SDF-1α Induction in Mature Smooth Muscle Cells by Inactivation of PTEN Is a Critical Mediator of Exacerbated Injury-Induced Neointima Formation

Raphael A. Nemenoff, Henrick Horita, Allison C. Ostriker, Seth B. Furgeson, Peter A. Simpson, Vicki VanPutten, Joseph Crossno, Stefan Offermanns, Mary C.M. Weiser-Evans

Objective—PTEN inactivation selectively in smooth muscle cells (SMC) initiates multiple downstream events driving neointima formation, including SMC cytokine/chemokine production, in particular stromal cell-derived factor-1α (SDF-1α). We investigated the effects of SDF-1α on resident SMC and bone marrow–derived cells and in mediating neointima formation.

Methods and Results—Inducible, SMC-specific PTEN knockout mice (PTEN iKO) were bred to floxed-stop ROSA26-β-galactosidase (βGal) mice to fate-map mature SMC in response to injury; mice received wild-type green fluorescent protein–labeled bone marrow to track recruitment. Following wire-induced femoral artery injury, βGal(+) SMC accumulated in the intima and adventitia. Compared with wild-type, PTEN iKO mice exhibited massive neointima formation, increased replicating intimal and medial βGal(+) SMC, and enhanced vascular recruitment of bone marrow cells following injury. Inhibiting SDF-1α blocked these events and reversed enhanced neointima formation observed in PTEN iKO mice. Most recruited green fluorescent protein(+) cells stained positive for macrophage markers but not SMC markers. SMC-macrophage interactions resulted in a persistent SMC inflammatory phenotype that was dependent on SMC PTEN and SDF-1α expression.

Conclusion—Resident SMC play a multifaceted role in neointima formation by contributing the majority of neointimal cells, regulating recruitment of inflammatory cells, and contributing to adventitial remodeling. The SMC PTEN-SDF-1α axis is a critical regulator of these events. (Arterioscler Thromb Vasc Biol. 2011;31:1300-1308.)

Key Words: macrophages ▪ vascular biology ▪ PTEN ▪ smooth muscle ▪ stromal cell-derived factor-1α

Restenosis, a wound healing response characterized by unchecked proliferation of resident smooth muscle cells (SMC) and vascular accumulation of inflammatory cells, is a major limitation of percutaneous angioplasty procedures. Resident SMC play a multifaceted role in the progression of this pathology. Under physiological conditions, SMC express a highly quiescent, differentiated phenotype. Activation of SMC in response to injury promotes a transition to a highly proliferative, inflammatory phenotype characterized by downregulation of smooth muscle (SM) contractile markers and increased production of multiple cytokines and chemokines (ie, activated phenotype).1 Many of these factors participate in the remodeling process through direct effects on SMC and through recruitment of inflammatory cells, which sustains progression of lesion formation.2,3 Therefore, existing evidence supports the concept that resident SMC are both initiators and effectors of the injury response.

The origin of SMC in intimal lesions has remained a controversial subject. Elegant studies from more than 20 years ago provided strong evidence that large numbers of differentiated, medial SMC enter the cell cycle in response to arterial injury, migrate, and proliferate, thereby contributing the majority of intimal SMC.4–6 This long-held theory recently has been challenged by several studies suggesting that transdifferentiation of bone marrow–derived progenitors to SMC during vascular repair contributes a significant percentage of intimal SMC.7,8 It is clear that bone marrow–derived cells are recruited to injured vessels, although their ability to transdifferentiate to SMC, typically defined by SM-α-actin expression, is disputed.9–12 In fact, a recent report that fate-mapped the time course of bone marrow cell accumulation on injured vessels showed that differentiation into SMC is a rare event.9 In contrast, these cells express a macrophage phenotype, although whether their contribution to neointima formation is good or bad remains unclear. From a clinical standpoint, the origin of intimal SMC, the role of resident, differentiated SMC vis-à-vis bone marrow–derived cells to vessel remodeling, and the underlying molecular signals regu-
lating pathological neointima formation are important issues for the design of more effective therapeutics to control restenosis.

