Runx2-Upregulated Receptor Activator of Nuclear Factor κB Ligand in Calcifying Smooth Muscle Cells Promotes Migration and Osteoclastic Differentiation of Macrophages

Chang Hyun Byon, Yong Sun, Jianfeng Chen, Kaiyu Yuan, Xia Mao, Jack M. Heath, Peter G. Anderson, Yin Tintut, Linda L. Demer, Deli Wang, Yabing Chen

Objective—Clinical and experimental studies demonstrate the important roles of vascular smooth muscle cells (VSMC) in the pathogenesis of atherosclerosis. We have previously determined that the osteogenic transcription factor Runx2 is essential for VSMC calcification. The present study characterized Runx2-regulated signals and their potential roles in vascular calcification.

Methods and Results—In vivo studies with atherogenic apolipoprotein E (apoE)−/− mice demonstrated that increased oxidative stress was associated with upregulation of Runx2 and receptor activator of nuclear factor κB ligand (RANKL), which colocalized in the calcified atherosclerotic lesions and were juxtaposed to infiltrated macrophages and osteoclast-like cells that are positively stained for an osteoclast marker, tartrate-resistant acid phosphatase. Mechanistic studies using RNA interference, a luciferase reporter system, chromatin immunoprecipitation, and electrophoretic mobility shift assays indicated that Runx2 regulated the expression of RANKL via a direct binding to the 5′-flanking region of the RANKL. Functional characterization revealed that RANKL did not induce VSMC calcification, nor was RANKL required for oxidative stress–induced VSMC calcification. Using a coculture system, we demonstrated that VSMC-expressed RANKL induced migration as well as differentiation of bone marrow-derived macrophages into multinucleated, tartrate-resistant acid phosphatase–positive osteoclast-like cells. These effects were inhibited by the RANKL antagonist osteoprotegerin and with VSMC deficient in Runx2 or RANKL.

Conclusion—We demonstrate that Runx2 directly binds to the promoter and controls the expression of RANKL, which mediates the crosstalk between calcifying VSMC and migration and differentiation of macrophages into osteoclast-like cells in the atherosclerotic lesions. Our studies provide novel mechanistic insights into the regulation and function of VSMC-derived RANKL in the pathogenesis of atherosclerosis and vascular calcification. (Arterioscler Thromb Vasc Biol. 2011;31:1387-1396.)

Key Words: calcification ■ RANKL ■ Runx2 ■ migration ■ osteoclastogenesis

Atherosclerosis is characterized by the formation of raised, often calcified lesions in the arterial intima, leading to narrowing of the vessel lumen. Vascular calcification reduces arterial compliance and therefore represents a key factor in the hemodynamic consequences of atherosclerosis. Accumulating evidence has demonstrated that vascular calcification is a cell-regulated process with many similarities to the mechanisms of embryonic osteogenesis, not simply a passive precipitation of crystals. Vascular calcification reflects an osteochondrogenic transformation of vascular smooth muscle cells (VSMC), which is associated with increased expression of growth factors, matrix proteins, and other bone-related markers.

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High levels of oxidative stress have been linked with increased prevalence of arterial calcification in hypercholesterolemia, hypertension, diabetes mellitus, and end-stage renal disease. Therefore, the generation of oxidative stress is considered to be a common final pathway by which these factors predispose to vascular calcification. Oxidative stress in atherosclerosis may lead to generation of reactive oxygen species by vascular cells, endothelial dysfunction, and plaque disruption. We have previously reported that H2O2, a cell-permeable reactive oxygen species, induces VSMC calcification in vitro. Receptor activator of nuclear factor-κB ligand (RANKL) is a member of the tumor necrosis factor superfamily, which is the key regulator for osteoclast formation. RANKL is highly expressed in lymphoid tissues and trabecular bone, particularly in areas associated with active bone remodeling or inflammatory osteolysis. In normal vessels and noncalcified arteries or valves, RANKL is frequently undetectable. By contrast, RANKL has been reported to be...
upregulated and expressed in calcified atherosclerotic lesions. Consistently, mice that lack of osteoprotegerin (OPG), the decoy receptor for RANKL, exhibit increased vascular calcification. Inhibition of RANKL by human osteoclast-like cell line and primary mouse bone marrow macrophages from wild-type (WT) C57BL6 mice. Details of materials and experimental procedures are given in the Supplemental Methods section, available online at http://atvb.ahajournals.org.

Methods
Primary mouse aortic smooth muscle cells (VSMC, passages 3 to 5) and atherogenic apolipoprotein E (ApoE) mice were used for in vitro and in vivo studies. In vitro coculture was performed with VSMC and bone marrow macrophages from wild-type (WT) C57BL6 mice. Details of materials and experimental procedures are given in the Supplemental Methods section, available online at http://atvb.ahajournals.org.

Results
Runx2 and RANKL Upregulation, Macrophage Infiltration, and Tartrate-Resistant Acid Phosphatase–Positive Cells Are Associated With Vascular Calcification In ApoE−/− Mice
We have previously demonstrated that oxidative stress induces the expression of Runx2, which is essential for VSMC calcification in vitro. Using an atherosclerosis model, ApoE−/− mice, we characterized the expression of Runx2 in vascular calcification in vivo. A significant increase in calcification was found in the aortic roots obtained from ApoE−/− mice fed a high-fat diet compared with those from chow-fed control animals (P=0.01, Figure 1A and 1B), which was associated with high-fat diet–induced increased oxidative stress (Supplemental Figure 1). Increases in Runx2- and RANKL-immunopositive areas were found concurrently with enhanced vascular calcification in the high-fat diet–fed animals compared with those from chow-fed mice (Figure 1C). Quantitative analysis confirmed significant upregulation of Runx2 and RANKL expression in the high-fat diet–fed mice (P=0.01 for each, Figure 1D).

