SUPPLEMENT MATERIAL

Fibronectin is an important regulator of flow-induced vascular remodeling

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SUPPLEMENTAL MATERIALS AND METHODS

Proteins

Human fibronectin was purified as described [1]. Recombinant vitronectin was produced as described [2]. Laminin was purchased from BD Biosciences (San Jose, CA), collagen type I from UBI (Lake Placid, NY) and fibrinogen from Enzyme Research Laboratories (South Bend, IN). pUR4 cDNA was a generous gift from Dr. Hanski [3], and was provided to us by Dr. Mosher (University of Wisconsin, Madison, WI). pUR4 (also known as FUD, functional upstream domain) is a 49-mer peptide derived from the bacterial F1 adhesin. We modified pUR4 cDNA to remove the coding region for a cys residue that was present in the N-terminal portion of the peptide. This cys is not part of the adhesin F1, but was added as a consequence of the original cloning strategy. The sequence of pUR4 that we used in this study is: **MRGSHHHHHHGS**KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNMVE TEDTK**LN**. Bold sequences were added as part of the cloning strategy, and to provide a his tag for purification purposes. The control peptide is a his tagged carboxyl terminal fragment (68-mer) of fibronectin's III-11 module (III-11C) and was produced as described [4]. III-11C has been used in in vivo studies by others [5, 6], and has no known biological effects in vitro or in vivo[4-8]. Endotoxin was removed from peptides using Detoxi-Gel (Pierce, Rockford, IL). Endotoxin levels were measured with a Limulus amebocyte lysate assay kit (QCL-1000, Lonza, Basel, Switzerland). Endotoxin levels were less than 0.1 endotoxin units (EU) per mg of peptide.

Enzyme linked immunosorbant assay (ELISA)

96-well tissue culture plates were coated with 10 μg/mL type I collagen, fibronectin, laminin, fibrinogen, or vitronectin overnight at 4°C. Denatured collagens were generated as described [9]. Plates were blocked with 1% bovine serum albumin (BSA) in Tris

buffered saline (TBS) for 1 hour, then washed with TBS. pUR4 was serially diluted into coated wells, then incubated at room temperature for 90 min. Wells were washed with TBS containing 0.1% Tween 20. An anti his antibody (HisG, Invitrogen, Carlsbad, CA) that recognizes the his tag on pUR4 was added for 1.5 hour at room temperature. Wells were washed, then incubated with an horseradish peroxidase (HRPO) conjugated secondary antibody. After washing, peroxidase activity was quantified by using 2,2'-azino-bis-(3-ethylbenthiazoline-g-sulfonic acid). Measurements were done at 405 nm on a Wallac 1420 multilabel counter.

Cell culture

Rat aortic smooth muscle cells (RASM) were obtained from Cell Applications (San Diego, CA) and maintained in serum containing media (Cell Applications). SMC were used at passages 4-6.

Animal Studies

Both sexes were used for the carotid ligation experiments, since no significant differences in the remodeling response were reported between male and female mice [10]. All procedures were approved by the University of Rochester Animal Care Committee, and were performed in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals.

Morphometry

Digital images of Verhoeff-van Gieson elastic stained cross-sections were captured and morphometric analysis was performed using Image-Pro Plus software (Media Cybernetics, MD).

The circumferences of the lumen, internal elastic lamina (IEL) and external elastic lamina (EEL) in the sections were identified using the automatic trace mode in Image-Pro. The accuracy of the automated tracing was verified by visual inspection of the images. Cross-sectional areas of the lumen, neointima, media, adventitia, and the area encompassed by the EEL were measured. Vessel compartment volumes were calculated as described [10]. Briefly, starting with the carotid bifurcation as the origin, a series of cross-sections of 5 μm were cut every 200 μm through the first mm length of the carotid artery. Morphometric analysis was perfomed at each point, and the average of each division was calculated, summed, and reported as the volume.

