CCN1 Knockdown Suppresses Neointimal Hyperplasia in a Rat Artery Balloon Injury Model

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Objective—CCN1 (Cyr61) is an extracellular matrix-associated protein involved in cell proliferation and survival. CCN1 is bound to vascular smooth muscle cells (VSMCs) via integrins and is expressed in VSMCs in atherosclerotic lesions, suggesting involvement in the regulation of vascular smooth muscle cell (VSMC) proliferation and atherosclerosis. We hypothesized that knockdown of CCN1 may inhibit VSMC proliferation and suppress neointimal hyperplasia.

Methods and Results—We examined the effect of the knockdown of CCN1 using rat cultured VSMCs and a rat balloon injury model. CCN1 stimulated adhesion and migration of VSMCs in a dose-dependent manner, and this was blocked by an antibody for integrin αβ1. Moreover, knockdown of endogenous CCN1 by lentiviral delivery of siRNA significantly inhibited proliferation of VSMCs and the uptake of 5-bromo-2′-deoxyuridine (BrdU). Replenishment with recombinant CCN1 reversed the effect of siRNA knockdown. Interestingly, knockdown of CCN1 significantly suppressed neointimal hyperplasia in a rat carotid artery balloon injury model at days 14 and 28 after injury. Gene transfer of CCN1 to smooth muscle reversed the effect of CCN1 knockdown on neointimal formation. These results suggest that endogenous CCN1 regulates proliferation of VSMCs and neointimal hyperplasia.

Conclusion—Inhibition of CCN1 may provide a promising strategy for the prevention of restenosis after vascular interventions. (Arterioscler Thromb Vasc Biol. 2008;28:1077-1083)

Key Words: CCN1 ■ neointimal hyperplasia ■ small interfering RNA ■ vascular smooth muscle cell

Percutaneous coronary intervention (PCI) is widely used for the treatment of coronary artery disease. Although drug eluting stents have shown considerable promise in reducing the incidence of restenosis,1 it remains a considerable medical challenge.2 Because in-stent restenosis is primarily caused by proliferation of vascular smooth muscle cells (VSMCs),3 a complete understanding of the biological stimuli that initiate and promote VSMC proliferation is important.

CCN1 (cyr61) is a member of the CCN (cyr61, ctgf, nov) protein family. CCN1 is associated with the extracellular matrix and mediates angiogenesis, cell growth, and cell survival through cell-type-specific binding to integrins.4–12 CCN1 is expressed in VSMCs during embryonic development, and its deletion by gene targeting suggested a critical role for CCN1 in vascularization of the placenta.13 CCN1 was shown to be upregulated in balloon-injured carotid arteries of adult rats, atherosclerotic aortas in apolipoprotein E–deficient mice14 and human carotid atherectomies.15 Moreover, CCN1 was shown to support VSMC adhesion and stimulate chemotaxis through αβ1 integrin.16 These results suggested that CCN1 may be involved in neointimal hyperplasia and the development of atherosclerosis through interaction with VSMCs.

In this study, we examined the effect of CCN1 on VSMC proliferation and investigated whether knockdown of CCN1 could suppress neointimal hyperplasia in a rat balloon injury model. The results suggested that inhibition of CCN1 may provide a novel strategy for the treatment of vascular diseases.

Methods

Proteins, Antibodies, and Reagents
Details of the additional methods and materials are provided in the online Data Supplement section (http://atvb.ahajournals.org).18,19

Cell Culture and Adhesion Assay
Details of the additional methods and materials are provided in the online Data Supplement section.

Real-Time Polymerase Chain Reaction
Total RNA isolation, cDNA synthesis, and real-time PCR were performed as previously described.20 Primer sequences used for quantification of CCN1 and GAPDH are listed in the online Data Supplement section.

