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Supplemental Information

FXR1 Is an IL-19-Responsive RNA-Binding Protein that Destabilizes Pro-inflammatory Transcripts in Vascular Smooth Muscle Cells

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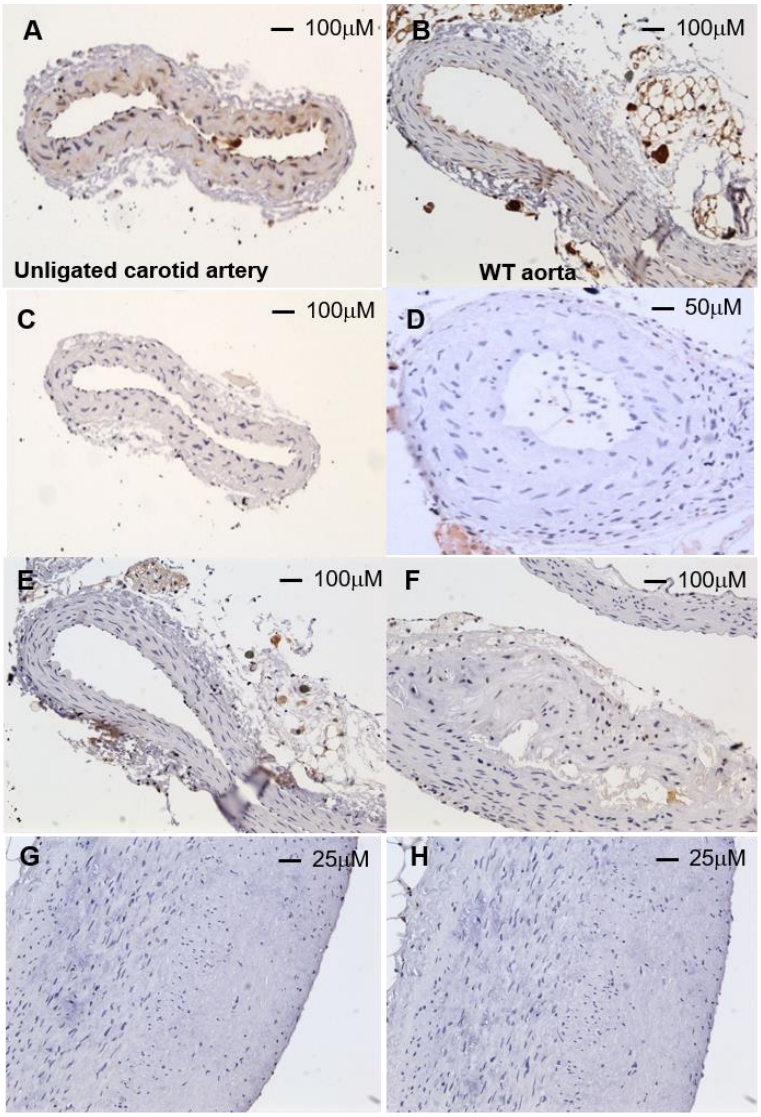

Figure S1. Normal tissue controls for Figure 2. Related to Figure 2. A. unligated normal mouse carotid artery. B. wild-type aorta. C-H matching isotype control antibody for all tissues shown.

