

Role of Osteoprotegerin and Its Ligands and Competing Receptors in Atherosclerotic Calcification

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Vascular calcification significantly impairs cardiovascular physiology, and its mechanism is under investigation. Many of the same factors that modulate bone osteogenesis, including cytokines, hormones, and lipids, also modulate vascular calcification, acting through many of the same transcription factors. In some cases, such as for lipids and cytokines, the net effect on calcification is positive in the artery wall and negative in bone. The mechanism for this reciprocal relation is not established. A recent series of reports points to the possibility that two bone regulatory factors, receptor activator of NF- κ B ligand (RANKL) and its soluble decoy receptor, osteoprotegerin (OPG), govern vascular calcification and may explain the phenomenon. Both RANKL and OPG are widely accepted as the final common pathway for most factors and processes affecting bone resorption. Binding of RANKL to its cognate receptor RANK induces NF- κ B signaling, which stimulates osteoclastic differentiation in preosteoclasts and induces bone morphogenetic protein (BMP-2) expression in chondrocytes. A role for RANKL and its receptors in vascular calcification is supported by several findings: a vascular calcification phenotype in mice genetically deficient in OPG; an increase in expression of RANKL, and a decrease in expression of OPG, in calcified arteries; clinical associations between coronary disease and serum OPG and RANKL levels; and RANKL induction of calcification and osteoblastic differentiation in valvular myofibroblasts.

Key words: vascular calcification, osteoprotegerin, biomineralization, atherosclerosis

Cardiovascular disease, most often due to atherosclerosis, is the leading cause of mortality and morbidity in the United States,¹ and osteoporosis is the most common, disease of bone.² Atherosclerosis, vascular calcification, and osteoporosis correlate in an age-independent manner with one another and with hyperlipidemia and osteoprotegerin levels.^{3–11} The mechanisms for these links are not understood.

Vascular calcification is present in half of middle-aged individuals and in 90% of those over age 70 years.¹² It particularly affects patients with athero-

sclerosis, diabetes, and end-stage renal disease.¹³ It contributes substantially to cardiovascular morbidity and mortality by destroying the artery's normal elastance, leading to systolic hypertension, left ventricular hypertrophy, diastolic hypotension, coronary insufficiency, congestive heart failure, aortic stenosis, and, potentially, plaque rupture.^{12,14–21}

Intimal and Medial Calcification

Intimal (atherosclerotic) calcification colocalizes with atherosclerosis and correlates with plaque burden and cardiovascular events.^{22–24} Medial calcification occurs independently of atherosclerosis, primarily in patients with end-stage renal disease and diabetes. Intimal calcification appears to be driven by inflammatory factors such as cytokines and atherogenic lipids.^{25,26} In contrast, medial calcification appears to be promoted by a variety of factors, including high leptin levels,²⁷ hyperphosphatemia,^{28–30} uremia,³¹ hypercalcemia,³² hyperparathyroidism,³³ warfarin,³⁴ 1- α ,25-dihydroxyvitamin D₃,^{21,34–36} and pyrophosphate deficiency.³⁷ In a given individual, both intimal and medial vascular calcification may occur, and more than one mechanism may be operative. The final common pathway, if any, is not known.

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Similarity to Osteogenesis

Atherosclerotic calcification shares many features with embryonic osteogenesis. It involves the same histologic transitional stages, including amorphous mineral remodeling, invasion of preosteoblasts, and, finally, formation of full bone tissue with trabecular architecture.^{38–41} The full complement of osteogenic factors and bone proteins is present in atherosclerotic calcification, including bone morphogenetic protein 2 (BMP-2), which governs mesenchymal lineage acquisition via Smad-dependent signals, *Msx-2*, *Cbfa1*, and downstream effector molecules such as osteopontin, matrix γ -carboxyglutamic acid protein (MGP), and bone sialoprotein.^{42–49} Thus, calcified atherosclerotic lesions resemble bone in many ways.

