Smooth Muscle Cell–Specific Runx2 Deficiency Inhibits Vascular Calcification

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Rationale: Vascular calcification is a hallmark of atherosclerosis, a major cause of morbidity and mortality in the United States. We have previously reported that the osteogenic transcription factor Runx2 is an essential and sufficient regulator of calcification of vascular smooth muscle cells (VSMC) in vitro.

Objective: To determine the contribution of osteogenic differentiation of VSMC to the pathogenesis of vascular calcification and the function of VSMC-derived Runx2 in regulating calcification in vivo.

Methods and Results: SMC-specific Runx2-deficient mice, generated by breeding SM22α-Cre mice with the Runx2 exon 8 floxed mice, exhibited normal aortic gross anatomy and expression levels of SMC-specific marker genes. Runx2 deficiency did not affect basal SMC markers, but inhibited oxidative stress-reduced expression of SMC markers. High-fat-diet-induced vascular calcification in vivo was markedly inhibited in the Runx2-deficient mice in comparison with their control littermates. Runx2 deficiency inhibited the expression of receptor activator of nuclear factor κB ligand, which was accompanied by decreased macrophage infiltration and formation of osteoclast-like cells in the calcified lesions. Coculture of VSMC with bone marrow–derived macrophages demonstrated that the Runx2-deficient VSMC failed to promote differentiation of macrophages into osteoclast-like cells.

Conclusions: These data have determined the importance of osteogenic differentiation of VSMC in the pathogenesis of vascular calcification in mice and defined the functional role of SMC-derived Runx2 in regulating vascular calcification and promoting infiltration of macrophages into the calcified lesion to form osteoclast-like cells. Our studies suggest that the development of vascular calcification is coupled with the formation of osteoclast-like cells, paralleling the bone remodeling process.

Key Words: vascular smooth muscle cells ■ osteogenic differentiation ■ Runx2 ■ vascular calcification ■ vascular osteoclasts ■ smooth muscle cells ■ vascular disease

The extent of vascular calcification, featuring calcium deposition—reduced elasticity, and decreased compliance of the vessel wall—predicts the cardiovascular outcome of atherosclerotic patients. Many studies have now supported the concept that vascular calcification is an active cell-driven process characterized by osteogenic differentiation of vascular cells. The origin of these cells has not been well defined. A variety of vascular cells—including vascular smooth muscle cells (VSMC), myofibroblasts, vascular mesenchymal progenitors, and endothelial cells—has been implicated in vascular calcification. It is not clear to what extent transdifferentiation of VSMC contributes to the pathogenesis of vascular calcification in vivo, although many in vitro studies have demonstrated that VSMC can undergo osteogenic differentiation and calcification.

The osteogenic transcription factor Runx2 plays an important role in VSMC calcification in vitro. Runx2 belongs to the runt-related transcription factor family and is essential for osteoblast differentiation and chondrocyte maturation. Multiple signals converge on the Runx2 transcription factor to regulate osteoblast differentiation. Using small interfering RNA, we have demonstrated that Runx2 is necessary for oxidative stress-induced VSMC calcification; and Runx2 by itself is sufficient to induce VSMC calcification in vitro. However, the function of Runx2 in regulating vascular calcification in vivo is unknown.
Gene ablation of Runx2 in mice completely blocks bone formation and results in neonatal lethality, which precludes further characterization of the role of Runx2 in regulating vascular calcification in vivo. Therefore, generating a mouse model with vascular cell-specific Runx2 deficiency is necessary. The SM22α-Cre transgenic mice have been shown to mediate specific and efficient recombination in the smooth muscle cell lineage at E8.0 during embryonic development. The C-terminus of Runx2 encoded by the exon 8 is critical for Runx2 function. Truncation of the Runx2 exon 8 results in complete absence of bone formation and embryonic lethality, a phenotype similar to those observed in Runx2 null mutant mice. The SMC-specific Runx2-deficient mouse model, generated by breeding SM22α-Cre mice with the Runx2 exon 8 floxed mice, is an ideal model to determine the role of SMC-derived Runx2 in regulating vascular calcification in vivo.

Mice lacking apolipoprotein E (ApoE), a ligand for receptors that clear the remnants of chylomicrons and very-low-density lipoproteins, have been used as a valuable model to investigate the genetic and environmental factors that regulate pathogenesis of atherosclerotic vascular calcification. Using the ApoE<sup>−/−</sup> model, we have demonstrated that high-fat diet (HFD) induces Runx2 expression in the calcified atherosclerotic lesions, which is associated with increased macrophage infiltration and the presence of osteoclast-like cells in the vicinity of these lesions. However, the function of increased Runx2 expression in VSMC upregulates the expression of ligand for receptor activator of nuclear factor κB (RANKL), which promotes macrophage migration and formation of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells. However, the function of increased Runx2 production in the calcification of VSMC in vivo and SMC-derived Runx2 in regulating macrophage infiltration and the formation of osteoclast-like cells in the calcified atherosclerotic lesions remain unknown. In the present report, we have generated SMC-specific Runx2-deficient mice in the ApoE<sup>−/−</sup> background, and characterized the functional contribution of SMCexpressed Runx2 to the development of vascular calcification and macrophage infiltration in vivo.

We have determined that deletion of the Runx2 exon 8 in SMC inhibits vascular calcification in vivo, and concurrently blocks the upregulation of RANKL, macrophage infiltration and the formation of osteoclast-like cells in the atherosclerotic lesions. These data have demonstrated the importance of SMC-derived Runx2 and Runx2-dependent osteogenic differentiation of VSMC in the pathogenesis of vascular calcification. Furthermore, our studies have revealed an intrinsic coupling between calcifying VSMC and the formation of vascular osteoclasts that parallels the bone-remodeling process. Therefore, targeting Runx2 or Runx2 signals in VSMC may represent a novel strategy for prevention and therapy-targeting vascular calcification.

### Methods

Details of materials and experimental procedures are in the Methods section in the Online Data Supplement.

The smooth muscle specific–Runx2 deficient mice were generated by crossing the Runx2 exon 8 floxed mice (Runx2<sup>f/f</sup>) with the SM22α-Cre transgenic mice. Characterization of vascular calcification in vivo was performed with mice crossed into ApoE<sup>−/−</sup> background.

