Response Gene to Complement 32 Promotes Vascular Lesion Formation Through Stimulation of Smooth Muscle Cell Proliferation and Migration

Jia-Ning Wang, Ning Shi, Wei-Bing Xie, Xia Guo, Shi-You Chen

Objective—The objectives of this study were to determine the role of response gene to complement 32 (RGC-32) in vascular lesion formation after experimental angioplasty and to explore the underlying mechanisms.

Methods and Results—Using a rat carotid artery balloon-injury model, we documented for the first time that neointima formation was closely associated with a significantly increased expression of RGC-32 protein. Short hairpin RNA knockdown of RGC-32 via adenovirus-mediated gene delivery dramatically inhibited the lesion formation by 62% compared with control groups 14 days after injury. Conversely, RGC-32 overexpression significantly promoted the neointima formation by 33%. Gain- and loss-of-function studies in primary culture of rat aortic smooth muscle cells (RASMCs) indicated that RGC-32 is essential for both the proliferation and migration of RASMCs. RGC-32 induced RASMC proliferation by enhancing p34CDC2 activity. RGC-32 stimulated the migration of RASMC by inducing focal adhesion contact and stress fiber formation. These effects were caused by the enhanced rho kinase II-α activity due to RGC-32-induced downregulation of Rad GTPase.

Conclusion—RGC-32 plays an important role in vascular lesion formation following vascular injury. Increased RGC-32 expression in vascular injury appears to be a novel mechanism underlying the migration and proliferation of vascular smooth muscle cells. Therefore, targeting RGC-32 is a potential therapeutic strategy for the prevention of vascular remodeling in proliferative vascular diseases. (Arterioscler Thromb Vasc Biol. 2011;31:e19-e26.)

Key Words: restenosis • migration • proliferation • response gene to complement 32 • vascular smooth muscle cells

Percutaneous coronary intervention has been widely used in patients with coronary artery disease. However, restenosis following percutaneous coronary intervention limits the long-term outcome of percutaneous coronary intervention, and patients frequently require repeated revascularization procedures. Systemic pharmacological approaches to reduce restenosis have not been successful in clinical use. Local treatment with a drug-eluting stent has demonstrated significant reduction in restenosis rate and the subsequent need for revascularization. However, a drug-eluting stent increases the risk of late stent thrombosis. Therefore, restenosis remains a medical challenge in cardiovascular field.

A large body of clinical and experimental studies have demonstrated that neointima formation contributing to restenosis is triggered by complex biological responses, including inflammation, thrombosis, cellular proliferation, and extracellular matrix production. Among these multiple factors, media-to-intima migration and proliferation of vascular smooth muscle cells (VSMCs) with subsequent synthesis of extracellular matrix is the most critical step in the pathogenesis of neointima formation. Although many factors are found to be involved in VSMC migration or proliferation, the endogenous regulators contributing to neointima formation remain largely unknown.

Emerging data suggest that the response gene to complement 32 (RGC-32) plays a role in VSMC function. RGC-32 can be activated by complement C5b-9, serum, and other growth factors. Functionally, RGC-32 has been shown to stimulate or suppress endothelial cell growth depending on the physiological or pathological conditions. In VSMCs, RGC-32 plays an important role in cell proliferation. RGC-32 physically associates with cyclin-dependent kinase p34CDC2, which increases the kinase activity to induce quiescent aortic smooth muscle cells to enter the S phase. However, it is unknown whether RGC-32 plays a role in neointima formation following vascular injury in vivo and whether RGC-32 contributes to VSMC migration.

In the present study, we used a well-defined rat carotid artery balloon-injury model and an adenoviral gene delivery approach to test the hypothesis that RGC-32 contributes to lesion formation after vascular injury. We found that RGC-32...
was activated along with the progression of neointima formation in balloon-injured carotid arteries. Knockdown of RGC-32 markedly inhibited the lesion formation, whereas RGC-32 overexpression exacerbated the injury-induced vascular remodeling. RGC-32 appeared to stimulate both migration and proliferation of VSMCs. RGC-32 promoted VSMC migration via enhancing the focal adhesion contact and stress fiber formation, which is due to RGC-32-mediated down-regulation of Rad GTPase. Our data indicate that targeting RGC-32 may be a promising therapeutic strategy for the prevention of lesion formation in proliferative vascular diseases.

