Molecular Cardiology

Requirement for p38 Mitogen-Activated Protein Kinase Activity in Neointima Formation After Vascular Injury

Brandon M. Proctor, PhD; Xiaohua Jin, MD; Traian S. Lupu, DVM; Louis J. Muglia, MD, PhD; Clay F. Semenkovich, MD; Anthony J. Muslin, MD

Background—Angioplasty and stent delivery are performed to treat atherosclerotic vascular disease but often cause deleterious neointimal lesion formation. Previously, growth factor receptor-bound protein 2 (Grb2), an intracellular linker protein, was shown to be essential for neointima formation and for p38 mitogen-activated protein kinase (MAPK) activation in vascular smooth muscle cells (SMCs). In this study, the role of vascular SMC p38α MAPK in neointimal development was examined.

Methods and Results—Compound transgenic mice were generated with doxycycline-inducible SMC-specific expression of dominant-negative p38α MAPK (DN-p38α). Doxycycline treatment resulted in the expression of DN-p38α mRNA and protein in transgenic arteries. Doxycycline-treated compound transgenic mice were resistant to neointima formation 21 days after carotid injury and showed reduced arterial p38 MAPK activation. To explore the mechanism by which p38α MAPK promotes neointima formation, an in vitro SMC culture system was used. Inhibition of p38α MAPK in cultured SMCs by treatment with SB202190 or small interfering RNA blocked platelet-derived growth factor–induced SMC proliferation, DNA replication, phosphorylation of the retinoblastoma protein, and induction of minichromosome maintenance protein 6.

Conclusions—SMC p38α MAPK activation is required for neointima formation, perhaps because of its ability to promote retinoblastoma protein phosphorylation and minichromosome maintenance protein 6 expression. (Circulation. 2008;118:658-666.)

Key Words: muscle, smooth ▪ restenosis ▪ signal transduction

The proliferation of arterial smooth muscle cells (SMCs) contributes to several pathological conditions, including restenosis after angioplasty, posttransplantation coronary artery disease, and hypertensive vasculopathy. Elaboration of neointima made up of extracellular matrix and SMCs that proliferate and migrate from the tunica media is known to contribute to the development of restenosis.1,2

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SMC proliferation may be triggered by the action of extracellular peptide growth factors and ligands that are released locally in blood vessels and that stimulate intracellular signaling cascades. In particular, platelet-derived growth factor (PDGF) and basic fibroblast growth factor are thought to be involved in the neointimal hyperplasia that develops after vascular injury.3 Other growth factors and ligands, including epidermal growth factor and thrombin, are released locally in vessels after injury.4,5 These ligands bind to transmembrane receptors on the surface of SMCs that initiate intracellular signaling cascades.

In previous work, the role of the intracellular linker protein growth factor receptor-bound protein 2 (Grb2) in the development of neointima after vascular injury was evaluated.6 Grb2 is an Src homology 2 and Src homology 3 domain-containing protein that facilitates the activation of the small GTPase ras and downstream mitogen-activated protein kinases (MAPKs) by growth factor receptor tyrosine kinases. Haploinsufficiency for Grb2-rendered mice resistant to neointimal lesion development after carotid injury.6 In addition, Grb2+/− cultured murine aortic SMCs were resistant to PDGF-induced cell proliferation. Reduction of Grb2 protein in cultured rat arterial SMCs caused defective p38 MAPK and c-Jun N-terminal kinase (JNK) MAPK activation in response to PDGF stimulation. These findings suggest that p38 MAPK or JNK MAPK, or both in combination, is a critical intermediary in growth factor signaling cascades downstream of Grb2 that are responsible for SMC proliferation and neointima development.

The p38 MAPK family of serine/threonine kinases consists of 4 members (p38α, p38β, p38γ, and p38δ); p38α MAPK is

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ubiquitously expressed in mammalian tissues. The activation and function of p38α MAPK have been studied in more detail than other family members, and in some cell types, activation of p38α MAPK antagonizes cell cycle progression, promotes inflammatory responses, and may also contribute to apoptosis. In particular, p38α MAPK activity in cardiomyocytes blocks cell cycle progression and cytokinesis and induces the expression of inflammatory cytokines such as tumor necrosis factor-α and interleukin-6. Furthermore, in immature thymocytes, p38α MAPK activation by V(D)J-mediated double-strand DNA breaks induces a G2/M cell cycle checkpoint via the phosphorylation and accumulation of p53. However, the role of p38α MAPK in cell proliferation and cell survival depends on cell type and physiological context.

The biological response of cultured mammalian SMCs to p38α MAPK activation contrasts with that observed in cardiomyocytes and immature thymocytes. In particular, recently published studies support the hypothesis that p38α MAPK plays an important role in growth factor–stimulated SMC proliferation. Work by Chen et al demonstrated that endothelin-dependent rat aortic SMC (RAOSMC) proliferation depends on p38α/β MAPK activity. Specifically, endothelin-1–stimulated p38α/β MAPK activation led to c-Myc and E2F gene expression, which contributed to cell cycle progression. In another study, Jacob et al demonstrated that human cultured vascular SMC proliferation in response to injury depends on p38α/β MAPK activation. Furthermore, Kavurma and Khachigian, using SB202190, showed that p38α/β MAPK is important for cultured SMC proliferation. However, the specific requirement for p38α MAPK in neointima formation in vivo has not been determined. In this work, the role of p38α MAPK in the pathogenesis of neointima formation was analyzed by use of compound transgenic mice with inducible SMC-specific expression of DN-p38α.

Methods

Generation of SM/TRE-DN-p38α Compound Transgenic Mice

SM22α-rTTA (SM) mice were originally generated in the C57Bl/6J×CBA mixed genetic background with DNA encoding the reverse tetracycline transactivator (rTTA) linked to a 455–base pair fragment of the SM22α promoter as previously described. More than 10 original transgenic lines were generated, and 1 line that exhibited persistent arterial expression of rTTA in adult mice was used in these studies. SM mice were crossed into the C57Bl/6J background for >7 generations.

TRE-flag-DN-p38α (TRE-DN-p38α) mice were generated with DNA containing a dominant-negative form of murine p38α MAPK in which the TxY activation loop is mutated to AxF. In addition, the DNA is linked to an N-terminus flag tag sequence and tetracycline response element (TRE). A linearized form of this DNA construct was injected into the male pronucleus of 1-cell murine C57Bl/6J embryos and used to generate TRE-DN-p38α founder mice. One line of TRE-DN-p38α transgenic mice in the C57Bl/6J genetic background was created by this method. TRE-DN-p38α transgenic mice were crossed with SM transgenic mice that were previously demonstrated to exhibit arterial SMC-specific expression of rTTA.

