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ANGIOTENSIN II REGULATION OF EXTRACELLULAR MATRIX PROTEINS: LYSYL OXIDASE AND OSTEOPONTIN

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by

Ebony Washington Remus

B.S., Haverford College, 2004

Advisor: W. Robert Taylor, MD, Ph.D.

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Abstract

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The renin angiotensin system has been implicated in the pathogenesis of cardiovascular disease through its primary effector molecule Angiotensin II (Ang II). Ang II is multifunctional hormone that acts as a potent vasoconstrictor, mediating its action on cells within the vasculature primarily through the angiotensin I receptor. Vascular smooth muscle cells (VSMCs) are a prominent cell type within the vasculature on which Ang II exerts its effects. Ang II bound to its receptor initiates intracellular signaling events that lead to hypertrophy, hyperplasia, migration, adhesion and an increased oxidative state of vascular smooth muscle cells. These changes are mediated in part by Ang II induced changes to the extracellular matrix environment, leading to the progression of atherosclerosis and abdominal aortic aneurysms. Within this thesis, we will explore Ang II regulation on two important extracellular matrix components implicated in vessel stability: lysyl oxidase and osteopontin. The lysyl oxidase family of extracellular matrix enzymes crosslink collagen and elastin, thereby enhancing vessel wall stability. Osteopontin is an extracellular matrix protein that facilitates adhesion, migration and inflammation in vascular smooth muscle cells. While lysyl oxidase contributes to the stability of the vessel wall, osteopontin represents an extracellular matrix protein that decreases vessel integrity. This thesis aims to understand the regulation of these extracellular matrix proteins using both *in vivo* disease models and primary cell culture. The results from these experiments support the notion that lysyl oxidase stabilizes vessel structure in a mouse model of abdominal aortic aneurysms; however Ang II does not directly regulate its activity or expression in vascular smooth muscle cells. Conversely, Ang II does increase osteopontin expression in a mouse model of hypertension that precedes atherosclerosis formation. Furthermore, by uncovering a previously unreported translation dependent induction of osteopontin protein expression by Ang II in VSMCs, we were able to identify a novel mechanism for inhibiting Ang II induced osteopontin expression using microRNAs.

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Abbreviations

3'UTR	3' untranslated region
AAA	Abdominal Aortic Anuersyms
ACE	Angiotensin converting enzyme
Ang II	Angiotesnin II
AP-1	Activator protein 1
AT1	Angiotensin type 1 receptor
BMP-1	Bone morphorgenic protein
Ca2+	Calcium
CRL	Cytokine receptor-like domain
DAG	Diacylglycerol
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ERK1/2	Extracellular-signal Regulated Kinase
ET-1	Endothelian 1
FAK	Focal adhesion kinase
GPCR	G-protein coupled receptor
H2O2	Hydrogen peroxide
H-caldesmon	Heavy-caldesmon
ICAM	Intracellular adhesion molecule
IP3	Inositol triphosphate
KOs	Knockout
LDL	Low density lipoproteins
LIMK	LIM kinase
LOX/LOXL	Lysyl Oxidase and Lysyl Oxidase Like
LOXL1	Lysyl Oxidase Like 1
LOXL2	Lysyl Oxidase Like 2
LOXL3	Lysyl Oxidase Like 3
LOXL4	Lysyl Oxidase Like 4
LTQ	Lysine tyrosylquinone cofactor
MCP-1	Monocyte Chemotactic Protein 1
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix Metalloproteinase
MMP-2	Matrix Metalloproteinase 2
MMP-9	Matrix Metalloproteinase 9
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NFκB	Nuclear factor kappa B

O_2^-	Superoxide
OPN	Osteopontin
PAI-1	Platelet activator inhibitor
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PIP2	Phophatidyl inositol 4,5 bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phopholipase C
RAS	Renin-angiotensin system
RGD	Arginine-glycine-aspartic acid
RISC	RNA induced silencing complex
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SM α-actin	Smooth muscle alpha actin
SMCs	Smooth muscle cells
SM-MHC	Smooth muscle myosin heavy chain
SP-1	Specificity protein 1
SRCR	Scavenger receptor cysteine-rich
VCAM	Vascular cell adhesion molecule
VSMCs	Vascular Smooth Muscle Cells
β-APN	ß-aminopropionitrile

Chapter 1: Background

Angiotensin II and Cardiovascular Disease

Accumulating evidence from both *in vivo* and *in vitro* studies supports the notion that Angiotensin II (Ang II) directly contributes to cardiovascular disease. Ang II is a multifunctional octapeptide hormone that promotes numerous actions within the vascular wall. It modulates vasoconstriction, cell growth and apoptosis, cell migration and extracellular matrix deposition; it is proinflammatory and it stimulates the production of growth factors (platelet derived growth factor (PDGF)) and vasoconstrictors (endothelin 1 (ET-1)). In essence, Ang

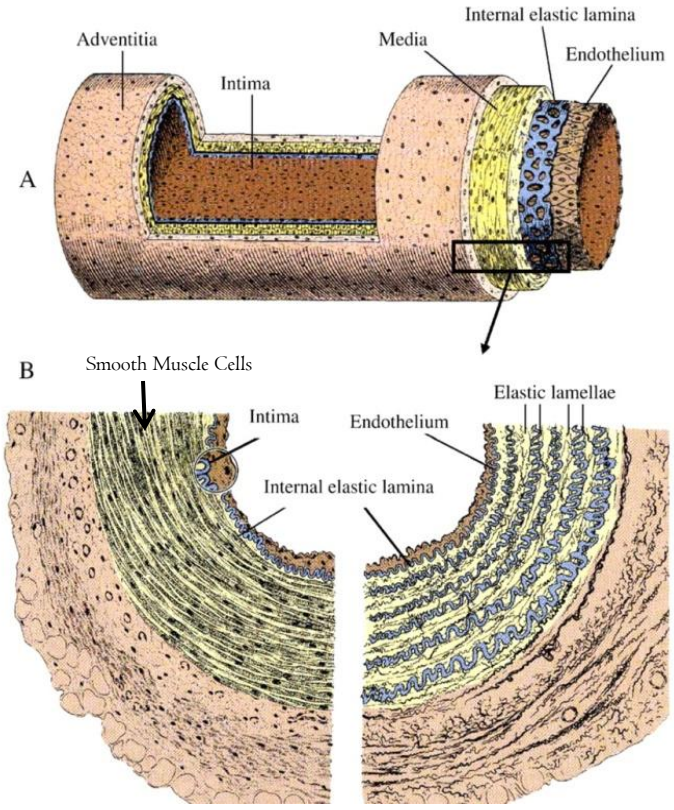

Figure 1. The structure of an artery.

The aorta is comprised of three distinct layers of cells including the intima (endothelial cells), the media (smooth muscle cells) and the adventitia (fibroblast and connective tissue) arranged in order of proximity to the lumen. Panel A shows a 3 dimensional view of the aorta while panel B is a cross sectional depiction of the aorta. Images are modified from (Patel*, et al.* 2006).

II plays a physiological role in the regulation of blood pressure, but also in the pathophysiological development of atherosclerosis and abdominal aortic aneurysms (AAAs).

The major role of Ang II is vasoconstriction for blood pressure homeostasis, however in excess Ang II mediates cell specific signaling events that shift a healthy blood vessel to a more diseased unhealthy vessel. Architecturally, blood vessels are composed of three cell layers: the adventitia, the media and the intima as shown in Figure 1. (Patel*, et al.* 2006) The intimal layer is composed of a single layer of endothelial cells in direct contact with the lumen or flow of blood. In large and medium sized blood vessels, the medial layer, comprised of smooth muscle cells, is the thickest layer, held in place by an orderly array of collagen and elastin. Lastly, the adventitia is composed of fibroblast and extracellular matrix components. Collectively, blood vessels maintain resistance and elasticity in response to blood flow. There are several factors that can disrupt the integrity of the vessel structure, leading to narrowed or blocked blood vessels and contributing to cardiovascular disease. In this thesis, we will discuss the contribution of the primary effector molecule angiotensin II on extracellular matrix components within the vessel wall, as it relates to the progression of hypertension, atherosclerosis and abdominal aortic aneurysms (AAAs), using both *in vivo* and *in vitro* approaches.

Hypertension

Hypertension is a term used to describe elevated blood pressure. Blood pressure is the measurement of the amount of force exerted against the wall of arteries as the heart pumps blood through the body. The pathogenesis of hypertension is a multifactorial process that also involves the interaction of genetic and environmental factors. In varying degrees, abnormalities of volume regulation, enhanced vasoconstriction, and remodeling of the arterial wall (decreasing lumen diameter and increasing resistance) contribute to the development of hypertension. (Alexander 1995) One of the key characteristics of hypertension is increased peripheral resistance, largely due to a reduced lumen diameter (vasoconstriction). An increase in peripheral resistance would be caused by an increase in vasoconstriction, which would decrease blood flow and increase blood pressure. The arteries that determine peripheral resistance undergo both structural and functional changes leading to hypertension. Examples of this include increased reactivity to contractile agents, such as Angiotensin II, impaired endothelial function, vascular smooth muscle cell growth and vascular inflammation. (Paravicini and Touyz 2006) Unfortunately, hypertension is a well-established cardiovascular condition that increases the risk of atherosclerosis. It is worth noting that atherosclerosis and hypertension are distinct disease entities; everyone who has hypertension does not manifest extensive atherosclerosis, nor is atherosclerosis always or even usually accompanied by hypertension.(Alexander 1995)

Atherosclerosis

Numerous clinical and epidemiological studies have identified systemic arterial hypertension as an independent and potent risk factor for the development of atherosclerotic disease. (Alexander 1995; Lithell 1994; Weiss*, et al.* 2001) Etiologically, atherosclerosis is widely accepted as an inflammatory disease characterized by the presence of monocytes/macrophages and T cells within the vascular wall, lesions localizing in large conduit or elastic arteries in areas of low shear stress, smooth muscle cell proliferation and migration from the media to the intima, the deposition of increased amounts lipids and fibrous elements, and increased levels of oxidative stress. (Alexander 1995) The collective contribution of the aforementioned characterizations, lead to the buildup of fat, cholesterol, and other substances in the arterial wall to form structures called plaques, resulting in arterial stiffness as illustrated in Figure 2. (2007)

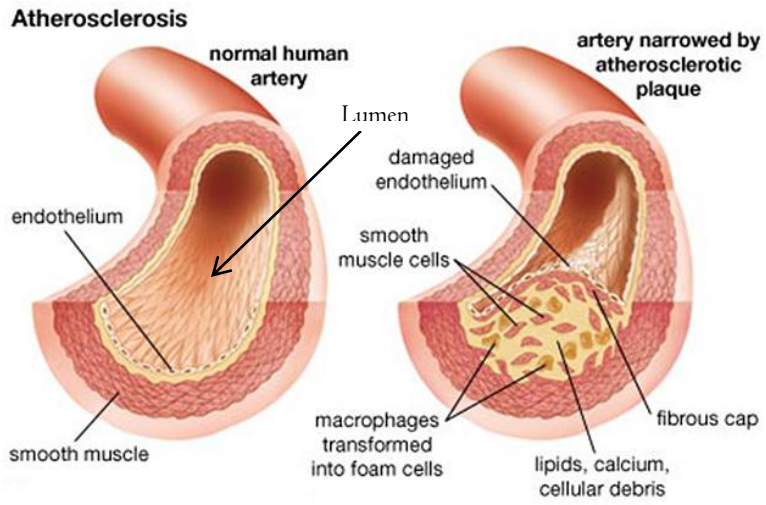

Figure 2. The progression of atherosclerosis.

A healthy aorta has an intact vessel wall composed of intact intimal, medial and adventitial layers. During the progression of atherosclerosis, the vessel wall undergoes significant remodeling leading to occlusion of the aorta and restricted blood flow. Image modified from Encyclopedia Britannica (2007).

In a more cell specific manner, one of the earliest events in atherosclerosis is the expression of adhesion molecules on the surface of endothelial cells, namely vascular cell adhesion molecule (VCAM-1), intracellular adhesion molecule (ICAM-1) and E-selectin. (Libby 2002) Several cytokines and vasoactive peptides, such as Ang II, can induce the expression of these adhesion molecules within the vascular wall initiating an inflammatory response. Under ordinary circumstances, the endothelial monolayer, which is in contact with the flow of blood, resists firm adhesion of leukocytes. However, in the presence of cell adhesion molecules, leukocytes such as the blood monocyte, the T lymphocyte and/or the mast cell can undergo diapedesis between intact endothelial cells to penetrate the intima of the arterial wall, forming a neointimal layer. Once resident in the intima, the blood monocyte acquires characteristics of a tissue macrophage. The macrophage expresses scavenger receptors that can bind oxidized low density lipoproteins (LDL). These processes give rise to the arterial foam cell, a hallmark of the arterial lesion. Infiltration of another leukocyte, the mast cell, leads to a series of coordinated events that further increase Ang II production and matrix metalloproteinase activation, allowing for smooth muscle migration and proliferation, as shown in Figure 3. (Libby 2002) Ang II mediated active remodeling of the vascular wall contributes to the progression of atherosclerosis and the restriction of blood flow. Ultimately, atherosclerosis is defined by remodeling of the vascular wall, mediated in part by extracellular matrix components that facilitate migration and adhesion and are actively produced in response to injury. Monitoring the availability and function of

extracellular matrix proteins is essential to decreasing atherosclerotic progression. Progressive atherosclerosis can be detrimental due to complications that include myocardial infarction, stroke, and the development of an aortic aneurysm. (Lithell 1994) In the next section, we will review abdominal aortic aneurysms.

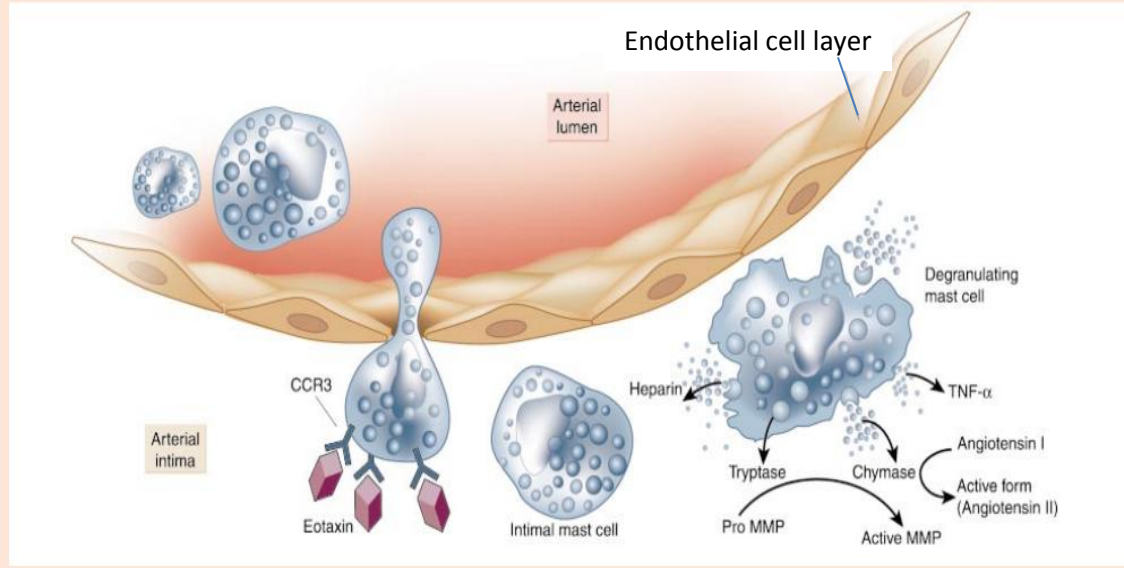

Figure 3. Infiltration of leukocytes into the vascular wall.

A mast cell can diapedese into the intima of the vessel wall and undergo degranulation releasing several molecules that promote smooth muscle cell migration and proliferation, such as tryptase and chymase. Tryptase promotes MMP activation leading to smooth muscle cell migration while chymase converts angiotensin I to angiotensin II, further mediating proliferation and migration of VSMCs. Image modified from (Libby*, et al.* 2002).

Abdominal Aortic Aneurysms

Abdominal Aortic Aneurysms (AAAs) are defined by a dilatation in the abdominal region of the aorta, the major blood vessel carrying blood away from the heart. This pronounced dilatation may exceed the normal diameter by more than 50%, resulting in significant remodeling of the normal blood vessel. Rupture and blood clots are major risks associated with AAA; both are life threatening. Risk factors for AAA are not completely understood, although elderly white men with a history of smoking and atherosclerosis are at the highest risk. (Golledge*, et al.* 2006) The current definitive therapy for AAA is surgery or stent placement performed when the risk of rupture outweighs the risk of surgical intervention.

Histologically, AAA is characterized by a loss of smooth muscle cells in the media, thinning and fibrous replacement of the media, medial elastin degeneration and loss of organization, accumulation of inflammatory cells in the adventitia (macrophages and T lymphocytes), and a generalized loss of architectural integrity in the vessel wall. (Nollendorfs*, et al.* 2001; Papalambros*, et al.* 2003; Pearce and Koch 1996) The exact etiology of AAA is not well understood but several logical theories are currently being developed. Although very controversial, the initial insult leading to the degeneration of the abdominal aorta has long been attributed to atherosclerosis, but additional factors may also contribute synergistically or independently. (Golledge*, et al.* 2006) AAA and atherosclerosis have been closely associated because patients tend to present with both diseases; however they have distinct epidemiologies. In addition to atherosclerosis, a genetic predisposition affecting collagen and/or elastin

synthesis (specifically mutations in genes encoding type III collagen) can cause a weakened aortic wall and ultimately lead to AAA. (Anderson*, et al.* 1996; Kuivaniemi*, et al.* 1991) Other causes of AAA include, but are not limited to infection, inflammation, and trauma. All of the aforementioned causes fail to adequately explain the continued development and expansion of AAA. The progression of AAA is more likely attributed to mechanical wall stress caused by hypertension, molecular genetics, immune responses that promote reactive oxygen species, and/or proteolytic degeneration of aortic wall matrix proteins, namely collagen and elastin. (McCormick*, et al.* 2007) Elastin degradation, a hallmark for AAA, allows previously fixed SMCs in the medial layer to migrate freely throughout the aortic wall. Migration in this capacity allows for vessel remodeling to occur, leading to dilation of the aorta and potentially rupture.

Previous studies have shown the impact of proteolytic degeneration of matrix components by implicating matrix metalloproteinases (MMPs) in the pathogenesis of AAA. (Davis*, et al.* 1998; Longo*, et al.* 2002; Papalambros*, et al.* 2003) MMPs are a family of endopeptidases that degrade elastin and collagen fibers. Several MMP family members aggravate matrix degeneration. (Longo*, et al.* 2002) Of the large family of MMPs, two have attracted particular interest: MMP-2 and MMP-9. MMP-9 is noteworthy because of its activity on insoluble elastin fibers. MMP-9 is abundantly expressed by aneurysm-infiltrating macrophages located at the site of tissue damage. (Papalambros*, et al.* 2003) MMP-9 is also the most abundant elastin proteinase produced by human AAA tissues as demonstrated *in vitro.* (Papalambros*, et al.* 2003) MMP-9 activity

directly correlates with increased aneurysmal diameter. (Papalambros*, et al.* 2003) Plasma levels of MMP-9 are highly elevated in patients with AAA. (Papalambros*, et al.* 2003) Equally important in AAA formation is MMP-2. MMP-2 is found primarily in SMCs and fibroblasts, and at very low levels in macrophages and monocytes. (Davis*, et al.* 1998) MMP-2 degrades fibrillar collagens (collagen types I, IV, V and VII) and elastin. MMP-2 expression is increased in AAA tissues as compared to normal aortic tissue; however MMP-2 activity has not been shown to correlate with AAA size. (Longo*, et al.* 2002; Papalambros*, et al.* 2003) Both MMP-2 and MMP-9 knockout mice are resistant to aneurysm formation. (Longo*, et al.* 2002) MMP-2 and MMP-9 work synergistically to promote rapid degeneration of the ECM. In essence, the composition of extracellular matrix components is essential to the integrity of the vascular wall. When a normal aorta becomes aneurysmal, matrix degeneration of the medial elastic laminae and adventitia occur, making it a hallmark of AAA. Previous studies have shown that in AAA, medial elastin degradation is responsible for weakening and dilatation of the aortic wall, while collagen degradation is connected to rupture (Papalambros*, et al.* 2003). Very little is known about the compensatory mechanism that may exist in the matrix to prevent AAA progression.

Angiotensin II

Renin Angiotensin System

The renin-angiotensin system (RAS) plays an essential role in the regulation of blood pressure and sodium-water balance *in vivo* through its primary effector molecule Ang II. Ang II acts on three systems to increase blood pressure: the hypothalamus inducing thirst and drinking, on the blood vessels to cause vasoconstriction and the adrenal cortex causing release of aldosterone which leads to salt and water retention in the body. (Touyz and Schiffrin 2000) Systemic Ang II is derived from the classic renal RAS, in which the production of Ang II is the result of the activity of several endopeptidases on the precursor, angiotensinogen. Renin, excreted from the kidneys, cleaves liver derived angiotensinogen to angiotensin I. In the presence of lung derived angiotensin converting enzyme (ACE), angiotensin I is then converted to angiotensin II. In addition to the systemic production of Ang II, Ang II can be produced locally in a tissue-specific manner. All of the cells within the vasculature, including VSMCs are able to produce all the components of the RAS system, with the exception of renin. (Touyz and Schiffrin 2000) Once in contact with the vasculature, Ang II interacts with the angiotensin type 1 (AT_1) receptor on vascular smooth muscle cells to activate cytoplasmic signaling pathways that contribute to increased hypertrophy, hyperplasia, migration, extracellular matrix formation, and inflammatory cytokine release. (Higuchi*, et al.* 2007; Kim and Iwao 2000; Mehta and Griendling 2007; Touyz and Schiffrin 2000) These known proinflammatory responses to Ang II have led to a series of studies that implicate Ang II in the

pathogenesis of hypertension, atherosclerosis and AAA. The sections below will detail what is known about Ang II signaling and how it promotes changes in vessel structure.

In vitro angiotensin II signaling

Because vascular smooth muscle cells (VSMCs) play a central role in the development of Ang II-mediated hypertension, atherosclerosis and AAAs, it is essential to understand the Ang II-induced intracellular signaling pathways that mediate changes in vessel structure within VSMCs. As mentioned previously, Ang II elicits its response primarily through binding of the angiotensin type 1 receptor (AT1). The AT1 receptor is a traditional seven transmembrane spanning heterotrimeric G-protein coupled receptor (GPCR). (Touyz and Schiffrin 2000) Initial binding of Ang II to AT1 results in the rapid activation of phospholipase C (PLC) through the dissociation of the Gα subunit of the heterotrimeric G protein from the βγ subunits. PLC cleaves membrane bound phosphatidyl inositol 4,5 bisphosphate (PIP2) into two molecules; the membrane bound diacylglycerol (DAG) and the soluble inositol triphosphate (IP3). (Lyle and Griendling 2006) IP3 facilitates the release of intracellular Ca^{2+} from the sarcoplasmic reticulum, allowing Ca^{2+} to act as a second messenger for further signal transduction. Intracellular increases in Ca^{2+} bind calmodulin, which further leads to the phosphorylation and activation of myosin light chain kinase (MLCK). (Touyz and Schiffrin 2000) Upon activation, MLCK phosphorylates myosin light chain (MLC) facilitating a cross bridge between actin and myosin, ultimately

leading to contraction within seconds of Ang II stimulation. Other actions of Ca^{2+} as a second messenger include assisting DAG in the activation of protein kinase C (PKC). (Lyle and Griendling 2006) PKC is a serine/threonine kinase capable of activating the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), resulting in the generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). O_2^- and H_2O_2 production, as well as the βγ subunits of the heterotrimeric G protein coupled receptor can activate the tyrosine kinase, c-Src and lead to the transactivation of several receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and the insulin receptor. (Touyz and Schiffrin 2000) Activation of several tyrosine and serine/threonine kinases, such as JAK/STAT and the MAP kinases, enable the phosphorylation and translocation of several transcription factors into the nucleus, which influence hypertrophy, proliferation, migration and adhesion as illustrated in Figure 4. Collectively, Ang II directly causes cell growth, regulates gene expression of various bioactive substances (vasoactive hormones, growth factors, extracellular matrix components, cytokines) and activates multiple intracellular signaling cascades (MAP kinases, tyrosine kinases and transcription factors) to promote migration and adhesion in vascular smooth cells. (Lyle and Griendling 2006) The goal of this thesis is to elucidate the effect of Ang II on extracellular matrix proteins as it relates to these processes in cardiovascular disease progression.

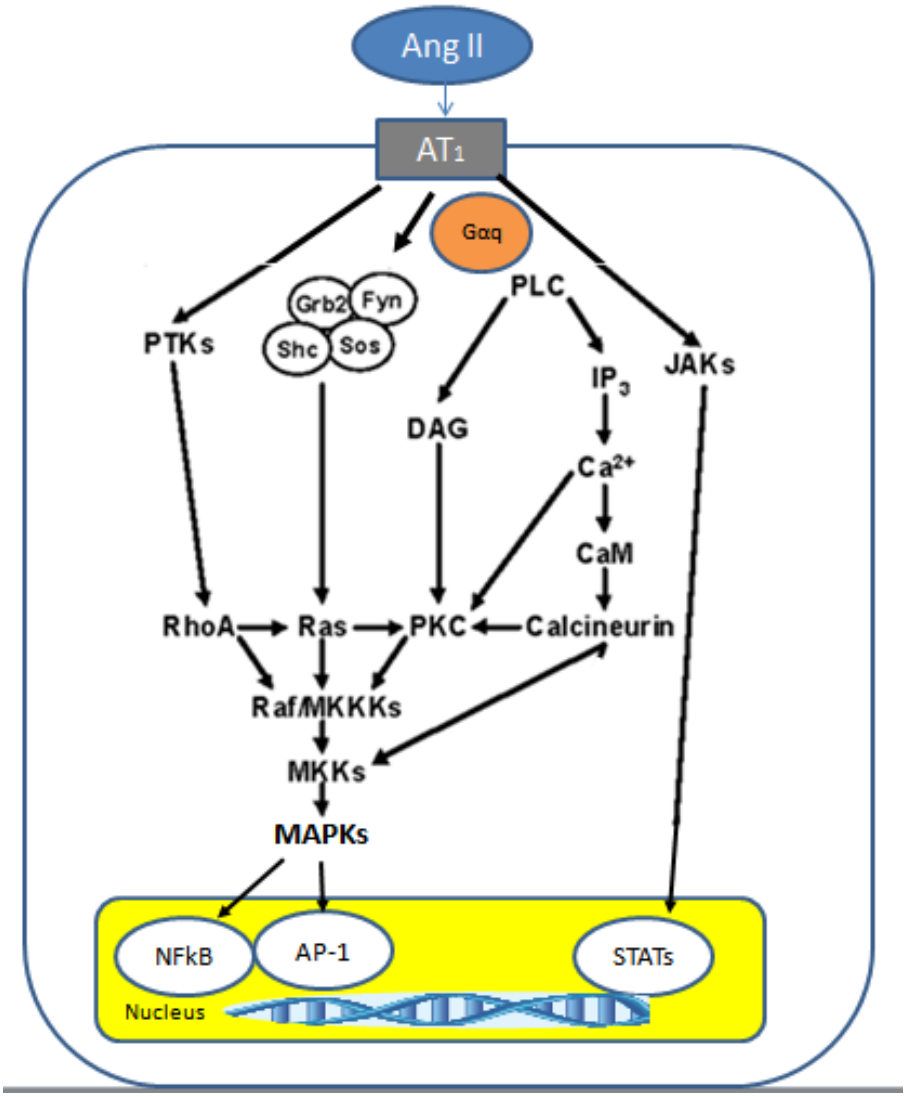

Figure 4. Ang II intracellular signaling events

Ang II can transduce intracellular signaling events through binding of the AT1 receptor to activate several second messengers (Ca^{2+}, PKC). In addition, activation of several protein tyrosine kinases (PTKs) further induces signaling. These events lead to hypertrophy, migration and adhesion of VSMCs and collectively contribute to vessel remodeling and disease progression.

Migration and adhesion of vascular smooth muscle cells

Ang II signaling pathways mediate various cell functions, in particular adhesion and migration, which are crucial for embryonic development, wound healing and inflammatory responses. (Horwitz and Parsons 1999) *In vivo*, VSMCs found in the medial layer can have considerable amounts of heterogeneity, depending on the state of the vessel wall. One population of VSMCs consists of a differentiated contractile phenotype that maintains vascular tone and resistance. These differentiated VSMCs highly express a number of pro-contractile proteins that include smooth muscle myosin heavy chain (SM-MHC), smooth muscle alpha actin (SM α-actin), and heavy-caldesmon (H-caldesmon). In healthy vessels, differentiated VSMCs are adhesive and non-migratory. (Gerthoffer 2007) These VSMCs differ in both signaling and function from their de-differentiated, pro-growth, pro-migratory and highly adhesive counterparts found in regions of cardiovascular injury and disease. (Moiseeva 2001) These de-differientated VSMCs are motile and express an altered set of adhesion receptors. In the sections below, we will discuss the events that lead to migration of VSMCs, with emphasis on adhesion, which is a necessary step for migration.

The process of cell migration can be divided into distinct sequential processes (Gerthoffer 2007; Horwitz and Parsons 1999) as illustrated in Figure 5. (Ananthakrishnan and Ehrlicher 2007) Migration begins with an altered cytoskeleton by cell surface receptors that lead to the formation of a leading edge. In VSMCs, in response to injury, a gradient is commonly established by

growth factors, such as PDGF. (Chen*, et al.* 2006; Lauffenburger and Horwitz 1996) The initial protrusion or extension of the plasma membrane at the leading edge of a cell, in response to a stimulus or environmental cue, is known as a lamellipodium. The formation of these protrusions requires the polymerization of a network of cytoskeletal actin filaments with the assistance of actin polymerizing proteins, which is a process regulated by the monomeric G protein Rac. (Nobes and Hall 1995) Rac stimulates actin polymerization via several mechanisms, including nucleation of new actin filaments (Machesky and Insall 1998; Miki*, et al.* 1998), extension of existing filaments (Hartwig*, et al.* 1995), and activation of LIM kinase (LIMK), which phosphorylates and inactivates the actin-capping protein cofilin, thus preventing actin depolymerization (Arber*, et al.* 1998; Yang*, et al.* 1998). After the formation of the leading edge, new focal contacts form behind the leading edge to increase adhesion of the cell to the matrix. These complexes are found in regions of the plasma membrane where integrin receptors, actin filaments, and other proteins such as talin, paxillin and focal adhesion kinase (FAK), cluster together to activate, and bind extracellular matrix components (Gerthoffer 2007; Nobes and Hall 1995; Nobes and Hall 1995). Integrin activation increases integrin binding to extracellular matrix components, thereby increasing adhesion through the formation of focal complexes. These focal complexes, mature, grow and strengthen into larger, more organized focal adhesions that serve as points of traction over which the cell body moves during the process of migration. (Nobes and Hall 1995; Nobes and Hall 1995) Contraction enables the cell to migrate, following establishment of the leading

edge and adhesion formation. The monomeric GTPases cdc42 and Rho regulate contractile forces by influencing myosin light chain (MLC) phosphorylation. (Dechert*, et al.* 2001) MLC phosphorylation promotes dimerization and interaction with actin to drive contraction. (Horwitz and Parsons 1999) Rho kinase, activated by Rho and ROS (Jin*, et al.* 2004), functions to inhibit the myosin phosphatase, allowing MLCs to remain in a contractile (phosphorylated) state (Horwitz and Parsons 1999). For the cell to make forward progression in response to contraction, it must release its rear adhesions to allow a net forward displacement. This is, in part, mediated by several proteases including the matrix metalloproteinases and calpain. (Gerthoffer 2007; Lauffenburger and Horwitz 1996; Regen and Horwitz 1992) The processes of adhesion and migration are highly influenced by components of the extracellular matrix, and as such implicate the ECM as critical to vessel remodeling and disease progression.

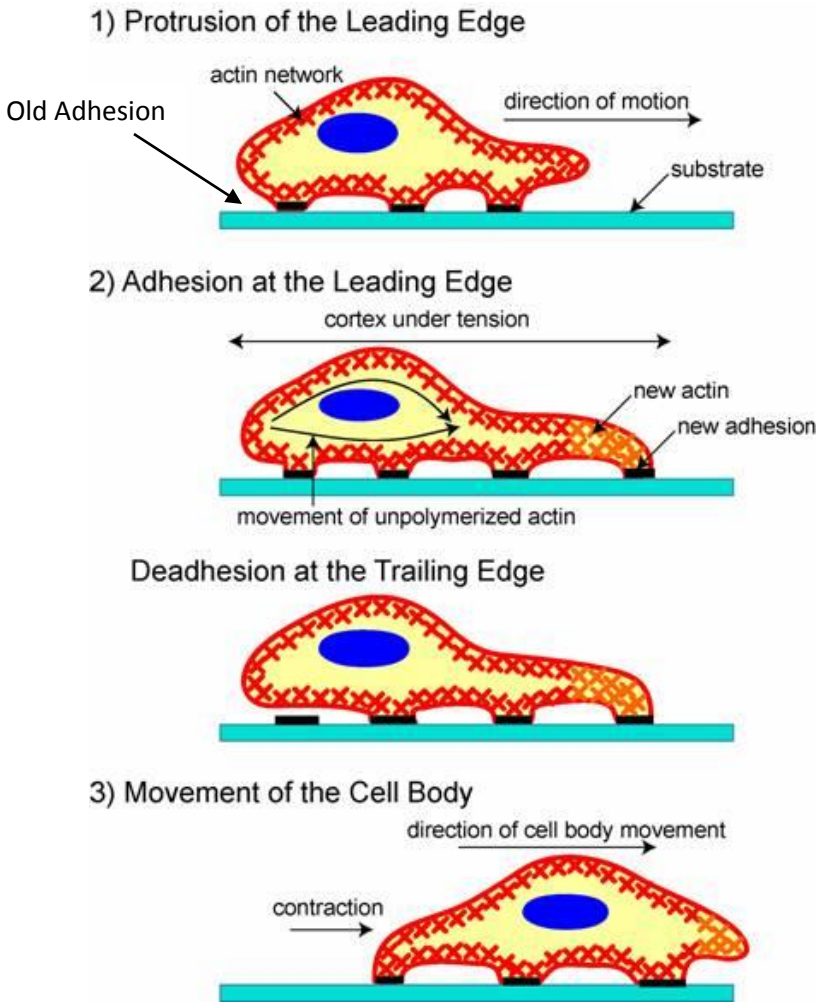

Figure 5. Adhesion and migration of VSMCs

Migration begins with an initial protrusion at the leading edge of the cell. The formation of these protrusions requires the polymerization of a network of cytoskeletal actin filaments. The protrusion is stabilized through the formation of new adhesive complexes within the protrusion. The focal complexes at the front of the cell mature into larger, more organized focal adhesions that serve as points of traction over which the cell body moves. Next, contraction occurs in the body of the cell to propel the cell forward. For the cell to make forward progression, it must release its rear adhesions to allow a net forward displacement. Image modified from (Ananthakrishnan and Ehrlicher 2007).

