

Inhibition of FOXO1/3 Promotes Vascular Calcification

Liang Deng,* Lu Huang,* Yong Sun,* Jack M. Heath, Hui Wu, Yabing Chen

Objective—Vascular calcification is a characteristic feature of atherosclerosis, diabetes mellitus, and end-stage renal disease.

We have demonstrated that activation of protein kinase B (AKT) upregulates runt-related transcription factor 2 (Runx2), a key osteogenic transcription factor that is crucial for calcification of vascular smooth muscle cells (VSMC). Using mice with SMC-specific deletion of phosphatase and tensin homolog (PTEN), a major negative regulator of AKT, the present studies uncovered a novel molecular mechanism underlying PTEN/AKT/FOXO (forkhead box O)-mediated Runx2 upregulation and VSMC calcification.

Approach and Results—SMC-specific PTEN deletion mice were generated by crossing PTEN floxed mice with SM22 α -Cre transgenic mice. The PTEN deletion resulted in sustained activation of AKT that upregulated Runx2 and promoted VSMC calcification in vitro and arterial calcification ex vivo. Runx2 knockdown did not affect proliferation but blocked calcification of the PTEN-deficient VSMC, suggesting that PTEN deletion promotes Runx2-dependent VSMC calcification that is independent of proliferation. At the molecular level, PTEN deficiency increased the amount of Runx2 post-transcriptionally by inhibiting Runx2 ubiquitination. AKT activation increased phosphorylation of FOXO1/3 that led to nuclear exclusion of FOXO1/3. FOXO1/3 knockdown in VSMC phenocopied the PTEN deficiency, demonstrating a novel function of FOXO1/3, as a downstream signaling of PTEN/AKT, in regulating Runx2 ubiquitination and VSMC calcification. Using heterozygous SMC-specific PTEN-deficient mice and atherogenic ApoE^{-/-} mice, we further demonstrated AKT activation, FOXO phosphorylation, and Runx2 ubiquitination in vascular calcification in vivo.

Conclusions—Our studies have determined a new causative effect of SMC-specific PTEN deficiency on vascular calcification and demonstrated that FOXO1/3 plays a crucial role in PTEN/AKT-modulated Runx2 ubiquitination and VSMC calcification. (*Arterioscler Thromb Vasc Biol.* 2015;35:175-183. DOI: 10.1161/ATVBAHA.114.304786.)

Key Words: AKT ■ FOXO1/3 ■ PTEN ■ Runx2 ■ ubiquitination ■ vascular calcification

Vascular calcification, namely aberrant calcium deposition in the vessel wall, reduces elasticity and compliance of the vessel wall. It is a well-known predictive risk factor of subsequent cardiovascular mortality.¹⁻³ Vascular calcification has now been recognized as an active cell-regulated process resembling bone modeling, rather than simply passive calcium deposition.⁴⁻¹⁰ Several cell types are involved in this process, including vascular smooth muscle cells (VSMC), which undergo osteogenic differentiation and calcification.⁴⁻¹⁰

We have demonstrated that runt-related transcription factor 2 (Runx2), the key osteogenic regulator for osteoblast differentiation and chondrocyte maturation,^{11,12} plays an essential role in regulating osteogenic differentiation of VSMC in vitro¹³ and vascular calcification in atherosclerosis in vivo.¹⁴ Furthermore, we have determined that activation of protein kinase B (AKT) is crucial for oxidative stress-induced VSMC calcification through upregulation of Runx2,¹³ and constitutively activated AKT promotes VSMC calcification.¹⁵ However, the molecular mechanisms underlying AKT-regulated upregulation of

Runx2 and VSMC calcification are unknown. Activation of AKT is regulated by 2 major upstream signals: phosphatidylinositol 3-kinase and phosphatase and tensin homolog (PTEN). PTEN is a protein/lipid phosphatase, which was originally discovered as a tumor repressor.^{16,17} PTEN inactivates AKT by hydrolyzing phosphatidylinositol-3,4,5-trisphosphate.¹⁸ PTEN has been implicated in regulating neointimal smooth muscle cell proliferation and migration.^{19,20} PTEN deficiency in smooth muscle cells activates AKT in the mouse vasculature,²¹⁻²³ which contributes to increased smooth muscle cell proliferation that leads to intimal hyperplasia in mouse vascular development^{21,23} and in response to injury.²² In atherosclerosis studies, partial inactivation of PTEN does not seem to affect high-fat diet-induced atherosclerosis of the atherogenic ApoE^{-/-} mice,²⁴ but chemical-induced expression of PTEN was found to be associated with inhibition of high-cholesterol diet-induced atherosclerosis in a rabbit model.²⁵ It is unknown whether PTEN plays a role in regulating vascular calcification. Using the SMC-specific PTEN deletion mice,

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Nonstandard Abbreviations and Acronyms	
AKT	protein kinase B
FOXO	forkhead box O
PTEN	phosphatase and tensin homolog
Runx2	runt-related transcription factor 2
VSMC	vascular smooth muscle cells

we determined a causative effect of the PTEN deficiency on vascular calcification, independent of cell proliferation, and elucidated the underlying molecular mechanisms.

One of the known downstream signals regulated by activated AKT is the family of forkhead box O (FOXO) proteins. The FOXO family includes FOXO1, FOXO3, FOXO4, and FOXO6 in mammalian cells. FOXO1, FOXO3, and FOXO4 are ubiquitously expressed, whereas FOXO6 is specifically expressed in brain and liver.^{26–29} Activated AKT phosphorylates FOXOs and leads to exclusion of the FOXOs from the nucleus, which blocks the transcriptional activity of FOXOs.³⁰ The role of the FOXOs in VSMC calcification is entirely unknown. Previous studies have suggested a potential link between FOXO and Runx2 expression in other cell types.^{31–34} In osteoblasts and human embryonic stem cells, FOXO1 or FOXO3 increases Runx2 expression, whereas other studies suggest that FOXO1 inhibits Runx2 activity in osteoblasts or prostate cancer cells.^{31–34} Therefore, the function of FOXOs in regulating Runx2

expression may be cell type-dependent. In this study, we have determined, for the first time, the function of FOXOs in regulating VSMC calcification and elucidated the role of FOXOs in AKT-regulated upregulation of Runx2 and VSMC calcification.

