Functional Characterization of a Putative Serine Carboxypeptidase in Vascular Smooth Muscle Cells

Ting-Hein Lee, Jiyuan Chen, Joseph M. Miano

Rationale: We previously identified a novel serine carboxypeptidase, SCPEP1, that undergoes cleavage across all tissues where it is expressed. SCPEP1 bears the signature catalytic triad found in all serine carboxypeptidases, but its biological function is completely unknown.

Objective: To begin elucidating the functions of SCPEP1 in vitro and in the vessel wall after injury.

Methods and Results: Cultured smooth muscle cells were transduced with adenovirus carrying wild-type Scpep1, a short hairpin RNA to Scpep1, or variants of Scpep1 with mutations that disrupt the catalytic triad domain or SCPEP1 cleavage. Western blotting of key growth regulators or growth and migratory responses were assessed following SCPEP1 gain- or loss-of-function in smooth muscle cells. Vascular injury--induced remodeling and cell proliferation were evaluated in wild-type or newly created Scpep1 knockout mice. Overexpression of wild-type or cleavage-defective SCPEP1, but not a catalytic triad mutant SCPEP1, promotes smooth muscle cell proliferation and migration in vitro. A short hairpin RNA to Scpep1 blunts endogenous growth, which is rescued on concurrent expression of Scpep1 carrying silent mutations that evade knockdown. SCPEP1 protein is highly expressed in the neointima of 2 models of vascular remodeling. Scpep1-null mice show decreases in medial and intimal cell proliferation as well as vessel remodeling following arterial injury.

Conclusions: SCPEP1 promotes smooth muscle cell proliferation and migration in a catalytic triad–dependent, cleavage-independent manner. SCPEP1 represents a new mediator of vascular remodeling and a potential therapeutic target for the treatment of vascular occlusive diseases. (Circ Res. 2009;105:00-00-00.)

Key Words: Scpep1 ■ smooth muscle ■ neointima ■ protease ■ knockout

Smooth muscle cells (SMCs) are critical for blood vessel homeostasis, but they also contribute to the pathogenesis of several vasculopathies. In response to arterial injury, SMCs shift from a quiescent, contractile phenotype to a proliferative, synthetic state that undermines normal arterial function leading to neointimal formation.1-3 Myriad factors and cytokines, as well as proteases and their associated substrates, have been implicated in vascular pathology,4-8 but additional proteases are likely involved in this process. For example, the serine carboxypeptidase cathepsin A (CTSA) cleaves a number of substrates (eg, endothelin-1) that effect pathological changes in the vessel wall.6-8 Recently, a mutant CTSA allele defective for enzyme activity was knocked into the wild-type (WT) locus of mice and shown to confer a decrease in the inactivation of endothelin-1, elevated arterial blood pressure, and altered elastogenesis.9

Serine carboxypeptidases belong to the family of serine proteases and are most prevalent in the plant kingdom, where they function in numerous processes related to growth and development.10 Three serine carboxypeptidases are found in mammals and each shares the same catalytic triad (serine, aspartic acid, and histidine) found in plant homologs.10,11 We previously reported a novel serine carboxypeptidase from cultured SMCs in a screen for retinoid-induced genes.12 We call this protease serine carboxypeptidase (SCPEP1) because it contains several conserved domains common to all members of the serine carboxypeptidase family, including a substrate-binding domain and the catalytic triad. Northern blotting and in situ hybridization studies demonstrated Scpep1 mRNA in SMCs of the aorta and proximal convoluted tubular epithelium of the kidney.12 More recently, we developed an antibody to SCPEP1 and showed its cleavage from a mature 55-kDa isoform to a 35-kDa isoform in all adult mouse tissues studied, including vascular SMCs and renal proximal convoluted tubular epithelium.13 The biological substrates for SCPEP1, however, remain a mystery. We therefore consider SCPEP1 an orphan protease. Here, we have performed gain- and loss-of-function studies in vitro and in vivo to provide the first biological insight into SCPEP1 function.

Materials and Methods
An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. Cultured SMCs transduced by adenovirus were used for growth curve studies, migration assays, and immunoblotting. Total RNA
was extracted from cultured SMCs and tissues with TRIzol reagent (Invitrogen). RT-PCR was performed using the ProSTAR System (Stratagene). Short hairpin RNAs were generated as described.14 SCPEP1 mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene). All constructs were then incorporated into adenovirus (Invitrogen). The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Rochester. Scepl knockout (KO) mice were generated through the University of Rochester Transgenic Core and back-crossed to C57BL/6 mice. Twelve-week-old Scepl KO mice or WT littermates were subjected to complete common carotid artery ligation as described.15