To induce expression of DN-p38α MAPK, SM/TRE-DN-p38α compound transgenic mice and control single transgenic SM mice were treated with doxycycline. After 1 week of doxycycline treatment, aortas were isolated for reverse-transcriptase polymerase chain reaction (RT-PCR) assays.

Results

Generation of Compound Transgenic Mice With Inducible SMC-Specific Expression of DN-p38α

In previous work, Grb2−/− mice were demonstrated to be resistant to neointima formation in response to carotid injury, and arterial p38 MAPK activation was found to be reduced in these animals. To directly test the role of p38α MAPK in neointima formation in vivo, we generated compound transgenic mice with doxycycline-inducible expression of dominant-negative p38α MAPK in arterial SMCs. A DNA construct was generated that contained a tetracycline response element (TRE) linked to cDNA encoding DN-p38α where the TxY kinase activation loop amino acid sequence is mutated to AxF. This DNA construct was linearized and injected into the male pronucleus of 1-cell murine C57Bl/6J embryos and used to generate TRE-DN-p38α founder mice. After 1 week of doxycycline, induction of DN-p38α MAPK mRNA was assessed. After 1 week of doxycycline, induction of DN-p38α MAPK mRNA was observed (Figure 1B). In addition, after 2 weeks of doxycycline treatment, carotid arteries, aortas, and other tissues were obtained for protein analysis. Western blot analysis of protein lysates revealed that DN-p38α protein was expressed in the aorta in the SM/TRE-DN-p38α compound transgenic background but not in the SM single transgenic background (Figure 1C). Furthermore, 2 weeks of doxycycline induction resulted in decreased activation of native p38 MAPK in the aorta and carotid arteries of SM/TRE-DN-p38α compound transgenic mice compared with arterial tissues from SM mice (Figure 1D). In contrast, DN-p38α
induction did not block extracellular signal-regulated kinase (ERK) MAPK activation (Figure 1D).

Requirement of p38α MAPK for Response to Carotid Arterial Injury in Mice

To evaluate the role of SMC p38α MAPK activity in the pathogenesis of neointima formation, doxycycline-treated compound transgenic SM/TRE-DN-p38α and single transgenic SM mice were subjected to carotid injury by the epoxy resin beaded-probe method. Both groups of mice tolerated the procedure well, and no postoperative mortality was observed. Evans Blue dye and von Willebrand factor staining demonstrated that the degree of denudation is similar from mouse to mouse (Figure I of the online-only Data Supplement).

Histological examination of carotid cross sections performed 3 weeks after injury revealed robust neointima formation in single transgenic SM mice (Figure 2A and 2C). In SM mice, the neointimal lesions were at least partially occlusive, and sometimes the lesions completely occluded the vessel lumen. In contrast, SM/TRE-DN-p38α compound transgenic mice exhibited markedly reduced lesion formation. An 84% reduction was noted in the mean neointima-to-media ratio of SM/TRE-DN-p38α mice compared with SM mice (Figure 2B). Specifically, the mean±SE neointima-to-media ratio of SM mice was 2.23±0.80 but was only 0.36±0.10 in SM/TRE-DN-p38α mice (P=0.029; Figure 2B). In addition, the mean neointimal area was less for SM/TRE-DN-p38α mice compared with SM mice (P=0.017; Figure 2C). However, the mean medial areas of SM and SM/TRE-DN-p38α mice were similar (Figure 2D).

The SMC component of neointimal lesions was assessed by immunohistochemical staining with an anti–SMC-actin primary antibody. Immunohistochemical analysis revealed that SMCs were a major component of neointimal lesions in SM mice (Figure 3). Although SMCs were present in the tunica media and intima of SM/TRE-DN-p38α mice, SMC-actin staining was markedly reduced compared with SM mice (Figure 3). Vascular signaling also was evaluated in carotid cross sections from mice. Immunohistochemical staining with anti–phospho p38 MAPK antibody showed that p38 MAPK activation was present in both the tunica media and neointima of single transgenic SM mice (Figure 3). In contrast, p38 MAPK activation was dramatically reduced in the tunica media and intima of SM/TRE-DN-p38α mice (Figure 3).

Role of Retinoblastoma Protein and Minichromosome Maintenance Protein 6 in p38α MAPK–Induced SMC Proliferation

To explore the molecular mechanism(s) by which p38α MAPK promotes carotid artery neointima formation in response to injury, an in vitro model system with a cultured rat vascular SMC-derived cell line was used. Subconfluent A10 cells were pretreated with a pharmacological inhibitor of both p38α and p38β MAPK, with SB202190, or with control diluent. PDGF treatment of A10 cells caused robust phosphorylation of the p38 MAPK activation loop (Figure 4A). Activation of p38 MAPK, but not ERK MAPK, was blocked by SB202190 (Figure 4A). When quiescent A10 cells were pretreated with control diluent and then stimulated with PDGF for 20 hours, a 1.85-fold
increase in cell number was observed ($P<0.05$; Figure 4B).

However, when A10 cells were pretreated with SB202190, PDGF treatment did not result in increased cell numbers (Figure 4B). [3H]-thymidine incorporation assays were performed to determine whether p38 MAPK function is required for PDGF-induced DNA synthesis. PDGF treatment of A10 cells resulted in an 11.4-fold increase in [3H]-thymidine incorporation ($P<0.05$; Figure 4C), but SB202190 dramatically reduced [3H]-thymidine incorporation (Figure 4C). Similarly, SB202190 blocked PDGF-induced primary RAOSMC proliferation and reduced [3H]-thymidine incorporation (online-only Data Supplement Figure IIA and IIB).