We have determined that SMC-specific PTEN deletion promoted VSMC calcification in vitro, aortic calcification ex vivo and in vivo, via increased activation of AKT that upregulates Runx2. VSMC calcification induced by the PTEN deficiency is independent of VSMC proliferation. Mechanistically, activation of AKT by PTEN deficiency induces inhibition of FOXO1/3 that led to the upregulation of Runx2. The upregulation of Runx2 occurred post-transcriptionally through inhibiting Runx2 ubiquitination. Using heterozygous SMC-specific PTEN-deficient mice and atherogenic ApoE^{-/-} mice, we have also demonstrated AKT activation, FOXO phosphorylation, and Runx2 ubiquitination in vascular calcification in vivo. Altogether, our studies have provided the first evidence demonstrating a causative effect of SMC-specific PTEN deficiency on vascular calcification and identified a novel function of FOXO1/3 in the regulation of the Runx2 ubiquitination and vascular calcification.

Materials and Methods

The smooth muscle-specific PTEN-deficient mice were generated by crossing PTEN exon 5 floxed mice (PTEN^{fl/fl})³⁵ with the SM22 α -Cre transgenic mice.^{14,36} Details of materials and experimental procedures are in the Materials and Methods section in the online-only Data Supplement.

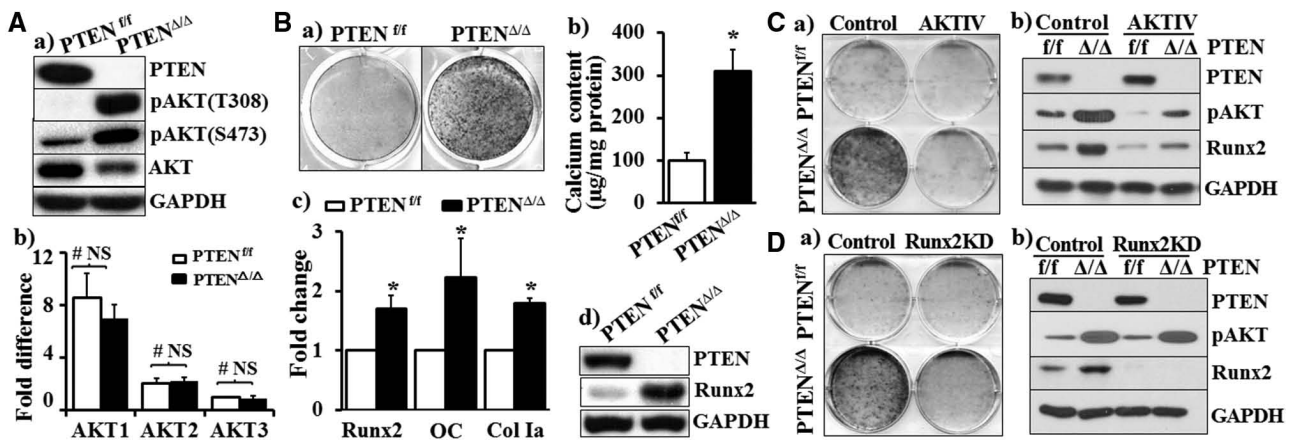


Figure 1. Increased protein kinase B (AKT) by phosphatase and tensin homolog (PTEN) deficiency promotes Runx2 upregulation and vascular smooth muscle cell (VSMC) calcification. **A**, Increased activation of AKT in PTEN-deficient VSMC. VSMC were isolated from PTEN^{fl/fl} and SMC-specific PTEN-deficient mice (PTEN^{Δ/Δ}) and cultured in growth media. **a**, Western blot analysis of the expression of AKT and its phosphorylated (activated) forms; and **b**, Real-time PCR analysis of AKT1, AKT2, and AKT3 in VSMC, normalized by the expression of GAPDH (n=5 mice per group, NS=not significant). The expression of AKT3 in PTEN^{fl/fl} VSMC is defined as 1. **B**, Calcification of VSMC from PTEN^{fl/fl} and PTEN^{Δ/Δ} mice. **a**, VSMC were cultured in osteogenic medium for 3 weeks, and calcification was determined by Alizarin Red staining. **b**, Calcification of VSMC in parallel experiments as in **a**, calcium content was quantified by Arsenazo III method. Results shown are normalized to total protein (n=3, *P=0.017). **c**, Real-time PCR analysis of the expression of Runx2 and osteogenic marker genes, osteocalcin (OC), and collagen type I (Colla) in parallel experiments as in **a**. The expression of each gene in PTEN^{fl/fl} VSMC is defined as 1 (n=3, *P<0.05). **d**, Western blot analysis of the expression of PTEN and Runx2 in parallel experiments as in **a**. **C**, Inhibition of AKT attenuates PTEN deficiency-promoted calcification. PTEN^{Δ/Δ} and control PTEN^{fl/fl} VSMC were exposed to H₂O₂-containing osteogenic media with/without an AKT inhibitor (AKTIV, 1.0 μM). **a**, Calcification was determined by Alizarin red staining; and **b**, Western blot analysis was performed to determine the AKT phosphorylation and Runx2 expression in the cells in **a**. **D**, Runx2 knockdown abolished PTEN deletion-promoted VSMC calcification. VSMC were stably infected with lentivirus carrying control scrambled small hairpin RNA (shRNA; Control) or shRNA specific for Runx2 and selected by puromycin. Stably infected VSMC were exposed to H₂O₂-containing osteogenic medium. **a**, Calcification was determined by Alizarin red staining; and **b**, Western blot analysis was performed to determine the expression of pAKT and Runx2. Representative results from 3 independent experiments are shown.