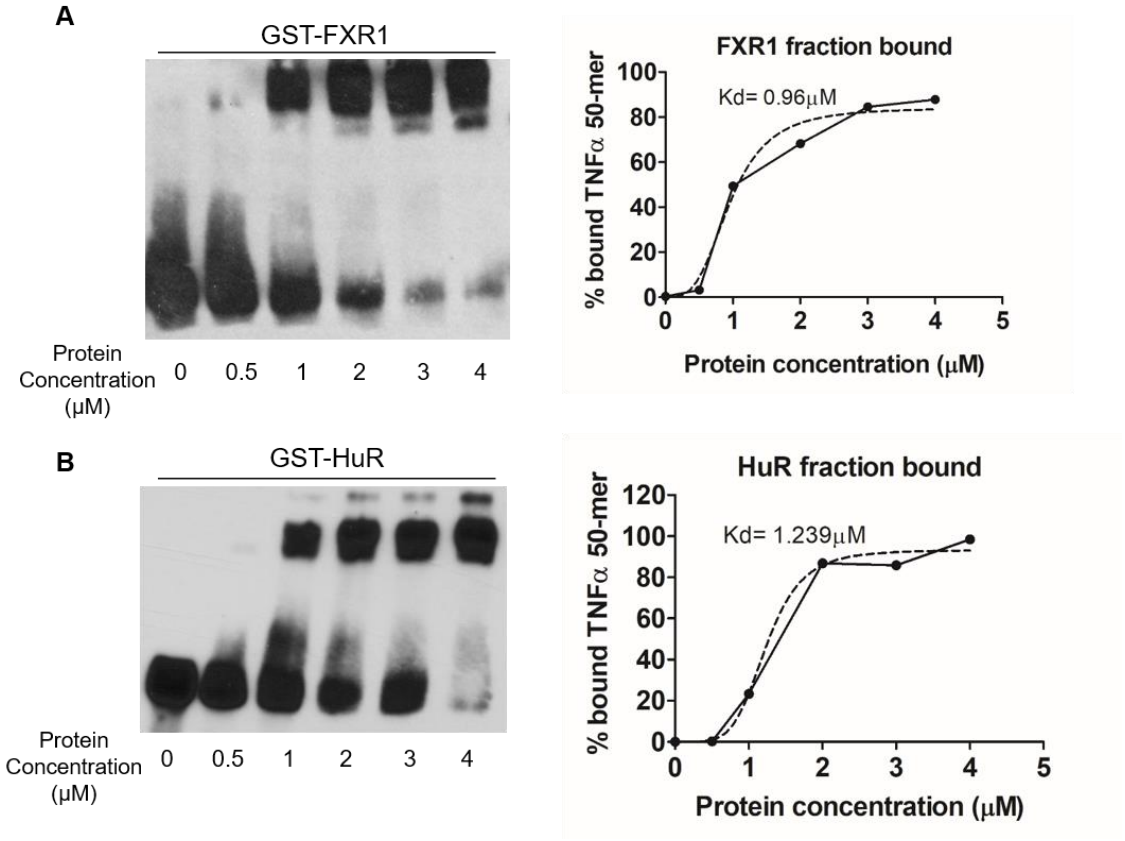

Figure S2. Binding affinities of A. FXR1 and B. HuR for the TNFα 3'UTR 50 mer. Related to Figure 6. Increasing concentrations of GST-fusion protein were incubated with TNFα 3'UTR 50 mer and fraction bound was calculated using non-linear fit, site-specific binding with Hill slope.

