

The effect of silicate ions on proliferation, osteogenic differentiation and cell signalling pathways (WNT and SHH) of bone marrow stromal cells†

Cite this: *Biomater. Sci.*, 2013, **1**, 379

Pingping Han,^a Chengtie Wu^{*b} and Yin Xiao^{*a}

Silicon (Si) is a trace element, which plays an important role in human bone growth. Si has been incorporated into biomaterials for bone regeneration in order to improve their osteogenic potential, both *in vitro* and *in vivo*. Little is known, however, as to how Si ions elicit their biological response on bone-forming cells. The aim of this study was to investigate the effect of Si ions on the proliferation, differentiation, bone-related gene expression and cell signalling pathways of bone marrow stromal cells (BMSCs) by comparing the BMSC responses to different concentrations of NaCl and Na₂SiO₃, while taking into account and excluding the effect of Na ions. Our study showed that Si ions at a concentration of 0.625 mM significantly enhanced the proliferation, mineralization nodule formation, bone-related gene expression (OCN, OPN and ALP) and bone matrix proteins (ALP and OPN) of BMSCs. Furthermore, Si ions at 0.625 mM could counteract the effect of the WNT inhibitor (W.I.) cardamonin on the osteogenic genes expression, (OPN, OCN and ALP), WNT and SHH signalling pathway-related genes in BMSCs. These results suggest that Si ions by themselves play an important role in regulating the proliferation and osteogenic differentiation of BMSCs, with the involvement of WNT and SHH signalling pathways. Our study provides evidence to explain possible molecular mechanisms whereby Si ions released from Si-containing biomaterials can acquire enhanced bioactivity at desired concentration.

Received 6th August 2012,
Accepted 15th November 2012

DOI: 10.1039/c2bm00108j

www.rsc.org/biomaterialsscience

1. Introduction

For the past thirty years, significant attention has been paid to silicon (Si), due to the studies of Carlisle and Schwarz, who showed that Si is an important factor in skeletal development and repair.^{1,2} Si has been found to be an important trace element in human body. Si is located at active calcification sites in the bones and is directly involved in the mineralization process of bone growth.¹ Due to this significant function of Si in human bone, Si has been widely incorporated into Ca–P bioceramics in order to enhance their bioactivity.^{3–8} Typically, Si-doped hydroxyapatite and tricalcium phosphate ceramics have been prepared. It has been found that Si-doped Ca–P bioceramics have improved biological performance compared to

pure Ca–P ceramics.^{6,9–11} Silicate-based bioactive glasses and ceramics have also been widely studied for bone regeneration applications.^{12–16} The significant advantages of this family of materials are that Si-containing ionic products from these biomaterials significantly enhance the proliferation and osteogenic differentiation of a series of stem cells, including bone marrow stromal cells, human dental pulp cells, periodontal ligament cells, and adipose-derived stem cells.^{17–23} Our recent study found that silicate ceramics have significantly enhanced *in vivo* bone formation in a form of lactide-co-glycolide microspheres.²⁴ Some silicate ceramics, CaSiO₃ and Ca₂MgSi₂O₇, have significantly improved *in vivo* osteogenesis compared to conventional β-tricalcium phosphate ceramics.^{25,26} These *in vitro* and *in vivo* studies seem to indicate that Si may play an important role in stimulating the biological response of bone-forming cells and further enhancing the osteogenic bioactivity of biomaterials. However, as reported by Bohner in his leading opinion paper, until now there has been no obvious and direct evidence linking the improved biological performance of Si-substituted calcium phosphates to Si release.²⁷ Similarly, for silicate bioactive glasses and ceramics, their ionic products contain not only Si, but also other important biological ions, such as Na, Mg, Zn, Ca, etc.^{22,28,29} It is difficult to claim that silicate-based bioactive glasses and ceramics have improved

^aInstitute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland 4059, Australia. E-mail: yin.xiao@qut.edu.au; Tel: +61-7-31386240; Fax: +61-7-31386030

^bState Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics, Chinese Academy of Sciences, Shanghai 200050, People's Republic of China. E-mail: chengtiewu@mail.sic.ac.cn; Tel: +86-21-52412249; Fax: +86-21-52413903

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c2bm00108j

in vitro and *in vivo* bioactivity due to the Si ions. It is therefore of great importance to investigate the role of Si ions themselves on the biological response of bone-forming cells. Previously, Zou *et al.* applied sodium silicate (Na_2SiO_3) to study the effect of Si ions on human osteoblast adhesion, proliferation and alkaline phosphatase (ALP) activity and found that 30 mM of Si ions could enhance the metabolic activity of osteoblasts.³⁰ However, in their study, Na ions were also incorporated into cell culture medium, which may also influence cell response.³⁰ The study has, therefore, not evaluated independently the effect of Si ions on cell response. Although Reffitt and Duivenvoorden found that orthosilicic acid (H_4SiO_4) could stimulate collagen type 1 synthesis and osteoblastic differentiation of human osteoblasts, it was still unclear how Si ions themselves influence cell response.^{31,32} To the best of our knowledge, there are few studies concerning the influence of Si ions on bone-related gene expression and cell signalling pathways of bone-forming cells. The aim of this study was therefore to systematically investigate the effects of Si ions on the proliferation, differentiation, nodule formation, bone-related gene expression and signalling pathways of BMSCs by comparing the response of BMSCs to various concentrations of NaCl and Na_2SiO_3 in order to exclude the effect of Na ions. Here we provide some evidence of the important role of Si ions in Si-containing biomaterials in influencing the cell response during osteogenic differentiation.

2. Experimental section

2.1 Preparation of cell culture medium containing different concentrations of Na_2SiO_3 and NaCl

In order to investigate the effect of Si ions on the proliferation, differentiation, bone-related gene expression and signalling pathways of BMSCs, two groups of studies were carried out with the same concentration of Na ions. Cells in group A were cultured with different concentrations of NaCl (A group: NaCl), while cells in group B were treated with a range of concentrations of Na_2SiO_3 (B group: Na_2SiO_3), as shown in Table 1. As group A and group B have the same concentration of Na ions in each corresponding concentration pair (Table 1), the effect of Si ions on cell response could be studied through the fold change by comparing group A and group B (fold change > 1: Si ions stimulating cell response; fold change < 1: Si ions down-regulating cell response). To prepare the stock solutions of Na_2SiO_3 and NaCl, Na_2SiO_3 and NaCl powders were dissolved into low glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Pty Ltd., Australia) at concentrations of 50 mM and 100 mM, respectively. After sufficient dissolution, serial

concentrations of NaCl and Na_2SiO_3 were prepared using DMEM medium. Subsequently, the original and diluted solutions were sterilized using 0.2 μm filter and supplemented with fetal calf serum (FCS, 10% by volume; Invitro Technology, Australia) and penicillin/streptomycin (P/S, 1% by volume; Invitrogen Pty Ltd., Australia) for further cell culture experiments.

2.2 Isolation and culture of BMSCs

Isolation and culture of BMSCs was performed as described before.^{24,33} BMSCs were isolated from bone marrow aspirates from patients (mean age: 65 years) undergoing elective knee and hip replacement surgery, with consent by all patients involved and ethical approval by the Human Ethics Committees of Queensland University of Technology and The Prince Charles Hospital. In brief, to prevent coagulation, 2 mL of heparin was mixed with 10 mL of bone marrow aspirate and then diluted with 12 mL of PBS. Then, using a serological pipette, 12 mL of a density gradient solution (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway) was carefully placed underneath the bone marrow solution and centrifuged at 800 rpm for 10 min. The resulting supernatants were cultured in DMEM supplemented with 10% v/v fetal bovine serum, 1% v/v P/S at 37 °C in a humidified 5% CO_2 incubator. The medium was then changed after five days to remove non-adherent cells. The cells were passaged at approximately 80% confluence and expanded through two passages to obtain a sufficient number of cells for the *in vitro* assays.

2.3 The effect of Si on the cell proliferation of BMSCs

The proliferation of BMSCs in two groups was carried out with a colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as described before.^{24,33} Briefly, BMSCs were cultured in 96-well plates at an initial density of 5000 cells per well. On days 1 and 7, 10 μL of 5 mg mL^{-1} of MTT solution (Sigma-Aldrich, Australia) were added to each well and incubated at 37 °C to form formazan crystals. After 4 h, the medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the MTT-formazan product. The absorbance for the MTT-formazan was measured at λ 495 nm with a microplate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA).

2.4 The effect of Si on alkaline phosphatase (ALP) activity of BMSCs

BMSCs were cultured in 24-well culture plates with a seeding density of 1×10^5 cells per well. ALP activity was assessed at day 7 and 14 in BMSCs cultured in different concentrations of NaCl and Na_2SiO_3 using the method as published before.^{24,33} Briefly, the cells were lysed in 200 μL of 0.2% Triton® X-100 and then centrifuged at 14 000 rpm for 15 min at 4 °C. 50 μL supernatants were mixed with 150 μL working solution according to the manufacturer's protocol (QuantiChrom™ Alkaline Phosphatase Assay Kit, BioAssay Systems, USA). The total protein content was measured by the bicinchoninic acid protein assay kit (Sigma-Aldrich, Australia). The relative ALP

Table 1 Corresponding concentrations of Na and Si ions in two groups (A: NaCl; B: Na_2SiO_3) (mM)

Group A	Na	10	5	2.5	1.25	0.125	0.0125
	Si	0	0	0	0	0	0
Group B	Na	10	5	2.5	1.25	0.125	0.0125
	Si	5	2.5	1.25	0.625	0.0625	0.00625

activity was then obtained as the changed optical density (OD) values divided by the reaction time and total protein content.

2.5 The effect of Si on nodule formation of BMSCs

In order to identify mineralization-like nodule formation, Alizarin Red S staining was performed on day 14 after the BMSCs were cultured in different concentrations of NaCl and Na₂SiO₃. The cells were fixed in cold 100% methanol for 10 min, then stained in a solution of 1% Alizarin Red S at pH 4.1 for 10 min and then washed with H₂O. The samples were air dried and photos were taken under a light microscope.