Results

Activation of AKT by PTEN Deficiency Promotes Runx2 Upregulation and VSMC Calcification

We have demonstrated that activation of AKT is associated with VSMC calcification *in vitro*.^{13,15} Using mice with SMC-specific ablation of PTEN,¹⁸ the key upstream negative regulator for AKT activation, we aimed to determine a direct effect of endogenous AKT activation on VSMC calcification. The SMC-specific PTEN deletion mice (PTEN^{ΔΔ}) were generated by crossing the PTEN exon 5 floxed mice with SM22Cre transgenic mice. Similar to previous observation,³⁷ the SM22Cre-mediated PTEN deletion resulted in early death of the PTEN^{ΔΔ} mice. We and others have reported that osteogenic differentiation of VSMC determines vascular calcification^{5,6,14}; therefore, the effect of the PTEN deletion on osteogenic differentiation of VSMC was first characterized using primary VSMC from the PTEN^{ΔΔ} mice and their control PTEN^{fl/fl} littermates.

The deletion of PTEN in VSMC was demonstrated by Western blot analysis (Figure 1Aa). AKT1 was found to be the predominant isoform of AKT in VSMC. The PTEN deletion did not affect the expression of AKT mRNA in VSMC (Figure 1Ab); however, it increased activation of AKT1, as indicated by AKT phosphorylation at both serine 473 (S473) and threonine 308 (T308) residues in the PTEN^{ΔΔ} VSMC cultured in growth media (Figure 1Aa). Marked increase in calcification was also observed in PTEN^{ΔΔ} VSMC cultured in osteogenic media for 3 weeks compared with the PTEN^{fl/fl} VSMC (Figure 1Ba), which was further confirmed by quantitative calcium measurement

(Figure 1Bb). Concurrently, the expression of osteogenic transcription factor Runx2 and Runx2-regulated osteogenic marker genes, including osteocalcin and collagen type I, was increased in the PTEN^{ΔΔ} VSMC (Figure 1Bc). Notably, a dramatic increase in the Runx2 protein level was evident in the PTEN^{ΔΔ} VSMC (Figure 1Bd). Accordingly, we conclude the PTEN deletion in VSMC constitutively activates AKT and promotes upregulation of Runx2 and osteogenic differentiation of VSMC.

Inhibition of AKT and Runx2 Attenuates Calcification of the PTEN-Deficient VSMC

The definitive role of AKT/Runx2 signaling axis in mediating the PTEN deficiency-induced VSMC calcification was further determined by loss of function studies. Inhibition of AKT activation by an AKT inhibitor abolished calcification of the PTEN^{ΔΔ} VSMC (Figure 1Ca), which was associated with inhibition of Runx2 upregulation (Figure 1Cb). Furthermore, we found that knockdown of Runx2 by specific small hairpin RNA blocked calcification of PTEN^{ΔΔ} VSMC (Figure 1Da). The Runx2 knockdown did not affect AKT phosphorylation (Figure 1Db). These data demonstrated that upregulation of Runx2 by activation of AKT is essential for the PTEN deficiency-induced VSMC calcification. Of note, the Runx2 knockdown blocked calcification but did not affect proliferation of the PTEN^{ΔΔ} VSMC (Figure I in the online-only Data Supplement), suggesting that the Runx2 upregulation-dependent calcification in the PTEN-deficient VSMC is independent of its effect on VSMC proliferation.

