differentiation factors in culture medium. For instance, Yoshizawa et al. proposed that Mg-induced upregulation of *Col10A1* and *VEGFA* is mediated by HIF in undifferentiated hBMSCs, and by PGC-1α in osteoblastic hBMSCs (Yoshizawa et al., 2014b). PKC-δ has been previously reported to stabilize HIF-1α and to regulate the expression of VEGF in human adenocarcinoma cells (Lee et al., 2007). However, there is no literature evidence supporting a relationship between PKC-δ and PGC-1α to the best of our knowledge. In the current study, we demonstrated that the 10 mM $MgSO_4$ medium enhanced the activity of PKC-δ. This PKC-δ activity might mediate the HIF dependent upregulation of *Col10A1* and *VEGFA* in undifferentiated hBMSCs. In light of our findings, it would be of interest to investigate whether the differentiation state of hBMSCs affects the activity of the CaSR and PKC-δ in the presence of increased Mg^{2+} concentrations.

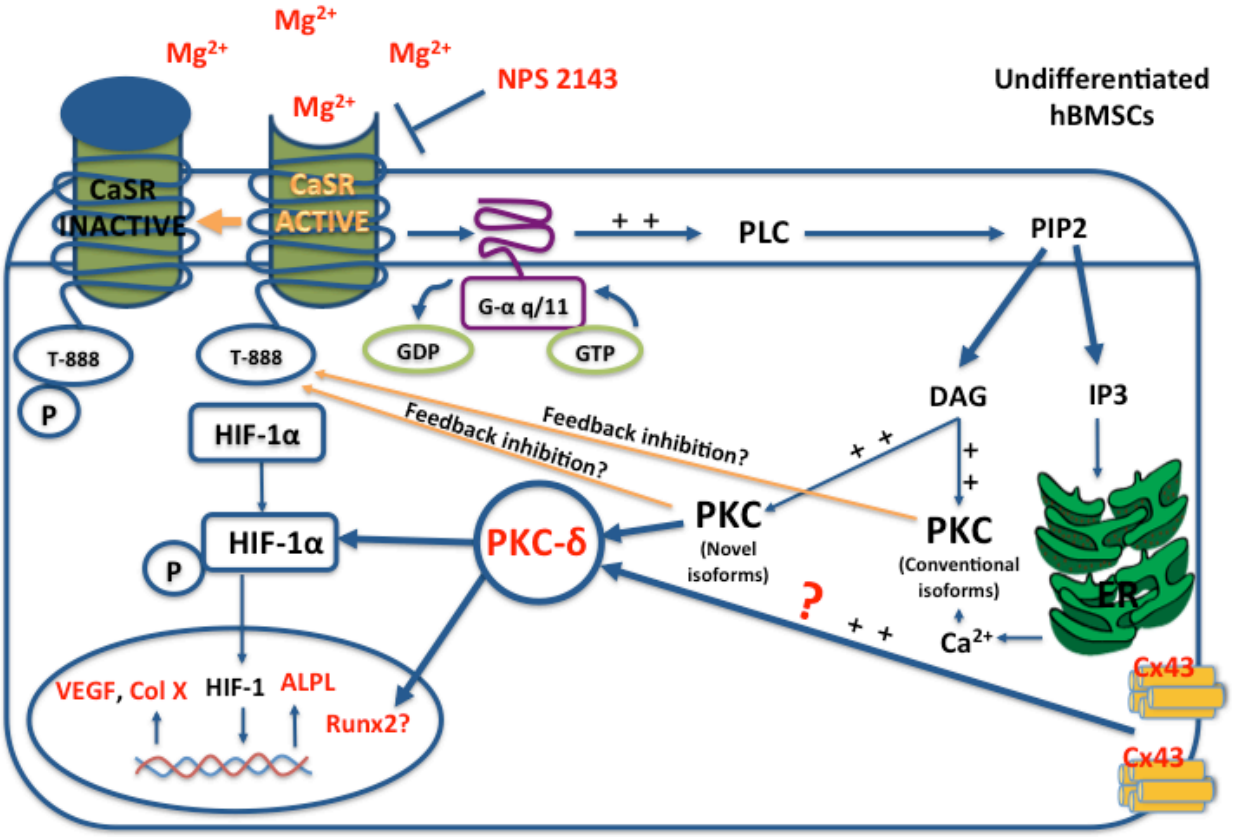


Figure 10. A diagrammatic representation of a hypothetical signaling mechanism activated by Mg^{2+} in hBMSCs. Stimulation of the CaSR by Mg^{2+} initiates a G-protein coupled receptor activation pathway that leads to $CaSR^{T888}$ phosphorylation in a PKC dependent manner. In addition, CaSR stimulation could activate the intracellular PKC-δ in a DAG-dependent manner. PKC-δ activation could promote osteogenesis through upregulating ALPL, Runx2, Collagen X and VEGF expression by hBMSCs. HIF-1α could link PKC-δ to VEGFA and Collagen X (Col10A1) expression. Finally, the gap junctional protein Cx43 could potentiate the osteogenic effects of PKC-δ.

6.0 CONCLUSION

This study sheds the light on the potential cellular mechanisms underlying Mg-mediated osteogenesis. We investigated the involvement of the CaSR and its downstream kinases PKC-δ and Erk1/2 in the osteogenic response of hBMSCs to Mg^{2+}. Our data suggest a possible role for PKC-δ and the gap junctional protein Cx43 in promoting neovascularization and bone matrix mineralization around Mg implants. This role of PKC-δ might be regulated by a CaSR upstream signaling mechanism. Further functional studies need to be conducted to confirm the findings presented in this work. However, those findings could be useful in designing future mechanistic studies aiming to understand the biological effects of Mg on the bone healing process.

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