SUPPLEMENTAL MATERIAL

Expanded Methods

Phenotypic and functional characterization of endothelial and smooth muscle cells seeded on fibronectin coated tubes

Endothelial (EC) and smooth muscle cell (SMC) coverage was determined using a MATLAB®-based image analysis program. Images were obtained by confocal microscopy of tube sections fixed and stained with specific antibodies (CD31 and smooth muscle cell α-actin, for EC and SMC, respectively). Inducible cell adhesion molecule expression markers (e-Selectin, VCAM-1, ICAM-1) were studied by flow cytometric analysis of cells in suspension. Briefly, cells were washed with PBS, treated with 0.05 % Trypsin / 0.53 mM EDTA for 5 min, and disrupted by gentle shaking. Cell-suspensions were washed and $3x10^5$ cells were resuspended in FACS buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide, Sigma Chemicals). EC were incubated with FITC-labeled mouse anti-human ICAM-1 (clone 15.2, Serotec), anti-human E-selectin (clone 1.2B6, Serotec), and mouse anti-human VCAM-1 (clone 1.G11B1; BD Pharmingen) for 30 min at 4°C. Cells were then washed, fixed in 1% paraformaldehyde, and 10^4 cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson). Surface tissue factor expression was quantified applying the MATLAB®-based image analysis program mentioned above to samples stained with anti-tissue factor from American Diagnostica. A 6-keto-prostaglandin F1a enzyme-immunoassay system (Amersham) was assayed for prostacyclin in serum-free perfusate collected after 24 h flow exposure to pulsatile arterial shear stress. Total glycosaminoglycan production was quantified by means of the dimethylmethylene blue assay. eNOS activity in cell lysates,

obtained by EDTA (1 mM)/Tris (25 mM) treatment followed by sonication immediately after cessation of 24 h flow exposure, was determined using the [3H]-L-arginine/L-citrulline assay kit (Cayman Chemical). Results were normalized to the total amount of protein determined by the BCA protein assay kit (Pierce).

Western Blot Analysis

Cell pellets were washed with cold PBS and immediately treated with 200µl lysis buffer (20mM tris buffer, 150mM NaCl, 1% triton 100X, 0.1% sodium dodecyl sulfate, 2mM sodium orthovanadate, 2mM PMSF, 50mM NaF, 1 complete protease inhibitor pellet) . Lysates were centrifugated at 10.000g for 10min at 4^{o}C and supernatants snap frozen until analysis. Protein was quantified using Biorad protein assay.

Confocal microscopy – staining, imaging and image quantification

A Perkin-Elmer spinning disk confocal microscopy system using a Zeiss Axiovert 200M microscope connected to a Hamamatsu Orca-ER camera was used to obtain fluorescence images of cells (EC and SMC) and proteins (p-S6RP, CD31 and Tissue Factor). A DAPI solution (1:500 in PBS/BSA) was used to stain the cells nuclei, while proteins were immunostained with their respective primary antibodies (diluted 1:50 in PBS/BSA) conjugated to Alexa Fluor 448 and/or 647 secondary antibodies. The laser wavelengths used were 405 nm for DAPI, 488 nm for Alexa Fluor 488 and 640 nm for Alexa Fluor 647. The emited light was discriminated Blue, Green and Red, respectively. The exposure times depended on the experiment, but were constant for each separate experiment. The results of this processing (exposing + filtering) are black and white multi-layer images of the light emited after exciting the samples. Those images were

artificially coloured red, green and blue (RGB) depending on the wavelength used and combined in order to obtain an RGB stack of every sample. Using a MATLAB-based analysis program, cells were counted and their fluorescence was analyzed. In the case of p-S6RP quantification (cf. Figures 3E, 3F, 1S), the analysis consisted in defining the area of the cell, the artificial colour of the protein of interest (Red, Green or Blue) and finally adding all the values of this colour intensity (0-255) for every pixel of the area. In cases with background fluorescence, this value was estimated and retrieved from the final result. In the case of stent recovery quantification (cf. Figures 1G, 4S), the area of the stent was determined with ImageJ and the number of cells on the stent strut were manually counted.

