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S100A4 inhibition by RNAi up-regulates osteoblast related genes in periodontal ligament cells

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Abstract

Periodontal ligament (PDL) is a thin fibrous connective tissue located between alveolar bone and cementum that remains unmineralized physiologically. It is thus thought that PDL cells possess mechanisms to inhibit mineralization. It has been demonstrated that S100A4, a member of the S100 calcium-binding protein family, is synthesized and secreted by PDL cells, and that it may act as an inhibitor of mineralization. However, the mechanisms of action of S100A4 in mineralization have not been thoroughly clarified. In the present study we investigated the effects of S100A4 inhibition by a short interfering RNA (siRNA) on the expression of osteoblast related genes by human PDL cells. Inhibition of S100A4 by siRNA resulted in increased expression of osteoblastic markers such as osteopontin and osteocalcin, and the osteoblast-specific transcription factors, Runx2/Cbfa1 and Osterix. These results indicate that S100A4 suppresses the expression of osteoblastic genes in PDL cells and may thus inhibit mineralization in the PDL.

Keywords: Periodontal ligament; S100A4; siRNA; Runx2/Cbfa1; Osterix; Osteoblastic differentiation

Periodontal ligament (PDL) is a thin fibrous connective tissue situated between two mineralized tissues, i.e., alveolar bone and cementum. Despite the mechanical stress of mastication or orthodontic forces during orthodontic movement, under physiological conditions, the PDL maintains its width unmineralized. It has thus been speculated that PDL cells possess regulatory mechanisms to inhibit mineralization. Interestingly, under certain conditions, some cells within the PDL exhibit properties of osteoblasts and are capable of forming a mineralized matrix [1,2].

S100A4 is a member of the S100 calcium-binding protein family [3]. It has two calcium-binding motifs; one canonical EF-hand and another specific to the S100 family. Most of the proteins in this family are synthesized and localized intra-cellularly, and play fundamental roles such as cell metabolism, motility, and intra-cellular signaling [4,5]. S100A4 is known to be involved in motility and metastasis of cancer cells by interacting with cytoskeletal components. It is, therefore, considered a prognostic marker for cancer progression [6–8]. It is also reported that S100A4 is expressed in normal tissues such as smooth muscle, liver, bone marrow, smooth muscle cell of arteries, kidney, and osteoblastic cell in humans [9,10]. However, physiological functions

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of S100A4 in normal tissues have not been thoroughly clarified.

Previous studies have shown that S100A4 was expressed by periodontal tissues and localized intra- and extra-cellularly in the PDL. Strutz et al. [11] demonstrated high gene expression of S100A4 in the periodontal mesenchyme during early stages of tooth development by in situ hybridization. It has been previously reported that the expression level of the S100A4 mRNA in bovine PDL is remarkably high when compared to other oral tissues such as dental follicle, dental papilla, and gingiva [12]. Furthermore, S100A4 is secreted from cultured bovine PDL cells and the addition of a recombinant mouse S100A4 protein inhibits mineralized nodule formation in cultured rat osteogenic cells in a dose-dependent manner [13]. These results suggest that S100A4 is localized in the PDL and may act as a negative regulator of mineralization. Moreover, in osteoblastic cell clones synthesizing low levels of S100A4, the formation of mineralized nodules was accelerated/increased and the mRNA expression of osteoblastic phenotypic markers was enhanced [14].

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene [15]. While the mechanism of RNAi is not yet fully understood, recent genetic and biochemical studies have revealed that RNAi is a useful technique for inhibition of specific gene expression. Elbashir et al. [15] showed that synthetic 21- to 23-nucleotide short interfering RNA (siRNA) could induce efficient RNAi in mammalian cells. Thus, RNAi has emerged as a powerful tool for silencing of gene expression in animals and plants.

In the present study, we employed the siRNA technique to inhibit S100A4 and investigated if S100A4 inhibition affects the expression of osteoblast-specific transcriptional factors and related genes in human PDL cells.

Materials and methods

Cell culture. Human PDL (hPDL) cells were obtained from healthy human teeth indicated for extraction for orthodontic treatment according to the report described by Somerman et al. [16]. Periodontal tissue was removed from the middle third of the root using a sterile scalpel. The tissue was rinsed five times with growth media (α-modified minimal essential medium, α-MEM; Gibco) and transferred to culture dishes.

