

**THE ROLE OF FXR1 IN CELL CYCLE CONTROL AND THE
INDUCTION OF SENESCENCE IN VASCULAR
SMOOTH MUSCLE**

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ABSTRACT

Despite the advent of stents, intimal hyperplasia subsequent to vascular interventional procedures remains a major obstacle. Vascular smooth muscle cells (VSMC) play a critical role in the pathogenesis of intimal hyperplasia; therefore, regulation of VSMC gene expression is a logical intervention point. FXR1 is a muscle-enhanced RNA binding protein and its expression is increased in injured arteries. We have shown that modulation of FXR1 levels affects stability and abundance of inflammatory transcripts in VSMC, suggesting that FXR1 is a negative regulator of inflammation. RNA-sequencing analysis in FXR1-depleted human VSMC (hVSMC) identified a number of transcripts with decreased abundance, the overwhelming majority of which were associated with proliferation and cell division. This drives our hypothesis that FXR1 is involved in mitigating vascular disease by regulating inflammatory and proliferative mRNA in VSMC.

The mRNA abundance and stability of a number of these transcripts was decreased in FXR1 depleted hVSMC, and RIP-sequencing demonstrated that FXR1 interacts with transcripts involved in cell cycle control, and stability of these transcripts is decreased with FXR1 depletion. FXR1-depleted cells showed decreased proliferation ($p < 0.05$), however, an increase in β -galactosidase ($p < 0.05$) and γ H2AX ($p < 0.01$), indicative of senescence was noted. Senescent cells exhibit a senescence associated secretory phenotype (SASP) with characteristic gene expression leading to increased inflammation in the tissue microenvironment. HVSMC depleted of FXR1 had increased transcripts abundance of many SASP genes, as well as an increase of both mRNA and protein expression of canonical senescence markers p53 and p21.

We developed a novel SMC-specific conditional knockout mouse (FXR1^{SMC/SMC}) to further study these results in a more translational context. In a carotid artery ligation model of intimal hyperplasia, FXR1^{SMC/SMC} mice have significantly reduced neointima formation (p<0.001) post-ligation compared to controls. qPCR analysis from FXR1 conditional knockout mouse VSMC (mVSMC) show increased transcripts associated with senescence (p21, p16, p53) as well as increased SASP-associated mRNA, a decrease in proliferation, and an increase in β -galactosidase staining.

Our results are the first to suggest that in addition to destabilization of inflammatory transcripts, FXR1 may stabilize cell cycle related genes in VSMC, and absence of FXR1 leads to induction of a senescent phenotype, an increase in SASP genes, and reduction of intimal hyperplasia.

DEDICATION

For Alex (human) and Tigger (cat), the loves of my life. Thank you for getting me through. I could never begin to repay you for all that you've done for me, but soon I'll be able to afford to buy us a nice dinner and some fancier cat treats.

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LIST OF ABBREVIATIONS

ARE	AU-Rich Element
ATM	ataxia-telangiectasia mutated
CVD	Cardiovascular Disease
FMRP	Fragile X Mental Retardation Protein
FXR1	Fragile X Related Protein 1
HuR	Human Antigen R
ICAM-1	intercellular adhesion molecule 1
IkB	inhibitory kB
Ikk	IkB Kinase Complex
IL-19	Interleukin 19
IL-6	Interleukin 6
ING1	Inhibitor of Growth Family 1
KLF4	Kruppel-Like Factor 4
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
m ⁶ a	N ⁶ -methyladenosine
MCP-1	macrophage chemoattractant protein-1
METTL14	methyltransferase-like 14
METTL3	methyltransferase-like 3
MI	Myocardial Infarction
miRNA	Micro RNA
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MRN	MRE11-RAD50-NBS1
MTC	Methyltransferase Complex
MYOCD	Myocardin
NFkB	Nuclear Factor-kB
NO	Nitric Oxide
oxLDL	Oxidized Low Density Lipoprotein
QKI	Quaking
RBD	RNA Binding Domain
RBP	RNA Binding Protein
RNP	ribonucleoprotein particles
SASP	Senescence Associated Secretory Phenotype
SCAPs	senescent cell anti-apoptotic pathways
siRNA	Short Interfering RNA
SM22a	Smooth Muscle 22 alpha
SMA	Smooth Muscle Actin

SMC	Smooth Muscle Cell
SOCS2	Suppressor of Cytokine Signaling 2
SRF	Serum Response Factor
TMEM38B	Transmembrane protein 38B
TNF α	Tumor Necrosis Factor alpha
UTR	Untranslated Region
VCAM-1	vascular cell adhesion molecule
VSMC	Vascular Smooth Muscle Cell
WTAP	Wills Tumor Associating Protein-1
ZNF706	Zinc Finger 706

CHAPTER 1

INTRODUCTION

1.1 Cardiovascular Health and Disease

Cardiovascular disease (CVD) is the leading cause of death worldwide, being responsible for over 30% of global mortality in 2019.¹ As individuals are living longer due to advances in science and medicine, more people will become affected by cardiovascular disease. One quarter of adults in the United States have high cholesterol and on average someone dies of cardiovascular disease every 34 seconds.² This is a massive healthcare and economic burden throughout the world and it is imperative that we focus on finding better therapeutics to treat these diseases. With the number of affected individuals increasing every year, it is crucial that scientists understand the molecular mechanisms underpinning cardiovascular diseases so that new treatments can be developed.

There are many different types of cardiovascular disease including coronary heart disease, hypertension, heart failure, and stroke, and the vast majority of the diseases making up this mortality category are diseases of the vasculature. The vasculature comprises the body's veins and arteries that carry blood throughout the body, and some of the main etiologies negatively affecting blood flow are vascular occlusive diseases including atherosclerosis and restenosis.³ Atherosclerosis accounts for about half of mortality in the US and is rising in the developing world.⁴ Atherosclerosis is a progressive, lipid-driven inflammatory vascular disease caused by the accumulation of fats and cholesterol in the subendothelial space of the arterial wall, which forms an atherosclerotic plaque.

1.1.1 Atherosclerosis: Atherogenesis starts with damage to the endothelium.

Smoking, obesity, infection, or high glucose consumption can cause an increase in free radicals which upset the nitric oxide (NO) balance. Appropriate NO balance is critical to maintaining vascular homeostasis by inhibiting platelet aggregation, maintaining vascular tone, and decreasing expression of macrophage chemoattractant protein-1 (MCP-1).⁵ When the bioavailability of NO is decreased, the endothelium becomes dysfunctional leading to a proinflammatory, prothrombotic phenotype and an increase in endothelial permeability. The increased permeability of the endothelium allows cells that are normally present in the blood, including monocytes and oxidized low density lipoprotein (oxLDL) to enter the intima of the artery.

OxLDL is a modification of low-density lipoprotein (LDL). LDL makes up the majority of cholesterol, and an excess amount of LDL can be pathological for the vasculature. OxLDL is taken up and internalized by macrophages through scavenger receptor CD36 as part of a feed-forward mechanism that causes accumulation of oxLDL and the transformation of macrophages into lipid-laden foam cells.⁶ These foam cells cannot effectively process the overload of oxLDL, and this begins the process of atherogenesis. OxLDL's presence in the intima allows for an increase in MCP-1 and other chemokines which, among other functions, regulates recruitment and infiltration of monocytes and macrophages into the artery. OxLDL also stimulates the release of pro-inflammatory cytokines and production of endothelial leukocyte adhesion molecules P- and E- selectin on the endothelium, allowing monocytes and neutrophils to adhere reversibly to the vessel wall.⁷

The Nuclear Factor-kB (NFkB) pathway is activated, increasing expression of inflammatory cytokines to contribute to the forming plaque. NFkB is a transcription

factor that interacts with inhibitory κ B (I κ B) proteins in the cytoplasm of the cell, keeping it inactive.⁸ In response to inflammatory stimuli such as those present during atherosclerotic plaque formation, the I κ B Kinase Complex (IKK) is activated, leading to the phosphorylation and polyubiquitination of I κ B. Free from the I κ B proteins, NF κ B translocates to the nucleus where it initiates transcription of its target genes including chemokines, cell adhesion molecules, and additional inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF α).⁹

Cell adhesion molecule transcription resulting from NF κ B activation includes intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which bind to integrins on the vessel wall and facilitate the firm adhesion of monocytes and neutrophils to the activated endothelium.^{10, 11} Following firm binding, leukocytes slowly roll along the endothelium to find optimal locations for extravasation through the endothelial layer. This leads to VSMC activation and infiltration into the growing plaque.¹² When VSMC are activated in this inflammatory environment, they become proliferative and migratory and begin to synthesize extracellular matrix, contributing to plaque progression.¹³ In an atherogenic environment, VSMC can take on the characteristics of other cell types and further exacerbate the growing plaque. Many VSMC take up lipid and become foam cells resembling macrophages, expressing macrophage surface markers such as CD68.¹⁴ These foam cells accumulate in the plaque and trigger programmed cell death pathways. The associated increase in cell death grows the necrotic core of the plaque, decreasing its stability and risking rupture and associated thrombus formation.¹⁵ When a plaque ruptures, it forms a thrombus and occludes the artery, leading to ischemic events including myocardial infarction (MI).

1.1.2 Restenosis: A common medical intervention following an ischemic event is balloon angioplasty with stent placement.^{16, 17} The balloon is inflated to compress the plaque against the artery wall, and the stent acts as a scaffold to hold open the previously occluded artery. This medical intervention which is necessary to restore blood flow can itself cause mechanical injury to the artery known as restenosis. The development of neointimal hyperplasia is a maladaptive response to injury and happens in 4 stages: 1) the mechanical injury causes platelet activation and 2) increased inflammation (minutes/hours post-injury), causing 3) VSMC to become activated cells that migrate into the intima, proliferate (days/weeks post-injury), and 4) start to synthesize extracellular matrix leading to vessel remodeling (months post-injury).¹⁸ Specifically, when the stent is inserted, it causes denudation of the endothelium resulting in exposure of the internal elastic lamina. This is the area of the artery wall in between the intima and the media, and when the internal elastic lamina is exposed to blood flow, circulating platelets begin to adhere to it. Platelets secrete growth factors such as platelet-derived growth factor (PDGF), as well as chemokines which exacerbate the inflammatory environment. Smooth muscle cells are stimulated by PDGF and the increase in cytokines to de-differentiate from their normal quiescent contractile phenotype to their synthetic form where they enter the cell cycle.^{19, 20} They migrate through the internal elastic lamina and proliferate in the intima of the artery to begin forming the neointima.^{19, 21} This leads to intimal hyperplasia and subsequent loss of lumen, known as restenosis (figure 1).

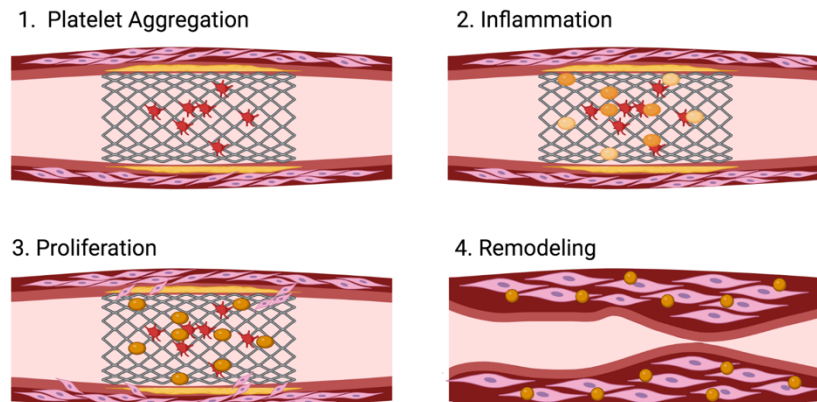


Figure 1. Step-wise development of restenosis following stent placement. Made with Biorender.com, adapted from Kraitzer et al., 2007.

To combat restenosis, researchers have developed stents that can deliver drugs localized to the area where they are deployed, including rapamycin and paclitaxel, with the goal of reducing VSMC activation and proliferation.²² Rapamycin is a lipophilic antibiotic that easily passes through cell membranes and can dampen the immune response after injury. It also inhibits the mTOR complex-1, heavily involved in proliferative processes, as well as reduces the activity of cyclin dependent kinases.²³ Paclitaxel induces cell cycle arrest in VSMCs by affecting microtubule assembly, and inhibits mitotic spindle formation and cell motility.²⁴ While both of these drugs have been successful in reducing neointima formation in clinical trials as well as in patient care, their mechanisms of action impair wound healing.^{25, 26}

With bare metal stents alone, the restenosis rate following angioplasty with stent placement is around 20%, while the advent of drug eluting stents has helped to reduce the incidence of restenosis following these procedures to around 5-10%.²⁷ Despite its reduction in incidence, restenosis is still a severe complication affecting many patients

and therefore is an important phenomenon to study and find better treatments for. VSMC play a major role in this pathology as they are involved in both proliferation and the secretion of inflammatory cytokine, and they are therefore the major cellular target for anti-restenotic therapies.

1.2 Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMC) make up the majority of the medial layer of arteries. They normally exist in a contractile, quiescent state and undergo phenotypic switching to an activated state in response to injurious and inflammatory stimuli.^{28, 29} In their physiological contractile state, VSMCs are responsible for regulating blood flow and pressure by regulating vessel diameter and vascular tone.³⁰ VSMC contractile function is modulated by cytoskeletal dynamics. Two of the main contractile components in smooth muscle cells are actin and myosin, which work together to create force.³¹ VSMC contraction is calcium-dependent. An increase in free cytoplasmic calcium leads to the activation of the myosin light chain kinase (MLCK), which activates the myosin light chain protein (MLC). Activation of the MLC allows myosin filaments to bind to and slide along actin filaments, causing cell contraction.³² If VSMC cytoskeletal dynamics are dysregulated, or if there is a pathological condition that the VSMC are responding to, VSMC undergo phenotypic switching to an activated, synthetic state where they lose their contractile properties and become migratory and proliferative.³² Synthetic VSMC display increased expression of fetal and proliferative genes such as cyclin D1, cyclin D2, and non-muscle myosin heavy chain B (SMemb), as well as decreased expression of contractile VSMC genes including smooth muscle alpha-actin (SMA) and smooth muscle 22 alpha (SM22 α). In this activated state, the VSMC proliferate, migrate, and begin to secrete chemokines and extracellular matrix leading to progression of vascular disease.³³

In most arteries, the blood flow is laminar with a higher rate of flow in the middle of the artery and a lower rate along the sides of the arteries, where there is more friction. There is direct communication between endothelial cells and VSMC that inhibits VSMC proliferation during laminar flow.^{34,35} However, in areas of the arteries such as branch points and the ascending aorta, fluid hemodynamics result in turbulent flow. The change in shear stress caused by turbulent flow impairs this communication and VSMC are able to switch to a proliferative phenotype.³⁶

There are a number of signaling pathways that have been identified that facilitate phenotypic switching of VSMC. Serum response factor (SRF), a ubiquitously expressed transcription factor important for the contractile function of VSMC, normally binds to CArG elements in VSMC.³⁷ The CArG elements exist in regulatory regions of smooth muscle specific genes such as smooth muscle actin (ACTA2), calponin (CNN1), and myosin-11 (MYH11), all responsible for the contractile phenotype of VSMC, and serve as DNA recognition sequences. Myocardin (MYOCD) can enhance SRF's binding to the aforementioned genes and helps to promote the VSMC contractile phenotype.³⁸ MYOCD is expressed exclusively in cardiac and smooth muscle, and is responsible for the VSMC-specificity of SRF. When NFkB and PDGF are upregulated in response to injury or in an atherosclerotic environment, MYOCD and SRF binding is interrupted, therefore decreasing SRF binding to VSMC contractile genes.³⁸ Another molecular switch implicated in the VSMC phenotypic transformation is Kruppel-like factor 4 (KLF4). MicroRNAs (miRNAs) miR-143 and miR-145 are upregulated by SRF and act on KLF4 to reduce its expression and promote the induction of a synthetic phenotype.³⁹ miRNAs such as miR-92a and miR-146a have the opposite effect and act to enhance transcription of KLF4, therefore inhibiting the phenotypic switch.

1.3 Cytokines in Cardiovascular Disease

Inflammation that is present during injury and disease is regulated by inflammatory cytokines. Inflammation is the first line of defense following injury and exists to clear out dead cells and begin the healing process, however when inflammation is not resolved in an appropriate timeframe it becomes pathogenic. As discussed previously, nuclear factor- κ B (NF κ B), the master switch in regulation of immune and inflammatory gene expression, is a family of transcription factors that are responsible for transcriptional activation of inflammatory mediators which in turn influence the function of immune cells such as macrophages.⁹ When macrophages are activated, they can differentiate into either of two phenotypes: M1 (inflammatory) or M2 (anti-inflammatory) macrophages. This M1 phenotype is highly implicated in cardiovascular disease pathogenesis, whereas M2 macrophages secrete cytokines that are protective in the vasculature such as Interleukin-19 (IL-19).⁴⁰

Interleukin-19 is a member of the IL-10 subfamily of cytokines and is expressed in immune cells like monocytes, T, and B lymphocytes. It binds to the IL-20 receptor complex and as opposed to a proinflammatory response to an antigen, IL-19 incites an anti-inflammatory response.⁴¹ Previous studies in the Autieri lab have shown that IL-19 protects against plaque formation, as well as neointima formation following carotid ligation in LDL receptor (LDLR)^{-/-} mice.⁴² This data supports the idea that IL-19 is an anti-atherosclerotic, anti-inflammatory cytokine that is protective in the vasculature. Characterizing the proteins and mechanisms which regulate inflammatory gene expression is key to finding therapeutic targets to reduce atherosclerosis, therefore addressing a major health epidemic. In order to characterize these mechanisms, we have aimed to elucidate mechanisms where cytokine regulation of inflammation crosses with

gene transcription of these pro-inflammatory messages. One such possible convergence is the control of RNA processing by RNA binding proteins (RBPs).

