Nuclear PTEN functions as an essential regulator of SRF-dependent transcription to control smooth muscle differentiation

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Vascular disease progression is associated with marked changes in vascular smooth muscle cell (SMC) phenotype and function. SMC contractile gene expression and, thus differentiation, is under direct transcriptional control by the transcription factor, serum response factor (SRF); however, the mechanisms dynamically regulating SMC phenotype are not fully defined. Here we report that the lipid and protein phosphatase, PTEN, has a novel role in the nucleus by functioning as an indispensible regulator with SRF to maintain the differentiated SM phenotype. PTEN interacts with the N-terminal domain of SRF and PTEN–SRF interaction promotes SRF binding to essential promoter elements in SM-specific genes. Factors inducing phenotypic switching promote loss of nuclear PTEN through nucleo-cytoplasmic translocation resulting in reduced myogenically active SRF, but enhanced SRF activity on target genes involved in proliferation. Overall decreased expression of PTEN was observed in intimal SMCs of human atherosclerotic lesions underlying the potential clinical importance of these findings.
Cardiovascular diseases are the leading causes of death in industrialized nations. Atherosclerosis is a chronic inflammatory disease that progresses to complex, unstable arterial lesions. Restenosis is an acute inflammatory vascular disease and a major limitation of percutaneous angioplasty procedures, especially in higher risk patient populations. Both are characterized by activation of vascular smooth muscle cells (SMCs) resulting in an inflammatory environment, neointimal hyperplasia and vessel occlusion. Under physiologic conditions, SMCs express a quiescent, differentiated phenotype distinguished by high levels of SMC-specific contractile proteins (for example, SM-alpha-actin (Acta2/sSMA) and SM myosin heavy chain (Myh11/SM-MHC)). SMC activation promotes a transition to a highly proliferative, inflammatory phenotype characterized by downregulation of SM genes and increased production of multiple cytokines and chemokines (that is, SMC dedifferentiation). Collectively, existing evidence supports the concept that resident SMCs serve as both initiators and effectors thereby playing a multifaceted role in the progression of vascular disease.

SMC differentiation is associated with serum response factor (SRF)-dependent transcriptional activation of SM contractile genes, thereby conferring the distinctive physiological characteristics of SMCs. SRF is a transcription factor that binds CArG box elements in promoter regions of target genes. In SMCs, SRF regulates two distinct gene programs, SM contractile genes and growth-related immediate early genes (IEG; for example, Fos/c-Fos). While seemingly paradoxical, identification of specific SRF co-factors that promote SM contractile (myocardin) or SM proliferative (ETS-like transcription factor 1 (Elk-1)) programs resolved the contradiction. Yet questions remain regarding the mechanisms and factors responsible for maintaining the SMC differentiation program particularly in vivo. Recent findings also demonstrated that the ability of SRF and co-factors to engage essential CArG sites of SM target genes requires epigenetic modifications of chromatin. Cellular spatial regulation may be another potential mechanism controlling SRF activity. SRF nuclear exclusion (NES) resulted in decreased SM gene expression in airway SMCs and we showed that PDGF represses SRF activity and translocates SRF out of the nucleus in vascular SMCs. SRF has a higher affinity for IEG promoters compared with SM gene promoters, likely due to degenerate CArG elements in SM gene promoters; thus, where SRF levels are a limiting factor, this may be a critical determinant for gene program activation. Despite the work in the field, however, a clear mechanistic understanding of SMC phenotype control, how phenotypic switching is dynamically regulated and mechanisms mediating cell specificity is lacking.

The concept of SMC phenotypic modulation is well-accepted and plays an essential role in vascular disease progression. The mechanisms regulating SM gene repression are complex yet a complete understanding, while a challenge, is critical to enable therapeutic advances in the treatment of vascular diseases. Although multiple stimuli dedifferentiate SMCs, the underlying molecular programs actively repressing dedifferentiation remain unclear. PTEN is a dual-specificity protein and lipid phosphatase that suppresses numerous signalling networks is involved in cell proliferation, survival and inflammation. We and others showed that PTEN inactivation promotes an activated SMC phenotype characterized by increased proliferation, increased inflammatory cytokine production, decreased SM gene expression and vascular disease progression. PTEN classically functions as a cytoplasmic lipid phosphatase to antagonize PI3-kinase/Akt-mediated signalling. Our previous work demonstrated that effects on SMC proliferation and cytokine production are phosphatase-dependent and mediated by Akt-induced NFκB and HIF-1α activity. In addition, we showed that PTEN is a downstream effector of SRF through a microRNA (miRNA)-dependent pathway. Loss of an SRF–PTEN axis promotes reprogramming of SMCs into a proliferative, inflammatory phenotype. However, inhibition of Akt signalling only partially restored SM gene expression in the setting of PTEN deletion. Since SM gene expression is under direct transcriptional control by SRF, the mechanism underlying PTEN’s regulation of the SMC differentiation program remained unclear. Emerging data in other cell systems support a role for nuclear PTEN both in a phosphatase-dependent and -independent manner thereby uncovering a function for PTEN independent of its cytoplasmic Akt-antagonizing effects. However, there is no information regarding the biological significance of nuclear PTEN in SMCs.

We describe here a novel and unanticipated function for PTEN in transcriptional control through association with SRF and its muscle-specific co-factor, myocardin. This association facilitates selective binding of SRF on the Myh11 and Acta2 promoters, but not the Fos promoter, thus activating SMC contractile gene expression. We used mouse genetic models and in vitro approaches to demonstrate that PTEN is an indispensable regulator of SRF that plays a key role in SRF transcriptional activity as a mechanism to dynamically regulate SM contractile genes. In addition, we report overall decreased expression of PTEN in intimal SMCs of a small cohort of human atherosclerotic lesions underlying the potential clinical significance of our findings.

Results

Aortic contractility is reduced in PTEN iKO mice. Abnormal contractility occurs in atherosclerotic and restenotic vessels. We showed that PTEN deficiency in vitro promotes a dedifferentiated phenotype characterized molecularly by decreased expression of SM contractile genes. Here to determine if normal vessel contractility is impaired by PTEN deficiency, we used an inducible SMC-specific PTEN knockout (PTEN iKO) mouse model previously generated in our lab. Vascular reactivity of intact aortic rings isolated from wild-type (WT) or PTEN iKO mice was measured as described in Methods. Aortic rings from PTEN iKO mice exhibited 9- and 5.8-fold decreases in maximal contractile force in response to KCl and phenylephrine (PE), respectively, compared with WT mice (Fig. 1a,b). Maximal contractile force in response to Ca++ was decreased 10-fold in aortic rings from PTEN iKO mice compared with WT mice (Fig. 1b), suggesting an intrinsic impairment of the contractile machinery by PTEN deficiency. Decreased vessel contractility was associated with decreased expression of SM-MHC in aortic media from PTEN iKO mice compared with WT mice (Fig. 1c), in agreement with our published in vitro data. Surprisingly, decreased SRF expression was also observed (Fig. 1c).