### Results

#### Generation of SMC-Specific Runx2 Exon 8 Deletion Mice (Runx2<sup>ΔE8/ΔE8 SMC</sup>)

Runx2<sup>ΔE8/ΔE8 SMC</sup> mice were generated by crossing the Runx2 exon 8 floxed mice (Runx2<sup>f/f</sup>) with SM22α-Cre transgenic mice. Cre-mediated SMC-specific recombination was validated by breeding the SM22α-Cre with ROSA26 reporter mice (Online Figure I). Mice from the F1 generation were heterozygous mice with Runx2<sup>ΔE8/+/Cre<sup>−/−</sup></sup> genetic backgrounds. These heterozygous mice were backcrossed with the Runx2<sup>f/f</sup> mice to generate Runx2<sup>f/f</sup> mice containing the Cre transgene. Deletion of the Runx2 exon 8 was verified by PCR analysis (Online Figure II).

The homozygous Runx2<sup>ΔE8/ΔE8 SMC</sup> mice were viable with appearance and growth similar to those of the Runx2<sup>f/f</sup> littermates. No differences in body weights were identified between Runx2<sup>f/f</sup> and Runx2<sup>ΔE8/ΔE8 SMC</sup> mice in both genders (data not shown). Furthermore, we analyzed aortas from Runx2<sup>ΔE8/ΔE8 SMC</sup> and the Runx2<sup>f/f</sup> littermates and did not observe any significant differences in aortic smooth muscle cell morphology or gene expression (Online Figure II).

#### SMC-Specific Runx2 Deficiency Inhibits VSMC Calcification In Vitro

To determine the effect of Cre-mediated Runx2 deficiency in SMC on vascular calcification, we characterized VSMC isolated from Runx2<sup>ΔE8/ΔE8 SMC</sup> mice and Runx2<sup>f/f</sup> littermates. Western blot analysis revealed the expression of a truncated Runx2 protein lacking the exon 8 in VSMC from Runx2<sup>ΔE8/ΔE8 SMC</sup> mice (Figure 1A), demonstrating highly
effective and efficient excision of the Runx2 exon 8 by the SM22α-Cre.

Using VSMC from Runx2ΔE8/ΔE8 SMC mice and the Runx2f/f littermates, we determined the effect of the Runx2 deletion on oxidative stress-induced calcification. Increased expression was evident in Runx2f/f VSMC, as determined by Alizarin Red–stained nodules and Von Kossa staining (Figure 1B, upper panels). In sharp contrast, deletion of the Runx2 exon 8 resulted in inhibition of oxidative stress–induced VSMC calcification (Figure 1B, lower panels). Furthermore, oxidative stress–induced total calcium content in Runx2f/f VSMC was blocked by the Runx2 deficiency (Figure 1C). Runx2 deficiency concurrently abolished oxidative stress–induced expression of stage-specific osteogenic markers, such as alkaline phosphatase (ALP), type I collagen (Col Ia), and osteocalcin (OC) (Figure 1D). On the other hand, oxidative stress–induced decrease in the expression of SMC markers was inhibited by Runx2 deficiency (Online Figure III). Further characterization determined that the Runx2 truncated protein exhibited markedly reduced transductional activity, despite of remaining DNA binding activity (Online Figure IV). Taken together, these data indicate that Runx2 transcriptional activity is essential for both osteogenic commitment and maturation during VSMC calcification.

SMC-Specific Runx2 Deficiency Inhibits Vascular Calcification In Vivo

To determine the in vivo requirement of Runx2 for vascular calcification, we utilized the atherogenic ApoE−/− mouse model. We have previously established that HFD-induced Runx2 expression is associated with increased oxidative stress and atherosclerotic calcification.22 HFD-induced vascular calcification was examined in Runx2Δf/ApoE−/− and Runx2ΔE8/ΔE8 SMC:ApoE−/− mice. The HFD-induced vascular calcification in the aortic root of the Runx2Δf/ApoE−/− mice (Figure 2A, upper panels) was inhibited by the SMC-specific deficiency of the Runx2 exon 8 (Figure 2A, lower panels). Quantitative analysis demonstrated a significant decrease (P<0.001) of calcified lesions in aorta from HFD-fed Runx2ΔE8/ΔE8 SMC:ApoE−/− mice in comparison with those from Runx2Δf/ApoE−/− mice (Figure 2B). In addition, the presence of the early osteogenic marker, ALP, was also inhibited by the Runx2 deficiency (Figure 2C), supporting the role of Runx2 in regulating early and late stages of osteogenic differentiation of the VSMC in vivo.

Furthermore, we determined the direct contribution of the SMC-specific Runx2 deficiency to HFD-induced calcification in the descending aorta. SMC-specific Runx2 deficiency inhibited HFD-induced increase in aortic calcium content measured in the descending arteries (Figure 2D). These data demonstrate the importance of SMC-specific Runx2 in the development of vascular calcification in vivo.

SMC-Specific Runx2-Deficiency Inhibits HFD-Induced RANKL Expression and Macrophage Infiltration

We have demonstrated that Runx2 regulates the expression of RANKL via directly binding to the RANKL promoter and that HFD-induced Runx2 is associated with RANKL upregulation and macrophage infiltration in the calcified atherosclerotic lesions in ApoE−/− mice.23 To determine the function of SMC-specific Runx2 in regulating RANKL expression and macrophage infiltration in vivo, the expression of RANKL and the presence of macrophages were compared in the aortic root sections from Runx2Δf/ApoE−/− and Runx2ΔE8/ΔE8 SMC:ApoE−/− mice fed HFD (Figure 3). Immunohistochemical staining of RANKL and CD68 (a macrophage marker) revealed decreases in both RANKL (Figure 3A, panel a versus panel b) and CD68-positive areas (Figure 3B, panel c versus panel d).
Quantitative analysis determined a significant decrease in RANKL expression and macrophage infiltration in the aortas from Runx2<sup>A8/ΔE8</sup> SMC:ApoE<sup>/−/−</sup> mice (P<0.005, Figure 3C). Images taken at a higher magnification further revealed that macrophage infiltration was restricted to the endothelial layer in the Runx2<sup>A8/ΔE8</sup> SMC: ApoE<sup>/−/−</sup> mice (Figure 3B, panel d1), whereas extensive infiltration into the neointima was apparent in the Runx2<sup>fl/fl</sup>:ApoE<sup>/−/−</sup> mice (Figure 3B, panel c1). These data indicate an important role of SMC-derived Runx2 in regulating macrophage infiltration via RANKL.