In Vitro Vascular Osteogenesis

Vascular smooth muscle cells (SMCs) and closely related cells, such as microvascular pericytes, calcifying vascular cells, adventitial myofibroblasts, and cardiac valvular myofibroblasts, express osteoblastic differentiation markers and produce the bone mineral hydroxyapatite, with a characteristic time course^{44,46,48,50–55} nearly identical to that in skeletal bone cell osteogenesis.⁵⁶ The process is governed by BMP-2 and master osteogenic transcriptional regulators, such as *Cbfa1* and *Msx-2*.⁵⁷ It is modulated by atherogenic lipids and lipoproteins,²⁵ lipoprotein receptor-related protein 5 and Wnt pathway members,⁴⁹ transforming growth factor (TGF)- β ,⁵⁸ cyclic adenosine monophosphate agonists,⁴⁸ 1- α ,25-dihydroxyvitamin D₃,⁵⁹ warfarin,³⁴ tumor necrosis factor (TNF)- α ,^{60,61} and monocyte coculture.⁶² It is inhibited by high-density lipoprotein,⁶³ collagen IV and laminin,⁶⁴ osteopontin,⁶⁵ extracellular phosphate,⁵¹ parathyroid hormone (PTH)-related peptide,⁵⁹ and PTH itself, which, in turn, down-regulates *Msx-2*.⁶⁶

Bone-Vascular Connection

In vivo, bone and vascular mineralization may be linked through endocrinologic mechanisms or they may be independent, depending on the circumstances. In metastatic vascular calcification, as occurs in patients with osteolytic cancer or vitamin D overdose, vascular calcification results from excess serum calcium. Extremely rapid bone resorption, beyond that of ordinary osteoporosis, can overcome the normally tight endocrine regulation of serum calcium. As serum calcium increases, the serum calcium-phosphate product eventually exceeds the threshold required for spontaneous crystallization in many tissues, including

arteries.⁶⁷ High-dose vitamin D has been used to produce experimental vascular calcification in rodents, and factors that arrest the bone resorption consequently inhibit the vascular calcification.^{68,69} Because of this potential effect of excess bone resorption on vascular calcification, genetic interventions to assess the direct effects of genes on vascular calcification may need to be vascular specific.

In many mouse models of vascular calcification, including mice deficient in fibrillin, MGP, and carbonic anhydrase, the skeleton is either normal or hypermineralized, indicating that vascular calcification does not require high bone-resorptive activity.^{70–72} Even some mouse models with both vascular calcification and osteoporosis have normal serum calcium, suggesting that the bone phenotype is not sufficiently severe to induce metastatic calcification.

In atherosclerotic calcification, serum calcium and phosphate are not elevated, and the association with osteoporosis may be attributable to a common cause such as hyperlipidemia and/or inflammation, which have reciprocal effects on osteogenic differentiation in bone versus vascular tissues.^{25,73,74} Although the reciprocal response to inflammation recapitulates the reciprocal response to chronic infection (calcification in soft tissue versus osteolysis in bone), the signaling mechanism is not known. One possibility is that the reciprocal response is mediated via RANKL activity, which has been shown to promote osteoclastogenesis in bone and osteogenesis in SMCs.

RANKL and OPG in Bone

Regulation of receptor activator of nuclear factor- κ B (RANK) is now considered the final common pathway for most mediators of bone catabolism.⁷⁵ Binding of RANK to its ligand, RANKL, is essential for the development and activation of osteoclasts.⁷⁶ In bone, RANKL is expressed as a transmembrane protein on osteoblasts and marrow stromal cells, and when it binds to RANK on the surface of preosteoclasts, it induces osteoclastic differentiation and maturation.⁷⁷ OPG blocks interaction of RANKL with RANK, thus preventing osteoclast differentiation and bone resorption.^{77–79} High RANKL states cause osteoporosis owing to excess resorption by osteoclasts.

RANKL expression in osteoblast lineage cells is low at baseline,⁸⁰ but it is up-regulated by osteolytic factors such as inflammatory cytokines, 1,25-dihydroxyvitamin D₃, and dexamethasone in regions of bone that are undergoing rapid turnover or osteolysis. These osteolytic factors also inhibit OPG expression.^{81–87} RANKL can be cleaved by the metalloproteinase TNF- α converting

enzyme to release a soluble form (sRANKL). Like other TNF receptor family members, RANK acts primarily via nuclear factor (NF)- κ B and Jun-N-terminal kinase activation in preosteoclasts.⁸⁴ It also activates p44 and p38 mitogen-activated protein kinases, as well as the phosphatidylinositol-3 kinase pathway.⁸⁸

As an aside, although RANKL, RANK, and OPG recently were assigned new nomenclature, “tumor necrosis factor superfamily member 11” and “TNF receptor superfamily members 11a and 11b,” respectively, the simpler nomenclature used in this review is generally recommended for the bone literature.⁸⁹

Mouse Models

In mouse models lacking RANKL/RANK signaling, such as RANKL-deficient,⁷⁶ RANK-deficient,⁹⁰ and OPG-overexpressing⁷⁸ mice, the lack of osteoclastic resorption results in rigid and overmineralized “osteopetrotic” bone with limited marrow cavities. No vascular phenotype has been reported, and no vascular findings are expected at baseline if RANKL is the operative factor.

