

SUPPLEMENTAL MATERIAL

Animals

All animal tests were approved by the Malmö/Lund regional ethical committee (Sweden). ApoE-null mice in a B16 background (B6.129P2-ApoEtm1UncN11) purchased from Taconic (Lille Skensved, Denmark) and fibromodulin-null mice generated by targeted disruption¹ were crossed and the resulting ApoE/fibromodulin-null mice were used in experiments. ApoE-null mice were used as controls.

In vivo alteration of shear stress

As described previously by Cheng et al.² standardized changes in shear stress were induced by a periaortic cast placed around the right carotid artery of ApoE- and ApoE/fibromodulin-null female mice. Mice were kept on a cholesterol-rich diet throughout the experiment, starting two weeks before surgery. Cast placement was performed on 18-week-old mice anaesthetized with isoflurane carried by oxygen (initiated at 4% isoflurane and kept at 2-3% during surgery). Mice were given buprenorphine (0.1 mg/kg) subcutaneously once before surgery and once five to seven hours post-surgery. Mice were sacrificed at 30 weeks of age.

Tissue preparation

Mice were sacrificed through administration of an overdose of ketamine, xylazine and acepromazine and blood was collected from the right chamber of the heart. The mice were then either perfused with HistoChoice tissue fixative (Amresco Inc) before carotid arteries, aortas and hearts were removed and fixed for (a minimum of) several days with Histochoice; or perfused with PBS before carotid arteries were embedded in optimal cutting temperature compound (OTC, Sakura Finetek, Japan), frozen directly (isopropanol on dry ice) and sectioned (7 µm). Carotid arteries fixed with HistoChoice were rinsed in PBS, dehydrated in series of rising ethanol concentrations, two changes of xylene, paraffin overnight, then embedded in paraffin and sectioned (5 µm). Hearts were incubated in 30% sucrose overnight and in OTC with 15% sucrose for 30 min. They were then embedded in OTC, frozen and sectioned (7 µm). Descending aortas were cleaned and mounted en face on slides with ovalbumin/glycerine (Sigma-Aldrich).

Immunohistochemistry and histology

Carotid artery sections were stained for macrophages and foam cells with a Mac-2 antibody (Cedarlane; Burlington, Ontario, Canada), for SMCs with an alpha smooth muscle actin (α -SMA) antibody (clone 1A4, Sigma-Aldrich) and for fibromodulin (antibody kindly provided by Prof. Dick Heinegård). Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ (Sigma-Aldrich) and heat induced antigen epitope retrieval, pH 6.0, was performed for 11 minutes (Mac-2 and α -SMA staining). The Vectastain Elite Kit (goat anti-rabbit) and the MOM-kit (both from Vector laboratories; Burlingame, California) were used according to the manufacturer's instructions, though the former with a goat anti-rat biotin-conjugated secondary antibody (Abcam; Cambridge, UK) during Mac-2 staining. Positive fibromodulin stains were visualized by incubation with ImmPACT NovaRed (Vector laboratories; Burlingame, California, USA) and counterstained with Harris' hematoxylin (Sigma-Aldrich). Positive Mac2-stains were visualized by 3,3'-Diaminobenzidine and counterstained with Vector Methyl Green nuclear counterstain (Vector laboratories; Burlingame, California, USA). Apoptotic cells were stained using the TUNEL kit TACS XLDAB In Situ Apoptosis Detection Kit from Trevigen according to the manufacturer's instructions.

PCNA stainings were performed using a PCNA antibody from AbCam and the "PCNA staining kit" from Invitrogen according to the manufacturer's instruction with the addition of quenching of endogenous peroxidase activity as described previously as well as heat induced

antigen epitope retrieval pH 6.0 for 20 minutes. Sequential double staining was performed using the Mac-2 or α -SMA antibody together with the PCNA antibody with positive stains visualized by 3,3'-Diaminobenzidine and streptavidin alkaline phosphatase with StayRed/AP (AbCam) and counterstained with Harris' hematoxylin.

Masson's trichrome staining of carotid artery sections was performed using the "Accustain trichrome stain (Masson)" (Sigma-Aldrich) according to the manufacturer's instructions. Frozen carotid artery and aortic root sections were incubated in 60% isopropanol, rinsed with dH₂O, stained with 0.3% Oil Red O (Sigma-Aldrich) for 20 minutes and then mounted with GVA mount (Zymed, San Francisco, California, USA). Flat preparations of aortas were stained with Oil red O for 50 minutes and mounted with Mountquick (Daido Sangyo Co. LTD, Tokyo, Japan).