In addition, increased macrophage infiltration was demonstrated in the aortic root of ApoE−/− mice fed the high-fat diet (P=0.02, Figure 1D), as determined by staining with a macrophage marker, CD68. The area that stained positive for macrophages in the high-fat diet–fed mice was closely associated with RANKL immunopositivity (Figure 1C). Tartrate-resistant acid phosphatase (TRAP)–positive cells were observed in the aortic roots of high-fat diet–fed animals (Figure 1C), which were mainly found in the areas of highest expression of RANKL and macrophage infiltration (Figure 1C). The greatest expression of Runx2/RANKL and of CD68/TRAP was in the aortic sinuses at base of valve leaflets, a common site of atherosclerotic lesions. With immunofluorescent staining, we further confirmed the colocalization of Runx2 and RANKL in the calcified atherosclerotic lesions from high-fat diet–fed ApoE−/− mice (Figure 1E). Importantly, TRAP-positive cells were found in close apposition to the RANKL-positive areas (Figure 1E), supporting a link between RANKL induction and formation of osteoclast-like cells.

Oxidative Stress Induces the Expression of RANKL in VSMC During Calcification
To determine the effects of oxidative stress on the expression of RANKL in calcifying VSMC, we characterized the effects of a series of concentrations of H2O2 (0.05 to 0.4 mmol/L) on RANKL expression by primary mouse VSMC. Based on quantitative real-time polymerase chain reaction (PCR), H2O2 dose-dependently induced expression of RANKL in parallel with VSMC calcification (Figure 2A and 2B). Similarly, oxidative stress induced the expression of RANKL in rat and human VSMC (Supplemental Figure III). Increased expression of RANKL protein by oxidative stress was identified predominantly in the cell lysates (Figure 2C), indicating that oxidative stress–induced RANKL is expressed primarily within VSMC. Furthermore, H2O2 increased RANKL mRNA in a time-dependent manner, concurrently with H2O2-induced Runx2 expression (Figure 2D).

H2O2-Responsive Element in the RANKL Promoter
To elucidate the molecular mechanism of oxidative stress–induced expression of RANKL, we examined the H2O2-responsive region on the RANKL gene using a series of luciferase reporter constructs containing deletion mutants of the RANKL 5′-flanking region: renila luciferase (RL) (full length [FL]) (−950), RL(−700), RL(−550), RL(−400), RL(−200), RL(−150), and RL(−50). H2O2 induced higher promoter activities in VSMC transfected with RL(FL),...
RL (–700), RL (–550), and RL (–400) compared with controls (Figure 2E), but not in VSMC transfected with RL (–200). Therefore, the −400 to −200 bp region is essential for the H$_2$O$_2$-induced transcription of the RANKL gene in VSMC.

Runx2 Regulates RANKL Transcription

Sequence analyses of the −400 to −200 bp region of the RANKL promoter identified multiple binding sites for the key osteogenic transcription factor Runx2 (Runx2-1, Runx2-2, Runx2-3, and Runx2-4; Figure 3A). We previously
The P3 putative Runx2 binding site was found to be conserved binding to Runx2, indicating binding specificity (Figure 3D).

Mutations of the Runx2 binding were detected predominantly with the P3 probe, and with other probes to a much lesser extent. Mutations of the Runx2 binding sites were amplified by PCR using the appropriate primer sets (whole, R1&2, R3&4, R3&4, −290/−85) and DNA templates extracted from protein/DNA cross-linked samples. Runx2 was found to bind specifically to the R3&4 region (−290/−85), as well as to the entire (−438/−85) region of the RANKL promoter (Figure 3B). With primer sets spanning each of the putative Runx2-binding regions of the RANKL promoter (R1 to R4), the Runx2 binding sites were located preferentially to the R3 region and, to a lesser extent, the R4 region (Figure 3C).

Furthermore, direct binding of Runx2 to the RANKL promoter was confirmed by electrophoretic mobility shift assay (EMSA) with oligonucleotides derived from putative Runx2-binding sites in the RANKL promoter (P1, −378/−354; P2, −337/−313; P3, −216/−192; P4, −190/−166). The previously reported Runx2 consensus binding probe was used as a positive control. As with the results of ChiP assay, binding complexes were detected predominantly with the P3 probe, and with other probes to a much lesser extent. Mutations of the Runx2 binding sites on all probes (P1m, P2m, P3m, and P4m) abolished their binding to Runx2, indicating binding specificity (Figure 3D). The P3 putative Runx2 binding site was found to be conserved among the mouse, rat, and human RANKL gene, but the P1, P2, and P4 sites were not (Supplemental Figure IV).

Runx2 binding to the P3 probe was blocked by excess amounts of cold P3 probe and Runx2 consensus probe but not by P3 probe with a mutation in Runx2 binding site (P3m) (Figure 3E). Moreover, antibody specific for Runx2 produced a supershift of the Runx2 binding complex, confirming the specificity of Runx2 in the DNA-protein complex (Figure 3F). In addition, mutation of P3 inhibited H2O2- and Runx2-induced luciferase activity driven by RANKL promoter (Supplemental Figure IV). Taken together, these results indicate a specific and predominant binding of Runx2 to the P3 region of RANKL promoter.