2.6 The effect of Si on bone-related gene expression of BMSCs

To investigate the effect of Si on the gene expression of *Alkaline Phosphatase (ALP)*, *osteocalcin (OCN)*, *osteopontin (OPN)*, the total RNA was extracted from BMSCs cultured in different concentrations of NaCl and Na₂SiO₃ using Trizol reagent (Invitrogen Pty Ltd., Australia) and complementary DNA was synthesized using Superscript III reverse transcriptase (Invitrogen Pty Ltd., Australia) from 1 µg of the total RNA, following the manufacturer's instructions. RT-qPCR primers (Table 2) were designed based on cDNA sequences from the NCBI Sequence database using the Primer Express® software, and the primer specificity was confirmed by BLASTN searches. RT-qPCR was performed on an ABI Prism 7300 Thermal Cycler (Applied Biosystems, Australia) with SYBR Green detection reagent following the protocol published previously.^{24,33} The relative mRNA expressions of *ALP*, *OPN* and *OCN* were assayed and normalized against the house keeping gene 18s. Each sample was performed in triplicate. The mean cycle threshold (Ct) value of each target gene was normalized against the Ct value of housekeeping gene 18s ribosomal RNA and the relative expression calculated using the following formula: $2^{-(\text{normalized average } C_t)} \times 10^4$.

2.7 The effect of Si on WNT and SHH signalling pathways of BMSCs

To investigate the effect of different concentrations of Si on the expression of WNT and SHH signalling related genes of BMSCs, measurements of the gene expression of *axins inhibition protein 2 (AXIN2)*, *beta-catenin (β-CATENIN or CTNNB)*, *sonic hedgehog homolog (SHH)* and *protein patched homolog (PTCH1)* were carried out. Cells were collected at two time

points (day 7 and 14) and RT-PCR was carried out using the same method as described in Section 2.6.

To further investigate the effect of Si ions on cell proliferation in relation to the WNT signalling pathway, cardamonin, an effective W.I., was added at a concentration of 10 µM directly to the culture medium containing a specific concentration of Si ions. After 1, 3 and 7 days, cells were collected and MTT assays were performed using the same method described in Section 2.3. When indicated, the effect of Si ions on cell response was displayed using the fold changes by comparing corresponding concentration of group A and group B (fold change > 1: Si ions stimulating cell response; fold change < 1: Si ions down-regulating cell response).

The effect of Si ions on osteogenic differentiation and WNT/SHH cell signalling pathways in the presence of a W.I. was also investigated. Cells were cultured in the presence of 10 µM of cardamonin in culture media containing NaCl and Na₂SiO₃. After 3 and 7 days, cells were collected and the expression of osteogenic genes (*ALP*, *OCN*, *OPN*), *AXIN2*, *CTNNB*, *SHH* and *PTCH1* was performed with RT-PCR using the same method described in Section 2.6. When indicated, the effect of Si ions on cell response was measured using the same method described in Section 2.1.

2.8 The effect of Si on bone-related protein of human BMSCs (hBMSCs) by western blotting analysis

Western blotting analysis was performed for the direct detection of the ALP, OPN and OCN protein expression. In brief, whole cell lysates were obtained from cells cultured with 0.625 mM Na₂SiO₃ and 1.25 mM NaCl for one week. 10 µg proteins from each sample were separated on SDS-PAGE gels and then transferred onto a nitrocellulose membrane (Pall Corporation, East Hills, NY, USA) and blocked in 5% non-fat milk. The membranes were incubated with primary antibodies against ALP (1 : 1000, rabbit anti-human; Abcam, Sapphire Bioscience Pty. Ltd., Australia), OPN (1 : 1000, rabbit anti-human; Thermo Fisher Scientific, USA), OCN (1 : 1000, rabbit anti-human; Thermo Fisher Scientific, USA) and GAPDH (1 : 1000, mouse anti-human; Abcam, Sapphire Bioscience Pty. Ltd., Australia) overnight at 4 °C. The membranes were washed three times in TBS-Tween buffer, and then incubated with anti-mouse/rabbit HRP conjugated secondary antibodies at 1 : 4000 dilutions for 1 h at room temperature. The protein bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific,

Table 2 Primer pairs used in qRT-PCR analysis

Gene	Forward primer	Reverse primer
OCN	5' GCAAAGGTGCAGCCTTTGTG 3'	5' GGCTCCCAGCCATTGATACAG 3'
OPN	5' TCACCTGTGCCATACCAGTTAA 3'	5' TGAGATGGGTCCAGGGTTTAGC 3'
ALP	5' TCAGAAGCTAACACCAACG 3'	5' TTGTACGTCTTGAGAGGGC 3'
AXIN2	5' CCCCAAAGCAGCGGTGC 3'	5' GCGTGGACACCTGCCAG 3'
β-CATENIN	5' GCTACTGTTGGATTGATTCGAAATC 3'	5' CCCTGCTCACGCAAAGGT 3'
SHH	5' CGCCAGCGGAAG GTATGAAG 3'	5' CAACTTGTCTTACACCTCTGAGTCA 3'
PTCH1	5' GAGTCCCTTTCGTTCATGCTAGGTC 3'	5' GCC CCTATGCCAAAGGTGACC 3'
18s	5' TTCGGAAGTGGCCATGAT 3'	5' CGAACCTCCGACTTCGTTC 3'

Australia) and exposed on X-ray films (Fujifilm, Australia). The relative intensity of protein bands was quantified using ImageJ software.

2.9 Statistical analysis

The experiments were carried out in triplicate. Analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). All the data were expressed as mean \pm standard deviation (SD) and were analysed using one-way ANOVA with a Post Hoc test. A p -value < 0.05 was considered statistically significant.

3. Results

3.1 The effect of Si on cell proliferation

An MTT proliferation assay showed that NaCl at high concentration (2.5–10 mM) decreased the cell number and proliferation of BMSCs at both day 1 and day 7; whereas there was no significant difference in the BMSC proliferation in other NaCl concentrations in comparison with that in normal culture media (blank control) (Fig. 1a). The concentrations of Na₂SiO₃,

on the other hand, had an obvious effect on cell proliferation (Fig. 1b). The cell viability at the low end of the Na₂SiO₃ concentration ranges (0.00625 to 2.5 mM) was much higher than at high concentrations (5 mM) and there was a trend of stimulation of cell proliferation with increased concentrations of Si (0.00625 to 2.5 mM) (Fig. 1b). By comparing the viability and proliferation of BMSCs between NaCl and Na₂SiO₃, it is clear that concentrations of Si of 1.25 mM and 2.5 mM had the effect of increasing cell viability at day 1 and proliferation at day 7 (fold change > 1), while Si at 5 mM inhibited cell proliferation of BMSCs (Fig. 1c).

3.2 The effect of Si on ALP activity and mineralization nodule formation of BMSCs

The NaCl concentration had no significant effect on the ALP activity of BMSCs (Fig. 2a). At the lower NaCl concentration of 0.0125 mM, ALP activity showed an increase over time (Fig. 2a). The ALP activity of BMSCs cultured in low concentrations of Na₂SiO₃ (0.00625–1.25 mM) showed no difference and was decreased at high concentrations (2.5 and 5 mM) at

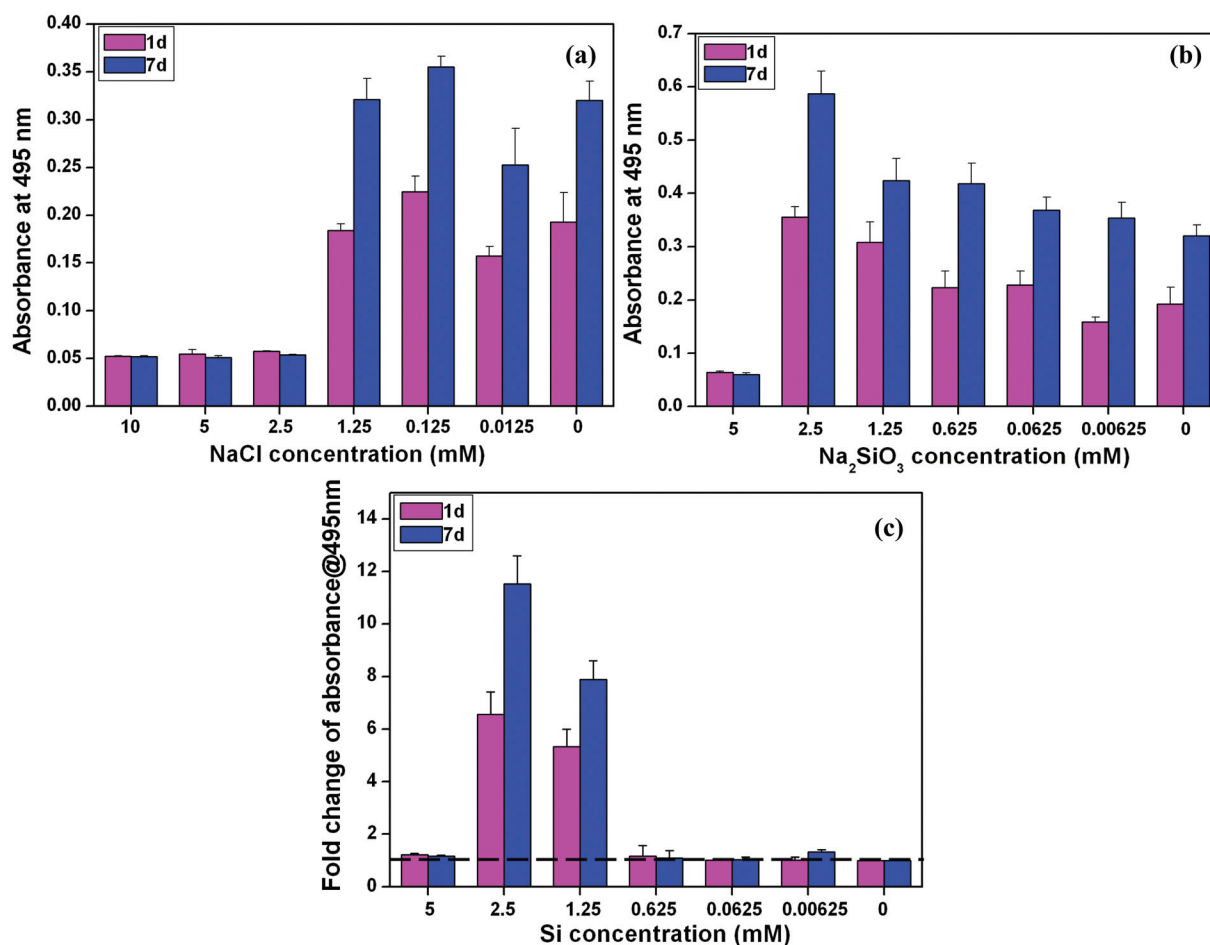


Fig. 1 BMSCs viability at day 1 and proliferation at day 7 in medium containing different concentrations of NaCl (a) and Na₂SiO₃ (b) and the fold change of cell proliferation with different Si concentrations (c) after comparing the groups of NaCl and Na₂SiO₃. At both day 1 and 7, Si ions at the concentration of 2.5 and 1.25 mM significantly enhanced cell viability and proliferation (fold change > 1) (c). The dotted line shows a fold change of 1 in cell proliferation between NaCl and Na₂SiO₃ groups, indicating there is no difference between these two groups.