The effect of the SMC-specific Runx2 deficiency on aortic expression of RANKL and CD68 was further determined in the descending aorta. Quantitative real-time PCR demonstrated that HFD induced aortic expression of glyceraldehyde-3-phosphate dehydrogenase, and is defined as 1 in Runx2<sup>fl/fl</sup>:ApoE<sup>/−/−</sup> mice (P<0.001). SMC indicates smooth muscle cells; HFD, high-fat diet; ApoE, apolipoprotein E; PCR, polymerase chain reaction; RANKL, receptor activator of nuclear factor kappa-B ligand.
RANKL and CD68 in aortas from Runx2<sup>f/f</sup>:ApoE<sup>−/−</sup> mice was significantly inhibited by the SMC-specific Runx2 deficiency in the Runx2<sup>ΔE8/ΔE8 SMC</sup>:ApoE<sup>−/−</sup> mice (Figure 3D). Together, these data demonstrate that SMC-derived Runx2 mediates macrophage infiltration into the calcified lesions.

**SMC-Specific Runx2-Deficiency Inhibits the Formation of TRAP-Positive Osteoclast-Like Cells**

The presence of TRAP-positive osteoclast-like cells has been identified in close apposition to the RANKL-positive areas in the calcified atherosclerotic lesions of HFD-fed ApoE<sup>−/−</sup> mice. With immunofluorescent staining, we determined HFD-induced RANKL expression in the calcified atherosclerotic lesions of Runx2<sup>f/f</sup>:ApoE<sup>−/−</sup> mice (Figure 4A). TRAP-positive cells were identified in the vicinity of RANKL-positive calcified lesions (Figure 4A, 2 upper panels), supporting a link between RANKL induction and formation of osteoclast-like cells. In contrast, fewer TRAP-positive cells were identified in the aortas from HFD-fed Runx2<sup>ΔE8/ΔE8 SMC</sup>:ApoE<sup>−/−</sup> mice, in which HFD-induced RANKL was inhibited (Figure 4A, bottom panel). Quantitative analysis determined that the Runx2 deficiency in SMC inhibited the formation of TRAP-positive cells (Figure 4B). To examine whether the presence of a greater number of TRAP-positive cells was associated with a larger calcified atherosclerotic lesion, we quantified Alizarin Red-positive lesion area and TRAP-positive area in the aortic root of HFD-fed Runx2<sup>f/f</sup>:ApoE<sup>−/−</sup> mice (Figure 4C). Spearman correlation analysis demonstrated a positive correlation between the amount of TRAP-positive area to the extent of calcification (Spearman correlation=0.697, *P*<0.0026), suggesting that the formation of osteoclast-like cells is coupled to the development of vascular calcification.

**SMC-Specific Runx2 Deficiency Inhibits the Formation of TRAP-Positive Osteoclast-Like Cells in Coculture**

We next assessed the functional contribution of Runx2-regulated RANKL in VSMC, and found that oxidative stress–induced RANKL expression was inhibited in the Runx2<sup>ΔE8/ΔE8 SMC</sup> VSMC in vitro (Figure 5A & B). Utilizing a coculture system of VSMC with bone marrow macrophages (BMM), we determined the effects of the Runx2 deficiency in VSMC on the differentiation of BMM into TRAP-positive osteoclasts. BMM cocultured with oxidative stress–treated Runx2<sup>f/f</sup> VSMC formed multinucleated TRAP-positive cells (Figure 5C, upper left panels). In contrast, BMM cocultured with oxidative stress–treated Runx2<sup>ΔE8/ΔE8 SMC</sup> VSMC failed to form multinucleated TRAP-positive cells (Figure 5C, upper right panels). The defect was restored by addition of soluble RANKL (25 ng/mL) (Figure 5C, lower panels). These data demonstrate a direct effect of Runx2-mediated RANKL expression by VSMC on the formation of osteoclast-like cells.

**Discussion**

Emerging studies have supported the finding that vascular calcification is an active cell-driven process characterized by the acquisition of osteogenic phenotype of vascular cells.
during atherosclerotic development.\textsuperscript{1–5} However, the precise vascular cell type and the molecular mechanisms underlying osteogenic differentiation of vascular cells during atherosclerosis remain undefined. Studies from our group and others have shown that Runx2 is involved in osteogenic differentiation and calcification of VSMC in vitro and that the upregulation of Runx2 is associated with arterial calcification in human and mouse models.\textsuperscript{4,7,14} But the regulation and upregulation of Runx2 is associated with arterial calcification in vivo.\textsuperscript{13,14} We found that the Runx2 deficiency inhibits formation of TRAP-positive cells in a SMC/macrophage coculture system. A, Real-time PCR analysis of RANKL expression in VSMC exposed to osteogenic media with or without H\textsubscript{2}O\textsubscript{2} (0.4 mmol/L) for 2 weeks. The expression of RANKL in Runx2\textsuperscript{−/−} VSMC in the control condition (first bar) is defined as 1 (n=3, \textit{P}<0.05 in comparison with control condition). B, RANKL protein expression in VSMC from separate sets of experiments as A. The expression of RANKL protein in cell lysates was quantified by ELISA (n=3, \textit{P}<0.05 in comparison with control). C, Coculture of H\textsubscript{2}O\textsubscript{2}-treated VSMC with BMMs. BMMs were plated on top of Runx2\textsuperscript{−/−} (a and c) or Runx2\textsuperscript{+/+} (b and d) VSMC that were preincubated in osteogenic media with H\textsubscript{2}O\textsubscript{2} (0.4 mmol/L) for 2 weeks in the absence (top, a and b) or presence of RANKL (25 ng/mL, c and d) for 1 week, and stained for TRAP. Representative images of stained wells from 2 independent experiments performed in duplicate are shown. Higher-magnification images of the boxed areas of a, b, c, and d are shown at the right of each image (panels a1, b1, c1, and d1). SMIC indicates smooth muscle cell; VSMC, vascular smooth muscle cell; RANKL, receptor activator of nuclear factor kappa-B ligand; PCR, polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase; BMM, bone marrow macrophage.

