Targeted Deletion of PTEN in Smooth Muscle Cells Results in Vascular Remodeling and Recruitment of Progenitor Cells Through Induction of Stromal Cell–Derived Factor-1α

Raphael A. Nemenoff, Peter A. Simpson, Seth B. Furgeson, Nihal Kaplan-Albuquerque, Joseph Crossno, Pamela J. Garl, James Cooper, Mary C.M. Weiser-Evans

Abstract—We previously showed that changes in vascular smooth muscle cell (SMC) PTEN/Akt signaling following vascular injury are associated with increased SMC proliferation and neointima formation. In this report, we used a genetic model to deplete PTEN specifically in SMCs by crossing PTEN flox/flox mice to mice expressing Cre recombinase under the control of the SM22α promoter. PTEN was downregulated with increases in phosphorylated Akt in major vessels, hearts, and lungs of mutant mice. SMC PTEN depletion promoted widespread medial SMC hyperplasia, vascular remodeling, and histopathology consistent with pulmonary hypertension. Increased vascular deposition of the chemokine stromal cell–derived factor (SDF)-1α and medial and intimal cells coexpressing SM-α-actin and CXCR4, the SDF-1α receptor, was detected in SMC PTEN-depleted mice. PTEN deficiency in cultured aortic SMCs induced autocrine growth through increased production of SDF-1α. Blocking SDF-1α attenuated autocrine growth and blocked growth of control SMCs induced by conditioned media from PTEN-deficient SMCs. In addition, SMC PTEN deficiency enhanced progenitor cell migration toward SMCs through increased SDF-1α production. SDF-1α production by other cell types is regulated by the transcription factor hypoxia-inducible factor (HIF)-1α. We found SMC nuclear HIF-1α expression in PTEN-depleted mice and increased nuclear HIF-1α in PTEN-deficient SMCs. Small interfering RNA–mediated downregulation of HIF-1α reversed SDF-1α induction by PTEN depletion and inhibition of phosphatidylinositol 3-kinase signaling blocked HIF-1α induction by PTEN depletion. Our data show that SMC PTEN inactivation establishes an autocrine growth loop and increases progenitor cell recruitment through a HIF-1α–mediated SDF-1α/CXCR4 axis, thus identifying PTEN as a target for the inhibition of pathological vascular remodeling. (Circ Res. 2008;102:1036-1045.)

Key Words: smooth muscle cell ■ PTEN ■ neointima ■ autocrine growth ■ conditional knockout mouse

Smooth muscle cell (SMC) accumulation in the arterial intima is a key event in the pathogenesis of atherosclerosis, postangioplasty/in-stent restenosis, and graft arteriosclerosis,1 with changes in the biological function and phenotype of SMCs contributing to the pathology.2 These conditions are characterized, to varying degrees, by dedifferentiation, migration, and proliferation of medial-derived SMCs to form the neointima. Recent data suggest that bone marrow–derived, circulating, and/or resident progenitor/proinflammatory cells are recruited to the injured vessel, differentiate down a SMC lineage, and proliferate, thereby contributing to neointimal lesion formation.3–6 Compelling evidence supports the contribution of both processes to intimal hyperplasia, and major advances have identified numerous factors involved in this complex pathobiology. However, the underlying mechanism(s) initiating lesion formation are not clearly defined. Increased SMC production of chemokines, such as stromal cell–derived factor (SDF)-1α (CXCL12), has been shown to be centrally involved in progenitor cell recruitment,7 although their role in inducing an autocrine growth pathway within the artery wall itself is unknown. We focused on the hypothesis that SMCs are central mediators of the injury response. Perturbations in SMC signaling, result in the production of soluble factors that regulate significant SMC hyperplasia and progenitor/proinflammatory cell recruitment through an autocrine/paracrine mechanism.

Under physiological conditions, the mature blood vessel is a highly quiescent tissue,5,9 suggesting that pathological vascular remodeling requires the inactivation of active growth inhibitory pathways before rendering SMCs permissive to growth stimulation. Several studies support the con-
Figure 1. Arterial remodeling and histopathology consistent with pulmonary hypertension in SMC-specific PTEN-null mice. A, Immunofluorescence for SM-α-actin in aorta and pulmonary artery (MPA) from WT and KO mice (red). Blue indicates 4',6-diamidino-2-phenylindole (DAPI) (nuclei). Images are oriented lumen side up. Two independent animals per genotype are shown for MPA. Arterial wall thicknesses were measured; means ± SD are presented in the graph; n=8; P<0.01. Arrowheads indicate internal elastic laminae; arrows, intimal SM-α-actin-positive cells. B, Left, Immunohistochemistry for BrdUrd on representative aorta and MPA showing increased vascular cell proliferation in KO mice (brown nuclei; arrows). Line indicates arterial media. The percentage of replicating aortic intimal, medial, and adventitial cells was determined; means ± SD are presented in the graph; n=3; P<0.01. Right, Double immunofluorescence for BrdUrd (green) and SM-α-actin (red). Arrows indicate double-positive; arrowheads, BrdUrd-positive and SM-α-actin-negative. C, Cardiac hypertrophy in KO mice. Top, Total heart weight per body weight. Bottom, Right ventricular hypertrophy in KO mice (right ventricle/left ventricle + septum). Values are the means ± SD; n=8. *P<0.01. D, Left and Middle, Representative hematoxylin/eosin staining of lung from WT (top) and KO (bottom) mice showing increased wall thickness of small pulmonary arteries (arrowheads) and reduced alveolarization. Right, Double immunofluorescence for SM-α-actin (green) and von Willebrand factor (red). Arrows indicate...
cept that neointimal SMCs exhibit a distinct growth phenotype similar to fetal-derived SMCs that is characterized by mitogen-independent proliferation and, despite the expression of functional growth factor receptors (e.g., platelet-derived growth factor [PDGF] receptor-β), a blunted mitogenic response to exogenous growth factors known to stimulate medialized SMCs. Our previous studies demonstrated that mitogen-independent growth is actively repressed in differentiated SMCs, suggesting that loss of growth suppressors likely leads to enhanced SMC growth potential in pathological conditions such as restenosis. Our subsequent work strongly implicated the tumor suppressor PTEN as a potent, endogenously produced inhibitor of mitogen-independent SMC proliferation.

