



## Orthosilicic acid, Si(OH)<sub>4</sub>, stimulates osteoblast differentiation *in vitro* by upregulating miR-146a to antagonize NF-κB activation



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### ABSTRACT

Accumulating evidence over the last 40 years suggests that silicate from dietary as well as silicate-containing biomaterials is beneficial to bone formation. However, the exact biological role(s) of silicate on bone cells are still unclear and controversial. Here, we report that orthosilicic acid (Si(OH)<sub>4</sub>) stimulated human mesenchymal stem cells (hMSCs) osteoblastic differentiation *in vitro*. To elucidate the possible molecular mechanisms, differential microRNA microarray analysis was used to show that Si(OH)<sub>4</sub> significantly up-regulated microRNA-146a (miR-146a) expression during hMSC osteogenic differentiation. Si(OH)<sub>4</sub> induced miR-146a expression profiling was further validated by quantitative RT-PCR (qRT-PCR), which indicated miR-146a was up-regulated during the late stages of hMSC osteogenic differentiation. Inhibition of miR-146a function by anti-miR-146a suppressed osteogenic differentiation of MC3T3 pre-osteoblasts, whereas Si(OH)<sub>4</sub> treatment promoted osteoblast-specific genes transcription, alkaline phosphatase (ALP) production, and mineralization. Furthermore, luciferase reporter assay, Western blotting, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence showed that Si(OH)<sub>4</sub> decreased TNFα-induced activation of NF-κB, a signal transduction pathway that inhibits osteoblastic bone formation, through the known miR-146a negative feedback loop. Our studies established a mechanism for Si(OH)<sub>4</sub> to promote osteogenesis by antagonizing NF-κB activation via miR-146a, which might be interesting to guide the design of osteo-inductive biomaterials for treatments of bone defects in humans.

### Statement of Significance

Accumulating evidence over 40 years suggests that silicate is beneficial to bone formation. However, the biological role(s) of silicate on bone cells are still unclear and controversial. Here, we report that Si(OH)<sub>4</sub>, the simplest form of silicate, can stimulate human mesenchymal stem cells osteoblastic differentiation. We identified that miR-146a is the expression signature in bone cells treated with Si(OH)<sub>4</sub>. Further analysis of miR-146a in bone cells reveals that Si(OH)<sub>4</sub> upregulates miR-146a to antagonize the activation of NF-κB. Si(OH)<sub>4</sub> was also shown to deactivate the same NF-κB pathway to suppress osteoclast formation. Our findings are important to the development of third-generation cell- and gene affecting biomaterials, and suggest silicate and miR-146a can be used as pharmaceuticals for bone fracture prevention and therapy.

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## 1. Introduction

The earliest work involving silicate and bone dates back to the early 1970s, when it was suggested that silicate may play an important role in skeletal development and repair [1–3]. Subsequently, the positive effect of silicate on bone has raised the interest of multiple research groups working on bone graft substitutes [4]. Silicate-based bioactive glasses [4–9], silicate-substituted ceramics [10–15], and natural silicate bioceramics [16–20] have been developed and, in most of cases, have shown better biological properties in orthopaedic applications when compared to their silicate-free counterparts. Systematic studies by Hench et al. [21–23] and De Aza [16,17,24] have identified silicate in bioactive glasses or glass-ceramics could be released as  $\text{Si}(\text{OH})_4$  by hydrolyzing Si–O–Si bridges and re-precipitate to form surface silanol groups. The concentration of silanol groups could be critical because they act as a nucleation agent for apatite formation [25,26]. A “passive” role for silicate has been proposed in which the presence of silicate in the materials results in changes of grain size, surface topography, etc., leading ultimately to a change of biological response [4,8,9,27]. There is evidence in the literature which indicates that inorganic dissolution products ( $\text{Si}(\text{OH})_4$ ,  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$ , etc.) from inorganic silicate materials can influence and control the cell cycle of osteogenic precursor cells and ultimately control the differentiated cell population [28,29]. An “active” role, as claimed by researchers and companies, is that silicate can affect bone cell metabolism [30]. Thus, the silicate materials are not simply bone graft substitutes but, effectively, can be cell- and gene-activating biomaterials [31].<sup>1</sup> However, none of the studies focused on the active roles have demonstrated yet that the positive biological effects can be attributed solely to soluble silicate, since  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$ , which are released from silicate-based materials, are known to have osteo-inductive effects on bone cells [32,33]. A number of *in vitro* studies using osteoblast-like cells have added to the evidence that silicate may be beneficial for bone formation. For example, silicic acid addition to MG63 osteosarcoma cells has been shown to increase collagen type I (Col I) levels 1.75-fold [34]. The authors proposed a structural role (stabilization of collagen) or a metabolic role (a co-factor for prolyl hydroxylase) for silicate [35]. In osteoblasts and mesenchymal stem cells, silicic acid also increased the gene expression of osteocalcin (OCN) and alkaline phosphatase (ALP) [36–38], which are excellent indicators of osteogenic differentiation and mineralization. Recently, silica ( $\text{SiO}_2$ ) nanoparticles and nanoplatelets were shown to mediate potent stimulatory effects on osteoblast differentiation [39,40]. The spherical silica nanoparticles of 50 nm were shown to enter the cells through caveolae-mediated endocytosis, which triggered the stimulation of the mitogen activated protein kinase (MAPK) ERK1/2 (p44/p42) pathway. These nanoparticles were likely released from the endosome to stimulate autophagy, which suggested a cellular mechanism for the stimulatory effects of silica nanoparticles on osteoblast differentiation and mineralization [41]. It should be noted, as the authors themselves point out, that subtle changes in surface charge, surface area, shape, size, and/or surface chemistry of the particles may play direct roles in determining specific cellular responses [39]. For example, hexagonal silica nanoparticles with size about 100 nm did not show any effect on differentiation of MSCs *in vitro* [42]. It is possible that not silicate chemistry but the nano-size particles could be responsible for the positive outcome in these studies. Therefore, the exact biological roles of soluble silicate on bone cells remain unclear and highly controversial [31].

