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High extracellular magnesium inhibits mineralized matrix deposition and modulates intracellular calcium signaling in human bone marrow-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) have the potential to differentiate into several cell types and provide an attractive source of autologous cells for regenerative medicine. However, their cellular biology is not fully understood. Similar to Ca^{2+}, extracellular Mg^{2+} plays an important role in the functions of the skeletal system. Here, we examined the effects of extracellular Mg^{2+} on the deposition of calcium phosphate matrix and Ca^{2+} signaling with or without ATP stimulation in human bone marrow-derived mesenchymal stem cells (hBMSCs). We found that high extracellular Mg^{2+} concentration ($[Mg^{2+}]_e$) inhibited extracellular matrix mineralization in hBMSCs in vitro. hBMSCs also produced a dose-dependent decrease in the frequency of calcium oscillations during $[Mg^{2+}]_e$ elevation with a slight suppression on oscillation amplitude. In addition, spontaneous ATP release was inhibited under high $[Mg^{2+}]_e$ levels and exogenous ATP addition stimulated oscillation reappear. Taken together, our results indicate that high $[Mg^{2+}]_e$ modulates calcium oscillations via suppression of spontaneous ATP release and inactivates purinergic receptors, resulting in decreased extracellular mineralized matrix deposition in hBMSCs. Therefore, the high magnesium environment created by the rapid corrosion of Mg alloys may result in the dysfunction of calcium-dependent physiology processes and be disadvantageous to hBMSCs physiology.

1. Introduction

Mesenchymal stem cells (MSCs) have the potential to differentiate into several cell types and provide an attractive alternative source of cells for regenerative medicine [1,2]. As they have become more available in recent years, various studies are providing new information about physiological processes of MSCs, including proliferation, differentiation and mineralization. However, their cellular biology is not fully understood, especially those cellular activities regulated by the cytosolic Ca^{2+}.

Calcium is a highly versatile intracellular messenger responsible for controlling numerous cellular functions [3]. The calcium signaling pattern of human MSCs occurs as repetitive spontaneous calcium oscillations. These oscillations in MSCs are initiated by autocrine/paracrine ATP via the activation of the inositol trisphosphate receptors (IP_3Rs) mediated Ca^{2+} release from intracellular stores and sustained by Ca^{2+} influx/extrusion through cell membrane. The role of the Ca^{2+} oscillation is still unknown but they have been conclusively shown to regulate cellular processes. Cells have frequency or amplitude coding and decoding properties [4,5]. The evidence for calcium oscillation involvement in cellular physiology is that Ca^{2+} spikes can initiate gene expression more effectively than a steadily maintained level of the same average intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Consistently, some recent studies revealed manipulating Ca^{2+} oscillations in hBMSCs can indeed regulate their proliferation and differentiation and mineralization [6–8].

Magnesium and calcium are crucial components of bone and play important roles in skeletal health. An appropriate balance between $[Mg^{2+}]$ and extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) is associated with normal cerebellar, cardiovascular and skeletal functions. Significant fluctuations in $[Mg^{2+}]_e$ can result in changes in $[Ca^{2+}]_i$ as well as a variety of cellular process dependent on it [9–11]. Epidemiology has linked insufficient magnesium intake with osteoporosis [12] while the effect of high magnesium on

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skeletal system still remains to be clarified. During the fast degrading process, local magnesium concentration on the surface of a biodegradable magnesium implant may be higher than physiological concentration in intercellular space in bone tissue. In addition, some puzzling clinic data have shown that high magnesium status leads to mineralization defects [13] and osteopenia [14], leading to a question on excess magnesium's detrimental effect on skeletal system. In this study, we aimed to investigate the effect of high $[Mg^{2+}]_e$ on extracellular mineralized matrix deposition in hBMSCs and the mechanism involved in its calcium signaling modulation. These findings provide a new perspective on the molecular mechanisms involved in hBMSCs physiology and novel information regarding the use of magnesium-based alloys as biomaterials.