PTEN is a dual-specificity lipid and protein phosphatase that inhibits cell proliferation, survival, and growth predominantly through dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate, thus antagonizing phosphatidylinositol 3 (PI3)-kinase-mediated signaling events. Regulation of the PI3-kinase/Akt/mTOR signaling pathway plays a pivotal role in SMC proliferation in culture and during the pathological conditions of restenosis. Importantly, inactivation of PTEN as an antiatherogenic protein. Our studies show that temporally controlled PTEN activity correlated with significant alterations in SMC growth rate during vascular development and after experimental vascular injury. Importantly, inactivation of PTEN in the setting of vascular injury, leading to constitutive Akt activation, is an early and critical event involved in neointima formation. The molecular events mediating the effects of PTEN inactivation on SMC hyperplasia and on progenitor cell recruitment, however, have yet to be identified. To define the role of PTEN signaling in this context, we examined the effects of conditional deletion of SMC PTEN.

By mating PTEN<sub>KO</sub> mice to transgenic mice expressing Cre recombinase under the control of the SM22α promoter, we show here that SMC-specific PTEN mutant mice (PTEN knockout [KO]) exhibit many features associated with pathological vascular remodeling, including remarkable medial and intimal SMC hyperplasia as well as vascular recruitment of progenitor cells. In particular, PTEN depletion results in hypoxia-inducible factor (HIF)-1α-mediated production of the chemokine SDF-1α, which induces an autocrine SMC growth loop and increases progenitor cell migration through a paracrine signaling mechanism. Our data thus suggest that an alteration in SMC PTEN signaling serves as one of the initiating determinants driving pathological vascular remodeling.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Animals and Generation of PTEN mutant mice

SMC-specific PTEN-null mutant mice were generated by crossing SM22α-Cre transgenic mice to PTEN<sup>floxed</sup> mice. PTEN<sup>floxed</sup>;SM22α-Cre<sup>breath</sup>; R26R<sup>red</sup> mice were generated by interbreeding PTEN<sup>floxed</sup> mice with R26R mice followed by crossing PTEN<sup>floxed</sup>;SM22α-Cre<sup>breath</sup> to PTEN<sup>floxed</sup>; R26R<sup>red</sup> mice. Animals were bred and maintained following guidelines approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver Health Sciences.

Additional Methods

An expanded Materials and Methods section containing details regarding animals and generation of PTEN mutant mice; cell culture; generation of stable short hairpin (sh)RNA-expressing SMCs and small interfering (si)RNA transfections; quantitative RT-PCR, Western blot analysis, and ELISA; immunohistochemistry, immunofluorescence, LacZ staining; peripheral blood mononuclear cell (PBMC) isolation, flow cytometry, and cell labeling; growth and transmigration assays; and statistical analysis is available in the online data supplement.

Results

SMooth Muscle–Specific PTEN Depletion Promotes Spontaneous Development of Arterial Remodeling and Histopathology Consistent With Pulmonary Hypertension

Using a rat angioplasty model, we established that PTEN inactivation was localized to the developing neointima and exclusively in replicating neointimal SMCs strongly implicating inactivation of PTEN in the pathogenesis of injury-induced intimal hyperplasia. Therefore, to define the role of PTEN specifically in smooth muscle (SM) tissues using a genetic model, PTEN<sup>floxedP<sup>LoxP</sup></sup> mice were mated to transgenic mice expressing Cre recombinase (Cre) under the control of the SM22α promoter (Figure IA in the online data supplement and Materials and Methods). Cre specificity was verified by crossing SM22α-Cre transgenic mice to ROSA26 Reporter (R26R) mice, as described previously (supplemental Figure IB). Crossing SM22α-Cre mice with PTEN<sup>floxed</sup> mice generated control mice (PTEN<sup>floxed</sup>; +/-, PTEN<sup>floxed</sup>; +/+), and PTEN<sup>floxed</sup>; Cre<sup>breath</sup>) and homozygous mutant mice (PTEN<sup>floxed</sup>; Cre<sup>breath</sup>; PTEN KO). Normal Mendelian ratios of all genotypes were observed in embryos and at birth (data not shown). PTEN KO mice, however, were smaller (supplemental Figure ID) and weaker, and the majority died by 3 weeks of age (all before 6 weeks); no differences among the other 3 groups were observed. All studies reported hereafter compared 20-day-old PTEN KO mice with PTEN<sup>floxed</sup>; +/- control littermates (WT). PCR for the PTENα allele (indicating Cre activity) in DNA extracted from aortic, carotid artery, heart, and lung tissues revealed the expected PCR product in PTEN KO but not WT mice (supplemental Figure IC). Western blotting showed significant reductions in total PTEN protein in large arteries, hearts, and lungs of PTEN KO mice.

Figure 1 (Continued), occluded precapillary arterioles. E, Immunohistochemistry for BrdUrd (brown nuclei) on representative lung sections showing increased cell proliferation in KO mice (left and middle). Aw indicates airway; PA, pulmonary artery; arrows, representative BrdUrd-positive. Right, Double immunofluorescence for BrdUrd (green) and smooth muscle α-actin (red) on representative KO lung sections. Arrows indicate double-positive; arrowheads, BrdUrd-positive and smooth muscle α-actin-negative.
compared with WT, with an accompanying increase in phospho-Akt levels, demonstrating a functional loss of PTEN activity (supplemental Figure ID). PTEN KO mice exhibited significant SMC hyperplasia in major vessels, leading to increased medial thickness along with aortic intimal accumulation of SM-α-actin–positive cells (Figure 1A). Increased numbers of SM-α-actin–positive, 5-bromodeoxyuridine (BrdUrd)–positive replicating cells were observed throughout the aortic and pulmonary arterial walls in PTEN KO mice (Figure 1B). In addition, PTEN KO mice spontaneously developed right ventricular hypertrophy and pulmonary vascular remodeling at Denver, Colo, ambient air (1609 meters). RV/LV+S ratios, a measure of right ventricular hypertrophy, were significantly higher in PTEN