PTEN, a dual-specificity lipid and protein phosphatase, functions to suppress multiple signaling networks involved in cellular proliferation, survival, and inflammation. Tight regulation of PTEN levels and activity is essential for the maintenance of normal physiological states. PTEN is susceptible to positive and negative transcriptional regulation, posttranscriptional inhibition through specific micro-RNAs, and posttranslational regulation by phosphorylation and oxidation leading to inactivation of its phosphatase activity. PTEN oxidation is particularly important in pathological situations characterized by chronic vascular oxidative stress (eg, diabetes), which would lead to persistent inactivation of PTEN. Importantly, although multiple stimuli have been reported to promote an activated, inflammatory SMC phenotype, the underlying molecular programs actively repressing this remain unclear. Our group and others have demonstrated that regulation of PTEN signaling in SMC plays a critical role in pathological vascular remodeling. Notably, PTEN negatively regulates SMC phenotypic modulation with loss of PTEN in SMC associated with multiple downstream events regulating neointima formation, including promotion of an activated SMC phenotype. In addition, our previous work demonstrated that molecular depletion of PTEN in SMC establishes an autocrine growth loop through induction of the chemokine stromal cell-derived factor-1α (SDF-1α). Others have demonstrated a critical role for SDF-1α induction in the progression of neointima formation. However, direct in vivo effects of increased SDF-1α production by SMC as a consequence of SMC PTEN loss on resident SMC, and their precise role in injury-induced neointima formation has not been examined. Here we used an innovative in vivo approach to fate-map the contribution of highly differentiated resident SMC to injury-induced vessel remodeling and enhanced neointima formation in PTEN iKO mice. A, Immunofluorescence staining for βGal and α-SMA on 7-day injured femoral arteries from WT and PTEN iKO mice. Arrows indicate representative intimal βGal(+);α-SMA(+) SMC; filled arrowheads, representative adventitial βGal(+) SMC; open arrowheads, representative intimal βGal(−);α-SMA(−) SMC; lines delineate the arterial media. B (top), Time course of experimental protocol. Hematoxylin/eosin staining of representative arterial lesions in WT and PTEN iKO mice. Medial and intimal areas were measured using SPOT software. Intima-to-media ratios (left) and percentage of stenotic areas (right) are presented in the graphs. C, Total numbers of BrdU(+) compared with βGal(+);BrdU(+) SMC in the arterial intima, media, and adventitia at 3 weeks postinjury were counted separately, and the data are reported in the graphs. *Different from WT; n=8.
SMCs compared with bone marrow–derived progenitor cells to wire-induced neointima formation and to define the role of SMC PTEN-induced SDF-1α production in this response.

Materials and Methods
PTEN<sup>flox/flox</sup> mice (Dr Tak Mak, Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada),<sup>27</sup> smooth muscle myosin heavy chain (SMMHC)-CreER<sup>T2</sup> transgenic mice (Dr Stephen Ofemanns, University of Heidelberg, Heidelberg, Germany),<sup>28</sup> and ROSA26 reporter (R26R) mice (Jackson Laboratory) were bred to generate tamoxifen-inducible Cre recombinase fused to a mutant ligand-binding domain of the estrogen receptor (CreER<sup>T2</sup>) under the control of the murine SMMHC promoter.<sup>28</sup> WT controls expressed CreER<sup>T2</sup> but were WT for PTEN<sup>+/−</sup>. To fate-map differentiated SMC in response to vascular injury, mice were crossed with floxed-stop ROSA26-LacZ reporter mice. Before tamoxifen and injury, mice were transplanted with bone marrow from enhanced green fluorescent protein transgenic mice to track the accumulation of bone marrow–derived cells to neointima formation. Flow cytometry of peripheral blood mononuclear cells 10 weeks after transplantation showed that >90% of circulating mononuclear cells were green fluorescent protein (GFP)(+) (not shown). Mice received tamoxifen once daily for 5 days before vascular injury, which genetically and permanently marked SMMHC-expressing SMC by tamoxifen-induced Cre-mediated β-galactosidase (βGal) knock-in. Because tamoxifen was administered before injury and then stopped, SMC expressing SMMHC at the time of injections and before injury and their progeny were the only cells labeled with βGal throughout the experimental time period. This allowed fate mapping of differentiated SMC in response to vascular injury even if these cells subsequently lost SM markers. Following tamoxifen administration and before injury, βGal expression was induced in virtually 100% of arterial medial SMC and was specific to SMC; no labeled cells were detected in the absence of tamoxifen (Supplemental Figure IA to IC). Tamoxifen resulted in efficient depletion of PTEN and a corresponding increase in phospho-Akt from major arteries of PTEN iKO mice compared with WT (Supplemental Figure ID).