2. Materials and methods

2.1. hBMSCs isolation and expansion

All experimental protocols involving bone marrow collection were approved by the Ethics Committee of Shanghai Tongji University School of Medicine, China. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were isolated and expanded according to the methods reported by Pittenger [15] with some modifications. The bone marrow aspirates were placed on tissue culture dishes in α-MEM containing 10% fetal bovine serum and 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA, USA) in a 37 °C, 5% CO_2 environment. At the end of the second passage, the hBMSCs were cryopreserved until use. We used hBMSCs with less than five passages in the following experiments.

2.2. Alizarin Red staining

hBMSCs were grown in a 24-well plate (six wells per group) in control medium (0.8 mM Mg^{2+}), osteoinductive medium (OM, with 0.8 mM Mg^{2+}) or osteoinductive medium supplemented with different magnesium (1.05 mM Mg^{2+}, 1.3 mM Mg^{2+}, 1.8 mM Mg^{2+} or 3.8 mM Mg^{2+}) for 21 days. For all osteoinductive medium was supplemented with a cocktail of 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate (Sigma–Aldrich, St. Louis, MO, USA). The medium was replaced every 3 days. After 21 days, Alizarin Red staining was performed as described elsewhere [16] to quantify the mineralization. Cells were observed and recorded with an inverted epifluorescence microscope (Olympus 1 × 2-ILL 100).

2.3. Calcium dye loading and fluorescence imaging

Ca^{2+} imaging was conducted using confocal microscopy scanning technique as described previously [8]. Briefly, Cells were loaded with 10 μM fluo-4 acetoxymethyl ester (AM) (Molecular Probes, Invitrogen) dissolved in HBSS (HEPES-buffered saline) solution for 30 min at 37 °C in the dark. HBSS solution contained (in mM): 121 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 25 HEPES, 1.8 $CaCl_2$, and 6.0 $NaHCO_3$ (pH = 7.3). The concentration of magnesium in α-MEM culture medium and standard bath solution is 0.8 mM. Thereafter, cells were rinsed with HBSS twice and the loaded dye was allowed to de-esterify for 20 min at 37 °C in the dark. Coverslips were then mounted on a temperature-controlled perfusion chamber mounted on an inverted microscope (Leica, Wetzlar, Germany) and fluorescence data were recorded through a 40× quartz objective lens. Fluorescence images of $[Ca^{2+}]_i$ of calcium oscillations and after ATP stimulation were acquired in real time at 10 s and 2 s interval, respectively. In some studies, HBSS with different magnesium (1.3 mM Mg^{2+}, 1.8 mM Mg^{2+}, 3.8 mM Mg^{2+}, 5.8 mM Mg^{2+} and 10.8 mM Mg^{2+}) was achieved by magnesium addition.

2.3. Analysis of Ca^{2+} responses

We used pseudo-ratio $\delta F/F0$: $\delta F/F0 = (F - F_{base})/F_{base}$ to analysis calcium response in hBMSCs. F: measured fluorescence intensity of Fluo-4, F_{base}: the lowest level of fluorescence intensity in the cell. Ca^{2+} responses elicited by ATP showed characteristic biphasic decays with rapid and slow components. The amplitude of this secondary component was the distance from the well-defined intersection point between the rapid and slow phases of $[Ca^{2+}]_i$ and baseline level.

2.4. Adenosine 5′-triphosphate assay

The concentrations of ATP were measured using a luciferase-based ATPlite-M kit (Perkin–Elmer) as reported previously [17]. The ATP values were determined by an ATP standard calibration curve calculated on the basis of corresponding HBSS (i.e., matched $[Mg^{2+}]$ and $[Ca^{2+}]_e$) and expressed in absolute values (nM).

2.5. Statistical analysis

All statistical data are expressed as the mean ± one standard deviation (SD). Paired and/or unpaired Student's t-tests were used as appropriate to evaluate the statistical significance of differences between two group means (using SPSS 18.0 software). Significant differences were defined as $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

3. Results

3.1. High magnesium inhibit mineralization in vitro

To evaluate the effect of high magnesium on the matrix mineralization in vitro, hBMSCs were treated with different concentrations of $[Mg^{2+}]_e$ while osteoinduction. Extracellular calcium deposition was quantified after 21 days, mineralization was significantly inhibited when cultured in the presence of the OM with $\geqslant$1.3 mM $[Mg^{2+}]_e$ in a dose-dependent manner as compared to that observed in OM. But there is no significance between the 1.05 mM $[Mg^{2+}]_e$ group and the control group (Fig. 1). These results indicate that high $[Mg^{2+}]_e$ inhibits hBMSCs mineralization in vitro.