Figure 2. Circulating PBMCs traffic to the vasculature in SMC-specific PTEN-null mice. A, PBMCs were isolated, stained for indicated cell surface markers, and sorted by flow cytometry. Left, Representative fluorescence-activated cell sorting profiles. Right, Percentages of gated progenitor marker–positive cells; data are from 1 of 3 representative experiments. B, Donor PBMCs from WT or KO mice were labeled with fluorescing nanocrystals and reinjected into WT or KO recipients. Tissues from recipient mice were analyzed for labeled PBMC accumulation (positive indicates cytoplasmic nanocrystal [green] plus a nucleus [DAPI, blue]). Top, Representative aorta from KO-to-KO mice. Arrows indicate labeled PBMCs on the intimal and adventitial surfaces. Bottom, Representative lung from controls (WT-to-WT, WT-to-KO, KO-to-WT) and from KO-to-KO mice. Arrows indicate labeled PBMCs. Labeled cells in lung tissue were quantified by counting 20 high-power fields per section (graph). C, Immunofluorescence for SM-α-actin (red) on lung sections from KO-to-KO mice. Arrows indicate SM-α-actin–positive labeled PBMCs; arrowhead, SM-α-actin–negative labeled PBMCs. D, Immunohistochemistry for CD45 (brown) on representative aorta (left) and lung (right) showing increased vascular and perivascular accumulation in KO mice. Arrows indicate CD45-positive cells; PA, pulmonary artery.
KO compared with WT mice, suggesting the development of pulmonary hypertension (Figure 1C). Compared with WT, PTEN KO mice exhibited increased wall thickness of small pulmonary arteries, occluded precapillary arterioles, and reduced alveolarization (Figure 1D). Occluded precapillary vessels stained positive for von Willebrand factor (an endothelial marker) and SM-α-actin, characteristic of plexiform lesions observed in human primary pulmonary hypertension.28 Increased numbers of replicating SM-α-actin–positive and –negative cells were observed throughout the lungs of PTEN KO mice, including small, muscularized pulmonary arteries (Figure 1E). In addition, increased numbers of replicating coronary artery SMCs, coronary perivascular cells, cardiomyocytes, and interstitial fibroblasts were observed in hearts of PTEN KO mice (supplemental Figure II). Cardiomyocyte proliferation was consistent with early developmental cardiac expression of SM22α and, therefore, inactivation of PTEN. Enhanced extracellular matrix deposition was detected in major arteries and in perivascular locations in lungs and coronary vessels of mutants (supplemental Figure III), demonstrating vascular, lung, and cardiac interstitial fibrotic changes. The observed myocardial fibrosis is a key pathological feature of heart failure, the likely cause of death of mutants.

**Recruitment of Circulating Progenitor Cells and Increased SDF-1α Expression in PTEN KO Mice**

In addition to the above, PTEN KO mice consistently exhibited significant splenomegaly (supplemental Figure IVA). Increased bone marrow hyperplasia and hyperplasia of splenic red pulp, consistent with increased extramedullary hematopoiesis, were detected in PTEN KO mice (supplemental Figure IVB through IVD); no changes were observed in livers or kidneys (not shown). Because enhanced bone marrow progenitor/hematopoietic cell mobilization can result in extramedullary hematopoiesis, we hypothesized that SMC-specific PTEN deletion results in production of systemic factors involved in mobilization and trafficking of bone marrow–derived progenitors. To determine whether histological changes were accompanied by increases in peripheral blood progenitor cells, PBMCs were analyzed by 2-color flow cytometry for the hematopoietic leukocyte marker CD45; the progenitor markers CD34, c-Kit, Sca-1, and CXCR4; the endothelial cell progenitor marker FLK-1; or the monocyte progenitor marker CD14. Higher numbers of circulating CD34+, c-Kit+, CD14+, and CXCR4+ hematopoietic cells were detected in PTEN KO mice compared with WT (Figure 2A); no changes in FLK-1+ or Sca-1+ cells were detected (supplemental Figure VA). In PTEN KO mice expressing the ROSA26 reporter allele, where LacZ is expressed in cells lacking PTEN, no Cre activity was detected in bone marrow, therefore direct inactivation of PTEN in bone marrow cells did not mediate progenitor cell mobilization (supplemental Figure IVE).

To determine whether mutant-derived PBMCs traffic to the vasculature, PBMCs isolated from 3-week-old WT or PTEN KO donor mice were labeled with fluorescent quantum dot nanocrystals, and reinfected retroorbitally to either 3-week-old WT or PTEN KO recipient mice. Target tissues were analyzed for fluorescence 24-hour postinjection; cells were scored positive if they contained significant amounts of cytoplasmic, granular fluorescent nanocrystals plus a nucleus. No labeled cells were detected on the aorta of control animals (WT donor-to-WT recipient; WT donor-to-KO recipient; KO donor-to-WT recipient). Only single positive cells were detected in the lungs (Figure 2B), and these were not associated with recipient lung alveolar or vascular surfaces. In contrast, labeled cells were identified on aortic intimal and adventitial surfaces and in lungs of KO donor-to-KO recipient mice; large numbers of labeled progenitors were identified in the lungs (Figure 2B). SM-α-actin–positive and

**Figure 3.** SDF-1α deposition and vascular accumulation of CXCR4–positive cells in SMC-specific PTEN-null mice. A, Serum ELISA measurements for SDF-1α levels. Shown are the means±SD; n=6. B, Immunofluorescence for SDF-1α (green, aorta; red, lung) and SM-α-actin (red, aorta; green, lung) on aortic (left) and lung (right) sections from WT and KO mice. Blue indicates DAPI (nuclei). Aorta: merged images (top); SDF-1α staining (bottom). Merged images are shown only for lung sections. C, Immunofluorescence for CXCR4 (green) and SM-α-actin (red) on representative aortic sections showing CXCR4 expression in SM-α-actin–positive medial SMCs in WT and KO mice and in SM-α-actin–positive intimal cells (arrows) in KO mice. Arrowheads indicate internal elastic lamina. Right images are nonspecific rat IgG control.
negative PBMCs were detected often in a perivascular location (Figure 2C), suggesting some of the recruited PBMCs differentiate toward SMCs. Increased numbers of labeled cells were also found in spleens (supplemental Figure VIA), but not livers (not shown), of KO donor-to-KO recipient mice. Consistent with increased trafficking of PBMCs, accumulation of CD45<sup>+/H11001</sup> and c-Kit<sup>+/H11001</sup> cells was observed on major arteries and in perivascular locations in lungs of PTEN KO mice (Figure 2C and supplemental Figure VB). Taken together, these results support the concept that SMC-specific PTEN depletion produces a local microenvironment favorable for trafficking and accumulation of circulating progenitor cells.