Cultures were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco), in an atmosphere of 5% CO_2 in a humidified incubator. The medium was changed twice weekly. The cells were subcultured before reaching confluency.

siRNA transfection. siRNA, a double-stranded RNA (21 base pairs), was designed according to the siRNA design program si-Search [17] and modified for human S100A4 gene sequence as follows:

5′-GGA CAG AUG AAG CUG CUU UTT-3′,
5′-AAA GCA GCU UCA UCU GUC CTT-3′.

A mismatch siRNA (non-sense siRNA) of 21 base pairs was used as a negative control. Transfection of siRNA and non-sense siRNA was performed by the Oligofectamine kit (Invitrogen) according to the manufacturer's instructions. Briefly, hPDL cells were plated at 30% confluency with α-MEM without antibiotics. Before transfection, culture medium was changed to Opti-MEM Reduced Serum Medium (Gibco), and then siRNA and non-sense siRNA were transfected into the cells at the concentrations of 50–200 nM. Oligofectamine reagent alone (0 nM) was applied to the cells as a negative control. After 4 h, the medium was replaced with medium containing serum for further 72 h.

Immunostaining. Immunostaining was performed by the HISTOFINE kit (Nichirei). Briefly, hPDL cells were washed with phosphate-buffered saline (PBS), fixed with 10% formalin in PBS for 15 min at room temperature, and washed again with PBS. The cells were then treated with acetone/ethanol (50:50, vol/vol) for 1 min to permeate the cell membrane. After washing with PBS, the cells were incubated with rabbit polyclonal anti-mouse S100A4 antibody [7] (1:5 dilution with 1% BSA in PBS) or pre-immune rabbit serum for 3 h at room temperature. After the incubation, the cells were washed with PBS and further incubated with polyclonal anti-mouse IgG labeled with peroxidase for 1 h at room temperature. After washing with PBS, peroxidase substrate (diaminobenzidine) was applied to the hPDL cells for 3 min to develop color. Nuclei of hPDL cells were counterstained with hematoxylin. The cells were soaked in Carazzi's Hematoxylin solution for 30 min and washed with water three times. The staining was then observed under a light microscope. Total cells and positive stained cells were counted at randomly selected four fields per well and the average was used as the number of cells.

Reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from cultured hPDL cells using the acid guanidinium thiocyanate–phenol–chloroform method. Cultured hPDL cells were washed two times with PBS and collected using sterile scrapers after the addition of RNAzol B (Cosmo Bio). RNA was recovered by phenol–chloroform extraction, precipitated with isopropanol, washed with 75% ethanol, and suspended in distilled water. UV absorbance at 260 and 280 nm was then measured and purity of RNA was confirmed by calculating the 260/280 ratio. Three micrograms of sample RNA was used for reverse transcription-polymerase chain reaction (RT-PCR) using SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen) and AccuPrimer SuperMix I (Invitrogen). Primers specific for human S100A4, Runx2/Cbfa1, Osterix (Osx), osteopontin (OPN), osteocalcin (OCN), and GAPDH were designed as follows:

S100A4	5′-GGCCCTGGATGTGATGGTGT-3′
	5′-TCCACCACCCTGTTGCTGTA-3′
Runx2/Cbfa1	5′-GTGGACGAGGCAAGAGTTTCA-3′
	5′-TGGCAGGTAGGTGTGGTAGTG-3′
Osx	5′-CTTCAGTCTTCCCAACTTCTTACAC-3′
	5′-ACAAATTGGGTTAGCTACATCTCTG-3′
OPN	5′-TGACCTCTGTGAAAACAGCGT-3′
	5′-TGTACATTGTGAAGCTGTGAA-3′
OCN	5′-TTGTGTCCAAGCAGGAGGGCA-3′
	5′-ACATCCATAGGGCTGGGAGGT-3′
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′
	5′-TCCACCACCCTGTTGCTGTA-3′

PCR was performed with annealing temperature of 60 °C (Runx2/Cbfa1), 51 °C (Osx), and 55 °C (others). After 30 (S100A4, GAPDH), 40 (Runx2/Cbfa1, Osx), and 45 (OPN, OCN) cycles, aliquots of the

PCR products were resolved on 1% TAE-agarose gels, stained with ethidium bromide, and photographed under ultraviolet light. To ensure that there are no artifacts in the RT-PCR procedure, we analyzed RT(−) sample as a negative control.