Previous studies have shown that IL-19 reduces inflammatory gene expression by reducing RNA stability. Following pre-treatment with IL-19, mRNA stability of pro-inflammatory cytokines binding RNA stability protein Human antigen R (HuR) is decreased.⁴³ mRNA half-life of these inflammatory cytokines is also significantly decreased following pretreatment with IL-19. This suggests that inflammation present in vascular disease affects mRNA processing, possibly by influencing the activity of RNA binding proteins.

1.4 RNA Binding Proteins

Transcription and translation are tightly controlled at all levels by multiple mechanisms. One of the most important mechanisms is the post-transcriptional control of mRNA by RNA binding proteins (RBPs). RNA binding proteins regulate mRNA splicing, transcription, translation, trafficking, decay, and modification.⁴⁴ These proteins contain RNA binding domains (RBDs) that assemble with mRNAs to form ribonucleoprotein particles (RNPs) and affect the behavior of mRNA transcripts.⁴⁵ RNP formation is dynamic and context-dependent, and many play housekeeping roles in the cell. Loss of function of RBPs often result in tissue-specific disease etiology even if the RBP does not have tissue-specific expression, likely due to the tissue specificity of targets modified by the RBP.⁴⁶

RNA binding proteins bind to cis-acting regulatory elements in their target transcripts. These include AU-rich elements (AREs) in the 3' untranslated region (UTR) of mRNAs. These AREs are areas where transcript fate is determined by binding of RBPs to stabilize or destabilize the mRNA transcript and therefore influence protein expression

and cell behavior.⁴⁷ Generally, RBPs that bind to AREs are able to translocate between the nucleus and the cytoplasm, with location-specific functions. RBPs have a multitude of functions. Some bind transcripts and destabilize them by targeting them for degradation, while others bind and stabilize transcripts, which can lead to increased methylation, acetylation, translation, and increased gene expression (figure 2).

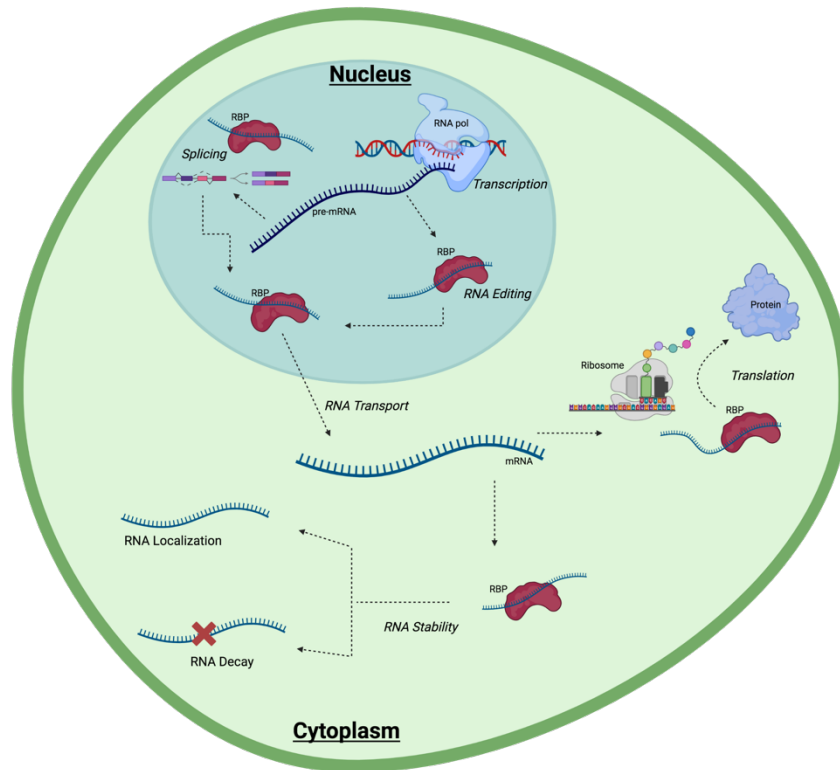


Figure 2. RNA binding protein (RBP) roles in post-transcriptional gene regulation. Made with Biorender.com, adapted from Cornelius et al., 2022.

A well-studied RNA stability factor binding AREs is the RNA binding protein Human antigen R (HuR). HuR is one of the most widely studied RNA binding proteins and is expressed ubiquitously. HuR regulates a host of cellular behaviors including tumorigenesis, cell cycle progression, apoptosis, and inflammation.^{48, 49} It is predominantly present in the nucleus, but when stimulated, HuR translocates to the cytoplasm to stabilize inflammatory mRNA.⁵⁰ HuR has previously been shown to be an

important regulator of TNF α mRNA stability. This mechanism is imperative in initiating pro-inflammatory events such as the formation of an atherosclerotic plaque. Pre-treatment of hVSMC with IL-19 decreases abundance of HuR, and IL-19 has been shown to destabilize inflammatory mRNA by inhibiting the translocation of HuR out of the nucleus.⁵¹

Just as there are RBPs that stabilize mRNA, there are RBPs that destabilize mRNA transcripts. Using mass spectrometry, it was found that HuR interacts with the RBP Fragile X-Related Protein 1 (FXR1), which is the focus of this dissertation. FXR1 appears to destabilize inflammatory transcripts that would normally be stabilized by HuR, and its expression is induced by IL-19.⁴³ We hypothesize that RNA-binding proteins play a crucial role in regulating inflammatory transcript stability and abundance in VSMCs during vascular inflammatory diseases, and that RBPs are to some degree regulated by anti-inflammatory cytokine IL-19.

The induction of an inflammatory response is tightly regulated and so requires the coordinated expression and control of hundreds of genes. These genes are not only controlled transcriptionally, but post-transcriptionally by RNA binding proteins. The ability to intermittently stabilize and destabilize transcripts responsible for cell cycle control, proliferation, and inflammation allows the cell to rapidly modify gene expression as needed during disruptions to homeostasis.⁴⁶ This is particularly important in VSMCs, which make up the majority of the artery and are directly involved in the pathogenesis of vascular disease when these processes go awry.

Quaking (QKI), an RBP involved in regulation of the cardiovascular network, may be involved in VSMC phenotypic switching. QKI is increased in neointimal VSMC in a mouse model of restenosis and its attenuation decreases the proliferative properties

of VSMC and promotes contractile protein expression.⁵² It's thought that QKI is upregulated following vascular injury and interacts with MYOCD to push VSMC to a proliferative phenotype, and when vascular injury is resolved QKI expression subsides, allowing for VSMC to return to their normal quiescent, contractile phenotype. HuR also unsurprisingly is implicated in cardiovascular dysfunction, given its role in regulation of the inflammatory response. HuR is upregulated in proliferative vascular diseases and interacts with mRNAs encoding angiotensin receptor, vascular endothelial growth factor (VEGF), and other proteins involved in progression of atherosclerosis, diabetes, hypertension, and heart failure.⁴⁸

RNA binding proteins play a part in a number of vascular diseases and researchers are currently looking into ways that they can be exploited as therapeutics. As previously noted, dysregulation of RBPs often cause tissue-specific disease etiology. One group was able to inhibit post-myocardial infarction (MI) left ventricle inflammation by knocking down HuR, often implicated in dysregulated inflammatory responses.⁵³ This attenuated left ventricle stress and ultimately decreased the infarct size in a mouse model. The involvement of RBPs in inflammatory and disease processes highlights RBPs as a valid avenue for therapeutic studies.

1.5 Fragile-X Related Protein 1 (FXR1)

FXR1 is a muscle-specific homolog of Fragile X Mental Retardation Protein (FMRP) which is a protein implicated in fragile X syndrome. FMRP plays many roles in the CNS, specifically in synaptic plasticity underlying learning and memory, axonal development, synapse formation, and development of neuronal circuits.⁵⁴ FXR1 consists of 5 functional domains. It has an agenet-like domain for regulating protein-protein interaction, a nuclear localization signal, 2 mRNA binding domains KH1 and KH2, and

an RNA specificity domain RGG.^{54, 55} Based on this structure, FXR1 may be involved in post-transcriptional regulation and RNA processing. The overwhelming majority of work on FXR-related proteins has been done in neurons, and very little is known about the family of proteins in VSMCs or vascular pathophysiology, or their responsiveness to inflammation.

A previous study from the Autieri Lab found that loss of FXR1 exacerbates inflammatory mediator expression in VSMCs while overexpression of FXR1 suppresses inflammatory mediator expression.⁴³ The same study showed that FXR1 is an IL-19 responsive RNA binding protein, leading us to believe that FXR1 is in some way contributing to reduced inflammation. When FXR1 is knocked out in hVSMC using siRNA, IL-19 is no longer able to reduce the abundance or stability of HuR. These results suggest that FXR1 is necessary for IL-19-induced destabilization of HuR.⁴³

To investigate the function of FXR1 further, the Nelson group at Baylor College of Medicine made a total FXR1 knockout mouse. They found that total knockout of FXR1 is neonatal lethal with a striated muscle phenotype.⁵⁶ These mice have delocalized costameres and significantly impaired muscle development and died a few hours post-birth. Because of the lethality of a total knockout, an FXR1 floxed mouse was generated for a conditional knockout of the FXR1 gene. We crossed this mouse with a SMC-specific cre-driver mouse to knock out FXR1 in SMC only and the offspring of these mice are used in the forthcoming studies.

While the majority of work with FXR-related proteins has been done in neurons, there is a growing body of evidence for FXR1's role in cancer progression. Numerous studies point to the idea that FXR1 may be an oncogene⁵⁷⁻⁶⁰, with one stating that an increased amount of FXR1 expression correlates with poorer overall survival in multiple

cancer types including breast and non-small cell lung cancer.⁶¹ Researchers found that under normal circumstances in a human squamous cell carcinoma (HSCC) cell line, FXR1 destabilizes p21 which allows for subversion of the senescence pathway and subsequent tumor growth. This may be a mechanism by which FXR1 allows cells to evade the compensatory senescence response by cells to control tumorigenesis. In HSCC cells treated with shRNA to knock down FXR1, cells accumulate in the G0/G1 phase of the cells cycle and β -galactosidase and γ H2AX staining is increased, consistent with an increase in cellular senescence.⁶¹ In a later study to examine how FXR1 plays a role in the senescence response in HSCC, they found that FXR1 stabilizes a micro RNA, miR301a-3p, which targets p21 for degradation. It was therefore determined that the FXR1-miR301a-3p axis is how cancer cells evade the senescence response that would otherwise be triggered by DNA damage.⁶²

1.6 Senescence in Vascular Disease and Aging

Cellular senescence is stable, irreversible cell cycle arrest. Cellular senescence can be positive, as it is a compensatory mechanism so that damaged cells do not continue to propagate; however, senescence is also pro-survival, meaning that instead of going through apoptosis, the cells accumulate and can contribute to age-related diseases. The signaling pathway that most often leads to senescence is that DNA damage signals activation of p53, which leads to the transcriptional activation of CDK inhibitor p21, and this inhibits cyclin-dependent kinases leading to cell cycle arrest.⁶³ Senescent cells exhibit phenotypic changes called the senescence associated secretory phenotype (SASP) which includes increased secretion of inflammatory cytokines, growth factors, and chemokines which all lead to increased inflammation in the tissue microenvironment.⁶⁴ It is widely accepted that the upregulation of cyclin dependent kinase inhibitor p21 as well

as the tumor suppressor p53 are obligate events in the induction and maintenance of senescence.

Tumor suppressor protein p53 has been called the “guardian of the genome” due to its significant involvement in preserving the integrity of the genome. p53 is heavily and intricately involved in DNA damage and repair pathways. In response to DNA damage, the MRE11-RAD50-NBS1 (MRN) complex is recruited to the site of the break. The MRN complex is one of the first complexes to detect double stranded breaks in DNA and it sets in motion the processes of DNA repair including non-homologous end joining and homologous end joining, and it is able to recruit protein kinases such as ataxia-telangiectasia mutated (ATM) to begin other DNA damage responses.⁶⁵ ATM phosphorylates H2AX to γ H2AX (a common senescence-associated marker), which sets up a positive feedback loop to amplify ATM’s signal, recognize the DNA break, and arrest the cell cycle in order to repair the damage.⁶⁶

Transcriptional activation of p53 is a necessary step in the process of cell cycle arrest and DNA damage repair. Under physiological conditions, p53 is complexed to the MDM2 protein through their N-terminal domains, where MDM2 is an E3 ubiquitin ligase and degrades p53 mRNA. Activation of ATM phosphorylates MDM2, inhibiting its ability to ubiquitinate p53 mRNA, and leading to transcriptional activation of the gene.⁶⁶ Activated p53 targets many genes involved in cell cycle arrest, but one of the most notable is p21. p21 is a cyclin dependent kinase inhibitor and its induction by p53 pauses progression of the cell cycle so that DNA damage can be resolved. If upregulation of p21 persists, however, p16^{INK4A}, another cyclin dependent kinase inhibitor, will become upregulated, which leads to maintenance of the senescence response through the retinoblastoma (RB) pathway.⁶⁷

Senescent cell accumulation is seen in many aging-related diseases including cardiovascular diseases. While there are many disordered cell types in vascular diseases, senescent cells are a specific type of issue because they are non-proliferative and secrete large amounts of inflammatory factors, meaning that their presence inhibits repair of damaged tissues.⁶⁸ Senescence is a normal process of aging, as cells lose proliferative capacity over time as their telomeres shorten with progressive replications, but it can also be triggered by DNA damage from exogenous sources. There are a number of CVD risk factors that lead to DNA damage and accumulation of senescent cells, such as increased angiotensin II expression, and heightened insulin signaling.^{69, 70} As VSMCs specifically become senescent, they lose their contractile functions and are unable to proliferate. This leads to aberrant regulation of blood flow, an increase in calcification, and arterial and aortic stiffness.

There is also ample evidence that senescent VSMC are involved in the progression of CVDs such as atherosclerosis, though it's difficult to pinpoint to what extent given that there are no singular markers of senescent cells. VSMC from atherosclerotic plaques proliferate more slowly⁷¹ and have increased expression of senescence-associated genes such as p16 and p21 compared with normal artery VSMCs.⁷² Furthermore, VSMCs from advanced human atherosclerotic plaques have significantly smaller telomeres than control VSMCs.⁷³ Senescent cells also secrete a multitude of inflammatory factors as part of the SASP, which contribute to the already inflammatory, atherogenic environment. Along with inflammatory cytokines, the SASP includes secretion of matrix degrading proteases⁷⁴, which can weaken the fibrous cap of the plaque and increase risk of rupture.