Methods

Animals
Male Sprague-Dawley rats weighing 450 to 500 g were purchased from Harlan. All rats were housed under conventional conditions in the animal care facilities. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the institutional animal care and use committee of the University of Georgia.

Cell Culture
Rat aortic smooth muscle cells (RASMCs) were cultured by the explant method from rat thoracic aorta as described previously.18,17 Rat thoracic aortas were removed and washed with Dulbecco’s modified Eagle’s medium. Aortic media were carefully dissected from the vessels, cut into pieces (<1 mm³), and explanted onto a modified Eagle’s medium. Aortic media were carefully dissected from the vessels, cut into pieces (<1 mm³), and explanted onto a modified Eagle’s medium supplemented with 20% fetal bovine serum (FBS), penicillin, and streptomycin was slowly added, and cells were allowed to grow at 37°C in a humidified atmosphere of 5% CO₂ for 2 weeks. RASMCs were confirmed by expression of smooth muscle α-actin and SM22α.

Construction of Adenovirus
RGC-32 cDNA was subcloned into the XhoI site of pShuttle-IRES-GFP vector. The pcDNA3.1 vector was used as a control. Recombinant adenovirus was produced by homologous recombination in AD-1 competent cells following the manufacturer’s instructions (Agilent).18–20 The resultant recombinant adenovirus was infected into 293 cell or tissue debris by centrifugation, 20

Western Blot Analysis
RASMCs or rat carotid arteries were homogenized in homogenization buffer (50 mmol/L Tris-HCl, pH 7.5/150 mmol/L NaCl/1% SDS/protease inhibitor cocktail [Sigma-Aldrich]). After removal of cell or tissue debris by centrifugation, 20 μg of proteins were separated by 10% SDS-PAGE and were transferred to polyvinylidene difluoride membrane (Bio-Rad). Antibodies against RGC-32, Rad GTPase, and α-tubulin were used for immunoblotting. Western blotting was performed as described previously.

Wound Healing Assay
RASMC migration was evaluated by wound healing assay using the CytoSelect Wound Healing Assay Kit (Cell Biolabs).9,29 Wound healinginserts were put into 24-well cell culture plates coated with fibronectin. Cell suspension (250 μL) was added to either side of the insert and incubated overnight to form a monolayer. The inserts were then removed to allow the cells to migrate. Images of wound healing were captured using a dissection microscope at a magnification of 40X. Cell migration was quantified by blind measuring the migration distances.

Cell Proliferation Assay
RASMC cell proliferation was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay using a TACS MTT Cell Proliferation Assay Kit (Trivegen).30 The optical density at 570 nm was measured.
Immunoprecipitation and ROK Kinase Assay

RASMCs was transfected with myc-tagged rho kinase II (ROKα) expression plasmid using Lipofectamine LTX plus (Invitrogen) and infected with Ad-expressing GFP, dominant-negative Rad GT-Pase, RGC-32 shRNA, or a combination thereof. Forty-eight hours after transduction, the cells were scraped off and lysed in an immunoprecipitation buffer (Pierce). The cell lysates were subjected to immunoprecipitation with anti-myc monoclonal antibody (clone 4A6, Upstate). Immunoprecipitates were washed 5 times with kinase-assay buffer and resuspended in 50 μL of kinase-assay buffer. ROKα kinase activity was measured by using a ROCK Assay Kit following the manufacturer’s recommended protocols (Cell Biolabs, Inc). Phosphorylated MYPT-1 (ROKα substrate) was detected by Western blot using p-MYPT-1 antibody provided in the kit.

Statistical Analysis

All data were evaluated with a 2-tailed, unpaired Student t test or compared by 1-way ANOVA followed by the Fisher test and are expressed as mean±SD. A value of P<0.05 was considered statistically significant.