PTEN regulates SRF protein levels and transcriptional activity. To determine the mechanism underlying PTEN’s effect on SM gene expression, PTEN-specific short hairpin RNA (shRNA) was used to selectively reduce PTEN in cultured SMCs. We observed reduced levels of SRF protein in PTEN-deficient SMCs compared with controls (Fig. 1d), in agreement with the in vivo data. There were no changes in SRF mRNA indicating SRF was not regulated at the level of transcription (Fig. 1e). Treatment of PTEN-deficient SMCs with the PI3K inhibitor, LY294002, had no effect on SRF levels (Fig. 1f) indicating a lipid phosphatase-independent effect of PTEN on SRF expression. In contrast, inhibition of proteasomal degradation restored SRF protein levels.
Figure 1 | PTEN-dependent vessel contractility and serum response factor (SRF) activity. Wild-type (WT) and inducible PTEN knockout (PTEN iKO) mice were generated and treated with tamoxifen as described in Methods. (a,b) Isometric force normalized to vessel length was measured in isolated aortic rings from WT and PTEN iKO mice exposed to the indicated concentrations of potassium chloride (K\(^+\)), phenylephrine (PE) or calcium chloride (Ca\(^{++}\)). (a) Representative tracing from potassium-stimulated aortic rings. (b) Quantification of force generation. Data represent averages ± s.e.m. from six (K\(^+\), PE) or four (Ca\(^{++}\)) vessels per group. *P<0.01 versus WT. (c) Western blot analysis for PTEN, phospho-Akt, SRF and SM myosin heavy chain (SM-MHC) in whole cell lysates (WCL) of aortic media from WT or PTEN iKO mice. β-Actin was used as a loading control. Left—representative blot from two mice per genotype; each lane represents an individual mouse. Right—fold changes in densitometry measurements ± s.e.m. N = 12; *P = 0.016; **P = 0.000056 versus WT. (d,e) Smooth muscle cells (SMCs) stably expressing control (Ctrl) or PTEN-specific shRNA were serum-restricted for 24 h (RNA) or 48 h (protein). (d) WCL were analysed for total PTEN, phospho-Akt, SRF and αSMA. (e) Total RNA was analysed by qPCR for SRF mRNA. Shown are fold changes in SRF mRNA copy number ± s.e.m. from six independent experiments. (f) Ctrl and PTEN-deficient SMCs were serum-restricted in the presence or absence of the PI3-kinase inhibitor, LY294002 (10 μM) for 24 h. WCL were analysed for total PTEN, phospho-Akt, total Akt and SRF levels. N = 3 independent experiments. Molecular weight markers were cropped out for final SRF blots; please see Supplementary Fig. 8. qPCR, quantitative PCR.
in PTEN-deficient SMCs, suggesting PTEN loss results in posttranslational SRF degradation (Supplementary Fig. 1a). To determine if PTEN overexpression is sufficient to enhance SRF transcriptional activity, SMCs were transduced with adenoviruses expressing empty vector (EV), WT PTEN or lipid/protein phosphatase-inactive PTEN (MT). Compared with EV, overexpression of both WT and MT PTEN increased SRF protein and, importantly, increased expression of the SRF target gene, αSMA (Supplementary Fig. 1b) supporting a role for PTEN in regulation of SRF transcriptional activity. In contrast, overexpression of PTEN in HEK 293 cells or L929 fibroblasts had no effect on SRF protein levels or SM gene induction (Supplementary Fig. 2a–c) suggesting a selective effect of PTEN in SMCs. As inhibition of PTEN-regulatable Akt activity did not restore SRF in PTEN-deficient SMCs and both WT and MT PTEN increased SRF transcriptional activity, our data suggest a novel role for PTEN independent of its known lipid or protein phosphatase activity.

PTEN, SRF, and myocardin form a multi-protein complex. The lipid phosphatase, PI3K–Akt-antagonizing activity of PTEN occurs in the cytoplasm. Recent data, however, support important biological roles for nuclear PTEN through direct interactions with nuclear factors and independent of its phosphatase activity²⁰ or as a nuclear protein phosphatase that directly targets transcription factors to activate them.⁴⁶,⁵¹ To determine if PTEN regulates SRF through interaction between the proteins, reciprocal co-immunoprecipitation (co-IP) assays were performed. We found PTEN immunoprecipitated SRF and, similarly, SRF immunoprecipitated PTEN in both human- and rat-derived aortic SMCs (Fig. 2a,b); complex formation was specific as no interaction was observed with the use of a non-specific IgG. Protein–protein binding was independent of PTEN’s phosphatase activity as rescue of PTEN levels in PTEN-depleted SMCs using either WT or MT PTEN restored PTEN–SRF interactions (Fig. 2c). Cell fractionation showed that PTEN localizes to both the cytoplasm and nucleus in SMCs, with the majority present in the cytoplasm (Fig. 2d). However, under basal conditions PTEN interaction with SRF occurred predominantly in the nucleus (Fig. 2e). In vitro binding assays using bacterially expressed and purified amino terminal-tagged His–PTEN and glutathione-S-transferase (GST)–SRF were conducted to determine whether this interaction is direct (Supplementary Fig. 3). Compared with a non-specific IgG negative control, reciprocal co-IPs demonstrated that in in vitro conditions PTEN directly interacted with SRF (Fig. 2f). SRF controls SM gene or EIG expression through selective interactions with the muscle-restricted co-factor, myocardin or the ETS-domain family member, Elk-1, respectively. Co-IPs were performed to determine if PTEN forms a higher order protein complex selectively with myocardin to regulate SRF-dependent SM gene expression. Consistent with SMCs expressing a differentiated phenotype, interactions between myocardin and SRF and myocardin and PTEN were readily detected, but not between Elk-1 and SRF or Elk-1 and PTEN (Fig. 2g).