Lentivirus-Mediated Delivery of Small Interfering RNA (siRNA)
Lentivirus mediated siRNA construct was designed as previously described.17 Briefly, annealed oligonucleotides encoding sense
and antisense strands linked by the loop sequence were subcloned into pSiNsi-mU6 vector (Takara Bio, Shiga, Japan). We designed the siRNA sequences (CR1-sense: 5'-GUGGAGUUAACAGAAC-A3'; CR1-antisense: 5'-UGUUUCUGUUAACUCCAC-3'; CR2-sense: 5'-AGGAGGAGUUAACAGAAC-3'; CR2-antisense: 5'-UUCUUGUUAACUCCAC-3') with 100% homology to the full length CCN1 using siDirect (University of Tokyo, Japan) and confirmed that there was no significant homology with other known sequences using the database of the National Center for Biotechnology Information. A nonsilencing control (NSC) sequence was designed according to a negative-control siRNA purchased from B-Bridge International. The siRNA producing construct was introduced into a lentivirus vector, pLenti6/V5-d-TOPO (Invitrogen Life Technologies), and the recombinant lentiviruses were propagated in 293T cells. Transfection of VSMCs was performed by replacing the culture medium with virus-containing medium followed by centrifugation of the culture plates at 2500 rpm for 30 minutes at 30°C. After incubation in virus-containing medium for 24 hours and in serum-free medium for 48 hours, the cells were used for analysis. For in vivo transfection, the supernatant was changed with 0.5% FBS. The number of VSMCs was counted after 72 hours of lentivirus-mediated GFP transfection.

Transwell Migration Assay and Western Blotting
Details of the additional methods and materials are provided in the online Data Supplement section.

Proliferation Assay and BrdU Uptake
Rat VSMCs were plated at 1.0×10^4/well into a 24-well plate coated with collagen type I (cellmatrix type I-C; Nitta Gelatin) and incubated in DMEM containing 10% FBS for 24 hours. Recombinant lentivirus generated in 293T cells were transfected into VSMCs as described above, and proliferation assay was performed in DMEM with 0.5% FBS. The number of VSMCs was counted after 72 hours using a cell counter (COULTER particle counter, GMI), and BrdU uptake by VSMCs was quantified using a Cell Proliferation ELISA BrdU kit (Roche Diagnostics GmbH).

Rat Carotid Artery Balloon Denudation Injury
Adult male Sprague–Dawley rats weighing 400 to 450 g (Japan SLC) were anesthetized with pentobarbital (Dainippon Sumitomo Pharma) and heparinized with 100U/kg heparin sodium. Balloon denudation was performed 6 times in the left common carotid artery with a 2-French catheter as previously described. Six rats were used in each group. In a preliminary experiment, we performed balloon injury in 10 rats and the success rate of intimal hyperplasia was 100%. After balloon injury, a 24G indwelling needle was inserted into the balloon injured region from the distal portion of left external carotid artery. Fifty picograms (p24 antigen) of indicated lentiviral vectors diluted to a total volume of 100 μL was instilled into the arterial segment that was isolated by vascular clamps and incubated for 30 minutes. After removal of this solution and needle, the external carotid artery was ligated and blood flow to the common carotid artery was restored. Rats were euthanized 3, 14, and 28 days after injury. The blood vessels were rinsed with PBS and embedded in OCT compound (Tissue Tek) for immunohistochemical analysis. Animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Morphometry
Morphometry was performed as previously described. Briefly, cryosections (6-μm thickness, 300 μm apart) were taken from the middle portion of the balloon injured segment, and 5 slices of each sample were analyzed (Zeiss, Axioskop2 plus) for neointima area and total intimal cells. Cell number was analyzed by counting nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI, Boehringer). Morphometric analysis of areas was performed on these sections after visualization of arterial elastic laminae with Evans Blue (0.3%) staining and fluorescent light. Sections were then photographed, digitalized, and analyzed with Zeiss KS400 software (Feldlicht Switzerland).

Immunohistochemistry
The method for immunohistochemistry is available in the online supplement section.

Statistical Analysis
Data were analyzed by 1-factor ANOVA followed by Fisher PLSD as a posthoc test. A probability value <0.05 was considered statistically significant.

Results
Effect of CCN1 on VSMC Adhesion In Vitro
We first examined the effect of CCN1 on adhesion of VSMCs. A solid-phase binding assay showed that immobilized CCN1 was bound to VSMCs in a dose-dependent and saturable manner (Supplemental Figure IA). Moreover, blocking antibodies against integrin α6 or β1 (GOH3 or P4C10) significantly inhibited adhesion of VSMCs to CCN1, but blocking antibodies against integrins αvβ3 had no effect on VSMC adhesion (Supplemental Figure IB).

