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### Authors

Varanasi, Venu G  
Leong, Kelly K  
Dominia, Lisa M  
et al.

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# Si and Ca Individually and Combinatorially Target Enhanced MC3T3-E1 Subclone 4 Early Osteogenic Marker Expression

Venu G. Varanasi<sup>1\*</sup>  
Kelly K. Leong<sup>2</sup>  
Lisa M. Dominia<sup>2</sup>  
Stephanie M. Jue<sup>3</sup>  
Peter M. Loomer<sup>3</sup>  
Grayson W. Marshall<sup>2</sup>

This study tests the hypothesis that silicon and calcium ions combinatorially target gene expression during osteoblast differentiation. MC3T3-E1 subclone 4 osteoblast progenitors (transformed mouse calvarial osteoblasts) were exposed to Si<sup>4+</sup> (from Na<sub>2</sub>SiO<sub>3</sub>) and Ca<sup>2+</sup> (from CaCl<sub>2</sub>·H<sub>2</sub>O) ion treatments both individually (0.4 mM each + control treatment) and combinatorially (0.4 mM Si<sup>4+</sup> + 0.4 mM Ca<sup>2+</sup> + control treatment) and compared to control treated ( $\alpha$ -minimum essential medium, 10% fetal bovine serum, and 1% penicillin-streptomycin) cells. Cell proliferation studies showed no significant increase in cell density between treatments over 5 days of culture. Cellular differentiation studies involved addition of ascorbic acid (50 mg/L) for all treatments. Relative gene expression was determined for collagen type 1 (Col(I) $\alpha$ 1/Col(I) $\alpha$ 2), core-binding factor a (cbfa1/Runx2), and osteocalcin (OCN), which indicated osteoblast progenitor differentiation into a mineralizing phenotype. Increased Si<sup>4+</sup> or Ca<sup>2+</sup> ion treatments enhanced Col(I) $\alpha$ 1, Col(I) $\alpha$ 2, Runx2, and OCN expression, while increased Si<sup>4+</sup> + Ca<sup>2+</sup> ion treatments enhanced OCN expression. Moreover, it was found that a Si<sup>4+</sup>/Ca<sup>2+</sup> ratio of unity was optimal for maximal expression of OCN. Collagen fiber bundles were dense, elongated, and thick within extracellular matrices (ECM) exposed to Si<sup>4+</sup> and Si<sup>4+</sup> + Ca<sup>2+</sup> treatments, while collagen fiber bundles were sparse, short, and thin within Ca<sup>2+</sup> and control treated ECM. These results indicated that individual ions enhance multiple osteogenic gene expression, while combined ion treatments enhance individual gene expression. In addition, these results indicated that Si<sup>4+</sup> enhanced osteoblast gene expression and ECM formation at higher levels than Ca<sup>2+</sup>. These results support the larger concept that ions (possibly released from bioactive glasses) could control bone formation by targeting osteoblast marker expression.

**Key Words:** bioactive glass, combinatorial, ions, silicon, calcium

<sup>1</sup> Department of Biomedical Sciences, Texas A&M Health Science Center Baylor College of Dentistry, Dallas, Tex.

<sup>2</sup> Division of Biomaterials and Bioengineering, University of California, San Francisco, Calif.

<sup>3</sup> Division of Periodontology, University of California, San Francisco, Calif.

\* Corresponding author, e-mail: venu.varanasi@ucsf.edu

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## INTRODUCTION

**B**iomaterials, traditionally used in bone healing, are required to facilitate osteoblast attachment and eventual mineralized tissue formation. Biomaterials are either bioinert or bioactive. Bioinert materials such as titanium tend to mechanically support bone attachment to their surfaces but do not directly bond with bone. Bioactive materials are a super family of materials that are bioconductive and/or bioinductive. Bioconductive materials (eg, hydroxyapatite [HA]) facilitate osteoblast movement, growth, ingress, and chemical bonding to their surfaces. Bioinductive materials (eg, growth factors, drugs) alter cellular function by influencing cellular gene expression.

Bioactive glasses have traditionally been considered bioconductive because they release ions (eg,  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$ ,  $\text{Si}^{4+}$ ) to cells as raw materials for mineralized tissue formation, while forming an HA surface

layer for direct mineralized tissue attachment. Our work,<sup>1</sup> along with that of others<sup>2-5</sup> has found that the ions released from bioactive glasses played an inductive role during osteogenesis. These ions enhanced osteogenic marker expression (eg, collagen type 1 [Col(I) $\alpha$ 1]; core binding factor a [cbfa1/Runx2]; and osteocalcin [OCN] (Table). For example,  $\text{Si}^{4+}$  and  $\text{Ca}^{2+}$  (as products of 53 wt%  $\text{SiO}_2$ -based bioactive glass dissolution) increased the expression of Col(I) $\alpha$ 1, Col(I) $\alpha$ 2, Runx2, and OCN,<sup>1</sup> which were consistent with the results from Hench and colleagues<sup>4</sup> using 58 wt%  $\text{SiO}_2$ -based sol-gel derived glass corrosion products on Runx2 and OCN expression.