Immunohistochemistry and quantitative analysis

Sections selected from the first mm of the carotid artery in each group were used for immunohistochemistry (IHC). Endogenous peroxidase activity was blocked with 3% H_2O_2, followed by incubation with Dako serum-free blocking solution (Dako; Glostrup, Denmark). The primary antibodies used were: polyclonal anti-fibronectin (Chemicon/Millipore, Billerica, MA), polyclonal anti-collagen type I (LF-67; a gift from Dr. Fisher, NIH, Bethesda, MD), monoclonal anti-proliferating cell nuclear antigen (PCNA) (Sigma, St. Louis, MO), polyclonal anti-smooth muscle myosin heavy chain (SMMHC) (Biomedical Technologies Inc., Stoughton, MA), monoclonal smooth muscle α-actin (SM α-actin) (Dako), anti-leukocyte common antigen, CD45 (BD Pharmingen, San Jose, CA), monoclonal anti-intercellular adhesion molecule-1 (ICAM-1) (BD Pharmingen), and polyclonal anti-vascular cell adhesion molecule-1 (VCAM-1) (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen retrieval for anti-fibronectin and anti-collagen type I antibodies was performed by incubating sections with proteinase K in 0.05M Tris-HCL buffer. Anitigen retrieval for CD 45 antibody was performed by incubating sections with 10 mM citrate

buffer (pH=6) at 120°C. Sections were then incubated with appropriate biotinylated secondary antibodies followed by avidin-biotin immunoperoxidase system (Vector Laboratories, Burlingame, CA). Liquid DAB Substrate Chromogen system (Dako) or Vector Red (Vector) was used for detection. PCNA, CD45, ICAM-1, and VCAM-1-immunostained sections were counterstained with hematoxylin.

Quantitative IHC analysis was performed using Image-Pro Plus software. Data from 3-5 mice were averaged, and the average values ± s.e.m. determined. For fibronectin, collagen type I, SMMHC, and SM α-actin, color digital images were captured using a 40X objective and transformed into gray scale. 16 fields of view of equal size per section in the media or adventitia were randomly chosen, and the mean of the optical densities from the 16 fields of view was determined. 3 sections were analyzed per animal. For quantitative comparison of leukocyte infiltration and cell adhesion molecule expression, images were acquired using a 60X objective. The CD45, ICAM-1, and VCAM-1-positive areas were obtained using an automated programmed segmentation procedure in ImagePro. The intima plus media or adventitial regions were traced manually. The percent of the positively stained area to the total traced area was determined. For evaluation of proliferating cells, the percent of PCNA (+) cells to total cells was determined by counting cell numbers in PCNA-stained sections that had been counterstained with hematoxylin. The percent positive cells is reported as the proliferation index. To determine the SMC density, sections were stained with hematoxylin, and cells present in the media were counted. The data is expressed as number of cells/μm^2.

Detection of peptides in the vessel wall

pUR4 and III-11C were conjugated to Texas-Red, as per the manufacturer's instructions (Molecular Probes/Invitrogen). 20 μM Texas-Red (TR) conjugated proteins were embedded in pluronic gel and applied to the vessel following ligation surgery. Mice were sacrificed 1, 3, or 7 days after surgery. Longitudinal frozen sections (20 μm thickness) were cut and immunostained with a polyclonal anti-TR antibody (Molecular Probes/Invitrogen). For quantitatively evaluating the efficacy of peptide delivery to the common carotid artery, 15-18 fields of view in the media from 2-3 mice at each time point were analyzed. The TR (+) areas were quantitated using an automated programmed segmentation procedure in ImagePro. The percentage of TR (+) area to the total medial area was determined.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). mRNA was isolated from RASMs 24 hours after incubation with 500 nM pUR4 or III-11C control peptides using RNAzol as per the manufacturer's instructions (Invitrogen). cDNA was prepared using a Superscript First Strand cDNA kit as per the manufacturer's instructions (Invitrogen). Mouse carotid arteries were harvested and frozen in liquid nitrogen. RNA was isolated using Trizol and purified using a QIAgen RNeasy Micro Kit. RNA integrity was examined with an Agilent 2100 Bioanalyser using RNA6000 NanoAssay (Agilent Technology). Whole Transcriptome Amplification was performed using Qiagen's QuantiTect Whole Transcriptome Amplification Kit. Quantitative RT-PCR analyses were performed using ABI Prism 7900HT sequence detection system (Applied Biosystems). The qRT-PCR primers for 3 mouse genes were obtained from ABI: GADPH (4352932E), fibronectin (Mm01256744_m1), and collagen Ia1 (Mm00801666_g1). TaqMan probe chemistry was used according to the manufacturer's

instructions. qRT-PCR reactions were run in triplicate for each sample. The data was normalized to the levels of GAPDH in each sample, and the results were averaged.

Statistical analysis

For animal studies, data are presented as the mean ± s.e.m. Comparisons were made with an unpaired, 2-tailed Student's *t* test, or one-way ANOVA with GraphPad Prism software (San Diego, CA). A difference between the means was considered significant when $p < 0.05$.