Enhanced Neointima Formation in PTEN iKO Mice and Role of Resident Mature SMC
To assess the contribution of differentiated SMC to vessel remodeling, the appearance of βGal(+) cells was examined in the intima, media, and adventitia of injured arteries. βGal(+) cells populated the media and accumulated in the intima and adventitia at 7 days postinjury in both WT and PTEN iKO mice (Figure 1A and Supplemental Figure II), establishing that migration of differentiated medial SMC is a major contributor to neointima formation and furthermore that these cells are surprisingly found in the adventitia. Double staining for βGal and α-smooth muscle actin (α-SMA) revealed that most βGal(+) intimal SMC coexpressed α-SMA, although some βGal(+); α-SMA(−) SMC were detected (Figure 1A). Levels of α-SMA were generally lower in the PTEN iKO mice. In addition, we consistently detected βGal(+) cells in the adventitia after injury; all βGal(+) adventitial cells were
α-SMA(−) (Figure 1A). To test whether loss of PTEN selectively in mature SMC enhances neointima formation, WT and PTEN iKO mice were subjected to wire-induced femoral artery injury following tamoxifen (Figure 1B). Injury to PTEN iKO mice resulted in the development of a larger neointima at 3 weeks postinjury compared with WT mice (Figure 1B). Morphometric measurements confirmed 5-fold and 3-fold increases in intima-to-media ratio and percentage of stenosis, respectively, in PTEN iKO mice compared with controls (Figure 1B). The increase in neointima size in PTEN iKO mice was associated with increased numbers of replicating cells in the arterial intima and media (Figure 1C and Supplemental Figure IIIA). Double staining for 5-bromo-2'-deoxyuridine (BrdU) and βGal showed that the majority of replicating intimal and medial cells in WT and iKO mice were βGal(+) (Figure 1C and Supplemental Figure IIIIB), indicating that mature SMC contribute to the bulk of replication in these arterial compartments. Replication of mature SMC accounted for only a percentage of replicating adventitial cells (Figure 1C). These data thus confirm that mature SMC contribute to intimal, medial, and adventitial remodeling and that loss of PTEN exacerbates their proliferation.

**Bone Marrow–Derived Progenitors Are Recruited to Injured Vessels but Do Not Differentiate Into SMC**

To determine the contribution of bone marrow–derived progenitors to neointima formation, all mice received bone marrow from enhanced GFP transgenic mice 6 weeks before tamoxifen injections and vascular injury. At 3 weeks postinjury...
jury, we detected abundant numbers of GFP(+) cells in all arterial compartments of WT and PTEN iKO mice. Consistent with a previous report, double staining for GFP and α-SMA revealed few, if any, GFP(+)α-SMA(+) cells (Figure 2A); no GFP(+)SMMHC(+) cells were detected (not shown). Spatially distinct regions of either GFP(+) or α-SMA(+) cells were detected, indicating that recruited bone marrow cells do not differentiate into SMC. In addition, although an occasional GFP(+) bone marrow cell was found in the media of uninjured arteries, none of these medial GFP(+) cells coexpressed βGal following tamoxifen administration (Figure 2B), confirming that bone marrow cells do not serve as SMC progenitors. In contrast to α-SMA, the large majority of GFP(+) cells coexpressed the macrophage marker, Mac3 (Figure 2C and Supplemental Figure IV). GFP(+) macrophage recruitment to injured vessels was increased in the intima and media of injured vessels from PTEN iKO mice compared with WT (Figure 3A); however, macrophage replication accounted for only a small number of replicating cells in these vessel areas (Figure 3B). In contrast, increased numbers of replicating macrophages were detected in the adventitia of PTEN iKO mice compared with WT (Figure 3B). Therefore, bone marrow–derived macrophages contribute to injury-induced vascular remodeling, likely through crosstalk with SMC rather than through differentiation into SMC. PTEN loss in mature SMC augments macrophage recruitment and retention in injured vessels.

Enhanced Neointima Formation in PTEN iKO Mice Is Dependent on SDF-1α

We previously showed that loss of PTEN in SMC results in induction of the chemokine SDF-1α, and others have implicated SDF-1α in the mobilization and recruitment of bone marrow–derived smooth muscle progenitor cells following vascular injury. Compared with WT, SDF-1α mRNA and protein were increased in injured arteries (Figure 4A), as well as in serum (Figure 4B) from PTEN iKO mice. Mice were treated with neutralizing antibodies against SDF-1α to determine its role in mediating increased neointima formation. Blocking SDF-1α reversed increases in intima-to-media ratio and percentage of stenosis observed in PTEN iKO mice compared with WT mice (Figure 4C). Inhibition of neointima formation was associated with attenuated proliferation of resident βGal(+) SMC in the intima, media, and adventitia of PTEN iKO mice (Figure 5A). These in vivo findings are consistent with our earlier cell culture studies showing that SDF-1α induction establishes an autocrine growth loop driving SMC proliferation. In addition to effects on SMC, SDF-1α neutralization also blocked accumulation of GFP(+) bone marrow–derived cells in the intima and media and proliferation of adventitial GFP(+) cells (Figure 5B and 5C and Supplemental Figure V). These findings support the concept that induction of SDF-1α is a major regulator of exacerbated neointima formation mediated by loss of PTEN signaling through profound effects on resident SMC proliferation combined with inflammatory cell accumulation.

