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# Perlecan-Induced Suppression of Smooth Muscle Cell Proliferation Is Mediated Through Increased Activity of the Tumor Suppressor PTEN

Pamela J. Garl, Janet M. Wenzlau, Heather A. Walker, John M. Whitelock, Mercedes Costell, Mary C.M. Weiser-Evans

**Abstract**—We were interested in the elucidation of the interaction between the heparan sulfate proteoglycan, perlecan, and PTEN in the regulation of vascular smooth muscle cell (SMC) growth. We verified serum-stimulated DNA synthesis, and Akt and FAK phosphorylation were significantly reduced in SMCs overexpressing wild-type PTEN. Our previous studies showed perlecan is a potent inhibitor of serum-stimulated SMC growth. We report in the present study, compared with SMCs plated on fibronectin, serum-stimulated SMCs plated on perlecan exhibited increased PTEN activity, decreased FAK and Akt activities, and high levels of p27, consistent with SMC growth arrest. Adenoviral-mediated overexpression of constitutively active Akt reversed perlecan-induced SMC growth arrest while morpholino antisense-mediated loss of endogenous PTEN resulted in increased growth and phosphorylation of FAK and Akt of SMCs on perlecan. Immunohistochemical and Western analyses of balloon-injured rat carotid artery tissues showed a transient increase in phosphoPTEN (inactive) after injury, correlating to high rates of neointimal cell replication; phosphoPTEN was largely limited to actively replicating SMCs. Similarly, in the developing rat aorta, we found increased PTEN activity associated with increased perlecan deposition and decreased SMC replication rates. However, significantly decreased PTEN activity was detected in aortas of perlecan-deficient mouse embryos, consistent with SMC hyperplasia observed in these animals, compared with E17.5 heterozygous controls that produce abundant amounts of perlecan at this developmental time point. Our data show PTEN is a potent endogenously produced inhibitor of SMC growth and increased PTEN activity mediates perlecan-induced suppression of SMC proliferation. (*Circ Res.* 2004;94:175-183.)

**Key Words:** smooth muscle cell proliferation ■ restenosis ■ vascular injury  
■ vascular development ■ basement membrane

Vascular smooth muscle cells (SMCs) demonstrate high rates of replication during embryonic development significantly contributing to the maturation of the vessel wall.<sup>1</sup> In addition, SMCs are capable of marked increases in replication after injury to the mature vessel wall, a major component of the vessel remodeling observed in a variety of vascular pathologies.<sup>2,3</sup> In the absence of vascular trauma, the mature blood vessel remains a highly quiescent tissue and SMCs are resistant to stimulation by most mitogens,<sup>2-5</sup> suggesting the existence of active growth-suppressive mechanisms. SMCs in mature arteries are surrounded by a basement membrane matrix.<sup>6</sup> Our previous studies showed the accumulation of a perlecan-rich basement membrane actively inhibits SMC growth.<sup>7-10</sup>

Perlecan, a large multidomain heparan sulfate proteoglycan, is essential for the assembly and maintenance of a functional basement membrane.<sup>11-14</sup> Homozygous perlecan-null mice die in utero and, important to our studies, display hyperplasia of

SMC-specific  $\alpha$ -actin-positive mesenchymal cells.<sup>13-15</sup> We showed perlecan expression is initiated in early fetal life when the first postreplicative SMCs appear and expression is limited to nonreplicating SMCs.<sup>7,9</sup> Decreased SMC-derived perlecan production is associated with increased SMC replication rates after vascular injury.<sup>16</sup> However, accumulation of perlecan in the later stages of injury repair is associated with the attenuation of neointimal SMC proliferation. Our recent studies show perlecan-induced SMC growth suppression is mediated, at least in part, through the active upregulation of focal adhesion kinase-related nonkinase (FRNK), which subsequently suppresses FAK-dependent growth signals.<sup>10</sup> In the present study, we sought to identify factors downstream of perlecan capable of mediating these molecular events.

PTEN, first discovered as a potent tumor suppressor, is a dual-specificity lipid and protein phosphatase<sup>17-20</sup> and a negative regulator of PI3K- and FAK-mediated signaling.

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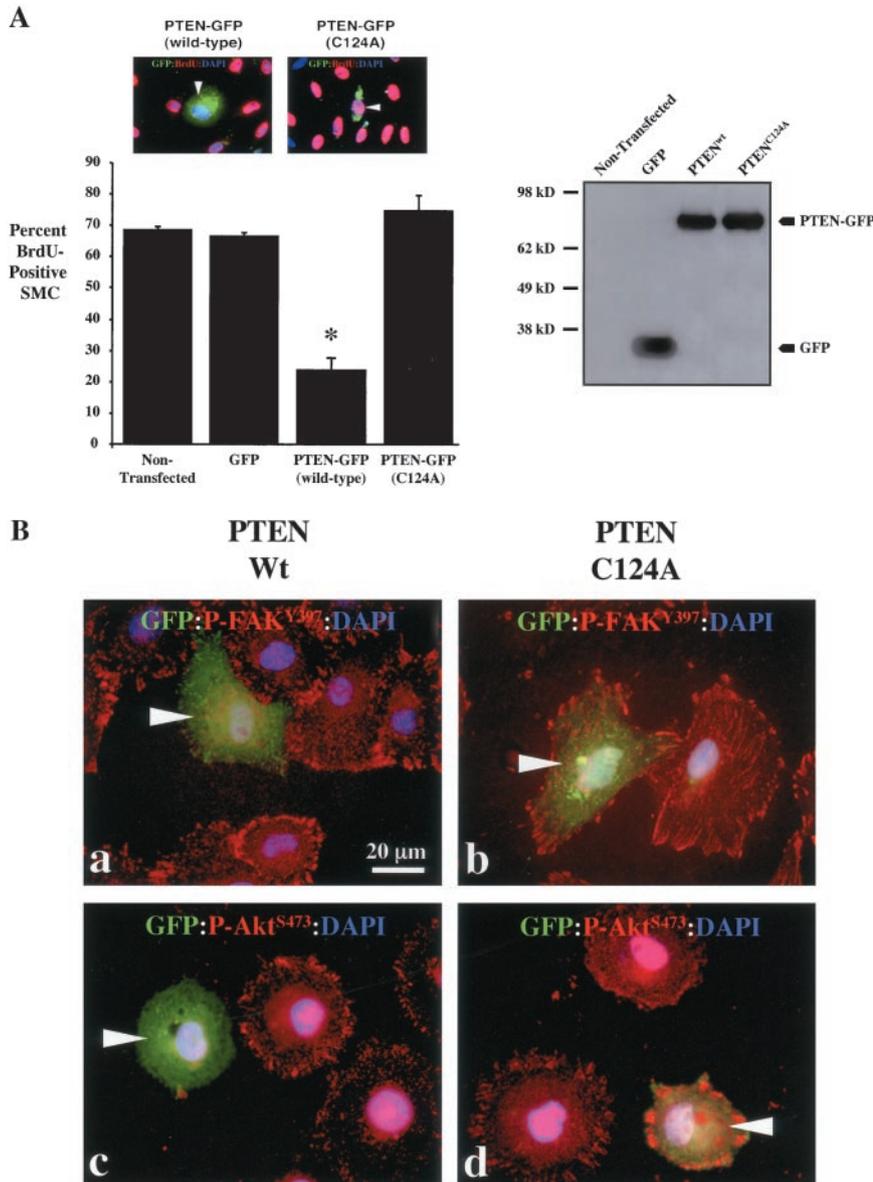
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**Figure 1.** PTEN overexpression inhibits SMC growth and Akt and FAK phosphorylation. **A**, Left, A10 rat aortic SMCs were transfected with wild-type GFP-tagged PTEN, dominant-negative, phosphatase-inactive GFP-tagged PTEN<sup>C124A</sup>, or an empty GFP plasmid, allowed to recover for 24 hours, then replated on coverslips precoated with fibronectin in the presence of 10% CS and 10 mmol/L BrdU. Cells were fixed 24 hours later and immunofluorescently stained for BrdU and DAPI. The percentage of BrdU-positive cells was determined independently for nontransfected and transfected SMCs by counting a minimum of 200 SMCs per condition. The data are presented as the mean ± SE. \**P* < 0.05, different than nontransfected SMCs. Top panel shows a representative immunofluorescent stain. Arrowheads indicate GFP-positive SMCs. Red nuclei indicate BrdU-positive SMCs. Right, Cell lysates were collected from parallel cultures and analyzed by Western analysis for GFP expression to verify transfection. **B**, A10 SMCs transfected as above were replated on fibronectin-coated coverslips for 4 hours. SMCs were fixed and immunofluorescently stained for phosphoFAK (top panels) or phosphoAkt (bottom panels) using phosphospecific antibodies. Arrowheads indicate GFP-positive SMCs.