Runx2 Is Essential for Oxidative Stress–Induced RANKL Expression and Sufficient to Induce RANKL

To determine whether Runx2 is required for oxidative stress–induced expression of RANKL, we used lentivirus-mediated short hairpin RNA (shRNA) to knock down the expression of Runx2 in VSMC. As expected, H2O2-induced Runx2 expression in VSMC was not affected by control virus (lenti–green fluorescent protein); however, Runx2 expression was inhibited in VSMC infected with shRNA against Runx2 (Figure 3G). The inhibitory effect of lentiviral shRNA on Runx2 expression resulted in blockage of H2O2-induced RANKL expression in VSMC (Figure 3H), confirming that Runx2 is essential for H2O2-induced expression of RANKL in VSMC.
Adenovirus-mediated overexpression of Runx2 alone was sufficient to induce the expression of RANKL in VSMC (Figure 3I), which is consistent with our previous finding that overexpression of Runx2 induces VSMC calcification. Therefore, increased expression of Runx2, either induced by H2O2 or mediated by viral infection, upregulates the expression of RANKL in calcifying VSMC.

RANKL Does Not Induce VSMC Calcification and Is Not Required for Oxidative Stress–Induced VSMC Calcification

To assess whether RANKL directly induces VSMC calcification, recombinant RANKL protein (100 ng/mL) was added to VSMC in osteogenic media with or without 0.4 mmol/L H2O2. As expected, H2O2 induced calcification of VSMC (Figure 4A), whereas RANKL did not (Figure 4A and 4B). OPG (50 ng/mL), a soluble decoy receptor of RANKL, did not block calcification induced by oxidative stress (Figure 4A). In addition, H2O2-induced calcification was not inhibited in VSMC from RANKL-deficient mice compared with WT VSMC, as determined by von Kossa cytachemical staining, as well as by quantification of calcium levels in cell lysates (Figure 4C and 4D), indicating that RANKL signaling is not essential for oxidative stress–induced VSMC calcification.

Oxidative Stress–Stimulated VSMC Promote Migration of Bone Marrow Macrophages in a Runx2/RANKL-Dependent Manner

Soluble RANKL protein has been shown to exhibit chemotactic properties toward human monocytes. To test
whether RANKL expression by VSMC may affect bone marrow macrophage (BMM) migration, freshly isolated mouse BMM were indirectly exposed to VSMC using the Transwell migration assay. Soluble RANKL protein at 100 ng/mL was used as a positive control. As shown in Figure 5A, oxidative stress–stimulated VSMC increased the migration of BMM by 5.2-fold compared with serum-free control, whereas unstimulated VSMC had no effect on BMM migration. The effects of oxidative stress–stimulated VSMC on BMM migration was blocked in VSMC from RANKL-deficient mice or in VSMC with Runx2 knockdown, demonstrating the requirement of Runx2–regulated RANKL production by VSMC. Furthermore, addition of OPG abolished the effect of oxidative stress–stimulated VSMC on BMM migration (Figure 5B), supporting a role of RANKL expressed by VSMC in regulating BMM migration.

**Oxidative Stress–Stimulated VSMC Promote Osteoclastic Differentiation of Macrophages in a Runx2/RANKL-Dependent Manner**

RANKL is a central regulator of osteoclast formation, and RANKL produced by osteoblasts induces osteoclast differentiation of osteoclast precursors. The presence of TRAP-positive osteoclast-like cells in areas of high RANKL expression prompted us to test the hypothesis that oxidative stress–induced RANKL in VSMC during calcification may induce osteoclast formation. With the use of a coculture system of VSMC and BMM as a source of osteoclast precursors, we determined that \( \text{H}_2\text{O}_2 \)-stimulated VSMC induced the formation of multinucleated TRAP-positive cells from BMM, compared with unstimulated VSMC (Figure 6A). However, in VSMC from RANKL-deficient mice, \( \text{H}_2\text{O}_2 \) treatment did not induce any multinucleated TRAP-positive cells in coculture with BMM (Figure 6A), indicating a direct effect of VSMC-derived RANKL. Furthermore, osteoclastic differentiation was restored by addition of soluble RANKL (100 ng/mL) to the coculture of RANKL−/− VSMC with BMM (Figure 6A). Addition of OPG, however, completely blocked the stimulatory effects obtained with \( \text{H}_2\text{O}_2 \) (Figure 6B).

Furthermore, VSMC with Runx2 knockdown by lentiviral shRNA failed to promote the formation of multinucleated TRAP-positive cells in a coculture system when compared with WT VSMC (Figure 6C), confirming that Runx2 is essential for oxidative stress–induced RANKL expression in VSMC and that it functions in regulating osteoclastic differentiation.