Morphometric measurements

Lesion size was expressed as area and intima-media ratio and represents the mean value of four sections 15 μ m apart where the lesions were at their largest. Positively stained areas of Oil red O-stained descending aortas mounted en face were quantified and expressed as the percentage of the inner arterial lining covered by lesions. Aortic root sections were collected between first appearance and disappearance of the aortic valves. One section every 56 μ m was stained with Oil red O. Lipid content was expressed as the mean percentage of lesion area which stained positive for lipids.

Transmission electron microscopy

Low and oscillatory shear stress carotid plaques as well as control common carotid arteries and descending aortas (segments cut immediately below the aortic arch; n=3) from ApoE- and ApoE/fibromodulin-null mice were fixed in 0.15 M sodium cacodylate-buffered 2% glutaraldehyde, postfixed in 0.1 M-collidine-buffered 2% osmium tetroxide, and embedded in epoxy resin¹. Ultrathin sections were analyzed in Philips CM-10 electron microscope (Philips, Amsterdam, The Netherlands). Collagen fibril thickness was measured with ImageJ (NIH).

RNA extraction and quantitative real time PCR (qRT-PCR)

Uninjured carotid arteries (as control) and low and oscillatory shear stress carotid plaques from ApoE- and ApoE/fibromodulin-null (n=6 per genotype) were isolated after saline perfusion of the mice. Due to the small sample size, low and oscillatory shear stress plaques from 3 mice were pooled yielding 2 groups per genotype. In addition, uninjured carotid arteries from 2 mice were pooled, yielding 3 groups per genotype. Samples were homogenized with TissueLyser (Qiagen, Valencia, CA) in 75 μ l extraction buffer and total RNA was extracted with RNeasy Micro Kit (Qiagen) according to the manufacturers' instructions. Fifty ng total RNA was subjected to first strand cDNA synthesis with Superscript VILO cDNA Synthesis Kit (Invitrogen). QRT-PCR were performed on LightCycler (Roche Applied Science) in duplicates by mixing cDNA with MaximaTM SYBR Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany) and gene specific primer pairs (available upon request). Amplification results were analyzed using LightCycler software Version 3. The calculated threshold cycle values for each gene were normalized to the threshold cycle value of the internal standard hypoxanthine phosphoribosyltransferase 1 (Hprt1).

Analysis of plasma cholesterol and cytokines

The colorimetric assay Infinity Total Cholesterol (Thermo Scientific, Liverpool, U.K.) was used to quantify total plasma cholesterol and the mouse Th1/Th2 9-Plex (IFN- γ , IL-1 β , TNF- α , IL-2, IL-12, IL-4, IL-5, IL-10, and KC) Ultra-Sensitive Kit (Meso Scale Discovery) was used to quantify plasma cytokine concentrations. Both analyses were performed according to instructions from the manufacturer

***In vitro* assays with RAW264.7 cells**

Aortic SMCs were isolated³ from fibromodulin-null and wild type C57BL/6 mice. The aortas were digested in 0.3% collagenase (type II, Gibco) and then seeded in plastic cell culture flasks and kept for up to six passages in Ham's F-12 medium (Gibco) supplemented with 50 µg/ml gentamicin (Sigma-Aldrich), 5 mg/ml ascorbic acid (Sigma-Aldrich) and 10% NCS (Gibco).

Mouse RAW264.7 cells (gift from Prof. Fredrik Ivars) were grown in DMEM GlutaMax (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and 10% FBS Gibco).

Native human LDL (NatLDL; provided by the Experimental Cardiovascular Research Unit at the Clinical Research Center, Lund University) at a concentration of 1 mg/ml was oxidized with 5 µM CuCl₂ for 18h at 37°C. Oxidation was stopped by 1 mM EDTA and protein concentration was determined with the Pierce BCA protein assay kit (Thermo Scientific).