PTEN directly antagonizes growth factor receptor- and integrin-stimulated signaling, thus promoting cell cycle arrest, decreased cell migration, and apoptosis. Compared with its role in cancer biology, there is a smaller, but growing accumulation of data regarding PTEN's role in normal physiological processes. PTEN-null mice die between E6.5 and E9 and exhibit poorly organized ectodermal and mesodermal layers, demonstrating PTEN's importance in embryonic development.<sup>21,22</sup> However, little is known still about upstream regulators of PTEN phosphatase activity or its role in vascular SMC biology. Our previous work demonstrated high *in vivo* SMC growth rates during vascular development and after vascular injury are associated with decreased perlecan expression and PTEN inactivation.<sup>23</sup> In the present study, we hypothesized perlecan:SMC interactions upregulate PTEN phosphatase activity mediating perlecan-induced SMC growth suppression.

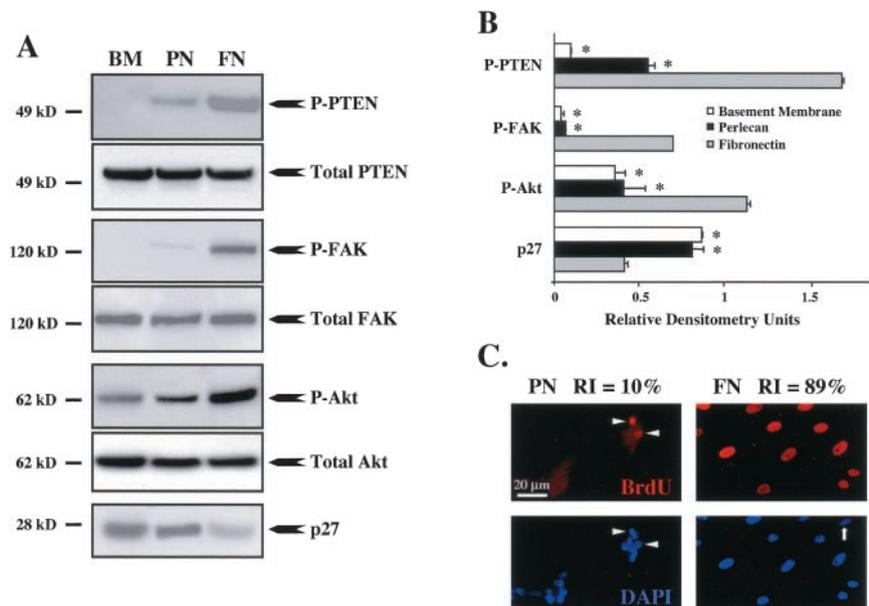
### Materials and Methods

An expanded Materials and Methods section can be found in the online data supplement available at <http://www.circresaha.org>.

### Results

#### PTEN Overexpression Inhibits SMC Growth and Activation of Akt and FAK

To verify PTEN inhibits SMC growth and Akt and FAK activities, SMCs were transiently transfected with a CMV promoter-based vector encoding wild-type GFP-tagged PTEN or phosphatase-dead GFP-tagged mutant PTEN. Transfected SMCs were replated on fibronectin-coated coverslips in the presence of 10% CS and bromodeoxyuridine (BrdU) for 24 hours. SMCs were immunofluorescently stained for BrdU, and the percentage of BrdU-positive, GFP-positive SMCs was determined. SMCs transfected with vector alone or with the mutant PTEN construct demonstrated high rates of DNA synthesis in response to serum stimulation (Figure 1A). In contrast and consistent with a previous study,<sup>24</sup> DNA synthesis was significantly decreased in SMCs overexpressing wild-type PTEN suggesting PTEN is a very potent inhibitor of SMC growth.



**Figure 2.** Perlecan:SMC interactions increase endogenous PTEN activity. A and B, Rat aortic SMCs were growth-arrested for 72 hours in serum-free medium and replated in the presence of 10% CS on basement membranes (BM) or on culture dishes precoated with perlecan (PN) or fibronectin (FN). Whole-cell lysates were prepared 24 hours after replating and analyzed by Western analysis for the indicated proteins. Western analyses from 3 independent experiments were scored for relative densitometry (phosphoproteins normalized to total proteins; p27 to  $\beta$ -actin); the data are presented in the graph. \* $P < 0.05$ , different than FN. C, Rat aortic SMCs were cultured on precoated dishes as above in the presence of 10 mmol/L BrdU. Cells were fixed 24 hours later and immunofluorescently stained for BrdU and DAPI. Arrowheads indicate BrdU-positive SMCs on PN; arrow, BrdU-negative SMCs on FN.

