

# The effect of Mg–Ca–Sr alloy degradation products on human mesenchymal stem cells

Ida S. Berglund,<sup>1</sup> Elliott W. Dirr,<sup>2</sup> Vidhya Ramaswamy,<sup>1</sup> Josephine B. Allen,<sup>1,3</sup> Kyle D. Allen,<sup>2</sup> Michele V. Manuel <sup>1</sup>

<sup>1</sup>Department of Materials Science and Engineering, University of Florida, Gainesville, Florida 32611

<sup>2</sup>Department of Biomedical Engineering, University of Florida, Gainesville, Florida 32611

<sup>3</sup>Institute of Cell and Tissue Science and Engineering, University of Florida, Gainesville, Florida 32611

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**Abstract:** Biodegradable Mg alloys have the potential to replace currently used metallic medical implant devices, likely eliminating toxicity concerns and the need for secondary surgeries, while also providing a potentially stimulating environment for tissue growth. A recently developed Mg–Ca–Sr alloy possesses advantageous characteristics over other Mg alloys, having a good combination of strength and degradation behavior, while also displaying potentially osteogenic properties. To better understand the effect of alloy degradation products on cellular mechanisms, *in vitro* studies using human bone marrow-derived mesenchymal stem cells were conducted. Ionic products of alloy dissolution were found to be nontoxic but changed the proliferation profile of stem cells. Furthermore, their presence changed the progress of osteogenic

development, while concentrations of Mg in particular appeared to induce stem cell differentiation. The work presented herein provides a foundation for future alloy design where structures can be tailored to obtain specific implant performance. These potentially bioactive implants would reduce the risks for patients by shortening their healing time, minimizing discomfort and toxicity concerns, while reducing hospital costs. © 2017 The Authors Journal of Biomedical Materials Research Part B: Applied Biomaterials Published by Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 106B: 697–704, 2018.

**Key Words:** magnesium, strontium, osteogenic, stem cell, proliferation, gene expression, bioabsorbable

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## INTRODUCTION

Degradable magnesium (Mg) alloys have been identified as candidates for biomedical implants. These materials have been shown to possess high specific strength and a nearly ideal stiffness for bone fracture fixation.<sup>1,2</sup> Magnesium–calcium–strontium (Mg–Ca–Sr) alloys are potentially safer biomaterials than other Mg-based alloys, as the human body can easily metabolize their degradation products.<sup>3,4</sup> Previous research on the Mg–1 wt%Ca–0.5 wt%Sr alloy has identified improved properties as compared to alloys with similar chemistry, such as slower degradation and possible osteogenic characteristics.<sup>5,6</sup> A recent study showed this alloy was well tolerated by rats and the local tissue surrounding the implant, and furthermore, the healing rate paired well with the degradation rate of the alloy.<sup>7</sup> These results suggest the alloy may provide a stimulating environment for bone growth, which would be beneficial for applications involving bone repair.

Mg and Ca ions are essential to every cell.<sup>8,9</sup> They regulate hundreds of biochemical reactions in the body, including signals involved in stem cell differentiation.<sup>10</sup> Bone

marrow stem cells have the ability to differentiate into bone forming cells, and thus elucidating mechanisms involved at early stages of differentiation is crucial in understanding the effect on bone regeneration. Previous research has shown a beneficial effect of increased extracellular ion concentrations of Mg and Ca. For example, various additions of Ca ions can enhance proliferation and induce osteogenesis in human mesenchymal stem cell (hMSC) cultures in the absence of serum.<sup>11</sup> In hMSC cultures, in both the absence and presence of serum, addition of certain Mg concentrations can upregulate different gene expressions involved in tissue regeneration or osteogenic differentiation.<sup>12</sup> Multiple *in vitro* studies have also reported on the enhanced effects of Mg or Ca on osteoblast activity, such as improved growth<sup>13–15</sup> and alkaline phosphatase (ALP) expression.<sup>14,15</sup>

Both oral administration and *in vitro* studies confirm Sr can pharmacologically increase bone formation and reduce bone resorption.<sup>16–19</sup> At cellular level, Sr or Sr-containing compounds can activate different signaling pathways affecting both osteoblast and osteoclast activity.<sup>20</sup> Sr has also

**Correspondence to:** M. V. Manuel; e-mail: mmanuel@mse.ufl.edu

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been shown to enhance osteogenic differentiation of stem cells; upregulation of several osteogenic genes as well as an increase in ALP protein expression has been observed when culturing rat MSCs in Sr.<sup>21</sup> Increase in both gene- and protein expression of various osteogenic markers in Sr-treated osteogenic hMSC cultures has also been detected.<sup>22</sup> Furthermore, increased proliferation in both osteogenically-induced and nonosteogenic cultures of hMSCs has been seen, as well as increased *ALPL* protein activity in the osteogenic cultures containing various amount of Sr.<sup>23</sup> Recent studies have also acknowledged potential benefits of incorporating Sr into biomaterial surfaces and coatings to improve osseointegration and facilitate faster healing.<sup>24–26</sup>