In contrast, mouse models with unopposed RANKL activity develop osteoporosis. Soluble RANKL treatment induces overactive osteoclastic resorption in mice.⁷⁷ Mice lacking OPG develop osteoporosis.⁹¹ Mice with ubiquitous RANKL overexpression die at the late fetal stage.⁹² A transgenic mouse expressing soluble RANKL in the liver (driven by a serum amyloid P promoter, *SAP-rankl^{tg}*) and released in the circulation has osteoporosis, hypercalcemia, and abnormalities of lymphocytes, monocytes, and dendritic cells.⁹² In these mice, the indirect effects of the bone phenotype would also confound interpretation of a vascular phenotype.

RANKL and OPG in the Artery Wall

All three members of the RANKL/OPG/RANK system are expressed in vascular cells:

1. RANK is expressed in cultured umbilical and microvascular endothelial cells (EC).^{88,93} Although not found in normal arteries, it is expressed in the calcified arteries of *opg*^(-/-) mice.⁹⁴
2. RANKL is not expressed at baseline in cultured endothelial cells (ECs) or SMCs,^{88,95} but it is induced in ECs by inflammatory cytokines and factors from actively remodeling bone such as TGF- β .^{11,96} RANKL immunoreactivity is not found in normal mouse arteries,⁹⁴ but it is present in calcified vascular tissue in humans^{47,55,97} and in *opg*^(-/-) mice.⁹⁴

3. OPG is expressed by cultured arterial ECs^{11,93,98} and SMCs.^{11,95,99} OPG immunoreactivity is found in calcified human atherosclerosis,⁹⁷ but, in contrast with RANKL, it is expressed at higher levels in the normal artery wall.^{94,100}

RANKL and OPG in Vascular Calcification

The mechanism of vascular calcification in *opg*^(-/-) mice is not known. Given that these mice have normal serum calcium and the vascular calcification is not reversed by correction of the osteoporosis,^{91,94} it is most likely not attributable (directly) to the osteoporosis. Interestingly, in vitro evidence has shown that RANKL induces osteoblastic differentiation and mineralization of cultured cardiac valvular (myofibroblastic) cells.⁵⁵ The molecular regulatory mechanism for this phenomenon is unknown. In osteoclasts, NF- κ B is a major signaling mechanism for RANKL activation of RANK. Interestingly, NF- κ B response elements have been identified in the 5' regulatory region of BMP-2, and NF- κ B induces BMP-2 in chondrocytes.^{101,102} Chondrocytes from p50/p52 double mutants showed decreased BMP-2.¹⁰¹ One possibility is that RANKL induces vascular osteogenesis by activating NF- κ B signaling and up-regulating BMP-2 in SMCs.

Whereas OPG deficiency leads to mineralization in the artery wall, it leads to demineralization in bone. A possible explanation for this reciprocal effect is the difference in the availability of osteoclast progenitor cells. Normal bone has a rich supply of preosteoclasts from the marrow, whereas normal artery wall has few, if any. Thus, in bone, but not artery wall, unopposed RANKL activity stimulates osteoclastic differentiation and resorption. Given that the net result is an osteoporotic phenotype, the osteoclastogenic effect of RANKL apparently overrides any possible promineralization effect of RANKL in bone, where preosteoclasts are abundant. But, in the artery wall, a promineralization effect of RANKL may be unmasked by the lack of preosteoclasts.

TRAIL in Vascular Calcification

OPG also serves as a decoy receptor for another ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is expressed in the artery wall, together with its receptor (TRAILR), which is encoded by the gene *dr5*.¹⁰³ Previous studies have not linked TRAIL to vascular calcification. However, TRAIL is known to induce apoptosis in certain cells, including ECs.¹⁰⁴ It is possible that TRAIL promotes vascular calcification by inducing apoptosis. Apoptotic bodies may serve as nucleation sites for crystal formation similar to matrix vesicles, and Proudfoot

and colleagues showed evidence of apoptosis in vascular cell calcification.¹⁰⁵ Another mechanism by which TRAIL could contribute to vascular calcification is by occupying OPG sites, which would indirectly increase the effective availability of RANKL.

