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INHIBITION OF FOXO1/3 PROMOTES VASCULAR CALCIFICATION

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Abstract

Objective—Vascular calcification is a characteristic feature of atherosclerosis, diabetes and end-stage renal disease. We have demonstrated that activation of AKT upregulates Runx2, a key osteogenic transcription factor that is crucial for calcification of vascular smooth muscle cells (VSMC). Using mice with SMC-specific deletion of PTEN, a major negative regulator of AKT, the present studies uncovered a novel molecular mechanism underlying PTEN/AKT/FOXO-mediated Runx2 upregulation and VSMC calcification.

Approaches and Results—SMC-specific PTEN deletion mice were generated by crossing PTEN floxed mice with SM22α-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-depedent 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.

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DISCLOSURES
None.

Keywords

FOXO1/3; AKT; PTEN; Vascular calcification; Runx2; ubiquitination

INTRODUCTION

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 vitro*[13] and vascular calcification in atherosclerosis *in vivo*[14]. Furthermore, we have determined that activation of AKT is crucial for oxidative stress-induced VSMC calcification through upregulation of Runx2[13], and enforced expression of 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 two major upstream signals: phosphatidylinositol 3-kinase (PI3K) and phosphatase and tensin homolog (PTEN). PTEN is a protein/lipid phosphatase, which was original 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 appear 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, 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, while 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.

METHODS

The smooth muscle specific-PTEN deficient mice were generated by crossing PTEN exon 5 floxed mice ($PTEN^{f/f}$)[35] with the SM22α-Cre transgenic mice[14, 36]. Details of materials and experimental procedures are in the Methods section in the Online Data Supplement.

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 ($PTEN^{\Delta/\Delta}$) 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 $PTEN^{\Delta/\Delta}$ 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 $PTEN^{\Delta/\Delta}$ mice and their control $PTEN^{f/f}$ littermates.

The deletion of PTEN in VSMC was demonstrated by Western blot analysis (Fig. 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 (Fig 1Ab), however, it increased activation of AKT1, as indicated by AKT phosphorylation at both serine 473 (S473) and threonine 308 (T308) residues in the $PTEN^{\Delta/\Delta}$ VSMC cultured in growth media (Fig 1Aa).

Marked increase in calcification was also observed in PTEN$^{\Delta/\Delta}$ VSMC cultured in osteogenic media for 3 weeks compared with the PTEN$^{f/f}$ VSMC (Fig 1Ba), which was further confirmed by quantitative calcium measurement (Fig 1Bb). Concurrently, the expression of osteogenic transcription factor Runx2 and Runx2-regulated osteogenic marker genes, including osteocalcin (OC) and collagen type I (Col Ia), was increased in the PTEN$^{\Delta/\Delta}$ VSMC (Fig 1Bc). Notably, a dramatic increase in the Runx2 protein level was evident in the PTEN$^{\Delta/\Delta}$ VSMC (Fig 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$^{\Delta/\Delta}$ VSMC (Fig 1Ca), which was associated with inhibition of Runx2 upregulation (Fig 1Cb). Furthermore, we found that knockdown of Runx2 by specific shRNA blocked calcification of PTEN$^{\Delta/\Delta}$ VSMC (Fig 1Da). The Runx2 knockdown did not affect AKT phosphorylation (Fig 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$^{\Delta/\Delta}$ VSMC (Online Supplemental Fig I), suggesting that the Runx2 upregulation-dependent calcification in the PTEN deficient VSMC is independent of its effect on VSMC proliferation.

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$^{\Delta/\Delta}$ 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 (Fig 2A, PTEN arrows). Histological analysis demonstrated increased medial thickness and decreased smooth muscle α-action (SMA), a SMC marker in the PTEN$^{\Delta/\Delta}$ aortas (Fig 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$^{\Delta/\Delta}$ aorta (Fig 2A). Similar to the observation with isolated VSMC, aortas from PTEN$^{\Delta/\Delta}$ mice exhibited intensive calcification after cultured in osteogenic media for 2 weeks (Fig 2B, Alizarin red and Von Kossa), which was associated with decreased SMA and elevated Runx2 (Fig 2B). By contrast, vascular calcification was not evident in aortas from the control PTEN$^{f/f}$ littermates under the same conditions (Fig 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.

The 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$^{\Delta/\Delta}$ VSMC. The amount of the Runx2 protein was increased during calcification of

the PTEN$^{\Delta/\Delta}$ VSMC (Fig 1Bd), which was associated with a modest increase in expression of the Runx2 mRNA (Fig 1Bc). These observations led us to examine the amount of both Runx2 mRNA and protein in PTEN$^{\Delta/\Delta}$ 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$^{\Delta/\Delta}$ VSMC (Fig 3Aa), while expression of the Runx2 mRNA is not altered significantly (Fig 3Ab). Inhibition of *de novo* protein synthesis by cycloheximide decreased the amount of Runx2 in the control PTEN$^{f/f}$ VSMC (Fig 3B) in a time dependent manner. In contrast, the amount of the Runx2 protein was sustained in the PTEN$^{\Delta/\Delta}$ 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 (Fig 3C, input). However, modification of Runx2 by poly-ubiquitin was markedly decreased in the PTEN$^{\Delta/\Delta}$ VSMC (Fig 3C, IP:Runx2), indicating that ubiquitination-mediated degradation of Runx2 was inhibited, which rendered Runx2 stable in the PTEN$^{\Delta/\Delta}$ VSMC.