3.2. Effects of elevated $[Mg^{2+}]_e$ on frequency/amplitude of Ca^{2+} oscillations

Intracellular calcium is a key component in regulating extracellular mineralization [18]. As an antagonist for calcium, magnesium has been reported to modulate intracellular calcium signaling [19]. To test our hypothesis that high magnesium inhibit mineralizing capacity through interfering calcium signaling in hBMSCs, confocal imaging were used. Spontaneous and repetitive Ca^{2+} oscillations was successfully observed and was sustained in the HBSS containing 0.8 mM $[Mg^{2+}]_e$. About 56% of cells displayed robust and long lasting calcium oscillations. Fluorescence imaging revealed typical changes in $[Ca^{2+}]_i$ from hBMSCs (Fig. 2). Also, the quantitative data for the effects of high $[Mg^{2+}]_e$ on Ca^{2+} oscillatory frequency and amplitude are summarized. Increasing $[Mg^{2+}]_e$ from 0.8 to 1.3 mM did not evoke any statistically significant change in the average oscillatory amplitude or frequency. $[Mg^{2+}]_e$ at 1.8 mM and 3.8 mM significantly decreased calcium oscillation frequency by 37% ± 14% and 59% ± 18%, respectively. In addition, a substantial fraction of hBMSCs (32.5% and 64% respectively) no longer showed $[Ca^{2+}]_i$ oscillations. The average Ca^{2+} spike amplitude did not change significantly until hBMSCs were exposed to 3.8 mM $[Mg^{2+}]_e$ and only a slight reduction (10.5%) was observed (Fig. 3).

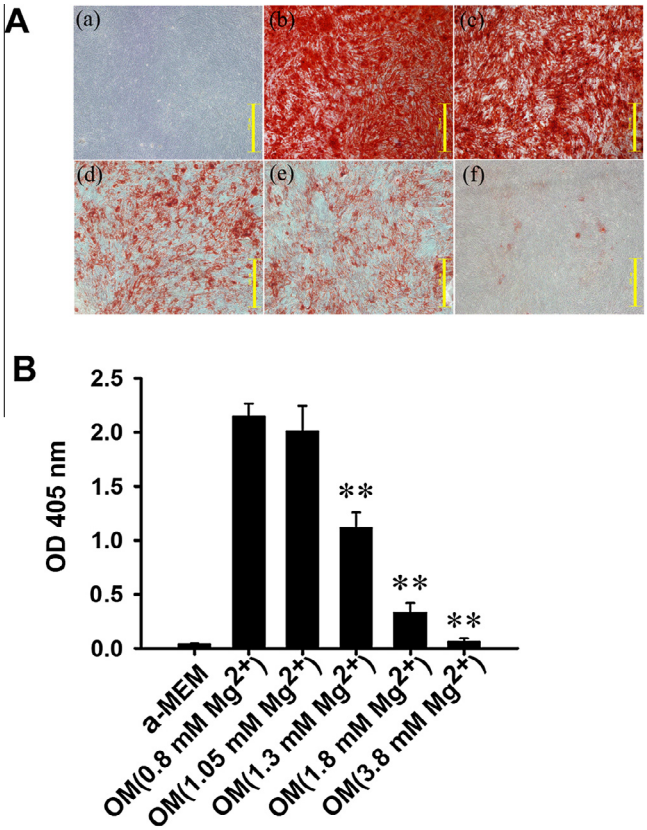

Fig. 1. High magnesium inhibits hBMSCs matrix mineralization in vitro. hBMSCs were cultured in α-MEM (a) or osteoinductive medium (OM) with 0.8 mM (b), 1.05 mM (c), 1.3 mM (d), 1.8 mM (e) and 3.8 mM (f) Mg^{2+} for 21 days. Matrix mineralization was evaluated by Alizarin Red staining (A) and quantified by acid extraction and the absorbance measured at 405 nm (B). $^{**}P < 0.01$ versus control group (OM 0.8 mM Mg^{2+}).