In vivo studies; animal preparation, surgery details and hystopathological analysis

To prevent or reduce the occurrence of thrombotic events, animals were treated on Day -1 or Day -2 with aspirin (650 mg, per os [PO]) and clopidogrel (300 mg, PO). The animals were then treated with aspirin (81 mg, PO) and clopidogrel (75 mg, PO) daily thereafter. Telazol® (4–6 mg/kg intramuscularly [IM]) was administered as a pre-anesthetic. Pre-operative nifedipine (10 mg, sublingual) was also given. An intravenous (IV) catheter was placed in an ear vein for administration of IV fluids at a rate sufficient to maintain patency of the catheter, or as needed. After induction of anesthesia, an incision was made in the neck to expose the carotid artery. An arterial sheath was introduced and advanced into the artery. Heparin (50–200 U/kg, IV) was administered on Day 0 (after placement of the introducer sheath) to prolong activated clotting time (ACT) to a target range of approximately 300 seconds during device deployment. Arterial blood samples

(< 0.5 mL each) were collected within 5–10 minutes of heparin administration, then at intervals of up to 45 minutes to measure ACT. Under fluoroscopic guidance, a guide catheter was advanced through the sheath into the ascending aorta and to the coronary arteries. Angiographic images of the vessels were obtained with contrast media to identify the proper location for the deployment site. Stents were introduced into the coronary arteries by advancing the stent delivery system through the guide catheter and over the guide wire to the deployment site within the coronary artery. The balloon was then inflated at a steady rate to a pressure sufficient to target a visually assessed balloon-artery ratio of 1.1–1.3:1 and held for 30 seconds (from initiation of inflation). Confirmation of this balloon-artery ratio was made when the angiographic images were quantitatively assessed. After the target balloon-artery ratio was achieved, vacuum was applied to the inflation device in order to deflate the balloon and slowly remove the delivery system.

Histopathological scoring was used to grade endothelialization. The scores generally reflected the degree and extent of injury. Scoring was performed using light microscopy. Endothelialization score depends on the extent of the circumference of the artery lumen showing coverage with endothelial cells (0, absent; 1, <25%; 2, 25-75%; 3, >75%; 4 = 100%).

Supplemental Table 1. Phenotype and function of EC and SMC seeded on silicone-rubber tubes.

Characteristic/function (cell type)	Measured
Coverage (EC*)	Monolayer (267±12cells/mm^2)
Coverage (SMC†)	Multilayer (369±84 cells/mm^2)
CD31‡ (EC)	>99% positive
α-actin (SMC)	>99% positive
Surface Tissue Factor (SMC)	83 ±1.23%
E-Selectin (EC)	1.2±0.3 % (unstimulated) 58.3±3.4 %(TNFα** stimulation)
VCAM-1§ (EC)	24.7±5.85 (unstimulated) 89.7±5.4% (TNFα stimulation)
ICAM-1‖ (EC)	18.34±2.28% (unstimulated) 98.7±0.15% (TNFα stimulation)
Prostacyclin (EC)	$1.68*10^{-3}\pm 8.05*10^{-4}$ pg/cell
eNOS# (EC)	$4.40*10^{-3}\pm 1.78*10^{-4}$ pg/cell
Glycosaminoglycans (EC)	0.56±0.34pg/cell
Soluble tissue factor (SMC)	0.33±0.12ng/cell

*Endothelial Cells, †Smooth Muscle Cells, ‡Platelet Endothelial Cell Adhesion Molecule-1, §Vascular Cell Adhesion Molecule-1, ||Inter-Cellular Adhesion Molecule 1, #Endothelial Nitric Oxide Synthase, **Tumor Necrosis Factor Alpha

Supplemental Figure Legends

Figure 1S. Expression of phospho-S6ribosomal protein (p-S6RP) in endothelial cells (EC) depends upon flow exposure (F) and is abrogated by the sirolimus analog, temsirolimus (Tems). Representative immunofluorescent images of phospho-S6RP expression in EC (green) after flow exposure and after treatment with the inhibitor (A-D). Cell nuclei were labelled with DAPI (blue).

