Supplemental Materials

1. Supplemental Methods

Rat carotid artery balloon angioplasty model

Carotid artery balloon angioplasty was performed in male Sprague-Dawley rats (Charles River, 250-300g) as previously described[1]. Briefly, rats were anesthetized through inhalation of 2.5% isoflurane (the same throughout this study). The left common carotid artery was exposed through a midline cervical incision. A 2F Fogarty catheter (Edwards Lifesciences) was inserted into the common carotid artery *via* an arteriotomy in the external carotid artery. To produce arterial injury, the balloon was inflated and withdrew to the carotid bifurcation and this action was repeated three times. The external carotid artery was then permanently ligated, and blood flow was resumed. The animal studies conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, 1996 revision). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin.

Local perivascular HF delivery to injured arteries

In order to avoid potential systemic side effects, we treated balloon-injured arteries with HF using an established local drug delivery strategy as described in our previous report[1]. Briefly, to ensure complete solubility, 30 μg of HF from a DMSO stock was first dissolved in 30 μl of 10% DMSO and then mixed with 270 μl of 25% F-127 pluronic gel (Sigma-Aldrich) that was kept on ice. Immediately after balloon angioplasty in the left common carotid artery, the gel containing HF was applied to the outside of the injured segment of the carotid artery. In the control group, equal volume of vehicle (30 μl of 20% DMSO) mixed with pluronic gel was applied.

Morphometric analysis of adaptive remodeling, intimal hyperplasia, and restenosis

Two weeks after balloon angioplasty, common carotid arteries were collected from anesthetized animals (under 2.5% isoflurane) following perfusion fixation at a physiological pressure of 100 mmHg[1], the animals were then euthanized in a CO_2 chamber. Paraffin sections (5 μm thick) were excised at equally spaced intervals and stained with hematoxylin-eosin (H&E) for morphometric analysis, as described in our previous reports[1, 2]. Nine sections from each of six either vehicle (DMSO) or HF-treated animals were used and an average parameter was calculated using total 54 sections. Planimetric parameters as follows were measured on the sections and calculated using Image J: the area inside external elastic lamina (EEL area) or internal elastic lamina (IEL area), lumen area, intima area (= IEL area- lumen area), and media area (= EEL area – IEL area). Measurements were performed by a student blinded to the experimental conditions. In this report, adaptive remodeling is defined as an increase of EEL area; intimal hyperplasia is quantified as a ratio of intima area versus media area; restenosis is evaluated as a decrease in lumen area.

Immunostaining for assessment of collagen and pSmad3 levels and PCNA-positive proliferating cells in the arterial wall

Immunostaining was performed on carotid artery sections collected on day 14 after angioplasty following our published method[1, 2]. Briefly, the sections were first incubated with each of the primary antibodies for 1h with a dilution ratio as follows: rabbit anti-Col-1 (Mybiosource), 1:100; rabbit anti-Col-3 (Fitzgerald), 1:750; rabbit anti-pSmad3 (Invitrogen), 1:100; rabbit anti-Smad3 (Invitrogen), 1:100; rabbit anti-PCNA (Santa Cruz), 1:200. The sections were then incubated with the ImmPRESS HRP-conjugated goat-anti-rabbit secondary antibody (Vector Laboratories, 1:200), followed by visualization with 3, 3-diaminobenzidine (DAB). The slides were counterstained with hematoxylin. Intensity of stained proteins on the sections was quantified using Image J. In each experimental group (DMSO control or HF treatment), at least 8 sections from each of 4 animals were used. For quantification of PCNA-positive cells, cell number was counted on 8-bit binary

images converted (by Image J) from the pictures of immunostained sections and normalized by the microscopic field. Cell counting was performed by a student blinded to experimental conditions.