Despite these and other studies that showed enhanced osteogenic effects by bioactive glass ionic products, no study has shown that these ions are involved in the combinatorial control of osteoblast gene expression. The combinatorial principle involves control over cellular gene expression by gene regulatory proteins

TABLE  
Various ion sources and their effects on osteogenic marker expression

Ion Source	Change in Ion Concentrations*	Effect on Osteogenic Marker Expression ( $\times$ control)	Reference
45S5	$\text{Si}^{4+} = 0.4 \text{ mM}$ $\text{Ca}^{2+} = 0.16 \text{ mM}$ $\text{PO}_4^{3-} = 0.04 \text{ mM}$ $\text{Na}^+ = 0 \text{ mM}$	Collagen type 1 (4 $\times$ control, 1 day) Alkaline phosphatase (2 $\times$ control, 10 days) OCN (6 $\times$ control, 3 days) Runx2 (2 $\times$ control, 7 days)	Varanasi et al <sup>1</sup>
6P53-b	$\text{Si}^{4+} = +33 \text{ ppm}$ $\text{Ca}^{2+} = 0.07 \text{ mM}$ $\text{PO}_4^{3-} = 0.03 \text{ mM}$ $\text{Na}^+ = 0 \text{ mM}$ $\text{Mg}^{2+} = 0 \text{ mM}$ $\text{K}^+ = 0 \text{ mM}$	Collagen (3 $\times$ control, 1 day) Alkaline phosphatase (2 $\times$ control, 10 days) OCN (14 $\times$ control, 3 days) Runx2 (2 $\times$ control, 7 days)	Varanasi et al <sup>1</sup>
6P55	N/A	Runx2 (2 $\times$ control, 7 days)	Foppiano et al <sup>2</sup>
45S5	N/A	Alkaline phosphatase, 1.25 $\times$ control, 6 days	Bielby et al <sup>4</sup>
45S5	$\text{Si}^{4+} = 0.14 \text{ mM}$ $\text{Ca}^{2+} = 0.08 \text{ mM}$ $\text{PO}_4^{3-} = 0.03 \text{ mM}$ $\text{Na}^+ = 0 \text{ mM}$	Bone derived growth factor (3 $\times$ control, 2 days) Decorin; bone proteoglycan II precursor (2.2 $\times$ control, 2 days) Vascular endothelial growth factor (2 $\times$ control)	Xynos et al <sup>3</sup>
$\text{Na}_2\text{SiO}_3$	$\text{Si}^{4+} = 0.01\text{--}0.05 \text{ mM}$	Collagen type 1 (1.8 $\times$ control, 3 days) OCN (1.5 $\times$ control, 3 days)	Reffitt et al <sup>6</sup>
$\text{CaCl}_2$	$\text{Ca}^{2+} = +0.2\text{--}20 \text{ mM}$	OCN (3D culture, 2.25 $\times$ control, 7 days) OCN (2D culture, 2 $\times$ control, 7 days)	Maeno et al <sup>7</sup>
45S5	N/A	Runx2 (1.5 $\times$ control) Alkaline phosphatase (1.6 $\times$ control) OCN (2 $\times$ control, day 13)	Bielby et al <sup>4</sup>

\*N/A indicates not applicable; OCN, osteocalcin.

(GRPs). Each GRP targets many genes, while multiple GRPs target specific genes. Analogously, the ions released by bioactive glasses could influence osteoblast gene expression along these conceptual lines. This novel approach is aimed at decoupling the individual and combined roles of  $\text{Si}^{4+}$  and  $\text{Ca}^{2+}$  ions on osteoblast gene expression during early bone development of extracellular matrices (ECM). This concept is applied by simulating a specific combination and concentration of these ions that may be released from bioactive glass surfaces.

A variety of  $\text{Si}^{4+}$  and  $\text{Ca}^{2+}$  ion concentrations have shown enhanced effects. At dietary levels, increased  $\text{Si}^{4+}$  (0.01–0.05 mM) led to relatively moderately increased Col(I) and OCN expression (Reffitt et al,<sup>6</sup> Table), while relatively high levels of additional  $\text{Ca}^{2+}$  (0.2–20.0 mM) led to increased Col(I) and OCN expression (Mae-no et al,<sup>7</sup> Table). Bioactive glasses showed similar effects on osteogenesis for  $\text{Si}^{4+}$  and  $\text{Ca}^{2+}$  concentrations below 1 mM.<sup>8</sup> For example, bioactive glass release of  $\text{Ca}^{2+}$  (0.2–1.25 mM) and  $\text{Si}^{4+}$  (0.14–0.4 mM) increased Col(I) and OCN expression (Varanasi et al,<sup>1</sup> Xynos et al,<sup>3</sup> Table). Besides individual ion effects, the combined ion effect could also influence cellular gene expression through a combined ion ratio. Although not tested in osteoblasts, Waters and Eide<sup>9</sup> showed that ions (iron and zinc) combinatorially targeted yeast cellular gene expression. They found that concentrations of these ions (0.01  $\mu\text{M}$ –1 mM) added to yeast cells at an  $\text{Fe}^{2+}/\text{Zn}^{2+}$  ratio of 1 was optimal for the regulation of FET4 and ZAP1 expression and plasma membrane flux of  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$ . Thus, we will use a range of ion concentrations (0.1–0.4 mM) to study the individual and combinatorial effects of  $\text{Si}^{4+}$  or  $\text{Ca}^{2+}$  on osteoblast differentiation.