Persistent Inflammatory Environment Is Promoted Through Crosstalk Between SMC and Macrophages and Is Dependent on SDF-1α

Our data suggest that SMC-macrophage crosstalk contributes to enhanced vascular remodeling. To examine this in vitro, bone marrow–derived cells isolated from WT mice were cultured in the presence of macrophage colony stimulating factor to promote macrophage maturation as previously described. These cells have the morphology of macrophages and are >95% F4/80 positive (not shown). Cocultures of WT or PTEN-depleted SMC with WT macrophages were used to analyze the effects of interactions on macrophage adhesion to SMC, cytokine production by SMC, and macrophage-mediated SMC proliferation. As shown previously, PTEN-depleted SMC exhibit an activated, inflammatory phenotype,
characterized by increased Akt phosphorylation and cytokine production (Supplemental Figure VI). We examined whether this activated phenotype enhances macrophage adhesion and found increased numbers of WT macrophages adhered to PTEN-depleted SMC compared with WT SMC (Figure 6A). To determine whether SMC-macrophage interactions perpetuate an inflammatory response, macrophages were cocultured with WT or PTEN-depleted SMC using Transwells, allowing diffusible mediators to act on each cell type. Coculture of macrophages with WT SMC resulted in induction of SDF-1/H9251, monocyte chemotactic protein-1 (MCP-1), and keratinocyte chemoattractant (KC) mRNAs, which correlated with decreased PTEN mRNA expression by SMC (Figure 6B); cytokine production was further enhanced in cocultures of macrophages with PTEN-depleted cells (Figure 6B). Coculture of macrophages with WT SMC increased SMC proliferation under basal conditions (Figure 6C). Although PTEN-depleted SMC cultured under basal conditions exhibited higher rates of proliferation compared with WT, coculture with macrophages significantly enhanced their proliferation (Figure 6C).

SMC PTEN inactivation promotes induction of several cytokines18 (Supplemental Figure VI). However, despite this, the present in vivo data suggest that SDF-1α is a critical mediator of both the proliferative and inflammatory events associated with SMC PTEN loss. To determine whether SDF-1α regulates SMC cytokine production and macrophage adhesion, WT SMC were stimulated with recombinant SDF-1α. MCP-1, interleukin-6 (IL-6), and KC mRNAs were induced in WT SMC stimulated with SDF-1α (Figure 6D), and this was associated with protein release into culture media (Supplemental Figure VIIA). Consistent with these in vitro findings, serum levels of MCP-1, IL-6, and KC were reduced in PTEN iKO mice treated with neutralizing anti-SDF-1α antibodies compared with control IgG-treated mice (Supplemental Figure VIIB). No change in macrophage adhesion was observed in SDF-1α-treated SMC compared with controls (data not shown). Collectively, these data suggest that SMC-macrophage crosstalk creates a persistent inflammatory SMC phenotype that is dependent on SDF-1α induction. Chronic SMC PTEN inactivation enhances these responses, which likely contribute to
the exacerbated neointima formation observed in PTEN iKO mice.

**Discussion**

Molecular changes in SMC initiate multiple events associated with restenosis. Although numerous stimuli activate SMC, restenosis reflects an underlying failure in the regression of wound repair due to defects in antiproliferative and anti-inflammatory processes. The central importance of PTEN in SMC in restricting neointima formation is supported by our findings using the inducible system described here. We show that genetic depletion of PTEN in SMC, which physiologically recapitulates events associated with vascular injury, was sufficient to promote massive neointima formation in a genetic strain of mice that are normally low-to-moderate responders to vascular injury. Enhanced neointima formation was associated with increased resident SMC proliferation combined with increased accumulation of inflammatory cells. Importantly, inhibition of SDF-1α, induced in SMC by PTEN loss, reversed resident SMC proliferation and blocked the accumulation of inflammatory cells, supporting previous studies suggesting that SDF-1α could be an important pharmacological target to limit restenosis.

Local production of SDF-1α has been shown to be an important factor in mediating injury-induced neointima formation. Previous studies using apolipoprotein E–null mice showed that hypoxia-inducible factor-1α–mediated induction of SDF-1α in SMC after injury promotes progenitor cell recruitment and neointima formation. Using culture systems, we demonstrated that PTEN inactivation in SMC promotes an autocrine growth loop through hypoxia-inducible factor-1α–dependent induction of SDF-1α, thus establishing that SDF-1α directly effects SMC function. Consistent with our in vitro data, enhanced neointima formation in PTEN iKO mice was accompanied by increased local production of SDF-1α. Surprisingly, despite induction of many cytokines/chemokines in the setting of PTEN loss, blocking SDF-1α in vivo resulted in complete reversal of neointima formation. Our findings provide the first evidence that SDF-1α has significant direct effects in vivo on resident SMC proliferation. Thus, PTEN operates as an upstream regulator of SDF-1α, which functions as a final common mediator of both the proliferative and inflammatory events that are fundamentally important to restenosis. It should be noted, however, that at this time, we cannot rule out the...
possibility that the pronounced sensitivity observed in this study to anti-SDF-1α treatment is unique to the SMC PTEN-null background.

