

UCLA

UCLA Previously Published Works

Title

Regulation of RANKL-induced osteoclastic differentiation by vascular cells

Permalink

<https://escholarship.org/uc/item/98g6x1b7>

Journal

Journal of Molecular and Cellular Cardiology, 39(2)

ISSN

0022-2828

Authors

Tintut, Yin

Abedin, Moeen

Cho, John

et al.

Publication Date

2005-08-01

DOI

10.1016/j.yjmcc.2005.03.019

Copyright Information

This work is made available under the terms of a Creative Commons Attribution-NonCommercial-NoDerivatives License, available at

<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Peer reviewed

Brief communication

Regulation of RANKL-induced osteoclastic differentiation by vascular cells

Yin Tintut ^{a,*}, Moeen Abedin ^a, John Cho ^a, Andrea Choe ^a, Jina Lim ^a,
Linda L. Demer ^{a,b}^a Department of Medicine, David Geffen School of Medicine at UCLA, 10833 LeConte Avenue, Los Angeles, CA 90095-1679, USA^b Department of Physiology, David Geffen School of Medicine at UCLA, 10833 LeConte Avenue, Los Angeles, CA 90095-1679, USA

Received 10 November 2004; received in revised form 10 March 2005; accepted 25 March 2005

Available online 12 May 2005

Abstract

Vascular calcification is a regulated process of biomineralization resembling osteogenesis. Many bone-related factors, including resorptive osteoclast-like cells, although in low abundance, have been found in calcified atherosclerotic lesions. The regulatory mechanisms governing them in the vasculature, however, are not clear. Previously, we found that calcifying vascular cells (CVC), a subpopulation of bovine aortic smooth muscle cells (BASMC), undergo osteoblastic differentiation and form mineralized nodules. Since osteoblasts and marrow stromal preosteoblasts regulate osteoclastic differentiation in bone, we hypothesized that vascular cells also regulate differentiation of osteoclastic precursors in the artery wall. Peripheral blood monocytes, which are osteoclast precursors, were co-cultured with CVC or BASMC. Results showed that monocytes co-cultured with both of the vascular cells yielded fewer resorption pits than monocytes cultured alone. Furthermore, monocytes co-cultured with CVC had fewer resorption pits than those co-cultured with BASMC. Conditioned media from the vascular cells also inhibited resorptive activity of monocytes suggesting that the inhibitory effect was mediated in part by soluble factors. Compared with BASMC, CVC had lower mRNA expression for osteopontin, which promotes osteoclast attachment, but greater mRNA expression for the soluble inhibitory cytokine, IL-18. Increased osteoclastic differentiation was observed when neutralizing antibody to IL-18 receptor was added to the cultures of preosteoclasts with CVC conditioned media. Osteoprotegerin, another osteoclast inhibitory cytokine, was expressed at similar levels in both cultures. These results suggest that vascular cells inhibit osteoclastic differentiation, and that CVC have greater inhibitory effects than BASMC.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Smooth muscle cells; Osteoclasts; Vascular calcification; Resorption; RANKL

1. Introduction

Vascular calcification is a significant predictor for cardiovascular events and is present in the majority of patients with clinically significant coronary artery disease [1]. Key functional aspects of bone formation and remodeling are recapitulated in vascular calcification, and many bone-related factors such as bone matrix proteins and regulatory factors are found in atherosclerotic lesions [2–4]. Osteoclast-like cells have also been identified by histopathology in calcified human atherosclerotic plaques [5]. Previous studies have provided evidence for a functional role of osteoclast-like cells in regres-

sion of ectopic calcification. Steitz et al. [6] studied ectopic calcification in cardiac valves transplanted subcutaneously in mice. They showed osteopontin-dependent local upregulation of carbonic anhydrase II in neighboring osteoclast-like cells and regression of the ectopic mineral. In addition, Asou et al. [7] showed resorption of ectopic bone in muscle, which was inhibited in osteopontin null mice and rescued by topical application of recombinant osteopontin.