Extracellular Matrix

The vascular extracellular matrix (ECM) is a collection of many different macromolecules organized by cross-linking into a biomechanically active polymer that imparts tensile strength, elasticity, and compressibility to the vascular wall. Each component of the extracellular matrix possesses unique structural properties that determine its separate roles during the development of hypertension, atherosclerosis and AAA. Not only does this matrix provide the architectural framework that influences the structural integrity of the vessel wall, but it also provides the milieu for vascular cells and participates in the adhesive, proliferative, and migratory events that characterize disease progression. Therefore the ECM is subdivided into two classes of proteins, those that subserve a primarily structural function and those that direct other roles, such as regulating intracellular communication, migration and adhesion, termed matricellular proteins.

Lysyl Oxidase and Lysyl Oxidase Like Isoenzymes

Lysyl Oxidase (LOX) is a copper containing monoamine oxidase that initiates the covalent crosslinking of collagen and elastin in the ECM, serving a primarily structural role. These enzymes catalyze the conversion of lysine residues in collagen and elastin via an oxidation deamination reaction to an aldehyde. (Kagan and Li 2003) The resulting aldehyde is highly reactive and spontaneously condenses to form highly stable intra- and intermolecular

crosslinks with other peptidyl lysines and/or aldehydes to impart great mechanical strength to the arterial wall. The byproducts of this reaction are ammonia and hydrogen peroxide. (Lucero and Kagan 2006)

Five different LOX enzymes exist in both humans and mice: Lysyl Oxidase (LOX) and Lysyl Oxidase Like proteins (LOXL)-1, -2, -3 and -4 derived from five different, yet closely related genes. After translation, all of the LOX/LOXL family members undergo glycosylation and secretion into the extracellular space, where several proteases, including bone morphorgenic protein (BMP-1) initiate LOX/LOXL activation, through cleavage of the propeptide region. (Molnar*, et al.* 2003) All five family members are capable of performing the catalytic crosslinking activity. Characteristic domains of the LOX/LOXL family members include a highly conserved C terminus that encodes this catalytic domain. (Molnar*, et al.* 2003) The C terminus is more than 95% similar between the five isoforms and is necessary and sufficient for enzyme function. (Csiszar 2001) The C terminus includes a cytokine receptor like domain, a copper binding domain and a lysyl-tyrosyl quinone (LTQ) cofactor region as illustrated in Figure 6. (Mäki 2002) The five isoforms have unique N terminal sequences that may determine individual roles, mediate protein-protein interactions and/or define cellular localization (Csiszar 2001; Molnar*, et al.* 2003). For example, LOXL2, LOXL3 and LOXL4 have scavenger receptor cysteine rich (SRCR) motifs that have been suggested to mediate protein-protein interactions that facilitate adhesion. In addition to secretion, there is further evidence that the LOX/LOXL family members can reenter the cell and concentrate within the nuclei where they

are thought to modify gene expression. (Lucero and Kagan 2006; Rodriguez*, et al.* 2008) There is evidence of catalytic activity in the nucleus and the cytoplasm of cells, although most LOX/LOXL activity appears to be present in the ECM.

LOX/LOXL family members have diverse biological roles and as such, have been implicated in the pathogenesis of several medical conditions. The byproduct of LOX activity, H_2O_2 is associated with cell motility and migration in breast cancer. (Payne*, et al.* 2005) LOX has also been implicated in cellular senescence, developmental regulation, and tumor suppression. Losses of heterozygosity in the LOX and LOXL2 genes have been associated with colonic and esophageal neoplasms, identifying these genes as tumor suppressors. (Kagan and Li 2003) Increased LOX activity has been linked to conditions of fibrosis, such as atherosclerosis. (Kagan and Li 2003; Kagan*, et al.* 1981) Decreased LOX activity has been associated with impaired copper metabolism.

LOX activity is crucial for *in vivo* development of elastic vessels. Rat pups weaned from their mothers and fed a general LOX inhibitor, ß-aminopropionitrile (β-APN), form aneurysms. (Siegel 1979) In addition, LOX knockout mice die soon after birth. (Maki*, et al.* 2002) The cause of death is impaired vascular development that leads to aneurysmal dilatation of the aorta and subsequent rupture. (Hornstra*, et al.* 2003; Maki*, et al.* 2002) LOXL1 KOs are not lethal but have elastin regeneration problems. (Liu*, et al.* 2004) LOX has been extensively studied in the progression of atherosclerosis mediating endothelial dysfunction, plaque rupture and smooth muscle cell migration. (Rodriguez*, et al.* 2008) However, little is known about the regulation of LOX in the setting of AAA. In the

setting of AAA, elastin and collagen medial degeneration is prominent and facilitated by MMPs, particularly

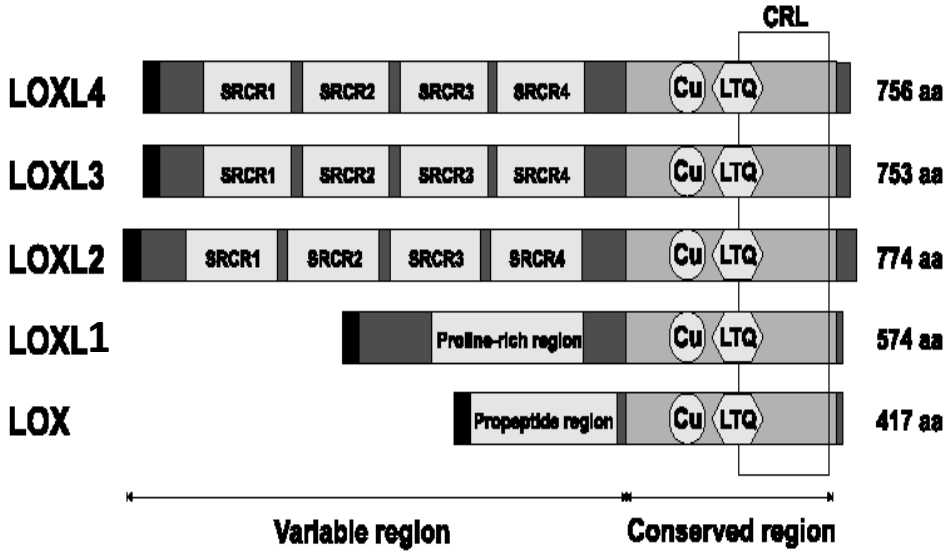

Figure 6. The lysyl oxidase family

The predicted signal peptides are represented by black boxes, and four scavenger receptor cysteine-rich regions (SRCR) in LOXL2, LOXL3, and LOXL4, the propeptide region in LOX, and the proline-rich region in LOXL are also indicated. The sites of the putative copper-binding region (Cu), the lysine tyrosylquinone cofactor formation (LTQ), and the cytokine receptor-like domain (CRL) are highly conserved between all lysyl oxidase proteins. The number of amino acids in each protein is indicated on the right. Image modified from (Mäki 2002).

MMP-2 and MMP-9. Certain cell types, such as VSMCs can secrete collagen and elastin into the ECM as illustrated in Figure 7. (Mitchell and Niklason 2003) Under normal conditions, elastin and collagen fibers are able to resist spontaneous breakdown due to their ability to form stable crosslinks with one another. However, in the presence of activated MMPs, collagen and elastin degeneration is present in the medial layer. Emerging evidence demonstrates that Ang II increases MMP expression and activation. (Guo*, et al.* 2008; Jimenez*, et al.* 2009; Luchtefeld*, et al.* 2005) LOX/LOXLs represent one of the counter compensatory mechanisms to matrix degeneration and MMP activity, by crosslinking to stabilize ECM components. Our goals are to examine the effect of Ang II on LOX/LOXL, as we examine a potential role for LOX/LOXL in the stabilization of AAAs.

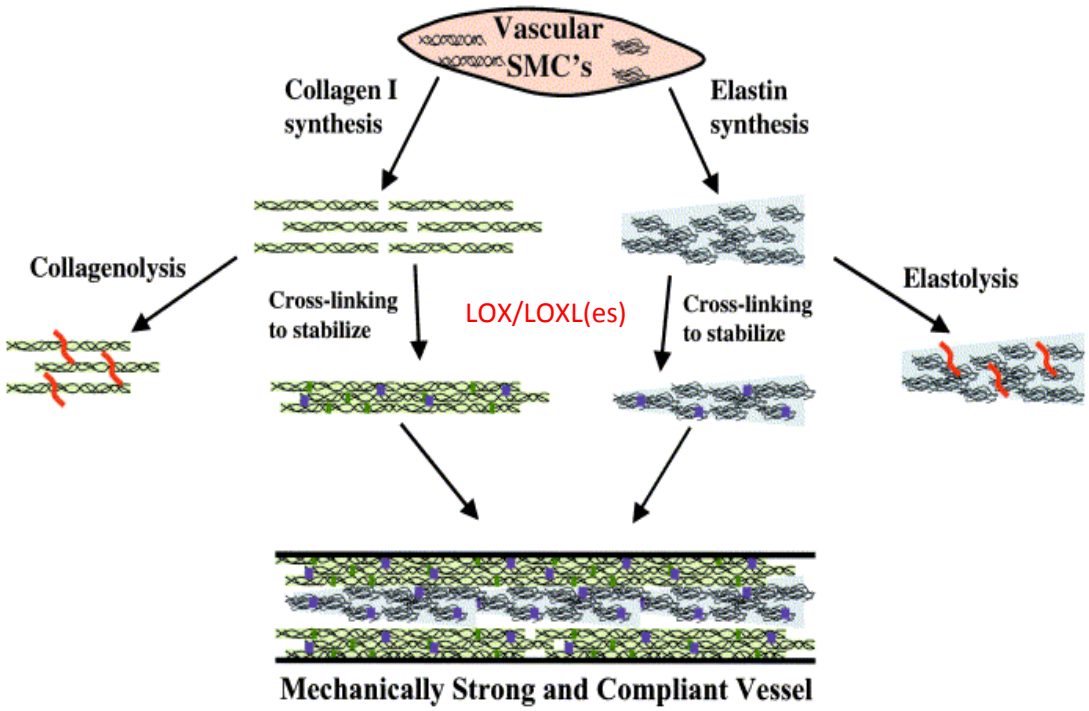

Figure 7. Achieving Vessel Stability

Vascular Smooth Muscle Cells (VSMCs) can secrete extracellular matrix components, such as collagen and elastin, in response to stimuli. Stable crosslinking of ECM components contribute to a compliant vessel wall. Proteolysis of newly synthesized ECM components impairs vessel integrity. Crosslinking, by the LOX/LOXL family members can stabilize vessel structure. Image modified from (Mitchell and Niklason 2003)

Osteopontin

Osteopontin (OPN) is a negatively charged, secreted glycosylated phosphoprotein that is abundant in atherosclerotic plaques and in the injured arterial wall, suggesting a role in the development of atherosclerosis. (Rangaswami*, et al.* 2006) OPN primarily functions as a matricellular protein facilitating cell attachment, migration, immune cell activation and mediating signal transduction via a number of receptors from within the ECM. (Denhardt*, et al.* 2001) OPN is expressed in many tissues including bone, kidney, arterial smooth muscle cells, and a plethora of mucosal membranes. (Rangaswami*, et al.* 2006) OPN's extensive posttranslational modifications (5 glycosylation sites 30+ phosphorylation sites), illustrated in figure 8, are suspected to contribute to its

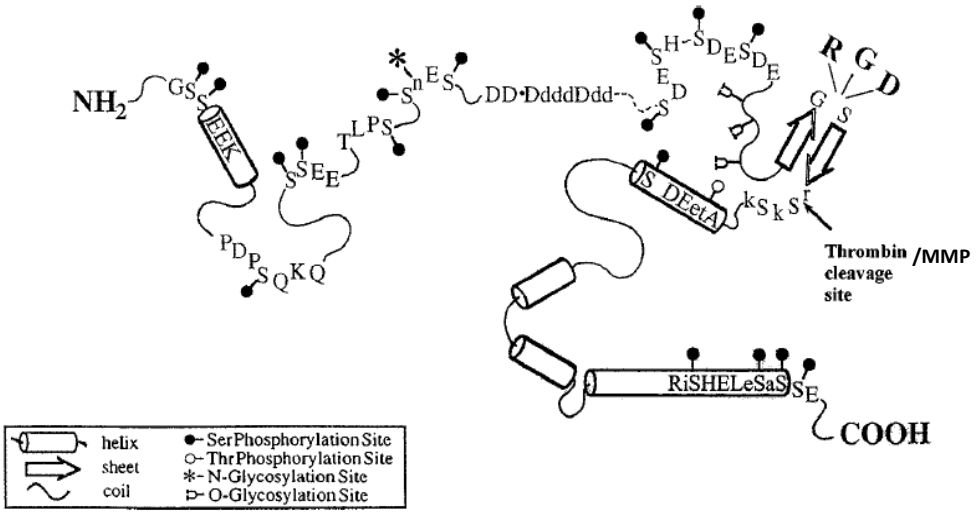

Figure 8. Osteopontin has extensive posttranslation modifications.

A representative image of the secondary structure of OPN across several species (human, mouse, rat, pig, and chicken). Conserved residues are capitalized. OPN is highly phosphorylated, glycosylated and has a conserved RGD motif and thrombin cleavage site. Image modified from (Denhardt*, et al.* 2001).

diverse roles in adhesion, migration, bone reabsorption, and cell activation. (Rangaswami*, et al.* 2006) OPN is expressed by several cell types in response to stimuli including VSMCs, endothelial cells and macrophages. Structurally, OPN contains an arginine-glycine-aspartic acid (RGD) motif, which upon thrombin cleavage allows the N terminal domain to be recognized by integrins (primarily the αvβ3 integrin, as well as αvβ1 and αvβ5) to mediate adhesion, migration and other signal transduction pathways. (Denhardt*, et al.* 2001; Standal*, et al.* 2004) In addition to facilitating adhesion via integrin receptors, OPN can also directly bind ECM components such as type I collagen. (Denhardt*, et al.* 2001) After thrombin cleavage, the C terminal fragment of OPN can also bind to CD44, a cell surface glycoprotein, through a non-RGD dependent mechanism to also initiate intracellular signaling pathways. (Rangaswami*, et al.* 2006) Under certain conditions, OPN can act as an inflammatory protein by activating macrophages, as well as serving as a chemoattractant for VSMCs, endothelial cells and macrophages at sites of injury.(Standal*, et al.* 2004) OPN is resolved by SDS gel electrophoresis as two species that differ by ~ 2kDa in molecular weight at 66kDa and 64kDa based on post translational modifications. The OPN protein is encoded by a single gene and its promoter region is responsive to many transcription factors including activator protein 1 (AP-1), specificity protein 1 (SP-1), nuclear factor kappa B (NFkB) and Protein C-ets-1 (Ets-1). (Abe*, et al.* 2008; Denhardt and Guo 1993; Standal*, et al.* 2004) Largely, OPN is secreted into the extracellular space; however emerging evidence demonstrates its presence at the cell surface and as well as localization at the

leading edge of cells. (Denhardt*, et al.* 2001; Standal*, et al.* 2004) It was previously shown that Ang II induces OPN expression in cultured smooth muscle cells via an ERK1/2 mediated pathway; however mechanisms to regulate Ang II-induced OPN expression remain to be elucidated.(Abe*, et al.* 2008)

Scope of Dissertation

In totality, the extracellular matrix is no longer viewed as an inert structural mass, but rather as a collection of molecules that possesses the capacity to drive events central for remodeling the vascular wall. A more thorough understanding of Ang II regulation of extracellular matrix proteins is a reasonable approach towards a better understanding of cardiovascular disease. In this thesis we will demonstrate the effect of Ang II on two distinct types of ECM proteins as they relate to the progression of atherosclerosis and abdominal aortic aneurysms 1) The Lysyl Oxidase Family Members, enzymes that contribute to structural stability of the ECM and 2) Osteopontin, a matricellular protein that facilitates inflammation, adhesion and migration from within the ECM. Understanding the regulation of lysyl oxidase by Ang II may improve its ability to crosslink collagen and elastin, preventing medial degradation and reversing a crucial hallmark of AAA. In this thesis, we propose that LOX/LOXL family members represent a counter regulatory mechanism for AAA formation and investigate the role of Ang II in this hypothesis. On the other hand, inhibiting osteopontin's ability to increase adhesion, migration and inflammation to preserve the integrity of the smooth muscle cell layer may reduce atherosclerosis. Collectively, *in vivo* and *in*

vitro approaches will be used to study Ang II regulation of extracellular matrix proteins. Also within this dissertation we will discuss novel mechanisms for regulating Ang II-induced expression of extracellular matrix proteins as a potential mechanism for reducing cardiovascular disease.

Chapter 2: The role of Angiotensin II on Lysyl Oxidase Family Members in the regulation of Abdominal Aortic Aneurysms and in cultured Vascular Smooth Muscle Cells.

Part 1. The Role of Lysyl Oxidase Family Members in the Stabilization of Abdominal Aortic Aneurysms

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Abstract

Abdominal Aortic Aneurysms (AAAs) are a major cause of morbidity and mortality in the United States today. We employed a model for AAA development using an Apolipoprotein E knock out mouse ($ApoE^{-/-}$) fed a high fat diet and treated with Angiontensin II (Ang II) and ß-aminopropionitrile (β-APN) for 4 weeks. Ang II induces hypertension and atherosclerotic disease while β-APN inhibits the activity of the Lysyl Oxidase (LOX/LOXL) family members. LOX/LOXL family members crosslink collagen and elastin in the extracellular matrix and therefore contribute to the integrity and stabilization of a healthy vessel wall. In this model, co-treatment with Ang II and β-APN caused a 90% AAA incidence and increased atherosclerotic lesion formation from less than 5% to greater than 25% after 4 weeks. In more atheroprotected mouse strains (C57BL/6 and BalbC), co-treatment with Ang II and β-APN caused 50% and 40% AAA incidence, respectively. These data demonstrate the importance of LOX/LOXL to the stability of the vessel wall. Therapeutic strategies to overexpress LOX/LOXL enzymes or to support the crosslinking of soluble matrix proteins in a polymeric scaffold are a promising opportunity to achieve stabilization of AAAs.

Introduction

Abdominal Aortic Aneurysms (AAAs) are a major cause of morbidity and mortality in the United States today. In the US, the incidence of AAA is estimated to be 4-8% in elderly men. (Lederle*, et al.* 2000) Rupture occurs in 1-3% of men aged 65 and older and the mortality rate is approximately 95%. (Lindholt*, et al.* 2005) Furthermore, the prevalence of AAA will likely increase as the number of people in the susceptible age group continues to rise**.** Risk factors for AAA are not completely understood, although elderly white men with a history of smoking and/or hypertension are at the highest risk. (Golledge*, et al.* 2006; Kuivaniemi*, et al.* 1991) The current definitive therapy for AAA is surgery or percutaneous stent placement performed when the risk of rupture outweighs the risk of intervention.

Several animal models of AAA formation have been developed to better understand its pathogenesis including calcium chloride injury of the aorta, elastase infusion and systemic Angiotensin II (Ang II) infusion. (Daugherty and Cassis 2004; Freestone*, et al.* 1997; Liu*, et al.* 2004) Previous studies have demonstrated the importance of proteolytic degeneration of matrix components by matrix metalloproteinase's (MMPs) in the pathogenesis of AAA. (Davis*, et al.* 1998; Longo*, et al.* 2002; Papalambros*, et al.* 2003) MMPs are a family of endopeptidases that degrade elastin and collagen fibers. MMP knockout mice 2, 3, 9, and 12 are resistant to aneurysm formation, which points directly to the critical importance of matrix stabilization in AAA pathogenesis. (Davis*, et al.* 1998; Longo*, et al.* 2005; Longo*, et al.* 2002; Papalambros*, et al.* 2003; Silence*, et al.* 2001)

The Lysyl Oxidases (LOX) are a family of enzymes that stabilize matrix components. LOX family members crosslink collagen and elastin fibers to create insoluble proteins resistant to proteolytic degeneration. This crosslinking activity stabilizes the vessel wall and imparts mechanical strength. The LOX enzymes are a family of copper-containing monoamine oxidases found in the extracellular matrix. (Kagan and Li 2003) These enzymes catalyze the conversion of lysine residues in collagen and elastin via a deamination reaction to allysine. The allysine residue spontaneously condenses to form highly stable crosslinks that impart additional mechanical strength to collagen and elastin.

Five different LOX enzymes are known to exist in both humans and mice: Lysyl Oxidase (LOX) and the Lysyl Oxidase Like proteins: LOXL1, LOXL2, LOXL3 and LOXL4, collectively termed LOX/LOXL. All five LOX/LOXL family members possess catalytic crosslinking activity (Molnar*, et al.* 2003). The C terminus demonstrates 95% homology among the five isoforms which is necessary and sufficient for enzyme function (Csiszar 2001). The five isoforms have unique N terminal sequences that may confer additional roles, mediate protein-protein interactions and/or define cellular localization (Csiszar 2001; Molnar*, et al.* 2003).

LOX activity has been shown *in vivo* to be crucial for the development of elastic vessels. Rat pups weaned from their mothers and fed the general LOX/LOXL inhibitor, ß-aminopropionitrile (β-APN), form aneurysms (Narayanan*, et al.* 1972; Siegel 1979). In addition, LOX knockout mice die soon after birth (Maki*, et al.* 2002). The cause of lethality in the LOX KO mice is impaired

vascular development that leads to an aneurysmal dilatation of the aorta and subsequent rupture (Hornstra*, et al.* 2003; Maki*, et al.* 2002). LOXL1 KOs are not lethal but have defects in elastin regeneration (Liu*, et al.* 2004). To date there are no publications on LOXL2, LOXL3 or LOXL4 knockout mice.

Our study investigates whether the LOX/LOXL family members provide a protective and/or compensatory mechanism against AAA formation. The expression and functional contribution of LOX/LOXL to AAA formation were analyzed in a model of accelerated atherosclerosis and AAA formation consisting of Apolipoprotein E knockout ($ApoE^{-/-}$) mice fed an atherogenic diet and infused with Ang II (Daugherty and Cassis 2004; Weiss*, et al.* 2001; Weiss*, et al.* 2001).

Methods

Animals

$ApoE^{-/-}$, C57BL/6, and BalbC mice age six to eight weeks (Jackson Laboratory, Bar Harbor, ME) were fed either a control low fat diet (standard low fat rodent chow Purina 5001) or a high fat diet using modified Paigen's atherogenic diet (Research Diets Inc. New Brunswick, NJ) and treated either with or without Ang II (0.75 mg/kg/day) via an osmotic mini-pump. Some animals were also treated with β-APN at a dose of 100 mg/kg/day via subcutaneous osmotic mini-pumps. (Narayanan*, et al.* 1972) All housing, surgical procedures, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Emory University. Animals had free access to chow and water during all

experiments. Noninvasive systolic blood pressure measurements were obtained using the Visitech Systems BP 2000 tail-cuff system as previously described. (Weiss*, et al.* 2001) Aortic atherosclerotic lesion area was measured as previously described by pressure perfusing the vasculature at approximately 100mmHg with normal saline and subsequent fixation with 10% formalin. (Weiss*, et al.* 2001) The aortic length was cleaned of periadventitial fat and connective tissue and then removed with the heart and kidneys attached. The thoracic-abdominal aorta was pinned on a wax dish and dissected *en face* longitudinally. Dissected aortas were digitally imaged and analyzed for lesion area and aortic circumference using Image J 1.61 software (NIH, Bethesda Maryland).

Immunostaining

Pressure fixed aortas were embedded in paraffin and sections were obtained at 5μm intervals. Immunostaining for the LOX/LOXL family members was carried out using rabbit polyclonal antibodies (supplied by Drs. Fong and Csiszar from University of Hawaii At Manoa) and goat anti-rabbit biotinylated secondary antibodies. Imaging was accomplished using streptavidin tagged quantum dots (emission 605 nM) as previously reported. (Ferrara*, et al.* 2006)

Quantitative Real Time - PCR

RNA was extracted from snap frozen aortic tissue 7 days after treatment using the RNeasy kit (Quiagen, Valencia, CA) according to their protocol and quantified using a spectrophotometer. cDNA was prepared and purified from aortic RNA

using standard protocol with primers unique for each of the five lysyl oxidase isoforms (Table 1).

Table 1. LOX/LOXL and 18s primer sequences and annealing temperatures

cDNA Targets	Primer Sequences	Annealing (^{o}C)
LOX	5’ TAGCGAAGCACATAGCATTG 3’ 5’ TGCAGCAATGAGTCTACAGC 3’	60
LOXL1	5’ CTATGCCTGCACCTCTCACA 3’ 5’ TGGACGATTTTGCAGTTTGT 3’	61
LOXL2	5’ GAGTGGAGGTGCTAACAGAG 3’ 5’ AGTCTGCTGGACAATCTCAG 3’	59
LOXL3	5’ ACTGTACCCATGATGAGGAT 3’ 5’ GCCATGTTGACTCTCTTTTC 3’	57
LOXL4	5’ CTGGCGTTGCTTGTATGAACA 3’ 5’ AGACAGAAGCTGGCCTTGTGT3’	64
PAI-1	5’ ACAGCCAACAAGAGCCAATC 3’ 5’ GACACGCCATAGGGAGAGAA 3’	64
18S	5’ GAACGTCTGCCCTATCAACT 3’ 5’ CCAAGATCCAACTACGAGCT 3’	64

Western Blot

Mouse aortas were harvested and snap frozen in liquid nitrogen. After homogenization in RIPA buffer (Santa Cruz Biotechnology Inc, Santa Cruz, CA), protein was extracted, sonicated and quantified using the Bradford assay. Samples were then boiled for 5 min at 95°C and aliquots of the lysate were subjected to 8% SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane by electroblotting. The membranes were treated with LOX/LOXL antibodies procured from Novus (LOX) and Santa Cruz (LOXL1, LOXL2 and GAPDH) or made in house (LOXL3). Anti-rabbit and anti-goat secondary antibodies conjugated to horse-radish peroxidase were procured from Santa Cruz and Biorad. Immunoreactive proteins were detected by an enhanced chemiluminescence system (GE).

Additional Reagents

ß-aminopropionitrile (β-APN), Ang II, and PCR primers were obtained from Sigma Chemicals Co. St Louis, MO. Osmotic mini-pumps were obtained from Alzet, Cupertino, CA.

Statistical Analysis

Systolic blood pressure, cholesterol, and percent atherosclerotic lesion area are presented as mean ± SEM. ANOVA was performed using Graph Pad Prism

Software. Post hoc analyses were performed using the Bonferroni test. *P* values <0.05 were considered to be significant.

Results

LOXL3 mRNA expression is significantly upregulated in the aortas of Ang II treated ApoE$^{-/-}$ mice fed a high fat diet.

To quantitatively assess if LOX/LOXL family member expression was regulated in our model of AAA formation, LOX/LOXL mRNA levels were measured by quantitative real-time PCR using mRNA extracted from the aortas of control ApoE$^{-/-}$ mice fed a low fat diet mice and compared to those fed a high fat diet and treated with Ang II for 7 days. Mice treated with Ang II infusion and fed a high fat diet exhibited significant increases in blood pressure. (Figure 9) At baseline LOX expression in aortic tissue was highest compared to the other family members, with expression of LOXL1, LOXL2 and LOXL3 much lower and LOXL4 expression undetectable. (Figure 10A-D) Comparing mRNA from our AAA mouse model to the low fat control mice after 7 days resulted in significantly increased expression of LOXL3 mRNA. (Figure 10D) LOXL4 expression remained undetectable after treatment. Interestingly, western blot analysis on protein harvested from aortic tissue 7 days after treatment with Ang II on a high fat diet failed to show any significant difference in the detected isoforms (LOX and LOXL3) over control tissue (low fat diet). (Figure 11) In addition, other family members that were previously detected by qRT-PCR and immunohistochemistry (LOXL1 and LOXL2), were not detected at 7 days in aortic tissue from either low

fat or high fat + Ang II treated mice by western blot, suggesting temporal or spatial regulation at the protein level.

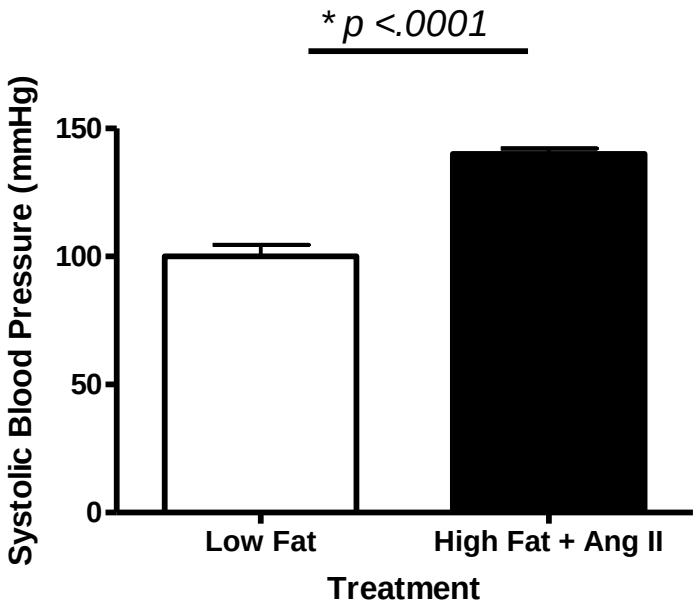


Figure 9. Ang II infusion leads to increased systolic blood pressures.

Mean systolic blood pressures were taken in ApoE$^{-/-}$ mice prior to treatment. Animals were then fed a high fat diet and treated with Ang II for two weeks resulting in a significant increase in blood pressure. **p=.0001*

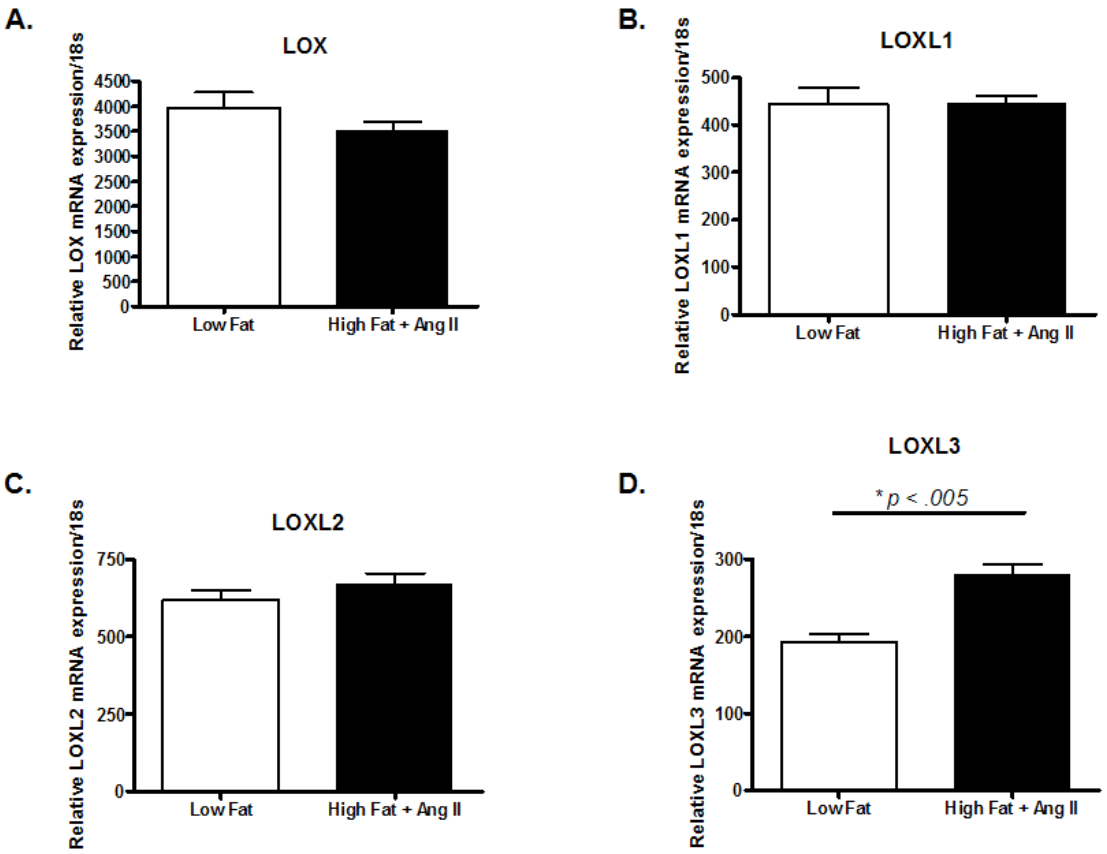


Figure 10. LOX/LOXL family member expression 7 days after Ang II infusion in the presence of a high fat diet.

qRT-PCR was performed on cDNA from aortic tissue of ApoE$^{-/-}$ mice. (A-C) LOX, LOXL1 and LOXL2 expression was not affected by Ang II in the presence of a high fat diet. D.) LOXL3 expression increased with Ang II on an atherogenic diet. *$p=.005$.

