Materials and Methods

Cell Culture. Human Coronary Artery Smooth Muscle Cells (HCASMC) were obtained from Lonza (Walkersville, MD) and cultured in optimized Smooth Muscle Cell Medium (Clonetics^{R,} Lonza), supplemented with hEGF, Insulin, hFGF and 10% FCS. Cells were characterized by staining positive for smooth muscle cell α-actin, calponin, myosin and smoothelin.

Western Blot Analysis. Proteins from cell lysates (12-15 µg) were resolved on SDS-PAGE (12% resolving, 5% stacking) prior to transfer onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were stained with Ponceau S and probed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure equal protein loading and transfer and rinsed in wash buffer (PBS containing 0.05% Tween-20) before being probed as described previously [1].

Quantitative real-time RT-PCR. Total RNA (0.5-1 µg), isolated from cells using Qiagen RNeasy kit (Valencia, CA) was reverse-transcribed using iscript^{TM} cDNA Synthesis kit from BIO-RAD (Carlsbad, CA). The gene-specific oligonucleotide sequences were as previously described [1]. Real-Time RT-PCR was performed using the Stratagene MX3005 machine and the SYBER green jumpstart PCR kit (Sigma, St. Louis, MO) as described by the manufacturer.

Cell Proliferation. Changes in HCASMC cell number were determined by cell counting following exposure to pulsatile flow. Cells were seeded at 1×10^4 cells/well onto 6-well plates in 10% growth media. Cells were counted each day where the average of three wells was quantified by using a hemocytometer. In parallel studies, proliferating cell

nuclear antigen (PCNA) protein expression was determined by western blot following exposure to pulsatile flow.

Perfused transcapillary co-culture system. The perfused transcapillary culture apparatus (Cellmax Quad artificial capillary culture system, Spectrum Laboratories) was used to expose cells *in vitro* to different flow rates corresponding to normal and pathological low flow as previously described [2]. In the current study, pathologic low flow was modeled by a flow rate of 0.3 ml/min, corresponding to a pulse pressure of 24/18 mmHg, a frequency of 0.2 Hz, and amplitude of 6 mmHg in the extracapillary space. A normal flow rate of 25 ml/min was used corresponding to a pulse pressure of 64/14 mmHg, a frequency of 2 Hz, and an amplitude of 50 mmHg in the extracapillary space for 1-6 days as described. Cellular mRNA was harvested using TRIzol reagent (Invitrogen) and protein using lysis buffer from BIO-RAD (Carlsbad, CA) according to the manufacturer's specifications.

Mouse Carotid Artery Partial Ligation. The carotid artery ligation model of vascular injury and remodeling was performed essentially as described utilizing 6-8 week male C57BL/6 mice [3]. All procedures were approved by the University of Rochester Animal Care Committee. After buprenorphine analgesia and induction of anesthesia using inhalational isoflurane, the mouse was positioned on a clean operating table, with a warming pad to maintain body temperature. The animal was clipped and the surgical site prepped using betadine solution and alcohol. A midline cervical incision was made. With the aid of a dissecting microscope, the left external and internal carotid arterial branches were isolated and ligated with 6-0 silk suture reducing left carotid blood flow to flow via the patent occipital artery. The neck incision (2 layers, muscle and skin) was closed with

a running suture using 4-0 coated Vicryl. Partial ligation of the left carotid artery in this manner resulted in a decrease in blood flow in the left carotid artery, concomitant with an increase in the right carotid artery, with an intact endothelial monolayer, when compared to sham-operated control.

siRNA delivery *in vivo.* Select *in vivo* ready siRNAs (Life Technologies, Grand Island, NY) were pre mixed with lipofectamine based transfection reagents and added to the pluronic gel. Pluronic gel solutions (Sigma, St. Louis, MO) at 1mg/ml were prepared and kept at 4°C. Following carotid ligation, 200µL of this solution was applied to the carotid artery. On contact with tissues at 39°C the pluronic gel solidifies instantaneously, generating a translucent layer that envelops the treated region. The wound was then closed immediately after the application of the gel. Treated vessels were removed at 14 days post ligation for analysis. siRNA transfection efficiency by pluronic gel was verified by *Alexa Flour 555*-tagged control siRNA (A kind gift from Nitin Puri, Life Technologies, Grand Island, NY) showing localized delivery throughout the vessel (Supplemental Figure 5).

Preparation of Carotid Artery RNA. Mice were perfused with heparin/saline solution. Carotid arteries were collected in Trizol (Invitrogen) and homogenized using an Ultra-Turrax tissue disperser and RNA was prepared according to the manufacturers specifications.

Immunohistochemistry and Histomorphometry. Mice were perfusion fixed with 10% paraformaldehyde in sodium phosphate buffer (pH 7.0), 14 days post ligation, and the carotids harvested and embedded in paraffin. Starting 500 µM below the carotid

bifurcation landmark, a series of cross-sections (10 x 5 μm) were made, every 200 μm through 2 mm length of carotid artery. Cross-sections were stained with Verhoeff-Van Gieson stain for elastic laminae and sections were imaged using a Nikon TE300 microscope equipped with a Spot RT digital camera (Diagnostic Instruments). Digitized images were analyzed using MCID image software. Assuming a circular structure *in vivo*, the circumference of the lumen was used to calculate the lumen area, the intimal area was defined by the luminal surface and internal elastic lamina (IEL), the medial area was defined by the IEL and external elastic lamina (EEL) and the adventitial area was the area between the EEL and the outer edge. All histomorphometric analysis was performed " blind analysis" by the same observer. Immunofluorescence for smooth muscle actin (Sigma; A2547), activated Notch1 (NICD; Abcam, ab8925), Patched-1 (Abcam ab39266), and proliferating cell nuclear antigen (PCNA) (Laboratory Vision; RB-9055) was performed by standard procedures.

Data Analysis. Results are expressed as mean ± SEM. Experimental points were performed in triplicate, with a minimum of 3 independent experiments (VSMC), or a minimum of 5 animals per group. An ANOVA test was performed on cell count data and a Wilcoxon Signed rank test was used for comparison of two groups when compared to normalized control. A value of $p \leq 0.05$ was considered significant.

Bibliography

1) Sweeney C, Morrow D, Birney YA, Coyle S, Hennessy C, Scheller A, Cummins PM, Walls D, Redmond EM, Cahill PA. Notch 1 and 3 receptor signaling modulates vascular smooth muscle cell growth, apoptosis, and migration via a cbf-1/rbp-jk dependent pathway. *Faseb J*. 2004;18:1421-1423

2) Morrow D, Sweeney C, Birney YA, Guha S, Collins N, Cummins PM, Murphy R, Walls D, Redmond EM, Cahill PA. Biomechanical regulation of hedgehog signaling in vascular smooth muscle cells in vitro and in vivo. *American Journal of Physiology-Cell Physiology*. 2007;292:C488-C496

3) Morrow D, Cullen JP, Liu W, Guha S, Sweeney C, Birney YA, Collins N, Walls D, Redmond EM, Cahill PA. Sonic hedgehog induces notch target gene expression in vascular smooth muscle cells via vegf-a. *Arterioscler Thromb Vasc Biol*. 2009;29:1112-1118