SUPPLEMENTAL FIGURES

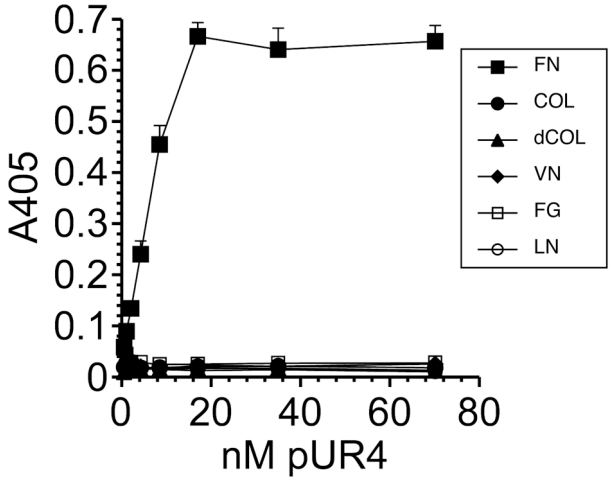

Figure I. *Binding of pUR4 to ECM Proteins.* 96-well plates were coated with 10 μg/mL type I collagen (COL), denatured type I collagen (dCOL), fibronectin (FN), laminin (LN), vitronectin (VN), or fibrinogen (FG) at 4°C overnight. pUR4 was added to the wells and serially diluted. Bound pUR4 was detected as described in the Supplemental Methods. Measurements were done at 405 nm on a Wallac 1420 multilabel counter. Data represents the average of duplicate determinations, and the error bars the range.

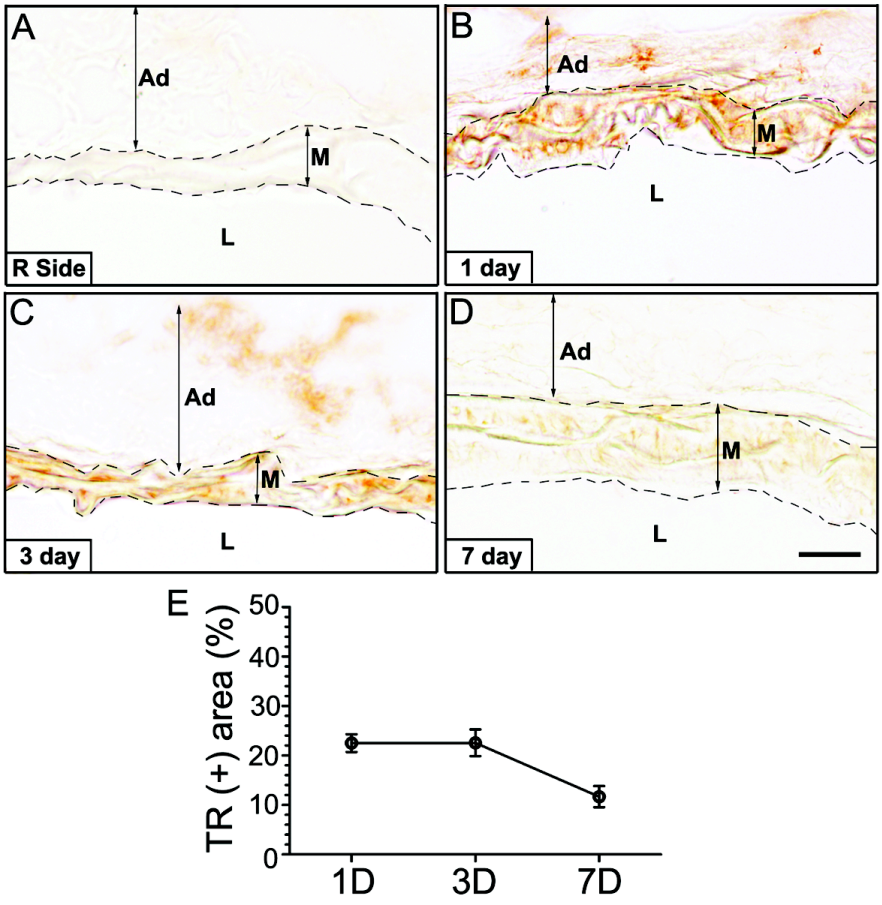

Figure II. *Visualization of pUR4 in the vessel wall.* Texas-Red (TR) conjugated pUR4 (20 μm) was embedded in pluronic gel and peri-adventitially delivered to the left carotid artery following ligation surgery as described in the Methods. The right carotid artery from the same animal was used as a control. Mice were sacrificed 1, 3, and 7 days after surgery, and the carotid arteries were harvested and flash frozen. Longitudinal sections (20 μm) were immunostained with an anti-TR antibody. Representative images from the right common carotid artery (A), and left common carotid artery of 1 day (B), 3 day (C) and 7 day (D) animals are shown. (E) Percentage of the area which is TR (+) was assessed in the media of the vessels. Media=M; adventitia=Ad; lumen=L. Bar, 20 μm.