Figure 5. SMC-specific Runx2 deficiency inhibits formation of TRAP-positive cells in a SMC/macrophage coculture system. A, Real-time PCR analysis of RANKL expression in VSMC exposed to osteogenic media with or without H\textsubscript{2}O\textsubscript{2} (0.4 mmol/L) for 2 weeks. The expression of RANKL in Runx2\textsuperscript{−/−} VSMC in the control condition (first bar) is defined as 1 (n=3, \textit{P}<0.05 in comparison with control condition). B, RANKL protein expression in VSMC from separate sets of experiments as A. The expression of RANKL protein in cell lysates was quantified by ELISA (n=3, \textit{P}<0.05 in comparison with control). C, Coculture of H\textsubscript{2}O\textsubscript{2}-treated VSMC with BMMs. BMMs were plated on top of Runx2\textsuperscript{−/−} (a and c) or Runx2\textsuperscript{+/+} (b and d) VSMC that were preincubated in osteogenic media with H\textsubscript{2}O\textsubscript{2} (0.4 mmol/L) for 2 weeks in the absence (top, a and b) or presence of RANKL (25 ng/mL, c and d) for 1 week, and stained for TRAP. Representative images of stained wells from 2 independent experiments performed in duplicate are shown. Higher-magnification images of the boxed areas of a, b, c, and d are shown at the right of each image (panels a1, b1, c1, and d1). SMIC indicates smooth muscle cell; VSMC, vascular smooth muscle cell; RANKL, receptor activator of nuclear factor kappa-B ligand; PCR, polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase; BMM, bone marrow macrophage.

The SMC-specific Runx2 deficiency did not affect basal vascular smooth muscle phenotype, since the gross anatomy of vessel walls and SMC marker gene expression was similar in control and Runx2 deficiency mice (Online Figure IIB). This observation is consistent with the finding that Runx2 global knockout mice appear to have a normal cardiovascular system.\textsuperscript{8,9} The expression of Runx2 is very low in normal arteries\textsuperscript{13,14} and in isolated VSMC.\textsuperscript{7} We found that the Runx2 deficiency did not affect the basal expression of SMC marker genes, including SMA and SM22\textalpha (Online Figure III), which is consistent with our previous observation that Runx2 knockout by shRNA in VSMC does not result in a compensatory increase in expression of smooth muscle marker genes.\textsuperscript{7} In contrast, the impact of the Runx2 deficiency on oxidative stress–induced expression of SMC marker genes is dramatic, as the decrease in expression of the SMC markers under the oxidative stress condition was significantly inhibited in Runx2-deficient VSMC (Online Figure III). The molecular mechanisms underlying Runx2-regulated expression of SMC markers under oxidative stress might be attributed to its interaction with the key SMC regulators, myocardin and serum response factor (SRF). Overexpression of Runx2 in C3H10T1/2 mesenchymal cells was found to directly interact with SRF and disrupt the formation of myocardin/SRF complex, which in turn repressed myocardin-induced expression of the SMC marker genes.\textsuperscript{26} Accordingly, in addition to a direct inhibition of myocardin by oxidative stress,\textsuperscript{7} upregulation of Runx2 by oxidative stress (in vitro and in vivo) during vascular calcification may interrupt the formation of the SRF/myocardin complex, and thus contribute to the decreased expression of the SMC gene markers. Therefore, in the Runx2-deficient VSMC, oxidative stress–induced repression of SMC markers was inhibited. Our finding of an inverse correlation of the expression of Runx2 and SMC markers is consistent with the observations that downregulation of the SMC markers is linked to the upregulation of Runx2 in the calcified media from the mice deficient in Matrix Gla protein, a well-studied calcification mouse model.\textsuperscript{27,28} Our studies demonstrate a definitive role of SMC-derived Runx2 in regulating SMC markers and VSMC dedifferentiation under oxidative stress conditions.

Figure 5. SMC-specific Runx2 deficiency inhibits formation of TRAP-positive cells in a SMC/macrophage coculture system. A, Real-time PCR analysis of RANKL expression in VSMC exposed to osteogenic media with or without H\textsubscript{2}O\textsubscript{2} (0.4 mmol/L) for 2 weeks. The expression of RANKL in Runx2\textsuperscript{−/−} VSMC in the control condition (first bar) is defined as 1 (n=3, \textit{P}<0.05 in comparison with control condition). B, RANKL protein expression in VSMC from separate sets of experiments as A. The expression of RANKL protein in cell lysates was quantified by ELISA (n=3, \textit{P}<0.05 in comparison with control). C, Coculture of H\textsubscript{2}O\textsubscript{2}-treated VSMC with BMMs. BMMs were plated on top of Runx2\textsuperscript{−/−} (a and c) or Runx2\textsuperscript{+/+} (b and d) VSMC that were preincubated in osteogenic media with H\textsubscript{2}O\textsubscript{2} (0.4 mmol/L) for 2 weeks in the absence (top, a and b) or presence of RANKL (25 ng/mL, c and d) for 1 week, and stained for TRAP. Representative images of stained wells from 2 independent experiments performed in duplicate are shown. Higher-magnification images of the boxed areas of a, b, c, and d are shown at the right of each image (panels a1, b1, c1, and d1). SMIC indicates smooth muscle cell; VSMC, vascular smooth muscle cell; RANKL, receptor activator of nuclear factor kappa-B ligand; PCR, polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase; BMM, bone marrow macrophage.
The inverse correlation of the expression of Runx2 and SMC markers under oxidative stress conditions is consistent with the vascular calcification phenotype we observed in the Runx2-deficient VSMC, supporting a functional contribution of Runx2 to vascular calcification under pathological conditions, such as oxidative stress. Oxidative stress–induced calcification was significantly inhibited in VSMC from Runx2E8/Δ mice, despite the presence of the truncated Runx2 protein (Figure 1). The truncated Runx2 protein contains intact DNA-binding runt homology, nuclear localization signal, and a partial transactivation domain, and thus maintains its DNA binding activity (Online Figure IV). However, its transcriptional activity was markedly ablated. These results are consistent with previous observation in other cells. In the Runx2 exon 8–deleted VSMC, oxidative stress–induced upregulation of Runx2-dependent osteogenic marker genes, including OC and Colla, was also inhibited (Figure 1), further demonstrating that oxidative stress induces VSMC calcification via a Runx2-dependent pathway in vitro. These in vitro results are consistent with in vivo observations that SMC-specific Runx2 deficiency blocked HFD-induced vascular calcification in atherosclerotic lesions (Figure 2). Of note, some Alizarin Red–positive punctate calcified nodules were identified in the aortic sections from Runx2E8/Δ;ApoE−/− mice (Figure 4A, indicated by arrows), which may be explained by the residual activity of the truncated Runx2 in VSMC or Runx2-independent alternative osteogenic pathways. Nevertheless, we also found that the Runx2 deficiency inhibited oxidative stress–induced expression of osteirx and Msx2 in VSMC (data not shown), suggesting a key role of Runx2 in regulating multiple signals that contribute to oxidative stress–induced osteogenic differentiation of VSMC.