Because primary human osteoblast populations have heterogenic phenotypes (due to multiple types of cells extracted from bone), MC3T3-E1 subclone 4 cells are used since they exhibit a homogenous

osteoblast progenitor phenotype<sup>1</sup> and differentiate upon the addition of ascorbic acid.<sup>10,11</sup> Col(I) $\alpha$ 1 and Col(I) $\alpha$ 2 expression occurs within a few hours to a few days upon the onset of differentiation and forms the base matrix for mineralized tissue during osteogenesis.<sup>12–17</sup> Runx2 is a transcription factor that indicates the mineralizing phenotype and is expressed after 3 to 6 days after the onset of differentiation. Both Col(I) $\alpha$ 1 and Runx2 affect the expression of OCN. OCN is a noncollagenous Ca-binding protein involved in bone matrix formation that is typically expressed within 6–10 days after the onset of differentiation. During proliferation, MC3T3-E1 subclone 4 cells have a doubling time of 38 hours, which suggests changes in the growth phase of this cell line can occur within 3 days. In comparing our previous work using MC3T3-E1 subclone 4 cells<sup>1</sup> and human periodontal ligament fibroblasts (another osteoblast progenitor),<sup>18</sup> both types of cells had similar timelines for the above mentioned osteogenic markers. Thus, the MC3T3-E1 subclone 4 cell will be used to study the individual and combined ion effects of  $\text{Si}^{4+}$  and  $\text{Ca}^{2+}$ .

Thus, we test the hypothesis that  $\text{Si}^{4+}$  and  $\text{Ca}^{2+}$  ions combinatorially target enhanced expression of osteoblast-specific markers during early bone matrix development. Our aim is to demonstrate that (1) individual ion treatments enhance key osteogenic marker expression, and (2) combined ion treatments enhance individual osteogenic marker expression.

## MATERIALS AND METHODS

### Study design

For this experiment, differentiating osteoblasts were exposed to increased  $\text{Si}^{4+}$  (0.4 mM),  $\text{Ca}^{2+}$  (0.4 mM), and  $\text{Si}^{4+}$  (0.4 mM) +  $\text{Ca}^{2+}$  (0.4 mM) ion concentrations and compared to control treated cells. The combined ion treatment and its effect on osteoblast gene expression was also varied at  $\text{Si}^{4+}/\text{Ca}^{2+}$  ratios of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1, 2, and 4 (fits

within range of ion concentrations given above). For proliferation studies, cells were treated with individual ion, combined ion, and control treatments over a 5-day period (no ascorbic acid). For differentiation, the treatment period was 6 days, with cell lysates analyzed for relative gene expression after 1 and 6 days (early-term effect). A control treatment was used for relative comparison of gene expression. ECM staining for collagen fiber bundle formation was used to determine the effect of these ions on early stage development of bone matrix. Note that gene knockout studies were not conducted as they do not reflect on the application of implant materials that release these ions to bone tissues.

#### ***Treatment and control media preparation***

For proliferation studies, 4 different treatments were used: 0.4 mM Si + control media; 0.4 mM Ca + control media; 0.4 mM Si + 0.4 mM Ca + control media; and control media ( $\alpha$ -minimum essential medium,  $\alpha$ -MEM; 10% fetal bovine serum, FBS; and 1% penicillin-streptomycin, pen-strep). Additional supplementation of ascorbic acid (50 mg/L) was used with these treatments for differentiation studies. All solutions were sterile filtered (0.2  $\mu$ m) prior to FBS and pen-strep supplementation and prior to use in experiments. The Si ion source (sodium meta-silicate,  $\text{Na}_2\text{SiO}_3$ ), Ca ion source (calcium chloride,  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ ), and ascorbic acid (AA) were commercially purchased (Sigma Inc, St Louis, Mo). To vary the  $\text{Si}^{4+}/\text{Ca}^{2+}$  ion ratio, the concentration of Si and Ca ions was adjusted within the prescribed concentration range of  $\text{Si}^{4+}/\text{Ca}^{2+} = 1/4, 1/2, 1, 2, \text{ and } 4$ .

#### ***Cell culture***

Osteoblasts (MC3T3-E1 subclone 4, ATCC, Manassas, Va, passages 25–30) were cultured in 150  $\text{cm}^2$  flasks. Cells were pelleted and counted (using a standard hemacytometer and inverted optical light microscope). Cells were then seeded (50 000 cells/ $\text{cm}^2$ ) into 6-well plates and cultured

for 1 and 6 days during osteoblast differentiation. Cells were seeded at a density of 15 000 cells/ $\text{cm}^2$  in 96-well plates and treated for 5 days in each treatment (no AA) for proliferation studies. Well plates were arranged such that each treatment was administered in triplicate. Cells had their media changed every 2 days.

#### ***Cell proliferation assay***

Measurement of cell density was performed using the MTS assay (Promega Inc, Madison, Wis). This assay is colorimetric and the intensity and hue of the color is measured using a spectrophotometer (490 nm, SpectraMax Plus, Molecular Devices, San Jose, Calif). All treatments were administered in triplicate. The complete experiment was also repeated for statistical robustness.

