Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 246–254
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JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01227.x

Localization of *SOST*/sclerostin in cementocytes *in vivo* and in mineralizing periodontal ligament cells *in vitro*

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Jäger A, Götz W, Lossdörfer S, Rath-Deschner B. Localization of SOST/sclerostin in cementocytes in vivo *and in mineralizing periodontal ligament cells* in vitro. *J Periodont Res 2010; 45: 246–254. © 2009 John Wiley & Sons A/S*

Background and Objective: Cementum and bone are rather similar hard tissues, and osteocytes and cementocytes, together with their canalicular network, share many morphological and cell biological characteristics. However, there is no clear evidence that cementocytes have a function in tissue homeostasis of cementum comparable to that of osteocytes in bone. Recent studies have established an important role for the secreted glycoprotein sclerostin, the product of the *SOST* gene, as an osteocyte-derived signal to control bone remodelling. In this study, we investigated the expression of sclerostin in cementocytes *in vivo* as well as the expression of *SOST* and sclerostin in periodontal ligament cell cultures following induction of mineralization.

Material and Method: Immunolocalization of sclerostin was performed in decalcified histological sections of mouse and human teeth and alveolar bone. Additionally, periodontal ligament cells from human donors were cultured in osteogenic conditions, namely in the presence of dexamethasone, ascorbic acid and β-glycerophosphate, for up to 3 wk. The induction of calcified nodules was visualized by von Kossa stain. *SOST* mRNA was detected by real-time PCR, and the presence of sclerostin was verified using immunohistochemistry and western blots.

Results: Expression of sclerostin was demonstrated in osteocytes of mouse and human alveolar bone. Distinct immunolocalization in the cementocytes was shown. In periodontal ligament cultures, following mineralization treatment, increasing levels of *SOST* mRNA as well as of sclerostin protein could be verified.

Conclusion: The identification of SOST/sclerostin in cementocytes and mineralizing periodontal ligament cells adds to our understanding of the biology of the periodontium, but the functional meaning of these findings can only be unravelled after additional *in vitro* and *in vivo* studies.

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Key words: human cementocyte; human periodontal ligament cell; *SOST gene*; sclerostin; *in vitro*; *in vivo*

Accepted for publication March 30, 2009

The main function of tooth cementum is to ensure that the tooth is in its functional position within the alveolar bone by providing attachment to collagen fibres of the periodontal ligament. In addition, it is known to be involved in tooth repair and regeneration. Cementum and bone share some structural features. but cementum does

not have the lamellar organization found in bone, is avascular in most species studied, is non-innervated, does not contain bone marrow and does not undergo physiological remodelling (1). Compared with osteocytes in bone, cementocytes are more widely dispersed and more randomly arranged. There is a lower canalicular density in the cellular cementum than in bone, and areas devoid of canaliculi are found (2). Although the canalicular system transporting tissue fluid into the deep regions seems to be well developed, the deep cementocytes have less endocytotic ability than those close to the surface (3). Furthermore, the canaliculi are preferentially oriented towards the periodontal ligament, their chief source of nutrition.

For proper functioning of bone, a system consisting of osteoblasts, osteocytes, bone-lining cells and osteoclasts is required. Via their dendritic processes, osteocytes, which make up more than 90% of all bone cells, communicate with each other but also with cells on the bone surface and in the bone marrow. For example, mechanosensing in bone is thought to be primarily co-ordinated by osteocytes, and the signals resulting from bone loading are translated into osteoclast- and osteoblast-recruiting signals (4–6).

Among the proteins by which osteocytes possibly influence the function and number of the cells responsible for remodelling, recent studies point out the important role of sclerostin, which is the product of the *Sost* gene (7–10). The amino acid sequence of sclerostin shares similarity with the cystine knot, containing factors belonging to the differential screening-selected gene aberrative in neuroblastoma family of secreted glycoproteins (11).

Only osteocytes and no other cells in bone express sclerostin. High levels of sclerostin were detected in their lacunae and canaliculi system. Sclerostin has been demonstrated to antagonize several members of the bone morphogenetic protein (BMP) family (7–10) and also binds to lipoprotein receptor related protein 5/lipoprotein receptor related protein 6, preventing canonical Wnt (combination of Wg gor wingless and Int-1) signalling (12,13). Both BMPs and Wnts are critical for osteoblastogenesis because they provide the initial and essential stimulus for commitment of multipotential mesenchymal progenitors to the osteoblast lineage (14–16). Loss of SOST in humans causes the high bone mass disorders of van Buchem's disease (17) and sclerosteosis (18). In addition, administration of an antisclerostin antibody increases bone formation and restores the bone loss upon ovariectomy in rodents (19). Conversely, transgenic mice overexpressing *Sost* exhibit low bone mass (17).