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 (Online Supplemental Fig II). The PTEN deficiency in VSMC did not affect the expression of FOXOs mRNA (Online Supplemental Fig II) or production of FOXO1/3 proteins (Fig 4A). In contrast, phosphorylation of FOXO1 and 3 was dramatically increased in the PTEN$^{\Delta/\Delta}$ VSMC (Fig 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$^{\Delta/\Delta}$ VSMC and control PTEN$^{f/f}$ 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 (Fig 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 (Fig 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 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 shRNA. Knockdown of FOXO1 decreased the expression of FOXO3 in VSMC, and vice versa (Fig 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 (Fig 5A & 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 (Fig 5C). Furthermore, knockdown of FOXO1 or FOXO3 promoted VSMC calcification, as determined by Alizarin red staining (Fig 5D) and quantitative calcium measurement (Fig 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 $PTEN^{\Delta/\Delta}$ mice prevented us from carrying out long-term *in vivo* vascular calcification studies, the heterozygous SMC-specific PTEN deficient mice ($PTEN^{\Delta/+}$) were utilized. The $PTEN^{\Delta/+}$ mice appeared normal as their control littermates ($PTEN^{f/+}$). Decreased PTEN expression was evident in the vasculature of the heterozygous mice (Fig 6A). Importantly, the heterozygous SMC-specific PTEN mice fed on a normal chow diet spontaneously developed vascular calcification at 6 months of age (Fig 6A&B). Alizarin red staining revealed apparent calcium deposition in aortas from the $PTEN^{\Delta/+}$ mice, whereas no calcification was detected in the aortas from control littermates (Fig 6A). Quantitative measurement of total calcium content in the aortic tissues further demonstrated increased vascular calcification in the $PTEN^{\Delta/+}$ mice (Fig 6B). Moreover, increased vascular calcification in the $PTEN^{\Delta/+}$ mice was associated with increased AKT activation, FOXO phosphorylation and Runx2 upregulation in the vasculature, as determined by immunostaining (Fig 6C) as well as Western blot analyses (Fig 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 (supplemental Figure IV). Similar to the observation with the $PTEN^{\Delta/+}$ mice (Fig 6), Runx2 upregulation was associated with increased AKT activation and FOXO phosphorylation in calcified vasculature of the $ApoE^{-/-}$ mice (supplemental Figure IV, A&Ba). Furthermore, decreased Runx2 ubiquitination was also evident in aortas from the HFD-fed $ApoE^{-/-}$ mice (supplemental Figure IV, Bb). Taken together, results from these two 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 very 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 $PTEN^{\Delta/\Delta}$ VSMC cultured in osteogenic medium without any additional stimuli. Runx2-regulated osteogenic marker genes, including OC and Col1a[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 due to 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 $PTEN^{\Delta/\Delta}$ VSMC. Studies in osteoblasts have identified several molecules that contribute to Runx2 degradation, including Smad ubiquitin regulatory factor 1 (Smurf1) and cyclin-D1[45, 46]. Smurf1 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

Smurf1-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 Smurf1 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 uncreased 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 shRNA, we determined that FOXO1/3 regulates upregulation of Runx2 and subsequent calcification of VSMC. FOXO1 and/or 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.

MATERIALS AND METHODS

The smooth muscle specific-PTEN deficient mice were generated by crossing PTEN exon 5 floxed mice ($PTEN^{f/f}$)[1] with the SM22α-Cre transgenic mice[2, 3]. Details of materials and experimental procedures are in the Methods section in the Online Data Supplement.

Online Supplemental Methods

Generation of SMC-specific PTEN deletion mice—PTEN exon5 floxed mice ($PTEN^{f/f}$) mice[1] and SM22α-Cre transgenic mice[2, 3] were obtained from The Jackson Laboratory. The SM22α-Cre mice were bred with $PTEN^{f/f}$ to generate smooth muscle-specific PTEN deletion mice ($PTEN^{\Delta/\Delta}$). Exon 5 encodes the phosphatase domain of PTEN[1], which is the functional domain. As most of the smooth muscle-specific PTEN deletion mice died around 4–5 weeks of age, all experiments were performed with aortas or smooth muscle cells isolated from 25–30 days old littermates. Primer sets for genotyping are: Cre: F-5'-GCGGTCTGGCAGTAAAAACTATC-3' and R-5'-GTGAAACAGCATTGCTGTCACTT-3'; PTEN: F-5' - CAAGCACTCTGCGAACTGAG-3' and R-5'-AAGTTTTTGAAGGCAAGATGC- 3'.

In vivo calcification of the heterozygous SMC-specific PTEN deletion mice—The effects of smooth muscle-expressed PTEN on vascular calcification was determined in 6-months old heterozygous SMC-specific PTEN deletion mice and their control littermates on normal chow diet. Aortic calcification was determined by Alizarin red staining as well as calcium content measurement Arsenazo III method as described before[3, 4].