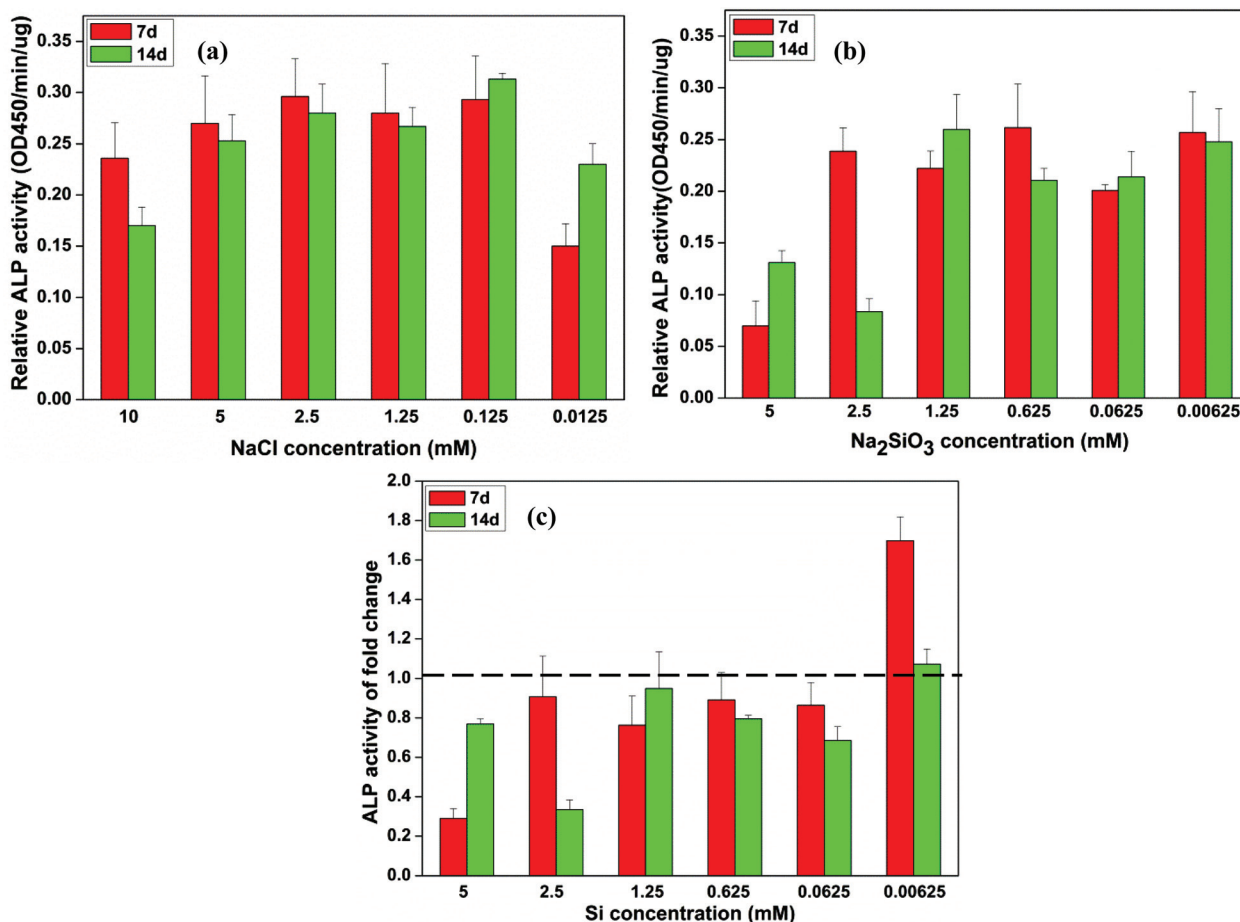


Fig. 2 The relative ALP activity of BMSCs observed in culture with different concentrations of NaCl (a) and Na₂SiO₃ (b) and the independent Si effect shown by the fold change of ALP activity between the treatment groups of NaCl and Na₂SiO₃ (c). At days 7 and 14, a concentration of Si ions of 0.00625 mM significantly increased the ALP activity of BMSCs (~1.7 and 1.1 fold change, respectively); in contrast, ALP activity of BMSCs decreased at other concentrations of Si (fold change < 1) (c). The dotted line indicates a fold change of ALP activity of 1 between NaCl and Na₂SiO₃ groups.

both 7 and 14 days (Fig. 2b). A comparison of ALP activities between cells cultured in NaCl and Na₂SiO₃ revealed that the relative ALP activity increased significantly at an Si concentration of 0.00625 mM on day 7 (fold change = 1.7) and day 14 (fold change = 1.1) (Fig. 2c). There were no stimulatory effects at other Si concentrations; in contrast, ALP activity was reduced (Fig. 2c).

Alizarin Red S staining showed evidence of nodule formation and calcium deposition. It was noted that mineralized nodules were seen at all concentrations of Na₂SiO₃ (Fig. 3) and the nodules became more distinct with increased concentration of Na₂SiO₃ (Fig. 3 right column). There was no obvious effect on Ca mineralization seen in the NaCl groups (Fig. 3 left column).

3.3 The effect of Si on bone-related gene expression of BMSCs

At day 7, the mRNA expression of *OPN* in BMSCs increased as NaCl concentration decreased. At day 14, *OPN* expression was increased in NaCl concentrations of 0.0125 and 5 mM compared to the other concentrations and also had increased over

time (Fig. 4a). *OPN* expression increased over time with most Na₂SiO₃ concentrations. A comparison of *OPN* expression between the NaCl and Na₂SiO₃ groups showed that, at day 7, Si significantly enhanced *OPN* expression across the concentration range (fold change > 1) and 0.625 mM Si in particular stood out with a 25 fold increase of *OPN* expression (Fig. 4c). At day 14, *OPN* expression was reduced at high Si concentrations (2.5 and 5 mM) and expression was highest at 0.625 mM Si (~5 fold change) compared to the other concentrations (Fig. 4c).

The relative mRNA expression of *OCN* in BMSCs at day 7 was higher at 1.25 mM NaCl than at the other concentrations and, at day 14, *OCN* expression was significantly increased at 0.0125 and 5 mM of NaCl (Fig. 4d). The *OCN* expression level of cells treated with 0.625 mM of Na₂SiO₃ was significantly higher than in other concentrations at both days 7 and 14 (Fig. 4e). Comparing the *OCN* expression of the NaCl and Na₂SiO₃ groups revealed a reduced expression of *OCN* at day 7 in 0.0625, 1.25 and 2.5 mM of Si (fold change < 1); at the other Si concentrations *OCN* expression increased (fold change > 1). At day 14, *OCN* expression increased strongly at 0.625 mM of Si by an 8 fold change compared to the other concentrations (Fig. 4f).

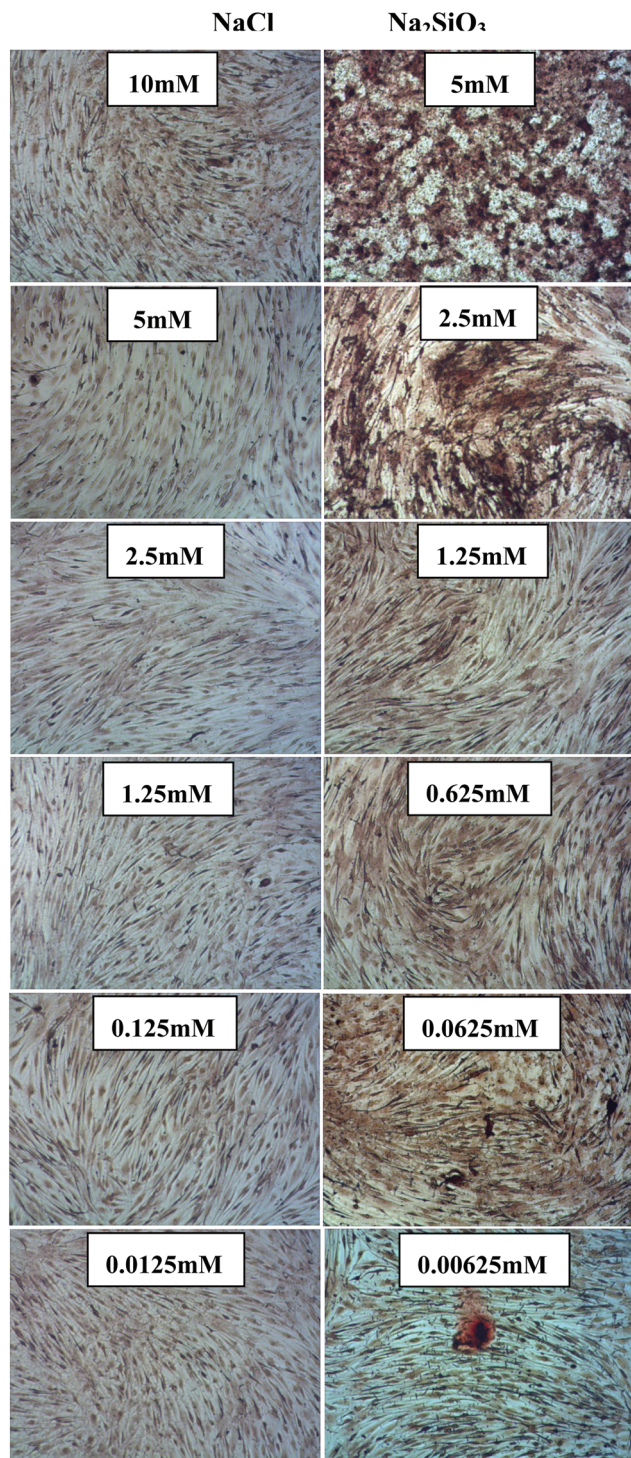


Fig. 3 Alizarin Red S staining of BMSCs treated with different concentrations of NaCl (left column) and Na_2SiO_3 (right column). There is no obvious nodule formation and calcium deposition in NaCl group, however, Na_2SiO_3 increased nodule formation and slightly enhanced the calcium deposition in BMSC cultures.

