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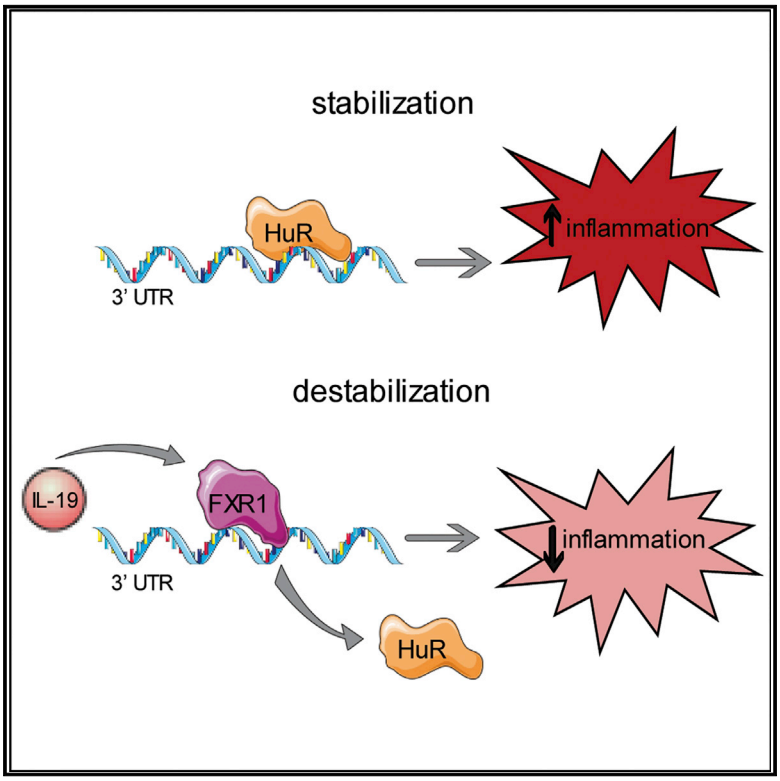
FXR1 Is an IL-19-Responsive RNA-Binding Protein that Destabilizes Pro-inflammatory Transcripts in Vascular Smooth Muscle Cells

Graphical Abstract



Authors

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In Brief

FXR1 is a muscle-enhanced and IL-19-inducible RNA-binding protein. Herman et al. show that FXR1 acts as a negative regulator of inflammatory gene expression by competing with HuR on the 3′ UTR of inflammatory mRNA transcripts. FXR1 is required for IL-19-dependent reduction of HuR mRNA stability and abundance.

Highlights

- FXR1 interacts with HuR via mRNA tethering on the 3′ UTR of inflammatory transcripts
- FXR1 is a negative regulator of inflammatory transcript mRNA stability
- FXR1 binds canonical and non-canonical sequences in the 3′ UTR of TNFα
- FXR1 is required for IL-19-dependent reduction of HuR mRNA stability and abundance

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

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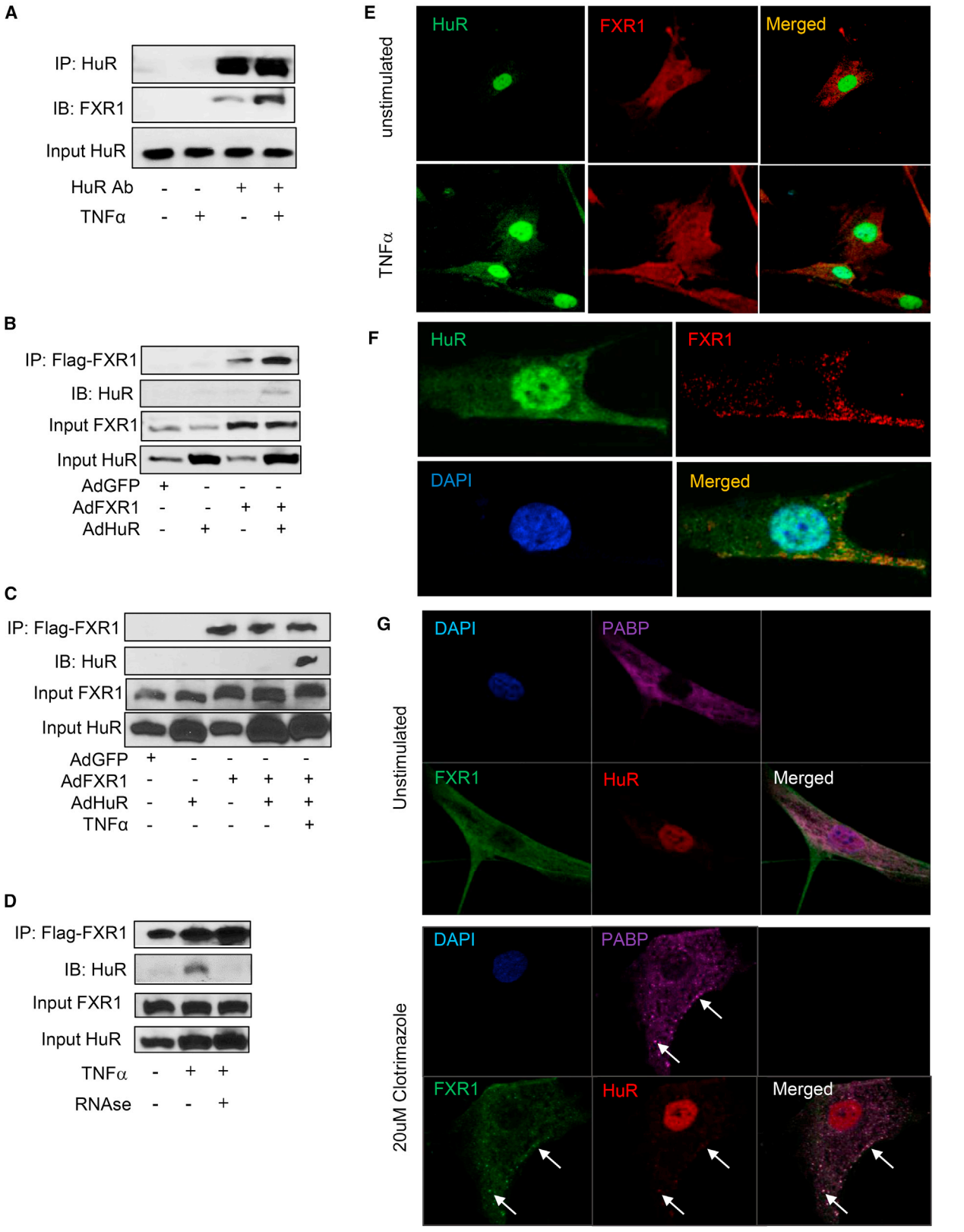
SUMMARY

This work identifies the fragile-X-related protein (FXR1) as a reciprocal regulator of HuR target transcripts in vascular smooth muscle cells (VSMCs). FXR1 was identified as an HuR-interacting protein by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The HuR-FXR1 interaction is abrogated in RNase-treated extracts, indicating that their association is tethered by mRNAs. FXR1 expression is induced in diseased but not normal arteries. siRNA knockdown of FXR1 increases the abundance and stability of inflammatory mRNAs, while overexpression of FXR1 reduces their abundance and stability. Conditioned media from FXR1 siRNA-treated VSMCs enhance activation of naive VSMCs. RNA EMSA and RIP demonstrate that FXR1 interacts with an ARE and an element in the 3′ UTR of TNFα. FXR1 expression is increased in VSMCs challenged with the anti-inflammatory cytokine IL-19, and FXR1 is required for IL-19 reduction of HuR. This suggests that FXR1 is an anti-inflammation responsive, HuR counter-regulatory protein that reduces abundance of pro-inflammatory transcripts.

INTRODUCTION

Despite nutritional modification and lipid-reducing medications, atherosclerotic and other vascular syndromes account for 50% of all mortality and is increasing in the developing world. The injurious effects of pro-inflammatory cytokines resulting in vascular smooth muscle cell (VSMC) activation and development of multiple vascular diseases are well described (Allahverdian et al., 2014; Ross, 1999). Results from the recent CANTOS trial support the preeminent role of inflammation in vascular disease (Weber and von Hundelshausen, 2017). VSMCs respond to and synthesize pro-inflammatory immune modulators (Doran et al., 2008; Hansson and Libby, 2006; Singer et al., 2004) and promulgate the recruitment of leukocytes to the lesion (Hansson and Libby, 2006; Libby et al., 1997), leading to a localized vascular inflammatory lesion. In many vascular diseases, VSMCs migrate into the intima, where they proliferate and synthesize cytokines and matrix proteins leading to loss of lumen and subsequent tissue ischemia. Resolution of inflammation is a dynamic and tightly regulated process, and much attention has been aimed at identification of countervailing mechanisms that modulate inflammatory processes (Fredman and Tabas, 2017; Libby et al., 2014). A better understanding of countervailing mechanisms that modulate inflammatory processes, and identification of proteins and pathways that modulate the VSMC response to injury is key to the development of therapeutics to combat multiple vascular diseases.