#### ***Quantitative reverse transcription polymerase chain reaction***

All cells in their respective treatments were cultured in well plates for gene expression studies. Two-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) was implemented. Total RNA was extracted (RNEasy Mini Kit, Qiagen, Valencia, Calif), converted to cDNA (Reverse Transcription System, Promega, Madison, Wis), measured for cDNA concentration (using a full-spectrum UV/Vis nanodrop volume analyzer, ND-1000, Nanodrop Technologies, Wilmington, Del), and amplified using a qPCR machine (ABI 7900, Applied Biosystems Inc, Foster City, Calif). qPCR amplification results were analyzed using the delta-delta  $C_T$  method and the sigmoidal curve fitting method of Qiu et al<sup>19</sup> and the SigmaPlot software package (Systat Inc, San Jose, Calif).

#### ***Statistics***

All experiments were conducted with triplicate ( $n = 3$ ) sampling for each experiment. Each experiment was repeated to confirm results. Gene expression

results were analyzed using analysis of variance (SPSS statistical software package, SPSS Inc, Chicago, Ill) with  $P < .05$  for statistical significance.

### **Histology**

As described above, cells (MC3T3-E1 subclone 4) were seeded onto glass cover slips that were placed in 6-well plates. Cells were allowed to adhere for 4 hours prior to administration of synchronization media ( $\alpha$ -MEM, 1% FBS, 1% pen-strep for 2 days). Synchronization media were then exchanged with individual ion, combined ion, and control treatments along with ascorbic acid to induce differentiation. Cells were allowed to culture for 6 days. Cells on cover slips were then removed from well plates and transferred to a fresh 6-well plate. These samples were then fixed in Bouin fixative and stained in Fast Green (green-blue background tissue stain) and Picrosirius red (yellow collagen stain) as described previously.<sup>1</sup> These samples were imaged for collagen fiber bundles using polarized light microscopy (BX51, Olympus Inc, Tokyo, Japan). Images were captured and annotated using ImagePro. Collagen fiber bundles were counted within several areas of samples. Although a convention has not been established regarding a threshold length that defines elongated and short fiber bundles, we used a 0.1-mm threshold as a basis for counts of elongated and short fiber bundles.

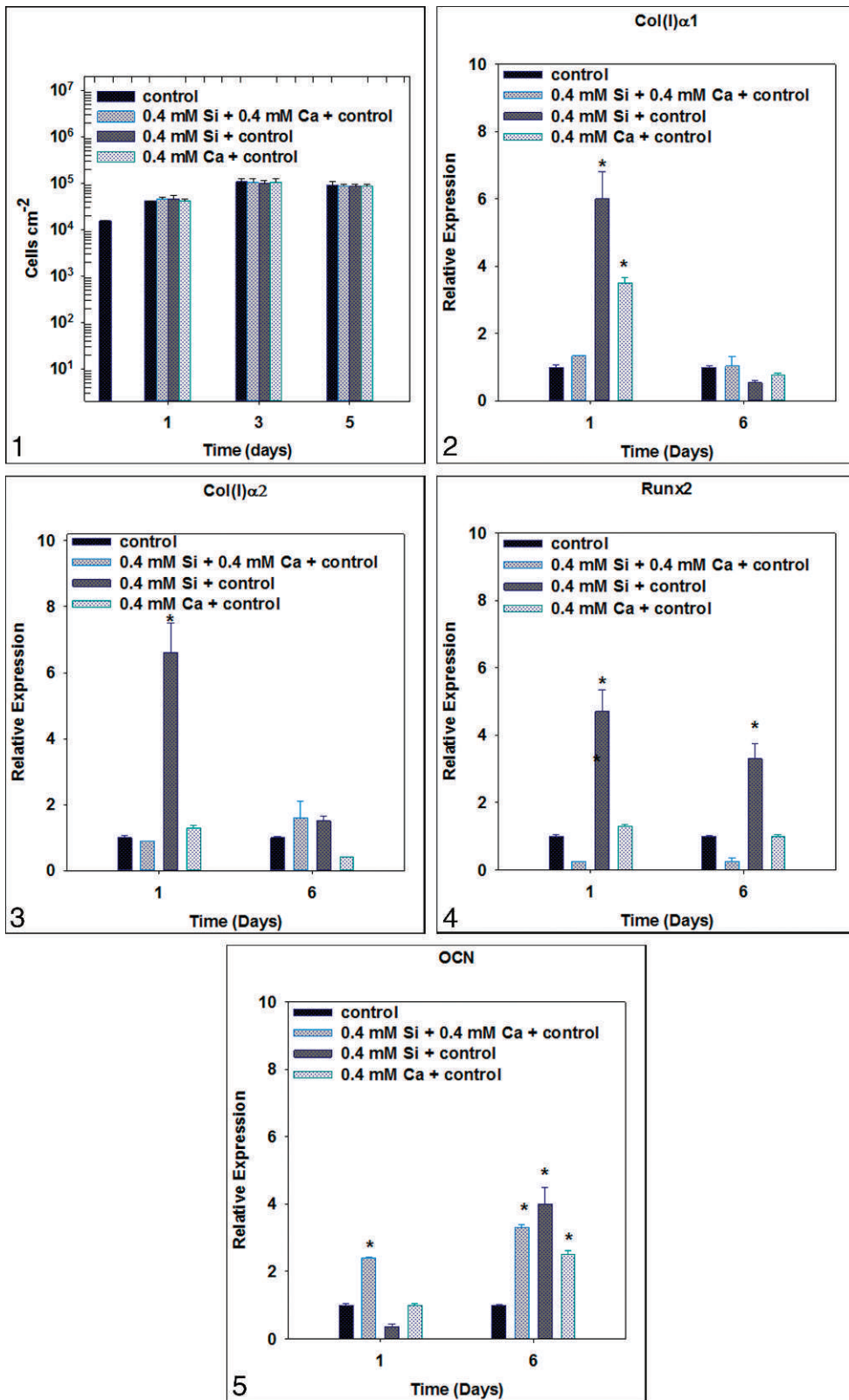
### **RESULTS**

Results of cell proliferation are given in Figure 1. Cells were treated with individual ion, combined ion, and control treatments without the addition of ascorbic acid. Si and Ca (either individually or combinatorially) did not alter cell proliferation within the first 5 days of culture. The slight decrease in cell number between days 3 and 5 was not significant and was attributed to the limitation of the assay reagent because of contact inhibition in

