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Original Article

S100A4 upregulation suppresses tissue ossification and enhances matrix degradation in experimental periodontitis models

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Aim: S100A4, also known as fibroblast-specific protein 1 or metastasin 1, is not only highly expressed in growth-stimulated cultured cells and metastatic tumor cells, but also in the periodontal ligament. The aim of this study was to investigate the roles of S100A4 in the pathogenesis of periodontitis and its regulatory mechanisms in inflammatory milieu.
Methods: Experimental periodontitis was induced in rats by submarginal silk ligatures. TRAP activity and S100A4 expression in periodontal ligaments were examined using immunohistochemistry and immunofluorescence methods. IL-1β-treated human periodontal ligament cells (hPDLCs) were used as *in vitro* model of experimental periodontitis. S100A4 mRNA and protein were assessed using qRT-PCR and Western blot, respectively. hPDLCs were transfected with either S100A4 overexpression plasmids or shRNAs plasmids. The mineralization in hPDLCs was evaluated with a 12-d osteogenic induction assay, and the expression of ALP, OCN, MMP-2 and MMP-13 was analyzed by qRT-PCR.
Results: In the periodontal ligaments of rats with experimental periodontitis, TRAP activity and S100A4 protein staining were considerably more intense compared with those in the control rats. Treatment of hPDLCs with IL-1β (10, 50 and 100 ng/mL) dose-dependently increased the mRNA and protein levels of S100A4. Transfection with shRNAs markedly increased mineralized nodule formation and the osteogenic-related markers ALP and OCN levels in hPDLCs, whereas the overexpression of S100A4 significantly reduced mineralized nodule formation, and increased the matrix degradation enzymes MMP-2 and MMP-13 levels in hPDLCs.
Conclusion: S100A4 is upregulated in the experimental rat periodontitis and in IL-1β-treated hPDLCs, where S100A4 suppresses osteogenic differentiation and enhances matrix degradation. Thus, S100A4 is a potential target for the treatment of periodontitis.

Keywords: S100A4; periodontitis; periodontal ligament; human periodontal ligament cells; IL-1β; matrix metalloproteinases; osteogenic differentiation

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Introduction

The highly common illness periodontitis includes a set of inflammatory diseases that affect the periodontium and involve the progressive loss of the alveolar bone around the teeth. If left untreated, periodontitis can lead to the loosening and subsequent loss of teeth. As the key structure of periodontium, the periodontal ligament is critical for homeostasis and regeneration because of its instant response to bacterial insults and its osteogenic differentiation potential. Several studies have reported impaired osteogenic differentiation abilities and increased proteolytic enzyme synthesis (*eg*, matrix metalloproteinases) in periodontitis-affected periodontal ligament cells (most of which are fibroblasts)[1-4], but the mechanisms of these alterations in biologic behaviors remain unclear.

S100A4, which is also known as fibroblast-specific protein (FSP1), metastasin (Mts1) and other names, is strongly expressed in the periodontal ligament[5, 6]. This protein has been extensively studied in the field of cancer over a long period. Initial cloning efforts identified S100A4 as a highly expressed transcript in growth-stimulated cultured cells and metastatic tumor cell lines[7, 8].

Although S100A4 expression in dental tissues has also received wide attention in the oral field, the physiological functions of S100A4 in these tissues have not been thoroughly

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clarified[9-11]. In mouse embryos, S100A4 is highly expressed in the region of periodontal mesenchyme at day E13.5[12], and its expression is dominant in the periodontal ligament and the pulp tissues[11]. Inhibitions of the expressions of osteoblastic genes by S100A4 have also been demonstrated in several studies, and these results indicate that S100A4 is a negative regulator of mineralization[9, 10, 13]. Moreover, S100A4 contributes to the pathogenesis of chronic inflammation and cartilage destruction[14]. The S100A4 protein can activate the immune response and inflammation in immune-mediated diseases because it induces the production of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, in mononuclear cells[15].

Despite the high level of the expression of S100A4 in periodontal ligament cells, we are still far from achieving a comprehensive understanding of its role in the pathogenesis of chronic periodontitis. We attempted to investigate the functional role of S100A4 periodontitis and whether it is regulated by IL-1β, which is the key proinflammatory cytokine in chronic periodontitis[4, 16].

