Biological Responses and Mechanisms of Human Bone Marrow Mesenchymal Stem Cells to Zn and Mg Biomaterials

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ABSTRACT: Zn biomaterials attract strong attentions recently for load-bearing medical implants because of their mechanical properties similar to bone, biocompatibility, and degradability at a more matched rate to tissue healing. It has been shown previously that Zn alloys are beneficial for bone regeneration, but the supporting mechanisms have not been explored in detail. Here, we studied the biological responses of human bone marrow mesenchymal stem cells (hMSC) to Zn and the underlying cellular signaling mechanisms. Typical Mg material AZ31 was used as a comparative benchmark control. Direct culture of cells on the materials revealed that cell adhesion, proliferation, and motility were higher on Zn than on AZ31. Significant cytoskeletal reorganizations induced by Zn or AZ31 were also observed. Mineralization of extracellular matrix (ECM) and hMSC osteogenic differentiation, measured by Alizarin red and ALP staining and activities, were significantly enhanced when cells were cultured with Zn or AZ31. Quantitative PCR also showed the increased expression of bone-related genes including ALP, collagen I, and osteopontin. Using small RNA interference to knockdown related key molecules, we illustrated the mechanisms of Zn-induced cellular signaling. TRPM7 and GPR39 appear to be the major cellular receptors facilitating Zn2+-entry into hMSC. The intracellular Zn2+ then activates the cAMP-PKA pathway and triggers intracellular Ca2+ responses, leading to activation of MAPK. In addition, Zn2+ activates the Gαq-PLC-AKT pathway as well. Eventually, all of this signaling would lead to enhanced differential regulation of genes, cell survival/growth and differentiation, ECM mineralization, osteogenesis, and other cellular activities.

KEYWORDS: biodegradable metal, magnesium alloy, AZ31, cell proliferation, cell differentiation, osteogenesis, zinc receptor

INTRODUCTION

Biodegradable metals have attracted a great amount of attention in the field of biomaterials and tissue engineering in recent decades. The biodegradable metallic implants are expected to degrade gradually in vivo, with minimal host response caused by released corrosion products, then dissolve completely after fulfilling the mission to help tissue healing without implant residues.1,2 Three main biodegradable metals are magnesium (Mg), ferrous (Fe), and zinc (Zn). Research has mainly focused on Mg- and Fe-based materials in the past decades. However, both materials have clinical disadvantages. Major issues with Mg materials are their fast degradation, rapid loss of mechanical strength, and harmful hydrogen evolution.3–8 For Fe materials, on the contrary, their degradation rates are generally too slow to be applied clinically, and the long retention of the degradation residues would lead to long-term complications in human body.1,2,9

Recently, Zn-based metallic materials have emerged as an alternative candidate for cardiovascular and orthopedic applications.10–16 Their mechanical and physical properties are closer to that of human bone than stainless steels or titanium alloys.14,15 Compared to Fe- and Mg-based biodegradable alloys reported so far, Zn-based alloys do not possess the limitations of unmatched corrosion rate to tissue healing and lack of ductility. In addition, Zn is one of the most abundant essential elements in human body, playing essential roles in human health.16–19 Zn is a cofactor of over 300 enzymes and other macromolecules.19 Furthermore, it is reported that Zn exhibited good biocompatibility to promote vascular and new bone formation around the implants.16–19 This feature can greatly increase the possible health benefits of a biodegradable Zn implant. Unfortunately, the related research on this new type of promising biodegradable materials, especially the cell–Zn material interactions, is still largely missing in the literature.

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The objective of the present research was to study the biological responses of human bone marrow mesenchymal stem cells (hMSC) to Zn, and the underlying cellular signaling pathways through which Zn enhances tissue regeneration. Typical Mg material AZ31 was used as a comparative benchmark control. We hypothesized that direct culture of hMSC with Zn materials would lead to an increased exposure of cells to Zn ions, which would improve the osteogenic gene expression, ECM production, and mineral deposition. The use of hMSC in this study was justified by their important roles in early implant osteointegration and bone regeneration in vivo.20 This study provides some first evidence on the biological responses of hMSC at the interface including direct cell adhesion, proliferation, motility, cytoskeletal reorganization, stem cell differentiation, matrix protein gene expression and production, and mineral deposition, and so forth. More importantly, first evidence on the mechanistic insight of hMSC cellular signaling pathways triggered by Zn ion was also reported. The identification of these responses and pathways will lead to improved Zn-based implant design in the future and other possible therapeutic applications of Zn.