Thus, one way for osteocytes to regulate bone remodelling would be by altering the secretion of sclerostin. Accordingly, it was shown that mechanical stimulation *in vivo* reduced the expression of sclerostin by osteocytes (20). Also, it is possible that osteocyte death is a signal for bone formation because the level of sclerostin would decrease. Sevetson *et al.* (21) demonstrated that the osteoblast differentiation factor Cbfa1/Runx2 increases SOST expression. Up to now, there is some disagreement on the effect of parathyroid hormone (PTH) on SOST expression. One study (22) showed that intermittent PTH treatment reduced SOST expression. In contrast, Bellido *et al.* (23,24) found that PTH given intermittently to mice did not alter the levels of SOST, but PTH given continuously did decrease SOST expression.

In contrast to the situation in bone, it is widely held that cementum functions without such a complex cell system. According to a recent review by Bosshardt (25), there has been no clear evidence that cementocytes have a comparable function in tissue homeostasis of cementum. However, signals from the adjacent periodontal ligament probably influence cementocyte function and vice versa. As an example, following compressive forces, Kagayama *et al.* (26) observed increased expression of chondroitin 6-sulphate in the lacunae and canaliculi of osteocytes as well as of cementocytes of cellular cementum. In addition, both osteocytes and cementocytes showed an intense signal for mRNA of matrix metalloproteinase-1 in response to orthodontic tooth movement (27).

In this study, for the first time, immunolocalization of sclerostin in mouse and human cementocytes is presented. In addition, we identified an increase in the expression of the mRNA and the translation of the protein by human periodontal ligament cells in the course of *in vitro* mineralization.

Material and methods

Tissues for histology

Clinically healthy premolars and molars were obtained from five patients aged between 11 and 60 years. Following extraction and with written informed consent, the teeth were halved sagittally for better fixation and decalcification. Additionally, paraffin blocks of decalcified edentulous mandibles of three patients aged between 50 and 76 years resected due to oral squamous carcinoma at the Department of Oral, Maxillofacial and Plastic Surgery were provided by the Institute of Pathology, both at the University of Bonn (Germany).

Five male NMRI mice (Swiss-type mouse; Charles River Laboratories International, Hannover, Germany), aged 11 mo, were killed by cervical dislocation after induction of general anaesthesia with ether, and the upper jaws dissected and isolated. Teeth and jaws were fixed by immersion in 4% buffered formaldehyde and then decalcified in disodium ethylene-diamino-tetraacetic acid (EDTA) solution. After hydration, tissues were dehydrated in an ascending series of ethanol and embedded in paraffin, with the pulp facing the cutting surface for human teeth and in the sagittal direction for mouse jaws. Serial sagittal sections of 6–8 μm thickness were cut on a microtome (HM 355s; Microm Int., Walldorf, Germany), and selected sections showing root cementum for human teeth and the roots of the first maxillary molar for rodent jaws were stained with haematoxylin and eosin. Sections of the human mandibles were cut in the sagittal direction and also stained with haematoxylin and eosin for orientation purposes. Only sections free of tumour were used.

Cell culture and differentiation of periodontal ligament cells in a mineralizing medium

Human periodontal ligament cells were obtained from premolars of three donors between 12 and 15 years of age. Approval was obtained from the Ethics Committee of the University of Bonn before experiments. The teeth had to be extracted for orthodontic reasons and were diagnosed to be free of caries and periodontal disease. Periodontal ligament cells were harvested only from the middle third of the root surface to avoid contamination by gingival cells. Cells were obtained by the explant-outgrowth technique and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin: diluted from a stock solution containing 5000 U/mL penicillin and 5000 U/mL streptomycin; (GIBCO, Grand Island, NY, USA), in a humidified atmosphere of 5% CO_2 in air. Periodontal ligament cells were used between the third and fourth passage after documentation that they expressed typical markers, such as alkaline phosphatases, osteocalcin and collagen type I, using RT-PCR (28; data not shown).

At the onset of the experiments, the cells were grown in the growing medium noted above until reaching about 80% confluency for 5 d. Thereafter, to induce differentiation, the cells were grown in a mineralizing medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin: diluted from a stock solution containing 5000 U/mL penicillin and 5000 U/mL streptomycin, 50 mg/mL ascorbic acid, 10 mM sodium β-glycerophosphate and 5 μM dexamethasone; all from Sigma-Aldrich (Hamburg, Germany); 'differentiation treatment'). Once experiments had started, medium was changed every third day for up to 3 wk. Results were obtained on day 0 and consecutively after 1 and 3 wk.

von Kossa stain

Mineralization activity of the cell cultures was analysed using the von Kossa stain. After coverslips had been added to the culture dishes, cells were seeded. At the end of the experiments, the medium was removed, and cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min. After being washed with distilled water, cells were incubated with 5% silver nitrate at 4°C for 40 min. Subsequently, cells were washed again, and 1% pyrogallole solution was added at room temperature for 5 min. After removal of the pyrogallole solution and a washing, cells were fixed in a 5% sodium sulphate solution. Finally, cells were washed and counterstained with a 0.1% nuclear fast red solution for 10 min, dehydrated with 100% ethanol and cleared with xylene.