The regulation of mRNA stability and translation are two levels of post-transcriptional regulation that permit VSMCs to rapidly respond to inflammatory stimuli (Barreau et al., 2006). AU-rich elements (AREs) in the 3′ UTR of mammalian mRNA appear to be the target sequence for degradation or stabilization of transcripts. Most of the transcripts targeted for rapid degradation encode key regulatory proteins involved in cell growth, inflammation, and other responses to external stimuli (Bakheet et al., 2001). Importantly, most inflammatory cytokines contain conserved or semi-conserved AU-rich elements in their 3′ UTR, imparting target specificity for a potential anti-inflammatory modality (Peng et al., 1996). Controlling mRNA decay allows the cell to fine-tune mRNA abundance and translation for rapid adaptation to environmental conditions, especially inflammation (Schoenberg and Maquat, 2012). An essential regulatory protein involved in this process is human antigen R (HuR), a member of the Elav protein family and one of the best characterized, ARE-recognizing, RNA-binding proteins (RBPs) involved in mRNA stability and regulation of pro-inflammatory gene expression (Doller et al., 2008; Palanisamy et al., 2012). While HuR is ubiquitously expressed, it is activated in response to inflammatory signals to stabilize inflammatory mediators (Doller et al., 2008; Palanisamy et al., 2012). Since most pro-inflammatory transcripts contain AREs in their 3′ UTR, this is a crucial and specific mechanism for the initiation and maintenance of the pro-inflammatory phenotype observed in vascular diseases. The exact mechanisms of HuR regulation have yet to be characterized; however, they could represent key targets in regulating inflammation



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(Gallouzi and Steitz, 2001). Even though modulation of mRNA stability has been posited as a possible therapeutic strategy (Eberhardt et al., 2007), surprisingly, there is negligible literature exploring the concept that it could be directly regulated or possibly reduced by anti-inflammatory stimuli. We posit that the regulation of HuR and other RBPs is a critical, and understudied, step in the regulation of vascular inflammation.

We previously reported that interleukin (IL)-19, an anti-inflammatory cytokine, reduced inflammatory transcript mRNA stability in VSMCs (Cuneo et al., 2010) and reduced HuR abundance in several cell types (Ellison et al., 2013). In this report, we identify and characterize one protein, termed fragile X-related protein (FXR1), a muscle-enhanced, autosomal homolog of the FMR (fragile X mental retardation) neural protein, which interacts with HuR in inflammatory, but not basal, conditions. We report here that FXR1 expression is induced by IL-19 in VSMCs and that modulation of FXR1 regulates ARE-containing transcripts in VSMCs. RNA EMSA (electrophoretic mobility shift assay) and RNA immunoprecipitation demonstrate that FXR1 interacts with the canonical AREs and a previously uncharacterized element in the 3′ UTR of tumor necrosis factor alpha (TNFα. This work implicates FXR1 as a previously unrecognized negative regulator of inflammation and suggests that IL-19 induction of FXR1 expression is a negative compensatory, counter-regulatory mechanism used by VSMCs to respond to and resolve inflammation.

RESULTS

HuR Interacts with FXR1

It is presumed that HuR activity is regulated by interacting proteins (Doller et al., 2008; Gallouzi and Steitz, 2001; Pullmann et al., 2005). Human vascular smooth muscle cells (hVSMCs) were transfected with a FLAG-tagged HuR or FLAG-tagged empty control vector and then starved for 48 hr in 0.1% fetal bovine serum (FBS) before stimulation with TNFα. HuR pull-down was followed by un-biased liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the proteins that immunoprecipitated with HuR. HuR-interacting candidates were identified by eliminating any protein with a raw peptide count below ten (Table S1). Interacting proteins were also scrutinized in the Contaminant Repository for Affinity Purification (CRAPome) database (Mellacheruvu et al., 2013) to determine the occurrence of proteins in control experiments to eliminate "sticky proteins" that may non-specifically interact with HuR. The final list of proteins that met these criteria were examined for Gene Ontology (GO) annotation. A number of interacting proteins were identified; most were involved in various aspects of mRNA processing (Table S2). The last row of Table S2 includes Elav1 (HuR), as it was the bait protein used to perform LC-MS/MS, although nothing is known about HuR and the FLAG-tag epitope according to the CRAPome database. FXR1 was chosen for further study because of the novelty of its interaction; because FXR1 expression is muscle enhanced (Garnon et al., 2005; Mientjes et al., 2004); and because no literature exists on FXR1 inducibility by inflammatory stimuli or VSMC, making FXR1 a particularly attractive target to study in the context of vascular disease. Finally, similar to HuR, FXR1 is presumed to be an RBP (Adinolfi et al., 1999). FXR1 exists in several isoforms in mouse and is predicted to have three isoforms in human (Dubé et al., 2000). We were only able to detect isoform 1 in human VSMCs by western blot and transcript-specific qRT-PCR (data not shown). Subsequent experiments utilized isoform 1 to ensure that all domains were represented.

A series of immunoprecipitations were performed in order to confirm the interaction between HuR and FXR1. First, we performed a co-immunoprecipitation of endogenous FXR1 for HuR in hVSMCs that were either serum starved or serum starved and stimulated with TNFα for 8 hr (Figure 1A). We also overexpressed FXR1 using a FLAG-tagged adenovirus (adeno-FXR1 [AdFXR1]) and concurrently overexpressed HuR also using an adenovirus (AdHuR) in hVSMCs and performed immunoprecipitation using anti-FLAG-conjugated beads (Figure 1B). Next, hVSMCs were treated as described, but after serum starvation, they were stimulated with TNFα for 8 hr. Figure 1C shows that the HuR/FXR1 interaction was enhanced in TNFα-stimulated cells. Figure 1D shows that the FXR1-HuR interaction is abrogated by the addition of RNase A, suggesting that the interaction we identified via proteomics may be mediated by RNA tethering. The increased FXR1-HuR interaction observed in TNFα-stimulated VSMCs may be due to an increase in transcripts that harbor both FXR1 and HuR binding elements.

We next used confocal microscopy to determine HuR and FXR1 localization under basal and inflammatory conditions in hVSMCs. HuR nucleocytoplasmic shuttling has been reported (Wu et al., 2016), and consistent with the literature, in TNFα-stimulated VSMCs, HuR translocated from the nucleus to the cytoplasm. FXR1 remained predominantly cytoplasmic in both unstimulated and stimulated conditions. Interestingly, HuR and FXR1 co-localized in the cytoplasm following 8-hr TNFα

Figure 1. FXR1 and HuR Interact

(A) Co-immunoprecipitation of endogenous FXR1 with HuR in unstimulated or TNFα-stimulated VSMCs. IP, immunoprecipitation; IB, immunoblot.

(B) hVSMCs transduced with adeno-GFP-control vector, FLAG-tagged adeno-FXR1, and HuR adenovirus, followed by immunoprecipitation by anti-FLAG-conjugated beads.

(C) The FXR1-HuR interaction increases in TNFα-stimulated conditions.

(D) FXR1-HuR interaction is mediated by mRNA. The addition of RNase A to the immunoprecipitation reaction disrupted TNFα-driven FXR1-HuR interaction.

(E) HuR and FXR1 co-localization in hVSMCs. HuR remained predominantly nuclear, while FXR1 localized to the cytoplasm in unstimulated VSMCs. Upon stimulation with TNFα, HuR shuttled to the cytoplasm where it co-localized with FXR1.

(F) High-resolution confocal microscopy of HuR-FXR1 interaction in the cytoplasm of TNFα-stimulated hVSMCs.

(G) FXR1 and HuR co-localize to stress granules. HVSMCs were untreated or stimulated with 20 μM clotrimazole for 45 min and then stained for stress granule marker poly(A)-binding protein (PABP), FXR1, and HuR, and with DAPI, and were imaged using confocal microscopy to determine co-localization.

Magnification is 630× for (E) and (G) and 1,260× for (F).

See also Tables S1 and S2.