The presence of Mg, Ca, or Sr ions can clearly affect various cellular responses; however, the effect of co-administration and potential synergistic and antagonistic responses are uncertain. We hypothesize cells in the bone are affected by the Mg–Ca–Sr alloy and/or its degradation products, and in particular, there are beneficial effects on osteogenic activity. To test this hypothesis, alloy degradation extracts were used to study their effects on stem cell toxicity, proliferation, and osteogenic differentiation.

Previous work by the authors showed that the degradation rate of the alloy varies with microstructure<sup>6</sup>; this work suggests that the microstructure of the alloy can be altered to obtain implants with tailorable degradation behavior and thereby tailorable ionic elutions. In this study, different concentrations (ionic ratios) were obtained, through microstructural modification, which directly correlate to the ionic elutions of alloy components.

The aim of the work presented here was to assess the effect of alloy degradation products on cellular activities involved in bone repair; to obtain a better understanding of their effect on potential mechanisms involved in tissue repair. Understanding these processes is crucial for improving the bioactivity and functional utility of Mg–Ca–Sr implants, and assessing whether Mg–Ca–Sr implants are appropriate for use in humans.

## MATERIALS AND METHODS

### Alloy preparation

Casting of the alloy has been described elsewhere.<sup>5</sup> Cast ingots were homogenized by wrapping in steel foil and heat-treating in an Ar atmosphere at 450°C for 24 h followed by air-cooling. To obtain microstructures that produced varying degradation rates,<sup>6</sup> samples were deformed by hot rolling. Specimens of approximately 100 × 20 × 5 mm were prepared by sectioning homogenized casts using a low-speed saw with diamond-coated blades (Allied HighTech Products, Inc, Rancho Dominguez, CA) followed by grinding the surfaces down to 1200 grit using silicon carbide article and a planar. The samples were then deformed between steel rolls (Fenn Rolling Mill, SPX Corporation, Charlotte, NC) by repeatedly passing them unidirectionally. The samples were preheated at 300°C for 10 min and reheated for 5 min after each pass. Each pass resulted in a thickness reduction of 0.254 mm, and rolling was continued until approximately 45% total reduction in thickness was achieved.

### Cell culture

Frozen adult bone-marrow-derived hMSCs were obtained commercially (Lonza Group Ltd., Walkersville, MD) and thawed immediately before use. The cells were cultured using standard procedures and kept in an incubator maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. All tests were performed on cells of passage 4. The culture medium consisted of  $\alpha$ -minimal essential medium (dMEM, HyClone, Thermo Scientific, Waltham, MA) containing 4 mM L-glutamine, 5 mM glucose, and 1% sodium pyruvate, and supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.2% fungizone. For osteogenic differentiation of hMSCs, osteogenic media was prepared by the addition of 50  $\mu$ M ascorbic acid, 10 mM  $\beta$ -glycerophosphate, and 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO). Throughout the study, phase contrast images were obtained using an optical microscope (Nikon Eclipse TE2000-U, Nikon Instruments Inc., Melville, NY).

### Preparation of extracts

Alloy extracts were prepared according to ISO standard 10993-12.<sup>27</sup> Homogenized and rolled alloy specimens were ground to a surface finish of 1200 grit using silicon carbide paper, cleaned in ethanol followed by sterilization under UV light for 15 min. Alloy specimens were then placed in 15 mL conical tubes, immersed in complete cell culture media at a ratio of 1 mL per cm<sup>2</sup>, and incubated for 72 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The culture media contained all the supplements as described previously, except osteogenic supplements which were added after extraction. After 72 h, the specimens were removed from the conical tube and the alloy extracts were filtered through a 0.22  $\mu$ m pore size filter, and one extract was serially diluted to make 10% concentrate with fresh culture media to obtain a range of dissolution product (ion) ratios for investigation. Extraction media were refrigerated at 4°C until utilized. The composition of dissolved ions in culture media and alloy extracts was measured using inductively coupled plasma-mass spectrometry (ICP-MS, PerkinElmer; PerkinElmer Corp., Norwalk, CT). In addition to alloy extracts, Sr-only extracts were prepared by dissolving strontium salt, SrCl<sub>2</sub> (99.9% Sigma-Aldrich, St. Louis, MO), in cell culture media.