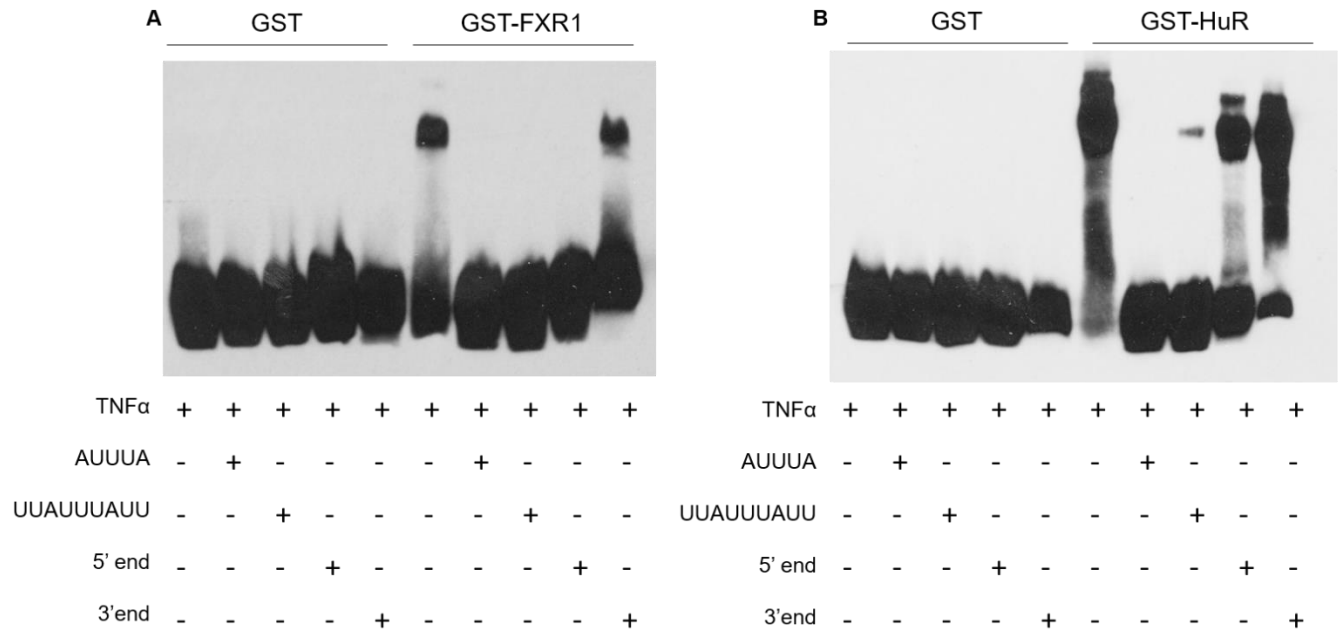

Figure S3. RNA-EMSA cold competition GST-controls. Related to Figure 6. A. Non-biotinylated probes consisting of a 25-mer of $(AUUUA)_5$, a 27-mer of $(UUAUUUAUU)_3$, a 36- mer of $(CUUGUGAUU)_4$, a 40-mer of $(CAGAGAUGAA)_4$ (40 bases) were added to the reaction to compete with the biotinylated TNF αprobe, prior to the addition of the biotinylated TNFα 50mer, and RNA EMSA performed using GST-control and GST-FXR1. The same experimental design was used in B, using GST-control and GST-HuR.