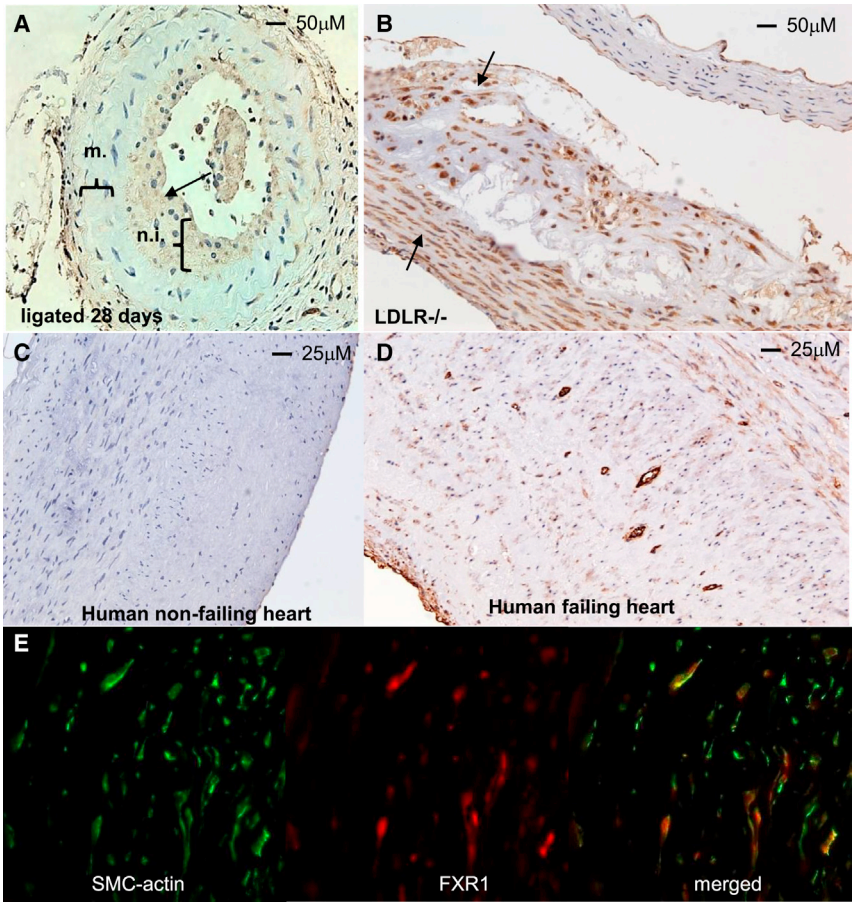


Figure 2. FXR1 Protein Expression in Vascular Injury and Disease Models

(A) FXR1 expression in ligated murine carotid artery. Mouse carotid arteries were harvested 28 days after ligation, and immunohistochemistry was performed. FXR1 primarily stains in the neointima (n.i.), but not the media (m.) in the ligated artery.

(B) FXR1 expression in mouse atherosclerotic plaque. Cross-section from an LDLR$^{-/-}$ mouse aorta fed an HFD for 12 weeks to develop atherosclerotic plaque. VSMCs in plaque and smooth muscle cell cap are enriched for FXR1 expression.

(C) FXR1 expression in normal human coronary artery from a non-failing heart.

(D) FXR1 expression in myleofibroid atherosclerotic plaque from a failing human artery. FXR1 expression is enriched in myleofibroid VSMCs in the plaque as compared to healthy human control artery.

(E) Fluorescent co-staining of fibro-atherosclerotic cap from human atherosclerotic plaque using antibody to SMC-actin and FXR1. See also Figure S1 for normal mouse artery and negative controls for immunohistochemistry.

Magnification is 200× for all panels. The arrows point to areas of enhanced FXR1 expression in (A) VSMCs of the neointima or in (B) VSMC of the cap of the atherosclerotic plaque.

See also Figure S1.

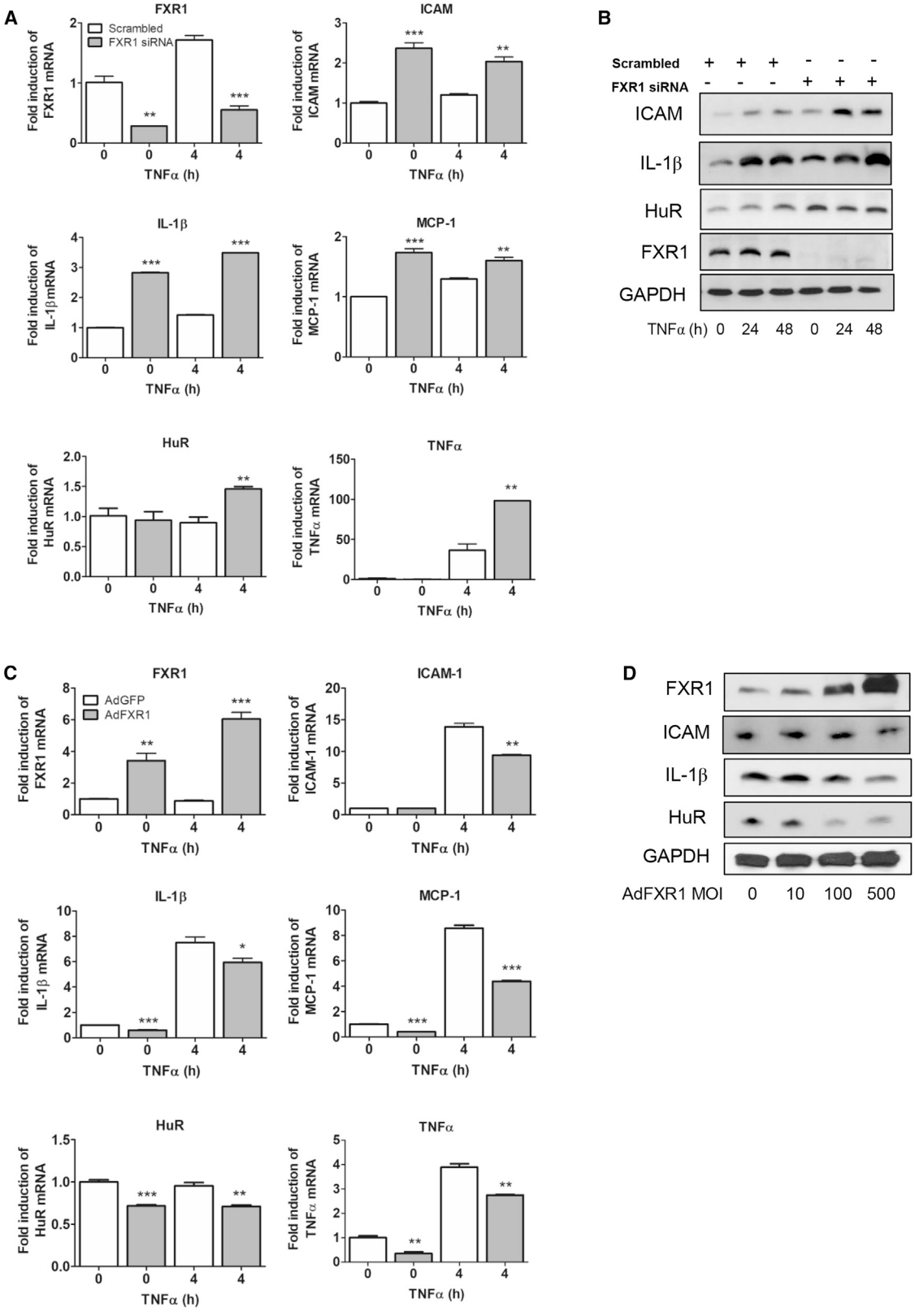
stimulation, which is consistent with literature showing HuR nucleocytoplasmic translocation upon inflammatory stimuli (Figures 1E and 1F). RNA processing often occurs in phase-dense structures that form in the cytoplasm of eukaryotic cells in response to environmental stresses. The composition of stress granules suggests that regulation of labile ARE-containing inflammatory transcripts could be occurring there. To further associate a relationship between FXR1 and HuR in RNA processing, we used immunostaining and confocal microscopy to co-localize HuR and FXR1 within punctate stress granules in hVSMCs. Clotrimazole was used to induce stress granule formation, and poly(A)-binding protein (PABP) was used as a marker for stress granules (Kedersha et al., 2008). Figure 1G shows that hVSMCs stimulated with 20 μM clotrimazole demonstrated well-defined, punctate co-localization of FXR1 and HuR in stress granules, which further suggests a role for FXR1 along with HuR in RNA processing in VSMCs.

FXR1 Expression Is Induced in Diseased VSMCs

There is no literature describing FXR1 induction in VSMCs or models of vascular injury. We examined FXR1 expression in mouse and human atherosclerotic and restenotic tissue and detected inducible FXR1 expression in VSMCs in multiple models of vascular injury. Figure 2A indicates that FXR1 expression is increased in neointimal, compared with medial, VSMCs in the carotid artery from ligated mice. Similarly, FXR1 expression is increased in VSMCs in atherosclerotic plaque and cap, but much lower in non-diseased medial VSMCs in the aortic arch from LDLR$^{-/-}$ mice fed an atherogenic diet (Figure 2B). FXR1 expression is negligible in normal, non-diseased arteries from these mice (Figures S1A and S1B). Importantly, FXR1 expression is barely detectible in a coronary artery from a non-failing human heart, but expression is enhanced in myofibrous atherosclerotic plaque from a human coronary artery (Figures 2C and D). Figure 2E shows dual-color immunohistochemistry indicating that FXR1 expression co-localizes in plaque SMCs in the human coronary artery. Together, these data suggest that FXR1 induction is a VSMC response to inflammatory stimuli *in vivo*.