Immunohistochemistry—Frozen aortic sections were processed for histology and immunohistochemistry as we described[3, 4]. In brief, Hematoxylin & Eosin (H&E) and Verhoeff-Van Gieson (VVG) was used for histological analysis. Antibody for PTEN, SMA, Runx2 and phosphorylated-AKT was applied to acetone-fixed cryosections. The sections were washed and exposed to a secondary antibody (horseradish peroxidase-conjugated antibodies), and antibody binding was visualized with diaminobenzidine. Sections were counterstained with hematoxylin.

Aortic ring culture—Descending aortas were cut into 2–3 mm rings, which were cultured in osteogenic medium containing 1% FBS with 0.3mM H_2O_2 for 2 weeks with medium changed every 3 days. At the end of experiments, aortic rings were harvested, fixed and embedded in paraffin.

Aortic calcification—Consecutive sections from the cultured aortic rings were stained with H&E for histology. Alizarin Red staining or Von Kossa staining (Sigma Aldrich) were used to detect calcification as we previously described[3, 4]

VSMC culture—Primary VSMC were isolated from mouse aorta and cultured in growth medium as we described previously[5]. All experiments were performed with VSMC at passages 3 to 5.

In vitro VSMC calcification[5]—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 or without H_2O_2 (0.3 mmol/L). Calcification was determined by Alizarin Red staining or quantified by measuring total calcium in the cell lysates by the Arsenazo III method[4].

In vitro VSMC proliferation[6]—Proliferation was assayed by the incorporation of 5-bromo-2-deoxyuridine (BrdU Proliferation Assay Kit, Calbiochem) as we reported[6]. VSMC were cultured in growth media in 96 well plates. BrdU incorporation in 24 hours was identified with a fluorescein-labeled anti-BrdU antibody (Calbiochem) and fluorogenic peroxidase secondary antibody using the Synergy 2 plate reader (BioTek).

Lentivirus transduction of VSMC—Lentiviral constructs carrying short hairpin RNA (shRNA) for Runx2, FOXO1 or FOXO3 were purchased from Open Biosystems and packaged into lentiviral particles as previously described[5]. Lentiviral vector expressing constitutively active AKT protein was obtained from Dr. Hongju Wu (Tulane University). Viral transductions were performed by incubating VSMC with recombinant lentiviruses in growth media supplemented with 10μg/mL Polybrene (Sigma). After 12 hours, virus-containing medium was changed to fresh medium and cultured for another 36 to 48 hours. 5μg/ml puromycin was used to select stably infected cells.

Real-time polymerase chain reaction (PCR)[4]—Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed into cDNA. SYBR Green-based PCR was performed using specific primers for mouse Runx2, osteocalcin (OC), type I collagen (Col Ia), AKT1/2/3 and FOXO1/3 or 4 (Primer sequences are listed in table 1), using iQ SYBR Green Supermix on an iCycler Thermal Cycler (Bio-Rad).

Table 1

Primer sequences (mouse) for real-time PCR

Name	Chain	Sequence	Gene ID
Runx2	Forward	5’- CGGCCCTCCCTGAACTCT-3’	NM_001145920.1
	Reverse	5’- TGCCTGCCTGGGATCTGTA-3’	
OC	Forward	5'-TGCTTGTGACGAGCTATCAG-3'	NM_007541.2
	Reverse	5'-GAGGACAGGGAGGATCAAGT-3'	
Col1	Forward	5'-CTGGCGGTTCAGGTCCAAT-3'	NM_007742.3
	Reverse	5'-TTCCAGGCAATCCACGAGC-3'	
AKT1	Forward	5'-GACCCACGACCGCCTCTG-3'	NM_009652.3
	Reverse	5'-GACACAATCTCCGCACCATAGAAG-3'	
AKT2	Forward	5'-GAGGACGCCATGGATTACAAG-3'	NM_007434.3
	Reverse	5'-GACAGCTACCTCCATCATCTCAGA-3'	
AKT3	Forward	5'-GAGTACCTGGCACCAGAGGT-3'	NM_011785.3
	Reverse	5'-AGAAAGGCAACCTTCCACAC-3'	
FOXO1	Forward	5'-TACGCCGACCTCATCACCAAG-3'	NM_019739.3

Name	Chain	Sequence	Gene ID
	Reverse	5'-GCACGCTCTTCACCATCCACT-3'	
FOXO3	Forward	5'-TCACTGAGGAAAGGGGAAATGG-3'	NM_019740.2
	Reverse	5'-AAAGGTGTCAAGCTGTAAACGGA-3'	
FOXO4	Forward	5'-CAAGAAGAAGCCGTCTGTCC-3'	NM_018789.2
	Reverse	5'-CTGACGGTGCTAGCATTTGA-3'	
	Reverse	5'-GCAGGAGAGGAAGTTGTTGG-3'	
Smurf1	Forward	5'-AGCATCAAGATCCGTCTGACA-3'	NM_001038627.1
	Reverse	5'-CCAGAGCCGTCCACAACAAT-3'	
Cyclin-D1	Forward	5'-TCCTCTCCAAAATGCCAGAG-3'	NM_007631.2
	Reverse	5'-GCAGGAGAGGAAGTTGTTGG-3'	
GAPDH	Forward	5'-AACACGGAAGGCCATGCCAG-3'	NM_008084.2
	Reverse	5'-TGCATCCTGCACCACCAACT-3'	