Results

RGC-32 Was Activated in Carotid Artery Following Balloon Withdrawal Injury

As an initial step to define the role of RGC-32 in vasculature, we established a highly characterized rat balloon withdrawal injury model. Normal and balloon-injured rat carotid arteries at 1, 3, 7, and 14 days after surgery were harvested and paraffin embedded. Vessel sections were stained with hematoxylin-eosin (Figure 1A). Neointima was first observed at 3 days and progressively increased at 7 and 14 days after the injury, as reported previously.21,22

To determine the role of RGC-32 in neointima formation, RGC-32 expression in normal and injured vessels was examined by immunohistochemistry staining (Figure 1B). RGC-32 expression was negligible in the uninjured arteries and the injured vessels at 1 day after surgery. In contrast, strong expression of RGC-32 was observed in the developing neointima and media at 3, 7, 14 days after the injury. To quantify RGC-32 expression following vascular injury, we performed Western blot analysis using proteins extracted from balloon-injured rat carotid arteries at different time points. RGC-32 protein expression was upregulated at 3 days, and reached its peak at 7 days, and remained at the elevated level at 14 days after the injury (Figure 1C). Quantitative analysis showed that RGC-32 protein expression was upregulated by 2.4-fold at 1 day, 4.9-fold at 3 days, 16-fold at 7 days, and 8.1-fold at 14 days after the injury compared with uninjured vessels (Figure 1D). These data suggest that RGC-32 is involved in vascular lesion formation.

RGC-32 Promoted Neointima Formation In Vivo

To investigate the role of RGC-32 in neointimal formation in vivo, recombinant Ads Ad-GFP, Ad-RGC32, and Ad-shRGC32 were generated. We knocked down or overexpressed RGC-32 in the injured vessels via Ad-mediated gene transfer. The distal segments of injured left carotid arteries were incubated with saline, Ad-GFP, Ad-RGC32, or Ad-shRGC32 for 20 minutes immediately after the balloon injury and were harvested, fixed, paraffin-embedded, and sectioned 14 days later. Immunohistochemistry staining showed no significant change in RGC-32 expression between saline-treated and Ad-GFP-transduced vessels. However, RGC-32 expression was markedly increased in Ad-RGC32-transduced balloon-injured vessels but was greatly inhibited in Ad-shRGC32–transduced vessels as compared with the saline or Ad-GFP-incubated vessels (Figure 2A). These results indicate that exogenously introduced Ad-RGC32 was effectively expressed in the distally injured carotid artery and that Ad-shRGC32 successfully blocked RGC-32 expression in the neointima in vivo (Figure 2A).

Importantly, balloon-injured carotid arteries transduced with Ad-RGC32 exhibited a marked increase in neointima formation compared with arteries transduced with Ad-GFP (Figure 2B). Morphometric analysis of elastic-stained sec-
tions showed that the expression of Ad-RGC32 increased neointima area by 33% compared with Ad-GFP-transduced vessels (0.180 ± 0.005 versus 0.135 ± 0.003 mm²; \( P < 0.05 \), \( n = 6 \), Figure 2C). Conversely, arteries transduced with Ad-shRGC32 exhibited a significantly reduced neointima compared with those treated with Ad-GFP or saline (Figure 2B). Quantitative analysis showed that RGC-32 knockdown inhibited neointimal formation by 62% compared with Ad-GFP-transduced vessels (0.051 ± 0.002 versus 0.135 ± 0.003 mm²; \( P < 0.01 \), \( n = 6 \), Figure 2C). Similar results were obtained when intima/media area ratios were compared (Figure 2D). No significant difference in neointima formation was observed between Ad-GFP- and saline-treated groups (Figure 2C), consistent with the RGC-32 expression (Figure 2A).

**RGC-32 Promoted VSMC Proliferation**

VSMC proliferation plays an important role in neointima formation after vascular injury. To delineate the mechanism by which RGC-32 promoted neointimal formation, we first evaluated RGC-32 function in VSMC proliferation in vitro using the adenoviral vectors that were used in the in vivo studies. Quiescent RASMCs did not express RGC-32. Ad-RGC32 transduction, however, caused robust expression of RGC-32 in the RASMCs (Figure 3A, left). RGC-32 expression is induced in RASMCs by serum. Serum-induced RGC-32 was effectively blocked by Ad-shRGC32 transduction, but not by Ad-GFP transduction (Figure 3A, right). To determine the effect of RGC-32 on RASMC proliferation, MTT proliferation assay was performed. As shown in Figure 3B, RGC-32 overexpression by Ad-RGC32 transduction significantly promoted the proliferation of RASMCs as compared with control or Ad-GFP-transduced RASMCs (\( P < 0.01 \), \( n = 6 \)). On the other hand, shRNA knockdown of serum-induced RGC-32 by Ad-shRGC32 significantly inhibited the proliferation of RASMCs as compared with that of Ad-GFP or control (\( P < 0.01 \), \( n = 6 \), Figure 3C). These data demonstrate that RGC-32 plays an important role in VSMC proliferation. RGC-32 appeared to stimulate VSMC proliferation by regulating p34\(^{CDC2} \) (Cdk1) activity because RGC-32 overexpression by Ad-RGC32 significantly increased Cdk1 activation (phosphorylation at Thr161) (Figure 3D), consistent with previous study showing that RGC-32 regulates cell cycle via modulating Cdk1 activity.\(^{12} \) Because RGC-32 also regulates Akt signaling in endothelial cells, finding out whether or not RGC-32-mediated Cdk1 activity directly regulates VSMC cell cycle requires further investigation.\(^{16} \)