PTEN antagonizes Akt- and FAK-mediated signaling, and we showed FAK phosphorylation is essential for SMC replication.<sup>10</sup> SMC cultures transfected as above were fixed for immunofluorescent analysis of activated Akt and FAK using phosphospecific antibodies. Nontransfected SMCs and mutant PTEN-transfected SMCs exhibited high levels of phosphoAkt and phosphoFAK (Figure 1B). In contrast, although PTEN wild-type overexpressing SMCs attached on fibronectin (FN), there was little, if any phosphoAkt and phosphoFAK present.

### Perlecan Heparan Sulfate:SMC Interactions Increase Endogenous PTEN Activity

As shown above, PTEN overexpression suppresses SMC proliferation likely, as with other cell systems, due to swamping a normally fine-tuned signaling system. PTEN is a protein phosphatase whose activity is decreased by phosphorylation<sup>25,26</sup>; however, little is known of endogenous upstream regulators of PTEN phosphorylation. Recent data from other cell systems suggest PTEN dephosphorylation, and a subsequent increase in activity is the regulated event.<sup>25,26</sup> We demonstrated perlecan heparan sulfate:SMC interactions result in sustained inactivation of FAK activity and inhibition of serum-stimulated SMC growth.<sup>10</sup> We next examined the ability of perlecan and perlecan-rich basement membranes and growth inhibitory matrices to increase endogenous PTEN activity in cultured SMCs. SMCs were plated in the presence of 10% CS on intact basement membranes or on individual matrices of perlecan or fibronectin (a growth-promoting matrix) for 24 hours. Equal amounts of total PTEN, FAK, and Akt proteins were expressed under all conditions (Figure 2A). Very low levels of phosphoPTEN were detected in SMCs plated on perlecan or basement membranes (Figures 2A and 2B). In contrast, abundant phosphoPTEN was detected in SMCs plated on fibronectin. Phosphorylation of FAK and Akt was suppressed in SMCs plated on perlecan and intact basement membranes, compared with SMCs plated on fibronectin (Figures 2A and 2B). In addition, SMCs plated on growth inhibitory matrices exhibited increased p27 levels (Fig-

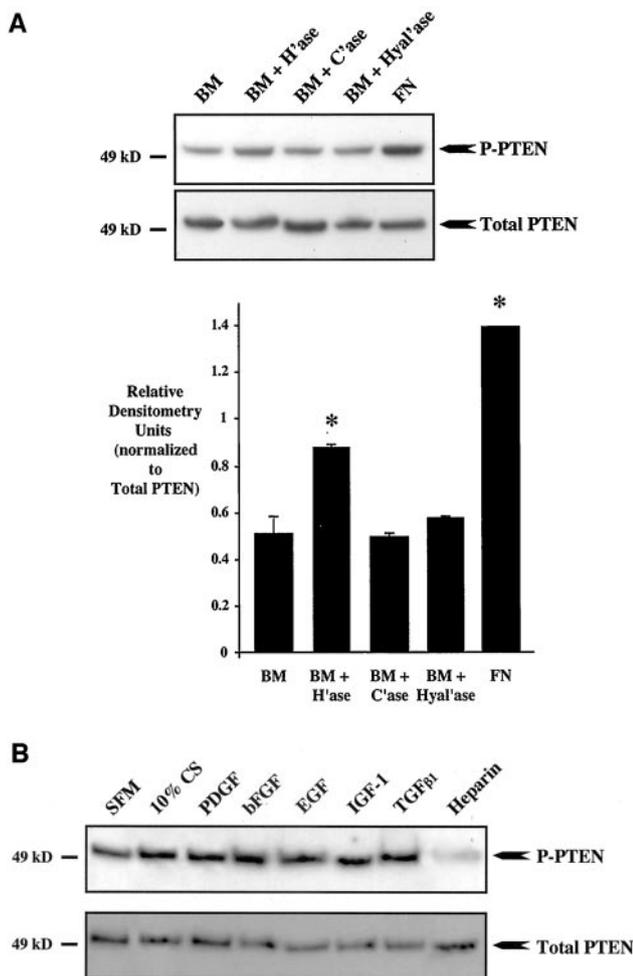
ures 2A and 2B), a protein previously shown to be upregulated by PTEN.<sup>27</sup> These data are consistent with the low growth potential of SMCs on these matrices, despite the addition of serum (Figure 2C).

To examine the role of glycosaminoglycan chains in mediating perlecan's effect on PTEN, intact basement membranes were treated with heparinase I/II, chondroitinase ABC, or hyaluronidase. SMCs were then plated on untreated or digested membranes in the presence of 10% CS. In contrast to untreated, chondroitinase-, or hyaluronidase-treated membranes, SMCs plated on heparinase-treated basement membranes exhibited higher levels of phosphoPTEN (Figure 3A), suggesting heparan sulfate side chains contribute to perlecan-induced upregulation of PTEN activity. The increase in phosphoPTEN, however, was considerably less than observed in SMCs plated on fibronectin (Figure 3A).

Since loss of heparan sulfate moieties in part decreased PTEN activity, we examined the effect of exogenous heparin in regulating PTEN under standard culture conditions. SMCs were cultured on tissue culture plastic, growth-arrested in serum-free conditions, and then stimulated with various growth factors and inhibitors. Equal amounts of total PTEN protein were expressed under all conditions (Figure 3B). SMCs plated on plastic exhibited high levels of phosphoPTEN in serum-free medium and after serum, PDGF, FGF2, EGF, IGF1, or TGF $\beta$ 1 stimulation (Figure 3B). We found decreased phosphorylation (ie, increased activity) only after incubation of serum-deprived SMCs with exogenous heparin.

### Akt Activation Reverses Perlecan-Mediated SMC Growth Suppression

Our previous work showed growth inhibition by perlecan is partially reversed by overexpressing constitutively active FAK.<sup>10</sup> Therefore, we determined whether overexpression of constitutively active Akt could also reverse perlecan-induced SMC growth inhibition. SMCs were transduced with a replication-defective adenovirus expressing either GFP (pAd-GFP) or an active form of Akt (pAd-MyrAkt) and replated in



**Figure 3.** Perlecan heparan sulfate chains and exogenous heparin upregulate PTEN activity. A, Basement membranes (BM) were digested with 10 U/mL heparinase I/II (H'ase), 1 U/mL chondroitinase ABC (C'ase), or 0.1% hyaluronidase (Hyal'ase) for 4 hours at 37°C. Rat aortic SMCs were plated on untreated or treated membranes or on fibronectin (FN) in the presence of 10% CS. Cell lysates were prepared after 24 hours, and equal protein concentrations were analyzed by Western analysis for phosphoPTEN or total PTEN. Western analyses from 3 independent experiments were scored for relative densitometry, and the data are presented in the graph (phosphoPTEN normalized to total PTEN). \* $P < 0.05$ , different than basement membrane. B, Rat aortic SMCs were plated on tissue culture plastic, growth-arrested for 48 hours in serum-free DMEM, then stimulated with the indicated factors. Cell lysates were prepared after 24 hours, and equal protein concentrations were analyzed by Western blotting as described in A.

the presence of 10% CS on basement membranes, fibronectin, or tissue culture plastic for 4 days (Figure 4). Compared with SMCs plated on fibronectin or plastic, GFP-transduced SMCs plated on basement membranes showed no increase in cell number in response to serum stimulation. In contrast, MyrAkt-transduced SMCs exhibited significant increases in cell number in response to serum stimulation even when plated on basement membranes, suggesting Akt inactivation is a significant factor in perlecan-mediated SMC growth suppression. Western analysis confirmed pAd-MyrAkt-transduced SMCs exhibited increased phosphoAkt even when plated on basement membranes (Figure 4).