For ECM preparation wild type and fibromodulin-null SMCs were seeded on cover slips in 24 well-plates and cultured seven to nine days. Cells were then extracted with 0.5% Triton X-100 in PBS and 25 mmol/litre NH₄OH in PBS leaving cover slips covered with cell-free ECM.⁴ The effect of fibromodulin-null ECM on LDL-uptake was assessed through seeding RAW264.7 cells on the ECM-covered cover slips and adding 50 µl/ml native or oxidized LDL (in DMEM GlutaMax supplemented with 10% lipoprotein-deficient human serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco)) to each well. RAW264.7 cells were then incubated for four days – fresh LDL was added after two days – and then fixed in 4% formaldehyde and stained with 0.3% Oil Red O for 10 minutes. Cells were counterstained with Methyl Green (Trevigen) and the intracellular lipid content was quantified as Oil Red O-positive area relative to total cell area (values added from 20 micrographs per cover slip).

For immunoblotting, the cell extract from the above-mentioned cultures was precipitated with 50% ethanol, and the pellet was dissolved in SDS-PAGE reducing loading buffer. The samples were run on 4-20% Bis-Tris SDS-PAGE gels and the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 4% milk in PBS, and incubated with home-made anti-fibromodulin, or anti-calreticulin (Abcam ab14234) in 0.5% milk and 0.1% Tween-20 in PBS, and detected with HRP-conjugated secondary antibodies followed by ECL (SuperSignal West Pico Substrate, Thermo Scientific). All membrane washes were done in PBS with 0.1% Tween-20.

The effect of wild type and fibromodulin-null ECM on cytokine production in RAW264.7 cells was assessed using the Mouse ProInflammatory 7-Plex Tissue Culture Kit (IFN-γ, IL-1β, IL-10, IL-12 p70, IL-6, KC/GRO/CINC (CXCL1), TNF-α; Meso Scle Discovery). Cells were seeded on wild type and fibromodulin-null ECM-covered cover slips and native or oxidized LDL was added as described for LDL-uptake studies. After two days 30 ng/ml PMA and 1 µg/ml ionomycin was added to the wells for 24 hours and cells were then lysed using the CelLytic MT reagent according to the manufacturer's instructions (Sigma-Aldrich).

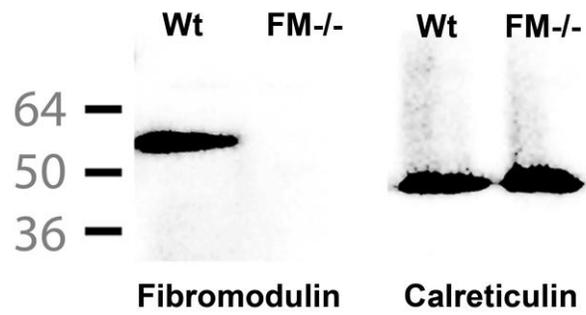
Software and statistical methods

Sample size is expressed as n and error bars represent standard deviation (S.D.). Stained sections were scanned and digitalized using an Aperio ScanScope digital slide scanner (Scanscope Console v8.2.0.1263, AperioTechnologies, Inc., Vista, California, USA) and positively stained areas (fibromodulin, Mac2, Oil Red O and Masson's Trichrome stain) were quantified using BioPix iQ software (BioPixAB, Gothenburg Sweden). Two-tailed t-test (electron microscopy image analysis) was performed using Microsoft Excel. Mann-Whitney t-test (image analysis) and Student's t-test and one-way ANOVA analysis with Bonferroni post-test (qRT-PCR) were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

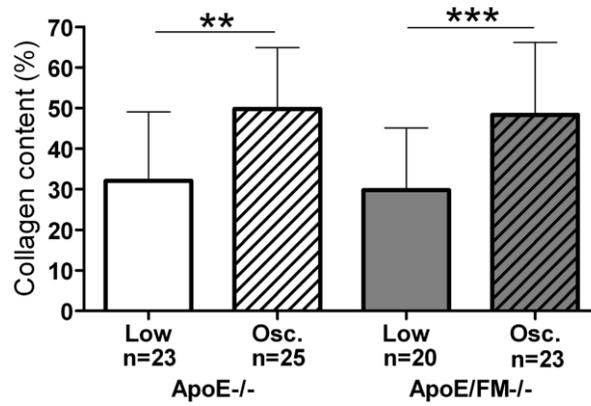
References

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2. Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJ, Krams R, de Crom R. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. *Circulation.* 2006;113:2744-2753
3. Hultgardh-Nilsson A, Lovdahl C, Blomgren K, Kallin B, Thyberg J. Expression of phenotype- and proliferation-related genes in rat aortic smooth muscle cells in primary culture. *Cardiovasc Res.* 1997;34:418-430
4. Tran-Lundmark K, Tran PK, Paulsson-Berne G, Friden V, Soininen R, Tryggvason K, Wight TN, Kinsella MG, Boren J, Hedin U. Heparan sulfate in perlecan promotes mouse atherosclerosis: Roles in lipid permeability, lipid retention, and smooth muscle cell proliferation. *Circ Res.* 2008;103:43-52