MATERIALS AND METHODS

Material Preparation. Mg alloy AZ31 (Mg96/Al3/Zn1) and pure Zn (99.9998%) rods with 10 mm diameter were purchased from Goodfellow and Alfa Aesar, respectively. Metal rods were cut (Techcut 5, Allied High Tech Products, US) into 10 mm × 2 mm disc and polished (EcoMet 250 Grinder, Buehler, Lake Bluff, IL, U.S.A.) with SiC paper. The metal discs were cleaned by ultrasonically (M2510 Ultrasonic cleaner, Branson, U.S.A.) in acetone bath for 5 min. Before test, the metal discs were sterilized by UV light for at least 30 min on each side.

Protein Adsorption Assay. The protein adsorption on AZ31 and Zn were studied by Pierce BCA protein assay kit (Thermo Fisher Scientific, U.S.A.). The metal discs were put into 24-well plates and 100 μL bovine serum albumin (BSA, 1 mg/mL) was pipetted onto the disc surface. The plate was incubated in a hydrated incubator at 37 °C for 1, 2, and 4 h. After the incubation, nonadherent BSA was washed gently with 1× DPBS. Then, 200 μL 2% SDS solution was added to each well and the plate was incubated overnight at 37 °C. The solutions were collected and 0.1 mL of those solutions were put into a tube. Then 2.0 mL working reagent was added to each tube and mix thoroughly. The tubes were incubated at 60 °C water bath for 30 min and then cooled down to room temperature. Subsequently, absorbance was read at 562 nm (UV−vis, Thermo Fisher Scientific, U.S.A.) within 10 min. The absorbed BSA was determined from a prepared standard curve.

Cell Culture. Human mesenchymal stem cell (hMSC) derived from bone marrow were purchased from PromoCell (Germany). The culture medium was α-MEM without nucleotides (Thermo Fisher Scientific, U.S.A.) containing 10% FBS (Thermo Fisher Scientific, US) and 1% penicillin/streptomycin (Thermo Fisher Scientific, US). Cells were maintained at 37 °C, 5% CO2, and 95% relative humidity and the medium was changed every 2 days. After reaching ~80% confluence, cells were detached by trypsin/EDTA solution (Thermo Fisher Scientific, U.S.A.). Culture medium was used to stop the cell detachment and solutions were centrifuged at 500g for 5 min. Supernatant was removed and cell pellet was resuspended in culture medium. Cell solution was diluted to specific density for different assays.

To verify MSC phenotypes after culture with Zn or Mg materials in normal culture conditions, cells were stained for specific surface markers. Cells exhibited ~95% positive for CD73, CD105, and CD90, and ~95% negative for CD45, CD34, CD14, and CD19, suggesting no phenotype changes of hMSC. For alkaline phosphatase staining and alizarin red staining, the differentiation medium used was hMSC Osteogenic BulletKit (Lonza, U.S.A.). In some specific testing, cells were exposed to certain concentrations of media extract from degraded samples according to the extract culture methods (ISO 10993-5 and -12). Extract medium was prepared by soaking metal discs in culture medium at a ratio of 1.25 mL/cm² for up to 1 week at 37 °C, 5% CO2, and 95% relative humidity. Then extract solution was collected and diluted with culture medium to specific concentrations. The measured Zn ion concentration in the extract solutions we used for cells treatment was 20–30 μM, in line with a previous study.19

CyQUANT Direct Proliferation Assay. Metal samples were soaked in medium with serum for 4 h before cell culture. hMSCs were seeded onto the surface of AZ31 and Zn discs in 24-well plate at the density of 3000 cells/cm². Cells were incubated in humidified incubator at 37 °C, 5% CO2 for 7 days and 14 days. After incubation, the culture medium was replaced by 1× detection reagent (diluted with culture medium), and the plate was incubated at 37 °C for 1 h. The fluorescence was read from the bottom at 480/520 nm (Molecular Devices, U.S.A.). Culture media with and without cells were used as the positive and negative control, respectively. The cell proliferation rate was then determined by following the manufacture’s instruction.