1.7 M6a Modifications in the Control of mRNA Transcript Fate

The fate of many transcripts can be determined by the binding of RPBs to the AREs in their 3'UTR. Modifications can be made to mRNA transcripts to make them more recognizable to certain RBPs. N⁶-methyladenosine (m6a) is the most prevalent modified nucleotide in mRNA and is overwhelmingly found in the 3'UTR. This modification involves the addition of a methyl group at position N6 of adenosine by a complex of the methyl transferases methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14).⁷⁵ These methyltransferases are considered “m6a writers.” Just as there are methyltransferases that “write” the m6a modifications, there are RBPs that serve as the “readers” of these modified transcripts. The “readers” play a part in the biological functions of m6a mRNA, recognizing the m6a modifications and playing a role in determining the fate of the transcripts.⁷⁶ M6a modified mRNA is susceptible to dynamic regulation both throughout development and in response to specific cellular stimuli, meaning that it can be transient and context-dependent. M6a enhances the separation of mRNA into compartments such as P-bodies and stress granules, and m6a RNA is typically less stable than unmodified mRNA.⁷⁶

METTL3 and METTL14 act as a complex with Wilms' tumor 1-associating protein (WTAP) to form the methyltransferase complex (MTC), which is necessary for the nuclearization of the METTL3-METTL14 complex. WTAP cannot catalyze the m6a modification, but its absence significantly affects m6a modification levels as the binding capacity of METTL3 is significantly reduced.⁷⁷ This complex plays a role in the cellular stress response, especially in response to DNA damage. The METTL3/14 complex is recruited to the sites of DNA damage induced by radiation.⁷⁸ WTAP plays a role in many cellular functions, with cell-type specific behavior. In many types of cancers, WTAP is

upregulated, serving as an oncogene. In human umbilical vein endothelial cells (HUVECs), WTAP is proliferative, while in smooth muscle cells WTAP is anti-proliferative.⁷⁹ Senescence may also to some extent be regulated by these RNA modifications. A study from 2017 suggests that METTL3 and METTL14 methylate p21 mRNA in a colorectal cancer cell line and enhances its translation in HeLa cells.⁸⁰

METTL3 and METTL14 are involved in senescence through m6a-independent mechanisms as well. METTL3 and METTL14 transcriptionally drive expression of transcripts that are a part of the senescence-associated secretory phenotype (SASP), but this is not regulated through m6a modifications. SASP expression is decreased with the absence of either METTL3 or METTL14, or in absence of all 3 complex members METTL3/14 and WTAP.⁸¹ This decrease in SASP transcript expression was not associated with any change in senescent cell behavior such as growth arrest, increased β -galactosidase, or increased p21/p16. This suggests that the MTC does not affect the induction or maintenance of the senescence response, but it is implicated in the degree of SASP expression. Further investigation revealed that METTL3 and METTL14 redistribute in senescent cells.⁸¹ METTL3 binds near transcription start sites of genes, many of which are direct target genes of NF κ B, and METTL14 redistributes to bind near gene bodies where it co-localizes with H3K27ac, an enhancer of transcription. Binding of H3K27ac with METTL14 enriched a number of genes implicated in the SASP including chemokines CXCL5 and CXCL6 and inflammatory cytokines IL6 and IL1- β .⁸¹

A recent study showed that METTL3 is upregulated in a mouse model of atherosclerosis and silencing METTL3 using shRNA alleviates atherosclerosis progression and could stabilize plaques in this same model.⁸² Further experiments from the same study suggest that METTL3 is involved in the regulation of VSMC phenotypic

switching, and that this might happen through a signaling pathway that increases expression of miR-375-3p, leading to inhibition of pyruvate dehydrogenase kinase 1 (PDK1), thus promoting the phenotypic switching of VSMC. There is also evidence that m6a modification can inhibit the proliferation and migration of VSMC in a mouse model of intimal hyperplasia. M6a methylation increases SM22 α expression, decreasing cell proliferation, and a recent study identified that the m6a demethylase Fat mass and obesity-associated protein (FTO) was elevated in a diabetic mouse model of intimal hyperplasia. When these mice were treated with lenti-sh-FTO to knock down demethylase FTO, neointima formation was decreased, and there was an increase in m6a modification and overall expression of SM22 α .⁸³

There is evidence that FXR1 is an m6a reader, binding preferentially to transcripts with this modification. Global m6a interactome profiling has revealed that FXR1 interacts with m6a mRNA and the most highly enriched pathways binding to m6a mRNA are mRNA processing and metabolism related.⁸⁴ This study further found that G3BP1, a protein involved with formation of stress granules, is repelled by m6a, leading them to believe that G3BP1 is involved in stabilization rather than degradation of m6a modified transcripts. Our lab has found that FXR1 co-localizes with stress granule marker poly-a-binding protein (PABP).⁸⁵ It remains to be studied how FXR1 interacts with m6a mRNA in the vasculature, which is one of the gaps that the present study aims to fill.

1.8 Summary

Vascular diseases account for one of the most significant categories of illness and mortality worldwide. Atherosclerosis, an inflammatory vascular disease characterized by the oxidation of low-density lipoprotein (LDL) and buildup of plaque in the subendothelial space of the artery, is one of the main contributors. Atherosclerosis has an

inflammatory and proliferative profile, where vascular smooth muscle cells (VSMC) switch from a quiescent, contractile phenotype to a synthetic, proliferative phenotype, driving disease progression. This proliferative profile is not unique to atherosclerosis. Other vascular diseases such as restenosis, the formation of a neointima that often occurs following stent placement, follow this same etiology – an increase in inflammation and otherwise injurious environment leads to VSMC proliferation and ultimately progression of vascular pathophysiological conditions.

This increase in inflammation and proliferation can be mediated by a multitude of factors including through RNA binding proteins (RBPs). RBPs bind to and modulate the stability and translation of mRNA transcripts that are involved in cellular function and disease. Many RBPs have been shown to play a role in vascular disease pathogenesis and affecting the expression of some RBPs ameliorates the disease states that they are associated with. This suggests that RBPs are an area that may be exploited for therapeutic advancements in vascular disease research.

The focus of the present study is on the RBP FXR1, a muscle-enhanced homolog of FMRP, which plays a role in the cellular response to inflammation and injury. We have previously reported that FXR1 expression is upregulated in injured arteries, is induced by the anti-inflammatory cytokine IL-19, and binds to and destabilizes inflammatory mRNAs. Emerging evidence in immortal cancer cell lines suggests that FXR1 may be an oncogene that plays a part in the evasion of the senescence response, allowing for increased tumorigenesis. A recent study from the lab reported that FXR1 is involved in the regulation of cytoskeletal dynamics and that smooth muscle-specific FXR1 knockout mice are hypotensive compared to controls. These findings suggest a

role for FXR1 in regulating vascular disease and possibly control of the cell cycle, though more research is needed into these mechanisms.

1.9 Central Hypothesis

The central hypothesis of this dissertation is that FXR1 regulates vascular occlusive disease by acting as a crucial regulator of inflammatory and proliferative transcript stability in VSMC.

1.10 Specific Aims

Aim 1 - Determine if FXR1 expression can regulate restenosis *in vivo*. We will utilize our novel tamoxifen-inducible, SMC-specific FXR1 knockout mice subjected to carotid artery ligation to test the hypothesis that FXR1 expression can modulate the VSMC response to injury and intimal hyperplasia indicative of restenosis. We will perform morphometric analysis of intimal hyperplasia and quantitate abundance of genes related to senescence and cell cycle control. Findings will be confirmed by IHC using carotid artery tissue sections.

Aim 2 – Determine the molecular mechanisms by which FXR1 regulates inflammatory and proliferative mRNA abundance. Using RNA-sequencing and RIP-sequencing we have identified transcripts involved in inflammation and control of the cell cycle whose expression are changed by FXR1 knockout and who associate directly with FXR1. We will determine if changes in FXR1 expression affect the stability of these mRNAs and will perform *in vitro* and *ex vivo* experiments to determine how modulation of FXR1 affects VSMC proliferation, gene expression, and senescence response.

CHAPTER 2

METHODS

2.1 Generation of FXR1^{SMC/SMC} Mice.

FXR1^{flox/flox} mice on the C57Bl/6 background were obtained from the FMR research resource at Baylor University and described in⁵⁶. Total knockout of the gene is lethal shortly after birth. Female FXR1^{flox/flox} mice were crossed with congenic male C57BL/6 Myh11-CreERT2 (a smooth muscle-specific cre promoter) mice described in⁸⁶ as the allele is carried only on the Y chromosome, and appropriate offspring identified by PCR-based genotyping. Because Myh11-CreERT2 resides on the Y chromosome only male mice were used for these studies. Cre-Lox-mediated knockout of FXR1 was induced in 6-wk-old male mice after intraperitoneal injection of 1 mg tamoxifen (T-5648, Sigma) per mouse per day for 5 consecutive days.

Tamoxifen-mediated cre recombinase expression is one of the most widely used tools for gene modification⁸⁷. Briefly, the male FXR1^{SMC/SMC} mice have loxP sites flanking the FXR1 gene and carry a smooth muscle-specific cre recombinase coupled to a mutated estrogen receptor on the Y chromosome. Under physiological conditions, this mutated estrogen receptor does not bind its ligand, but when the mouse is injected intraperitoneally with tamoxifen (a synthetic hormone), the receptor is activated and cre translocates from the nucleus to interact with the loxP sites and cleave out the FXR1 gene in smooth muscle cells.⁸⁸ SMC-specific excision of FXR1 was validated by western blot and immunohistochemistry. No gross differences between FXR1 knockout and wild type mice were observed in any offspring.

2.2 Carotid Artery Ligation.

Age -matched male mice (ages 8-12 weeks) were used for these studies. The ligation model of injury was performed as described previously.⁸⁹ Briefly, mice were anesthetized by inhalation of isoflurane (induction: 3-5%, maintenance: 1-2%). The left common carotid artery was dissected and ligated near the bifurcation. After 28 days mice were euthanized, and tissue prepared for immunohistochemistry and morphological analysis. All animal procedures followed Temple University-IACUC approved protocols.

2.3 VSMC Culture, Proliferation Assays.

Primary human coronary artery vascular smooth muscle cells were obtained as cryopreserved secondary culture from LifeLine, Inc. (Oceanside, CA), and maintained as we described.^{42, 43, 90} VSMC were used from passage 2–4. For mouse VSMC, abdominal aorta from control and FXR1^{SMC/SMC} mice were excised, endothelial layer removed, and VSMC isolated as described.^{89, 91} VSMC were cultured in DMEM supplemented with 20% FCS. Greater than 95% of isolated cells were SMC actin positive from initial passage. Two different proliferative assays were performed. Proliferation was assayed by the BrdU kit from Cell Signaling, Inc, according to manufacturer's instructions. Flow cytometry was performed as we described.⁹² Briefly, cells were transfected with FXR1 siRNA or a scrambled control and cultured for 3 days before they were trypsinized, fixed in ice cold 70% ethanol, and stained with propidium iodide (PI) using the FxCycle PI/RNase staining solution from Invitrogen (Waltham, MA; cat#2110506) according to manufacturer's instructions. Samples were analyzed on a BD LSR II flow cytometer. Finally, proliferation was measured by counting a timecourse. Cells were transfected with FXR1 siRNA or a scrambled control, of transduced with AdCre or AdGFP virus and seeded into 12-well trays at 10,000 cells/well. Cell counts were taken on days 3 and 7.

2.4 β -galactosidase (β -gal) and γ H2AX Staining.

Human VSMC were transfected with FXR1 siRNA or a scrambled control and seeded into chamber slides. 72 hours after transfection cells were fixed and stained for common senescence markers γ H2AX (Novus, Littleton, CO; cat# NB100-384) and β -gal using a kit from Cell Signaling, Inc (cat# 6813S) according to manufacturers instructions. VSMC were also isolated from FXR1^{SMC/SMC} mice, seeded into chamber slides, transduced with 100moi of GFP or Cre adenovirus, and stained for β -gal after 72 hours using the kit referenced above.

2.5 Immunohistochemistry and Quantitative Morphology.

Digitized images of hematoxylin-eosin stained carotid artery cross-sections were measured and averaged from at least three representative 5 micron-thick stained tissue sections at least 75-100 microns apart per carotid artery using Image Pro Plus (Media Cybernetics) as previously described.^{90, 91, 93} At least 6 mice per group were used for morphology and IHC. The circumference of the lumen, the area encircled internal elastic lamina (IEL), and the external elastic lamina (EEL) were quantitated. The medial area was calculated by subtracting the area defined by the IEL from the area defined by the EEL, and intimal area calculated as the difference between the area inside the IEL and the luminal area. Tissue fixation, processing, and immunohistochemical staining was performed as described.^{51, 91, 94}

2.6 Transfection, RNA Extraction, and Quantitative RT-PCR.

FXR1 and WTAP depletion in primary hVSMC was performed using 10nM ON-TARGET plus SMARTpool FXR1 siRNA and WTAP siRNA containing a mixture of four siRNAs that target human FXR1 and WTAP, purchased from Dharmacon

(Lafayette, CO, USA). Cells were transfected using an AMAXA Nucleofector 2b device from Lonza (Walkersville, Md) as described previously.⁹⁵ 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^{42, 88, 95} and described in detail in table 1. Multiple mRNAs (Ct values) were quantitated simultaneously by the PCR machine software. Primer pairs were purchased from Integrated DNA Technologies (IDT), (Coralville, IA) and SYBR green was used for detection. For RNA stability, cells were stimulated for 16hr with TNF α (10ng/nL) and then treated with 10ng/ml Actinomycin D as we described^{43, 95}, and RNA was extracted at various times post actinomycin D addition (0, 2, 4 hours).

Table 1. List of human primers used for qPCR in the present thesis.

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
<i>GAPDH</i>	CGAGAGTCAGCCGCATCTT	CCCCATGGTGTCTGAGCG
<i>FXR1</i>	AGTTACCGCCATTGAGCTAG	ACTTTTCCAACGAGATTCCTAGG
<i>TNFα</i>	GGTCTACTTTGGGATCATTGC	GAAGAGGTTGAGGGTGTCTG
<i>CDK1</i>	ACAAAGGAACAATTAAGCTGGCTG	CTGGAGTTGAGTAACGAGCTG
<i>LIN9</i>	GACTGAACAAGGACCTAAACAAAG	CATGCCGAACAATTCCTGTG
<i>BUB1</i>	CCGAGTCTCAGAAAATACCAGG	AAATGGAGAAAGGTACTACTGGG
<i>GJA5</i>	GTTTTGGCATCTGTTCCCTG	CGGAATATGAAGAGGACAGTGAG
<i>CD93</i>	CAGACAGTTACTCCTGGGTTC	GTGGCTGGTGACTCTAGTG
<i>Ki67</i>	GACCCAGCACTCCAAAGAAA	TCT GCG CTC TAC CTA CTA CAA
<i>CDKN3</i>	GCCAGCTGCTGTGAAATAATG	GTAGGAGACAAGCAGCTACAAG
<i>HMGB1</i>	GAATCTCTATGTTGCCAGGT T	CAGTGGTGTGTCCCTGTAATC
<i>IL8</i>	CCAGGAAGAAACCACCGGA	GAAATCAGGAAGGCTGCCAAG

Table 1. (continued).

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
<i>IL1a</i>	CTGAAGGAGATGCCTGAGATAC	GATGGGCAACTGATGTGAAATAG
<i>IL6</i>	CCAGGAGAAGATTCCAAAGATGTA	CGTCGAGGATGTACCGAATTT
<i>IL1B</i>	TCCCCAGCCCTTTTGTGTA	TTAGAACCAAATGTGGCCGTG
<i>MMP9</i>	GAACCTTGACAGCGACAAGAAG	CGGCACTGAGGAATGATCTAA
<i>CXCL11</i>	GTGTGAAGGGCATGGCTATAG	CACTTTCCTGCTTTTACCCC-
<i>HMGB2</i>	GAAGTGTTTCGGAGAGATGGAAG	CACCTTTGGGAGGAACGTAAT
<i>MCP1</i>	AGCAGAAGTGGGTTTCAGGATT	TGTGGAGTGAGTGTTCAAGTCT
<i>ING1</i>	AAGTGGTACTGTCCCAAGTGCC	GCCCTCTCTTTTTTGGATTCTCC
<i>SOCS2</i>	GCCTTGCCTTCTTAGGTTCT	CTGGTTCCTTCCCACTTCTT
<i>WTAP</i>	AGTCAGAGGCCACAAGTAAAG	ATTCCCTGGAGAAGAAGGAAAG
<i>ZNF706</i>	GCAGTATGACCTGGCTCTAAC	ACAAGGGCTCACTGACAATC
<i>TMEM38B</i>	CTGCATTGGCATGGAAGAATC	TGCAAGCAGTAGACAGGATAAA

Table 2. List of mouse primers used for qPCR in the present thesis.

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
<i>GAPDH</i>	GGAGAAACCTGCCAAGTATGA	TCCTCAGTGTAGCCCAAGA
<i>FXR1</i>	GAACGCATGGCACTAACATAC	CACAAATTCCAAGAAACCTCTAGC
<i>CDK1</i>	TTGAAGAGGCAACCGAGTAAG	GGCGTTAGGTCATCCATCAA
<i>CDKN3</i>	GACTTGGAAGATCCTGTCTTGT	GACATCTCGAAGGCTGTCTATG
<i>BUB1</i>	CCCTGGAGTAGGAAGCTAGT	ATGGTGGTCTCACAACAAGAA
<i>Ki67</i>	GACCCAGCACTCCAAAGAAA	TCTGCGCTCTACCTACTACAA
<i>p16</i>	GTGTGCATGACGTGCGGG	GCAGTTCGAATCTGCACCGTAG
<i>p21</i>	AACATCTCAGGGCCGAAA	TGCGCTTGGAGTGATAGAAA
<i>p53</i>	GTATTTACCCCTCAAGATCC	TGGGCATCCTTTAACTCTA
<i>HMGB1</i>	CCCTACTAAAGAAGACCTGAGAATG	AGCCAGCGTTCCTTGTGATAG

2.7 Adenoviral Knockout of FXR1.

Primary abdominal aortic vascular smooth muscle cells were isolated from FXR1^{SMC/SMC} mice as described previously⁸⁷ and cultured in DMEM supplemented with 20% fetal calf serum. Cells were transduced with 100moi of a control GFP adenovirus or 100moi of a Cre adenovirus to knock out FXR1. Subsequent procedures were carried out following 72 hours of transduction to ensure knockout of the gene.

2.8 RNA Sequencing.

Library preparation and unbiased RNA sequencing was performed by GeneWiz, (South Plainfield, NJ). Total RNA was isolated from primary hVSMC transfected with FXR1 siRNA (5nM) or scrambled control, serum starved or stimulated with 10ng/mL TNF α for 6 hours, libraries were sequenced using a 1 \times 100-bp single end rapid run on the HiSeq2500 platform, and a comparison of gene expression using DESeq2. The Wald test was used to generate p-values and log₂ fold changes. Genes with a p-value < 0.05 and absolute log₂ fold change > 1 were called as differentially expressed genes. Significantly differentially expressed genes were clustered by their gene ontology and the enrichment of gene ontology terms was tested using Fisher exact test (GeneSCF v1.1-p2).