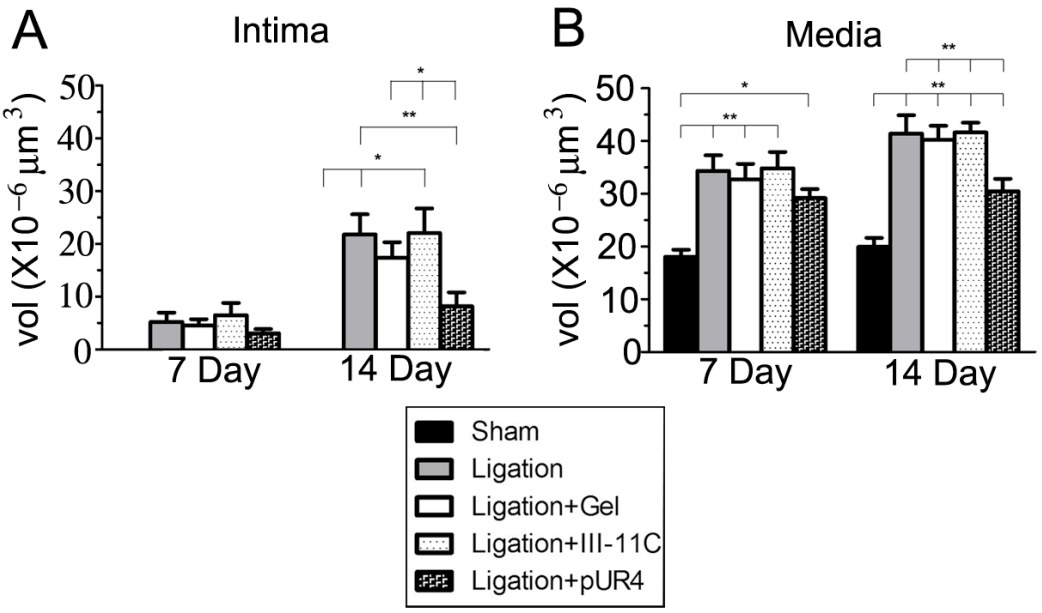

Figure III. *The pUR4 fibronectin inhibitor decreases intima and media thickening.* Morphometric analysis of the intima (A) and media (B) 7 and 14 days after ligation. * indicates $p<0.05$, and ** indicates $p< 0.01$.

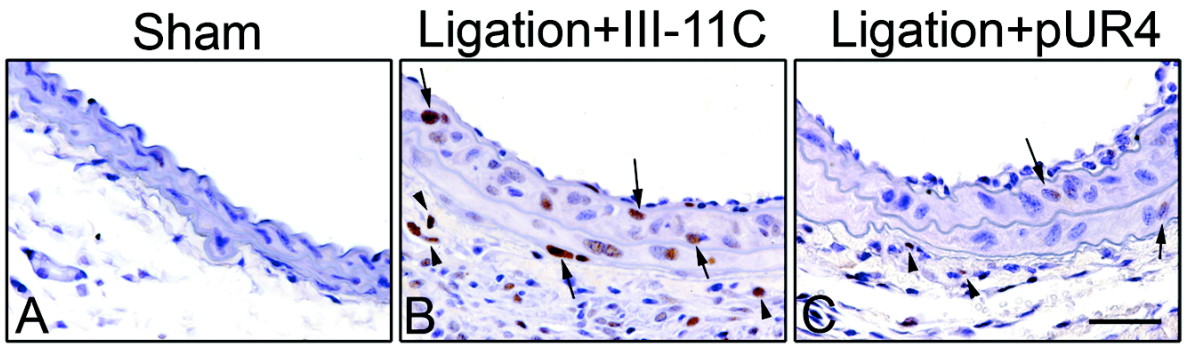

Figure IV. *The pUR4 fibronectin inhibitor decreases cell proliferation in the carotid artery.* Sections of the left carotid artery were stained with antibodies to PCNA seven days post ligation. Sections were counterstained with hematoxylin. Representative photomicrographs of PCNA-stained sections from sham-operated animals (A), ligated animals with III-11C treatment (B) and ligated animals with pUR4 treatment (C). Arrows indicate PCNA (+) cells in the media, and arrowheads indicate PCNA (+) cells in the adventitia. Bar, 50 µm.