These results suggest that high magnesium suppress calcium oscillations in hBMSCs, primarily on oscillatory frequency.

3.3. Effects of $[Mg^{2+}]_e$ on ATP release

Spontaneous ATP release has been identified to be responsible for the generation of calcium oscillations [20] and its release is modulated by extracellular divalent cations such as $[Ca^{2+}]_e$ and $[Mg^{2+}]_e$. Thus, we investigated if $[Mg^{2+}]_e$ elevation affects spontaneous ATP release in culture. One hour after incubation with HBSS, the ATP concentration measured in the medium containing hBMSCs was 8.22 ± 0.72 nM. This value was significantly higher than that measured in the medium without cells, which was 0.38 ± 0.11 nM. ATP release from hBMSCs was significantly enhanced after incubated without Mg^{2+} (10.05 ± 0.44 nM). Increasing $[Mg^{2+}]_e$ from 0.8 to 1.3 mM lead to a statistically significant change in the average ATP level, 7.18 ± 0.53 nM, subsequent elevation of $[Mg^{2+}]_e$ 1.8 mM and 3.8 mM lead to a further reduction of ATP level to 5.95 ± 0.36 and 5.47 ± 0.25 compared to control (Fig. 4A). These results indicate that high $[Mg^{2+}]_e$ suppress spontaneous ATP release in vitro.

3.4. Effects of exogenous ATP on $[Ca^{2+}]_i$ oscillations under elevated $[Mg^{2+}]_e$

The receptor targets of extracellular ATP belong to one of two classes: ionotropic P2X receptors (P2XRs) and metabotropic G-protein coupled P2Y receptors (P2YRs) [21]. To further test our hypothesis that high $[Mg^{2+}]_e$ inhibits oscillatory frequency through ATP release inhibition, we applied exogenous ATP into incubation medium containing 0.8, 3.8, 5.8, and 10.8 mM $[Mg^{2+}]_e$. A concentration of 50 μM was used because it was previously reported to elicit calcium spikes [22]. ATP addition evoked a biphasic increase in $[Ca^{2+}]_i$ with a rapid initial phase followed by a secondary decay component with a time constant of decay. Calcium oscillation disappeared after the application of ATP and reappeared immediately or after minutes (Fig. 4B). In addition, as the amplitude of the initial phases under ATP stimulation is mainly due to the activation of P2Rs and were significantly decreased with $[Mg^{2+}]_e$ elevation. These results also indicated a dose-dependent depressive effect of increased $[Mg^{2+}]_e$ on the amplitude of the initial phases under ATP stimulation (Fig. 4C and D). 50 μM exogenous ATP effectively stimulated the reappearance of calcium oscillations among hBMSCs (Fig. 3E). In those hBMSCs which did not show oscillations after incubated with 3.8 mM $[Mg^{2+}]_e$, almost all (95%) the cells showed oscillations after ATP addition (Fig. 4F). These results suggest that exogenous ATP stimulates calcium oscillations reappear.

4. Discussion

Magnesium alloys have attracted much attention in orthopedics due to its biodegradability and good mechanical properties while there have been few studies questioned and investigated the side effects of high Mg^{2+} on bone cells. It should be noted that during the fast degrading process of magnesium alloy in the body, gas and alkaline environment are produced and local Mg^{2+} concentration on the implant surface may be higher than physiological concentration in the intercellular space in bone tissue, which could results in a detrimental environment for bone regeneration. Though our previous study did not find a suppression effect of high Mg^{2+} (3–10 mM) on hBMSCs osteogenic differentiation [23], we failed to find an increase in OPN level, a prominent component of mineralized matrices of bone and was secreted during osteogenic differentiation. Here we demonstrate that high magnesium (⩾1.3 mM) significantly suppressed mineralizing capacity of hBMSCs, which is consistent with those results that high $[Mg^{2+}]_e$ inhibits the matrix mineralization of VSMCs [24], ATDC5 cells [25], U2-OS cells [26] SaOS-2 cells and human osteoblasts [27]. This finding explains some puzzling clinic data that in patients with chronic renal failure or in individuals undergoing dialysis, serum Mg^{2+} concentrations are frequently elevated and correlate with mineralization defects [13]. Another intriguing set of studies also showed that premature infants would develop osteopenia and multiple fractures secondary to prolonged $MgSO_4$ maternal administration for preterm labor [14,28].