Cell Viability. The MTT assay (Thermo Fisher Scientific, U.S.A.) was used to determine the cell viability as described.18,19,21–23 30 000/cm² cells were seeded onto the surface and incubated at 37 °C, 5% CO2, and 95% relative humidity for 1, 7, and 14 days. Culture medium with and without cells were used as positive and negative control, respectively. The cell viability was calculated according to the following formula: Cell viability = (Aexperiment − Anegative)/(Apositive − Anegative).18 Cell population doubling time was also calculated based on the MTT data.

Calcein AM Staining. For each metal disc, 10,000/cm² cells were seeded onto the surface and incubated at 37 °C, 5% CO2, 95% relative humidity for 1, 7, and 14 days. After incubation, and cell debris was washed away gently using DPBS (Thermo Fisher Scientific, U.S.A.). 500 μL calcein AM (Thermo Fisher Scientific, U.S.A.) solution was added and incubated for 30 min at 37 °C. And then cell debris was washed away gently using DPBS. The metal discs were inversely put into the plate and images were taken by a fluorescent microscopy (EVOS, AMG, U.S.A.).

Cytoskeleton Staining and Cell Morphology. Cytoskeleton staining and cell morphology were determined as described before.19 Briefly, hMSCs were seeded into the cover glass (Thermo Fisher Scientific, U.S.A.) in a 24-well plate at the density of 10 000 cell/well and allowed to attach for 24 h. Cell medium was replaced by specific extract medium (25%) and cultured for 3 days. After incubation, and cell debris was washed away gently using DPBS. Cells were fixed with 4% formaldehyde for 15 min at room temperature and then solution was removed and cells were washed with 1× DPBS for 3 times. After that, cells were permeabized by 0.5% Triton X-100 for 15 min and then washed by 1× DPBS for 3 times. Following that, cells were blocked by 1% BSA (Thermo Fisher Scientific, U.S.A.) solution for 1 h. After blocking, and cell debris was washed away gently using DPBS. One drop of ActinGreen 488 ReadyProbes Reagent (Thermo Fisher Scientific, U.S.A.) was added to each well, and the plate was incubated at room temperature for 30 min, protected from light. Cells were washed by 1× DPBS for 3 times, and the cover glass was inversely put on a clean glass slide (Thermo Fisher Scientific, U.S.A.) containing one drop of SlowFade Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, U.S.A.). The cover glass was sealed by CoverGrip Covership Sealant (Biotium, U.S.A.), and cells were incubated in the dark for 30 min. Images were taken by the fluorescent microscope (EVOS, AMG, U.S.A.) and cell morphology was analyzed by ImageJ (NIH, U.S.A.). For cell morphology characterization, at least 50 cells were used for each sample as a previous study.24

Cell Migration Rate Analysis. Cell migration analysis was performed as described before.24 Briefly, a gap in a cell monolayer was created by scratching the surface using a fine pipet tip, and cell debris was washed away gently using DPBS. Fresh medium was added, and cells were incubated for 24 h. Then images were taken (Advanced Microscopy, U.S.A.) and the width of the gap was measured using
Quantiﬁcation of hMSCs was assessed by Alizarin Red Staining. A BCA assay was performed to determine the total protein in the supernatant. Supernatant was put into a tube and equal volume of DMEM was added to each well and the plate was incubated at room temperature for 30 min. After centrifugation, 200 μL supernatant was collected and 200 μL ammonium hydroxide was added to neutralize the acid. Then the absorbance of the supernatant was read at 405 nm (UV−vis, Thermo Fisher Scientiﬁc, U.S.A.).

Alkaline Phosphatase Staining and Activity. Alkaline phosphatase (ALP) staining assay was performed with StemTAG Alkaline Phosphatase Staining and Activity Assay Kit (Thermo Fisher Scientiﬁc, U.S.A.) as described before.19 Brieﬂy, hMSCs were seeded into the 6-well plate at the density of 50 000 cells/plate and allowed to attach for 24 h. The cell differentiation medium was replaced by extract medium which was changed every 3 days. After incubation, cell medium was removed and cells were washed by 1× DPBS for 3 times gently. Cells were harvested after 7 days. For ALP activity assay, the cells were lysed by Cell Lysis Buffer and incubated for 10 min at 4 °C. The cell lysate was collected and 200 μL supernatant was mixed with 1× Stop Solution and mixed well. The absorbance was read at 405 nm (UV−vis, Thermo Fisher Scientiﬁc, U.S.A.). The average adsorbed BSA on surfaces of AZ31 and Zn materials for 1, 2, and 4 h (scale bar = 50 μm).