The observation of the function of the Runx2 exon 8 in regulating VSMC calcification is consistent with previous findings that the Runx2 exon 8 is essential for commitment and progression of the osteoblast and chondrocytes phenotype, and the Runx2 exon 8 truncation results in complete absence of bone formation and embryonic lethality. These in vitro studies suggest that the Runx2 exon 8 is required for vascular calcification in vivo directly regulating osteogenic differentiation of VSMC. In addition to a direct effect on promoting osteogenic differentiation of VSMC, we have determined that Runx2 mediates oxidative stress–induced expression of RANKL in VSMC by direct binding to the RANKL promoter. RANKL has been reported to be upregulated in atherosclerotic lesions, calcified vessels, and valves. In ApoE−/− mice, we have found that HFD induces the expression of RANKL, which is associated with increased expression of Runx2 in the calcified lesions. The present studies further determined that HFD-induced RANKL expression was inhibited in the Runx2-deficient mice (Figure 3), demonstrating a direct regulation of RANKL by Runx2 in vivo. It was proposed that RANKL may be a procalcification molecule during the development of atherosclerosis. However, inhibition of RANKL per se does not directly inhibit the calcification process, since VSMC from RANKL knockout mice still undergo calcification in response to oxidative stress. In addition, recombinant RANKL protein does not directly promote calcification of mouse primary VSMC in culture.

Our studies suggest that Runx2-regulated RANKL expression in VSMC promotes macrophage infiltration into the calcified atherosclerotic lesions. Monocyte/macrophage recruitment plays a critical role in the pathogenesis of atherosclerosis. In the aortas of the Runx2-deficient mice, macrophage infiltration was blocked, and the majority of macrophages were retained in the endothelium layer (Figure 3). The accumulation of macrophages in the arterial wall follows adhesion of circulating monocytes to the endothelium. Once the firm adhesion is established, monocytes transmigrate across the endothelium into the intima along a chemotactic gradient secreted by endothelial cells and SMC. The blockage of macrophage infiltration into the (neo) intima SMC layer in the Runx2-deficient mice (Figure 3) suggests inhibition of production of SMC-derived chemotactic molecules. We have previously demonstrated that SMC-derived RANKL promotes macrophage migration in vitro, finding similar to the observation that soluble RANKL protein exhibits chemotactic properties toward monocytes/macrophages. Therefore, inhibition of HFD-induced expression of RANKL in the Runx2-deficient VSMC directly contributes to impaired macrophage infiltration in the (neo) intimal areas (Figure 3). Other chemokines have also been reported to promote macrophage infiltration. A decrease in monocytes/macrophages in arterial walls has been found in mice deficient in monocyte chemotactic protein-1 (MCP-1) or its primary receptor in vivo, CCR2. RANKL can induce the migration of MonoMac-6 mononuclear cells as well as peripheral blood mononuclear cells in a dose-dependent manner with an efficacy similar to that of MCP-1. In addition, RANKL induces the expression of MCP-1, which contributes to its effect on macrophage recruitment. Here we found that inhibition of RANKL was associated with decreased expression of MCP-1 and CCR2 in the aortas of Runx2-deficient mice (Online Figure V). The expression of MCP-1 has been found in neointimal VSMC and intimal macrophages in atherosclerotic lesions of ApoE−/− mice. It is likely that SMC-derived RANKL directly regulates MCP-1/CCR2 to induce macrophage infiltration further into the (neo) intimal/medial layers after initial transendothelial migration of macrophages into the subendothelium. Alternatively, Runx2-upregulated RANKL may work in concert with MCP-1/CCR2 to mediate macrophage infiltration. Taken together, our data support the role of SMC-derived Runx2 in regulating the expression of these chemokine/cytokines that are key to the interplay between VSMC and macrophages.

As a key regulator for osteoclast formation, the major function of RANKL, highly expressed in lymphoid tissues and trabecular bone, is to promote development of multinucleated bone-resorptive osteoclasts from monocyctic precursors. Therefore, upregulation of RANKL in VSMC during calcification may contribute to the formation of TRAP-positive multinucleated osteoclast-like cells, which have been observed in close apposition to the mineralized bone-like structures within atherosclerotic plaques of human and mouse arteries. We have previously determined in vitro that SMC-derived RANKL promotes differentiation of macro-
phages into TRAP-positive multinucleated osteoclast-like cells in a coculture of VSMC and bone marrow–derived macrophages. The present studies demonstrated that Runx2-regulated RANKL in VSMC is important for the formation of TRAP-positive osteoclast-like cells in the calcified lesions in vivo. The type(s) of macrophages that develop into osteoclast-like cells is not clear, considering the heterogeneity of the macrophages identified in the atherosclerotic lesions. By coupling the calcification of VSMC and the formation of vascular osteoclasts, therefore, targeting Runx2 or Runx2-regulating signals in VSMC may represent a novel strategy for prevention and therapy of vascular calcification.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- Atherosclerotic calcification reflects an osteochondrogenic transformation of vascular cells, which is associated with increased expression of bone-related markers.
- Runx2 regulates calcification of vascular smooth muscle cells (VSMC) in vitro.
- TRAP-positive osteoclast-like cells are present in calcified atherosclerotic plaques, in the vicinity of ectopic mineralization sites.
- Increased RANKL, a key regulator for osteoclast differentiation, is associated with vascular calcification.