**Discussion**

Using atherogenic ApoE−/− mice, we determined that increased Runx2 and RANKL were colocalized in the calcified atherosclerotic lesions, in close apposition to TRAP-positive osteoclast-like cells. We demonstrated for the first time that oxidative stress enhanced expression of RANKL in primary murine VSMC, which was mediated by the osteogenic transcription factor Runx2 via a direct binding to the RANKL promoter. VSMC-derived RANKL was not essential for oxidative stress induced VSMC calcification, and RANKL did not induce VSMC calcification when added to the culture media. Instead, VSMC-derived RANKL was found to promote migration and osteoclastic differentiation of BMM. These observations provide novel evidence for the molecular mechanism underlying RANKL upregulation in atherosclerotic calcification and the function of Runx2/RANKL in regulating the crosstalk between calcifying VSMC and macrophages in the genesis of TRAP-positive osteoclast-like cells in atherosclerotic lesions.
Increased expression of RANKL was found to colocalize with Runx2 in the calcified atherosclerotic lesions (Figure 1). Such an observation is consistent with previous reports that RANKL expression is increased in calcified atherosclerotic lesions but not in normal vessels, and mice deficient in OPG develop arterial calcifications. RANKL is a transmembrane protein expressed in the bone marrow microenvironment and stimulates osteoclast progenitor cells to differentiate into osteoclasts. Under normal steady-state conditions in adults, mineral deposition by osteoblasts and mineral resorption by osteoclasts are delicately balanced to maintain bone homeostasis. Similar to the observation in human atherosclerotic arteries, we identified TRAP-positive multinucleated osteoclast-like cells in close apposition to the calcified areas. The high levels of RANKL and the presence of TRAP-positive osteoclast-like cells in calcified atherosclerotic lesions support the notion that molecular mechanisms similar to bone remodeling processes are manifest in mineralized atherosclerotic artery walls.

Increased oxidative stress in the atherosclerotic lesions may contribute to the increase in RANKL expression. Oxidative stress–stimulated VSMC promote BMM migration in a Runx2/RANKL-dependent manner. After VSMC were cultured in osteogenic media for 2 weeks with or without 0.4 mmol/L H$_2$O$_2$, cells were coated on the lower side of a Transwell filter, and Dil (Invitrogen)-labeled BMM were added to the upper chambers. After 24 hours, migrated BMM were measured using a microplate fluorescence reader. Results from 3 independent experiments performed in duplicate are shown (P=not significant for unstimulated WT VSMC vs serum-free, P=0.01 for oxidative stress–stimulated WT VSMC vs serum-free). B, WT VSMC were preincubated in osteogenic media for 2 weeks with or without 0.4 mmol/L H$_2$O$_2$ and then coated on the lower side of Transwell. Effects of conditioned media from the preincubation or addition of OPG (50 ng/mL) on macrophage migration was determined. Results from 3 independent experiments performed in duplicate are shown (P=0.005 for oxidative stress–stimulated VSMC vs serum-free control).

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expression of RANKL (Figure 3H) and sufficient to induce RANKL in VSMC (Figure 3I), findings that are consistent with the inhibition of RANKL expression in Runx2-deficient osteoblast cells. By contrast, calcitriol-induced RANKL expression was not affected in Runx2-deficient calvarial cells. In the ST2 mouse bone marrow stromal cells, decreased Runx2 expression by protein kinase A activation was associated with increased RANKL expression. Accordingly, the effects of Runx2 on RANKL expression appear to differ among cell types and their response to different stimuli.

The Runx2 binding domains were identified within −400 to −200 bp in the RANKL promoter in VSMC, with a predominant binding of Runx2 to the −206 to −201 bp region (P3, Figure 3), which is the most conserved sequence among human, rat, and mouse RANKL gene (Supplemental Figure IV). Mutation of P3 was found to inhibit Runx2 binding (Figure 3E and 3F), as well as H2O2-induced and Runx2-induced luciferase activity of the RANKL promoter (Supplemental Figure IV). Similar to our finding, deletion or mutation of Runx2 binding sites within the RANKL promoter or overexpression of a dominant negative Runx2 abolished bone morphogenetic protein-2- and Smad1-mediated activation of RANKL promoter activity in chondrocytes. Despite structural similarity to the core Runx2-binding sequences (5′-ACCPuCPu-3′), the other putative Runx2 binding site showed significantly less binding activity, suggesting the importance of overall genomic context containing the core sequences for Runx2 activity. In the ST2 bone marrow stromal cells, mutation of each of the 3 putative Runx2 binding sites on the RANKL promoter (corresponding to P1, P3, and P4 in the present study), especially the P3 and P4 sites, decreased the basal promoter activity of RANKL and inhibited protein kinase A-induced RANKL expression.

Therefore, our study is consistent with the study in ST2 cells with respect to supporting the requirement for direct binding of Runx2 on the RANKL promoter, especially the P3 region, in regulating RANKL expression.

Unlike previous reports that RANKL promotes an osteogenic phenotype in aortic myofibroblast and rat aortic VSMC calcification, we did not find a direct effect of RANKL on mouse VSMC calcification (Figure 4A), excluding the possibility of autocrine or paracrine effects of VSMC-derived RANKL on calcification. Similar results were also found in calcifying vascular cells (CVC); treatment of RANKL (100 ng/mL) in osteogenic media did not induce calcification of these cells (Supplemental Figure II). Differences in model cell systems may explain the differences between the present results and prior reports. The difference in expression of RANK, the receptor for RANKL, in mouse and rat VSMC was not statistically significant, which may not contribute to the different effects of RANKL on the calcification of mouse and rat VSMC (Supplemental Figure V). In addition, patterns of mineral deposition seen by von Kossa staining are more diffuse in rat cells and more nodular in mouse cells, suggesting that RANKL induction of calcium positive stains in rat aortic VSMC may occur via a distinct mechanism of biomineralization or via increased intracellular calcium. In any case, oxidative stress promoted calcification in RANKL-deficient VSMC, supporting the concept that VSMC-derived RANKL is not essential for direct regulation of VSMC calcification in vitro (Figure 4B).