Materials and methods

Animals and experimental design

The experimental periodontitis model was induced by placing ligatures submarginally around the cervix of the right first maxillary molars of eight-week-old male SPF Sprague-Dawley rats (n=6, weight 200 g±20 g) for 14 d. The left first maxillary molars received no experimental intervention and were used as the control group. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the USA. The protocols were approved by the Ethics Committee for Experimental Research of Tongji University. After the experimental period, the animals were sacrificed under general anesthesia. For histological analyses, the 1st molar regions were resected from each rat and fixed in 4% paraformaldehyde for 48 h. The tissues were subsequently processed with STP 120 Tissue Processor (Thermo Scientific, Germany), embedded in paraffin, and then sectioned in the bucal-lingual plane at 5 microns. A rabbit polyclonal antibody for S100A4 (1:200, ZSbio, China) was used to assess the expression of S100A4 with a Streptavidin-Biotin Complex kit (Biotech Well, China) and was stained with a DAB Horseradish Peroxidase Color Development kit following the manufacturer's instructions (Biotech Well, China). For the immunofluorescence test, we incubated the sections with rabbit polyclonal S100A4 antibody (1:250, Abcam, UK) diluted in PBS-1% goat serum overnight in a humidified chamber at 4°C. The binding sites of the primary antibodies were revealed with Cy3-conjugated goat anti-rabbit IgG (1:1000, CST, USA) for 60 min at room temperature. The nuclei were stained using DAPI (0.5 μg/mL, CST, USA) in PBS for 10 min. To identify the osteoclast activity, tartrate-resistant acid phosphatase (TRAP) activity was detected using the Leukocyte Acid Phosphatase kit (Sigma-Aldrich, USA). The slides were examined, and the images were captured using a Nikon ECLIPSE 80i (Nikon, Japan).

Cell culture and treatments

Human periodontal ligament cells (hPDLCs) were isolated from the periodontal ligaments of the healthy orthodontic extracted bicuspids of four different individuals between the ages of 25 and 35 years as previously described[17]. The cells were pooled and used in this study at passage 4-5. The hPDLCs were treated with increasing concentrations of IL-1β (0, 10, 50, 100 ng/mL, Peprotech, USA) for 6, 12, 24 and 48 h in αMEM medium (Gibco, USA) supplemented with 10% FBS.

Total RNA extraction and real-time fluorescence quantitative PCR analysis

The total cellular RNA from each incubation was isolated using TRIzol Reagent (Invitrogen, CA) according to the manufacturer's instructions. First-strand cDNA was then synthesized using a PrimeScript First Strand cDNA Synthesis Kit (Takara, China). A LightCycler DNA Master SYBR Green I kit (Roche, Germany) was used for the quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Light Cycler 96, Roche, Switzerland) in accordance with the manufacturer's protocols. The primers used in this study were as follows:

S100A4 forward 5′-TCAGAACTAAAGGAGCTGCTGACC-3′, reverse 5′-TTTCTTCCTGGGCTGCTTATCTGG-3′; osteocalcin (OCN) forward 5′-GGTGCAGCCTTTGTGTCCAAGC-3′, reverse 5′-GGCAAGGGGAAGAGGAAAGAAGG-3′; alkaline phosphatase (ALP) forward 5′-CATGAAATACGAGATCCACCGAGAC-3′, reverse 5′-ATGCGACCACCCTCCACGAAG-3′; matrix metalloproteinase-2 (MMP-2) forward 5′-TGATCTTGACCAGAATACCATCGA-3′, reverse 5′-GGCTTGCGAGGGAAGAAGTT-3′; matrix metalloproteinase-13 (MMP-13) forward 5′-TTGTTGCTGCGCATGAGTTCG-3′, reverse 5′-GGGTGCTCATATGCAGCATCA-3′; β-actin forward 5′-TGGCACCCAGCACAATGAA-3′, reverse 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′.

The relative mRNA expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The mean values were calculated from triplicate qRT-PCR reactions. The amount of the target gene was normalized to β-actin.