The identification of myocardin significantly advanced our understanding of how SM gene transcription is controlled by SRF, a widely expressed transcription factor. However, myocardin, exclusively expressed in cardiac and smooth muscle, is essential for both cardiac and SM contractile gene transcription.⁴⁶,⁵⁴ To determine if multi-protein complex formation was specific to SMCs or more general to muscle gene expression, neonatal rat ventricular cardiomyocytes (NRVMs) were cultured under basal conditions or in response to hypertrophic stimuli (PE). As expected, both PTEN and SRF were readily detectable in NRVMs under both conditions; compared with basal, no differences in expression were observed in response to PE (Supplementary Fig. 4a). In contrast to SMCs, PTEN and SRF did not interact in NRVMs under basal conditions, although PE promoted a strong interaction between PTEN and SRF similar to that observed in SMCs (Supplementary Fig. 4b) suggesting a role for PTEN in agonist-mediated cardiomyocyte hypertrophy. While PTEN overexpression in L929 fibroblasts was not sufficient to promote SM gene induction (Supplementary Fig. 2c), similar to SMCs and PE-stimulated NRVMs, SRF immunoprecipitated with PTEN in these cells indicating an uncoupling of PTEN-dependent SRF-mediated SM gene transcription in non-muscle cells (Supplementary Fig. 4c).
Figure 2 | PTEN forms a nuclear multi-protein complex with SRF and myocardin. (a,b) PTEN (top) or SRF (bottom) proteins were immunoprecipitated (IP) from WCL of cultured human (a) or rat (b) aortic SMCs serum restricted for 48 h. About 10% of input WCL and co-immunoprecipitating SRF or PTEN were detected by immunoblotting (IB). A non-specific IgG was used for IPs as a negative control. (c) SMCs stably expressing control (Ctrl) or PTEN-specific shRNA were transiently transduced with empty vector adenovirus (EV) or adenoviruses encoding wild-type PTEN (WT) or phosphatase-inactive PTEN (MT) (multiplicity of infection = 100). PTEN was immunoprecipitated (IP) and co-immunoprecipitating SRF was detected by immunoblotting (IB). (d) Cytoplasmic (cyto) and nuclear (nuc) SMC extracts were analysed by western blotting for SRF and PTEN levels. β-Tubulin and Lamin A/C were used as cytoplasmic and nuclear loading controls, respectively. (e) PTEN was immunoprecipitated from cytoplasmic and nuclear SMC extracts and co-immunoprecipitating SRF was detected by immunoblotting. A non-specific IgG was used for IPs as a negative control. (f) Recombinant His-tagged PTEN and GST-tagged SRF were purified, incubated together, and PTEN (top) or SRF (bottom) were immunoprecipitated. Co-immunoprecipitating SRF or PTEN was detected by immunoblotting. About 10% of protein mixture was immunoblotted to control for input. A non-specific IgG was used for IPs as a negative control. (g) Myocardin (left), Elk-1 (middle) and SRF or PTEN (right) proteins were immunoprecipitated from WCL of SMCs serum restricted for 48 h. About 10% of input WCL and co-immunoprecipitating PTEN, SRF or Elk-1 were detected by immunoblotting. Shown are representative blots from a minimum of three independent experiments. *, heavy chain IgG. Molecular weight markers were cropped out for final SRF blots; please see Supplementary Fig. 8.
Figure 3 | PTEN–SRF interact with CArG boxes of SM genes. (a) Chromatin immunoprecipitation (ChIP) analyses for protein binding to the Myh11 or Acta2 promoters. DNA from serum-restricted rat (left) or human (right) SMCs was cross-linked with formaldehyde and recovered from immunoprecipitated samples using SRF, PTEN or Elk-1 (rat SMCs only) antibodies or a no-antibody negative control. Immunoprecipitated DNA was subjected to qPCR amplification using primers flanking essential CArG boxes in the Myh11 and Acta2 promoters. About 2% genomic DNA input was used as a positive control. (b–e) Electrophoretic mobility shift assays (EMSAs) were conducted as described in Methods using the indicated amount of a fluorescently labelled 20-bp DNA fragment containing CArG ‘B’ of the Acta2 promoter (b) or a 95-bp DNA fragment consisting of CArGs ‘A’ and ‘B’ of the Acta2 promoter (c–e) and the indicated volumes of purified recombinant SRF and PTEN. (b) EMSA with 20-bp DNA fragment. Positions of SRF-containing complexes are labelled 1 and 2 (lane 2); positions of SRF–PTEN-containing complexes are labelled 3 and 4 (lane 3); unbound DNA not shown. (c) EMSA with 95-bp DNA fragment. DNA plus rSRF alone (lane 2) compared with DNA plus rSRF and increasing amounts of rPTEN (lanes 3–6). Lane 7 shows DNA plus rPTEN alone. (d) EMSA with 95-bp DNA fragment. DNA plus 1 μl rSRF (lane 2) or saturating amounts (3 μl) of rSRF (lane 5) compared with DNA plus 3 μl rPTEN and 1 μl rSRF (lane 3) or saturating amounts (3 μl) of rSRF (lane 4). Position of SRF-containing complexes are labelled ‘2’ (lane 5); position of SRF–PTEN-containing complexes are labelled ‘1’ (lane 4). (e) EMSA with 95-bp DNA fragment. DNA plus 1 μl rSRF (lane 2), DNA plus 3 μl rPTEN and 1 μl rSRF (lane 3) supershifted with a PTEN-specific antibody (lane 4), and DNA plus 3 μl rPTEN and 1 μl rSRF (lane 3) supershifted with an SRF-specific antibody (lane 5). Position of SRF–PTEN-containing complexes are labelled ‘1’; position of SRF–PTEN-containing complexes supershifted with a PTEN antibody are labelled ‘3’ and position of SRF–PTEN-containing complexes supershifted with an SRF antibody are labelled ‘2’. Shown for each panel are representative images from a minimum of three independent experiments.
Figure 4 | PTEN is essential for SRF binding to CArG boxes of SM genes. SMCs stably expressing control (Ctrl) or PTEN-specific shRNA were serum-restricted for 48 h. (a) ChIP assays for SRF binding to CArG elements in the Myh11 (left), Acta2 (middle) and Fos (right) promoters were performed as described in Fig. 3. Data represent average fold changes ± s.e.m. \( N = 3 \) independent experiments; *\( P < 0.01 \) versus Ctrl shRNA. (b) SMCs stably expressing control (Ctrl) or PTEN-specific shRNA were serum-restricted for 48 h. WCL were analysed for total PTEN, SRF, c-fos, and \( \alpha \)SMA. \( \beta \)-Actin was used as a loading control. (c) PTEN (top), Myocardin (MyoCD; middle) or Elk-1 (bottom) proteins were immunoprecipitated from WCL of SMCs serum restricted for 48 h. 10% of input WCL and co-immunoprecipitating SRF were detected by immunoblotting. Left—representative blots. Right—fold changes in densitometry measurements ± s.e.m. for SRF–MyoCD and SRF–Elk-1 versus \( N = 3; * P < 0.01 \) versus Ctrl shRNA. *, heavy chain IgG. (d) Left—schematic diagram of wild-type HA-tagged SRF (top) and the mutant forms of HA–SRF used to map the PTEN binding domain. (e) Right—L929 fibroblasts were transiently transfected with expression plasmids encoding wild-type HA-tagged SRF or HA-tagged SRF deletion mutant proteins. Top blots—western blot showing expression of wild-type and mutant HA-tagged SRF proteins (anti-HA antibody) and endogenous PTEN (anti-PTEN antibody) used for co-immunoprecipitation assays. \( \beta \)-Actin was used as a loading control. Bottom blot—SRF proteins were immunoprecipitated from WCL with an anti-HA antibody and co-immunoprecipitating endogenous PTEN was detected with an anti-PTEN antibody. *, heavy chain IgG. Shown is a representative blot from three independent experiments. Molecular weight markers were cropped out for final SRF blots; please see Supplementary Fig. 8.
elements in the Myh11 and Acta2 promoters was markedly reduced in PTEN-depleted SMCs (Fig. 4a); this was associated with enhanced SRF binding to CArG elements of the Fos promoter (Fig. 4a). Greater SRF binding to the Fos promoter correlated to increased cFos protein expression in PTEN-depleted SMCs compared with controls (Fig. 4b). Myocardin selectively increases SRF binding to CArG elements in SM genes, while Elk-1 enhances SRF binding to the Fos promoter. Repression of SRF gene transcription occurs at least in part through disruption of SRF–myocardin interactions thus promoting SRF–Elk-1 association17. Using co-IP assays, compared with control SMCs, SRF–myocardin interactions decreased, but SRF–Elk-1 interactions increased in PTEN-depleted SMCs (Fig. 4c), consistent with the ChIP and western blot data and loss of SRF-dependent SM gene transcription. Myocardin and Elk-1 compete with each other for a common binding site on SRF17. A series of SRF deletion mutants were used to map the domain responsible for PTEN–SRF interaction and determine if this site is distinct from the myocardin/Elk-1-binding domain (Fig. 4d). Deletion of the central MADS box, that consists of the SRF DNA-binding domain, SRF dimerization domain and myocardin/Elk-1-binding site, had no effect on PTEN–SRF interaction. In contrast, deletion of the N terminus amino acids 16 through 132 resulted in loss of PTEN–SRF interaction (Fig. 4e). Therefore, sequences within the N terminus of SRF and separate from myocardin/Elk-1 interaction sequences are necessary and sufficient for interaction with PTEN.

PDGF blocks PTEN binding to CArG elements in SM genes. PDGF-BB is a known physiological regulator of SMC phenotypic switching5 that represses SM gene transcription at least in part through regulating SRF binding to CArG boxes of SM genes17. We therefore investigated whether PDGF-BB regulated PTEN binding to SM promoters and therefore SM gene transcription. As shown in Fig. 5a, compared with vehicle control, PDGF-BB stimulation reduced expression of PTEN, SRF, and αSMA. This was associated with loss of PTEN binding and reduced SRF binding to CArG boxes on SM gene promoters (Fig. 5b,c; Acta2 shown), but increased SRF binding to Fos CArG boxes (Fig. 5d), similar to shRNA-mediated molecular depletion of PTEN and consistent with PDGF-mediated SMC dedifferentiation.