Clinical Cardiovascular Disease

Recent animal and clinical studies suggest that serum OPG and RANKL levels are associated with cardiovascular disease. Serum OPG correlates positively with cardiovascular disease and mortality,^{106–109} whereas serum RANKL levels correlate inversely with cardiovascular disease.¹⁰⁹ Although this may appear to be paradoxical, it may reflect an incomplete compensatory response. It is not unusual for serum levels of protective factors to be elevated in compensatory response to the diseases they prevent. For example, matrix Gla protein, which is recognized as an inhibitory factor in vascular calcification, is increased in calcified lesions, and serum OPG is elevated in a variety of conditions involving persistent immune activation as a partial compensatory response to proinflammatory activity of other members of the TNF family.¹¹⁰ Similarly, natriuretic factors are elevated in heart failure, yet they are successfully used as treatment for heart failure. As another consideration, OPG could be selectively increased in the circulation and not in the artery wall by factors that selectively induce OPG expression in the endothelium. This is because ECs segregate and store OPG in their Weibel-Palade bodies, which are selectively exocytosed into the circulation. Thus, the serum levels of OPG may be increased independently of levels in the medial layer of the artery wall.

Summary

The RANKL/OPG/RANK system governs biomineralization of the skeleton, and growing evidence suggests that it has a role, possibly reciprocal, in biomineralization of the artery wall. Overall, the evidence clearly and consistently indicates that RANKL stimulates differentiation and maturation of osteoclast progenitor cells and that the net effect of high RANKL activity on bone in vivo is excess resorption and osteoporosis. Consequently, deficiency of its decoy receptor, OPG, would have the same effects, and the net effect of reduced OPG activity on bone in vivo is osteoporosis. In contrast, recent evidence now suggests that RANKL also stimulates osteoblastic differentiation and mineralization in vascular cells. Atherosclerotic calcification may result in part from the unopposed activity of RANKL. Given that normal arteries lack osteoclast progenitor cells, high RANKL activity would

be expected to stimulate calcification in the vasculature. This would explain the finding of aortic calcification in the OPG-deficient mice. Given that inflammatory factors, as are present in atherosclerotic lesions, promote RANKL and inhibit OPG, it is possible that the reciprocal effects of hyperlipidemia on bone and artery are mediated through disturbance of the RANKL/OPG balance toward a RANKL-dominant state.

References

1. Glass CK, Witztum JL. Atherosclerosis. The road ahead. *Cell* 2001;104:503–16.
2. Brown SA, Rosen CJ. Osteoporosis. *Med Clin North Am* 2003;87:1039–63.
3. Hirose K, Tomiyama H, Okazaki R, et al. Increased pulse wave velocity associated with reduced calcaneal quantitative osteo-sono index: possible relationship between atherosclerosis and osteopenia. *J Clin Endocrinol Metab* 2003;88:2573–8.
4. Tanko LB, Bagger YZ, Christiansen C. Low bone mineral density in the hip as a marker of advanced atherosclerosis in elderly women. *Calcif Tissue Int* 2003;73:15–20.
5. Yamaguchi T, Sugimoto T, Yano S, et al. Plasma lipids and osteoporosis in postmenopausal women. *Endocr J* 2002;49:211–7.
6. Jie KG, Bots ML, Vermeer C, et al. Vitamin K status and bone mass in women with and without aortic atherosclerosis: a population-based study. *Calcif Tissue Int* 1996;59:352–6.
7. Pennisi P, Signorelli SS, Riccobene S, et al. Low bone density and abnormal bone turnover in patients with atherosclerosis of peripheral vessels. *Osteoporos Int* 2004;15:389–95.
8. Hak AE, Pols HA, van Hemert AM, et al. Progression of aortic calcification is associated with metacarpal bone loss during menopause: a population-based longitudinal study. *Arterioscler Thromb Vasc Biol* 2000;20:1926–31.
9. Adami S, Braga V, Zamboni M, et al. Relationship between lipids and bone mass in 2 cohorts of healthy women and men. *Calcif Tissue Int* 2004;74:136–42.
10. Allison MA, Wright M, Tiefenbrun J. The predictive power of low-density lipoprotein cholesterol for coronary calcification. *Int J Cardiol* 2003;90:281–9.
11. Collin-Osdoby P, Rothe L, Anderson F, et al. Receptor activator of NF- κ 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.
12. Newman AB, Naydeck BL, Sutton-Tyrrell K, et al. Coronary artery calcification in older adults to age 99: prevalence and risk factors. *Circulation* 2001;104:2679–84.
13. Moe SM, O'Neill KD, Reslerova M, et al. Natural history of vascular calcification in dialysis and transplant patients. *Nephrol Dial Transplant* 2004;19:2387–93.
14. Ohtsuka S, Kakiyama M, Watanabe H, Sugishita Y. Chronically decreased aortic distensibility causes deterioration of coronary perfusion during increased left ventricular contraction. *J Am Coll Cardiol* 1994;24:1406–14.