The proliferation, differentiation and survival of osteoclasts and their function in bone resorption require support from osteoblasts and marrow preosteoblasts. These cells secrete stimulatory cytokines, monocyte-colony stimulating factor (M-CSF) and receptor activated NF- κ B ligand (RANKL), as well as inhibitory cytokines, such as osteoprotegerin (OPG), IL-12 and IL-18 [8]. IL-18 not only inhibits

* Corresponding author. Tel.: +1 310 206 9964; fax: +1 310 825 4963.

E-mail address: ytintut@mednet.ucla.edu (Y. Tintut).

osteoclasts but it also regulates atherosclerosis. IL-18 deficiency reduces atherosclerosis in *apoE* null mice [9], and IL-18 treatment induces a fourfold increase in atherosclerotic lesions in *apoE* null mice through an IFN- γ -dependent mechanism [10]. Osteoblasts also secrete extracellular matrix proteins such as osteopontin, which promote osteoclast attachment and activity [11]. The importance of osteoblasts in regulation of osteoclasts is underscored by the large number of factors, such as parathyroid hormone and calcitriol that control osteoclast function only in an indirect manner through effects on osteoblasts [8].

The artery wall contains preosteoclasts in the form of monocytes, as well as cells and factors that regulate osteoclastic differentiation. Vascular smooth muscle cells express OPG [12], monocyte-macrophages express IL-18, and endothelial and smooth muscle cells express the receptor for IL-18 [13]. In addition, well-established osteoclast differentiation factors, M-CSF and RANKL [8], have been detected in calcified atherosclerotic lesions [4,14]. Previously, we isolated a subpopulation of osteoblast-like cells, calcifying vascular cells (CVC), from primary cultures of bovine aortic smooth muscle cells (BASMC) [2]. CVC have some distinct features from its parent population of BASMC [16]. In particular, they undergo the distinctive molecular sequence of osteoblastic differentiation, typical of skeletal and bone marrow-derived osteoblasts [15]. In bone, marrow-derived osteoblasts regulate osteoclastic differentiation. However, in the artery wall, it is not known whether either or both of these vascular cells regulate osteoclastic differentiation of osteoclast precursors, peripheral blood monocytes.

In this study, we hypothesized that vascular cells regulate osteoclastic differentiation of peripheral blood monocytes. Results from the co-culture and conditioned media experiments showed that vascular cells inhibit osteoclastic differentiation of monocytes, and that both BASMC and CVC express osteoclastic inhibitory factors. There was slightly greater inhibition of osteoclast differentiation by CVC compared with BASMC, possibly explained by the fact that CVC expressed less osteopontin and more IL-18 than BASMC.

2. Methods

2.1. Materials

Recombinant human M-CSF, human RANKL and neutralizing antibody to mouse IL-18 receptor were obtained from R&D Systems, Inc. Osteologic discs were from BD Bioscience. Peripheral blood monocytes were from the Atherosclerosis Research Unit at UCLA. The monocytes, purified to 85–95% from peripheral blood mononuclear cells (PBMC) were collected from healthy volunteers [17]. The Northern probe for human osteopontin was from American Type Culture Collection (ATCC). Anti-osteopontin antibody was purchased from NeoMarkers (Freemont, CA).

2.2. Cell culture

CVC, isolated from bovine aortic explants [2], were cultured as previously described [15]. CVC and BASMC were maintained in DMEM (Cellgro) supplemented with 15% FBS (Hyclone, Logan, UT), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 units/ml). Both CVC and BASMC were used at similar passage number (15–20) in these experiments. To collect the conditioned media, fresh media were placed on confluent cell cultures. After 6 days, the medium was centrifuged to remove cell debris, and stored in aliquots at -80°C until used.