Western blot analysis—Cytosolic and nuclear extracts were prepared and protein concentration was measured as we described[3,5]. Western blot analyses were performed with specific antibody for Runx2 (MBL, D130-3), PTEN (Cell Signaling Technology, CST, 9552), Total AKT (CST, 4685), pAKT(Ser473, CST 9271 and Thr308, CST 9275), FOXO1 (CST, 2880), pFOXO1(CST, 9461), FOXO3 (CST, 2497), pFOXO3 (CST, 2599), poly-ubiquitin (Abcam, ab7780), lamin B (Santa Cruz) and GAPDH (Fitzgerald, 10R-G109a), and detected with a chemiluminescence detection kit (Millipore).

Determination of Runx2 stability—VSMC at 90% confluence were treated with cycloheximide (50 μM) for 0, 4, 6 and 12 hours. Western blot analysis was performed to detect the expression of Runx2. The expression of PTEN was verified in these cells, and the expression of GAPDH was used as a loading control.

Determination of Runx2 ubiquitination—VSMC were pretreated with 50 μM MG132 (Calbiochem) for 6 hours to inhibit proteasomal degradation. Then protein extracts were collected with non-denaturing lysis buffer (20mM Tris.HCL pH 8, 150mM NaCl, 10% Glycerol and 1% Triton X-100). 1000 μg lysates were incubated with 2 μg Runx2 antibody for at least 1h. Immune complexes were recovered from the supernatant by incubation with 50 μl of 1:1 slurry of protein G-agarose beads (Invitrogen) overnight at 4 °C. Beads were washed by cold 1×PBS for 5 times, and resuspended in 20μl 2×loading buffer. Then, the mixture was boiled at 100°C for 10min. After brief centrifugation, proteins in the supernatant were analyzed by Western blot with Runx2 and poly-ubiquitin antibodies.

Characterization of FOXO phosphorylation and Runx2 ubiqutination in atherogenic ApoE$^{-/-}$ mice—8-week old ApoE$^{-/-}$ mice were fed a normal chow or a high-fat diet for 30 weeks[7, 8]. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Consecutive aortic sections were stained with Alizarin Red and antibodies for pAKT, pFOXO1/3/4 and Runx2. Western blot analysis was performed in aortic protein extracts using specific

antibodies Aortic Runx2 ubiqutination was determined as described above by immunoprecipitation with Runx2 antibody, followed by Western blot using poly-ubiqutin antibody.

Statistical analysis—All the data are expressed as means ± SD. Differences between two groups were compared with Student's paired 2-tailed *t* test. A *p* value less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

AKT	Protein kinase B
ALP	alkaline phosphatase
BMP2	bone morphogenetic protein 2
Col I	type I collagen
FOXO	Forkhead box O
OC	osteocalcin
PTEN	phosphatase and tensin homolog
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol-3,4,5-trisphosphate
Runx2	runt-related transcription factor 2
shRNA	small hairpin RNA
VSMC	vascular smooth muscle cells

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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 Runx2 in regulating vascular calcification. The present studies have determined a new causative effect of SMC-specific 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 since FOXO1/3 knockdown phenocopies the PTEN deficiency, including Runx2 ubiquitination and VSMC calcification. Using heterozygous SMC-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.

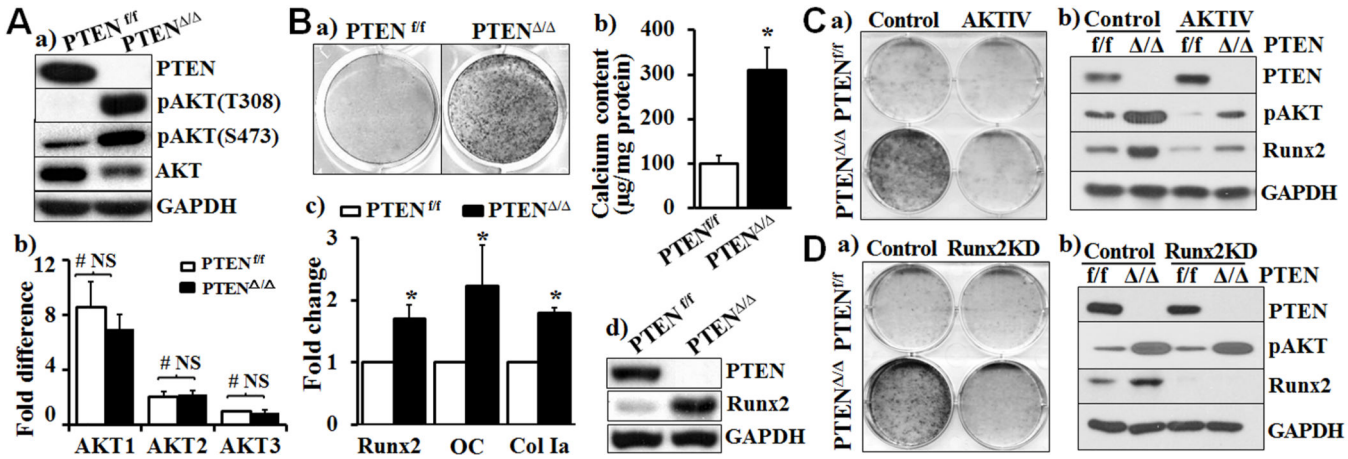

Fig. 1. Increased AKT by PTEN deficiency promotes Runx2 upregulation and VSMC calcification

