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Effects of Silicon on Osteoblast Activity and Bone Mineralization of MC3T3-E1 Cells

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Abstract Previous studies have reported that dietary silicon (Si) intake is positively associated with bone health including bone mineral density. Although the amount of Si intake is high among trace elements in humans, how dietary Si affects bone formation at the cellular level is not well addressed. The purpose of this study was to investigate the role of Si in osteoblast activity and bone mineralization. MC3T3-E1 was cultured as mature osteoblasts and treated with sodium metasilicate (0, 1, 5, 10, 25, 50, and 100 μM) as a source of Si. After 7 days of treatment, 5 and 10 μM of sodium metasilicate significantly increased intracellular alkaline phosphatase activity ($p<0.05$) when compared to the control. Additionally, all doses of sodium metasilicate (1, 5, 10, 25, 50, and 100 μM) increased mineralized nodule formation at 14 days of differentiation as evidenced by increased Alizarin Red S staining. In the analysis of gene expression, 50 μM of sodium metasilicate upregulated type I collagen (COL-I) compared to the control group. However, the increase of COL-I gene expression as a result of treatment with 1, 10, 25, and 100 μM of sodium metasilicate did not reach statistical significance. mRNA expression of insulin-like growth factor-I and receptor activator of NF-κB ligand was not significantly changed at any dose of sodium metasilicate (0, 1, 5, 10, 25, 50, and 100 μM). In light of the results, we conclude that Si has a positive effect on bone metabolism by enhancing osteoblast mineralization activity.

Keywords Silicon · Proliferation · Osteoblast · Mineralization · Gene expression

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Introduction

Silicon (Si) is one of the most abundant elements in nature and an important trace nutrient in human physiology, including bone metabolism [1]. There is evidence that Si affects the increase of bone density and decrease of bone loss. A cross-sectional study showed that Si intake positively correlated with bone mineral density (BMD) at the hip in men and premenopausal women, indicating that higher dietary Si intake in men and younger women has beneficial effects on skeletal health [2]. Previous animal studies demonstrated that Si depletion attenuates the development of the extracellular matrix and the formation of hydroxylapatite [3, 4]. Si has also been shown to play a structural role in the crosslinking and stabilization of collagen and glycosaminoglycans [5]. Although these studies suggest that Si depletion favorably modulates bone metabolism, this beneficial effect of Si is only shown in a nonbiological setting, and the effect of dietary supplementation of Si in bone metabolism appears to be somewhat limited.

Bone metabolism is mainly determined by the bone formation activity of osteoblasts and the bone resorption activity of osteoclasts [6]. Osteoblasts synthesize enzymes involved in bone matrix formation and mineral accumulation and produce osteoclast differentiation factors including receptor activator of NF-κB ligand (RANKL) [7]. Osteoblast activity and the process of bone mineralization are regulated by growth factors which include insulin-like growth factor (IGF)-I [8], alkaline phosphatase (ALP) [9],

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an enzyme involved in the formation of hydroxylapatite, and lysyl oxidase, an enzyme responsible for collagen cross-linking [10, 11]. The increase of production of type I collagen (COL-I), a bone matrix protein, is also closely associated with enhanced osteoblast activity [12]. Furthermore, osteoblast-specific transcription factors, Osterix and Runx2, were reported to regulate the expression of ALP and IGF-I as well as several bone matrix proteins including COL-I [13, 14]. Hence, these growth factors, enzymes, and proteins could be indicating factors for assessing the ability of pharmaceuticals and nutrients to enhance osteoblast activity.

Based on the findings from animal and clinical studies [2–4, 15], we hypothesized that Si positively affects bone metabolism by altering osteoblast activity and maturation. Hence, the purpose of this study was to investigate whether Si stimulates osteoblast activity and mineralized nodule formation and how Si affects bone metabolism in vitro.

