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TNF-α mediates the stimulation of sclerostin expression in an estrogen-deficient condition

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ABSTRACT

Although recent clinical studies have suggested a possible role for sclerostin, a secreted Wnt antagonist, in the pathogenesis of postmenopausal osteoporosis, the detailed mechanisms how estrogen deficiency regulates sclerostin expression have not been well-elucidated. Bilateral ovariectomy or a sham operation in female C57BL/6 mice and BALB/c nude mice was performed when they were seven weeks of age. The C57BL/6 mice were intraperitoneally injected with phosphate-buffered serum (PBS), 5 μg/kg β-estradiol five times per week for three weeks, or 10 mg/kg TNF-α blocker three times per week for three weeks. Bony sclerostin expression was assessed by immunohistochemistry staining in their femurs. The activity and expression of myocyte enhancer factors 2 (MEF2), which is essential for the transcriptional activation of sclerostin, in rat UMR-106 osteosarcoma cells were determined by luciferase reporter assay and western blot analysis, respectively. Bony sclerostin expression was stimulated by estrogen deficiency and it was reversed by estradiol supplementation. When the UMR-106 cells were treated with well-known, estrogen-regulated cytokines, only TNF-α, but not IL-1 and IL-6, increased the MEF2 activity. Consistently, TNF-α also increased the nuclear MEF2 expression. Furthermore, the TNF-α blocker prevented the stimulation of bony sclerostin expression by ovariectomy. We also found that there was no difference in sclerostin expression between ovariectomized nude mice and sham-operated nude mice. In conclusion, these results suggest that TNF-α originating from T cells may be at least in part responsible for stimulating the sclerostin expression observed in an estrogen-deficient condition.

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1. Introduction

Postmenopausal osteoporosis is a highly prevalent disease caused primarily by the decline in estrogen levels. Although estrogen deficiency increases the bone remodeling intensity, a considerable quantitative gap between bone resorption and formation has been noted. This imbalance results primarily from increased bone resorption [1]. However, there may also be a relative defect of bone formation in an estrogen deficiency [2].

Since the establishment of the central role of estrogen deficiency in the pathogenesis of postmenopausal osteoporosis, an enormous effort has been focused on elucidating the mechanisms by which estrogen may modulate bone formation and resorption. While there is substantial evidence for the direct action of estrogen on bone cells [3], indirect mechanisms involving various factors from immune cells have also been implicated. Tumor necrosis factor-α (TNF-α), produced mainly by T cells in estrogen-deficiency, directly enhances the activity of mature osteoclasts and indirectly stimulates osteoclastogenesis by activating receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) in osteoblasts and stromal cells [4,5]. Activated T cells are also an important source of RANKL [6] and are thus regarded as key immune cells mediating estrogen-deficient bone loss. Interleukin (IL)-1 and IL-6 are other potent stimulators of osteoclast differentiation and activation that have been linked to the accelerated bone resorption seen in postmenopausal osteoporosis [7,8].

Sclerostin, which is expressed almost exclusively in osteocytes, is a secreted Wnt antagonist that acts as a negative regulator of bone formation by binding to the Wnt co-receptor, low-density lipoprotein receptor-related protein-5/6 (LRP5/6) [9,10]. In vivo studies have shown that knockout mice deleted for *SOST*, which encodes sclerostin, have abnormally high bone mass and bone strength [11], whereas mice over-expressing *SOST* exhibit an osteoporotic phenotype [12,13]. The clinical relevance of sclerostin in

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bone metabolism was initially recognized in sclerosteosis and van Buchem's disease [14,15], both of which are characterized by excessive osteosclerosis with high bone mineral density (BMD) resulting from defective sclerostin production. Therefore, anti-sclerostin neutralizing antibodies have been developed as novel anabolic agents increasing bone mass [16].