We found that in VSMC, the majority of RANKL protein induced by oxidative stress was cell-associated (Figure 2C), unlike in activated murine T lymphocytes, which secrete an active soluble form of RANKL into the culture medium. In MC3T3-E1 cells, mechanical strain has been shown to increase membrane-bound RANKL at the expense of soluble RANKL, via a reduction in ectodomain shedding. The dominance of VSMC-associated RANKL over soluble RANKL in the context of oxidative stress may explain why the RANKL protein is concentrated in areas of atherosclerosis, whereas serum levels of soluble RANKL are inversely correlated with presence of coronary artery disease. VSMC exposed to oxidative stress induced the migration of BMM in a Runx2/RANKL-dependent manner, supporting the role of oxidative stress–induced RANKL from VSMC in regulating macrophage infiltration (Figure 5). Macrophage infiltration plays a significant role in the formation of atherosclerotic lesions. Aikawa et al found that macrophage infiltration and inflammation precede the osteogenic conversion of VSMC. Deficiency of OPG in ApoE−/− mice accelerates atherosclerotic lesion progression and increases atherosclerotic calcification and macrophage infiltration, suggesting a role of RANKL in upregulating calcification and macrophage infiltration in vivo. Consistently, we demonstrated increased macrophage infiltration in close association with Runx2/RANKL expression in calcified vessel walls (Figure 1C and 1E). RANKL has been reported to induce migration of MonoMac-6 mononuclear cells, as well as peripheral blood mononuclear cells, in a dose-dependent manner with an potency similar to that of monocyte chemotactant protein-1, a chemotactic factor that contributes to atherogenesis. The effect of RANKL on macrophage recruitment may be attributed to its induction of several cytokines, including CCL2 (macrophage-derived chemokine), monocyte chemotactant protein-1, and interleukin-8. The chemokines, in turn, increase RANKL expression, amplifying the inflammatory process. In addition, RANKL has been found to upregulate RANK expression on monocytes, to promote cell survival, and activate the capacity of monocytes for antigen presentation through induction of costimulatory molecules.

In bone, RANKL expression by osteoblastic and/stromal cells is essential for the complete development of multinucleated bone-resorptive osteoclasts from mononuclear precursors, and for the resorptive activity and survival of mature osteoclasts as well. Consistently, we found that VSMC-derived RANKL induced the differentiation of BMM into multinucleated TRAP-positive cells, an effect that was antagonized by exogenous OPG (Figure 6). These results are consistent with the observation by Collin-Osdoby et al that tumor necrosis factor-α-activated human microvascular endothelial cells, which express RANKL on the cell surface, promote osteoclastogenesis in cocultures with human monocyte precursors via a RANKL-mediated mechanism. In contrast, calcifying vascular cells and conditioned media from calcifying vascular cells were previously shown to inhibit osteoclastic differentiation in a coculture system.
however, calcifying vascular cells expression of RANKL expression during osteoblastic differentiation and mineralization was not determined. The role of osteoclasts in atherosclerotic calcification is still not clear. In keeping with the hypothesis that the process of vascular calcification resembles that observed in bone tissue, net calcium deposition in vessel walls might result from focal perturbation of the balance between osteoblast-like cells and osteoclast-like cells. Thus, our observations support the notion that osteoclast-like cells in the vascular wall may represent important cellular mediators of mineral resorption in arteries. Nevertheless, our observation that oxidative stress promotes VSMC induction of osteoclastogenesis may shed light on the origin and potential role of osteoclast-like cells in atherosclerotic plaque. Additional studies are warranted to characterize the pathological function of osteoclast-like cells in the regulation of vascular calcification.

Taken together, our studies demonstrate the molecular mechanisms underlying Runx2-regulated expression of RANKL and its function during oxidative stress–induced VSMC calcification. The results indicate that expression of Runx2 and RANKL and vascular calcification are associated with macrophage infiltration and TRAP-positive cells in atherosclerotic lesions of ApoE−/− mice; that VSMC-derived RANKL, induced by Runx2-dependent signaling, increases migration and osteoclastic differentiation of macrophages; that RANKL does not directly regulate osteogenic differentiation of VSMC; and that osteoclast-like cells arise in the proximity to mineralized areas of atherosclerotic lesions, making it likely that osteoclastic cells indirectly regulate vascular calcification, as seen in the interplay of osteoblasts and osteoclasts in regulating bone homeostasis.

Acknowledgments
We thank Dr Jay M McDonald (University of Alabama at Birmingham) for helpful discussion and critical review and Dr Xu Feng (University of Alabama at Birmingham) for providing the RANKL-reporter constructs.