Through our fate-mapping approach, we definitively showed that the large majority of replicating neointimal cells are derived from SMMHC-expressing, mature SMC originally residing in the medial wall, consistent with concepts proposed more than 20 years ago.4–6 In addition and in a finding not previously reported, we consistently detected resident SMC βGal(+)—derived cells in the adventitia of injured vessels. Adventitial βGal(+) SMC-derived cells did not express SM marker proteins, demonstrating a previously unidentified role for SMC in vessel repair through their contribution to the cellular component of the adventitia. The precise fate and subsequent role of adventitial βGal(+) α-SMA(−) SMC-derived cells is a topic of our current ongoing research. Importantly, however, most studies have used α-SMA to define the role of SMC to injury-induced vessel remodeling. The use of α-SMA to identify SMC is somewhat unreliable, as it has been demonstrated that infiltrating cells and myofibroblasts often transiently express α-SMA.10 In addition, our data suggest that sole use of α-SMA as a marker for SMC will lead to underestimating the numbers of mature SMC contributing to vessel remodeling and therefore to underestimating the overall importance of SMC in vessel repair.

Although large numbers of bone marrow cells accumulated in injured vessels, we detected very few that coexpressed SM markers, supporting a growing number of reports that suggest that these cells do not serve as definitive SMC progenitors. In further support, an occasional GFP(+) cell was detected in the arterial media of uninjured vessels. However, none of these medial GFP(+) cells coexpressed βGal following tamoxifen treatment, which would be anticipated if bone marrow progenitors differentiated into SMMHC-expressing SMC. Therefore, in agreement with recent reports,9,11,12 our data suggest that although recruitment of these cells is an important contributor to neointima formation, this is not through differentiation into SMC but rather through crosstalk with SMC and persistence of the inflammatory microenvironment. We propose, consistent with a well-accepted cascade model,31 that interactions of these circulating cells with vascular cells leads to sustained neointima formation in part through continual production of various growth factors and cytokines.

Macrophage infiltration, adhesion, and retention have been shown to predict the severity of restenosis.32 Our coculture studies demonstrate that macrophage-SMC crosstalk results in inactivation of PTEN, which is associated with SMC production of several cytokines, thus indicating that the inflammatory environment can mediate sustained PTEN loss. Consistent with this, PTEN-depleted SMC, which exist in an activated, inflammatory state, promote enhanced macrophage adhesion and the synergistic induction of cytokines and enhanced SMC proliferation observed in cocultures with macrophages. Although SDF-1α did not enhance macrophage adhesion, it did mediate production of MCP-1, IL-6, and KC, thus contributing to the inflammatory environment. Consistent with the in vitro data, blocking SDF-1α in vivo reduced circulating levels of MCP-1, IL-6, and KC, which likely underlies some of the efficacy on neointima formation. Importantly, the functional changes mediated by SMC PTEN deletion phenocopies the behavior of SMC isolated from experimental diabetic animal models.33 Therefore, our findings have potential clinical importance, especially related to populations of high-risk patients (eg, patients with type 2 diabetes) who exhibit augmented inflammation and accelerated rates of restenosis. Therapeutically, it appears equally important to prevent SMC activation and macrophage infiltration to successfully limit lesion formation. Our data indicate that inhibiting SDF-1α will affect both of these processes. However, additional signaling pathways are likely to contribute, and additional studies will be required to define the molecular signals underlying the functional effects observed in response to SMC-macrophage interactions.

Neointimal hyperplasia continues to be a major obstacle to the long-term success of percutaneous interventions as well as surgical procedures (eg, bypass grafting). Although existing drug-eluting stents have reduced the rates of in-stent restenosis, they have proven less effective in high-risk patient populations. Defining the origin of neointimal SMC, the functional implications of interactions between resident SMC and infiltrating macrophages and other circulating cells and the molecular signals that regulate pathological neointima formation remain important issues for the design of more effective therapeutics to control restenosis. Our data implicate mature SMC as critical participants in this process, by contributing the majority of neointimal cells, regulating the recruitment of inflammatory cells, and contributing to adventitial remodeling. Targeting the PTEN pathway represents a powerful approach to affecting the complex biology associated with restenosis.

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**Disclosures**

None.