**What New Information Does This Article Contribute?**

- Osteogenic differentiation of VSMC plays an important role in the pathogenesis of vascular calcification in vivo.
- Smooth muscle cell–derived Runx2 regulates osteogenic differentiation of VSMC and atherosclerotic calcification in vivo.
- The Runx2 exon 8 is essential for its function to regulate osteogenic differentiation of VSMC; however, deletion of the Runx2 exon 8 does not affect basal SMC markers.
- SMC-specific Runx2 deficiency inhibits high-fat diet induced-expression of RANKL in calcified atherosclerotic lesions, and blocks the formation of TRAP-positive osteoclast-like cells and infiltration of CD68-positive macrophages.
- The amount of TRAP-positive area in the calcified atherosclerotic lesions is positively correlated with the extent of calcification, suggesting that the formation of osteoclast-like cells is coupled with the development of vascular calcification in vivo.
- Runx2 deficiency inhibits RANKL production by VSMC, which abolishes osteoclastic differentiation of bone marrow–derived macrophages in a coculture system.

Atherosclerotic calcification reflects an osteochondrogenic transformation of vascular cells. We have previously identified that the osteogenic transcription factor, Runx2, is essential for oxidative stress–induced VSMC calcification in vitro. The present study demonstrates, for the first time, the importance of osteogenic differentiation of VSMC in the pathogenesis of vascular calcification in vivo. With a novel SMC-specific knock-out mouse model, we have determined a critical role of SMC-derived Runx2 and Runx2-dependent osteogenic differentiation of VSMC in regulating vascular calcification in vivo. Our studies reveal intrinsic coupling between calcifying VSMC and the formation of vascular osteoclasts in the calcified atherosclerotic lesions, which parallels the bone remodeling process.
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SMOOTH MUSCLE CELL-SPECIFIC RUNX2 DEFICIENCY INHIBITS VASCULAR CALCIFICATION

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Online Supplemental Methods

Generation of SMC-specific Runx2 exon 8 deletion mice. The SM22α-Cre transgenic mice were obtained from The Jackson Laboratory and crossed into C57BL/6 background for 10 generations. The Runx2 exon 8 floxed (Runx2f/f)1 were crossed into C57BL/6 background for 10 generations. In the Runx2f/f mice, exon 8 of the Runx2 gene was floxed based on previous studies demonstrating that the C-terminus of Runx2 encoded by exon 8 is critical for Runx2 function and truncation of Runx2 exon 8 results in complete absence of bone formation and embryonic lethality7. The truncated Runx2 protein lacks the C-terminus, but contains intact DNA binding runt homology, nuclear localization signal and a partial transactivation domain. The SM22α-Cre mice were bred with Runx2f/f to generate Runx2ΔE8/+:Cre+/- mice, which were backcrossed with Runx2f/f mice to generate smooth muscle-specific Runx2 deletion mice (Runx2ΔE8/ΔE8 SMC). The Runx2ΔE8/ΔE8 SMC mice were further crossed with ApoE -/- mice (B6 background). After interbreeding of the heterozygotes, polymerase chain reaction (PCR) analysis of tail-tip DNA was used to identify Runx2ΔE8/ApoE-/- and Runx2ΔE8/ΔE8 SMC:ApoE-/- mice. Primer sets for genotyping are: Cre: F-5'-GCGGTCTGGCAGTAAAAACTATC-3' and R-5'-GTGAAACAGCATTGCTGTCACTT-3'; Runx2: F-5'-GCAAGATCATGACTAGGGATTG-3' and R-5'-ATCAGTTCCCAATGGTACCCG-3'; ApoE: F-5'-GCCGCCCCGACTGCATCT-3', R-5'-TGTTGACTTGGGAGCTCTGCAGC-3' and R-5'-GCCGCCCCGACTGCATCT-3'.

VSMC culture. Primary VSMC were isolated from mouse aorta and cultured in growth media as we described previously3. All experiments were performed with VSMC at passages 3 to 5.

In vitro VSMC calcification3. VSMC calcification was induced as we previously described in osteogenic media containing 0.25 mmol/L L-ascorbic acid and 10 mmol/L β-glycerophosphate (Sigma Aldrich) with H2O2(0.4 mmol/L) for 3 weeks3. Calcification was determined by Von Kossa and Alizarin Red staining or quantified by measuring total calcium in the cell lysates by the Arsenazo III method4.

Real-time polymerase chain reaction (PCR). The expression of smooth muscle markers and osteogenic factors in aortic tissues and VSMC was determined by real-time PCR4. Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed into cDNA. SYBR Green-based real-time PCR was performed using specific primers for smooth muscle specific α-actin (SMA), SM22α, murine alkaline phosphatase (ALP), type I collagen (Col Ia), osteocalcin (OC), RANKL and CD683, using iQ SYBR Green Supermix (Bio-Rad) on an iCycler Thermal Cycler (Bio-Rad)4.

Western blot analysis of Runx2. Nuclear extracts from VSMC were prepared and protein concentration was measured as we described3. Western blot analyses were performed with specific antibody for Runx2 (MBL D130-3) or lamin B (Santa Cruz), and detected with a chemiluminescence detection kit (Millipore).

Electrophoresis mobility shift assay (EMSA). EMSA was performed to determine Runx2 DNA binding activity. Nuclear extracts were extracted and analyzed using 32P-labeled probe containing Runx2-binding oligonucleotide from the Bglap2 promoter (underline, Runx2 consensus): 5’-
CAGAAACCAACCACGGACCCAAACCACAGCTCCAC-3’ as we previously described5. For supershift, nuclear extracts were pre-incubated with anti-Runx2 antibody or control mouse IgG for 20 minutes before the addition of the labeled probe. For competition experiments, an excessive amount of unlabeled probe (100-fold) was added to binding reactions.

Transfection and luciferase-reporter assay5. To determine the transcriptional activity of Runx2 proteins, VSMC were transfected with pGL3-6×OSE2 luciferase reporter plasmid and a Renilla luciferase expression plasmid (control for transfection efficiency) using FuGene HD transfection reagent (Roche). Cells were then incubated for 72 hours and harvested to determine luciferase activity. The luciferase reporter activity was normalized by Renilla luciferase activity (Promega).