Figure 5 | PDGF blocks PTEN and SRF binding to CArG elements in SM genes. SMCs were serum-restricted for 48 h followed by stimulation with vehicle control or 20 ng ml⁻¹ platelet-derived growth factor-BB (PDGF-BB) for an additional 48 h. (a) Representative western blot for total PTEN, phospho-Akt, SRF and αSMA; N > 3 independent experiments. (b–d) ChIP assays for PTEN (b) and SRF (c,d) binding to CArG elements in the Acta2 (b,c) and Fos (d) promoters were performed as described above. Data represent average fold changes ± s.e.m. N = 3 independent experiments; unless otherwise noted, *P < 0.01 versus vehicle control; ** denotes statistical analysis not conducted due to complete loss of PTEN interactions in PDGF-stimulated SMCs (consistent undetectable values). Molecular weight markers were cropped out for final SRF blots; please see Supplementary Fig. 8.

PTEN–SRF interaction on CArG boxes of SM genes in vivo. To establish the in vivo relevance of nuclear PTEN, we analysed SMC-rich aortic media from WT mice. Co-IP analyses revealed that PTEN and SRF interacted with each other (Fig. 6a), consistent with the in vitro cell data. ChIP analyses demonstrated that PTEN selectively interacted with CArG boxes of the Myh11 promoter, but not the Fos promoter (Fig. 6b) in mature, uninjured aortic media. Vascular injury promotes SMC dedifferentiation through loss of SRF binding to SM genes27. To determine if injury-induced SMC dedifferentiation is associated with loss of PTEN binding to SM genes, we subjected mice to carotid artery ligation-induced injury. In agreement with previous findings27, compared with uninjured vessels, arterial injury assessed at 48 h resulted in reduced SRF binding to Myh11 and Acta2 CArG promoter regions (Fig. 6c,d), but enhanced SRF binding to Fos CArG elements (Fig. 6e). Importantly and consistent with a role for PTEN in facilitating SRF-dependent SM gene expression, injury resulted in complete loss (Myh11) or reduced (Acta2) PTEN binding to CArG boxes of SM gene promoters (Fig. 6c,d). To establish that nuclear PTEN is essential for SRF-dependent SM gene expression, ChIP assays were performed on aortic media from WT compared with PTEN iKO mice. Similar to vascular injury, SRF binding to CArG boxes in the Myh11 and Acta2 promoters was markedly reduced, but was increased on CArG elements of the Fos promoter in PTEN iKO mice compared with WT mice (Fig. 6f–h). Consistent with the in vitro data, enhanced SRF binding to the Fos promoter correlated to increased levels of c-Fos protein in aortic medial SMCs from PTEN iKO mice compared with WT mice (Fig. 5i). Collectively, these results reveal a novel function for nuclear PTEN as an essential regulator of SRF activity that is necessary for SM gene transcription. SMC PTEN loss promotes SRF co-factor switch, decreased SRF binding to SM gene promoters, but enhanced SRF interactions with Fos promoter elements.

Loss of SM genes by PDGF is blocked by nuclear PTEN. We next tested whether loss of nuclear PTEN plays a role in SMC phenotypic switching. PDGF treatment resulted in NES of PTEN (Fig. 7a), consistent with PDGF repression of SM gene expression and loss of SRF and PTEN binding to SM gene promoters (Fig. 5). Notably, cell fractionation and co-IP analyses showed that PDGF stimulation resulted in reduced nuclear, but increased...
PTEN deficiency in vivo promotes loss of SRF binding to SM gene promoters. (a) PTEN was immunoprecipitated from WCL of smooth muscle-rich intact aortic media of wild-type mice. About 10% of input WCL and co-immunoprecipitating SRF were detected by immunoblotting. Representative blot showing co-IPs from two separate mice; N = 6 independent mice analysed. *, heavy chain IgG. (b) ChIP assays for PTEN and SRF binding to CArG elements in the Myh11 (left) and Fos (right) promoters were performed on intact aortic media using pooled arteries from five individual wild-type mice. (c–e) Mice underwent carotid artery ligation-induced injury as described in Methods. Chromatin was isolated from 48-h injured and contra-lateral uninjured arteries and analysed by ChIP for SRF (c) and PTEN (d) binding to CArG elements in the Myh11 (c), Acta2 (d) and Fos (e) promoters. Data represent average fold changes ± s.e.m. N = 3 independent experiments using pooled arteries from 9 to 11 individual mice; *P < 0.01 versus uninjured control; ** denotes statistical analysis not conducted due to complete loss of PTEN interactions in injured vessels (consistent with undetectable values). (f–h) Smooth muscle-rich aortic media from wild-type (WT) and PTEN iKO (iKO) mice was analysed by ChIP for SRF binding to CArG elements in the Myh11 (f), Acta2 (g) and Fos (h) promoters. Data represent average fold changes ± s.e.m. N = 3 independent experiments using pooled arteries from five individual mice; *P < 0.01 versus WT. (i) Western blot analysis for c-fos levels in whole cell lysates (WCL) of aortic media from WT or PTEN iKO mice. β-Actin was used as a loading control (western blot is from the same samples shown in Fig. 1c using the same β-Actin image). Representative blot from two mice per genotype with each lane representing an individual mouse. Molecular weight markers were cropped out for final SRF blot; please see Supplementary Fig. 8.

cytoplasmic PTEN–SRF interactions (Fig. 7b). This was associated with translocation of a pool of SRF out of the nucleus in response to PDGF treatment (Fig. 7c). To test the significance of nuclear PTEN on SRF localization and SRF-dependent SM gene expression, WT SMCs were transfected with EV, haemagglutinin (HA)-tagged WT PTEN or HA-tagged WT PTEN harbouring artificial nuclear localization (NLS) or NES sequences. Under basal conditions, WT HA–PTEN localized to both the cytoplasm and nucleus, whereas NLS–HA–PTEN and NES HA–PTEN were enriched selectively in the nucleus or the cytoplasm, respectively (Fig. 7d). In response to PDGF, WT HA–PTEN was shuttled out of the nucleus, but NLS–HA–PTEN was retained in the nucleus (Fig. 7d). Co-transfection with green fluorescent protein-tagged SRF and WT PTEN demonstrated that a large percentage of SMCs overexpressing WT PTEN remain susceptible to PDGF-mediated SRF cytoplasmic shuttling (Fig. 7e). In contrast, overexpression of NLS–HA–PTEN blocked SRF nucleo-cytoplasmic translocation (Fig. 7e). Promoter-reporter assays were conducted by co-transfecting SMCs with the various PTEN constructs and an αSMA-luciferase reporter construct. As anticipated, PDGF treatment promoted loss of αSMA promoter activity in SMCs co-transfected with EV, WT HA–PTEN and NES HA–PTEN (Fig. 7f), consistent with PDGF-induced SMC dedifferentiation. Importantly, overexpression of nuclear localized PTEN prevented PDGF-dependent repression of αSMA promoter activity (Fig. 7f). Collectively, these data support the concept that nuclear PTEN prevents SRF nucleo-cytoplasmic translocation, and spatial regulation of a PTEN–SRF complex may be critical to control SMC dedifferentiation.
Decreased PTEN expression in human atherosclerotic lesions.

A recent study used DNA microarray to identify differentially expressed genes in human atherosclerotic coronary arteries combined with meta-analysis to compare expression profiles of atherosclerotic coronaries with existing expression profiles from human atherosclerotic carotid arteries\(^5\). PTEN was identified among the common downregulated genes in human atherosclerotic coronary and carotid arteries. Therefore, to assess the relevance of nuclear PTEN to human atherosclerosis, we analysed atherosclerotic coronaries from a small cohort of patients compared with normal aorta and non-atherosclerotic control coronary arteries for expression of PTEN. By confocal immunofluorescent imaging, we found PTEN uniformly expressed in the cytoplasm and nucleus of aortic and coronary arteries.