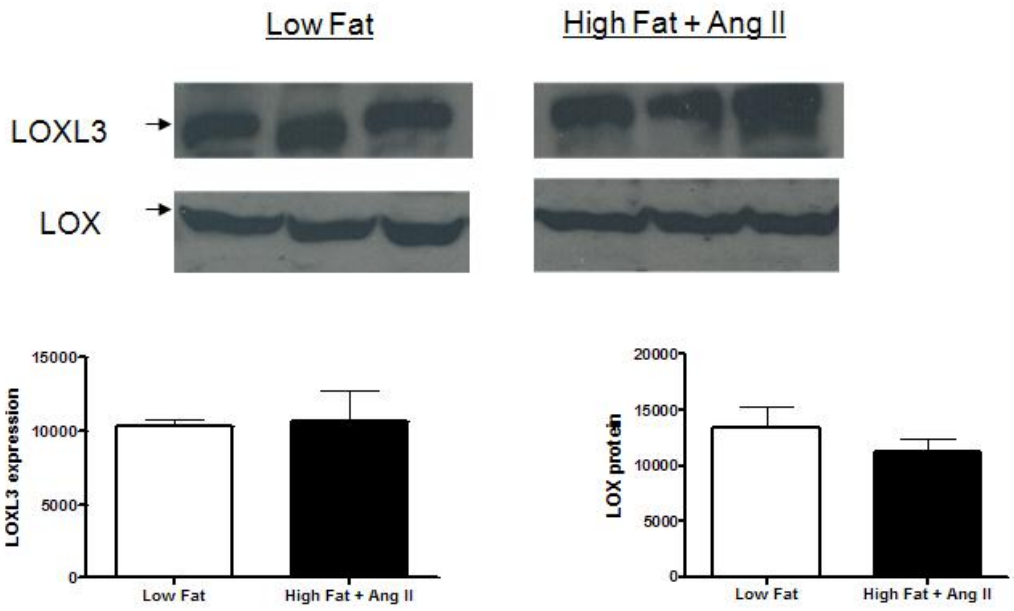


Figure 11. LOX/LOXL family member protein expression 7 days after Ang II infusion in the presence of a high fat diet.

Western Analysis on ApoE$^{-/-}$ mice 7 days after treatment with low fat diet or high fat diet + Ang II. By Western analysis, LOX and LOXL3 were the only detectable family members in the low fat and high fat + Ang II setting 7 days post treatment. Neither LOX nor LOXL3 showed a statistically significant change in expression with treatment. *n=3*

LOX/LOXL proteins are expressed in the aortic wall

Immunostaining was performed to determine the cellular localization of individual LOX/LOXL family members within the aortic wall of ApoE$^{-/-}$ mice fed a high fat diet and treated with Ang II for 8 weeks. LOX, LOXL1, LOXL2 and LOXL3 expression was diffuse throughout the aortic wall (Figure 12A-D). LOX, LOXL1 and LOXL3 displayed robust expression in the medial and intimal layers of the aortic wall. (Figure 12A, 12B and 12D) LOXL2 expression was most prominent in the endothelial cell layer (Figure 12C). Interestingly, LOXL1 and LOX3 had significant expression in the neointimal layer, suggesting certain LOX/LOXL family members are expressed in migratory smooth muscle cells. Differential LOX/LOXL family member localization and expression profiles further illustrate the importance of this family of matrix crosslinking enzymes to the structural integrity of the vessel wall. Each member was clearly expressed in the vessel wall, underscoring the potential role of the LOX/LOXL family in maintaining vascular stability.

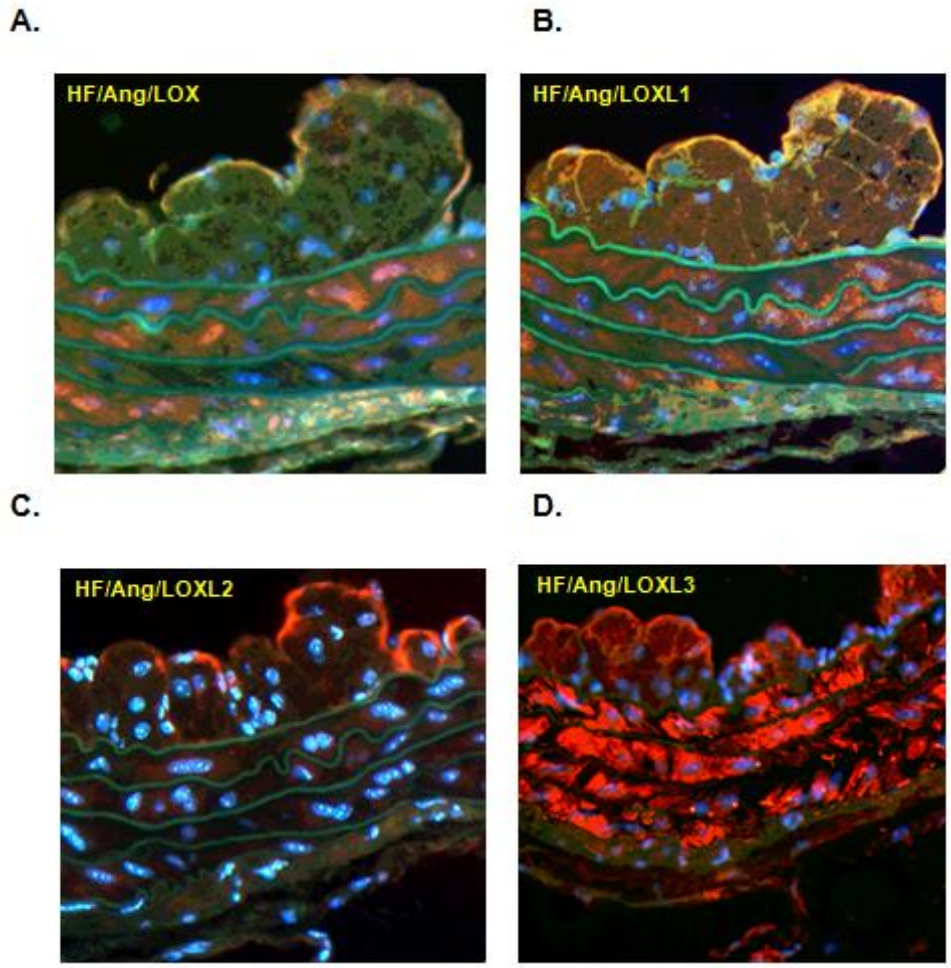


Figure 12. Localization of LOX/LOXL proteins in aortic tissue from ApoE$^{-/-}$ mice after 8 weeks.

Protein expression is concentrated in the media, neointimal, and endothelial layers for LOXL1 and LOXL3 (panel B&D). Expression of LOX is present in the medial layer and appears in the endothelial cell layer, but is absent from the neotintima. (panel A) LOXL2 staining is present most abundantly within the endothelial cell layer as shown in panel C. Antibody staining in red.

LOX/LOXL activity protects against abdominal aortic aneurysm formation

To determine the contribution of total LOX/LOXL activity to the stabilization of the aortic wall during AAA formation, we measured AAA incidence 4 weeks after treatment in the $ApoE^{-/-}$ mice in the following settings: 1) Vehicle alone 2) β-APN alone 3) Ang II + high fat diet and 4) Ang II + high fat diet and β-APN. β-APN is a known general LOX/LOXL inhibitor and did not affect blood pressure (data not shown). Aneurysmal dilation, defined as suprarenal circumference doubling, was most pronounced in animals treated with Ang II + high fat and β-APN. (Figure 13 and 14A) Within these groups the frequency of large aneurysm formation was greater than 90% and the high fat/Ang II/β-APN group was associated with 60% mortality. (Figure 13 and 14A) Necropsies revealed aortic rupture as the cause of death. Mice fed a low fat diet alone for 4 weeks +/- vehicle or β-APN did not demonstrate an increased frequency of aneurysm formation. Longer time points were not studied because of the high rate of mortality in the animals treated with β-APN. These data suggest that LOX/LOXL activity is critical for the crosslinking mechanisms that preserve vessel wall integrity during Ang II infusion.

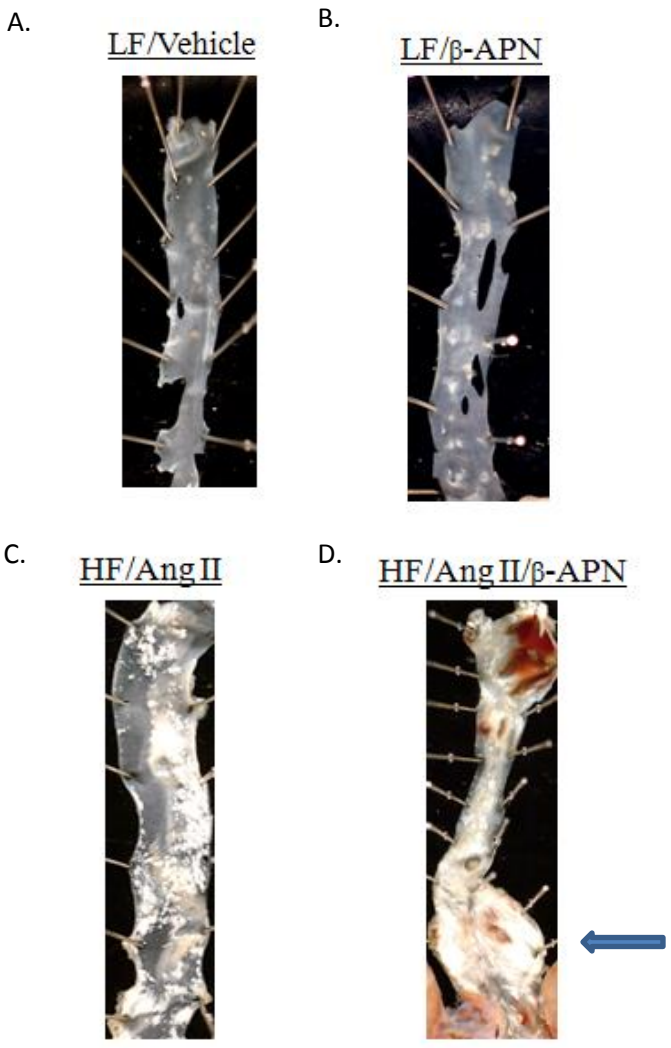
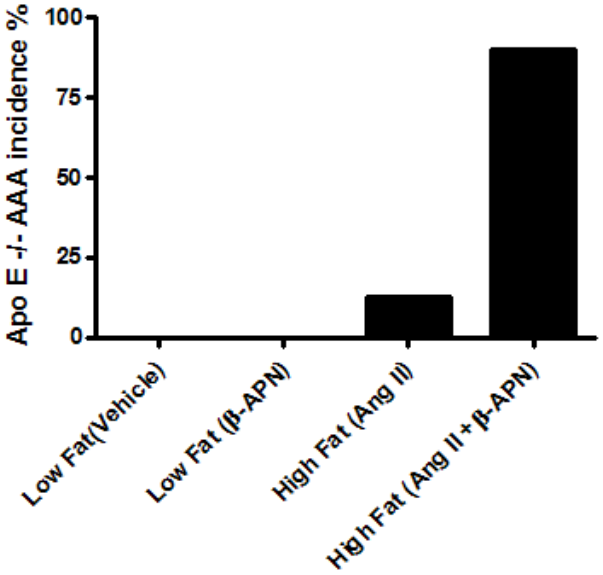


Figure 13. Effect of β-APN on AAA incidence and atherosclerotic lesion area in ApoE$^{-/-}$ mice on a high fat diet with Ang II infusion.

Representative examples of ApoE$^{-/-}$ mice treated with the lysyl oxidase inhibitor β-APN + Ang II. Panel A is a low fat aorta treated with vehicle. Panel B is a low fat aorta treated with β-APN. There is no lesion formation or AAA. Panel C is a high fat aorta treated with Ang II and there is significant lesion formation. Panel D is a high fat aorta with Ang II and β-APN co-treatment depicting a prominent AAA.

A.



B.

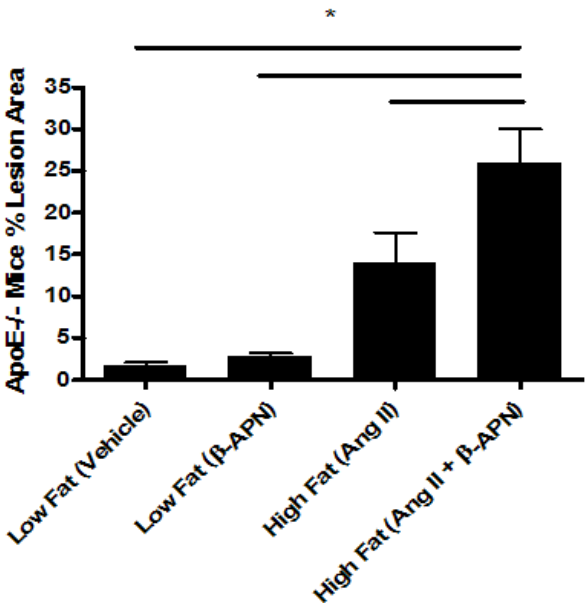


Figure 14. Effect of β-APN on AAA incidence and atherosclerotic lesion area in ApoE$^{-/-}$ mice on a high fat diet with Ang II infusion.

A.) Incidence of AAA in all groups of ApoE$^{-/-}$ mice. B.) Percent lesion area per total *en face* aortic area. *$p<.001$

LOX/LOXL activity protects against Ang II induced atherosclerotic lesion formation

To determine the functional importance of lysyl oxidase family member activity in maintaining the structural organization of the vasculature and in protection against disease, we measured the atherosclerotic lesion area in the aorta after 4 weeks of control diet and after treatment with Ang II + high fat with and without β-APN in ApoE$^{-/-}$ mice. Ang II+ high fat treatment induced a significant increase in atherosclerotic lesion formation above control (Figure 14B). Conversely, mice infused with β-APN alone did not demonstrate an increase in lesion formation. However, when β-APN infusion was combined with Ang II + a high fat, lesion formation increased significantly over control mice, covering nearly 35% of the aortic surface area. This dramatic increase in lesion formation demonstrates the deleterious effects of a damaged matrix on vascular disease and illustrates the protective action of LOX/LOXL against atherosclerotic lesion formation driven by an Ang II induced mechanism.

We also investigated the less atheroprone but not atheroprotected C57BL/6 mice in the same manner as the atherogenic ApoE$^{-/-}$ mice. The results of these experiments follow a similar trend as the ApoE$^{-/-}$ mice in that Ang II was sufficient to cause AAAs in some of the mice on a high fat diet, but dual treatment with Ang II and β-APN caused further induction of AAAs, although not to the same degree as in the ApoE$^{-/-}$ mice. (Figure 15 and 16A) Lesion area was collectively much lower in these mice in all treatment groups, as compared to the ApoE$^{-/-}$ mice and was not affected by β-APN treatment. (Figure 16B)

To further investigate the mechanism by which LOX/LOXL family members protect against AAA, the previously established atheroprotected BalbC mouse model (Stein*, et al.* 2002) was treated using the same experimental strategy as described above for the atherogenic ApoE$^{-/-}$ mice. As expected, neither Ang II alone, nor β-APN alone induced lesion formation. Ang II alone was not sufficient to produce any AAAs in the BalbC mice. However, the combination of both Ang II and β-APN induced significant aortic dilation above control (Figure 17 and 18) suggesting further that LOX/LOXL activity strongly counteracts Ang II induced vascular remodeling at the matrix level and is protective against AAA formation. These data also suggest that while the presence of atherosclerotic lesion formation greatly increases the incidence of AAA formation, it is not necessary, as AAAs were induced in C57Bl/6 and BalbC mouse models which have minimal or no atherosclerotic disease.

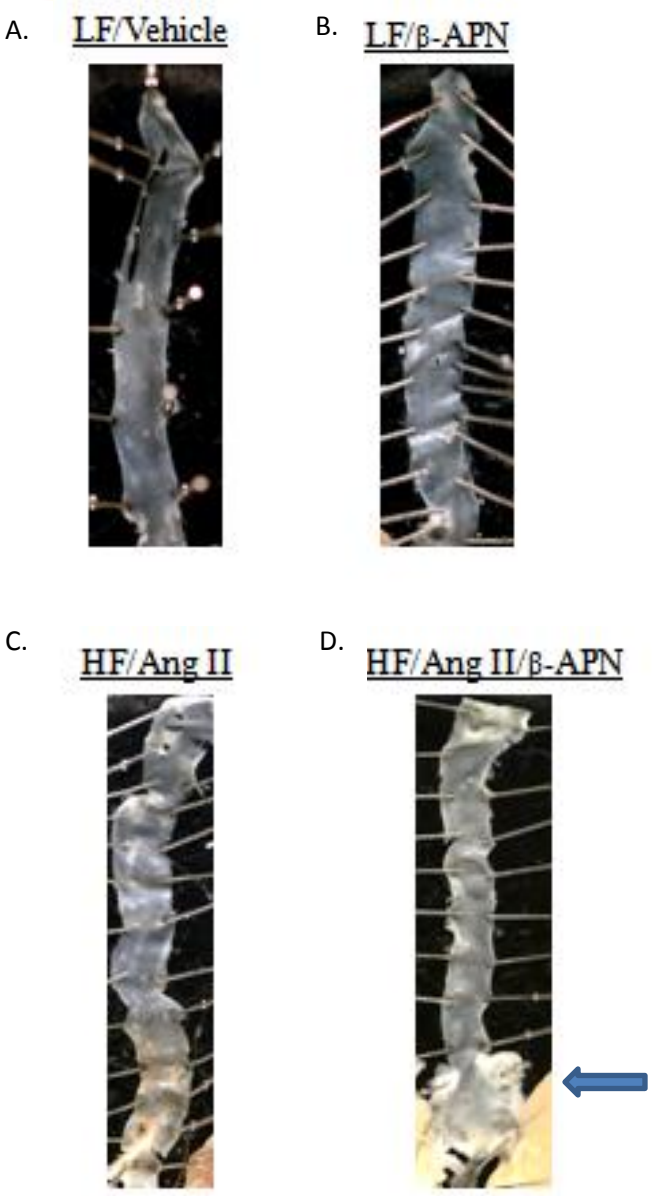
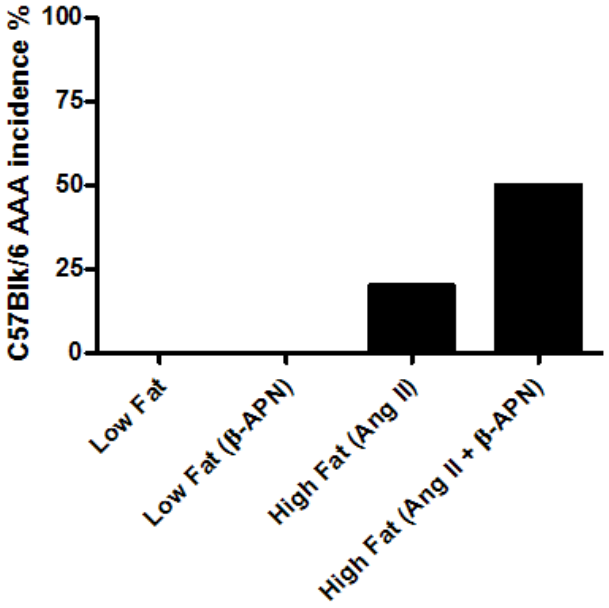


Figure 15. Effect of β-APN on AAA incidence and atherosclerotic lesion area in C57BL/6 mice on a high fat diet with Ang II.

Representative examples of C57BL/6 mice treated with the lysyl oxidase inhibitor β-APN + Ang II. Panel A is a low fat aorta treated with vehicle. Panel B is a low fat aorta treated with β-APN. Panel C is a high fat aorta treated with Ang II. There is no lesion formation or AAA. Panel D is a high fat aorta with Ang II and β-APN co-treatment showing a small AAA.

A.



B.

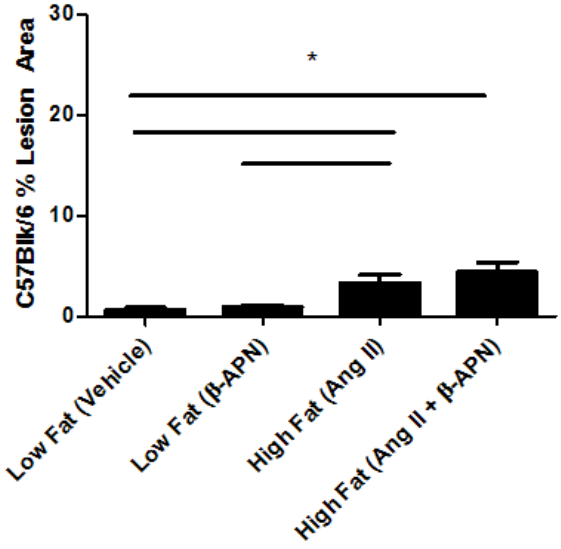


Figure 16. Effect of β-APN on AAA incidence and atherosclerotic lesion area in C57BL/6 mice on a high fat diet with Ang II.

A.) Incidence of AAA in all groups of C57BL/6 mice. B.) Percent lesion area per total en face aortic area *$p=.05$

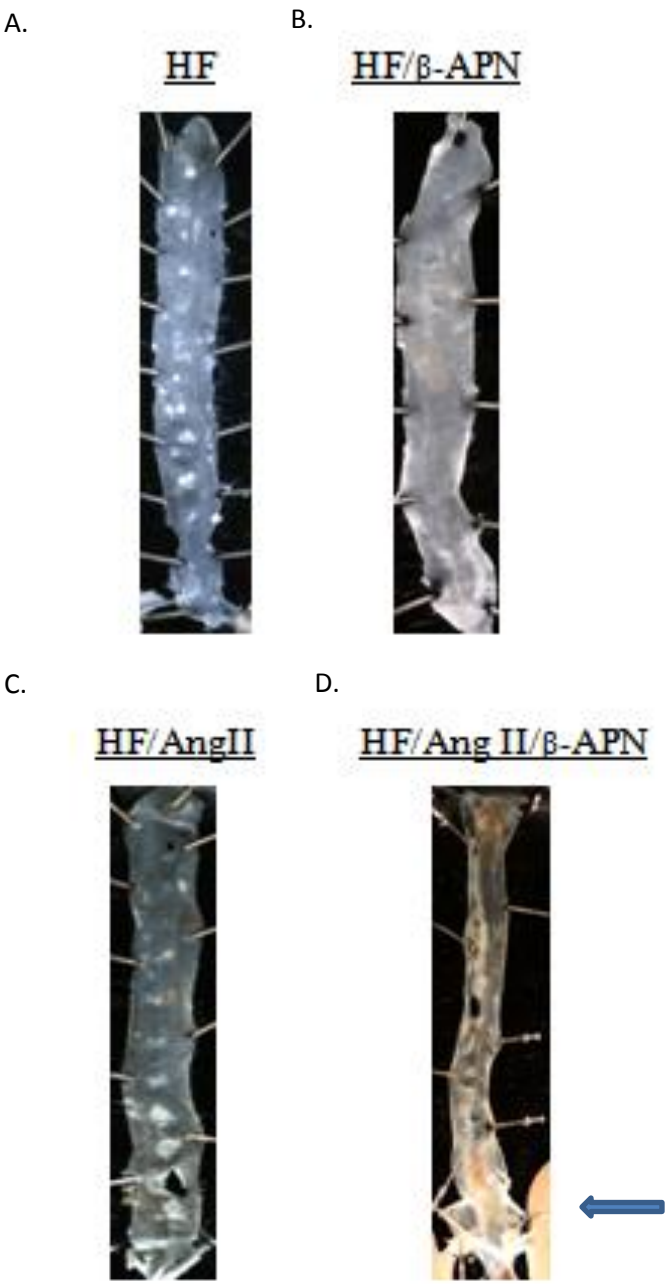


Figure 17. Effect of β-APN on AAA incidence and atherosclerotic lesion area in BalbC mice on a high fat diet with Ang II.

Representative examples of BalbC mice treated with the lysyl oxidase inhibitor β-APN + Ang II. Panel A is a low fat aorta treated with vehicle. Panel B is a low fat aorta treated with β-APN. Panel C is a high fat aorta treated with Ang II. There is no lesion formation or AAA. Panel D is a high fat aorta with Ang II and β-APN co-treatment showing a small AAA.

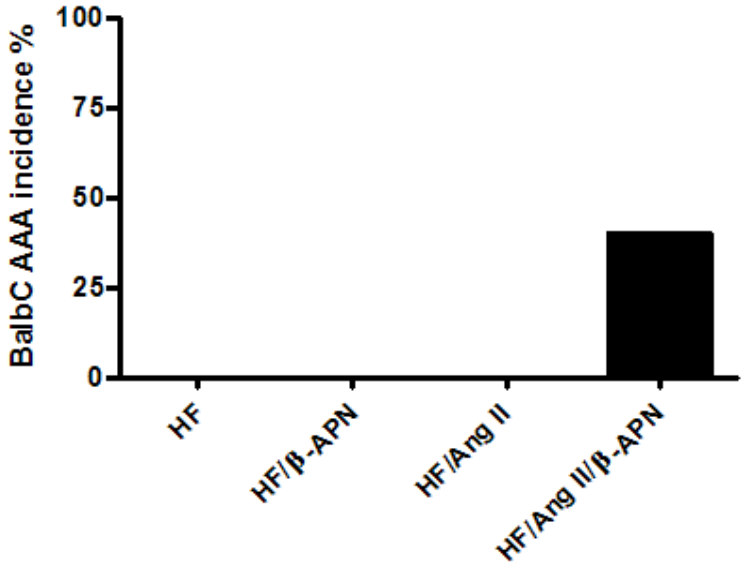


Figure 18. Effect of β-APN on AAA incidence and atherosclerotic lesion area in BalbC mice on a high fat diet with Ang II.

Incidence of AAA in all groups of BalbC mice. Lesion area was low/to undetectable.

Discussion

We have shown that LOX/LOXL family members are expressed in the aortic wall of normal and Ang II treated mice. We have further demonstrated that while there may be spatial differences in protein expression, global LOXL3 mRNA is upregulated at the mRNA level but not at the protein level in ApoE$^{-/-}$ mice fed an atherogenic diet 7 days after treatment with Ang II. To elucidate the functional significance of the LOX/LOXL family members in the ApoE$^{-/-}$ mice fed a high fat diet + Ang II, we inhibited LOX/LOXL activity with β-APN and observed accelerated AAA development in the setting of atherosclerosis. This suggests that the crosslinking activity of the LOX/LOXL family members is a necessary compensatory mechanism for vessel stability during vascular disease. To further demonstrate that LOX/LOXL family members are crucial for maintaining matrix stability, we recapitulated this model in less atherogenic prone C57BL/6 and BalbC mice. These mice developed AAAs, although their atherosclerotic lesion area was limited. These results point to the critical significance of LOX/LOXL family members in vessel stability independent of atherosclerotic disease.

The connection between Ang II, LOX/LOXL and AAA formation has yet to be completely elucidated. However, we can speculate on how these factors work synergistically to promote AAA formation. It is noteworthy that lesion area in the C57BL/6 and BalbC mice treated with Ang II was low (<10%) compared to the ApoE$^{-/-}$ mice treated with Ang II (20-30%). It is plausible that Ang II's contribution to AAA is through the induction of atherosclerotic lesion formation which ultimately destabilizes the vessel wall leading to dilation. This instability was

more pronounced in our most atheroprone mice, which could explain why more ApoE$^{-/-}$ mice develop AAAs with Ang II treatment alone. The rank order of atherosclerotic potential in our mouse models is ApoE$^{-/-}$ > C57BL/6 > BalbC. As such, we saw AAA formation with Ang II alone only in more atheroprone models and when lesion area was more severe. Inhibition of LOX/LOXL was also capable of causing instability in the vessel wall, but not in healthy adult mice. The combination of these factors (Ang II + high fat diet and β-APN) promoted AAA formation regardless of the atherosclerotic potential of the mouse model.

It is possible that the Ang II effect on LOX/LOXL mRNA expression is a compensatory mechanism invoked to stabilize aortic structure. The catalytic function of LOX/LOXL in modifying proteins responsible for tensile and elastic strength of aortic tissue suggests a potentially important role in aneurysm formation. It is likely that inhibition of LOX/LOXL family members by β-APN induced AAA in our animal model because there is a balance that exists between ECM proteolytic activity and LOX/LOXL activity, and we altered that early on. We suggest that tipping the balance in favor of MMPs can lead to expansion of AAA while tipping the balance in favor of LOX/LOXL can stabilization of the aortic wall. It has been reported that Ang II causes the activation and/or release of MMPs that are capable of degrading collagen and elastin fibers (Arenas*, et al.* 2004; Wang*, et al.* 2005). β-APN treatment in combination with Ang II prevented LOX/LOXL crosslinking activity, shifting the balance in favor of aortic dilation.

To date, LOX/LOXL family members are the only known enzymes responsible for the covalent crosslinking of soluble collagen and elastin into

protease resistant fibers. LOX activity has been demonstrated to be crucial for matrix development during gestation and post-natal development (Maki*, et al.* 2002; Siegel 1979). Although synthesis of elastin into concentric laminae during development is defined, synthesis of mature elastin during adulthood is quite limited (Perrin and Foster 1997; Shapiro*, et al.* 1991). Furthermore, our studies show that inhibition of LOX/LOXL activity in healthy adult animals is not associated with cardiovascular disease, implying that LOX/LOXL is crucially important during times of growth and remodeling. These results were previously suggested by Kanematsu using C57BL/6 mice that were treated with Ang II and β-APN which demonstrated that the hypertensive/hemodynamic effects of Ang II along with LOX/LOXL inhibition via β-APN contribute to the formation of AAAs. (Kanematsu*, et al.*)

Using immunohistochemistry, we have shown that LOX, LOXL1, LOXL2 and LOXL3 are expressed at the protein level and are regulated at the mRNA level by Ang II in the aortic wall. Western blot analysis did not suggest regulation in the global amount of LOX/LOXL family members after 7 days of Ang II treatment. We have further demonstrated that LOX/LOXL activity is crucial in the setting of Ang II induced vascular disease independently of atherosclerotic lesion formation. As such, therapeutic strategies to overexpress LOX/LOXL enzymes or to support the crosslinking of soluble matrix proteins in a polymeric scaffold are a promising opportunity to achieve stabilization of AAA.

In conclusion, these data in $ApoE^{-/-}$, C57BL/6 mice and BalbC mice establish that the activity of the LOX/LOXL family imparts critical structural

integrity to the vascular wall. Inhibition of LOX/LOXL in these models generates a novel insight to study AAA development from the standpoint of LOX/LOXL activity.

Grants

This work is supported by NIH R01 HL 70531 and HL 090584

Part II. The Regulation of LOX/LOXL family member expression in Vascular Smooth Muscle Cells by Angiotensin II

Ebony Washington Remus, Aaron Fan, Diane Sutcliff, Robert W. Taylor

Introduction

As described in part one of Chapter 2, one of the Abdominal Aortic Aneurysm (AAA) models used in our lab is an Apolipoprotein E knockout ($ApoE^{-/-}$) mouse fed an atherogenic diet and infused with Ang II (0.75mg/kg/day) via an osmotic mini-pump. This model was originally developed to study atherosclerosis. (Weiss*, et al.* 2001) ApoE is essential for the transport and catabolism of triglyceride rich lipoproteins; therefore, $ApoE^{-/-}$ mice have elevated levels of circulating cholesterol. Ang II treatment causes vasoconstriction and hypertension while inducing the expression of a wide range of inflammatory genes involved in atherosclerosis (e.g. IL-6, PAI-1, VCAM-1, and MCP-1) (Modlinger*, et al.* 2006). In an unexpected observation, we showed that approximately 20% of these atherosclerotic mice developed AAA after 8 weeks of treatment as demonstrated in Figure 14A. The aneurysms were supra-renal in location and occurred more commonly in male mice 16 weeks and older. Histologically, these aneurysms were very similar to those seen in human specimens. Microarray analysis on the aortas of mice treated with Ang II and a high fat diet for 2 weeks showed upregulated LOX expression when compared to control mice (unpublished lab data). The microarray data did not discern which LOX/LOXL family members were upregulated in this model. After infusing these mice with β-APN (a potent inhibitor of LOX activity), we found that 90% of the mice had AAA by 28 days, as shown in Figure 14 of chapter 2. This finding indicates that LOX/LOXL family members are essential for maintaining vascular wall integrity and protection from aneurysmal dilatation and may represent an

important compensatory mechanism for the stabilization of AAA. Furthermore, these data suggest that the regulation of LOX/LOXL family members may represent an important therapeutic strategy for preventing expansion and rupture of AAA. Therefore, it is of critical importance to better understand the regulation of LOX/LOXL family members in the setting of AAA formation.

Five different LOX enzymes in both humans and mice: Lysyl Oxidase (LOX) and Lysyl Oxidase Like isoenzymes (LOXL)-1, -2, -3 and -4, collectively termed LOX/LOXL. LOX/LOXL enzymes are a family of copper-containing monoamine oxidase enzymes found in the extracellular matrix (ECM). LOX/LOXL catalyzes the conversion of soluble collagen and elastin to insoluble forms resistant to proteolytic degradation. Aortic wall ECM degeneration has been shown to occur during the development of AAA. Ang II-induced upregulation of LOX/LOXL may represent a counter-regulatory mechanism against matrix degeneration and subsequent formation of AAA.