confluent cultures.<sup>1</sup> Thus, cell density increased significantly with time but did not change with treatment.

Individual ion treatments induced increased expression of all 4 genes on days 1 and 6 during differentiation. For example, cultures exposed to increased Si or Ca ion concentrations during differentiation (ascorbic acid addition) had increased expression of Col(I) $\alpha$ 1 (Figure 2, 0.4 mM Ca + control: 4 times control; 0.4 mM Si + control: 7 times control, after 1 day of culture), Col(I) $\alpha$ 2 (Figure 3, 0.4 mM Si + control: 6.5 times control after 1 day of culture), Runx2 (Figure 4, 0.4 mM Ca + control: 2.5 times control; 0.4 mM Si + control: 5 times control, after 6 days of culture), and OCN (Figure 5, 0.4 mM Ca + control: 2.5 times control; 0.4 mM Si + control: 4 times control, after 6 days of culture). Thus, individual ions enhanced the expression of multiple genes during the time period studied.

Interestingly, when these ions were added in combination, the expression of these genes was not uniformly increased and showed specificity to the increased expression of one gene. On one hand, when Si and Ca ions were added together after 1 day of culture, only a slight increase in Col(I) $\alpha$ 1 was observed (Figure 2, 0.4 mM Si + 0.4 mM Ca + control media, 1.2 times control, day 1). A similar result was observed for Col(I) $\alpha$ 2 (Figure 3, 0.4 mM Si + 0.4 mM Ca + control media, 1.5 times control, day 6). This result was not as marked an increase when compared to the individual ion treatments. On the other hand, when Si and Ca ions were added together, a significant increase in OCN expression was observed after 1 day of culture (Figure 5, 0.4 mM Si + 0.4 mM Ca + control media, 2.5 times control). This result was markedly higher than the effect that the individual ions had on this cell line after 1 day in culture. After 6 days of culture, Col(I) $\alpha$ 1 expression remained unchanged, while OCN expression increased. Runx2 expression never increased above control levels in the combined ion treat-



ment. These results indicate that OCN expression was selectively enhanced by the combination of Si and Ca addition on earlier days than their expected time points (typically 6–10 days<sup>10–17,20</sup>). These results indicate that combinations of ions target enhanced expression of individual genes.

ECM collagen fiber bundle formation was also affected by these ion treatments (Figure 6). Cells treated with 0.4 mM Si + control appeared to have relatively dense tissue with orthogonal planes of collagen fiber bundles (Figure 6a). These collagen fiber bundles appeared to be elongated and relatively thick (Figure 6a), while 0.4 mM Ca + control treated cells (Figure 6b) showed similar ECM formation as control treated cells. Combined ion treatments (0.4 mM Si + 0.4 mM Ca + control treated cells, Figure 6c) showed a mix of short and elongated collagen fiber bundles. Control + AA treated cells showed sparse presence of collagen fiber bundle formation (Figure 6d). Visualized counts of short and elongated fiber bundles (Figure 6e) showed a greater density of elongated collagen fibers in Si ion treatments (0.4 mM Si + control, 0.4 mM Si + 0.4 mM Ca + control) as compared to 0.4 mM Ca + control and control treatments.

To determine the optimal  $\text{Si}^{4+}/\text{Ca}^{2+}$  ion ratio on osteocalcin that maximizes its expression, we investigated the effect of varying the  $\text{Si}^{4+}/\text{Ca}^{2+}$  ion ratio on osteocalcin expression (all results normalized and compared to the 1:1  $\text{Si}^{4+}/\text{Ca}^{2+}$  ion ratio effect on osteocalcin, Figure 7). It was found that a 1:1 ratio maximally enhanced the expression of osteocalcin. When the  $\text{Si}^{4+}/\text{Ca}^{2+}$  ratio was less than unity, the osteocalcin expression decreased signifi-

cantly by 20%–30%. Although the decrease in osteocalcin expression at a  $\text{Si}^{4+}/\text{Ca}^{2+}$  ratio of 2 was not significant, this trend was significant at a 4:1  $\text{Si}^{4+}/\text{Ca}^{2+}$  ion ratio. These results indicate that a 1:1 ion ratio of  $\text{Si}^{4+}/\text{Ca}^{2+}$  treatment of MC3T3-E1 subclone 4 cells maximally increases the expression of osteocalcin after 1 day of culture during differentiation.