### Results

#### Ectopic SCPEP1 Enhances SMC Growth and Migration

Although SCPEP1 bears the catalytic triad common to all serine carboxypeptidases, we and others have been unable to demonstrate enzymatic cleavage activity. We therefore adopted different approaches to begin understanding the function of SCPEP1 in vascular SMC biology. As a first step, we transduced rat aortic SMCs (RASMCs) with adenovirus harboring full-length SCPEP1 (Ad-SCPEP1) and show both the 55-kDa and 35-kDa SCPEP1 products are expressed in a dose-dependent manner (Figure 1A). Similar expression is seen in a pulmonary artery SMC line (data not shown). Adenoviral-mediated expression of SCPEP1 in RASMCs and pulmonary artery SMCs increases cell number (Figure 1B and data not shown). Importantly, such levels of SCPEP1 are comparable to those present in vivo after arterial injury (see below). To further elucidate the mechanisms underlying the growth-promoting effect of SCPEP1, we screened several signaling pathways in serum-stimulated SMCs and found that cyclin D1 is upregulated with Ad-Scpepl overexpression (Figure 1C and 1D). In addition, downregulation of the negative growth regulator p27kip1 is exaggerated in Ad-Scpepl-transduced SMCs (Figure 1C and 1D).

To determine whether SCPEP1 is involved in SMC migration, a scratch wound assay was performed in SMCs stimulated with platelet-derived growth factor (PDGF)-BB. As shown in Figure 2A, cells migrate faster in the presence of SCPEP1 than in controls as early as 0.5 days post-PDGF stimulation. A quantitative analysis shows Ad-Scpepl significantly increases SMC migration over controls at every time point examined (Figure 2B). To exclude a cell growth effect in the wound assay, we evaluated cell migration in Ad-Scpepl-transduced SMCs by a modified Boyden chamber assay. SCPEP1 significantly elevates SMC migration in this assay as well (Figure 2C).

#### Knockdown of Endogenous Scpepl Reduces SMC Growth

To further assess the effects of SCPEP1 on SMC growth and migration, we generated adenovirus containing either a short hairpin RNA to Scpepl or a rescue Scpepl designed to evade knockdown through the introduction of silent mutations that preserve primary amino acid sequence (Online Figure I, A). Because endogenous SCPEP1 is very low in rat SMCs (Figure 1A and data not shown), we turned to a mouse aortic SMC line (MOVAS)16 that expresses higher levels of endogenous SCPEP1 (Figure 3A and data not shown). Importantly, such levels of SCPEP1 are indicated adenoviral transgenes. Densitometric quantitation (D) of cyclin D1 and p27kip1 expression (Figure 3A). Cells transduced with the short hairpin RNA to Scpepl show dramatic decreases in serum-stimulated growth (Figure 3B). Importantly, this finding does not appear to be attributable to off-target effects because restoring SCPEP1 levels with the rescue transgene normal-
izes SMC growth (Figure 3B). Similar changes in SMC growth and migration are observed in RASMCs when ectopically expressed SCPEP1 is knocked down (data not shown). Taken together with the above results, we conclude that elevated SCPEP1 induces vascular SMC growth and migration in vitro.

SCPEP1-Mediated SMC Growth and Migration Is Catalytic Triad-Dependent
To date, enzymatic activity of SCPEP1 has not been demonstrated despite the presence of a conserved catalytic triad, which is highly homologous to the catalytic triad of other serine carboxypeptidases (Online Figure I, B). To determine whether the growth and migratory effects of SCPEP1 require an intact catalytic triad, we generated SCPEP1S167A and SCPEP1H431Y point mutants (Figure 4A). Because SCPEP1S167A is expressed and cleaved properly (Figure 4A), we proceeded to study its effect on SMC growth and migration. Adenoviral delivery of SCPEP1S167A fails to increase cyclin D1 and decrease p27kip1 expression (Figure 4B). In addition, overexpression of SCPEP1S167A does not elicit increases in SMC growth (Figure 4C) or migration (Figure 4D). These results suggest that the stimulatory effects of SCPEP1 on SMC growth and migration require an intact catalytic triad providing the first evidence to support enzymatic activity for this novel protease.

SCPEP1 Cleavage Is Dispensable for SMC Growth and Migration
To determine whether SCPEP1 cleavage is important for SMC growth and migration, we first analyzed expression of several random SCPEP1 point mutants. We used the SCPEP1T103A mutant for growth and migration studies because, among mutants displaying a lack of cleavage, its amino acid change was least aggressive (Figure 5A). SCPEP1T103A is shown to be as effective as WT SCPEP1 in accentuating serum-mediated reductions in p27kip1 (Figure 5B). In contrast, SCPEP1S167A did not lower p27kip1 levels (Figure 5B). We next generated stable SMC lines expressing WT SCPEP1, SCPEP1T103A, or empty vector. As with WT SCPEP1, SCPEP1T103A exhibits stimulatory effects on SMC growth and migration (Figure 5C and 5D). These findings suggest that cleavage of SCPEP1 is not necessary for mediating SMC growth and migration.