A) Increased activation of AKT in PTEN deficient VSMC. VSMC were isolated from $PTEN^{f/f}$ and SMC-specific PTEN deficient mice ($PTEN^{\Delta/\Delta}$) 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 $PTEN^{f/f}$ VSMC is defined as 1. **B)** Calcification of VSMC from $PTEN^{f/f}$ and $PTEN^{\Delta/\Delta}$ mice. **a)** VSMC were cultured in osteogenic medium for 3 weeks, and calcification was determined by Alizarin Red staining. Results from three independent experiments performed in duplicate are shown. **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, OC and ColIa in parallel experiments as in **a)**. The expression of each gene in $PTEN^{f/f}$ 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)**. Representative blots from three independent experiments are shown. **C)** Inhibition of AKT attenuates PTEN deficiency-promoted calcification. $PTEN^{\Delta/\Delta}$ and control $PTEN^{f/f}$ VSMC were exposed to H_2O_2-containing osteogenic media with/without an AKT inhibitor (AKTIV, 1.0uM). **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)** and **b)**. Results from 3 independent experiments are shown. **D)** Runx2 knockdown abolished PTEN deletion-promoted VSMC calcification. VSMC were stably infected with lentivirus carrying control scrambled shRNA (Control) or shRNA specific for Runx2 and selected by puromycin. Stably infected VSMC were exposed to H_2O_2-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. Results from 3 independent experiments are shown.