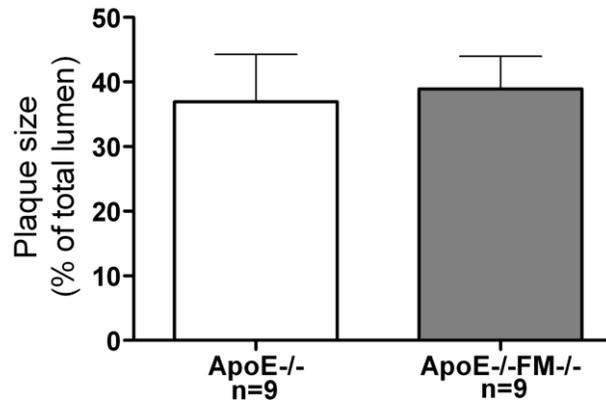
SUPPLEMENTAL FIGURES



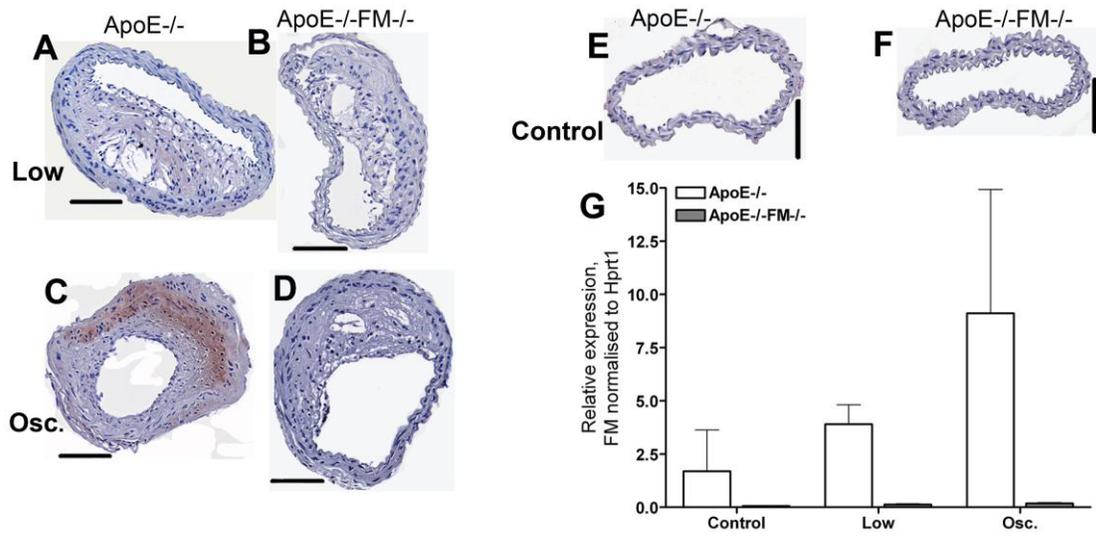
Supplemental figure I: Fibromodulin expression in SMCs *in vitro*.