Materials and Methods

Reagents

MC3T3-E1 (American Type Culture Collection (ATCC) no. CRL-2593) mouse calvarial pre-osteoblastic cells, which were originally purchased from ATCC (Manassas, AV, USA), were kindly provided by Dr. In-Sook Kwun at Andong National University (Andong, South Korea). Minimum essential medium (α-MEM), fetal bovine serum (FBS), and a mixture of penicillin and streptomycin were purchased from GIBCO-BRL (Grand Island, NY, USA). Sodium metasilicate (9 hydrate $Na_2SiO_3 \cdot 9H_2O$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ascorbic acid, β-glycerophosphate, and Alizarin Red S were purchased from Sigma-Aldrich (St. Louis, MO, USA). For ALP activity measurements, an ALP substrate kit from Bio-Rad (Hercules, CA, USA) was used. Unless otherwise listed, all other chemicals were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) or Junsei Chemicals (Tokyo, Japan).

Preparation and Verification of Si Treatment

Sodium metasilicate was used for Si treatment, and various concentrations of sodium metasilicate (0, 5, 10, 25, 50, 100, and 1,000 μM) in cell culture media were prepared by serial dilution of 10 mM stock solution. The sodium metasilicate concentrations that we used in this study corresponded to 0.0, 0.14, 0.28, 0.70, 1.4, 2.8, and 28.1 mg/L of Si, respectively. The sodium metasilicate solutions were pretreated and then subjected to inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Optima 5300 DV, PerkinElmer, MA, USA). The determined concentrations of Si at each dose of sodium metasilicate were 0.0, 0.1, 0.3, 0.6, 1.5, 2.7, and 23.5 mg/L of Si, respectively (Fig. 1).

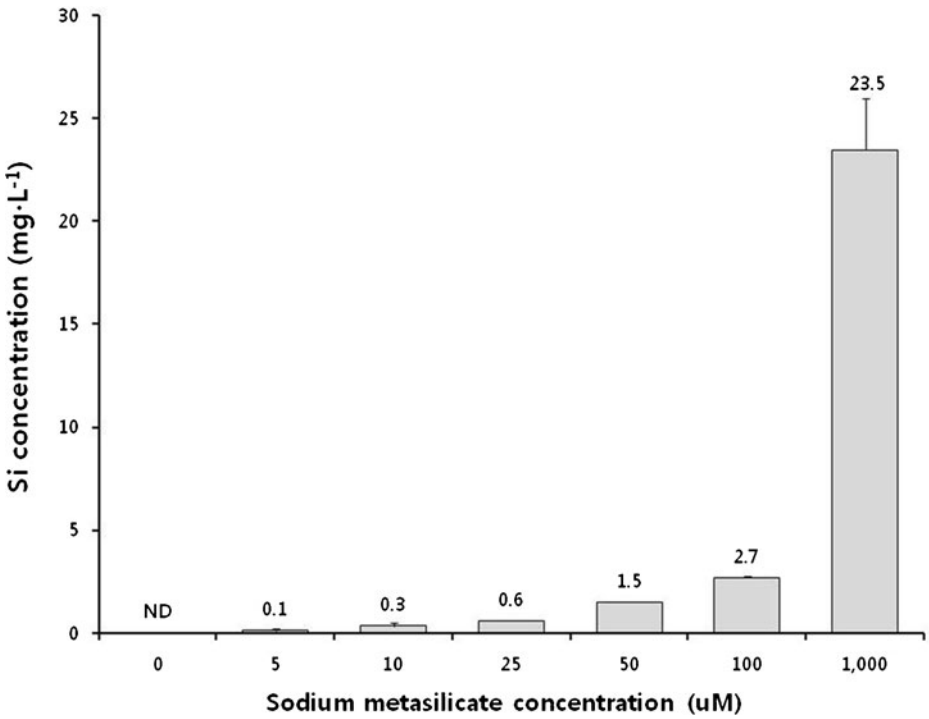

Fig. 1 Si concentration at each dose of sodium metasilicate in cell culture media detected by ICP-AES. Data are presented as mean ± SD (n=2). *ND* not detected

Osteoblast Culture

MC3T3-E1 cells were plated at a density of 1×10^5 cells/mL in 12-well plates ($n=3$) and cultured in α-MEM containing 10 % FBS, 2 mM L-glutamine, and a mixture of 100 U/L penicillin G and 100 mg/L streptomycin, at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. After cells reached confluence, the medium was replaced with α-MEM containing 10 mM β-glycerophosphate and 25 μg/mL ascorbic acid to facilitate in vitro mineralization. Cells were then treated with several doses of sodium metasilicate as indicated in the "Results" section. The culture media, which included sodium metasilicate with doses described above, were replaced every 3 days. For ALP activity measurement, culture media and cell monolayers were harvested at 7 and 14 days after confluence. For analysis of mineralized nodule formation, cells were fixed at 14 days and stained with Alizarin Red S as described below.