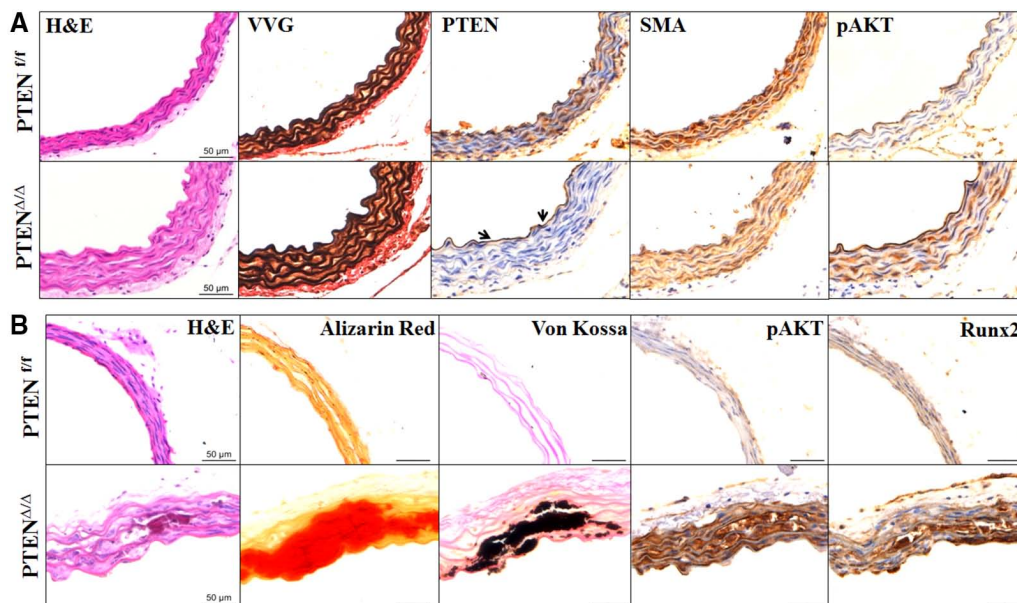


Figure 2. Smooth muscle cell (SMC)-specific phosphatase and tensin homolog (PTEN) deletion promotes *ex vivo* aortic calcification. **A**, SMC-specific PTEN deficiency increased phosphorylated AKT in the media. Consecutive sections from descending aortas from PTEN^{fl/fl} and PTEN^{ΔΔ} mice were stained with H&E (histology), VVG (Verhoeff-Van Gieson; elastin), or specific antibodies for PTEN, smooth muscle α -action (SMA), and phosphorylated protein kinase B (pAKT). Representative images from 5 pairs of littermates are shown. **B**, SMC-specific PTEN deficiency promoted aortic calcification. Aorta rings from PTEN^{fl/fl} and PTEN^{ΔΔ} mice were cultured in osteogenic medium containing H₂O₂ (0.3 mmol/L) for 2 weeks. Consecutive sections were stained by H&E, Alizarin Red (calcium), Von Kossa (calcium phosphate), and specific antibodies for pAKT and runt-related transcription factor 2 (Runx2). Representative images from 5 independent experiments are shown.

SMC-Specific PTEN Deletion Promotes Ex Vivo Aortic Calcification

The effect of SMC-specific PTEN deletion on calcification of VSMC in their natural milieu was characterized in an ex vivo aortic ring culture system using descending aortas from PTEN^{ΔΔ} mice and their control littermates. Immunohistochemical staining with a PTEN-specific antibody demonstrated specific deletion of PTEN in the media, but not in the endothelium (Figure 2A, PTEN arrows). Histological analysis demonstrated increased medial thickness and decreased smooth muscle α -action, a SMC marker in the PTEN^{ΔΔ} aortas (Figure 2A), which is consistent with the previous findings that PTEN regulates proliferation of SMCs.^{19–23,25} On the other hand, sustained increased phosphorylation of AKT was demonstrated in the PTEN^{ΔΔ} aorta (Figure 2A). Similar to the observation with isolated VSMC, aortas from PTEN^{ΔΔ} mice exhibited intensive calcification after cultured in osteogenic media for 2 weeks (Figure 2B, Alizarin red and Von Kossa), which was associated with decreased smooth muscle α -action and elevated Runx2 (Figure 2B). By contrast, vascular calcification was not evident in aortas from the control PTEN^{fl/fl} littermates under the same conditions (Figure 2B). These results demonstrated a direct effect of endogenous activation of AKT by the PTEN deletion in VSMC on upregulation of Runx2 and VSMC calcification.

PTEN Deficiency Upregulates Runx2 by Inhibiting Runx2 Ubiquitination

To determine the molecular mechanism underlying activation of AKT in promoting upregulation of Runx2 and VSMC calcification, we first characterized Runx2 expression in the PTEN^{ΔΔ} VSMC. The amount of the Runx2 protein was increased during calcification of the PTEN^{ΔΔ} VSMC (Figure 1Bd), which was associated with a modest increase in expression of the Runx2 mRNA (Figure 1Bc). These observations led us to examine the amount of both Runx2 mRNA and protein in PTEN^{ΔΔ} VSMC cultured in normal growth media, a basal condition. Under the basal condition, marked increase in the Runx2 protein level was also evident in the PTEN^{ΔΔ} VSMC (Figure 3Aa), whereas expression of the Runx2 mRNA is not altered significantly (Figure 3Ab). Inhibition of de novo protein synthesis by cycloheximide decreased the amount of Runx2 in the control PTEN^{fl/fl} VSMC (Figure 3B) in a time-dependent manner. In contrast, the amount of the Runx2 protein was sustained in the PTEN^{ΔΔ} VSMC after exposure to cycloheximide for 12 hours, indicating that the PTEN deletion stabilized Runx2 by preventing Runx2 protein from degradation. As ubiquitination mediates degradation of Runx2,^{38,39} we determined the effect of the PTEN deficiency on the Runx2 ubiquitination. The PTEN deletion did not affect the overall expression profile of poly-ubiquitin in VSMC (Figure 3C, input). However, modification of Runx2 by poly-ubiquitin was markedly decreased in the PTEN^{ΔΔ} VSMC (Figure 3C, IP:Runx2), indicating that ubiquitination-mediated degradation of Runx2 was inhibited, which rendered Runx2 stable in the PTEN^{ΔΔ} VSMC.

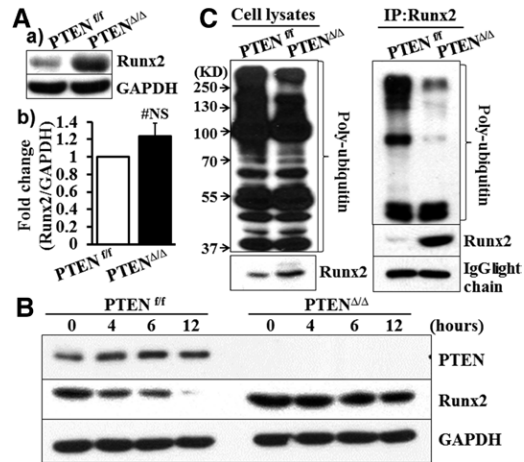


Figure 3. Phosphatase and tensin homolog (PTEN) deficiency increases runt-related transcription factor 2 (Runx2) protein level in vascular smooth muscle cells (VSMC) by inhibiting Runx2 ubiquitination. **A**, Expression of Runx2 in VSMC from PTEN^{fl/fl} and PTEN^{ΔΔ} mice cultured in growth media (basal conditions) determined by (a) Western blot analysis of Runx2 protein and (b) real-time PCR analysis of Runx2 mRNA. Representative results from VSMC from 3 pairs of littermates are shown (n=3, #NS, not significant). **B**, Effects of PTEN deletion on Runx2 stability. PTEN^{fl/fl} and PTEN^{ΔΔ} VSMC were cultured in growth media with cycloheximide (50 μ mol/L) for \leq 12 hours. The expression of PTEN and Runx2 protein was determined by Western blot. Representative blots from 3 independent experiments are shown. **C**, Runx2 ubiquitination determined by Western blot analysis. PTEN^{fl/fl} and PTEN^{ΔΔ} VSMC were pretreated with MG132 (50 μ mol/L). Proteins were extracted, and the expression of Runx2 and poly-ubiquitin was determined by Western blot analysis (Input). Immunoprecipitation was performed with Runx2 antibody; Runx2-bound poly-ubiquitin was determined by Western blot analysis (IP: Runx2). Representative images from 3 independent experiments are shown.