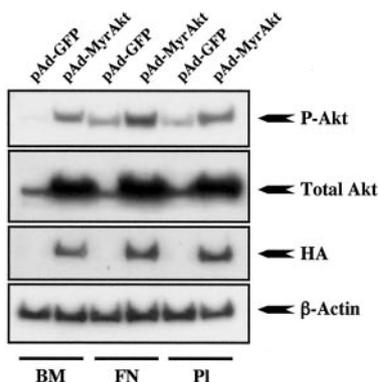
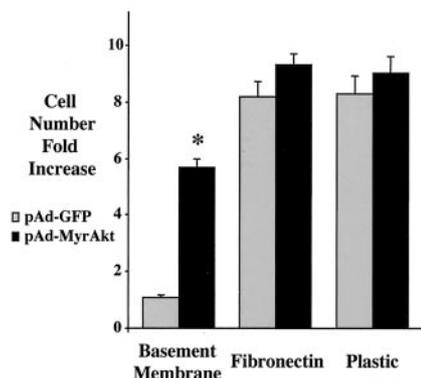
### Morpholino-Mediated Inhibition of PTEN Increases Growth and Akt and FAK Activation of SMCs Plated on Perlecan

To determine if the SMC growth inhibitory effects of perlecan are mediated through PTEN, SMCs were transfected with PTEN antisense morpholino oligonucleotides to reduce endogenous PTEN. Inverse antisense morpholino oligonucleotides were used as controls. SMCs were replated on perlecan or fibronectin matrices in the presence of 10% CS and BrdU for 24 hours. Antisense treatment resulted in a significant loss of endogenous PTEN protein compared with inverse antisense-treated or untreated controls (Figure 5B). Consistent with our previous studies, untreated and inverse antisense-treated SMCs demonstrated low growth rates when plated on perlecan. In contrast, PTEN antisense treatment resulted in significant increases in SMC replication on perlecan with growth rates comparable to those observed in SMCs plated on fibronectin (Figure 5A). Loss of endogenous PTEN resulted in increased phosphorylation of Akt and FAK in SMCs plated on perlecan (Figure 5B). Our previous studies showed perlecan-induced upregulation of FRNK contributes to SMC growth inhibition.<sup>10</sup> Morpholino-mediated loss of endogenous PTEN also resulted in decreased FRNK levels in SMCs plated on perlecan (Figure 5B), implying the presence of PTEN-responsive regulatory elements in the FRNK promoter. Ongoing studies in our laboratory are exploring this possibility. Our data strongly suggest perlecan is an upstream regulator of endogenous PTEN activity and increased PTEN activity inhibits SMC proliferation through the downregulation of FAK- and Akt-dependent signaling.

### High Levels of PhosphoPTEN Correlate to High In Vivo SMC Growth Rates, and PTEN Activity in the Developing Aorta Is Decreased in E17.5 Perlecan-Deficient Embryos

Our previous work showed increased levels of phosphoPTEN are observed in 7-day postinjured compared with sham-injured carotid artery tissues consistent with increased growth rates at this time.<sup>23</sup> Immunohistochemistry was used on a series of sham- and balloon-injured carotid arterial tissues to determine the temporal and spatial pattern of expression of phosphoPTEN (Figure 6A). Abundant levels of total PTEN protein were detected in the arterial media of sham and injured vessels and significantly elevated levels were found in the growing neointima. In contrast, very little phosphoPTEN was detected in sham-injured vessels corresponding to low SMC growth rates. However, at 2 days after injury, elevated phosphoPTEN was detected in medial SMCs, particularly in cells nearest the vessel lumen. By 4 to 10 days after injury, abundant phosphoPTEN was detected predominantly in the neointima. Fourteen days after injury, expression of phosphoPTEN was greatly diminished and was virtually undetectable by 28 days after injury. Western analysis of vessel protein expressions confirmed the immunohistochemistry data (Figure 6B).

Since the pattern of phosphoPTEN correlated to high rates of SMC replication and, in particular, neointimal SMC replication, we examined the relationship between SMC replication and phosphoPTEN expression on a cell-to-cell



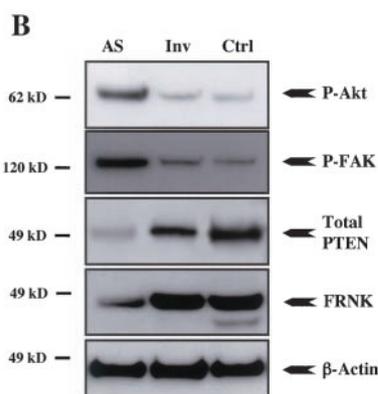
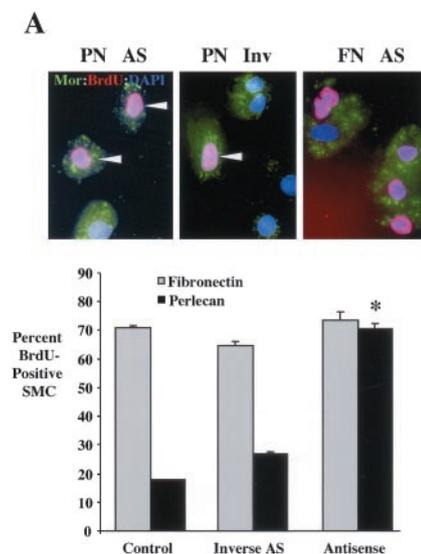
**Figure 4.** Constitutively active Akt reverses perlecan-mediated SMC growth suppression. Rat aortic SMCs were transfected overnight with pAd-GFP or pAd-Myr-Akt (MOI=100), allowed to recover for 24 hours, then plated on basement membranes, fibronectin, or tissue culture plastic in the presence of 10% CS. Total cell numbers were determined in triplicate after 4 days of serum stimulation. The data are presented as the mean ± SE of 3 independent experiments. \**P*<0.05, different than pAd-GFP-transduced SMCs plated on basement membranes. B, Whole-cell lysates were collected from SMC cultures treated as described in A and analyzed for phospho- and total Akt, HA (for transduction efficiency), and β-actin (control for protein loading) using specific antibodies.