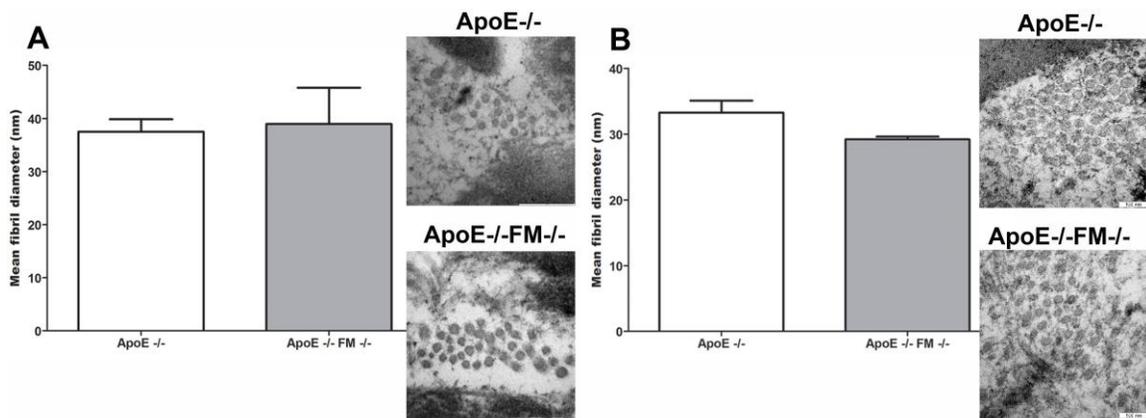
Supplemental figure II: Reduced collagen content in low shear stress carotid lesions from ApoE⁻ and ApoE/fibromodulin-null mice. Image analysis on Masson's trichrome-stained sections from low and oscillatory shear stress lesions from ApoE⁻ and ApoE/fibromodulin-null mice.



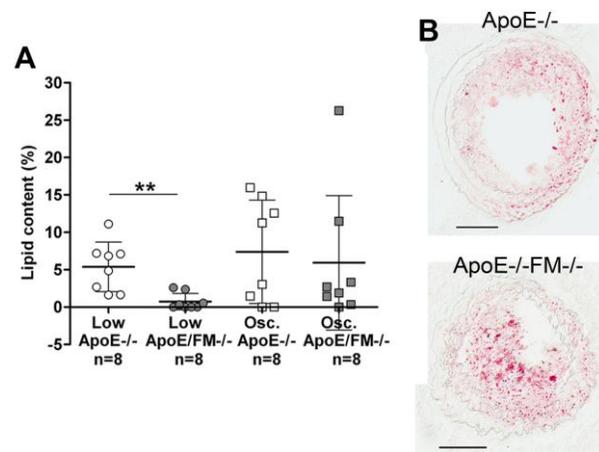
Supplemental Figure III: Size of aortic root lesions from ApoE- and ApoE/fibromodulin-null mice.



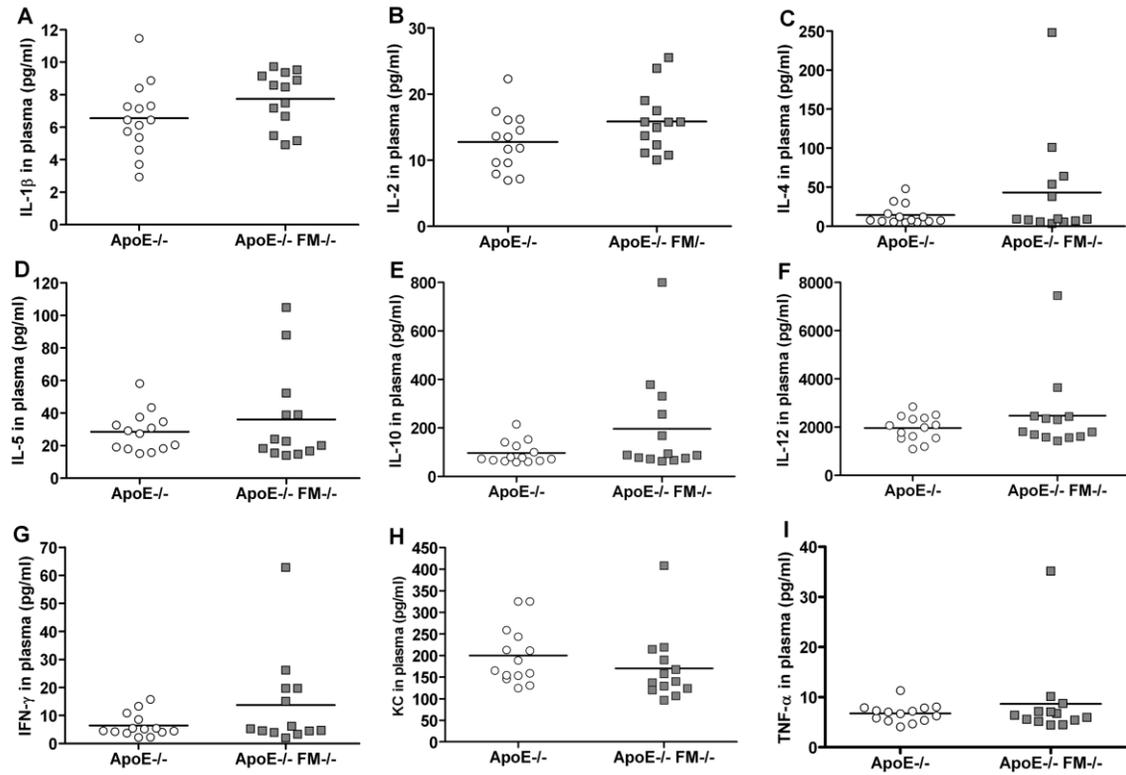
Supplemental Figure IV: Fibromodulin immunoreactivity in carotid lesions. Representative sections of fibromodulin-stained carotid lesions and control arteries from ApoE^{-/-} (A, C and E) and ApoE/fibromodulin-null (B, D and F) mice. Quantitative PCR results showing mean expression levels of fibromodulin mRNA in carotid low and oscillatory shear stress lesions and control arteries isolated from ApoE^{-/-} and ApoE/fibromodulin^{-/-} mice (G). Scale bars = 100 μ m.



