

OPG and RANKL mRNA and protein expressions in the primary and secondary metaphyseal trabecular bone of PTH-treated rats are independent of that of SOST

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Abstract Sclerostin, encoded by the SOST gene, is a recently identified protein which seems to affect bone remodeling by inhibiting bone formation via Wnt pathways. A previous study on OPG and RANKL, two cytokines involved in the control of osteoclastogenesis, showed that the anabolic effect produced by intermittent treatment with parathyroid hormone was characterized by an increase in OPG/RANKL mRNA ratio in the primary spongiosa of metaphyseal bone of rat femur, and by its falling in the secondary spongiosa, in comparison to controls (Silvestrini et al. (2007a)). Considering that Wnt pathway components seem to regulate osteoclast formation and bone resorption by repression of RANKL transcription and by positive regulation of OPG gene in osteoblastic cells, we have evaluated, in the same rats, whether and how SOST mRNA and protein in the primary and secondary metaphyseal bone are affected by PTH. SOST mRNA and protein significantly fell in both primary and secondary spongiosa where only a few osteocytes were positive to sclerostin. These data show that in the two metaphyseal areas no relationship does exist between the trends of OPG and RANKL mRNA and that of SOST, suggesting that there are no direct links between the effects induced by PTH on these molecules, at least in terms of gene expression.

Keywords Sclerostin · Osteocytes · Parathyroid hormone · Osteoclastogenesis · Osteoblastogenesis

Introduction

Bone remodelling, due to osteoblast formation and osteoclast resorption, is affected by several factors, such as hormones, cytokines, components of extracellular matrices and mechanical stimuli. Some important regulators of osteoclastogenesis or osteoblastogenesis have been recently identified. Osteoprotegerin (OPG), a decoy receptor for the receptor activator of nuclear factor- κ B ligand (RANKL), expressed by bone marrow stromal cells and osteoblasts, acts as an inhibitor of osteoclast differentiation, by preventing RANKL from binding to the receptor activator of nuclear factor- κ B (RANK) located on the pre-osteoclastic cell surface. Moreover, the SOST gene and its protein product, sclerostin, strongly expressed in mature osteocytes, identified as negative regulators of bone formation (Winkler et al. 2003; van Bezooijen et al. 2006; Poole et al. 2005; Silvestrini et al. 2007b). Sclerostin is structurally related to the bone morphogenetic protein (BMP) antagonist DAN/Cerberus family (Noda 2006) and seems to act by binding to BMPs or to LRP5/LRP6 of Wnt signaling cascade, so inhibiting bone formation (Winkler et al. 2003; van Bezooijen et al. 2006; Li et al. 2005), being both BMPs and Wnts critical for osteoblastogenesis (Noda 2006; Kolpakova and Olsen 2005; Ott 2005).

The anabolic effects on bone metabolism of some hormones, for instance, intermittently administered parathyroid hormone (PTH), or the catabolic effects of others, for instance, glucocorticoids, are mediated by different OPG/RANKL ratio (Hofbauer et al. 2000; Hofbauer and Shopped 2004; Silvestrini et al. 2007a; Khosla 2001). At present, only

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not well-defined or controversial results are available on the *in vivo* effects of PTH on OPG, RANKL (Ikeda et al. 2001; Ma et al. 2001) and SOST mRNA expressions (Bellido et al. 2005; Keller and Kneissel 2005). In order to clarify the mechanisms of the anabolic action of PTH on bone, in the present study we have evaluated the SOST mRNA and protein expression in the primary metaphyseal bone of the femur in a group of rats intermittently treated with PTH. The anabolic effects of the hormone had previously been ascertained in these animals by histomorphometry in metaphyseal secondary trabecular bone; in this same bone site, the effects on OPG, RANKL and SOST mRNA and protein had been studied by real time PCR and immunohistochemistry (Silvestrini et al. 2007a, b).