As an antagonist for calcium, it is feasible to propose that high $[Mg^{2+}]_e$ may alter intracellular Ca^{2+}/Mg^{2+} balance, thus leading to dysregulated Ca^{2+}-dependent cellular processes, such as extracellular matrix mineralizing [18]. Accordingly, high magnesium (5 mM) have been reported to inhibit osteoblast mineralizing activity in vitro, a phenomenon can be explained by that high magnesium reduce intracellular calcium by competing for the same transporters, for example, TRPM7 channel [9,27]. Also, high phosphate calcium-containing medium-induced vascular calcification was negatively regulated by high $[Mg^{2+}]_e$ (2–3 mM) through restored TRPM7 activity and increased expression of anticalcification proteins [24]. It should be noted that TRPM7 is required for MSCs survival and its expression is up-regulated during osteogenesis, suggesting its important role in hBMSCs physiology.

Cells often vary their frequency of Ca^{2+} waves in response to changes in stimulus intensity. In our study, the oscillatory frequency were suppressed immediately upon $[Mg^{2+}]_e$ reached 1.8 mM while amplitude didn't show reduction until $[Mg^{2+}]_e$ reached 3.8 mM. This is consistent with the idea that the specific information provided by repetitive Ca^{2+} spikes appears to be

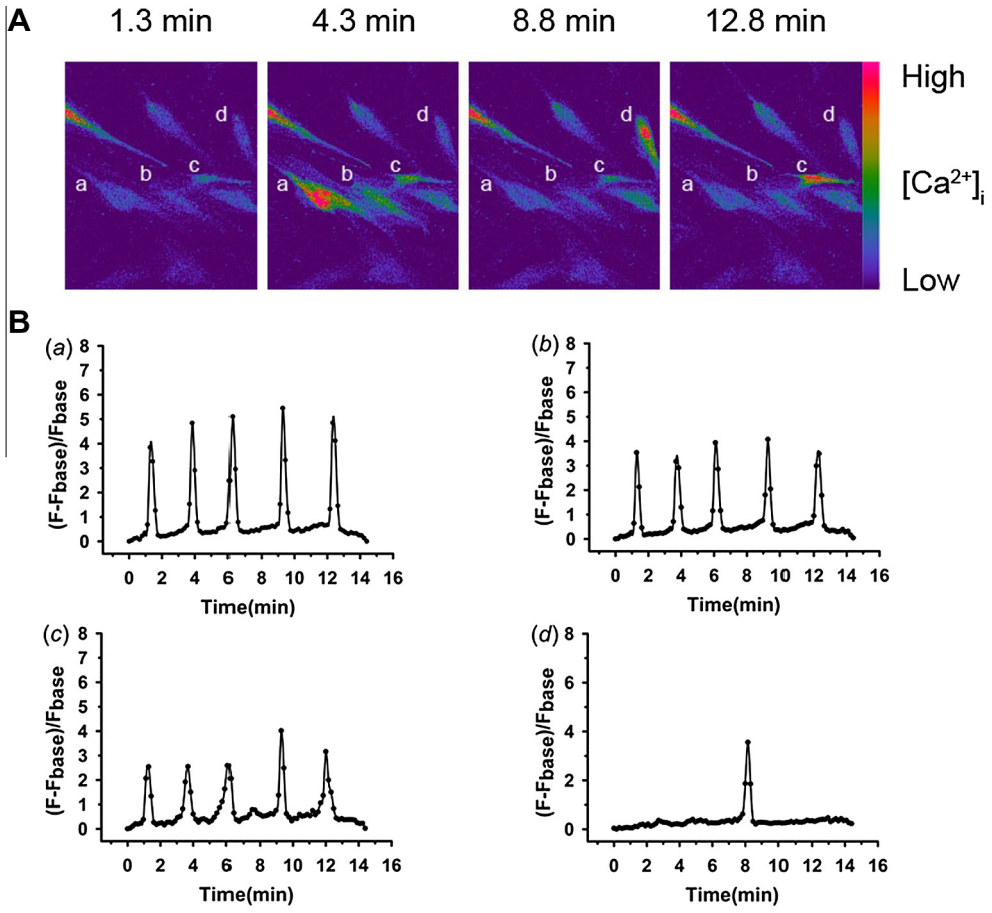

Fig. 2. Images of spontaneous $[Ca^{2+}]_i$ oscillations in hBMSCs. Images taken at 1.3, 4.3, 8.8, 12.8 min after beginning of the recording are shown (A). The time course of $[Ca^{2+}]_i$ in each cell indicated a–d in the image were plotted (B).