Recent clinical studies have highlighted the biological importance of sclerostin in patients with estrogen-induced bone loss. It was reported that postmenopausal women had a significantly higher concentration of circulating sclerostin than premenopausal women, and that serum sclerostin levels were inversely correlated with the free estrogen index in postmenopausal women [17]. Circulating sclerostin levels are known to correlate with bone marrow plasma levels [18]. In another study, four weeks of estrogen treatment decreased serum sclerostin concentrations in estrogen-deficient men and women [19]. Most recently, we observed that raloxifene, but not bisphosphonates, reduced circulating sclerostin levels, suggesting that sclerostin may mediate the action of estrogen on bone metabolism, independently of its anti-resorptive effects [20]. Taken together, these findings imply that estrogen deficiency may stimulate bone marrow and circulating sclerostin concentrations, which in turn contribute to increased bone resorption and decreased bone formation. However, the detailed mechanisms of how estrogen deficiency regulates sclerostin expression have not been well elucidated. In the present study, we investigated whether and how estrogen deficiency may regulate bony sclerostin expression using in vitro and in vivo experiments.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice and BALB/c nude mice were purchased from Orient Bio Inc. (Seongnam, Geonggi, Korea). All mice were kept in cages under standard laboratory conditions at a constant temperature of 25 °C and with a 12/12 h light–dark cycle. Mice were given a standard, rodent diet and water. Bilateral ovariectomy (OVX) or a sham operation was performed under anesthesia with an intraperitoneal injection of ketamine (48 mg/kg) and xylazine (5.6 mg/kg). Mice were sacrificed by cardiac puncture, and the success of OVX was confirmed by the absence of ovaries and atrophy of uteri. All experiments and protocols were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences (Seoul, Korea).

2.2. Experimental design to evaluate the effect of estrogen

Bilateral OVX or a sham operation was performed when the mice were seven weeks of age. Study groups consisted of sham-operated mice (SHAM control), estrogen-deficient mice by OVX (OVX control), and OVX mice with β-estradiol (Sigma–Aldrich Co., St.Louis, MO, USA) supplementation (OVX + E2). Each group consisted of four mice, and the experiments were performed independently three times. The mice were injected intraperitoneally five times per week for three weeks with phosphate-buffered serum (PBS) or 5 μg/kg β-estradiol. Mice were sacrificed by cardiac puncture under anesthesia at the end of the experiments, and their femurs were taken for immunohistochemical staining.

2.3. Immunohistochemistry and semiquantitative analysis

Immunohistochemical staining of sclerostin was performed on 7 μm frozen sections of 4% paraformaldehyde (PFA)-fixed femurs. Sections were quenched in 3% H_2O_2 in MeOH for 5 min in order to inhibit endogenous peroxidase. They were then washed in PBS for five min and pre-incubated with goat serum blocking reagent and avidin/biotin blocking reagent for 15 min in each case. After being washed in PBS for five min, the sections were incubated with anti-goat sclerostin antibody (R&D System Inc., Minneapolis, MN, USA) at a dilution of 1:100 at room temperature for 1 h. The sections were then further incubated with anti-goat secondary antibody in 0.01 M PBS containing 1% NaN_3 for 30–60 min and with streptavidin conjugated to horseradish peroxidase (HRP) in 0.01 M PBS containing 1% carrier protein for 30 min. Antibody binding was visualized with diaminobenzidine (DAB) substrate followed by counterstaining with methyl green. The sections were stained using a Cell & Tissue Staining Kit (R&D System Inc.) according to the manufacturer's instructions.

Bone tissue sections were qualitatively evaluated under a bright-field microscope (Olympus, Tokyo, Japan) at 200× magnification. Within these areas, five visual fields per section for each sample were digitized at 200× magnification with a digital camera (DP71; Olympus) and DP controller software (Olympus). The number of sclerostin-positive cells was calculated as the number of labeled nuclei per visual field. All analyses were performed in a blinded fashion.

2.4. Cell culture

Rat UMR-106 osteosarcoma cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daejen, Korea) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2.