Quantitative real-time PCR. Quantitative real-time PCR was performed with the Light Cycler FastStart Master Sybr Green I (Roche Molecular Biochemicals) in a standard PCR using the Light Cycle-Primer Set Human GAPDH, and Human CBFA1 (Search GmbH). Each PCR was carried out in a total volume of 20 μl in glass capillaries containing 10 μl cDNA sample diluted with H_2O and 10 μl PCR mix. The cDNA sample was denatured at 95 °C for 10 min and added to the capillaries. The reaction was carried out using the following conditions for GAPDH and Runx2/Cbfa1: 35 cycles of 95 °C for 10 s, 68 °C for 10 s, and 72 °C for 16 s with a single fluorescence detection point at the end of the relevant annealing or extension segment. One cycle of melting curve from 58 to 95 °C by a transition rate of 0.1 °C/s with continuous detection of fluorescence was performed. The temperature transition rate for all amplifications was 20 °C/s. Analysis was carried out with the LightCycler Software Ver. 3.5 (Roche). The amount of the target gene was normalized to GAPDH. As a negative control, 10 μl of water without the cDNA template was also subjected to real-time PCR.

Statistical analysis. Unpaired Student's *t* test was used to detect statistical significance in the comparison of the percentage of S100A4 positive cells by immunostaining. Data are expressed as means ± standard deviation (SD).

Results

Expression of S100A4 after siRNA transfection in hPDL cells

mRNA expression of S100A4 in cultured hPDL cells 72 h after siRNA transfection was detected by semi-quantitative RT-PCR methods. Non-sense siRNA was also transfected as a control. The results of the semi-quantitative RT-PCR analysis of S100A4 are shown in Fig. 1. S100A4 mRNA was expressed in cultured hPDL cells. The expression was down-regulated after siRNA transfection, while no change was observed after non-sense siRNA transfection.

Immunostaining of S100A4 in cultured hPDL cells with or without siRNA transfection is shown in Fig. 2A. All the staining procedures were performed 72 h after the transfection. None of the cells were stained in the control experiments using pre-immune serum (Fig. 2A-a). Many hPDL cells were positively stained for S100A4 in the culture without siRNA transfection (Fig. 2A-b). The positive cells showed flatter and larger appearance that clearly distinguished from the negative cells. It is evident that the staining of S100A4 protein localized only in the cytoplasm as shown in higher magnification (Fig. 2B). The cultures with siRNA transfection showed fewer positive cells and the intensity of the staining decreased dose-dependently up to 100 nM (Fig. 2A-c, d). Fig. 2C shows the percentage of positive cells in cultures with different concentrations of siRNA. The percentage in the cultures with siRNA transfection decreased to 20–30% of that in the culture without transfection, indicating that siRNA transfection reasonably reduced S100A4 production.

mRNA expression of osteoblast phenotypic markers

mRNA expression of osteoblast phenotypic markers in cultured hPDL cells after siRNA transfection was detected by semi-quantitative RT-PCR and quantitative real-time PCR methods. The results of the semi-quantitative RT-PCR analysis of osteoblast phenotypic markers are shown in Fig. 3. S100A4 mRNA was constitutively expressed in control culture (0 nM). The expression of S100A4 was significantly down-regulated, when the cells were transfected with siRNA. In contrast, the expression of Osx, OPN, and OCN was clearly up-regulated. RT-PCR analysis for Runx2/Cbfa1 exhibited three bands. Intensity of the upper band was most intensive and appeared not to be varied by the siRNA transfection. Lower two bands (middle and lower bands) showed relatively lower intensity compared to that of the upper band, but the intensity appeared to be gradually increased by