basis. Using a double-labeling PCNA-phosphoPTEN immunohistochemistry technique, we simultaneously determined DNA synthesis and phosphoPTEN expression in a single SMC in day 7 and day 10 postinjured carotid arteries. High levels of phosphoPTEN were expressed almost exclusively by replicating cells (PCNA-positive) of the neointima (Figure 6C). Collectively, these data suggest high rates of SMC replication after vascular injury are associated with PTEN inactivation. In addition, the observed expression pattern of phosphoPTEN after vascular injury correlates inversely to that reported for perlecan.<sup>15</sup>

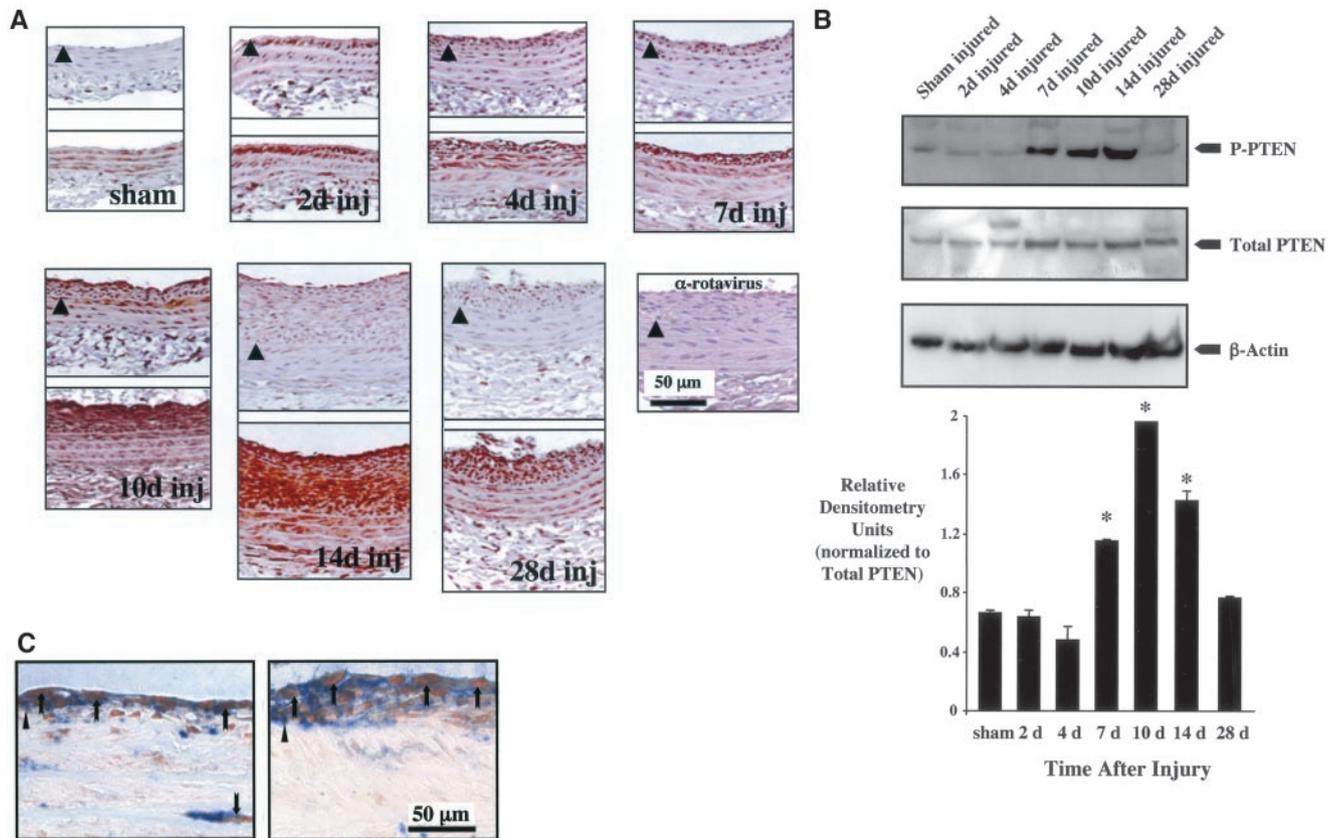
The above data demonstrate the *in vivo* pattern of active PTEN expression is similar to the pattern of perlecan mRNA and protein expression and inversely correlated with the degree of SMC replication observed after vascular injury. Similarly, we demonstrated the temporal expression of perlecan during vascular development is directly related to SMC quiescence.<sup>7</sup> We therefore determined the relationship between endogenous perlecan expression and PTEN activity in the developing aorta by first examining a developmental series of rat aortic tissues for the pattern of phosphoPTEN expression (Figure 7A). High levels of phosphoPTEN were

detected in E15 and E16 aortas corresponding to a period of rapid SMC replication. Loss of phosphoPTEN expression was observed at E19 and remained undetectable at E21. The temporally regulated increase in PTEN activity correlates with increased deposition of perlecan and occurs at a period when rat aortic SMC replication significantly declines.

Since the developmental patterns of active PTEN and perlecan are similar, we examined the expression pattern of phosphoPTEN in the developing aorta of perlecan-null mouse embryos. E17.5 heterozygous controls produce abundant amounts of perlecan at this developmental time point (comparable time when rat aortic perlecan deposition is high) (Figure 7C) and phosphoPTEN was virtually undetectable (Figure 7B). In contrast, PTEN activity was significantly decreased (increased phosphorylation) in aortas of age-matched perlecan-deficient embryos, consistent with SMC hyperplasia observed in these animals (Figure 7B). Collectively the present data suggest PTEN is less active in developing SMCs, contributing to a highly proliferative phenotype. At distinct times during the developmental process, increases in perlecan production induce an upregulation of PTEN activity thus contributing to SMC quiescence.



**Figure 5.** Antisense-mediated reduction of PTEN increases SMC growth on perlecan. A10 rat aortic SMCs were transfected with FITC-labeled PTEN-specific morpholino antisense or inverse antisense oligonucleotides and allowed to recover for 30 hours. Control SMCs were treated with morpholino delivery solution only. A, Transfected SMCs were replated on coverslips pre-coated with perlecan (black bars) or fibronectin (gray bars) in the presence of 10% CS and 10 mmol/L BrdU. Cells were fixed 24 hours later and immunocytochemically stained for BrdU (red nuclei). The data are presented as the mean ± SE. Top panel shows a representative immunofluorescent stain of transfected SMCs plated on PN (antisense, left panel; inverse antisense, middle panel) or FN (antisense, right panel). B, Cell lysates were prepared from transfected SMCs plated on PN as described above and analyzed by Western analysis for total PTEN, phosphoAkt, phosphoFAK, FRNK, and β-actin (control for protein loading).