15. Iribarren C, Sidney S, Sternfeld B, Browner WS. Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke and peripheral vascular disease. *JAMA* 2000;283:2810–5.
16. Mitchell JR, Adams JH. Aortic size and aortic calcification. A necropsy study. *Atherosclerosis* 1977;27:437–46.
17. Dart AM, Lacombe F, Yeoh JK, et al. Aortic distensibility in patients with isolated hypercholesterolaemia, coronary artery disease, or cardiac transplant. *Lancet* 1991;338:270–3.
18. Niederhoffer N, Bobryshev YV, Lartaud-Idjouadiene I, et al. Aortic calcification produced by vitamin D3 plus nicotine. *J Vasc Res* 1997;34:386–98.
19. Watanabe H, Ohtsuka S, Kakihana M, Sugishita Y. Coronary circulation in dogs with an experimental decrease in aortic compliance. *J Am Coll Cardiol* 1993;21:1497–506.
20. Simonson E, Nakagawa K. Effect of age on pulse wave velocity and “aortic ejection time” in healthy men and in men with coronary artery disease. *Circulation* 1960;22:126–9.
21. Demer LL. Effect of calcification on in vivo mechanical response of rabbit arteries to balloon dilation. *Circulation* 1991;83:2083–93.
22. Rumberger JA, Simons DB, Fitzpatrick LA, et al. Coronary artery calcium area by electron-beam computed tomography and coronary atherosclerotic plaque area. A histopathologic correlative study. *Circulation* 1995;92:2157–62.
23. Yamamoto H, Imazu M, Hattori Y, et al. Predicting angiographic narrowing $>$ or $=$ 50% in diameter in each of the three major arteries by amounts of calcium detected by electron beam computed tomographic scanning in patients with chest pain. *Am J Cardiol* 1998;81:778–80.
24. Arad Y, Spadaro LA, Goodman K, et al. Prediction of coronary events with electron beam computed tomography. *J Am Coll Cardiol* 2000;36:1253–60.
25. Parhami F, Morrow AD, Balucan J, et al. Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. *Arterioscler Thromb Vasc Biol* 1997;17:680–7.
26. Rajamannan NM, Sangiorgi G, Springett M, et al. Experimental hypercholesterolemia induces apoptosis in the aortic valve. *J Heart Valve Dis* 2001;10:371–4.
27. Reilly MP, Iqbal N, Schutta M, et al. Plasma leptin levels are associated with coronary atherosclerosis in type 2 diabetes. *J Clin Endocrinol Metab* 2004;89:3872–8.
28. Jono S, McKee MD, Murry CE, et al. Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 2000;87:E10–7.
29. Giachelli CM. Vascular calcification: in vitro evidence for the role of inorganic phosphate. *J Am Soc Nephrol* 2003;14(Suppl 4):300–4.
30. Giachelli CM, Jono S, Shioi A, et al. Vascular calcification and inorganic phosphate. *Am J Kidney Dis* 2001;38(Suppl 1):S34–7.