To specifically reduce p38α MAPK protein levels and activity in cultured cells, a short interfering (siRNA) approach was used. Subconfluent A10 cells were transfected with a rat p38α MAPK siRNA construct or with control, nontargeting siRNA. After 24 hours of transfection in serum-free media, A10 cells were stimulated with PDGF or control diluent for 20 hours. After stimulation, some A10 cells were used to generate lysates for Western blotting. Western blot analysis revealed that p38α MAPK protein levels were dramatically reduced in p38α MAPK siRNA–transfected A10 cells compared with control siRNA–transfected cells (Figure 5A). Specifically, densitometric analysis demonstrated an $76\%$ reduction in total p38α MAPK protein (Figure 5B). When stimulated with PDGF for 20 hours, A10 cells transfected with p38α MAPK siRNA proliferated at a much slower rate than control siRNA–transfected cells (Figure 5C). A10 cell number increased by 1.9-fold after PDGF stimulation in control siRNA-transfected cells ($P<0.05$). An increase in A10 cell number was blocked with p38α MAPK siRNA. After 20 hours of PDGF stimulation, [3H]-thymidine incorporation was reduced $56\%$ in p38α MAPK siRNA–transfected A10 cells compared with control siRNA–transfected cells (data not shown; $P=0.007$).

To address the downstream target(s) of p38α MAPK action responsible for growth factor–stimulated SMC proliferation,
the phosphorylation status of a key regulator of mammalian cell cycle progression, retinoblastoma protein (Rb), was evaluated. In nonproliferating cells, Rb is hypophosphorylated and is able to bind to and sequester members of the E2F family of transcription factors. Activation of cyclin-dependent kinases results in the hyperphosphorylation of Rb, the subsequent release of E2F proteins, and the transcription of genes involved in cell cycle progression. In previous work, inhibition of human vascular SMC proliferation by salicylate administration correlated with reduced Rb hyperphosphorylation. PDGF treatment of A10 cells for 20 hours resulted in a robust increase in Rb phosphorylation that was blocked by pretreatment of cells with SB202190 (Figure 6A). In addition, PDGF induced hyperphosphorylation of Rb in RAOSMCs that was blocked by SB202190 pretreatment (online-only Data Supplement Figure III).

One target of E2F activity during cell cycle progression is minichromosome maintenance protein 6 (MCM6). MCM6 is a regulator of DNA replication and DNA helicase activity. In previous work, liver X receptor ligand inhibition of human vascular SMC proliferation correlated with reduced expression of MCM6. PDGF treatment of subconfluent A10 cells for 24 hours resulted in a robust increase in MCM6 protein levels that was blocked by pretreatment of cells with SB202190 (Figure 6A). In addition, PDGF induced hyperphosphorylation of Rb in RAOSMCs that was blocked by SB202190 pretreatment (online-only Data Supplement Figure III).

We assessed the requirement for MCM6 in RAOSMC proliferation and DNA replication by transfecting RAOSMCs with MCM6-specific siRNA. Subconfluent RAOSMCs were transfected with a rat MCM6 siRNA construct or with control, nontargeting siRNA. After 2 days of transfection in serum-free media, RAOSMCs were stimulated with PDGF or control diluent for 15 hours. After stimulation, some RAOSMC lysate was generated for Western blotting. Western blot analysis revealed that MCM6 protein levels were dramatically reduced in MCM6 siRNA–transfected RAOSMCs compared with control siRNA–transfected cells (Figure 7A and 7B). In RAOSMCs transfected with control siRNA, overnight stimulation with PDGF induced a 2-fold increase in MCM6 protein (P < 0.05; Figure 7A and 7B). MCM6-specific siRNA reduced baseline MCM6 protein to ≈55% compared with control siRNA–transfected RAOSMCs (P < 0.05). In addition, MCM6-specific siRNA blocked PDGF induction of MCM6 protein (Figure 7A and 7B, P < 0.05). Moreover, MCM6-specific siRNA reduced both PDGF-induced proliferation (Figure 7C) and thymidine incorporation (data not shown). Specifically, in control siRNA–transfected RAOSMCs, cell number increased by 2.5-fold after 40 hours of PDGF stimulation (P < 0.05; Figure 7C). However, the increase in RAOSMC number was blocked with MCM6 siRNA.

MCM6 expression was evaluated in carotid cross sections of SM and SM/TRE-DN-p38α mice by immunohistochemical staining. Immunohistochemical analysis revealed that carotid cross sections from SM mice isolated 3 weeks after injury had increased MCM6 expression compared with sections from compound transgenic SM/TRE-DN-p38α mice (Figure 8A). Sections also were stained for PCNA. Carotid cross sections from SM mice had increased medial PCNA staining compared with SM/TRE-DN-p38α mice (Figure 8B). Specifically, 6.69 ± 0.45% of SM medial nuclei were PCNA positive. However, this value was reduced to 1.04 ± 1.80% for SM/TRE-DN-p38α mice (P = 0.006; Figure 8B).

To evaluate whether increased apoptosis was responsible for the phenotype observed in SM/TRE-DN-p38α mice 3 weeks after carotid injury, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on carotid cross sections. Infrequent TUNEL-positive cells were detected in the media and neointima of SM carotid arteries (n = 3), but no TUNEL-positive cells were detected in the media or neointima of SM/TRE-DN-p38α carotid arteries (n = 3; data not shown).

**Discussion**

The MAPKs are important effectors of growth factor and ligand action in eukaryotic cells. The p38 family of MAPKs consists of 4 proteins encoded by separate genes that regulate...
important aspects of cell physiology, including cell growth, proliferation, survival, and differentiation.7 In this work, the role of p38MAPK in the formation of neointima after arterial injury was analyzed. In previous work, the role of the ubiquitously expressed intracellular linker protein Grb2, which links growth factor receptor activation to MAPK activation, was analyzed.6 Grb2-haploinsufficient mice were resistant to neointima formation after carotid injury. Furthermore, Grb2-
haploinsufficient cultured SMCs exhibited reduced p38 and JNK MAPK activation in response to PDGF stimulation.

To address the specific role of p38 MAPK in neointima formation, SM/TRE-DN-p38 mice were generated in the present study. Doxycycline treatment of these animals resulted in the expression of DN-p38 mRNA and protein in aortas and carotid arteries. Next, doxycycline-treated SM/TRE-DN-p38 compound transgenic mice were found to be resistant to carotid injury-induced neointima formation compared with control SM mice. Furthermore, immunohistochemical analysis revealed that p38 MAPK activation was dramatically reduced in the tunica media and intima of SM/TRE-DN-p38 mice after carotid injury. These results strongly suggest that p38 MAPK activity is required for neointima formation after arterial injury.