The SOST mRNA and protein expressions in the primary metaphyseal bone of PTH treated animals have been compared with those previously obtained in the secondary metaphyseal spongiosa of the same rats (Silvestrini et al. 2007b). Aim of the present study was also that of investigating whether a direct relationship exists between SOST, OPG and RANKL mRNA and protein after PTH stimulation in the primary and secondary metaphyseal bone.

Materials and methods

Materials and methods have been described in two previous studies (Silvestrini et al. 2007a and b). Briefly, two groups of eight, three months old, male Wistar rats were treated for three weeks as follows: PTH (1–34) s.c. 80 µg/Kg, three times a week; Control (C), s.c. PBS. Control and treated animals were sacrificed 24 h after the last PBS or PTH administration, respectively. One half of left femurs was paraffin embedded for SOST, OPG and RANKL immunodetection and the other half was frozen for RNA extraction from both secondary and primary metaphyseal bone. Moreover, one half of the proximal ends of right tibiae were fixed in methacarn [methanol-chloroform-glacial acetic acid, 6:3:1 (v/v/v)] and embedded in paraffin for SOST mRNA and protein isolation.

Immunohistochemistry

Sections were incubated at 4°C overnight in 1:150 diluted goat polyclonal anti-mouse sclerostin (R&D Systems, Minneapolis, MN, USA), anti-human OPG (1:60 diluted) and RANKL (1:80 diluted) antibodies (Santa Cruz Biotechnol, CA, USA), using Vectastain Universal Quick kit (Vector Lab. Inc.) and diaminobenzidine as chromogen. Negative control: primary antibody was omitted or substituted by normal goat IgG (Santa Cruz Biotechnology Inc., CA, USA) diluted as the primary antibody, at 4°C overnight.

mRNA isolation from frozen samples and real-time-PCR

For each animal, the half of left femurs was immersed in OCT compound (Tissue-Tek, Zoeterwoude, Netherlands), frozen by liquid nitrogen, and stored at –80°C until mRNA analysis was carried out. A few sections had previously been cut using a cryostat (Leica Instruments, Nussloch, Germany) in order to visualize the two areas of interest. Specifically, samples of primary spongiosa corresponded to a rectangular area of about 1 mm height and 3 mm length located under the growth plate cartilage, deprived of periosteal areas and characterized by thin trabeculae with a calcified cartilage core covered with bone, while secondary spongiosa corresponded to an area of about 3 mm length and 2 mm height located under the primary spongiosa, deprived of periosteal areas and characterized by a network of larger trabeculae. These two areas were manually dissected from each frozen sample by using a razor blade, pooled for each group and ground in a porcelain mortar in liquid nitrogen, before being dissolved in TRIzol reagent (RNA-Fast, Molecular System, San Diego, CA).

Sequences were obtained from the GenBank database: OPG: (gi: 52138596) forward, ATGTACGCACTCAAGCACTT, reverse, AAAGAGTTTCTGATACAATCGGTAC; RANKL (gi: 16924011) forward, TTCAAGGGGCCGTGCAAAG, reverse, AGCCACGAACCTTCCATCATA; GAPDH (gi: 62647963) forward ATGGCCTTCCGTGTTCTTAC, reverse, CACCTTCTTGATGTCATCATACTTG; SOST: NM 030584 forward, GCCTCCTCAGGAAGTGAAGAAC, reverse, TACTCGGACACGTCTTTGGTG. Real-time PCR was carried out as shown previously (Silvestrini et al. 2007a, b).

SOST mRNA and protein isolation from methacarn-fixed, paraffin embedded specimens

Primary and secondary metaphyseal spongiosa areas, manually selected from each rat tibia (four rats/groups) by following the same method as above, were pooled and treated for mRNA and protein isolation as previously reported (Silvestrini et al. 2007b). Real-time PCR of extracted RNA was carried out as reported above and results were compared with those previously obtained from frozen samples. Since insufficient amounts of protein were extracted from each sample, pooling of samples was decided for carrying out western blot analysis, as previously described (Silvestrini et al. 2007b).

Statistical analysis of OPG, RANKL and SOST mRNA results was carried out using unpaired Student's t-test.