Sources of Funding
This work was supported by National Institutes of Health Grants HL092215 and AR055339 (to Y.C.), HL081202 (to L.L.D.), and DK081346 (to Y.T.); VA Merit Review Award BX000369 (to Y.C.); and American Heart Association Award 0865081E (to Y.C.). Chang Hyun Byon was supported by a National Institutes of Health T32 Pre-doctoral Training Grant.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2011;31:1387-1396; originally published online March 31, 2011;
doi: 10.1161/ATVBAHA.110.222547

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RUNX2-UPREGULATED RANKL IN CALCIFYING SMOOTH MUSCLE CELLS PROMOTES MIGRATION AND OSTEOCLASTIC DIFFERENTIATION OF MACROPHAGES

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Running title: Regulation and function of RANKL in calcification

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Supplemental Materials and Methods

**VSMC culture.** Primary VSMC were isolated from the aorta of C57BL/6 mice and cultured in growth media as we described previously\(^1\). All experiments were performed with VSMC at passages 3 to 5.

**Reverse transcriptase-polymerase chain reaction and real-time PCR analysis.** The effect of oxidative stress on the expression of RANKL was determined by RT-PCR and real-time PCR. Total RNA was isolated from VSMC using Trizol (Invitrogen) and reverse transcribed into cDNA. PCR was performed using specific primers for murine Runx2\(^2\), RANKL\(^3\), and GAPDH\(^4\) as control. The primers for the receptor RANK are: 5'-GTGTGTCTGCCCTGTGGCCC-3' and 5'- CGTGTTCCGACGGCAGCACTC-3', which are conservative sequences among mouse, rat and human RANK genes. SYBR Green-based real time PCR was performed in a 96-well plate format using SYBR Premix Ex Taq (TaKaRa) on an iCycler Thermal Cycler (Bio-Rad).

**Enzyme linked immunosorbent assay (ELISA).** VSMC were cultured in a 6-well plate in osteogenic media and treated with 0.4 mM H\(_2\)O\(_2\) for 2 weeks. RANKL protein content was measured using the murine RANKL Quantikine ELISA kit (R & D Biosystems) in culture supernatants and cell lysates. Protein concentrations of all samples were measured using a BCA protein assay kit.

**In vitro calcification.** Calcification of mouse aortic VSMC was induced by the addition of 0.4 mM H\(_2\)O\(_2\) in osteogenic media containing growth media with 0.25 mM L-ascorbic acid and 10 mM β-glycerophosphate (Sigma Aldrich) for 2 weeks. Calcification was determined either by von Kossa staining or by Alizarin Red S staining; or quantified by measuring total calcium in the cell lysates by o-cresolphthalein complexone method. For von Kossa staining, cells were fixed by 2% paraformaldehyde in PBS for 15 min and washed with deionized water 3 times. After adding 5% silver nitrate (Sigma Aldrich) in deionized water, plates were placed on aluminum foil under a 60-watt white lamp until it turned black. Plates were washed with deionized water 3 times and 5% sodium thiosulfate (Sigma Aldrich) was added for 5 min to remove unreacted silver. For Alizarin Red S staining, cells were fixed in 2% formaldehyde in PBS for 45 minutes at 4°C. The cultures were then washed in deionized water and exposed to Alizarin Red S (2% aqueous, Sigma Aldrich) for 5 minutes and then washed again with deionized water.

**Luciferase reporter assay.** VSMC in 6-well plates were transiently transfected with 1 µg of serial deletion constructs of murine RANKL promoter-driven luciferase reporter (kindly provided by Dr. Xu Feng, University of Alabama at Birmingham)\(^6\) or RL(-400) luciferase reporter with the P3 mutation, using FuGENE6 (Roche) according to the manufacturer’s protocol. A plasmid encoding Renilla luciferase gene
downstream of a minimal SV40 promoter was used to normalize for transfection efficiency. 24 hours after transfection, VSMC were washed and treated with 0.4 mM H$_2$O$_2$ for an additional 48 h. Luciferase activities were determined with the Dual Luciferase Assay Kit (Promega).

**Chromatin immunoprecipitation assay (ChIP).** ChIP assay was performed using a kit from Upstate Corp. (Lake Placid, New York) with the following modifications: After exposing cells to 0.4 mM H$_2$O$_2$ for 1 or 2 weeks, VSMC were treated with 1% formaldehyde at 37°C in the presence of 5% CO$_2$ for 15 minutes. Cells were sonicated on ice 25 times using a Vibra-Cell ultrasonic processor (Sonics) for 25 seconds with a 60 seconds interval between each sonication. Anti-Runx2 antibody (10μg; Santa Cruz sc-10758) were used with 100μg of chromatin per ChIP. The amounts of DNA fragment in immunoprecipitates were determined by PCR reactions. The primers used for this analysis were:

1. 5'-GAGTTCTAGAATTTCCCAAG-3' (-438/-418)
2. 5'-CTCAAAATCTTAGAGGACC-3' (-328/-348)
3. 5'-GGTTCTCCTCAGATTGGAG-3' (-348/-328)
4. 5'-TTTGCATCTCTTGGGCTCAG-3' (-240/-259)
5. 5'-GGAGTTTCACTAAGTGACT-3' (-290/-271)
6. 5'-GGAGCCTTGGGCTTGAG-3' (-182/-199)
7. 5'-ACCCAACCCACGCTCC-3' (-199/-182)
8. 5'-GTGCGCTTTTCAAAGCCAC-3' (-85/-105)

**Electrophoretic mobility shift assay (EMSA).** VSMC were exposed to 0.4 mM H$_2$O$_2$ for 1 week and nuclear extracts were prepared as described previously. Custom synthesized oligonucleotides designed to encompass putative Runx2 binding sites in the RANKL promoter were:

- **P1** (-378/-354): 5'-CTGAGGCTAATCTCCGATTCTTG-3'
- **P2** (-337/-313): 5'-AGATTTGAGAGTTGGTGTACAGGAA-3'
- **P3** (-216/-192): 5'-CCAGAAACCCACACTGGACCACAAAC-3'
- **P4** (-190/-166): 5'-CACAGCCTCCACCTCAGAGGCCGCT-3'
- **P1 mutant** (-378/-354): 5'-CTGAGGCTAgCtTgATCATTCTTG-3'
- **P2 mutant** (-337/-313): 5'-AGATTTTGAGAaTaGcGTACAGGAA-3'
- **P3 mutant** (-216/-192): 5'-CCAGAAACCAgCtAtTGGACCACAAAC-3'
- **P4 mutant** (-190/-166): 5'-CACAGCCTCCgCtTgAGAGGCCGCT-3'

For the competition assay, 100-fold molar excess amounts of unlabeled oligonucleotides were added to the binding reaction.

**Lentiviral shRNA transduction for VSMC.** Lentiviral vector encoding a 21-nucleotide Runx2 short hairpin RNA (shRNA) was purchased from Open Biosystems and packaged into lentiviral particles as previously described. Viral transductions were performed by incubating VSMC with recombinant lentiviruses in growth media supplemented with 10 μg/mL diethylaminoethyl (DEAE)-dextran. After 24 hours, cells were washed with PBS and kept in growth media containing puromycin for 2 weeks to select stable transfectants.

**Adenovirus-mediated transduction of Runx2.** Adenovirus encoding murine wild-type Runx2 (Ad-Runx2) or control virus (Ad-GFP) transduction into VSMC was performed as we previously described. Briefly,
VSMC grown in six-well plates were infected with adenoviral particles for 2 h in serum-free media and 16 h in serum-containing media. Subsequently, cells were cultured in osteogenic media for 2 weeks.

**Western blot analysis.** VSMC cell extracts were prepared and protein concentration was measured as we previously described. Western blot analyses were performed with the use of specific antibody for Runx2 (MBL D130-3), RANK (H-300 Santa Cruz), and detected with a Western blot chemiluminescence detection kit (Millipore).

**Macrophage migration assay.** The bottom side of a Transwell (8 µm pore size; Corning Inc.) was coated with human plasma fibronectin (Chemicon) at 10 µg/ml for 1 hour at 37º C. Then, it was inverted and a collar, which fits exactly to the Transwell, was put on top as described previously. After exposing to 0.4 mM H₂O₂ for 2 weeks, VSMC were allowed to adhere to the lower side of a transwell filter in the inverted position at 37º C and 10% CO₂. After 4 hours, the collars were carefully removed and the transwells were placed in an upright position with VSMC upside-down in 12-well plates and subsequently cultured for 2 days. BMM were labeled with Vybrant DiI cell-labeling solution (Invitrogen) for 30 min at 37º C. After labeling, cells were washed and resuspended in serum-free medium to a final concentration of 1 x 10⁶/ml. The transwells were washed with serum-free medium just before the start of the experiment. DiI-labelled BMM (1 x 10⁵/transwell) were seeded on top of the filter and cultured for 24 h. For a positive control, the lower compartment contained RANKL as a chemoattractant. After cells from the lower side of transwell filter and lower chamber were lysed and pooled, the released fluorescence was measured at 620 nm upon excitation at 540 nm on a microplate fluorescence reader (Synergy2; BioTek). The culture medium used for the transwell migration assay was serum-free BMM medium.

**Osteoclastogenesis of BMM co-cultured with VSMC.** Bone marrow cells were isolated from femora and tibiae of C57BL/6 mice. Briefly, the bone ends were cut and the bone marrow cavity flushed into a petri dish using a sterile 23-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single cell suspension. The bone marrow cells were washed twice, resuspended in α-MEM with 10% Hi-FBS (Sigma Aldrich), and incubated for 24 h. Then, non-adherent cells were harvested and kept in α-MEM with 10% Hi-FBS/10 ng/ml M-CSF. After VSMC were cultured in osteogenic media for 2 weeks with or without 0.4 mM H₂O₂, BMM were resuspended in α-MEM with 10% Hi-FBS/10 ng/ml M-CSF and added to the 24-well dish containing VSMC. After co-culture for 1 week, the cells were subjected to TRAP staining. Briefly, cells were washed with PBS and fixed in buffered formalin. After incubated for 1 h at 37ºC in a Naphthol AS-BI phosphoric acid/Fast Garnet/tartrate solution (Sigma Aldrich), cells were rinsed in distilled water and kept in PBS.

**Experimental animals.** 8-wk-old ApoE/- mice, which were homozygous for the disrupted ApoE gene on a C57BL/6J genetic background, were treated with either a chow or a high-fat, high-cholesterol diet containing 21.2% fat and 0.15% (by weight) cholesterol (Harlan Teklad diet TD88137) for 8 months. Both food and fluid intake were ad libitum. All the protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**Tissue processing.** After euthanasia, the vasculature was perfused with sterile PBS. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium (Tissue-Tek) for serial cryosectioning covering 0.7 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Two sections of 10 µm thickness were harvested per slide, yielding 35 slides per mouse.