The relative mRNA expression of *ALP* rose gradually and peaked at 2.5 mM of NaCl at day 7. 5 mM of NaCl caused a significant increase in *ALP* expression at day 14 (Fig. 4g). *ALP* expression increased at 0.625 mM of Na_2SiO_3 at both days 7 and

14 (Fig. 4h). The effect of Si ions alone showed that, at day 7, 5 mM of Si had the greatest effect of increasing *ALP* expression in BMSCs (fold change > 1). Other Si concentrations had the effect of decreasing *ALP* expression (fold change < 1). At day 14, Si concentrations at 0.625 and 5 mM significantly enhanced *ALP* expression (fold change > 1), but other concentrations led to decreased expression (fold change < 1) (Fig. 4i).

3.4 The effect of Si on WNT and SHH signalling pathway-related genes expression of BMSCs

Quantitative RT-PCR analysis indicated that relative *AXIN2* mRNA expression peaked at 2.5 and 5 mM of NaCl at days 7 and 14, respectively (Fig. 5a). Na_2SiO_3 dramatically enhanced *AXIN2* expression across the range of concentrations over time (Fig. 5b). The effect on *AXIN2* expression of Si ions alone was deduced by comparing the NaCl and Na_2SiO_3 groups. At day 7, Si concentration of 1.25 and 2.5 mM slightly decreased *AXIN2* expression (fold change < 1), whereas the other Si concentrations enhanced *AXIN2* expression (fold change > 1). At day 14, *AXIN2* expression rose across the Si concentration range (fold change > 1), especially so at 0.625 mM, which had a 32 fold expression change (Fig. 5c).

The expression of β -*CATENIN* (*CTNNB*) rose significantly at day 7 in 0.0125 and 2.5 mM of NaCl compared to the other concentrations (Fig. 5d). Na_2SiO_3 at 5 mM increased *CTNNB* gene expression of BMSCs (Fig. 5e) and the fold change of *CTNNB* expression as the result of Si alone confirmed that Si at 5 mM had the greatest influence on *CTNNB* expression of BMSCs over time. At days 7 and 14, the Si concentration of 0.625 mM also induced a distinct increase in *CTNNB* expression (Fig. 5f).

The relative mRNA expression of *sonic hedgehog* (*SHH*) in BMSCs gradually decreased with decreasing concentrations of NaCl from 10 mM to 1.25 mM at day 7, but expression of *SHH* was increased by NaCl at the concentrations of 0.125 and 0.0125 mM (Fig. 6a). The relative *SHH* expression increased across the whole Na_2SiO_3 concentration range over time (Fig. 6b). The effect of Si alone when comparing the NaCl and Na_2SiO_3 groups showed that Si at 0.625 mM dramatically enhanced *SHH* gene expression 38 and 93 fold at days 7 and 14, respectively (Fig. 6c).

At day 7, the mRNA expression of *patched-1* (*PTCH1*) increased as NaCl concentrations decreased. *PTCH1* expression reached a maximum at day 14 at a concentration of 5 mM NaCl (Fig. 6d). Na_2SiO_3 at 5 mM induced the highest *PTCH1* expression at both day 7 and 14 (Fig. 6e). Allowing for the effect of Na^+ ions, Si ions at 5 mM increased *PTCH1* gene expression 200 and 20 fold at days 7 and 14, respectively. Si at 0.625 mM also enhanced *PTCH1* expression at both day 7 and 14 by a ~4 and 17 fold change, respectively (Fig. 6f).

3.5 The effect of Si on BMSCs proliferation, osteogenic gene expression, and signalling pathway-related genes in the presence of W.I.

The effect of cardamomin (W.I.) at a concentration of 10 μM on the BMSC proliferation (A) and WNT-related gene expression

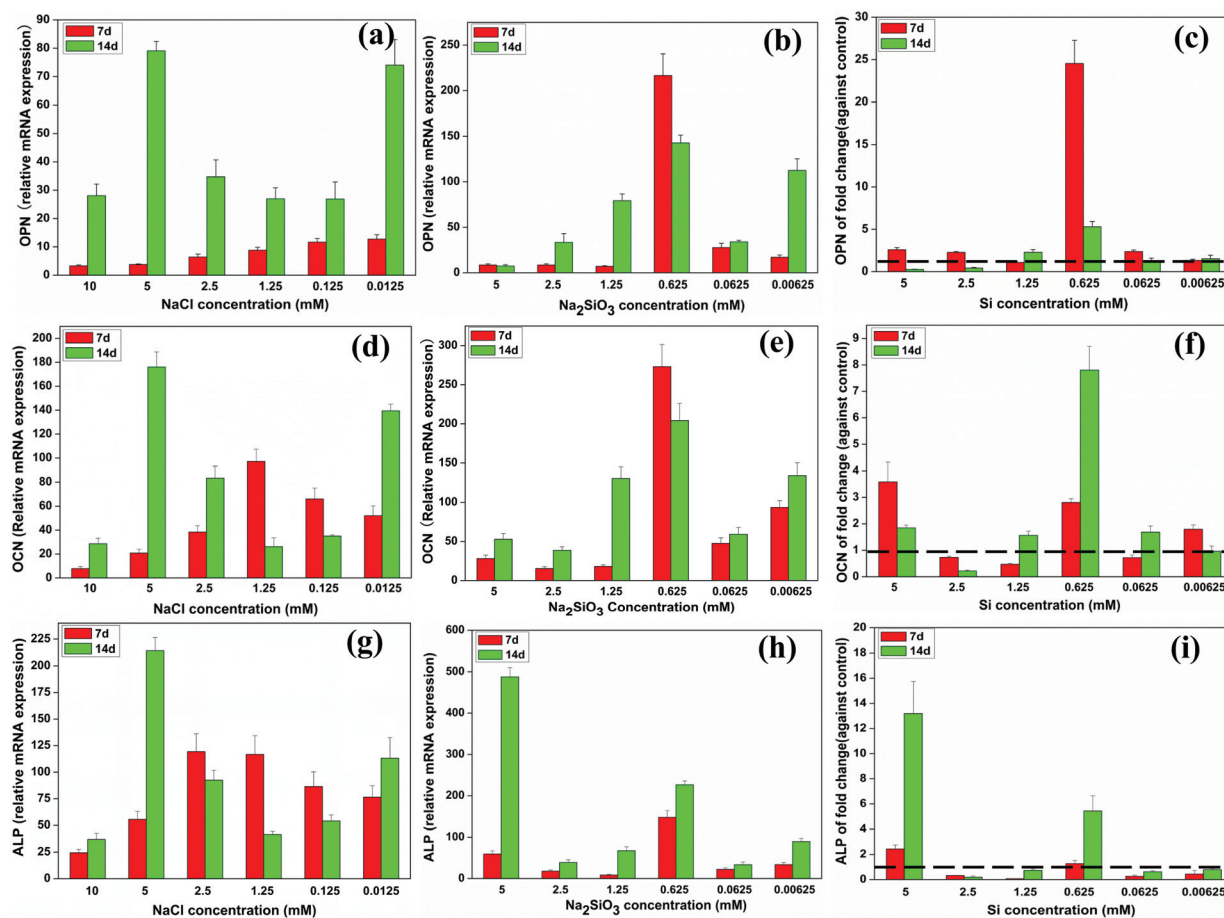


Fig. 4 The expression of osteogenic genes of BMSCs cultured with a range of concentrations of NaCl (a, d, g) and Na_2SiO_3 (b, e, h). The effect of Si ion on osteogenic genes expression by comparing treatments with NaCl and Na_2SiO_3 (c, f, i). At both days 7 and 14, Si ions significantly enhanced *OPN* (c), *OCN* (f) and *ALP* (i) gene expression at a concentration of 0.625 mM (fold change > 1). The dotted line indicates a fold change of gene expression of 1 between NaCl and Na_2SiO_3 groups.

(B) was evaluated (ESI, Fig. S1†). The cell proliferation of BMSCs was significantly decreased in the presence of cardamomin (A). The fold change of WNT related genes of *AXIN2* and *CTNNB* was also significantly inhibited by the W.I. cardamomin (B) (fold change < 1).

Interestingly, it was noted that when the culture media contained Si ions at a concentration of 0.625 mM, the number of BMSCs cultured with the W.I. was higher than that without W.I. (Fig. 7a). However, at both day 3 and 7, the fold change of mRNA expression of *OPN*, *OCN* and *ALP* was significantly enhanced by 0.625 mM of Si ions in the presence of cardamomin at 10 μM (fold change > 1) (Fig. 7b–d). Furthermore, when the culture media contained both W.I. and Si ions, the mRNA expression of *AXIN2*, *CTNNB*, *SHH*, and *PTCH1* was significantly enhanced at day 3 (fold > 1), then decreased at day 7 (Fig. 7e–h).

3.6 The effect of Si on bone-related protein expression of hBMSCs by western blotting analysis

Western blotting analysis showed that ALP and OPN were elevated when hBMSCs were cultured with 0.625 mM Na_2SiO_3

compared to those cultured with 1.25 mM NaCl (Fig. 8a). The relative intensity analysis demonstrated that the expression of ALP and OPN for hBMSCs cultured with 0.625 mM Na_2SiO_3 was higher than that in the 1.25 mM NaCl group (Fig. 8b and c). OCN protein expression for hBMSCs cultured with 0.625 mM Na_2SiO_3 is comparable to that of 1.25 mM NaCl (Fig. 8d).