AKT Activation Promotes Cytosolic Translocation of FOXO1/FOXO3

The FOXO protein family, one of several known downstream targets of activated AKT, has been associated with expression of Runx2 in osteoblasts, human embryonic stem cells, and prostate cancer cells, although data from these studies are not compelling.^{31–34} We found that FOXO1 and FOXO3a are highly expressed in VSMC compared with FOXO4 (Figure II in the online-only Data Supplement). The PTEN deficiency in VSMC did not affect the expression of FOXOs mRNA (Figure II in the online-only Data Supplement) or production of FOXO1/3 proteins (Figure 4A). In contrast, phosphorylation of FOXO1 and 3 was dramatically increased in the PTEN^{ΔΔ} VSMC (Figure 4A). As phosphorylation of FOXO has been shown to be associated with nuclear exclusion,⁴⁰ we examined the cellular localization of FOXO1/3 in PTEN^{ΔΔ} VSMC and control PTEN^{fl/fl} VSMC. The PTEN deletion in VSMC increased cytosolic translocation and decreased nuclear localization of FOXO1/3, which was associated with increased Runx2 in the nuclear (Figure 4B). Furthermore, activation of AKT using a lentivirus carrying a constitutively activated AKT similarly promoted cytosolic translocation of FOXO1/3 and abolished their nuclear localization (Figure 4C), which was again associated with increased amount of Runx2. These data demonstrate that activation of AKT, either by PTEN deficiency or by overexpression of

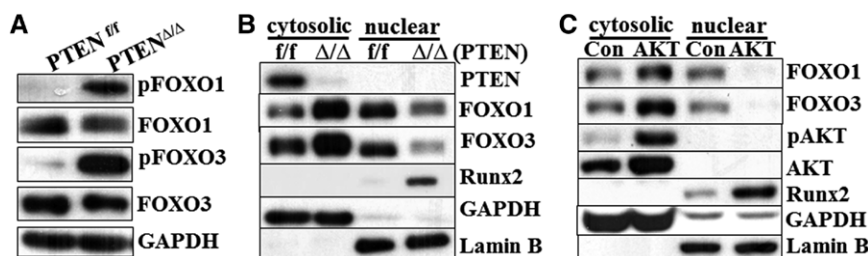


Figure 4. Protein kinase B (AKT) activation promotes nuclear exclusion of forkhead box O (FOXO)1 and 3. **A**, Increased phosphorylation of FOXO1/3 in phosphatase and tensin homolog (PTEN)-deficient vascular smooth muscle cells (VSMC). Western blot analysis of the expression of FOXO1/3 and their phosphorylated forms in PTEN^{fl/fl} and PTEN^{Δ/Δ} VSMC cultured in growth media. **B**, Increased Runx2 expression and nuclear exclusion of FOXO1/3 in PTEN-deficient VSMC. Western blot analysis of Runx2 and FOXO1/3 in the cytosolic and nuclear fractions of the PTEN^{fl/fl} and PTEN^{Δ/Δ} VSMC. **C**, Constitutively activated AKT promoted nuclear exclusion of FOXO1/3 and upregulated Runx2. Wild-type VSMC overexpressing constitutively active AKT (AKT) or control green fluorescent protein (Con) were cultured in growth media. The expression of AKT, FOXO1/3, and Runx2 in cytosolic and nuclear fractions was determined by Western blot analysis. Representative blots from 3 independent experiments are shown.

constitutively activated AKT, leads to nuclear exclusion of FOXO1/FOXO3 in VSMC, which was associated with upregulation of Runx2.

FOXO1/3 Knockdown Inhibits Runx2 Ubiquitination and Promotes VSMC Calcification

We further determined whether inhibition of FOXO1/3 directly affect Runx2 and VSMC calcification by knocking down FOXO1 or FOXO3 in VSMC using specific small hairpin RNA. Knockdown of FOXO1 decreased the expression of FOXO3 in VSMC, and vice versa (Figure 5A). Such an observation is consistent with a previous report that FOXO1 and FOXO3 regulate each other in human fibroblasts.⁴¹ Knockdown of FOXO1 or FOXO3 markedly increased the amount of Runx2 detected, without affecting expression of the Runx2 mRNA (Figure 5A and B). The effects of FOXO1/3 knockdown on ubiquitination of Runx2 were further determined. Similar to the PTEN deletion, FOXO1

or FOXO3 knockdown in VSMC did not affect the general expression profile of poly-ubiquitin, but inhibited Runx2-bound poly-ubiquitin (Figure 5C). Furthermore, knockdown of FOXO1 or FOXO3 promoted VSMC calcification, as determined by Alizarin red staining (Figure 5D) and quantitative calcium measurement (Figure 5E). These data demonstrate that FOXO1/3 negatively regulates the Runx2 stability in VSMC and knockdown of FOXO1/3 phenocopies the PTEN deficiency.