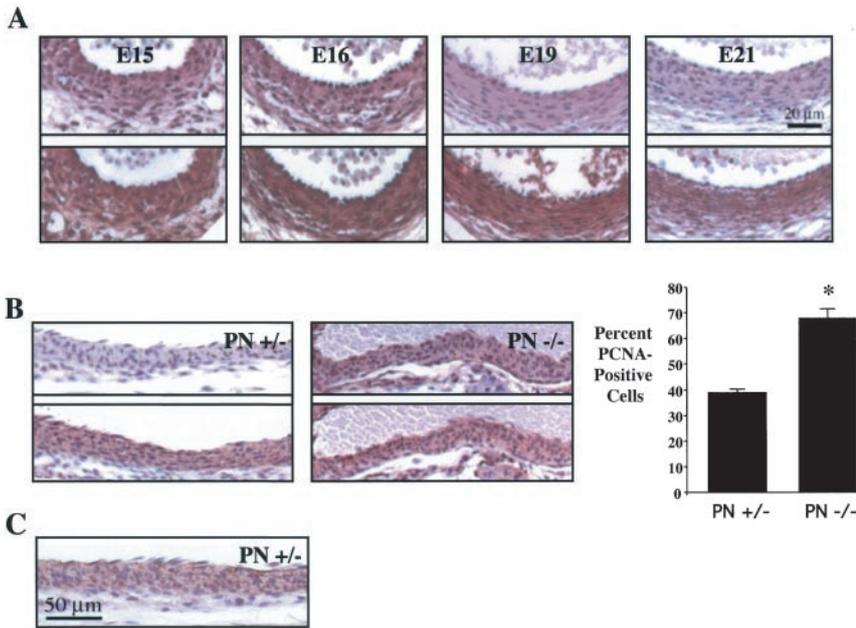
**Figure 6.** PTEN activity is inversely correlated to SMC replication after vascular injury. A, Immunohistochemistry was used to analyze total PTEN (lower photomicrographs) and phosphoPTEN (upper photomicrographs) expression in rat carotid artery tissue sections from sham- or balloon-injured arteries (2, 4, 7, 10, 14, and 28 days after injury); the reaction color is reddish-brown. Serial sections from each time point were stained. The arterial lumen is oriented toward the top of each panel. Arrowheads mark the internal elastic lamina. B, Arteries as described above were harvested at the indicated times, stripped of adventitia, and equal protein concentrations from whole-cell lysates were analyzed by Western analysis for phosphoPTEN (P-PTEN), total PTEN, and  $\beta$ -actin (control for protein loading). Western analyses from 3 independent experiments were scored for relative densitometry, and the data are presented in the graph (phosphoPTEN normalized to total PTEN). \* $P < 0.05$ , different than sham-injured. C, Sections of 7-day (left panel) and 10-day (right panel) injured carotid arteries were examined by double-labeling immunohistochemistry for expression of phosphoPTEN concurrently with PCNA to detect replicating SMCs. Brown nuclei indicates PCNA-positive SMCs; blue reaction color, phosphoPTEN. Arrowheads indicate the internal elastic lamina; arrows, representative PCNA-positive SMCs expressing significant levels of phosphoPTEN.

### Discussion

During vascular morphogenesis, SMCs undergo a phase of rapid proliferation, during which time the vessel wall acquires its complement of SMCs.<sup>1</sup> Replication significantly decreases as the vessel matures, and SMCs remain highly quiescent in the mature, uninjured artery. The ability of these cells to rapidly, but transiently, proliferate after vascular injury suggests active inhibitory and stimulatory mechanisms coordinately regulate SMC growth.<sup>2,3</sup> Mechanisms of endogenous SMC growth inhibition are poorly understood. Previous studies from our laboratory have shown timed accumulations of perlecan heparan sulfate into the SMC basement membrane contributes to SMC growth suppression at least in part via the upregulation of FRNK, which subsequently downregulates FAK-mediated growth signals.<sup>7,10</sup> In this study, we extend these findings and report perlecan:SMC interactions increase PTEN activity, mediating perlecan-induced SMC growth arrest via decreased FAK and Akt signaling despite serum stimulation. Morpholino antisense-mediated reduction of endogenous PTEN reversed growth and Akt and FAK

activities of SMCs plated on perlecan. In addition, we report increases in PTEN activity directly correlate to perlecan deposition and inversely correlate to SMC replication during vascular development and after vascular injury. Accordingly, in the developing aorta of perlecan-null mice, we found decreased PTEN activity associated with SMC hyperplasia.

The central finding of this study is SMC quiescence is actively controlled through temporally regulated increases in activity of the growth suppressor PTEN via upregulation of SMC-derived perlecan expression. Perlecan expression and PTEN activity are associated with developmental and late postinjury decreases in SMC replication.<sup>7,16</sup> PTEN inactivation is associated specifically with actively dividing SMCs during early to mid stages of lesion formation. In the early stages of injury repair, SMCs proliferate in response to increased release of growth factors.<sup>28,29</sup> In addition, vascular injury is associated with upregulation of several proteolytic and glycolytic enzymes, contributing to basement membrane and extracellular matrix (ECM) degradation and remodeling.<sup>30,31</sup> Degradation of inhibitory matrix proteins, such as



**Figure 7.** PTEN activity in the developing aorta is decreased in E17.5 perlecan-deficient embryos. **A**, Immunohistochemistry was used to localize phosphoPTEN (upper photomicrographs) and total PTEN protein (lower photomicrographs) in the aortas of E15, E16, E19, and E21 rat embryos; the reaction color is reddish-brown. Serial sections were stained. The arterial lumen is oriented toward the top of each panel. **B**, E17.5 perlecan heterozygous (PN +/-) and homozygous mutant (PN -/-) embryos were harvested, fixed, and processed for immunohistochemistry. Serial sections were stained for total PTEN (bottom photomicrographs) and phosphoPTEN (upper photomicrographs); the reaction color is reddish-brown. Separate sections were stained for PCNA to identify replicating SMCs. The percentage of PCNA-positive cells was determined per condition, and the data are presented in the graph as the mean  $\pm$  SE. \* $P < 0.05$ , different than PN +/- . **C**, Monoclonal perlecan-specific antibody was used to localize perlecan protein in aortas of mouse E17.5 PN +/- embryos; the reaction color is reddish-brown.

perlecan, and increased deposition of growth stimulatory matrix proteins, such as fibronectin, are observed at these stages. Our data show SMC interactions with perlecan increase PTEN activity and block mitogen-stimulated SMC proliferation, suggesting the induction of SMC proliferation after injury requires a balance between loss of active growth inhibitory signals and gain of active growth stimulatory signals.