Recent data support a role for the chemokine SDF-1<sup>+/H9251</sup> and its receptor expressed by hematopoietic progenitor cells, CXCR4, in promoting vascular remodeling. Changes in the SDF-1<sup>+/H9251</sup> gradient from bone marrow to blood result in movement of precursor cells into the circulation. Increased plasma levels and local vascular accumulation of SDF-1<sup>+/1α</sup> in response to vascular injury recruit CXCR4<sup>+/</sup> bone marrow cells to remodeling vessels. Because circulating CXCR4<sup>+/</sup> PBMCs were detected in PTEN KO mice, SDF-1<sup>+/1α</sup> expression in tissues from WT and PTEN KO mice was examined. There was a nonsignificant trend toward increased serum SDF-1<sup>+/1α</sup> levels (Figure 3A) and increased vascular and perivascular deposition of SDF-1<sup>+/1α</sup> in aortae and lungs of PTEN KO mice (Figure 3B). This was associated with intimal accumulation of CXCR4<sup>+/</sup> cells in the vasculature (Figure 3C) and in spleens of PTEN KO mice (supplemental Figure VIB). CXCR4 expression was also detected on medial SMCs of WT and KO mice (Figure 3C), suggesting SMCs express functional SDF-1<sup>+/1α</sup> receptors. These results support a model in which SMC-specific PTEN inactivation indirectly promotes vascular recruitment of progenitor cells through induction of

Figure 4. PTEN depletion induces autocrine growth of SMCs through increased SDF-1<sup>+/1α</sup> production. A, Cultured aortic SMCs stably expressing CTRL or PTEN-specific shRNA were analyzed for total PTEN and phospho-Akt expression under basal and PDGF-stimulated conditions in the presence or absence of LY294002 to inhibit PI3-kinase activity (LY). B, CTRL and PTEN shRNA-expressing SMCs were analyzed by BrdUrd immunohistochemistry for SMC proliferation under basal and PDGF- or serum-stimulated conditions. Left, Means±SD percentage of BrdUrd-positive SMCs of triplicates from 1 of 3 representative experiments. Right, Fold changes in BrdUrd-positive SMCs from basal. *Different from CTRL in the same condition (P<0.01). C, CTRL and PTEN-deficient SMCs were analyzed by BrdUrd immunohistochemistry for SMC proliferation in response to media conditioned by CTRL or PTEN-depleted SMCs (source). Graph shows means±SD percentage of BrdUrd-positive SMCs of triplicates from 1 of 3 representative experiments. Note that PTEN-depleted SMC conditioned media drives proliferation of CTRL SMCs, whereas CTRL SMC conditioned media attenuates mitogen-independent proliferation of PTEN-deficient SMCs. D, CTRL and PTEN-deficient SMCs were serum-restricted for 24 hours. Left, Quantitative RT-PCR for SDF-1<sup>+/1α</sup> mRNA. β-Actin was used for normalization of cDNA. Shown are fold changes in mRNA copy number±SD from control SMCs from 3 independent experiments. *P<0.05. Right, Conditioned media were analyzed by ELISA, and SDF-1<sup>+/1α</sup> levels were normalized to total cell number. Shown are fold changes±SD from CTRL SMCs from 3 independent experiments. *P<0.05. E, SMCs were maintained in serum-free media (SFM) in the presence or absence of recombinant SDF-1<sup>+/1α</sup>, recombinant PDGF-BB, neutralizing anti–SDF-1<sup>+/1α</sup> or anti–PDGF-BB, or PTEN-deficient SMC conditioned media with or without anti–SDF-1<sup>+/1α</sup> or anti–PDGF-BB and analyzed for proliferation. Shown are means±SD percentage of BrdUrd-positive SMCs of triplicates from 1 of 3 representative experiments. Inset, Western blot analysis for CXCR4 and total PTEN on CTRL and PTEN-deficient SMCs maintained under basal conditions.
SDF-1α. Because CXCR4 was detected on medial SMCs, increased SMC-derived SDF-1α could also directly affect the biological function of SMCs.

PTEN Depletion Induces SMC Autocrine Growth and Enhances Progenitor Cell Migration Through Increased SDF-1α Production

To verify the direct effect of PTEN depletion on SMC function in vitro, aortic SMCs stably expressing PTEN-specific shRNA were generated. PTEN depletion resulted in enhanced Akt activity under basal conditions and in response to PDGF stimulation compared with SMCs transfected with empty vector (CTRL). Increased Akt phosphorylation was blocked by treatment with LY294002 to inhibit PI3-kinase. PTEN depletion increased Akt activity under basal conditions and in response to PDGF stimulation compared with SMCs transfected with empty vector (CTRL). Increased Akt phosphorylation was blocked by treatment with LY294002 to inhibit PI3-kinase