Despite the fact that immunohistochemistry revealed the most abundant expression of LOX/LOXL family members in the media and neointima of atherosclerotic mice (Figure 12), we did not observe any changes in LOX/LOXL protein expression in mouse aortas after Ang II treatment by Western blot. During the development of atherosclerosis, a neotintimal layer is formed between the smooth muscle cell (medial) layer and the endothelial (intimal) layer, termed the neotintima. The neointima is distinct from the preexisting intima because of its composition of migratory SMCs from the medial layer. (Majesky 1994) Because both the media and the neointima contain primarily smooth muscle

cells, we sought to determine the direct effect of Ang II on VSMC using an *in vitro* cell culture approach.

Using aortic vascular smooth muscle cells (VSMCs) as an experimental system, we attempted to identify basal and Ang II regulated expression of LOX/LOXL family members, as well as any changes in LOX/LOXL activity in response to Ang II. We choose this simplified cell culture system, to better elucidate whether Ang II could directly upregulate LOX/LOXL family members, without the complication of other cell types and modulators.

Methods

Cell culture

VSMCs generously provided by Dr. Kathy K. Griendling PhD, Emory University, Atlanta GA, were obtained as previously described. (Griendling*, et al.* 1991) Briefly, VSMCs were enzymatically extracted from rat thoracic aortas and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 units/mL penicillin and 100ug/mL streptomycin and seeded into 75 cm2 flasks. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells were used between passages 6 and 15 for all experiments and were seeded into 100mm dishes, fed on alternate days. Prior to experimental stimulation, cells were serum deprived for 48hrs to induce quiescence (growth arrest) and stimulated with 100ng/ml Ang II (Sigma) for indicated time points.

Quantitative Real Time-PCR

RNA was purified using a commercial kit (Qiagen - Valencia, CA). RNA was reverse transcribed into cDNA using the SuperScript™ III First Strand Synthesis System (Invitrogen – Grand Island, NY), which was subsequently purified using a commercially available kit (Qiagen - Valencia, CA). Primers for all of the LOX/LOXL family members and 18s were designed using PrimerQuest software (IDT DNA technologies). Our lab developed and confirmed a panel of primers for detection of LOX/LOXL family members in RASMs. (Table 2) SYBR Green intercalating dye (Applied Biosystems – Carlsbad, CA) was used to perform real time PCR with using the StepOnePlus PCR (Applied Biosystems – Carlsbad, CA). Standards for each gene were amplified from cDNA and purified. Standard concentrations were determined using spectrophotometric measurement at 260 nm, and standards were serially diluted to an appropriate range of concentrations. Transcript concentration in template cDNA solutions was quantified from the linear standard curve, normalized to 18s rRNA copy number, and expressed as copies per 10^6 18s. Ang II treatment has been shown to stimulate expression of Plasminogen Activator Inhibitor type 1 (PAI-1), the main inhibitor of the fibrinolytic system. Northern blot analysis demonstrated a several fold increase in PAI-1 mRNA 3 to 8 hours after stimulation with 300 nmol/L Ang II in RASMs. (van Leeuwen*, et al.* 1994) Therefore, PAI-1 will be used as a positive control for Ang II treatment and quiescence of RASMs.

Western blotting

Cell culture protein samples were lysed in radio-immunoprecipitation assay buffer (RIPA) lysis buffer (Santa Cruz - Santa Cruz, CA) with supplied protease inhibitors and rocked with end-over-end rotation at 4°C for 15min to allow for complete cell lysis. This was followed by centrifugation at 10,400xG at 4°C for 10 min. Protein concentration was determined using a standard Bradford Assay (Biorad – Hercules, CA). Samples containing 50ug of protein were loaded onto 10% SDS polyacrylamide gels and subsequently blotted onto nitrocellulose (Amersham - Piscataway, NJ). Membranes were blocked with 5% milk (Santa Cruz – Santa Cruz, CA). LOX/LOXL protein expression was assessed with antibodies from Novus - Littleton, CO (LOX - NB110-59729) Santa Cruz (LOXL1 – SC48720 and LOXL2 - SC48724) or an antibody previously developed in our lab (LOXL3). HRP conjugated Anti-goat or anti rabbit secondary was used with an ECL (GE Healthcare) Western blotting system for signal detection. Data was normalized to β-actin (Cell Signaling - Beverly, MA).

LOX/LOXL Activity Assay

General LOX/LOXL activity of whole cell lysates was measured using a modified Amplex Red Florescence Assay, as previously described (Molecular Probes, Eugene, OR). (Payne*, et al.* 2005) Proteins in their native confirmation were isolated from VSMCs using mammalian protein extraction reagent (MPER)

(Thermo Scientific, USA) and then 50ug was added to a reaction mix and incubated at 37˚C for measurement of activity. The assay reaction mixture consisted of 50 nmol/L sodium borate (pH 8.2), 1.2 mol/L urea, 50umol/L Amplex Red, 0.1 units/mL horseradish peroxidase, and 10mmol/L 1,5-diaminopentane as substrate. In this assay, the substrate dictates specificity for LOX/LOXL family members. To further evaluate specific LOX/LOXL activity, samples were treated with .5M β-APN (a specific inhibitor of LOX/LOXL activity) to calculate β-APN inhibitable activity. The fluorescent product was excited at 560 nm and the emission read at 590 nm.

Table 2. LOX/LOXL primer sequences

	Forward	Reverse	Size
LOX	cctggtacccgatccctact	gcggcttggtaagaagtcag	202
LOXL1	acttgcctgtgcgaaactct	cctgcacgtagttgggatct	149
LOXL2	caaccccaaagcctacaaaa	ctggctaggcacacaactga	190
LOXL3	atgactggggaacactggag	ttgtggtgagcacattggtt	175
LOXL4	ccaacaacatgattcgatgc	tggctccagtaagctgaggt	200
18S	gaacgtctgccctatcaact	ccaagatccaactacgagct	345

Results

Ang II has variable effects of LOX/LOXL family members at the mRNA level in VSMCs

Contrary to the *in vivo* model, where only LOXL3 is upregulated in the whole aorta at the mRNA level, we show *in vitro* that, LOXL1 and LOXL2 mRNA levels were upregulated in response to Ang II at 24 hours (Figure 20) as measured by qRT-PCR and normalized to 18S. LOXL2 expression increased by 2-fold in response to Ang II, while LOXL1 increased by just under 20% at 24 hours. Prior to 24 hours, LOX/LOXL family member mRNAs were unchanged in response to Ang II. (Figure 19) It is worth noting that among the family members, LOX is the most abundant family member in quiescent cultured VSMCs, followed by LOXL3, LOXL1 and LOXL2, as shown in Figure 21. LOXL4 was undetectable by qRT-PCR. To verify that the cells were responding to Ang II, we measured the expression of PAI-1 and MCP-1 mRNA, 4 hours after Ang II stimulation and found increases in their mRNA levels, as expected based on previous literature.(Touyz and Schiffrin 2000; van Leeuwen*, et al.* 1994) (Figure 22)

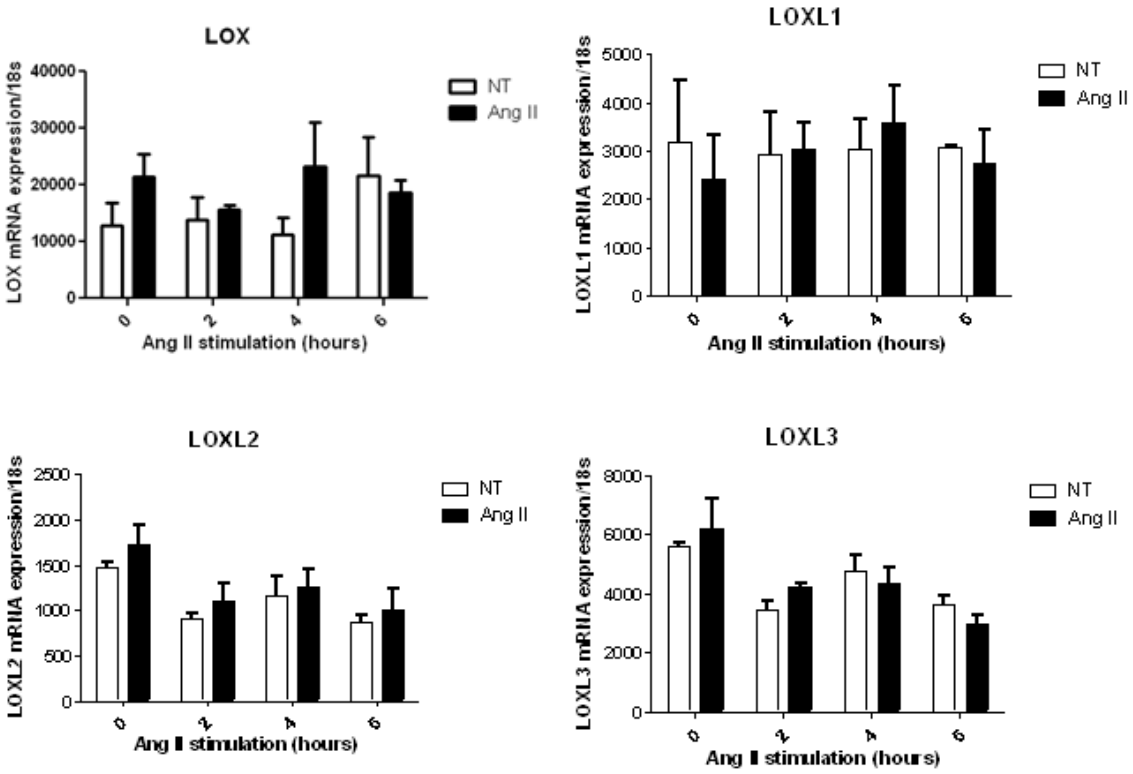


Figure 19. Ang II has no effect of LOX/LOXL family members mRNA steady state levels in VSMCs early after stimulation.

Ang II did not increase expression of any LOX/LOXL family members at early time points. NT represents non treated samples. (*n = 3). Differences are not significant*. LOXL4 was undetectable by qRT-PCR in VSMCs.

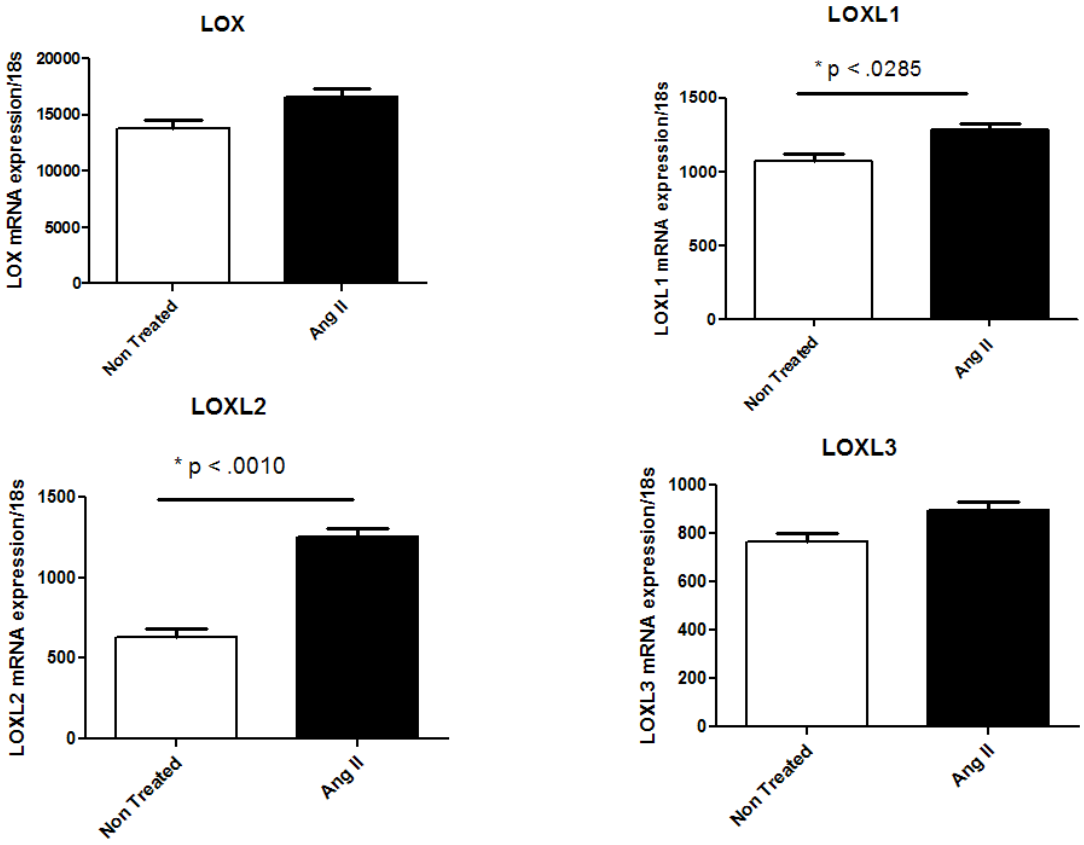


Figure 20. Effect of Ang II on LOX/LOXL family members steady state levels at 24 hrs.

Ang II increased LOXL1 and LOXL2 mRNA expression 24 hours post stimulation. Ang II had no effect of the LOX or LOXL3 expression. *n = 3. p < .05*

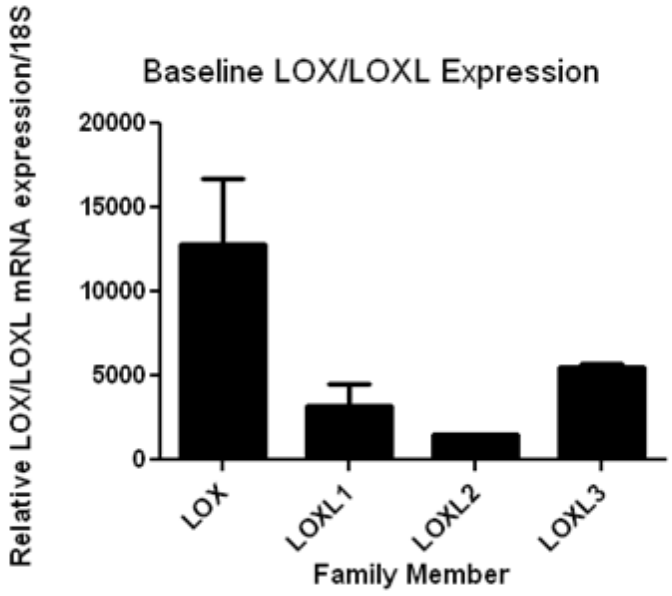


Figure 21. LOX/LOXL expression in quiescent VSMCs.

LOX expression was the most abundant mRNA expression at baseline with 3 times as many copies as compared the other LOX/LOXL family members. LOXL3, LOXL1 and LOXL2 followed from highest to lowest in copy. These relative expression patterns were similar to those observed *in vivo*. LOXL4 was undetectable in VSMCs by qRT-PCR. *n = 3*

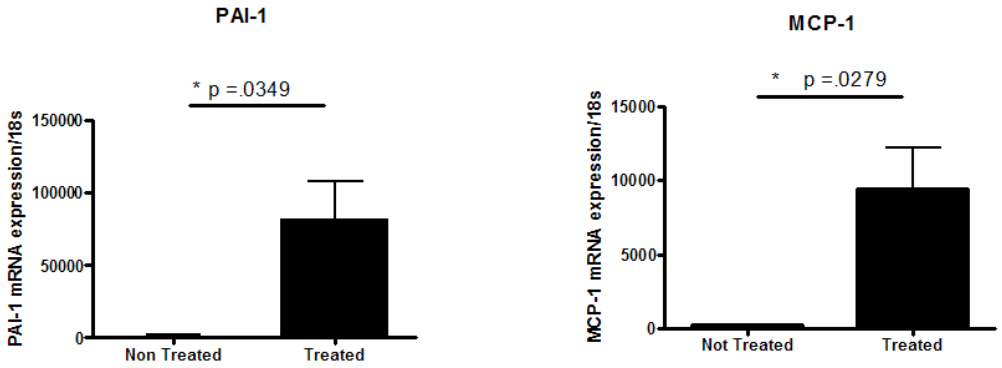


Figure 22. Ang II induced expression of PAI-1 and MCP-1 mRNA after 4 hours of Ang II treatment compared to non-treated cells.

This demonstrated that the VSMCs were quiescent and responded to Ang II stimulation. *n = 3 p < .05*

Ang II has no effect of LOX/LOXL protein expression in cultured VSMCs

We next sought to determine whether Ang II alters protein levels of the LOX/LOXL family members. We showed that in VSMCs Ang II had no effect on protein expression of any of the LOX/LOXL family members at 24hrs or 48hrs, as measured by Western blot (Figure 23). In addition, the levels of LOX/LOXL in the concentrated media were not affected by Ang II (data not shown).

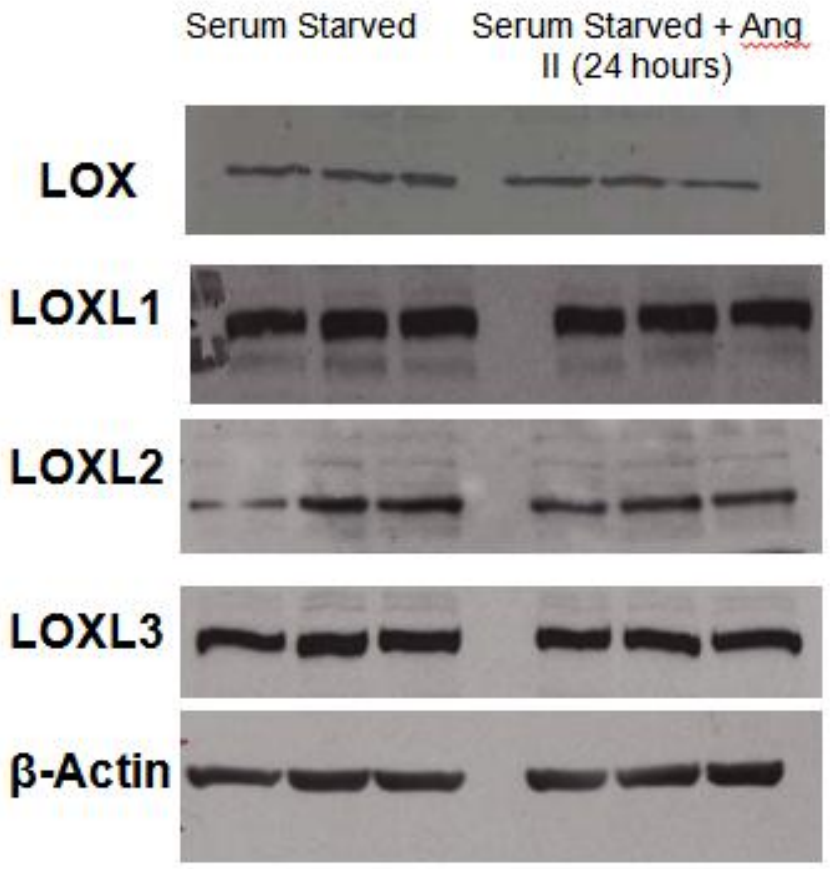


Figure 23. Ang II had no effect on LOX/LOXL protein expression as detected by Western blot after 24 hrs.

Lanes represent individual LOX/LOXL family member expression in 3 independent experiments for each treatment group (serum starved vs Ang II treated cells). All bands were normalized to β-actin.

Ang II does not change LOX/LOXL activity

Next we sought to determine whether treatment with Ang II modulates the function of LOX/LOXL isoenzymes. We showed that Ang II had no effect on total LOX/LOXL activity after 24 hours of stimulation, by using a specific LOX/LOXL activity assay that measures H_2O_2 production as a byproduct of LOX/LOXL activity. (Figure 24) The LOX/LOXL family members are secreted and packaged into intracellular vesicles for secretion. However there is evidence that the LOX/LOXL enzymes can reenter the cell and concentrate within the cell nuclei and cytoplasm. (Lucero and Kagan 2006) Because of this, we measured LOX/LOXL activity in cell lysates from membrane (insoluble) and non-membrane (soluble) fractions. While we observed a trend for an increase in LOX/LOXL activity in the insoluble fraction, this result was not statistically significant. ($p=.095$) We did not measure activity of secreted LOX/LOXL in response to Ang II in the cultured media. Based on these data, we concluded that treatment with Ang II does not change the activity of the LOX/LOXL isoenzymes.

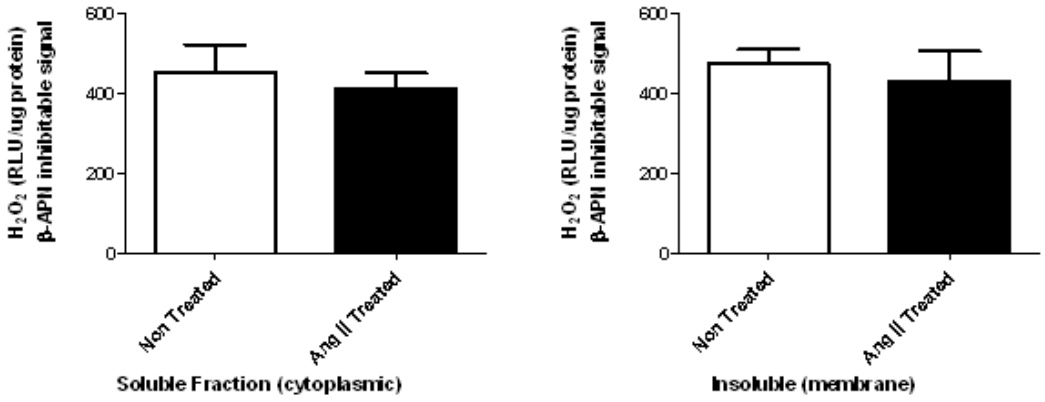


Figure 24. Ang II had no effect on total LOX/LOXL activity as measured using an Amplex Red based assay for quantification of H_2O_2 production.

The LOX/LOXL specific substrate 1,5-diaminopentane, as well as a specific inhibitor of LOX/LOXL: β-APN was used to quantify H_2O_2 production as relative light units (RLU) per 50µg of total protein.

Discussion

Ang II exerts many effects on the vasculature, as discussed in Part I of Chapter II. Among the pathophysiological effects of Ang II are atherosclerotic lesion formation and vascular remodeling, leading to the development of atherosclerosis and AAA formation in $ApoE^{-/-}$ mice. A rapid and more robust increase in AAA development was discovered when treating the same mouse model with β-APN, a specific inhibitor of all LOX/LOXL isoenzymes, thus suggesting a role for LOX/LOXL in maintaining vascular structure and integrity. Our goal was to understand LOX/LOXL expression and regulation, as a mechanism for increasing the expression and activity of LOX/LOXL within the vasculature in order to stabilize the vascular wall and prevent AAA rupture. Data from our lab suggest that LOXL3 expression is upregulated early after treatment, as measured by qRT-PCR on aortas of $ApoE^{-/-}$ mice treated with Ang II and a high fat diet. This finding led to the hypothesis that the upregulation of LOX/LOXL family members may be a compensatory mechanism in response to injury in order to maintain the integrity of the vascular wall. We set out to examine this upregulation directly in cultured VSMCs in response to Ang II, as a more direct approach to studying LOX/LOXL regulation. *In vivo*, there are multiple effectors and cell types that may be responsible for changes in LOX/LOXL expression. As such, we choose VSMCs because of their abundance in the vascular wall and Ang II because of its major role in the formation of AAAs. We show that Ang II has no effect on LOX/LOXL protein expression or activity in VSMCs. Interestingly, we also find that Ang II increased the steady state level of

LOXL1 and LOXL2 mRNA. This is comparable to what was found *in vivo*, in that Ang II had no effect on protein expression, but upregulated LOXL3 mRNA levels. The discrepancy may be due to a more complex regulation *in vivo* that comes from multiple cell types and/or effector molecules. *In vivo,* the aortic wall is composed of VSMCs, endothelial cells, fibroblasts and immune cells, such as macrophages that can infiltrate the site of injury. From these data, we conclude that Ang II does not change LOX/LOXL protein expression *in vivo* or *in vitro.* Future directions will seek to investigate mechanism to increase LOX/LOXL protein expression in response to Ang II.

In addition to evaluating LOX/LOXL protein expression in VSMCs, we investigated LOX/LOXL protein expression in other cultured cell types present within the vascular wall, including macrophages and endothelial cells. We found expression of the LOX/LOXL family members in both cell types. In actively dividing macrophages, we found expression of LOX and LOXL3, and in endothelial cells isolated from human umbilical veins we found expression of LOX, LOXL1, LOXL2 and LOXL3 as shown in Figure 25. From these results we speculate that Ang II may affect LOX/LOXL protein expression in other cells types such as macrophages or endothelial cells.

The simultaneous evaluation of LOX/LOXL expression with an *in vivo* and *in vitro* approach allowed us to exploit the strengths of each model system. *In vivo* it is difficult to completely define a direct relationship between a stimulus and a protein. An *in vitro* system allows direct interactions between a stimulus and a protein to become highly amplified within a specific setting. The limitation of an

in vitro system is that it is an artificial representation of a more complex model and sometimes multiple effectors or cells types are necessary to observe changes in protein, or the wrong cell type was evaluated for *in vitro* studies.

Altogether our *in vivo* and *in vitro* models, allowed us to conclude that Ang II has no effect on the protein expression of LOX/LOXL family members in the vascular wall. However, expression may be spatially regulated in response to Ang II or a combination of other factors. Evidence for spatial regulation comes from immunohistochemistry on mouse aortas showing differential expression of LOX/LOXL within the vascular wall after Ang II treatment. For example, LOX and LOXL3 were the only family members expressed in the neointimal layer, suggesting that they are involved in the migratory actions of VSMCs.

In addition to spatial regulation, we also postulated that LOX/LOXL activity is altered in the vascular wall in response to injury, resulting in AAA formation. This is evident because β-APN treatment with Ang II amplifies AAA formation. More specifically, Ang II may not be the correct or only stimulus that affects LOX/LOXL activity, as is suggested by our *in vitro* data. Reevaluating the stimulus responsible for changes in LOX/LOXL activity would be critical in further evaluating the regulation of LOX/LOXL family members. One approach would be to return to the *in vivo* model and measure total LOX/LOXL activity over time after stimulation with Ang II and a high fat diet, alone and in combination, in $ApoE^{-/-}$ mice. We speculate that there are modulations in LOX/LOXL activity over time, making it difficult to evaluate the activity of the LOX/LOXL isoenzymes *in vitro.*

Lastly, it is plausible that LOX/LOXL activity is not changed but rather imbalanced *in vivo*. As mentioned within the introduction, LOX/LOXL isoenzymes crosslink collagen and elastin and stabilize their presence with the ECM. The ECM is essential for the integrity of the arterial wall, including the abdominal aorta. Certain cell types, such as VSMCs can secrete collagen and elastin into the ECM. Under normal conditions, elastin and collagen fibers resist spontaneous breakdown due to their ability to form stable crosslinks with one another in the presence of LOX/LOXL. When a normal aorta becomes aneurysmal, matrix degeneration occurs. Previous studies have shown that in AAA, medial elastin degradation is responsible for weakening and dilatation of the aortic wall, while collagen degradation is connected to rupture. (Papalambros*, et al.* 2003) Several matrix metalloproteinase's (MMPs) aggravate matrix organization. (Longo*, et al.* 2002) Of the large family of MMPs, two are crucial to AAA formation: MMP-2 and MMP-9. Aneurysmal diameter and plasma levels of MMP-9 are elevated in patients with AAA. Equally important in AAA formation is MMP-2. MMP-2 is found primarily in SMCs and fibroblasts and is capable of degrading fibrillar collagens (collagen types I, IV, V and VII) and elastin. (Davis*, et al.* 1998) MMP-2 expression is increased in AAA tissues as compared to normal aortic tissue. (Longo*, et al.* 2002; Papalambros*, et al.* 2003) Both MMP-2 and MMP-9 knockout mice are resistant to aneurysm formation. (Longo*, et al.* 2002) MMP-2 and MMP-9 work synergistically to promote rapid degeneration of the ECM. A delicate balance seems to exist between matrix degeneration and matrix regeneration. Tipping the balance in favor of either may

cause AAA rupture or AAA stabilization. Therefore, another possible explanation is that matrix stability and regeneration may be mediated by the imbalance of activity between LOX/LOXL and MMPs. Because the activities of these two enzymes are measured using different assays, it would be difficult to compare activity amongst the two enzyme families in order to determine whether or not they are balanced.

Lastly, it is plausible that the LOX/LOXL family members are important yet not regulated in adult aortas. It is clear that the lack of regulation of the LOX/LOXL family members does not underscore its importance in the vascular wall, as seen upon inhibition of LOX/LOXL activity in an *in vivo* model. From these data we are able to separate regulation from importance and conclude that not every gene/protein/function is regulated, however, its presence may still be important.

Overall, we conclude that LOX/LOXL protein expression is unchanged in response to treatment with Ang II in aortas and in VSMCs. Future experiments need to investigate whether the observed increase in LOX/LOXL mRNA levels by Ang II infusion can result in increased LOX/LOXL protein levels. Our primary focus will be microRNAs, as they have been recently implicated in cardiovascular pathophysiologies. Ultimately, we postulate that increased LOX/LOXL expression and activity can stabilize AAAs and prevent rupture.

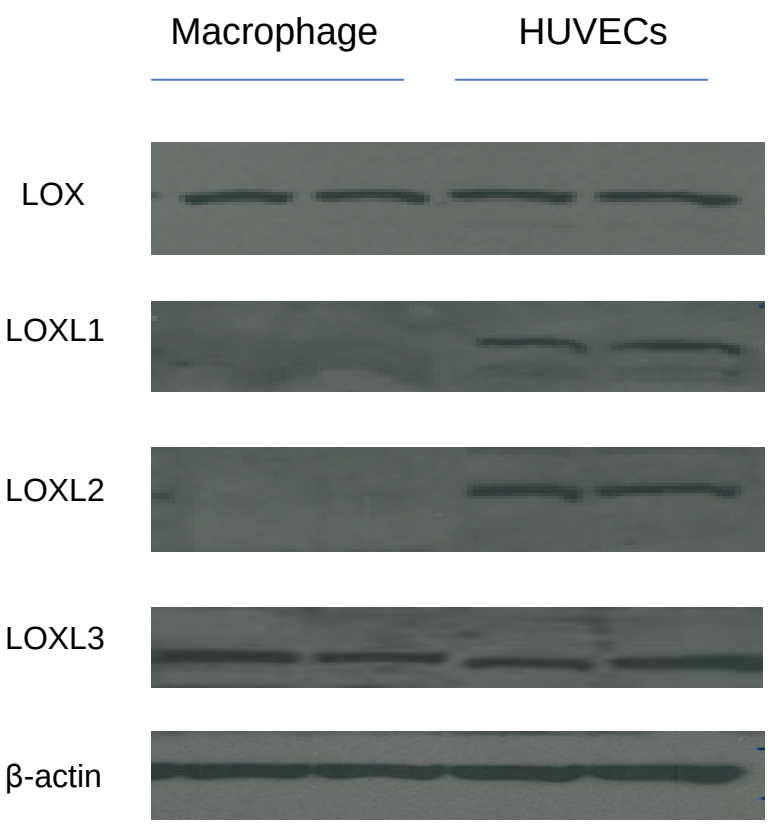


Figure 25. LOX family members are expressed in HUVECs and macrophages

Lanes represent 2 individual experiments for each cell type. By Western blot, all of the LOX/LOXL family members are detected in endothelial cells (HUVECs). Only LOX and LOXL3 are detected in macrophages by Western blot suggesting spatial regulation as a likely mode of regulation.

Chapter 3: The regulation of miRNAs by Angiotensin II in VSMCs

Ebony Washington, Diane Sutcliff, Martina Weber, Charles Searles, W. Robert Taylor

Abstract

Angiotensin II (Ang II) is a potent regulator of vascular disease and mediates its effects by promoting hypertrophy, migration and reactive oxygen species production in vascular smooth muscle cells (VSMCs). Recently miRNAs have been identified as critical regulators of protein expression in many disease processes, including cardiovascular disease. However, a link between Ang II and miRNAs in VSMCs has yet to be established. We identified candidate miRNAs that were regulated by Ang II 24 hours after treatment in VSMCs using microRNA expression profiling. Further qRT-PCR validation confirmed that Ang II induces expression of mir222, mir30b and mir125b greater than 1.5 fold while suppressing the expression of mir181a, and mir26b. mir26b is predicted to target Lysyl Oxidase like 2 (LOXL2), a gene whose steady state mRNA levels are increased by Ang II, with no subsequent increase in protein in VSMCs. Through the transfection of miRNA antagonist and overexpressors, we modulated the expression of mir26b, and explore that effect on protein expression of LOXL2. We report no significant change in protein expression of LOXL2 with miRNA manipulation. Future directions include further exploration of validated miRNAs and their regulation of protein expression. Using bioinformatics web based tools mir181a has been identified as a potential target for osteopontin (OPN); an Ang II regulated extracellular matrix protein, but has yet to be confirmed in VSMCs.

Introduction

We previously demonstrated in part 2 of Chapter 2 that in vascular smooth muscle cells (VSMCs) angiotensin II (Ang II) mediates an increase in certain Lysyl Oxidase (LOX/LOXL) family members (LOXL1 and LOXL2) at the mRNA level; however we observed no subsequent change in protein expression. This discrepancy in LOX modulation in response to Ang II, led us to further investigate potential mechanisms by which increases in mRNAs do not translate into changes in protein expression in response to a stimulus. It is to be noted that there are many layers of regulation at both the transcriptional and translational levels. Here we present three major categories. At the transcriptional level cell signaling events that lead to changes in transcription factor activation, mRNA splicing events, and poly-adenylation are some of the primary ways in which a transcriptional activation is silenced. At the translational level mechanisms of protein localization, modification and degradation are ways the cell silences protein expression. Lastly, in between transcription and translation are mechanisms by which a gene is still transcribed, yet not translated to protein, as described by microRNAs (miRNAs). (Chen and Rajewsky 2007) The effect of Ang II on many of these control mechanisms has been previously investigated; however, one potentially important and novel modulating mechanism that has not been investigated is the effect of Ang II on miRNAs within the vasculature, specifically VSMCs.