Alizarin Red Staining and Activity. The osteogenic differentiation of hMSCs was assessed by Alizarin Red Staining Quantification Assay (ARS, ScienCell, U.S.A.). Cells were seeded into the 6-well plate at the density of 50 000 cells/well and attached for 24 h. hMSCs were cultured for 21 days with extract medium which was changed every 3 days. After incubation, cell medium was removed and cells were washed with 1× DPBS for 3 times gently. Cells were ﬁxed by 4% formaldehyde solution (Santa Cruz Biotecnology, U.S.A.) for 15 min at room temperature and then ﬁxing solution was removed, and the cells were washed by deionized water. The deionized water was removed and 40 mM ARS was added to each well. The plate was kept at room temperature for 30 min with gentle shaking. After that, ARS were removed and washed with deionized water for 5 times. Images were taken by microcopy (Zeiss, U.S.A.) and at least 5 different ﬁelds were taken for each sample.

To quantify the mineral deposit, 800 μL of 10% acetic acid was added to each well and the plate was incubated at room temperature for 30 min with gently shaking. Cells were lysed by cell scraper, and the lysate was collected to a small tube. The tube was sealed with parafilm and the cell lysate was heated at 85 °C for 10 min. Then the tube was put on ice for 5 min and cell lysate was centrifuged at 20 000×g for 15 min. After centrifugation, 200 μL supernatant was collected and 200 μL ammonium hydroxide was added to neutralize the acid. Then the absorbance of the supernatant was read at 405 nm (UV−vis, Thermo Fisher Scientiﬁc, U.S.A.). ARS concentration was determined according to a prepared standard curve.

Real Time-PCR. Real time-PCR was used to examine gene expression using a CFX96 Touch RT-PCR Detection System (Bio-Rad, U.S.A.) as described before.23 Brieﬂy, cells were harvested after treatment and total RNA was extracted with an RNasy Mini Kit (Qiagen, U.S.A.). Amount of RNA was quantiﬁed using a spectrophotometer (Nanodrop 2000, U.S.A.) with OD260/OD280 ratios between 1.9 and 2.1. An RT2 First Strand Kit (Qiagen, U.S.A.) for reverse transcription was used in a thermo cycler (T100, Bio-Rad, U.S.A.). The mRNA levels of different genes/proteins were then estimated. Levels of mRNA of interest were normalized to the endogenous control, β-actin. Gene expression data were then analyzed in Bio-Rad CFX Manager 3.1 software using the 2−ΔΔCt method to calculate the level of changes in gene expression.

Gene Silencing. Different siRNA constructs were purchased from Sigma-Aldrich, and were utilized for silencing of proteins of interest. Cells were cultured to subconfluent in standard culture media without antibiotics, and then were transfected with siRNA using Lipofectamine 2000 (Thermo Fisher Scientiﬁc, U.S.A.) according to the manufacturer’s instruction.

Calcium Imaging. Cells were cultured in cover sliders chambers, and were incubated with 3 μM Fura-2 acetoxyethyl ester for 30 min in Ringer’s solution with 0.1 BSA. The cells were then washed in Ringer’s solution, and cover sliders chambers were mounted into the
microscope for imaging. All treatments with Zn$^{2+}$ were applied in without serum to avoid chelation. Furo-2 was excited at 340 and 380 nm, and imaged with a s10 nm filter. Furo-2 fluorescence change was determined by the rate of initial normalized fluorescence change (ΔR/Δt) as previously described.24

**Western blot Analysis.** Western blot was performed as described before.25 Briefly, cells lysates were collected and protein concentrations were determined using the Bradford assay. Then equal amounts of protein for each sample were subjected to SDS/PAGE electrophoresis, and proteins were subsequently transferred onto nitrocellulose membranes (0.45 μm, Bio-Rad). After blocking of nonspecific binding with 5% (w/v) nonfat dried skimmed milk TBST solution, primary antibodies against protein of interest and corresponding secondary antibodies conjugated with horseradish peroxidase were applied. The β-actin or total forms of proteins of interest was used as loading control.