While medial SMCs proliferate in response to growth factors, such as PDGF and bFGF, in the early stage after vascular injury, the mechanisms regulating continual cell replication during intimal thickening appear to be growth factor-independent.<sup>28,29</sup> We and others have shown transient increases in Akt activity associated with peak intimal cell replication.<sup>23,32</sup> In addition, we reported neointimal SMCs transiently express a unique, highly proliferative phenotype characteristic of embryonic SMCs rather than traditional adult-derived vascular SMCs.<sup>3,23,33</sup> This phenotype is associated with growth factor-independent cell replication, low perlecan expression, PTEN inactivation, and constitutive Akt signaling. Elevated levels of phosphatidylinositol 3,4,5-trisphosphate, the predominant PTEN substrate, are observed in PTEN-deficient cells and are sufficient to activate Akt to transduce proliferative signals in the absence of other stimuli.<sup>19,20</sup> Morpholino-mediated knockdown of endogenous PTEN not only reverses SMC growth inhibition by perlecan but also induces a mitogen-independent, embryonic-like phenotype.<sup>23</sup> Likewise, inhibition of perlecan increases serum-stimulated<sup>10</sup> and mitogen-independent (M.C.M.W.-E.; unpublished results, 2003) growth of adult-derived SMCs. We therefore propose rapid intimal cell growth during neointimal formation is driven, in large part, via loss of perlecan-mediated PTEN activation resulting in constitutive Akt signaling. Constitutive FAK signaling may likewise contribute

to rapid SMC growth due to decreased perlecan-induced FRNK expression.<sup>10</sup>

The molecular mechanism mediating perlecan's effect on PTEN remains undefined. The present data and our previous work<sup>8,10</sup> indicate at least some of the growth inhibitory activity of perlecan resides in its highly sulfated HS chains, consistent with a large body of evidence implicating heparin-like molecules in the regulation of SMC growth.<sup>34-38</sup> In this respect, HS chains of perlecan may bind heparin-binding growth factors, thus attenuating growth factor-mediated SMC replication. In agreement, a previous study showed proliferative responses to growth factors of intimal SMCs in late lesions is increased after heparinase treatment.<sup>16</sup> Additionally, heparin increases SMC-derived perlecan core protein production, which may, in turn, promote SMC growth inhibition.<sup>7</sup> However, we have consistently shown heparin or HS chains alone do not elicit the same degree of growth inhibition as fully sulfated perlecan, especially under serum-stimulated conditions. This suggests the efficacy of perlecan on SMC growth inhibition may derive from the coordinate actions of perlecan HS chains and binding of the core protein to the SMC surface.

In addition, interaction of perlecan with various ECM proteins, including collagens, fibronectin, and laminin,<sup>39</sup> could interfere with ECM-integrin interactions that otherwise facilitate growth factor-stimulated SMC replication. The present results show SMC adhesion to fibronectin results in robust phosphorylation, and therefore inactivation, of PTEN associated with increased integrin- and growth factor-mediated signaling events supporting this hypothesis. In addition, Lundmark et al<sup>40</sup> demonstrated a combined matrix of perlecan and fibronectin inhibits SMC adhesion to fibronectin. In this scenario, perlecan could passively activate PTEN by blocking growth factor receptor- and/or integrin-induced phosphorylation of PTEN.

Alternatively, or in addition to the effects of perlecan HS chains and a hypothesis we favor, much of the growth inhibitory signals could arise from perlecan core protein interactions with cell surface receptors. Perlecan specifically binds membrane-bound proteins, including dystroglycan and integrins, which could be involved in cell signaling.<sup>41,42</sup> Receptor-mediated perlecan-induced increases in PTEN activity would block downstream of growth factor receptor and integrin signals that would otherwise coordinately increase cell proliferation. In addition to the effects on growth, morpholino-mediated knockdown of endogenous PTEN also reduced endogenous FRNK levels, an SMC-specific endogenous inhibitor of FAK activity.<sup>43</sup> Along with direct antagonism of proliferative signals, increased PTEN activity could also signal to transcription factors involved in FRNK mRNA expression.<sup>27</sup> Loss of SMC-perlecan interactions, as observed in rapid SMC proliferative phases during vascular development and after vascular injury, would release active growth inhibitory signals and essentially tip the balance toward proliferative signals. Identification of a perlecan receptor capable of eliciting growth inhibitory signals and the phosphatase responsible for regulation of PTEN activity should therefore provide further insights into how perlecan activates PTEN to induce SMC growth arrest.

Surprisingly, little is known of transcriptional/posttranslational signals associated with SMC-specific perlecan expression. The antiatherogenic factor apolipoprotein E increases endothelial cell-derived perlecan protein synthesis/secretion and sulfation of heparan sulfate chains contributing to perlecan-induced SMC growth arrest<sup>44</sup> and TGF- $\beta$ 1 induces fibroblast-specific perlecan expression.<sup>45</sup> In contrast, atherogenic factors, such as oxidized LDL, decrease perlecan core protein synthesis/secretion and increase heparan sulfate degradation via increased heparanase secretion. Whether these factors have similar effects on SMC-derived perlecan has yet to be determined. Clearly, selectively targeting perlecan, PTEN, or factors modulating perlecan expression holds promise for the development of antirestenotic and/or antiatherosclerotic therapies.

Despite major advances in vascular biology, mechanisms regulating continual SMC replication during pathological lesion formation remain unknown thus compounding the challenge of successful clinical treatment. Defining endogenous SMC growth-suppressive mechanisms in the mature vessel wall could lead to a targeted molecular approach focusing on such mechanisms to reduce lesion formation. Based on the present study and our previous work, we propose PTEN is less active in developing SMCs, thus contributing to a highly proliferative growth phenotype. At distinct developmental times, differentiating SMCs produce increasing amounts of perlecan, which contribute to upregulation of PTEN activity and subsequent loss of constitutive proliferative signals, both associated with SMC quiescence. Vascular injury, however, results in local perlecan proteolysis, decreased PTEN activity, and increased proliferative signaling events. In addition, SMC precursors recruited to the site of injury pass through a series of developmental events including low perlecan production and PTEN activity asso-

ciated with rapid growth. Our ongoing studies are addressing several aspects of this overall proposal.