2.3. Resorption pit assays in co-cultures

Vascular cells (CVC and BASMC at 17,500 cells per cm^2) were plated in separate 24 well dishes containing osteologic discs, and at confluence (approximately 2 days), monocytes (125,000 per cm^2) were co-cultured on both cell types along with hM-CSF (25 ng/ml) and hRANKL (40 ng/ml). Cultures were fed with fresh media and agents every 3–4 days. Resorption assays were performed after 7 days in culture as described previously [17].

2.4. Conditioned media experiment

Monocytes (100,000 cells per well) were cultured in the conditioned media (collected from either CVC or BASMC cultures as described above), supplemented with 10% FBS, hM-CSF and hRANKL (at concentrations above). After 7 days of culture, resorption pit formation was assessed.

2.5. TRAP stain

Murine RAW264.7 cells (1 K per well: ATCC) were plated in 10% DMEM containing 100 ng/ml hRANKL. After 4 days in culture, media was replaced with the conditioned media collected from CVC containing hRANKL and either buffer or neutralizing antibody to mouse IL-18 receptor. TRAP staining was performed after 8 days in culture as described previously [18].

2.6. Northern analysis

CVC and BASMC were plated at 600,000 cells per plate in separate 60-mm dishes. At the indicated time points, RNA was isolated. RNA (10 μg) was run on a gel and probed for osteopontin, osteoprotegerin and IL-18. OPG and IL-18 Northern probes were generated by PCR and purified by gel electrophoresis.

OPG forward primer 5'-GTGTGACAAATGTNCTCCTG-3', and

reverse primer 5'-CAGCAAACCTGAAGAANGCCTG-3',

IL-18 forward primer 5'-GCTGCCGTCTTCTGTAAGGAAAAG-3' and

reverse primer 5'-GGTCTGGGGTGCATTATCTGAACA-3'.

2.7. Western analysis

Cells were lysed in a cold lysis buffer containing protease inhibitor cocktail (Calbiochem) along with phosphatase inhibitors. Western analysis was performed as previously described [16].

2.8. Data analysis

Data were expressed as mean $\pm$ S.D., and means were compared using one-way ANOVA, with comparison of different groups by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered significant.

3. Results

To investigate whether vascular cells affect differentiation of preosteoclasts in the artery wall, peripheral blood monocytes were cultured alone or co-cultured with either BASMC or CVC in the presence of hRANKL (40 ng/ml) and hM-CSF (25 ng/ml), which are required for osteoclastic differentiation. Osteoclastic formation was assessed after 7 days in culture by formation of resorption pits on osteologic discs. Results showed that resorption pits were observed in the control monocyte monoculture treated with hRANKL and hM-CSF (304 ± 52 pits per well; Fig. 1A). Reduced numbers of resorption pits were observed in monocytes co-cultured with BASMC (22.7 ± 11 pits per well; Fig. 1B) and no resorption pits were observed in the CVC co-culture. (Fig. 1C). In control cultures when RANKL was not added, no resorption pits were observed (data not shown).

To investigate whether the observed inhibitory effect of vascular cells requires cell–cell contact or is mediated by soluble factors, monocytes were cultured for 7 days with hM-CSF and hRANKL in conditioned media collected from CVC and BASMC cultures. Results showed that monocytes cultured in the conditioned media from CVC produced fewer resorption pits per well than those cultured with the conditioned media from BASMC (7.75 ± 2.63 vs. 74.75 ± 14.20 pits per well, $P < 0.0001$).

Ek-Rylander et al. [19] have previously shown that post-translational modification of osteopontin is required for adhesion of osteoclasts. Hence, we determined the expression of osteopontin and its post-translational modification in these vascular cells. As shown in Fig. 2A, BASMC expressed higher levels of osteopontin mRNA than CVC at 3 days of culture. Western analysis from whole-cell lysates, in the presence of phosphatase inhibitors, showed higher levels of lower molecular weight (i.e. less modified by phosphorylation or glycosylation) osteopontin in CVC than BASMC (Fig. 2B).