### Toxicity testing

Toxicity testing was carried out using the lactate dehydrogenase (LDH) cytotoxicity assay (Pierce, Thermo Scientific, Waltham, MA), which measures the amount of released LDH enzyme in the cell culture media. The LDH present in the culture medium directly correlates to the amount of dead cells, as LDH is normally sequestered inside the cell but rapidly released into the culture media when the cell membrane is damaged. hMSCs were seeded onto 24-well polystyrene plates at a density of 3000 cells per cm<sup>2</sup> and incubated for 24 h to allow full attachment and spreading. The culture medium was then replaced with 1 mL of alloy extraction medium per well. After 3 days of culture, the LDH assay was performed on the cell culture media from

TABLE I. Primer Sequences Used in the RT-PCR

Gene	Forward	Reverse
GAPDH	5'-ACATCGCTCAGACACCATG-3'	5'-TGTAGTTGAGGTCAATGAAGG-3'
RUNX2	5'-CTTCACAAATCCTCCCAAGT-3'	5'-AGGCGGTGAGAGAACAAAC-3'
ALPL	5'-GCTCATGCATAACATCAGGGA-3'	5'-TCGTCACTCTCATACTCCACA-3'

each well per the manufacturer's protocol. Untreated cells grown in culture medium devoid of any alloy components and cells treated with a lysis buffer for complete membrane damage were used as negative and positive controls, respectively. These controls are referred to as 0% toxicity and 100% toxicity, respectively.

### Cell proliferation

For proliferation testing, the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, Grand Island, NY) was used to determine the amount of DNA present in cells, which directly correlates to cell number. hMSCs were seeded onto 24-well plates at a density of 3000 cells per cm<sup>2</sup> and incubated for 24 h to allow for attachment. The culture medium was then replaced with 0.5 mL of extraction medium or fresh culture medium (control) per well. After 3, 6, and 9 days of culture with extraction media or control media, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed using 200 μL of 1% Triton X-100 in PBS while kept on ice for 15 min. The lysates were then collected and kept frozen at -20°C until utilized. The assay was performed per the manufacturer's protocol utilizing standards of known DNA concentration.

### Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

For qRT-PCR, hMSCs were seeded at a density of 7100 cells per cm<sup>2</sup> in 12-well tissue culture polystyrene plates and allowed to attach for 24 h. After 24 h, the media was replaced with 800 μL of dMEM extraction medium, with and without osteogenic supplements, fresh complete culture medium (negative control), or osteogenic medium (osteogenic control), per well.

The medium was replaced every 3–4 days. On day 21, the cells were washed with PBS and then 300 μL of trypsin was added to each well. After 15 min at room temperature, 900 μL of 2% FBS in PBS was added, the suspension was collected and then centrifuged at 250g for 5 min. The supernatant was then aspirated and the pellet was stored at -80°C until utilized. RNA was isolated using the RNeasy

mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany).

First-strand cDNA was synthesized from total RNA using iScript™ cDNA synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instruction. A maximum of 1 μg of total RNA was used for reverse transcription. Amplification occurred in a 20 μL reaction consisting of 10 μL Maxima™ SYBR™ green qPCR Master Mixes (Thermo Scientific, Waltham, MA); 2 μL of forward and reverse primer mix (Integrated DNA Technologies, Coralville, IA), resulting in a final concentration of 500 nM per primer; 7 μL of RNase, DNase-free water; and 1 μL of template. Forward and reverse primers used are listed in Table I. Quantitative reverse transcriptase-PCR (qRT-PCR) was performed using CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA). First, a denature step was performed for 10 min at 95°C followed by 40 cycles of 15 s denature at 95°C, 60 s at 60°C for annealing and extension. Expression of target genes was normalized by expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Statistical analyses

All cell culture conditions were performed in triplicate, and each assay run in triplicate, to make  $n = 9$  for each condition assayed. Statistical analyses were performed using the Kruskal-Wallis one-way analysis of variance (ANOVA) with Mann-Whitney post-hoc tests ( $\alpha = 0.05$ , Minitab 14, Minitab Inc., State College, PA).

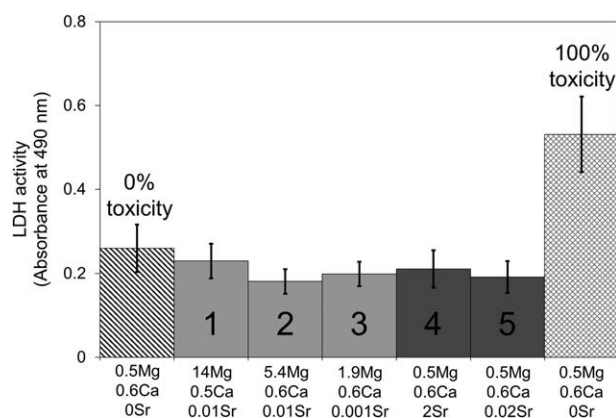
## RESULTS

### Alloy extract concentrations

The test matrix and respective ion concentrations are enlisted in Table II. All measured values have standard deviation within 10%, as determined by five independent readings during ICP measurements. In addition to different alloy extracts, Sr-only extracts were included, to determine potential differences between Sr-only and Mg-Ca-Sr co-administration. Two different concentrations of the Sr compound was selected for testing, one corresponding to expected Sr amounts eluded from the alloy, and one concentration in the order of