Assay of Cell Viability

The proliferation of MC3T3-E1 cells was measured according to the colorimetric MTT method by following commercial protocols. The yellow-colored MTT reagent enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark purple-colored formazan product and measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [16]. MC3T3-E1 cells were seeded on 96-well plates (1×10^5 cells/mL) and incubated overnight. Then, sodium metasilicate at different concentrations (0, 5, 10, 25, 50, 100, 1,000, and 10,000 μM) was added. Control wells were prepared by addition of α-MEM. Wells containing α-MEM without cells were set as blanks. At each indicated time point (1, 2, and 3 days) after plating, 20 μL of MTT (5.0 mg/mL) was added and incubated for another 4 h at 37 °C. Then, the supernatant was removed and dimethyl sulfoxide was added as a stop solution. Absorbance (optical density, O.D.) at 570 nm was measured on a microplate spectrophotometer (Bio-Tek, Model ELX 808, CA, USA). Cell viability was calculated according to the formula: [(O.D. sample−O.D. blank)−(O.D. control−O.D. blank)]/(O.D. control−O.D. blank)×100, and indicated as percent of viability of control cells.

Intracellular and Extracellular ALP Activity

Since cytotoxic effects from sodium metasilicate at 10,000 μM were observed, this high dose of sodium metasilicate was excluded, and a low dose (1 μM) of sodium metasilicate was added in the remaining experiments. After 7 and 14 days of treatment with sodium metasilicate (0, 1, 5, 10, 25, 50, and 100 μM), media were collected and the cell monolayer was gently washed twice with ice-cold phosphate-buffered saline (PBS). Cells were lysed with 0.2 % Triton X-100, the lysate was centrifuged at 14,000×*g* for 5 min, and supernatant was used for the measurement of ALP activity. ALP was determined by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at 405 nm according to the manufacturer's instruction (Bio-Tek, Model ELX 808, CA, USA). Total protein was assessed using the bicinchoninic acid method [17] and ALP activity was expressed in nanomole of *p*-nitrophenol produced per minute per microgram of protein.

Nodule Formation

Bone mineralization was determined by Alizarin Red S (AR-S) staining at 14 days. Cells were washed twice with PBS and then fixed in ice-cold 70 % ethanol for 1 h at room temperature. Following the PBS wash, cells were stained with 40 mM Alizarin Red S (pH 4.2) for 10 min at room temperature. Digital images of the stained matrix were acquired using a digital camera (Canon, Tokyo, Japan). For the quantification of staining density, AR-S staining was released from the cell matrix by incubation with 10 % cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 20 min. The AR-S concentration was determined by measuring the absorbance at 562 nm (Bio-Tek, Model ELX 808, CA, USA).

Analysis of Gene Expression Using Real-Time PCR

For analysis of gene expression, MC3T3-E1 cells were differentiated as indicated above and then treated with 0, 1, 5, 10, 25, 50, and 100 μM of sodium metasilicate for 7 days. Total cellular RNA was isolated using Trizol following the manufacturer's guidelines (Invitrogen, Rockville, MD, USA). The concentration and quality of the RNA were determined by O.D.s measured at 260 and 280 nm by BioSpec-nano (Shimadzu, Tokyo, Japan). The expression of mRNA was quantified by real-time RT-PCR using a CFX-96 (Bio-Rad, Hercules, CA, USA) with real-time PCR Master Mix based on SYBR green (Toyobo, Osaka, Japan). Denatured RNA (50 ng) from cells was reverse transcribed at 58 °C for 10 min for cDNA synthesis using CFX-96 (Bio-Rad, Hercules, CA, USA). cDNAs were amplified with gene-specific primers (Table 1) over 45 cycles at 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. Post-PCR melting curves confirmed the specificity of single-target amplification. The amount of mRNA for each gene was