To determine whether RGC-32 promotes VSMC proliferation in neointima formation in vivo, we examined the expression of a cell proliferation marker PCNA in the artery sections treated with or without Ad-RGC32 or Ad-shRGC32. We found that vascular injury caused PCNA expression in VSMCs (Figure 3E). RGC-32 overexpression increased the number of VSMCs expressing PCNA. RGC-32 knockdown, however, markedly decreased the number of PCNA-positive VSMCs (Figure 3E to 3F). These data suggest that RGC-32 may play a role in VSMC proliferation in vivo, which contributes to the neointima formation following vascular injury.

**RGC-32 Promoted VSMC Migration**

VSMC migration from the media into the intimal surface of blood vessels is an important step during neointima formation.
after vascular injury. To further explore the mechanisms by which RGC-32 promotes intimal hyperplasia, we used a wound healing assay to evaluate the effect of RGC-32 on VSMC migration. RASMCs transduced with Ad-RGC32 were cultured in FBS-free Dulbecco’s modified Eagle’s medium, whereas those transduced with Ad-shRGC32 were cultured in medium containing 10% FBS. Immunostaining showed that RGC-32 was highly expressed in Ad-RGC32–transduced RASMCs, whereas it was effectively suppressed in Ad-shRGC32–transduced serum-treated RASMCs (Figure 4A, FBS groups). The wound healing assay showed that RGC-32 overexpression promoted migration of quiescent RASMCs as compared with Ad-GFP-treated cells (273/11006 31 versus 92/11006 28 m; P < 0.01, n = 6; Figure 4A and 4B, left panel). RGC-32 knockdown, however, inhibited serum-induced RASMC migration as compared with Ad-GFP-transduction (212/11006 19 versus 315/11006 20 m; P < 0.01, n = 6, Figure 4A and 4B, right panel). These data demonstrate that RGC-32 stimulates VSMC migration in vitro.

Platelet-derived growth factor (PDGF) is a potent regulator for VSMC migration. 32 To determine whether RGC-32–induced VSMC migration is related to PDGF activity or whether RGC-32 is a downstream target of PDGF, we treated RASMC with PDGF-BB and detected RGC-32 expression. We found that RGC-32 overexpression promoted migration of quiescent RASMCs as compared with Ad-GFP-treated cells (273±31 versus 92±28 μm; P < 0.01, n = 6; Figure 4A and 4B, left panel). RGC-32 knockdown, however, inhibited serum-induced RASMC migration as compared with Ad-GFP-transduction (212±19 versus 315±20 μm; P < 0.01, n = 6, Figure 4A and 4B, right panel). These data demonstrate that RGC-32 stimulates VSMC migration in vitro.

To investigate the mechanism by which RGC-32–induced VSMC migration, we examined whether RGC-32 affects cytoskeletal organization. Ad-GFP–, Ad-RGC32–, or Ad-shRGC32–transduced RASMC cells were cultured on fibronectin-coated glass coverslips. The focal adhesion contacts and actin stress fibers were then labeled by anti-vinculin and anti-α-actin antibodies, respectively. As shown in Figure 5, overexpression of RGC-32 in RASMCs enhanced both focal adhesion contacts (Figure 5A) and stress fiber formation (Figure 5B), whereas knockdown of RGC-32 inhibited them. These data document that RGC-32 stimulates VSMC migration via enhancing focal adhesion and stress fiber organization.