SMC-Specific PTEN Reduction Promotes Vascular Calcification in Mice

The role of AKT/FOXO/Runx2 signaling axis in vascular calcification was further determined *in vivo*. As the early death of the homozygous SMC-specific PTEN^{Δ/Δ} mice prevented us from carrying out long-term *in vivo* vascular calcification studies, the heterozygous SMC-specific PTEN-deficient mice (PTEN^{Δ/+}) were utilized. The PTEN^{Δ/+} mice appeared normal

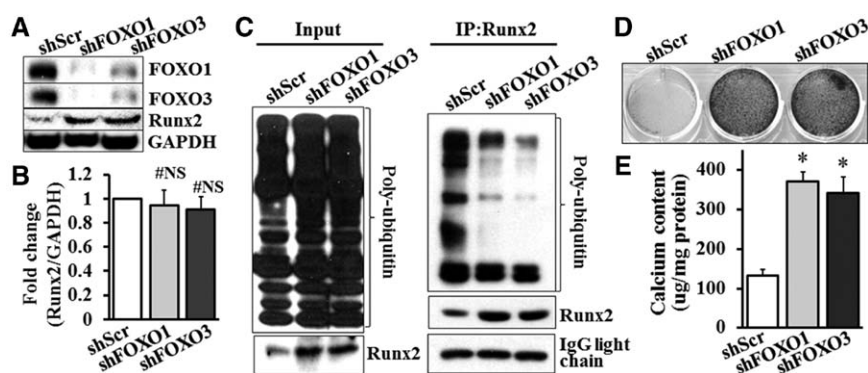


Figure 5. Forkhead box O (FOXO)1 or 3 knockdown inhibits runt-related transcription factor 2 (Runx2) ubiquitination and promotes vascular smooth muscle cells (VSMC) calcification. Wild-type VSMC stably infected with lentivirus carrying control scrambled small hairpin RNA (shRNA; shScr) or shRNA specific for FOXO1 (shFOXO1) or FOXO3 (shFOXO3) were generated and characterized. **A**, FOXO1/3 knockdown in VSMC increased Runx2 protein expression. Stably infected VSMC were cultured in growth media, and the expression of FOXO1/3 and Runx2 proteins was determined by Western blot analysis. **B**, FOXO1/3 knockdown in VSMC does not affect Runx2 mRNA expression. Real-time PCR analysis was performed to determine the expression of Runx2 mRNA in FOXO1 or 3 knockdown VSMC (n=3, #NS indicating not significant). **C**, Effects of FOXO1/3 knockdown on Runx2 ubiquitination. VSMCs with FOXO1 or FOXO3 knockdown and control VSMC were pretreated with MG132 (50 μmol/L). Proteins were extracted, and the expression of Runx2 and poly-ubiquitin was determined by Western blot analysis (Input). Immunoprecipitation was performed with Runx2 antibody, and Runx2-bound poly-ubiquitin was determined by Western blot analysis (IP: Runx2). **D** and **E**, FOXO1/3 knockdown promotes VSMC calcification. Stably infected VSMC from **A** were cultured in osteogenic medium with H₂O₂ (0.3 mmol/L) for 3 weeks. Calcification was determined by **(D)** Alizarin red staining and **(E)** calcium quantification with Arsenazo III, in parallel experiments (n=3, *P<0.002). Representative results from 3 independent experiments are shown.