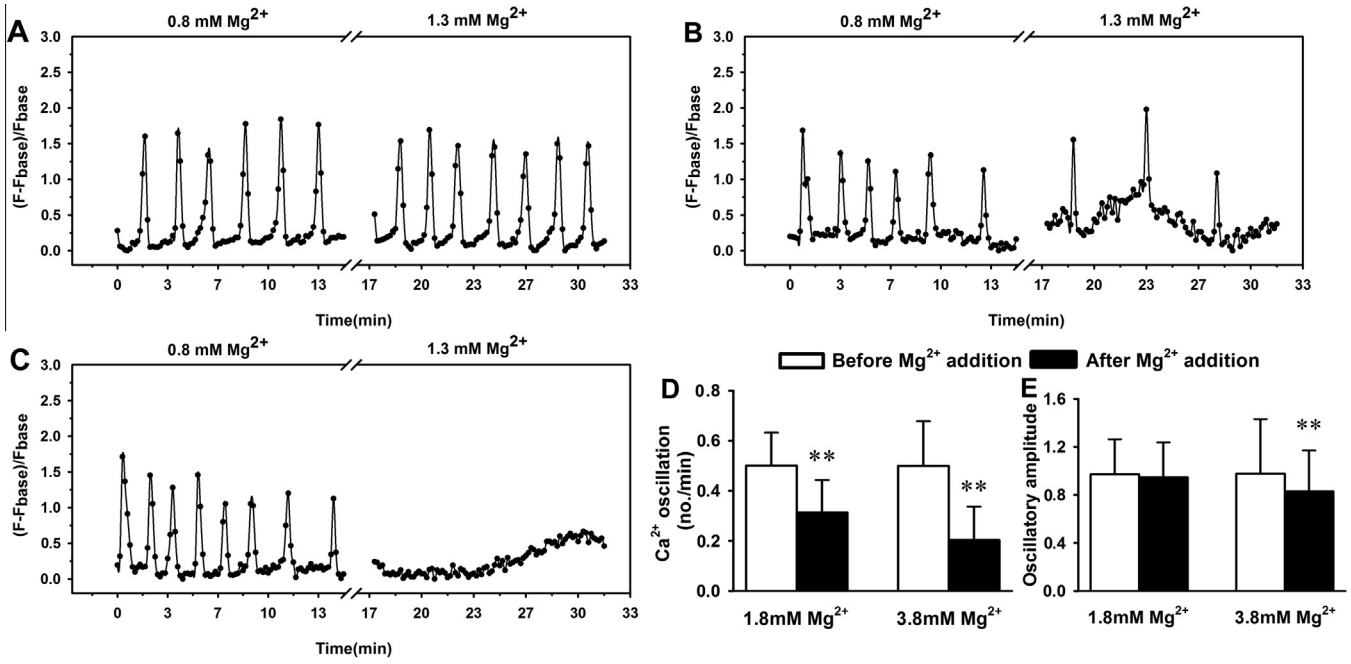

Fig. 3. Effects of high $[Mg^{2+}]_e$ on calcium oscillations. Representative time course of typical calcium oscillations of three hBMSCs before and after 1.3 mM (A), 1.8 mM (B) and 3.8 mM (C) Mg^{2+} treatment. Mean values of Ca^{2+} oscillation frequency and amplitude in the absence or presence of 1.8 mM and 3.8 mM Mg^{2+} (n = 24–30 cells) (D). Exogenous ATP induces calcium oscillations' reappearance under 3.8 mM $[Mg^{2+}]_e$ (E). $^{**}P < 0.01$ versus control group (0.8 mM Mg^{2+}).