**References**


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SUPPLEMENTAL MATERIAL

COMPLETE METHODS

Animals

Generation of inducible smooth muscle-specific PTEN iKO mice. PTEN\textsuperscript{floxfloxp} mice were generously provided by Dr. Tak Mak (Ontario Cancer Institute, University of Toronto, Toronto, Ontario)\textsuperscript{1} and SMMHC-\textsuperscript{CreER\textsuperscript{T2}} transgenic mice by Dr. Stephen Offermanns (U. Heidelberg, Heidelberg, Ger)\textsuperscript{2}. Mice were fully backcrossed (>10 generations) to the C57BL6/J background. The ROSA26 reporter (R26R) line was purchased from Jackson Laboratory. PTEN\textsuperscript{floxfloxp}, R26R, and SMMHC-\textsuperscript{CreER\textsuperscript{T2}/+} transgenic mice were bred together to generate tamoxifen-inducible SMC-specific PTEN knockout mice carrying the R26R allele to fate-map SMC by β-galactosidase knock-in (PTEN iKO: PTEN\textsuperscript{floxfloxp};R26R/R26R;SMMHC-\textsuperscript{CreER\textsuperscript{T2}/+}). Wild type controls also expressed \textsuperscript{CreER\textsuperscript{T2}} and R26R, but were wild type for PTEN (WT: PTEN\textsuperscript{+/+};R26R/R26R;SMMHC-\textsuperscript{CreER\textsuperscript{T2}/+}). 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{1,2}.

Bone marrow transplant. For bone marrow transplant, transgenic UBI-\textsuperscript{EGFP} donor mice on a C57BL/6 background were sacrificed, femurs and tibias were aseptically removed, and bone marrow obtained by aspiration. Cells were suspended in sterile Hank’s buffered salt solution. Recipient WT and PTEN iKO mice were irradiated (900–1,200 RAD split doses) by X-ray source at 5 wks of age. One hour following the second dose, isofluorane anesthetized, irradiated recipients were injected with donor marrow via retro-orbital injection (5 x 10\textsuperscript{6} BM MNC/mouse). Mice were allowed to recover and fully engraft donor bone marrow for 6 weeks prior to experimentation. Mice were reconstituted with wild type GFP-labeled bone marrow to track recruitment of bone marrow-derived cells in response to arterial injury.

Femoral artery wire injury. Twelve-week old male mice were used for all vascular injury experiments. One week prior to injury, all mice received 1 mg I.P. tamoxifen injections for 5
consecutive days to induce PTEN knock-out and βGal knock-in followed by a four-day wash-out period. To induce neointima formation, right femoral arteries underwent wire-induced injury using a 0.015-inch diameter fixed core wire guide (Cook, Inc., Bloomington, IN) as previously described. Three weeks following injury, right injured and left uninjured femoral arteries were harvested, perfusion fixed in 4% paraformaldehyde, and embedded in paraffin or frozen in OCT. Mice were injected with 100 mg of BrdU (Sigma) per kg body weight 18 and 2 hours before death. For SDF-1α neutralization experiments, mice received 100 µg I.P. control IgG or neutralizing SDF-1α antibody (clone 79014; R&D Systems) 24 hours before and immediately following injury; mice received additional 50 µg I.P. injections twice weekly for the duration of the experiment. All mice were maintained in the Center for Laboratory Animal Care at the University of Colorado Denver. Animals were bred and maintained and all procedures performed under a protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

**Morphometric Analysis and Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissues were analyzed by H&E staining for morphology. Arterial specimens were sectioned to identify the region with maximal luminal narrowing. These sections were selected for morphometric measurements of medial area, intimal area, intima-media ratio, and percent stenotic area as described previously. Sections were visualized using an Olympus light microscope and measurements made using SPOT software. A minimum of 8 mice per genotype were analyzed for morphometric measurements. 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). Antigen:antibody complexes were visualized using kits from Vector Laboratories and sections lightly counterstained with hematoxylin. For immunofluorescence, paraffin-embedded tissue sections were pre-treated as above. For frozen sections, tissues were rinsed in PBS, saturated with 30% sucrose, and frozen in OCT. Frozen sections were fixed in 100% MeOH for 5 minutes
and permeabilized in 0.05% Tween-20/PBS for 2 minutes. Following incubations with primary antibodies, antigen:antibody complexes were visualized using Alexa Fluor-568-coupled or Alexa Fluor-488-coupled secondary antibodies (Molecular Probes). Sections immunohistochemically or immunofluorescently stained for BrdU incorporation were pretreated with 2N HCl prior to antibody steps. To quantify in vivo replication rates, intimal, medial and adventitial cells were analyzed independently for BrdU-positive nuclei; total numbers of BrdU-positive nuclei were determined by counting a minimum of 200 cells per compartment per tissue section from a minimum of six animals per genotype. 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). Sections were imaged using a Nikon inverted fluorescence microscope equipped with Metamorph software or using a laser-scanning confocal microscope (510 META NLO, Carl Zeiss, Thornwood, NY) with a 63x or 100x oil immersion objective. Images were analyzed using LSM 510 software. Antibodies used include monoclonal anti-BrdU (1:100; BD Pharmingen), monoclonal anti-SM-α-actin-Cy3-conjugated (1:2000; Sigma), polyclonal anti-SM-α-actin (1:1000; Abcam), polyclonal anti-β-Galactosidase (1:100; Abcam), polyclonal anti-GFP-FITC-conjugated (1:200; Abcam), polyclonal anti-SMMHC (1:100; Biomedical Technologies, Inc), rat anti-Sca1 (1:100; BD Pharmingen), and monoclonal anti-Mac-3 (1:50; BD Pharmingen). Negative controls included the use of rat, chicken, or rabbit IgG. 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.