Figure 2S. A) Digitonin extracts cytosol from EC not from SMC. Digitonin (Dig) and RIPA extracts of EC and SMC grown alone and from EC monolayer co-cultured on top of SMC (SMC/EC co-culture). Cells were treated with digitonin and RIPA for indicated time. Digitonin extracts the cytosol of the cells it comes in direct contact with, while RIPA produces lysate of the entire cell layers. Vascular smooth muscle actin is the marker of SMC. Tubulin is used for loading control. Very little actin is seen in SMC/EC co-culture system indicating that digitonin extracts cytosol from the uppermost EC monolayer with which the detergent comes in direct contact and not the underlying SMC. B) Flow increases abundance of phosphorylated S6RP in EC. Endothelial cells were seeded on fibronectin-coated silastic tubing and exposed to flow and digitonin-extracted fractions were probed for p-S6RP and tubulin. Flow increased the amount of p-S6RP normalized to cell tubulin content. Representative Western blots are displayed from three individual samples. C) SMC inhibit flow-induced increased phosphorylation of S6RP in EC. EC or SMC/EC-vessel like constructs were exposed to flow (+F) or no flow (-F) and

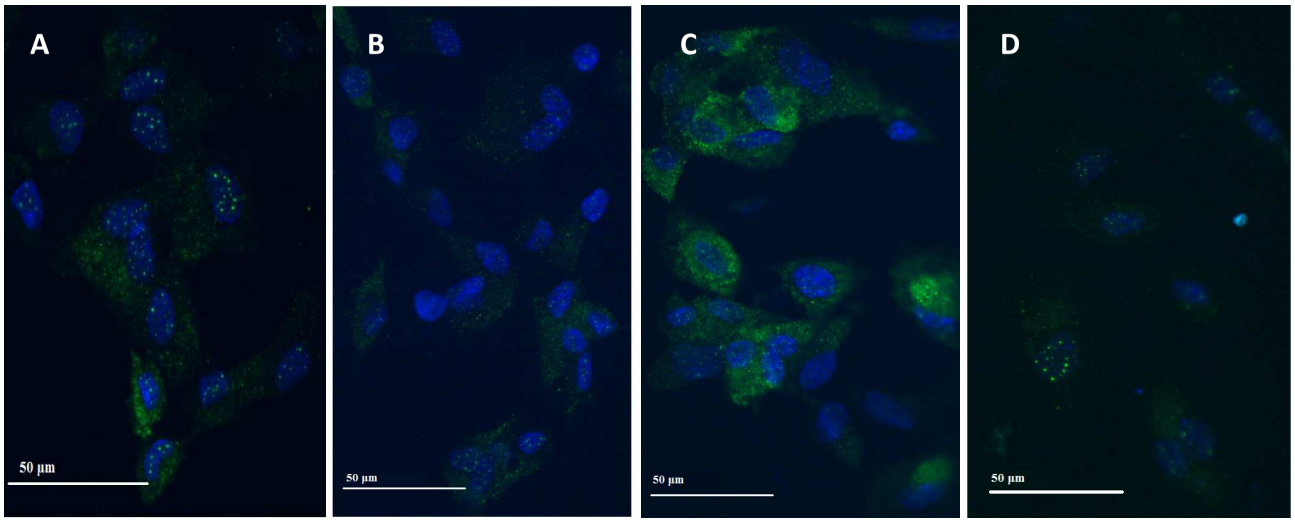
digitonin-extracted fractions were probed for p-S6RP and tubulin. Flow increased abundance of p-S6RP in ECs, while SMC reduced it.

Figure 3S. Immunohistochemical images of consecutive slices of the same bare metal stent strut injury. Cells were stained with antibodies against p-S6RP (A), SMC α-actin (B), and CD45RA (C). Photomicrographs suggest that the unstable matrix under the EC in the neointima, poor in SMC and rich in fibrin, is responsible for the upregulation of p-S6RP. The absence of CD45RA positive cells (C) implies that the upregulation of p-S6RP cannot be explained by presence of inflammatory cells in the neointima.

Figure 4S. Density of stent coverage on stent struts with and without inhibitor in the presence or absence of flow. After 24 hours flow exposure in the perfusion bioreactor, stents were carefully removed of the vessel-like construct, cut open and imaged using epifluorescence microscopy. EC on stent struts and stent strut surface were quantified using using ImageJ®. When EC were cultured alone without underlying SMC, flow increased the response to the injury by promoting rapid re-endothelialization of the stent strut. Static conditions and tensirolimus slowed the repair process. When in co-culture, SMC diminished the ability of EC to re-endothelialize after stent injury.

Legend Video 1S. Representative EC and SMC image stack obtained by confocal imaging of multilayered SMC/EC vessel-like constructs. EC and SMC are identified by surface CD31 (green) and surface tissue factor (red), respectively.