2.5. MEF2 luciferase reporter assay

Myocyte enhancer factors 2 (MEF2) plasmid (CCS-7024L; SABiosciences, Frederick, MD, USA) was transfected using Lipofectamine™ Reagent (Invitrogen, Rockville, MD, USA) according to the manufacturer's instructions. Briefly, rat UMR-106 osteosarcoma cells were incubated with MEF2 plasmid DNA-reagent mixtures in OPTI-MEM (Invitrogen) for 6 h. The medium was then replaced with fresh complete DMEM and the cells were further cultured for two days with IL-1 10 ng/ml, IL-6 10 ng/ml, and TNF-α 10 ng/ml (R&D System Inc.). Cells were lysed and luciferase activity was assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Transcriptional activity was expressed as the ratio of firefly to renilla luciferase activity.

2.6. Cell fractionation and western blot analysis

Detailed information is described in the Supplementary Materials and Methods.

2.7. Experimental design to evaluate the effect of TNF-α

Bilateral OVX or a sham operation was performed when the mice were seven weeks old. Study groups consisted of sham-operated mice (SHAM control), estrogen-deficient mice by OVX (OVX control), and OVX mice with TNF-α blocker (infliximab; Janssen Biotech Inc., Horsham, PA, USA) supplementation (OVX + TNF-α blocker). Each group consisted of four mice, and the experiments were performed independently three times. The mice were injected intraperitoneally three times per week for three weeks with PBS or with a 10 mg/kg TNF-α blocker. Mice were sacrificed by cardiac puncture under anesthesia, and their femurs were obtained for immunohistochemical staining.

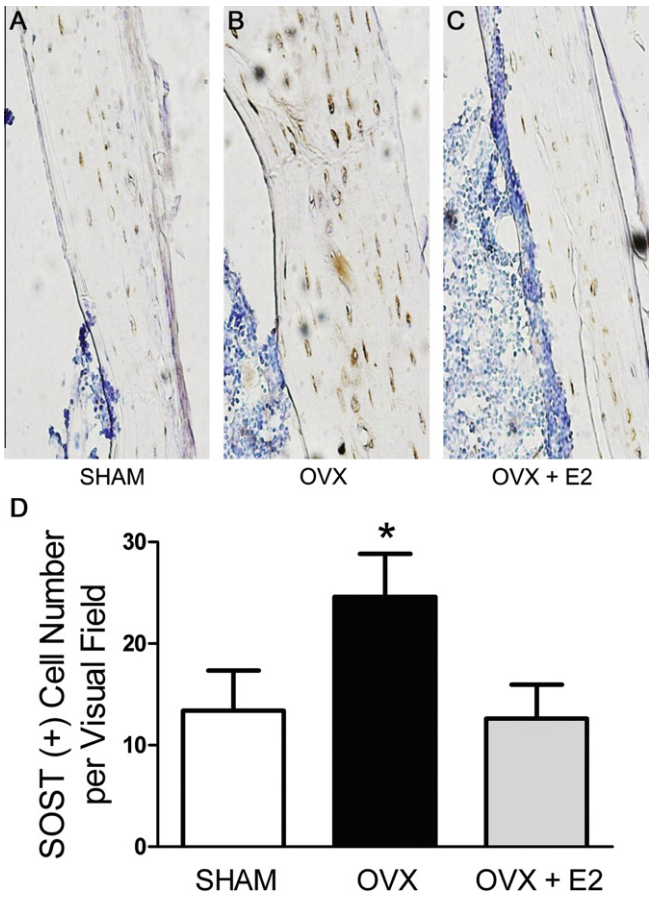

Fig. 1. Immunohistochemistry staining for sclerostin in femur frozen sections of SHAM (A), OVX (B), and OVX + E2 (C) at high (200×) magnification. Immunohistochemistry demonstrates pervasive staining for sclerostin (brown) in the femur of OVX and scant staining in the femur of SHAM and OVX + E2. (D) *Bars* represent mean and SDs of sclerostin-positive cells per visual field of each sample. $^{*}P < 0.05$ compared with SHAM control. SHAM, sham-operated mice; OVX, ovariectomized mice; OVX + E2, ovariectomized mice with estradiol supplementation.