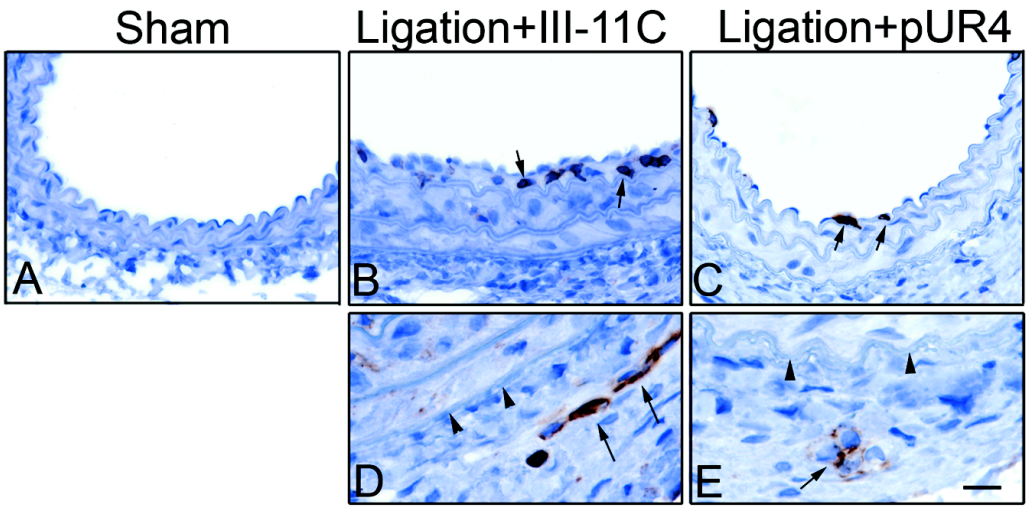

Figure V. *The pUR4 fibronectin inhibitor decreases leukocyte infiltration into the vessel.* Sections of the left carotid artery were stained with antibodies to CD45 seven days after ligation. Sections were counterstained with hematoxylin. Representative photomicrographs of CD45 (+) staining in the media (A,B,C) and adventitia (D,E) of the carotid artery from sham-operated animals (A), ligated animals with III-11C treatment (B and D) and ligated animals with pUR4 treatment (C,E). Arrows indicate CD45 (+) cells in the media and adventitia. Arrowheads indicate the location of the external elastic lamina. Bar, 25 μm in (A-C) and 12.5 μm in (D,E).