## DISCUSSION

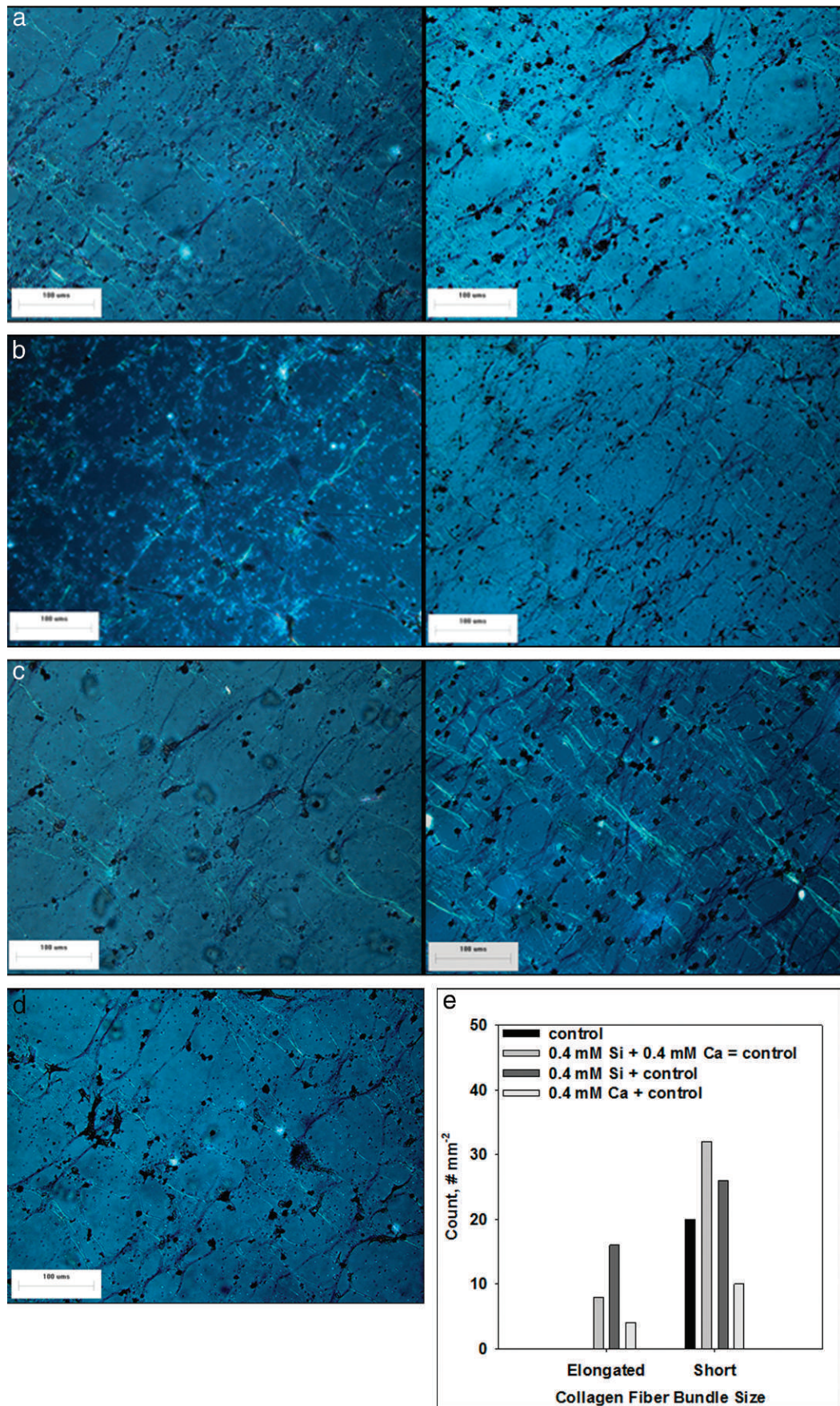
As expected, the timeline of enhanced expression of Col(I) $\alpha$ 1 and Col(I) $\alpha$ 2 (1 day), OCN (6 days), and Runx2 (6 days) was consistent with the established timeline of MC3T3-E1 subclone 4 expressions of these markers.<sup>21</sup> These results indicated that the individual ion effect fits into the first concept of combinatorial gene expression control in that each ion enhanced the expression of multiple genes and followed the expected time course of osteoblast differentiation.

An interesting trend was observed in which Si ion treatments had a greater impact on osteoblast gene expression than Ca ion treatments. Individual Si ion treatments showed increased expression of Col(I) $\alpha$ 1 (1.5 times), Col(I) $\alpha$ 2 (5 times), Runx2 (3 times), and OCN (1.5 times) as compared to individual Ca ion treatments (Figure 5). Moreover, this increased collagen expression by Si ion treatments probably led to the increased density of elongated collagen fiber bundles within ECM (Figure 6) as compared to Ca, Ca + Si, and control treated cells. The differences in ECM collagen fiber bundle structure between individual Si ion treatments and the other treatments could be attributed to the relative difference in collagen type 1

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**FIGURES 1–5.** **FIGURE 1.** Effect of Si, Ca, and Si + Ca ion treatments on MC3T3-E1 subclone 4 proliferation (ANOVA,  $P > .05$ ). **FIGURE 2.** Effect of Si and Ca ions on MC3T3-E1 subclone 4 expression of collagen type 1 [Col(I) $\alpha$ 1] after 1 and 6 days of culture. \*indicates statistical significance relative to control, ANOVA,  $P < .05$ . **FIGURE 3.** **FIGURE 4.** Effect of Si and Ca ions on MC3T3-E1 subclone 4 expression of collagen type 1 [Col(I) $\alpha$ 2] after 1 and 6 days of culture. \*indicates statistical significance relative to control, ANOVA,  $P < .05$ . **FIGURE 5.** Effect of Si and Ca ions on MC3T3-E1 subclone 4 expression of osteocalcin after 1 and 6 days of culture. \*indicates statistical significance relative to control, ANOVA,  $P < .05$ .