Protein isolation and Western blotting

The total proteins were extracted with RIPA Lysis Buffer and 1% PMSF (Beyotime, China) for 30 min on ice. Next, the cell lysates were centrifuged at 12000 ×*g* at 4°C, and the protein concentrations were determined with a BCA Protein Assay Kit (Thermo, USA). Forty micrograms of the cell proteins was denatured for 5 min at 100 °C and then loaded on a 15% SDS-PAGE supplemented with 8 mol/L urea for S100A4 detection and transferred onto a 0.22-μm PVDF membrane. After blocking for 30 min with 7% skim milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T), the membranes were incubated with an anti-human S100A4 antibody (1:1,000, Abcam, USA) or an anti-human β-actin antibody (1:1,000, Cell Signaling, USA) overnight at 4 °C followed by a 1-h incubation with the appropriate horseradish peroxidase (HRP)–conjugated IgG antibodies (Proteintech, USA). The protein expressions were detected by chemiluminescence using an Immobilon™ Western Chemilu-

minescent HRP Substrate (Millipore, USA), and the bands were then visualized with a SmartChemi™ Image Analysis System (Sagecreation, China). All immunoblotting experiments were repeated at least 3 times and produced similar results.

In vitro osteogenic differentiation assay

The hPDLCs were analyzed for their capacities to differentiate toward osteogenic lineages. The osteogenic induction began when the cells reached 70%-80% confluence. The osteogenic medium was α-MEM supplemented with 10% FBS, 100 nmol/L dexamethasone, 5 mmol/L β-glycerophosphate and 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St Louis, MO, USA) and was changed every 3 d. At d 12, the samples were subjected to alizarin red staining (ARS) (Sigma-Aldrich, St Louis, MO, USA), as reported previously[18]. To quantify the staining, the cultures were destained using 10% cetylpyridinium chloride (CPC) at pH 7.0 for 15 min at room temperature, and the ARS concentrations were then determined via absorbance measurements at 562 nm (Tecan Infinite M200, Austria).

Cell transfection

To investigate the function of S100A4, the hPDLCs were transfected with either overexpression plasmids for S100A4 (NM_002961) or short hairpin RNA (shRNA) (Genechem, China). The hPDLCs were divided into the following three groups: the first group was parental (no transfection), the second group was transfected with vector only or scrambled sequence plasmids, and the experimental group was transfected with either overexpression plasmids (NM_002961) or S100A4 shRNA plasmids (the target gene: CTGCTTTCCAGAAGCTGAT). The plasmids or vectors were added at 4 μg/well in 6-well Costar® plates.

The S100A4 shRNA sequences were as follows:

S100A4-RNAi-a: 5′-CCGGAGCTGCTTTCCAGAAGCTGATCTCGAGATCAGCTTCTGGAAAGCAGCTTTTTTG-3′; S100A4-RNAi-b: 5′-AATTCAAAAAAGCTGCTTTCCAGAAGCTGATCTCGAGATCAGCTTCTGGAAAGCAGCT-3′.

The vectors contained enhanced green fluorescent protein (EGFP) and the puromycin resistance cassette (GeneChem, China) and were transfected with Lipofectamine® 3000 (Invitrogen, USA) and then selected with 1 μg/mL puromycin for 2 weeks to obtain stably transduced cells.

Statistical analyses

The quantitative data and statistical analyses were processed with SPSS 19.0. The means and standard deviations (SD) are illustrated in the figures. For comparisons between groups, one-way analyses of variance (ANOVAs) were performed and followed with Dunnett's *post hoc* tests. The basal group (IL-1β=0 ng/mL) was used as a single control. $P<0.05$ was considered statistically significant.

Results

S100A4 was upregulated in the experimental periodontitis models

Experimental alveolar bone loss and inflamed gingiva with red edema signs were induced in the ligatured group. The presence of inflammatory cell infiltration, epithelial attachment loss and absorption of the lacuna were also observed in the experimental group. Additionally, the TRAP staining was remarkably different between the control (Figure 1A) and the experimental groups (Figure 1B), and the arrows in the figure indicate bone resorption lacuna caused by inflammation. Immunohistochemistry analysis revealed that S100A4 was dominantly expressed in the periodontal ligaments and blood and that the intensity of the staining was much stronger in the experimental periodontitis group (Figure 1D) than in the control group (Figure 1C). A greater number of S100A4-positive cells with staining in the cytoplasm and nucleus were observed in the experimental group than in the control group. In the immunofluorescence test, the S100A4 antibody revealed much stronger staining in the periodontal ligament in the experimental periodontitis group (Figure 1F) than in the control (Figure 1E); these results were similar to those of the immunohistochemistry analysis.