autocrine growth of PTEN-depleted SMCs and blocked by treatment with LY294002 to inhibit PI3-kinase/Akt signaling. PTEN depletion increased cell proliferation under basal conditions and reduced responsiveness to PDGF-BB, a known mitogen for medial-derived SMCs (Figure 4B), similar to what is observed in fetal and neointimal SMCs.10–12 As with fetal and neointimal SMCs, reduced mitogenic responsiveness to PDGF was not attributable to a loss of functional PDGF receptors (data not shown). Conditioned media from PTEN-depleted SMCs stimulated CTRL SMC proliferation, indicating that PTEN depletion results in secretion of a mitogen, which may act in an autocrine fashion (Figure 4C). Consistent with results in PTEN KO mice, PTEN deficiency in vitro increased SDF-1α mRNA and secretion into the cell media (Figure 4D). Recombinant SDF-1α stimulated proliferation of CTRL SMCs that was blocked with an anti–SDF-1α neutralizing antibody (Figure 4E). A neutralizing anti–SDF-1α antibody also attenuated autocrine growth of PTEN-depleted SMCs and blocked growth of CTRL SMCs induced by conditioned media from PTEN-depleted SMCs (Figure 4E). This effect was specific for SDF-1α, because neutralization of PDGF had no effect on growth responses to conditioned media from PTEN-depleted SMCs (Figure 4E). Consistent with in vivo data, CXCR4 was expressed by CTRL and PTEN-depleted SMCs (Figure 4E, inset), thus supporting the concept that SDF-1α upregulation is a key event in the activation of SMCs in PTEN KO mice and SMCs.

To determine whether increased production of SDF-1α by PTEN-deficient SMCs results in enhanced migration of circulating progenitor cells in vitro, CTRL and PTEN-depleted SMCs were cocultured with PBMCs from WT or PTEN KO mice using a Transwell system (Figure 5A). PBMCs from both WT and PTEN KO mice showed increased migration toward PTEN-depleted SMCs compared with CTRL SMCs, consistent with enhanced SDF-1α levels mediating chemotaxis of CXCR4+ progenitor cells (Figure 5B). Higher numbers of migrating PBMCs were observed from PTEN KO mice compared with WT mice, consistent with higher numbers of circulating CXCR4+ cells in PTEN KO mice. Migration toward PTEN-depleted SMCs was attenuated by the addition of neutralizing anti–SDF-1α and increased toward CTRL SMCs in response to recombinant SDF-1α (Figure 5B), suggesting that enhanced secretion of SDF-1α induced by PTEN depletion drives progenitor cell migration through a paracrine signaling mechanism.

Discussion

We previously demonstrated that vascular injury drives increased SDF-1α expression in SMCs.13,14 The goal of the present study was to examine the consequences of PTEN inactivation using a genetic model in which PTEN is specifically deleted in SMCs. Because changes in SMC PTEN signaling are observed in the early stages following vascular injury, we postulated that PTEN loss would initiate key events involved in pathological vascular remodeling. Our data indicate that...
deletion of PTEN is sufficient to induce proliferation of medial SMCs and accumulation of SMCs in the vascular intima. Our findings are consistent with a previous study that used a similar genetic inactivation strategy and showed SMC PTEN loss resulted in widespread SMC hyperplasia associated with the initiation of leiomyosarcoma development by 2 months of age.36 We did not detect leiomyosarcomas because our mutants rarely survived past 21 days, considerably shorter than what was reported in this study (mean, 70.3 days). Differences are likely attributable to genetic background, because our mice were fully backcrossed to the C57BL/6 background. Nevertheless, in both instances, PTEN depletion specifically in SMCs induced significant SMC hyperplasia in the absence of other stimuli. In addition, we report that SMC PTEN depletion is sufficient to recruit hematopoietic progenitor cells to the vasculature. Based on both our in vivo and in vitro data, a critical mediator of PTEN inactivation is increased HIF-1α–mediated production of SDF-1α by SMCs.

Several studies have demonstrated that vascular remodeling is associated with increased chemokine production by SMCs, as well as other cell types.7,37 Increased SMC production of SDF-1α has been implicated in the recruitment of bone marrow–derived progenitor cells expressing the SDF-1α receptor CXCR4, which contribute to neointima formation. Vascular accumulation of SDF-1α is critical for targeting CXCR4-positive cells to the site of injury. Consistent with these studies, our data show that in PTEN KO mice, CXCR4-expressing intimal cells and PBMCs homing to the vasculature express SM-α-actin, suggesting recruitment of SMC progenitors. The role of SDF-1α on SMC proliferation, however, has been less studied. Consistent with previous studies,38 our data indicate that SMCs express CXCR4, and therefore production and release of SDF-1α would be anticipated to establish an autocrine growth loop. Our in vitro studies demonstrate that PTEN silencing is sufficient to induce SDF-1α expression and increase autonomous growth of SMCs. In fact, blocking SDF-1α reduced autonomous proliferation of PTEN-depleted cells to control levels, suggesting that, in addition to its effects on progenitor cell recruitment, increased SMC-derived SDF-1α may be a major regulator of enhanced SMC proliferation.

Although we have not defined the downstream effectors leading to SDF-1α induction, our data suggest that Akt-dependent upregulation of HIF-1α is likely to be important. Hypoxia-induced SDF-1α upregulation in endothelial cells was shown to be HIF-1α–dependent,39 and forced overexpression of PTEN in glioma cells was shown to significantly reduce HIF-1α expression.40 Karshovska et al44 demonstrated induction of HIF-1α in SMCs following vascular injury; HIF-1α inhibition resulted in reduced neointimal area and

Figure 6. SMC PTEN inactivation increases HIF-1α expression, leading to SDF-1α upregulation. A, Immunohistochemistry for HIF-1α (brown) on representative aorta from WT (top) and PTEN KO (middle) mice showing increased SMC nuclear staining in PTEN KO mice. Bottom, Nonspecific IgG control. Arrows indicate representative HIF-1α–positive cells; lines, arterial media. B, Quantitative RT-PCR for HIF-1α mRNA on CTRL and PTEN-deficient SMCs. β-Actin was used for normalization of cDNA. Shown are fold changes in mRNA copy number±SD from CTRL SMCs. *P<0.05. C, Cytoplasmic and nuclear proteins from CTRL and PTEN-deficient SMCs were analyzed for HIF-1α and total PTEN expression under basal conditions. D, CTRL and PTEN-deficient SMCs were transfected with HIF-1α–specific siRNA oligonucleotides and analyzed by quantitative RT-PCR for HIF-1α (left) and SDF-1α (right) mRNA. β-Actin was used for normalization of cDNA. E, CTRL and PTEN-deficient SMCs were analyzed by quantitative RT-PCR for HIF-1α (left) and SDF-1α (right) mRNA under basal conditions in the presence or absence of LY294002 to inhibit PI3-kinase activity. β-Actin was used for normalization of cDNA.
increased SDF-1α expression. Our studies show that inactivation of PTEN in SMCs is sufficient to upregulate HIF-1α in an Akt-dependent manner; blocking HIF-1α upregulation reverses the induction of SDF-1α. Studies are ongoing to determine the molecular pathways responsible for the regulation of HIF-1α and SDF-1α production mediated by PTEN inactivation.