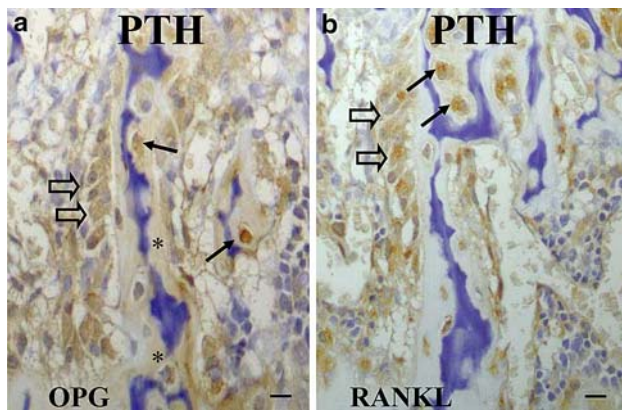


Fig. 1 Sequential sections of primary metaphyseal trabecular bone immunostained with OPG (a) and RANKL (b) antibodies. Most of the plump osteoblasts (a, b, empty arrows) and young osteocytes (a, b, black arrows) are stained. A light diffuse OPG immunolabeling is present in trabecular bone matrix (asterisks)

Results

Immunohistochemistry

The distribution of cellular OPG, RANKL and sclerostin immunolabeling in the primary metaphyseal trabecular bone was similar to that previously obtained in the secondary metaphyseal bone (Silvestrini et al. 2007a, b) in the same C and PTH groups of rats. No evident staining in the osteocyte canaliculi was visible in the metaphyseal trabecular bone, although some canaliculi were sclerostin positive in the cortical bone of the diaphysis in the same rats, using the same antibody and immunohistochemical procedure (Silvestrini et al. 2007b). OPG and RANKL proteins were co-located in most osteoblastic cells, in some lining cells, in a few osteoclasts, in young and a few mature osteocytes (Plate 1-Figs. 1a, b).

Only a few osteocytes were sclerostin positive in the primary metaphyseal spongiosa (Plate 1-Figs. 2a, b). They were too few to be counted, but appeared numerically similar to those in the secondary spongiosa (Silvestrini et al. 2007b). They were mainly located in the deepest areas of trabeculae and, particularly, near the unresorbed mineralized cartilage. Negative controls did not show any aspecific labeling.

OPG, RANKL and SOST mRNA expressions

Real-time PCR analysis

The measurements of OPG, RANKL and SOST mRNA expression in the primary and secondary metaphyseal spongiosa in control and PTH-treated groups (frozen samples) are shown in Figs. 3 a–c, respectively.

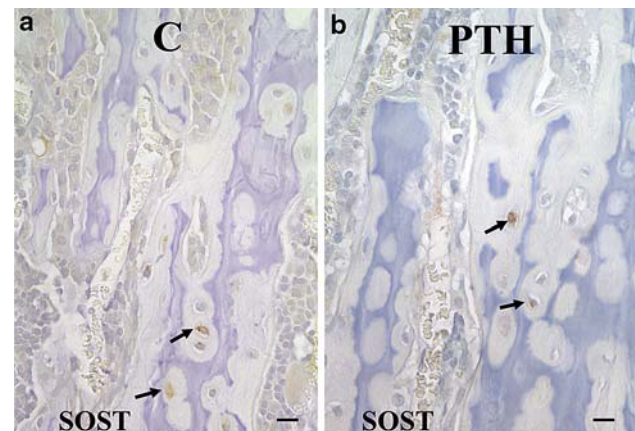


Fig. 2 Control (a) and PTH-treated group (b). Sections of primary metaphyseal trabecular bone immunostained with SOST antibody. The distribution of positive-osteocytes (black arrows) is similar in the control and PTH-treated group. Nuclear Mayer's hemalum counterstaining. Bars = 10 μ m

In the primary spongiosa, after PTH administration, OPG mRNA rose vs controls ($P < 0.05$) (Fig. 3a) while RANKL mRNA significantly fell (Fig. 3b); in the secondary spongiosa, OPG mRNA expression significantly fell (Fig. 3a) while, contrary to OPG, RANKL mRNA expression rose in the PTH treated rats vs controls ($P < 0.05$) (Fig. 3b).