Bone marrow-derived human mesenchymal stem cells (hMSCs) are a population of self-renewing multipotent cells and are excellent candidates for cell-based therapeutic strategies to regenerate injured or damaged tissue. They can differentiate into multiple lineages, including osteogenic lineage, in response to appropriate stimuli [43]. It is now evident that osteogenic differentiation of MSCs is tightly controlled by several regulators including microRNAs (miRNAs) [44,45]. MiRNAs are small endogenous RNA molecules that govern gene expression by targeting mRNA at post-transcriptional level. miRNAs have emerged as key regulators of diverse cellular processes, including growth, apoptosis, development, metabolism, stress adaptation, differentiation, etc. [46–48]. For example, miR-138 was shown to inhibit osteoblast differentiation of hMSCs by targeting FAK (focal adhesion kinase) translation. Consequently, reduced phosphorylation of FAK and ERK1/2 (Extracellular signal-related kinase) resulted in a decrease of Runt-related transcription factor 2 (Runx2) phosphorylation [49]. miR-29b has been reported to promote osteogenesis by directly down-regulating known inhibitors of osteoblast differentiation, HDAC4, TGF $\beta$ 3, ACVR2A, CTNBP1, and DUSP2 proteins, through binding to target 3'-UTR sequences in their mRNAs [50]. Various miRNAs that positively or negatively regulate osteoblast differentiation were summarized by Vimalraj and Selvamurugan [51]. However, no miRNAs have been identified to bridge the biological functions of silicate with bone formation.

In the physiological pH and at concentration below 2 mM (56 ppm Si), silicate exists predominantly as orthosilicic acid ( $\text{Si}(\text{OH})_4$ ). This tetrahedral  $\text{Si}(\text{OH})_4$  is uncharged at neutral pH, but has the tendency to polymerize to polysilicate at silicate concentration above 2–3 mM (56–84 ppm Si) [52]. To clarify the direct effects of silicate on bone formation, we examined the effect of ( $\text{Si}(\text{OH})_4$ ) on bone cell proliferation, differentiation, and intracellular signaling events. We demonstrated that soluble  $\text{Si}(\text{OH})_4$  stimulated hMSCs to proliferate in growth medium and enhance osteogenic differentiation in osteogenic medium. We also sought to ascertain at least one mechanism by which  $\text{Si}(\text{OH})_4$  accomplishes these bioactivities by regulating microRNAs expression.

## 2. Materials and methods

### 2.1. $\text{Si}(\text{OH})_4$ preparation

Final concentrations of  $\text{Si}(\text{OH})_4$  solution with 0–50 ppm Si were prepared by adding sodium metasilicate ( $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ ) in  $\alpha$ MEM and then adjusting pH to 7.2 ~ 7.4 with 1 M HCl before adding FBS or any other supplements (Equation below). Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis was performed to establish the ion concentration for Si in the medium using an Agilent Technologies 710 ICP-emission spectrometer. Standard solutions (SQCSTD27-100B) were purchased from RICCA Chemical Company for the calibration of the instrument. All the reagents, unless stated specially, were obtained from Sigma-Aldrich and were of the highest available purity.



### 2.2. Cell culture

Bone marrow-derived hMSCs (Lonza<sup>®</sup>) at passage 4 were grown in regular growth medium ( $\alpha$ MEM, 10% FBS, and 1% penicillin-streptomycin). The cells were cultured until 70–75%

<sup>1</sup> To avoid a conflict of interest, Dr. Marc Bohner acted as the Editor for this manuscript.

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