$\text{Log}_2(\text{Group 2 mean normalized counts}/\text{Group 1 mean normalized counts}) =$
 $\text{Log}_2\text{FoldChange}.$

2.9 RNA Immunoprecipitation (RIP).

For RIP, hVSMCs were transduced with AdenoFXR1 prior to serum starvation for 48 hours. Cells were treated with TNF α for 24 hours 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 hours at 25 degrees C. The beads were then centrifuged and washed 5X in IP buffer. Trizol was added to the pelleted beads and RNA was

extracted and reverse transcribed to cDNA. Pellets were sent for unbiased RNA sequencing as described above, or for qRT-PCR for the transcripts indicated.

2.10 Western Blotting and Protein Determination.

Human and mouse VSMC extracts were prepared as described.⁹⁰ Membranes were incubated with a 1:2000–9000 dilution of primary antibody, and a 1:2000 dilution of secondary antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and FXR1 were from Cell Signaling (Danvers, MA, USA; cat# 2118S and 2295S, respectively), HSC70 and p53 from Santa Cruz Biotechnology (Dallas, TX, USA; cat# sc-7298 and sc-126), BUB1 and CDKN31 from Abcam, Inc., (Cambridge UK; cat# ab195268 and ab175393, respectively), CDK1 from Boster, Inc., (Pleasanton, CA, USA; cat# PB9533), and Inhibitor of Growth Family 1 (ING1) from R&D Systems (Minneapolis, MN, USA; cat#MAB5758). Secondary antibodies were from Cell Signaling (anti-rabbit IgG cat#7074S, anti-mouse IgG cat# 7076S). Reactive proteins were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) according to manufacturer's instructions. Relative intensity of bands were normalized to GAPDH or HSC70, and quantitated by scanning image analysis and the ImageJ densitometry program software version 1.53a (NIH, Bethesda, MD; <http://imagej.nih.gov.libproxy.temple.edu/ij>; last accessed 10/24/22).

2.11. Generation of FXR1 Mutant Plasmids.

Amino acid substitution mutations were made for 1) KH1 and KH2 domains, 2) KH1 domain only, 3) KH2 domain only, 4) nuclear localization signal, 5) RGG box. These plasmids were flag-tagged and ordered from IDT (Coralville, IA). 2-5ug plasmid was transfected into NIH 3T3 cells.

2.12 M6a mRNA Epitranscriptomic Microarray.

For the m6a microarray, hVSMC were stimulated with TNF α for 0, 2, 6, 18, and 24 hours after which RNA was extracted and sent to Arraystar (Rockville, MD, USA) where the microarray was carried out using Arraystar's standard protocols. Briefly, after the samples arrived the RNA was immunoprecipitated with an anti-m6a antibody and immunoprecipitated with magnetic beads. The modified RNAs were eluted from the immunoprecipitated magnetic beads as the "IP". The unmodified RNAs were recovered from the supernatant as "Sup". The arrays were scanned in two-color channels by an Agilent Scanner G2505C and Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. "m6A methylation level" was calculated for the percentage of modification based on the IP (Cy5-labelled) and Sup (Cy3-labelled) normalized intensities.

2.13 Statistics.

Results are expressed as mean \pm SE. Differences between groups were evaluated with the use of ANOVA to evaluate differences between individual mean values or by t tests where appropriate.

CHAPTER 3

RESULTS

Results in Chapter 3 were published in and edited from Corbett et al., *Am J Pathol*, 2023⁹⁶ <https://doi.org/10.1016/j.ajpath.2023.01.006>.

3.1 Depletion of FXR1 Modifies Abundance of Proliferation-Associated Transcripts.

FXR1 is an mRNA binding protein and presumed to be an mRNA de-stability factor, so our initial goal was to identify inflammation-responsive genes whose abundance might be modified by deletion of FXR1. First, it was determined that FXR1 expression in cultured primary human VSMC was responsive to inflammatory stimuli. Figures 3A-C show that FXR1 mRNA and protein abundance in VSMC are increased in response to TNF α stimulation.

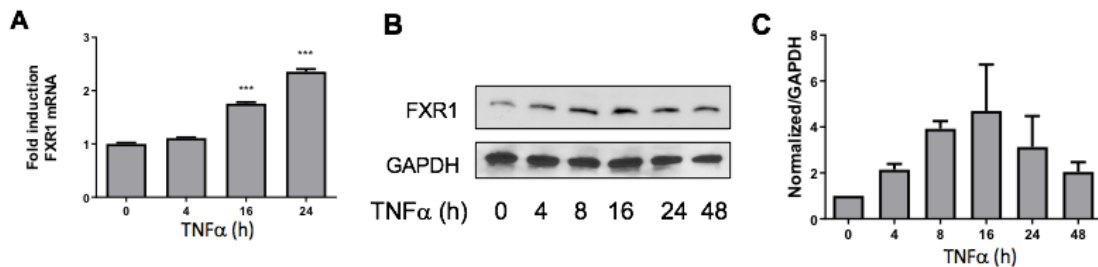


Figure 3. FXR1 expression is induced by TNF α stimulation in hVSMC. A. FXR1 mRNA expression is induced by TNF α stimulation in hVSMC (***)= $p < 0.001$). B, C., FXR1 protein expression is induced by TNF α stimulation.

Because TNF α is a potent pro-inflammatory cytokine and a major driver of vascular inflammation, and TNF α induces FXR1 mRNA and protein expression, we aimed to identify genes whose mRNA abundance would be altered by FXR1 knockdown in primary human VSMC in the presence and absence of TNF α . Figure 4A shows the

effectiveness of FXR1 siRNA transfection in knock down of the FXR1 protein. Figures 4B and 4C are volcano plots of genes whose expression was significantly altered (P value of less than or equal to 0.05) by the loss of FXR1 in starved and TNF α -stimulated VSMC, respectively. It was apparent from these plots that in both serum-starved and TNF α -stimulated VSMC, the loss of FXR1 resulted in more transcripts with increased abundance compared with those with reduced abundance, which is consistent with an mRNA de-stability protein.

Figures 4D and 4E are Gene Ontology (GO) profiles from both unstimulated and TNF α -stimulated VSMC. Categories were enriched for genes that participate in cell division, mitosis, proliferation, and as expected, mRNA metabolism. Considering that FXR1 is an mRNA binding protein and a putative mRNA de-stability factor, it was surprising that there were a number of genes whose abundance were decreased by FXR1 knockdown, suggesting that FXR1 participated in stabilization of those transcripts. It was particularly interesting that in FXR1-depleted VSMC, over 90% of these transcripts were associated with cell division and proliferation (Table 2). This suggested a function for FXR1 in regulation of VSMC proliferation, perhaps by stabilization of transcripts important in cell division.

Table 3. Genes reduced between the maximal amount of 2.47 and 1.75-fold whose abundance were decreased by FXR1 knockdown.

GeneID	GeneName	log2FoldChange	P value
ENSG00000169607	<i>CKAP2L</i>	-1.81	0.18
ENSG00000274290	<i>HIST1H2BE</i>	-1.83	0.01
ENSG0000024526	<i>DEPDC1</i>	-1.83	0.009
ENSG00000142945	<i>KIF2C</i>	-1.85	0.011
ENSG00000072571	<i>HMMR</i>	-1.87	0.01
ENSG00000123485	<i>HJURP</i>	-1.9	0.02
ENSG00000100526	<i>CDKN3</i>	-1.91	0.04
ENSG00000164109	<i>MAD2L1</i>	-1.92	0.01
ENSG00000138160	<i>KIF11</i>	-1.93	0.01
ENSG00000129195	<i>PIMREG</i>	-1.93	0.03
ENSG00000170312	<i>CDK1</i>	-1.94	0.01
ENSG00000137812	<i>KNL1</i>	-1.95	0.01
ENSG00000066279	<i>ASPM</i>	-1.97	0.01
ENSG00000276903	<i>HIST1H2AL</i>	-1.97	0.02
ENSG00000124575	<i>HIST1H1D</i>	-2.02	0.01
ENSG00000274641	<i>HIST1H2BO</i>	-2.03	0.01
ENSG00000278272	<i>HIST1H3C</i>	-2.05	0.01
ENSG00000197153	<i>HIST1H3J</i>	-2.05	0.02
ENSG00000276410	<i>HIST1H2BB</i>	-2.07	0.01
ENSG00000138778	<i>CENPE</i>	-2.08	0.01
ENSG00000183598	<i>HIST2H3D</i>	-2.1	0.01
ENSG00000080986	<i>NDC80</i>	-2.11	0.01
ENSG00000125810	<i>CD93</i>	-2.15	0.01
ENSG00000183814	<i>LIN9</i>	-2.18	0.04
ENSG00000073849	<i>ST6GAL1</i>	-2.19	0.01
ENSG00000117399	<i>CDC20</i>	-2.2	0.01
ENSG00000175063	<i>UBE2C</i>	-2.22	0.02
ENSG00000169679	<i>BUB1</i>	-2.27	0.01
ENSG00000163535	<i>SGO2</i>	-2.28	0.01
ENSG00000112984	<i>KIF20A</i>	-2.29	0
ENSG00000158402	<i>CDC25C</i>	-2.32	0.04
ENSG00000148773	<i>MKI67</i>	-2.36	0.01
ENSG00000112742	<i>TTK</i>	-2.47	0

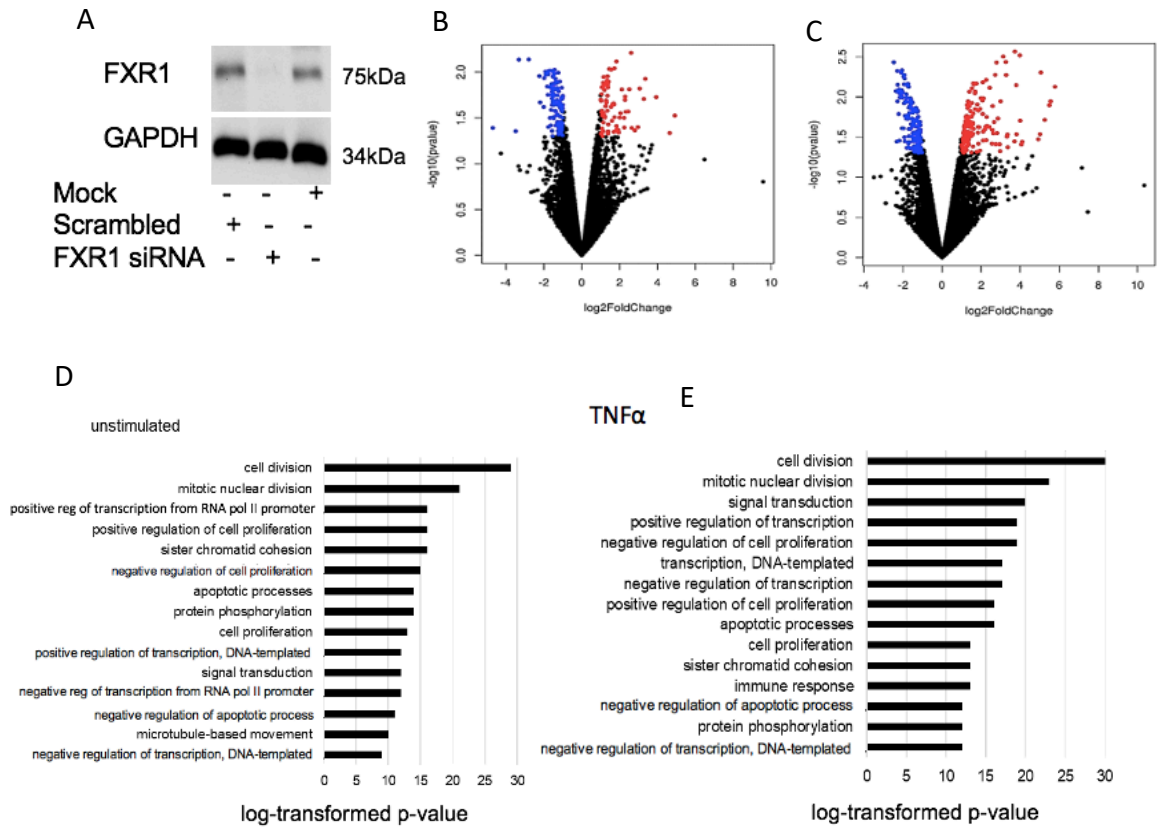


Figure 4. Expression and FXR1-dependent gene expression in stimulated hVSMC. A. FXR1 smart-pool siRNA effectively knocks down FXR1 protein in hVSMC 72 hours post-transfection. Volcano plots comparing mRNA abundance detected from RNA sequencing of FXR1 siRNA and control siRNA transfected hVSMC serum-starved 48 hours B., and stimulated with TNF α for 6 hours C. Gene ontology analysis of RNAseq in unstimulated D, and TNF α -stimulated hVSMC E. shows enhanced expression of transcripts associated with cell division.

Expression of a number of these proliferation-associated transcripts were validated by quantitative RT-PCR in response to TNF α stimulation (Figure 5). Since FXR1 is generally considered to be an RBP with effects on mRNA stability, we next determined the mRNA stability of transcripts modified by FXR1 abundance using the RNA polymerase inhibitor Actinomycin D. Figure 6A shows that the mRNA stability of CDK1, BUB1, MKI67, and CDKN3, all genes associated with cell division, are significantly decreased when FXR1 is knocked down. The decrease in mRNA abundance was reflected in reduced protein abundance by western blot, and figure 6B shows that the protein abundance of these genes is significantly decreased in TNF α stimulated hVSMC when FXR1 is knocked down.

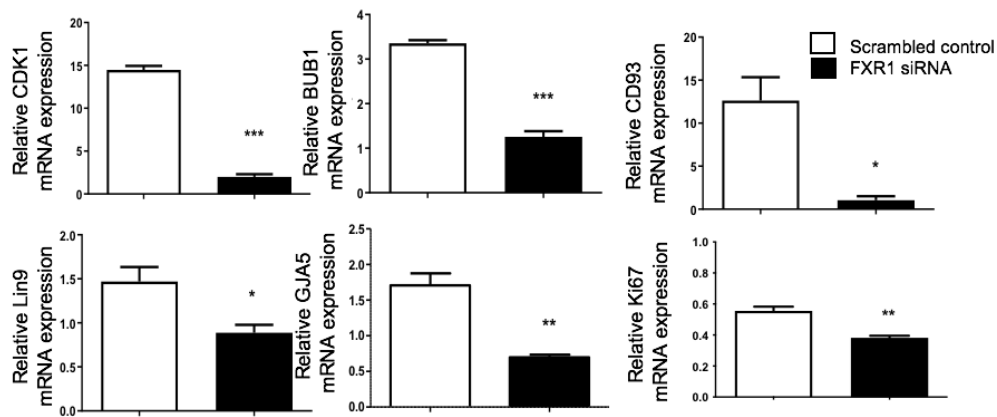


Figure 5. FXR1 deletion results in decreased cell cycle gene expression. Quantitative RT-PCR of mRNA abundance of cell-division associated genes in TNF α -stimulated hVSMC transfected with scrambled control or FXR1-specific siRNA. *=P<0.05, **=P<0.01, ***=P<0.001 from at least three experiments.