Ang II is involved in the pathogenesis of cardiovascular disease promoting hypertrophy and cell migration through the production of several second

messengers including increased intracellular Ca^{2+} produced as a consequence of phospholipase C activation, as well as reactive oxygen species (ROS) produced as a result of NADPH oxidase activation in VSMCs. (Mehta and Griendling 2007) Ultimately, these and other second messengers lead to phosphorylation events and transcription factor translocation into the nucleus for subsequent gene transcription. We, and others, have established a role for Ang II in modulating gene expression that contributes to vascular dysfunction. Campos *et al* identified a subset of six matricellular proteins, including osteopontin (OPN) and plasminogen activator inhibitor-1 (PAI-1) that are coordinately upregulated by Ang II, using microarray expression profiling in VSMCs. (Campos*, et al.* 2003) Campos's data, as well as recent data from our lab suggest that Ang II induces changes in vessel structure through its capacity to modulate the expression and/or activity of extracellular matrix proteins such as the family of LOX/LOXL and OPN. Current methods of regulating Ang II-mediated effects include the use of ACE inhibitors, to block the production of Ang II, as well as the use Angiotensin type 1 (AT1) receptor antagonists. Identification of novel methods for regulating Ang II induced ECM protein expression and/or function, in a more specific manner than currently employed, may prove to be useful in altering the pathogenesis and progression of cardiovascular disease. One potential target for new therapeutic development may be microRNAs (miRNAs), proposed to modulate expression at the post-transcriptional level by binding to target mRNAs and inhibiting translation in animal and plant genomes. (Chen and Rajewsky 2007)

Recently, miRNAs were identified as critical regulators of protein expression in various disease processes, including cardiovascular disease. More specifically, miRNAs have been implicated in cardiac and skeletal myogenesis, apoptosis, regeneration, hypertrophy, fibrosis and general cardiac function. (Daubman 2010) miRNAs are defined as family of small non protein coding single stranded RNAs that play an important role in the regulation of gene expression at the post-transcriptional level. (Slezak-Prochazka*, et al.* 2010) The first discovered miRNA was lin -4, which downregulates lin-14 protein expression in *Caenorhabditis elegans* during development. (Lee*, et al.* 1993) Currently 474 human miRNAs have been characterized; however, recent reports estimate that well over 1000 miRNAs exist in the human genome. (Catalucci*, et al.* 2009; Kuhn*, et al.* 2008) MicroRNAs are transcribed as long hairpin forming RNAs found within introns and exons of protein coding and non-coding genes in the nucleus as shown in Figure 26. (He and Hannon 2004) Once miRNAs are expressed, their maturation is mediated by two RNase III endonucleases: drosha and dicer. (Urbich*, et al.* 2008) Drosha cleaves pri-miRNAs into long double stranded precursor miRNAs (pre-miRNAs), which are then exported from the nucleus. (Urbich*, et al.* 2008; Zhu*, et al.*) (Figure 26) Dicer further processes these pre-miRNAs into small double stranded miRNAs in the cytoplasm. Subsequently, one strand of the complex, denoted “passenger strand”, is removed to leave a single stranded miRNA capable of interacting with the RNA induced silencing complex (RISC), as illustrated in Figure 26. (Catalucci*, et al.* 2009; Kuhn*, et al.* 2008; Slezak-Prochazka*, et al.* 2010; Zhu*, et al.*) Upon

interacting with RISC, mature miRNAs recognize their target mRNAs by base pairing 2-8 nucleotides of the miRNA (seed region) with complimentary nucleotides in the 3'UTR (untranslated region) of target mRNAs, using standard Watson and Crick base pairing. (Catalucci*, et al.* 2009; Daubman 2010; Kuhn*, et al.* 2008) miRNAs are able to inhibit gene expression by targeting mRNAs for translational repression or cleavage. (Kuhn*, et al.* 2008; Urbich*, et al.* 2008) mRNA degradation requires a high/exact miRNA-mRNA complementation, whereas translational repression is mediated by low miRNA-mRNA complementation. As such, miRNA mediated cleavage of mRNAs seems to be the dominant mode for plants, whereas translational repression appears to be the preferred mechanism in animals. (Chen and Rajewsky 2007) It has become increasingly evident that miRNAs play crucial roles in the control of many biological processes including development, differentiation, proliferation and apoptosis. (Bushati and Cohen 2007; Plasterk 2006) The notion that miRNAs are well conserved across species suggests an important evolutionary role for these small RNAs in gene regulation. (Chen and Rajewsky 2007) Furthermore, specific regulation of miRNAs is thought to be necessary, as each molecule of miRNA can regulate hundreds of target mRNAs. (Catalucci*, et al.* 2009)

Genes modulated by Ang II within the vasculature have been extensively investigated. (Campos*, et al.* 2003; Haendeler and Berk 2000; Mehta and Griendling 2007; Touyz 2004; Touyz and Schiffrin 2000) However, potential mechanisms to regulate the expression of these genes remain largely unexplored, especially as it relates to preventing or reversing vascular

dysfunction. The relationship between Ang II and miRNAs in VSMCs has yet to be determined. Furthermore, little is known about the regulation of miRNAs by Ang II. The goal of this study was to explore these relationships using microRNA expression profiling coupled with bioinformatics approaches. In this chapter, we investigate miRNA's that specifically target LOX/LOXL isoforms as well as identify multiple miRNA's whose levels change in response to Ang II in VSMCs.

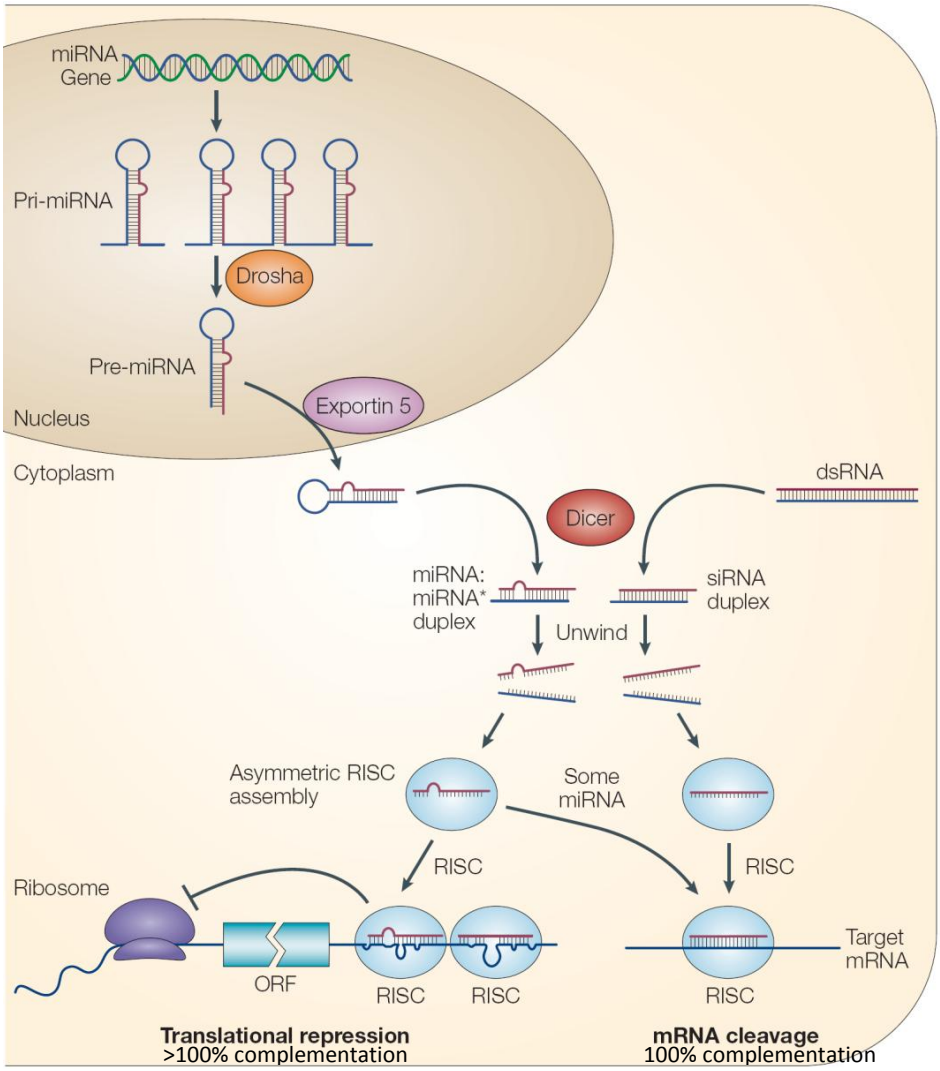


Figure 26. The current model for the generation of miRNAs and their post-transcriptional function.

microRNAs are endogenously found within the nucleus and processed by nuclear enzyme drosha before export into cytoplasm. Dicer further processes miRNAs into mature single stranded molecules that can interact with the RISC complex for mRNA surveillance. The result is translational repression on mRNA cleavage. Image modified from (He and Hannon 2004).

Methods

Cell culture

VSMCs were generously provided by Dr. Kathy K. Griendling, Emory University, Atlanta GA. Briefly, VSMCs were enzymatically extracted from rat thoracic aorta and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 units/mL penicillin and 100ug/mL streptomycin and seeded into 75 cm^2 flasks, per protocol described previously by Griendling *et. al.* (Griendling*, et al.* 1991) Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells were used between passage 6 and 15 for all experiments, and were seeded into 100mm dishes, media was replaced on alternate days. Appropriate confluence was determined according to experimental protocol and previous data. Prior to experimental stimulation, cells between 50-70% were serum-deprived using serum-free DMEM for 48hrs and stimulated with Ang II (Sigma) for designated time points.

microRNA Array and Analysis

Control and Ang II stimulated cells were pelleted, frozen in liquid nitrogen and sent to Miltenyi Biotec for miRNA analysis. RNA was then isolated using standard RNA extraction protocols (Trizol) and samples were quality-checked via the Agilent 2100 Bioanalyzer platform. The results of the Bioanalyzer run were visualized in a gel image and an electropherogram using the Agilent 2100 Bioanalyzer expert software to confirm the integrity of our RNA. (Figure 27A and

B) In addition to this visual control Miltenyi generated a RNA Integrity Number (RIN) to check integrity and overall quality of total RNA samples. The RIN value is calculated by a proprietary algorithm that takes several QC parameters into account, for example, 28S RNA/18S RNA peak area ratios. A RIN number of 10 indicate a high RNA quality, while a RIN number of 1 indicates low RNA quality. According to published data, RNA with a RIN number >6 is of sufficient quality for miRNA expression profiling experiments.(Fleige and Pfaffl 2006; Fleige*, et al.* 2006) All RNA samples exhibited RIN values between 9.5 and 10. High-throughput miRNA expression profiling was performed on four pairs of cell samples with or without Ang II stimulation for 24hr. The miRNA microarray design of the miRXplore microarrays (Miltenyi Biotec) as well as their analysis of the microarrays has been described in detail previously. (Bissels*, et al.* 2009) Briefly, sample labeling was performed and then hybridized overnight to miRXplore microarrays. Control samples were labeled with Hy3 and experimental samples were labeled with Hy5. Fluorescence signals of the hybridized miRXplore Microarrays were detected using a laser scanner from Agilent (Agilent Technologies). Red color indicates that the Hy5 signal intensity is higher than the Hy3 signal intensity. Therefore, the corresponding gene is overexpressed in the Hy5-labeled sample. Green spots indicate that the fluorescence intensity in the control sample is stronger than in the experimental sample. Yellow spots indicate that the signal intensities are equal for both samples. By using a sample:control ratio greater than 1.5 for all expressed

miRNAs as a threshold, we identified the strongest candidate miRNAs for subsequent qRT-PCR validation.

miRNA Extraction and qRT-PCR Validation

Total RNA was extracted from cultured cells using miRVana miRNA isolation kit (Ambion, Invitrogen) per company instructions and reverse transcribed for semi-quantification by standard real-time qRT-PCR (Applied Biosystems). First strand cDNA was synthesized using gene-specific stem-loop primers. All primers were obtained from Applied Biosystems. All reagents were included in the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction was performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) with samples incubated at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. An RT-negative control was included in each batch of reactions. The semi qRT-PCR reactions were performed using the StepOnePlus qRT-PCR System from Applied Biosystems and initiated with a 10 minute incubation at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All reactions were performed in triplicate and normalized to an internal control RNU6B. Fold change was calculated using $2^{(-\Delta\Delta CT)}$ as previously described (Livak and Schmittgen 2001) where :

$\Delta\Delta C_t = (C_{t,Target} - C_{t,control})_{Time\ X} - (C_{t,Target} - C_{t,control})_{Time\ 0}$ *and* C_t = threshold cycle

Overexpressing and Antagonizing mir26b

To overexpress mir26b, double-stranded, human/mouse/rat specific mir26b mimic RNA oligonucleotides were synthesized by Applied Biosystems - Carlsbad, CA and prepared according to the manufacturer's instructions. The mature sequence of the mir26b mimic is -AACAUUCAACGCUGUCGGUGAGU-. To antagonize mir26b, single stranded h/m/r specific 3' FAM labeled antimer for mir26b was synthesized by Exiqon Woburn, MA (ACCTATCCTTGAATTACTTGAA) which uses a locked nucleic acid (LNA) approach for stable inhibition of miRNAs. For transfection, VSMCs were grown to 60% confluence with DMEM (Hyclone) supplemented with 10% calf serum, Penicillin/Streptomycin and L-glutamine and maintained at 37 degrees with 5% CO_2. Cells were transfected with 25nm of a mir26b mimic or 25nm of mir26b antimers using oligofectamine (Invitrogen – Grand Island, NY) and recovered in OptiMEM (Invitrogen – Grand Island, NY) according to the manufacturer's specifications. 24hrs post transfection, cells were serum deprived for 48hrs and then treated with 100 nM of Ang II (Sigma – St Louis, MO). Protein was then harvested, quantified as described and analyzed for LOXL2 expression by Western blot analysis.

Flow Cytometry

Transfected adherent cells were isolated via trypsinization, washed with fully supplemented media to neutralize the trypsin, and then resuspended in cold PBS prior to analysis. Expression of FITC labeled antimer was quantitatively assessed using a BD FACSort flow cytometer. 5,000-50,000 events were recorded, and fluorescence was measured using a 488 nm laser with 530nm +/- 15nm detectors.

Protein Isolation

Cell culture protein samples were lysed in radio-immunoprecipitation assay buffer (RIPA) lysis buffer (Santa Cruz) with supplied protease inhibitors (sodium orthovanadate, PMSF and a cocktail inhibitor) and rocked with end-over-end rotation at 4°C for 15min to allow for complete cell lysis. This was followed by centrifugation at 10,400xG at 4°C for 10 min. Protein concentration was determined using a standard Bradford Assay (Biorad).

Western blotting

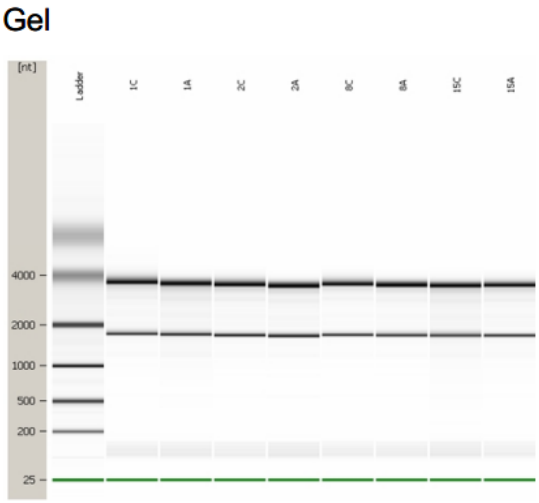
Samples containing equal amounts of protein (20µg) were loaded onto 7.5% SDS polyacrylamide gels (BioRad) and subsequently blotted onto nitrocellulose membrane (Amersham). Membranes were blocked with 5% milk (Santa Cruz) and incubated with primary antibodies: Drosha (Abcam - Cambridge, MA), Dicer

(Santa Cruz) and LOXL2 (Abcam - Cambridge, MA). HRP conjugated secondary was used alongside ECL (GE Healthcare) Western blotting system for signal detection. All protein was normalized to β-actin (Cell Signaling –Beverly, MA).

Statistical Analysis

All data are reported as mean **±** SEM for at least three independent experiments. Statistical significance for quantitative experiments was assessed with analysis of variance (ANOVA, 2-tailed) and Bonferonni's post-hoc test. In some cases, the Students t-test was used to compare between groups. Statistical significance was defined as $p<0.05$.

A.



B.

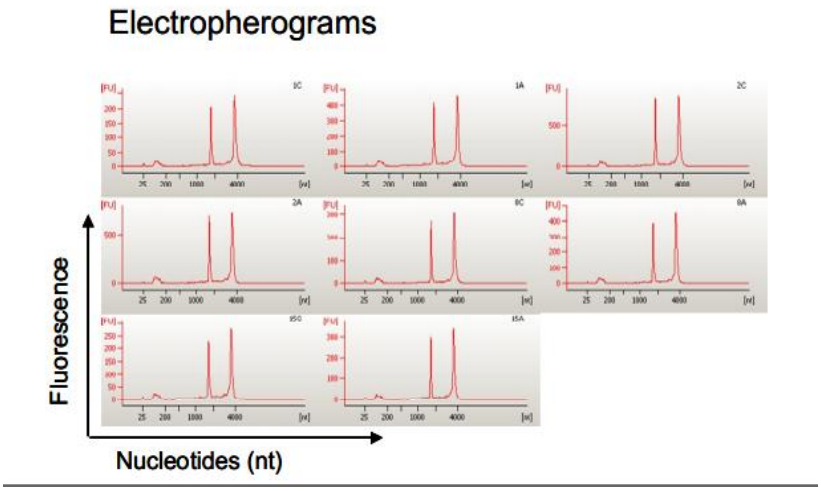


Figure 27. Integrity of microRNA for microarray analysis as shown by gel image and electropherogram.

(A) and electropherogram (B) of total RNA samples from 4 control samples and 4 Ang II treated samples. A. As a reference, the RNA molecular weight ladder (in nucleotides, nt) is shown in the first lane. All lanes show the presence of two bands, the 28S and 18s ribosomal RNA. The lowest migrating, green band represents an internal standard. B. The two prominent peaks within the electropherograms represent ribosomal RNA: left 18S RNA, right 28S RNA. Scaling of the y-axis is done automatically, relative to the strongest signal within a single run.

Results

Ang II modulates several miRNAs in VSMCs

Using miRNA expression profiling, we identified candidate miRNAs that are either positively or negatively regulated after stimulation with Ang II for 24 hours in VSMCs from 442 miRNAs probed across human, mouse and rat species. (Table 3) We tested each of these candidate miRNA's and other miRNA's implicated in cardiovascular disease, such as mir21 and mir222, by qRT-PCR and analyzed by $2^{-(\Delta\Delta CT)}$. (Catalucci*, et al.* 2009; Daubman 2010; Roy*, et al.* 2009; Urbich*, et al.* 2008) We observed some clear discrepancies between the qRT-PCR and microarray analysis both in terms of fold change and direction of the changed miRNA level. As qRT-PCR analysis is less prone to error since the probes are typically of better design, we focused our investigation on those miRNA that showed a significant change according to qRT-PCR. The fidelity of miRNA arrays, as subsequently reported in the literature is low, and as such we and others accept the qRT-PCR results as the gold standard. (Git*, et al.* 2010) To accommodate miRNAs that may have been overlooked in our miRNA analysis, we evaluated other miRNAs involved in cardiovascular disease based on the literature, as shown in Table 4. Table 3 and 4 show that Ang II significantly upregulated the expression of mir222, mir125b and mir30b greater than 1.5-fold while suppressing the expression of mir181a and mir26b compared to non-stimulated cells. miRNAs with (♦) denote the biologically inactive or non-binding miRNA sequence from the dsRNA molecule. The asterisks symbol (*) in

the qRT-PCR column denotes statistically significant qRT-PCR data, where p values are less than 0.05. Values from the array are represented as mean fold change ± SEM compared to control and the number of miRNA arrays with detectable values is represented in parenthesis (maximum = 4). Altogether, we evaluated a total of 18 miRNA candidates with qRT-PCR and analyzed data as fold increase using $2^{-\Delta\Delta CT}$.

Table 3 and 4. microRNA array as compared to qRT-PCR. qRT-PCR validation and analysis using $2^{-(\Delta\Delta CT)}$ show that Ang II significantly upregulated the expression of mir222, mir125b and mir30b greater than 1.5 fold while suppressing the expression of mir181a and mir26b compared to control. n/a represents miRNAs not detected on the array.

Table 3. miRNAs that are upregulated in the array and the qRT-PCR validation data

MicroRNAs	*MicroRNA Array*	*qRT-PCR*
181a	*2.11 +/- 0.06 (4)*	*0.52 +/- 0.07**
30b	*2.11 +/- 0.39 (4)*	*1.80 +/- 0.29**
15a	*2.26 +/- 0.33 (3)*	*0.93 +/- 0.22*
26b	*2.59 +/- 0.51 (2)*	*0.76 +/- 0.08**
21	*1.73 +/- 0.15 (4)*	*1.75 +/- 0.57**
347	*3.38 +/- 0.79 (4)*	*0.98 +/- 0.18*
222	*2.43 +/- 0.38 (4)*	*1.68 +/- 0.23**
29b	*3.79 +/- 0.89 (4)*	*1.10 +/- 0.18*
30e	*2.25 +/- 0.6 (4)*	*1.38 +/- 0.40*

Table 4. Additional list of candidates probed by qRT-PCR

miRNA candidates not detected and/or downregulated by miRNA array analysis.

MicroRNAs	*MicroRNA Array*	qRT-PCR
26b♦	*n/a*	0.53 +/- 0.06
143	*0.83 +/- 0.06 (4)*	1.30 +/- 0.27
664	*n/a*	1.41 +/- 0.18
10b	*1.69 +/- 0.15 (3)*	1.11 +/- 0.15
494	*1.61 +/- 0.43 (4)*	5.61 +/- 3.48
125b	*0.96 +/- 0.03 (4)*	1.78 +/- 0.23*
145	*0.85 +/- 0.03 (4)*	0.99 +/- 0.14
98/let	*n/a*	1.08 +/- 0.14
30b♦	*n/a*	0.51 +/- 0.08

Several miRNAs are predicted to target LOXL1 and LOXL2

Using several widely used miRNA web-based bioinformatics tools including targetscan.org, mirwalk.org, and microRNA.org, we identified potential miRNAs that target LOXL1 and LOXL2 as referenced in Table 5. The algorithms within these databases rely on several criteria for target prediction such as conservation among species, complimentary seed region, ΔG of target mRNA binding site, and the presence of multiple miRNA binding sites within the targets 3'UTR. (Catalucci*, et al.* 2009) Several of the miRNAs predicted to target LOXL1 and LOXL2 are also regulated by Ang II, as presented in Table 5. Table 5 shows the miRNAs from the array that target LOXL1 and LOXL2 alongside qRT-PCR analysis. We were most interested in miRNAs that targeted LOXL2 (mir26a, mir26b, mir29b) because LOXL2 had a greater increase in mRNA expression in response to Ang II as compared to LOXL1. We therefore overexpressed and antagonized mir26b (the only validated Ang II regulated LOXL2 targeting miRNA as defined by qRT-PCR) in the presence and absence of Ang II. Ang II downregulated mir26b by qRT-PCR. We expected to see an increase in LOXL2 expression by overexpressing mir26b. We did not detect any changes in LOXL2 expression with mir26b modulation in the presence of Ang II. We noted minimal increases in LOXL2 expression from baseline through both overexpression and inhibition of mir26b. (Figure 28) However, it is noteworthy that these data were neither reliable nor consistent throughout the course of experimentation, suggesting LOXL2 expression is regulated through a very complicated network of variables. We confirmed that mir26b antimers and mimics were indeed

transfected into VSMCs using confocal microscopy (to visualize FITC labeled antimer) and flow cytometry in Figure 29, as well as qRT-PCR to quantify the level of overexpression of mir26b with a mimic, as shown in Figure 30. Because we were unable to confirm the effect of Ang II on mir26b and LOXL2, we decided to focus instead on other Ang II regulated miRNAs with new protein targets relevant to Ang II mediated vascular dysfunction.

Table 5. miRNAs targeting LOXL1 and LOXL2.

(+) denotes an upregulation of the miRNA and (-) denotes a downregulated miRNA in the presence of Ang II.

Target	Predicted miRNA	Ang II regulated miRNAs (array)	Ang II regulated miRNAs (qRT-PCR)
LOXL1	mir125a, mir125b		mir125b(+)
LOXL2	mir26a, mir26b, mir29b	mir26b(+), mir29b (+)	mir26b(-)

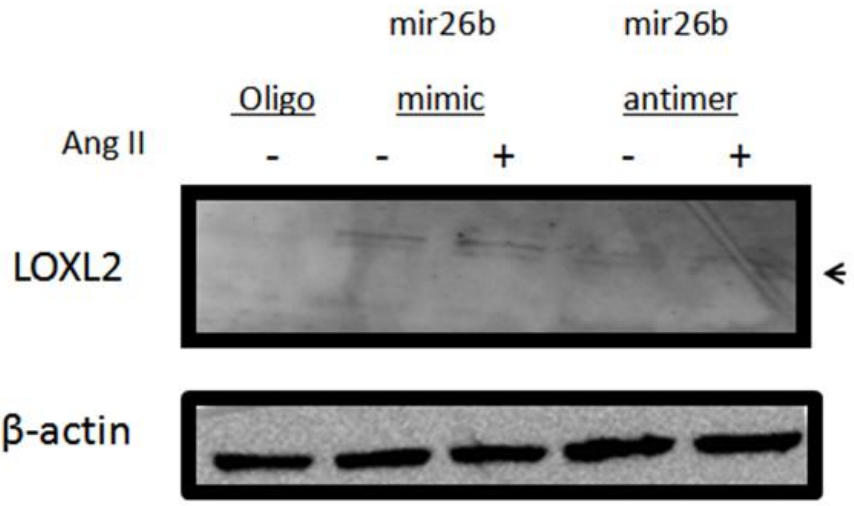


Figure 28. LOXL2 expression with mir26b modulation in the presence of Ang II.

Mir26b mimic overexpresses mir26b and mir26 antimer inhibits mir26b. In both settings, LOXL2 expression was not increased significantly upon Ang II treatment. Oligofectamine (oligo) represents control – oligofectamine only transfected cells.

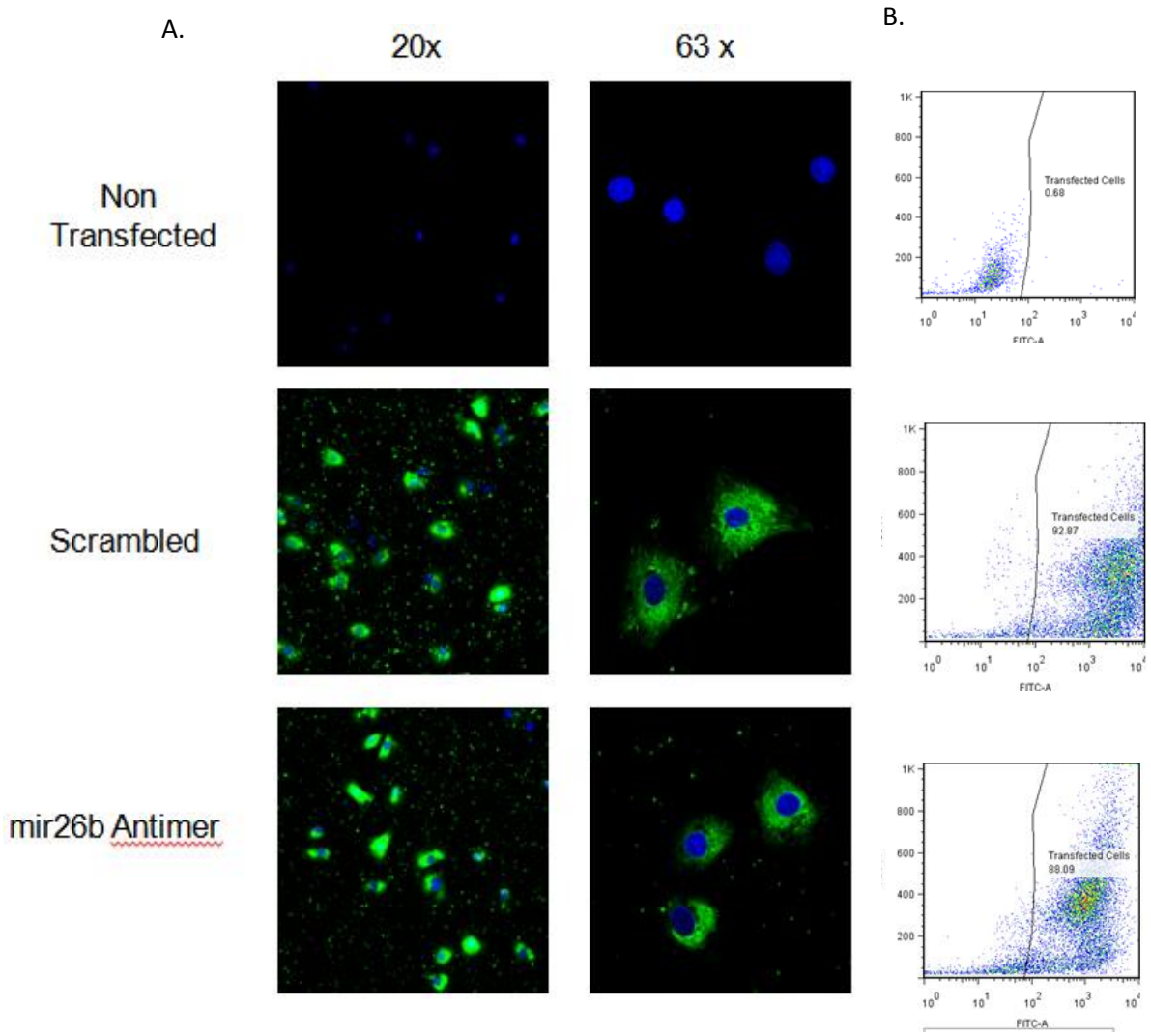
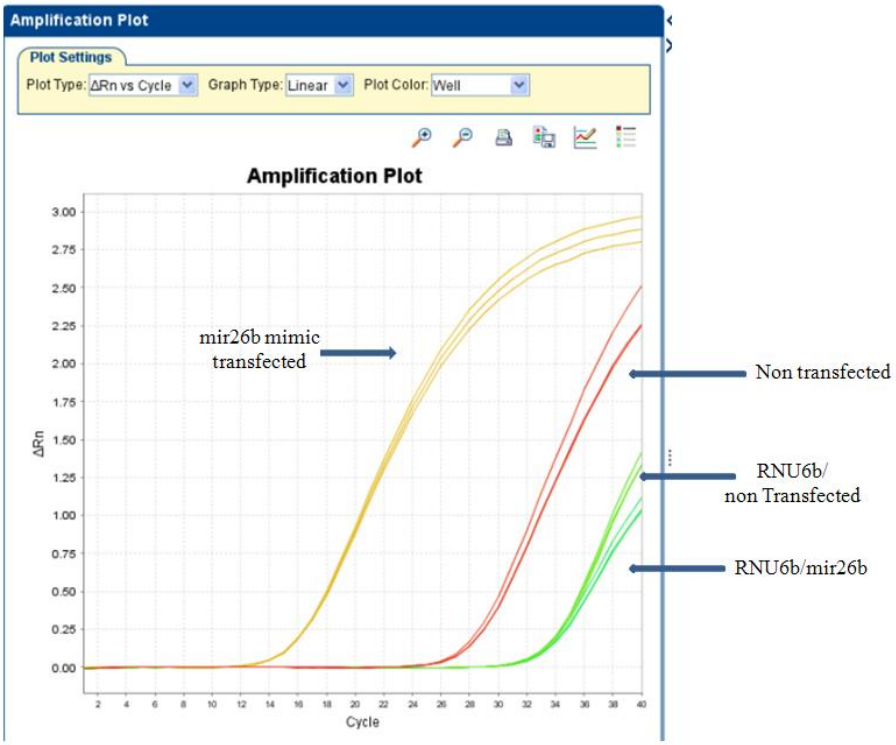


Figure 29. mir26b antimer transfection efficiency

A. mir26b antimer was transfected into VSMCs as shown by confocal microscopy (to visualize FITC labeled antimer) at 20X and 63X. The blue represents DAPI labeled nuclei and the green shows FITC labeled miRNA found within the cytoplasm. B. Flow cytometry was performed on unsorted VSMCs transfected with mir26b antimer (containing 3'FITC label). Flow cytometry shows the relative percentage of antimer transfected cells. Approximately 90% of VSMCs were transfected with mir26b antimer.