To investigate the IL-18 expression in both CVC and BASMC, RNA was isolated at 3, 7 and 10 days of culture, and Northern analysis was performed. Results showed that IL-18 expression was higher in CVC than in BASMC at all time points (Fig. 2C). Expression of OPG was similar in both

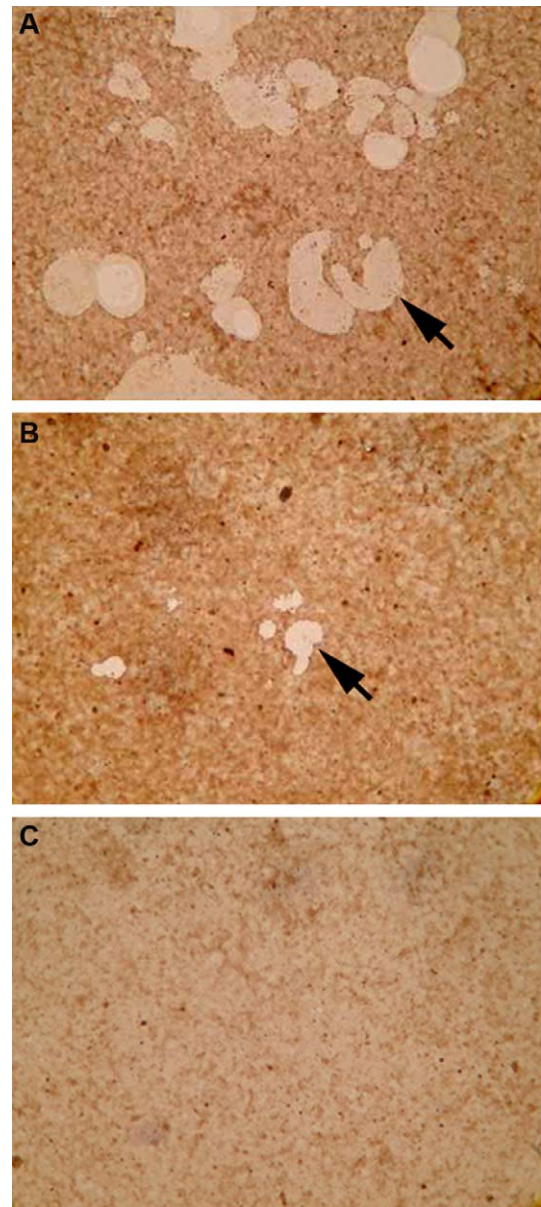


Fig. 1. Mineral resorption by peripheral blood monocytes cocultured with vascular cells. Resorption pit formation (arrow, 200 $\times$) by peripheral blood monocytes alone (A), co-cultured with BASMC (B) or CVC (C) in the presence of hM-CSF (25 ng/ml) and hRANKL (40 ng/ml) for 7 days ($n = 3$).

CVC and BASMC (data not shown). To investigate the inhibitory role of IL-18 in CVC conditioned media, preosteoclasts (RAW264.7 cells) were cultured in CVC conditioned media in the presence or absence of neutralizing antibody to mouse IL-18 receptor. Results showed increased numbers of TRAP positive cells in the presence of the neutralizing antibody (4.4 ± 1.6 vs. 2.5 ± 1.2 for control, $P < 0.05$).

4. Discussion

In the vasculature, multinucleated osteoclast-like cells are found in close association with calcified atherosclerotic lesions [5]. The source of osteoclasts is not established, how-