2.8. Statistics

All in vitro data are expressed as mean ± SD of at least three independent experiments with triplicate measurements, unless otherwise specified. The significance of differences between the two groups was assessed using the Mann–Whitney *U*-test, and differences between three or more groups were tested by analysis of variance (ANOVA) with post hoc analysis using Duncan's multiple-range test. A *P* value less than 0.05 was considered statistically significant. The SPSS 17.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

3. Results

3.1. Estrogen-deficiency stimulates sclerostin expression in mouse bone

We examined whether estrogen deprivation stimulates sclerostin expression using in vivo experiments. After bilateral OVX or a sham operation, the mouse femurs were stained in order to evaluate sclerostin expression (Fig. 1). Compared with the SHAM control, the OVX control had markedly intense stain for sclerostin. However, only scant stain for the protein was observed in OVX mice with estradiol supplementation, thus suggesting that estrogen deprivation stimulates bony sclerostin expression with reversal by estrogen therapy.

3.2. TNF- α, but not IL-1 and IL-6, stimulates MEF2 activity

IL-1, IL-6 and TNF-α are known as key cytokines mediating estrogen-deficient bone loss [4,5,7,8]. In particular, the TNF-α and TNF-related weak inducer of apoptosis (TWEAK) stimulated sclerostin expression in vitro [21]. Therefore, we tested the effect of these cytokines on MEF2 activity which is essential for the transcriptional activation of sclerostin [22] in rat UMR-106 osteosarcoma cells (Fig. 2A). TNF-α significantly increased the MEF2 activity, as determined by luciferase reporter assay, whereas IL-1 and IL-6 had no effect on the MEF2 activity. Consistently, TNF-α also increased the nuclear MEF2 expression in western blot analysis (Fig. 2B). These in vitro data suggest that TNF-α may mediate the activation of MEF2 in estrogen deficiency.

3.3. TNF-α blocker prevents the increase of sclerostin in bone of ovariectomized mice

To confirm the role of TNF-α on increased sclerostin levels related to estrogen-deficiency, we further performed an in vivo study. Immunohistochemistry staining for sclerostin was performed in femurs of OVX mice in the presence or absence of TNF-α blocker (Fig. 3). As described in Fig. 1, the OVX control had more intense stain for sclerostin than the SHAM control. However, supplementation with TNF-α blocker in OVX mice prevented the increase of sclerostin expression induced by ovariectomy.