**cAMP Direct Immunoassay.** The cAMP concentration was measured using a cAMP Direct Immunoassay Kit (Abcam, U.S.A.) by following the manufacturer’s protocol. Briefly, cell supernatants after treatment were collected. Samples were then neutralized and acetylated with Acetylating Reagent and Neutralizing Buffer, respectively. Samples were added to the wells precoated with Protein G in a 96-well plate. Next, cAMP antibody was added, followed by incubation at room temperature for 1 h with gentle shaking. After that, cAMP-HRP was added with additional 1 h incubation. After washing with 1× cAMP Assay Buffer, the amount of cAMP-HRP bound to the plate was determined by reading HRP activity at OD 450 nm. The intensity of OD 450 nm was inversely proportional to the concentration of cAMP in samples.

**PKA Activation.** PKA activation was determined using PKA kinase activity assay kit (ab139435, Abcam, U.S.A.) by following manufacture’s protocol. Cell lysates were collected, and protein concentration was adjusted to 4 mg/mL using BCA protein assay. About total of 2 μg protein of each sample were analyzed according to the manufacturer’s protocol.

**Statistical Analysis.** All data were presented as mean ± standard deviation. Each assay had three replicates and repeated 3 times independently. One-way ANOVA or Student t test was used to analyze the statistical significance. P < 0.05 was considered as statistically significant.

**RESULTS**

**hMSC Adhesion on AZ31 and Zn Materials.** The direct culture method takes the dynamic degradation processes of materials into account and makes it possible to probe cell behaviors directly on the material surface. Thus, we cultured hMSC on top of the bare AZ31 and Zn materials for a time course up to 14 days (Figure 1). First, we measured the plasma protein adsorption on both materials for a time course up to 4 h (Figure 1C), because plasma protein adsorbed on the material surface could facilitate cell attachment by serving as the bridge between the materials and cells. We found that the overall adsorbed BSA increased over time. The amount of adsorbed BSA on Zn was significantly higher than that on AZ31 after 4 h. To obtain a maximum adhesion, we seeded cells with a relatively high initial density (30 000 cells/cm²). Although the initial seeding density of cells was same for AZ31 and Zn, the viable cell adhesion density on Zn was significantly higher than that on AZ31 for the entire experimental period. The overall cell density on Zn did not change significantly over time, and the material surface was almost completely covered by cells from day 1 to day 14. On the contrast, the cell density on AZ31 was significantly less than that on Zn after 1 day indicating only a small portion of the seeding cells established themselves on the AZ31 surface 1 day later, and then the cell density increased gradually over time, and reached about 70% of that on Zn after 14 days.

**hMSC Viability and Proliferation on AZ31 and Zn Materials.** To quantitatively measure the survival and growth of hMSC after cultured directly on the surface of AZ31 and Zn materials, we examined the cell proliferation rate and viability over a time course up to 14 days. To reduce the potential contact inhibition, we used a lower seeding density of 3000 cell/cm² than that in the adhesion assay. Cell proliferation on Zn was significantly higher than that on AZ31 after 1 and 7 days of culture, but not after 14 days (Figure 2A).

**hMSC Morphology, Motility, and Cytoskeletal Organization on AZ31 and Zn Materials.** To gain further understanding of the interactions between hMSC and the materials at the interface, we examined the cell morphology and cytoskeletal organization after exposure to AZ31 and Zn materials (Figure 3). Cell morphology and cytoskeletal organization play key roles in cell survival/growth and