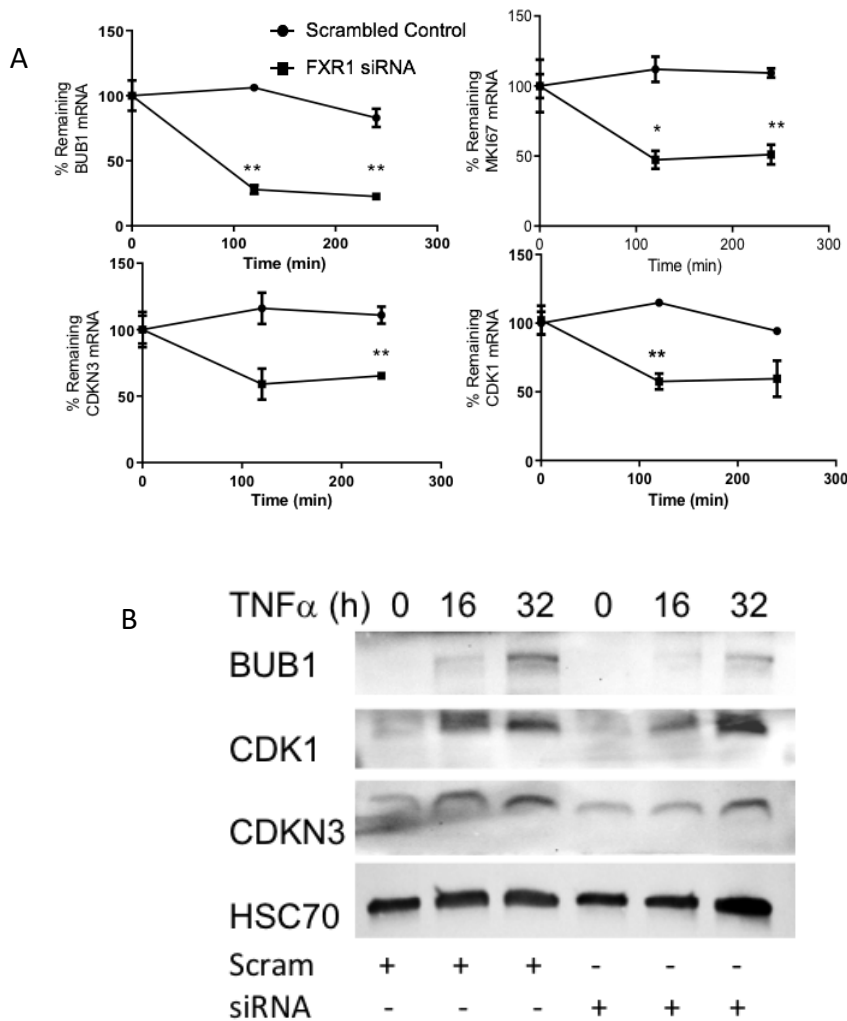


Figure 6. FXR1 deletion results in decreased cell cycle gene mRNA stability. A. Deletion of FXR1 decreases mRNA stability of cell-division associated genes. RNA was isolated from hVSMC stimulated with TNF α , then treated with Actinomycin D. RNA was quantitated at the indicated times post-addition of Actinomycin D. B. Representative western blot of cell-division associated genes in TNF α -stimulated hVSMC. Protein was extracted at the indicated times post-stimulation and immunoblotted with the indicated antibodies. *=P<0.05, **=P<0.01, ***=P<0.001.

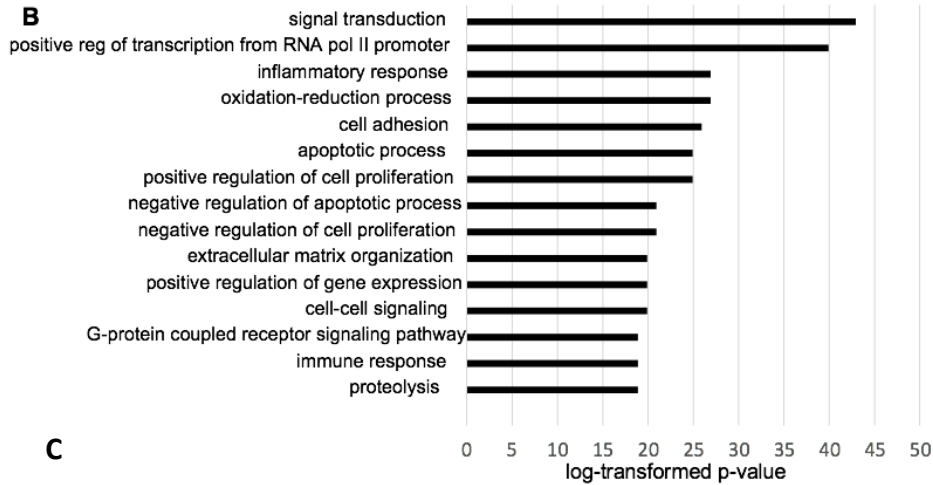
3.2 FXR1 Interacts with Proliferation-Associated mRNA Transcripts.

Many labile mRNAs are regulated at the post-transcriptional level by adenine and uridine-rich (AU-rich) elements (AREs) in the 3' untranslated regions (UTR).^{97, 98} The ARE provides a binding site for trans-acting RNA-binding proteins (RBPs), and it has been previously shown that FXR1 interacts with AREs.⁴³ One commonality shared by BUB1, CDK1, CDKN3, and MKI67 is the presence of AREs in the 3'UTR or intronic regions of their mRNA (Figure 7A), which may provide an explanation as well as potential mechanism for FXR1 interaction with these transcripts.^{99, 100} With this in mind, RNA immunoprecipitation sequencing (RIPseq) was performed to identify mRNAs which might specifically interact with FXR1. For these experiments, FLAG-tagged FXR1 was transduced into hVSMC which were serum starved, then stimulated with TNF α . GO analysis of transcripts which immunoprecipitated with FXR1 after TNF α -stimulation compared with unstimulated VSMC revealed an informative view of FXR1 mRNA transcript interactions in response to TNF α . GO analysis demonstrated enhancement of transcripts associated with regulation of cell proliferation, transcription from RNA, and inflammatory responses (Figure 7B). Importantly, mRNA for BUB1, CDK1, MIK67, and CDKN3 all contain AREs in their 3' UTR and were all identified as pulled down with FXR1. FXR1's interactions with these mRNAs were validated by an independent standard RIP-RT-PCR (Figure 7C), indicating that FXR1 interacted with these mRNA transcripts.

A

<u>ARE</u>	<u>3' UTR</u>	<u>total intronic</u>
CDK1	12	68
CDKN3	0	54
BUB1	1	102
MKi67	2	47

RIP 0 vs 24



C

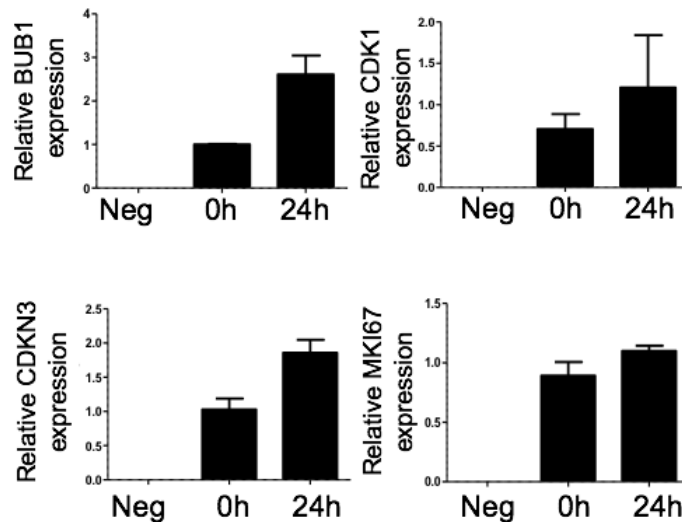


Figure 7. RNA-immunoprecipitation sequencing. hVSMC were infected with FLAG-tagged FXR1 adenovirus, serum-starved and stimulated with TNF α for 24 hours. A. Table showing number and location of ARE in CDK1, CDKN3, BUB1 and MKI67 mRNA. B. FXR1 RIP-seq. Gene Ontology of RIP-seq comparing transcripts immunoprecipitated in unstimulated with TNF α -stimulated hVSMC. C. Cell-division associated transcripts detected by RNAseq independently validated by immunoprecipitation with FLAG-tagged FXR1 in unstimulated and stimulated hVSMC.

3.3 Depletion of FXR1 Induces Senescence and SASP Expression in VSMC.

Because FXR1 expression modified the mRNA stability and protein abundance of several critical cell cycle and proliferation-associated proteins, experiments were performed to test the hypothesis that FXR1 depletion would negatively affect hVSMC cell cycle progression and proliferation. First, BrdU incorporation as well as standard cell counting assays determined that cell proliferation of FXR1-depleted hVSMC was significantly reduced compared with scrambled control cells (Figure 8).

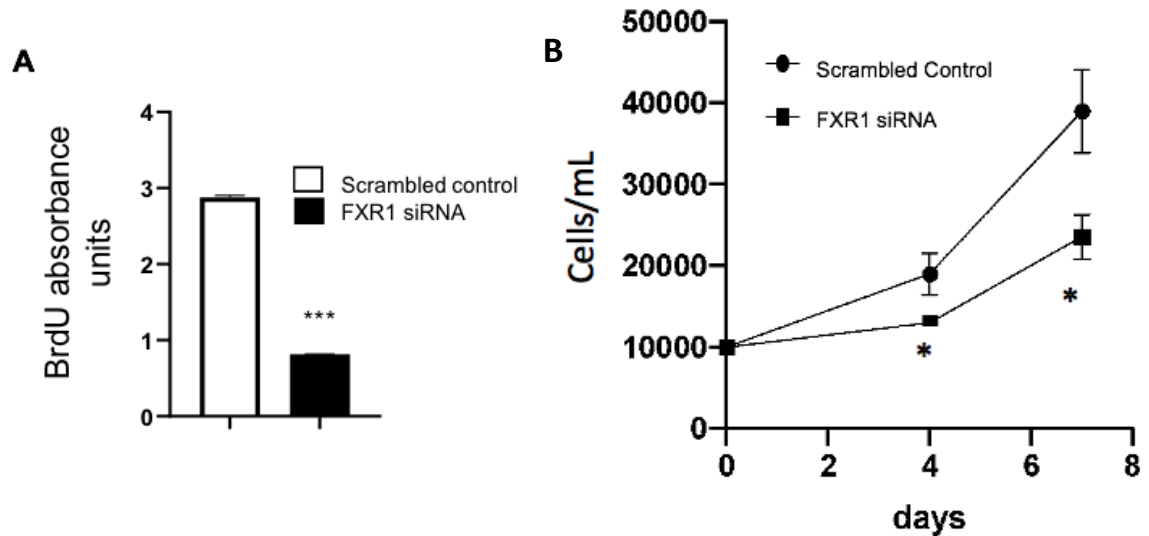


Figure 8. FXR1 knockdown reduces hVSMC proliferation. A. hVSMC transfected with FXR1 siRNA incorporate significantly less BrdU compared with control siRNA. B. hVSMC transfected with FXR1 siRNA and seeded at equal concentration show significantly less proliferation when counted in a 7-day time-course. *=P<0.05, ***=P<0.001

Next, hVSMC were subject to cell cycle analysis by PI staining and flow cytometry. Figure 9A is a representative experiment showing that in asynchronously growing hVSMC, a significantly greater number of FXR1-depleted cells are in G1, and there are significantly fewer proliferating cells (S and G2/M phase) in FXR1-depleted cells compared with scrambled control cells. A compilation of four asynchronous experiments and five experiments where hVSMC were synchronized by fetal calf depletion are depicted in Figure 9B and together, demonstrate that hVSMC depleted of FXR1 are less proliferative compared with control cells.

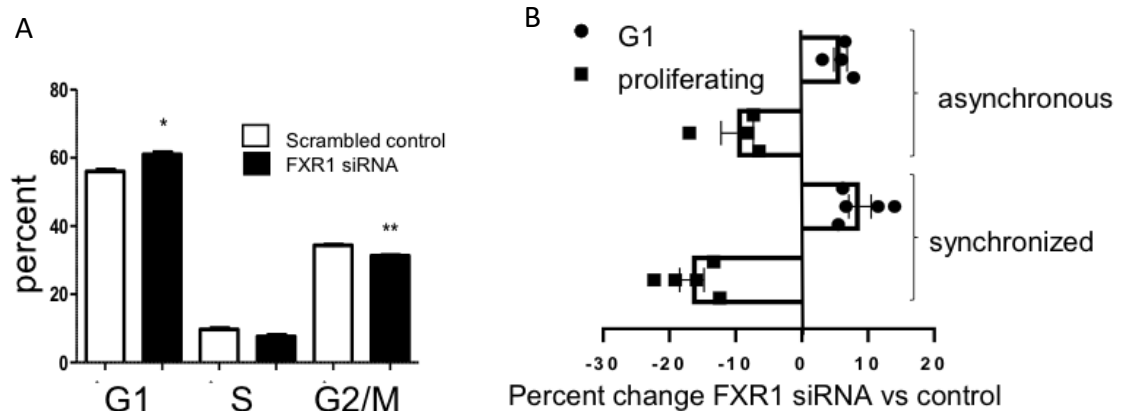


Figure 9. Loss of FXR1 leads to accumulation of cells in G1 phase of the cell cycle. A. Representative cell cycle analysis of asynchronously growing FXR1-depleted and scrambled control hVSMC analyzed by PI staining and flow cytometry. There are significantly more cells in the G1 phase of the cell cycle and significantly fewer cycling cells overall among cells treated with FXR1 siRNA compared with controls. B. Compilation of four asynchronously growing and five synchronized FXR1-depleted and scrambled control hVSMC analyzed by PI staining and flow cytometry, all independent experiments. *=P<0.05, **=P<0.01, ***=P<0.001, for panel A %cells in S phase P=0.067.

The possibility existed that impairment of cell cycle progression may predispose the cells to senescence, and several cellular and molecular approaches were used to test this possibility. First, after transfection with FXR1 siRNA or scrambled control, hVSMC were seeded on chamber slides, and after 72 hours, stained for β -galactosidase activity, a common readout for cellular senescence.⁶³ Figure 10A shows that FXR1 depleted hVSMC had significantly more β -galactosidase positive cells compared with control cells (10.85 +/-1.42 versus 33.17 +/-1.12 positive cells, $P < 0.001$ for scrambled control and FXR1 siRNA, respectively). In a second experiment, FXR1 depleted and control hVSMC were immunostained with phospho γ H2AX, a marker of DNA damage associated with senescence.¹⁰¹ Nuclear punctate staining was significantly increased in FXR1 hVSMC compared with control cells (12.82 +/-2.58 versus 33/70 +/-6.05 cells/HPF, $P < 0.001$, for scrambled versus FXR1 siRNA, respectively), (Figure 10B). When staining hVSMC with alpha smooth muscle actin to visualize the cellular structure, pyknotic nuclei were present in the FXR1 depleted cells (Figure 10C). Though most frequently associated with apoptosis, pyknotic nuclei are also the result of DNA damage and associated with aging.

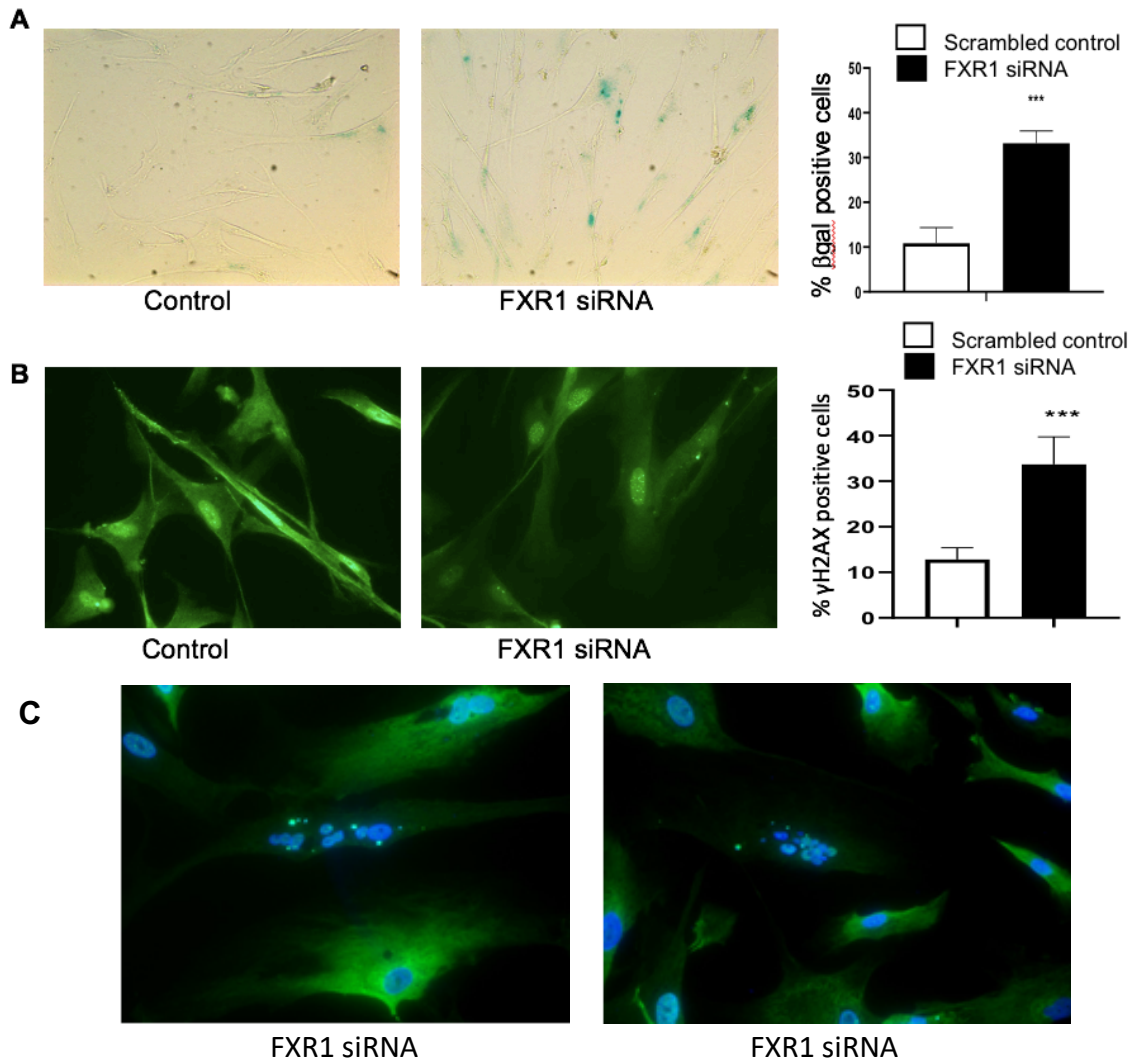


Figure 10. FXR1 knockdown induces senescence. A. FXR1-depleted hVSMC become senescent. hVSMC transfected with FXR1 or control siRNA were seeded on glass chamber slides and stained for β -galactosidase. Percent positive cells were quantitated by counting four HPF from three independent experiments. Pictures taken at 20x magnification. B. FXR1-depleted and scrambled control hVSMC were immunostained with γ H2AX. Punctate nuclear staining indicates positivity. Percent positive nuclei were quantitated from four HPF from three independent experiments. C. Representative images showing pyknotic nuclei in FXR1-depleted hVSMC stained with DAPI and alpha-smooth muscle actin. Pictures taken at 40x magnification. ***= $P < 0.001$

Expression of a number of cytokines and related genes of the senescence-associated secretory phenotype (SASP) is indicative of cellular senescence.¹⁰² hVSMC transfected with FXR1 siRNA or a scrambled control were tested for expression of genes considered to be part of the SASP. Figure 11A indicates that numerous recognized SASP markers, including HMGB1, HMBG2, CXCL11, IL-1 β , IL-6, and MCP1 were all significantly increased in FXR1-depleted hVSMC. Increased p53 expression is an obligate event in senescence and Figure 11B demonstrates that p53 abundance is significantly increased in FXR1-depleted hVSMC, indicative of senescence. Together, these data strongly suggest that depletion of FXR1 results in decreased hVSMC cell proliferation, and may induce a senescent phenotype in these cells.