In the PTH group, SOST mRNA fell in both the primary and secondary spongiosa vs controls ($P < 0.05$) (Fig. 3c).

The results of SOST mRNA expression in frozen specimens, shown in Fig. 3c, were comparable with those in methacarn-fixed specimens (results not shown).

SOST protein expression

In agreement with *real-time* PCR results, western blot analysis (Figs. 4a, b) showed a PTH-induced fall of sclerostin protein values, although less evident in the primary than in the secondary metaphyseal spongiosa.

Discussion

The molecular mechanism of the anabolic effect of PTH on bone is not fully understood. To our knowledge, no other studies are available on the effects of intermittent PTH administration on OPG, RANKL and SOST mRNA and protein in different skeletal areas of the rat, such as the primary and secondary metaphyseal bone. Previous histomorphometrical analysis in the same rats used in the present study showed that the PTH treatment has an anabolic effect on bone remodeling (Silvestrini et al. 2007a). This appears to be in line with the significant fall in SOST

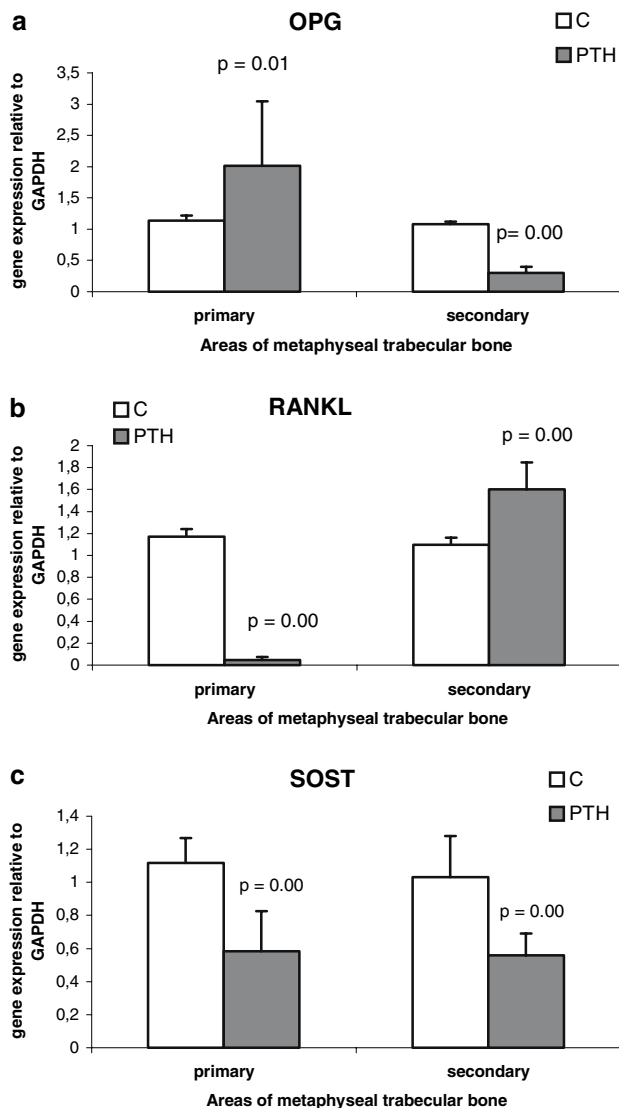


Fig. 3 OPG (a), RANKL (b) and SOST (c) gene expression in control rats (white columns) and rats treated with intermittent PTH (grey columns) evaluated by quantitative real-time PCR analysis of RNA extracted from primary and secondary metaphyseal trabecular bone of femur. Data are presented as the mean \pm standard deviation, and are calculated from four experiments for each group ($n =$ five rats/group for OPG and RANKL and $n =$ eight rats/group for SOST). Comparison between PTH and C groups are carried out by Student's t test

mRNA. This fall, previously demonstrated in three different bone segments (epiphyseal and secondary metaphyseal trabecular bone and diaphyseal cortical bone (Silvestrini et al. 2007b), also occurs in the primary metaphyseal bone, supporting a negative role of SOST in bone formation also in this area.