SCPEP1 Expression Is Increased in the Neointima of Vascular Lesions
As a first step toward understanding SCPEP1 function in an in vivo setting, we investigated its expression by immunohistochemistry of mouse and rat carotid arteries subjected to different modes of arterial injury.15,17,18 We note intense SCPEP1 expression in the neointima of the mouse carotid artery following ligation injury (Figure 6A, a and d). Blocking studies establish the specificity of SCPEP1 staining (Figure 6A, b and e) as previously reported.13 That SCPEP1 is expressed in phenotypically modulated SMCs is evident from immunostaining studies using one of the most specific markers for the SMC lineage, the smooth muscle isoform of myosin heavy chain (MYH11) (Figure 6A, c and f).19 We also found that SCPEP1 expression is enriched at the luminal border of the balloon-injured rat carotid artery where SMCs display strong immunostaining for growth factors (Figure 6B, a and c).20 Importantly, Western blotting reveals a clear increase in SCPEP1 protein 28 days after injury (Figure 6C), with similar increases seen at 7 days (data not shown). Neither platelets nor plasma appears to express SCPEP1 (data not shown), suggesting the major source of elevated SCPEP1 stems from vascular cells. These data demonstrate injury-
induced SCPEP1 expression in the vessel wall where modulated SMCs are known to be proliferative and migratory.

Development and Initial Characterization of Scpep1 KO Mice

We generated a Scpep1 KO mouse by replacing exons 1 and 2, encoding the N-terminal signal peptide and a portion of the putative substrate-binding domain, with a nuclear LacZ-floxed Neo cassette (Online Figure II, A). Targeting of the Scpep1 allele was validated by Southern blotting, as well as PCR genotyping, RT-PCR of the endogenous mRNA, and Western blotting of adult tissues (Online Figures II and III and data not shown).

Scpep1 KO mice are born in the expected Mendelian ratios (eg, +/−/+, n=57; +/-/+, n=114; −/−, n=61). Scpep1-null mice appear normal, are fertile, and exhibit no histopathology across all organ systems analyzed (Online Figure IV and data not shown); similar findings were reported by an independent group during manuscript submission of this study.20 Scpep1 KO mice show no detectable SCPEP1 protein expression in aorta and kidney (Online Figure II, C and D). No obvious differences are seen between KO and WT littermates with respect to blood pressure, heart rate, and body weight (Online Figure V).

Given 2 additional serine carboxypeptidases in mammals,11 we considered that one or both paralogs may compensate for loss in Scpep1. However, we found no differences in mRNA expression of the 2 serine carboxypeptidase paralogs between KO and WT mice (Online Figure VI). Collectively, these results reveal that genetic deletion of Scpep1 is compatible with embryonic development and does not elicit an overt phenotype under normal conditions in the adult mouse.

Vascular Remodeling and Intimal/Medial Cell Growth Are Attenuated in Scpep1 KO Mice

To directly test the role of SCPEP1 in a proliferative/migratory model of neointimal formation, we evaluated the
extent of neointimal formation in the presence or absence of Scpep1 following ligation injury.15 Medial and intimal areas are similar in the contralateral, uninjured vessels of WT and Scpep1-null mice (Figure 7A and 7B). The ligated arteries of WT mice reveal significant neointimal formation (Figure 7C). Scpep1 KO mice, however, show a dramatic attenuation in injury-induced neointimal formation (Figure 7D and Online Figure VII). Morphometric analyses reveal significantly reduced intimal area (D and E), medial area (F), and intimal/medial ratio (G) vs WT controls (n=5/group). Area bound by the external elastic lamina (EEL) was elevated in WT mice (C vs D), and this increase was significantly greater than that observed in Scpep1 KO vessels at day 28 (H). Results are shown as means±SD. *P<0.05; **P<0.01; ***P<0.001.

Discussion

Since we first identified Scpep1 8 years ago,12 virtually nothing has been published on this presumptive serine carboxypeptidase. The primary amino acid sequence of SCPEP1 contains the classic catalytic triad common to all members of this protease family,21 yet we have been unable to identify substrates for SCPEP1, even though many of the substrates tested can be cleaved by the serine carboxypeptidase, CTSA.6,7 A recent report also failed to reveal intrinsic protease activity for SCPEP1.20 Nevertheless, we hypothesize that SCPEP1-induced increases in SMC growth and migration require catalytic activity because SCPEP1S167A was ineffective in mediating these processes. Loss-of-function studies in vitro demonstrate that SCPEP1 is necessary for SMC growth and migration, a finding substantiated in vivo following vascular injury in Scpep1 KO mice. These results, together with the demonstrated increase in SCPEP1 following acute arterial injury, strongly support an important role for SCPEP1 in vascular remodeling accompanying damage to the vessel wall.