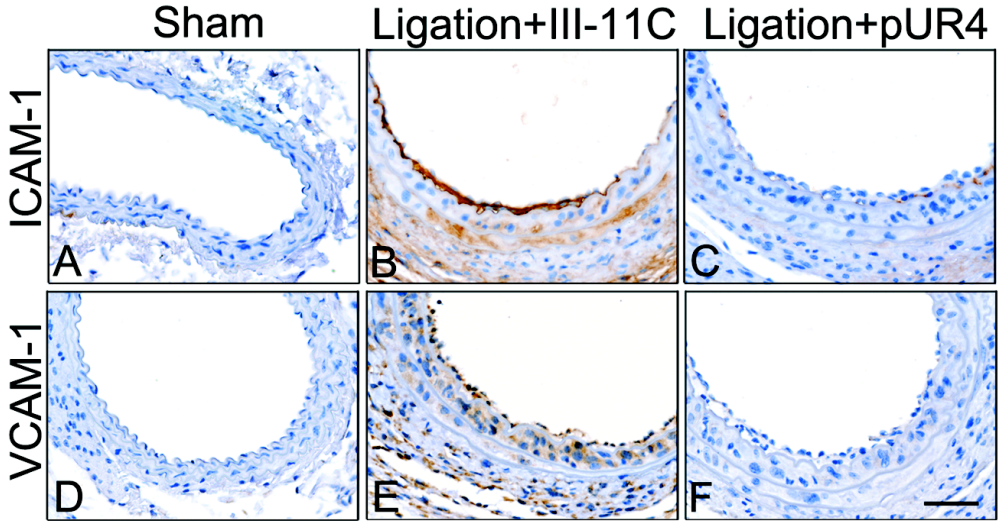

Figure VI. *The pUR4 fibronectin inhibitor decreases ICAM-1 and VCAM-1 levels.* Sections of the left carotid artery were stained with antibodies to ICAM-1 (A-C) or VCAM-1 (D-F) seven days after ligation. Sections were counterstained with hematoxylin. Representative photomicrographs of sections from sham-operated animals (A and D), ligated animals with III-11C treatment (B and E) and ligated animals with pUR4 treatment (C and F). Bar, 20 μm.

Sample	Treatment	FN	Col Ia1
SMC	PBS	1.00 ± 0.28	1.00 ± 0.08
	111-11C	1.16 ± 0.12	1.27 ± 0.13
	pUR4	1.43 ± 0.02	1.42 ± 0.06

Table I. *qRT-PCR of fibronectin and collagen I in SMC.* Confluent cultures of rat aortic SMCs were incubated in the presence of 500 nM pUR4, control III-11C (C,D) peptide or an equivalent volume of PBS for 24 h. qRT-PCR was performed in triplicate for each sample using TaqMan chemistry as described in Methods. Data represent the average of duplicate samples, and the error bars the range. The relative levels of fibronectin and collagen are shown; the PBS control was set equal to 1.

Sample	Treatment	FN		Col Ia1	
		Day 4	Day 7	Day 4	Day 7
Carotid Arteries	111-11C	1.00 ± 0.34	1.00 ± 0.09	1.00 ± 0.32	1.00 ± 0.16
	pUR4	0.70 ± 0.23	0.41 ± 0.23	0.60 ± 0.30	0.58 ± 0.36

Table II. *qRT-PCR of fibronectin and collagen I in carotid arteries.* Mouse carotid arteries were harvested from pUR4 and III-11C treated animals 4 or 7 days post surgery. qRT-PCR was performed as described in Methods. qRT-PCR reactions were run in triplicate for each arterial sample. Three arteries were analyzed for each condition. The data represent the average of these 3 samples, and the error bars the s.e.m. The relative levels of fibronectin and collagen are shown; III-11C was set equal to 1. A two tailed homoscedastic (two sample equal variance) t-test was used to analyze the data. No statistically significant differences ($p < 0.05$) were found between the groups.

SUPPLEMENTAL REFERENCES

1. Miekka SI, Ingham KC, Menache D. Rapid methods for isolation of human plasma fibronectin. *Thromb Res.* 1982;27:1-14.
2. Sottile J, Shi F, Rublyevska I, Chiang HY, Lust J, Chandler J. Fibronectin-dependent collagen I deposition modulates the cell response to fibronectin. *Am J Physiol Cell Physiol.* 2007;293:C1934-1946.
3. Ozeri V, Tovi A, Burstein I, Natanson-Yaron S, Caparon MG, Yamada KM, Akiyama SK, Vlodavsky I, Hanski E. A two-domain mechanism for group A streptococcal adherence through protein F to the extracellular matrix. *Embo J.* 1996;15:989-998.

4. Morla A, Zhang Z, Ruoslahti E. Superfibronectin is a functionally distinct form of fibronectin. *Nature.* 1994;367:193-196.
5. Yi M, Ruoslahti E. A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis. *Proc Natl Acad Sci U S A.* 2001;98:620-624.
6. Yi M, Sakai T, Fassler R, Ruoslahti E. Antiangiogenic proteins require plasma fibronectin or vitronectin for in vivo activity. *Proc Natl Acad Sci U S A.* 2003;100:11435-11438.
7. Sottile J, Chandler J. Fibronectin matrix turnover occurs through a caveolin-1-dependent process. *Mol Biol Cell.* 2005;16:757-768.
8. Bourdoulous S, Orend G, MacKenna DA, Pasqualini R, Ruoslahti E. Fibronectin matrix regulates activation of RHO and CDC42 GTPases and cell cycle progression. *J.Cell Biol.* 1998;143:267-276.
9. Engvall E, Ruoslahti E, Miller EJ. Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J.Exp.Med.* 1978;147:1584-1595.
10. Korshunov VA, Berk BC. Flow-induced vascular remodeling in the mouse: a model for carotid intima-media thickening. *Arterioscler Thromb Vasc Biol.* 2003;23:2185-2191.