TABLE II. Measured Extract Ion Concentrations Used in the *in vitro* Testing

Extract Name	Mg <sup>2+</sup> (mM)	Ca <sup>2+</sup> (mM)	Sr <sup>2+</sup> (mM)	Description
Ctrl: 0.5Mg-0.6Ca-0Sr	0.48	0.59	-	hMSC growth media
1: 14Mg-0.5Ca-0.01Sr	14.44	0.51	0.01	100% extract (homogenized alloy)
2: 5.4Mg-0.6Ca-0.01Sr	5.39	0.60	0.01	100% extract (rolled alloy)
3: 1.9Mg-0.6Ca-0.001Sr	1.87	0.59	0.001	10% extract (homogenized alloy)
4: 0.5Mg-0.6Ca-2Sr	0.48	0.59	2.00	(SrCl <sub>2</sub> salt)
5: 0.5Mg-0.6Ca-0.02Sr	0.48	0.59	0.02	(SrCl <sub>2</sub> salt)



**FIGURE 1.** Toxicity, as presented by LDH activity, of various metal extracts to hMSCs after 3 days of culture. No extract culture is significantly different from the “0% toxicity” control representing untreated cells. The 100% toxicity is shown for reference and represents the amount of LDH released upon lysing all cells in culture.

magnitude shown in literature to affect bone cell expression.<sup>13,28</sup> In the following sections, results are presented by Mg/Ca/Sr content (expressed in mM), as well as extract numbers, as denoted in Table II, for ease of navigation.

### Toxicity

The toxicity results as shown by the LDH assay from 3 days of culture are shown in Figure 1. The results are presented as the absorbance at 490 nm, along with positive (lysed cells) and negative (untreated cells) controls for easy comparison. The “0% toxicity” control corresponds to the amount of LDH released from healthy cultures, including background intensity from the culture media, representing normal healthy cell turnover. Optical densities falling within this region are considered nontoxic. The “100% toxicity” control corresponds to the amount of LDH released from all cells present in the culture. To ensure that the different culture media did not interfere with the optical readings, each alloy extract was tested separately. There was no significant difference between any of the extracts and the basal media in terms of optical density in the LDH assay (data not shown). All three alloy-treated cultures (extracts 1–3) and the Sr-only cultures (extracts 4 and 5) are similar to the negative control (Figure 1), with none of the extracts causing immediate toxicity to hMSC. Optical viewing of the cell cultures supports these results; there was no evidence of cell detachment from the surface of the culture dish, and no rounded/dead cells present in the media.

### Proliferation

The results from the proliferation study are shown in Figure 2, presented as total cellular DNA content at three different time points (day 3, 6, and 9) for the different alloy extracts and the control conditions. Significant differences, either an increase or decrease, compared to the untreated control at each day are indicated (\*).

Control cells, which were cultured in growth media absent of alloy extraction products, showed an increase in

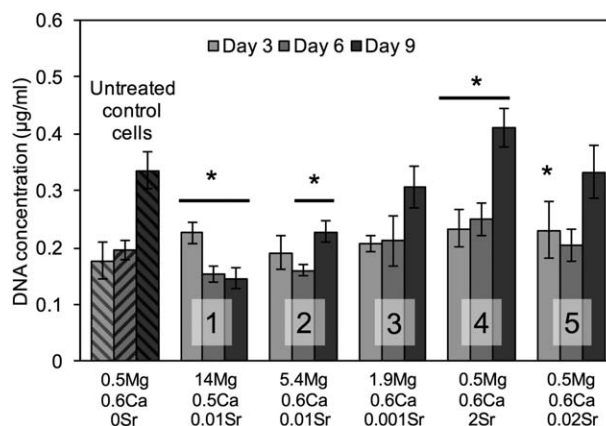
DNA content with time, as expected from proliferating cells (Figure 2), with significant increase at the 9 day time point. Extracts 1 and 2, which contain the higher concentrations of Mg, show a negative trend in proliferation when compared to the control. While extract 1 shows a significant decrease in DNA content compared to the control at day 6 and day 9, respectively, as well as with time, extract 2 shows no significant change in DNA content with time, but a significant decrease at days 6 and 9 compared to control cells. Extract 3 and the two Sr-only-treated cultures (extracts 4 and 5) show exponential proliferation, where the DNA content increases with culture time. The highest proliferation rate is seen in the 2 mM Sr-treated cultures (extract 4).