On the basis of recent data indicating that Wnt signaling regulates the expression of RANKL in osteoblasts, and inhibits osteoclastogenesis (Spencer et al. 2005), and that SOST might therefore be supposed to affect both

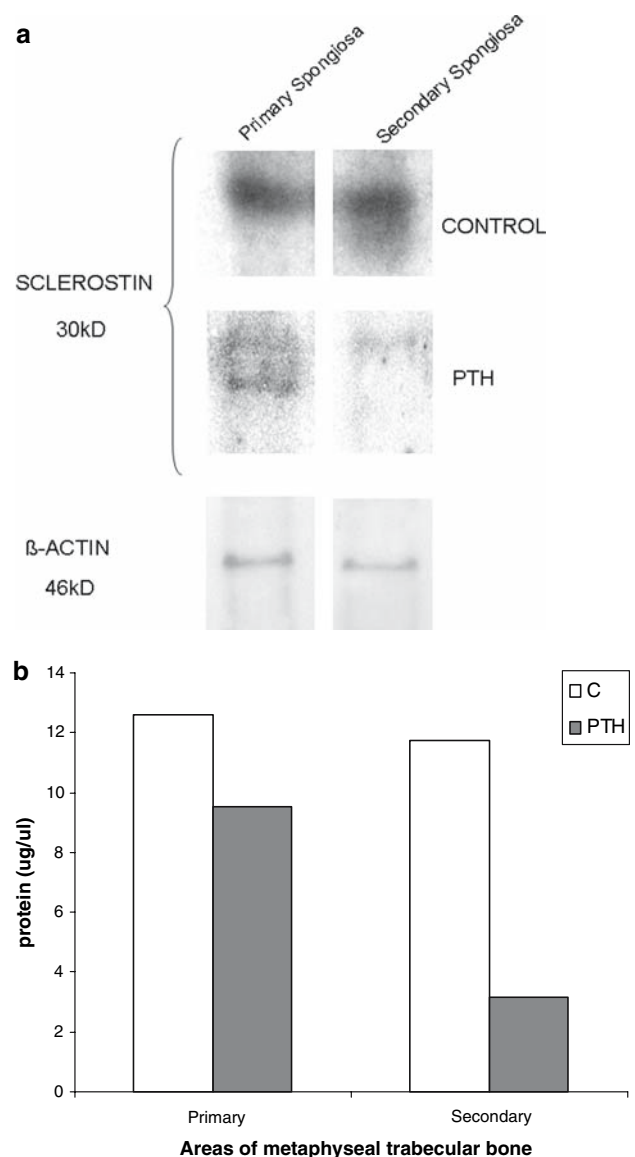


Fig. 4 Representative western blot assay (a) and graphic representation of densitometric analyses (b) of sclerostin in pooled extracts (four rats/group) of methacarn-fixed, paraffin embedded Control (white columns) and PTH-treated (grey columns) primary and secondary spongiosa. The same goat anti-mouse sclerostin antibody was used for the western blotting and immunohistochemistry. A marked reduction of sclerostin was detected in both the bone segments, with a higher extent in the secondary spongiosa as shown in a and b. β -actin is used as loading control. Data from the primary and secondary metaphyseal spongiosa were separately shown, as they were obtained from a unique experiment where protein extracts from the secondary metaphyseal spongiosa, diaphyseal and cortical bone of the same rats were analyzed

osteoblastogenesis and osteoclastogenesis via Wnt signaling, the possibility has been analysed of a direct relationship between the patterns of OPG, RANKL and SOST mRNA and proteins in the primary and secondary metaphyseal bone. Two important results have been obtained: first, no direct relationship exists in the primary

and secondary metaphyseal trabecular bone between the PTH-induced trends of OPG and RANKL mRNA and that of SOST; second, SOST mRNA falls in both primary and secondary spongiosa with a similar pattern after PTH treatment. In contrast to SOST expression data, sclerostin protein reduction, in the PTH group, was less evident in the former than in the latter bone compartment although these results could not be statistically compared being drawn from pooled extracts. Anyway, all these data cannot exclude the possibility that intermittent PTH administration, which down-regulates SOST mRNA, could impact osteoclastogenesis via modulated Wnt signalling, in a dose and/or time-dependent manner until a reduction of SOST protein levels occurs. To test this hypothesis it could be crucial to evaluate OPG, RANKL and SOST mRNA levels at the identical time point following intermittent PTH administration.