To determine the molecular mechanism(s) that contribute to p38 MAPK-mediated neointima formation, an in vitro SMC culture system was used. PDGF-induced cell proliferation was blocked in cultured SMCs after treatment with SB202190, an inhibitor of p38α/β MAPK. To examine more specifically the role of p38α MAPK in SMC proliferation in vitro, an siRNA approach was used. PDGF-induced SMC proliferation was blocked in cells treated with SB202190. Furthermore, siRNA reduction of MCM6 blocked PDGF-induced SMC proliferation and DNA replication.

Taken together, our results show that p38α MAPK activity is required for SMC proliferation in vitro and neointima formation in vivo. This activity of p38α MAPK contrasts with its role in cardiomyocytes, in which in certain situations p38α MAPK blocks cell proliferation, promotes apoptosis, and antagonizes hypertrophic growth.8,22–25 However, one of the hallmarks of MAPKs is that the biological effects of kinase activation are highly dependent on the cell type and physiological context.7 One molecular mechanism of p38α MAPK action in SMCs may...
be to promote the hyperphosphorylation of Rb, the subsequent activation of E2F family members, and the stimulation of MCM6 expression that results in cell proliferation and arterial neointima formation.

The results of this study provide preliminary evidence that p38α MAPK inhibitors may be useful for the treatment of arterial restenosis and transplant vasculopathy. Our previous work and the work of other groups also support the use of p38α MAPK inhibitors to limit pathological cardiac remodeling after myocardial infarction.24–26 Although numerous p38α MAPK inhibitors have been developed, none are currently approved for use in human patients, and clinical trials evaluating the efficacy of these agents in cardiovascular disease have not been published.27

Figure 7. siRNA-mediated reduction of MCM6 protein inhibits PDGF-BB-induced RAOSMC proliferation. A, Analysis of MCM6 protein levels after siRNA treatment. Subconfluent RAOSMC monolayers were transfected with MCM6-specific siRNA or siCONTROL Non-Targeting siRNA No. 2. Two days after transfection, the cells were treated in triplicate wells with PDGF-BB (5 ng/mL) or 0.1% BSA, as a vehicle control, for 15 hours. Lysates were generated, and Western blotting was performed with anti-MCM6 antibody. Reprobing with anti-actin antibody was conducted to control for protein loading. B, Densitometric analysis of Western blot from A was conducted (MCM6 siRNA without PDGF-BB vs control siRNA without PDGF-BB, *P < 0.05; MCM6 siRNA plus PDGF-BB vs MCM6 siRNA without PDGF-BB, **P < 0.05; MCM6 siRNA plus PDGF-BB vs control siRNA plus PDGF-BB, ***P < 0.05). C, Analysis of PDGF-stimulated cell proliferation after MCM6 siRNA transfection. Subconfluent RAOSMC monolayers were transfected and stimulated as described in A for 40 hours. Then, determination of cell number was conducted (control siRNA without PDGF-BB vs control siRNA plus PDGF-BB, *P < 0.05). For bar graphs in B and C, results are expressed as mean ± SD of experiments conducted in triplicate.

Figure 8. SM/TRE-DN-p38α compound transgenic mice exhibit reduced arterial MCM6 and PCNA staining. A, SM and SM/TRE-DN-p38α mice were given doxycycline, and mechanical injury of the left carotid artery was performed. Three weeks after injury, immunohistochemical analysis of carotid cross sections was performed with anti-MCM6 antibody. The sections were counterstained with hematoxylin. Micrographs were collected with a ×63 objective. Red arrows depict areas exhibiting enriched MCM6 staining. B, Mice were given doxycycline and injured as in A. Three weeks after injury, immunohistochemical analysis of carotid cross sections was performed with anti-PCNA antibody. The sections were counterstained with hematoxylin. Micrographs were collected with a ×40 objective. The PCNA-positive nuclei are depicted with diaminobenzidine (brown) staining. Red arrows highlight the media-adventitia and media-neointima borders. The bar graph shows the mean ± SD percentage of PCNA-positive medial nuclei per group (SM mice, n = 3; SM/TRE-DN-p38α mice, n = 3; *P = 0.006). N indicates neointima; L, lumen; and M, media.
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Disclosures
None.

References

CLINICAL PERSPECTIVE
Atherosclerotic vascular disease is commonly treated by percutaneous arterial interventions. Neointima formation often occurs at the site of intervention and may lead to significant restenosis. The use of drug-eluting stents to treat atherosclerotic vascular disease results in a markedly reduced incidence of restenosis, but delayed in-stent thrombosis can occur that leads to myocardial infarction. A cause of delayed thrombosis may be inadequate endothelialization of the inner surface of the drug-eluting stent resulting from the toxic effects of the embedded agents, sirolimus or taxol. This issue has led to a search for agents that inhibit the smooth muscle cell migration and proliferation required for neointima formation without inhibiting endothelialization. In this work, mice expressing a dominant-inhibitory form of p38 mitogen-activated protein kinase (p38 MAPK) only in smooth muscle cells were resistant to neointima formation after carotid artery injury. Knockdown of p38 MAPK in cultured smooth muscle cells blocked cell proliferation. In previous work, inhibition of p38 MAPK in endothelial cells promoted cell survival and proliferation. Therefore, p38 MAPK inhibition is predicted to inhibit smooth muscle cell proliferation while promoting endothelial cell survival and growth. The use of agents that block p38 MAPK may block restenosis without preventing stent endothelialization.
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Supplemental Figure I

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Supplemental Figure III

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- phospho-Rb (110 kDa)
- phospho-p38 (38 kDa)
- actin (42 kDa)
Expanded Methods

Carotid Injury

Prior to carotid injury, adult SM and SM/TRE-DN-p38α mice were administered doxycycline (200 ppm) rodent chow (Research Diets Inc., New Brunswick, NJ) and doxycycline (1 mg/ml) drinking water (Sigma-Aldrich, Inc., Saint Louis, MO) containing sucrose (50 mg/ml) *ad libitum* for three weeks. Then, mechanical injury of the left carotid artery was performed as previously described by our group.\(^1\) Briefly, carotid injury was performed via modification of the guidewire probe method first described by Lindner and altered by Kuhel.\(^2,3\) Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg of body weight) and xylazine (16 mg/kg body weight). Per animal, the entire length of the left carotid artery was exposed and the proximal common carotid artery was occluded with a microvascular clamp.\(^1\) Another microvascular clamp was placed at the internal carotid arterial branch. A 7-0 suture was placed around the external carotid artery immediately distal to the point of bifurcation. After a transverse arteriotomy was made in the external branch, an epoxy resin-beaded probe was introduced into the branch and advanced toward the aortic arch and withdrawn with rotation 3 times. This procedure over-stretched the artery of each animal and denuded the endothelium, and thus, mimicked the mechanisms of balloon angioplasty. A series of epoxy resin-beaded probes were formed by attaching epoxy resin beads of different diameters (0.5 mm – 0.6 mm) to 0.30 mm guidewires.\(^1\) A probe that was slightly larger than the diameter of the left carotid artery was selected for use in each animal by measuring the diameter of each artery under a microscope. Per animal, after the probe was removed, the proximal external carotid suture was ligated,
and blood flow through the common carotid and internal carotid arteries was restored. After surgery, all animals were allowed to recover on a 37° C heating pad. Animals were maintained on doxycycline rodent chow and doxycycline drinking water, as described above, prior to performing morphometry.