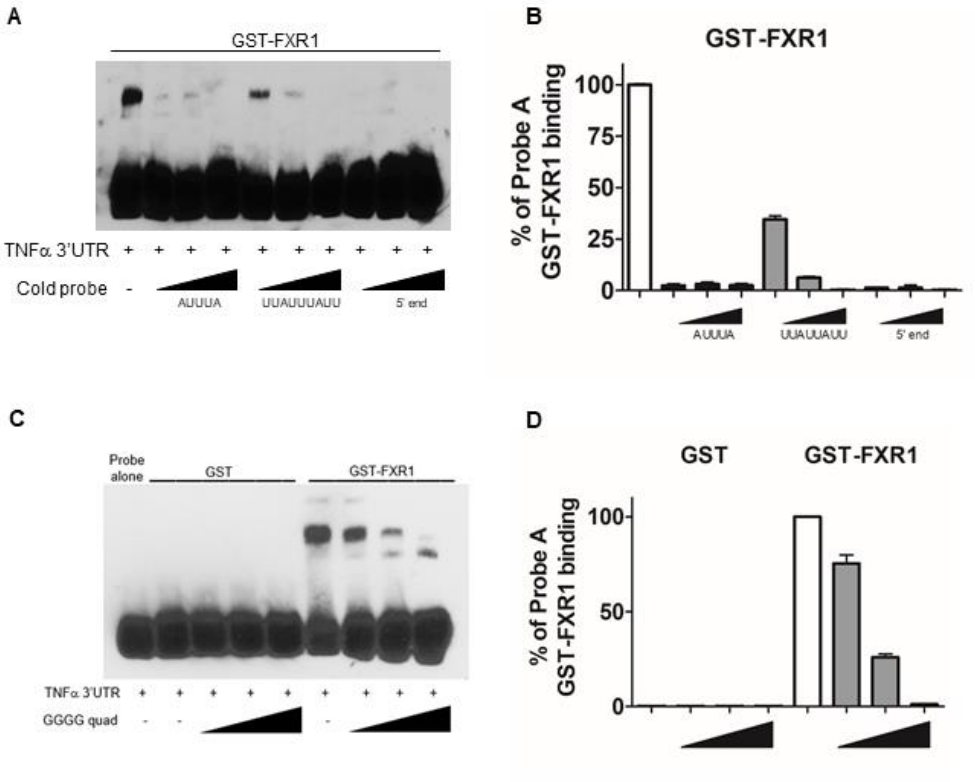

Figure S4. RNA-EMSA cold competition. Related to Figure 6. A. Non-biotinylated probes consisting of a 25-mer of $(AUUUA)_5$, a 27-mer of $(UUAUUUAUU)_3$, a 36- mer of $(CUUGUGAUU)_4$, a 40-mer of $(CAGAGAUGAA)_4$ (40 bases) were added to the reaction to compete with the biotinylated TNF αprobe at increasing concentrations of 0.01mM, 10mM, and 1000mM, prior to the addition of the biotinylated TNF α50mer, and RNA EMSA performed using GST-FXR1. B. Densiometric analysis of percent binding of cold probes to GST-FXR1. C. G quadruplex (GGGG) cold probe was incubated with GST and GST-FXR1 at increasing concentrations 0.01mM, 10mM, and 1000mM for 30 minutes prior to the addition of the biotinylated TNFα 50-mer probe. D. Densiometric analysis of G quadruplex binding to GST-FXR1.

Protein	-	+	% occurrence Agarose	% occurrence FLAG epitope	GO Analysis
HELZ2	0	50	1.78	1.28	Poly(A) RNA binding, ligand-dependent nuclear receptor transcription coactivator activity
UPF1	0	45	3.91	8.33	Poly(A) RNA binding
FAM120A	0	43	8.19	2.56	Poly(A) RNA binding
EIF461	0	37	N/A	N/A	RNA binding
EIF	0	36	N/A	N/A	RNA binding
EIF4G1	0	36	16.73	32.05	RNA binding
LARP1	0	35	16.73	10.26	Poly(A) RNA binding
ILF3	0	33	33.10	41.67	Poly(A) RNA binding and double-stranded RNA binding
IGF2BP3	0	27	17.44	19.23	RNA binding, nucleic acid binding
MOV10	0	26	5.69	5.77	RNA binding, helicase activity
IGF2BP2	0	24	14.95	16.67	RNA binding, nucleic acid binding
PABP14	0	24	N/A	N/A	RNA binding
RRBP1	0	24	13.17	10.26	Poly(A) RNA binding and receptor activity
PABC4	0	24	N/A	N/A	RNA binding
SYNCRIP	0	23	38.43	53.85	Nucleic acid binding and RNA binding
PRRC2C	0	23	14.59	8.33	Poly(A) RNA binding and protein C-terminus binding
OASL	0	20	N/A	N/A	Poly(A) RNA binding and double-stranded RNA binding
FAM120C	0	20	N/A	N/A	Poly(A) RNA binding
ELAV I	0	18	N/A	N/A	Nucleic acid binding and RNA binding
TROVE2	0	18	2.92	2.56	RNA binding and U2 snRNA binding.
HNRNPU	0	18	58.72	83.97	Poly(A) RNA binding and kinase activity
DHX9	0	18	40.21	56.41	Nucleic acid binding and helicase activity
HNRNPA1	0	17	53.38	75.64	Nucleic acid binding and RNA binding
NUFIP2	0	17	23.49	11.54	Poly(A) RNA binding and RNA binding
HNRNPK	0	16	56.94	93.59	Nucleic acid binding and RNA binding
HNRNPL	0	16	42.35	44.87	Nucleic acid binding and RNA binding
STAU2	0	16	6.41	1.92	Poly(A) RNA binding, double-stranded RNA binding
STAU1	0	16	12.10	7.69	Poly(A) RNA binding, double-stranded RNA binding
CCAR2	0	16	21.71	41.03	Poly(A) RNA binding, enzyme inhibitor activity
DDX3X	0	16	33.10	65.38	nucleic acid binding and RNA binding
FUBP3	0	16	9.96	8.33	Nucleic acid binding and RNA binding
DDX5	0	16	50.18	67.95	Nucleic acid binding and enzyme binding
FLJ94229	0	16	N/A	N/A	Nucleic acid binding and RNA binding
DDX3Y	0	15	30.60	61.54	Nucleic acid binding and RNA binding
RPS9	0	15	32.03	46.79	Poly(A) RNA binding, structural constituent of ribosome
ATXN2L	0	15	37.72	8.33	Poly(A) RNA binding and structural constituent of ribosome
CSDA	0	15	N/A	N/A	Nucleic acid binding and transcription factor activity, sequence-specific DNA binding
RPL4	0	14	46.98	41.67	Poly(A) RNA binding and structural constituent of ribosome
ILF2	0	14	29.89	44.87	Poly(A) RNA binding and double-stranded RNA binding
HNRNPC	0	14	38.43	33.97	Nucleic acid binding and identical protein binding
AGO2	0	14	6.05	11.54	Nucleic acid binding and RNA binding
FXR1	0	13	6.05	6.41	Nucleic acid binding and RNA binding
ATXN2	0	13	9.61	0.64	Poly(A) RNA binding and protein C-terminus binding
HNRNPA2B1	0	13	55.16	66.67	Nucleic acid binding and RNA binding
HNRNPH1	0	12	49.47	89.10	Nucleic acid binding and RNA binding
HNRNPA3	0	12	39.86	44.87	Nucleic acid binding and RNA binding
DDX1	0	12	18.86	41.03	Nucleic acid binding and chromatin binding
MYO5A	0	12	4.27	8.33	Nucleic acid binding and chromatin binding
EIF4G3	0	12	10.32	5.13	Poly(A) RNA binding and binding