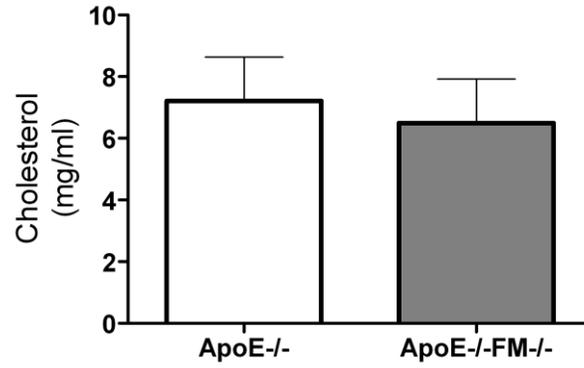
Supplemental Figure V: Collagen fibril diameter similar in healthy arteries from ApoE^{-/-} and ApoE^{-/-}/fibromodulin-null mice. Mean fibril diameter was calculated for each carotid artery or aorta from at least three electron micrographs per sample. Average fibril diameter in carotid artery vessel walls were 33 ± 1.8 nm from ApoE^{-/-} mice and 29 ± 0.4 nm from ApoE^{-/-}/fibromodulin-null mice (A; $n = 3$ for each genotype; $p > 0.09$). Average fibril diameter in vessel walls from aorta were 37.5 ± 2.4 nm in ApoE^{-/-} mice and 39 ± 6.8 nm in ApoE^{-/-}/fibromodulin-null mice (B; $n = 3$ for each genotype; $p > 0.85$). Scale bars = 200 nm in A and 100 nm in B, *error bars* represent standard error of the mean.



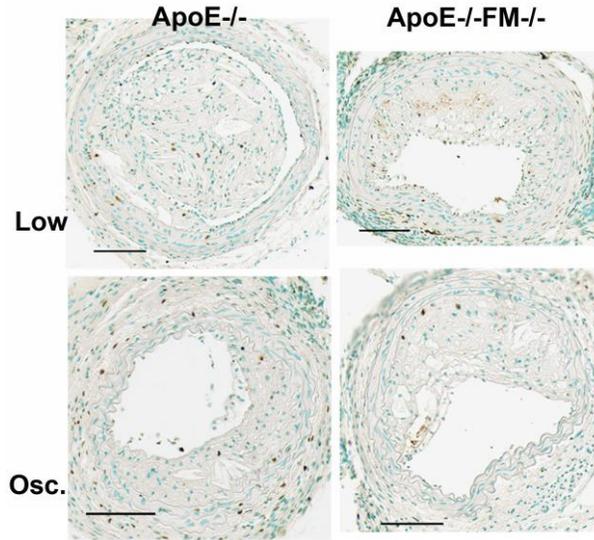
Supplemental Figure VI: Decreased lipid content in the media in ApoE/fibromodulin-null mice. Image analysis (A) and representative Oil Red O-stained sections from oscillatory shear stress carotid lesions from ApoE^{-/-} and ApoE/fibromodulin-null mice. Scale bars = 100 μ m.