Experimental animals. 8-wk-old Runx2\textsuperscript{f/f}:ApoE\textsuperscript{−/−} and Runx2\textsuperscript{ΔE8/ΔE8 SMC}:ApoE\textsuperscript{−/−} mice were fed a normal chow or a high-fat, high-cholesterol diet containing 21.2% fat and 0.2% cholesterol (Harlan Teklad diet TD88137) for 30 weeks. Both food and fluid intake were given ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Tissue harvest and processing. After euthanization, the heart and vasculature were perfused with sterile PBS. The heart and the aorta were dissected under a microscope and frozen in OCT embedding medium (Tissue-Tek) for serial cryosectioning. The initial sections were collected when the first cusp became visible in the lumen of the aorta. Serial sections (8μm in thickness) were collected from the aortic roots and the innominate arteries. Descending aorta was dissected for calcium quantification and PCR analysis of gene expression.

Immunohistochemistry. Frozen sections were processed for immunohistochemistry as we described previously4. In brief, 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). All quantitative analyses in this study were performed blindly by at least two investigators.

Immunofluorescent staining. Frozen aortic root sections were processed and immunofluorescently stained as described previously4. Antibodies for RANKL (Santa Cruz) were applied to fixed cryosections. Slides were washed extensively before the addition of species-specific fluorescently labeled secondary antibody (Alexa Fluor® 594, Invitrogen). For TRAP staining, slides were incubated with ELF97 TRAP (Invitrogen) diluted in acetate buffer. 4’, 6-diamidino-2-phenylindole (DAPI) was used for nuclear localization.

Aortic calcification. Calcium deposits in aortic sections were stained using Alizarin Red (Sigma Aldrich) as described previously5. Alkaline phosphatase activity was detected by NBT/BCIP Reagent Kit (Invitrogen). Stained specimens were examined microscopically (Leica M165 FC), and the percentage of positively stained area for each section was quantified using ImageJ software (NIH Bethesda, MD).

Aortic calcium content in descending aortas was measured as previously described5. Descending aortas were lyophilized to constant weight and decalcified with 0.6 mmol/L HCl at 37°C for 48 hours. Calcium released from the lyophilized tissues was determined colorimetrically by Arsenazo III (3, 6-bis(2-Arsonophenyl) Azo]-4,5-dihydroxy-2,7-naphthalenedisulfonic acid with calcium diagnostic kit; Stanbio Laboratory). The amount of vascular calcium was normalized by the dry weight of the tissues and expressed as millimolar/gram dry weight.
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. The RANKL protein level was measured using the murine RANKL Quantikine ELISA kit (R & D Biosystems) in cell lysates. Protein concentrations of all samples were measured using a BCA protein assay kit.

Osteoclastogenesis of bone marrow macrophages co-cultured with VSMC. Bone marrow macrophages (BMMs) were isolated from femora and tibiae of 8-10 week old C57BL/6 mice and grown in α-MEM with 10% Hi-FBS/10 ng/ml M-CSF (Sigma Aldrich) as we described.$^7$ For co-culture experiments, BMMs were added on top of VSMC that were cultured in osteogenic media for 2 weeks with H$_2$O$_2$ (0.4 mM). After 1 week, cells were stained with a Naphthol AS-BI phosphoric acid/Fast Garnet/tartrate solution (Sigma Aldrich) to determine the formation of osteoclast-like cells TRAP-positive cells.

Statistical analysis. All the data are expressed as means ± SD. Differences in data between groups were compared with Student’s paired 2-tailed $t$ test or 1-way ANOVA. For correlation analysis, normality was tested with the Shapiro–Wilk normality test. When normality passed ($p$≥0.05), Pearson correlation was used. When normality failed ($p$<0.05), Spearman correlation was used.$^8$ A $p$ value less than 0.05 was considered statistically significant.

Online Supplemental Data

Characterization of Cre-mediated recombination by SM22Cre mice: To determine specificity and efficiency of Cre-mediated excision of the SM22Cre mice, SM22Cre mice were bred with ROSA reporter mice (R26R). X-gal staining was demonstrated in aorta and VSMC from the R26R:SM22Cre mice (Suppl. Fig IA,b, &B,d) but not in those from the R26R mice (Suppl. Fig IA,a &B,c). Sections of the aortas from R26R:SM22Cre mice demonstrated X-gal expression in the media SMC of aorta vessels (Suppl. Fig IC, h &k), which is co-localized with the SMC-specific α-actin staining (SMA, Suppl. Fig IC, i&l). Focal positive staining of SMA, but not x-gal staining was not detected in adventitial myofibroblasts (Suppl. Fig IC, i&l), indicating Cre-mediated recombination did not occur in adventitia myofibroblasts. These results demonstrated SM22Cre-mediated specific and efficient gene excision in SMC of the aorta.
Suppl. Fig I. X-gal staining of A) aortas and B) VSMC from ROSA reporter mice with or without SM22Cre transgene. Aorta (a, b) and VSMC (c, d) explanted from 4 week old R26R control mouse (a,c) or ROSA:SM22Cre+/mouse (b, d) were stained with X-gal. X-gal staining (blue) indicates effective SM22Cre-mediated excision. C) Immunostaining of cross sections of aortas that pre-stained with X-gal and then stained for H&E (a, d, g, j) or SMA (c, f, i, l). a-f, from ROSA mice; g-l, from ROSA:SM22Cre mice. Photomicrographs of bright field microscopy, magnification at medium (a-c and g-h) and high (d-f and j-l) are shown. M: media, A: adventitia.

Characterization of vascular phenotype of SMC-specific Runx2 deficient mice: The expression of Runx2 is low in normal arteries\textsuperscript{9,10} and in isolated VSMC\textsuperscript{3}. To determine whether deletion of Runx2 exon 8 in smooth muscle cells affects normal vascular phenotype, we analyzed aortas from Runx2\textsuperscript{\AER/AES} SMC mice and Runx2\textsuperscript{Ef}\ mice (Suppl. Fig II). PCR analysis determined that Runx2 deletion in VSMC from Runx2\textsuperscript{\AER/AES} SMC mice (Suppl. Fig IIA). Histological analyses did not identify any gross structural differences (H&E, Suppl. Fig. IIB). Immunohistochemical analysis further revealed that there was no difference in the development of the smooth muscle layer positively stained with SMC-specific \(\alpha\)-actin (SMA, Suppl. Fig IIB). In addition, Runx2 deletion did not affect the aortic expression levels of the smooth muscle marker genes, SMA and SM22\(\alpha\) (Suppl. Fig IIC).