TRIM25	0	11	7.12	5.13	Poly(A) RNA binding and acid-amino acid ligase activity.
AGO3	0	11	2.85	5.13	Nucleic acid binding and RNA binding
PTBP1	0	10	35.94	51.92	Nucleic acid binding and RNA binding
HNRNPD	0	10	45.91	56.41	Nucleic acid binding and RNA binding
HNRNPUL2	0	10	11.74	6.41	Poly(A) RNA binding and kinase activity
HNRNPUL1	0	10	18.86	17.31	Nucleic acid binding and RNA binding
ZC3HAV1	0	10	14.95	5.77	Poly(A) RNA binding and NAD+ ADP-ribosyltransferase activity
FLJ58199	0	10	N/A	N/A	Nucleic acid binding and RNA binding

Table S1. Complete table of putative HuR-interacting proteins identified by LC-MS/MS ranked in order peptide sequences. Related to Figure 1. Columns Flag empty-vector (-) and the Flag-immunoprecipitated (+) provide the raw spectral counts from VSMCs following IP with anti-Flag antibody. Proteins were identified by LC MS/MS and were included in the table if the spectral count was above 10. The additional columns provide the percent occurrence of an identified protein in agarose bead or Flag epitope samples found in the Contaminant Repository for Affinity Purification (CRAPome) database. Elav1 was the bait protein used to pull-down proteins for LC MS/MS. CRAPome database provided no additional information about Elav1.

Protein	-	+	% occurrence FLAG epitope	GO Analysis
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UPF1	0	45	8.33	Poly(A) RNA binding
PRRC2C	0	23	8.33	Poly(A) RNA binding and protein C-terminus binding
FUBP3	0	16	8.33	Nucleic acid binding and RNA binding
ATXN2L	0	15	8.33	Poly(A) RNA binding and protein C-terminus binding
MYO5A	0	12	8.33	Nucleic acid binding and chromatin binding
ELAV1	0	18	N/A	Nucleic acid binding and RNA binding

Table S2. Putative HuR-interacting proteins identified by LC-MS/MS ranked in order of percent occurrence by FLAG epitope found in the Contaminant Repository for Affinity Purification (CRAPome) database. Related to Figure 1. Columns Flag empty-vector (-) and the Flag-immunoprecipitated (+) provide the raw peptide sequences from VSMCs following IP with anti-Flag antibody. Proteins were identified by LC MS/MS. *Indicates Elav1 was the bait protein used to pull-down proteins for LC MS/MS. CRAPome database provided no additional information about Elav1.