Immunohistochemistry and immunocytochemistry

Selected sections were deparaffinized, rehydrated and rinsed for 10 min in Tris-buffered saline (TBS). Endogenous peroxidase was blocked in a methanol–H_2O_2 solution (Merck, Darmstadt, Germany) for 10 min in the dark. After having been rinsed, sections were pretreated with PBS containing 1% bovine serum albumin (BSA) for 20 min at room temperature and digested with 0.4% pepsin for 30 min at 37°C. Following further rinsings, sections were incubated with the primary antibodies in a humid chamber. Human teeth and mandibles were incubated with anti-human mouse monoclonal SOST antibody (R&D Systems, Wiesbaden, Germany) diluted 1:25 in 1% TBS–BSA for 1 h at 37°C, rodent jaws with anti-mouse goat polyclonal SOST antibody (R&D Systems) diluted 1:50 in 1% TBS–BSA at 4°C overnight. The specificities of the antibodies were given by blots and data supplied by the company. For the detection of the monoclonal SOST antibody binding, sections were washed three times for 10 min in TBS and incubated with the EnVision™ peroxidase anti-mouse system (Dako-Cytomation, Hamburg, Germany) for 30 min at room temperature. After a rinse, peroxidase activity was visualized with diaminobenzidine (Pierce, Rockford, IL, USA) yielding a brown staining product. Polyclonal goat Sost antibody binding was detected using a peroxidase-conjugated anti-goat secondary antibody (DakoCytomation) diluted 1:50 in 1% TBS–BSA for 30 min at room temperature following diaminobenzidine visualization. After immunohistochemistry, all slides were rinsed and then counterstained with Mayer's haematoxylin, dehydrated and coverslipped for microscopic analysis.

The specificity of the immunohistochemical reactions was assessed by the following: (1) replacement of the primary antibody with buffer; (2) its substitution with non-immune mouse or goat immunoglobulin G (1:10 dilution); (3) incubation with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity; and (4) preadsorption tests involving excess of the corresponding antigens (Human SOST; R&D Systems; 15 μg/mL). Complete lack of detected staining in the control sections demonstrated the specificity of the reactions Fig. 1C.

In the cell culture experiments, sclerostin protein synthesis was also documented by immunocytochemistry. Coverslips were added to the culture dishes and, subsequently, cells were seeded. At the end of the experiments, the periodontal ligament cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.05% Triton X-100, washed, and blocked with 2% goat serum for 2 h. Cells were incubated with a mouse monoclonal anti-human SOST antibody (R&D Systems) for 4 h at 37°C. For detection, a horseradish peroxidase-conjugated anti-mouse immunoglobulin G (DAKO, Glostrup, Denmark) was applied for 1 h. Thereafter, cells were stained with diaminobenzidine for 5 min. After having been washed with PBS, nuclei were counterstained with haemalum solution. Finally, cells were dehydrated with 100% ethanol and cleared with xylene. Images were captured with a Zeiss microscope (Axiophot 2, Zeiss, Goettingen, Germany). The control method for specificity of the immunohistochemical reactions was performed as described above (Fig. 2D).