Suppl. Fig II. SMC-specific Runx2 deletion does not affect smooth muscle gene expression. (A) PCR analysis of genomic DNA to detect Cre-mediated recombination of exon 8 of Runx2 gene in VSMC from wild type (+/+), Runx2\textsuperscript{Ef}/(Ef) and Runx2\textsuperscript{\AER/AES} SMC (\(\Delta/\Delta\)) mice. PCR reaction was performed using primers spanning the coding region of exon 8 F-5'-TTCGGGAGTTAGACGAGA-3' and R-5'-GCAAGATCATGACTAGGGATTG-3; or Cre gene. (B) Histological analysis of aortas from Runx2\textsuperscript{Ef} and Runx2\textsuperscript{\AER/AES} SMC (Runx2\textsuperscript{\A/A}) mice. Cross sections of descending aorta were stained with hematoxylin and eosin (a & b, upper panels) and an antibody for smooth muscle specific \(\alpha\)-actin (c & d, lower panels). Higher magnification images of boxed areas are shown under each image (a1, b1, c1 & d1). M: media, A: adventitia. (C) Expression of SMC-specific markers SMA and SM22\(\alpha\) was determined by real-time PCR analysis of aorta from Runx2\textsuperscript{Ef} and Runx2\textsuperscript{\AER/AES} SMC (Runx2\textsuperscript{\A/A}) mice (n=4 mice for each genotype).
Effects of SMC-specific Runx2 deficiency on the expression of SMC markers in response to oxidative stress: To determine whether SMC-specific Runx2 deficiency affects the expression of SMA in response to oxidative stress, real-time PCR analyses were performed. The basal levels of the expression of SMC markers, SMA and SM22α, were similar in VSMC from Runx2^ff or Runx2^ΔΔ mice (Suppl. Fig III). However, oxidative stress-induced decrease in the expression of SMC markers (n=3, \( p<0.05 \)) was inhibited, at least partially, in the Runx2^ΔΔ VSMC (n=3, \( p<0.05 \)).

Suppl. Fig III. Runx2 deficiency inhibits oxidative stress-induced decreases in the expression of SMC markers. VSMC from Runx2^ff and Runx2^ΔΔ mice were exposed to control condition or H\(_2\)O\(_2\) (0.4 mM) for 10 days. Real-time PCR analysis was performed to determine the expression of SMA and SM22α. The expression of SMA and SM22α, normalized by the levels of GAPDH mRNA, in Runx2^ff under control conditions are defined as 1.

Characterization of the function of Runx2 truncated protein in VSMC: VSMC from the Runx2^ΔE8/ΔE8 SMC mice expressed Runx2 truncated protein (Fig 1A & Suppl. Fig IVA). The DNA binding activity and transcriptional activity of the truncated Runx2 protein was further determined. EMSA demonstrated that the truncated Runx2 protein was able to bind to Runx2-specific consensus elements similarly to the full-length Runx2 protein (Suppl. Fig. IVB, lanes 7-11). However, transcriptional activity of the truncated Runx2 protein was dramatically ablated compared to that of the full-length Runx2 (Suppl. Fig. IVC).

Suppl. Fig IV. Effects of the exon 8 truncation on Runx2 DNA binding and transcriptional activities. VSMC from Runx2^ff and Runx2^ΔΔ mice were exposed to H\(_2\)O\(_2\) (0.4 mM) for 10 days. (A) Western blot analysis of nuclear expression of Runx2 in these VSMC (indicated by the arrows). The expression of lamin B was used as a loading control. (B) Runx2 DNA binding activity determined by EMSA. Lanes 1-5, verification of Runx2-specific binding activity by supershift assays with Runx2 antibody and competitive binding assays, using nuclear extract from osteogenic MC3T3 cells (positive controls). fp: probe alone; Lanes 2-5: probe and nuclear extracts with Runx2 (R2) or control (IgG) antibody, or cold probe (Cp). Lanes 7&8 and 10&11 (with Runx2 antibody): Runx2 DNA binding activity in nuclear extracts from Runx2^ΔΔ (ΔΔ) and Runx2^ff (ff) VSMC. Representative images from four independent experiments are shown. (C) VSMC were seeded on 24-well plates and co-transfected with Runx2 luciferase reporter (pGL3-6×OSE) and Renilla luciferase reporter (control for transfection efficiency). Luciferase activities were analyzed after 48 hours. Data shown are relative luciferase activities (normalized by Renilla luciferase activity) in Runx2^ΔΔ VSMC compared with those in Runx2^ff VSMC, which is defined as 100 (n=6, \( p<0.001 \)).
Effects of SMC-specific Runx2 deficiency on HFD-induced aortic expression of MCP-1 and CCR2:

The effect of SMC-specific Runx2 deficiency on aortic expression of MCP-1 and its receptor CCR2 was determined in descending aortas from Runx2^{f/f}:ApoE^{-/-} mice and the Runx2^{AE/AE_SMC}:ApoE^{-/-} mice. Quantitative real-time PCR demonstrated that aortic expression of MCP-1 and CCR2 was significantly inhibited by SMC-specific Runx2 deletion in the Runx2^{AE/AE_SMC}:ApoE^{-/-} mice (Suppl. Fig V).

**Suppl Fig V.** SMC-specific Runx2 deficiency inhibits HFD-induced aortic expression of MCP-1 and CCR2. Real-time PCR analysis of the expression of MCP-1 and CCR2 in descending aortas from high-fat fed Runx2^{f/f}:ApoE^{-/-} (n=4 mice) and Runx2^{AE/AE_SMC}:ApoE^{-/-} mice (Runx2^{AE/}:ApoE^{-/-}, n=3 mice). The expression of MCP-1 and CCR2, normalized by the levels of GAPDH mRNA, in Runx2^{f/f}:ApoE^{-/-} is defined as 1. *p<0.05.

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