These results, compared with those previously detected (Silvestrini et al. 2007b), confirm that the fall of SOST mRNA is less evident in the metaphyseal bone (primary and secondary) than in the epiphysis or diaphysis, where a different bone response to the hormone, probably related to the bone metabolic rate (higher in metaphysis; minor in cortex and epiphysis), has been suggested. The absence of staining in the osteocyte canaliculi of the metaphyseal spongiosa is possibly related to the higher bone turnover occurring in this bone compartment. In this connection, Bellido et al (2005) have shown canalicular staining in the mouse vertebra, a bone compartment characterized by lower bone turnover than metaphysis and where the majority of osteocytes are mature, in opposite to the higher portion of younger osteocytes in the metaphyseal spongiosa.

An inverse trend of OPG/RANKL mRNA ratio between primary and secondary metaphyseal spongiosa was found in PTH-treated rats with respect to controls, rising in the primary, while falling in the secondary metaphyseal bone. It has been proposed (Silvestrini et al. 2007a) that the PTH-mediated opposite trend of OPG and RANKL mRNA in these two bone segments could be related to their different metabolic processes (Gorski 1998) corresponding to modeling in primary metaphyseal bone and remodeling in secondary metaphysis. Anyway, the anabolic response to PTH appeared similar in both metaphyseal areas, also in terms of osteoclast surface, suggesting that there was no evident differential impact of PTH on osteoclastogenesis.

The fact that, after intermittent PTH treatment, no differences in the cellular immunolabeling distribution of OPG and RANKL are detectable in the primary metaphyseal bone, similarly to the secondary metaphysis (Silvestrini et al. 2007a), supports the hypothesis of their coupled action in bone formation and resorption mechanisms. Sclerostin is confirmed to be normally expressed by

mature osteocytes, especially those located near the unresorbed cartilage (van Bezooijen et al. 2006; Poole et al. 2005).

Until now, OPG, RANKL and SOST mRNA expressions have never been individually evaluated in primary and secondary metaphyseal bone after intermittent PTH treatment, and controversial results of PTH effect on these factors are available in the literature. Our data confirm a PTH-induced fall of SOST mRNA which is in line with the data of Keller and Kneissel (2005). Other authors have shown that SOST mRNA and protein fall after continuous infusion of PTH in vivo and in vitro, and that only a transient fall in SOST mRNA occurs after each PTH injection (Bellido et al. 2005). A SOST decrease, and not necessarily a complete absence of SOST, can have consequences on bone formation, as suggested by bone markers in Sclerostosis and Van Buchem disease patients and carriers (Wergedal et al. 2003; Gardner et al. 2003).

After PTH, low numbers of sclerostin-positive osteocytes are visible in the primary metaphyseal bone. This is in agreement with lower percentage and numbers of sclerostin-positive osteocyte/mm² of bone area (Silvestrini et al. 2007a) counted in the secondary metaphyseal bone of the same rats in comparison with the diaphyseal and epiphyseal bone. Thus, in the primary and secondary spongiosa of the metaphysis, both characterized by higher bone turnover, the lower amount of sclerostin positive cells could be due to the increased portion of younger osteocytes.

In conclusion, the fall in SOST mRNA after intermittent PTH administration could represent a novel mechanism for the hormonal control of bone remodeling mediated by osteocytes. This mechanism could not be strictly related to the trends of OPG and RANKL mRNA, at least in the metaphysis of the rat. SOST protein and mRNA reduction could be influenced by bone site and turnover, as well as PTH dose, and could be time-dependent.

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