S100A4 levels are elevated in hPDLCs in response to IL-1β

To gain further insights into S100A4 regulation in the inflammatory milieu, we investigated the time and dose effects of IL-1β on S100A4 transcription. Cells were exposed to increasing doses of IL-1β for 6, 12, 24 and 48 h, and the S100A4 mRNA levels were then determined by RT-PCR. As shown in Figure 2A, the S100A4 mRNA levels were markedly increased following exposure to IL-1β in dose- and time-dependent manners within 24 h. However, the stimulatory effect of IL-1β on S100A4 transcription decreased slightly at the 48 h time point. The basal group (IL-1β=0 ng/mL) served as the single control for the multiple comparison procedure ($P<0.05$). Similarly, the S100A4 protein levels in the hPDLCs increased significantly compared with the levels of the basal group (Figure 2B).

S100A4 was negatively correlated with osteogenic differentiation and positively related to matrix degradation-related genes

The 12-day *in vitro* osteogenic induction assay revealed that the S100A4-RNAi cells formed obvious mineralized nodules (Figure 3B); however, the control group (no transfection) exhibited limited few calcified deposits (Figure 3A), and the S100A4-overexpressed group displayed very few calcified deposits in the cultures (Figure 3C). The *OD* values at the different ARS concentrations exhibited significant differences in each of the pairwise comparisons of the 3 groups ($P<0.05$, Figure 3D).

Following transfection with either the overexpression or shRNA plasmids, the S100A4 mRNA and protein levels were significantly altered (Figure 4B, 4D; the S100A4 mRNA changes are not shown). The mRNA expressions of the osteoblast phenotypic markers (*ie*, ALP and OCN) and matrix degradation markers (*ie*, MMP-2 and MMP-13) in the cultured hPDLCs were investigated 48 h after the plasmid transfections. The ALP and OCN mRNA expressions were markedly and significantly up-regulated following shRNA transfection compared to the parental and scrambled sequence groups