Real-time PCR

In additional cell culture experiments, mRNA expression for SOST and

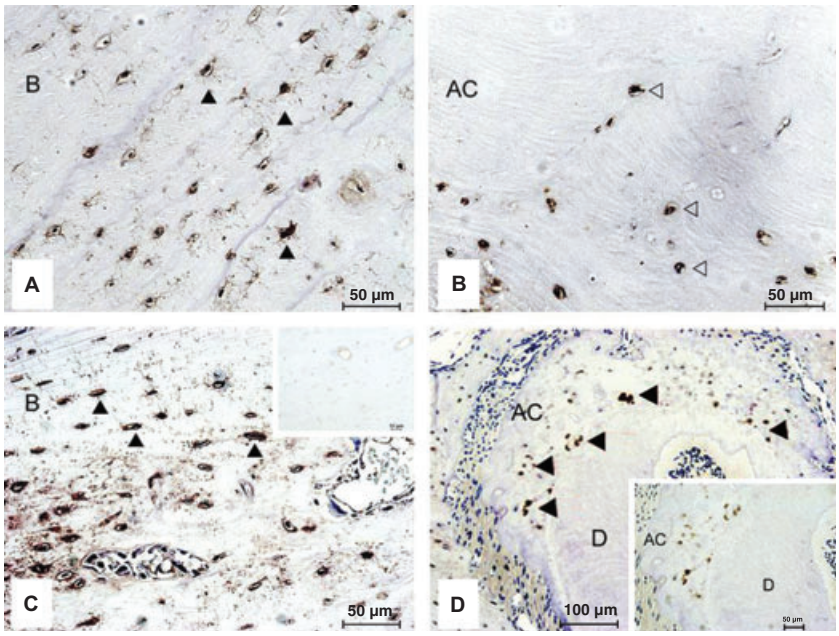

Fig. 1. Expression of the sclerostin protein by osteocytes in alveolar bone (A and C) and cementocytes in cellular cementum (B and D) determined by immunohistochemistry in human (A and B) and mouse tissues (C and D). Serial sections were stained with anti-human mouse monoclonal SOST antibody (R&D Systems; A and B) and anti-mouse goat polyclonal SOST antibody (R&D Systems; C and D). The inset in (C) depicts a negative control experiment in which an isotype-matched immunoglobulin G was substituted for the primary antibody in a section of alveolar bone. The presented images are representative of those obtained from different human and mouse material. In the alveolar bone (A and C), canaliculi and/or lacunae of osteocytes are positive for sclerostin in human and mouse samples (arrowheads). In the cementum (B and D), cementocytes stained positively (arrowheads), canaliculi were hardly observed. Sclerostin expression was not found in osteoblasts, cementoblasts or periodontal ligament cells.

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed by real-time RT-PCR. The RNA was extracted according to the manufacturer's recommended protocols with an RNA extraction kit (Qiagen, Valencia, CA, USA). A total of 1.0 μg RNA was reverse transcribed with 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 42°C for 50 min followed by 70°C for 15 min. Quantification of the gene expression was performed using a LightCycler detection system (Roche, Basel, Switzerland). The PCR was performed using a QuantiTect primer assay (QT00219968, Entrez Gene identification 50964, amplicon length 96 bp) and a QuantiTect SYBR Green PCR Kit (Qiagen). Each PCR reaction was carried out in 20 μL mixture containing 2 μL of cDNA,10 μL of 2× QuantiTect SYBR Green PCR Master Mix, 500 nM of each primer and deionized water. After an initial step at 95°C for 15 min to activate HotStarTaq DNA polymerase, there followed 40 cycles with denaturation at 94°C for 15 s, annealing at 50–60°C for 30 s and extension at 72°C for 30 s. The GAPDH was used as a housekeeping gene. To analyse the data, the comparative threshold cycle (CT) method was applied (29). The SOST mRNA expression was measured on day 0 and after 3 wk, three wells per donor being analysed at each time point. Statistical comparison of the two time points was performed using (SPSS software; SPSS Inc., Chicago, IL, USA). The experiment was repeated and revealed similar results. The results of one representative experiment are presented (Fig. 3A).

Western blots

In addition, synthesis of the sclerostin protein by the periodontal ligament cells following differentiation treatment was determined by western blot analysis on day 0 and after 1, 2 and 3 wk. Proteins were extracted and resolved on SDS–10% PAGE in reducing conditions. Following electrophoresis, proteins were electrotransferred to nitrocellulose membranes, blocked with 5% non-fat milk and probed with the 'mouse anti-human monoclonal antibody' (R&D Systems). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (DAKO) and Lightening Chemiluminescence reagent (Pierce) were applied for detection. A mouse monoclonal anti-β-actin antibody (ABCAM, Cambridge, MA, USA) was used for standardization. For the calibration of the size of the molecular mass of the proteins after SDS–PAGE, a standard from Bio-Rad (Bio-Rad Precision Plus Protein Kaleidoscope Standards no. 161-0375; Hercules, CA, USA) was applied. The western blots from the three donors revealed nearly identical results, and the results of one representative experiment are presented.

For the specificity control, decreasing amounts of the antibody were applied. In addition, a peptide neutralization assay using a mixture of the antibody and the corresponding antigen (Human SOST protein; R&D Systems) showed disappearance of the ~26 kDa bands in the blots (Fig. 3B).

Results

Immunohistochemical detection of the SOST protein in human and mouse alveolar bone and cellular cementum

In mouse and human lamellar alveolar bone, osteocytes were the only cell type expressing sclerostin. Osteoblasts and lining cells as well as osteoclasts were consistently negative (Fig. 1A,C). Images captured at higher power demonstrated the location of sclerostin in canaliculi and lacunar walls.