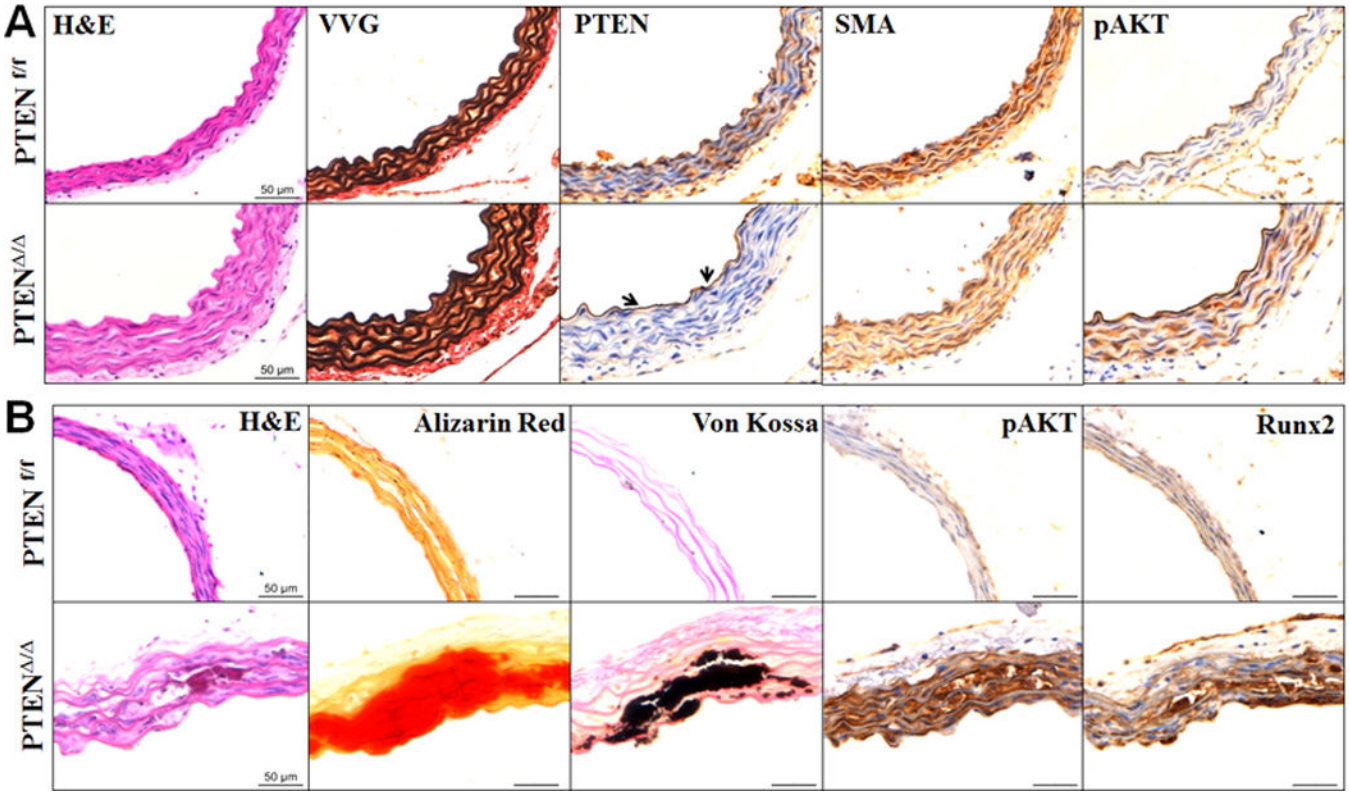

Fig. 2. SMC-specific PTEN deletion promotes ex vivo aortic calcification

A) SMC-specific PTEN deficiency increased phosphorylated AKT in the media. Consecutive sections from descending aortas from $PTEN^{f/f}$ and $PTEN^{\Delta/\Delta}$ mice were stained with H&E (histology), VVG (elastin) or specific antibodies for PTEN, SMA, and phosphorylated AKT (pAKT). Representative images from 5 pairs of littermates are shown. **B)** SMC-specific PTEN deficiency promoted aortic calcification. Aorta rings from $PTEN^{f/f}$ and $PTEN^{\Delta/\Delta}$ mice were cultured in osteogenic medium containing H2O2 (0.3mM) for 2 weeks. Consecutive sections were stained by H&E, Alizarin Red (calcium), Von Kossa (calcium phosphate), and specific antibodies for pAKT and Runx2. Representative images from 5 independent experiments are shown.

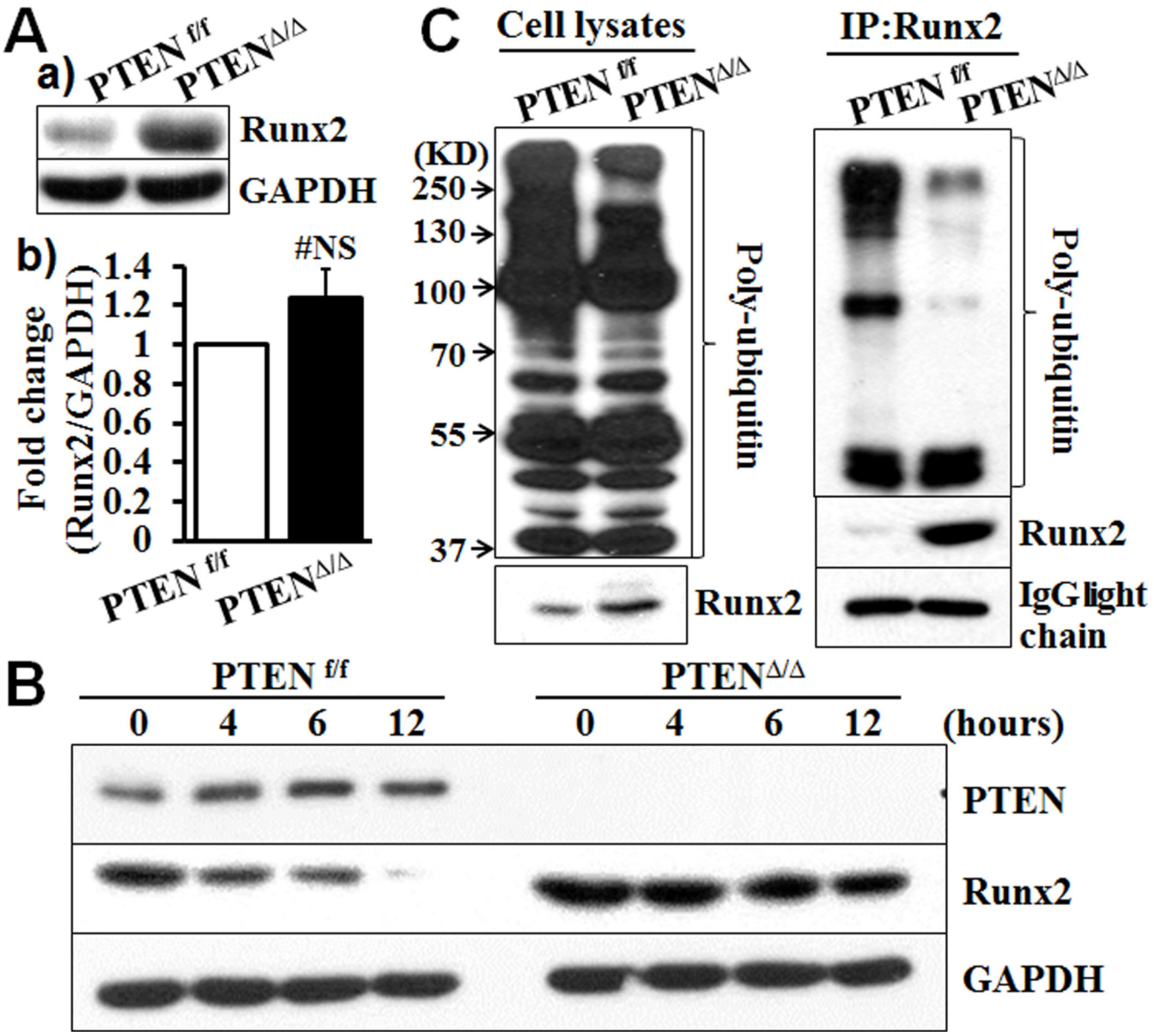

Fig. 3. PTEN deficiency increases Runx2 protein level in VSMC by inhibiting Runx2 ubiquitination

A) Expression of Runx2 in VSMC from PTEN$^{f/f}$ and PTEN$^{\Delta/\Delta}$ 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$^{f/f}$ and PTEN$^{\Delta/\Delta}$ VSMC were cultured in growth media with cycloheximide (50 μM) for up to 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$^{f/f}$ and PTEN$^{\Delta/\Delta}$ VSMC were pretreated with MG132 (50 μM). 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 three independent experiments are shown.