Table 1 Primer sequences for real-time polymerase chain reaction

Target gene	Probe	Primers
GAPDH	F	5′-GTG AAG GTC GGA GTC AAC-3′
	R	5′-TGG AAT TTG CCA TGG GTG-3′
IGF-I	F	5′-TCA CAT CTC TTC TAC CTG GCA CTC-3′
	R	5′-CAG TAC ATC TCC AGC CTC CTC AGA-3′
COL-I	F	5′-CCC AAG GAA AAG AAG CAC GTC-3′
	R	5′-AGG TCA GCT GGA TAG CGA CAT C-3′
RANKL	F	5′-TGA AGA CAC ACT ACC TGA CTC CTG-3′
	R	5′-CCA CAA TGT GTT GCA GTT CC-3′

GAPDH glyceraldehyde-3-phosphate dehydrogenase, *IGF-I* insulin-like growth factor-I, *COL-I* type I collagen, *RANKL* NF-κB ligand

calculated using a delta–delta CT method, and expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis

Statistical analysis was performed using SAS Version 9.2 (SAS Institute, Cary, NC, USA). The significance of sodium metasilicate treatment effects was analyzed using the Duncan's multiple-range test. Values were expressed as means ± standard deviation (SD) and difference between treatments was declared to be significant at $p<0.05$. Each experiment was performed three to four times and the data from one representative experiment are presented.

Results

Cell Viability

Cell viability of control cells and cells treated with all doses of sodium metasilicate (5, 10, 25, 50, 100, 1,000, and 10,000 μM) increased in a time-dependent manner (1, 2, and 3 days). In MC3T3-E1 cells, cell viability was slightly (5~10 %) decreased after 1 and 2 days of Si treatment, but after 3 days, cell viability in 5, 10, and 25 μM of sodium metasilicate treatment increased up to control levels. However, remarkable cytotoxic effects at 10,000 μM of sodium metasilicate were still observed in the MC3T3-E1 (Fig. 2).

Intracellular and Extracellular ALP Activity

ALP activity in cell lysates and media under 0, 1, 5, 10, 25, 50, and 100 μM of sodium metasilicate treatment was further analyzed. At 7 days after differentiation, 5 and 10 μM of sodium metasilicate showed higher ALP activities than the control treatment, but at day 14, only 100 μM sodium metasilicate showed higher ALP activity than control cells (Fig. 3a). The activity of ALP released by the cells into the media was also analyzed. Both at days 7 and 14, no alteration of the extracellular ALP activity was observed in any of the doses of sodium metasilicate (Fig. 3b).

Nodule Formation

To determine the effect of Si on osteoblast function, cells were stained with AR-S at 14 days post-confluence for the