RGC-32 Suppressed Rad GTPase Expression in VSMCs
Rad (Ras associated with diabetes) is the prototypic member of Ras-related GTPase family. 33 It has been reported that Rad inhibits VSMC migration through inhibition of focal adhesion and stress fiber formation, leading to suppression of neointima formation following vascular injury. 11 Because RGC-32 promotes focal adhesion and stress fiber organization, we hypothesized that RGC-32 regulates Rad GTPase expression. To test this hypothesis, we examined the effect of RGC-32 on the expression of Rad GTPase in RASMCs. Western blot analysis showed that overexpression of RGC-32 completely blocked whereas RGC-32 knockdown by shRNA significantly increased Rad GTPase expression as compared with the expression in Ad-GFP-transduced RASMCs (Figure 6A and 6B). These data indicate that RGC-32 may promote...
VSMC migration and neointima formation, at least partially, through inhibition of Rad GTPase expression.

Rad GTPase inhibits VSMC migration by inhibiting the ROKα singling pathway. To determine whether downregulation of Rad GTPase by RGC-32 affects ROKα activity, we performed ROKα kinase assays. We found that RGC-32 knockdown by shRNA significantly inhibited ROKα kinase activity (Figure 6C and 6D), suggesting that RGC-32 may enhance ROK signaling to induce VSMC migration. Importantly, RGC-32 shRNA-attenuated ROKα activity can be restored by dominant-negative Rad GTPase (Figure 6C and 6D), suggesting that Rad GTPase mediated RGC-32-induced ROKα activity.

Discussion

RGC-32 has pleiotropic effects in diverse cellular processes, such as cell proliferation, differentiation, and immunity. RGC-32 overexpression causes quiescent human aortic smooth muscle cells to enter S-phase and G2/M in vitro. However, the role of RGC-32 in proliferative vascular dis-
eases has not been reported. Our studies demonstrate for the first time that RGC-32 plays a critical role in vascular lesion formation after experimental angioplasty. RGC-32 is progressively expressed in the neointima following vascular injury. Importantly, RGC-32 silencing dramatically inhibits the neointima formation, whereas RGC-32 overexpression markedly increases the size of the vascular lesion compared with the controls.

Accumulation of VSMCs in the vascular lesion is attributed to their proliferation and migration. RGC-32 appears to stimulate both VSMC proliferation and migration. Although RGC-32 function in endothelial cell proliferation could be stimulatory or inhibitory depending on physiological conditions,15,16 our data strongly support the possibility that RGC-32 is a positive regulator for VSMC proliferation. RGC-32 stimulates VSMC proliferation by enhancing the activation of cycle cell regulator p34CDC2, consistent with previous report.12,13 RGC-32 not only induces VSMC proliferation in vitro but also appears to play a role in VSMC proliferation in vivo. Vascular injury causes marked expression of PCNA, which is blocked by RGC-32 shRNA, suggesting that RGC-32 is involved in stimulating PCNA expression, leading to VSMC proliferation in the neointima formation.

In addition to its role in VSMC proliferation, our data demonstrate for the first time that RGC-32 also stimulates VSMC migration. Focal adhesion formation and cytoskeletal organization are key processes in cell locomotion and migration. RGC-32 appears to induce VSMC migration by enhancing focal adhesion and stress fiber organization. Mechanistically, RGC-32 regulates VSMC migration by altering the expression of Rad GTPase. Rad GTPase has been shown to inhibit VSMC migration, resulting in an attenuation of vascular lesion formation.11 RGC-32 overexpression inhibits Rad GTPase expression in RASMCs, whereas RGC-32 knockdown markedly increases it. Downregulation of Rad GTPase by RGC-32 causes an enhanced ROCK kinase activity, which ultimately induces VSMC focal adhesion and stress fiber formation, resulting in VSMC migration.


Response Gene to Complement 32 Promotes Vascular Lesion Formation Through Stimulation of Smooth Muscle Cell Proliferation and Migration
Jia-Ning Wang, Ning Shi, Wei-Bing Xie, Xia Guo and Shi-You Chen

Arterioscler Thromb Vasc Biol. 2011;31:e19-e26; originally published online June 2, 2011;
doi: 10.1161/ATVBAHA.111.230706
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/31/8/e19

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/06/02/ATVBAHA.111.230706.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Materials

Response Gene to Complement 32 Promotes Vascular Lesion Formation through Stimulation of Smooth Muscle Cell Proliferation and Migration

Jia-Ning Wang, Ning Shi, Wei-bing Xie, Xia Guo, Shi-You Chen

Manuscript ID#: ATVB/2011/230706

Detailed Methods:

Animals
Male Sprague-Dawley rats weighing 450-500 g were purchased from Harlan. All rats were housed under conventional conditions in the animal care facilities. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Care and Use Committee of The University of Georgia.