CCN1–Integrin Interaction on VSMC Migration and Proliferation In Vitro
To investigate the effect of CCN1 on VSMC migration, we performed a transwell migration assay. VSMCs migrated to soluble CCN1 in a dose-dependent manner (Figure 1A). Interestingly, treatment of VSMCs with a blocking antibody against integrin α6 or β1 (GOH3 or P4C10) significantly inhibited the VSMC migration, whereas treatment with anti-αV/β3 antibody (LM609) had no effect (Figure 1B). This result indicated that the effect of CCN1 on VSMC migration was mediated via integrin α6β1. Moreover, the effect of CCN1 on BrdU incorporation was significantly blocked by the treatment of VSMCs with GOH3 or P4C10 antibody, but not by the treatment with LM609 antibody (Figure 1C), indicating that the integrin α6β1 mediated the proliferative effect of CCN1 on VSMCs.

Effect of Knockdown of CCN1 on VSMC Proliferation In Vitro
We found that CCN1 was involved in VSMC adhesion, migration, and proliferation in human VSMCs and that this effect was mediated through an interaction with integrin α6β1. To determine whether endogenous CCN1 had an effect on VSMC proliferation, we attempted knockdown of CCN1 using siRNA delivered by lentivirus. We generated 2 siRNA constructs directed against different regions of CCN1 (CR1 and CR2). CR1 and CR2 decreased the expression of CCN1 both at mRNA and protein levels in rat VSMCs (Figure 2A). Results of real-time PCR are shown as the means±SD of 6
Knockdown of CCN1 Suppressed Neointimal Hyperplasia in a Rat Balloon Injury Model

We investigated whether knockdown of CCN1 could suppress VSMC proliferation and neointimal hyperplasia in vivo using a rat carotid artery balloon-injured model. At 14 days after balloon-injury, immunohistochemical analysis revealed that CCN1 expression was upregulated in the media and neointima (Supplemental Figure IIA). Upregulation of CCN1 in balloon-injured arteries was also shown by Western blotting both in total lysate and membrane fraction of carotid arteries (Supplemental Figure IIB). Double immunofluorescence staining revealed that most of the cells expressing CCN1 in these areas were VSMCs (Supplemental Figure IIC). Upregulation of CCN1 expression in VSMCs in the neointima of injured arteries suggested that CCN1 may be involved in neointimal formation in vivo.

We first examined the efficiency of lentiviral transfection in rat carotid arteries by delivering lentivirus expressing GFP to balloon-injured carotid arteries. We evaluated the efficiency of the lentivirus-mediated delivery system using anti-GFP antibody and diaminobenzidine (DAB) immunostaining to avoid chromogenic reaction of the elastic lamina. Immunohistochemical studies revealed GFP expression in the neointima, suggesting that the lentivirus-mediated delivery system was effective in rat carotid arteries (Supplemental Figure III). The percentage of GFP-positive nuclei was 76.5±9.9%. We then delivered lentiviral constructs expressing nonsilencing control (NSC), CCN1, and siRNAs for CCN1 (CR1 and CR2) to rat carotid arteries. Downregulation of CCN1 protein (whole artery and membrane compartment) by transfection with CR1 or CR2 was confirmed by Western blotting (Figure 3A). Cotransfection with CCN1 encoding vectors reversed the downregulation of CCN1 by CR2. The effects of CR1 and CR2 on CCN1 expression were confirmed in vivo in rat carotid arteries by delivering lentivirus expressing GFP containing CCN1 (CR1 and CR2) to rat carotid arteries. Downregulation of CCN1 protein (whole artery and membrane compartment) by transfection with CR1 or CR2 was confirmed by Western blotting (Figure 3A). Cotransfection with CCN1 encoding vectors reversed the downregulation of CCN1 by CR2. The effects of CR1 and CR2 on CCN1 expression were confirmed in vivo in rat carotid arteries by delivering lentivirus expressing GFP containing CCN1 (CR1 and CR2) to rat carotid arteries. Downregulation of CCN1 protein (whole artery and membrane compartment) by transfection with CR1 or CR2 was confirmed by Western blotting (Figure 3A). Cotransfection with CCN1 encoding vectors reversed the downregulation of CCN1 by CR2. 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regulation of CCN1 by CR2 (Figure 4B). Neointimal area on Day 28 was also significantly reduced by transfection with lentiviral constructs expressing CR1 or CR2 compared with transfection with NSC (3.29 \times 10^5 \text{m}^2 vs 1.48 \times 10^5 \text{m}^2 [CR1], 1.26 \times 10^5 \text{m}^2 \text{[CR2]; P}<0.05; n=6). When lentiviral constructs expressing CCN1 were cotransfected with those expressing CR2, neointimal formation area was restored to near the control level (3.09 \times 10^5 \text{m}^2; n=6, P<0.05 compared with CR2 group; Figure 4C). Data from 6 animals are given as mean±SE (*P<0.05).