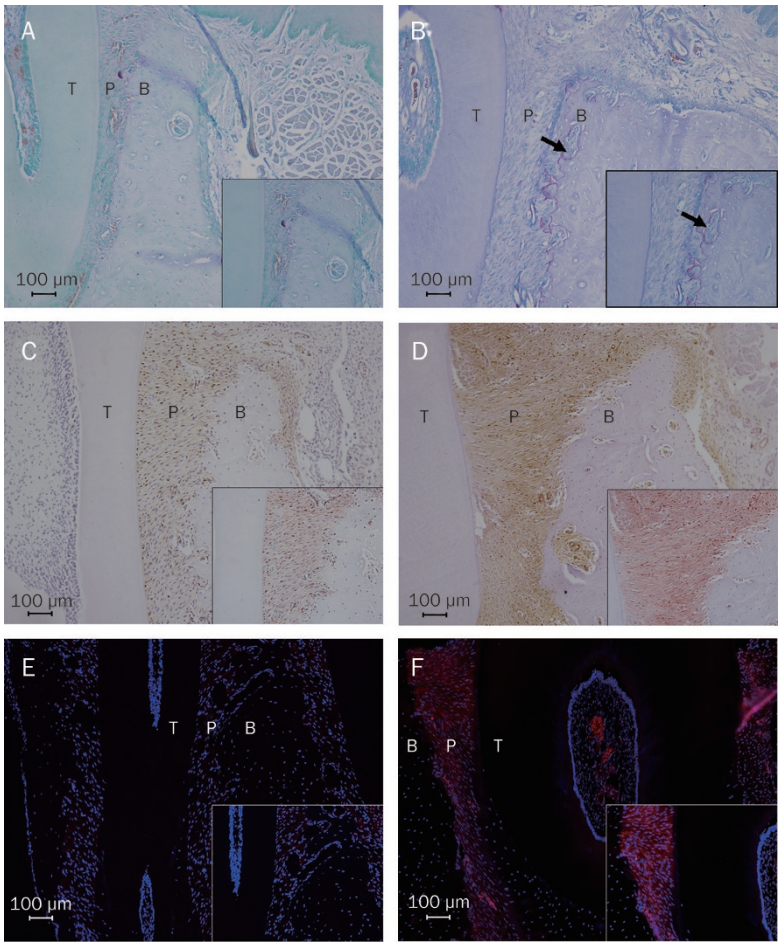

Figure 1. S100A4 upregulation in the experimental periodontitis models. The TRAP activity was higher in the experimental group (B) than in the control (A). The alveolar bone exhibited significant lacuna resorption (indicated by the arrows) and blunt crests in the experimental group in contrast to the thinner alveolar crests of the control (A, B). The S100A4 protein staining was intensely and evenly distributed in the periodontal ligaments of the experimental periodontitis group (D, F), whereas the control group exhibited many fewer positively stained cells without signs of inflammation (C, E). The images were magnified by 100×, and 200× magnifications are shown in the boxed areas. B, alveolar bone; T, tooth; P, periodontal ligament.

(P<0.01, Figure 4A). Moreover, the overexpression of S100A4 was associated with significant increases in MMP-2 and MMP-13 mRNA expressions (P<0.01, Figure 4C).

Discussion

To our knowledge, this paper is the first to illustrate that S100A4 can participate in the pathogenesis of periodontitis using both *in vitro* and *in vivo* models. We not only observed intense S100A4 staining in the cytoplasm and nuclei of the PDLCs in the 14-day tooth ligature-induced experimental rat periodontitis models but also discovered that the critical proinflammatory cytokine of periodontitis, *ie*, IL-1β, played a significant role in the regulation of S100A4 levels, further hindered hPDLC osteogenic differentiation and promoted the expressions of MMP-2 and MMP-13.

Although periodontitis is initiated and perpetuated predominantly by gram-negative, anaerobic bacteria and some pathogenic molecules, such as lipopolysaccharide (LPS), the presence of these bacteria or molecules is insufficient to cause the disease to occur or worsen. Indeed, host factors may even outweigh bacteria as determinants of whether the disease occurs and the severity of clinical outcome[19]. During the progress of periodontitis, periodontal ligament cells could contribute to tissue destruction via the significant upregulation of matrix metalloproteinases, which are functionally related to extracellular matrix degradation.

Periodontitis pathogens (*eg*, *A. actinomycetemcomitans* and *P. gingivalis*), as well as proinflammatory cytokines (IL-1α and IL-1β) and LPS can all upregulate MMP-2 levels in periodontal ligament cells[20-23]. MMP-13 is another important tissue destruction marker in periodontitis because chronic periodontitis is characterized by increased MMP-13 expression. During disease progression, MMP-13 production tends to increase in the active sites[24] and is promoted by TNF-α and LPS in human periodontal ligament cells[25, 26]. Several studies have demonstrated that MMPs production can be upregulated by S100A4[27-29], and these findings are consistent with those of the present study. The elevation of S100A4 in periodontitis may

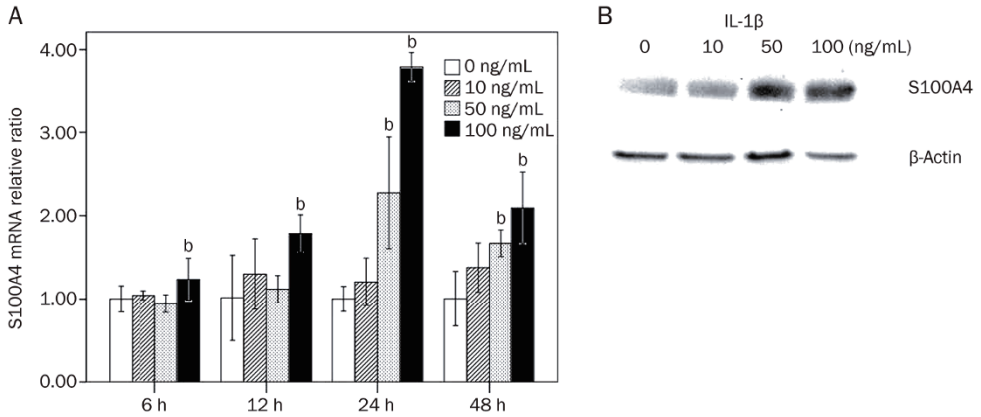

Figure 2. S100A4 levels were elevated in the hPDLCs in response to IL-1β. S100A4 mRNA levels were markedly increased following exposure to IL-1β in dose- and time-dependent manners within 24 h. However, the stimulatory effect of IL-1β on S100A4 transcription decreased slightly at the 48 h time point compared with that at the 24 h time point. $^{b}P<0.05$; the basal group (IL-1β=0 ng/mL) was the single control (A). Western blot analysis revealed that the S100A4 protein levels increased after 48 h of incubation with IL-1β (B).