31. Chen NX, O’Neill KD, Duan D, Moe SM. Phosphorus and uremic serum up-regulate osteopontin expression in vascular smooth muscle cells. *Kidney Int* 2002;62:1724–31.
32. Farzaneh-Far A, Proudfoot D, Weissberg PL, Shanahan CM. Matrix gla protein is regulated by a mechanism functionally related to the calcium-sensing receptor. *Biochem Biophys Res Commun* 2000;277:736–40.
33. Goodman WG. Vascular calcification in chronic renal failure. *Lancet* 2001;358:1115–6.
34. Price PA, Faus SA, Williamson MK. Warfarin-induced artery calcification is accelerated by growth and vitamin D. *Arterioscler Thromb Vasc Biol* 2000;20:317–27.
35. Eisenstein R, Ellis H, Rosato J. In vitro studies of vitamin D-induced aortic calcification. *Proc Soc Exp Biol Med* 1969;132:58–62.
36. Fleckenstein-Grun G, Thimm F, Frey M, Matyas S. Progression and regression by verapamil of vitamin D3-induced calcific medial degeneration in coronary arteries of rats. *J Cardiovasc Pharmacol* 1995;26:207–13.
37. Rutsch F, Ruf N, Vaingankar S, et al. Mutations in ENPP1 are associated with ‘idiopathic’ infantile arterial calcification. *Nat Genet* 2003;34:379–81.
38. Towler DA. Angiogenesis and marrow stromal cell fates: roles in bone strength. *Osteoporos Int* 2003;14(Suppl 5):46–53.
39. Hunt JL, Fairman R, Mitchell ME, et al. Bone formation in carotid plaques: a clinicopathological study. *Stroke* 2002;33:1214–9.
40. Mohler ER III, Gannon F, Reynolds C, et al. Bone formation and inflammation in cardiac valves. *Circulation* 2001;103:1522–8.
41. Feldman T, Glagov S, Carroll JD. Restenosis following successful balloon valvuloplasty: bone formation in aortic valve leaflets. *Cathet Cardiovasc Diagn* 1993;29:1–7.
42. Tanimura A, McGregor DH, Anderson HC. Calcification in atherosclerosis. I. Human studies. *J Exp Pathol* 1986;2:261–73.
43. Giachelli CM, Bae N, Almeida M, et al. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest* 1993;92:1686–96.
44. Bostrom K, Watson KE, Horn S, et al. Bone morphogenetic protein expression in human atherosclerotic lesions. *J Clin Invest* 1993;91:1800–9.
45. Schluesener HJ, Meyermann R. Immunolocalization of BMP-6, a novel TGF- β -related cytokine, in normal and atherosclerotic smooth muscle cells. *Atherosclerosis* 1995;113:153–6.
46. 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.
47. Dhore CR, Cleutjens JJP, Lutgens E, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 2001;21:1998–2003.
48. Tintut Y, Parhami F, Bostrom K, et al. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. *J Biol Chem* 1998;273:7547–53.
49. Shao JS, Cheng SL, Pingsterhaus JM, et al. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. *J Clin Invest* 2005;115:1210–20.