In summary, we report that SMC PTEN-deficient mice spontaneously develop features in both the systemic and pulmonary vasculature associated with pathological vascular remodeling that are mediated, at least in part, through the induction of the chemokine, SDF-1α. It should be noted, however, that although SDF-1α/CXCR4 signaling has been shown to promote pathological vascular remodeling, recent data also suggest SDF-1α/CXCR4 signaling exerts protective effects in primary atherosclerosis.41 Unfortunately, early lethality in the present model precludes the ability to fully examine molecular events associated with the pathogenesis of remodeling on PTEN inactivation. Although our data suggest PTEN inactivation promotes medial SMC proliferation and the potential recruitment of SMC progenitors, these mice cannot be used as recipients for bone marrow transplant studies. It is, therefore, difficult to determine whether recruited cells are pathological or protective in the present system. We are in the process of developing an inducible SMC-specific system to more clearly answer these questions. Nevertheless, our data are consistent with the proposal that sustained SMC PTEN signaling serves as a central regulator of the vascular injury response and, therefore, have important clinical implications in pathologies associated with vascular remodeling, including restenosis and pulmonary hypertension.

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**Disclosures**

None.

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METHODS

Animals and Generation of PTEN mutant mice

PTEN\textsuperscript{flox/flox} mice, generated as previously described and generously provided to us by Dr. Tak Mak (Ontario Cancer Institute, University of Toronto, Toronto, Ontario\textsuperscript{1}), were fully backcrossed (>10 generations) to the C57BL6/J background. SM22\textalpha-Cre transgenic mice were generated as previously described and generously provided to us by Dr. J. Miano (U. Rochester, Rochester, NY\textsuperscript{2}). SM22\textalpha-Cre activity was verified by crossing SM22\textalpha-Cre transgenic mice with the ROSA26 reporter (R26R) line, as described\textsuperscript{2}. PTEN\textsuperscript{flox/flox} mice were mated to SM22\textalpha-Cre transgenic mice to generate control mice that do not express Cre, but have both (PTEN\textsuperscript{flox/flox};+/+) or only one (PTEN\textsuperscript{flox/+};+/+) PTEN floxed alleles, heterozygous mice that express Cre and have only one PTEN floxed allele (PTEN\textsuperscript{flox/+};Cre/+), and homozygous mutant mice that express Cre and have both PTEN alleles floxed (PTEN\textsuperscript{flox/flox};Cre/+; PTEN KO). PTEN\textsuperscript{flox/flox}, SM22\textalpha-Cre/+; R26R/+ mice were generated by interbreeding PTEN\textsuperscript{flox/flox} mice with R26R mice followed by crossing PTEN\textsuperscript{flox/+};SM22\textalpha-Cre/+ to PTEN\textsuperscript{flox/flox};R26R/+ mice. Animals were bred and maintained following guidelines approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver and Health Sciences Center. For genotyping, genomic DNA from mouse tails was isolated and amplified using the REDExtract-N-Amp tissue PCR kit (Sigma) and primer sequences, as previously described\textsuperscript{1,3}.

Cell culture, generation of stable shRNA-expressing SMC, and siRNA transfections

Primary rat aortic SMC were isolated and cultured as previously described\textsuperscript{4}. A short hairpin RNA (shRNA) sequence based on the mouse PTEN gene (NCBI accession #NM008960) was designed and cloned into a retroviral expression vector driven by the U6 promoter (Open Biosystems, Huntsville, AL). To generate stable SMC clones, control or PTEN shRNA vectors were packaged into replication-
defective retrovirus, as described previously. SMC were incubated with secreted virus for 48 hrs and cells expressing control or PTEN shRNA were selected by culturing in medium containing puromycin. Individual clones were screened by immunoblotting with total PTEN and phosphoAkt antibodies. Transient transfections were performed on primary rat aortic SMC (passage 3) using Oligofectamine and 0.2 µg of PTEN-specific or control mismatched siRNA according to protocols provided. To deplete HIF-1α from control or PTEN shRNA-expressing SMC, transient transfections were performed using 0.2 µg of HIF-1α-specific or control mismatched siRNA. siRNA oligonucleotides were obtained through Dharmacon (Lafayette, CO).

**Quantitative RT-PCR**

To assay for SDF-1α mRNA expression, total RNA was isolated from control or PTEN shRNA-expressing SMC using the QIAshredder and RNeasy Plus kits (Qiagen) and first strand cDNA was made using the iScript cDNA synthesis kit (BioRad). Sequence-specific primers were designed using the MacVector program: SDF-1α: sense (5’-TTTGAGAGCCATG TCGCCAG-3’), antisense (5’-TGTTGTTGCTTTTTCAGCCTTG-3’); β-Actin: sense (5’-AGGGTGTGATGGTGTTGGGATGG-3’), antisense (5’-AATGCCGTGTTCAATGGGG-3’); HIF-1α: sense (5’-TAGACTTTGAAAT GCTGGCTCCCT-3’), antisense (5’-TGGCAGTGACAGTGATGGT). Quantitative real-time PCR was performed as previously described and β-Actin was used for normalization.