Immunohistochemical staining of cellular cementum using the same antisclerostin antibodies revealed exclusive expression of the protein in the lacunae of cementocytes. This held true for the specimens obtained from humans as well as from mice (Fig. 1B,D).

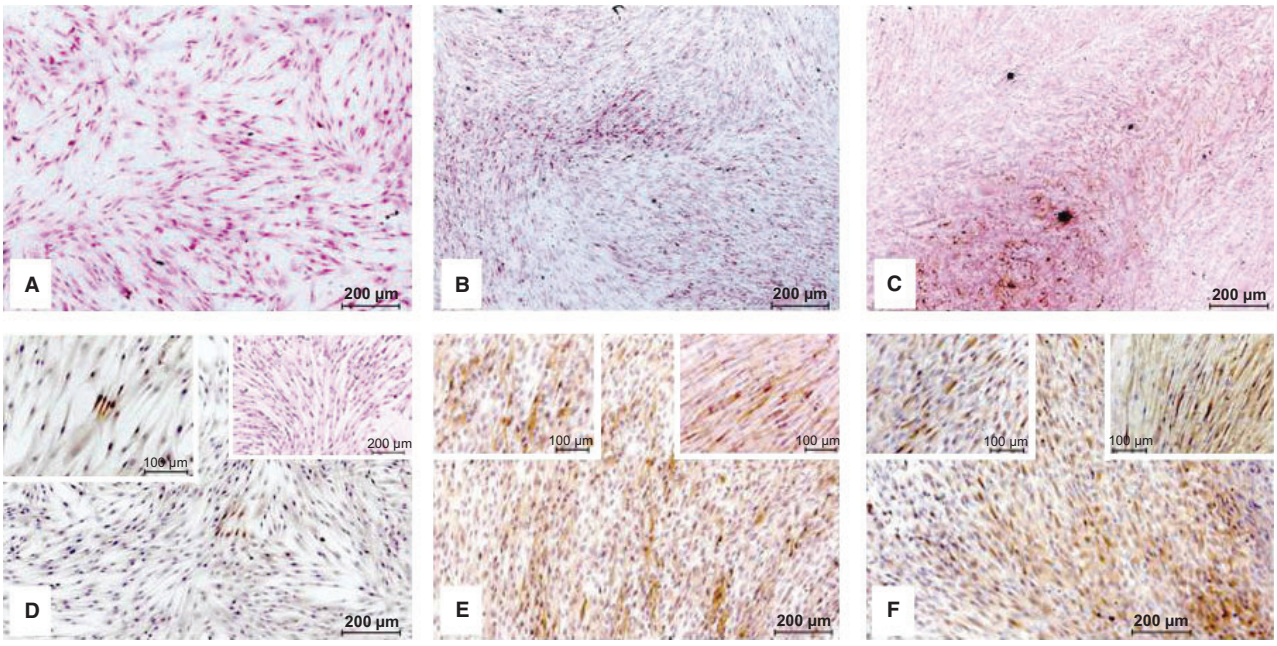

Fig. 2. Documentation of mineral deposits using von Kossa staining (A–C) and immunocytochemical detection of sclerostin using a mouse anti-human monoclonal antibody (R&D Systems; D–F) in periodontal ligament cultures. Periodontal ligament cells from different human donors were cultured in osteogenic conditions, namely with dexamethasone, ascorbic acid and β-glycerophosphate for up to 3 wk. The results on day 0 (A and D) and those after 1 (B and E) and 3 wk (C and F) are presented. The experiments were performed with cell cultures of three different donors and revealed comparable results. The results of one representative experiment are presented. In (D), the inset on the right represents the result of a control experiment using substitution of the antibody with non-immune mouse immunoglobulin G. After 1 wk, mineralization was still negligible within the culture using von Kossa staining (B). An increasing level of mineralization was observed after 3 wk (C). At the same time, the number of cells expressing the sclerostin protein increased significantly after 1 wk (E) and protein expression was even more obvious and concentrated in the regions of the nodules after 3 wk (F).

Canaliculi connectin neighbouring cementocytes and containing sclerostin could hardly be found. Cementoblasts covering the surface of cementum as well as the cells of the periodontal ligament were always negative in the immunoreaction.

No specific staining was seen in the negative control sections, where non-immune mouse immunoglobulin G was used instead of the primary antisclerostin antibodies, either for alveolar bone or for cellular cementum. In addition, no obvious difference in sclerostin expression in the different specimens under investigation was observed.