Figure 2. Cell proliferation and viability on AZ31 and Zn materials. (A) Normalized cell proliferation after direct culture of 1, 7, and 14 days on AZ31 and Zn as compared to control. (B) Normalized cell viability after direct culture of 1, 7, and 14 days on AZ31 and Zn as compared to control. (C) Cell doubling time after culture on AZ31 and Zn biomaterials.
functions. Thus, the effect of Zn or Mg on cells can be revealed by examining cell morphology and cytoskeleton, serving as an indirect indicator. The overall cell area was significantly higher on Zn while no obvious difference on cell perimeter was observed (Figure 3B, C). There was significantly more actin polymerization as shown by the amount of F-actin (Figure 3D) on AZ31 and Zn than control. More F-actin was distributed at the cell edge when cultured with AZ31 and Zn (Figure 3A), indicating the occurrence of cytoskeletal reorganization. Additionally, cell motility was significantly enhanced on AZ31 and Zn compared with that of control (Figure 3E).

hMSC Differentiation on AZ31 and Zn Materials. MSCs can differentiate into multiple cell lineages that resemble osteoblasts, chondrocytes, myoblasts, adipocytes, and fibroblasts, as well as express some of the key cell markers typical of endothelial cells, neuron-like cells, and cardiomyocytes. Here, we explored the effect of Zn or Mg on MSC differentiation toward bone cells because most applications of these biomaterials are in the field of orthopedic and bone fixations due to its own physical and mechanical properties. Effect of AZ31 and Zn materials on hMSC differentiation was first assessed by ALP analysis (Figure 4). It is interesting to find that both materials induced significant ALP activity of hMSC as compared to control. Next, the osteogenesis potency of hMSC was also examined by Alizarin red staining (Figure 5). Similar to ALP, both materials induced significant calcific deposition of hMSC as shown by the levels of Alizarin red staining. To further evaluate the osteogenic property of hMSC, we next evaluated the mRNA levels of different genes related to osteogenesis. The bone-related gene expressions of ALP, collagen I, and osteopontin were determined using quantitative PCR (Figure 6). Generally, both materials induced significantly higher levels of ALP, collagen I, and osteopontin as compared to control. Zn also had an even more obvious trend than AZ31 on promoting hMSC differentiation.

Molecular and Cellular Mechanisms of hMSC Responses to Zn. Results showed that both AZ31 and Zn materials could induce different hMSC responses and alter their behavior. The major components and degradation products of

![Figure 3. Cell morphology, cytoskeleton and motility on AZ31 and Zn materials. (A) Cell F-actin staining showing the morphology after 3 days of culture. (B) Average cell area. (C) Average cell perimeter. (D) Normalized F-actin intensity as compared to control. (E) Cell motility as measured by its migration rate (scale bar = 5 μm).](image)

![Figure 4. ALP staining and activity in hMSC. (A) ALP staining of hMSC after 1 day of culture in differentiation medium. (B) Quantitative measurements of ALP activity in hMSC after 1 day of culture in differentiation medium (scale bar = 20 μm).](image)

![Figure 5. Alizarin red staining and activity in hMSC. (A) Alizarin red staining of hMSC after 1 day of culture in differentiation medium. (B) Quantitative measurements of Alizarin red activity in hMSC after 1 day of culture in differentiation medium (scale bar = 20 μm).](image)
these two materials are Mg and Zn ions, respectively. The mechanistic cellular signaling for Mg has been illustrated recently,25−27 but not for Zn. Hence, we explored the molecular and cellular signaling pathways by culturing the Zn extract with hMSC directly. Given that TRPM7 and GPR39 are reported to transport Zn ion across cell membranes,24,28,29 we examined their role in Zn entry into hMSC. First, we controlled TRPM7 and GPR39 expression in hMSC as shown by immunofluorescence (Figure 7A, C) and RT-PCR (Figure 7B, D) using siRNA against TRPM7 and GPR39, respectively. Both cellular receptors are present on the cell surface, and the small RNA interference could effectively knock them down. Next, we explored the Ca^{2+} signaling and the downstream pathways involving cAMP, PKA, AKT, and MAPK (Figure 8). Extracellular Zn addition induced a significant Ca^{2+} response in hMSC (Figure 8A). Silencing of GPR39 or TRPM7 lead to partially diminished Ca^{2+} response triggered by Zn, while silencing both receptors at the same time led to a more complete blockage of Ca^{2+} response, indicating both receptors are important for Zn entry into hMSC. To further verify the response is triggered via the Gαq-PLC pathway as suggested before,24 we used pharmacological inhibitors and monitored the Zn-dependent Ca^{2+} response. Inhibition of the Gαq using YM254890 (1 μM) almost completely inhibited the Zn-dependent Ca^{2+} response (Figure 8B). The PLC/β inhibitor u73122 (10 μM) largely attenuated the signaling triggered by Zn, yet to a lower extent than the Gαq inhibitor or because of the fact that the PLC is downstream to the Gαq, thus inhibition of the latter suppresses the Ca^{2+} response more efficiently. Moreover, to further understand the mechanism of Zn on cell survival/growth, we examined the intracellular cAMP level and the activation of PKA as well as the AKT and MAPK pathways because they are the important modulators in cell growth and differentiation. The level of cAMP was significantly enhanced (Figure 8C), followed by increased PKA activity after Zn treatment (Figure 8D). Zn also induced significant phosphorylation of AKT and MAPK proteins suggesting the activation of such signaling molecules (Figure 8E–G). Similar to Ca^{2+} responses, silencing TRPM7 or GPR39 largely alleviated the activations of these kinases, but not completely. Knockdown of both receptors, however, had an additive effect on blocking the activation of these signaling molecules triggered by Zn ion.