Fig 1S



-F-Tems -F+Tems +F-Tems +F+Tems

Fig 2S

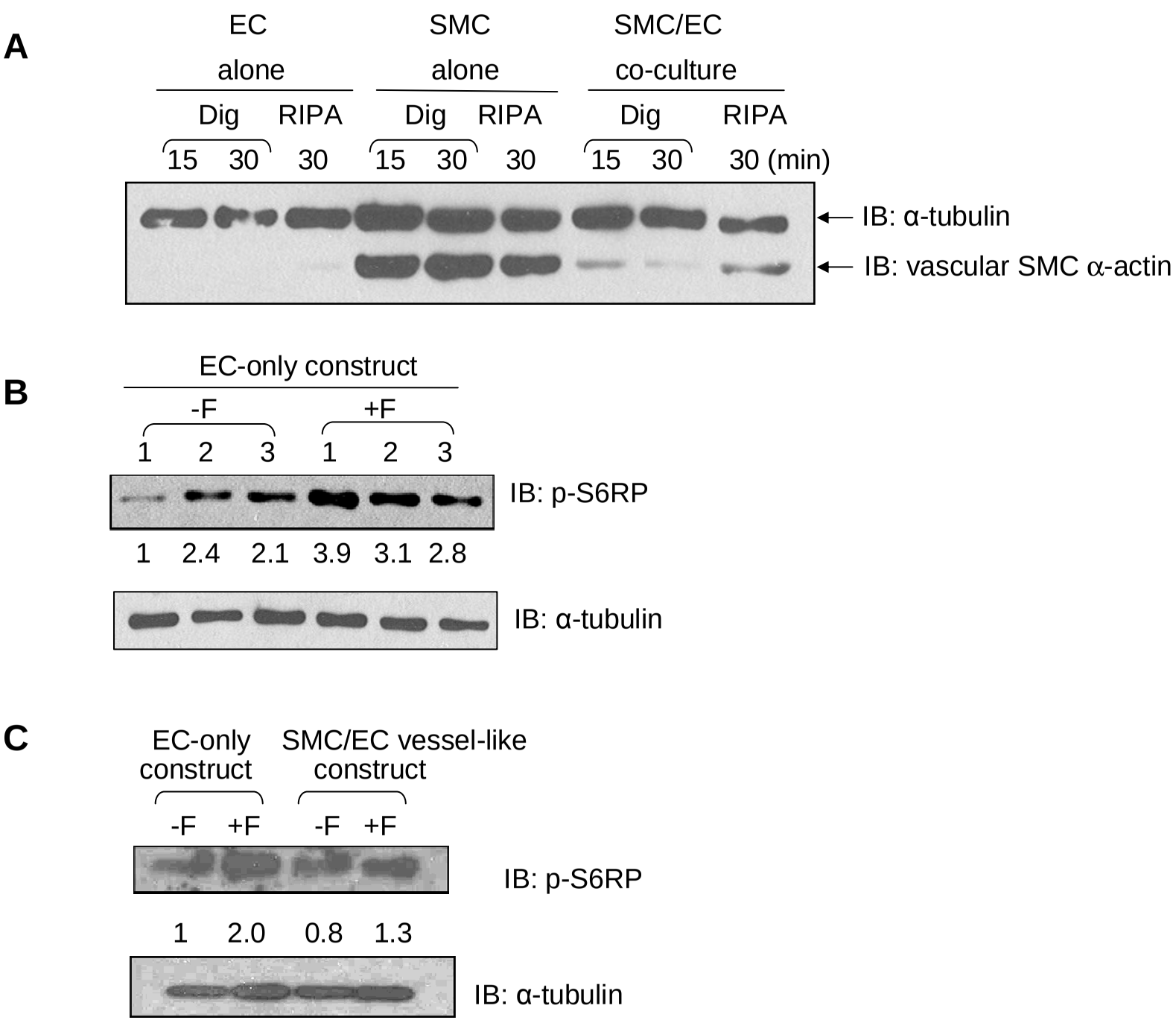