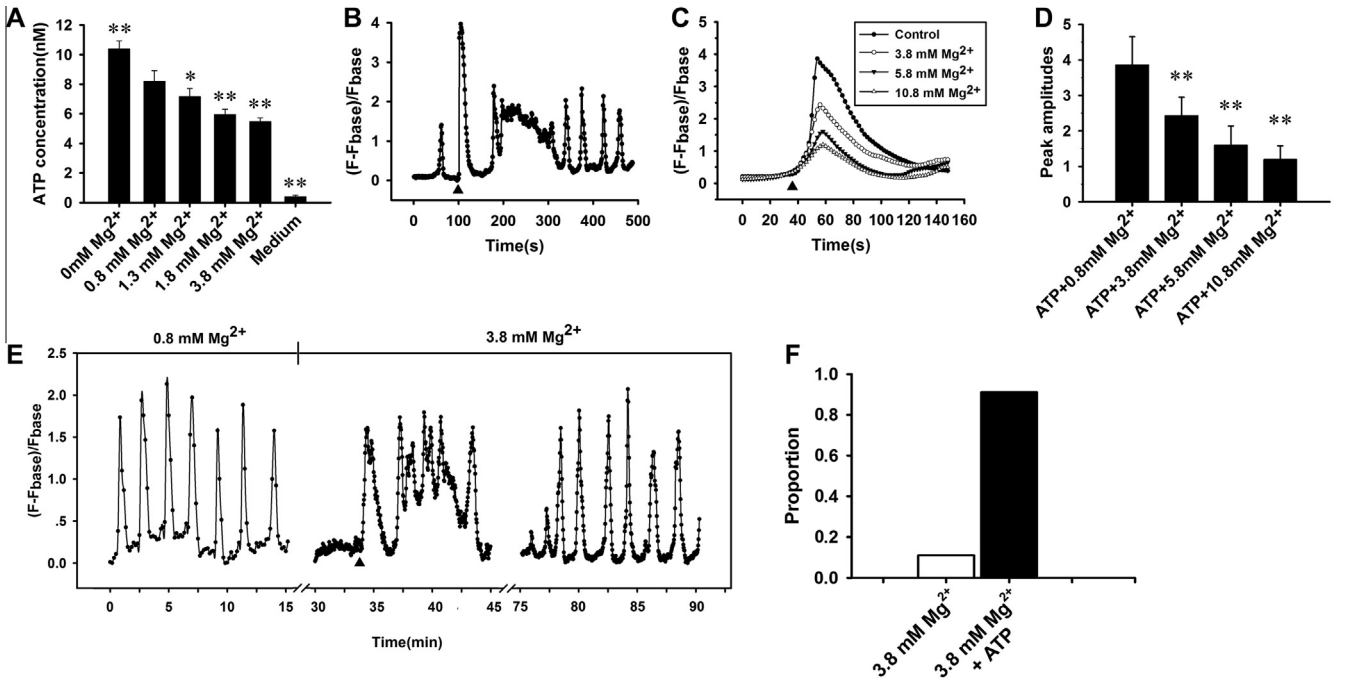

Fig. 4. Peak amplitudes suppression and ATP release inhibition. Extracellular ATP concentrations were measured in HBSS containing hBMSCs with different $[Mg^{2+}]_e$ 1 h after a medium change and in control medium not containing cells (A). * $P < 0.05$, ** $P < 0.01$ versus control group (0.8 mM Mg^{2+}). Representative time course of $[Ca^{2+}]_i$ evoked by 50 μM ATP application (black arrowhead) (B). Effects of high $[Mg^{2+}]_e$ on the response evoked by ATP ($n = 50$ cells) (C). Overall responses induced by ATP for the different cell treatments ($n = 5$ per treatment group) (D). ** $P < 0.01$ versus control group (ATP + 0.8 mM Mg^{2+}). Exogenous ATP induces calcium oscillations' reappearance under 3.8 mM $[Mg^{2+}]_e$ (E) and a higher proportion of hBMSCs exhibiting calcium oscillations were observed (F).