### Differentiation

The qRT-PCR results of osteogenically supplemented (OS+) and nonosteogenically treated (OS-) cultures are presented in Figure 3. The nonosteogenically treated cultures and those supplemented with osteogenic factors are denoted (OS-) and (OS+), respectively, to distinguish them.

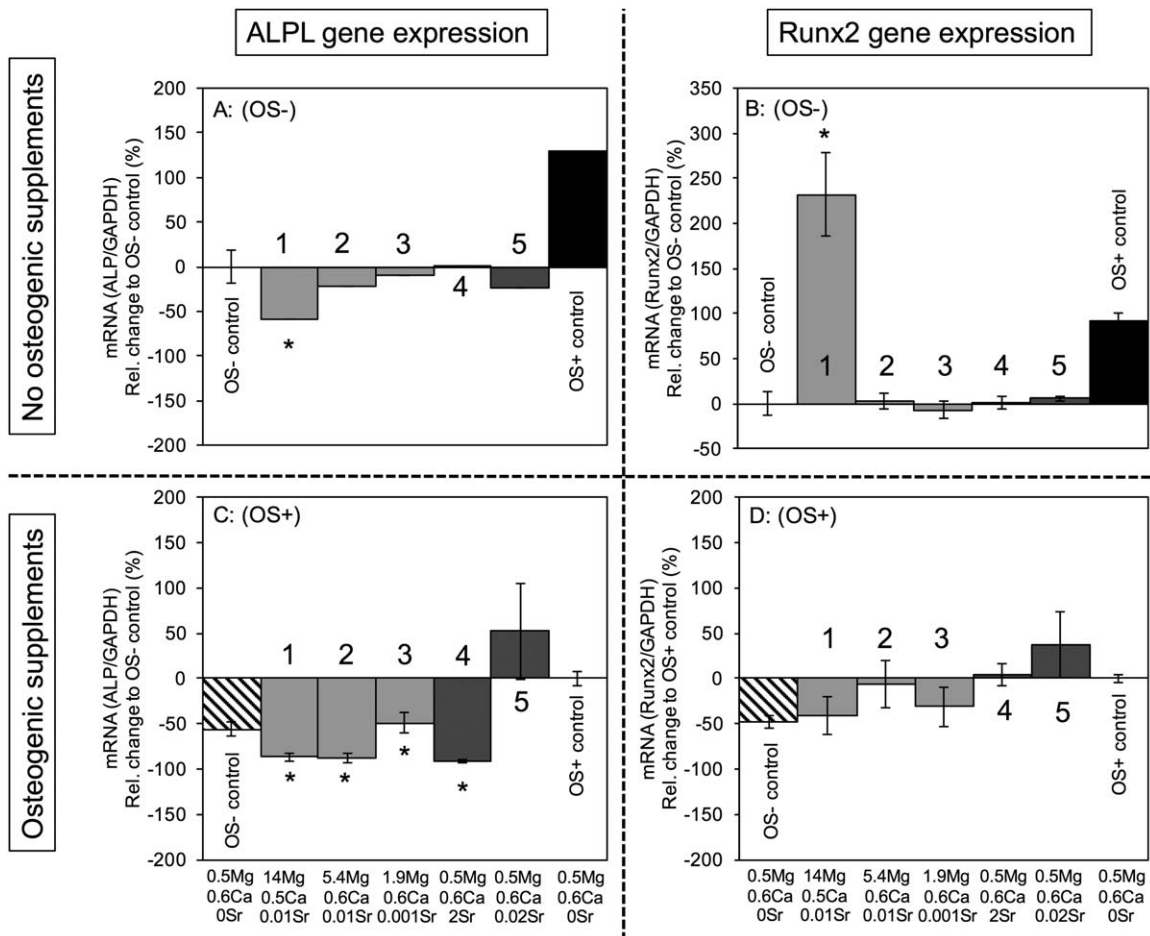
**(OS-) cultures.** Figure 3(A,B) shows the *ALPL* and *RUNX2* gene expression of hMSC cultures (OS-) assessed after 21 days of culture. Note, these extract cultures do not contain osteogenic supplements; however, for reference and easy interpretation, an osteogenically treated culture is included in each of these studies. The *ALPL* gene expression has shown to be nonlinear throughout the progression of osteogenesis; therefore, in Figure 3(A) (and 3C), an expression different from the untreated cell control, either an increase or decrease, is considered positive for osteogenic differentiation.<sup>29,30</sup> Extract 1 showed a positive response for osteogenic differentiation. This extract is high in Mg content, and contains similar concentrations of Ca and Sr as extracts 2 and 5, which have significantly lower Mg content. Both extracts 2 and 5 showed a negative response.