50. Doherty MJ, Ashton BA, Walsh S, et al. Vascular pericytes express osteogenic potential in vitro and in vivo. *J Bone Miner Res* 1998;13:828–38.
51. Shioi A, Nishizawa Y, Jono S, et al. Beta-glycerophosphate accelerates calcification in cultured bovine vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1995;15:2003–9.
52. Mohler ER III, Chawla MK, Chang AW, et al. Identification and characterization of calcifying valve cells from human and canine aortic valves. *J Heart Valve Dis* 1999;8:254–60.
53. Rajamannan NM, Subramaniam M, Rickard D, et al. Human aortic valve calcification is associated with an osteoblast phenotype. *Circulation* 2003;107:2181–4.
54. Steitz SA, Speer MY, Curinga G, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circ Res* 2001;89:1147–54.
55. Kaden JJ, Bickelhaupt S, Grobholz R, et al. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulate aortic valve calcification. *J Mol Cell Cardiol* 2004;36:57–66.
56. Lian JB, Stein GS. Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Crit Rev Oral Biol Med* 1992;3:269–305.
57. Towler DA, Bidder M, Latifi T, et al. Diet-induced diabetes activates an osteogenic gene regulatory program in the aortas of low density lipoprotein receptor-deficient mice. *J Biol Chem* 1998;273:30427–34.
58. Watson SA, Robinson KE, McWilliams D, et al. Transforming growth factor-alpha-mediated growth pathways in human gastro-intestinal cell lines in relation to the gastrin autocrine pathway. *Int J Cancer* 2000;87:20–8.
59. Jono S, Nishizawa Y, Shioi A, Morii H. Parathyroid hormone-related peptide as a local regulator of vascular calcification. Its inhibitory action on in vitro calcification by bovine vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1997;17:1135–42.
60. Tintut Y, Patel J, Parhami F, Demer LL. Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. *Circulation* 2000;102:2636–42.
61. Shioi A, Katagi M, Okuno Y, Mori K, et al. Induction of bone-type alkaline phosphatase in human vascular smooth muscle cells: roles of tumor necrosis factor-alpha and oncostatin M derived from macrophages. *Circ Res* 2002;91:9–16.
62. Tintut Y, Patel J, Territo M, Saini T, et al. Monocyte/macrophage regulation of vascular calcification in vitro. *Circulation* 2002;105:650–5.
63. Parhami F, Basseri B, Hwang J, et al. High-density lipoprotein regulates calcification of vascular cells. *Circ Res* 2002;91:570–6.
64. Watson KE, Parhami F, Shin V, Demer LL. Fibronectin and collagen I matrixes promote calcification of vascular cells in vitro, whereas collagen IV matrix is inhibitory. *Arterioscler Thromb Vasc Biol* 1998;18:1964–71.
65. Wada T, McKee MD, Steitz S, Giachelli CM. Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. *Circ Res* 1999;84:166–78.
66. Shao JS, Cheng SL, Charlton-Kachigian N, et al. Teriparatide (human parathyroid hormone (1–34)) inhibits osteogenic vascular calcification in diabetic low density lipoprotein receptor-deficient mice. *J Biol Chem* 2003;278:50195–202.
67. Abe M, Segami H, Wakasa H. Hypercalcemia and metastatic calcification in adult T-cell leukemia—pathogenesis of hypercalcemia. *Fukushima J Med Sci* 1985;31:85–97.
68. Price PA, June HH, Buckley JR, Williamson MK. Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. *Arterioscler Thromb Vasc Biol* 2001;21:1610–6.
69. Price PA, Faus SA, Williamson MK. Bisphosphonates alendronate and ibandronate inhibit artery calcification at doses comparable to those that inhibit bone resorption. *Arterioscler Thromb Vasc Biol* 2001;21:817–24.
70. Qiao JH, Fishbein MC, Demer LL, Lusis AJ. Genetic determination of cartilaginous metaplasia in mouse aorta. *Arterioscler Thromb Vasc Biol* 1995;15:2265–72.
71. Qiao JH, Xie PZ, Fishbein MC, et al. Pathology of atheromatous lesions in inbred and genetically engineered mice. Genetic determination of arterial calcification. *Arterioscler Thromb* 1994;14:1480–97.
72. Spicer SS, Lewis SE, Tashian RE, Schulte BA. Mice carrying a CAR-2 null allele lack carbonic anhydrase II immunohistochemically and show vascular calcification. *Am J Pathol* 1989;134:947–54.
73. Parhami F, Jackson SM, Tintut Y, et al. Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. *J Bone Miner Res* 1999;14:2067–78.
74. Parhami F, Tintut Y, Beamer WG, et al. Atherogenic high-fat diet reduces bone mineralization in mice. *J Bone Miner Res* 2001;16:182–8.
75. Hofbauer LC, Khosla S, Dunstan CR, et al. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res* 2000;15:2–12.
76. Kong YY, Yoshida H, Sarosi I, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999;397:315–23.
77. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;93:165–76.
78. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;89:309–19.
79. Yasuda H, Shima N, Nakagawa N, et al. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* 1998;139:1329–37.
80. Bu R, Borysenko CW, Li Y, et al. Expression and function of TNF-family proteins and receptors in human osteoblasts. *Bone* 2003;33:760–70.
81. Nakagawa N, Yasuda H, Yano K, et al. Basic fibroblast growth factor inhibits osteoclast formation induced by 1alpha,25-dihydroxyvitamin D(3) through suppressing the