There is evidence indicating that neointimal cells originate, in part, from medial SMCs that switch from a quiescent and sessile phenotype (contractile) to a proliferative and migra-
tory state (synthetic) following various perturbations to the vessel wall.2 Our in vivo data show that SCPEP1 is highly expressed in the neointima, where SMCs display reduced expression of MYH11, the gold standard marker for SMC lineages.19 Western blotting data suggest that cells of the vessel wall, most likely modulated SMCs of the media and intima, account for the increase in SCPEP1 following injury, although we cannot exclude circulating cells or plasma itself as additional sources of SCPEP1. Interestingly, levels of SRF, which is a major transcription factor for differentiation markers such as Myh11, do not appreciably change in the neointima22 or in normal medial SMCs from Scpep1 KO mice (Online Figure II, D). Thus, knockout of SCPEP1 does not appear to alter the SMC differentiation program. Loss of SCPEP1 does, however, have an effect on vascular SMC proliferation, as evidenced by decreases in Ki-67 immunostaining and significant increases in the negative growth regulator p27kip1. Whether the elevation of p27kip1 is a direct or indirect consequence of loss in SCPEP1 awaits further study.

Our previous data showed that full-length SCPEP1 undergoes cleavage to a 35-kDa isoform.13 Whereas another serine carboxypeptidase enriched in macrophages does not appear to undergo cleavage,23 CTSA is proteolytically processed to 32- and 20-kDa fragments, each of which harbors a portion of the catalytic triad.8 Although SCPEP1 is cleaved into a 35-kDa protein, the precise boundaries of this cleavage product are currently unknown. Nevertheless, our data suggest that SCPEP1 cleavage is not necessary for SCPEP1 to mediate SMC growth and migration responses. This result is somewhat unexpected because many proteases involved in vascular remodeling, such as matrix metalloproteinases, are synthesized and secreted as inactive proenzymes that subsequently undergo cleavage and activation either through an autocatalytic process or via other extracellular proteases.4,24 In this context, secreted SCPEP1 exists as a mature 55-kDa protein with no detectable 35-kDa species (data not shown), suggesting that this protease primarily undergoes intracellular proteolytic cleavage, perhaps within the lysosomal compartment of the cell. Evidence supporting the latter include colocalization of SCPEP1 with a lysosomal marker,13,25 as well as loss in SCPEP1 cleavage on treatment with the lysosomotropic agent chloroquine (data not shown).

Mutation of the active site residue (serine) is known to extinguish catalytic activity in other serine carboxypeptidases.9 Here, we show that mutation of serine 167 of the catalytic triad of SCPEP1 confers an inability for SCPEP1 to stimulate SMC growth and migration. Thus, although the substrate(s) for SCPEP1 remains undefined, disruption of the catalytic triad results in loss of SCPEP1 function, suggesting that SCPEP1 cleaves and activates proteins that may be of relevance for SMC growth and migration. Alternatively, SCPEP1 may cleave and inactivate substrates that normally function to maintain a quiescent, sessile SMC phenotype. It
will be interesting to determine whether SCPEP1S167A is able to rescue the vascular phenotype in Scpep1 KO mice. Moreover, cells expressing SCPEP1S167A or Scpep1 KO cells will be useful tools to identify SCPEP1 substrates using a variety of biochemical and genetic approaches.\textsuperscript{26}

There are several highly homologous domains between SCPEP1 and CTSA (Online Figure I, A).\textsuperscript{11} Moreover, each has similar patterns of expression in adult tissues, and both are localized to lysosomes.\textsuperscript{8,13} These findings suggest that SCPEP1 and CTSA may have overlapping functions. Evidence to date, however, suggests otherwise. CTSA has 2 distinct functions: a structural protective function in the lysosomes and extralysosomal catalytic activity.\textsuperscript{8} CTSA deficiency in humans and mice results in the lysosomal storage disease galactosialidosis, which is related to the loss of its structural protective function for \( \beta \)-galactosidase and neuraminidase.\textsuperscript{8,11} Apparently, SCPEP1 cannot compensate for this protective function because gene inactivation of CTSA in both humans and mice is incompatible with normal life. Moreover, studies in transgenic mice that carry a catalytically inactive CTSA reveal normal structural protection of the \( \beta \)-galactosidase/neuraminidase lysosomal complex; however, the degradation rate of endothelin-1, a known CTSA substrate, is significantly reduced, resulting in elevated arterial blood pressure.\textsuperscript{9} If SCPEP1 cleaved endothelin-1, the hypertensive phenotype would not likely be manifest. In this context, our data show that Scpep1 KO mice and WT littersmates appear to have similar systolic blood pressure. Although CTSA does not compensate for loss in SCPEP1 with respect to the reduced neointimal phenotype, it is possible that there is compensation in other organ systems (eg, kidney). Pending further investigation, our results suggest that SCPEP1 and CTSA have distinct functions and thus nonoverlapping pools of substrates that function in cardiovascular homeostasis.

The in vivo remodeling and proliferation data from Scpep1 KO mice point to an important role for SCPEP1 in the response to injury of the vessel wall, although we cannot at this time definitively rule out an effect of the mixed genetic background. Further evidence for the physiological activity of SCPEP1 is highlighted by a sharp increase in the negative regulation of p27\textsuperscript{kip1} in injured Scpep1 KO vessels. We speculate that one mechanism for the growth stimulatory effects of SCPEP1 may be through direct or indirect downregulation of p27\textsuperscript{kip1}, a notion supported by the SCPEP1S167A data (see Figure 4B). The reduced caliber of injured Scpep1 KO vessels is also suggestive of SCPEP1 acting on substrates that, following proteolytic cleavage, may effect cell–cell and/or cell–matrix changes accompanying outward remodeling of injured blood vessels. The development of new mouse models using recombinant Scpep1 alleles offers a unique opportunity to directly assess the importance of the catalytic triad domain of SCPEP1 in these and other pathological contexts.