Extract 1 has a significantly increased *RUNX2* expression [Figure 3(B)]. The *RUNX2* expression of extract 1 is also higher than the positive/osteo-induced control. Extracts 2–5



**FIGURE 2.** Proliferation presented as total DNA content at days 3, 6, and 9 (left to right) for each condition (as labeled 1–5). Significant differences ( $p < 0.05$ ) compared to untreated cells at each day are indicated (\*).





**FIGURE 3.** *ALPL* and *RUNX2* gene expression in hMSC metal extract cultures at day 21. (A) *ALPL* expression in OS<sup>-</sup> cultures, (B) *RUNX2* expression in OS<sup>-</sup> cultures, (C) *ALPL* expression in OS<sup>+</sup> cultures, and (D) *RUNX2* expression in OS<sup>+</sup> cultures. Significant differences ( $p < 0.05$ ) compared to untreated cells (OS<sup>-</sup>, A and B) or osteogenically supplemented control cells (OS<sup>+</sup>, C and D) are indicated (\*).

showed no significant difference in either *ALPL* or *RUNX2* expression when compared to the negative controls.

**(OS<sup>+</sup>) cultures.** The *ALPL* and *RUNX2* gene expressions in osteogenically induced cultures at day 21 of culture are shown in Figure 3(C,D). Untreated cells cultured in osteogenic media (OS<sup>+</sup>), as well as noninduced cultures (OS<sup>-</sup>) taken to day 21, are shown as reference points. The *ALPL* expression is different between many of the cultures [Figure 3(C)], and furthermore, all but extract 3 are different than the noninduced control (statistical analysis results not shown). All the alloy extracts and one of the Sr-only extracts (extract 4) are different than the osteogenic control (as indicated by \*).

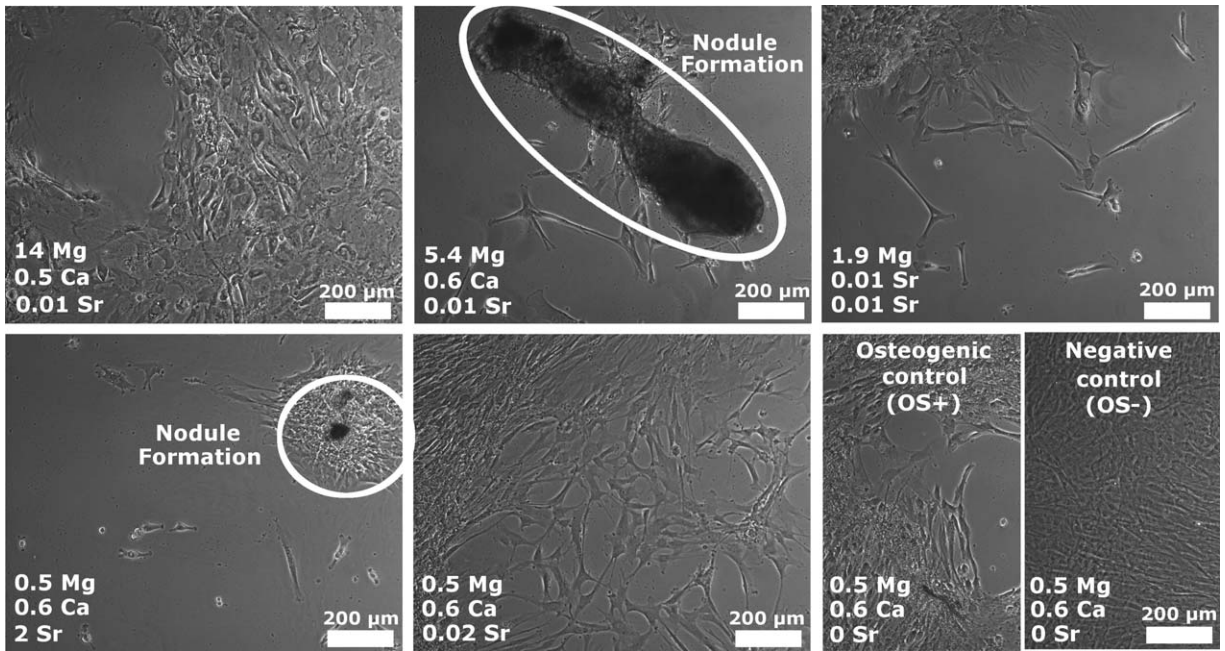
*RUNX2* gene expression varies between the extracts and the control [Figure 3(D)]. However, no statistically significant differences were observed. The Sr-only cultures show the largest upregulation in *RUNX2* gene expression.

The osteogenically stimulated cultures were optically imaged to show progression of the differentiation, as shown in Figure 4. A nonstimulated control (OS<sup>-</sup>) is included for comparison. The negative (nonosteogenic) control is densely populated, as expected from proliferating cells.