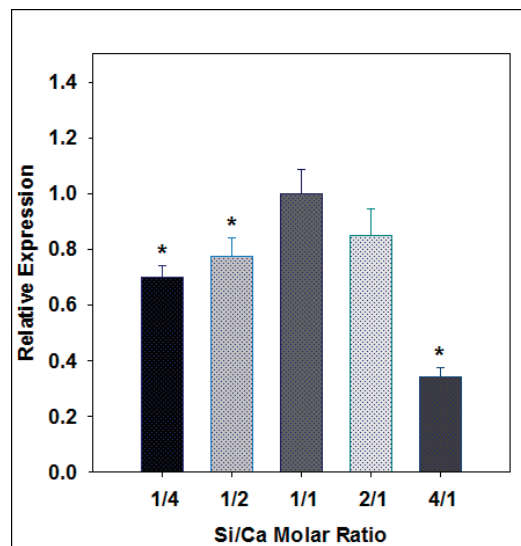




gene expression. This enhanced effect by Si on osteoblast differentiation was also observed at relatively low concentrations (0.01–0.05 mM),<sup>6</sup> whereas similar enhanced effect on osteoblast differentiation by Ca ions occurred at relatively higher concentrations (2–20 mM).<sup>7</sup> These results illustrate the impact that Si ions have on early MC3T3-E1 subclone 4 differentiation and suggest that Si ion release from bioactive glasses may enhance the bone healing process.

Unexpectedly, the combined ion treatment solely enhanced OCN gene expression but did not markedly increase collagen gene expression. According to Xiao et al<sup>21</sup> and Yu et al,<sup>22,23</sup> OCN expression is partially affected by the upstream expression of Col(I) $\alpha$ 1 and Runx2. Surprisingly, OCN expression increased despite a lack of increased Col(I) $\alpha$ 1 and Runx2. Furthermore, increased OCN expression occurred after 1 day of treatment and continued onto day 6 without the increased expression of Runx2. Moreover, a Si<sup>4+</sup>/Ca<sup>2+</sup> ion ratio of unity was found to be the most effective at maximizing OCN gene expression (Figure 7). These results are different from our previous work and that of Hench and colleagues<sup>3–5</sup> in that the ionic products of bioactive glasses (which contain Si and Ca) (and other ions such as PO<sub>4</sub><sup>3-</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>2+</sup>) enhanced Runx2 expression after 6 days by osteoblasts (Table). These differences and their implications are discussed below.

The lack of increased Runx2 expression in the combined ion treatment suggested an alternative mechanism may be involved. Besides Runx2, activating transcription factor 4 (ATF4) is involved in the regulation of OCN,<sup>24,25</sup> and its repression does not significantly affect collagen type 1 expression.<sup>25</sup> Since ATF4 is independent



**FIGURE 7.** Effect of Si and Ca ions on MC3T3-E1 subclone 4 expression of osteocalcin after 1 and 6 days of culture. \*indicates statistical significance relative to control, ANOVA,  $P < .05$ .

of Runx2 expression,<sup>26</sup> it is possible that the observed increase in OCN expression could have been owed to enhanced expression of ATF4. The effect that these ions have on other osteogenic transcription factors will help elucidate these potential alternative pathways in future studies.

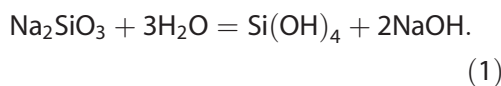
Despite the combined ion effect not markedly increasing collagen gene expression, the increased presence of elongated collagen fiber bundles was still observed. The increased presence of collagen fiber bundles in combined ion treatments was not as dense as those in Si ion treatments. This result, along with results showing Si ion treatments resulting in higher levels of OCN expression as compared to combined ion treatments, suggests that collagen gene expression could have been enhanced sometime between day 1 and 6. Yet, this increased expression could not have been as high as that in Si ion

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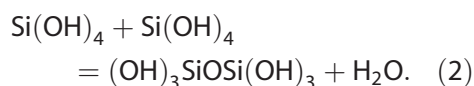
**FIGURE 6.** Effect of individual ion (0.4 mM Si + control + AA [a]), 0.4 mM Ca + control + AA [b]), and combined ion (0.4 mM Si + 0.4 mM Ca + control + AA [c]), and control (+ AA [d]) treatments on MC3T3-E1 subclone 4 collagen fiber bundle formation after 6 days of culture. (Elongated fiber bundles indicated with orange stars.) Collagen fiber bundle counts [e] confirmed more elongated fiber bundles in the treatments incorporating Si as compared to the treatments that did not incorporate Si.

treatments since the density of elongated collagen fibers was higher in Si ion treatments as compared to combined ion treatments. These results confirm that collagen gene and matrix formation is enhanced by elevated levels of Si ions.<sup>27</sup>