In summary, we provide the first documented biological activity of a putative vascular protease (SCPEP1) that we show enhances SMC proliferation and migration in a catalytic triad–dependent and cleavage-independent manner. SCPEP1 is elevated within the injured vessel wall and genetic inactivation of Scpep1 results in a marked reduction of SMC growth and vascular remodeling. Together, these results lay a critical foundation for future identification of SCPEP1 substrates and the further delineation of the mechanisms of action of SCPEP1 in vascular biology.

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Disclosures

None.

References

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Supplement Material

Extended Materials and Methods

Antibodies

Anti-SCPEP1 rabbit polyclonal antibody was generated by a synthetic C-terminally His-tagged protein corresponding to the C-terminal fragment of mouse SCPEP1 (aa 173-452, Genbank accession no. NP_083299) (Proteintech Group Inc., Chicago, IL). Anti-β-actin and anti-α-tubulin antibodies were obtained from Sigma (St. Louis, MO); anti-cyclin D1 and p27kip1 antibodies were purchased from Cell Signaling Technology (Danvers, MA); anti-MYH11 antibody was from Biomedical Technologies (BT-562, Stoughton, MA); anti-SRF antibody (sc-335) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Ki-67 and anti-smooth muscle α-actin (SM α-actin) were purchased from DAKO (Glostrup, Denmark); and anti-collagen I was a gift from Dr. Jane Sottile (University of Rochester, NY). To examine the specificity of SCPEP1 staining, anti-SCPEP1 was incubated with 1mg/ml full-length SCPEP1 overnight at 4°C prior to application as described.

Cell culture

Pulmonary artery smooth muscle cells, Cos-7 cells (ATCC, Rockville MD), rat aortic smooth muscle cells (RASMC, Cascade Biologics Inc., Portland, OR), and mouse vascular smooth muscle cells (MOVAS) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing high glucose, supplemented with 10% fetal bovine serum. Antibiotics and antimycotics were not included in the medium. Cells were grown to sub-confluency prior to each experiment.
**cDNA cloning, mutagenesis and transfection**

Total RNA was extracted from mouse BC3H1 cells with TRIzol reagent (Invitrogen). RT-PCR was performed using the ProSTAR Ultra HF RT-PCR System (Stratagene) employing a pair of Scpep1-specific primers (forward primer: 5'-gagctctcgcggcggatctgtctcgtgc-3', and reverse primer: 5'-ctcctgcttggtcaccagcttcatcatct -3'). The amplification product, corresponding to the open reading frame of Scpep1 less the initiating methionine and stop codon, was subcloned into pCMV-FLAG (Stratagene).

To generate a rescue Scpep1 construct to evade Scpep1 knockdown and two Scpep1 constructs containing the catalytic triad mutants, S167A and H431Y, mutation of wildtype Scpep1 was performed using a QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer's directions. All of the mutants were sequenced to confirm mutation of only the desired sequences and then subcloned into a new pCMV-FLAG backbone (Stratagene). All constructs were then incorporated into adenovirus per manufacturer’s specifications (Invitrogen).

For transfection studies, cells were grown to sub-confluence and transfected using Lipofectamine Plus reagents (Invitrogen). The vector without inserts was also transfected in parallel as a control. Cells were then allowed to grow for 24 or 48 hrs in complete medium. After washing with cold PBS, the cells were analyzed by Western blotting or immunostaining.

**Short hairpin RNA design and testing**

To generate the short hairpin RNA to Scpep1, potential RNAi oligo sites within Scpep1 were predicted using the RNAi Oligo Retriever (http://katahdin.cshl.org:9331/RNAi/html/rnai.html). Pairs of oligonucleotides were synthesized by a commercial distributor (Integrated DNA Technologies), annealed, and then ligated into BseRI-BamHI cut pSHAG (a generous gift of Dr.
In this vector, the short hairpin RNA to \textit{Scpep1} is under the control of the U6 RNA polymerase III promoter. Sequence was confirmed by in-house sequencing and correct clones were tested for silencing by transient transfections in Cos-7 cells followed by Western blotting. The most effective construct was then incorporated into adenovirus.