SOST mRNA and protein expression during periodontal ligament cell differentiation *in vitro*: immunocytochemistry and von Kossa stain

Within the 3 wk of the experiment, the periodontal ligament cell cultures showed the morphologically distinct stages well known from other *in vitro* mineralization studies. On day 0, before the start of the induction of differentiation, periodontal ligament cells formed a monolayer and showed a typical fibroblast-like polygonal elongated shape and, using von Kossa staining, no mineralized deposits were visible (Fig. 2A). At the same time, only single cells presented with positive cytoplasmatic reaction with the anti-SOST antibody (Fig. 2D). After 1 wk, the cell cultures had reached a multilayer stage. Cell proliferation started to concentrate in focal areas, forming distinct clusters of cells with a fibroblast shape but, at this time, von Kossa staining was still only very slightly positive (Fig. 2B). In contrast, within the cell clusters, increased reactivity with the anti-SOST antibody became obvious (Fig. 2E). After 3 wk, the cells appeared to be even more densely packed, and the cultures exhibited many distinct clusters of varying size and shape. Within these clusters, von Kossa staining revealed mineralized deposits presenting as brown or black amorphous precipitates (Fig. 2C). Worthy of note, even throughout the nodules, the cells maintained their fibroblastic elongated shape although, in contrast, many of these cells demonstrated intense staining with the anti-SOST antibody (Fig. 2F).

Real-time PCR

The results of the comparison of SOST mRNA expression measured on day 0 and after 3 wk of 'differentiation treatment' is presented in Fig. 3A. There was a significant, on average threefold, increase after 3 wk.

Western blots

The western blot analyses of protein extracts prepared from the periodontal ligament cultures on day 0 and after 1, 2 and 3 wk using the 'mouse anti-human monoclonal antibody' are presented in Fig. 3C. A continuous increase of the sclerostin protein from day 0 to 3 wk of 'differentiation treatment' is obvious.

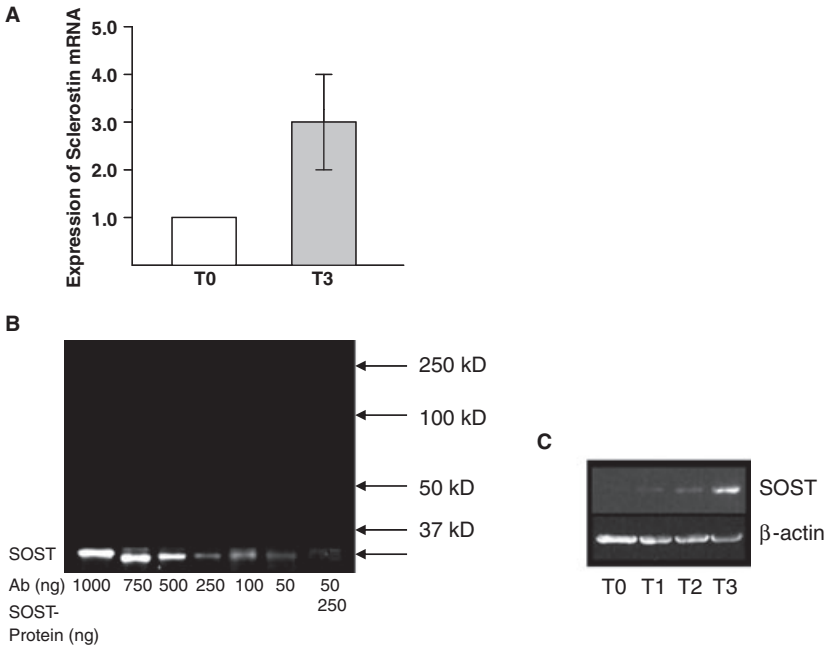

Fig. 3. Real-time PCR and western blot analyses of the expression of sclerostin in human periodontal ligament cell cultures. Cells were cultured in osteogenic conditions, namely with dexamethasone, ascorbic acid and β-glycerophosphate for up to 3 wk. The experiments were performed with cell cultures of three different donors. (A) SOST mRNA expression measured on day 0 (T0) and after 3 wk (T3), three wells per donor being analysed at each time point. GAPDH was used as house-keeping gene. For analysis of the data, the comparative threshold cycle (CT) method was applied. The experiment was repeated and revealed similar results. The results of one representative experiment are presented. The SOST mRNA expression was significantly increased after 3 wk. (B) Peptide neutralization assay for specificity control of the SOST antibody (R&D Systems). Proteins were electrophoretically transferred to nitrocellulose membranes and probed with different concentrations of the antibody against SOST (lanes 1–6) and an antibody–SOST–protein mixture (1:5; lane 7). For the calibration of the size of the molecular mass of the proteins after SDS–PAGE, a standard from Bio-Rad (Bio-Rad Precision Plus Protein Kaleidoscope Standards no. 161-0375) was applied. After neutralization of the SOST antibody with the peptide, the specific band disappeared. (C) Western blot analyses of protein extracts prepared from the periodontal ligament cultures using the 'mouse anti-human monoclonal antibody' (R&D Systems) are presented on day 0 (T0) and after 1 (T1), 2 (T2) and 3 wk (T3). A mouse monoclonal anti-β-actin antibody (ABCAM) was used for standardization. The western blots from the three donors revealed nearly identical results, and the results of one representative experiment are presented. There was a continuous increase in the SOST protein from day 0 to 3 wk of 'differentiation treatment'.