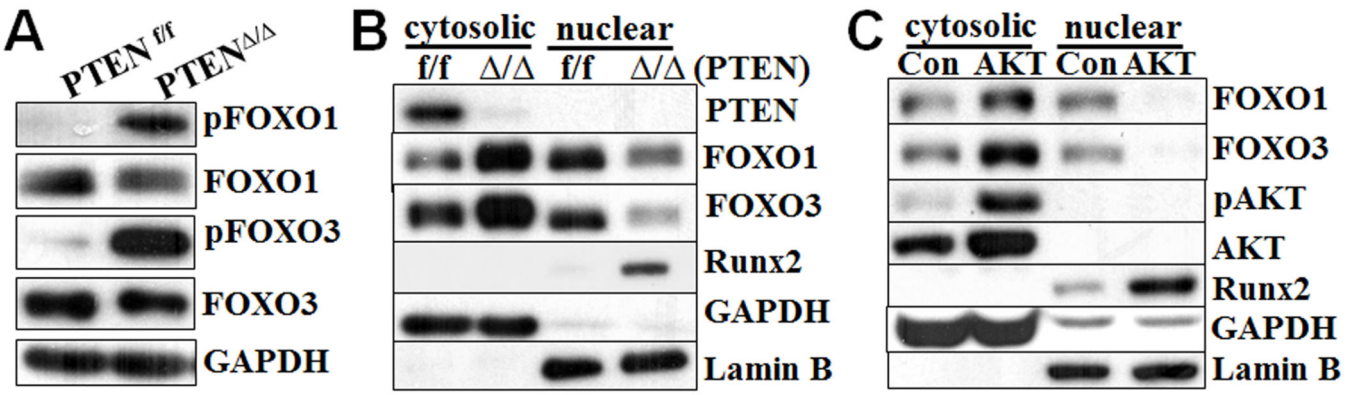

Fig. 4. AKT activation promotes nuclear exclusion of FOXO1/3

A) Increased phosphorylation of FOXO1/3 in PTEN deficient VSMC. Western blot analysis of the expression of FOXO1/3 and their phosphorylated forms in $PTEN^{f/f}$ and $PTEN^{\Delta/\Delta}$ 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 $PTEN^{f/f}$ and $PTEN^{\Delta/\Delta}$ VSMC. **C)** Constitutively activated AKT promoted nuclear exclusion of FOXOX1/3 and upregulated Runx2. Wild type VSMC overexpressing constitutively active AKT (AKT) or control GFP 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 three independent experiments are shown.

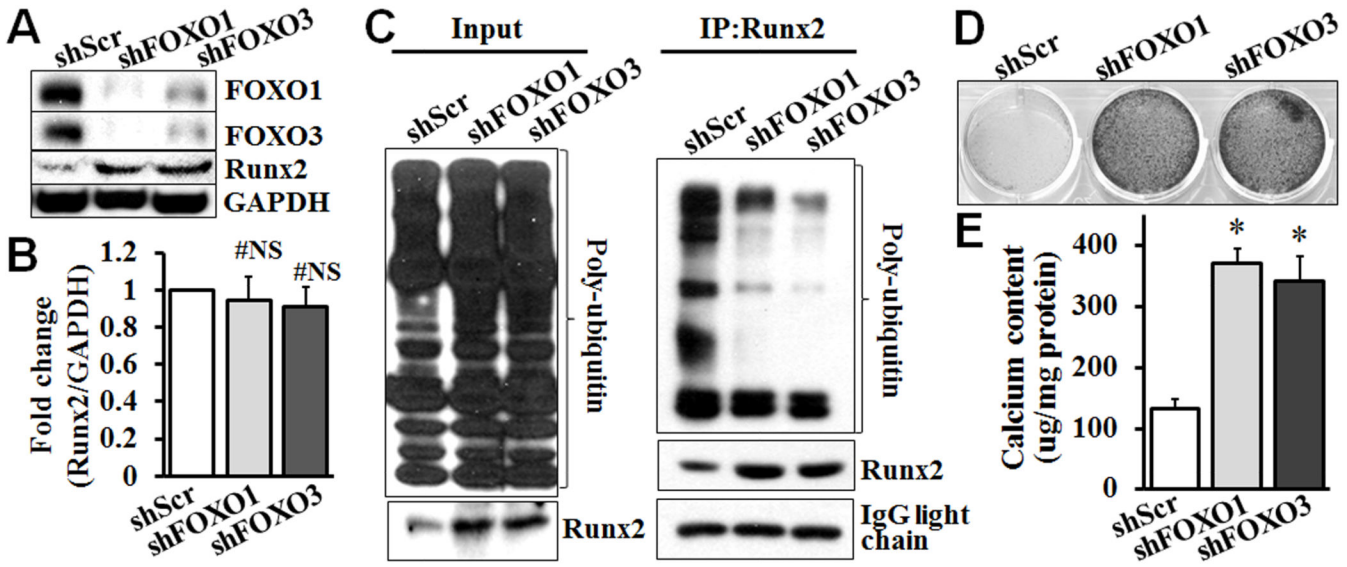

Fig. 5. FOXO1/3 knockdown inhibits Runx2 ubiquitination and promotes VSMC calcification