**Western analysis and ELISA**

Whole tissues were harvested, snap frozen in liquid nitrogen, and crushed into a fine powder under liquid nitrogen. Tissues or SMC were lysed with ice-cold RIPA buffer, pH 7.4, equal amounts of solubilized proteins were separated by SDS-PAGE and transferred for Western analysis, as described
previously⁴, ⁷, ⁸. The NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology) was used for separation and preparation of nuclear and cytoplasmic proteins from cultured SMC. Total PTEN, total Akt, phosphoSer⁴⁷³ Akt (Cell Signaling), and CXCR4 (Capralogics) antibodies were used at 1:1000 dilutions. A HIF-1α antibody (Novus Biologicals) was used at 1:500 and β-actin (Sigma) was used at 1:5000; β-actin served as a loading control. To detect secreted SDF-1α, conditioned media from control or PTEN-expressing shRNA SMC was assayed using a mouse SDF-1α-specific solid-phase ELISA kit according to the protocols provided (R&D Systems). Values were normalized to total cell protein from individual cell cultures.

**Immunohistochemistry/immunofluorescence/LacZ staining**

Formalin-fixed, paraffin-embedded tissues were analyzed by H&E staining for morphology. Metamorph software was used to measure vessel wall thickness; six measurements per vessel were obtained from 5 animals per genotype. For immunohistochemistry, formalin-fixed, paraffin-embedded tissues were deparaffinized, rehydrated and underwent antigen retrieval by heating for 20 min at 115°C in a decloaking chamber (Biocare). Sections were then exposed to specific antibodies overnight at 4°C. Antigen:antibody complexes were visualized using kits from Vector Laboratories and sections lightly counterstained with hematoxylin. Negative controls included the use of mouse or rabbit IgG. For BrdU analysis, mice were injected with 100 mg of BrdU (Sigma) per kg body weight 12 and 2 hours before death. Sections stained for BrdU incorporation were pretreated with 2N HCl prior to antibody steps. Sections were visualized using an Olympus light microscope equipped with SPOT software. To quantify in vivo replication rates, intimal, medial, and adventitial cells were analyzed independently for BrdU-positive nuclei; the percentages of BrdU-positive nuclei were determined by counting a minimum of 200 intimal, medial, or adventitial cells per tissue section from three animals per genotype⁴, ⁹. For
immunofluorescence, tissue sections were treated as above. Following incubations with primary antibodies, antigen:antibody complexes were visualized using rhodamine (Alexa Fluor-568)-coupled or fluorescein isothiocyanate (Alexa Fluor-488)-coupled secondary antibodies (Molecular Probes). For double labeling, sections were sequentially incubated with specific primary and secondary antibodies. Coverslips were mounted with VectaShield medium containing DAPI to detect all cell nuclei (Vector Laboratories), and sections were visualized using a Nikon inverted fluorescence microscope equipped with Metamorph software. Antibodies used include BrdU (1:100; BD Pharmingen), SM-α-actin-Cy3-conjugated (1:1000; Sigma), SM-α-actin (1:1000; Abcam), vWF (1:1000; Dako), CD45 (1:50; BD Pharmingen), CXCR4 (1:50; R&D Systems), c-Kit (1:100; Dako), SDF-1α (1:200; Abcam), and HIF-1α (1:25; Novus Biologicals). To stain for LacZ activity, tissues were fixed in glutaraldehyde and whole mount staining was performed at 37°C overnight using a kit from GTS, Inc according to the protocols provided. Tissues were then paraffin-embedded for histological analysis.

**Peripheral blood mononuclear cell (PBMC) isolation, flow cytometry, and cell labeling**

PBMCs from WT or PTEN KO mice were isolated from equal volumes of peripheral blood by differential centrifugation with Histopaque (Sigma) and resuspended in PBS containing 5% FBS. Hemolyzed PBMCs were stained with fluorescein isothiocyanate- (FITC), phycoerythrin (PE), or APC-conjugated antibodies (BD Pharmingen): CD45-PE (30-F11), CD45-FITC (30-F11), CXCR4-FITC (2B11), cKit-FITC (2B8), Sca-1-FITC (D7), CD34-PE (581), Flk-1-APC (12α1), or CD14-PE (rmC5-3). Cells were analyzed by two-color flow cytometry using a Beckman Coulter FC500 with CXP software. For cell trafficking experiments, purified PBMCs from donor WT or PTEN KO mice were aseptically labeled with 5nM green fluorescent Qdot 525 nanocrystals using the Qtracker 525 cell labeling kit (Invitrogen) according to the protocols provided. Labeled cells were washed extensively and equal
numbers re-injected retro-orbitally to WT or PTEN KO recipient mice. Tissues from recipient mice were harvested 24 hr post-injection, fixed, stained with DAPI, and analyzed for fluorescence as described above.

**Growth and transmigration assays**

Cell replication was analyzed by BrdU immunocytochemistry, as previously described

Briefly, SMC were plated in triplicate, maintained in serum-free conditions for 72 hrs, and then stimulated with 10 ng/ml PDGF-BB or 10% CS or left in serum-free medium for 24 hrs in the presence of 100 µM BrdU. Some wells received neutralizing antibodies against SDF-1α or PDGF (R&D Systems), 100 ng/ml recombinant SDF-1α (R&D Systems), or 20 ng/ml recombinant PDGF-BB (R&D Systems). Cells were fixed in methanol and stained as described above. To determine the effect of SMC-specific PTEN depletion on progenitor cell recruitment, PBMCs from WT or PTEN KO mice were isolated as described above. Control or PTEN-shRNA expressing SMC were plated in the bottom chamber of a Transwell chamber and maintained under serum-free conditions or in the presence of 10% CS. Some wells received a neutralizing antibody against SDF-1α or 100 ng/ml recombinant SDF-1α. Equal numbers of PBMCs, suspended in serum-free DMEM, were added to the top chamber and migration measured 24 hrs later. Cells that had migrated to the bottom of the Transwell membrane were fixed, stained with DAPI, and quantified by counting five high power fields (400x) per well.