Discussion

Immunohistochemical detection of the SOST protein in human and mouse alveolar bone and cellular cementum

In the first part of this study, using immunohistochemistry, the expression of sclerostin, the product of the SOST gene, was demonstrated in osteocytes of human and mouse alveolar bone. In addition, evidence of the deposition of the protein in cementocytes could be shown for the first time.

Owing to the critical role of dental cementum in physiology and pathology of the periodontium, there is great interest in the biology of cementum. Although cementum is considered to share many characteristics with bone, the two hard tissues deviate in several important aspects. While bone is richly vascularized, cementum is avascular and physiologically shows no obvious remodelling activity (1). In addition, the cementocytes with their cytoplasmic processes inside the canaliculi are distributed less densely than the osteocytes (25).

Recent literature assumes that sclerostin is expressed almost exclusively in osteocytes (7,30–32). Osteocytes in mineralized cortical and cancellous bone were found positive for sclerostin, with positive staining also along the cytoplasmatic processes in the osteocyte canaliculi (31). In our immunohistochemical studies, the morphological results for cementum resembled in many respects those of alveolar bone. As for the osteocytes, we demonstrated expression of the sclerostin protein in the lacunae of cementocytes from humans as well as from mice. At the same time, cementoblasts covering the surface of cementum and the cells of the periodontal ligament were always negative for the immunoreaction.

The detection of the sclerostin within the cementocytes might give rise to speculation about the functional importance of this finding but, up to now, in contrast to the increasing evidence for a major role of the osteocytes in bone biology, there is no clear-cut evidence of a comparable role of cementocytes in cementum. Like osteocytes, cementocytes express the markers CD44 (33) and E11/gp38 (34) and, in addition, different growth factors (e.g. insulin-like growth factors, fibroblast growth factors and epidermal growth factor) and prostaglandines were found (35).

For a possible effect on cementum homeostasis, the sclerostin which is produced by cementocytes would have to be transported to the periodontal space. Kagayama *et al.* (2) using confocal microscopy demonstrated that the cell processes of cementocytes branch and anastomose with those of neighbouring cells. Gap junctions have been shown to be present between the cytoplasmic processes of adjacent cementocytes (36). Using microperoxidase as a tracer, Ayasaka *et al.* (3) found that cementum fluid diffuses from capillaries in the periodontal ligament to the deep cementum but the circulation is more irregular than that of bone fluid. As in bone, the

cementum fluid is localized in the pericellular spaces of the lacunae and canaliculi, and the pericellular spaces are the transport pathway of this fluid.

Before the further implications of our results can be thought about, additional functional analysis of a possible role of sclerostin in cementum and/or periodontium physiology and pathology has to be performed. Studies on sclerostin knockout mice or on transgenic mice overexpressing the protein, as well as clinical studies, should be of help in analysing possible diseases of the periodontium in patients with van Buchem's disease or sclerosteosis.

SOST mRNA and protein expression during periodontal ligament cell differentiation *in vitro*

Within human adult iliac bone, newly embedded osteocytes were negative for sclerostin staining but became positive after mineralization (31). According to Irie *et al*. (37), mineralization of the matrix is the trigger for cytodifferentiation of the mature osteocyte and, at the same time, the osteocyte starts to secrete sclerostin in the mineralized bone matrix only after maturation. Thus, to study the differentiation-dependent expression of the gene in the cells of the periodontium, in the second part of this study we applied differentiation treatment of human periodontal ligament cell cultures using ascorbic acid, sodium β-glycerophosphate and dexamethasone.