- production of osteoclast differentiation factor. *Biochem Biophys Res Commun* 1999;265:45–50.
82. Yasuda H, Shima N, Nakagawa N, et al. A novel molecular mechanism modulating osteoclast differentiation and function. *Bone* 1999;25:109–13.
 83. Matsuzaki K, Udagawa N, Takahashi N, et al. Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochem Biophys Res Commun* 1998;246:199–204.
 84. Hsu H, Lacey DL, Dunstan CR, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A* 1999;96:3540–5.
 85. Tsukii K, Shima N, Mochizuki S, et al. Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha,25-dihydroxyvitamin D₃, prostaglandin E₂, or parathyroid hormone in the micro-environment of bone. *Biochem Biophys Res Commun* 1998;246:337–41.
 86. Brandstrom H, Bjorkman T, Ljunggren O. Regulation of osteoprotegerin secretion from primary cultures of human bone marrow stromal cells. *Biochem Biophys Res Commun* 2001;280:8315.
 87. Penno H, Silfversward CJ, Frost A, et al. Osteoprotegerin secretion from prostate cancer is stimulated by cytokines, in vitro. *Biochem Biophys Res Commun* 2002;293:451–5.
 88. Kim HH, Shin HS, Kwak HJ, et al. RANKL regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. *FASEB J* 2003;17:2163–5.
 89. American Society for Bone and Mineral Research President's Committee on Nomenclature. Proposed standard nomenclature for new tumor necrosis factor family members involved in the regulation of bone resorption. *J Bone Miner Res* 2000;15:2293–6.
 90. Dougall WC, Glaccum M, Charrier K, et al. RANK is essential for osteoclast and lymph node development. *Genes Dev* 1999;13:2412–24.
 91. Bucay N, Sarosi I, Dunstan CR, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998;12:1260–8.
 92. Mizuno A, Kanno T, Hoshi M, et al. Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. *J Bone Miner Metab* 2002;20:337–44.
 93. Kim YM, Kim YM, Lee YM, et al. TNF-related activation-induced cytokine (TRANCE) induces angiogenesis through the activation of Src and phospholipase C (PLC) in human endothelial cells. *J Biol Chem* 2002;277:6799–805.
 94. Min H, Morony S, Sarosi I, et al. Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. *J Exp Med* 2000;192:463–74.
 95. Zhang J, Fu M, Myles D, et al. PDGF induces osteoprotegerin expression in vascular smooth muscle cells by multiple signal pathways. *FEBS Lett* 2002;521:180–4.
 96. Ishida A, Fujita N, Kitazaqa R, Tsuruo T. Transforming growth factor-beta induces expression of receptor activator of NF-kappa B ligand in vascular endothelial cells derived from bone. *J Biol Chem* 2002;277:26217–24.
 97. Schoppet M, Al-Fakhri N, Franke FE, et al. Localization of osteoprotegerin, tumor necrosis factor-related apoptosis-inducing ligand, and receptor activator of nuclear factor-kappaB ligand in Monckeberg's sclerosis and atherosclerosis. *J Clin Endocrinol Metab* 2004;89:4104–12.
 98. Chikatsu N, Takeuchi Y, Fukumoto S, et al. Clonal endothelial cells produce humoral factors that inhibit osteoclast-like cell formation in vitro. *Endocr J* 2002;49:439–47.
 99. Hofbauer LC, Heufelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J Mol Med* 2001;79:243–53.
 100. Tyson KL, Reynolds JL, McNair R, et al. Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification. *Arterioscler Thromb Vasc Biol* 2003;23:489–94.
 101. Feng JQ, Xing L, Zhang JH, et al. NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. *J Biol Chem* 2003;278:29130–5.
 102. Li X, Massa PE, Hanidu A, et al. IKKalpha, IKKbeta, and NEMO/IKKgamma are each required for the NF-kappa B-mediated inflammatory response program. *J Biol Chem* 2002;277:45129–40.
 103. Michowitz Y, Goldstein E, Roth A, et al. The involvement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in atherosclerosis. *J Am Coll Cardiol* 2005;45:1018–24.
 104. Pritzker LB, Scatena M, Giachelli CM. The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell* 2004;15:2834–41.
 105. Proudfoot D, Skepper JN, Hegyi L, et al. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res* 2000;87:1055–62.
 106. Browner WS, Lui LY, Cummings SR. Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. *J Clin Endocrinol Metab* 2001;86:631–7.
 107. Kiechl S, Schett G, Wenning G, et al. Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004;109:2175–80.
 108. Jono S, Ikari Y, Shioi A, et al. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. *Circulation* 2002;106:1192–4.
 109. Schoppet M, Sattler AM, Schaefer JR, et al. Increased osteoprotegerin serum levels in men with coronary artery disease. *J Clin Endocrinol Metab* 2003;88:1024–8.
 110. Ueland T, Bollerslev J, Godang K, et al. Increased serum osteoprotegerin in disorders characterized by persistent immune activation or glucocorticoid excess—possible role in bone homeostasis. *Eur J Endocrinol* 2001;145:685–90.