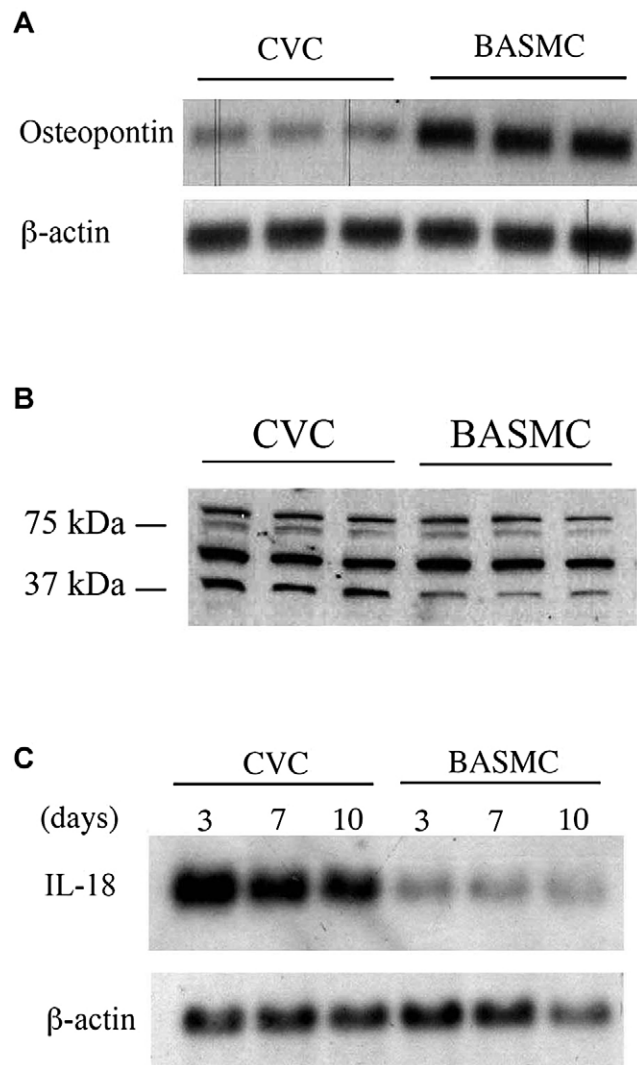


Fig. 2. Expression of osteopontin and IL-18 in vascular cells. Northern analysis of RNA from CVC and BASMC for osteopontin (A) and IL-18 (C) ($n = 3$). β -Actin gene expression was used as a control. (B) Western analysis of whole-cell lysates from CVC and BASMC for osteopontin showing multiple bands representing different degrees of post-translational modification.

ever they are most likely to derive from peripheral blood monocytes, which migrate abundantly into atherosclerotic plaque and differentiate into bone resorbing osteoclasts in the presence of M-CSF and RANKL in vitro. Collin-Osdoby et al. [20] have shown that osteoclastic differentiation of human PBMC co-cultured with microvascular endothelial cells is regulated by inflammatory cytokines. The present results suggest that aortic smooth muscle cells inhibit osteoclastic differentiation and resorption of monocyte-derived osteoclasts, and the inhibition is in part via soluble secreted factors. Udagawa et al. [21] have shown that expression of IL-18, which inhibits osteoclastic differentiation by 50% even at very low concentrations (1 ng/ml), was detected in osteoblasts and that osteoclast non-inductive cells express higher levels of IL-18 than osteoclast inductive cells. Our present results suggest that CVC have a greater inhibitory effect on osteoclasts than do their parental cell population, BASMC. In addition, CVC express more mRNA for the osteoclast inhibitory fac-

tor, IL-18, compared with BASMC. As evidence of its functional relevance, neutralizing antibody to IL-18 receptor reversed the osteoclast inhibitory effects of CVC conditioned media.

OPG and IL-18, osteoclastic regulatory cytokines, are also known to regulate atherosclerosis and vascular calcification. OPG deficient mice develop not only osteoporosis but also vascular calcification [22]. In the vasculature, IL-18 enhances atherosclerosis in *apoE* null mice [10], and double knockout mice (*il-18/apoE*) have reduced lesions compared with *apoE* null mice [9]. The present results indicate that osteopontin mRNA expression and the extent of post-translational modification, which promote osteoclastic function, are lower in CVC than BASMC.