**Figure 7** | Nuclear PTEN blocks PDGF-mediated repression of SM gene transcription. (a, b) SMCs were serum-restricted for 48 h followed by stimulation with vehicle control or 20 ng ml\(^{-1}\) PDGF-BB for 24 h (a) or 48 h (b). (a) SMCs were fixed, immunofluorescently stained for PTEN (green) and analysed for PTEN localization using confocal microscopy; nuclei were stained for DAPI (blue). (b) PTEN was immunoprecipitated (IP) from cytoplasmic (cyto) and nuclear (nuc) fractions of vehicle- or PDGF-stimulated SMCs. Co-immunoprecipitating SRF was detected by immunoblotting (IB). Representative western blot from three separate experiments. (c) SMCs were transfected with a construct expressing SRF-GFP, maintained in serum-restricted conditions or stimulated with 20 ng ml\(^{-1}\) PDGF-BB, fixed and analysed for GFP localization; nuclei were stained for DAPI (blue). Shown are representative images (two serum-restricted and four PDGF-stimulated cells are shown); arrows indicate cytoplasmic localized SRF-GFP; nuclei are outlined with white lines. (d) SMCs were transfected with HA-tagged wild-type PTEN (WT), nuclear localized PTEN (NLS) or nuclear excluded PTEN (NES). SMCs were maintained in serum-restricted conditions or stimulated with 20 ng ml\(^{-1}\) PDGF-BB, fixed, immunofluorescently stained for HA (red) and analysed for PDGF localization; nuclei were stained for DAPI (blue). Arrowheads, HA-PTEN-transfected SMCs. (e) SMCs were transfected with GFP-SRF (ctrl) or co-transfected with GFP-SRF and WT PTEN or nuclear localized PTEN (NLS) then maintained in serum-restricted conditions or stimulated with 20 ng ml\(^{-1}\) PDGF-BB. Transfected SMCs exhibiting cytoplasmic GFP expression were scored as described in Methods. Data represent average per cent positive ± s.e.m. N = 3 independent experiments; *P < 0.01 versus control 0.1% CS; **P < 0.01 versus control PDGF. (f) SMCs were serum-restricted for 48 h followed by stimulation with vehicle control or 20 ng ml\(^{-1}\) PDGF-BB for an additional 24 h. Lucifierase activity normalized to β-galactosidase was determined; shown are fold changes from Ctrl serum-restricted (0.1% CS) SMCs. Data represent average fold changes ± s.e.m. N = 3 independent experiments; *P < 0.01 versus Ctrl 0.1% CS; **P < 0.01 versus Ctrl PDGF. Molecular weight markers were cropped out for final SRF blot; please see Supplementary Fig. 8. Scale bars for all images, 20 μm.
artery medial SMCs (Fig. 8 and Supplementary Fig. 6a–c). Compared with medial SMCs, confocal immunofluorescence demonstrated loss of nuclear PTEN that was associated with loss of αSMA expression in intimal SMCs of human atherosclerotic lesions (Fig. 8b and Supplementary Fig. 6d). In contrast, αSMA-rich intimal SMCs in fibrous cap regions of atherosclerotic lesions exhibited nuclear expression of PTEN (Supplementary Fig. 6e). Immunofluorescence and immunohistochemical analysis of a limited number of human tissues representing various stages of atherosclerosis suggested the extent of SMC PTEN loss correlates to lesion severity (Supplementary Figs 6f and 7), indicating atherosclerosis progression may involve a chronic deficiency of SMC PTEN and subsequent loss of SRF transcriptional activity.

**Discussion**

Here we describe a novel and previously unknown function for nuclear PTEN as an indispensable regulator of SRF transcriptional activity and demonstrate that its interaction with SRF is independent of its phosphatase activity. PTEN’s canonical function is to dephosphorylate PIP3, which antagonizes cytoplasmic PI3-kinase/Akt signalling events, as well as function as a phosphatase for several defined protein substrates; however, recent studies identified nuclear and phosphatase-independent functions for PTEN as well. In the setting of cancer, NLS of PTEN is essential for its tumour suppressive function and a recent study demonstrated that PTEN interacts with chromatin. However, to our knowledge...
our study is the first report demonstrating association of PTEN with a transcription factor on promoter elements critical for regulation of SM contractile genes. EMSA data using rPTEN and SRF suggest that PTEN does not bind to this DNA by itself, but rather associates with SRF to facilitate SRF-dependent transcription. Moreover, PTEN–SRF interactions favour selective DNA binding of SRF on SM gene promoters, but not SRF-dependent IEG promoters, suggesting a mechanism actively repressing SMC phenotypic switching. Inactivation of PTEN both in cultured SMCs and in in vivo mouse models results in loss of SRF binding to SM gene promoters, but enhanced SRF binding to proliferation-associated gene promoters demonstrating its critical role in maintenance of SMC contractile gene expression.

We demonstrate that physiological agents that drive SMC dedifferentiation (for example, PDGF) promote NES of PTEN and a pool of SRF, resulting in lower levels of nuclear SRF. This was associated with enhanced SRF activity on target genes involved in cell proliferation (for example, Fos). These findings are consistent with a previous study demonstrating enhanced SRF binding to the Fos promoter in response to PDGF and vascular injury. While seemingly contradictory, the paradoxical regulation of differentiation- and growth-associated genes by SRF is complex and likely dependent on additional mechanisms not yet identified. Of potential interest, SRF, first identified as a regulator of IEG expression in fibroblasts, is found at much lower levels in fibroblasts compared with SMCs (Supplementary Fig. 4). Despite lower SRF expression, fibroblasts induce SRF-dependent c-Fos expression in response to growth signals. Combined with our ChIP data demonstrating loss of SRF binding to SMC promoters with PTEN loss, this could suggest that despite a reduction of SRF–PTEN NES results in an overall bioactive nuclear pool of SRF available to bind alternative promoters (for example, Fos). As decreased expression of PTEN was observed in intimal cells in atherosclerotic lesions from human patients, this process is implicated as a possible mechanism in vascular disease progression and underlies the important clinical relevance of our findings.

Since a nuclear localized PTEN construct blocked PDGF-mediated repression of αSMA expression, our data suggest that PDGF actively targets PTEN for NES as a mechanism to dynamically suppress SM gene expression. Alternatively, it remains possible that nuclear PTEN blocks PDGF-mediated repression of SM genes independent of a direct role on PTEN localization. PTEN does not contain classic nuclear import motifs. Recent work identified two lysine residues that are mono-ubiquitinated to transport it between the nucleus and cytoplasm and studies in the setting of cancer demonstrated that mono-ubiquitination is critical to control NLS. Once in the nucleus, PTEN is de-ubiquitinated by USP7 and subsequently remains nuclear localized. It will be of significant interest and the focus of our future studies to define whether alterations in USP7 activity and thus the mono-ubiquitination status of PTEN underlie SMC dedifferentiation and vascular disease progression, which could lead to novel therapeutics that selectively target this system. Alternative mechanisms of PTEN nucleo-cytoplasmic shuttling include simple diffusion, RAN-mediated nuclear import and the potential use of non-traditional NLS-like sequences.