4. Discussion

In this study we have explored the effect of Si ions on the proliferation, differentiation and mineralization, as well as the gene expression of bone, WNT and SHH-related pathways, of BMSCs cultured in two groups with defined concentrations of either NaCl or Na_2SiO_3 . Given that both salts contain Na^+ , it was therefore possible to account for the effect of Si ions alone by comparing the results from both groups. It was found that Si ions showed a mixed effect on BMSC osteogenic gene expression and that, at certain concentrations, they significantly enhanced the proliferation, mineralization nodule formation, bone-related gene expression, and WNT and SHH

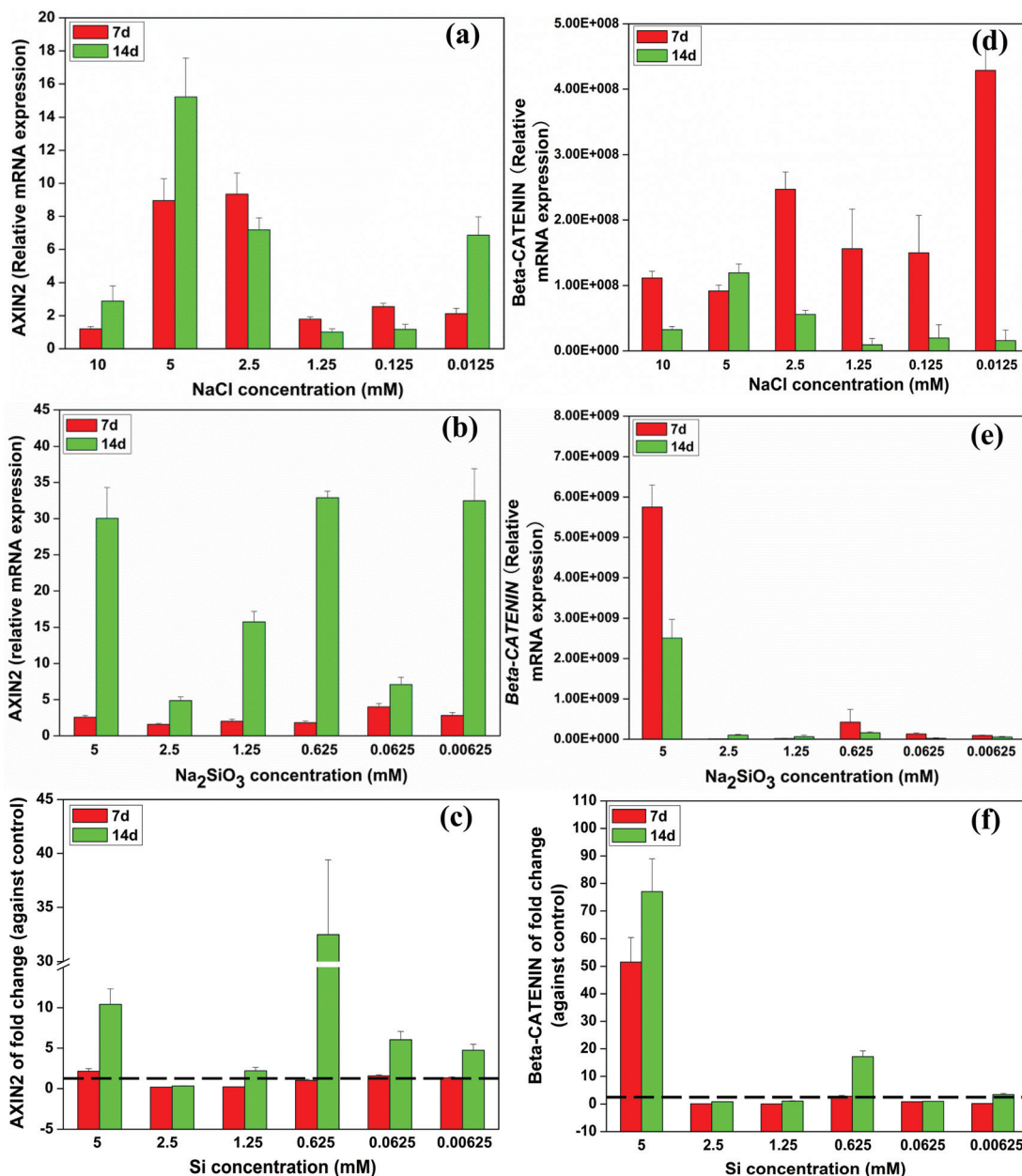


Fig. 5 Relative expression of WNT-related genes of BMSCs cultured with a range of concentrations of NaCl (a, d) and Na₂SiO₃ (b, e). Comparing groups with NaCl and Na₂SiO₃, effect of different concentrations of Si ions on the fold change of *AXIN2* and *CTNNB* gene expression of BMSCs (c, f). At day 14, Si ions at a concentration of 0.625 mM increased *AXIN2* (c), and *CTNNB* (f) expression (fold change > 1). The dotted line indicates a fold change of gene expression of 1 between NaCl and Na₂SiO₃ groups.

signalling pathways in BMSCs. These results demonstrate that Si ions play an important role in regulating cellular responses, including WNT and SHH signalling pathways, in BMSCs. Our study may therefore provide direct evidence to explain the stimulatory effect on osteogenesis of Si-containing biomaterials and may potentially provide a guide for the design of Si-containing biomaterials with desirable Si ion release in order to achieve osteogenesis.

There has been longstanding interest in the osteogenically stimulatory effects of Si-containing biomaterials on bone-derived cell types,^{4,34} but until now the mechanism of the

biological role of Si ions on bone-forming cells has been unclear. Recent studies have sought to investigate the effect of Si ions on cell functions by two separate means. In one method, human osteoblastic cell lines were treated with different concentrations of orthosilicic acid (H₄SiO₄) and it was found that H₄SiO₄ could enhance osteogenic differentiation.^{31,32} The other method investigated the effect of silicate ions on the proliferation, adhesion and differentiation of osteoblast or osteoblast-like cells by culturing these in a range of concentrations of Na₂SiO₃.^{30,35} Both approaches have disadvantages for exploring the effect of Si ions themselves on cell

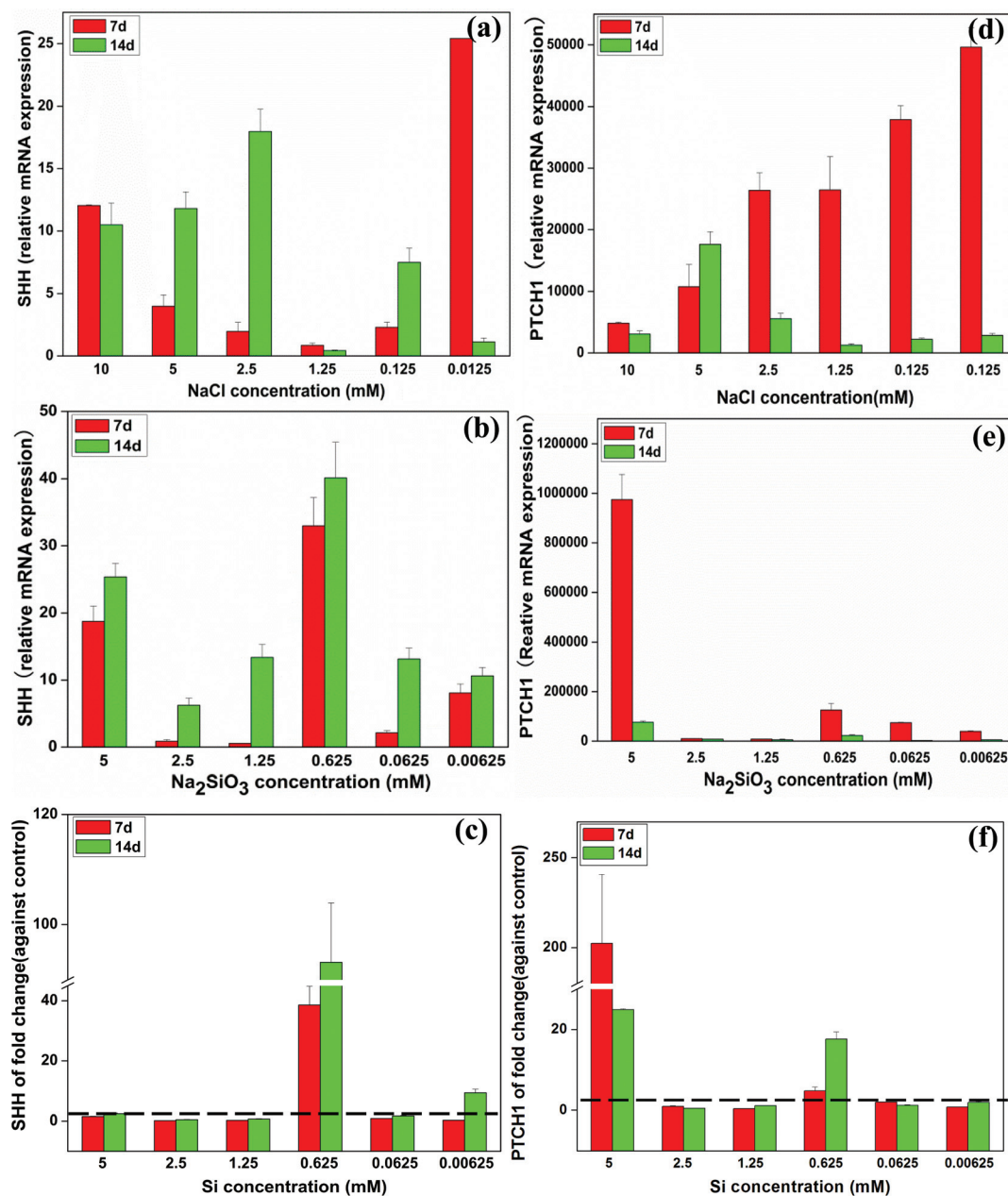


Fig. 6 The expression of SHH-related genes of BMSCs in the presence of a range of concentrations of NaCl (a, d) and Na_2SiO_3 (b, e). After comparing groups containing NaCl and Na_2SiO_3 , effect of different concentrations of Si ions on the fold change of *SHH* and *PTCH1* gene expression of BMSCs (c, f). At day 14, Si ions at a concentration of 0.625 mM enhanced *SHH* (c) and *PTCH1* (f) expression (fold change > 1). The dotted line indicates a fold change of gene expression of 1 between NaCl and Na_2SiO_3 groups.

proliferation and differentiation. For example, the effect on the cell response of the low pH caused by the release of H^+ ions from H_4SiO_4 was not taken into account in that approach³¹ and, in the approach using Na_2SiO_3 , the effect of Na ions has not been factored in.³⁵ As a result, the actual cellular effect of Si ions on their own remains unclear from either of these two methods. In addition, the cell molecular effect of Si ions on the bone-related gene expression and cell signaling pathways is largely unknown. In the present study, we have investigated the effect of Si ions on cell proliferation, differentiation and the bone-related gene expression and signalling pathways of

BMSCs by implementing two treatment groups which were both exposed to the same relative concentrations of Na ions, therefore accounting for the effect of Na ions. The fold change attributable to Si ions alone could thus be estimated. This approach gave a more accurate picture of the real impact of Si ions on cell responses than those methods used previously.