Carotid injury was solely performed by our murine vascular surgeon who was blinded to the genotypes of the mice for the entire study. To evaluate the consistency of endothelial denudation achieved by the carotid injury method, adult, C57Bl/6J mice were injured as stated above.

**Histological analysis**

Two hours or 21 days after carotid injury, animals were anesthetized with ketamine and xylazine as described above. Then, an angiocatheter was placed in the left ventricle of each animal and the left carotid arteries were perfusion-fixed in situ with 10% buffered formalin (pH 7.0) for 5 minutes with constant pressure of 100 mm Hg. Two hours after surgery, some mice were infused with Evans blue dye prior to fixation. The whole carotid arteries were harvested and visualized via en face or embedded in paraffin. For morphometric analysis of the left carotid arteries, per left carotid artery, 10 sections, 5µm thick, were obtained at 150 μm intervals. For morphometry, 10 serial sections were stained with hematoxylin and eosin, and with Verhoeff’s von Giesen stain to visualize cells and elastic laminae, respectively. For immunohistochemical analysis, representative serial sections were probed with rabbit anti-SMC-actin primary antibody (Spring Bioscience, Fremont, CA), rabbit anti-phospho (Thr180/Tyr182) p38 MAPK primary antibody (Cell Signaling Technology, Inc., Danvers, MA), goat anti-MCM6
primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and normal goat IgG (Santa Cruz Biotechnology, Inc.) and normal rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) as negative controls. Vectastain® Elite ABC and DAB substrate kits (Vector Laboratories, Inc.) were used for probing and visualization of staining, respectively. In addition, the DDRCC Morphology Core Facility at Washington University in St. Louis, School of Medicine conducted TUNEL, and stained sections for Proliferating Cell Nuclear Antigen (PCNA) and von Willebrand Factor.

**Morphometry**

The neointimal and medial areas of left carotid artery sections were analyzed after staining of the cellular and elastic laminae contents.¹ Morphometry was performed by an individual who was blinded to the genotypes of the mice. A micrograph for each section was obtained with a Zeiss Axioskop microscope equipped with a Zeiss AxioCam HRc camera (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Morphometric measurements of the luminal, intimal, and medial areas of sections were determined as described by Kuhel using Axio Vision 4.6 software (Carl Zeiss MicroImaging, Inc.).³ The total neointimal area, total medial area, and neointimal area/medial area ratio of each mouse was determined from the mean of serial sections, described above.

**Quantification of PCNA-positive medial nuclei**

Carotid cross-sections were stained for PCNA and counterstained with hematoxylin by the DDRCC Morphology Core Facility at Washington University in St. Louis, School of Medicine. Then, using a Zeiss Axioskop microscope equipped with a
Zeiss AxioCam HRc camera (Carl Zeiss MicroImaging, Inc.), a representative micrograph was collected for each mouse using a 40x objective. The percentage of PCNA-positive medial nuclei was determined for each micrograph. Results are expressed as the mean ± SD of three mice per group.

**Cell culture**

A10 rat vascular SMCs were obtained from American Type Culture Collection (Manassas, VA) and rat aortic smooth muscle cells (RAOSMCs) were obtained from Cell Applications, Inc. (San Diego, CA). A10 cells and RAOSMCs were cultured in monolayers in Dulbecco’s Modification of Eagle's Medium containing L-glutamine (4.5 g/L), sodium pyruvate, penicillin (100 units)/streptomycin (100 µg/ml; Washington University in St. Louis, School of Medicine, Tissue Culture Support Center), and fetal calf serum (10% (v/v); Invitrogen Corporation, Carlsbad, CA). They were maintained in a 37º C cell culture incubator with 5% CO₂ and used in methods described below.

**PDGF-BB stimulation of A10 and RAOSMCs**

To induce quiescence, cells were cultured in serum-free media. Then, they were pretreated with the p38 MAPK inhibitor SB202190 (EMD Chemicals, Inc., San Diego, CA) or DMSO (vehicle control). In other experiments, cells were transfected with siRNA, as described below. Following pretreatment or transfection, A10 and RAOSMCs were stimulated with 60 ng/ml and 5 ng/ml, respectively, recombinant rat platelet-derived growth factor-BB (PDGF-BB; R&D Systems, Minneapolis, MN) or 0.1%
BSA (v/v)/water (vehicle control) at 37º C for Western blotting, [³H]-thymidine incorporation, and proliferation assays.

**Western blotting**

Sub-confluent A10 cells and RAOSMCs were transfected with siRNA constructs in triplicate, as described below. In other experiments, A10 cells and RAOSMCs were pre-treated with SB202190 or DMSO (vehicle control). Following transfection or pharmacological pre-treatment, A10 and RAOSMCs were treated with PDGF-BB or 0.1% BSA (vehicle control) in duplicate or triplicate culture wells. Then, the cells were placed on ice and washed with ice cold PBS. Next, cell lysates were prepared with 1% (v/v) Triton® x-100 (Sigma-Aldrich, Inc., Saint Louis, MO)/PBS containing phosphatase (Pierce Biotechnology, Inc., Rockford, IL) and protease (Roche, Basel, Switzerland) inhibitor cocktails.

For analysis of murine arterial tissue, *SM* and *SM/TRE-DN-p38α* mice were euthanized via CO₂ asphyxiation in accordance with institutional guidelines. Then, whole carotid arteries and aortae were dissected from each mouse. Adventitial fat and blood was thoroughly removed from each artery by dissection and washing in ice cold PBS, respectively, prior to homogenization in the lysis buffer described above.