While it is well-established that bone undergoes continuous turnover, it is not clear whether significant spontaneous regression or remodeling occurs in calcified mineral deposits in the artery wall, despite the presence of osteoclastogenic factors, including M-CSF and RANKL in atherosclerotic lesions [4,14]. The present findings raise the possibility that vascular cells restrict osteoclast function through modulating release of matrix proteins such as osteopontin and soluble factors such as OPG and IL-18. Teleologically, vascular mineralization may serve as a defense mechanism in response to chronic inflammation, producing a wall of bone to isolate the noxious process. If so, simultaneous restriction of mineral resorption would be an additional mechanism for pathologic progression of vascular calcification.

Acknowledgements

This research was supported by NIH grants HL/AR69261 and HL30568, the Guthman Endowment, and the Laubisch and Cohen Funds.

References

- [1] Wayhs R, Zelinger A, Raggi P. High coronary artery calcium scores pose an extremely elevated risk for hard events. *J Am Coll Cardiol* 2002;39:225–30.
- [2] Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL. Bone morphogenetic protein expression in human atherosclerotic lesions. *J Clin Invest* 1993;91:1800–9.
- [3] Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest* 1994;93:2393–402.
- [4] Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 2001;21:1998–2003.
- [5] Jeziorska M, McCollum C, Wooley DE. Observations on bone formation and remodelling in advanced atherosclerotic lesions of human carotid arteries. *Virchows Arch* 1998;433:559–65.
- [6] Steitz SA, Speer MY, McKee MD, Liaw L, Almeida M, Yang H, et al. Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. *Am J Pathol* 2002;161:2035–46.

- [7] Asou Y, Rittling SR, Yoshitake H, Tsuji K, Shinomiya K, Nifuji A, et al. Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. *Endocrinology* 2001;142:1325–32.
- [8] Roodman GD. Cell biology of the osteoclast. *Exp Hematol* 1999;27:1229–41.
- [9] Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, et al. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res* 2003;59:234–40.
- [10] Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein E(–/–) mice through release of interferon-gamma. *Circ Res* 2002;90:E34–E38.
- [11] Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, et al. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. *J Biol Chem* 1993;268:9901–7.
- [12] Hofbauer LC, Shui C, Riggs BL, Dunstan CR, Spelsberg TC, O'Brien T, et al. Effects of immunosuppressants on receptor activator of NF-kappaB ligand and osteoprotegerin production by human osteoblastic and coronary artery smooth muscle cells. *Biochem Biophys Res Commun* 2001;280:334–9.
- [13] Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U. Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J Exp Med* 2002;195:245–57.
- [14] Rosenfeld ME, Yla-Herttuala S, Lipton BA, Ord VA, Witztum JL, Steinberg D. Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. *Am J Pathol* 1992;140:291–300.
- [15] Tintut Y, Parhami F, Boström K, Jackson SM, Demer LL. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. *J Biol Chem* 1998;273:7547–53.
- [16] Tintut Y, Alfonso Z, Saini T, Radcliff K, Watson K, Bostrom K, et al. Multilineage potential of cells from the artery wall. *Circulation* 2003;108:2505–10.
- [17] Seager Danciger J, Lutz M, Hama S, Cruz D, Castrillo A, Lázaro J, et al. Method for large scale isolation, culture and cryopreservation of human monocytes suitable for chemotaxis, cellular adhesion assays, macrophage and dendritic cell differentiation. *J Immunol Methods* 2004;288:123–34.
- [18] Tintut Y, Parhami F, Tsingotjidou A, Tetradis S, Territo M, Demer LL. 8-Isoprostaglandin E2 enhances receptor-activated NFkappa B ligand (RANKL)-dependent osteoclastic potential of marrow hematopoietic precursors via the cAMP pathway. *J Biol Chem* 2002;277:14221–6.
- [19] Ek-Rylander B, Flores M, Wendel M, Heinegard D, Andersson G. Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. *J Biol Chem* 1994;269:14853–6.
- [20] Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. *J Biol Chem* 2001;276:20659–72.
- [21] Udagawa N, Horwood NJ, Elliott J, Mackay A, Owens J, Okamura H, et al. Interleukin-18 is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast formation. *J Exp Med* 1997;185:1005–12.
- [22] Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998;12:1260–8.