**Discussion**

The expression of CCN1 in VSMCs was first identified in the analysis of genes induced by angiotensin II (Ang II) stimulation in VSMCs. CCN1 transcripts were rapidly induced by Ang II in vitro and in vivo, and CCN1 expression was colocalized with Ang II in atherosclerotic arteries of apoE-deficient mice and in humans with atherosclerosis, suggesting that CCN1 may be involved in the development of plaque neovascularization under control of Ang II. In the present study, we focused on the function of CCN1 in cell proliferation and a potential role of CCN1 in neointimal hyperplasia. Here we provide the first evidence that CCN1 may be involved in VSMC proliferation and neointimal hyperplasia after balloon injury. Knockdown of endogenous CCN1 by lentiviral delivery of siRNA significantly suppressed neointimal hyperplasia, which was reversed by CCN1 replenishment, demonstrating that inhibition of CCN1 could suppress...
neointimal hyperplasia after vascular injury. Moreover, we showed that the effect of knockdown of CCN1 on neointimal hyperplasia persisted up to 28 days after CCN1-siRNA transfection, suggesting that it is enough to knockdown CCN1 at a single early time point for the inhibition of neointimal hyperplasia.

Recently, Lee et al reported that adenoviral expression of the forkhead transcription factor FOXO3a resulted in a significant reduction in neointima in a rat balloon injury model.23 Constitutively active FOXO3a gene transduction suppressed CCN1 expression in cultured VSMCs and adenoviral expression of both FOXO3a and CCN1 resulted in reversal of neointimal hyperplasia to near the control level, suggesting that suppression of CCN1 may be one of the mechanisms by which FOXO3a inhibits neointimal hyperplasia. In the present study, we demonstrated that direct inhibition of CCN1 could suppress VSMC proliferation and neointimal hyperplasia.

Neointimal hyperplasia is a major cause of restenosis after angioplasty.24–26 In this process, growth factors released from platelets, leukocytes, and VSMCs regulate the proliferation and migration of VSMCs from the media into the neointima.27,28 VSMCs synthesize and secrete growth factors including platelet-derived growth factor (PDGF)28 and tumor necrosis factor (TNF)-α.29 In this study, we found the expression of CCN1 in VSMCs in balloon-injured rat carotid arteries and demonstrated that endogenous CCN1 has a critical role in VSMC proliferation and neointimal hyperplasia. We have found that inhibition of endogenous CCN1 does not affect the expression level of PDGF or TNF-α in VSMCs (HM and TM, 2007 unpublished observation). These results indicated that CCN1 is an independent and critical regulator of VSMC proliferation and neointimal hyperplasia. Moreover, CCN1 is associated with the extracellular matrix and mediates a wide variety of biological function through cell-type specific binding to integrins, described above. Grzeszkiewicz et al reported that CCN1 supported VSMC adhesion and stimulated chemotaxis through αβ1 integrin.16 To specify the underlying mechanism of the effect of CCN1 on SMC proliferation, it was also necessary to study CCN1-integrin interaction in our study. We demonstrated that CCN1 support VSMC adhesion and migration through αβ1 integrin, and it is noteworthy that CCN1 also has a function on VSMC proliferation through αβ1 integrin. In endothelial cells, CCN1 can promote tubule formation via αβ1 integrin.6,30–32 CCN1 has also been shown to promote endothelial adhesion, migration, and proliferation in culture7 and to induce angiogenesis in corneal implants.33 Although the precise mechanisms involved in neointimal formation are still unknown, our data indicate that CCN1-αβ1 integrin interaction may participate in the VSMC proliferation and migration. Additionally, Moulton et al reported that angiogenesis inhibitors, endostatin and TNP-470, reduce intimal neovascularization and plaque growth in Apo-E deficient mice.34 Suppression of neointimal hyperplasia by knockdown of CCN1 may be produced by inhibition of proangiogenic effect of CCN1.