**SMC-Bone Marrow Macrophage Co-culture**

Bone marrow–derived cells were isolated from femurs and tibias of wild type C57BL/6 mice and cultured in the presence of M-CSF to promote macrophage maturation as previously described. After 4 d in culture, these cells have the morphology of macrophages, and are >95% F4/80
positive (not shown). Primary rat or mouse aortic SMC were used for experiments. Rat aortic SMC were transduced with lentiviral particles expressing control or PTEN-specific shRNA as previously described\(^7\). Primary mouse aortic SMC were isolated and cultured from PTEN\(^{\text{floxfloxflox}}\) mice. Cells from the same isolation were treated with control, empty vector adenovirus or adenovirus expressing Cre recombinase to delete PTEN. For adhesion assays, macrophages were labeled with 5µg/ml BCECF-AM (A.G. Scientific, Inc.) for 15min at 37\(^\circ\), trypsinized, and equal numbers added to 24-hr growth-arrested (0.1% FCS media) control or PTEN-depleted SMC (5 \times 10^5 per well). At the indicated times, non-adherent macrophages were rinsed off the plate. To quantify numbers of adherent macrophages, total numbers of fluorescent cells in five 10x-fields per well were counted. As a secondary quantitative measure of adherence, cells were washed, lysed (0.1M Tris-Cl + 0.1% Triton X-100; pH8.0), and lysates assayed for fluorescence in triplicate (excitation 485, emission 535). Transwell systems were used to assess cytokine production and cell proliferation in co-culture. Bone marrow–derived macrophages were grown on the bottom of Transwell inserts. WT or PTEN-depleted SMC were plated on Transwell filters and growth-arrested for 24 hours prior to co-culture. After 24 h of co-culture in 0.1% FCS media, total RNA was isolated from SMC and analyzed by qRT-PCR. For SMC proliferation, co-cultures were maintained in 0.1% FCS media for 48hr. BrdU (100 µM) was added to co-cultures for the final 24-hr and cell replication analyzed by BrdU immunocytochemistry, as previously described\(^5\).

**Quantitative RT-PCR**

To assay for PTEN and cytokine mRNA expression, total RNA was isolated from WT or PTEN-depleted SMC or arterial tissues 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: PTEN: forward (5'-CTTTTGAAGACCATAACCCAC-3'), reverse (5'-TTACACCAGTCCGCTCTTTCC-3'); SDF-1\(\alpha\): forward (5'-CTTGTCTGGTTGGCTTTTCGAC-3'), reverse (5'-GCCAGACGCAACGTCAACAC-3');
MCP-1: forward (5’-TGCTGTCTCAGCCAGATGCAGTTA-3’), reverse (5’-TACAGCTTCTTTGGACACCTGCT-3’); KC: forward (5’-GAGACCACAAGGTGTC-AACCA-3’), reverse (5’-CCCACATGCTCTACACCGTAA-3’); IL-6: forward (5’-GGTCTTAGCC- ACTCTTCTGTG-3’), reverse (5’-GATGCTACAAAATGGTATAATC-3’); β-Actin: forward (5’- AGGGTGTGATGGTGGGTATGG-3’), reverse (5’-AATGCCGTGTTCAATGGGG-3’).

Quantitative real-time PCR was performed as previously described\(^8\) and β-actin was used for normalization.

**Western Analysis and ELISA**

Whole arterial tissues were harvested, snap frozen in liquid nitrogen, crushed into a fine powder under liquid nitrogen, and lysed in ice-cold M-PER mammalian protein extract reagent (Thermo Scientific); cultured 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 previously\(^7,8\). Total PTEN and phospho\(^{\text{Ser}473}\)Akt antibodies (Cell Signaling) were used at 1:1000 and β-actin (Sigma) was used at 1:5000; β-actin served as a loading control. To detect serum levels of SDF-1\(\alpha\), MCP-1, IL-6, and KC, serum from WT or PTEN iKO mice was assayed using a mouse-specific solid-phase ELISA kits according to the protocols provided (R&D Systems). To detect secreted cytokines, conditioned media from SMC was assayed using rat IL-6-, MCP-1-, and CXCL1/KC-specific sandwich immunoassay kits according to the protocols provided (R&D Systems). Values were measured in duplicate using a Sector Imager 1200 reader and normalized to total cell protein.