\textbf{Adenoviral construction, titering, and transduction}

According to the manufacturer's protocol, the short hairpin RNA \textit{Scpep1}, wildtype \textit{Scpep1}, Rescue \textit{Scpep1} and \textit{Scpep1}^{S167A} mutant vectors were recombined with pAd plasmids (Invitrogen) using LR clonase (Invitrogen) to create the adenoviral constructs. Following linearization with \textit{PacI} (New England Biolabs), each construct was transfected separately into HEK-293 cells with Lipofectamine 2000. The supernatant was harvested when adenoviral lysis was observed. A crude viral lysate was purified from the supernatant and tested by Western blot. Large scale adenoviral preparations were provided through the Maine Medical Center Research Institute’s Viral Vector Core Facility (\url{http://www.mmeri.org/facility/vector.html}). We then titered purified adenovirus with the Adeno-X Rapid Titer kit (BD Clontech).

For adenoviral transductions, cells were seeded in 100-mm or 6 well plates and allowed to adhere overnight. The cells from one plate were detached by trypsinization and counted with a hemocytometer to determine the number of adherent cells. The cells were transduced with a viral load of 10-100 MOI as shown in the text or figures and incubated overnight.
Generation of stable cell lines

To create stable cell lines expressing Scpep1 and the Scpep1T103A under the control of the CMV promoter, a HindIII/XbaI fragment encoding each was subcloned from pCMV-Scpep1 or pCMV-Scpep1T103A into pcDNA3.1 (Invitrogen) and subsequently verified by sequencing. The pcDNA3.1-Scpep1, Scpep1T103A or pcDNA3.1 vector constructs were transfected into a pulmonary artery SMC line with Lipofectamine 2000. Stably-expressing clones were selected for 2 weeks with 600 µg/ml G418 and tested for SCPEP1 expression by immunoblotting.

Immunoblotting

Adenovirus-transduced, transiently-transfected, or stably-transfected cells were washed in cold PBS and lysed with “crack buffer” [50 mM Tris-HCl (pH, 6.8), 100 mM DTT, 100 µg/ml PMSF, 2% SDS, 10% glycerol, and 1 µg/ml each of pepstatin A, leupeptin, and aprotinin]. Tissues from Scpep1 WT and KO mice were homogenized with the same lysis buffer. Cell or tissue lysates were electrophoresed on 10% SDS–PAGE gels under reducing conditions and transferred to nitrocellulose. The membranes were probed with antiserum to SCPEP1 (1:1000), cyclin D1 (1:3000), p27kip1 (1:2500), β-actin (1:4000) or α-tubulin (1:3500) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies (1:4000, Amershan Bioscience, UK) for one hour at room temperature. Signal was detected by enhanced chemiluminescence (Pierce Biotechnology Inc., Rockford IL). Some blots were imaged and quantitated using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln NE).
SMC growth assay

Virally-transduced SMC or stably-transfected cells were growth-arrested and then stimulated with 10% FBS over a 5-day period. Trypan Blue-excluding cells were counted with a hemocytometer (triplicate wells) and data were analyzed by ANOVA with Tukey’s post-hoc testing for significance between groups using GraphPad Prism Software (San Diego, CA) as in previous studies. A second observer performed a similar analysis to verify initial findings.

SMC migration assays

A scrape wound assay was used to assess the effect of SCPEP1 on SMC migration as described. SMC were grown until 90% confluent, followed by transducing with adenoviruses overnight and then serum starvation for 24 hrs. At this point, several scrape wounds were performed across the dish with a sterile yellow pipet tip. The underside of the dish was carefully marked over the wound areas at three discrete fields of view to denote the areas of the wound to sample each day. We initiated experiments with treatment of cells with 10 ng/ml PDGF-BB. Photographs of the wound area were taken immediately after PDGF-BB addition (day 0) and at various times (0.5-2 days) post-wounding with an Olympus IX-70 microscope connected to a digital camera. At least three photographs were taken per well at each time point. Wound areas were determined using Image-Pro Software (Media Cybernetics, Inc., Bethesda, MD). Measurement of migration was determined by subtracting the cell-free area at different time points from the cell-free area at day 0. Data were presented as means ± SD of several independent experiments and analyzed by two-way ANOVA with Tukey’s post-hoc testing for significance between groups using Graphpad Prism software (GraphPad, San Diego, CA). A
modified Boyden-chamber assay was used to confirm the results of SMC migration as per the manufacturer's directions (Neuro Probe, Inc., Gaithersburg MD).

**Immunohistochemistry**

Tissues were fixed with 10% neutral buffered formalin overnight and then embedded in paraffin and sectioned. Sections were quenched with 3 % H₂O₂ and blocked with 5% normal goat serum in 0.1M PBS. Sections were incubated with rabbit antiserum to SCPEP1 (1:300), SRF (1:500), MYH11 (1:300) or collagen I (1:2000) overnight at 4°C. After rinsing in 0.1M PBS, sections were incubated with biotinylated goat anti-rabbit IgG (1:100) for one hour and the avidin–biotin complex (Vector, Burlingame, CA) for an additional one hour. Finally, sections were stained by DAB kit (Dako Cytomation, Carpinteria, CA), and then observed under an Olympus BX-41 light microscope (Olympus, Melville, NY). For detection of proliferating SMC in vessel wall, sections were incubated with rat antiserum to Ki-67 and mouse antiserum to SM α-actin at the same time and then liquid DAB Substrate Chromagen system and Vector Red (Vector) were applied per manufacturer’s directions. To test the specificity of the antiserum, the primary antibody was replaced with preabsorbed anti-SCPEP1 or non-specific rabbit IgG (Dako Cytomation, Carpinteria, CA) in some experiments. To assess SMC apoptosis, we used Apotag Peroxidase Apoptosis Detection Kit (Chemicon) as per manufacturer’s protocol and counterstained sections with hematoxylin.