SMCs and cardiac myocytes express unique sets of SRF- and myocardin-dependent contractile proteins that confer their contractile ability. Despite common transcriptional machinery, why SM genes are not activated in cardiac myocytes remains an intriguing question. While our findings reveal a critical role for PTEN in maintaining SM contractile gene expression, association of SRF with PTEN in cardiac myocytes was only observed under hypertrophic conditions. This finding suggests that PTEN is dispensable for regulating physiological cardiac contractile genes, but possibly plays a role in pathological cardiac hypertrophy. Moreover, lack of interaction under physiological settings suggests that blocking PTEN–SRF nuclear association may be a unique mechanism repressing SM gene activation in cardiomycocytes. We propose this could be due to differences in nucleo-cytoplasmic trafficking of PTEN in cardiomycocytes or expression of a cardiomycocyte-specific co-repressor that prevents PTEN–SRF association with SM gene promoters. SRF-dependent induction of the cardiac ‘fetal’ gene program is a characteristic response during pathological cardiac hypertrophy. Moreover, lack of interaction under physiological settings suggests that blocking PTEN–SRF nuclear association may be a unique mechanism repressing SM gene activation in cardiomycocytes. We propose this could be due to differences in nucleo-cytoplasmic trafficking of PTEN in cardiomycocytes or expression of a cardiomycocyte-specific co-repressor that prevents PTEN–SRF association with SM gene promoters. SRF-dependent induction of the cardiac ‘fetal’ gene program is a characteristic response during pathological cardiac hypertrophy. While beyond the scope of the current study, it will be of interest to determine whether PTEN regulates cardiac fetal genes in the setting of pathological cardiac hypertrophy and determine the role of agonist-induced PTEN nucleo-cytoplasmic shuttling in this context. Finally, while PTEN–SRF interaction was also detected in fibroblasts, PTEN overexpression to levels comparable in SMCs was not sufficient to activate SM genes. Ectopic expression of myocardin in fibroblasts, which normally lack myocardin, promotes SM gene induction; reviewed in refs 20,22. As discussed by these authors, if additional co-factors other than SRF–myocardin are necessary for SM gene activation, they must be expressed by the fibroblasts. Our ongoing studies are analyzing SM gene expression by myocardin overexpression in fibroblasts lacking PTEN, which will demonstrate the essential function of PTEN on SM gene transcription.

Regulated co-factor switching combined with the ability of SM gene DNA to appropriately bend have been proposed as potential mechanisms controlling which co-factor interacts with SRF to coordinate SM gene or IEG expression; reviewed in ref. 19. While our data suggest that PTEN does not specifically interact with DNA, results from our study demonstrate that PTEN and SRF together form a higher order protein–DNA complex. PTEN associates with the N terminus of SRF, a domain distinct from the myocardin- and Elk-1-binding domain. Therefore it is unlikely that there is direct competition between PTEN and Elk-1 for binding to SRF. In addition, using a longer, multi-CArG 95-bp DNA probe, intriguing EMSA results suggest that PTEN potentially promotes a conformational change in the PTEN–SRF–DNA complex, resulting in increased electrophoretic mobility under non-denaturing conditions compared with SRF–DNA alone. These data could imply a role for PTEN in altered DNA conformation that results in the stabilization of interactions or facilitation of contact between essential regulatory factors required for SM gene transcription (for example, SRF–myocardin). Our studies focused on αSMA and SM-MHC expression, two SM genes harbouring multiple CArGs in their promoter regions and previously shown to require cooperativity among the CArG boxes for activation of gene expression. Alternatively, we cannot rule out the possibility that PTEN alters SRF dimerization and/or DNA binding, which could result in the increased mobility. While we favour the former model, future studies will address these questions in more detail.

Co-IPs demonstrating PTEN–SRF interaction in the nucleus, ChIP data demonstrating that PTEN binds to CArG-containing chromatin, EMSA data using purified rPTEN and SRF demonstrating that PTEN directly interacts with SRF on CArG elements of SM genes, and loss of SRF binding to SM genes, loss of SRF–myocardin interaction and a functional loss of SMC contractility in the setting of PTEN deficiency, all hallmarks of SMC dedifferentiation, support the concept that PTEN serves as an SRF transcriptional co-factor. However, to demonstrate the importance of direct PTEN–SRF interaction for control of SMC gene transcription, attempts were made to show that WT SRF add-back, but not add-back using the non-binding SRF mutant (Fig. 4), restores αSMA promoter activity in SRF-depleted SMCs. Two independent investigators conducted a total of seven
PTEN partially restored SM gene expression. It is possible that inhibition of Akt activity in SMCs expressing reduced levels of PTEN supported a phosphatase-dependent effect of PTEN given that increases SRF and signaling; and (3) Overexpression of phosphatase-dead PTEN did not restore in PTEN-reduced SMCs by inhibition of Akt activity. In addition, our findings. For instance, while we demonstrated direct interaction between SRF and PTEN using recombinant proteins in an in vitro pull down assay (Fig. 2f), it is possible that direct interaction is not essential for PTEN’s effect on SMC gene transcription and that other ‘bridging’ proteins facilitate PTEN’s effect. We also demonstrated that PTEN interacts with myocardin in whole cell lysates. Whether this is direct is yet to be determined, but is an area we are exploring.

Our studies suggest a phosphatase-independent effect of PTEN on SRF–PTEN interaction, which is supported by the following data: (1) Overexpression of phosphatase-dead PTEN in PTEN-reduced SMCs restores PTEN–SRF interactions; (2) SRF levels are not restored in PTEN-reduced SMCs by inhibition of Akt signalling; and (3) Overexpression of phosphatase-dead PTEN increases SRF and αSMA levels. Our previous data, however, supported a phosphatase-dependent effect of PTEN given that inhibition of Akt activity in SMCs expressing reduced levels of PTEN partially restored SM gene expression. It is possible that phosphatase-dependent and -independent effects are not mutually exclusive. For instance, nucleo-cytoplasmic shuttling of PTEN could be dependent on Akt activity. In addition or alternatively, PTEN could function as a nuclear protein phosphatase that directly interacts with SRF and dephosphorylates sites required for SRF SM gene expression. Phosphorylation of SRF at S162 was shown to block binding to SM gene promoters while promoting binding to the Fos promoter, suggesting a novel switch-directing SM gene or IEG expression. PTEN-mediated dephosphorylation of this site could serve as a means of maintaining SM gene transcription and would identify SRF as a novel protein target of PTEN. Thus, it is likely that PTEN possesses both phosphatase-independent (for example, interaction with SRF) and -dependent functions essential for regulating SM gene expression. Our ongoing studies are designed to clarify these findings in more detail.

Our recent published work identified a miRNA network utilized by SRF to regulate PTEN-dependent SMC proliferation and inflammatory mediator production. The current data support a positive feedback loop between PTEN and SRF that underlies maintenance of a contractile SMC phenotype (Fig. 9). Our data link loss of endogenous PTEN, which antagonizes multiple pathological pathways linked to vascular disease, as a major mechanism facilitating disease progression. In contrast to the many individual and likely redundant growth factors/cytokines found upregulated in diseased vessels, PTEN therefore represents a potentially significant therapeutic target. Activated SMCs contribute to lesion formation through proliferation, inflammatory cytokine production and abnormal vessel contractility. Thus, targeting PTEN–SRF nuclear interactions has the potential to develop novel therapeutics critical to preserve the mature differentiated SMC phenotype for such purposes as stabilization of atherosclerotic lesions, inhibition of in-stent restenosis and perhaps stabilization of a neovascularature in the setting of ischaemic tissue injury or tumour progression.

Methods

Cell culture and reagents. Primary rat aortic SMCs were isolated and cultured as previously described. Briefly, the aggregate population of aortic medial SMC from adult Sprague Dawley rats was aseptically dissected and SMCs were obtained by
digestion in Eagle’s MEM Medium (EMEM) containing collagenase and elastase. Isolated cells were maintained in EMEM containing 10% calf serum (CS) and were used at passages 3–6. Cells through passage 10 were transfected with PTEN-specific shRNA were performed on primary rat aortic SMC (passage 3–6) generated using lentiviral shRNA plasmids (OpenBiosystems, AL), as described previously.