**Statistical Analysis**

Data are expressed as means+/-SD and were determined using either two-tailed t-test analyses or 1-way ANOVA followed by Fisher’s exact test analyses. P values less than 0.05 were considered statistically significant.
**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure S1. Targeted deletion of PTEN in smooth muscle.** (A). Schematic of the strategy used to generate SMC-specific PTEN null mice (PTEN KO). (B). LacZ activity in smooth muscle tissue in SM22α-Cre transgenic mice carrying a ROSA reporter (R26R) allele (blue reaction color due to Cre-mediated excision of a floxed neo cassette upstream of the lacZ gene within the ROSA26 locus). (C). DNA was extracted from whole aortae, carotid arteries, hearts, and lungs from WT (PTEN^fl/fl; +/+) and PTEN KO (PTEN^fl/fl; SM22α-Cre/+ ) mice and analyzed by RT-PCR for the PTENΔ allele; positive band = Cre-mediated excision of floxed PTEN alleles. (D). Right: Western analysis of whole aorta, carotid artery, heart, and lung showing reduced total PTEN levels with accompanying increased phosphoAkt in tissues from SMC-specific PTEN KO mice. Left: SMC-specific PTEN KO mice exhibit reduced body size (graph); all die before 6 weeks of age. Values are presented as the means+/−SD; n=8; p<0.01.

**Supplemental Figure S2. Increased cell replication in hearts of PTEN KO mice.**
Immunohistochemical staining for BrdU on representative heart sections from WT (top panel) and PTEN KO (bottom panel) mice showing increased cell proliferation in PTEN KO mice (brown nuclei). CoA = coronary artery; arrows = representative BrdU-positive cells.

**Supplemental Figure S3. Increased perivascular extracellular matrix accumulation in PTEN KO mice.** Representative Masson’s Trichrome staining of aortic (top left), heart (bottom left), and lung (right) tissues from WT and PTEN KO mice. Note increased vascular ECM deposition in PTEN KO tissues (blue reaction color). Arrowheads = internal elastic laminae; Arrows in aortic sections = ECM-rich intimal cell accumulation in PTEN KO mice; Arrows in heart sections = peri-vascular ECM accumulation; * = coronary artery; PA = pulmonary arteries; Aw = airway.


Supplemental Figure S4. Bone marrow hypercellularity and splenomegaly in PTEN KO mice. (A). Macroscopic image showing splenomegaly in PTEN KO mice. Spleen weights were obtained and shown in the graph as spleen size per body weight. * = different from WT; p<0.01. (B). Representative H&E staining of tibiae (left; cross sections shown) and spleen (middle and right) sections from WT (top panels) and PTEN KO (bottom panels) mice. Note increased bone marrow cellularity in PTEN KO and expansion of red pulp in PTEN KO spleen tissue. (C). Immunohistochemical staining for BrdU on representative spleen sections showing increased cell proliferation in PTEN KO mice (brown nuclei). (D). Immunohistochemical staining for BrdU on representative sections of tibiae showing increased bone marrow cell proliferation in PTEN KO mice (brown nuclei; longitudinal sections shown). Arrows = trabecular bone. (E). LacZ activity in PTEN KO mice carrying a ROSA reporter (R26R) allele (blue reaction color due to SM22α-Cre-mediated excision of a floxed neo cassette upstream of the lacZ gene within the ROSA26 locus). PA = pulmonary artery; CA = carotid artery; CoA = coronary artery; M = bone marrow; B = bone. Note uniform SM22α-Cre activity in SMC of major arteries and the myocardium of PTEN KO mice, but no SM22α-Cre activity in bone marrow of PTEN KO mice.

Supplemental Figure S5. Circulating FLK-1+ or Sca-1+ cells in SMC-specific and vascular accumulation of c-Kit+ cells in PTEN null mice. (A). PBMCs were isolated, stained with antibodies against the indicated cell surface markers and sorted by flow cytometry. Left: Representative FACS profiles. Right: Percents of gated progenitor marker-positive cells; data from one of three representative experiments. (B). Immunohistochemistry for c-Kit on representative aortic sections; brown reaction color. Arrows = c-Kit-positive cells. Arrowheads = internal elastic laminae.
Supplemental Figure S6. Trafficking of labeled PBMCs to the spleen. (A). Donor PBMCs from WT or PTEN KO mice were labeled with fluorescing nanocrystals and re-injected into WT or PTEN KO recipients. Spleen tissues from recipient mice were analyzed for labeled PBMC accumulation (positive score = significant quantities of cytoplasmic nanocrystal [green] plus a visible nucleus [DAPI, blue]). Arrows = representative labeled PBMCs; * = background fluorescence from red blood cells. (B). Immunofluorescence staining for CXCR4 (red) in spleen tissues from WT and PTEN KO mice. Blue = DAPI (nuclei).

Supplemental Figure S7. Transient siRNA-mediated depletion of PTEN in primary cultured SMC. Cultured aortic SMC were transfected with control (CTRL) or PTEN-specific siRNA oligonucleotides and were analyzed by Western for total PTEN and phosphoAkt expression under basal and PDGF- or serum-stimulated conditions. Note that PTEN depletion results in enhanced phosphorylation of Akt under basal conditions and increased and sustained activation of Akt following stimulation.

REFERENCES


A. PTEN Flox E3 LoxP → E4 LoxP E5 LoxP → E6

SM22α-Cre transgenic

PTEN Δ4/5 LoxP → E6

SM22α: embryonic expression in primitive heart tube, dorsal aortae, cephalic mesenchyme, and yolk sac vasculature; postnatal expression limited to arterial, venous, and visceral SMC

B. SM22α-Cre activity in the R26R mouse

aorta

carotid artery

bronchial SMC

pulmonary artery

C. Deletion of the PTEN gene

Deletion of the PTEN gene

PTEN allele

Aorta Carotid Artery Heart Lung

D. Total PTEN pAkt Total Akt βActin

Body Weight at 3 Weeks

Supplemental Figure 1
Supplemental Figure 2

WT PTEN^fl/fl.;+/+

KO PTEN^fl/fl;Cre/+
Supplemental Figure 3
Supplemental Figure 4
Supplemental Figure 5
Supplemental Figure 6

A. WT donor to WT recipient

WT donor to KO recipient

KO donor to WT recipient

KO donor to KO recipient

B. WT PTEN<sup>fl/fl</sup>; +/+  KO PTEN<sup>fl/fl</sup>; Cre/+  CXCR4  DAPI
Supplemental Figure 7