**Scpep1-KO mouse**

The *Scpep1*-targeting vector was designed and constructed by Dr. Jiyuan Chen. The targeting vector was submitted to the University of Rochester Transgenic Core for ES cell targeting and blastocyst microinjections. Chimeric mice were obtained and bred to the F1 generation.
Heterozygous mice were then interbred to obtain homozygous null mice and wildtype littermate control mice.

**Southern blotting and PCR genotyping**

Mice were properly anesthetized prior to tail-snipping for genomic DNA isolation to do Southern blotting. Briefly, mouse genomic DNA from tail tissues was extracted by Puregene® DNA purification kit (Stratagene) and digested by EcoRI. Southern blotting was done with standard methods as described 6 using a probe external to the 3’ homology arm of mouse Scpep1 DNA as shown (Red P4, Online Figure IIA).

Genomic tail DNA was isolated for PCR genotyping from weaned mice. Adult mice were properly anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) prior to tail-snipping for genomic DNA isolation. Briefly, two separate PCR reactions were performed: one for the presence of LacZ and the other for the presence (if heterozygous) or absence (if homozygous) of exon 1 in Scpep1 (forward primer: 5’-acccactctttgtcataacccac-3’, reverse primer for LacZ: 5’-tcgccattcaggctgcgcaa-3’, and reverse primer for exon 1 of Scpep1: 5’-gcgctgaagcccagtaagaacgatagg-3’).

**Quantitative RT-PCR**

Total RNA was extracted from kidney using a commercial kit (TRIzol, Invitrogen) and, following the first strand cDNA synthesis (Invitrogen), quantitative RT-PCR was performed with a BioRad MyIQ cycler. Primers flanking introns of Ctsa and Cpvl were designed (Ctsa forward 5’-aatctcagctggctgctgtg-3’ and reverse 5’-taagtggcagggcaggtgagatgtgt-3’, 230 bp product; Cpvl forward 5’-atacagaaaaggcagtcggag-3’ and reverse 5’-tcattggctggtcatagggca-3’, 140 bp
product) and synthesized by a commercial distributor (Integrated DNA Technologies). Quantitative PCR was done with Syber Green and normalized to an internal control (Hprt). Fold changes in the relative gene expression of each gene in Scpep1-KO versus WT control mice were calculated by the standard $2^{-\Delta\Delta C_t}$ method as described. 7

**Blood pressure tests**

We used a tail-cuff method (BP2000) to assess systolic blood pressure in WT versus Scpep1-KO mice. Systolic blood pressures and pulses were taken without knowledge of the genotype result.

**Carotid artery ligation**

Scpep1-KO mice used for ligation injury were backcrossed to C57BL/6J mice for 4 generations (the mixed genetic background: 6.25% SV129svev/ 93.75% C57BL/6J). 12-week-old Scpep1-KO or wildtype littermate mice were subjected to complete common carotid artery ligation. 8, 9 Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was dissected free from the surrounding connective tissues and was completely ligated with 6-0 silk just proximal to the carotid bifurcation. Two groups of operated animals were processed for morphometric studies at 4 weeks after the operation (5 mice per group).

**Morphometric analysis**

Both injured and contralateral carotid arteries were isolated 1 or 4 weeks after ligation for morphometric analyses. Mice were euthanized by sodium pentobarbital and perfused with
normal saline and 10% neutral buffered formaldehyde. The carotid arteries were isolated, fixed with 10% neutral buffered formalin for 24 hours, embedded in paraffin, and sectioned to 5-6 μm thickness. The entire length of the injured and contralateral carotid arteries was sectioned from the ligation site right below the bifurcation of common carotid artery, and 0.6 mm away from the ligation site, 6 sections located at 200-μm intervals were examined per mouse. Digital images of vessels were captured and vessel areas were measured in a blind manner using Image Pro Plus software (Media Cybernetics Inc, Silver Spring, MD). In brief, the intimal area was calculated by subtracting the luminal area from the internal elastic lamina area, and the medial area was calculated by subtracting the internal elastic lamina area from the external elastic lamina area. EEL area was defined as the area included with external elastic lamina.

**Statistical analysis**

All data shown as means ±SD are representative of at least three independent experiments.