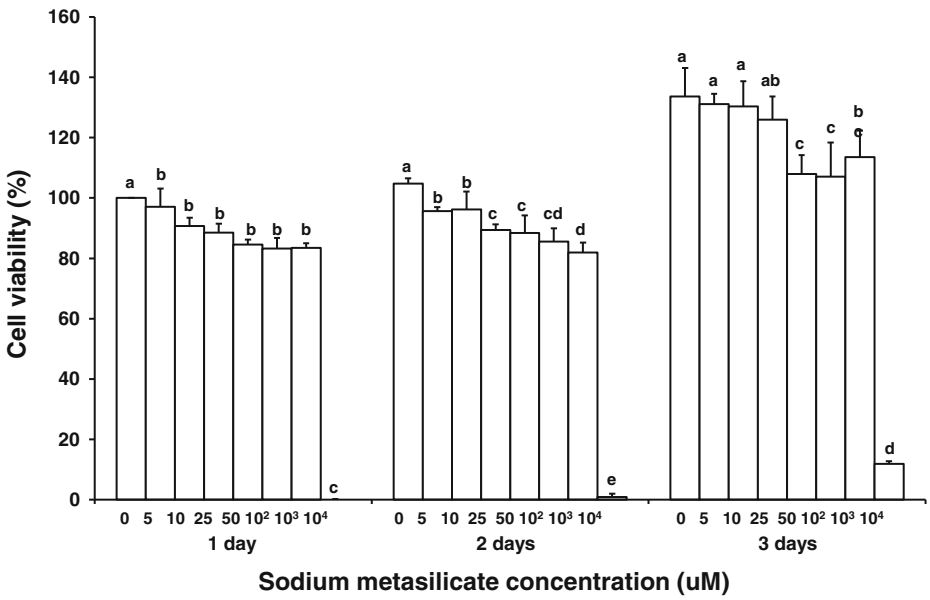

Fig. 2 Cell viability of osteoblastic MC3T3-E1 cells by silicon treatment. *Different letters* indicate significant difference computed by Duncan's multiple-range test at $\alpha=0.05$. Data are presented as mean ± SD ($n=3$)