FXR1 Regulates Abundance and mRNA Stability of Pro-inflammatory mRNA and Protein

Literature on FXR1 function is inconsistent and appears to be cell type specific. To more definitively link FXR1 function with vascular inflammation, we transfected hVSMCs with FXR1-specific small interfering RNA (siRNA) and then stimulated the cells with TNFα. Knockdown of FXR1 resulted in a dramatic and significant increase in the abundance of inflammatory transcripts in hVSMCs (IL-1 β, ICAM1, and MCP-1 are shown as examples). Interestingly, these transcripts have been previously shown to be stabilized by HuR in other cell types (Aguado et al., 2015; Chen et al., 2006; Krishnamurthy et al., 2010; Wu et al., 2016) (Figure 3A). Correspondingly, Figure 3B shows that siRNA reduction of FXR1 also increases the abundance of inflammatory proteins as well as HuR in TNFα-stimulated human VSMCs. As FXR1



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knockdown increased inflammatory transcripts, we reasoned that FXR1 overexpression would reduce abundance of inflammatory mRNA and protein. Adenoviral overexpression of FXR1 decreases the abundance of inflammatory mRNA (Figure 3C) and protein (Figure 3D) in a dose-dependent fashion compared with AdGFP control. The siRNA knockdown and overexpression data are complementary and strongly suggest that FXR1 expression may regulate abundance of inflammatory proteins as well as HuR in VSMCs.

FMR1 family members are putative RBPs (Adinolfi et al., 1999), and we next determined whether modulation of FXR1 would affect mRNA stability. Using the transcription inhibitor actinomycin D in TNFα-stimulated VSMCs, we determined that the mRNA stability of ARE-containing transcripts IL-1 β, ICAM1, and HuR is significantly increased when FXR1 is knocked down and, importantly, significantly decreased when FXR1 is overexpressed (Figures 4A and 4B). The mRNA stability of peroxisome proliferator-activated receptor alpha (PPARα), the expression of which is not regulated by AREs in its 3′ UTR, was not affected by FXR1 knockdown or overexpression, demonstrating target transcript specificity for FXR1 activity. Of particular importance was the finding that FXR1 appears to have a reciprocal relationship with HuR abundance, suggesting important, possibly competitive roles for these proteins in regulation of mRNA stability. Together, these results suggest a previously unrecognized function for FXR1 in regulation of mRNA stability and subsequent abundance of pro-inflammatory proteins.

FXR1 Regulates VSMC Proliferation and Inflammation through Paracrine Signaling

Maladaptive VSMC proliferation is a hallmark of several vascular pathologies and is driven by inflammatory gene expression (Hansson and Libby, 2006; Libby, 2002; Libby et al., 2014; Ross, 1999). Knockdown of FXR1 in hVSMCs significantly increased cell proliferation compared to scrambled control cells (Figure 5A). Concordantly, VSMC proliferation was significantly decreased in a manner inversely proportional with FXR1 expression, confirming that FXR1 can regulate VSMC proliferation (Figure 5B).

VSMC paracrine signaling is also a characteristic of many vascular diseases. Since FXR1 appeared to regulate the abundance of cytokines, it was important to determine whether this participated in paracrine signaling. First, serum-starved VSMCs were stimulated with conditioned media collected from VSMCs transfected with FXR1 siRNA or scrambled controls for 4 hr; then, inflammatory transcript mRNA was quantitated. Figure 5C shows that hVSMCs treated with FXR1 siRNA knockdown conditioned media had increased inflammatory gene expression compared to scrambled control.

Next, using conditioned media from scrambled control or FXR1 siRNA knockdown VSMCs, we performed a proliferation assay to demonstrate the autocrine and paracrine effects on cell growth (Figure 5D). hVSMCs treated with FXR1 siRNA conditioned media had significantly increased proliferation compared to scrambled media control. These data suggest that the reduction of FXR1 results in increased cytokine production that has potential autocrine and paracrine effects on other hVSMCs.

FXR1 Binds RNA via Canonical ARE and Non-ARE Sequences

Various complementary methods were used to determine whether FXR1 binds mRNA. First, glutathione S-transferase (GST) and human GST-FXR1 fusion proteins were used in RNA EMSAs (cRNA EMSAs) with a biotinylated probe consisting of a 50-bp region of the human TNFα 3′ UTR (position 1,333–1,380). The addition of recombinant FXR1 to this probe suggested that it bound to RNA (Figure 6A). The AUUUA monomer (as negative control probe) did not form a complex with FXR1. Interaction specificity was demonstrated by super-shift of the FXR1-probe complex by the addition of anti-FXR1 antibody (Figure 6B). FXR1 binding affinity for this region was calculated, and, in our hands, FXR1 has similar affinity to the TNFα probe as HuR (Figures S2A and S2B).

To determine the site or sites on the TNFα 3′ UTR recognized by FXR1, four probes representing different regions of the 50-bp TNFα 3′ UTR (probe A) were synthesized; a 25-mer of $(AUUUA)_5$ (probe B), a 27-mer of $(UUAUUUAUU)_3$ (probe C), a 36-mer of $(CUUGUGAUU)_4$ (probe D), and a 40-mer of $(CAGAGAUGAA)_4$ (probe E), were added to the cRNA EMSAs to compete with the biotinylated 50-bp TNFα 3′ UTR probe. The GST negative control protein did not interact with any probes (Figures S3A and S3B). FXR1 binding with various amounts of cold competitor probes was performed to determine probe input concentrations (Figures S4A and S4B). Figure 6C shows that probes B and C, which contained recognized AREs, were capable of competing with the full-length probe for FXR1 binding and nearly ablated the gel shift. Interestingly, probe D, which does not contain a

Figure 3. FXR1 Regulates Abundance of Pro-inflammatory mRNA and Proteins in VSMCs

(A) FXR1 knockdown increases mRNA abundance of inflammatory mediators in VSMCs. hVSMCs were transfected with FXR1 siRNA or scrambled control siRNA, serum starved, and then stimulated with TNFα for 4 hr to induce mRNA expression that was quantitated by qRT-PCR and normalized to GAPDH expression. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

(B) FXR1 knockdown increases protein abundance of inflammatory mediators in VSMCs. Cell extracts were prepared from VSMCs treated as described above and stimulated with TNFα for 24 hr, and proteins were identified by western blot analysis. Image shown is representative from 3 independently performed experiments.

(C) Overexpression of FXR1 reduces abundance of pro-inflammatory mRNA and proteins. hVSMCs were transduced with 100 MOI of adeno-FXR1 (AdFXR1) or AdGFP, serum starved, and then stimulated with TNFα for 4 hr to induce mRNA expression that was quantitated by qRT-PCR and normalized to GAPDH expression. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

(D) FXR1 overexpression increases protein abundance of inflammatory mediators in VSMCs. Cell extracts were prepared from VSMCs treated as described above and stimulated with TNFα for 24 hr, and proteins were identified by western blot analysis. Image shown is representative of 3 independently performed experiments.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