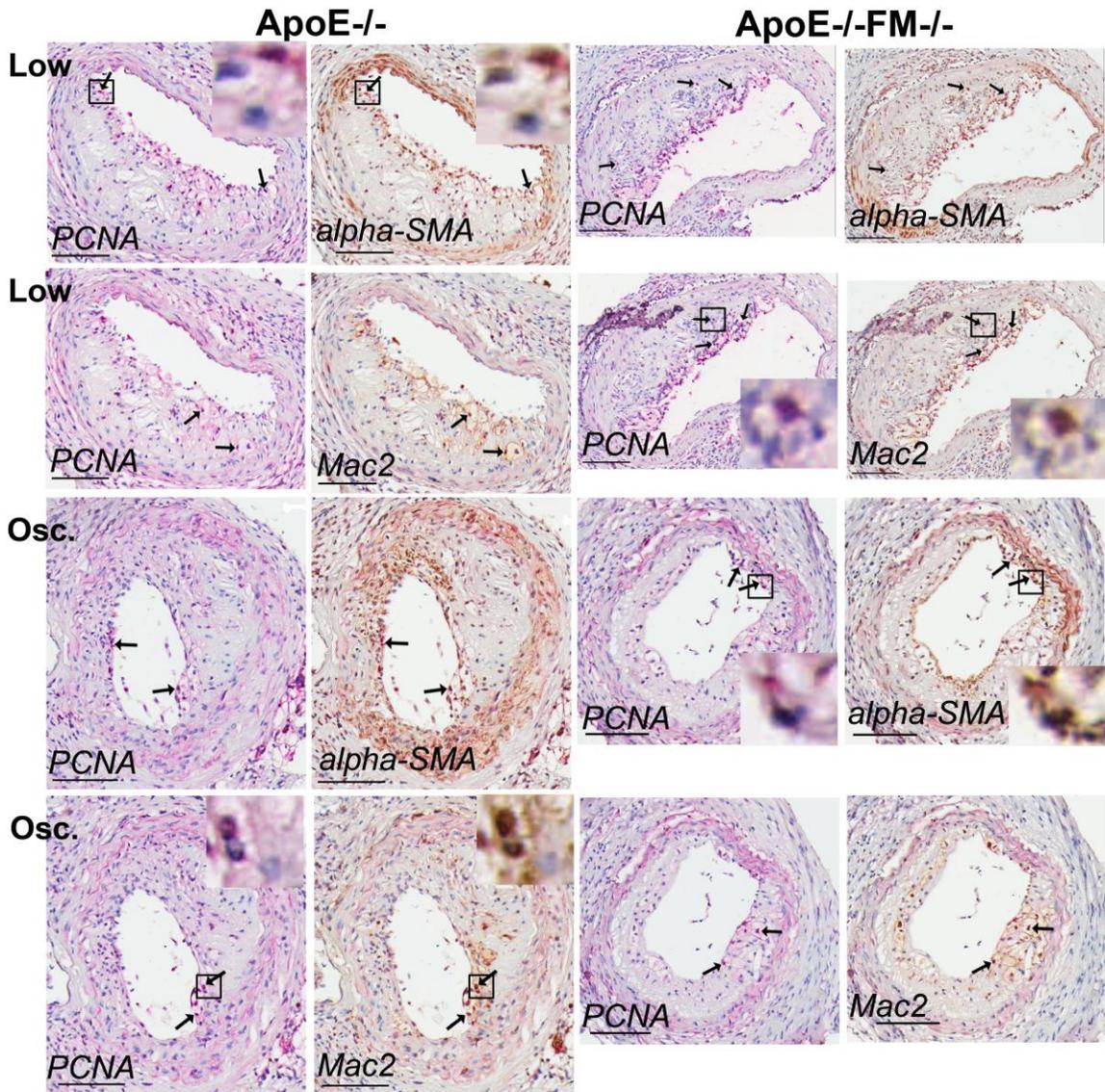
Supplemental Figure VII: Plasma levels of interleukin-1 β (A), -2 (B), -4 (C), -5 (D), -10 (E), -12 (F), interferon- γ (G), KC (H) and tumor necrosis factor- α (I). N=14 and 13 for ApoE-null and ApoE/fibromodulin-null mice, respectively.