Biodegradable metals are breaking the current paradigm in biomaterial design to develop only corrosion-resistant metals. Particularly, metals which consist of trace elements existing in the human body are promising candidates for biomedical implants. Zn has been recently studied sporadically as a new kind of biodegradable metallic materials. We investigated the cellular response and provided mechanistic insight of hMSC to Zn with Mg material as a comparative benchmark control. The data show that both Zn and Mg biomaterials supported hMSC attachment, proliferation, and induced cytoskeletal reorganization and ECM mineralization in vitro. The results support the hypothesis that exposure to Zn enhances hMSC growth, differentiation, osteogenic gene expression, matrix production, and mineral deposition through pathways involving Zn^{2+} entry through TRPMP7 and GPR39 receptors, intracellular cAMP and PKA activation, and subsequent AKT and MARK activation.

In this study, we found that hMSC attached to the Zn surface much more strongly and faster than AZ31. The overall cell density on Zn remained similar over the time course and maintained at a subconfluent level (Figure 1). However, the initial cell attachment on AZ31 was significantly lower, and the cell density increased over time. A possible explanation could be that Zn can adsorb more plasma proteins which facilitate cell adhesion as suggested by our data (Figure 2B). Also, hMSC proliferated faster on Zn than the controls (Figure 2A). This is in agreement with previous reports that Zn ion promoted vascular cells growth.18,19 Cell morphology and cytoskeleton are important indicators for cell health and function. Direct culture on Zn induced a larger cell area indicating a stronger attachment between the cells and the materials at the interface. In addition, the cell motility was higher on the Zn surface, which is supported by the observation that more F-actin is present in cells (Figure 3). These findings are in agreement with previous reports on vascular cells due to Zn or Mg ion treatment.18,19,21,23 MSCs can differentiate into multiple cell lineages that resemble osteoblasts, chondrocytes, myoblasts, adipocytes, and fibroblasts as well as express some of the key cell markers typical of endothelial cells, neuron-like cells, and

![Figure 6](image-url). Real-time PCR measurements of mRNA levels of ALP, collagen I, osteopontin of hMSC cultured with AZ31 and Zn materials. (A) Fold change of ALP mRNA level as compared to control. (B) Fold change of collagen I mRNA level as compared to control. (C) Fold change of osteopontin mRNA level as compared to control.
cardiomyocytes. In this study, we explored the effect of Zn or Mg on hMSC differentiation toward bone cells because most applications of these biomaterials are in the field of orthopedic and bone fixations due to its own physical and mechanical properties. As shown in the ALP and Alizarin red staining and activity data (Figures 4 and 5), the culture of hMSC on Zn and AZ31 resulted in an increase in ECM mineralization compared to control. Previous reports have shown similar effects of Mg ion on bone marrow stromal cell differentiation.26,27 Our qPCR data also reveal that culture of hMSC with Zn or AZ31 promoted expression of ALP and osteopontin (Figure 6), which represent the early and later marker of osteogenic differentiation, respectively. This is consistent with previous research on the ionic product of related Mg- or Zn-containing biomaterials which could support the osteogenesis of bone-forming cells.30 Our data also support the observations of in vivo bone regeneration promoted by Mg or Zn.13,25