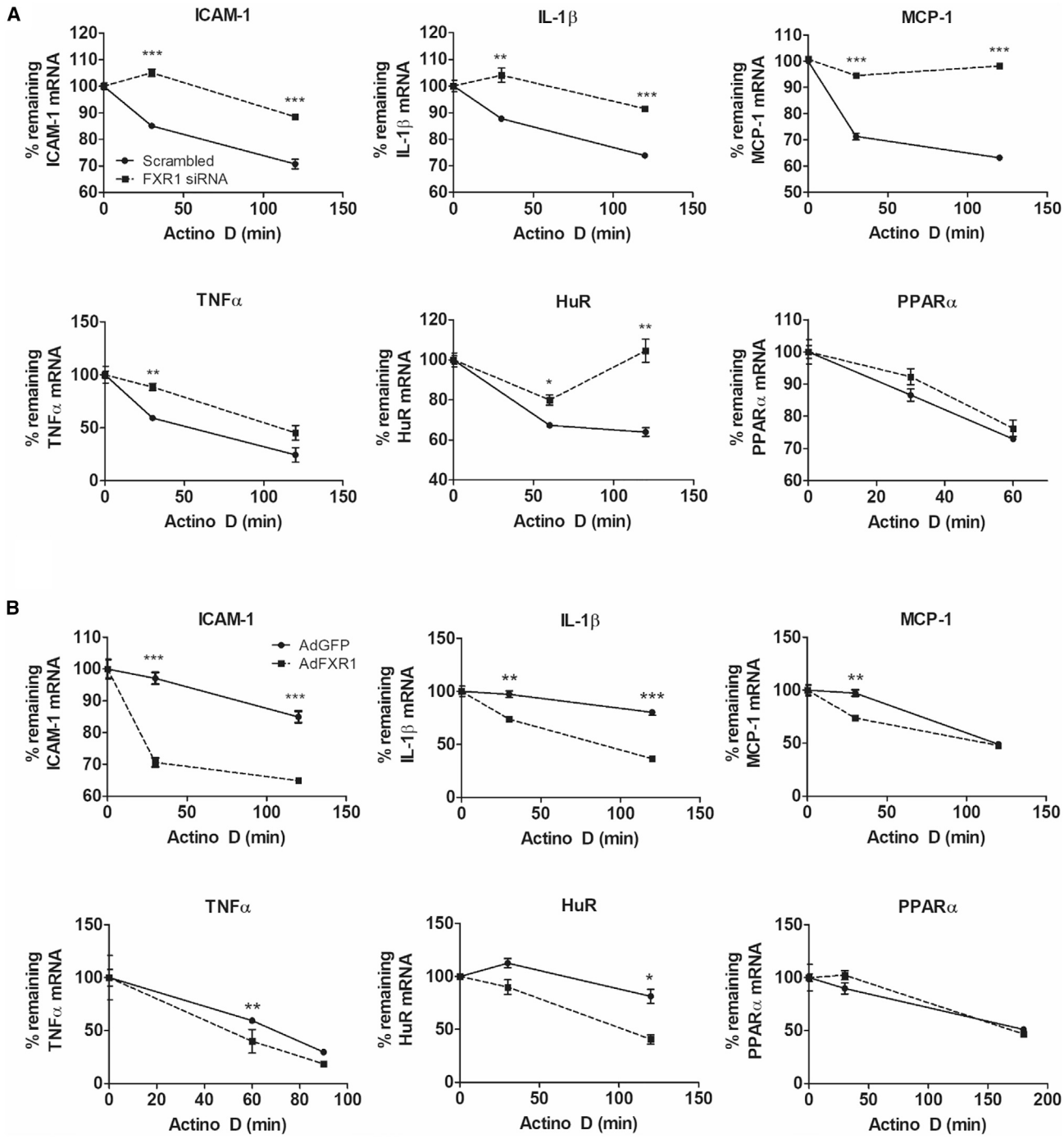

Figure 4. FXR1 Regulates mRNA Stability of Pro-inflammatory mRNA

(A) FXR1 knockdown increases inflammatory mRNA stability. hVSMCs were transfected with FXR1 siRNA or scrambled control siRNA, serum starved, and stimulated with TNFα for 4 hr, at which point actinomycin D (10 ng/mL) was added to halt transcription. RNA was isolated at indicated times post-addition of actinomycin D, and mRNA abundance was quantitated by qRT-PCR. Percent mRNA remaining was determined by normalizing each time point to beta-2-microglobulin (B2M). Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

(B) FXR1 overexpression reduces inflammatory mRNA stability. hVSMCs were transduced with adeno-FXR1 and adeno-GFP, serum starved, and stimulated with TNFα for 4 hr, at which point actinomycin D (10 ng/mL) was added. RNA was isolated at the various time points post-addition of actinomycin D, and mRNA abundance was quantitated by qRT-PCR. Percent mRNA remaining was determined by normalizing each time point to B2M. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

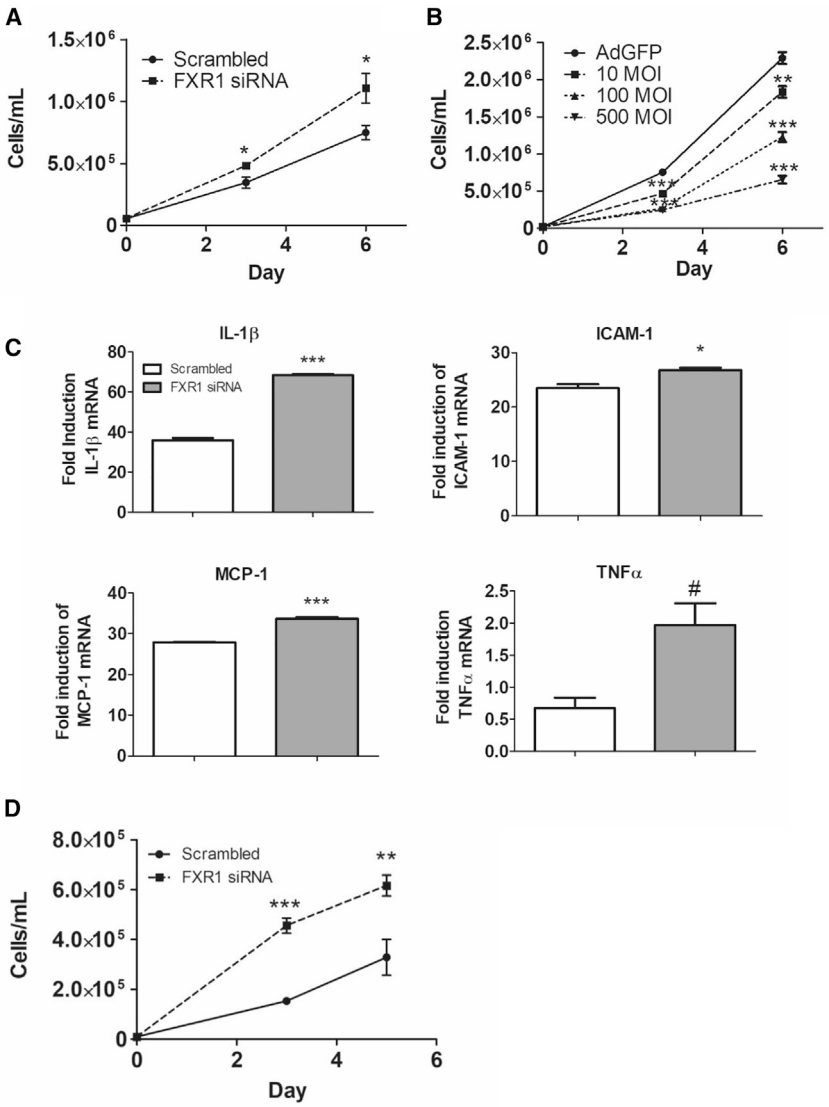

Figure 5. FXR1 Regulates VSMC Proliferation

(A) hVSMCs transfected with FXR1 siRNA or scrambled control siRNA were seeded at 10,000 cells per well and counted at days 3 and 6.

(B) hVSMCs were transduced with 100 MOI of adeno-FXR1 or AdGFP, seeded at 10,000 cells per well, and counted at days 3 and 6. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

(C) Conditioned media from VSMCs in which FXR1 is deleted can induce inflammatory and proliferative responses from naive VSMCs. hVSMCs were transfected with scrambled control or FXR1 siRNA and washed, and media were collected after 48 hr. Conditioned media from each of these groups was added to serum-starved hVSMCs for 4 hr, RNA was isolated and reverse-transcribed for qRT-PCR analysis. #$p < 0.07$.

(D) HVSMCs were seeded at 20,000 cells per well in FXR1 siRNA-conditioned media or scrambled control media. Cells were counted at days 3 and 5. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

canonical ARE, also successfully competed with the full-length TNFα 3′ UTR for FXR1 binding but did not compete with the full-length probe for HuR, suggesting an additional, previously unrecognized region on the TNFα 3′ UTR recognized by FXR1. These results were quantified using densitometry as a percentage of GST-protein bound to probe A (Figure 6C, lower panel). An additional binding element termed the G quadruplex has been implicated as a binding site for FXR1 (Bechara et al., 2007). Using this element as a cold competitor to the biotinylated TNFα 3′ UTR probe, we found that the G quadruplex was able to bind FXR1 (Figures S4C and S4D). Using RNA immunoprecipitation (RIP), we determined whether FXR1 directly binds RNAs that were shown to be regulated by FXR1. VSMCs were transduced with FLAG-tagged adeno-FXR1, serum starved for 48 hr, and then stimulated with TNFα for 8 hr. Figure 6D shows that several transcripts were identified as interacting with FXR1 compared to immunoglobulin G (IgG) control antibody. Importantly, mRNA transcripts not regulated by AREs in 3′ UTR such as PPARα were not amplified. It was possible that FXR1 could compete with HuR for occupancy on 3′ UTR of transcripts that contained these regions. A constitutively driven luciferase reporter representing the TNFα 3′ UTR and containing ARE tandem repeats was transfected into HEK cells and also transduced with plasmid encoding FXR1 cDNA or a control empty-vector plasmid. Figure 6E shows that FXR1 reduced luciferase activity, suggesting that FXR1 may compete with HuR for ARE occupancy. We also used an adenovirus expressing the TNFα 3′ UTR luciferase construct to perform the experiment in hVSMCs. Adeno-FXR1 and AdHuR were co-transduced with the adeno-expression TNFα 3′ UTR luciferase, as well as an AdGFP for control. Figure 6F demonstrates that, in hVSMCs, FXR1 overexpression reduced TNFα 3′ UTR luciferase activity, while HuR was able to increase it, supporting the concept that FXR1 is a negative regulator of inflammatory transcripts.