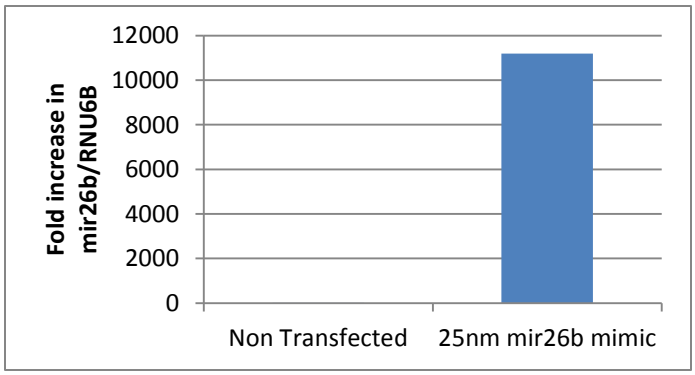


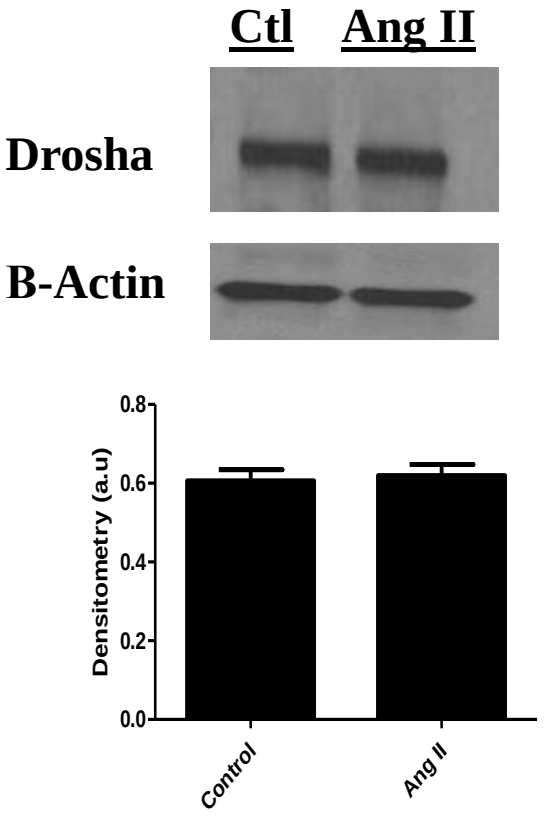
Figure 30. mir262b overexpression in vascular smooth muscle cells as demonstrated by qRT-PCR.

qRT-PCR shows mir26b is overexpressed in VSMCs by greater than 10,000 fold compared to non-transfected controls 24hrs after transfection. A) Representation of the difference in cycle number between cells overexpressing mir26b by qRT-PCR and B) fold change as calculated using $2^{-\Delta\Delta CT}$.

Ang II has differential effects on the expression of miRNA processing enzymes drosha and dicer

As discussed previously, drosha and dicer are critical for miRNA processing. In endothelial cells, genetic silencing of dicer and drosha significantly reduced capillary sprouting of endothelial cells and tube forming activity. (Kuehbacher*, et al.* 2007) Additionally the migration of endothelial cells was significantly decreased in the presence of dicer siRNA, whereas drosha siRNA had no effect. (Kuehbacher*, et al.* 2007) *In vivo*, the silencing of dicer but not of drosha has been shown to reduce angiogenesis. (Kuehbacher*, et al.* 2007) In VSMCs, we show that stimulation with Ang II for 24 hours did not affect the expression of drosha (nuclear processing of miRNAs), but it did decrease the expression of dicer (cytoplasmic processing of miRNAs). (31A and B) Ang II decreases dicer expression by approximately 30% in VSMCs (Figure 31B). However, there was not a global decrease in total miRNA expression in response to Ang II, as noted in Tables 3 and 4. This suggests that changes in miRNA expression are specific and not influenced by a small decrease in dicer expression in response to Ang II.

A.



B.

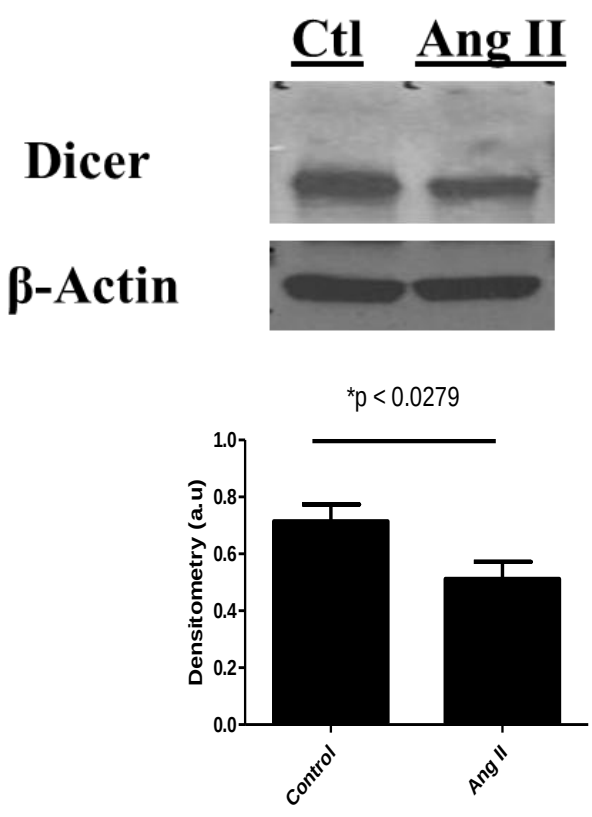


Figure 31. The effect of Ang II on miRNA processing enzymes drosha and dicer.

A. Ang II for 24 hours did not affect the expression of drosha. B. Ang II decreased dicer expression by approximately 20%. *p < .03*

miRNAs and potential mRNA targets

Cross referencing our list of miRNAs that are affected by Ang II treatment with genes that are known to be regulated by Ang II yielded one miRNA worth investigating further, mir181a. This miRNA is predicted by various miRNA target prediction servers, such as targetscan.org, mirwalk.org, microRNA.org and mirbase.org, to target the matricellular protein, osteopontin (OPN). Ang II regulates OPN expression in VSMCs (Abe*, et al.* 2008; Campos*, et al.* 2003) and in human liver cells mir181a modulates OPN expression. (Bhattacharya*, et al.*) In Chapter 4, we propose a novel mechanism by which Ang II regulates OPN expression through the modulation of mir181a. Tables 6 and 7 show a list of genes regulated by two of our five Ang II regulated miRNAs, mir181a and mir222. These tables are shown to demonstrate that there are additional interesting targets relevant to Angiotensin II mediated signaling that may be worth investigating in the future, such as ERK1/2, AKT and PAI-1.

Table 6. Predicted mir181a targets acquired using bioinformatic tools.

181 targets	Functions	Testable Biological Function	Ang II mediated	Ang II source	miRNA/tagret prediction source	Species containing predicted 3'UTR target
Osteopontin	functions in growth and migration (antiapoptotic factor)	Cell Migration	yes (upregulated) smooth muscle cells, endothelial cells	1.) Abe, K et al. Hypertension Research 2008 2.) Xie, Z et al Journal of Cellular Physiology 2001	Ang II array, targetscan.org, mirwalk, microRNA.org	Human and Mouse
PAI-1 (Serpine 1)	inhibits tissue plasminogen activator (tPA) and urokinase (uPA), inhibits MMP activity, accererlates Atherosclerosis	Development of Atherosclerosis	yes (upregulated) smooth muscle cells at mRNA level	Feener, EP et al Journal of Clinical Investigation 1995	Ang II array, targetscan.org, microRNA.org	Human and Mouse
PDGFRA	platelet-derived growth factor receptor alpha implicated in proliferation, differientiation, growth and development	Cell Proliferation	PDGFR is upregulated in response to Ang II	Mondorf et al Federation of European Biochemical Societies Letters 2000	targetscan.org and Ang II array	Human and Mouse
osteoprotegerin/TNFR super family	cytokine receptor involved in inflammation, innate immunity, cell survival and differentiation		yes (upregulated)	Moran, Cs et al Journal of Vascular Research 2009	target scan	
TGFRBR1	single pass serine/threonine kinase receptors that can control cell growth, cell proliferation, cell differentiation and apoptosis.	Cell proliferation, Apoptosis	Ang II upregulates TGF-beta. Receptor expression unknown	Marie J et al. Federation of European Biochemical Societies Letters 1983	targetscan.org	Human, Mouse and Rat
AP-1/fos	heterodimeric transcription factor that controls differentiation, proliferation and apoptosis	Cell proliferation, Apoptosis	Ang II increases AP1 DNA binding	Ahn, JD et al Diabetologia 2001	microRNA.org	Human, Mouse and Rat
GATA 6	zinc finger trancription factor - proliferation/apoptosis	Cell proliferation, Apoptosis	Ang II stimulates GATA6 DNA binding	Tan, NY et al Circulation Research 2009	targetscan.org and microRNA.org	Human and Mouse
bcl2	an anti-apoptotic protein; involved in inhibiting cell death in many different cell types [RGD]	Apoptosis	yes (downregulated) in endothelial cells	1.)Fabris, B et al. Journal of Hypertension 2007 2.)Dayuan, L et al Cardiovascular Research 1998	targetscan, mirwalk	Human and Mouse
PTEN	inhibits migration	Cell Migration			mirwalk and mircoRNA.org	Human and Mouse
Erk1/2 (MAPk3)	regulates cellular processes such as proliferation, differentiation, and cell cycle progression	Cell Proliferation	Ang II increases Phospo-erk1/2	Godeny, M et al American Journal of Physiology Cell Physiology 2006	mirwalk	Human, Mouse and Rat
p53	responds to diverse cellular stresses to induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism	Apoptosis	yes (upregulates)	Liu, Q et al Toxicology Letters 2009	mirwalk	Human, Mouse and Rat

Table 7. Predicted mir222 targets acquired using bioinformatic tools

222 targets	Functions	Testable Biological Function	Ang II mediated	Ang II source	miRNA/tagret prediction source	Species containing predicted 3'UTR target
akt	inhibits JUN kinase activation and mediates inhibition of apoptosis	Apoptosis			targetscan	
Erk1/2 (MAPk3)	regulates cellular processes such as proliferation, differentiation, and cell cycle progression	Cell Proliferation	Ang II increases Phospo-erk1/2	Godeny, M et al American Journal of Physiology Cell Physiology 2006	mirwalk	Human, Mouse and Rat
AP-1/fos	heterodimeric transcription factor that controls differentiation, proliferation and apoptosis	Cell proliferation, Apoptosis	Ang II increases AP1 DNA binding	Ahn, JD et al Diabetologia 2001	microRNA.org	Human, Mouse and Rat
PTEN	inhibits migration	Cell Migration			mirwalk and mircoRNA.org	Human and Mouse
Hif1a	hypoxia inducible factor; regulates transcription in response to low oxygen; may play a role in vascular biology				targetscan	
bcl2	an anti-apoptotic protein; involved in inhibiting cell death in many different cell types [RGD]	Apoptosis	downregulated in endothelial cells	1.)Fabris, B et al. Journal of Hypertension 2007 2.)Dayuan, L et al Cardiovascular Research 1998	targetscan, mirwalk	Human and Mouse
p53	responds to diverse cellular stresses to induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism	Apoptosis	yes (upregulates)	Liu, Q et al Toxicology Letters 2009	mirwalk	Human, Mouse and Rat

Discussion

miRNAs have been established as upstream mediators of gene expression and are involved in various physiological and pathological processes. As such, understanding their role in vascular remodeling, and in particular Ang II mediated vascular remodeling of VSMCs, may provide novel insight for alternate ways of regulating gene expression.

We confirm that 5 miRNAs are indeed regulated by Ang II in VSMCs using qRT-PCR: mir222, mir125b and mir30b are upregulated greater than 1.5 fold in response to Ang II, while the expression of mir181a and mir26b were suppressed. We and others recently confirmed that while miRNA arrays provide some useful information, they are not the best tool for evaluating miRNA expression, due to low fidelity. (Chen*, et al.* 2009; Git*, et al.* 2010) miRNA arrays present a distinct disadvantage from traditional microarrays because of the short length (19-22nt) of mature miRNA sequences, making probe design difficult. Often, the entire miRNA sequence must be used as a probe. (Git*, et al.* 2010) miRNA binding essentially requires only the seed region (~7nt) leading to many false positives and negative results when probing with full length probes. qRT-PCR avoids this issue by allowing for small miRNA probes using a quencher conjugated system. By using quencher conjugated probes, the short miRNA sequence is maintained and the quencher is not released until it binds with high affinity to a very specific sequence. The caveat to using qRT-PCR for identifying globally regulated miRNAs is that it is not high throughput. We conclude that miRNA arrays can be used as a prediction tool alongside primary literature and

bioinformatics tools but not as a standalone tool. In addition, it must be understood that their fidelity is extremely low compared to traditional microarrays. We conclude that a variety of methodologies should be employed cautiously to identify regulated miRNAs.

We have shown that Ang II has no effect on drosha and negligible effects on the expression of dicer. We conclude that the changes in the expression of dicer do not globally effect miRNA processing. We have identified 5 miRNAs confirmed by qRT-PCR that are regulated by Ang II in VSMC's. We demonstrate that modulation of mir26b, one of our regulated miRNAs predicted to target LOXL2, does not increase LOX2 expression. Recent literature demonstrates that miRNA manipulation is a promising tool for alleviating cardiovascular disease, as demonstrated with mir29b and the development of AAA. *In vivo* administration of locked nucleic acid anti–miR-29b greatly increased collagen expression, leading to an early fibrotic response in the abdominal aortic wall and resulting in a significant reduction in AAA progression over time. (Maegdefessel*, et al.* 2012) Future studies include evaluating the relationship between another Ang II regulated miRNA, mir181a, and OPN protein expression as it relates to the progression of vascular disease.

Chapter 4. mir181a Protects Against Ang II-Induced Osteopontin Expression in Vascular Smooth Muscle Cells

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**manuscript in preparation for submission*

Abstract

Angiotensin II (Ang II) promotes reactive oxygen species production, migration and hypertrophy in vascular smooth muscle cells (VSMCs). MicroRNAs (miRNAs) are critical regulators of protein expression in cardiovascular disease. However, the relationship between Ang II and miRNAs in VSMCs has yet to be determined. Osteopontin (OPN) is a multifunctional protein found in abundance in atherosclerotic plaques and in the injured arterial wall. In a mouse model of hypertension, we found that aortic OPN protein expression was increased 2-fold after 7 days of 0.75mg/kg/day Ang II treatment in C57Bl/6 mice *($p < 0.001$). In vitro*, we found Ang II increased OPN protein expression 4hrs after treatment by 420%±54 (*$p < 0.03$*) in cultured VSMCs. Literature searches revealed OPN mRNA as a potential target of mir181a. We found that Ang II decreased mir181a expression by 52%±7 *($p < 0 .0001$)* 24hrs post stimulation in VSMCs. We observed that over expression of mir181a inhibited Ang II induced increases in OPN protein expression at 4hrs in VSMCs by 69%±9 *($p < 0.05$).* We conclude that Ang II decreases mir181a expression while increasing OPN protein expression, and that over expressing mir181a blocks Ang II induced OPN protein translation. Furthermore, we demonstrate that mir181a over expression has a functional impact on VSMCs as evidenced by the inhibition of adhesion of VSMCs to collagen in response to Ang II compared to controls by 36% ± 4. *($p <0 .05$)* These results suggest that mir181a may be a novel therapeutic approach to alter OPN protein expression during Ang II induced vascular dysfunction.

Introduction

Atherosclerosis is widely accepted as a chronic inflammatory disease characterized by endothelial dysfunction, vascular inflammation, and the accumulation of lipids and cellular debris within the vessel wall, resulting in arterial stiffness. (Tesanovic*, et al.* 2010) The renin-angiotensin system (RAS) has been implicated in the pathogenesis of atherosclerosis through its primary effector molecule Angiotensin II (Ang II). (Mehta and Griendling 2007) The classic source of Ang II is circulating plasma-derived Ang II. During pathological conditions, chronic exposure of cardiovascular tissues to Ang II affects the growth, migration and adhesion of smooth muscle cells and fibroblasts. This in turn, leads to endothelial dysfunction and differentiation of monocytes into macrophages. (Faries*, et al.* 2002; Weiss*, et al.* 2001) Several *in vivo* studies have demonstrated that Ang II can induce the expression of adhesion molecules and increase production of reactive oxygen species (ROS), which initiates a potent inflammatory response resulting in the release of growth factors, cytokines and chemokines within the aortic wall. (Hansson*, et al.* 2006; Libby 2002; Libby*, et al.* 2002; Weiss*, et al.* 2001)

In the setting of atherosclerosis osteopontin (OPN) is abundantly expressed in atherosclerotic plaques and in the injured arterial wall. (Abe*, et al.* 2008; Asou*, et al.* 2001; Chakraborty*, et al.* 2006; Cho*, et al.* 2009; Giachelli*, et al.* 1993; Ikeda*, et al.* 1993) Furthermore, Ang II-induced atherosclerosis is attenuated in $OPN^{-/-}$ mice, suggesting that OPN is a potential mediator in the development of Ang-II-induced pathologies. (Bruemmer*, et al.* 2003; Cho*, et al.*

2009) OPN is a negatively charged, secreted, glycosylated phosphoprotein expressed in multiple tissues and cell types including osteoblasts, macrophages, VSMCs and T cells. (Abe*, et al.* 2008) OPN's extensive post-translational modifications are suspected to contribute to its diverse roles in adhesion, migration, bone morphogenesis, and immune cell activation.(Rangaswami*, et al.* 2006) OPN contains an arginine-glycine-aspartic acid (RGD) motif that is recognized by integrins (primarily αvβ3 integrin, as well as αvβ1 and αvβ5), which mediate adhesion, proliferation, and migration in VSMCs. (Li*, et al.* 2007; Rangaswami*, et al.* 2006; Standal*, et al.* 2004; Weintraub*, et al.* 2000; Yue*, et al.* 1994) Furthermore, OPN has been implicated as a pro-inflammatory protein, serving as a chemoattractant for both SMCs and macrophages, while inducing activation in the latter. Given the pleiotropic response of OPN signaling in a vast array of cells, it is important to fully understand the mechanisms responsible for the regulation of OPN by Ang II.

MicroRNAs (miRNAs) are important posttranslational regulators of gene expression. miRNAs are a class of endogenous single stranded RNA nucleotides (~22nt) that bind preferentially to the 3' untranslated region (3' UTR) of target eukaryotic mRNA and inhibit protein translation in mammalian cells. These small, endogenous RNAs play a crucial role in many biological processes including development, differentiation, proliferation, and apoptosis. (Bushati and Cohen 2007; Plasterk 2006) The goal of the present study is to understand the relationship between Ang II, mir181a and OPN protein levels in VSMCs as it relates to vascular dysfunction. We propose a model whereby overexpression of

mir181a inhibits Ang II-induced OPN expression. Ang II induced increases in OPN promote cell adhesion, contributing to the migratory actions of VSMCs, which is crucial in the pathophysiology of atherosclerosis.

Methods

Animals

C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) aged six to eight weeks were treated with or without Ang II (0.75 mg/kg/day) for 7 days via an osmotic mini-pump, as previously described. (Weiss*, et al.* 2001) All housing, surgical procedures, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Emory University. Animals had free access to chow and water during all experiments. Noninvasive systolic blood pressure measurements were obtained using the Visitech Systems BP 2000 tail-cuff system as previously described. (Weiss*, et al.* 2001)

Cell Culture

VSMCs generously provided by Dr. Kathy K. Griendling PhD, Emory University, Atlanta GA, were obtained as previously described.(Griendling*, et al.* 1991) Prior to experimental stimulation, cells were serum-starved for 48hrs and stimulated with Ang II (Sigma) for indicated time points. Mouse aortic vascular smooth muscle cells (MASMs) were prepared from $OPN^{-/-}$ or $OPN^{+/+}$ mice (age 6-8 weeks) obtained from Jackson Laboratories as previously described. (Ohmi*, et*

al. 1997) Sub-cultured MASMs were maintained in DMEM containing 100 units/mL penicillin, 100 µg/mL streptomycin and 10% FBS.

Quantitative Real Time-Polymerase Chain Reaction

RNA was extracted from either snap frozen mouse aortic tissue 7 days after treatment and/or VSMCs after treatment, using the RNeasy kit (Qiagen, Valencia, CA) according to their protocol and quantified using a spectrophotometer. cDNA was prepared and purified from using standard Invitrogen protocol with primers unique for rat or mouse OPN (Qiagen). All RNA was normalized to 18S (Invitrogen) unless otherwise noted.

Western blotting and ELISA

Twenty micrograms of protein lysed in radio-immunoprecipitation assay buffer (RIPA) were loaded onto 7.5% SDS polyacrylamide gels (BioRad –Hercules, CA) and subsequently blotted onto nitrocellulose (Amersham – Piscataway, NJ). Membranes were blocked with 5% milk (Santa Cruz – Santa Cruz, CA). OPN protein expression was assessed with an OPN antibody (R&D Systems Minneapolis, MN) at a 1:1000 dilution. HRP conjugated anti-goat secondary was used alongside an ECL (GE Healthcare) Western blotting system for signal detection. Data was analyzed using densitometry (U-Scan-It) and normalized to GAPDH (Santa Cruz) or β-actin (Cell Signaling – Beverly, MA) as indicated. ELISAs to measure secreted OPN were obtained from Enzo Life Sciences -

Farmingdale, NY and used according to manufacturer's protocol and specifications.

miRNA isolation

Total RNA was extracted from cultured cells using miRVana miRNA isolation kit (Ambion, Invitrogen – Grand Island, NY) and reverse transcribed for semi-quantification by standard real-time qRT-PCR (Applied Biosystems – Carlsbad, CA). MicroRNA expression was determined using the TaqMan microRNA assay kit for hsa-miR181a (Applied Biosystems), with RNU6B RNA used as an internal control. Fold change was calculated using the previously reported $2^{-\Delta\Delta CT}$ method. (Livak and Schmittgen 2001)

mir181a Overexpression

Double-stranded, human/mouse/rat specific mir181a mimetic RNA oligonucleotides were synthesized by Applied Biosystems and prepared according to the manufacturer's instructions. For transfection, VSMCs were grown to 80% confluence with DMEM (Hyclone) supplemented with 10% calf serum. $2x10^6$ cells were transfected with 50 pmoles of a mir181a mimetic -AACAUUCAACGCUGUCGGUGAGU- or negative controls using electroporation (AMAXA, Lonza) and recovered in OptiMEM (Invitrogen) according to the manufacturer's specifications. 24hrs post transfection, cells were serum deprived for 48hrs and then treated with 100 nM of Ang II (Sigma). Protein was

then harvested, quantified as described and analyzed for OPN expression by Western blot analysis.

Adhesion Assay

VSMCs were seeded at a density of $5x10^4$ cells/plate on pre-coated collagen type I coverslips (Fisher) for 1hr at 37° C in serum free media. Non-adherent cells were aspirated and the adherent cells were washed in phosphate-buffered solution (PBS) and fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized using 0.2% Triton X and quenched with 50nm NH_4Cl, followed by immunostaining with phalloidin (Invitrogen) and 4'-6-diamidino-2-phenylindole (DAPI/vectasheild) to visualize the nuclei. Images were acquired using an Olympus microscope at 10X magnification or confocal microscopy at 20X as indicated. Images were taken of 5 random fields per dish per experiment and quantified.

Statistical Analysis

All data were reported as mean **±** SEM for at least three independent experiments. Statistical significance for quantitative experiments was assessed with either a student t-test or analysis of variance (ANOVA, 2-tailed) with Bonferonni's post-hoc analysis. Statistical significance was defined as $p<0.05$.

Results

Ang II increases OPN protein expression in vivo and in vitro

To determine if OPN expression was regulated by Ang II *in vivo*, we employed the mouse model of Ang II-induced hypertension. Mice were treated with Ang II for 7 days and blood pressures were measured. Ang II treated animals became hypertensive with systolic blood pressures of 156.8mmHg ± 5 compared to non-treated animals at 107.9mmHg ± 5 ($p < .0001$). Animals were sacrificed and mouse aortas were analyzed for protein and mRNA expression. We found that aortic OPN protein expression was increased ~2-fold after 7 days of Ang II treatment as compared to control mice (Figure 32A). OPN mRNA was also increased in response to Ang II treatment in C57Bl/6 mice (Figure 32B). These data demonstrate that OPN is upregulated in response to Ang II stimulation in a mouse model of hypertension.

To further explore the potential mechanisms of regulation of OPN by Ang II, we used cultured VSMCs and found that OPN mRNA expression is not increased until at least 12hrs after Ang II administration (Figure 33A). Interestingly, OPN cellular protein expression began increasing as early as 2 hours after Ang II treatment and continued to increase by 420% ± 54 as soon as 4 hours after Ang II treatment ($p < 0.03$, Figure 33B). OPN expression continued to increase for at least 24 hours after treatment as shown in Figure 33B.

Taken together, these data demonstrate that Ang II increases OPN expression in aortic tissue *in vivo* and in cultured vascular smooth muscle cells *in*

vitro. Interestingly, the cell culture studies demonstrated that OPN protein expression was increased prior to any detectable change OPN mRNA expression suggesting that a mechanism exists for Ang II to increase OPN that is independent of changes in mRNA expression.

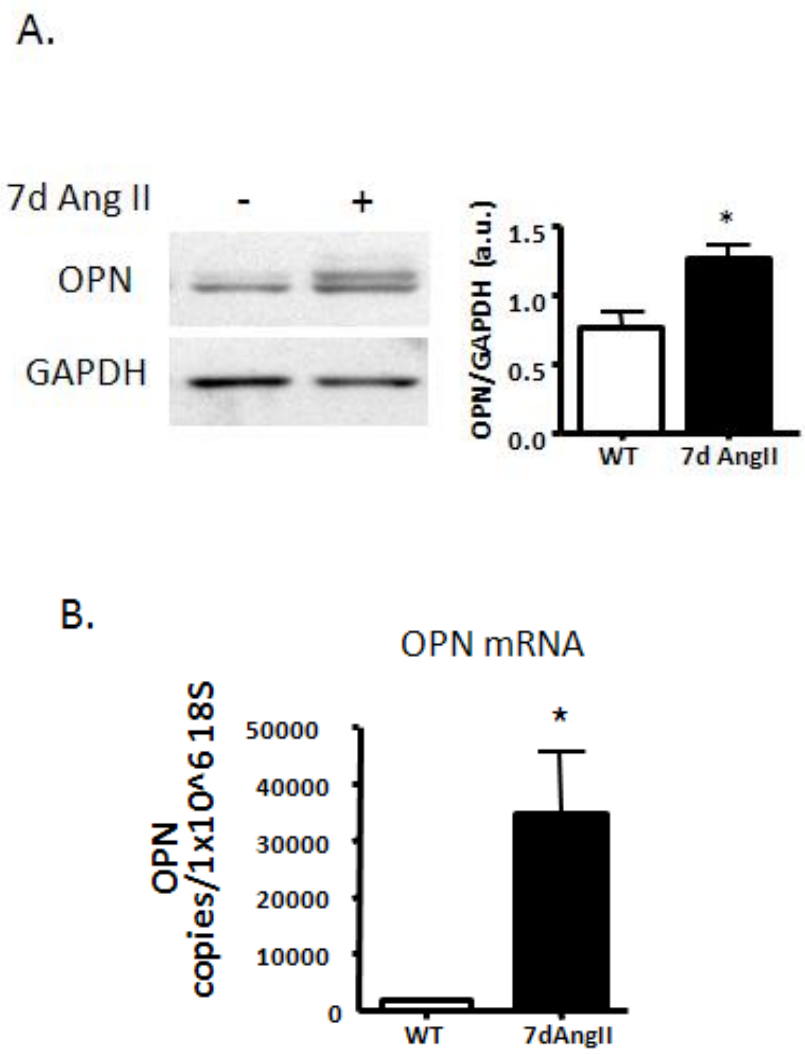
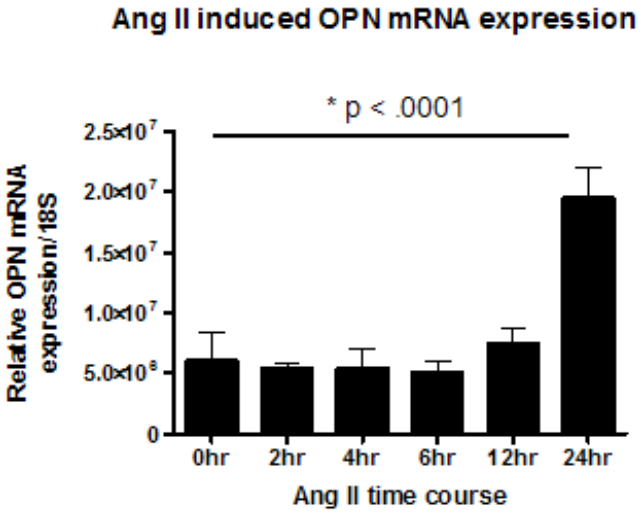


Figure 32. Ang II induces OPN protein and mRNA expression *in vitro*.

A. Ang II induces OPN protein expression by 2-fold after 7 days of 0.75mg/kg/day Ang II treatment in C57Bl/6 mice *($p < 0.001$).* B. Ang II increases OPN mRNA expression in vivo.

A.



B.

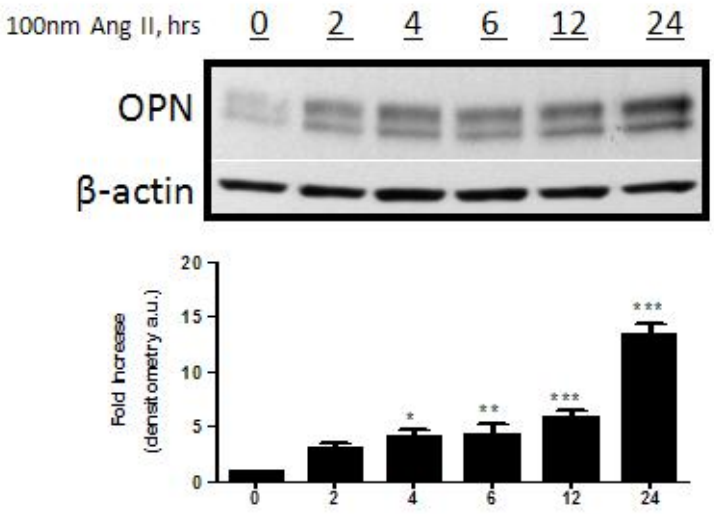


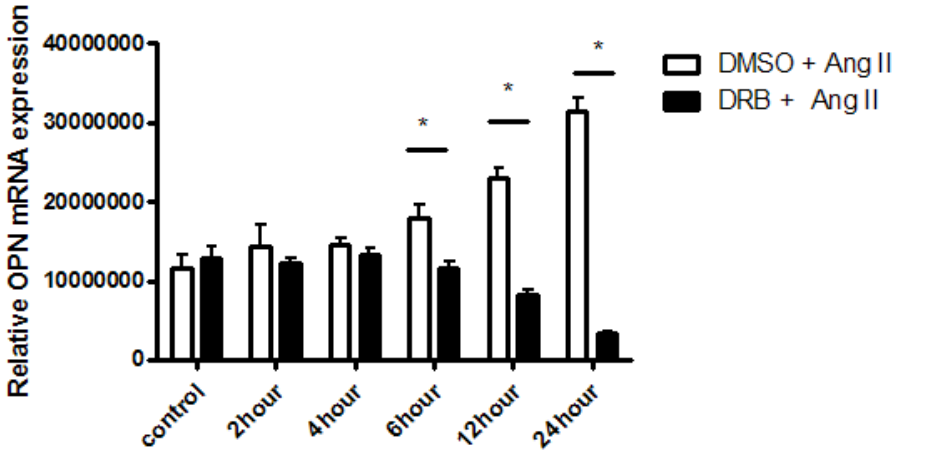
Figure 33. Ang II induces OPN protein expression *in vivo* and *in vitro*.

A. In vitro, Ang II increases OPN mRNA expression after 12hrs ($p < .001$) B. *In vitro*, Ang II increases OPN protein expression 4hrs after treatment by 420%±54 ($p < 0.0001$) in cultured VSMCs and continued throughout 24hr time course.

Early increases in Ang II-induced OPN expression are transcriptionally independent

To test whether Ang II-induced increases in OPN protein levels observed at the earlier time points after Ang II treatment are mediated through a post-transcriptional mechanism, we used DRB (a RNA polymerase II transcriptional inhibitor) to block transcription prior to stimulation with Ang II. We treated VSMCs with Ang II in the presence of 100μM DRB or DMSO (vehicle control). We found that Ang II-induced increases in OPN mRNA begin at 6hrs and were sustained throughout the 24hr time course in DMSO controls, whereas DRB treated cells exhibited no significant increase in OPN mRNA in response to Ang II (Figure 34A). This suggests that there is a pool of OPN mRNA at baseline that is readily translatable to protein and that transcriptional regulation of OPN by Ang II occurs at later time points. Furthermore, Ang II did not increase OPN mRNA stability as shown by comparing DRB treated cells in the presence and absence of Ang II prior to 6hrs (Figure 34B). These data suggest that OPN mRNA expression is unchanged at these time points in the presence of Ang II, and that early Ang II-induced increases in OPN protein levels may be the result of increased translation of pre-existing OPN mRNA. This represents a novel mechanism for Ang II regulation of early increases in OPN protein expression.

A.



B.

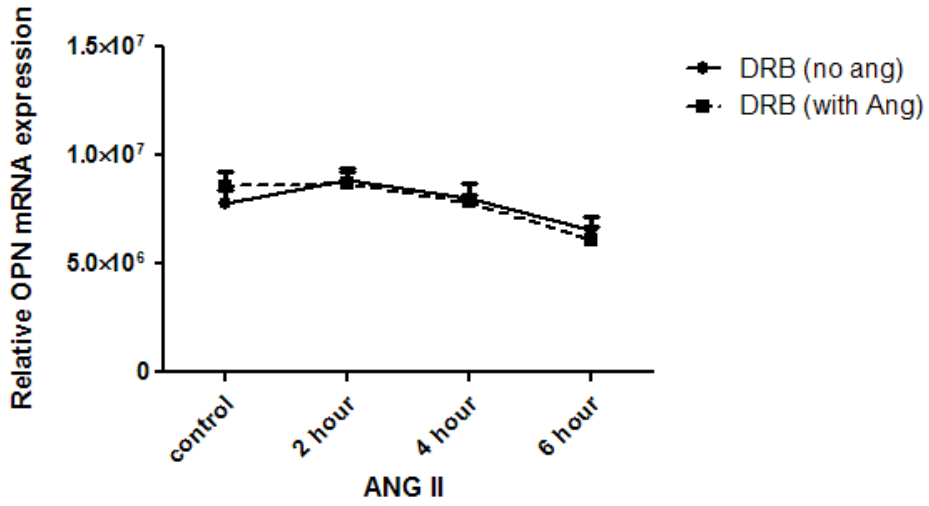


Figure 34. Transcriptional Repression reveals a Translation Dependent mechanism.