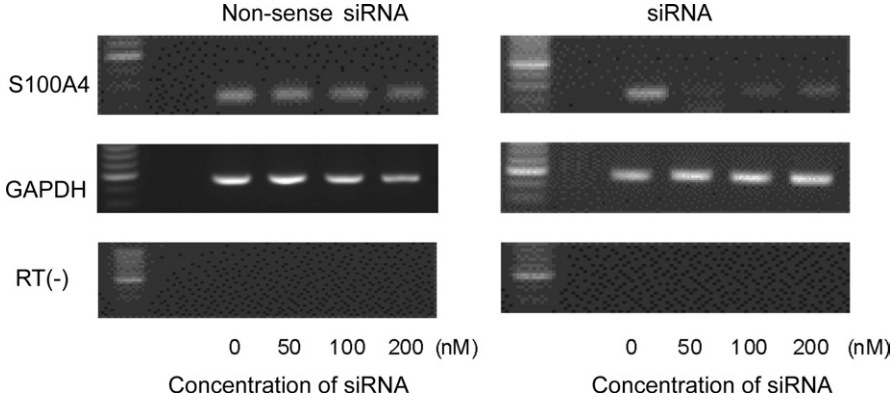

Fig. 1. mRNA expression of S100A4 72 h after siRNA or non-sense siRNA transfection. siRNA or non-sense siRNA was transfected into the cells at the concentrations of 50–200 nM. Oligofectamine reagent without siRNA (0 nM) was applied to the cells as a control. The expression of S100A4 mRNA in the human PDL cells was detected using semi-quantitative RT-PCR. The expression of S100A4 was down-regulated after siRNA transfection, whereas no change was observed after non-sense siRNA transfection. RT(−) sample was analyzed as a negative control.