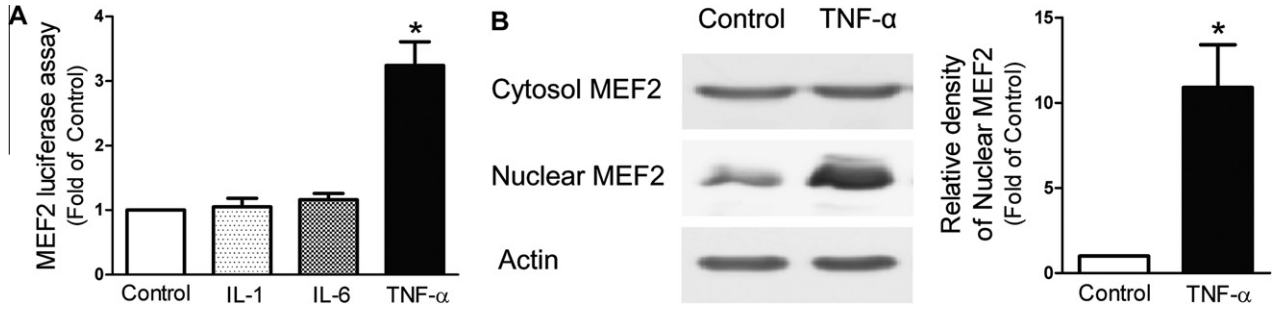

Fig. 2. TNF-α stimulates the activity and expression of MEF2 which is an essential transcription factor for sclerostin expression in rat UMR-106 osteosarcoma cells. (A) Rat UMR-106 osteosarcoma cells were treated for two days with well-known estrogen-regulated cytokines, such as IL-1 10 ng/ml, IL-6 10 ng/ml, and TNF-α 10 ng/ml. The MEF2 activity was determined by luciferase reporter assay. (B) The MEF2 expression in rat UMR-106 osteosarcoma cells after TNF-α 10 ng/ml treatment for two days was determined by western blot analysis. The band intensities were quantified by densitometry. The means and SDs of three to four, independent experiments are shown. $^{*}P < 0.05$ compared with control. TNF-α, tumor necrosis factor α; MEF2, myocyte enhancer factors 2; IL, interleukin.

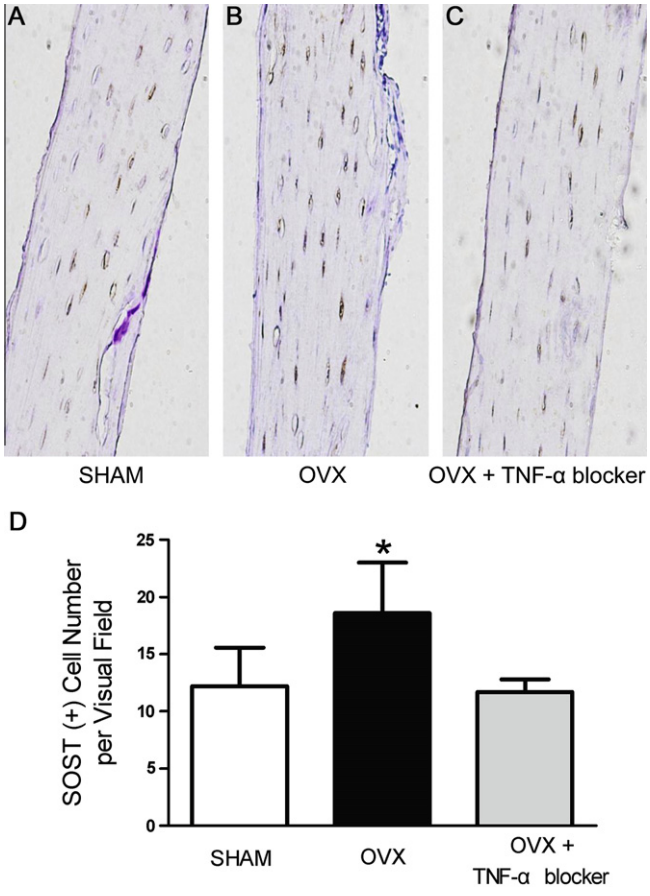

Fig. 3. Immunohistochemistry staining for sclerostin in femur frozen sections of SHAM (A), OVX (B), and OVX + TNF-α blocker (C) at high (200×) magnification. Immunohistochemistry demonstrates more intense expression of sclerostin (brown) in the femur of OVX than in the femur of SHAM and OVX + TNF-α blocker. (D) *Bars* represent mean and SDs of sclerostin-positive cells per visual field of each sample. $^{*}P < 0.05$ compared with SHAM control. SHAM, sham-operated mice; OVX, ovariectomized mice; OVX + TNF-α blocker, ovariectomized mice with TNF-α blocker supplementation.

3.4. Sclerostin expression is not increased in bone of ovariectomized nude mice

A nude mouse has a genetic mutation that causes a deteriorated or absent thymus, resulting in a greatly reduced number of T cells [23]. In addition, recent studies have demonstrated the importance of activated T cells in estrogen-deficiency-driven TNF-α production [4]. Therefore, we performed bilateral OVX or a sham operation in seven-week-old nude mice and compared the sclerostin expression of femurs using immunohistochemistry. As shown in Fig. 4, there was no significant difference in the expression of sclerostin between the two groups, thus suggesting that TNF-α contributing to the sclerostin expression in estrogen-deficiency may originate at least partially from T cells.