IL-19 Induces FXR1 Expression in VSMCs

IL-19 is anti-proliferative for VSMCs and reduces inflammatory transcript mRNA stability (Tian et al., 2008). Stimulation of VSMCs with IL-19 significantly induces FXR1 mRNA and protein expression (Figures 7A–7C). Long-term treatment of VSMCs with IL-19 also reduces HuR protein abundance (Cuneo et al., 2010; Ellison et al., 2013). To determine whether FXR1 mediated the IL-19 decrease in HuR abundance, VSMCs were transfected with FXR1 siRNA or scrambled control siRNA and serum starved. VSMCs were then treated with IL-19 for various time

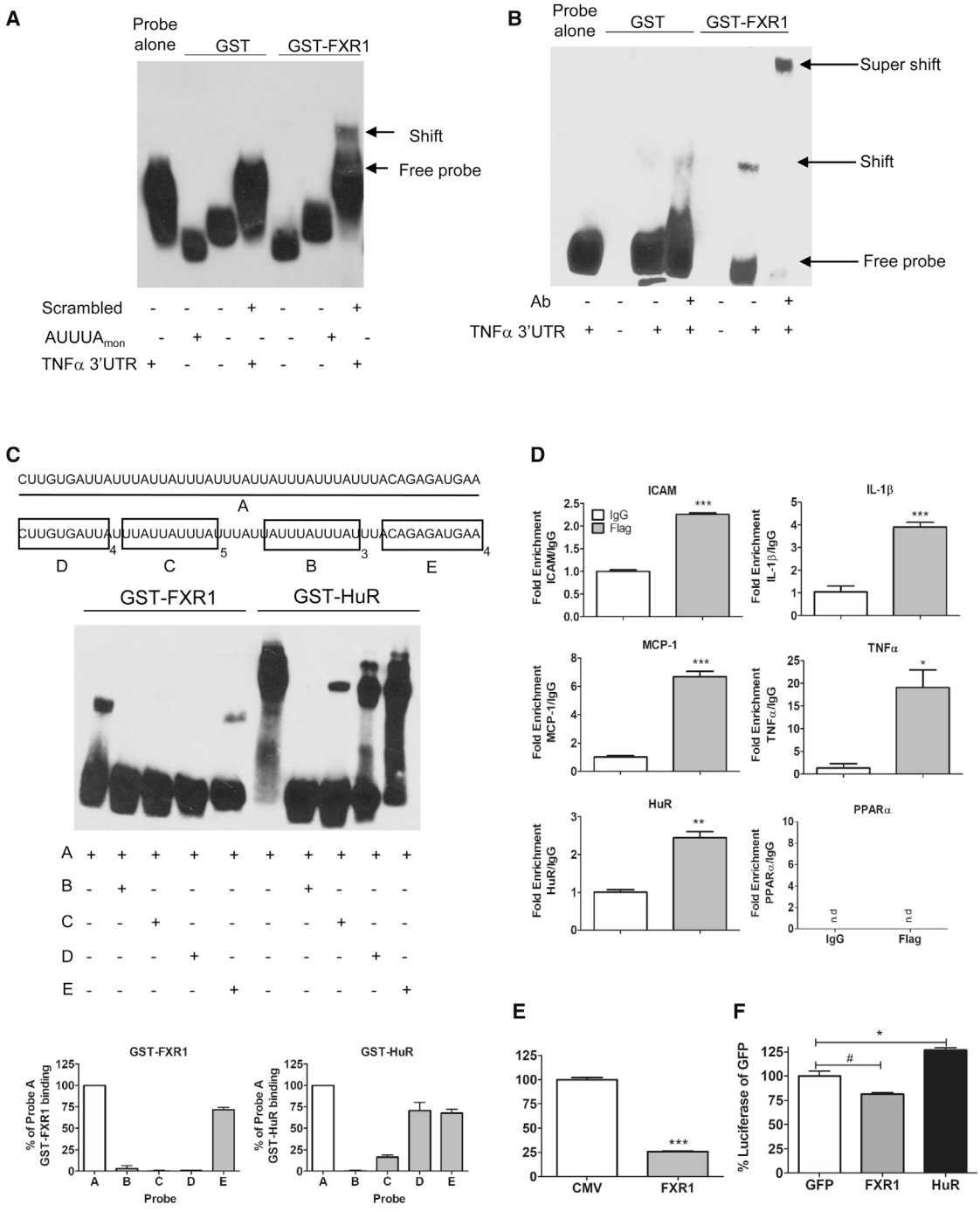

Figure 6. FXR1 Binds mRNA

(A) RNA EMSA. Biotinylated RNA probes containing a scrambled control, a 50-mer of human TNFα 3′ UTR, or $(AUUUA)_{monomer}$ were incubated with GST or GST-FXR1 and membrane blotted to demonstrate a shift indicating a protein-RNA complex.

(B) GST or FXR1 antibody was added to the EMSA reaction; supershift of the complex demonstrates specificity. Image shown is representative of 3 independently performed experiments.

(C) FXR1 binds ARE and a novel element. Non-biotinylated probes consisting of a 25-mer of $(AUUUA)_5$, a 27-mer of $(UUAUUUAUU)_3$, a 36-mer of $(CUUGUGAUU)_4$, and a 40-mer of $(CAGAGAUGAA)_4$ (40 bases) were added to the reaction prior to the addition of the biotinylated TNFα 50-mer, and RNA EMSA was performed using GST-FXR1 and GST-HuR. Densitometry of protein-probe complex was calculated as a percentage of binding to Probe A for both GST-FXR1 and GST-HuR. Image shown is representative of 3 independently performed experiments. Densitometry was calculated from at least 3 independently performed experiments.

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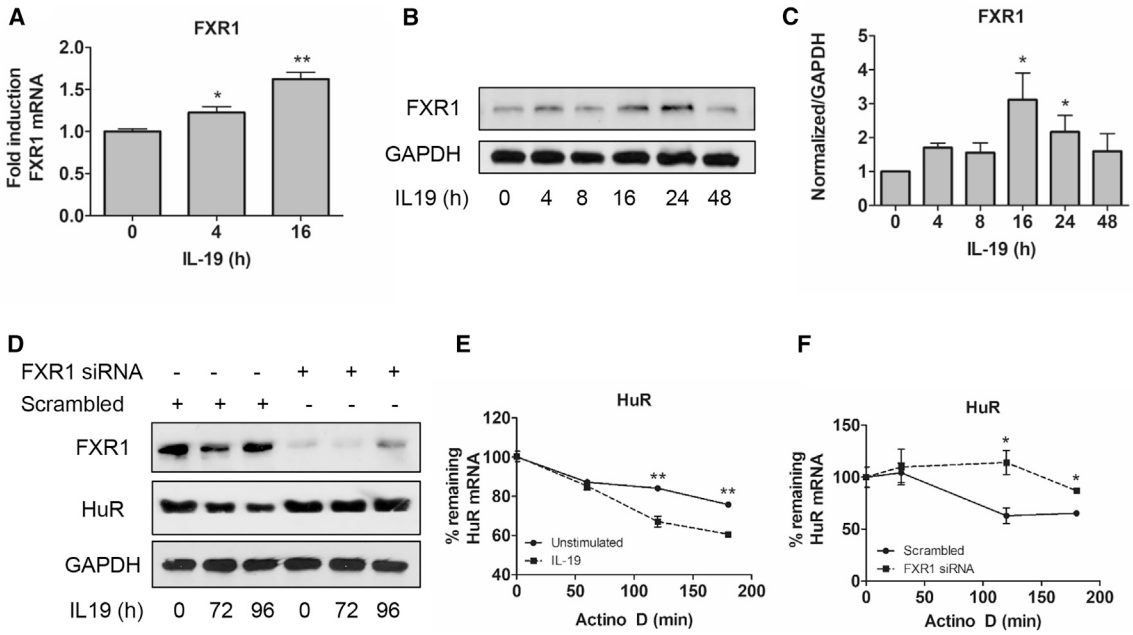

Figure 7. Anti-inflammatory Cytokine IL-19 Induces Expression of FXR1

(A) IL-19 induces FXR1 mRNA. hVSMCs were serum starved for 48 hr and then treated with IL-19 for the times indicated, and FXR1 mRNA was quantitated by qRT-PCR normalized to GAPDH.

(B) IL-19 induces FXR1 protein expression. hVSMCs were treated as described in (A), and FXR1 protein was detected by western blot analysis.

(C) Densiometric analysis of FXR1 protein expression in IL-19-treated VSMCs normalized to GAPDH. Image shown is representative of 3 independently performed experiments. Densitometry was calculated from at least 3 independently performed experiments.

(D) FXR1 mediates IL-19 reduction in HuR protein abundance. VSMCs were transfected with FXR1 siRNA or scrambled control siRNA and treated with IL-19 for the times indicated, and extracts were blotted to detect FXR1, HuR, and GAPDH proteins.