As a result, we observed the repeatedly described time-dependent developmental changes with the following stages: (1) a confluent stage; (2) a multilayer stage; (3) a nodule formation stage; and (4) finally, after 3 wk, a mineralization stage which could be verified by von Kossa staining. Using immunohistochemistry, the number of sclerostin-positive cells was shown to increase over the duration of the experiment. The cells of the periodontal ligament consist of fibroblastic subpopulations, osteoblasts, cementoblasts, endothelial cells, perivascular cells and epithelial cells. In addition, the periodontal ligament contains progenitor cells that can differentiate into the above-mentioned specialized cell types (25). Lallier & Spencer (38) found out that cells isolated from the periodontal ligament and grown in culture were different from those that populate the ligament *in vivo*. The cells in culture expressed decreased levels of the proteins associated with mature mineralized tissue (alkaline phosphatase, osteopontin and bone sialoprotein 2 (BSP-2). In contrast, these cells expressed more of the factors that inhibit differentiation and promote proliferation (follistatin, gremlin, stanniocalcin-2 and fibroblast growth factor 5) and more of the bone marrow stromal stem cells marker. Thus, the authors concluded that periodontal ligament cells grown in culture represent an immature form of periodontal ligament fibroblasts, possibly even a progenitor cell population.

In vitro, periodontal ligament cells have been shown to possess osteoblast-like properties (28,39,40) as well as the capacity to form mineralized nodules when treated with ascorbic acid, sodium β-glycerophosphate and dexamethasone (28,41–43). In their studies with rat periodontal ligament cells, Lin *et al*. (44) found that the expression of mRNA for osteopontin was induced at the multilayer stage while mRNA for BSP, alkaline phosphatase and Osteocalcin were induced at the nodule-formation and mineralization stages. In addition, the nodules produced by periodontal ligament cells were shown to be morphologically different from those formed by osteoblastic cells (45,46). In the nodules formed by rat periodontal ligament cells, fibroblastic cells were the only cell type identified, and no differentiation, either into osteoblasts or osteocytes, was observed. Thus, it was suggested that periodontal ligament cells may be different from osteogenic cells and that the nodules formed by periodontal ligament cells may be cementum-like nodules rather than bone nodules (42,47). To our knowledge, up to now an involvement of sclerostin in the differentiation and mineralization process of periodontal ligament cells has not been studied.

Using real-time PCR and the western blot technique, we were able to demonstrate increasing levels of SOST at the mRNA level as well as of sclerostin at the protein level following differentiation treatment of human periodontal ligament cells with MSCs and dexamethasone.

Van Bezooijen *et al*. (8) studied the onset of SOST mRNA expression during osteoblastic differentiation of mouse and human bone marrow-derived stemcell and of mouse preosteoblastic KS483 cells. All these cells differentiated into mature osteoblasts and formed a mineralized matrix when cultured in osteogenic conditions, including ascorbic acid and sodium β-glycerophosphate and, in the case of human specific cell surface antigen-1, also dexamethasone. Mouse MSCs formed alkaline phosphatase-positive bone nodules after 7 d in culture, and these nodules mineralized starting on day 12. The authors analysed SOST mRNA expression in relation to the osteoblast differentiation marker osteocalcin. In the mouse MSCs, at day 0, neither osteocalcin nor SOST mRNA were expressed. Induction of osteocalcin mRNA expression was seen on day 11 and increased further after day 14. SOST mRNA expression was not observed until the onset of osteocalcin mRNA expression and was found from day 14 onwards. Likewise, the preosteoblastic KS483 cells formed alkaline phosphatase-positive bone nodules during the first week of culture. Mineralization started on day 11, and osteocalcin mRNA expression was observed first on day 10 of culture and was further elevated from day 17 onwards. Again, for these cells, SOST mRNA expression was induced only after the onset of osteocalcin mRNA expression and was found from day 17 onwards. Interestingly, the human MSCs did not form real bone nodules, but a rather homogeneous layer of alkaline phosphatase-positive cells with areas containing various amounts of condensed mineralized matrix was observed. Osteocalcin mRNA was already expressed in undifferentiated cells, but the expression was elevated after day 14, which coincided with the onset of mineralization. SOST mRNA expression was not detected in undifferentiated cells but was always detected in late differentiated cells between 10 and 21 d of culture. In summary, van Bezooijen *et al*. (8)

demonstrated that SOST mRNA was expressed only after alkaline phosphatase and osteocalcin expression in all three culture systems, which is consistent with a differentiation-dependent expression. The results of our study with periodontal ligament cells mostly resembled those obtained with the human MSCs in the study of van Bezooijen *et al*. (8), once more emphasizing the progenitor-like character of these cells.

The identification of SOST/sclerostin in cementocytes and in mineralizing periodontal ligament cells adds to our understanding of the biology of the periodontium, but the question of the functional importance and meaning of these findings can only be answered after additional *in vitro* as well as *in vivo* studies.

Acknowledgements

The authors thank K. Reifenrath for expert technical assistance and C. Maelicke for her help in preparing the manuscript. This research was supported by a research grant from the Deutsche Forschungsgemeinschaft (DFG, KFO 208).

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