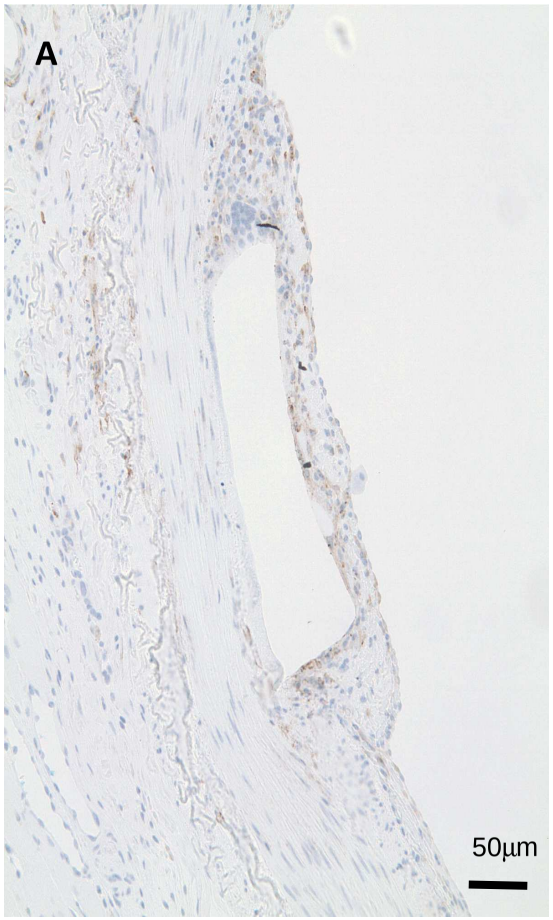
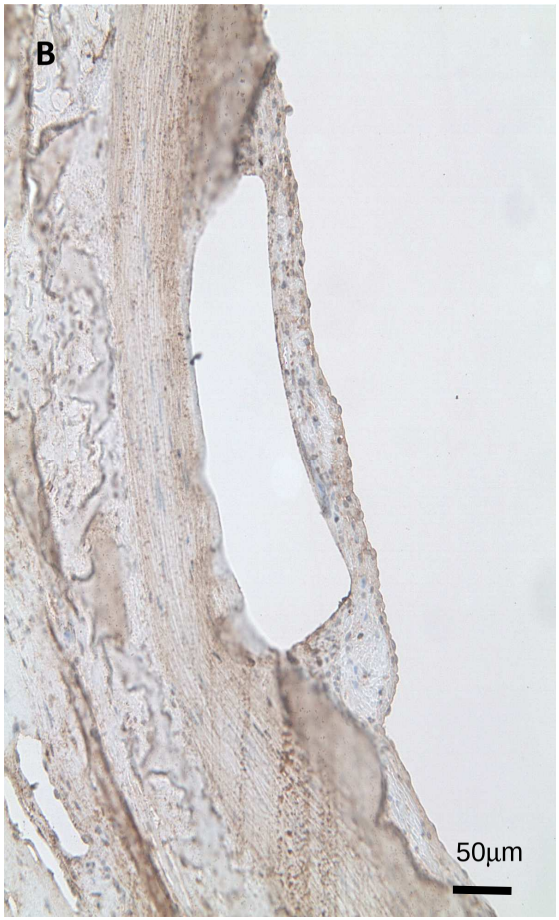