**Pharmacomechanical coupling**

Pharmacomechanical coupling defects and vessels were examined to determine maximal Ca^2+ concentration changes and Ca^2+ pharmacomechanical coupling. Maximal Ca^2+ concentration changes and Ca^2+ pharmacomechanical coupling were determined by immunoblotting with PTEN antibodies as described below. Human primary aortic SMCs (ATCC PCS-100-012) were maintained in vascular cell basal medium (ATCC PCS-100-030; ATCC PCS-100-042) according to ATCC specifications.

**Animals.** Inducible smooth muscle-specific PTEN iKO mice. PTENlox/lox mice (Dr Mak, Ontario Cancer Institute, the University of Toronto, Toronto, Ontario, Canada) and smooth muscle myosin heavy chain (Myh11)-CreERT2 transgenic mice (Dr Offermanns, the University of Heidelberg, Heidelberg, Germany) were bred to generate tamoxifen-inducible SMC-specific PTEN iKO mice, as described previously (89). Controls expressed Myh11-CreERT2 were but were WT for PTEN.

**Western blotting.** SMCs were lysed with ice-cold mPER lysis buffer and protease inhibitors. Tissues were harvested for contractility experiments, western immunoblot analysis or ChIP analysis.

**Actinostar** assay. Maximal force generation response to KCl was examined to determine pharmacochemical coupling defects and vessels were examined to determine maximal Ca^2+–induced vasoconstriction as a measure of Ca^2+ sensitivity of myofilaments, as described previously. Briefly, aortic rings were excised, placed in ice-cold physiological saline, and debrided of loose fat and connective tissue as previously described. Tissues were mounted between two plates and fixated at one end, and the other end was attached to a force transducer (AE 801, Denver). maximal force generation in response to KCl was examined. Contractility assay. Maximal force generation in response to KCl was examined. Contractility assay. Maximal force generation in response to KCl was examined. Contractility assay. Maximal force generation in response to KCl was examined.

**Quantitative RT-PCR.** Total RNA was isolated from SMCs using the Qiagen RNeasy Plus kit (Qiagen, Valencia, CA) and used 18000 μg/ml PE for 48 h for 48 h. HEK 293 cells, L929 fibroblasts, and L929 fibroblasts were obtained from ATCC (CRL-1573 and CRL-1573 respectively) and cultured in MEM containing 10% CS and penicillin-streptomycin. TRPSin/EDTA, Hank’s Balanced Salt Solution (HBSS), MEM and DME were from Invitrogen, CA. Unless otherwise noted, chemicals and reagents were obtained from Sigma Chemical Co. (Sigma, MO). PDGF-BB (Upstate, Millipore) was used at 20 ng/ml. Six-1221-1242 (Calbiochem, Millipore) was used at 20 μM.

**Contractility assay.** Maximal force generation response to KCl was examined to determine pharmacochemical coupling defects and vessels were examined to determine maximal Ca^2+–induced vasoconstriction as a measure of Ca^2+ sensitivity of myofilaments, as described previously (89). Briefly, aortic rings were excised, placed in ice-cold physiological saline, and debrided of loose fat and connective tissue as previously described. Tissues were mounted between two plates and fixated at one end, and the other end was attached to a force transducer (AE 801, AIME, Horten, Norway) and incubated in a well on a bubble plate for measurement of isometric force. Aortic rings (≥ 12 tissues for each condition) were contracted with 108 mM KCl for two contraction/relaxation cycles or until reproducible forces were obtained. Isometric force was normalized to tissue cross-sectional area. Arterial rings were measured in response to both depolarization (KCl 30 mM) and penicillin–streptomycin. To promote hypertrophy, NRVMs were maintained in the Center for Comparative Medicine, and procedures were performed under compliance with ethical regulations and approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

**Co-IP assay.** SMCs were lysed with ice-cold mPER lysis buffer (Thermo Fisher, PA) and protease inhibitors. Solubilized proteins were centrifuged at 14,000g for 4°C for 10 min. Supernatants were separated using 10% SDS−polyacrylamide gel electrophoresis and transferred to Immobilon P membranes (Millipore, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) containing 0.1% Tween-20 (TBST) and 5% BSA (Sigma), and then incubated with 5% BSA in TBST solution containing primary antibodies for 12−16 h at 4°C. Membranes were washed in TBST, and bound antibodies were visualized with alkaline phosphatase-coupled secondary antibodies and Lumi-Phos WB (Pierce, IL). Lysates were separated on a 3−12% NuPage gel and transferred to Immobilon P membranes (Millipore, MA). Membranes were probed with PTEN antibodies (Cell Signaling, MA), phosphoSer473Akt (1:1,000; Cell Signaling), total Akt (1:1,000; Cell Signaling), SM-MHC (1:100; Biomedical Technologies, MA), HA (1:100; Santa Cruz Biotechnologies, CA), c-fos (1:100; Santa Cruz Biotechnologies), SRF (1:50; Santa Cruz Biotechnologies), β-SMA (1:10,000; Sigma), Elk-1 (1:100; Santa Cruz Biotechnologies), β-actin (1:12,000; Sigma), β-tubulin (1:500; Santa Cruz Biotechnologies), Lamin A/C (1:500; Cell Signaling) anti-Rabbit light chain specific (Cell Signaling), horseradish peroxidase (HRP)−anti-mouse secondary (Cell Signaling), and AP-anti-Rabbit secondary, and HRP-anti-Rabbit secondary (Santa Cruz Biotechnologies). Changes were quantitated by densitometry using Image J software (rsb.info.nih.gov). Full uncropped western blots can be found in Supplementary Fig. 8.

**Bacterial expression of PTEN and SRF.** GST−SRF. E. coli Rosetta (DE3) pLySs cells transformed with the pGEX-5×3 plasmid carrying the GST−SRF gene. An overnight culture was grown in terrific broth (supplemented with 0.1% glucose in the presence of 34 μg/ml 1 chloramphenicol and 100 μg/ml ampicillin). About 15% of the pre-culture was used to inoculate 500 ml of terrific broth media supplemented with 0.1% glucose and 100 μg ml−1 ampicillin. Bacterial cells were grown to an OD600 of 1.3 at 37°C. The cells were chilled on ice to lower the temperature to 18°C prior to induction by addition of IPTG (final concentration 0.5 mM) and then incubated for 2.5 h. The cells were pelleted at 10,000 g for 20 min, flash-frozen, and stored at −80°C. The GST−SRF purification was carried out at 4°C. Cells were lysed using sonication in lysis buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.01% TTBS with a protease inhibitor cocktail tablet (Roche, EDTA-free) and 0.2 mM PMSF. The insoluble fraction was cleared via centrifugation at 40,000 g for 60 min. The soluble fraction was mixed with GST beads that were pre-equilibrated with lysis buffer. The beads were washed with 500 μl of lysis buffer and the protein was eluted with elution buffer (50 mM sodium phosphate, pH 8.0, 5 mM BME, 500 mM NaCl, 20 μl imidazole, 0.01% TTBS and a protease inhibitor cocktail tablet. The insoluble fraction was cleared via centrifugation at 40,000 g for 60 min. The soluble fraction was mixed with Ni-NTA agarose beads that were pre-equilibrated with lysis buffer for 2–4 h. The beads were washed with 500 ml of lysis buffer and the protein was eluted with elution buffer (50 mM sodium phosphate, pH 8.0, 5 mM BME, 300 mM NaCl, 250 mM imidazole, 0.01% Tween-20). The protein was dialyzed into 20 mM sodium phosphate, pH 7.8, 1 mM DTT, 150 mM NaCl, 5% glycerol, 0.01% Tween-20 and stored at −20°C until further use.