encoded in the frequency rather than in the oscillatory amplitude [29]. Furthermore, cells seem to have evolved sophisticated "decoding system" in order to use this frequency-modulated signaling system to decode the information embedded in the Ca^{2+} spikes. To date, several molecules such as NFAT, NF-κB, CaMKII, MAPK and calpain [5], have been reported to have frequency decoding properties. Kawano et al. reported that $[Ca^{2+}]_i$ oscillations promote the activation and nuclear translocation of NFAT in hBMSCs [20]. cADPR (cyclic ADP-ribose), a Ca^{2+} messenger, increases the frequency of Ca^{2+} oscillations and stimulates hBMSCs proliferation by enhancing phosphorylation of ERK1/2 [7] without altering adipogenesis and osteogenesis. Besides, adipogenesis and osteogenesis lead to cessation of Ca^{2+} oscillations [7], and manipulation calcium oscillations by electrical stimulation facilitates differentiation and mineralization [8]. Therefore, though it should be expected that this specific Ca^{2+} signaling pathway participates in multiple cellular processes regulation in hBMSCs including differentiation and matrix mineralization, this issue is insufficiently investigated and the underlying signaling pathways need to be clarified in further studies.

Magnesium's modulation on calcium oscillations can be explained by an inhibition on ATP release. Calcium oscillations in hBMSCs depend on spontaneous ATP release via gap-junction hemichannels [20]. A decrease in $[Mg^{2+}]_e$ and $[Ca^{2+}]_e$ have been reported to modulate spontaneous calcium signaling in numerous cell lines, an effect that result from increased connexin hemichannel opening and greater ATP release [10,19,30]. High $[Mg^{2+}]_e$ and $[Ca^{2+}]_e$ have a blocking effect on the open probability of connexin hemichannels [31,32]. In our work, the mean ATP value in the supernatant is 8.22 nM, similar to that from Coppi et al. (~7.5 nM) [17]. It experienced a 1.2-fold increase after incubation without $[Mg^{2+}]_e$, smaller than that after incubated without $[Ca^{2+}]_e$ [10] as Mg^{2+} has a less effective blocking effect than Ca^{2+}. In contrast, ATP level was reduced by $[Mg^{2+}]_e$ elevation (from 1.8 mM to 3.8 mM). Further, exogenous ATP stimulated oscillations reappear in cells whose oscillations were suppressed by elevated $[Mg^{2+}]_e$, indicating that high $[Mg^{2+}]_e$ modulate calcium oscillations through ATP release suppression. It should be noted that some of the "new" oscillations were more "strong" – a higher frequency and amplitude were observed, which can be attributed to higher local ATP concentrations.

In addition, as the initial phase Ca^{2+} increase under ATP stimulation is mainly due to the activation of P2Rs and extracellular Ca^{2+} influx [22]. Our experiments also discovered that high $[Mg^{2+}]_e$ (3–10 mM) suppresses P2Rs in hBMSCs. Recently, a variety of P2XR and P2YR subtypes have been detected in MSCs and were involved in various MSCs physiology [33]. Magnesium has been recognized as a dose-dependent inhibitor of P2Rs [34,35]. These reports are consistent with our results obtained through confocal imaging in which the rapid phases were effectively diminished by $[Mg^{2+}]_e$ ($\geqslant$3 mM) (Fig. 4C).

Collectively, the present study demonstrates that high $[Mg^{2+}]_e$ negatively regulates extracellular matrix mineralization and intracellular calcium signaling in hBMSCs, an effect can be explained by spontaneous ATP release inhibition. Thus, if the molecular imprint for cell lineage commitment is encoded in a specific code of calcium spiking, and oscillations represent a molecular code for the control of stem cell physiology programs, magnesium alloy implantation and stem cell therapies should be designed to ensure that the local environment between stem cells is modified because this interaction may be a prerequisite for successful implantation and proper cell commitment to organ-specific cell types. The elucidation of the physical roles of calcium oscillation and magnesium's role in calcium modulation could help identify novel approaches in understanding hBMSCs' physiology and could be useful for stem cell-based therapeutic applications and engineering tissue constructs. Further studies are required to elucidate how local environmental factors change during magnesium alloy corrosion in vivo, and also to address the specific downstream signaling of high magnesium's effect on calcium oscillatory frequency to

provide guidelines for the allowable concentration of magnesium ions produced during degradation in the future use of Mg as an orthopedic implant material.

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