(E) IL-19 reduces HuR mRNA stability. HVSMCs were unstimulated or treated with IL-19 for 16 hr prior to the addition of actinomycin D, and HuR mRNA abundance was quantitated by qRT-PCR. Percent mRNA remaining was determined by normalizing each time point to B2M. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

(F) hVSMCs were transfected with FXR1 siRNA or scrambled control prior to treatment with IL-19 for 16 hr. Actinomycin D was added, and HuR mRNA abundance was quantitated by qRT-PCR. Percent mRNA remaining was determined by normalizing each time point to B2M. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

*p < 0.05; **p < 0.01; ***p < 0.001.

points, and cell extracts were used for western blot analysis (Figure 7D). As reported, IL-19 treatment can reduce HuR protein abundance; however, in the absence of FXR1, IL-19 is unable to reduce HuR, indicating that IL-19 reduction of HuR requires FXR1. To further dissect the mechanism by which IL-19 reduces HuR abundance, we determined that IL-19 is able to reduce HuR mRNA stability in hVSMCs following actinomycin D treatment (Figure 7E). However, in the absence of FXR1, IL-19 was unable to reduce HuR mRNA stability compared to scrambled control, suggesting that FXR1 is necessary for IL-19-induced destabilization of HuR (Figure 7F). Since FXR1 is induced by IL-19 and is necessary for IL-19 destabilization and reduction of HuR, we conclude that FXR1 expression is a negative compensatory, counter-regulatory mechanism used by VSMCs to respond to and dampen inflammation.

DISCUSSION

The major findings of this study are that FXR1 expression in VSMCs reduces mRNA stability and the abundance of pro-inflammatory proteins, is induced by the anti-inflammatory cytokine IL-19, and acts as an effector of IL-19 anti-inflammatory activity in VSMCs. This has important implications for the resolution of inflammation in general, and in the attenuation of severity

(D) RNA immunoprecipitation. VSMCs were transduced with FLAG-tagged adeno-FXR1, serum starved for 48 hr, and then stimulated with TNFα. RNA-protein complexes were immunoprecipitated with FLAG or IgG control beads. PPARα, lacking AREs in its 3′ UTR, was not amplified.

(E) The TNFα 3′ UTR luciferase construct was co-transfected into HEK293 cells with either a vector control or pFXR1. The cells were seeded in triplicate for 48 hr before harvesting. Luminescence was measured using an Infinite M1000 Pro plate reader and graphed as a percentage of GFP luciferase control.

(F) Adenoviral expression of TNFα 3′ UTR luciferase construct was co-transduced into hVSMCs with AdGFP, adeno-FXR1, or AdHuR, and the results were graphed as a percentage of luciferase AdGFP. Data are expressed as mean ± SEM; the experiment was performed in triplicate. Data shown are representative of 3 independently performed experiments.

*p < 0.05, **p < 0.01, ***p < 0.001, #p < 0.07. See also Figures S2, S3, and S4.

of vascular inflammatory syndromes such as atherosclerosis, restenosis, and allograft vasculopathy in particular. Investigation into vascular inflammation primarily focuses on the role of immune cells, but in this study, we show that a non-immune cell can respond to anti-inflammatory stimuli through post-transcriptional mechanisms. Most inflammatory cytokines contain conserved or semi-conserved AU-rich elements in their 3′ UTR (Peng et al., 1996), imparting target specificity to allow the cell to fine-tune mRNA abundance and translation for rapid response to inflammation (Schoenberg and Maquat, 2012). An essential regulatory protein involved in this process is HuR (Peng et al., 1996; Schoenberg and Maquat, 2012), and proteins and pathways that regulate HuR may be key targets in the regulation of inflammation. We previously reported that IL-19 reduces HuR activity in VSMCs, leading to a decrease in mRNA stability in a HuR-mediated but un-characterized mechanism. A goal of this study was to identify proteins that would interact with HuR and regulate its activity.

An unbiased pull-down experiment using FLAG-tagged HuR and LC-MS/MS to identify protein constituents of HuR complexes in VSMCs uncovered a number of interesting proteins recognized to participate in various aspects of mRNA processing. Studies have extensively characterized the messenger ribonucleoprotein (mRNP) complexes that form to regulate mRNA transcripts and translation, while the profile of proteins known to directly interact with HuR, particularly in an inflammation responsive fashion, is more limited. Interaction with one candidate identified in our analysis, FXR1, was validated by co-immunoprecipitation and cellular co-localization assays, validating the LC-MS/MS result, and is a previously unrecognized observation of this study. FXR1 is an autosomal homolog of FMR protein, the prototypical and best studied member of the FXR (fragile X-related) family of neuronal proteins (Bardoni et al., 2001). The vast majority of work on FMR1-, FXR1-, and FXR-related proteins are performed in neurons and focuses on their role in cognitive ability; nothing is known about this family of proteins in VSMCs or vascular pathophysiology. FXR1 is the only fragile X protein family member significantly expressed in muscle cells (Garnon et al., 2005; Mientjes et al., 2004) and has been described as the "muscle homologue" of the FMR family (Mientjes et al., 2004). In addition, our data suggest that FXR1 is upregulated in VSMCs specifically in inflamed regions of arteries from disease states. For these reasons, we focused on the role of FXR1 in VSMCs for further study.

HuR is characterized as an mRNA-stabilizing RBP, while FXR1 function is less understood and appears to be cell type specific. When we knocked down FXR1 using siRNA in TNFα-stimulated hVSMCs, we were initially surprised to observe that inflammatory transcripts were increased at both the transcript and protein levels. Knockdown of FXR1 in VSMCs in the presence of actinomycin D demonstrated significantly increased mRNA stability of several pro-inflammatory transcripts, in direct contrast to a study in macrophages showing that absence of FXR1 did not affect TNFα mRNA half-life (Garnon et al., 2005). Stability of PPARα mRNA, a transcript not regulated by AREs in its 3′ UTR, was unaffected by FXR1 knockdown. RBPs have the ability to be cell type specific in their function, especially those that have cell-specific expression (Musunuru, 2003). Since knockdown of FXR1 increased inflammatory transcripts, we reasoned that FXR1 overexpression would reduce the abundance of inflammatory mRNA and protein. Indeed, adenoviral overexpression of FXR1 significantly decreased the abundance of several inflammatory mRNAs and proteins. Concordant with knockdown, overexpression did not affect PPARα mRNA stability in any way. This study indicates that FXR1 knockdown results in increased HuR protein abundance mRNA stability, and overexpression results in the opposite, suggesting a previously unrecognized mechanism in regulation of pro-inflammatory transcripts. HuR abundance is auto-regulated by a positive-feedback loop involving HuR interaction with the 3′ UTR of its own mRNA (Dai et al., 2012). These authors suggested that HuR mRNA is destabilized through an ARE-dependent but unidentified mechanism, which we posit could be FXR1 interaction with the HuR 3′ UTR. FXR1 knockdown also increased hVSMC proliferation, and FXR1 overexpression reduced hVSMC proliferation. This proliferative effect may be due to a decrease in autocrine expression of cytokines and growth factors, as VSMCs are known to proliferate in an autocrine fashion. Concordantly, conditioned media from FXR1 knockdown VSMCs increased inflammatory cytokine expression and proliferation of naive VSMCs as well. FXR1 expression is critically important, so much so that FXR1 knockout mice are postnatally lethal (Mientjes et al., 2004), and, in conjunction with its upregulation in injured arteries, this supports a role for FXR1 in the maintenance of the quiescent VSMC phenotype. Overall, siRNA knockdown and overexpression data are complementary, and are associated with the presence of AREs in transcript 3′ UTR. These data strongly suggest that, in VSMCs, FXR1 functions as an mRNA de-stability factor to reduce inflammatory transcripts. In this regard, FXR1 appears to function similarly to other destabilizing RBPs, such as Tristetraprolin (TTP) and AUF1 (hnRNP D), which function to alter the transcript stability of TNFα via AREs in the 3′ UTR (Carballo et al., 1998). Because FXR1 expression is enhanced in muscle; is induced by IL-19, an anti-inflammatory stimulus; and can regulate inflammatory protein abundance in VSMCs, FXR1 can potentially have key regulatory effects in the modulation of vascular inflammatory diseases.

It was plausible that both FXR1 and HuR would bind the same region in the 3′ UTR. The addition of RNase A abrogated the FXR1-HuR interaction, suggesting that the FXR1-HuR interaction was indirect, a result of tethering by occupancy on the same mRNA. This would also explain why we observed increased FXR1-HuR interaction in TNFα-stimulated VSMCs, as TNFα would induce expression of inflammation-inducible transcripts with AREs in their 3′ UTR, thus increasing the availability of transcripts for each protein to bind.

Many assumptions on the functions of FXR family members are based on the much better characterized FMR protein. Similar to the better characterized FMR protein, FXR1 contains two KH domains for RNA binding and an RGG box, which is the preferred binding domain of FMRP. Using regions of the TNFα 3′ UTR, two different but complementary methods were used to determine that FXR1 binds mRNA and also validate that FXR1 tethers to mRNA. FXR1 mRNA recognition sites have not been identified.

Using biotinylated cRNA pentameric probes representing various sequences of this region as cold competitors, we identified canonical AU-rich elements as putative FXR1 binding sites, as well as a previously undescribed element comprising the sequence CUUGUGAUU. This corroborates experiments showing that FXR1 modulation affected the stability of transcripts that contained AREs in their 3′ UTR. FXR1 recognition of AREs also suggested that FXR1 could compete with HuR for occupancy on AREs of inflammatory transcripts. FXR1 also bound the G quadruplex complex, but because this region is not present in the TNFα 3′ UTR 50-mer probe, it does not exclude the possibility of competition for a common binding site with HuR on inflammatory transcripts.