Previous studies have shown that cellular responses are influenced by several factors (*e.g.* ionic compositions, ionic concentrations and pH values).^{36–39} We have previously found that ionic products with graded concentrations from silicate bioceramics, such as akermanite ($\text{Ca}_2\text{MgSi}_2\text{O}_7$),³⁶ bredigite

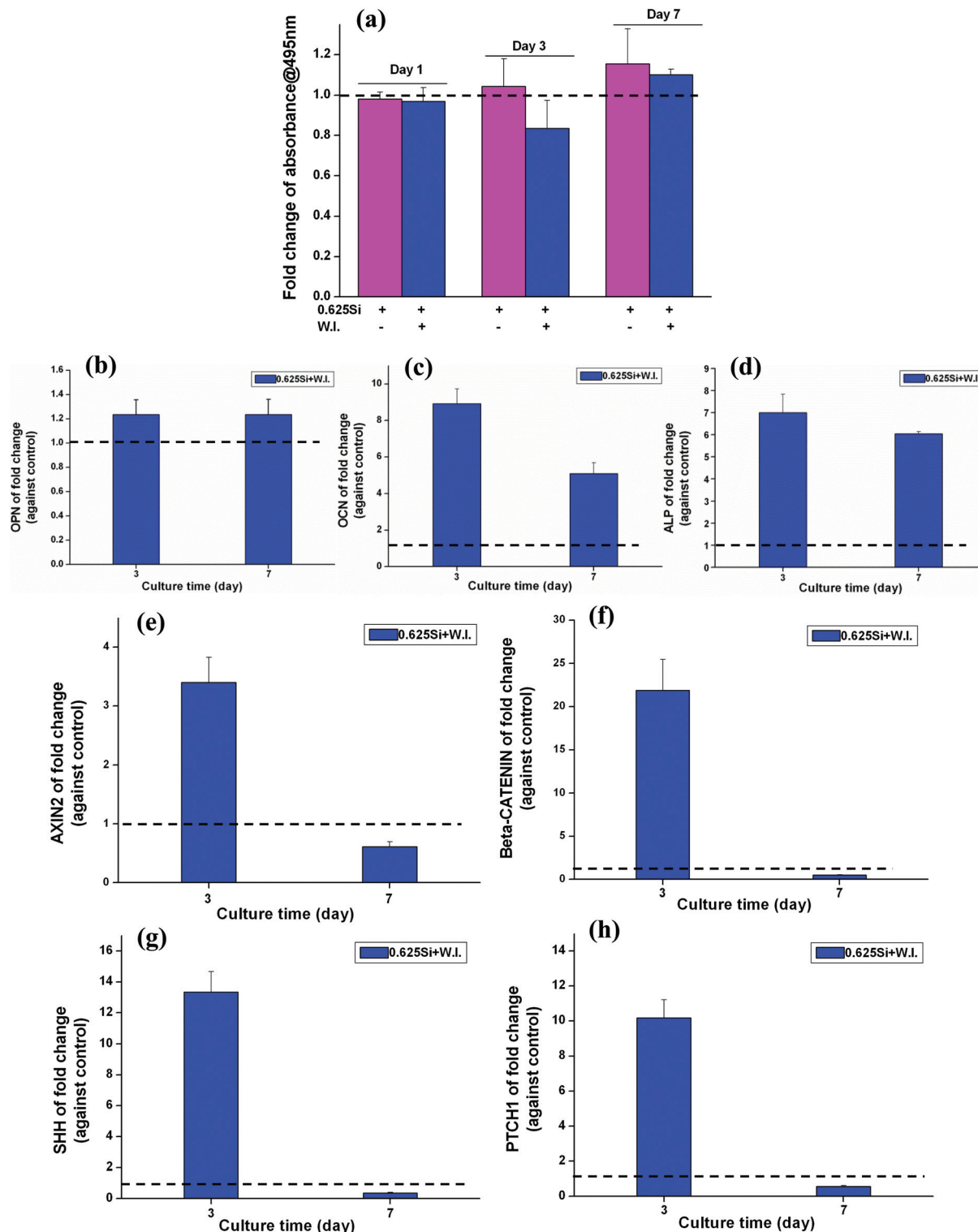


Fig. 7 The effect of Si ions on cell proliferation (a), osteogenic genes (b–d) and WNT, SHH-related genes expression (e–h) of BMSCs in the presence of cardamomin (W.I.) at a concentration of 10 μ M. At day 7, Si ions at a concentration of 0.625 mM stimulated the proliferation of BMSCs cultured with the W.I. at a concentration of 10 μ M (a). At both day 3 and 7, Si ions at a concentration of 0.625 mM enhanced the gene expression of *OPN*, *OCN* and *ALP* (b–d), *AXIN2*, *CTNNB* (e, f), *SHH* and *PTCH1* (g, h) with W.I. at a concentration of 10 μ M (fold change > 1). The dotted line shows a fold change of 1 between the NaCl and Na₂SiO₃ groups.

(Ca₇MgSi₄O₁₆)⁴⁰ and nagelschmidite (Ca₇P₂Si₂O₁₆)⁴¹ play an important role in influencing the proliferation and differentiation. Generally, at a certain concentration of ionic products,

the released silicate-based ionic products stimulate cell proliferation and differentiation. However, if the ionic concentrations are too high, this will result in a cytotoxic effect on

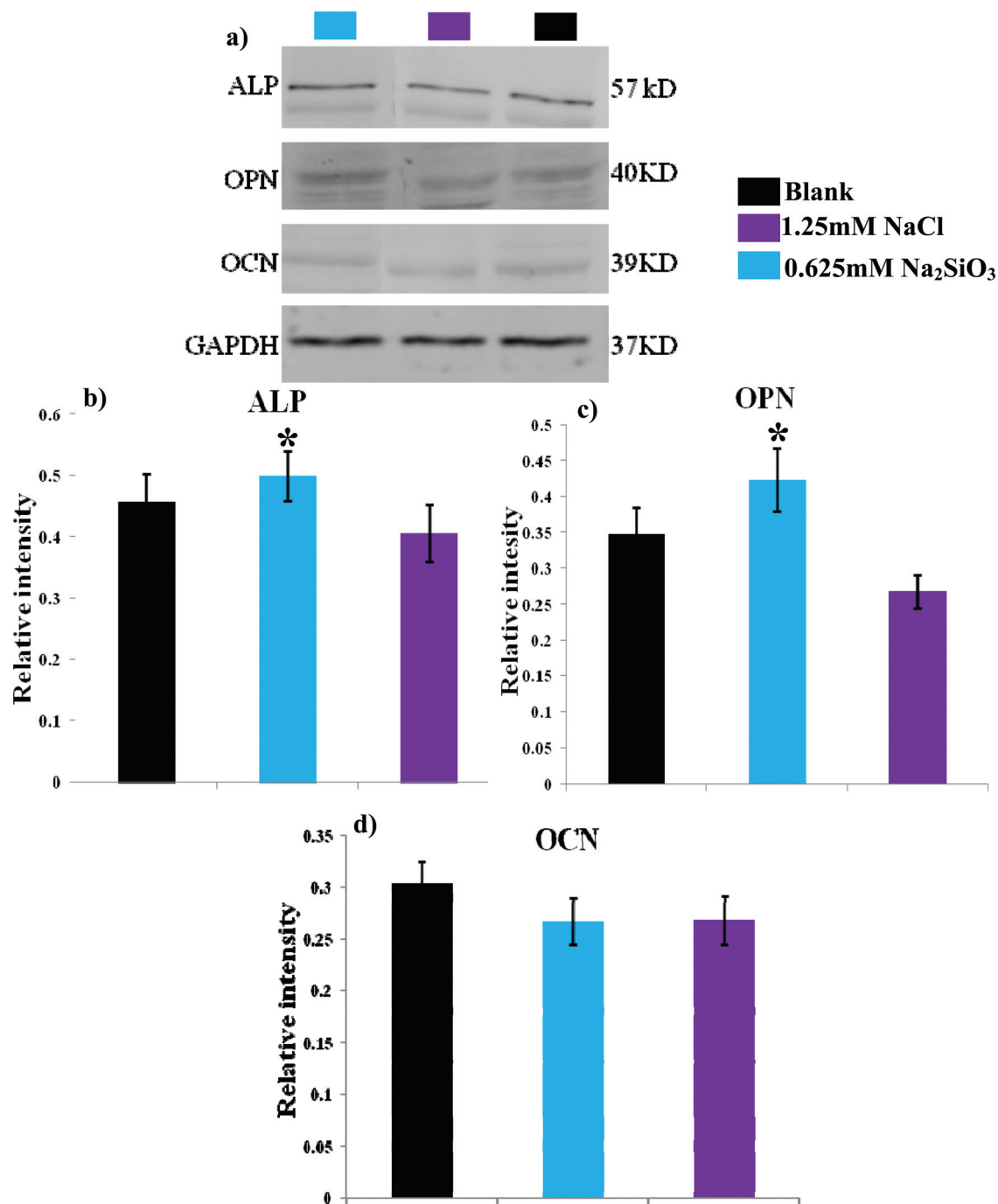


Fig. 8 The western blotting analysis of bone-related protein of ALP, OPN and OCN expression of hBMSCs after 7 days cultured at the concentration of 0.625 mM Na₂SiO₃ and 1.25 mM NaCl. * indicates a significant difference ($p < 0.05$) between 0.625 mM Na₂SiO₃ and 1.25 mM NaCl group.