Supplemental Experimental Procedures

Immunohistochemistry. Ligated mouse carotid arteries, plaque from LDLR-/- mice, and human coronary arteries were collected as part of studies described previously (Ellison et al., 2013, 2014). Tissue sections were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide in methanol for 15 min. Tissue sections were blocked in 10% goat serum, incubated with primary antibody at 0.5 μg/ml in 1%BSA/PBS and were applied for 1 h, followed by incubation with biotinylated secondary antibody (1:200), followed by avidin-biotin peroxidase complex each for 30 min. Non-specific identical IgG isotype control (Neomarkers # NC-100-P, and Biolegend #400601) antibodies were used as negative controls. The reaction product was visualized using DAB (Vector Labs, Burlingame, Ca, USA) as the chromogenic substrate, which produces a reddish-brown stain. The sections were counterstained with hematoxylin. Immunofluorescence co-staining on human coronary artery sections was performed as described (Autieri et al., 2002; Ellison et al., 2013). Briefly, anti-SMCα actin, (NeoMarkers, Inc, Burlingame, CA, USA) and anti-FXR1 antibody was followed by a 30-minute incubation with secondary antibody conjugated to AlexaFluor 568 (red) and AlexaFluor 488 (green) (Molecular probes, Inc., Eugene, OR, USA). For cellular stress granule staining, human VSMC were grown on glass cover slides and fluorescent stained with Anti-PABP antibody (AbCam, Cambridge, UK), Anti-FXR1 antibody (AbCam), were used. Will need to ask Sheri for HuR and DAPI.

LC-MS/MS. LC-MS/MS analysis was performed as described previously (Haines et al., 2012). Briefly, VSMC were transduced with recombinant adenovirus expressing FLAG-tagged HuR or empty vector control. Pellets were collected and lysed in maltoside based-lysis buffer containing 1 × protease inhibitor tablet (Roche) for 30 min on a nutator at 4 °C. The lysates were centrifuged at 16,600 × *g* for 15 min to remove cell debris, and the supernatant was incubated with anti-FLAG beads, followed by 5 washes in lysis buffer. Proteins were eluted from beads in 10 M freshly prepared urea. Digestion was performed in 100 mM Tris-HCl (pH 8.5) containing 8 M urea at 37 °C first with Lys-C (35 ng/mg lysate) for 4 h, and then the urea concentration was reduced to 2 M for trypsin (30 ng/mg lysate) digestion overnight. Following digestion, the tryptic peptides were desalted on a reversed-phase Vivapure C18 micro spin column (Sartorius Stedim Biotech, Gottingen, Germany) and concentrated using a SpeedVac. Dried samples were acidified by 0.2% formic acid prior to liquid chromatography-mass spectrometric analysis. All LC-MS/MS experiments were performed on a nanoflow LC system, EASY-nLC II (Thermo Scientific, Waltham, MA, USA) connected to a hybrid LTQ Orbitrap Classic (Thermo Scientific, Waltham, MA, USA) equipped with a nano-electrospray ion source. For the EASY-nLC II system, solvent A consisted of 97.8% H2O, 2% ACN, and 0.2% formic acid and solvent B consisted of 19.8% H2O, 80% ACN, and 0.2% formic acid. Samples were directly loaded onto a 16-cm analytical HPLC column (75 mm ID) packed in-house with ReproSil-Pur C18AQ 3 um resin (120A° pore size, Dr. Maisch, Ammerbuch, Germany). The column was heated to 45° C. The peptides were separated with a 160 min gradient at a flow rate of 350 nL/min. The gradient was as follows: 2–30% Solvent B (150 min), 30–100% B (1 min), and 100% B (9 min). Eluted peptides were then ionized using a standard coated silica tip (New Objective, Woburn, MA, USA) as an electrospray emitter and introduced into the mass spectrometer. The LTQ Orbitrap was operated in a data-dependent mode, automatically alternating between a full-scan (m/z 300-1700) in the Orbitrap and subsequent MS/MS scans of the 15 most abundant peaks in the linear ion trap (Top15 method). Data acquisition was controlled by Xcalibur 2.0.7 and Tune 2.4 software (Thermo Fisher Scientific, Waltham, MA, USA). For data analysis, peaks were generated from raw data files using MaxQuant (version 1.5.1.2) with default parameters and searched using the built-in search engine Andromeda. Peak lists were searched against the UniProt human database (148298 sequences including isoforms) and a contaminant database (262 sequences). Thresholds for peptide and protein scores were chosen to have at most a 1% FDR as estimated by a decoy database (Cox and Mann, 2008; Cox et al., 2011).