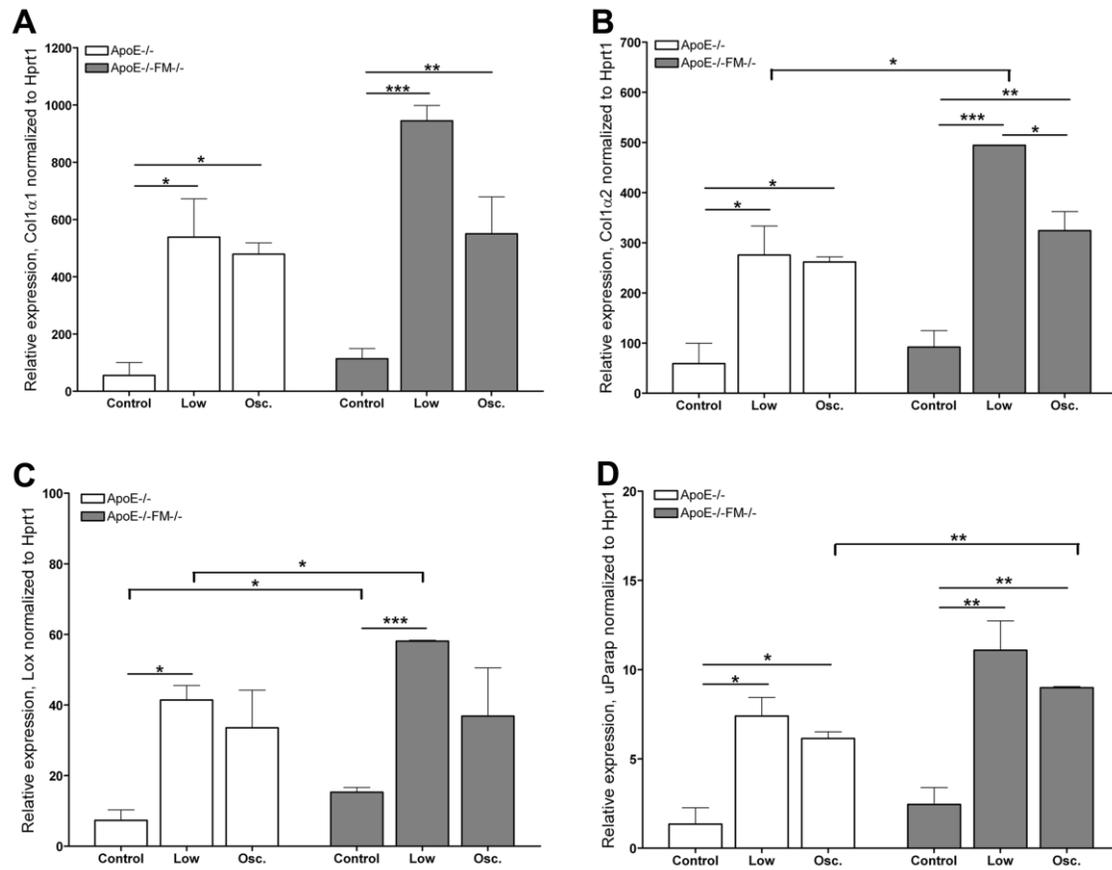
Supplemental Figure VIII: Cholesterol content in plasma from ApoE- and ApoE/fibromodulin-null mice. N=14 and 10 for ApoE-null and ApoE/fibromodulin-null mice, respectively.



Supplemental Figure IX: Fibromodulin deficiency does not affect the rate of apoptosis in lesions from low and oscillatory shear stress regions. Representative TUNEL-stained carotid artery plaques from ApoE^{-/-} and ApoE^{-/-}/fibromodulin-null mice. Scale bars = 100 μ m.



Supplemental Figure X: SMC and macrophage proliferation in low and oscillatory shear stress carotid artery lesions from ApoE- and ApoE/fibromodulin-null mice. Representative sections stained for PCNA only (first, third column; positive nuclei are red) and the same sections double stained (second, fourth column) for PCNA and α -SMA or Mac-2 (positive stains are brown). Black squares represent insets. Scale bars = 100 μ m.



Supplemental Figure XI: QRT-PCR results showing mean expression levels of Col1 α 1 (A), Col1 α 2 (B), Lox (C) and uParap (D) in carotid lesions (n=6). Relative transcript levels in control arteries and lesions were normalized to the reference gene Hprt1.