3.4 Genetic Deletion of FXR1 from VSMC Reduces Neointima Formation.

To test the hypothesis that FXR1 participates in the development of vascular disease, SMC-specific FXR1 conditional knockout mice were generated. Figure 12A shows that tamoxifen injection reduced FXR1 protein abundance in the aorta, and figure 12B is qRT-PCR showing tissue specific and tamoxifen-inducible FXR1 deletion. These mice were subject to carotid artery ligation to induce neointimal hyperplasia, which is primarily a VSMC-driven proliferative disease model.¹⁰³⁻¹⁰⁵ Figures 13A and 13B show that tamoxifen injected FXR1^{SMC/SMC} mice developed significantly less neointimal hyperplasia compared to control mice. There was no significant difference in I/M ratios among the control groups. However, FXR1^{SMC/SMC} mice injected with tamoxifen demonstrated a significantly lower I/M ratio compared with oil-injected and all other controls (0.93 \pm 0.11 vs 0.45 \pm 0.04, P<0.001 for oil and tamoxifen injected, respectively). Together, these data indicate that FXR1 participates in development of vascular restenosis, as absence of FXR1 reduces ligation-induced neointimal hyperplasia.

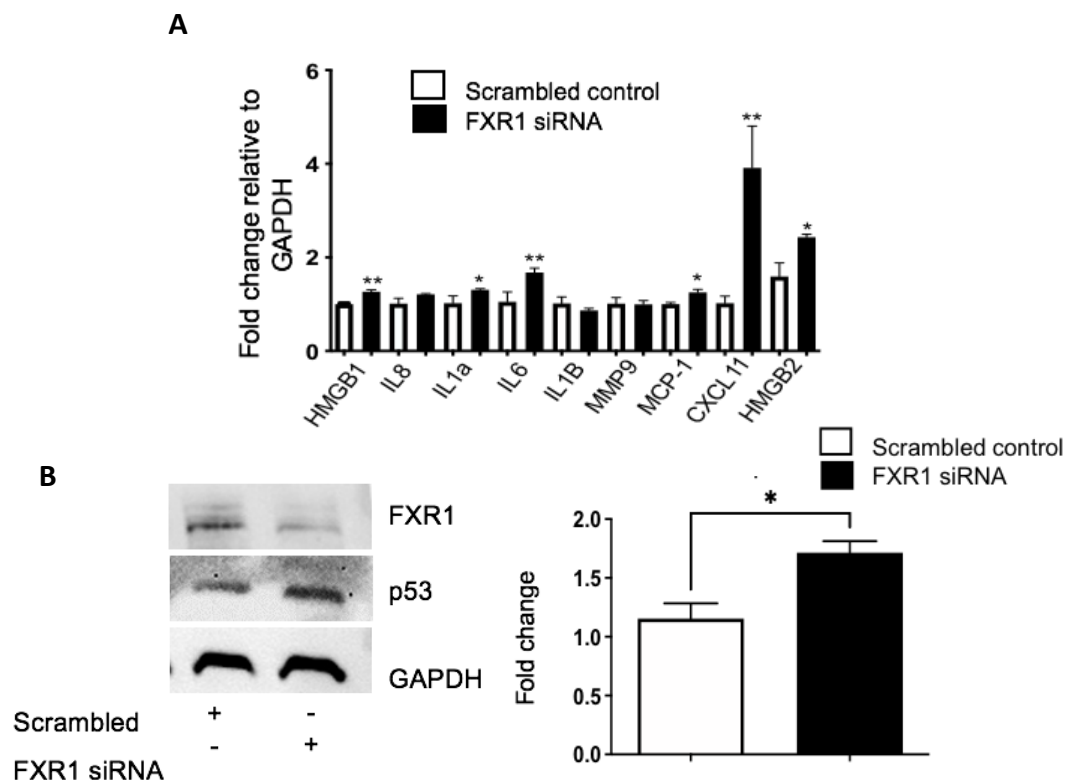


Figure 11. Loss of FXR1 increases senescence-associated gene expression. A. FXR1-depleted hVSMC display significantly increased SASP-associated gene expression compared with control cells. hVSMC transfected with FXR1 or control siRNA were serum-starved, then RNA was isolated, reverse transcribed, and amplified with primers to the genes shown. B. Representative western blot showing that there is significantly increased expression of p53 following knockdown of FXR1 in hVSMC. *=P<0.05, **=P<0.01, ***=P<0.001 from at least three experiments.

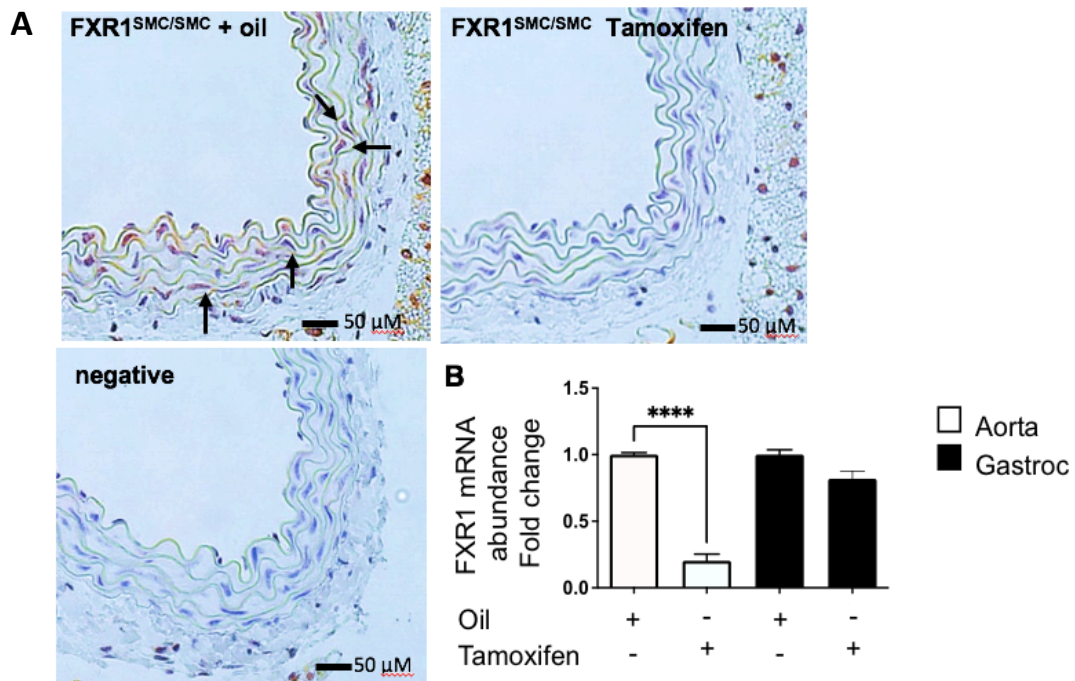


Figure 12. Validation of SMC-specific FXR1 knockout. A. VSMC specific FXR1 knockout express reduced FXR1 in smooth muscle cells. Immunohistochemical staining of mouse aorta with FXR1 specific antibody (brown stain) demonstrates absence of immunoreactivity in media of aorta in tamoxifen-injected mice. B. Quantitative RT-PCR from RNA isolated from various muscle tissue shows reduced expression of FXR1 in aorta in tamoxifen-injected mice (****= $P < 0.0001$), but not in oil-injected mice. There is no reduction of FXR1 in skeletal muscle (gastrocnemius).

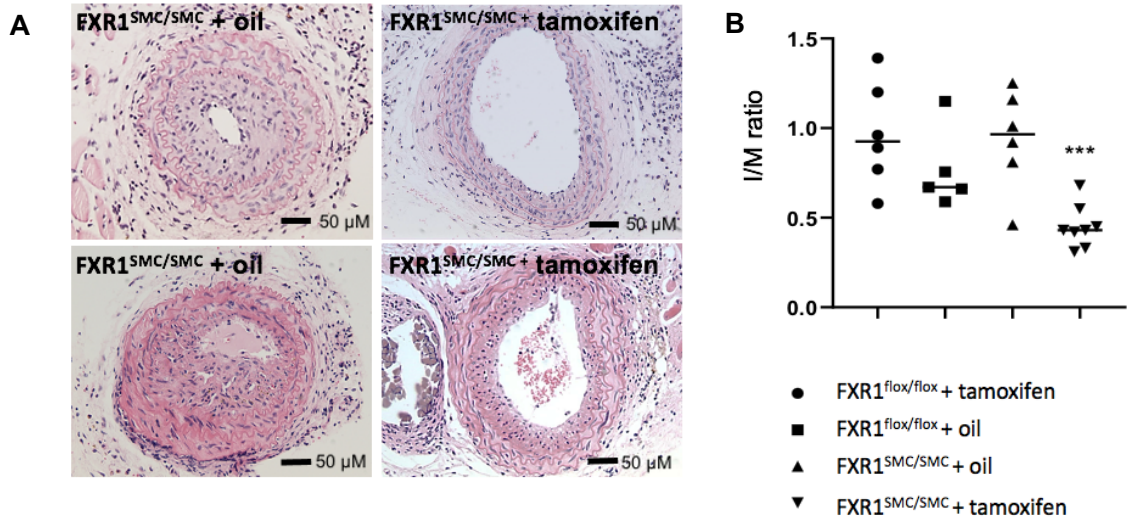


Figure 13. FXR1 knockout reduces neointima formation in a mouse model of restenosis. A. Representative cross-sections carotid arteries from oil and tamoxifen-injected mice 28 days post ligation stained with Hematoxylin-eosin showing decreased neointimal formation. B. quantitation of neointimal formation from oil and tamoxifen injected FXR1^{SMC/SMC} mice, as well as control mice injected with oil and tamoxifen. FXR1^{SMC/SMC} mice injected with tamoxifen have significantly less neointimal formation compared with all other control mice. ***= P<0.001 compared with oil injected cKO mice.

It was important to provide mechanistic insight to support the effects of FXR1 depletion on reduction of neointimal hyperplasia. Initially, we attempted to isolate and culture VSMC from tamoxifen-injected FXR1^{SMC/SMC} mice, normally a routine procedure, but the mouse VSMC (mVSMC) could not be passaged past their initial isolation to generate cell numbers that were sufficient to perform appropriate subsequent studies. Considering that senescence was induced in hVSMC depleted of FXR1 by siRNA, together with our inability to propagate aortic VSMC from tamoxifen injected FXR1^{SMC/SMC} mice, experiments were performed to test the possibility that VSMC isolated from these mice were senescent as well. One experiment that required a very limited number of VSMC was to quantitate β -galactosidase activity in mVSMC isolated from FXR1^{SMC/SMC} mice injected with tamoxifen and oil-injected control mice. Figure

14A shows that VSMC isolated from tamoxifen-injected mice had significantly more β -galactosidase activity compared with controls (1.94 \pm 1.23 versus 14.05 \pm 2.29 cells/HPF, $P < 0.01$ for FXR1^{flox/flox} and FXR1^{SMC/SMC} + tamoxifen, respectively).

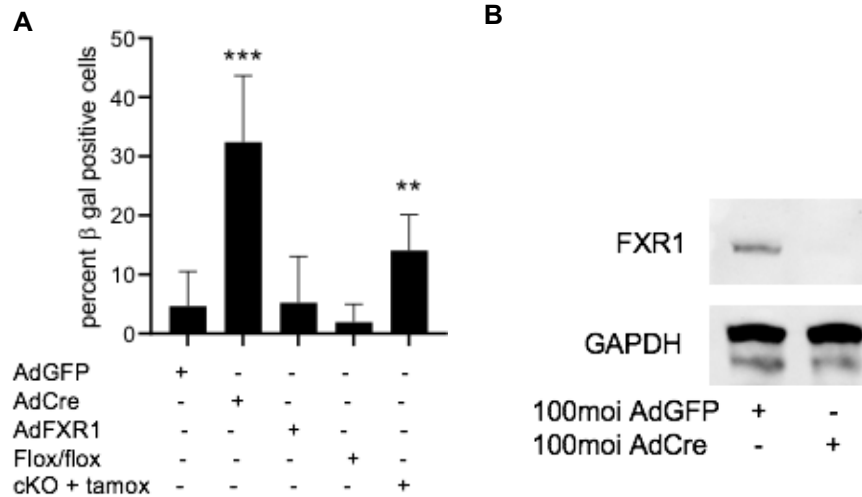


Figure 14. mVSMC depleted of FXR1 are senescent and have increased expression of β -galactosidase. A. mVSMC isolated from oil or tamoxifen-injected FXR1^{SMC/SMC} mice, and mVSMC isolated from FXR1^{fl/fl} mice transduced with AdGFP or AdCre were seeded on glass chamber slides, and stained for β -galactosidase activity. B. Protein extracts from mVSMC isolated from FXR1^{SMC/SMC} mice transduced with AdGFP or AdCre show reduction of FXR1 expression. **= $P < 0.01$, ***= $P < 0.001$.

In a second approach to garner additional cells to test this hypothesis, β -galactosidase activity was compared in VSMC isolated from un-injected FXR1^{SMC/SMC} mice transduced with AdCre, AdGFP or AdFXR1 viruses. Figure 14B shows that AdCre was effective at reducing FXR1 protein and mRNA expression. Figure 14A also shows that FXR1^{SMC/SMC} VSMC transduced with AdCre had significantly more β -galactosidase activity compared with AdGFP and AdFXR1 control cells (32.41 \pm 4.25 versus 4.64 \pm 2.22 cells/HPF, $P < 0.001$ for AdCre and AdGFP, respectively). In an attempt to

recapitulate our hVSMC phenotype, we subjected AdCre transduced mVSMC to proliferation assays and saw decreased proliferation with knockout of FXR1 (Figure 15).

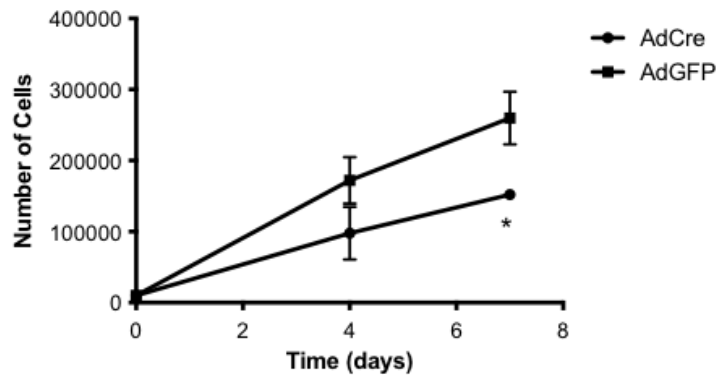


Figure 15. mVSMC explanted from FXR1^{SMC/SMC} mice and transduced with AdCre are less proliferative than controls. AdCre-transduced cells proliferate more slowly than AdGFP transduced control mVSMC. *p<0.05.

AdCre-transduced FXR1^{SMC/SMC} VSMC were then used to determine if decreased abundance of the cell division genes identified in RNA-sequencing of hVSMC were also decreased in AdCre-treated FXR1^{SMC/SMC} mVSMC. Figure 16A shows that expression of CDK1, CDKN3, and MKI67 were significantly decreased in these cells, which is what was observed in hVSMC. It was next determined that expression of senescence-associated genes p16, p21, and p53 were significantly increased in AdCre-transduced mVSMC (Figure 16B), supporting the β -galactosidase and γ H2AX results. Importantly, mRNA stability of these senescence-associated transcripts was not increased with FXR1 deletion, indicating that the increase in mRNA abundance was due to increased

senescence, not FXR1-mediated increase in mRNA stability (Figure 16C). The increase in multiple readouts of senescence was consistent with what was observed in human VSMC depleted of FXR1 by siRNA, and when taken together indicates that VSMC depleted of FXR1 assume a senescent phenotype.