**Co-IP assay.** SMCs were lysed with ice-cold mPER lysis buffer and protease inhibitors. Solubilized proteins were centrifuged at 14,000g in a microcentrifuge (4°C) for 10 min. Samples were immunoprecipitated with equal concentration and volumes. Samples were pre-cleared with 50 μl of a 50% slurry of protein A−Sepharose resin (Sigma) and rabbit IgG for 1 h at 4°C on an end-over-end turner. After incubation the resin from each sample was pelleted, and supernatant was transferred to new tubes and incubated with SRF−1:50 (Santa Cruz Biotechnologies), SRF-1:50 (Santa Cruz Biotechnologies), Myocardin 1:40 (Santa Cruz Biotechnologies). Elk-1:1:40 (Santa Cruz Biotechnologies), V5CAM 1:50 (Santa Cruz Biotechnologies) or rabbit IgG for 16−18 h, 4°C, on an end-over-end turner. After incubation, 45 μl of a 50% slurry of Protein A-Sepharose resin was added and allowed to incubate for 1 h at 4°C on an end-over-end turner. Resin from each sample was then washed with mPER Buffer and the bound proteins were eluted with 2% SDS−DTT sample buffer and
detected by western immunoblotting. For in vitro co-IP assays, rPTEN and SRF were incubated with 50 µl of a 50% slurry of protein A- Sepharose resin for 1 h at 4℃ on an end-over-end tumbler in Tris Buffer (10 mM Tris- HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT and 100 µg/ml bovine serum albumin). After incubation, the resin from each sample was pelleted, and supernatant was transferred to new tubes and incubated with 20 µg of a plasmid-encoding CMV-β-galactosidase (Clontech, CA) for 2 h on a rocking platform. Input controls were performed with either an empty vector, NES PTEN vector, NES PTEN vector, or a control vector. SMCs, HEK 293 cells and L929 fibroblasts were transiently transfected with Lipofectamine PLUS according to the manufacturer’s protocol. Plasmid-encoding human SRF tagged with GFP (RG229926) was obtained from OriGene. SMCs co-transfected with SRF–GFP and PTEN localization mutants were performed with Viafect transfection reagent (E4982) (Promega, WI). For PTEN overexpression experiments, SMCs were transduced in suspension with an EV adeno virus or adenoviruses expressing WT PTEN or phoshatase-inactive mutant PTEN (100 multiplicity of infection). Cells were then plated in EMEM with 10% CS. After 24 h, the SMCs were placed in fresh media containing 0.1% FCS for 24 h, whole cell lysates were harvested and analysed by western blot for expression of the indicated proteins.

Immunofluorescence and confocal microscopy. SMCs were transiently transfected with 4 µg of WT PTEN vector, NES PTEN vector, NES PTEN vector or a control vector, as described above. Transfected SMCs were plated on glass chamber slides, growth-arrested in Eagle’s MEM containing 0.1% FCS for 24 h, followed by stimulation with PDGF-BB (20 ng ml⁻¹) for 24 h. Cells were fixed in 4% PFA, permeabilized with MeOH and incubated with anti- HA 1:30 (Santa Cruz Biotechnologies) or PTEN (Novus, CO) antibody. Antibody/antibody complexes were visualized using Alexa Fluor-488 or -568-coupled secondary antibodies (Molecular Probes, NY). Coverslips were mounted with VectaShield medium containing DAPI to detect all cell nuclei (Vector Laboratories, CA) and cells imaged using a laser-scanning confocal microscope (510 META NLO, Olympus, NY). Images were taken using 63× oil immersion objectives and analysed using LSM 510 software. For SRF–GFP localization experiment, SMCs were transiently transfected with 0.5 µg WT PTEN vector, NES PTEN vector or a control vector, and 1 µg of SRF–GFP. Transfected SMCs were plated on glass chamber slides or 60 mm dishes, transfected, growth-arrested in Eagle’s MEM containing 0.1% FCS for 24 h, followed by stimulation with PDGF-BB (20 ng ml⁻¹) for 5 h. Experiments conducted on 60 mm dishes were analysed for SRF–GFP localization at 5 h with live cell imaging. Experiments conducted on glass chamber slides were fixed in 4% PFA and mounted with VectaShield medium containing DAPI to detect all cell nuclei. Cells were visualized using a Nikon inverted fluorescence microscope equipped with NIS element-BR software.

Human tissue sections and immunohistochemistry/fluorescence. Arterial tissues were obtained from explanted hearts from nine patients recruited as subjects for heart transplantation at the University of Colorado Anschutz Medical Campus. Tissues were collected under the Human Heart Tissue Bank protocol (COMIRB 01-568 PI P. Buttrick, Chief Cardiology Division; co-I for vascular studies K. Moulton). All appropriate informed consent was received from subjects and confirmation was obtained that they were aware that their samples would be used in research. For artery harvest, immediately after explant, left and right, left and right descending, left marginal, and coronary arteries as well as aortic tissues were dissected from diseased hearts, cleaned of fatty and cardiac muscle tissues, and processed for histology; a total of 24 individual vessels were analysed. After histological processing and haematoxylin and eosin staining, vascular tissue were reviewed by Dr Moulton and characterized based on relative size of atheroma, presence of plaque neovascularization, composition of cells, lipid and matrix and media attenuation. De-coded and de-identified arterial tissue sections were obtained from Dr Moulton and were immunohistochemically or immunofluorescently stained for PTEN; patient clinical data were not available for these tissues. For immunohistochemistry, formalin-fixed, paraﬁn-embedded tissues were deparafﬁnized, rehydrated and underwent antigen retrieval by heating for 20 min at 100°C in a decloaking chamber (Biocare). Antibody/antibody complexes were visualized using kits from Vector Laboratories and sections lightly counterstained with haematoxylin. Sections were imaged using an Olympus BX41 microscope equipped with SPOT software. Antibody used was monoclonal anti-PTEN (1:100; Cascade Bioscience, Winchester, MA). For immunofluorescent double staining, tissues were pre-treated as described above and subsequently incubated with a polyclonal anti-PTEN antibody (1:50; Millipore) followed by incubation with a cy3-conjugated monoclonal anti-PTEN antibody (Sigma). To detect SRF expression, a TSA Biotin amplification system was used (Perkin Elmer). Briefly, following incubation with anti-PTEN, sections were incubated with secondary biotinylated anti-rabbit IgG (1:400), HRP-conjugated Streptavidin (1:100), BiotinTyramide (1:50) and Alexa-488-conjugated Streptavidin. Sections were imaged using a laser-scanning confocal microscope (510 META NLO) and analysed using LSM 510 software.

Statistical analysis. Data were expressed as means ± s.e. and were determined using two-tailed t-test analysis. P values <0.05 were considered statistically significant. One-way analysis of variance (ANOVA) was performed to compare two or more groups. When a significant F value was obtained, post-hoc multiple comparisons between individual groups were performed with Tukey's test after Bonferroni correction. P values <0.05 were considered significant for the initial ANOVA and Tukey’s multiple comparison test was then used (P <0.05).
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**Author contributions**
M.C.M.W.-E. and H.H. designed all the studies; R.A.N. assisted with the design of the studies. H.H. and M.C.M.W.-E. performed most of the experiments. M.C.M.W.-E. performed the mouse vascular surgeries; A.O. assisted with mouse vascular surgeries. H.H. maintained, bred and genotyped the mouse lines. C.L.W. purified recombinant SRF and PTEN and assisted with EMSA experiments; M.E.A.C. helped design the EMSA experiments. L.A.W. performed the aortic contractility assays. K.S.M. is a co-investigator on the approved COMIRB protocol and obtained the human coronary artery specimens. M.L. carried out immunofluorescent studies of human coronary arteries. R.T. helped maintain and genotype mouse lines and conducted some of the transfection experiments. T.A.M. assisted with the cardiomyocyte experiments. M.C.M.W.-E. wrote the majority of the manuscript; H.H. wrote the Methods section of the manuscript. M.C.M.W.-E., H.H., R.A.N., M.E.A.C. and C.L.W. edited the manuscript.

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