Another interesting observation was the maximum increase in expression of osteocalcin at a 1:1 Si<sup>4+</sup>/Ca<sup>2+</sup> ratio. The decrease in OCN expression with increasing Si ion content (and increasing Si<sup>4+</sup>/Ca<sup>2+</sup> ion ratio) could be explained as follows. The dissolution of sodium meta-silicate results in the increased presence of ionic Si in the form of silanols (Si(OH)<sub>4</sub>);



These silanols can spontaneously polymerize into silica gel networks:



According to Le Chatelier's principle, increased silanol concentration shifts the reaction towards the formation of polymerized silanols, which can become insoluble at relatively moderate molecular weights. This could explain why cellular production of osteocalcin was dampened at Si<sup>4+</sup>/Ca<sup>2+</sup> ratios greater than unity (and thus greater than 0.4 mM Si). In the case of Si<sup>4+</sup>/Ca<sup>2+</sup> ratios less than unity, dampened osteocalcin expression could be owed to reduced Si ion availability as compared to Si<sup>4+</sup>/Ca<sup>2+</sup> ion ratio at unity. Concentrations of silanols approaching or above 2 mM have shown increased tendency for spontaneous polymerization.<sup>6</sup> Thus, the maximum increase in OCN expression at Si<sup>4+</sup>/Ca<sup>2+</sup> ratio of unity could be owed to decreased bioavailability of Si ions above or below this ratio.

Another ion that could be tested along with Si and Ca is phosphate. Phosphate ions have been shown to be directly involved in osteopontin expression,<sup>28</sup> which is a key marker involved in bone remodeling. It may be possible that combinations of Si, phosphate, and Ca

ions could potentially regulate the expression of osteopontin and bone matrix remodeling.

The concept of combinatorial control over cellular gene expression may provide an advantageous tool in aiding future implant designs. In this work, we used the combinatorial principle to optimize the Si<sup>4+</sup>/Ca<sup>2+</sup> ion ratio and found that a 1:1 ratio was most effective at enhancing osteocalcin expression. This optimized Si<sup>4+</sup>/Ca<sup>2+</sup> ion ratio could guide future implant coating structures that control ion release and regulate osteocalcin expression, which is involved in mineralized tissue formation.<sup>29</sup> However, increased OCN expression may impact other biomolecules that regulate other physiologic tissues or organs, for example, it has been found that increased OCN expression could impact insulin production in the spleen.<sup>30,31</sup> Such localized and systemic effects would need careful consideration when designing future implant coatings.

Although Hench<sup>32</sup> suggested that these ions could be used to design bioactive glasses that are genetically tailored to elicit specific cellular responses for varying outcomes, it has not been shown that the concept of combinatorial gene expression could be used to effect such genetic tailoring. Our work revealed that this may be a useful new strategy, and we will investigate this concept further by cataloging ion combinations and their effect on osteoblast gene expression and ECM development.

The results obtained in this study present a novel strategy for bone healing using bioactive glasses. For such a strategy to be of value, it must have clinical use. One aspect of the ionic effect relates to Si<sup>4+</sup> and collagen expression. It may be possible that Si<sup>4+</sup> can enhance other forms of collagen that alter the physical properties of the resultant ECM. This means that Si<sup>4+</sup> could increase or decrease the stiffness of the ECM, which in turn would increase the stiffness of the resultant bone matrix. In applications where weak bone is made (eg,

diseases in collagen formation), this strategy could help to strengthen and reinforce tissue-engineered bone to better serve the patient's bone loss needs. Other applications include the use of such materials for patients needing implants. The bioactive glass, as an implant coating, could truncate healing time or increase the rate of mineralized tissue formation around the implant. The potential of bioactive glass to meet the needs of patients for a variety of bone healing applications is broad, and this work presents a small window into its significance.

### CONCLUSIONS

In this study, we tested the combinatorial influence of Si and Ca on osteoblast (MC3T3-E1 subclone 4) gene expression during differentiation. Our results indicate that individual ions enhance the expression of multiple genes at their expected time points. Moreover, elevated Si ion concentrations appeared to enhance the expression of osteogenic markers at significantly higher levels as compared to elevated Ca ion concentrations and control treatments. Combinations of Si and Ca ions led to targeted and enhanced expression of OCN after 1 day and 6 days during differentiation. The enhanced Col(I) $\alpha$ 1 gene expression by elevated Si ion concentration also enhanced the presence of elongated collagen fiber bundle formation during early ECM development, while combined ion treatments, Ca ion treatments, and control treatments showed lower density of elongated collagen fiber bundles. The combined ion treatment of 1:1 for Si<sup>4+</sup>/Ca<sup>2+</sup> was found to be optimal for maximal up-regulation of OCN expression. These results indicate that the enhanced effect by ions on osteoblast differentiation depends on the individual or combined presence of Si and Ca.

### ABBREVIATIONS

AA: ascorbic acid

$\alpha$ -MEM:  $\alpha$ -minimum essential medium  
 cbfa1/Runx2: core binding factor a  
 Col(I) $\alpha$ 1/Col(I) $\alpha$ 2: collagen type 1  
 ECM: extracellular matrix  
 FBS: fetal bovine serum  
 GRP: gene regulatory protein  
 HA: hydroxyapatite  
 OCN: osteocalcin  
 pen-strep: penicillin-streptomycin  
 qRT-PCR: quantitative reverse transcription polymerase chain reaction

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