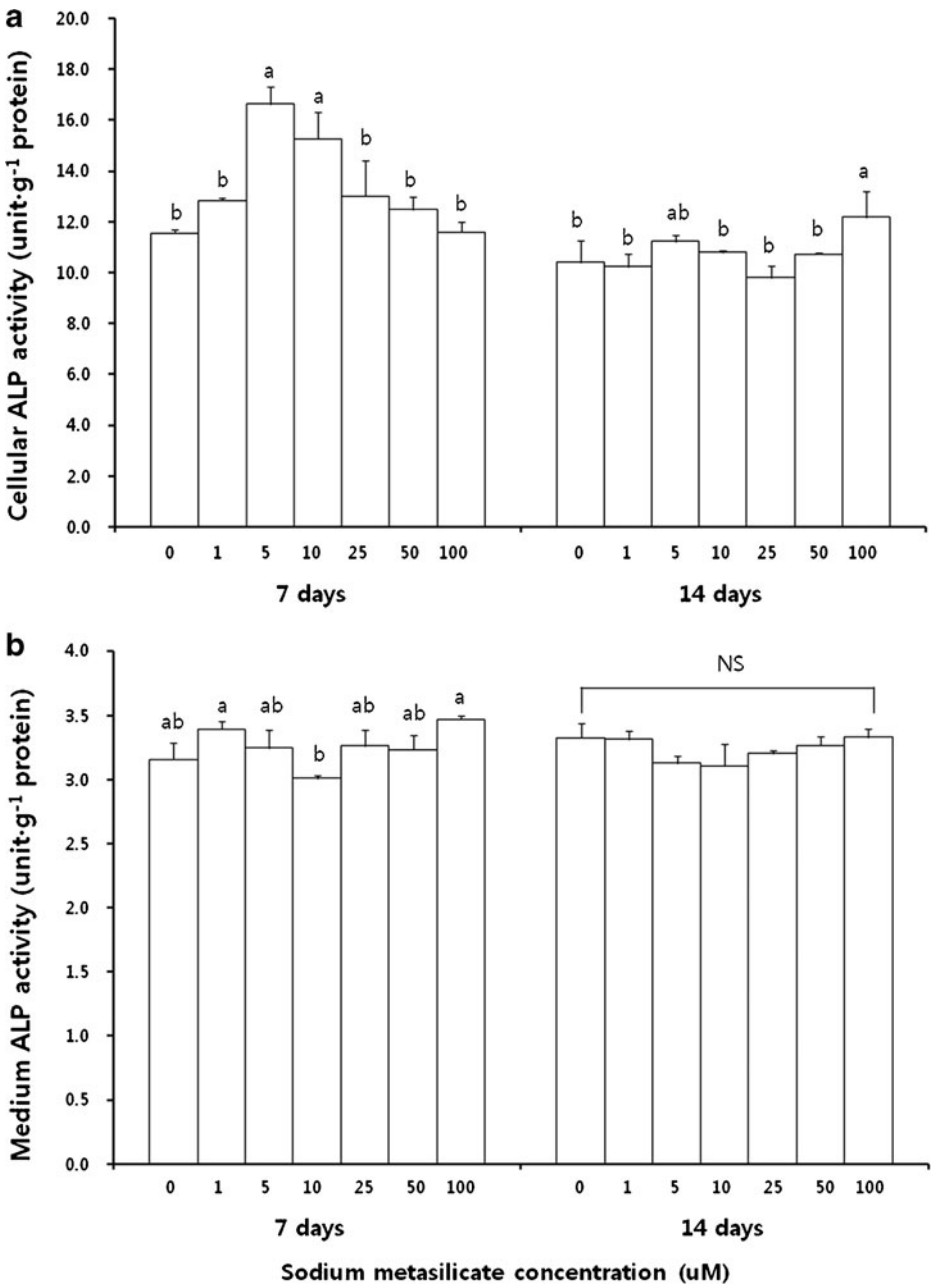

Fig. 3 ALP activity in osteoblastic MC3T3-E1 cells (**a**) and media (**b**) by silicon treatment. *Different letters* indicate significant difference computed by Duncan's multiple-range test at $\alpha=0.05$. Data are presented as mean ± SD ($n=4$). ALP one unit indicates nanomole of *p*-nitrophenol produced per minute per microgram of protein. *NS* not significant

identification of mineralized nodules (Fig. 4). After 14 days, all doses of sodium metasilicate (1, 5, 10, 25, 50, and 100 μM) significantly increased ($p<0.05$) the staining density of AR-S in quantification by spectrophotometer (Fig. 4a) and visible observation (Fig. 4b). An increase of nodule formation at lower doses of sodium metasilicate (1, 5 μM) was in the range of 10~20 %, and higher doses of sodium metasilicate (10, 25, 50, and 100 μM) further increased nodule formation (94 %), compared to the control cells. Increases in the level of nodule formation at each different dose of sodium metasilicate were significantly different from each other.

Gene Expression Involving Osteoblast Function

To identify the role of Si in intracellular production of growth factor, bone matrix protein, and signaling molecules, the gene expression of IGF-I, COL-I, and RANKL was measured (Fig. 5). Sodium metasilicate at 50 μM treatment significantly increased the expression of COL-I. The extent of the increase in COL-I expression was in the range of 221 to 562 %. However, other doses of sodium metasilicate (1, 5, 10, 25, and 100 μM) did not significantly increase COL-I expression. Additionally, changes in IGF-I expression at all doses of sodium metasilicate (1, 5, 10, 25, 50, and 100 μM)