A10, RAOSMC, and arterial lysates were clarified via microcentrifugation at 4º C and then protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) and fraction 5 BSA (Sigma-Aldrich, Inc.) for protein
concentration standards. Lysates were then diluted in Laemmli sample buffer containing 3.3% (v/v) 2-ME and boiled for 5 minutes. Using the Mini PROTEAN® 3 System (Bio-Rad Laboratories, Inc.) 8% and 10% resolving SDS-PAGE gels were poured with 5% stacking SDS-PAGE gels and these gels were used to fractionate the denatured and reduced protein samples. The fractionated samples were transferred to Hybond™-P membranes (Amersham Biosciences, Piscataway, NJ) and transfer efficiency was determined using Ponceau S. Membranes were washed with tris-buffered saline containing 0.05% (v/v) Tween® 20 (TBS/T) and then blocked with TBS/T containing 5% (w/v) non-fat dry milk. Primary antibodies were then used to probe the membranes and these included: anti-phospho (Thr180/Tyr182) p38 MAPK, anti-phospho (Thr202/Tyr204) ERK, anti-total ERK, anti-phospho (Ser807/811) Rb (Cell Signaling Technology, Inc), anti-total p38α MAPK, anti-14-3-3β, anti-total MCM6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-actin (Sigma-Aldrich, Inc.). Primary antibodies were then removed and the membranes were washed with TBS/T. Then, they were probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies which included: donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc), anti-rabbit IgG-HRP (GE Healthcare, UK), and anti-mouse IgG-HRP (Cell Signaling Technology, Inc.). Next, membranes were extensively washed with TBS/T, incubated with ECL™ (GE Healthcare) or Immobilon™ Western (Millipore Corporation, Billerica, MA) substrates, and exposed to film for visualization of probed proteins. For re-probing, membranes were stripped with 0.2 M NaOH or Restore™ Western Blot Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL). Densitometry
was performed with ImageJ (National Institutes of Health, Bethesda, MD) software according to the manufacturer's directions.

**Determination of $[^3]H$-thymidine incorporation**

A10 cells and RAOSMCs were pre-treated with SB202190 or DMSO (vehicle control), or transfected with siRNA, as described. Then, in triplicate culture wells, the cells were incubated with PDGF-BB (60 ng/ml or 5 ng/ml) or 0.1% BSA (vehicle control). Stimulation ensued for 15 hours in a tissue culture incubator and then the monolayers were pulsed with 1 $\mu$Ci/ml [6-$[^3]$H]-thymidine (PerkinElmer Life and Analytical Sciences, Waltham, MA) for 2.5 hours. Next, the cells were placed on ice and washed twice with ice cold PBS prior to incubation with ice cold 5% TCA (v/v)/water for 20 minutes and subsequent washing. Finally, the monolayers were harvested with 0.5M NaOH/0.5% SDS (w/v) and $[^3]$H-thymidine incorporation was determined with a scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Results are expressed as mean ± SD.

**Determination of cell number**

Sub-confluent A10 and RAOSMC monolayers were transfected with siRNA constructs in triplicate wells, as described below. In other experiments, serum-deprived, sub-confluent cells were pre-treated with SB202190 or DMSO (vehicle control) as described. Following transfection or pharmacological pre-treatment, cells were treated with PDGF-BB (60 ng/ml or 5ng/ml) or 0.1% BSA (vehicle control) in triplicate culture wells. Stimulation ensued for 20 or 40 hours in a tissue culture incubator. Then, cell numbers were determined via bright field microscopy with a Nikon TMS microscope.
Briefly, using 400x magnification, three randomly selected fields per well were counted. Then, the mean number of cells per 400x field for each well was determined. All mean cell numbers per treatment condition were calculated from determinations in triplicate wells. Results are expressed as mean ± SD.

**siRNA reduction of p38α MAPK and MCM6 protein**

A10 and RAOSMCs were cultured in complete growth media to sub-confluency in 12-well plates. ON-TARGET plus SMART pool MAPK14 (p38α MAPK), ON-TARGET plus SMART pool rat MCM6, and siCONTROL™ Non-Targeting siRNA #2 (Dharmacon, Lafayette, CO) were reconstituted to 20 µM with sterile, nuclease-free water. Then, the 20 µM siRNA constructs were prepared in serum-free, Dulbecco's Modification of Eagle's Medium with DharmaFECT™ 2 Transfection Reagent (Dharmacon), and the sub-confluent cells were transfected such that the final amounts of siRNA and transfection reagent were 200 nmol and 0.14% (v/v), respectively. Next, the transfected cells were returned to a tissue culture incubator and allowed to incubate for 24 hours to two days prior to stimulation with PDGF-BB (60 ng/ml or 5ng/ml) or 0.1% BSA (vehicle control). All transfection conditions were conducted in triplicate wells. Western blotting, determination of cell number, and determination of [³H]-thymidine incorporation was conducted as described above.

**Semi-quantitative RT-PCR**

For analysis of doxycycline-inducible DN-p38α mRNA expression in the arteries of SM-DN-p38α mice, SM and SM-DN-p38α mice were administered doxycycline
drinking water containing sucrose, as described above, or drinking water only containing sucrose ad libitum. After the durations of time indicated, SM and SM-DN-p38α mice were euthanized via CO₂ asphyxiation in accordance with institutional guidelines. Then, whole carotid arteries and aortae were dissected from each mouse. Adventitial fat and blood was thoroughly removed, as described above. Then, to isolate the total RNA population from arteries, each artery was thoroughly homogenized in TRIzol® Reagent (Invitrogen Corporation). Homogenates were clarified via centrifugation, and the supernatants were transferred to fresh tubes. Next, chloroform was added to assist phase separation. Following centrifugation, the aqueous phases were transferred to fresh tubes, and RNA was precipitated from each aqueous phase with 2-propanol. Then, each RNA pellet was washed with ethanol, air dried, and reconstituted in nuclease-free water. All RNA samples were treated with deoxyribonuclease I (Invitrogen Corporation) to remove any contaminating genomic DNA. Then, reverse transcription was performed with the SuperScript™ III Reverse Transcriptase kit (Invitrogen Corporation) and oligo (dT)₁₂-₁₈ primer (Invitrogen Corporation) to synthesize cDNA. For “minus reverse transcriptase (RT)” negative control reactions, the RT was replaced with nuclease-free water. After reverse transcription, all products were treated with ribonuclease H (Invitrogen Corporation) to degrade the RNA of RNA-DNA hybrids. Next, flag-DN-p38α and GAPDH were amplified via PCR with specific primers. Specifically, a 207 bp fragment of flag-DN-p38α was amplified with forward and reverse primers with 5'-ATGGATTACAAGGATGACGAC-3' and 5'-GGACTGAAACGGTCTCGACAG-3' sequences, respectively. The forward primer specifically anneals on the flag sequence
and the reverse primer anneals on the p38α MAPK sequence. GAPDH was amplified with commercially available primers supplied by Applied Biosystems (Foster City, CA).