Assays for post-angioplasty re-endothelialization

Re-endothelialization in balloon-injured arteries was evaluated on day 14 after angioplasty using Evans Blue assay according to the previously published method with minor modifications[3]. Briefly, 0.5 ml of 2% Evans Blue dye (Sigma-Aldrich) was injected into the saphenous vein of a rat anesthetized by 2.5% isoflurane. After 20 min, the rat was perfused with 20 ml of PBS buffer, the common carotid artery was then longitudinally opened and photographed on a white background. Remaining denuded areas were stained blue; unstained areas indicate re-endothelialization and were quantified using Image J. To further assess re-endothelialization, immunostaining of CD31 (an EC marker) was performed on carotid sections. Briefly, a goat anti-CD31 primary antibody (R&D Sytems,1:150) was incubated with the sections for 1h followed by an incubation with a biotinylated rabbit-anti-goat secondary antibody for 30 min. Immunostaining of CD31 was then visualized by using streptavidin-HRP and DAB. For quantification of re-endothelialization, we used Image J to measure the peri-luminal perimeter and the percentage of this perimeter that stained for CD31[4].

Cell culture and *in vitro* HF treatment

Rat aortic vascular smooth muscle cells (SMCs) and fibroblasts were isolated from the thoracoabdominal aorta of male Sprague-Dawley rats (anesthetized under 2.5% isoflurane) based on a protocol using an enzymatic dissociation method[2]. Cells were used at passages 5-7 for all experiments and were maintained in DMEM supplemented with 10% fetal bovine solution (FBS) at 37 °C with 5% CO_2. Cell viability was > 95% as indicated by Trypan Blue exclusion assay. For HF treatment, cells were first starved for 24h in DMEM containing 0.5% FBS, the medium was then replaced with fresh DMEM supplemented with 10% FBS in which HF was added to a desired

concentration. For control, equal volume of DMSO was added to a final concentration of 0.1 % (v/v versus medium).

Proliferation assay for cultured rat aortic SMCs

Proliferation of cultured SMCs was determined using cell number as a surrogate, for which Alamar Blue (Invitrogen) assay was performed following manufacturer's instructions. Cells were grown to 60% confluence, starved with 0.5% FBS, and then stimulated with 10% FBS in the presence of vehicle (DMSO) or HF. After 48h, Alamar Blue was added to the cell culture (1/10 of the medium volume), incubated for 24h, and then fluorescence was read using a Safir2 plate reader (Tecan, excitation/fluorescence: 530nm/590nm, bandwidth: 15 nm). A background from cell-free medium was subtracted.

Scratch assay for migration of cultured SMCs

The effect of HF on SMC migration was determined using the scratch wound assay as described previously[2, 3]. Rat SMCs were grown to 90% confluence in 6-well plates and then starved for 24h in DMEM containing 0.5% FBS. A sterile pipette tip was used to generate an ~1 mm cell-free gap, and dislodged cells were washed away with serum free DMEM. The medium containing either vehicle (DMSO) or 50 nM HF was added to the wells and incubated for 48h. Photographs were taken before (0h) and after (48h) the incubation, and cell migration was quantified by Image J based on the width of remaining cell-free gaps.

HF dose-response assay

To compare the effects of HF on SMCs and ECs, we performed dose-response experiments using primary human aortic SMCs and human aortic ECs as described previously[5]. Both cell types were purchased from Lonza simultaneously and then expanded in their respective optimal culture media (SMCs in SmGM-2 with 10% FBS and ECs in EGM-2 with 2% FBS, Lonza). Cells at passage 5

were seeded (2700 cells/200 μl medium/well) in a 96-well plate and incubated for 24h to allow cell attachment. Vehicle control (0.1 μl DMSO) or HF was added to generate different concentrations. After a 72 h incubation, Alamar Blue was added (1/10 of the medium volume) and incubated for another 24h, and then fluorescence was read as described above; a background from cell-free medium was subtracted. Curve fitting was performed with the Graph Pad Prizm software.

Western blotting for *in vitro* evaluation of protein levels

Rat SMCs were lysed in RIPA buffer containing protease inhibitors (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 10 μg/ml aprotinin). Alternatively, for detection of collagen secretion, culture media from fibroblasts were collected and concentrated using a Centricon filter (Millipore). Protein concentrations of either cell lysates or concentrated media were determined by Bio-Rad DC™ Protein Assay kit. Thirty micrograms of proteins from each sample were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes. Proteins of interest were detected by immunoblotting using the following primary antibodies and dilution ratios: Rabbit anti-Col-3 (1:1000) and anti-Col-1 (1:1000) from Fitzgerald, rabbit anti-phospho-Smad3 (1:1000) and anti-Smad3 (1:1000) from Invitrogen, and mouse anti-β-actin from Sigma-Aldrich. After incubation of the blots with HRP-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse, 1:5000, BioRad), specific protein bands on the blots were visualized by using enhanced chemiluminescence reagents (Pierce) and recorded using a LAS-4000 Mini imager. Intensity of the bands was quantified by Image J.