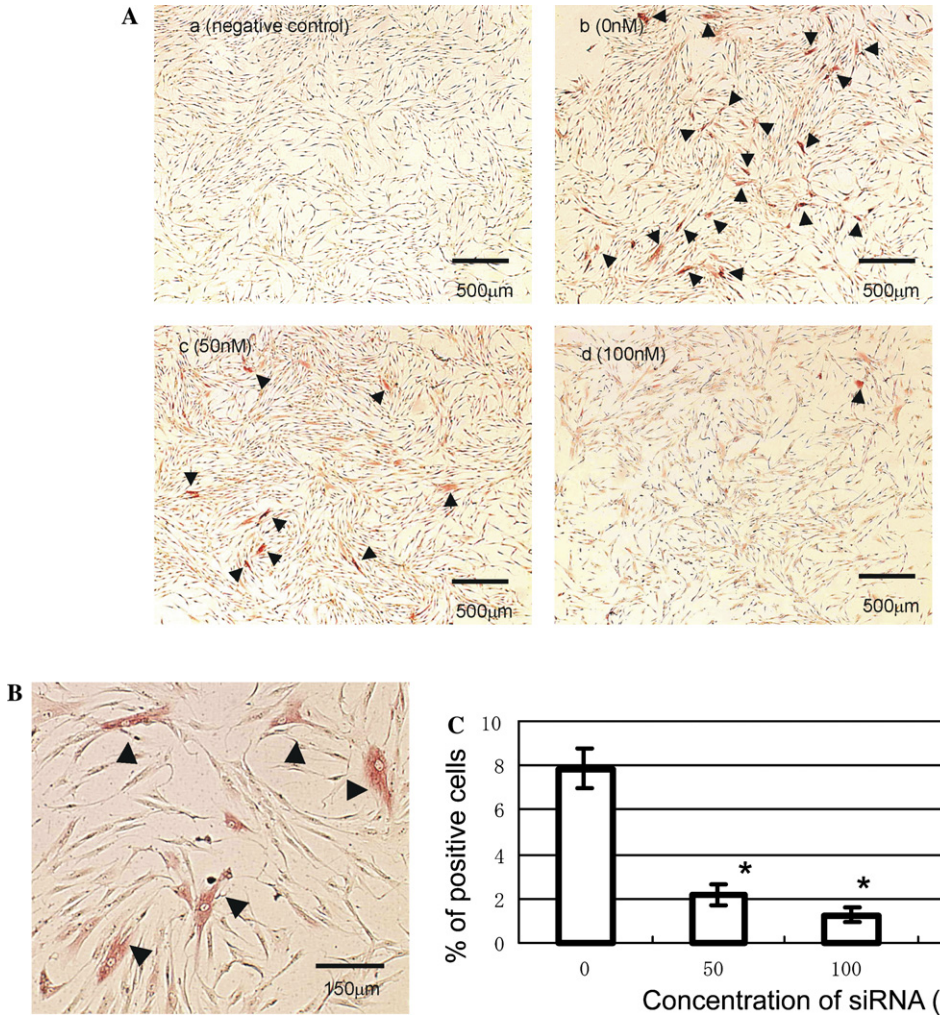

Fig. 2. (A) Immunostaining of S100A4 in cultured human PDL cells after siRNA transfection. All the staining procedures were performed 72 h after siRNA transfection. No staining was observed in the cells treated with pre-immune rabbit serum (a). In the culture without siRNA (b), many cells were positively stained. The positive cells (arrowheads) appeared flatter and larger comparing with negative cells. siRNA transfected cultures showed fewer positive cells (c,d). The intensity of the staining decreased dose-dependently up to 100 nM. Bars = 500 μm. Magnification 40×. (B) Cellular localization of S100A4 in hPDL cells (arrowheads). The staining of S100A4 localized only in the cytoplasm and no nuclear localization was detected. Bars = 150 μm. Magnification 100×. (C) The percentage of positive cells in cultures with different concentrations of siRNA. In the culture with siRNA transfection, the percentage of positive cells was significantly less compared to the control. Values are means ± SD ($n = 4$). *Significantly different from control ($P < 0.001$).

siRNA transfection in a dose-dependent manner. To analyze the expression level of Runx2/Cbfa1 more quantitatively, we performed quantitative real-time PCR analysis. The results revealed that the mRNA level of Runx2/Cbfa1 increased approximately 100- and 2000-fold after 50 and 100 nM siRNA transfection, respectively, at day 3 (Fig. 4). The increased Runx2/Cbfa1 expression could explain the up-regulation of Osx, OPN, and OCN, genes downstream to Runx2/Cbfa1.

Discussion

PDL cells are known to be a heterogeneous cell population and are indispensable for the regeneration of periodontal tissues including the unmineralized PDL and two mineralized tissues, i.e., cementum and bone [18].

S100A4 was detected in cultured hPDL cells at the protein and mRNA levels. Duarte et al. [14] have suggested that a decrease in S100A4 expression may be associated with a terminal osteoblastic differentiation and/or the initiation of mineralized matrix formation. Other reports have shown that the expression of S100A4 is high in osteoblast progenitors and decreases to undetectable levels in mature osteoblasts and in osteocytes [11,19]. These data suggest that S100A4 may play an important role both in PDL cells and osteoblasts in terms of regulation of osteoblastic differentiation/matrix mineralization.

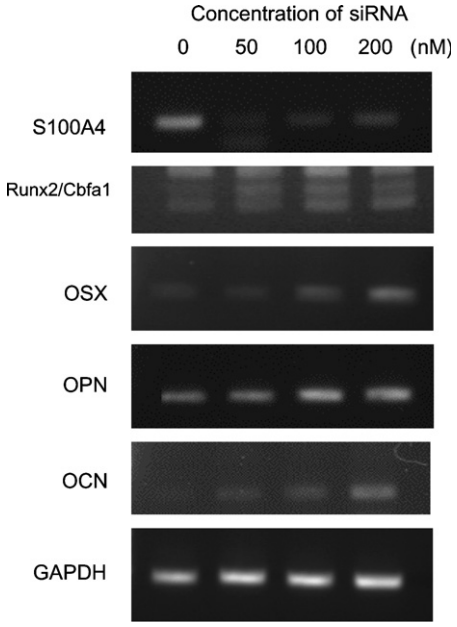

Fig. 3. mRNA expression of S100A4, Runx2/Cbfa1, Osterix (Osx), osteopontin (OPN), and osteocalcin (OCN) after siRNA transfection. siRNA was transfected into the cells at the concentrations of 50–200 nM. Oligofectamine reagent without siRNA (0 nM) was applied to the cells as a negative control. The expression levels of Runx2/Cbfa1, Osx, OPN, and OCN mRNA were up-regulated after siRNA transfection.

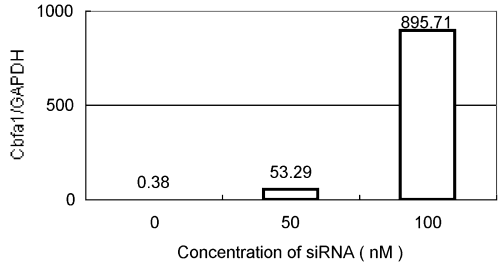

Fig. 4. Real-time PCR analysis of Runx2/Cbfa1 expression. *Y*-axis shows the ratio of Runx2/Cbfa1 to an internal control, GAPDH. mRNA level of Runx2/Cbfa1 increased approximately 100- and 2000-fold after 50 and 100 nM of siRNA transfection, respectively.