**In situ measurement of oxidative stress.** To evaluate levels of superoxide in the aortic root, staining for dihydroethidium fluorescence (DHE, Molecular Probes, Inc.) was used. Frozen aortic root sections were
incubated in 0.002 mM DHE and protected from light for 30 min at room temperature. Images were obtained using an Olympus BX51 fluorescent microscope equipped with a digital camera and analyzed using ImageJ software (NIH Bethesda, MD) as described previously with minor modifications. To evaluate the fluorescent intensity of positively stained area, the values for the background were subtracted from the values for total fluorescence.

**Immunohistochemistry.** Frozen aortic root sections were processed for immunohistochemistry as described previously with minor modifications. In brief, anti-Runx2 (Santa Cruz sc-10758, 1:100), anti-RANKL (Santa Cruz sc-9073, 1:100), and anti-CD68 (Serotec MCA1957, 1:250) antibodies were applied to acetone-fixed cryosections. The sections were washed and exposed to a secondary antibody (horseradish peroxidase-conjugated antibodies), and the antibody binding was visualized with diaminobenzidine. Sections were counterstained with hematoxylin. The percentage of positively stained areas for each aortic specimen was calculated using ImageJ software (NIH Bethesda, MD) as described previously.

**Immunofluorescent staining.** Frozen aortic root sections were processed and immunofluorescent staining performed as described previously. Antibodies for Runx2 (Santa Cruz) and RANKL (Santa Cruz) were applied to fixed cryosections. Slides were washed extensively before the addition of species-specific fluorescently labeled secondary antibodies (Alexa Fluor® 488 or 594, Invitrogen). For TRAP staining, ELF97 TRAP (Invitrogen) was diluted 1:1,000 in acetate buffer, and slides were incubated for 15 minutes at room temperature. 4′, 6-diamidino-2-phenylindole (DAPI) was used for nuclear localization.

**Aortic calcification.** Calcium deposits in aortic root sections were stained using Alizarin Red S (Sigma Aldrich) as described previously. Briefly, frozen sections were taken every 70 μm thereafter until a series of ten slides in total had been collected. After being air dried and submerged in distilled water, the sections were removed from the water and incubated with Alizarin Red S stain for five minutes. Excess stain was blotted away and slides were quickly washed in distilled water, dehydrated, cleared, and coverslipped. Stained specimens were examined microscopically (BX50; Olympus), and the percentage of positively stained surface area for each aortic specimen was calculated using ImageJ software (NIH Bethesda, MD) as described previously.

**Statistical analysis.** All the data in this study are expressed as means ± SD. Differences in data between the groups were compared with Student’s paired 2-tailed t test or 1-way ANOVA where appropriate. A p value less than 0.05 was considered statistically significant.

**Supplemental Figure 1**

Suppl. Figure 1. High-fat diet increased oxidative stress. (A) Aortic superoxide was measured by staining aortic root sections with DHE and imaging with fluorescence microscopy. (B) Images were analyzed using ImageJ software (NIH Bethesda, MD). A significant difference was found between chow and high-fat diet-fed animals. To evaluate the fluorescence intensity of positively stained areas, the values for background were subtracted from the values for total fluorescence. Values are expressed as mean ± SD with the number of mice shown above each bar.
Supplemental Figure 2

Suppl. Figure 2. RANKL does not induce calcification of CVC in culture. Calcifying vascular cells were plated at 20,000/cm². Cells were treated with control vehicle or RANKL (100 ng/ml) in DMEM supplemented with 10% FBS and 5 mM beta-glycerophosphate. The media were replaced every 3-4 days. Samples were taken at indicated days, and matrix calcium deposition was measured in 5 replicate samples and quantified by the o-cresolphthalein complexone method. Results shown are mean ± SD of three experiments.

Supplemental Figure 3

Suppl. Figure 3. H₂O₂ induces the expression of RANKL in rat and human VSMC. Expression of RANKL in VSMC exposed to 0.4 mM H₂O₂ in osteogenic media for 2 weeks was determined by A) Real-time PCR and B) quantitative real-time PCR. Results from two independent experiments in duplicates are shown (*p <0.05 compared with control conditions). C) Primers used for A and B.

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Supplemental Figure 4

Suppl. Figure 4. Mutation in the P3 putative Runx2 binding site inhibits luciferase activity-driven by RANKL promoter. A) Comparison of promoter sequence of mouse, rat and human RANKL gene at the P1-P4 region. B) Effect of P3 mutation on H₂O₂-induced RANKL promoter activity. C) Effect of P3 mutation on Runx2 overexpression-induced RANKL promoter activity. Results shown are relative luciferase activities in each of the conditions compared with that of the RL(-400) under control conditions, with the control defined as 1 (n=3, *p<0.05).

A. Mouse TTTCCCCAGCTTCCCGACGCAGGCTATATTGAGGGGTAAGCTCAA Human TGGACAGGGAGCTTCTTACAGAGAAGGCTATTGAGGCAGAA
Rat TTTCCCCAGCTTCCCGACGCAGGCTATATTGAGGGGTAAGCTCAA

B. Suppl. Figure 5. Expression of RANK in mouse, human and rat VSMC. Expression of RANK was determined by A) RT PCR, B) quantitative real-time PCR and C) Western blot analysis. Results from two independent experiments in triplicate are shown (*p <0.05 compared with mouse VSMC, defined as 1). The difference in RANK expression in mouse and rat VSMC was not statistically significant.
REFERENCES