3.5 Deletion of FXR1 Induces VSMC Senescence in Vivo.

Experiments were then done to test the hypothesis that the reduced neointimal hyperplasia observed in the ligated carotid arteries would be due to induction of senescence. RNA was isolated from ligated and unligated carotid arteries from tamoxifen and oil-injected FXR1^{SMC/SMC} mice and expression of cell division-related genes quantitated by qRT-PCR. Figure 17 shows that similar to cultured human and murine VSMC depleted of FXR1 in vitro, abundance of BUB1 and CDK1 mRNA was significantly reduced in ligated carotid arteries from tamoxifen-injected mice compared with control mice.

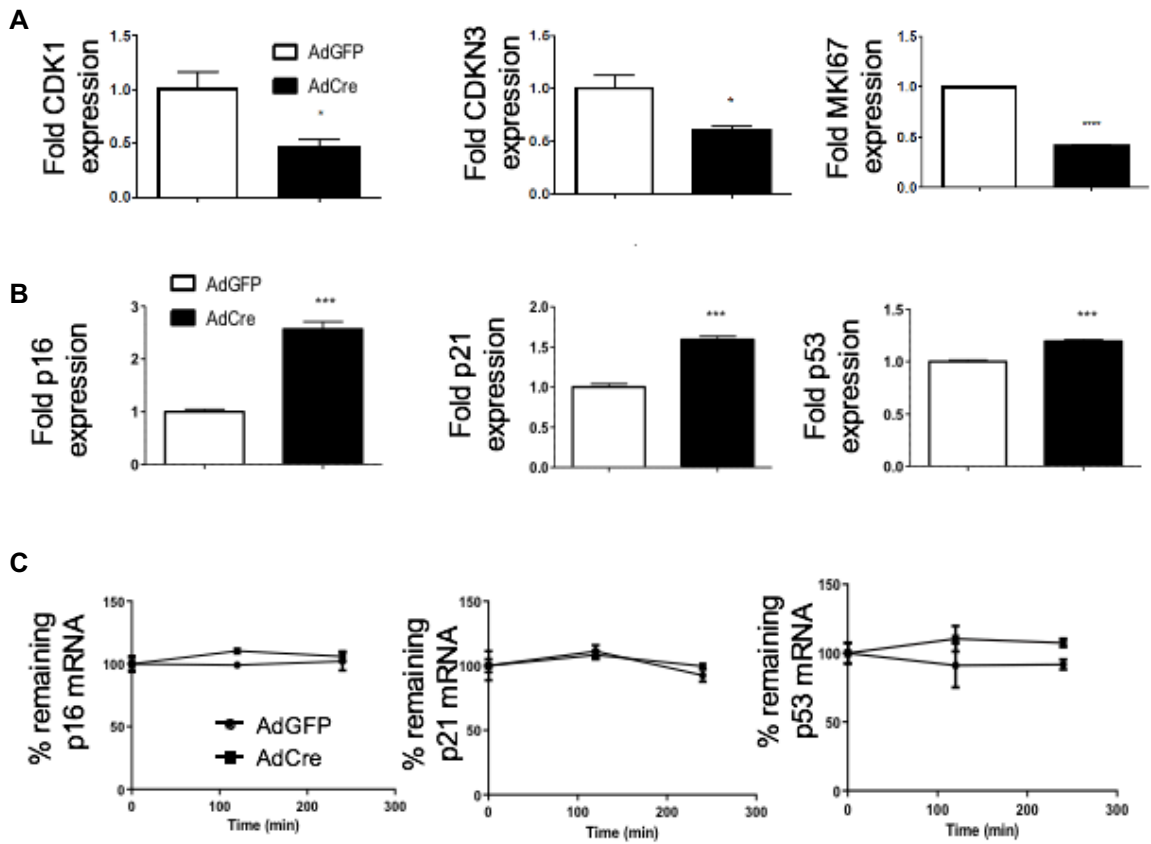


Figure 16. mVSMC depleted of FXR1 have reduced expression of cell cycle-associated genes. A. Abundance of cell division-associated genes identified by RNA-sequencing in FXR1 depleted hVSMC are also significantly decreased in AdCre-transduced FXR1^{SMC/SMC} mVSMC detected by qRT-PCR. B. Abundance of senescence-associated genes are significantly increased in AdCre-transduced FXR1^{SMC/SMC} mVSMC detected by qRT-PCR. C. Senescence-associated genes do not have reduced mRNA stability in FXR1-depleted mVSMC. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001.

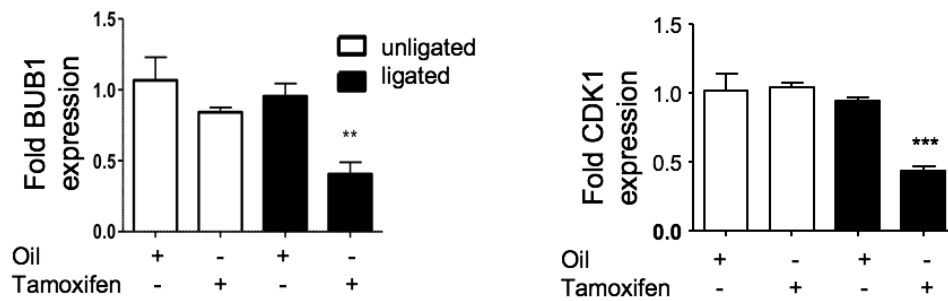


Figure 17. Decreased cell division-associated gene expression in ligated carotid arteries from $\text{FXR1}^{\text{SMC/SMC}}$ mice. Carotid arteries from tamoxifen and oil-injected mice were ligated as described in the methods section. Twenty eight days later, total RNA was extracted, and quantitative RT-PCR of select cell-division associated genes identified by RNAseq in FXR1 depleted hVSMC were identified as significantly decreased. **= $P < 0.01$, ***= $P < 0.001$.

Expression of senescence-associated genes were also increased in these arteries.

Figure 18A shows that p21, p53, and HMGB1 mRNA expression are significantly increased in carotid arteries from tamoxifen-injected mice compared with control mice. Importantly, p53 protein expression, an obligate event in p21 expression and cellular senescence, is significantly increased in FXR1 -depleted carotid arteries (Figure 18B), further validating a senescent state of aortic VSMC. Together, this suggests that at least one mechanism whereby $\text{FXR1}^{\text{SMC/SMC}}$ mice have reduced neointimal formation is due to reduced expression of cell cycle and proliferative transcripts as well as increased senescence-associated transcripts in arterial VSMC.

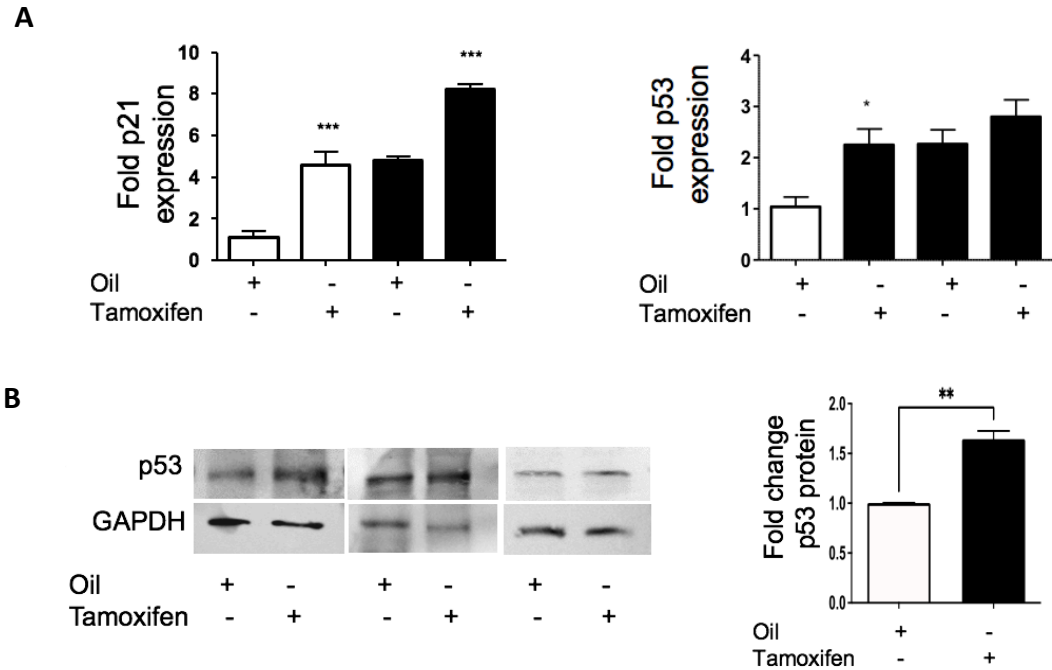


Figure 18. Increased expression of senescence-associated genes in tissue from FXR1^{SMC/SMC} mice. A. Abundance of senescence-associated genes are significantly increased in tamoxifen-injected mice compared with oil-injected control mice detected by qRT-PCR. B. p53 protein is increased in carotid arteries in tamoxifen injected mice detected by western blot, indicating senescence. Western blot shown is from three pairs of mice, and densitometry from these blots *=P<0.05, **=P<0.01, ***=P<0.001.

3.6 FXR1 Preferentially Interacts with Highly Methylated mRNA.

We next were interested in FXR1's potential role as an m6a reader. RNA-immunoprecipitations were performed using a flag-tagged FXR1 adenovirus and we observed that FXR1 tends to bind to highly methylated transcripts such as suppressor of cytokine signaling 2 (SOCS2) and inhibitor of growth family 1 (ING1) (figure 19A), in comparison with under-methylated transcripts such as Transmembrane protein 38B (TMEM38B) and Zinc finger protein 706 (ZNF706) (figure 19B). Further, ING1 protein expression is induced by stimulation with PDGF (figure 19C), similarly to FXR1.

WTAP, a member of the MTC, is necessary for nuclearization of the MTC, an obligate event in the m6a methylation of transcripts. We also found through mass spectrometry¹⁰⁶ that WTAP interacts with FXR1. We used WTAP siRNA (figure 20A) in order to determine how knockdown of this gene would affect both methylated and unmodified transcripts in hVSMCs. SOCS2 and ING1 were both significantly upregulated following WTAP knockdown (Figure 20B), as were BUB1, CDKN3, and FXR1 (figure 20C). It remains to be seen how WTAP knockdown affects FXR1's ability to bind methylated transcripts, but these results could be interpreted to mean that WTAP knockdown leads to decreased methylation, and therefore longer transcript longevity of normally less unstable, highly methylated transcripts.

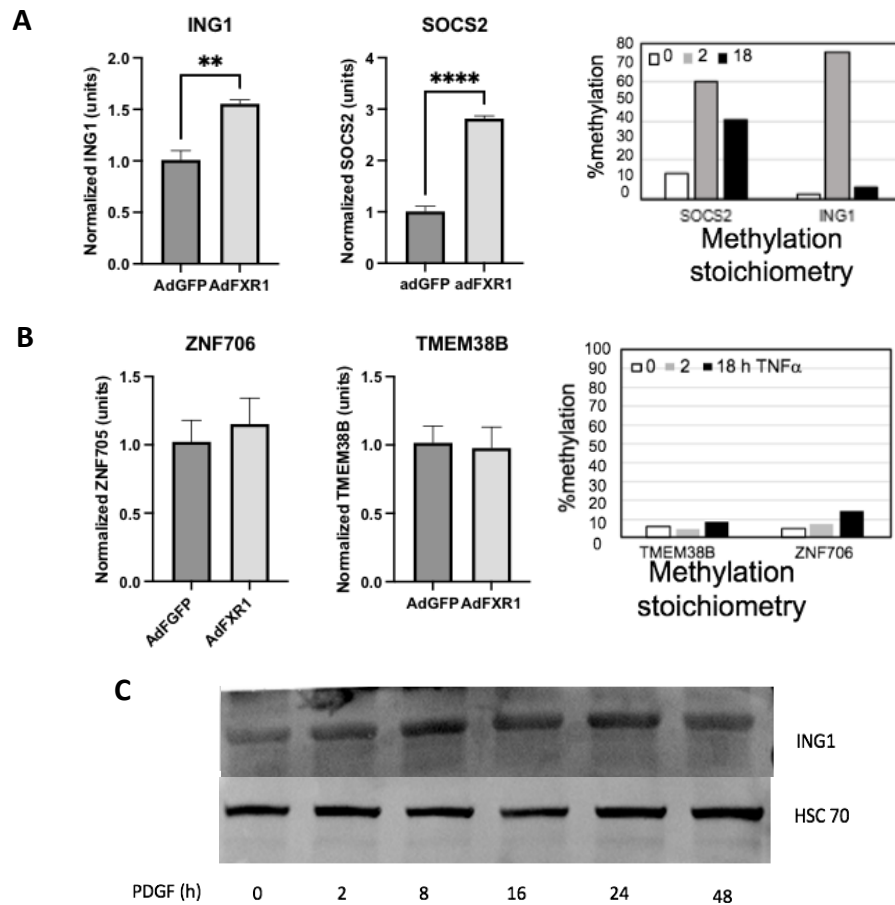


Figure 19. FXR1 binds to methylated mRNA transcripts. A. RIP-rt-pcr showing that FXR1 binds to highly methylated transcripts as evidenced by high %methylation stoichiometry from an m6a epitranscriptomic array. B. FXR1 does not bind to undermethylated transcripts, as evidenced by low %methylation stoichiometry from an m6a epitranscriptomic array. C. ING1 protein expression is induced by PDGF. **= $P < 0.01$, ***= $P < 0.001$.

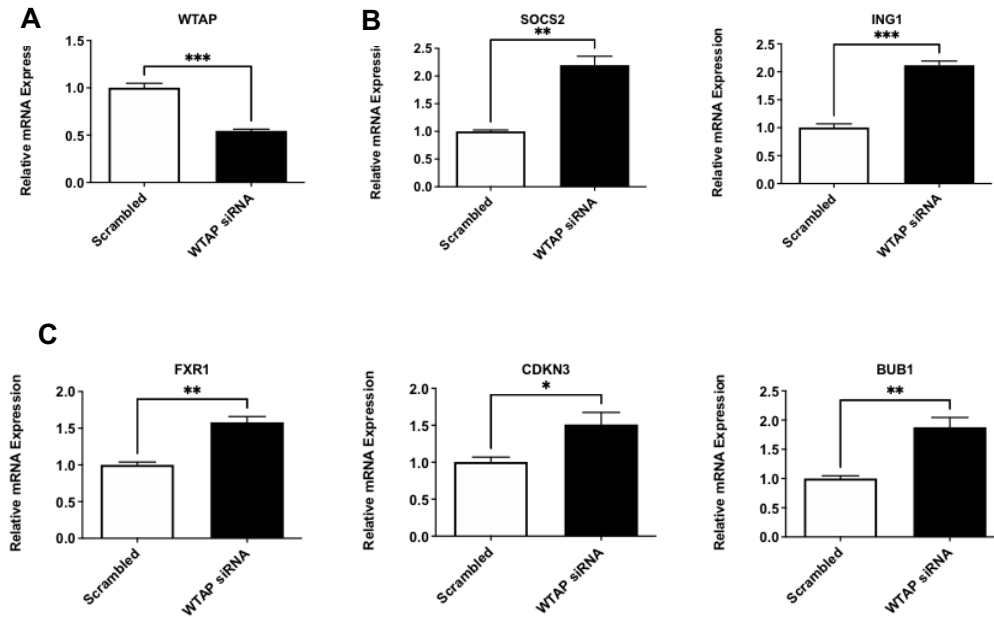


Figure 20. WTAP siRNA increases expression of highly methylated transcripts. A. SMARTpool WTAP siRNA effectively knocks down WTAP expression 72 hours post-transfection. B. mRNA abundance of SOCS2 and ING1 is increased in absence of WTAP. C. mRNA abundance of FXR1 and cell cycle control genes CDKN3 and BUB1 is increased in absence of WTAP. *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$

3.7 FXR1 Functional Domain Studies.

In an attempt to determine which functional domains of the FXR1 protein are responsible for its association with target transcripts and cellular behavior such as proliferation, mRNA stability, and migration, we had plasmids made with mutant versions of the FXR1 protein. These 5 flag-tagged plasmids contained amino acid substitution mutations of the functional domains of FXR1, rendering the domain null. Mutants were made to M1) KH1 and KH2 domains, M2) KH1 domain only, M3) KH2 domain only, M4) nuclear export signal, and M5) RGG box. Mutants were once successfully transfected into NIH 3T3 cells (figure 21), however when transfecting human and mouse VSMC as well as HEK cells, plasmids were not expressed, and we were unable to carry out the intended assays. We are in the process of having these

mutant plasmids made into adenoviruses so that we may be more easily able to express them and carry out the intended experiments.