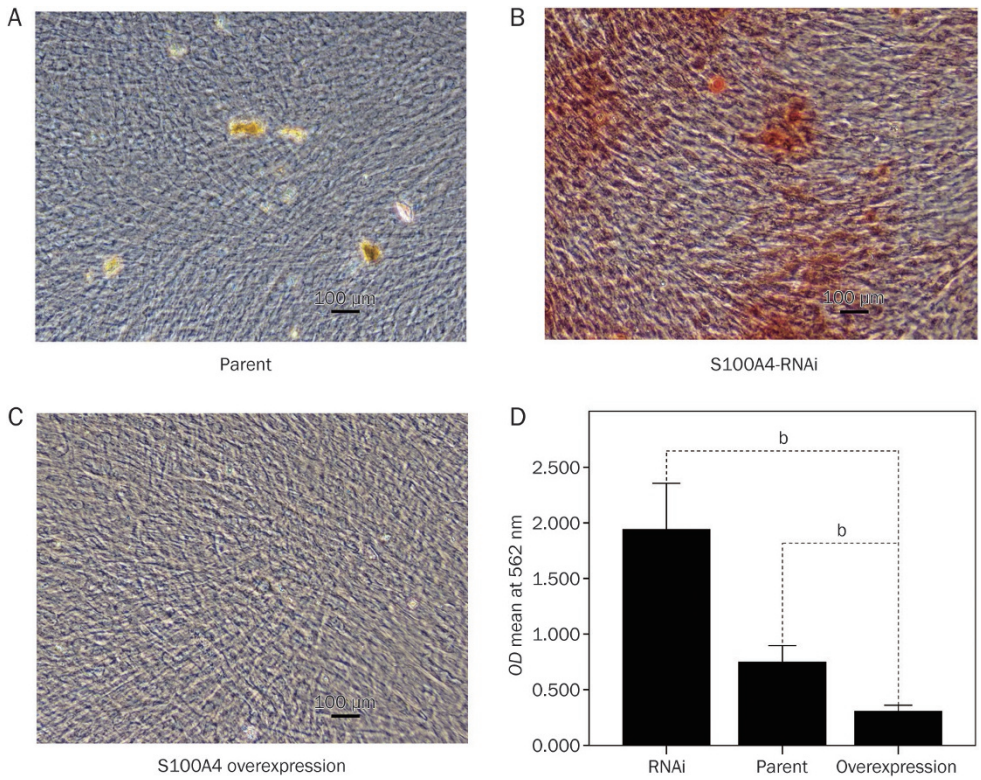

Figure 3. S100A4 was negatively correlated with mineralized nodule formation. The control group (no transfection) exhibited limited calcified deposits in the 12-d osteogenic induction assays (A), whereas the S100A4-RNAi cells formed obvious mineralized nodules (B). However, the overexpression of S100A4 resulted in very few calcified deposits in the culture (C). The *OD* values for the ARS concentrations indicated significant differences in each of the pairwise comparisons of the 3 ($^{b}P<0.05$) (D).

be the consequence of the upregulations of MMP-2 and MMP-13, which further promote tissue destruction.

Several studies have reported the depressed osteogenic potentials of periodontal ligament cells in the inflammatory milieu[2, 3, 30], which may be the reason for the compromised alveolar bone repair in periodontitis. As a negative regulator of mineralization, S100A4 can suppresses the expression of osteogenic genes in periodontal ligament cells and inhibit the mineralization of the periodontal ligament[9, 10, 13]. Moreover, it has been reported that the inhibition of S100A4 by siRNA results in the increased expression of osteoblastic markers, such as osteopontin and osteocalcin, and the osteoblast-spe-