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## ONLINE MATERIALS AND METHODS

***Reagents and Antibodies:*** Human plasma-derived fibronectin (FN) and mouse EHS-derived basement membrane extracts (BM; Matrigel™) were from Collaborative Biomedical Products (Bedford, MA). Human arterial endothelial-derived perlecan (PN) was purified as described previously (1). Bromodeoxyuridine (BrdU), heparin (used for cell number assays at 100 µg/ml), heparin lyase I and II, chondroitinase ABC, and hyaluronidase were from Sigma Chemicals (St. Louis, MO). Porcine platelet-derived growth factor (PDGF; 20 ng/ml), bovine basic fibroblast growth factor (bFGF; 20 ng/ml), human epidermal growth factor (EGF; 10 ng/ml), human insulin-like growth factor-I (IGF-I; 100 ng/ml), and porcine transforming growth factor-β1 (TGFβ1; 10 ng/ml) were from R&D Systems (Minneapolis, MN) and were used for cell number assays. Monoclonal anti-BrdU was from Becton Dickinson (Franklin Lakes, NJ). Monoclonal anti-perlecan antibody was from Zymed Laboratories (S. San Francisco, CA). Polyclonal anti-phosphoPTEN, anti-total PTEN, and anti-total Akt antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-FAK<sup>748-1053</sup>C (for detection of FAK and FRNK), monoclonal anti-PCNA, and polyclonal anti-p27 antibodies were from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-phosphoFAK (Y<sup>397</sup>) and anti-phosphoAkt (S<sup>473</sup>) antibodies were from Biosource International (Camarillo, CA). Rat monoclonal HRP-conjugated anti-HA antibody was from Roche Applied Science (Indianapolis, IN). Cy3- and FITC-conjugated antibodies to mouse and rabbit IgG's were from Jackson ImmunoResearch Laboratories (West

Grove, PA). Morpholino oligonucleotides were from Gene Tools, LLC (Philomath, OR).

Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham Life Science, Inc. (Arlington Heights, IL). All tissue culture supplies were from Gibco BRL (Gaithersburg, MD). The Bradford protein assay kit was from BioRad (Richmond, CA).

***Animals, In Vivo Tissue Preparation, Cell Culture and Growth Assays:*** Adult male Sprague-Dawley rats were subjected to balloon-injury, carotid artery tissues were harvested from sham injured or at 2, 4, 7, 10, 14, and 28 days after injury, and tissues were prepared as described previously (2). Whole embryos or aortic tissues from Sprague-Dawley rats were collected as previously described (2). The aortic media from adult Sprague-Dawley rats was aseptically dissected and SMC obtained by explant technique as previously described (2). The rat thoracic aorta A10 SMC cell line was obtained from American Type Culture Collection (Rockville, MD; ATCC CRL 1476) and used for transfection studies. Perlecan null and wild-type embryos were collected and processed as previously described (3). SMC replication was analyzed by BrdU or PCNA immunocytochemistry or immunofluorescence assays as described previously (2). Cell number assays were conducted as described previously (4).

***Immunohistochemistry:*** Formalin-fixed, paraffin-embedded tissues were deparaffinized, rehydrated and underwent antigen retrieval by incubating the sections in 10mM sodium citrate buffer at 180°F for 15 min. Sections were then exposed to specific antibodies overnight at 4°C. Antigen:antibody complexes were visualized as previously described using kits from Vector

Laboratories (Burlingame, CA)(5). Sections were lightly counterstained with hematoxylin. Negative controls included the use of mouse IgG or a polyclonal antiserum to human rotavirus (Dako, Inc., Carpinteria, CA) as a primary antibody. A mouse-to-mouse immunohistochemistry detection kit (Chemicon, Temecula, CA) was used on sections of mouse tissue that were stained with a monoclonal antibody according to the protocol provided. Selected samples were double-labeled by performing immunohistochemistry for PCNA and phosphoPTEN. Sections were pretreated as described above followed by exposure to monoclonal anti-PCNA. PCNA was visualized using an immunoperoxidase system with DAB as substrate. Sections were then exposed to polyclonal anti-phosphoPTEN followed by visualization using an avidin-biotin alkaline phosphatase system and Vector Blue as a substrate (Vector Laboratories). Double-labeled tissue sections were not counterstained.

***Preparation of Cell Lysates and Immunoblotting:*** For in vivo protein extraction, control or injured carotid arteries were isolated and immediately frozen in liquid N<sub>2</sub> prior to protein extraction. Total cell proteins were isolated from in vivo tissues or SMC cultures and equal amounts of protein were subjected to SDS-PAGE (4-12% gradient gels; NOVEX system, Invitrogen, Carlsbad, CA) followed by Western blotting as described previously (2). Ligand:antibody complexes were visualized using ECL chemiluminescence detection kits and Hyperfilm x-ray film. Radiographs were analyzed by scanning densitometry and normalized to  $\beta$ -actin, total PTEN, total FAK, or total Akt

signals (public domain NIH Image program; developed at the U.S. NIH and available at <http://rsb.info.nih.gov/nih-image/>).

**Plasmids, Transfections, Morpholino Assays, and Adenovirus Production:** CMV promoter-based plasmids containing wild-type GFP-tagged PTEN and dominant negative, phosphatase inactive GFP-tagged PTEN (C124A) were kind gifts from Dr. K.M. Yamada (Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). A10 SMC were transfected using the Effectene™ transfection reagent (Qiagen, Valencia, CA). Using this protocol, equal transfection efficiencies were obtained (30-40%). For Morpholino antisense assays, SMC were transfected for 3 h with FITC-conjugated PTEN-specific Morpholino oligonucleotides in the presence of a specialized osmotic delivery solution (Gene Tools, LLC; Philomath, OR), the media were changed and the cells allowed to recover for 30 h prior to the onset of growth assays. In parallel cultures, treated SMC were harvested and whole cell lysates were analyzed by Western blotting. The following oligonucleotides were used: PTEN antisense: 5'-TGGCTGAAGAAAAAGGAGGAGA-GAG-3', PTEN inverse antisense: 5'-GAGAGAGGAGGAAAAAGAAGTCGGT-3'. For adenovirus experiments, cells were transduced with replication-defective adenovirus vectors expressing constitutively active forms of murine Akt (Ad-MyrAkt) tagged with the hemagglutinin epitope (kind gift of Dr. K. Walsh, Tufts University School of Medicine) as described previously (5). Adenoviral vectors expressing the GFP transgene (Ad-GFP) were used as controls.

**Immunofluorescence Microscopy:** SMC were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were incubated with primary antibodies followed by Cy-3- or FITC-conjugated secondary antibodies to visualize antigen:antibody complexes. Cells were coverslipped with a DAPI mounting media (to detect all cells; Vector Laboratories) and were analyzed using fluorescent microscopy.

**Statistical Analysis:** Results are expressed as means $\pm$ SEM. Comparisons between groups were analyzed by a Student's *t* testing for two group comparisons or by 1-way ANOVA with Fisher's protected least-squares distribution post-testing for multiple comparisons. Differences were considered significant when  $P < 0.05$ .

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