RNA Extraction and Quantitative RT-PCR. VSMCs were serum starved in 0.1% FCS for 48 hours, then stimulated with 10 ng/mL TNFα for the indicated times. RNA from cultured VSMCs was isolated and reverse transcribed into cDNA, as we have described, and target genes were amplified using an Applied Biosystems

StepOne Plus Real-Time PCR System as we described (Gabunia et al., 2016, 2017). Multiple mRNAs (CT values) were quantitated simultaneously by the Applied Biosystems software. Primer pairs were purchased from Integrated DNA Technologies (Coralville, IA, USA), and SYBR Green was used for detection. The following primer pairs were used:

hGAPDH: F: CGAGAGTCAGCCGCATCTT, R: CCCCATGGTGTCTGAGCG,

hIL-1β: F: TCCCCAGCCCTTTTGTTGA, R: TTAGAACCAAATGTGGCCGTG,

hICAM1: F: CTCCAATGTGCCAGGCTTG, R: GAGTGGGAAAGTGCCATCCT

hFXR1: F: GAGTTACCGCCATTGAGCTAG, R: ACTTTTCCAACGAGATTCCTAGG

hHuR: F: CCGTCA CAAATGTGAAAGTG, R: TCGCGGCTTCTTCATAGTTT

hTNFα: F: GGTCTACTTTGGGATCATTGC, R: GAAGAGGTTGAGGGTGTCTG

hMCP1: F: AGCAGAAGTGGGTTCAGGATT, R: TGTGGAGTGAGTGTTCAAGTCT

hPPARα: F: GCTATCATTACGGAGTCCACG, R: TCGCACTTGTCATACACCAG

hB2M: F: GGCATTCCTGAAGCTGACAG, R: TGGATGACGTGAAACCTG

Transfection, siRNA knockdown and overexpression, and luciferase. Gene silencing was performed using ON-TARGET plus SMARTpool FXR1 siRNA, which contains a mixture of four siRNAs which target human FXR1 (10 nM) purchased from Dharmacon, Inc. (Lafayette, Co, USA) as we have described (Cuneo et al., 2010; Gabunia et al., 2016). Scrambled control siRNA was also purchased from Dharmacon, Inc. Transfection of VSMC was performed using the AMAXA Nucleofector™ Kit (Amaxa, Inc., Gaithersburg, MD, USA) following the manufacturer's instructions as we described (Gabunia et al., 2016, 2017). For overexpression studies, human AdenoFXR1 was purchased from Vigene Biosciences (Rockville, MD, USA). The AdenoFXR1 and control virus AdenoGFP were used at 100 MOI in the transduction of hVSMCs. Forty-eight hours after infections, VSMC were serum starved 24 hours, then treated as described in the legend. For luciferase experiments, 6μg of a constitutively-driven luciferase reporter vector (LightSwitch™ 3′UTR Reporter Vector, Switchgear Genomics, Menlo Park, Ca, USA) which contains multiple ARE tandem repeats was transfected into HEK using Fugene transfection reagent (Promega, Madison, WI, USA), and co-transduced with plasmid encoding FXR1 cDNA (6μg). Forty-eight hours after transfection, luciferase activity was quantitated as we have described using LightSwitch™ Luciferase Assay Kits (Gabunia et al., 2016).