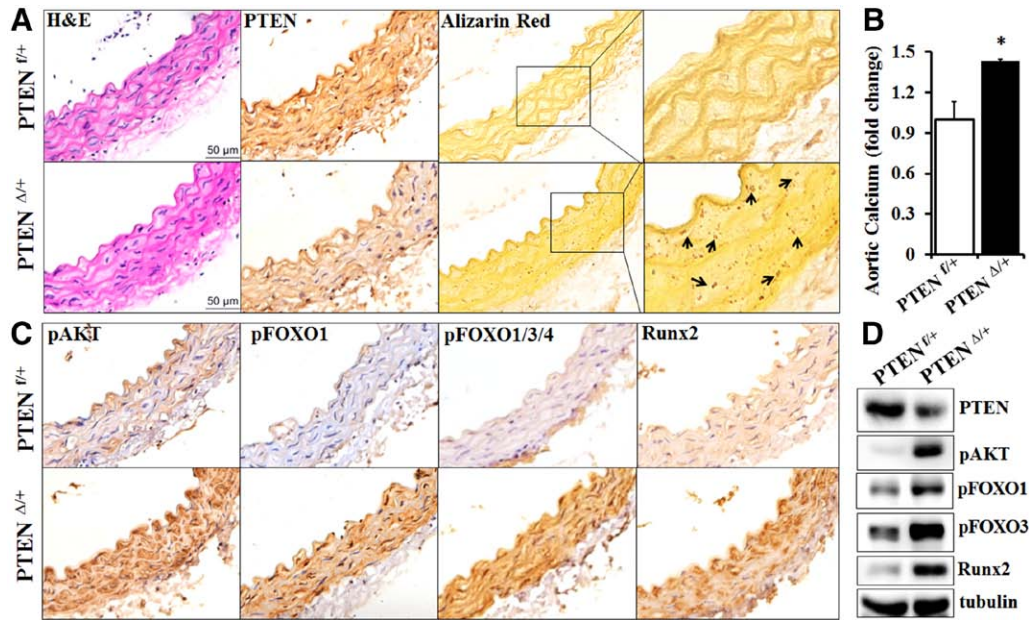


Figure 6. Decreased phosphatase and tensin homolog (PTEN) in smooth muscle cell (SMC) promotes vascular calcification. Descending aortas were isolated from 6-month-old heterozygous SMC-specific PTEN-deficient mice (PTEN^{Δ/+}) and their control littermates (PTEN^{fl/+}). **A**, Analysis of vascular calcification in aortic sections. Consecutive sections were stained by H&E, antibody for PTEN, and Alizarin Red. Higher magnification images of the boxed areas are shown to the right. Arrows indicate calcium deposition. **B**, Calcium content measured in descending aortas by Arsenazo III. The calcium content in aortas from the control mice was defined as 1 (n=3 mice/group, *P=0.03). **C**, Decreased PTEN in SMC increases protein kinase B (AKT) activation, forkhead box O (FOXO) phosphorylation, and runt-related transcription factor 2 (Runx2). Consecutive aortic sections from the heterozygous SMC-specific PTEN-deficient mice, and their control littermates were stained with specific antibodies for pAKT, pFOXO1, pFOXO1/3/4, and Runx2. Representative images from 3 mice/group are shown. **D**, Western blot analysis of the expression of PTEN, pAKT, pFOXO1, pFOXO3, and Runx2 in aortic lysates from PTEN^{Δ/+} and PTEN^{fl/+} mice.

as their control littermates (PTEN^{fl/+}). Decreased PTEN expression was evident in the vasculature of the heterozygous mice (Figure 6A). Importantly, the heterozygous SMC-specific PTEN mice fed on a normal chow diet spontaneously developed vascular calcification at 6 months of age (Figure 6A and B). Alizarin red staining revealed apparent calcium deposition in aortas from the PTEN^{Δ/+} mice, whereas no calcification was detected in the aortas from control littermates (Figure 6A). Quantitative measurement of total calcium content in the aortic tissues further demonstrated increased vascular calcification in the PTEN^{Δ/+} mice (Figure 6B). Moreover, increased vascular calcification in the PTEN^{Δ/+} mice was associated with increased AKT activation, FOXO phosphorylation, and Runx2 upregulation in the vasculature, as determined by immunostaining (Figure 6C) as well as Western blot analyses (Figure 6D).

In addition, we determined the AKT/FOXO/Runx2 signaling axis in the atherogenic ApoE^{-/-} mice, which developed atherosclerotic vascular calcification as we demonstrated previously.^{14,36} Consistently, HFD induced upregulation of Runx2 and vascular calcification (Figure IV in the online-only Data Supplement). Similar to the observation with the PTEN^{Δ/+} mice (Figure 6), Runx2 upregulation was associated with increased AKT activation and FOXO phosphorylation in calcified vasculature of the ApoE^{-/-} mice (Figure IVA and IVBa in the online-only Data Supplement). Furthermore, decreased Runx2

ubiquitination was also evident in aortas from the HFD-fed ApoE^{-/-} mice (Figure IVBb in the online-only Data Supplement). Taken together, results from these 2 animal models supported the important role of AKT/FOXO/Runx2 signaling axis in vascular calcification in vivo.

Discussion

Runx2 is a key transcriptional regulator required for vascular calcification in vitro and in vivo. Activation of AKT also promotes calcification of VSMC. To uncover underlying mechanisms connecting activation of AKT and upregulation of Runx2 in the development of vascular calcification, we generated a mouse model with sustained AKT activation in VSMC by selective deletion of PTEN, an upstream phosphatase that negatively regulates AKT activation. Our studies reveal a novel causative effect of the SMC-specific PTEN deficiency in promoting vascular calcification. Using VSMC from the SMC-specific PTEN deletion mice, we have also elucidated a new function of PTEN/AKT/FOXO1/3 signaling axis in regulating ubiquitination of Runx2 and vascular calcification. The important role of the AKT/FOXO/Runx2 signaling axis in vascular calcification in vivo has been further demonstrated with the heterozygous SMC-specific PTEN deletion mice and the atherogenic ApoE^{-/-} mice.

As a protein/lipid phosphatase, PTEN has diverse functions in different type of cells; most notable one is its tumor

repression activity. In the vasculature, PTEN has been implicated in proliferation, differentiation, and migration of smooth muscle cell^{19–23,37}; however, the role of PTEN in vascular calcification is entirely unknown. Our studies with comprehensive approaches using the SMC-specific PTEN deletion VSMC *in vitro*, aortas *ex vivo*, and the heterozygous SMC-specific PTEN deletion mice *in vivo* have strongly supported a causative effect of the PTEN inhibition on vascular calcification. Using an AKT inhibitor, we confirmed that activation of AKT is crucial for calcification of the PTEN-deficient VSMC. Furthermore, we demonstrated that upregulation of Runx2 was required for the calcification of VSMC from the PTEN-deficient mice. Importantly, the Runx2 knockdown was found to only block calcification but not proliferation of the PTEN-deficient VSMC, suggesting that PTEN deficiency-induced Runx2 upregulation promotes osteogenic differentiation of VSMC, which may be independent of its previously reported function in regulating VSMC proliferation, differentiation, and migration.^{19–23,37} In normal VSMC, the amount of Runx2 detected is low.^{42,43} In the PTEN-deficient VSMC, however, markedly increased Runx2 was evident even under the basal condition. Since we have shown that upregulation of Runx2 is essential and sufficient to induce VSMC calcification,^{13,14} AKT activation-induced elevation of the Runx2 protein in the PTEN-deficient VSMC may predispose the cells to undergo osteogenic differentiation. As a result, the PTEN deficiency promoted calcification of the PTEN^{ΔΔ} VSMC cultured in osteogenic medium without any additional stimuli. Runx2-regulated osteogenic marker genes, including osteocalcin and collagen type I,^{13,14,44} increased concurrently. This finding is also supported by the data from the *in vivo* studies with SMC-specific reduction of PTEN. Apparently, increased AKT activation by the PTEN reduction contributes to Runx2 upregulation and the spontaneous development of vascular calcification in these mice.