4. Discussion

Although recent clinical studies have implied the possible role of sclerostin in the pathogenesis of postmenopausal osteoporosis, we have not encountered any studies evaluating the mechanism by which estrogen deficiency leads to increased sclerostin expression. In the present study, we suggested that TNF-α originating from T cells in an estrogen-deficient condition may stimulate the expression of sclerostin by up-regulating the MEF2 transcription factor.

While estrogen has been proven to have direct effects on bone cells, many lines of recent evidence have identified additional regulatory effects of estrogen centered at the level of the adaptive immune response [5]. For example, estrogen exerts protective effects on bone via the suppression of several cytokines, such as IL-1, IL-6, and TNF-α, which are key mediators for the development of postmenopausal osteoporosis by augmenting bone resorption [3]. Among the cytokines, the pathological role of TNF-α in postmenopausal osteoporosis is particularly supported by animal model studies demonstrating that knockout mice which are deficient in either TNF or p55 TNF receptor are resistant to ovariectomy-induced bone loss [24]. Likewise, treating mice with TNF inhibitor completely prevented bone loss as well as an increase in both osteoclast formation and bone resorption induced by ovariectomy [25].

Regarding the association of cytokines with sclerostin, an in vitro study demonstrated that pro-inflammatory cytokines TNF-related weak inducer of apoptosis (TWEAK) and TNF-α induce the mitogen-activated protein kinase- (MAPK)-dependent expression of sclerostin in human osteoblasts [21]. Meanwhile, the expression of sclerostin in adult bone is known to crucially require a distant enhancer, and Leuptin et al., [22] showed that the MEF2 transcription factor is essential and sufficient for the enhancer activity and also mediates the inhibition of sclerostin expression by PTH. Based on these background factors, we tested the effect of estrogen-regulated cytokines on the MEF2 transcription factor.

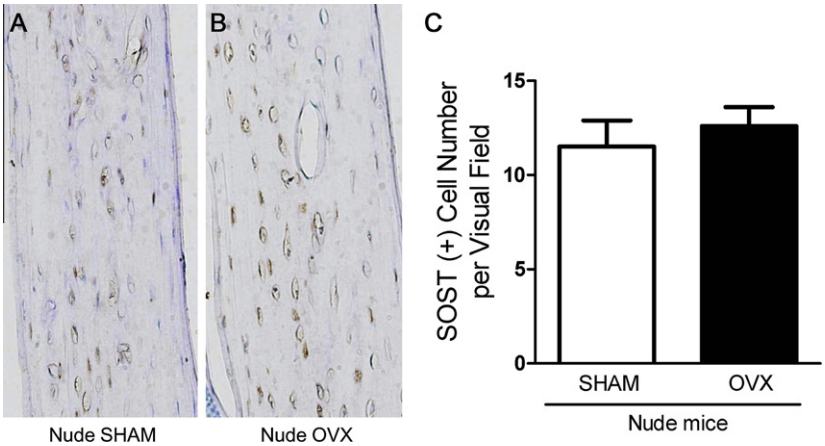

Fig. 4. Immunohistochemistry staining for sclerostin in femur frozen sections of SHAM (A) and OVX (B) nude mice at high (200×) magnification. There was no significant difference in the expression of sclerostin between the SHAM and OVX nude mice. (C) *Bars* represent mean and SDs of sclerostin-positive cells per visual field of each sample. SHAM, sham-operated mice; OVX, ovariectomized mice.

Only TNF-α, but not IL-1 and IL-6, significantly stimulated MEF2 activity, thereby suggesting the possibility that TNF-α may mediate the increased expression of sclerostin in the estrogen-deficient state. In accordance with this notion, an in vivo study using immunohistochemistry showed that treatment with TNF-α blocker prevented the increase in sclerostin expression induced by ovariectomy.