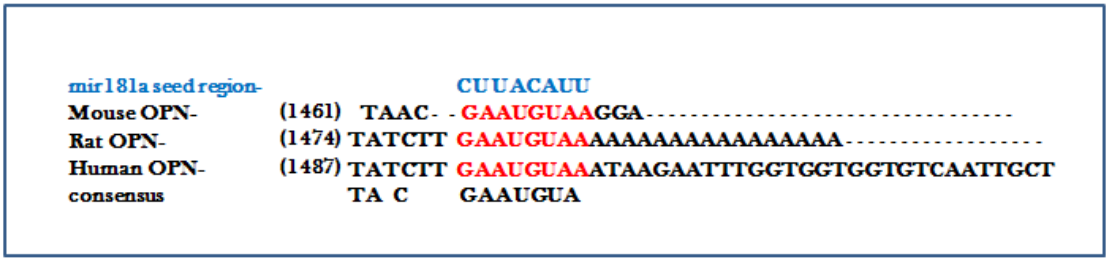
A. Ang II does increase OPN mRNA expression until after 6hrs as compared to DMSO controls. There is baseline OPN mRNA expression that does not increase with Ang II. B. Ang II does not further stabilize OPN mRNA before 6hrs as shown by comparing DRB in the presence and absence of Ang II at the indicated time points. $p < .05$ $n=3$

Ang II decreases mir181a expression

We next sought to determine a potential role for miRNAs in the regulation of OPN protein levels. We performed an extensive literature and database search for miRNAs that target the 3'UTR of OPN and result in translational repression, as a potential mechanism for modifying Ang II induced OPN expression. Search results from three miRNA databases (targetscan.org, miRWalk and microRNA.org) indicate that mir181a targets the 3'UTR of human OPN. Literature searches suggested human OPN as a potential target for mir181a by demonstrating that overexpressing and antagonizing mir181a affected OPN protein expression. Using human hepatocellular cancer cell lines expressing different amounts of endogenous OPN, mir181a was manipulated to show that OPN expression could be modulated by mir181a. (Bhattacharya*, et al.*) However, manipulating Ang II induced OPN expression with mir181a has not yet been determined in VSMCs or any other cell line. Therefore, our goal was to first determine whether regulation of OPN by mir181a occurs in VSMCs and secondly assess whether Ang II regulates mir181a expression. Alignment of OPN mRNA for human (NM_001040058.1), mouse (NM_001204201.1) and rat (NM_012881.2) species revealed a mir181a seed region binding site within the 3'UTR of all transcripts (–GAAUGUA- Figure 35A). This binding site was previously reported by Kazenwadel *et al.* in endothelial cells who showed that mir181a negatively regulates the expression of a homeobox transcription factor Prox1. (Kazenwadel*, et al.*)

In order to determine if mir181a is regulated by Ang II, we used qRT-PCR to determine if Ang II stimulation modulates mir181a expression. Indeed, we found that mir181a was decreased by 52% ± 7 ($p < 0.0001$) in VSMCs stimulated with 100nm Ang II for 24 hours compared to control cells (Figure 35B). These data suggest mir181a is a potential mediator through which Ang II increases OPN protein levels.

A.



B.

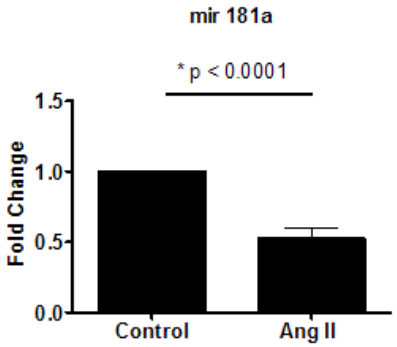


Figure 35. mir181a is predicted to target OPN and is regulated by Ang II.

A. The mir181a seed region binding site -GAAUGUA- is found within the 3'UTR of human, mouse and rat species. B. Ang II decreased mir181a expression by 52%±7 *(p < 0 .0001)* 24hrs post stimulation in VSMCs.

Overexpression of mir181a inhibits Ang II induced OPN protein expression

To test whether Ang II-induced increases in OPN protein expression at 4 hrs were mediated by decreased mir181a expression, we used a mir181a mimetic, which allows for overexpression of mir181a in VSMCs. As shown in Figure 36, overexpression of mir181a in VSMCs inhibited Ang II-induced increases in OPN protein expression at 4 hours by 69% ± 9 ($p<0.05$) compared to non-transfected control and negative miRNA treated cells stimulated with Ang II for 4 hours. The level of inhibition by mir181a reduced expression of OPN to levels similar to those observed at baseline in serum deprived VSMCs (control band, Figure 36). After 24 hours of Ang II stimulation, we still observed inhibition of OPN by overexpressing mir181a, suggesting a prolonged inhibitory effect. (Data not shown) To verify that signaling pathways in mir181a transfected cells maintained their ability to properly signal in response to Ang II stimulation of the AT1 receptor, we used a previously reported (Ishida*, et al.* 1998) assay to assess phosphorylation of Erk1/2 5mins after Ang II stimulation. As shown in Figure 37, mir181a overexpression does not interfere with Ang II signal transduction as evidenced by robust increases in phosphorylation of Erk1/2 at 5mins, suggesting that AT1 receptor signaling is intact.

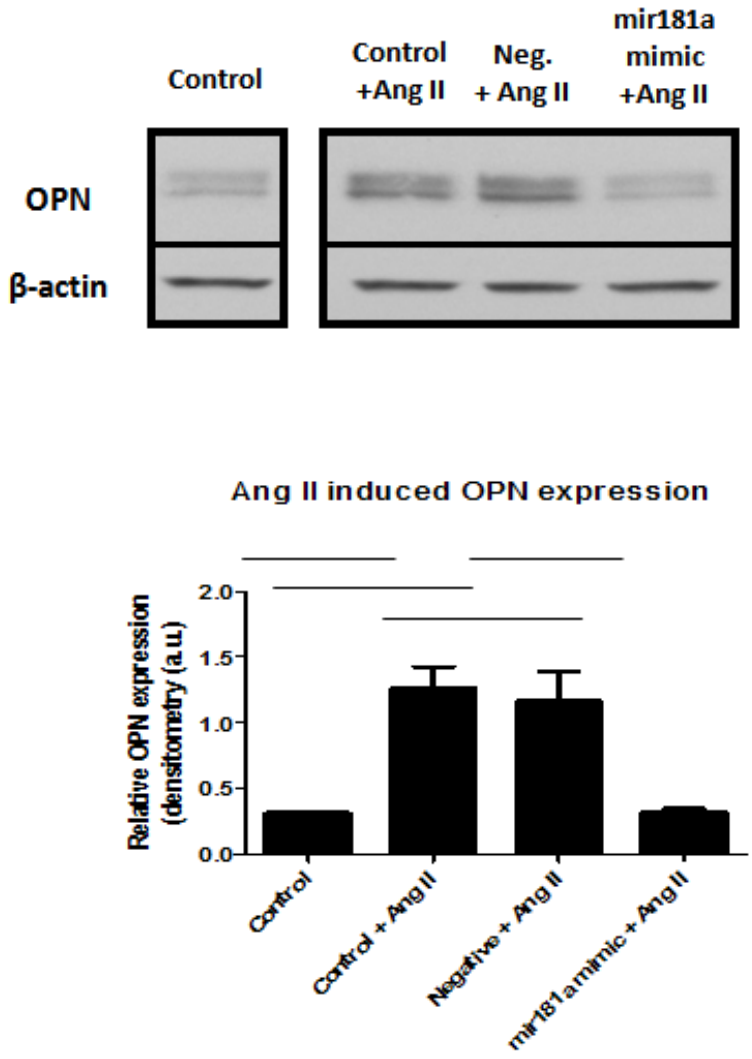


Figure 36. Overexpressing mir181a negatively regulates Ang II induced OPN expression.

Cells were stimulates with Ang II, alone or in combination with miRNA mimics. Ang II alone increases OPN expression. mir181a overexpression inhibits Ang II induced OPN expression at 4hrs in VSMCs by 69%±9 *($p < 0.001$)* The control lanes are from the same gel.

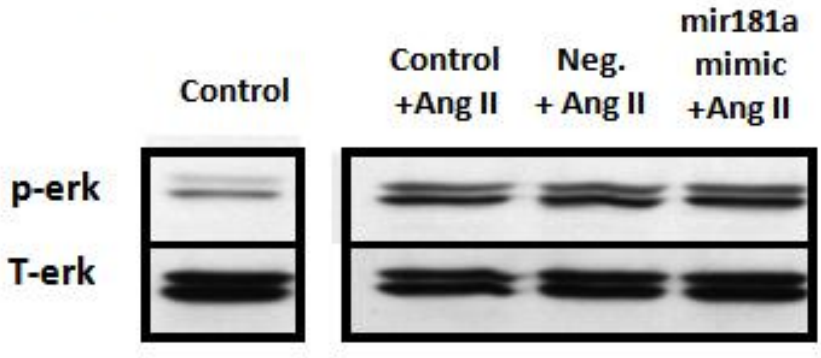


Figure 37. Overexpressing mir181a does not affect Ang II signaling.

Ang II is still capable of downstream signaling in miRNA overexpressed cells to the same degree as non-transfected Ang II stimulated cells. The control lanes are from the same gel.

mir181a overexpression inhibits Ang II-mediated VSMC Adhesion

To determine if mir181a modulation of OPN protein levels results in a physiologic impact, we examined the impact of modulation of mir181a expression on cell adhesion. Previous reports have suggested that the presence of OPN leads to increased adhesion of VSMCs to collagen substrates. (Weintraub*, et al.* 1996; Weintraub*, et al.* 2000) Therefore, we wanted to determine whether the inhibition of Ang II induced OPN with a mir181a mimetic resulted in changes in cellular adhesion. To assess the contribution of OPN to adhesion, we isolated and cultured MASMs from age matched $OPN^{-/-}$ or wildtype (WT) mice and plated them onto collagen coated coverslips. We observed that $OPN^{-/-}$ MASMs exhibited decreased adherence to collagen as compared to WT MASMs at 30 min and 1hr by 63.8%±9 and 61.9%±9, respectively. *($p<0.05$ and $p<0.001$, respectively;* Figure 38). The recovery of adhesion at later time points is consistent with previous literature suggesting that the cell is able to engage compensatory mechanisms to adjust for the lack of adhesion at early time points. (Weintraub*, et al.* 2000) These data demonstrate OPN contributes significantly to cell adhesion and thus provides a physiologic target to study the functional role of mir181a in a cell culture model.

We used an analogous strategy to assess the importance of mir181a dependent decreases in OPN expression on Ang II mediated VSMC adhesion to collagen. We found that in our negative and non-transfected control cells, Ang II increased cell adhesion by 68%±6 compared to non-stimulated controls (Figures 39 top panels). In contrast, we observed that VSMCs transfected with mir181a

mimetic and stimulated with Ang II for 24 hours adhere significantly less (36% ± 4) than VSMCs transfected with the negative control miRNA stimulated with Ang II (Figure 39 lower panels). When mir181a mimetic transfected VSMCs were treated with Ang II we observed a similar degree of adhesion as compared to baseline adhesion seen in non-stimulated VSMCs. As previously reported, reduced OPN results in reduced β1 integrin expression, leading to a decrease in adhesion to collagen substrates. We demonstrate a modest reduction in β1 integrin expression 24hrs after stimulation with Ang II, in the presence of a mir181a mimic. These data support our hypothesis that mir181a over expression has a functional impact on VSMCs as evidenced by the inhibition of adhesion of VSMCs to collagen in response to Ang II and the blocking of OPN protein translation.

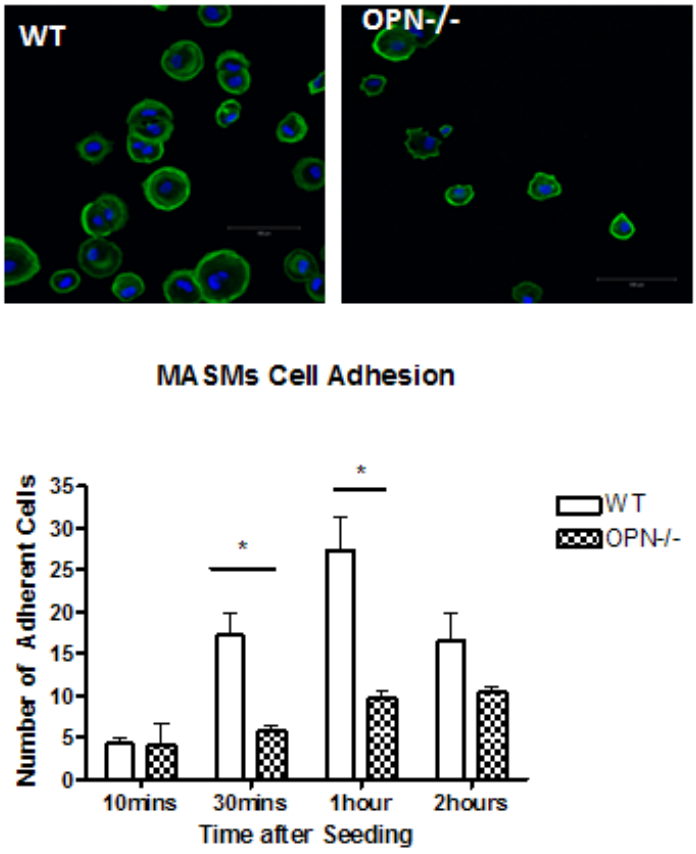
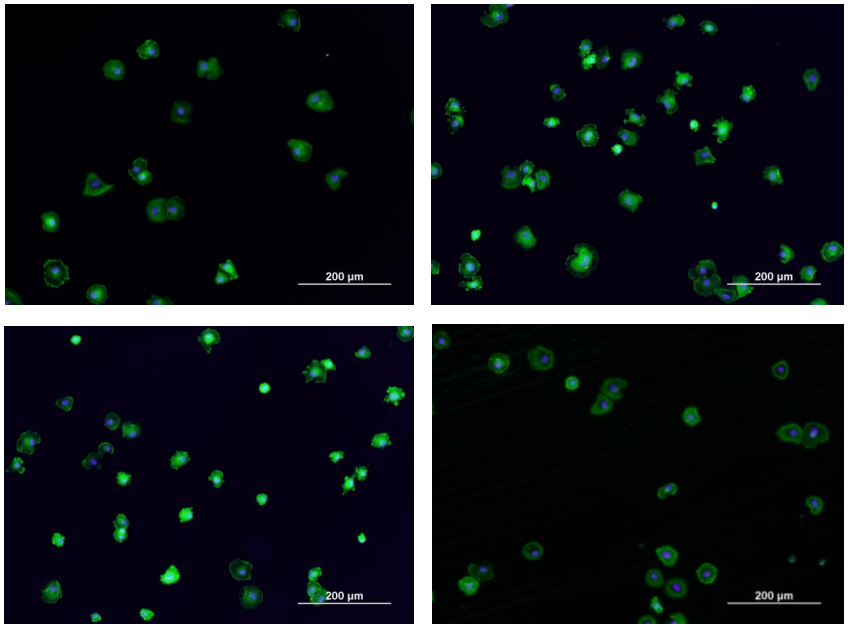


Figure 38. OPN is necessary for VSMCs adhesion.

OPN-/- MASMs adhere less to collagen coated coverslips than WT controls at 30 mins and 1 hr. *(p<.05 and p<.001 respectively.* Images are representative images from 5 random fields per experiments at 1hr, 20X confocal microscope. Green = Phalloidin Blue = DAPI



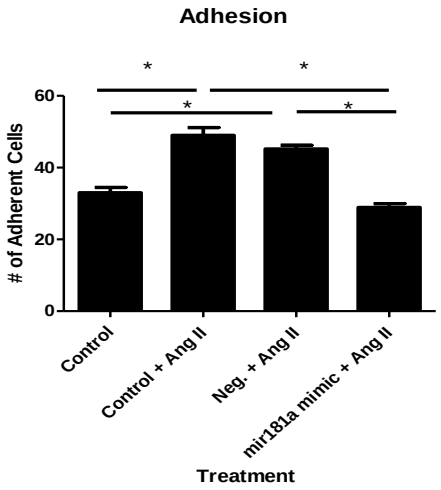


Figure 39. mir181a overexpression has a functional impact on VSMCs adhesion.

Ang II induces adhesion of VSMCs to type I collagen at 24 hours. (top panels) Ang II treated VSMCs with mir181a mimetic reduce adhesion of VSMCs to baseline levels. (Bottom panels) Green = Phalloidin Blue = DAPI

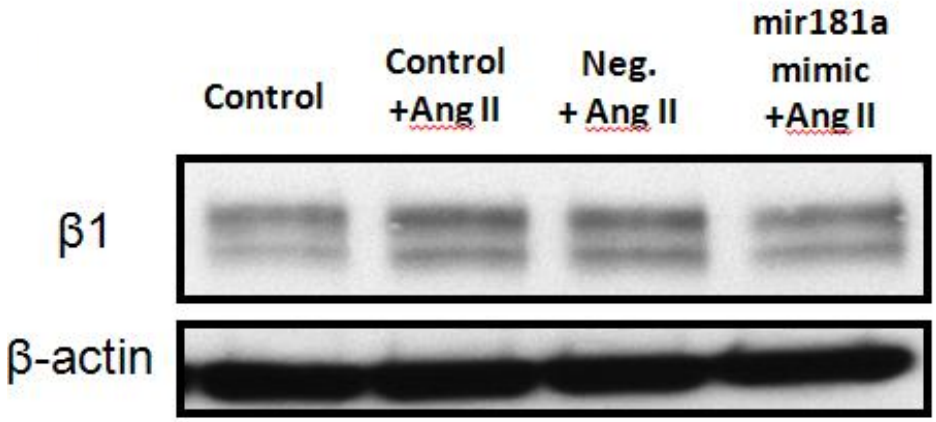


Figure 40. mir181a overexpression modestly reduced β1 integrin expression

Ang II increases β1 integrin expression in VSMCs and that expression is decreased after treatment with mir181a mimic suggesting a collaborative effort between β1 integrin and OPN in facilitating adhesion.

Discussion

Atherosclerosis is a well-documented inflammatory disease that exerts many of its effects on the vasculature via Ang II. One way Ang II promotes inflammation is through the upregulation of proinflammatory molecules, such as osteopontin. Micro RNAs function in part as critical regulators of protein expression by binding to the 3'UTRs of target mRNAs, resulting in inhibition of protein translation in mammalian cells. As such, miRNAs have been implicated in the pathogenesis of vascular disease and have been suggested as potential therapeutic targets. The goal of our study was to evaluate mir181a mediated regulation of Ang-II induced OPN protein expression in VSMCs. We found that Ang II increases OPN expression in a mouse model of hypertension at the mRNA and protein level compared to non-treated mice. Interestingly, *in vitro* we demonstrate that Ang II increases OPN protein expression in a translation-dependent manner. We have exploited this novel mechanism by identifying a miRNA, mir181a - previously demonstrated to regulate OPN protein expression in hepatocellular cell lines. We found that mir181a was expressed in VSMCs, and that its expression was downregulated by Ang II. We observed that overexpression of mir181a in VSMCs inhibits Ang II induced OPN induction in cultured VSMCs. We further demonstrate that mir181a overexpression had a functional impact on Ang II induced VSMCs adherence to collagen substrates. We therefore propose a novel mechanism for the regulation of Ang II induced OPN expression, and define a unique approach to regulating vascular dysfunction attributable to Ang II using a miRNA specific to OPN.

Clinical studies have previously reported that OPN is upregulated in atherosclerotic plaques and within injured arterial walls, indicating that OPN is detrimental in the setting of atherosclerosis. (Cho*, et al.* 2009) OPN transgenic mice that overexpress OPN protein, have medial thickening of the aorta without injury and in the presence of injury there is greater neointima formation. (Cho*, et al.* 2009) When mice that overexpress OPN are fed an atherogenic diet, they develop significantly larger lesions and have more activated macrophages within the atherosclerotic plaque than controls. (Cho*, et al.* 2009) Conversely, $ApoE^{-/-}/OPN^{-/-}$ mice have smaller atherosclerotic and inflammatory lesions compared to control counterparts. (Bruemmer*, et al.* 2003; Cho*, et al.* 2009) Collectively, these data suggest that OPN worsens atherosclerosis. These data, coupled with our data from hypertensive C57bl/6 mice where OPN is increased 7 days after treatment with Ang II, suggests that OPN protein expression exasperates vascular dysfuntion in mouse models of cardiovascular disease. This may be in part due to OPNs ability to promote macrophage and immune cell activation, as well as smooth muscle cell adhesion, migration and proliferation. It has previously been demonstrated that VSMCs derived from atherosclerotic human arteries exhibit greater adhesion, migration and proliferation than venous derived VSMCs. (Faries*, et al.* 2002) Since OPN mediates adhesion, migration and proliferation through binding and activation of integrin receptors, methods to regulate OPN protein expression are necessary.

Our *in vitro* studies suggest that Ang II-mediated transcriptional regulation of OPN occurs after 6hrs and the early increases in OPN cellular protein

expression by Ang II occur solely by translation dependent mechanisms. Ishida and colleagues have previously reported early increases in OPN mRNA, peaking between 8 and 12hrs, followed by later increases in secreted OPN protein in response to Ang II.(Abe*, et al.* 2008) Although we were able to confirm an increase at later time points in secreted OPN expression in response to Ang II, we did not observe an early increase in OPN mRNA expression prior to increases in cellular protein expression, which may be due to differences in experimental technique related to the culturing of VSMCs. The time differences we observed between increases in cellular and secreted OPN may be the result of intracellular packaging of OPN into vesicles for membrane targeting and/or secretion. Membrane bound and secreted OPN likely mediate adhesion and migration, as well as further downstream signaling events at later time points. Regardless, this is the first report that Ang II increases non-secreted OPN protein levels in VSMCs through a translation dependent manner following exposure to Ang II.

After confirming that Ang II treatment lead to an increase in the steady state level of OPN in VSMCs, we performed database searches for miRNAs capable of binding to the 3'UTR of OPN. Kuo and colleagues demonstrated that mir181a targets the human OPN 3'UTR using a luciferase based reporter system and serial deletion of components of the 3'UTR.(Bhattacharya*, et al.*) Using qRT-PCR, we were able to demonstrate that not only was mir181a expressed in unstimulated VSMCs, but also that Ang II reduces mir181a expression. Evaluating mir181a expression at early time points revealed decreases in

mir181a expression by less than ~20%. The developing nature of the miRNA field prevents us from establishing a threshold value for critical changes in expression that result in the loss or gain of function of miRNAs. Therefore, it is difficult to conclude what numerical decrease in mir181a expression would result in a dysfunctional miRNA.

Further investigation of the relationship between mir181a, OPN and Ang II, has allowed us to demonstrate a novel mechanism by which mir181a is capable of inhibiting OPN protein synthesis in the presence of Ang II in VSMCs. Our findings are consistent with previous observations demonstrating that mir181a plays an inhibitory role in the regulation of OPN protein expression in human hepatocellular cancer cell lines. (Bhattacharya*, et al.*) The conservation of this regulatory mechanism by which mir181a inhibits Ang II induced OPN protein expression in VSMCs and in non-stimulated hepatocellular cancer cells suggest that mir181a may be an important and necessary regulatory mechanism for OPN in all cell types. At present, the mechanism by which Ang II modulates mir181a expression remains to be elucidated. We speculate that Ang II regulates mir181a by decreasing the binding of mir181a to OPN's 3'UTR. A decrease of mir181a binding by Ang II could permit active translation of OPN mRNA through RNA conformational changes that alter the affinity of RNA binding proteins (RBPs) to the 3'UTR. RBPs and miRNAs work in concert to promote translation or repression of mRNA expression. (Ma*, et al.* 2010; van Kouwenhove*, et al.* 2011) mir181a may act to recruit RBPs that repress translation, but in the presence of Ang II, mir181a is no longer bound to OPN, RBPs are no longer

recruited, and translation occurs freely. Regardless of the mechanism, it is clear that overexpression of mir181a is a potential mechanism for the regulation of OPN translation.

Ang II promotes adhesion of cardiac fibroblast to collagen substrates through an integrin mediated mechanism in which PKC phosphorylates the β1 integrin, causing it to translocate to the cell surface. (Hein 2005) In addition, Weintraub *et al.* previously demonstrate that OPN facilitates adhesion to collagen through an OPN antisense transfection method to reduce OPN expression in VSMCs. (Weintraub*, et al.* 2000) Using these data as a precursor for our functional assessment of OPN, we have shown that mir181a overexpression in the setting of Ang II stimulation reduces the ability of VSMCs to adhere to a collagen substrate. As mentioned previously, OPN mediates adhesion of VSMCs to collagen substrates via integrin and CD44 binding. This binding can further activate downstream signaling events such as apoptosis, proliferation, migration etc. By evaluating the adhesive properties of VSMCs with reduced OPN protein expression, we can predict the efficacy of OPN to promote further signal transduction through integrin receptor activation. Weintraub *et al.* has further demonstrated that reduced adhesion of OPN deficient VSMCs to collagen is due to decreased expression of α1 and β1 collagen binding integrins. We observe a similar trend in that VSMCs deficient in OPN also have a decreased amount of integrin expression.

The fact that overexpressed mir181a inhibits Ang II induced increases in OPN protein in VSMCs suggests a role for mir181a in cardiovascular disease

through the ability of OPN to promote migration and proliferation of VSMCs. It should be noted that at baseline, intracellular OPN protein expression in VSMCs is low, making it difficult to observe a reduction in OPN levels when overexpressing mir181a. Additionally, no effect on OPN protein expression was noted in VSMCs transfected with a mir181a inhibitor, lending support to a RBP-based mechanism of de-repression. Without Ang II, translation will not be initiated, regardless of the miRNA/RBP relationship, potentially explaining why antagonizing mir181a has no effect on OPN protein expression in non-stimulated cells. Nonetheless, the mir181a mediated inhibition of Ang II induced OPN is valuable to the current understanding of miRNAs and vascular disease progression. The ability of mir181a to reduce OPN protein expression and function in the setting of Ang II may make mir181a a novel therapeutic target for changes to our cardiovascular disease outlook.

The ability of various miRNAs to inhibit the effect of Ang II on the vasculature has recently been evaluated in human endothelial cells, suggesting a role in inflammation and migration.(Zhu*, et al.* 2011) Overexpression of mir-155 and mir-221/222 was shown to block Ang II activation of Ets-1(endothelial transcription factor) and subsequent increases in inflammatory genes such as VCAM, MCP-1 and FLT-1.(Zhu*, et al.* 2011) miRNA regulation of Ang II induced protein expression in endothelial cells was successful, in part due to the downregulation of the AT1 receptor by mir155. Here we clearly demonstrate reduction of specific Ang II mediated effects through miRNA delivery. However,

in contrast to previous reports, the mechanism appears to be more specific and independent of AT1 receptor downregulation.

In conclusion, we have discovered a novel mechanism of OPN regulation in VSMCs; that of mir181a mediated post-transcriptional silencing. Our data indicate that mir181a activity is an important mechanism by which Ang II induced OPN expression is inhibited in the vasculature during exposure to Ang II. The ability of mir181a to have a functional impact on Ang II induced VSMC adhesion, underscores the contribution of miRNAs to disease pathology. Modulation of OPN levels using mir181a will have future implications for the treatment of cardiovascular disease.

Chapter 5: Summary and Future Directions

We and others have shown that angiotensin II (Ang II) promotes cardiovascular disease, including hypertension, atherosclerosis, and abdominal aortic aneurysms (AAA). The effect of Ang II on the vasculature is largely exerted by the modulation of extracellular matrix (ECM) components. In an effort to understand the direct effect of Ang II on ECM proteins as it relates to dysfunction in vascular smooth muscle cells (VSMCs), we focused on two types of ECM proteins, namely the Lysyl Oxidase isoenzymes and Osteopontin. These proteins are categorized based on their contribution to either the structural integrity of the matrix or their function as a modulator of cell matrix interactions. The lysyl oxidase isoenzymes fall into the subcategory of structural ECM proteins, based on their ability to crosslink and stabilize collagen and elastin in the ECM. This role of altering the stability of collagen and elastin fibers is shared with other enzymes, such as the well-studied matrix metalloproteinase (MMPs). Reasonable evidence supports the notion that the lysyl oxidase isoenzymes counter the activity of MMPs. In contrast, OPN falls into the category of an ECM protein that does not appear to subserve a primary structural role, but rather mediates adhesion, migration and other cell matrix interactions, making it a matricellular protein. Both structural ECM proteins and matricellular proteins have been implicated in the pathophysiology of cardiovascular disease as they facilitate matrix turnover and propagation of signaling pathways that lead to adhesion and migration of VSMCs.

We have found, using *in vivo* and *in vitro* models, that Ang II does not affect the expression or activity of the lysyl oxidase isoenzymes in VSMCs.

However, upon inhibition of LOX/LOXL family members with β-APN in the presence of Ang II *in vivo*, we observe a robust increase in AAA formation demonstrating that the LOX/LOXL family members are important for vessel integrity. It is plausible that the LOX/LOXL family members are important yet not regulated in adult aortas. It is clear that the lack of regulation of the LOX/LOXL family members does not underscore its importance in the vascular wall, as seen upon inhibition of LOX/LOXL activity with β-APN in an *in vivo* model of Ang II induced AAA. From these data we are able to separate regulation from importance and conclude that not every gene/protein/function is regulated, however, its presence may still be important, as in the case of the LOX/LOXL family members.

Conversely, we found that Ang II increases OPN expression in VSMCs, in a previously unreported translation dependent manner. This Ang II-induced increase is in agreement with increases observed *in vivo* in response to Ang II. We further identified a novel approach to regulate Ang II- induced OPN expression, through miRNA manipulation, illustrated in Figure 41. Based on our findings, we conclude that Ang II differentially regulates ECM proteins in VSMCs and that this regulation is most likely crucial in the progression of vascular dysfunction observed in Ang II-induced mouse models of hypertension, atherosclerosis and AAA. While we acknowledge that *in vivo*, the progression from a healthy aorta to a diseased aorta results from a series of coordinated events, we underscore the importance of understanding direct relationships between primary effectors such as Ang II and target ECM proteins. This

understanding provides the basis for more in-depth investigations that include other molecules, and opens the possibility to manipulate and regulate the response of ECM proteins with miRNAs.

Our future directions fall into four broad categories; 1) determine whether a direct relationship exist between Ang II, mir181a and OPN; 2) identify the mechanism by which Ang II modulates mir181a expression; 3) identify the signaling pathway by which OPN mediates β1 integrin expression as it relates to adhesion; and 4) determine whether mir181a manipulation has a functional role *in vivo*. The following paragraphs will discuss progress made towards each category and specific questions that should be addressed in each case.

In vivo, it is speculated that microRNAs work in concert with other miRNAs to promote changes in structure and function. Although, we see an effect on OPN when overexpressing mir181a alone, it is plausible that other miRNAs and/or proteins are collectively altered in response to Ang II. To tease out the direct relationship, we propose mutating the mir181a seed region within the 3'UTR of OPN to evaluate how precise mir181a is at influencing Ang II induced OPN. Mutations within the mir181a seed region (GAATGTA- to –ATCAGTA) result in loss of mir181a binding to its 3'UTR target.(Kazenwadel*, et al.* 2010) Therefore, we over-expressed a 6xHIS-tagged full length human OPN gene with a GAATGTA- to –ATCAGTA mutation in the mir181a binding site in VSMCs, using a lentiviral based transduction method. Future direction include overexpressing mir181a in the presence and absence of Ang II and evaluating the direct effect of mir181a binding on OPN expression in mutant cells.

Collectively these experiments will determine whether mir181a alone is sufficient for therapeutic intervention, or whether the relationship between Ang II, mir181a and OPN is more complex.

To identify the mechanisms by which Ang II modulates mir181a expression, we have made significant progress in demonstrating that Ang II downregulates mir181a expression. It is plausible that mir181a, which is endogenously expressed within a promoter driven gene, may be regulated by Ang II-induced changes in translocation of transcription factors and/or repressors. A likely scenario is that Ang II decreases the binding of a repressor through phosphorylation mediated events, and that repressor leads to a decrease in the steady state levels of mir181a. Currently, we are unaware of any report linking a transcription factor or repressor to mir181a expression. A BLAST search of the human, mouse and rat genome, using the mir181a miRNA sequence as a query, shows that in all species mir181a is located within introns of coding genes. There is no similarity in the location or gene that mir181a occupies across species with the exception that, mir181a consistently resides in a gene neighboring Ptprc (Protein Tyrosine Phosphatase Receptor C). The lack of similarity in mir181a gene location makes it difficult to analyze promoter regulation. In a counter approach, we attempted to identify a downstream Ang II effector that may more specifically alter mir181a expression. Ang II exerts many of its effects through second messengers such as increased intracellular Ca^{2+}, activated PKC, and increased H_2O_2 produced as a byproduct of an activated NADPH oxidase. We propose that altering these second messengers may

define a more specific pathway in which Ang II modulates mir181a expression. We have evaluated the effect of H_2O_2 on mir181a expression and observed no change over time. In an effort to map a mir181a signaling pathway in VSMCs, future research should focus on evaluating the effect of other candidate second messengers on mir181a expression.