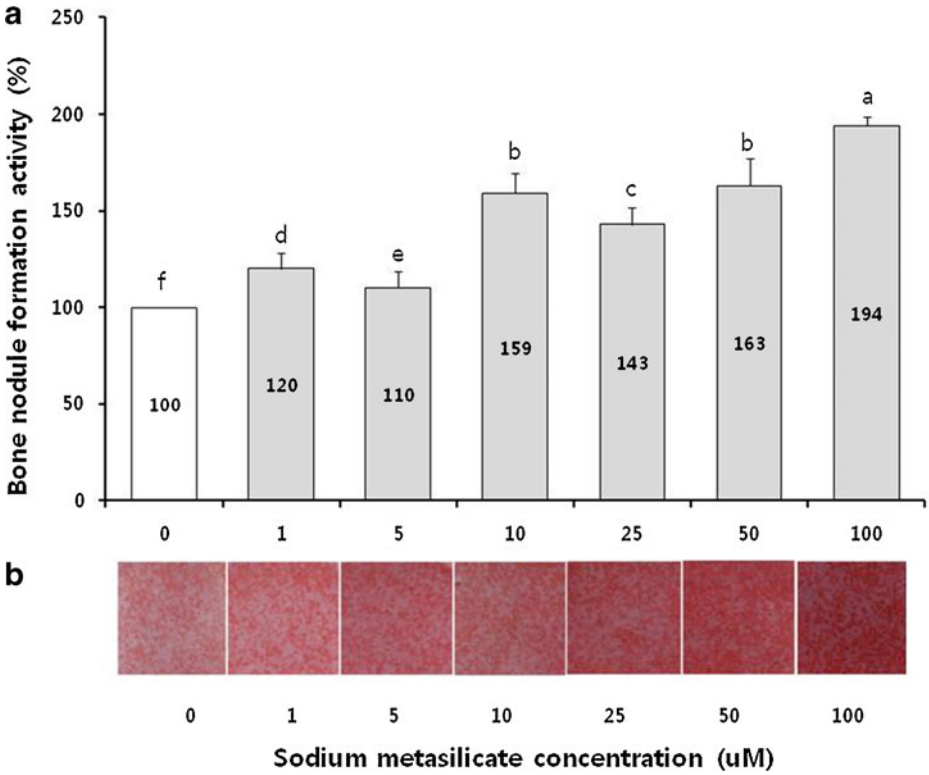

Fig. 4 Bone mineral nodule formation by silicon treatment in MC3T3-E1 cells at 14 days after differentiation. Quantification values (**a**) and digital images (**b**) of Alizarin Red S staining were presented. *Different letters* indicate significant difference computed by Duncan's multiple-range test at α=0.05. Data are presented as mean ± SD (n=3). Magnification rate in Alizarin Red S staining is ×2

treatment did not reach the level of significance compared to control cells. mRNA expression of RANKL, osteoclast differentiation factor produced by osteoblasts, was also measured, and no difference in RANKL expression at any dose of Si treatment was found.

Discussion

Results in this study suggest that Si has beneficial effects in osteoblasts and partially explain how Si was associated with the increase in bone mass in previous epidemiological studies [2, 18, 19] and osteoporotic animal models [15, 20, 21]. Si supplementation with monomethyl trisilanol resulted in increases in femoral and lumbar spine BMD in osteoporotic women [18]. In the Framingham Offspring cohort study, it was reported that higher intake of dietary Si was significantly and positively associated with BMD at the hip in men and premenopausal women, but not in postmenopausal women [2]. Spector et al. [22] also reported a trend for increased bone formation markers in serum, especially procollagen type I N-terminal propeptide, a marker COL-I synthesis, with increasing doses of ch-OSA in osteopenic subjects. In animal studies, Si supplementation for 1 month prevented OVX-induced bone loss at the fifth lumbar vertebrae and femur, and stimulated longitudinal bone growth, indicating the bone formation activity of Si [20].

In this study, Si enhanced osteoblast activity and the osteoblasts' ability to form mineralized nodules, and these positive changes were partially associated with the increase of ALP activity and upregulation of bone matrix proteins COL-I. Similarly to our findings with Si, drugs and other natural compounds which have anabolic properties have been shown to have positive effects on osteoblast activity via increases in growth factors (e.g., IGF-I) and proteins and increased enzymatic activity (e.g., ALP) involved in the formation of the mineralized bone matrix [23–25]. For example, PTH restored bone mass via an increase in ALP activity in postmenopausal women with osteoporosis [26] and by increasing IGF-I expression [27–29]. Another interesting finding in this study was that doses of Si (0.6~1.5 mg/L) decreased cell viability rather increased mineralized nodule formation and the data in this study indicate that cell cycle arrest by Si may halt the repression of the cellular differentiation process and finally lead to an increase in mineralized nodule formation.

There are a number of issues that remain related to the effect of Si in bone metabolism. For instance, it is unclear whether the effects of Si on osteoblasts reported here are specific to certain doses, due to little cytotoxic effects at high doses of Si. It is also unclear whether there are differences in the potency of Si in different methods of delivery and intake (e.g., diet, tap water vs. medical treatment). Also, the ability of Si to prevent bone loss or restore bone in preclinical and clinical studies still appears to be limited. Furthermore, the alterations in gene expression presented here were the result of alterations that occurred after 7 days of exposure to Si, and a change of mRNA expression may