cell growth. For this reason, in this study, we chose a wide ionic concentration range (from 0.00625 to 5 mM) to investigate the effect of Si ions on BMSC response. Our results showed that high concentrations of Si ions at 5 mM suppressed cell proliferation. One potential reason may be that high concentrations of Si ions themselves caused cytotoxicity. Another reason is that high concentrations of Si ions may influence pH and extracellular ionic environment and further inhibit cellular response. Our results demonstrate that

Na₂SiO₃ affects cell proliferation, differentiation and mineralization nodule formation in hBMSCs. Treatment with Na₂SiO₃ enhanced osteogenic differentiation of BMSCs *via* up-regulation of the bone-related genes *OPN*, *OCN* and *ALP*, as well as the expression of WNT and SHH signaling-related genes. Besides the effect of Si ions, we were also able to demonstrate that Na ions play an important role in regulating cell proliferation and differentiation. By allowing for the effect of Na ions in our study, the effect of Si ions themselves could be

effectively revealed in terms of the fold change after comparing the two groups. Interestingly, comparing Si ions at different concentrations, Si ions alone at certain concentrations (particularly 0.625 mM) significantly stimulated the proliferation, differentiation, Ca-mineralization, bone-related gene/protein expression and signaling pathways of the BMSCs. These results provide an insight into the importance of Si ions for differentiation and bone-related gene expression, as well as WNT and SHH signaling pathways, in BMSCs. This knowledge will make it easier to understand the biological role of Si, both *in vitro* and *in vivo*. Many earlier studies have shown that Si-containing biomaterials improve *in vitro* and *in vivo* osteogenesis,^{17–23,25} but the underlying mechanisms and the role of Si in biomaterials have remained unclear. The present study confirms the osteogenic effects of Si and offers some evidence to explain the molecular mechanisms responsible for the bioactivity of Si-containing biomaterials. It has been reported that Si ions at 4 mM can activate the MAPK-ERK pathway in osteoblast-like cells³⁵ and Ca and Si ions in Ca₂SiO₄ coatings enhanced cellular differentiation and collagen production *via* the TGF pathway by TGF-1 binding to its cognate receptors.⁴² To date there are no reports as to whether WNT and SHH signalling pathways are involved in the effect of Si ions on BMSC differentiation. The WNT canonical signalling pathway is known to play an important role in regulating cellular events, such as cell proliferation, differentiation and morphogenesis.^{26,43} AXIN2, a member of the WNT pathway, is recognized as a reporter gene of canonical WNT signalling pathway activity, since it is a direct target gene of the canonical WNT signalling pathway.^{44,45} β-Catenin is also a critical member of THE WNT/β-catenin signalling pathway involved in cell proliferation and differentiation.^{46,47} The Hedgehog-Gli signalling pathway also plays an important role in cell proliferation and differentiation.^{48–50} SHH is one of the ligands of this pathway and its cognate receptor, Patched-1 (Ptch1), is one of the target genes of the SHH-Gli signalling pathway.⁵¹ In this study, Si ions at 0.625 mM significantly stimulated these signalling pathway of BMSCs, as shown by upregulation of *AXIN2*, *β-catenin*, *SHH* and *PTCH1* gene expression. Furthermore, at a concentration of 0.625 mM, Si ions also stimulated bone-related gene expression (*OCN*, *OPN* and *ALP*) of BMSCs. Cardamonin has been identified as an inhibitor of the WNT signalling pathway by inactivating β-catenin, thereby blocking WNT/β-catenin signalling.⁵² Further experiments confirmed that the corresponding protein levels of ALP and OPN were increased by 0.625 mM Na₂SiO₃ compared to those with 1.25 mM NaCl. Our results demonstrated that Si ions at a concentration of 0.625 mM enhanced *OPN*, *OCN*, *ALP* and *WNT* and *SHH-related* gene expression, even when W.I. was added in the culture media, indicating that Si ions can rescue to a certain degree the effect induced by W.I. However, this counteracting effect of Si ions and cardamonin on BMSCs needs further investigation. Our results showed that soluble SiO₃²⁻ ions may stimulate cell proliferation and osteogenic differentiation of BMSCs through activation of WNT and SHH-related gene expression. The possible mechanisms have been summarized in Fig. 9. In brief, after

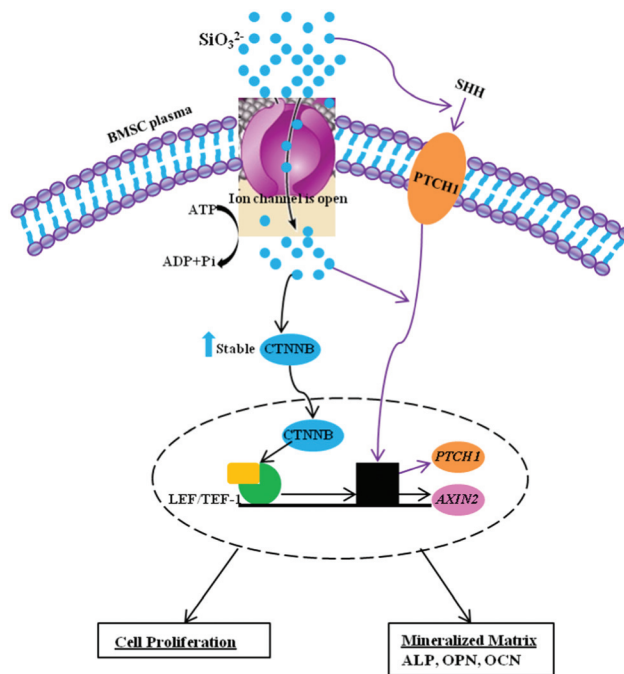


Fig. 9 Schematic model depicting the effect of SiO₃²⁻ on BMSCs differentiation.

travelling across the plasma membrane *via* ion channels, SiO₃²⁻ ions may play a role in stabilization of β-catenin and in leading its translocation into the nucleus. SiO₃²⁻ ions may also be associated with the LEF/TCF family to induce expression of the target gene *AXIN2*. On the other hand, SiO₃²⁻ ions could also induce SHH ligands binding with the receptors and subsequently promote the SHH target gene (*PTCH1*) expression. Activation of WNT and SHH-related genes leads to the upregulation of cell proliferation and osteogenic differentiation of BMSCs.

5. Conclusions

In the present study, the effect of Si ions on the proliferation, differentiation, mineralization and WNT and SHH-related gene expression of BMSCs has been systematically investigated by comparing the biological effects of NaCl and Na₂SiO₃. Si ions at a given concentration significantly enhanced the proliferation, mineralization nodule formation, bone-related gene expression and WNT and SHH signalling pathway of BMSCs. These results suggest that Si ions alone play an important role in regulating the cellular response of BMSCs and that the WNT and SHH signalling pathways are both involved in these cellular responses. Therefore, our results provide evidence for possible mechanisms whereby Si ions released from Si-containing biomaterials can exert their bioactivity.

Acknowledgements

Funding for this study was provided by the Recruitment Program of Global Young Talent, China (Dr Wu), Natural

Science Foundation of China (Grant 81201202), Shanghai Pujiang Talent Program (12PJ1409500) and ARC Discovery DP120103697.