References


Supplemental Figure Legends

Supplemental Figure I. Carotid injury induces consistent endothelial denudation.

A, Adult, C57Bl/6J mice were subjected to mechanical injury of the left carotid artery. Two hours after injury, six mice were infused with Evans blue dye prior to perfusion-fixation in situ with buffered formalin. A representative non-injured, right carotid artery and injured, left carotid artery is shown. The arteries were pinned by the en face method and images were collected with a digital camera. B, Adult, C57Bl/6J mice were
subjected to carotid injury as described in “A”. Two hours after surgery, three mice underwent perfusion-fixation in situ with buffered formalin. Then, carotid cross-sections were isolated and stained for von Willebrand factor. Micrographs were collected with a 63x objective. Two, representative non-injured, right and two representative injured, left carotid arteries are shown. Red arrows point at the vascular endothelium (VE, vascular endothelium; M, media).

**Supplemental Figure II. SB202190 blocks PDGF-induced cell proliferation and reduces DNA replication in RAOSMCs.** A, Evaluation of PDGF-stimulated cell proliferation after SB202190 treatment. Serum-deprived RAOSMCs were pre-treated with SB202190 (10 µM) or vehicle (DMSO) for 1 hour. Then, in triplicate wells, the monolayers were treated with PDGF-BB (5 ng/ml) or 0.1% BSA, as a vehicle control for PDGF-BB, in the continued presence of SB202190 or DMSO. After 40 hours cell number was determined. HPF, 400x high power field (DMSO, + PDGF-BB vs. DMSO, -PDGF-BB, *P<0.05; DMSO, + PDGF-BB vs. SB202190, + PDGF-BB, **P<0.05). B, Examination of PDGF-induced DNA replication after SB202190 treatment. Serum-deprived RAOSMCs were pre-treated with SB202190 (10 µM) or DMSO as in “A”. Then, the monolayers were treated with PDGF-BB (5 ng/ml) or 0.1% BSA for 15 hours in a tissue culture incubator in the continued presence of SB202190 or DMSO. Next, each well was pulsed with [6-³H]-thymidine (1 µCi/ml) for 2.5 hours and thymidine incorporation was determined; r.u., relative units (DMSO, + PDGF-BB vs. DMSO, -PDGF-BB, *P<0.05; DMSO, + PDGF-BB vs. SB202190, + PDGF-BB, **P<0.05).
Supplemental Figure III. SB202190 blocks PDGF-induced Rb hyper-phosphorylation in RAOSMCs cells. Analysis of PDGF-induced Rb phosphorylation after SB202190 treatment. Serum-deprived RAOSMCs were pre-treated with SB202190 (10 μM) or vehicle (DMSO) for 1 hour, and then monolayers were treated with PDGF-BB (5 ng/ml) or 0.1% BSA in the continued presence of SB202190 or DMSO. After 20 hours, cells were lysed and immunoblotting was performed with an anti-phospho (Ser 807/811) Rb antibody. Re-probing was conducted with anti-phospho (Thr180/Tyr182) p38 MAPK and anti-actin antibodies (SB, SB202190). Densitometric analysis of the Western blot is shown in parentheses; r.u., mean, relative units of phospho Rb signal for each group normalized to actin.
Supplemental Figure I

A. right (noninjured)
left (injured)

B. right (noninjured) left (injured)
Supplemental Figure II

A.  

- Mean cell number/HPF

- DMSO
- SB202190

- PDGF-BB

B.  

- [H]-thymidine incorporation (r.u.)

- DMSO
- SB202190

- PDGF-BB

* **
Supplemental Figure III

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Expanded Methods

Carotid Injury

Prior to carotid injury, adult SM and SM/TRE-DN-p38α mice were administered doxycycline (200 ppm) rodent chow (Research Diets Inc., New Brunswick, NJ) and doxycycline (1 mg/ml) drinking water (Sigma-Aldrich, Inc., Saint Louis, MO) containing sucrose (50 mg/ml) ad libitum for three weeks. Then, mechanical injury of the left carotid artery was performed as previously described by our group.² Briefly, carotid injury was performed via modification of the guidewire probe method first described by Lindner and altered by Kuhel.²,³ Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg of body weight) and xylazine (16 mg/kg body weight). Per animal, the entire length of the left carotid artery was exposed and the proximal common carotid artery was occluded with a microvascular clamp.¹ Another microvascular clamp was placed at the internal carotid arterial branch. A 7-0 suture was placed around the external carotid artery immediately distal to the point of bifurcation. After a transverse arteriotomy was made in the external branch, an epoxy resin-beaded probe was introduced into the branch and advanced toward the aortic arch and withdrawn with rotation 3 times. This procedure over-stretched the artery of each animal and denuded the endothelium, and thus, mimicked the mechanisms of balloon angioplasty. A series of epoxy resin-beaded probes were formed by attaching epoxy resin beads of different diameters (0.5 mm – 0.6 mm) to 0.30 mm guidewires.¹ A probe that was slightly larger than the diameter of the left carotid artery was selected for use in each animal by measuring the diameter of each artery under a microscope. Per animal, after the probe was removed, the proximal external carotid suture was ligated,
and blood flow through the common carotid and internal carotid arteries was restored. After surgery, all animals were allowed to recover on a 37º C heating pad. Animals were maintained on doxycycline rodent chow and doxycycline drinking water, as described above, prior to performing morphometry.

Carotid injury was solely performed by our murine vascular surgeon who was blinded to the genotypes of the mice for the entire study. To evaluate the consistency of endothelial denudation achieved by the carotid injury method, adult, C57Bl/6J mice were injured as stated above.