Recent in vivo and in vitro experiments emphasize the role of activated T cells in TNF-α production stimulated by estrogen deficiency, as demonstrated by Cenci et al., in a representative study using highly purified bone marrow cells [26]. Consistently, we also observed that estrogen treatment significantly decreased TNF-α expression in T cells (Supplementary Fig. 1). The mechanism of T cells activation elicited by estrogen deficiency is known to be primarily due to their increased export from the thymus [27]. Because nude mice, which result from alteration of the *FOXN1* gene, are born without a normal thymus and functionally mature T lymphocytes [23], they have been widely used in the study of T cell-related immune deficiency. When we compared the expression of sclerostin in sham-operated nude mice with that in ovariectomized nude mice, there was no significant difference between the two groups. These results suggest that the TNF-α, which may stimulate the expression of sclerostin in the estrogen-deficient state, is produced, at least partially, by T cells.

Although the importance and interest of sclerostin in bone metabolism is increasing, there is no well-established in vitro model system for studying the mechanisms affecting this protein because of the extreme difficulty of isolating primary osteocytes. Current procedures have very low yields and because primary osteocytes are terminally differentiated, they cannot be expanded without loss of phenotype. Furthermore, they are highly heterogeneous and thus may include cells at diverse stages of differentiation. Although we tried to isolate mature osteocytes expressing sclerostin from primary human bone marrow stromal cells (BMSCs), primary mouse BMSCs, and primary mouse calvarial osteoblasts in osteogenic conditions, we detected no sclerostin expression (data not shown), as observed by others. Therefore, researchers have tried to find other effective ways to study this bone cell. Keller and Kneissel [28] searched for a cell line that expressed high levels of sclerostin comparable to bone. However, none of the screened mouse bone and mesenchymal precursor cell lines, such as MLO-Y4 osteocytic cell line, expressed significant amounts of *SOST* transcript; surprisingly, only the rat osteosarcoma cell line UMR-106 expressed high levels of *SOST* expression (about 3.6-fold higher than in bone tissue). Moreover very little sclerostin expression was observed in MLO-A5 pre-osteocytic and MLO-Y4 osteocytic cell lines, whereas strong expression was again detected in UMR-106 cells [29]. These results suggest that the MLO-A5 and MLO-Y4 cell lines do not have the complete set of characteristics of osteocytes, and that UMR-106 cells seem to be a valid cellular system for studying *SOST* gene regulation at present.

As estrogen deficiency stimulates osteoclast differentiation in various ways [3], including by increasing RANKL production, some factor made by mature osteoclasts, or RANKL itself, might increase the expression of sclerostin in a paracrine manner. To investigate this possibility, the conditioned media (CM) collected from differentiated osteoclasts and pre-osteoclasts and the soluble RANKL were applied onto rat UMR-106 osteosarcoma cells for two days, after which the *SOST* mRNA expression and MEF2 activity were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and luciferase reporter assay, respectively. However, contrary to our hypothesis, sclerostin expression was unaffected by RANKL treatment and rather decreased by treatment with the CM of differentiated osteoclasts (Supplementary Fig. 2). Although the exact mechanisms inducing this observation are not fully understood at present, we assume that sclerostin may have a potential role in coupling phenomenon of bone remodeling, regardless of its contribution to the pathogenesis of estrogen-deficient bone loss. Recently, Pederson et al., verified that sphingosine 1-phosphate (S1P), bone morphogenic protein 6 (BMP6), and Wnt10b were significantly increased in mature osteoclasts, whereas sclerostin levels decreased during osteoclastogenesis [30], thus suggesting that these factors may mediate any changes in sclerostin expression. Considered along with our data, it seems possible that factors derived from differentiated osteoclasts during bone resorption may suppress sclerostin expression in osteoblast lineages and may consequently stimulate bone formation as a coupling mechanism.

In summary, our in vivo and in vitro studies suggest that TNF-α, which is increased in estrogen deficiency, may stimulate the expression of sclerostin via the MEF2 transcription factor. Our results provide the first evidence that the increased sclerostin mediated by TNF-α may at least partially contribute to the pathogenesis of postmenopausal osteoporosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.100.

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