Statistical analysis

Data are presented as mean ± standard error (SEM). Statistical analysis was conducted using two-tailed unpaired Student's t-test. Data are considered statistically significant when a *P* value is < 0.05.

2. Supplemental Figure

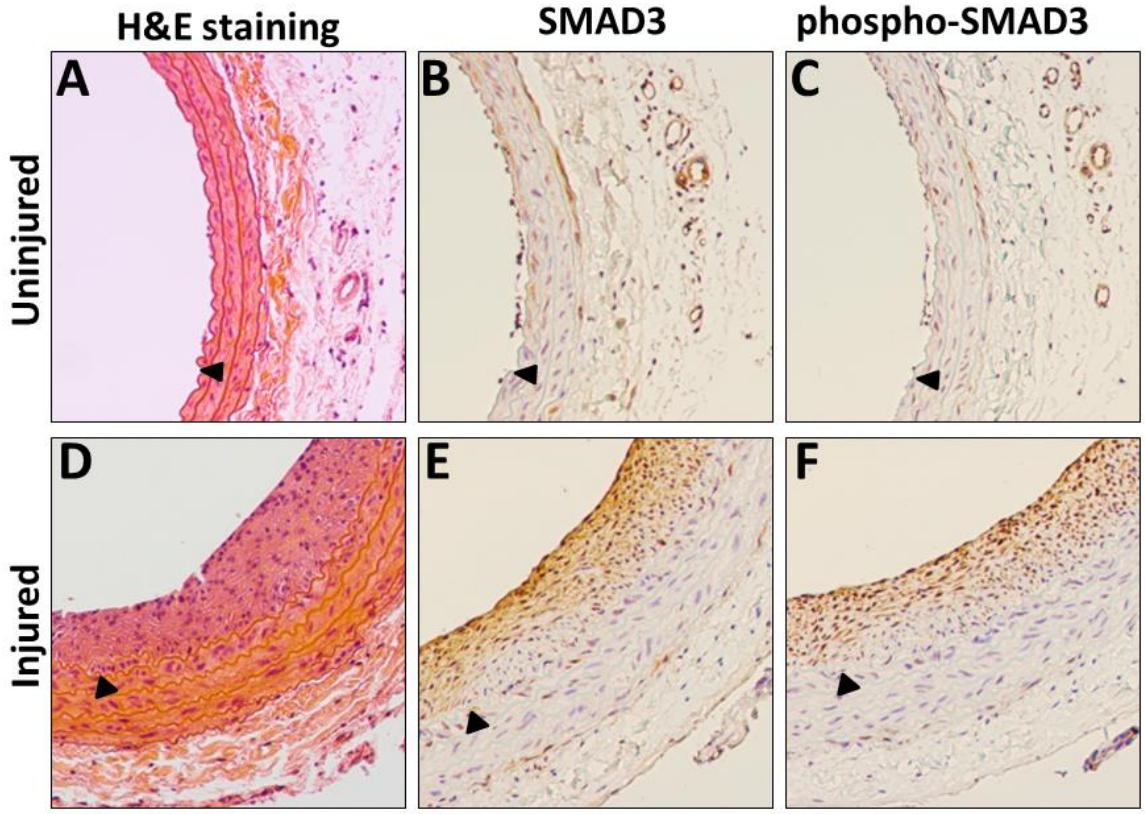

Figure S1. Smad3 and pSmad3 are elevated in rat carotid arteries after angioplasty

Balloon angioplasty was performed as described in Methods. Uninjured right carotid artery (A, B, and C) and injured left common carotid artery (D, E, and F) were collected from each rat on day 14 following angioplasty. Sections were prepared and stained with H&E (A and D), or immunostained for Smad3 (B and E) or pSmad3 (C and F). Shown are representative examples. Arrowhead marks IEL.

3. References

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