RBPs such as HuR can block endonucleolytic cleavage sites to prevent mRNA degradation and, therefore, increase mRNA stability (Hollams et al., 2002). FXR1 reduced luciferase activity in a reporter driven by the TNFα 3′ UTR, further suggesting that FXR1 may act as an mRNA de-stability protein by competing with HuR, which can also bind and stabilize the same region. Competition of FXR1 for HuR binding sites on the 3′ UTR as a mechanism to dampen the inflammatory response of select transcripts has not previously been reported. Considering FXR1's reciprocal relationship with HuR, FXR1 could potentially compete with HuR for binding to its own 3′ UTR, reducing HuR mRNA stability, decreasing HuR mRNA abundance, and thus repressing pro-inflammatory gene protein expression. While this study does not rule out the possibility that FXR1 binds to transcripts and targets them for degradation independently of HuR, it does strongly suggest that FXR1 has the potential to oppose HuR and thus act as an mRNA de-stability factor. Many factors involved in RNA stability, as well as transcriptional and translational regulatory proteins, localize in discrete cytoplasmic phase-dense stress granules. HuR and other RBPs, such as TTP and TIA-1, have been reported to localize to stress granules (Kedersha et al., 2008). The co-localization of FXR1 with HuR in cytoplasmic stress granules further points to an important role for these proteins in the post-transcriptional regulation of inflammatory mediators and resolution of the cellular inflammatory response.

IL-19 decreases atherosclerosis and vascular restenosis (Ellison et al., 2013, 2014) and reduces HuR protein abundance, inflammatory mRNA stability, and abundance of pro-inflammatory proteins in VSMCs (Cuneo et al., 2010; Tian et al., 2008). While some RBPs can be induced by pro-inflammatory stimuli, very little is known about the anti-inflammatory effects of RBPs as a countervailing mechanism to resolve inflammatory processes. In this report, we show that stimulation of hVSMCs with IL-19 increases the expression of FXR1, placing FXR1 expression as part of the IL-19 anti-inflammatory pathway. We also demonstrate that FXR1 is required for HuR reduction and destabilization by IL-19, further placing FXR1 as an effector protein and part of the anti-inflammatory pathway of IL-19. Our working hypothesis is that FXR1 expression in VSMCs is a counter-regulatory mechanism used by VSMCs to respond to and dampen vascular inflammation.

This study describes that FXR1, a muscle-enhanced protein best known as a homolog of the neuronal protein FMR, can be induced in VSMCs by anti-inflammatory stimuli. FXR1 can bind to mRNA at AREs and participate in inflammatory transcript processing by competing with HuR, resulting in a reduction in inflammatory mRNA stability.

The balance of RBPs in homeostasis and pathological conditions has not been well characterized, but, based on the opposing functions of HuR and FXR1 and the shared repertoire of transcripts they regulate, we posit that an equilibrium between stabilizing and destabilizing RBPs is critical to the maintenance of inflammatory and proliferative transcripts, particularly in VSMCs, a non-immune cell type. This work implicates FXR1 as a molecular mediator to resolve vascular inflammation.

EXPERIMENTAL PROCEDURES

VSMC Culture

Primary human coronary artery VSMCs were obtained as cryopreserved secondary culture from Lonza (Allendale, NJ, USA) and maintained as we described previously (Gabunia et al., 2017; Tian et al., 2008). Cells were used from passages 3–5.

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) and are described in detail in the Supplemental Information section.

LC-MS/MS

LC-MS/MS analysis was performed as described previously (Haines et al., 2012) and are provided in detail in the Supplemental Information section.

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 that target human FXR1 (10 nM) purchased from Dharmacon (Lafayette, CO, USA), as we have described previously (Cuneo et al., 2010; Gabunia et al., 2016), and that are described in detail in the Supplemental Information section.

For conditioned media transfer experiments, media was collected from hVSMCs transfected with scrambled control or FXR1 siRNA. Conditioned media from each of these groups was added to serum-starved hVSMCs for 4 hr, and RNA was isolated and reverse-transcribed for qRT-PCR analysis. For proliferation, hVSMCs were seeded at 20,000 cells per well in FXR1 siRNA conditioned media or scrambled control media. Cells were counted at days 3 and 5 on a Cellometer Auto T4 Bright Field Cell Counter (Nexcelom Bioscience, Lawrence, MA, USA) (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$).

RNA Extraction and qRT-PCR

VSMCs were serum starved in 0.1% fetal calf serum (FCS) for 48 hr and 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 previously, and target genes were amplified using an Applied Biosystems StepOne Plus Real-Time PCR System, as we described previously (Gabunia et al., 2016, 2017), and are described in detail in the Supplemental Information section.

Western Blotting and Protein Determination

Human VSMC extracts were prepared as described (Ellison et al., 2013; England et al., 2013; Gabunia et al., 2017). Membranes were incubated with a 1:5,000–9,000 dilution of primary antibody and a 1:5,000 dilution of secondary antibody. IL-1β, ICAM-1, FXR1, GAPDH, and HuR were from Santa Cruz Biotechnology (Dallas, TX, USA). HuR and FXR1 were from Abcam. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Cell Signaling Technology (Danvers, MA, USA). Reactive proteins were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA), according to the manufacturer's instructions. Relative intensity of bands was normalized to GAPDH,

and quantitated by scanning image analysis and the ImageJ densitometry program.

cRNA EMSA

FXR1 and GST control fusion proteins were generated using a GenScript kit for GST fusion protein purification, purified from *E. coli* lysates, and then separated using a protease kit from GE Life Sciences PreScission Protease (Piscataway, NJ, USA). Purified proteins (2 μM) were incubated with biotinylated probe (100 pM) consisting of nt 1,333–1,380 in the TNFα 3′ UTR (5′CUUGUGAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUACAGAGAUGAA-3′) in binding buffer and glycerol. The binding reaction was run on a 5% precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA). The gel was transferred to nylon membrane, cross-linked with UV light, and blocked. The membrane was then incubated with streptavidin antibody and developed using chemiluminescence. Five non-biotinylated probes were used in a cold competition experiment: $(UUAUUUAUU)_3$, $(AUUUA)_5$, $(CUUGUGAUU)_4$, $(CAGAGAUGAA)_4$, and scrambled control $(AUCG)_5$ were incubated with GST-protein and binding buffer and/or glycerol for 30 min before the addition of the biotinylated TNFα. In Figures S3C and S3D, the G quadruplex probe (GGGGUGGGUGGGGGGCAGUGGGGGCUGGGCGGGGGG) was used as a cold competitor to TNFα using the same experimental setup as previously described. Binding affinity was calculated as described previously (Bechara et al., 2007).

Co-Immunoprecipitation and RIP

For co-immunoprecipitation (coIP), hVSMCs were washed three times in PBS and scraped off the dish into a conical tube and centrifuged to form a cell pellet. hVSMC extracts were lysed in lysis buffer (50 mM HEPES [pH 7.5], 70 mM KOAc, 5 mM $Mg(OAc)_2$ in 0.1 M Tris-HCl [pH 8.5] with 0.1 g n-dodecyl-B-maltoside and protease inhibitor) and incubated on a nutator at 4°C for 30 min. Cells were centrifuged at 16,600 rpm for 15 min. Anti-FLAG M2 affinity beads (Sigma-Aldrich, St. Louis, MO, USA) were washed and added to each sample and incubated on the nutator overnight at 4°C. The samples were centrifuged at 13,000 rpm and washed three times in lysis buffer. Sample buffer was added, and samples were boiled and frozen or used for western blotting. For RIP, hVSMCs were transduced with adeno-FXR1 prior to serum starvation for 48 hr. Cells were treated with TNFα for 8 hr and lysed in IP buffer with RNase inhibitor. Samples were divided, and half were incubated with IgG control beads or FLAG-conjugated beads for 4 hr at 25°C. The beads were then centrifuged and washed 5× in IP buffer. TRIzol was added to the pelleted beads, and RNA was extracted and reverse transcribed to cDNA. We performed qRT-PCR for the transcripts indicated.

Statistical Analysis

Results are expressed as mean ± SEM. Differences between groups were evaluated with the use of Student's t test, or ANOVA, where appropriate, and performed using Prism software (GraphPad, La Jolla, CA, USA). Differences were considered significant when $p < 0.05$. All experiments using cultured cells were performed in triplicate, from at least three independent experiments. Quantifications of western blot and immunofluorescence images were performed using ImageJ software from at least three independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.002.

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AUTHOR CONTRIBUTIONS

A.B.H. performed the majority of the molecular biology experiments; C.N.V. performed cell culture and some molecular biology experiments; M.R. performed cell culture and some molecular biology experiments; S.E.K. performed immunohistochemistry; M.J.S. is the principal investigator of the mass spectrometry lab; A.M. worked in the mass spectrometry lab; D.S.H. interpreted mass spectrometry and other data; M.V.A. designed experiments, interpreted data, performed some western blots, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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