In summary, we showed that CCN1 is a critical regulator of VSMC proliferation and neointimal hyperplasia. CCN1 may...
represent a novel therapeutic target in the prevention of restenosis after vascular interventions.

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Disclosures
None.

References


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Supplemental data

Primer sequences used for real-time PCR

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<th>Gene Name</th>
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Supplemental Materials and Methods

Proteins, Antibodies, and Reagents

Recombinant human and murine CCN1 were produced as previously described\(^\text{17}\). Function-blocking monoclonal antibodies (mAbs) against integrins such as LM609 (anti-αvβ3), P4C10 (anti-β1) were purchased from Chemicon, Inc. (Temecula, CA). GoH3 (anti-α6) was purchased from R&D systems (Minneapolis, MN). Anti-CCN1 antibody for Western blotting and immunohistochemical analysis was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti β-actin antibodies were from Cell Signaling Technology (Danvers, MA).

Cell culture and adhesion assay

Rat VSMCs were obtained by the explant technique as described \(^\text{18}\). Briefly, the thoracic...
aorta was thoroughly dissected free from connective tissue and cut open longitudinally. Intimal and adventitial layers were scraped and the aorta was cut into small pieces. Aortic VSMCs were cultured in Dulbecco’s modified Eagle medium (DMEM, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (0.1mg/ml) in a humidified atmosphere of 5% CO2. Primary human aortic smooth muscle cells were obtained from Kurabo (Osaka, Japan) and maintained in smooth muscle basal medium (HuMedia-SB2, Kurabo) supplemented with 5% FBS, 0.5µg/ml human endothelial growth factor, 2µg/ml human fibroblast growth factor, 50mg/ml gentamicin, 50µg /ml amphotericin-B and 5mg/ml insulin. Since LM609 (anti-αvβ3), P4C10 (anti-β1), and GoH3 (anti-α6) are specific for human integrins, human VSMCs were used in the neutralization assays. Solid phase binding assay was performed as previously described with modifications19. Briefly, microtiter wells (Nunc [Danmark) were coated with 0.5%BSA and incubated at 4 °C overnight. Recombinant CCN1 (0.5-20µg/ml) was added to the wells and blocked with 1% BSA for 4 h at 4 °C. Non-treated VSMCs or VSMCs (2 × 10^5 cells/ml) that were pre-treated with function blocking mAbs for 1 h at room temperature were added and incubated at 37 °C for 1 h. After washing, adherent cells were fixed with 1% gluteraldehyde, stained with crystal violet, and quantified by dye extraction with measurement of absorbance at
Transwell migration assay

Effects of CCN1 on VSMCs migration were determined by a transwell migration assay as previously described\textsuperscript{21}. Briefly, VSMCs were added into the upper chamber of a seeded (5 × 10\textsuperscript{4} cells / 100µ l) transwell (Costar) containing serum free medium while the lower chamber was supplemented with serum free medium containing indicated recombinant CCN1 or PDGF-BB (15 ng/ml) as a positive control. VSMCs were subsequently allowed to migrate across a polycarbonate filter (8µm pore) for 8 h in a humidified atmosphere of 5% CO2/95% air at 37 °C. Cells on the top side of the filter (non-migrated cells) were removed by gentle scraping with a cotton swab and washing with PBS. Cells that had migrated on the bottom side of the filter were subsequently fixed with 100% methanol for 2 min. The filter was then washed with dH2O and permitted to air-dry before staining with Harris hematoxylin solution for 4 min, followed by two further washes in dH2O. Cells that had migrated were manually counted using a light microscope (Zeiss Axioskop2 plus). Cells in five random high power fields (hpf) were counted for each migration well in order to determine the average number of cells that migrated. Relative average numbers of VSMCs that
migrated compared with the controls (without CCN1) were calculated.