Cell culture
VSMCs were isolated by autogrowth of explant culture from the thoracic aortas of rat as described previously. Briefly, rat thoracic aortas were removed and washed with DMEM. Media were carefully dissected from the vessels, cut into pieces (<1 mm³). Tissue pieces were then explanted onto a 0.02% gelatin-coated cell culture flask. To get a firm attachment of tissue pieces, the flask was incubated upside-down for 1 hour and then DMEM supplemented with 20% FBS, penicillin and streptomycin was slowly added. Cells were allowed to autogrow for 2 weeks and then passaged until enough cells were obtained. VSMCs were maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO₂. VSMCs were characterized with the expression of smooth muscle α-actin and SM22α.

Construction of adenovirus
RGC32 cDNA was amplified from pT7-RGC32 and subcloned into the Xho I site of pShuttle-IRES-hrGFP-1 (Agilent) and was confirmed by sequencing. The sequences encoding rat RGC32 shRNA were as follows: top strand 5’ CGC GTC GAG CTC GAA GAC TTC ATC GCT GAT CTG GAT TCA AGA GAT CAG CGA TGA AGT CTT CGA GCT CTT TTT TCC AAA 3’; bottom strand 5’ AGC TTT TGG AAA AAA GAG CTC GAA GAC TTC ATC GCT GAT CTG GAT CTC TTG AAT CCA GAT CAG CGA TGA AGT CTT CGA GCT CGA 3’. Both strands were annealed and ligated into Mlu I and Hind III-digested pRNAT-H1.1/Adeno (Cat No.: SD1219, Genscript corporation). Recombinant adenoviral vectors were produced by homologous recombination within AD-1 competent cells according to commercial manufacturer’s manual of AdEasy XL system. The resultant recombinant vectors pAd-RGC32 and pAd-shRGC32 were
transfected into AD-293 cells, respectively, to package Ad-RGC32 or Ad-shRGC32 viral particles. The adenovirus was purified with gradient density ultracentrifugation of Cesium Chloride and dialyzed in dialysis buffer. The GFP adenovirus (Ad-GFP) was used as the control in this study.

**Rat carotid artery injury model and adenoviral gene transfer**

The rat carotid artery balloon injury model was based on a model described by Clowes et al. 7, 8 Briefly, rats were anesthetized by an intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (5 mg/kg). A 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare, Irvine, CA) was introduced through the left external carotid artery and advanced 4 cm toward the thoracic aorta. The balloon was inflated with 20 μL of 0.9% sodium chloride (saline) and then withdrawn through the common carotid artery to the carotid bifurcation with constant rotation between the fingers during denudation of the endothelia. This procedure was repeated two more times to ensure complete endothelial denudation. Heparin (200 u/kg) was then intraperitoneally injected to prevent thrombus formation. The protocol used for introducing adenovirus into rat balloon-injured carotid artery has been previously described. The injured carotid artery was washed with saline and incubated with 100 μL of saline or adenovirus (5x10⁹ pfu) expressing GFP, RGC32, or RGC32 shRNA, respectively, for 20 minutes. 14 days later, the balloon-injured segment of the artery from the proximal edge of the omohyoid muscle to the carotid bifurcation was perfused with saline and excised. The tissue was then fixed with 4% paraformaldehyde and paraffin-embedded. Subsequent morphometric analyses were performed in a double blinded manner.

**Histomorphometric analysis and immunohistochemistry staining**

Vessel segments were cut by serial sectioning (5 μm), and 10 sections that were evenly distributed in the vessel segment were collected for analysis. 9 The sections were stained with modified hematoxylin and eosin or Elastica van Gieson staining. Cross-sectional images were collected with a Nikon microscope and an Nikon camera (Nikon America Inc). The circumferences of the lumen, internal elastic lamina, and external elastic lamina were measured by image analysis (ImagePlus Softwares). For immunohistochemistry staining, sections were rehydrated, blocked with 5% goat serum and permeabled with 0.01% Triton X-100 in PBS, and incubated with RGC32 antibody overnight at 4°C. The sections were counterstained with hematoxylin.