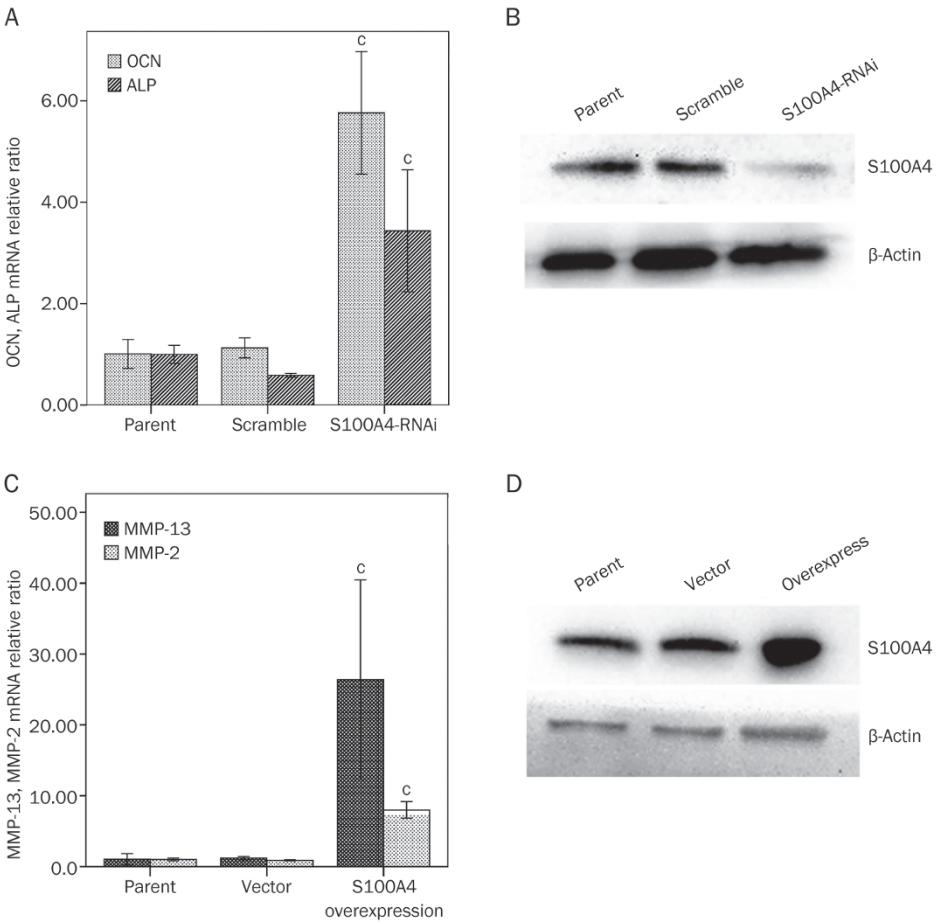

Figure 4. S100A4 was negatively correlated with osteogenic gene expression and positively related to matrix degradation gene expression. The S100A4-RNAi cells exhibited significantly decreased S100A4 protein levels and significantly increased ALP and OCN mRNA expression levels compared with the parental and scrambled sequence groups (A, B). The overexpression of S100A4 elicited increases in S100A4 protein levels and significant elevations in MMP2 and MMP13 mRNA expressions (C, D). $^{c}P<0.01$.

cific transcription factors Runx2/Cbfa1 and osterix[10]. Based on the above findings, we deduce that the impaired osteogenic differentiation abilities of periodontal ligament cells may be a consequence of S100A4 elevation in periodontitis.

In conclusion, the results of this study that S100A4 is upregulated in experimental periodontitis models and by IL-1β stimulation and that S100A4 further contributes the pathogenesis of periodontitis. S100A4 altered the biologic behavior of periodontal ligament cells via the downregulation of osteogenesis-related mRNAs (*ie*, ALP and OCN) and induced the expression of matrix degradation genes (*ie*, MMP-2 and MMP-13). However, the molecular mechanisms by which S100A4 inhibits mineralized nodule formation and promotes MMP gene expression remain unclear. In the future, drugs targeting S100A4 expression may represent a novel potential therapeutic approach against periodontitis.

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Author contribution

Min ZHOU performed the majority of the experiments, analyzed the data and drafted the manuscript; Zhuo-quan LI assisted in the experiments; Min ZHOU and Zuo-lin WANG designed the study and reviewed the manuscript.

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