**Western blotting**

Left and right common carotid arteries were harvested from rats at the indicated time points after balloon injury to prepare whole artery and membrane compartment samples. The vessels were pulverized in lysis buffer (50mM Tris, pH 7.4; 150mM NaCl; 1% Triton-X; 0.5% protease inhibitor) using a homogenizer, centrifuged at 100000rpm for 10min, and resuspended in sample buffer containing SDS. A protein of membrane compartment was prepared as follow. Common carotid arteries were harvested from rats and pulverized in lysis buffer without Triton-X using a homogenizer and centrifuged at 3000rpm for 5min. The supernatant was centrifuged at 100000rpm for 10min, and pellets were resuspended in sample buffer containing SDS. The protein extract was boiled for 5 min at 95 °C and then centrifuged at 14,000 rpm for 5 min at 4 °C. Protein concentrations were determined by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Protein expression was determined by Western blot analysis. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell, Middlesex, UK). Blots were incubated with primary antibody (anti-CCN1 or β actin) overnight at 4°C, subsequently incubated with horseradish
peroxidase-conjugated secondary antibody (DAKO, Glostrup Denmark), and
immunoreactive protein bands were detected by chemiluminescence (GE Amersham
Biosciences, St. Giles, UK). The intensity of the staining of the protein bands was
quantified by densitometry using an ImageJ analysis software for densitometric analysis.
The intensity of each band was normalized using the β-actin band intensity.

**Immunohistochemistry**

Cryosections and paraffin embedded sections were washed in PBS and blocked with 5%
normal goat serum in incubation buffer (0.2% Tween20 and 0.1% BSA in 0.15M PBS).
Incubation with primary antibodies was performed in a humidified chamber overnight at
4 ºC with the following antibodies: anti-human CCN1 antibody (1:500) (0.4µg/ml,
Santa Cruz Biotechnology) and anti-GFP antibody (1:200) (Clontech (Takara Bio)).
Sections were subsequently washed in washing buffer (0.5% Tween20 in 0.15M PBS)
and incubated with a biotinylated secondary antibody (Vectastain ABC Elite secondary
antibody, Vector Laboratories, Inc, Burlingame, CA ; 1:200 dilution), followed by
incubation with an avidin-biotin detection system (Vectastain ABC system). A
diaminobenzidine (DAB) stain kit (Vector Laboratories, Inc) was used to detect a
positive reaction by producing a brown color.
For immunofluorescent staining of CCN1, Alexa fluor® 546-conjugated IgG (1:200) (Invitrogen) was used as a secondary antibody. For immunofluorescent staining of smooth muscle actin, FITC-conjugated anti-smooth muscle actin antibody (1:250) (clone 1A4, FITC conjugate F3777, Sigma) was used.

**Supplemental figure legend**

Supplemental figure I. (A) CCN1 was bound to VSMCs in a dose-dependent and saturable manner. Data are mean ± SD of six dishes from three experiments. (B) Integrin α6β1 mediated VSMC adhesion to CCN1. Data are mean ± SD of six dishes from three separate experiments.

Supplemental figure II. (A) Immunohistochemical analysis of CCN1 in intact and balloon-injured rat carotid arteries. (Bar;100µm) (B) Immunoblot analysis showed that the expression of CCN1 at 14 days after balloon injury in whole artery (B upper line) and membrane compartment (B lower line) was significantly increased in balloon-injured arteries. (C) Images of double fluorescence staining are shown for CCN1 (left panel, red) and smooth muscle actin (middle panel, green). Yellow in a merged image (right panel) indicates colocalization of CCN1 and smooth muscle actin.
Supplemental figure III. (A) Immunohistochemical stains for GFP were performed on sections of injured rat carotid arteries with (right panel) or without (left panel) transfection with GFP expressing lentivirus. bar: 50µm.
Supplemental figure I

A

B

* p<0.05
Supplemental figure II

A

B whole artery

<table>
<thead>
<tr>
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<tr>
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membrane compartment

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C

CCN1-Alexa®546  SMactin-FITC  merge
Supplemental figure III

A

control  
lentivirus mediated GFP