Wild type VSMC stably infected with lentivirus carrying control scrambled 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. Representative blots from 3 independent experiments are shown. **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 μM). 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). Representative blots from three independent experiments are shown. **D)** and **E)** FOXO1/3 knockdown promotes VSMC calcification. Stably infected VSMC from **A** were cultured in osteogenic medium with H2O2 (0.3 mM) for 3 weeks. Calcification was determined by **D)** Alizarin red staining; and **E**) calcium quantification with Arsenazo III, in parallel experiments. Results from 3 independent experiments are shown (n=3, * $p \leq 0.002$).

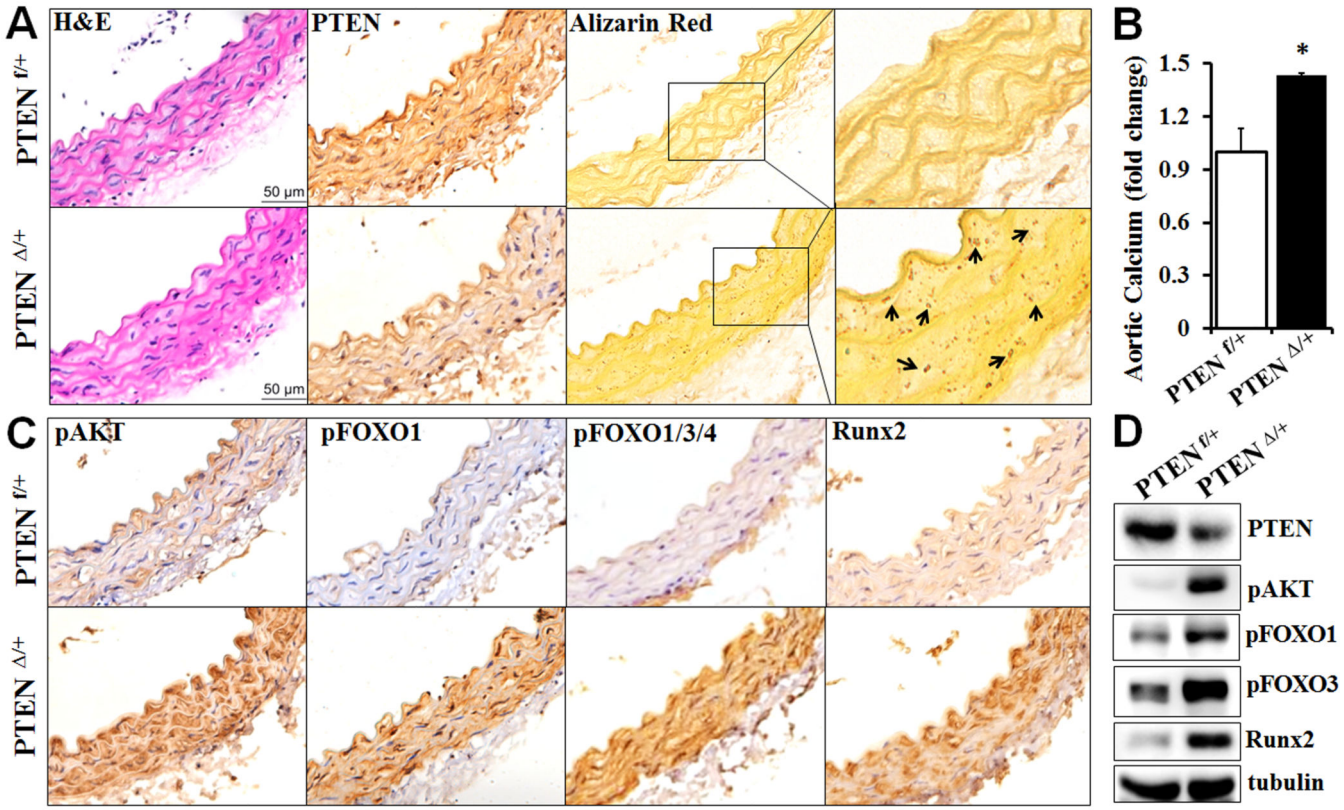

Fig. 6.

Decreased PTEN in SMC promotes vascular calcification. Descending aortas were isolated from 6 month-old heterozygous SMC-specific PTEN deficient mice (PTEN $^{\Delta/+}$) and their control littermates (PTEN $^{f/+}$). 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 AKT activation, FOXO phosphorylation and 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 $^{\Delta/+}$ and PTEN $^{f/+}$ mice.