The cellular mechanisms underlying osteogenesis properties of Mg have been illustrated both in vitro and in vivo recently.26,27 In a mouse animal model, it appears that Mg ion enters cells through TRPM7 and MAGT1 receptors, then triggers local neuronal production of CGRP, which triggers CALCRL- and RAMP1-dependent activation of cAMP-responsive CREB1 and SP7 to enhance osteogenic differentiation of the bone stem cells. In bone marrow stromal cells, Mg ion induces upregulation of COL10A1 and VEGF through HIF or NFAT pathways. In this study, our data show first evidence on the mechanisms involving Zn ion signaling (Figure 8), which are schematically represented (Figure 9). TRPM7 and GPR39 appear to be the major cellular receptors for Zn ion on hMSC.24,25 TRPM7 is a ubiquitous ion channel presenting on the cell membrane, which has a higher affinity for Zn2+ than Mg2+. GPR39 (or ZnR) is a specific Zn2+ receptor expressed on various cell types. After entry into cells, Zn triggers cAMP and PKA pathway, as well as the Gq signaling pathways. Then the Gq would subsequently activate the PLC and AKT signaling pathways. At the same time, Zn2+ may also induce intracellular Ca2+ response coupled with cAMP-PKA pathway, and the elevated Ca2+ level will next trigger many other Ca2+-dependent signaling cascades including MAPK and ERK. Taken together, all these pathways would lead to the differential regulation of genes, cell survival/growth, differentiation, ECM mineralization, and osteogenesis, and so forth.

It is noteworthy that Zn homeostasis and signaling are regulated by many different mechanisms and cellular receptors, including different Zn transporters, permeable channels, and metallothioneins, and are highly dependent on cell types.31 Thus, what we observed on Zn signaling in hMSC might be different from what has been reported in the literature. It has been suggested that Zrt-Irt-like proteins (ZIP) which have 14 mammalian members may be responsible for the movement of Zn into cytoplasm. Zn transporters (ZnT) which have 10 mammalian members may be responsible for Zn transport out of the cytoplasm. Recent studies have highlighted Zn’s dynamic activity and its role as a signaling mediator in a variety of cell types, capable of communicating between cells, converting extracellular stimuli to intracellular signals, and controlling cellular events. For example, ZIP13 was found to play an essential role in bone morphogenetic protein (BMP)/transforming growth factor β (TGF-β) signaling, and is involved in tooth, bone, and connective tissue development.32 ZIP14 can regulate G protein-coupled receptor (GPCR) signaling in the pituitary gland, liver, and cartilage, and is required for endocrine reactions and systemic growth.33 ZnT5 was shown to control protein kinase C (PKC) activity and nuclear factor kappa B (NF-κB)-mediated cytokine production in mast cells under Fc epsilon receptor I (FceRI) signaling.34

Our data show a possible Zn2+-stimulated intracellular signaling pathway in hMSC that may enhance the in vitro ECM mineralization and the in vivo bone regeneration. Our

Figure 7. TRPM7 and GPR39 protein and mRNA expression levels in hMSC. (A) Representative fluorescence staining of TRPM7 (green) and cell nuclei (blue) after siRNA silencing in hMSC. (B) Quantitative measurements of TRPM7 mRNA levels after siRNA silencing in hMSC. (C) Representative fluorescence staining of GPR39 (green) and cell nuclei (blue) after siRNA silencing in hMSC. (D) Quantitative measurements of GPR39 mRNA levels after siRNA silencing in hMSC (scale bar = 25 μm).
findings suggest that an appropriate concentration of Zn$^{2+}$ or Mg$^{2+}$ should be maintained in the healing bone tissue by controlling the degradation rate of those Mg- or Zn-based biomaterials in orthopedic applications. In addition, implant-derived Mg or Zn could be applied to treating defective bone diseases like Schmid-type metaphyseal chondrodysplasia, osteogenesis imperfecta, and osteoarthritis. When implants other than Mg- or Zn-based are used, it would be helpful to incorporate Mg or Zn as dopants to promote tissue regeneration.

**CONCLUSIONS**

Both Zn and Mg biomaterials can support hMSC adhesion and proliferation. Cell motility was higher on Zn than on AZ31.

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**Notes**

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