Histological analysis

Two hours or 21 days after carotid injury, animals were anesthetized with ketamine and xylazine as described above. Then, an angiocatheter was placed in the left ventricle of each animal and the left carotid arteries were perfusion-fixed in situ with 10% buffered formalin (pH 7.0) for 5 minutes with constant pressure of 100 mm Hg. Two hours after surgery, some mice were infused with Evans blue dye prior to fixation. The whole carotid arteries were harvested and visualized via en face or embedded in paraffin. For morphometric analysis of the left carotid arteries, per left carotid artery, 10 sections, 5µm thick, were obtained at 150 µm intervals. For morphometry, 10 serial sections were stained with hematoxylin and eosin, and with Verhoeff’s von Giesen stain to visualize cells and elastic laminae, respectively. For immunohistochemical analysis, representative serial sections were probed with rabbit anti-SMC-actin primary antibody (Spring Bioscience, Fremont, CA), rabbit anti-phospho (Thr180/Tyr182) p38 MAPK primary antibody (Cell Signaling Technology, Inc., Danvers, MA), goat anti-MCM6
primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and normal goat IgG (Santa Cruz Biotechnology, Inc.) and normal rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) as negative controls. Vectastain® Elite ABC and DAB substrate kits (Vector Laboratories, Inc.) were used for probing and visualization of staining, respectively. In addition, the DDRCC Morphology Core Facility at Washington University in St. Louis, School of Medicine conducted TUNEL, and stained sections for Proliferating Cell Nuclear Antigen (PCNA) and von Willebrand Factor.

**Morphometry**

The neointimal and medial areas of left carotid artery sections were analyzed after staining of the cellular and elastic laminae contents.\(^1\) Morphometry was performed by an individual who was blinded to the genotypes of the mice. A micrograph for each section was obtained with a Zeiss Axioskop microscope equipped with a Zeiss AxioCam HRc camera (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Morphometric measurements of the luminal, intimal, and medial areas of sections were determined as described by Kuhel using Axio Vision 4.6 software (Carl Zeiss MicroImaging, Inc.).\(^3\) The total neointimal area, total medial area, and neointimal area/medial area ratio of each mouse was determined from the mean of serial sections, described above.

**Quantification of PCNA-positive medial nuclei**

Carotid cross-sections were stained for PCNA and counterstained with hematoxylin by the DDRCC Morphology Core Facility at Washington University in St. Louis, School of Medicine. Then, using a Zeiss Axioskop microscope equipped with a
Zeiss AxioCam HRc camera (Carl Zeiss MicroImaging, Inc.), a representative micrograph was collected for each mouse using a 40x objective. The percentage of PCNA-positive medial nuclei was determined for each micrograph. Results are expressed as the mean ± SD of three mice per group.

**Cell culture**

A10 rat vascular SMCs were obtained from American Type Culture Collection (Manassas, VA) and rat aortic smooth muscle cells (RAOSMCs) were obtained from Cell Applications, Inc. (San Diego, CA). A10 cells and RAOSMCs were cultured in monolayers in Dulbecco's Modification of Eagle's Medium containing L-glutamine (4.5 g/L), sodium pyruvate, penicillin (100 units)/streptomycin (100 µg/ml; Washington University in St. Louis, School of Medicine, Tissue Culture Support Center), and fetal calf serum (10% (v/v); Invitrogen Corporation, Carlsbad, CA). They were maintained in a 37º C cell culture incubator with 5% CO₂ and used in methods described below.

**PDGF-BB stimulation of A10 and RAOSMCs**

To induce quiescence, cells were cultured in serum-free media. Then, they were pretreated with the p38 MAPK inhibitor SB202190 (EMD Chemicals, Inc., San Diego, CA) or DMSO (vehicle control). In other experiments, cells were transfected with siRNA, as described below. Following pretreatment or transfection, A10 and RAOSMCs were stimulated with 60 ng/ml and 5 ng/ml, respectively, recombinant rat platelet-derived growth factor-BB (PDGF-BB; R&D Systems, Minneapolis, MN) or 0.1%
BSA (v/v)/water (vehicle control) at 37º C for Western blotting, [³H]-thymidine incorporation, and proliferation assays.

**Western blotting**

Sub-confluent A10 cells and RAOSMCs were transfected with siRNA constructs in triplicate, as described below. In other experiments, A10 cells and RAOSMCs were pre-treated with SB202190 or DMSO (vehicle control). Following transfection or pharmacological pre-treatment, A10 and RAOSMCs were treated with PDGF-BB or 0.1% BSA (vehicle control) in duplicate or triplicate culture wells. Then, the cells were placed on ice and washed with ice cold PBS. Next, cell lysates were prepared with 1% (v/v) Triton x-100 (Sigma-Aldrich, Inc., Saint Louis, MO)/PBS containing phosphatase (Pierce Biotechnology, Inc., Rockford, IL) and protease (Roche, Basel, Switzerland) inhibitor cocktails.

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**Determination of $[^{3}\text{H}]-\text{thymidine incorporation}$**

A10 cells and RAOSMCs were pre-treated with SB202190 or DMSO (vehicle control), or transfected with siRNA, as described. Then, in triplicate culture wells, the cells were incubated with PDGF-BB (60 ng/ml or 5 ng/ml) or 0.1% BSA (vehicle control). Stimulation ensued for 15 hours in a tissue culture incubator and then the monolayers were pulsed with 1 μCi/ml $[6-^{3}\text{H}]$-thymidine (PerkinElmer Life and Analytical Sciences, Waltham, MA) for 2.5 hours. Next, the cells were placed on ice and washed twice with ice cold PBS prior to incubation with ice cold 5% TCA (v/v)/water for 20 minutes and subsequent washing. Finally, the monolayers were harvested with 0.5M NaOH/0.5% SDS (w/v) and $[^{3}\text{H}]-\text{thymidine incorporation}$ was determined with a scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Results are expressed as mean ± SD.

**Determination of cell number**

Sub-confluent A10 and RAOSMC monolayers were transfected with siRNA constructs in triplicate wells, as described below. In other experiments, serum-deprived, sub-confluent cells were pre-treated with SB202190 or DMSO (vehicle control) as described. Following transfection or pharmacological pre-treatment, cells were treated with PDGF-BB (60 ng/ml or 5ng/ml) or 0.1% BSA (vehicle control) in triplicate culture wells. Stimulation ensued for 20 or 40 hours in a tissue culture incubator. Then, cell numbers were determined via bright field microscopy with a Nikon TMS microscope.
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For analysis of doxycycline-inducible DN-p38α mRNA expression in the arteries of SM-DN-p38α mice, SM and SM-DN-p38α mice were administered doxycycline
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