Unpaired Student’s t test and 2-way ANOVA with post-hoc testing were used for analysis with GraphPad Prism software (GraphPad, San Diego, CA). Significance was determined at p < 0.05.
Supplemental References


Legends to Online Figures

**Online Figure I. Scpep1 shRNA targeted sequence and domain homologies**
(A), Schematic showing nucleotides 970-996 (amino acids 303-311) of mouse Scpep1 cDNA targeted by the shRNA to Scpep1 and silent mutations (red nucleotides in lower sequence) created in full-length Scpep1 to evade knockdown (Rescue transgene, Res). (B) Amino acid sequence comparisons of catalytic triad domain between human (Hsa), mouse (Mmu), and rat (Rno) SCPEP1 with CTSA and CPVL paralogs. Numbers reflect the amino acid positions of each species.

**Online Figure II. Generation and validation of Scpep1 KO mouse**
(A), Targeting strategy for the generation of Scpep1 null mice. Homologous recombination (HR) results in replacement of Exon 1-Exon 2 with a nuclear (n) LacZ-Neo cassette. Restriction enzyme sites are indicated by single letters. (B), Southern blotting of EcoRI digested tail genomic DNA from wildtype (WT), heterozygous (HET), and Scpep1 knockout (KO) mice. The external probe used is indicated by P4 (red heavy line) in panel A. (C), Immunoblotting of SCPEP1 protein in WT, HET and Scpep1- KO mouse kidney (Kid) and aorta (Ao). Note complete absence of the 35-kDa SCPEP1 protein (arrow) in KO tissues. (D), Immunostaining of SCPEP1, SRF and MYH11 in adult WT and KO kidney and aorta. Abbreviations are: (d) distal convoluted tubules; (g) glomerulus; (p) proximal convoluted tubules; NS: non-specific bands; B: BamHI; E: EcoRI; H: HindIII; N: NotI; RV: EcoRV; X: XhoI.
Online Figure III. Characterization of Scpep1-KO mice

(A), Genotyping of Scpep1-KO mice using primers described in Supplemental Materials and Methods.  (B), RT-PCR of Scpep1 mRNA in WT, HET and Scpep1-KO kidney samples.

Online Figure IV. Histology of Scpep1-KO mice

A. urinary bladder; B. coronal section of brain; C. cerebellum; D. cross section of esophagus; E. cross section of intestine; F. heart; G. liver; H. lung; I. skeletal muscle; J. spleen; K. stomach; L. cross section of testis taken from Scpep1 null mice. Hematoxylin and eosin stained sections were evaluated in blind format by two independent investigators (T-H L and JMM).

Online Figure V. Comparison of body weights, systolic pressures and heart rate between WT and Scpep1-KO mice

Body weights before (A in days, D) and after (B, in months, M) weaning were obtained in the indicated gender and genotype of mice (n=6 per condition). Moreover, no differences in blood pressure (C) and heart rate (D) were noted between WT and Scpep1-KO littermates (n = 4).

Online Figure VI. Quantitative RT-PCR assay for Ctsa and Cpvl in Scpep1-KO mice

Quantitative RT-PCR of whole kidney samples taken from WT control and Scep1 KO mice (n = 4). Expression data were normalized to Hprt according to the methods above. Please note, antibodies are not readily available to these proteins precluding a more definitive interrogation of the corresponding proteins.
Online Figure VII. Neointimal formation in wildtype versus Scpep1 KO vessels

Wildtype (A) and Scpep1 KO (B) carotid artery underwent ligation injury and 28 days later stained with Verhoeff-Van Gieson. Note reduced neointimal load and vessel caliber in Scpep1 KO vessel similar to that shown in Figure 7D.

Online Figure VIII. Evaluation of collagen I expression in injured carotid artery

Photomicrographs showing immunohistochemistry of collagen I in normal carotid artery (A) or in ligated carotid artery from WT (B) and Scpep1-KO (C) mice 7 days post ligation. (D), Quantification (optical density per mm²) shows no significant differences in intimal and medial collagen I staining between Scpep1-KO and WT mice 7 and 28 days post-ligation injury.

Online Figure IX. Apoptosis Rates in Scpep1 KO Versus WT Vessels

All cross sections of 7 day-ligated carotids from WT and Scpep1-KO mice were stained with TUNEL assay kit (see Methods). Quantification of SMC apoptosis (percent of total cells staining positive for TUNEL in 3 independent vessels) was ascertained in intima and media of WT and Scpep1-KO mice 7 days post-ligation. No significant difference was noticed between the two groups.
Online Figure I

A

Sc pep1: A CAC CTG CAA GGA GAC GCC TTA AGT CAA C
Res: A CAC CTG CAA GGA GAC GCC G CTC TCC CAG C

H L Q G D A L S Q

B

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Online Figure II

A 1 kb

WT Sccep1

Targeting Vector

B

WT HET KO

WT → Targeted

C

SCPEP1

Tubulin

NS

D

SCPEP1

SRF

MYH11

WT

KO

Kidney

Aorta

a b c d e f g h

25 μm

40 μm
A  Genotyping

B  RT-PCR

Scpep1

Gapdh
Online Figure VI

mRNA Expression (relative level)

WT  KO  WT  KO

Ctsa  Cpvf1