**Statistics**

Data are expressed as means+/-SE 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.
REFERENCES


**FIGURE LEGENDS**

**Supplemental Figure I. Generation of Inducible SMC-specific PTEN Null Mice.** (A) Representative staining for tamoxifen-induced βGal knock-in. (a&b) Cross sections of uninjured carotid arteries from WT (a) and PTEN iKO (b) mice were chemically stained for X-Gal (blue reaction color) 3 days after the last tamoxifen injection. (c-e) Representative double immunofluorescent staining for βGal (c) and α-SMA (d) on an uninjured aorta 3 days after the last tamoxifen injection. Panel (e) shows a merged image. (B) Uninjured femoral arteries from WT (a) and PTEN iKO (b) mice that did not receive tamoxifen injections were chemically stained for X-Gal. Note the absence of βGal knock-in. (C) Indicated tissues from tamoxifen-treated mice were whole mount stained for X-Gal (a,b,c,d). Histological sections from these stained tissues were analyzed for SMC-specific tamoxifen-induced βGal knock-in (a’,b’,c’,d’). Tr = trachea; Ao = aorta; MPA = main pulmonary artery; CoA = coronary artery; PA = pulmonary artery; Aw = airway; M = bone marrow; Sk Mu = skeletal muscle. Arrow in panel (c) = small artery; arrow in panel d’ = intestinal SMC. (D) Western analysis for total PTEN and phosphorylated Akt in whole aortae from WT or PTEN iKO mice harvested at the indicated times after the last tamoxifen injection. β-Actin was used as a loading control. n=2 per genotype 11- and 18-d post-tamoxifen; n=4 per genotype 25-d post-tamoxifen.

**Supplemental Figure II. Highly Differentiated SMC Contribute to Injury-Induced Vessel Remodeling.** Immunofluorescence staining on 7-d injured femoral arteries from WT and PTEN iKO mice for βGal (green). DAPI was used to stain all nuclei (blue). Arrows = representative intimal βGal(+) SMC; arrowheads = representative adventitial βGal(+) SMC.

**Supplemental Figure III. Staining for BrdU and βGal.** (A) Representative images of BrdU immunofluorescence on 3-w injured femoral arteries from WT and PTEN iKO mice. (B) (a&b)
Representative double histochemical staining for BrdU and X-Gal on injured femoral arteries. Arrowheads = internal (black) and external (white) elastic laminae. (c) Representative double immunofluorescence staining for BrdU and βGal on a 3-w injured femoral artery from a WT mouse. Arrowheads = internal (closed) and external (open) elastic laminae.

**Supplemental Figure IV. Recruited Bone Marrow-Derived Cells Express Macrophage Markers.** Representative double immunofluorescence staining for GFP and Mac3 on 2-w injured femoral arteries from WT (top panels) and PTEN iKO (bottom panels) mice. Right panels show merged images. Scale bars = 20 µm. N=neointima; M=Media; A=Adventitia.

**Supplemental Figure V. Increased Accumulation of Bone Marrow-Derived Macrophages in PTEN iKO mice is Dependent on SDF-1α.** Immunofluorescence staining for GFP on 3-w injured femoral arteries from WT and PTEN iKO mice treated with control or SDF-1α neutralizing antibodies. N=neointima; M=Media; A=Adventitia.

**Supplemental Figure VI. PTEN Depletion Promotes Increased phosphoAkt and Induction of Cytokines.** WT and PTEN-depleted SMC were serum-restricted. (A) Representative Western analysis for total PTEN and phosphoAkt. βActin was used as a loading control. (B) Representative qRT-PCR analysis for the indicated mRNAs. βActin was used for normalization of cDNA. Shown are fold changes in mRNA copy number from WT SMC.

**Supplemental Figure VII. Cytokine Production by SMC is Dependent on SDF-1α.** (A) Conditioned media from WT SMCs treated for 48 hr with vehicle or 100 ng/ml recombinant SDF-1α were analyzed by ELISA for MCP-1, KC, or IL-6 protein levels. Shown are the means from two independent experiments. (B) Serum from 3-w post-injured PTEN iKO mice treated with
control (CTRL) or SDF-1α (αSDF-1α) neutralizing antibodies were analyzed by ELISA for MCP-1, IL-6, and KC levels. * = different from CTRL; p<0.05.

**Supplemental Figure VIII. Negative Controls for Immunofluorescence Staining.** Negative controls for immunofluorescence staining were conducted by substituting primary antibodies with species- and isotype-matched antibodies (a = chicken + anti-chicken-488 secondary, b = rat + anti-rat-568 secondary, c = rabbit + anti-rabbit-488 secondary).
A. Uninjured Artery immediate post-Tamoxifen

WT (+/+, M-CreER/++; R/R) iKO (F/F, M-CreER/++; R/R)

X-Gal

β-Gal α-SMA Merge

B. No Tamoxifen X-Gal

C.

Heart

Lung

D.

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Total

Marrow

Esophagus

Bone
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