A
EC
alone
SMC
alone
SMC/EC
co-culture
Dig
RIPA
Dig
RIPA
Dig
RIPA
15
30
30
15
30
30
15
30
30 (min)
IB: α-tubulin
IB: vascular SMC α-actin
B
EC-only construct
-F
+F
1
2
3
1
2
3
IB: p-S6RP
1
2.4
2.1
3.9
3.1
2.8
IB: α-tubulin
C
EC-only construct
SMC/EC vessel-like construct
-F
+F
-F
+F
IB: p-S6RP
1
2.0
0.8
1.3
IB: α-tubulin

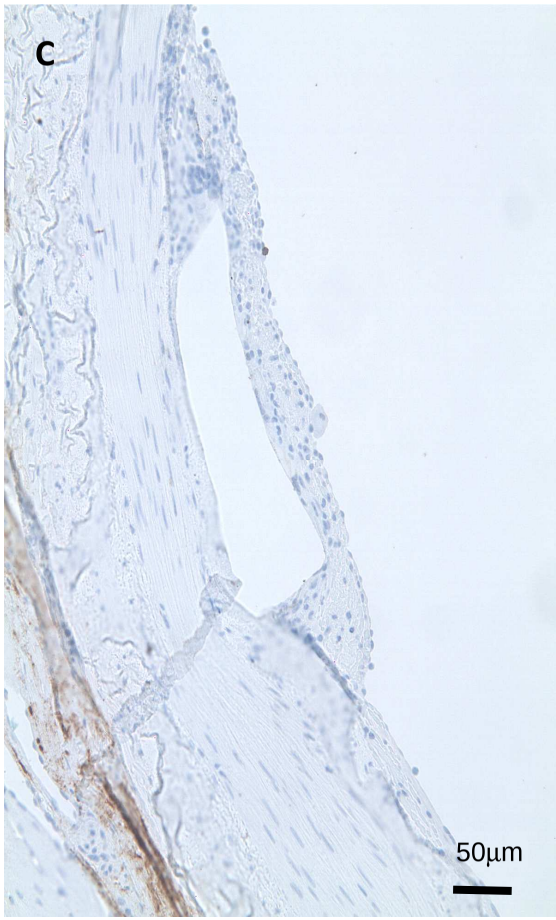
Fig 3S



p-S6RP

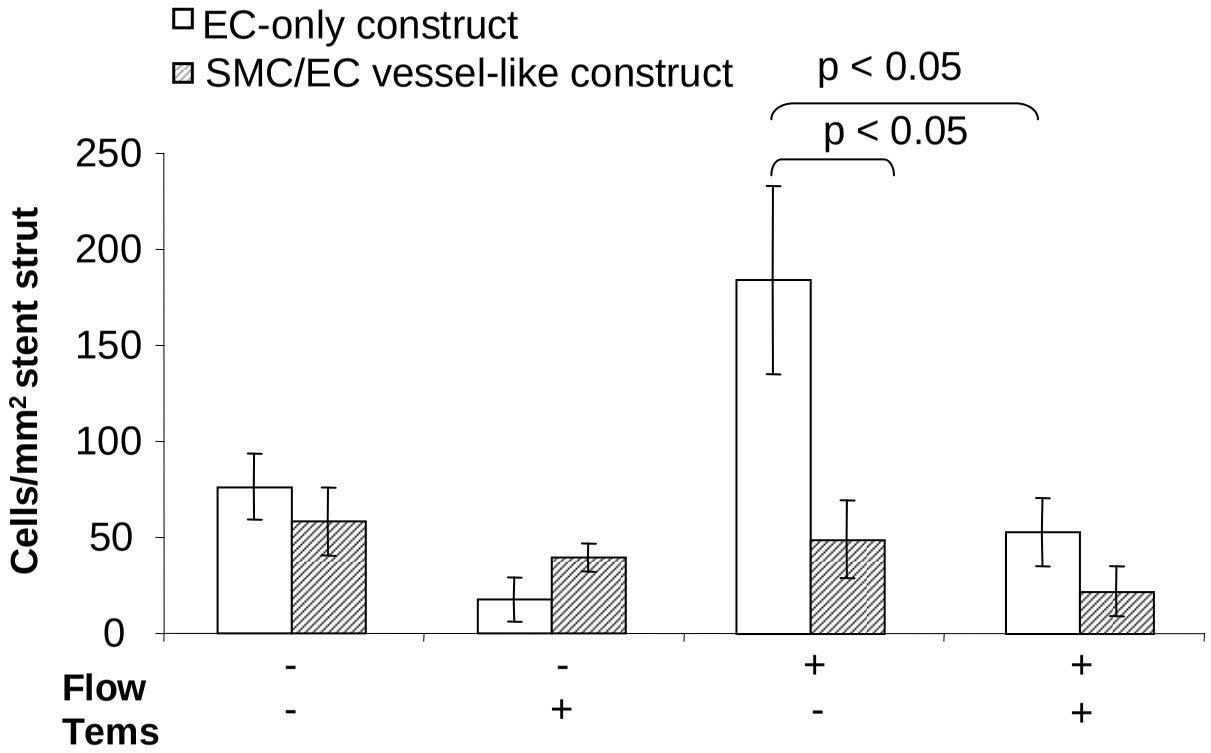


SMC α-actin



CD45RA

Fig 4S


EC-only construct
SMC/EC vessel-like construct
p < 0.05
p < 0.05
Cells/mm² stent strut
250
200
150
100
50
0
Flow
Tems
- -
- +
+ -
+ +