**Immunofluorescent Staining**

Exponentially growing cells were plated on glass coverslips in 6-well cell culture plates and incubated overnight at normal cell growth conditions. Immunofluorescent staining was performed as previously described. 10

**Western blot Analysis**

VSMCs or rat carotid arteries were homogenized in homogenization buffer (50 mmol/L Tris_HCl, pH 7.5/150 mmol/L NaCl/1% SDS/protease inhibitor cocktail [Sigma-Aldrich]). After removal of tissue debris by centrifugation, 20 μg of proteins were separated on 10% SDS-PAGE and were transferred to immobilon transfer membrane (Millipore). Antibodies against RGC32, Rad GTPase, α-Tubulin were used for immunoblotting followed by HRP-conjugated secondary antibodies. All samples were loaded equally for each gel based on protein concentration. Western blot was performed as described previously. 11, 12
Wound healing assay
Migration evaluation was performed with wound healing assay. It was carried out according to the manufacturer’s manual of CytoSelect 24-well Wound Healing Assay (Cell Biolabs, Inc). In brief, 24-well cell culture plates were coated with fibronectin and wound healing inserts were put into the wells with the inserts aligned with the same direction and firm contact with the bottom of the wells. Cell suspension (250 µL) was added to either side of the open ends at the top of the insert, and incubated overnight to form a monolayer. Then the inserts were removed to begin the wound healing assay. For each well, pictures were taken on a dissection microscope at a magnification of x40. Cell migration was quantified by blind measuring the migrated distance.

Cell proliferation
Cell proliferation was evaluated with MTT assay according to commercial manufacturer’s instructions of TACS MTT cell proliferation assays (Cat No.: 4890-25-K, Trivegen, Inc.). The optical density at 570 nm was measured and compared.

Immunoprecipitation and ROK Kinase Assay
RASMCs was transfected with myc-tagged ROCK II (ROKα) expression plasmid using Lipofectamine LTX plus (Invitrogen), and infected with adenovirus expressing GFP, dominant negative Rad GTPase (Ad-Rad DN), and/or RGC32 shRNA. 48 hours after the transduction, the cells were scraped off and lysed in an immunoprecipitation buffer (Pierce). The cell lysates were subjected to immunoprecipitation with anti-myc monoclonal antibody (clone 4A6, Upstate). Immunoprecipitates were washed five times with kinase-assay buffer and resuspended in 50 µl of kinase-assay buffer. ROKα Kinase activity was measured by using a ROCK Assay Kit following the manufacturer’s recommended protocols (Cell Biolabs, Inc). Phosphorylated MYPT-1 (ROKα substrate) was detected by western blot using p-MYPT-1 antibody provided in the kit.

Statistical analysis
All data were evaluated with a 2-tailed, unpaired Student t test or compared by 1-way ANOVA followed by Fisher t test and are expressed as mean ± SD. A value of P<0.05 was considered statistically significant.

Supplemental References:
4. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW,
Wang et al. RGC-32 Promotes Neointima Formation

Vogelstein B, He TC. A protocol for rapid generation of recombinant adenoviruses using the adeasy system. Nat Protoc. 2007;2:1236-1247

Supplemental Figures:

Supplemental Figure I: Adenovirus transduction efficiency (100%) in cultured RASMCs.
Supplemental Figure II: In vivo adenovirus transduction efficiency.

The balloon-injured arteries were transduced with adenovirus expressing GFP for 20 min. 1, 4, and 7 days after the surgery, the arteries were paraffin-embedded, sectioned, and then stained with anti-GFP monoclonal antibody (1:200 dilution) followed by incubation with HRP-conjugated secondary antibody and DAB reaction. Sections were counterstained with hematoxylin. Yellow-brown color shows GFP expression. The adventitia staining was probably due to the spill of adenovirus to the outside of the arteries during injection. Arrows indicate internal elastic lamina (Magnification: 200 x). Note that adenoviral vector can effectively deliver and express GFP in the balloon-injured carotid arteries, especially in the VSMCs.