References

- 1 E. M. Carlisle, Silicon: a possible factor in bone calcification, *Science*, 1970, **167**, 279–280.
- 2 K. Schwarz and D. B. Milne, Growth-promoting effects of silicon in rats, *Nature*, 1972, **239**, 333–334.
- 3 M. Vallet-Regi and D. Arcos, Silicon substituted hydroxyapatites. A method to upgrade calcium phosphate based implants, *J. Mater. Chem.*, 2005, **15**, 1509–1516.
- 4 I. R. Gibson, S. M. Best and W. Bonfield, Chemical characterization of silicon-substituted hydroxyapatite, *J. Biomed. Mater. Res.*, 1999, **44**, 422–428.
- 5 S. Langstaff, M. Sayer, T. J. Smith, S. M. Pugh, S. A. Hesp and W. T. Thompson, Resorbable bioceramics based on stabilized calcium phosphates. Part I: rational design, sample preparation and material characterization, *Biomaterials*, 1999, **20**, 1727–1741.
- 6 F. Balas, J. Perez-Pariente and M. Vallet-Regi, *In vitro* bioactivity of silicon-substituted hydroxyapatites, *J. Biomed. Mater. Res.*, 2003, **66**, 364–375.
- 7 S. Gomes, G. Renaudin, A. Mesbah, E. Jallot, C. Bonhomme and F. Babonneau, *et al.* Thorough analysis of silicon substitution in biphasic calcium phosphate bioceramics: a multi-technique study, *Acta Biomater.*, 2010, **6**, 3264–3274.
- 8 J. W. Reid, L. Tuck, M. Sayer, K. Fargo and J. A. Hendry, Synthesis and characterization of single-phase silicon-substituted alpha-tricalcium phosphate, *Biomaterials*, 2006, **27**, 2916–2925.
- 9 M. Mastrogiacomo, A. Muraglia, V. Komlev, F. Peyrin, F. Rustichelli and A. Crovace, *et al.* Tissue engineering of bone: search for a better scaffold, *Orthodont. Craniofacial Res.*, 2005, **8**, 277–284.
- 10 K. A. Hing, P. A. Revell, N. Smith and T. Buckland, Effect of silicon level on rate, quality and progression of bone healing within silicate-substituted porous hydroxyapatite scaffolds, *Biomaterials*, 2006, **27**, 5014–5026.
- 11 C. M. Botelho, R. A. Brooks, S. M. Best, M. A. Lopes, J. D. Santos and N. Rushton, *et al.* Human osteoblast response to silicon-substituted hydroxyapatite, *J. Biomed. Mater. Res., Part A*, 2006, **79**, 723–730.
- 12 C. Wu, W. Fan, J. Chang and Y. Xiao, Mussel-inspired porous SiO₂ scaffolds with improved mineralization and cytocompatibility for drug delivery and bone tissue engineering, *J. Mater. Chem.*, 2011, **21**, 18300–18307.
- 13 Y. Zhu, X. Li, J. Yang, S. Wang, H. Gao and N. Hanagata, Composition–structure–property relationship of the CaO–MxOy–SiO₂–P₂O₅ (M = Zr, Mg, Sr) mesoporous bioactive glass (MBG) scaffolds, *J. Mater. Chem.*, 2011, **21**, 9208–9218.
- 14 X. Li, X. Wang, D. He and J. Shi, Synthesis and characterization of mesoporous CaO–MO–SiO₂–P₂O₅ (M = Mg, Zn, Cu) bioactive glasses/composites, *J. Mater. Chem.*, 2008, **18**, 4103–4109.
- 15 Y. Ramaswamy, C. Wu, A. Van Hummel, V. Combes, G. Grau and H. Zreiqat, The responses of osteoblasts, osteoclasts and endothelial cells to zirconium modified calcium-silicate-based ceramic, *Biomaterials*, 2008, **29**, 4392–4402.
- 16 C. Wu, Y. Luo, G. Cuniberti, Y. Xiao and M. Gelinsky, Three-dimensional printing of hierarchical and tough mesoporous bioactive glass scaffolds with a controllable pore architecture, excellent mechanical strength and mineralization ability, *Acta Biomater.*, 2011, **7**, 2644–2650.
- 17 P. Valerio, M. M. Pereira, A. M. Goes and M. F. Leite, The effect of ionic products from bioactive glass dissolution on osteoblast proliferation and collagen production, *Biomaterials*, 2004, **25**, 2941–2948.
- 18 I. D. Xynos, A. J. Edgar, L. D. Buttery, L. L. Hench and J. M. Polak, Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis, *Biochem. Biophys. Res. Commun.*, 2000, **276**, 461–465.
- 19 H. Sun, C. Wu, K. Dai, J. Chang and T. Tang, Proliferation and osteoblastic differentiation of human bone marrow-derived stromal cells on akermanite-bioactive ceramics, *Biomaterials*, 2006, **27**, 5651–5657.
- 20 W. Peng, W. Liu, W. Zhai, L. Jiang, L. Li and J. Chang, *et al.* Effect of tricalcium silicate on the proliferation and odontogenic differentiation of human dental pulp cells, *J. Endod.*, 2011, **37**, 1240–1246.
- 21 L. Xia, Z. Zhang, L. Chen, W. Zhang, D. Zeng and X. Zhang, *et al.* Proliferation and osteogenic differentiation of human periodontal ligament cells on akermanite and beta-TCP bioceramics, *Eur. Cell. Mater.*, 2011, **22**, 68–82.
- 22 H. Gu, F. Guo, X. Zhou, L. Gong, Y. Zhang and W. Zhai, *et al.* The stimulation of osteogenic differentiation of human adipose-derived stem cells by ionic products from akermanite dissolution via activation of the ERK pathway, *Biomaterials*, 2011, **32**, 7023–7033.
- 23 C. Wu, J. Chang, S. Ni and J. Wang, *In vitro* bioactivity of akermanite ceramics, *J. Biomed. Mater. Res., A*, 2006, **76**, 73–80.
- 24 C. Wu, Y. Zhang, W. Fan, X. Ke, X. Hu and Y. Zhou, *et al.* CaSiO₃ microstructure modulating the *in vitro* and *in vivo* bioactivity of poly(lactide-co-glycolide) microspheres, *J. Biomed. Mater. Res., A*, 2011, **98**, 122–131.
- 25 S. Xu, K. Lin, Z. Wang, J. Chang, L. Wang and J. Lu, *et al.* Reconstruction of calvarial defect of rabbits using porous calcium silicate bioactive ceramics, *Biomaterials*, 2008, **29**, 2588–2596.
- 26 Y. Huang, X. Jin, X. Zhang, H. Sun, J. Tu and T. Tang, *et al.* *In vitro* and *in vivo* evaluation of akermanite bioceramics for bone regeneration, *Biomaterials*, 2009, **30**, 5041–5048.
- 27 M. Bohner, Silicon-substituted calcium phosphates – a critical view, *Biomaterials*, 2009, **30**, 6403–6406.
- 28 L. L. Hench and J. M. Polak, Third-generation biomedical materials, *Science*, 2002, **295**, 1014–1017.

- 29 L. L. Hench and I. Thompson, Twenty-first century challenges for biomaterials, *J. R. Soc. Interface*, 2010, **7**(Suppl 4), S379–S391.
- 30 S. Zou, D. Ireland, R. A. Brooks, N. Rushton and S. Best, The effects of silicate ions on human osteoblast adhesion, proliferation, and differentiation, *J. Biomed. Mater. Res., B: Appl. Biomater.*, 2009, **90**, 123–130.
- 31 D. M. Reffitt, N. Ogston, R. Jugdaohsingh, H. F. Cheung, B. A. Evans and R. P. Thompson, *et al.* Orthosilicic acid stimulates collagen type 1 synthesis and osteoblastic differentiation in human osteoblast-like cells *in vitro*, *Bone*, 2003, **32**, 127–135.
- 32 W. C. Duivenvoorden, A. Middleton and S. D. Kinrade, Divergent effects of orthosilicic acid and dimethylsilane-diol on cell survival and adhesion in human osteoblast-like cells, *J. Trace Elem. Med. Biol.*, 2008, **22**, 215–223.
- 33 J. Li, S. Mareddy, D. M. Tan, R. Crawford, X. Long and X. Miao, *et al.* A minimal common osteochondrocytic differentiation medium for the osteogenic and chondrogenic differentiation of bone marrow stromal cells in the construction of osteochondral graft, *Tissue Eng. A*, 2009, **15**, 2481–2490.
- 34 I. R. Gibson, J. Huang, S. M. Best and W. Bonfield, Enhanced *in vitro* cell activity and surface apatite layer formation on novel silicon-substituted hydroxyapatite, *Bioceramics*, 1999, **12**, 191–194.
- 35 M. Y. Shie, S. J. Ding and H. C. Chang, The role of silicon in osteoblast-like cell proliferation and apoptosis, *Acta Biomater.*, 2011, **7**, 2604–2614.
- 36 C. Wu, J. Chang, S. Ni and J. Wang, *In vitro* bioactivity of akermanite ceramics, *J. Biomed. Mater. Res., Part A*, 2006, **76**(1), 73–80.
- 37 C. Wu, Y. Ramaswamy, A. Soeparto and H. Zreiqat, Incorporation of titanium into calcium silicate improved their chemical stability and biological properties, *J. Biomed. Mater. Res., Part A*, 2008, **86**(2), 402–410.
- 38 I. A. Silver, J. Deas and M. Erecińska, Interactions of bioactive glasses with osteoblasts *in vitro*: effects of 45S5 bio-glass, and 58S and 77S bioactive glasses on metabolism, intracellular ion concentrations and cell viability, *Biomaterials*, 2001, **22**, 175–185.
- 39 D. A. Bushinsky, Metabolic alkalosis decreases bone calcium efflux by suppressing osteoclasts and stimulating osteoblasts, *Am. J. Physiol.*, 1996, **271**, F216–F222.
- 40 C. Wu, J. Chang, J. Y. Wang, S. Y. Ni and W. Y. Zhai, Preparation and characteristics of a calcium magnesium silicate (bredigite) bioactive ceramic, *Biomaterials*, 2005, **26**, 2925–2931.
- 41 Y. Zhou, C. Wu and Y. Xiao, The stimulation of proliferation and differentiation of periodontal ligament cells by the ionic products from Ca₇Si₂P₂O₁₆ bioceramics, *Acta Biomater.*, 2012, **8**, 2307–2316.
- 42 J. Li, L. Wei, J. Sun and G. Guan, Effect of ionic products of dicalcium silicate coating on osteoblast differentiation and collagen production *via* TGF-beta1 pathway, *J. Biomater. Appl.*, 2011, DOI: 10.1177/0885328211416393.
- 43 B. Zhang and J. X. Ma, Wnt pathway antagonists and angiogenesis, *Protein Cell*, 2010, **1**, 898–906.
- 44 E. H. Jho, T. Zhang, C. Domon, C. K. Joo, J. N. Freund and F. Costantini, Wnt/beta-Catenin/Tcf signalling induces the transcription of Axin2, a negative regulator of the signalling pathway, *Mol. Cell Biol.*, 2002, **22**, 1172–1183.
- 45 H. M. Yu, B. Jerchow, T. J. Sheu, B. Liu, F. Costantini and J. E. Puzas, *et al.* The role of Axin2 in calvarial morphogenesis and craniosynostosis, *Development*, 2005, **132**, 1995–2005.
- 46 J. Huelsken and W. Birchmeier, New aspects of Wnt signalling pathways in higher vertebrates, *Curr. Opin. Genet. Dev.*, 2001, **11**, 547–553.
- 47 N. Barker, The canonical Wnt/beta-catenin signalling pathway, *Methods Mol. Biol.*, 2008, **468**, 5–15.
- 48 P. N. De Aza, Z. B. Luklinska, A. Martinez, M. R. Anseau, F. Guitian and S. De Aza, Morphological and structural study of pseudowollastonite implants in bone, *J. Microsc.*, 2000, **197**, 60–67.
- 49 Y. Takahashi, S. Imazato, A. V. Kaneshiro, S. Ebisu, J. E. Frencken and F. R. Tay, Antibacterial effects and physical properties of glass-ionomer cements containing chlorhexidine for the ART approach, *Dent. Mater.*, 2006, **22**, 647–652.
- 50 S. V. Prykhozhij, In the absence of Sonic hedgehog, p53 induces apoptosis and inhibits retinal cell proliferation, cell-cycle exit and differentiation in zebrafish, *PLoS One*, 2010, **5**, e13549.
- 51 M. Varjosalo and J. Taipale, Hedgehog: functions and mechanisms, *Genes Dev.*, 2008, **22**, 2454–2472.
- 52 M. Cho, M. Ryu, Y. Jeong, Y. H. Chung, D. E. Kim and H. S. Cho, *et al.* Cardamonin suppresses melanogenesis by inhibition of Wnt/beta-catenin signaling, *Biochem. Biophys. Res. Commun.*, 2009, **390**, 500–505.