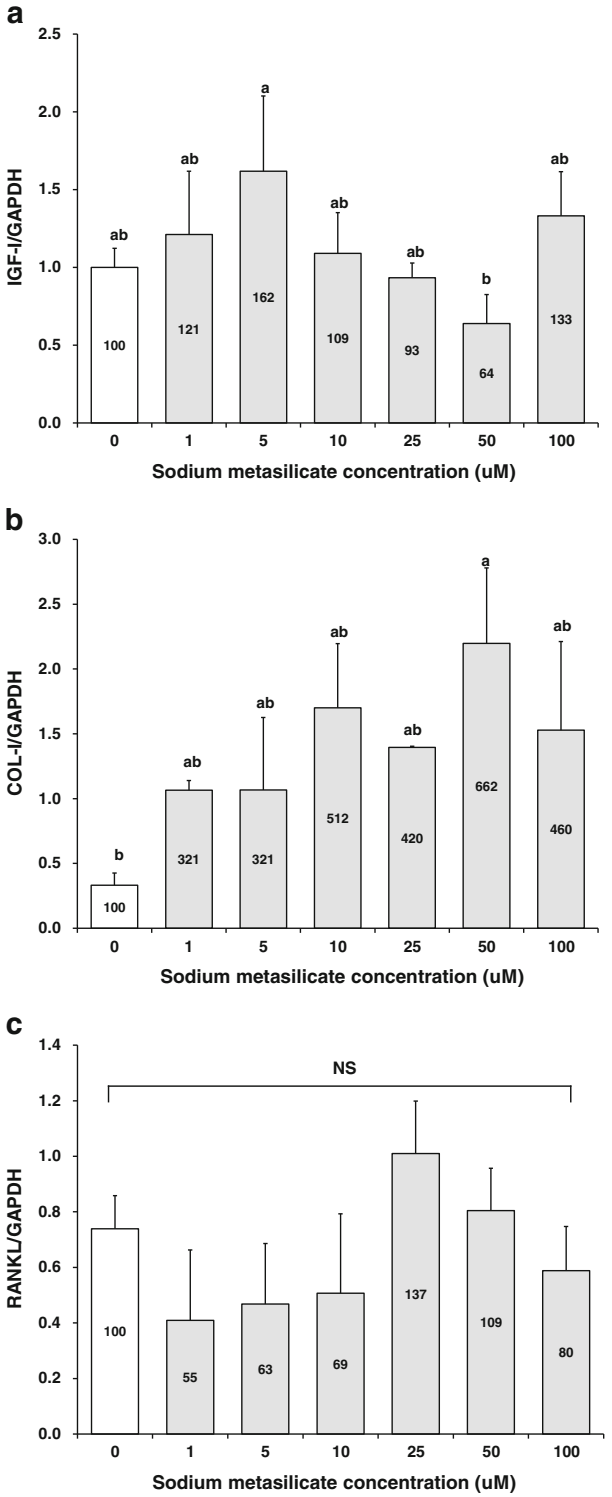

Fig. 5 Expression of mRNA related to osteoblast function in MC3T3-E1 cells by silicon treatment for 7 days (**a** insulin-like growth factor-I; **b** type I collagen; **c** RANKL). *Different letters* indicate significant difference computed by Duncan's multiple-range test at $\alpha=0.05$. Data are presented as mean ± SD ($n=3$). Data in *bar* indicate percentages of control. *NS* not significant

occur at a point prior to this. Due to the effect of Si on ALP activity and mineralized nodule formation, analysis of the effect of Si on gene expression over time may provide more insight.

In this study, Si effectively enhanced the osteoblastic mineralization activity and gene expression involved in bone matrix synthesis. The findings suggest that Si is partially responsible for the beneficial effects of Si in bone health reported in previous animal studies [15, 19, 21]. On the other hand, this study focused on the effect of Si only in osteoblasts and only investigated the effects of Si with no external stimulation. The effect of Si should be explored by challenging inflammatory cytokines such as TNF-α and interleukin-6 which decrease osteoblast activity and propagate osteoclast differentiation and activity [30, 31]. The importance of Si in osteoclast function and activity also needs to be investigated. In conclusion, Si increases ALP activity and mineral deposits in osteoblasts, and this indicates that in addition to Ca, Si could be a possible candidate mineral for enhancing bone health.

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