The elevated amount of Runx2 protein in the PTEN-deficient VSMC under basal conditions was not caused by increased expression of Runx2 at the transcriptional level. Instead, the PTEN deficiency in VSMC stabilizes the Runx2 protein by inhibiting Runx2 degradation, an important mechanism that has been demonstrated to stabilize the Runx2 protein in bone cells.³⁸ In bone cells, post-translational modulation by the ubiquitin-proteasome pathway regulates stability and degradation of Runx2 protein.³⁹ Our studies have provided the first evidence that the PTEN deficiency increases the Runx2 stability by inhibiting ubiquitination of Runx2 in the PTEN^{ΔΔ} VSMC. Studies in osteoblasts have identified several molecules that contribute to Runx2 degradation, including Smad ubiquitin regulatory factor 1 and cyclin-D1.^{45,46} Smad ubiquitin regulatory factor 1 is an E3-ligase that binds to Runx2 and mediates ubiquitin binding to Runx2 that leads to Runx2 degradation.⁴⁵ Cyclin-D1, on the other hand, contributes to proteasome-dependent degradation of Runx2 by cyclin D1/Cdk4-induced Runx2 phosphorylation in the C-terminus of Runx2, which is critical for Runx2 protein stability.⁴⁶ Additionally, other factors have been implicated in mediating Runx2 degradation by recruiting E3-ligases, such

as Smad 6, which binds to Runx2 and serves as an adaptor for Smad ubiquitin regulatory factor 1–induced Runx2 degradation.^{47,48} However, the PTEN deficiency in VSMC did not affect the expression of the previously characterized regulators of Runx2 ubiquitination in the bone cells, such as Smad ubiquitin regulatory factor 1 and cyclin-D1. Therefore, the molecular regulators that modulate Runx2 degradation and ubiquitination in VSMC may be novel, which warrant further exploration.

Our studies have also demonstrated that the FOXO1/3 signaling axis is responsible for the inhibition of the Runx2 ubiquitination, thereby upregulates Runx2 which then promotes VSMC calcification. Specifically, AKT-regulated phosphorylation and cytosolic translocation of FOXO1/3 increased Runx2 in VSMC. The phosphorylation and subsequent nuclear exclusion of the FOXO family of proteins has been associated with neointimal hyperplasia in a rat balloon injury model.⁴⁰ Upregulation of AKT/FOXO signaling has been found in the vasculature in an aging rat atherosclerosis model.⁴⁹ However, the function of SMC-expressed FOXO family proteins in regulating vascular calcification is unknown. Using FOXO1/3-specific small hairpin RNA, we determined that FOXO1/3 regulates upregulation of Runx2 and subsequent calcification of VSMC. FOXO1 and 3 have been found to increase Runx2 in osteoblasts and human embryonic stem cells; however, other studies indicate that FOXO1 inhibits the Runx2 activity in osteoblasts or prostate cancer cells.^{31–34} Our studies have delineated a novel function of FOXO1/3 in regulating the Runx2 protein stability in VSMC. Consistent with the cross-regulation of FOXO1/3 in human fibroblasts,⁴¹ knockdown of FOXO1 decreases the expression of FOXO3 in VSMC and vice versa, implying a positive feedback loop controlling the expression of FOXO1/3. Knockdown of FOXO1/3 phenocopied the PTEN deficiency in terms of Runx2 ubiquitination and VSMC calcification, which support a novel function of FOXO1/3 in the process. More importantly, increased FOXO phosphorylation and Runx2 upregulation are associated with AKT activation in vascular calcification in the heterozygous SMC-specific PTEN deletion mice as well as the atherogenic ApoE^{-/-} mice *in vivo*. Decreased Runx2 ubiquitination has been found in the vasculature of the HFD-fed ApoE^{-/-} mice, which may lead to decreased Runx2 degradation and thus increase Runx2 and promote vascular calcification. These animal studies have further supported an important role of AKT activation, FOXO phosphorylation, and Runx2 ubiquitination in vascular calcification *in vivo*.

In summary, the current studies have demonstrated a causative effect SMC-specific PTEN deficiency on vascular calcification via activation of AKT and upregulation of Runx2 *in vitro* and *in vivo*. We have further determined a novel function of the AKT/FOXO1/3 signaling axis in regulating Runx2 ubiquitination and stability. These studies have provided the first evidence demonstrating the role of FOXO1/3 in mediating activation of AKT-induced VSMC calcification and identified that FOXO1/3-modulated ubiquitination of Runx2 as a novel mechanism in regulating vascular calcification. The new molecular insights gained in the present

studies in the regulation of Runx2 in the development of VSMC calcification may lead to identification of novel therapeutic targets.

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Disclosures

None.

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Significance

Vascular calcification is emerging as an important risk factor that predicts outcome of cardiovascular diseases. Clinical and experimental studies from our group and others have demonstrated a critical role of the osteogenic transcription factor runt-related transcription factor 2 (Runx2) in regulating vascular calcification. The present studies have determined a new causative effect of smooth muscle cell-specific phosphatase and tensin homolog (PTEN) deficiency on Runx2 upregulation and vascular calcification. The PTEN deficiency upregulates Runx2 through inhibiting Runx2 ubiquitination. Our studies have revealed that FOXO1/3 is the link between AKT activation and Runx2 upregulation because FOXO1/3 knockdown phenocopies the PTEN deficiency, including Runx2 ubiquitination and vascular smooth muscle cell calcification. Using heterozygous smooth muscle cell-specific PTEN deletion mice and the atherogenic ApoE^{-/-} mice, we have demonstrated the important role of AKT/FOXO/Runx2 signaling axis in vascular calcification in vivo. Altogether, our studies have provided the first evidence demonstrating a new role of PTEN in regulating vascular calcification and identified FOXO1/3-regulated Runx2 ubiquitination as a novel underlying mechanism.