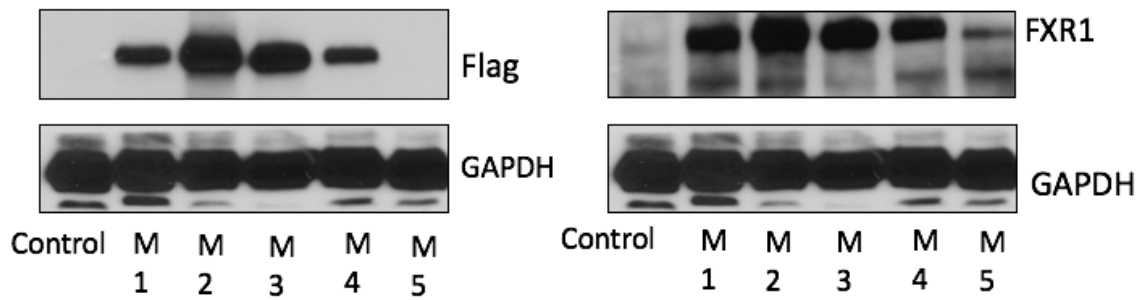


Figure 21. Expression of FXR1 mutant plasmids in NIH 3T3 cells. Cell extracts were western blotted for flag and corresponding FXR1 expression. Mutant 5 did not express, possibly due to the mutation being out of the reading frame, as FXR1 expression appears similar to control levels.

CHAPTER 4

DISCUSSION

In the present study we tested our over-arching hypothesis that FXR1, a muscle enhanced protein, participates in the VSMC response to vascular injury. Initially, it was determined that FXR1 mRNA and protein abundance are increased when primary hVSMC are challenged with TNF α , suggesting that FXR1 might participate in regulation of inflammation-responsive transcripts. RNA-sequencing in hVSMC depleted of FXR1 revealed a particularly large number of transcripts which were upregulated, consistent with our understanding of FXR1 as an mRNA stability RBP. However, intriguingly, a smaller number of transcripts appeared to be decreased in the absence of FXR1, which was unexpected. Importantly, in TNF α -stimulated VSMC, the overwhelming majority (29 of 32) of these transcripts were associated with regulation of cell division, including cell cycle progression, mitotic checkpoints, and chromosome assembly during mitosis.

BUB1 is a serine/threonine protein kinase which phosphorylates mitotic checkpoint complex proteins ensuring correct chromosome alignment¹⁰⁷, and it is required for ensuring proper chromosome segregation.¹⁰⁸ CDK1 is a serine/threonine protein kinase and a component of the M-phase promoting complex, which is essential for G1/S and G2/M phase transitions.^{109, 110} CDK1 also modulates association and phosphorylation of multiple cyclins which regulate S-phase and mitotic cyclins. CDKN3 is an inhibitor of cyclin-dependent kinases and is over expressed in many different cancers.^{111, 112} MKI67 is a marker for cellular proliferation and is necessary component for cell division as it associates with and is required to maintain mitotic chromosome separation during cell division.¹¹³ Knockdown of FXR1 in VSMC in the presence of the transcription inhibitor actinomycin D resulted in significantly decreased mRNA stability

of many of these transcripts, which suggested that their decreased mRNA abundance was likely due to reduced mRNA stability, counter to current thinking that FXR1 is an mRNA de-stability factor. FXR1 is noted for its ability to destabilize mRNA, most notably TNF α mRNA^{43, 114, 115}, but there are few reports of FXR1 acting to stabilize mRNA. One report in a prostate cancer cell line does suggest that FXR1 participated in, but was not solely responsible for stabilization of desmoplakin mRNA in a prostate cancer cell line.¹¹⁶ Overexpression of FXR1 in neonatal rat myocytes increased Cx45 mRNA levels, but neither mRNA stability nor effect of FXR1 knock down on Cx45 expression was examined.¹¹⁷

A recent publication from the Autieri lab identified FXR1 as binding to and stabilizing another subset of transcripts involved in cytoskeletal dynamics. FXR1 binds to transcripts involved in adhesion, contraction, and regulation of the cytoskeleton, and in absence of FXR1 these transcripts are decreased.¹⁰⁶ The study also showed that in hVSMC transfected with FXR1 siRNA to knock down the protein, the hVSMC have impaired contraction, migration, and actin polymerization. These findings, along with the findings in the present thesis, are some of the first reports of FXR1 stabilizing mRNA transcripts in primary cells.

The decrease in cell-division associated proteins provided impetus to determine if depletion of FXR1 would decrease TNF α -driven cellular proliferation. Knockdown of FXR1 in primary human VSMC led to decreased proliferation of these cells. Reduction of FXR1 in other cell systems has also been seen to influence cell proliferation. In a recent study, knockdown of FXR1 reduced keloid-derived proliferation, but these authors concluded this was due to induction of apoptosis in these cells.¹¹⁸ Another study determined that deletion of FXR1 in neural stem cells results in decreased generation of

new neurons, but mechanisms were not discussed.¹¹⁷ We observed in our hVSMC transfected with FXR1 siRNA the presence of pyknotic nuclei, which was not seen in the scrambled control. Pyknosis is a condensation of chromatin in the nucleus of cells undergoing apoptosis or necrosis. For our part, we saw no differences in abundance of transcripts associated with apoptotic or necrotic processes in our RNA sequencing or our RIP-sequencing results, but pyknosis is also associated with aging and DNA damage.^{119,}

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Cellular senescence is stable, irreversible cell cycle arrest and is closely associated with a decrease in proliferation.¹²¹ There are no reports in the literature associating FXR1 expression with senescence in primary cells. The present study found that two indices of senescence, increased β -galactosidase staining and γ H2AX positive nuclei were increased in primary hVSMC depleted of FXR1. This is consistent with one other report linking FXR1 with senescence in a murine cancer cell line¹²², and differs from that work in that we utilized primary human VSMC.

Senescent cells exhibit phenotypic changes called the senescence associated secretory phenotype (SASP) characteristic of a senescent cell, which includes increased secretion of inflammatory cytokines, growth factors, and chemokines which all lead to increased inflammation in the tissue microenvironment.⁷⁴ Many of these soluble factors contribute to vascular occlusive diseases in part because senescent cells are able to avoid clearance by senescent cell anti-apoptotic pathways (SCAPs).¹²³ They remain in the artery, viable and metabolically active, and secrete these factors which hinder tissue repair. With this in mind, we investigated the expression of transcripts to be associated with the senescent phenotype. Six of nine genes examined were significantly increased in siRNA treated hVSMC, indicating that depletion of FXR1 from primary human

VSMC initiates SASP expression. While SASP expression validates that hVSMC depleted of FXR1 are senescent, it cannot determine if these soluble factors are contributing to VSMC senescence *in vivo*.

Proliferation of intimal VSMC is a major cellular event in development of vascular restenosis and atherosclerosis.^{124, 125} VSMC senescence and death are hallmarks of advanced atherosclerotic lesions, and recent evidence supports a direct causative role of smooth muscle cell senescence in atherosclerotic plaque progression to the rupture-prone necrotic stage.¹²⁵ SASP factors drive immune cell chemotaxis and senescent VSMCs have significantly reduced collagen production, both of which make atherosclerotic plaques less stable.⁶⁸ We have previously shown that FXR1 expression is increased in injured arteries and acted as a de-stability factor for inflammatory mRNA, but direct causality for FXR1 expression in vascular disease needed to be established. Expression of FXR1 is muscle-enhanced, and FXR1 has been referred to as the muscle-specific FMR1 family member, suggesting a specialized function in muscle tissue.^{56, 114} Total knock out of FXR1 is postnatally lethal⁵⁶, displaying a striated muscle phenotype, providing rationale for our generation of SMC-specific conditional knock out mice. FXR1^{SMC/SMC} mice subject to carotid artery ligation demonstrated significantly reduced neointimal formation compared to controls, suggesting a central role for this RBP in VSMC pathophysiological processes.

To characterize the molecular mechanisms of reduced neointimal formation observed in the FXR1^{SMC/SMC} mice, we initially attempted to explant and culture aortic VSMC from FXR1 knockout mice. Although a routine procedure, we were surprised at being unable to culture these cells past the initial isolation, which prevented us from obtaining numbers sufficient for characterizing and validating our findings that primary

hVSMC depleted of FXR1 became senescent. Therefore, we turned to an alternative approach and transduced mVSMC cultured from FXR1^{SMC/SMC} mice with AdCre, and were successful at knocking out FXR1 expression. Interestingly, this approach was utilized by other investigators who were unable to grow freshly isolated neural cells from FXR1 knock out mice.¹¹⁷ Importantly, these mVSMC phenocopied hVSMC depleted of FXR1 by siRNA and showed decreased proliferation and increased indices of senescence, including β -galactosidase staining and increased expression of proteins associated with the senescent phenotype. These mVSMC also showed decreased expression of some of the same cell cycle-associated proteins that were destabilized in hVSMC.

We hypothesized that genetic deletion of FXR1 from VSMC results in significantly reduced neointima formation by decreased abundance of cell cycle proteins and induction of cellular senescence. Indeed, carotid arteries from FXR1^{SMC/SMC} mice injected with tamoxifen demonstrated significantly less expression of cell cycle-related genes initially identified in RNA-sequencing from hVSMC. Importantly, expression of p21 and p53, recognized markers of senescence, was significantly increased in FXR1^{SMC/SMC} arteries. Their increased expression is likely because of the senescent phenotype of the aortic VSMC, and not because FXR1 modulates their mRNA stability. Notably, these differences hold true in both ligated and unligated arteries, showing that not only is there a decrease in cell cycle control genes and an increase in senescence-associated genes during neointima formation, but that this difference exists at baseline in the knockout mice. This strongly suggests that one likely reason that deletion of FXR1 results in decreased neointima formation is through induction of senescence in medial VSMC in ligated carotid arteries. FXR1 has been reported to regulate p21 mRNA stability, affecting cancer cell line senescence.^{62, 126, 127} In our hands, however, we did

not observe any decrease in p21 mRNA stability, which may reflect cell type-dependent differences or distinctions between primary cells and transformed cell lines.

Further investigating our VSMC phenotype we looked at post-transcriptional regulation of mRNA transcripts. M6a methylated transcripts are the most common type of modified transcripts, with their mRNA being generally less stable than unmodified mRNA. FXR1 has been hypothesized to be an m6a reader⁸⁴ that recognizes m6a modified transcripts and binds to them to influence transcript fate. RNA-immunoprecipitation revealed that FXR1 does preferentially bind to highly methylated transcripts compared with under-methylated transcripts, which is consistent with this hypothesis. Recent studies have identified m6a methylation as being involved in the progression and maintenance of senescence. METTL3/METTL14 methylate the 3'UTR of p21 mRNA, and they work cooperatively with another methyltransferase, NOP2/Sun RNA methyltransferase 2 (NSUN2), to enhance p21 expression.⁸⁰ METTL3/METTL14 are also involved in SASP expression independently from m6a methylation.

Using an siRNA to knock down WTAP expression in the hVSMC, we observed an increase in highly methylated transcripts SOCS2 and ING1, and we also saw an increase in FXR1, BUB1, and CDKN3 expression. As transcripts with m6a modifications are typically less stable than unmethylated transcripts, it is possible that when the MTC is unable to localize to the nucleus and catalyze the m6a methylation, the abundance of those normally methylated transcripts such as SOCS2 and ING1 increases. There is also evidence that WTAP is anti-proliferative in VSMC⁷⁹, and an increase in BUB1 and CDKN3 in absence of WTAP corroborates this narrative. Furthermore, FXR1 has been identified as an oncogene, and its upregulation in absence of WTAP could be driving cell proliferation.

There are limitations in this work which future studies could address. The precise molecular mechanism(s) of how FXR1 regulates mRNA stability of cell division-related transcripts needs to be elucidated. It would also be informative to determine the percent of senescent VSMC in FXR1^{SMC/SMC} aorta, and the functionality of such aorta in which FXR1 was depleted. While likely that depletion of FXR1 results in decreased abundance of cell division proteins and decreased hVSMC cell proliferation resulting in a senescent phenotype in these cells, we cannot determine a direct effect of SASP factors in modulation and maintenance of this senescent phenotype. It also remains to be seen which domains of the FXR1 protein are responsible and essential to each aspect of its effects on cell behavior. If we could stably express our mutant plasmids in any cell type, we could carry out cell behavior assays including proliferation, migration, and assessing mRNA stability to determine this. Furthermore, determining the expression of FXR1 in aged and senescence-accelerated tissue could aid in our associating loss of FXR1 with a senescent phenotype.

In future studies, we aim to look more into the association of FXR1 with m6a methylation, and how this may regulate proliferation, control of the cell cycle, and cytoskeletal organization. Using WTAP and METTL3 siRNA, we will determine if FXR1's binding ability is impaired through RNA-immunoprecipitation. As previously stated, these proteins are core subunits of the MTC and without them m6a modifications cannot be effectively completed. We anticipate that if FXR1 binds preferentially to highly methylated transcripts, its binding to these transcripts such as ING1 and SOCS2 will be impaired with knockdown of WTAP or METTL3, as transcript methylation would be unable to proceed to its normal extent. Additionally, if FXR1 recognizes mRNA with m6a modifications, many of which are inflammatory mRNAs¹²⁸, and targets them for

degradation, overexpression of FXR1 may no longer reduce inflammatory mediator abundance as we have previously seen.⁴³

We are also particularly interested in the relationship of this work to atherosclerosis and would like to see whether SMC-specific knockout of FXR1 affects atherosclerosis in our mouse model. There is significant evidence supporting the role of senescent cells in contributing to an atherogenic environment and plaque progression, as well as accumulating in atherosclerotic plaques and contributing to rupture.¹²⁵ Given the state of research in the field, we hypothesize that smooth muscle knockout of FXR1 will increase atherosclerotic plaque size in a mouse model injected with a PCSK9 adenovirus and fed a high fat diet for 16 weeks. In addition to their non-proliferative capacity, senescent cells do not get cleared the way that dead cells do, and express “do not eat me” signals to avoid detection by the innate immune system.¹²⁵ Instead, they accumulate and secrete SASP factors which exacerbates the inflammatory environment and thus can lead to plaque progression and rupture.

In summary, this work shows that FXR1, a muscle-enhanced RBP recognized as a de-stabilizing RPB, stabilizes a select group of mRNA transcripts associated with control of the cell cycle. Depletion of FXR1 from VSMC reduces their proliferation and induces senescence, as evidenced by an increase in β -galactosidase and γ H2AX staining, upregulation of multiple transcripts considered to be part of the SASP, and an increase in mRNA and protein expression of canonical senescence genes p21 and p53. This induction of senescence is likely a mechanism for reduced neointimal formation observed in ligated carotid arteries from FXR1^{SMC/SMC} mice (figure 22).

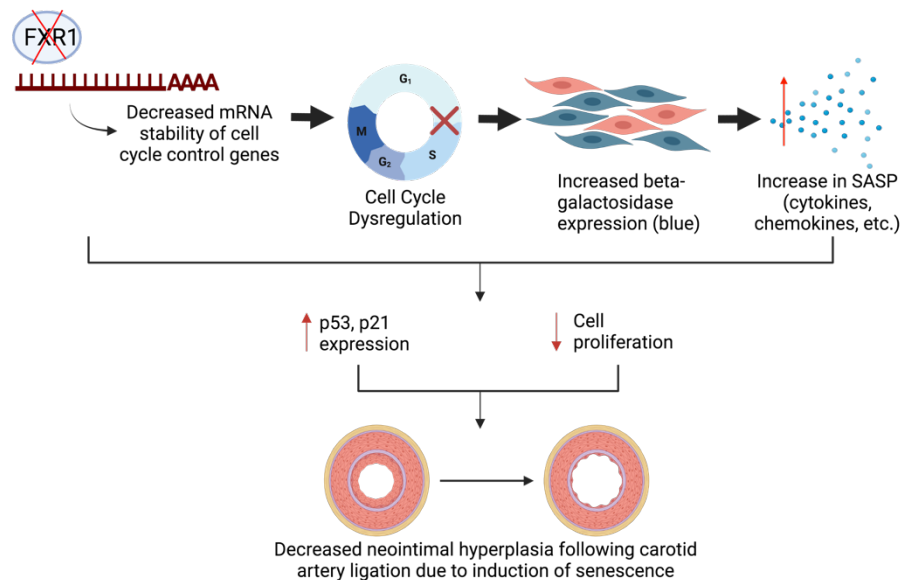


Figure 22. Mechanism of FXR1 knockout-induced cellular senescence. Knockout of FXR1 results in destabilization of cell cycle control genes, leading to dysregulation of the cell cycle and the induction of cellular senescence, resulting in reduced neointima formation following carotid artery ligation.

As an RBP, FXR1 preferentially binds to methylated transcripts and it remains to be seen exactly how this may play into the VSMC senescence response. In beginning to investigate FXR1's role as an m6a reader, we have seen that in absence of WTAP, some transcripts associated with cell cycle progression as well as FXR1 are increased. We also saw an increase in transcript abundance of SOCS2 and ING1, normally highly methylated. More studies need to be done to elucidate the precise interactions of FXR1 with m6a-modified mRNA and how this could play into the progression of vascular disease, but these data suggest that FXR1 expression and/or RNA binding activity would make an attractive target for modalities to combat vascular proliferative diseases such as atherosclerosis and restenosis.

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