## DISCUSSION

The overall goal of this study was to investigate the effect of degradation products of a novel Mg–Ca–Sr alloy on stem cell culture and differentiation for favorable design of implants. Alloy extracts were prepared, resulting in cell culture media containing different concentrations (ratios) of alloy elements, as listed in Table II. As a first step toward understanding the effect of alloy extract on stem cells, cytotoxicity of alloy extracts were assessed using LDH measurements. Note that media ion concentration changes may increase osmotic pressure to the cells, which was not directly measured; however, potential negative consequences were indirectly assessed by way of the toxicity study. While some alloy extracts were previously shown nontoxic to MC3T3-E1 mouse cells,<sup>5</sup> the current results confirm the more sensitive and clinically relevant primary human stem cells are not damaged by the presence of alloy elements.

After confirming the extracts were not toxic in culture, the effects of alloy extracts on the growth of hMSCs were studied. In all cases, the cell cultures show insignificant differences between the two earliest time points, which can be explained by the relatively slow growth of hMSC compared to other cell types and the exponential nature of proliferation.



**FIGURE 4.** Optical images of osteogenically treated alloy extract cultures and the control cultures at day 21. Some cultures show nodule formations and extracellular deposits, as indicated (circled), suggestive of late-stage osteogenesis.

The proliferation profiles of extracts 1 and 2 are different than the control. The higher amount of Mg (14.4 and 5.39 mM, respectively) in these extracts compared to the control cells (0.48 mM) could potentially impair the growth of hMSCs. 15 mM Mg concentration showed inhibited growth in hMSC cultures after 1 day in a study by Yang et al.<sup>31</sup> Contrarily though, a recent study showed media with Mg concentrations between 0.041 and 41.0 mM all enhanced the proliferation of hMSC after 1 day of culture compared to nontreated cells in media, with the largest effect (approximately 25% increase) seen at a concentration of 4.1 mM Mg.<sup>32</sup> In this study by Li et al.,<sup>32</sup> the proliferation may be increased at a time earlier than what was tested, explaining the higher DNA content of extract 1 at day 3 compared to the control, followed by a decrease in proliferation due to longer term Mg treatment. Unfortunately, the Mg content in the control cultures were not reported in the studies by Yang et al.<sup>31</sup> and Li et al.,<sup>32</sup> making direct comparisons in terms of proliferation rate difficult between the studies. For extracts 1 and 2 studied here, Correlating the proliferation results to the toxicity results, there are a few potential scenarios explaining the observed behavior: As no significant increase in extracellular LDH activity compared to the negative control cultures was seen and there is decrease in DNA content, these cells are either (1) growth inhibited, (2) differentiating (into osteoblasts or other phenotypes), or (3) exhibit a combination of growth inhibition and differentiation (with or without some small reduction due to cell death).

One of the alloy extract cultures (extract 3) and the two Sr-only treated cultures (extracts 4 and 5) display normal proliferation. Combined with the toxicity study that showed the extracts do not cause immediate cell death, the proliferation data for extract 3 suggests that the cells in these

cultures are either (1) not affected by the added ions or (2) they have enhanced proliferation while also differentiating, resulting in an overall balanced turnover. Increased proliferation compared to the control was seen in the two Sr-only treated cultures. This trend is in accordance with a study by Schumacher et al.<sup>23</sup> who saw an increase in cell number of hMSC cultures treated with 0.01 mM SrCl<sub>2</sub>; however, they did also notice reduced proliferation in cultures treated with 0.1 mM SrCl<sub>2</sub>, (although the difference was seen at day 21 of culture). Decreased viability was also observed in a study by Li et al.<sup>21</sup> in rat MSC cultures of similar Sr amount (0.1 and 1 mM) as currently tested; however, the source compound in their study was Sr ranelate (SR). The studies by Schumacher et al.<sup>23</sup> and Li et al.,<sup>21</sup> combined with the current results, suggest that Sr sourced from SrCl<sub>2</sub> could be more beneficial for MSC proliferation, implying potential synergistic/antagonistic effects of chlorine or ranelic salt.