The application of RNAi has the potential to allow the systematic analysis of gene expression and holds the promise of therapeutic gene silencing [20–22]. A few limitations in the use of RNAi in mammalian cells have been reported [20,23], however, the recent development of 21-nucleotide siRNA duplexes has circumvented these problems and allowed successful RNAi in cultures of several types of mammalian cells [15,24]. Therefore, we used siRNA technique to inhibit S100A4 expression and investigate the potential physiological roles of S100A4 in human PDL.

Runx2/Cbfa1 and Osx are transcription factors required for osteoblastic differentiation. They are necessary for the commitment of osteoblasts, the expression of osteoblastic phenotypic genes, and bone formation [25–27]. The transfection of siRNA targeting S100A4 resulted in reduction of S100A4 expression and a dramatic increase in the expression level of Runx2/Cbfa1 at day 3 after the transfection. Saito et al. [28] reported that PDL cell line expressed Runx2/Cbfa1 at the same level to or even higher than that in osteoblasts in vivo, but the PDL cell line never mineralized, suggesting the presence of a mechanism responsible for suppressing the Runx2/Cbfa1 activity in PDL cells. Since the inhibition of S100A4 by siRNA enhanced Runx2/Cbfa1 expression in PDL cells, S100A4 could be one of the explanations for the suppressing mechanism. RT-PCR analysis for Runx2/Cbfa1 showed three different bands as reported previously for osteoblastic cell line and two isoforms (middle and lower bands in Fig. 3) appeared to be up-regulated by inhibition of S100A4. Although, Runx2/Cbfa1 has three different isoforms, i.e., cbfa1-3, the function of each isoform was not well understood [29]. Further experiments to figure out the function of Runx2/Cbfa1 isoforms are needed. Osx was also up-regulated after the transfection with the siRNA. Conversely, Duarte et al. [14] reported that the inhibition of S100A4 by antisense did not affect the mRNA level of Runx2/Cbfa1 or Osx expression after 5 and 10 days of induction of osteoblastic differentiation in a mouse osteoblastic cell culture. We assume that this difference may be in part due to a different experimental design, different time points to investigate Runx2/Cbfa1 expression, period of time the cells remained in culture prior to induction of differentiation, and possibly inter-species differences. Lee et al. [30] have reported that a transient up-regulation of transcription factors such as Runx2/Cbfa1 was observed during initial stages of osteoblastic differentiation. Therefore, we focused on relatively early time points such as day 3 after the siRNA transfection. It has been reported that Runx2/Cbfa1 expression is differentially regulated in human and rodent cells [31]. The immunostaining of hPDL cells showed that S100A4 expression was detected only in cytoplasm. On the other hand, recent report demonstrates that S100A4 localizes in the nucleus [32]. Although it is not clear whether S100A4 directly or indirectly regulates Runx2/Cbfa1 gene expression in hPDL cells, it is possible that inter-species difference is a key determinant in such regulation. Further study is required to clarify the mechanism of the regulation.

Inhibition of S100A4 in hPDL cells by the siRNA transfection also resulted in up-regulation of OPN and OCN, markers of osteoblastic differentiation. This result is in agreement with previous findings [14]. PDL cells are known to be heterogeneous containing osteogenic as well as non-osteogenic cells at various levels of differentiation [2,33–35]. Therefore, certain cells within the PDL may have the potential to form mineralized tissues such as cementum and alveolar bone [18], and it is likely that there are factors regulating mineralization at

physiological conditions. Our results indicate that S100A4 may be one of these factors playing a role as a negative regulator of mineralization by modulating the commitment of some PDL cells to osteoblastic differentiation. The present study provides only a short-term evaluation of these phenomena, since the optimal inhibitory effect of siRNA is reported to last 72 h after transfection and approximately 21 days of the culture required to detect mineralization in cultures of PDL cells [36,37]. Thus, long-term evaluation may be required to clarify the precise function of S100A4 in mineralization.

We conclude that S100A4 inhibition using siRNA resulted in up-regulation of osteoblastic differentiation markers in hPDL cells in culture. S100A4 may play an important role in regulation of mineralization in the PDL by modulating the commitment of some PDL cells to osteoblastic differentiation. To our knowledge, this is the first report to show the involvement of S100A4 in the regulation of osteoblast-specific transcription factors, Runx2/Cbfa1 and Osx.

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