To investigate the effect of alloy degradation products on differentiation, cells cultured in alloy extracts were investigated by regulation of gene expression of two commonly studied osteogenic genes, *ALPL* and *RUNX2*. qRT-PCR was used to quantify the expression of these genes in both osteogenically supplemented (OS+) and nonsupplemented (OS-) cultures. Investigation of the OS- cultures revealed extract 1 upregulated genes for osteogenic differentiation. This extract is high in Mg content with minor reduction in Ca, and can be compared to extracts 2 and 5, which contain similar Sr but significantly lower Mg content. However, extract 2 and 5 both showed a negative response for gene regulation. This suggests that there is an uncertain threshold above which Mg induces differentiation, either by itself or in the presence of Sr (and minor reduction in Ca). *RUNX2* expression typically increases during osteogenic

differentiation,<sup>33,34</sup> but can also remain constant.<sup>35</sup> Extract 1 has a significantly increased *RUNX2* expression [Figure 3(B)], confirming signs of differentiation thus complementing the *ALPL* expression results [Figure 3(A)]. Interestingly, the *RUNX2* expression of extract 1 is also higher than the positive/osteogenic control. A potential explanation for this is enhanced differentiation. There is no significant difference in either *ALPL* or *RUNX2* expression of extracts 2, 3, 4, or 5 to the negative controls, indicating the ions present in these cultures do not induce differentiation of hMSCs.

To investigate if alloy degradation products change the kinetics of osteogenic differentiation, cells cultured with extracts supplemented with osteogenic factors were studied. OS+ cultures revealed the *ALPL* expression in many of the cultures are different from each other [Figure 3(C)], but all except extract 3 are statistically different than the non-induced control (statistical analysis results not shown). This confirms extract 1, 2, 4, and 5 cultures are differentiating, as expected. Also, all the alloy extracts and one of the Sr-only extracts (extract 4) showed significant difference when compared to the osteogenic control [as indicated by \* in Figure 3(C)]. This confirms alloy degradation products affect the progression of osteogenic differentiation. Further clarification is required to determine if this expression profile is shifted to enhance or reduce the kinetics. The extract 5 OS+ were not significant than the positive control. It is possible that the extract 5 OS+ cultures are unaffected by the small amount of Sr. For example, Schumacher et al.<sup>23</sup> noticed that for 0.01 mM SrCl<sub>2</sub>, the *ALPL* protein expression was not significantly different than the control, regardless of the differentiation time point. No statistically significant differences in *RUNX2* gene expression were observed between the OS+ extract cultures and controls. The largest increase in *RUNX2* gene expression appears to be in the Sr-only cultures. This correlates well with a study by Zhu et al.<sup>36</sup> who noticed an increased *RUNX2* expression at day 21 after culturing mouse stem cells in the presence of SrCl<sub>2</sub>. Optical viewing of the OS+ cells (Figure 4) suggests that these cultures are at different stages of differentiation; some cultures show high cell density and minor retraction of the cell layer (indicative of proliferation or early stage differentiation),<sup>37</sup> whereas other cultures have distinct nodule formations. Some of the nodule formations also display extracellular deposits, which is in accordance with advanced stages of differentiation.<sup>37</sup> Protein expression studies could provide further insight into the behavior of osteogenic differentiation upon ion stimulation.

Overall, the results from this study show that alloy degradation products can have positive effect on cell growth as well as differentiation of mesenchymal stem cells along an osteogenic pathway. This work will be useful for future development on smart alloy design to promote cell regeneration.

## CONCLUSIONS

A novel Mg–Ca–Sr alloy was investigated in terms of its effect on cellular mechanisms during degradation, as tested by *in vitro* studies using alloy degradation extracts.

The results presented herein show the alloy degradation products are nontoxic, but more importantly, have an effect on stem cell proliferation and gene expression.

Concentration of ions tested was found to have dual influence on human stem cells, increasing proliferation and likely inducing osteogenesis. The effect on proliferation is attributed to the presence of Sr, while the addition of Mg appeared to induce osteogenesis; hMSC differentiation was detected by changes in *ALPL* and *RUNX2* gene expression. It was further concluded that the alloy degradation products affect the osteogenic process. *ALPL* gene expressions in osteogenically induced cultures showed that alloy extracts had varied effect on osteogenesis. Further studies are required to determine specifically what the kinetic effects are for each extract.

Overall, it can be concluded Mg and/or Sr affect human stem cells in cultures. The results further suggest certain ratios of Mg and Sr are beneficial for bone growth. Additional studies are needed to deconvolute the different mechanisms taking place and potential cascading effects. While the ratios of ions used in the current tests directly correlates to alloy dissolution rates, it would be beneficial to determine the ionic amounts and ratios governing the observed expressions (proliferation and *ALPL*/*RUNX2* gene expressions) as well as their effect on individual cellular mechanisms.

In summary, it is likely ionic products from alloy dissolution improve tissue healing by promoting proliferation and/or differentiation of mesenchymal stem cells. The currently presented results thereby provide a foundation for future alloy design, where properties may be tailored to obtain specific implant performance.

## DISCLOSURE

Authors MVM and ISB are inventors on a patent application(s) relevant to the materials studied in this publication. Additionally, MVM serves as the Chief Scientific Officer, on the board of directors, and has shares and ownership in Element12Biotechnologies. All other authors have no conflict of interest relevant to this publication.

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