As mentioned previously, we only observed a modest decrease in mir181a expression after early stimulation with Ang II, leading us to conclude that the mechanism by which Ang II modulates the expression of mir181a is complex. Possibly, Ang II modulates binding of mir181a to the 3'UTR of the OPN gene. Previous literature reveals that miRNAs can regulate protein expression by directly recruiting RNA binding proteins (RBPs) that modulate translation of mRNA. Thus, a possible explanation is that mir181a is bound to the 3'UTR of OPN and attracts a repressor RBP. In the presence of Ang II, mir181a expression and binding may be reduced, thereby decreasing the affinity of the repressor for the mRNA. In this setting, Ang II would cause active translation of OPN mRNA. However, if mir181a is overexpressed, mir181a and the repressor would continually bind the 3'UTR of OPN, preventing Ang II from both reversing this repression and inducing translation of OPN. This scenario of interplay between a miRNA and a RBP has been previously reported in the literature.(Agami 2010; George and Tenenbaum 2006; Kedde and Agami 2008; Ma*, et al.* 2010) In order to test this potential explanation, we identified RBP binding sites within OPNs 3'UTR. Future directions include demonstrating the expression of RBPs in VSMCs, as well as their ability to bind to OPN mRNA in

the presence and absence of Ang II. To identify RBPs that bind the 3'UTR of OPN, we used a RBP database (http://rbpdb.ccbr.utoronto.ca/) which predicts potential proteins that bind to target RNA sequences, based on recognition motifs. Our database predicted approximately 60 RBPs to bind within the OPN 3'UTR including many translation initiation factors such as the poly A binding protein (PABP) and eukaryotic initiation factor 4B (eIF4B). Interestingly, when probing only the mir181a seed region sequence (7nt) for RBPs, we identified a repressor that is predicted to bind to the exact area of the mir181a region, named Pumilio 2 (PUM2). PUM2 interacts with the 3'UTR of RINGO/SPY mRNA to repress translation in Xenopus oocytes. (Cao*, et al.* 2010) Furthermore, a link between PUM2 and miRNAs has been previously established in regards to translation of E2F3 transcription factors. (Miles*, et al.* 2012) Future directions include determining the extent to which Ang II modulates the expression of PUM2 expression in VSMCs, as well as further evaluating the ability of PUM2 to bind OPN mRNA in all aforementioned scenarios. The experiments described would allow us to mechanistically identify Ang II-mediated regulation of mir181a, as well as determine whether mir181a works in concert with RBPs to promote translation.

To contribute to the understanding of the functional relationship between mir181a and Ang II, we have demonstrated that the presence of mir181a decreases Ang II-induced adhesion of VSMCs. We, as well as others, report that reduced OPN expression decreases integrin expression in VSMCs. Multiple studies report that an association exists between OPN expression and β1

integrin expression, but the pathway remains understudied. OPN primarily interacts with integrin αvβ3. Therefore, we postulate that this interaction propagates intracellular signaling pathways that lead to β1 integrin expression. Integrin αvβ3 is a distinct from other integrin's in its ability to upregulate intracellular Ca^{2+} and to transactivate several growth factor receptors, all known mechanisms for increasing protein expression.(Moiseeva 2001) The ligands for αvβ3 always contain a RGD motif. Treating cells with exogenous OPN and blocking αvβ3 activation with an RGD blockade such as a cyclic RGD peptide or an RGD mimetic would demonstrate the extent to which OPN induced β1 expression is dependent on αvβ3 activation. The aims and subsequent experiments proposed would contribute to mechanistically understanding how Ang II regulates mir181a, to defining the significance of mir181a in OPN protein translation, and to describing in further detail how reduced OPN expression leads to reduced adhesion via the β1 integrin.

Understanding mechanistically how mir181a is regulated and how this regulation contributes to OPN-mediated decreases in β1 expression and to reduced VSMCs adhesion to collagen is, arguably, only relevant in an *in vitro* setting. Identifying a relationship between mir181and Ang II in *vivo* would lend support to the use of miRNAs for therapies aimed at treating vascular disease. We propose conducting *in vivo* studies using a mouse model of hypertension, and evaluating how treatment with a mir181a mimic affects the expression of OPN. Based on our in vitro findings, we would expect that treatment would lead to a reduction in OPN expression. Other key components of an *in vivo* analysis

would include the evaluation of other markers of inflammation such as macrophage infiltration, VCAM and ICAM expression. One caveat of this experimentation would be targeting the mir181a mimic specifically to the cardiovascular system, our area of interest. In support of our proposed experimental setup, Bonauer *et al* has shown that systemic administration of a miR-92a inhibitor leads to enhanced blood vessel growth and functional recovery of damaged tissue.(Bonauer*, et al.* 2009) However, we are aware that miRNAs target hundreds of genes, which can lead to a wide range of off target effects. To limit the extent of our treatment to a specific site, we propose employing an approach similar to that of Ji and colleagues, where a gel based targeting system permitted the beneficial delivery of mir21 inhibitors to the coronary artery in a tissue specific manner after balloon angioplasty, resulting in decreased proliferation and increased apoptosis.(Ji*, et al.* 2007) This method is more specific and cost effective as compared to other approaches and would demonstrate the contribution of mir181a overexpression to Ang II induced cardiovascular disease.

In conclusion, the findings reported in this dissertation contribute to understanding and characterizing the evolving field of miRNA-based regulation of protein expression and function. The future directions we propose aim at expanding our findings to exploit the potential of miRNA-based therapies to treat cardiovascular disease.

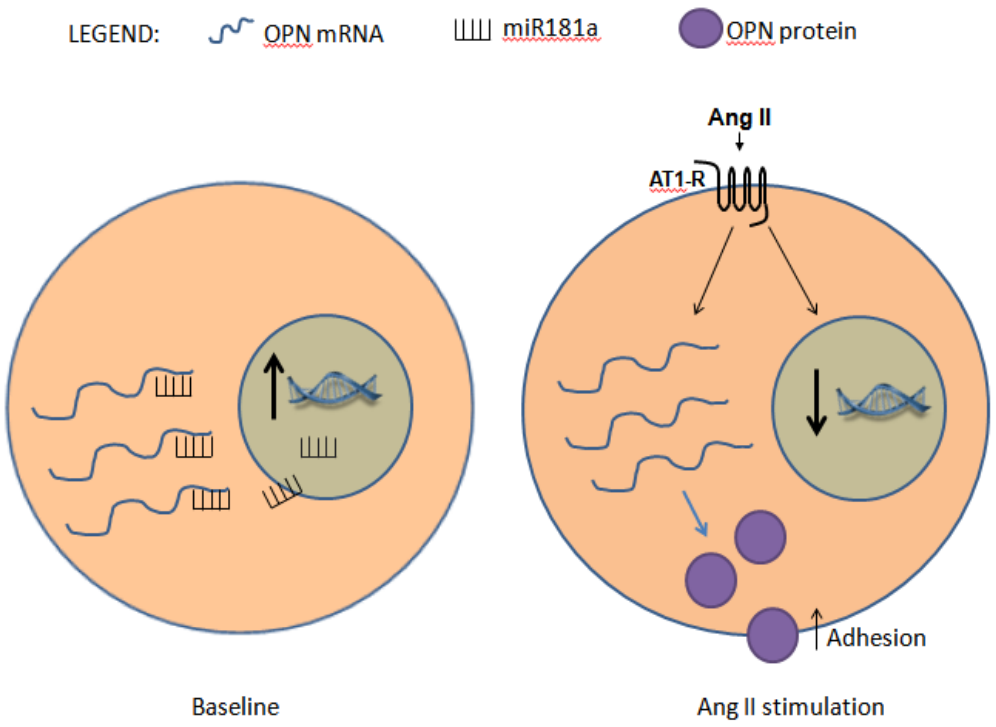


Figure 41. Model for mir181a regulation of OPN mRNA

At baseline, mir181a is actively transcribed and bound to the 3'UTR of preexisting OPN mRNA. In the presence of Ang II, mir181a expression is decreased and/or degraded and mir181a is no longer able to bind to OPNs' 3'UTR rendering Ang II capable of initiating translation of preexisting OPN mRNA. Ang II induced increases in OPN protein facilities adhesion of VSMCs to collagen substrates.

References

in *Encyclopædia Britannica, Inc.* (2007).

Abe, K., Nakashima, H., Ishida, M., Miho, N., Sawano, M., Soe, N. N., Kurabayashi, M., Chayama, K., Yoshizumi, M. & Ishida, T. Angiotensin II-induced osteopontin expression in vascular smooth muscle cells involves Gq/11, Ras, ERK, Src and Ets-1. *Hypertens Res* 31, 987-998, doi:10.1291/hypres.31.987 (2008).

Agami, R. microRNAs, RNA binding proteins and cancer. *Eur J Clin Invest* 40, 370-374, doi:10.1111/j.1365-2362.2010.02279.x (2010).

Alexander, R. W. Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* 25, 155-161 (1995).

Ananthakrishnan, R. & Ehrlicher, A. The forces behind cell movement. *Int J Biol Sci* 3, 303-317 (2007).

Anderson, D. W., Edwards, T. K., Ricketts, M. H., Kuivaniemi, H., Tromp, G., Stolle, C. A., Deak, S. B. & Boyd, C. D. Multiple defects in type III collagen synthesis are associated with the pathogenesis of abdominal aortic aneurysms. *Ann N Y Acad Sci* 800, 216-228 (1996).

Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. & Caroni, P. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393, 805-809 (1998).

Arenas, I. A., Xu, Y., Lopez-Jaramillo, P. & Davidge, S. T. Angiotensin II-induced MMP-2 release from endothelial cells is mediated by TNF-alpha. *Am J Physiol Cell Physiol* 286, C779-784 (2004).

Asou, Y., Rittling, S. R., Yoshitake, H., Tsuji, K., Shinomiya, K., Nifuji, A., Denhardt, D. T. & Noda, M. Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. *Endocrinology* 142, 1325-1332 (2001).

Bhattacharya, S. D., Garrison, J., Guo, H., Mi, Z., Markovic, J., Kim, V. M. & Kuo, P. C. Micro-RNA-181a regulates osteopontin-dependent metastatic function in hepatocellular cancer cell lines. *Surgery* 148, 291-297.

Bissels, U., Wild, S., Tomiuk, S., Holste, A., Hafner, M., Tuschl, T. & Bosio, A. Absolute quantification of microRNAs by using a universal reference. *RNA* 15, 2375-2384, doi:10.1261/rna.1754109 (2009).

Bonauer, A., Carmona, G., Iwasaki, M., Mione, M., Koyanagi, M., Fischer, A., Burchfield, J., Fox, H., Doebele, C., Ohtani, K., Chavakis, E., Potente, M., Tjwa, M., Urbich, C., Zeiher, A. M. & Dimmeler, S. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science* 324, 1710-1713, doi:10.1126/science.1174381 (2009).

Bruemmer, D., Collins, A. R., Noh, G., Wang, W., Territo, M., Arias-Magallona, S., Fishbein, M. C., Blaschke, F., Kintscher, U., Graf, K., Law, R. E. & Hsueh, W. A. Angiotensin II-accelerated atherosclerosis

and aneurysm formation is attenuated in osteopontin-deficient mice. *J Clin Invest* 112, 1318-1331, doi:10.1172/JCI18141 (2003).

Bushati, N. & Cohen, S. M. microRNA functions. *Annu Rev Cell Dev Biol* 23, 175-205, doi:10.1146/annurev.cellbio.23.090506.123406 (2007).

Campos, A. H., Zhao, Y., Pollman, M. J. & Gibbons, G. H. DNA microarray profiling to identify angiotensin-responsive genes in vascular smooth muscle cells: potential mediators of vascular disease. *Circ Res* 92, 111-118 (2003).

Cao, Q., Padmanabhan, K. & Richter, J. D. Pumilio 2 controls translation by competing with eIF4E for 7-methyl guanosine cap recognition. *RNA* 16, 221-227, doi:10.1261/rna.1884610 (2010).

Catalucci, D., Gallo, P. & Condorelli, G. MicroRNAs in cardiovascular biology and heart disease. *Circ Cardiovasc Genet* 2, 402-408, doi:10.1161/CIRCGENETICS.109.857425 (2009).

Chakraborty, G., Jain, S., Behera, R., Ahmed, M., Sharma, P., Kumar, V. & Kundu, G. C. The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. *Curr Mol Med* 6, 819-830 (2006).

Chen, K. & Rajewsky, N. The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet* 8, 93-103, doi:10.1038/nrg1990 (2007).

Chen, Y., Gelfond, J. A., McManus, L. M. & Shireman, P. K. Reproducibility of quantitative RT-PCR array in miRNA expression profiling and

comparison with microarray analysis. *BMC Genomics* 10, 407, doi:10.1186/1471-2164-10-407 (2009).

Chen, Z., Lee, F. Y., Bhalla, K. N. & Wu, J. Potent Inhibition of Platelet-derived Growth Factor-induced Responses in Vascular Smooth Muscle Cells by BMS-354825. *Mol Pharmacol* (2006).

Cho, H. J., Cho, H. J. & Kim, H. S. Osteopontin: a multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Curr Atheroscler Rep* 11, 206-213 (2009).

Csiszar, K. Lysyl oxidases: a novel multifunctional amine oxidase family. *Prog Nucleic Acid Res Mol Biol* 70, 1-32 (2001).

Daubman, S. MicroRNAs in angiogenesis and vascular smooth muscle cell function. *Circ Res* 106, 423-425, doi:10.1161/RES.0b013e3181d61a0d (2010).

Daugherty, A. & Cassis, L. A. Mouse models of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 24, 429-434, doi:10.1161/01.ATV.0000118013.72016.ea

01.ATV.0000118013.72016.ea [pii] (2004).

Davis, V., Persidskaia, R., Baca-Regen, L., Itoh, Y., Nagase, H., Persidsky, Y., Ghorpade, A. & Baxter, B. T. Matrix metalloproteinase-2 production and its binding to the matrix are increased in abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 18, 1625-1633 (1998).

Dechert, M. A., Holder, J. M. & Gerthoffer, W. T. p21-activated kinase 1 participates in tracheal smooth muscle cell migration by signaling to p38 Mapk. *Am J Physiol Cell Physiol* 281, C123-132 (2001).

Denhardt, D. T., Giachelli, C. M. & Rittling, S. R. Role of osteopontin in cellular signaling and toxicant injury. *Annu Rev Pharmacol Toxicol* 41, 723-749, doi:10.1146/annurev.pharmtox.41.1.723 (2001).

Denhardt, D. T. & Guo, X. Osteopontin: a protein with diverse functions. *FASEB J* 7, 1475-1482 (1993).

Faries, P. L., Rohan, D. I., Wyers, M. C., Marin, M. L., Hollier, L. H., Quist, W. C. & LoGerfo, F. W. Vascular smooth muscle cells derived from atherosclerotic human arteries exhibit greater adhesion, migration, and proliferation than venous cells. *J Surg Res* 104, 22-28, doi:10.1006/jsre.2002.6399 (2002).

Ferrara, D. E., Weiss, D., Carnell, P. H., Vito, R. P., Vega, D., Gao, X., Nie, S. & Taylor, W. R. Quantitative 3D fluorescence technique for the analysis of en face preparations of arterial walls using quantum dot nanocrystals and two-photon excitation laser scanning microscopy. *Am J Physiol Regul Integr Comp Physiol* 290, R114-123, doi:00449.2005 [pii]

10.1152/ajpregu.00449.2005 (2006).

Fleige, S. & Pfaffl, M. W. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* 27, 126-139, doi:10.1016/j.mam.2005.12.003 (2006).

Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J. & Pfaffl, M. W. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnol Lett* 28, 1601-1613, doi:10.1007/s10529-006-9127-2 (2006).

Freestone, T., Turner, R. J., Higman, D. J., Lever, M. J. & Powell, J. T. Influence of hypercholesterolemia and adventitial inflammation on the development of aortic aneurysm in rabbits. *Arterioscler Thromb Vasc Biol* 17, 10-17 (1997).

George, A. D. & Tenenbaum, S. A. MicroRNA modulation of RNA-binding protein regulatory elements. *RNA Biol* 3, 57-59 (2006).

Gerthoffer, W. T. Mechanisms of vascular smooth muscle cell migration. *Circ Res* 100, 607-621, doi:10.1161/01.RES.0000258492.96097.47 (2007).

Giachelli, C. M., Bae, N., Almeida, M., Denhardt, D. T., Alpers, C. E. & Schwartz, S. M. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest* 92, 1686-1696, doi:10.1172/JCI116755 (1993).

Git, A., Dvinge, H., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., Bertone, P. & Caldas, C. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA* 16, 991-1006, doi:10.1261/rna.1947110 (2010).

Golledge, J., Muller, J., Daugherty, A. & Norman, P. Abdominal aortic aneurysm: pathogenesis and implications for management. *Arterioscler Thromb Vasc Biol* 26, 2605-2613 (2006).

Griendling, K. K., Taubman, M. B., Akers, M., Mendlowitz, M. & Alexander, R. W. Characterization of phosphatidylinositol-specific phospholipase C from cultured vascular smooth muscle cells. *J Biol Chem* 266, 15498-15504 (1991).

Guo, R. W., Yang, L. X., Wang, H., Liu, B. & Wang, L. Angiotensin II induces matrix metalloproteinase-9 expression via a nuclear factor-kappaB-dependent pathway in vascular smooth muscle cells. *Regul Pept* 147, 37-44, doi:10.1016/j.regpep.2007.12.005 (2008).

Haendeler, J. & Berk, B. C. Angiotensin II mediated signal transduction. Important role of tyrosine kinases. *Regul Pept* 95, 1-7 (2000).

Hansson, G. K., Robertson, A. K. & Soderberg-Naucler, C. Inflammation and atherosclerosis. *Annu Rev Pathol* 1, 297-329, doi:10.1146/annurev.pathol.1.110304.100100 (2006).

Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A. & Stossel, T. P. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* 82, 643-653 (1995).

He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 5, 522-531, doi:10.1038/nrg1379 (2004).

Hein, L. Angiotensin II and cell-matrix adhesion: PKCepsilon is essential. *Cardiovasc Res* 67, 6-8, doi:10.1016/j.cardiores.2005.05.008 (2005).

Higuchi, S., Ohtsu, H., Suzuki, H., Shirai, H., Frank, G. D. & Eguchi, S. Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. *Clin Sci (Lond)* 112, 417-428, doi:10.1042/CS20060342 (2007).

Hornstra, I. K., Birge, S., Starcher, B., Bailey, A. J., Mecham, R. P. & Shapiro, S. D. Lysyl oxidase is required for vascular and diaphragmatic development in mice. *J Biol Chem* 278, 14387-14393 (2003).

Horwitz, A. R. & Parsons, J. T. Cell migration--movin' on. *Science* 286, 1102-1103 (1999).

Ikeda, T., Shirasawa, T., Esaki, Y., Yoshiki, S. & Hirokawa, K. Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. *J Clin Invest* 92, 2814-2820 (1993).

Ishida, M., Ishida, T., Thomas, S. M. & Berk, B. C. Activation of extracellular signal-regulated kinases (ERK1/2) by angiotensin II is dependent on c-Src in vascular smooth muscle cells. *Circ Res* 82, 7-12 (1998).

Ji, R., Cheng, Y., Yue, J., Yang, J., Liu, X., Chen, H., Dean, D. B. & Zhang, C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion

formation. *Circ Res* 100, 1579-1588, doi:10.1161/CIRCRESAHA.106.141986 (2007).

Jimenez, E., Perez de la Blanca, E., Urso, L., Gonzalez, I., Salas, J. & Montiel, M. Angiotensin II induces MMP 2 activity via FAK/JNK pathway in human endothelial cells. *Biochem Biophys Res Commun* 380, 769-774, doi:10.1016/j.bbrc.2009.01.142 (2009).

Jin, L., Ying, Z. & Webb, R. C. Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. *Am J Physiol Heart Circ Physiol* 287, H1495-1500 (2004).

Kagan, H. M. & Li, W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* 88, 660-672, doi:10.1002/jcb.10413 (2003).

Kagan, H. M., Raghavan, J. & Hollander, W. Changes in aortic lysyl oxidase activity in diet-induced atherosclerosis in the rabbit. *Arteriosclerosis* 1, 287-291 (1981).

Kanematsu, Y., Kanematsu, M., Kurihara, C., Tsou, T. L., Nuki, Y., Liang, E. I., Makino, H. & Hashimoto, T. Pharmacologically induced thoracic and abdominal aortic aneurysms in mice. *Hypertension* 55, 1267-1274.

Kazenwadel, J., Michael, M. Z. & Harvey, N. L. Prox1 expression is negatively regulated by miR-181 in endothelial cells. *Blood* 116, 2395-2401.

Kazenwadel, J., Michael, M. Z. & Harvey, N. L. Prox1 expression is negatively regulated by miR-181 in endothelial cells. *Blood* 116, 2395-2401, doi:10.1182/blood-2009-12-256297 (2010).

Kedde, M. & Agami, R. Interplay between microRNAs and RNA-binding proteins determines developmental processes. *Cell Cycle* 7, 899-903 (2008).

Kim, S. & Iwao, H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 52, 11-34 (2000).

Kuehbacher, A., Urbich, C., Zeiher, A. M. & Dimmeler, S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* 101, 59-68, doi:10.1161/CIRCRESAHA.107.153916 (2007).

Kuhn, D. E., Martin, M. M., Feldman, D. S., Terry, A. V., Jr., Nuovo, G. J. & Elton, T. S. Experimental validation of miRNA targets. *Methods* 44, 47-54, doi:10.1016/j.ymeth.2007.09.005 (2008).

Kuivaniemi, H., Tromp, G. & Prockop, D. J. Genetic causes of aortic aneurysms. Unlearning at least part of what the textbooks say. *J Clin Invest* 88, 1441-1444, doi:10.1172/JCI115452 (1991).

Lauffenburger, D. A. & Horwitz, A. F. Cell migration: a physically integrated molecular process. *Cell* 84, 359-369 (1996).

Lederle, F. A., Johnson, G. R., Wilson, S. E., Chute, E. P., Hye, R. J., Makaroun, M. S., Barone, G. W., Bandyk, D., Moneta, G. L. & Makhoul, R. G. The aneurysm detection and management study screening

program: validation cohort and final results. Aneurysm Detection and Management Veterans Affairs Cooperative Study Investigators. *Arch Intern Med* 160, 1425-1430 (2000).

Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75, 843-854 (1993).

Li, J. J., Han, M., Wen, J. K. & Li, A. Y. Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation. *Biochem Biophys Res Commun* 356, 13-19 (2007).

Libby, P. Inflammation in atherosclerosis. *Nature* 420, 868-874, doi:10.1038/nature01323 (2002).

Libby, P., Ridker, P. M. & Maseri, A. Inflammation and atherosclerosis. *Circulation* 105, 1135-1143 (2002).

Lindholt, J. S., Juul, S., Fasting, H. & Henneberg, E. W. Screening for abdominal aortic aneurysms: single centre randomised controlled trial. *BMJ* 330, 750, doi:bmj.38369.620162.82 [pii]

10.1136/bmj.38369.620162.82 (2005).

Lithell, H. Pathogenesis and prevalence of atherosclerosis in hypertensive patients. *Am J Hypertens* 7, 2S-6S (1994).

Liu, X., Zhao, Y., Gao, J., Pawlyk, B., Starcher, B., Spencer, J. A., Yanagisawa, H., Zuo, J. & Li, T. Elastic fiber homeostasis requires lysyl oxidase-like 1 protein. *Nat Genet* 36, 178-182 (2004).

Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408, doi:10.1006/meth.2001.1262 (2001).

Longo, G. M., Buda, S. J., Fiotta, N., Xiong, W., Griener, T., Shapiro, S. & Baxter, B. T. MMP-12 has a role in abdominal aortic aneurysms in mice. *Surgery* 137, 457-462, doi:S0039606004007263 [pii]

10.1016/j.surg.2004.12.004 (2005).

Longo, G. M., Xiong, W., Greiner, T. C., Zhao, Y., Fiotti, N. & Baxter, B. T. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest* 110, 625-632 (2002).

Lucero, H. A. & Kagan, H. M. Lysyl oxidase: an oxidative enzyme and effector of cell function. *Cell Mol Life Sci* 63, 2304-2316, doi:10.1007/s00018-006-6149-9 (2006).

Luchtefeld, M., Grote, K., Grothusen, C., Bley, S., Bandlow, N., Selle, T., Struber, M., Haverich, A., Bavendiek, U., Drexler, H. & Schieffer, B. Angiotensin II induces MMP-2 in a p47phox-dependent manner. *Biochem Biophys Res Commun* 328, 183-188, doi:10.1016/j.bbrc.2004.12.152 (2005).

Lyle, A. N. & Griendling, K. K. Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda)* 21, 269-280, doi:10.1152/physiol.00004.2006 (2006).

Ma, F., Liu, X., Li, D., Wang, P., Li, N., Lu, L. & Cao, X. MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by

antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. *J Immunol* 184, 6053-6059, doi:10.4049/jimmunol.0902308 (2010).

Machesky, L. M. & Insall, R. H. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr Biol* 8, 1347-1356 (1998).

Maegdefessel, L., Azuma, J., Toh, R., Merk, D. R., Deng, A., Chin, J. T., Raaz, U., Schoelmerich, A. M., Raiesdana, A., Leeper, N. J., McConnell, M. V., Dalman, R. L., Spin, J. M. & Tsao, P. S. Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development. *J Clin Invest* 122, 497-506, doi:10.1172/JCI61598 (2012).

Majesky, M. W. Neointima formation after acute vascular injury. Role of counteradhesive extracellular matrix proteins. *Tex Heart Inst J* 21, 78-85 (1994).

Mäki, J. *Lysyl oxidases*

Cloning and characterization of the fourth and the fifth human lysyl oxidase isoenzymes, and the consequences of a targeted inactivation of the first described lysyl oxidase isoenzyme in mice

University of Oulu, (2002).

Maki, J. M., Rasanen, J., Tikkanen, H., Sormunen, R., Makikallio, K., Kivirikko, K. I. & Soininen, R. Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. *Circulation* 106, 2503-2509 (2002).

McCormick, M. L., Gavrila, D. & Weintraub, N. L. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 27, 461-469 (2007).

Mehta, P. K. & Griendling, K. K. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 292, C82-97, doi:10.1152/ajpcell.00287.2006 (2007).

Miki, H., Suetsugu, S. & Takenawa, T. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *Embo J* 17, 6932-6941 (1998).

Miles, W. O., Tschop, K., Herr, A., Ji, J. Y. & Dyson, N. J. Pumilio facilitates miRNA regulation of the E2F3 oncogene. *Genes Dev* 26, 356-368, doi:10.1101/gad.182568.111 (2012).

Mitchell, S. L. & Niklason, L. E. Requirements for growing tissue-engineered vascular grafts. *Cardiovasc Pathol* 12, 59-64 (2003).

Modlinger, P., Chabrashvili, T., Gill, P. S., Mendonca, M., Harrison, D. G., Griendling, K. K., Li, M., Raggio, J., Wellstein, A., Chen, Y., Welch, W. J. & Wilcox, C. S. RNA silencing in vivo reveals role of p22phox in rat angiotensin slow pressor response. *Hypertension* 47, 238-244 (2006).

Moiseeva, E. P. Adhesion receptors of vascular smooth muscle cells and their functions. *Cardiovasc Res* 52, 372-386 (2001).

Molnar, J., Fong, K. S., He, Q. P., Hayashi, K., Kim, Y., Fong, S. F., Fogelgren, B., Szauter, K. M., Mink, M. & Csiszar, K. Structural and

functional diversity of lysyl oxidase and the LOX-like proteins. *Biochim Biophys Acta* 1647, 220-224 (2003).

Narayanan, A. S., Siegel, R. C. & Martin, G. R. On the inhibition of lysyl oxidase by -aminopropionitrile. *Biochem Biophys Res Commun* 46, 745-751 (1972).

Nobes, C. D. & Hall, A. Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem Soc Trans* 23, 456-459 (1995).

Nobes, C. D. & Hall, A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53-62 (1995).

Nollendorfs, A., Greiner, T. C., Nagase, H. & Baxter, B. T. The expression and localization of membrane type-1 matrix metalloproteinase in human abdominal aortic aneurysms. *J Vasc Surg* 34, 316-322 (2001).

Ohmi, K., Masuda, T., Yamaguchi, H., Sakurai, T., Kudo, Y., Katsuki, M. & Nonomura, Y. A novel aortic smooth muscle cell line obtained from p53 knock out mice expresses several differentiation characteristics. *Biochem Biophys Res Commun* 238, 154-158, doi:10.1006/bbrc.1997.7218 (1997).

Papalambros, E., Sigala, F., Georgopoulos, S., Menekakos, C., Giatromanolaki, A., Bastounis, E. & Sivridis, E. Immunohistochemical expression of metalloproteinases MMP-2 and MMP-9 in abdominal

aortic aneurysms: correlation with symptoms and aortic diameter. *Int J Mol Med* 12, 965-968 (2003).

Paravicini, T. M. & Touyz, R. M. Redox signaling in hypertension. *Cardiovasc Res* 71, 247-258, doi:10.1016/j.cardiores.2006.05.001 (2006).

Patel, A., Fine, B., Sandig, M. & Mequanint, K. Elastin biosynthesis: The missing link in tissue-engineered blood vessels. *Cardiovasc Res* 71, 40-49, doi:10.1016/j.cardiores.2006.02.021 (2006).

Payne, S. L., Fogelgren, B., Hess, A. R., Seftor, E. A., Wiley, E. L., Fong, S. F., Csiszar, K., Hendrix, M. J. & Kirschmann, D. A. Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide-mediated mechanism. *Cancer Res* 65, 11429-11436 (2005).

Pearce, W. H. & Koch, A. E. Cellular components and features of immune response in abdominal aortic aneurysms. *Ann N Y Acad Sci* 800, 175-185 (1996).

Perrin, S. & Foster, J. A. Developmental regulation of elastin gene expression. *Crit Rev Eukaryot Gene Expr* 7, 1-10 (1997).

Plasterk, R. H. Micro RNAs in animal development. *Cell* 124, 877-881, doi:10.1016/j.cell.2006.02.030 (2006).

Rangaswami, H., Bulbule, A. & Kundu, G. C. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol* 16, 79-87, doi:10.1016/j.tcb.2005.12.005 (2006).

Regen, C. M. & Horwitz, A. F. Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. *J Cell Biol* 119, 1347-1359 (1992).

Rodriguez, C., Martinez-Gonzalez, J., Raposo, B., Alcudia, J. F., Guadall, A. & Badimon, L. Regulation of lysyl oxidase in vascular cells: lysyl oxidase as a new player in cardiovascular diseases. *Cardiovasc Res* 79, 7-13, doi:10.1093/cvr/cvn102 (2008).

Roy, S., Khanna, S., Hussain, S. R., Biswas, S., Azad, A., Rink, C., Gnyawali, S., Shilo, S., Nuovo, G. J. & Sen, C. K. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. *Cardiovasc Res* 82, 21-29, doi:10.1093/cvr/cvp015 (2009).

Shapiro, S. D., Endicott, S. K., Province, M. A., Pierce, J. A. & Campbell, E. J. Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. *J Clin Invest* 87, 1828-1834, doi:10.1172/JCI115204 (1991).

Siegel, R. C. Lysyl oxidase. *Int Rev Connect Tissue Res* 8, 73-118 (1979).

Silence, J., Lupu, F., Collen, D. & Lijnen, H. R. Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. *Arterioscler Thromb Vasc Biol* 21, 1440-1445 (2001).

Slezak-Prochazka, I., Durmus, S., Kroesen, B. J. & van den Berg, A. MicroRNAs, macrocontrol: regulation of miRNA processing. *RNA* 16, 1087-1095, doi:10.1261/rna.1804410 (2010).

Standal, T., Borset, M. & Sundan, A. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. *Exp Oncol* 26, 179-184 (2004).

Stein, O., Thiery, J. & Stein, Y. Is there a genetic basis for resistance to atherosclerosis? *Atherosclerosis* 160, 1-10 (2002).

Tesanovic, S., Vinh, A., Gaspari, T. A., Casley, D. & Widdop, R. E. Vasoprotective and atheroprotective effects of angiotensin (1-7) in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 30, 1606-1613, doi:10.1161/ATVBAHA.110.204453 (2010).

Touyz, R. M. Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res* 37, 1263-1273, doi:/S0100-879X2004000800018 (2004).

Touyz, R. M. & Schiffrin, E. L. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev* 52, 639-672 (2000).

Urbich, C., Kuehbacher, A. & Dimmeler, S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 79, 581-588, doi:10.1093/cvr/cvn156 (2008).

van Kouwenhove, M., Kedde, M. & Agami, R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer* 11, 644-656, doi:10.1038/nrc3107 (2011).

van Leeuwen, R. T., Kol, A., Andreotti, F., Kluft, C., Maseri, A. & Sperti, G. Angiotensin II increases plasminogen activator inhibitor type 1 and tissue-type plasminogen activator messenger RNA in cultured rat aortic smooth muscle cells. *Circulation* 90, 362-368 (1994).

Wang, M., Zhang, J., Spinetti, G., Jiang, L. Q., Monticone, R., Zhao, D., Cheng, L., Krawczyk, M., Talan, M., Pintus, G. & Lakatta, E. G. Angiotensin II activates matrix metalloproteinase type II and mimics age-associated carotid arterial remodeling in young rats. *Am J Pathol* 167, 1429-1442 (2005).

Weintraub, A. S., Giachelli, C. M., Krauss, R. S., Almeida, M. & Taubman, M. B. Autocrine secretion of osteopontin by vascular smooth muscle cells regulates their adhesion to collagen gels. *Am J Pathol* 149, 259-272 (1996).

Weintraub, A. S., Schnapp, L. M., Lin, X. & Taubman, M. B. Osteopontin deficiency in rat vascular smooth muscle cells is associated with an inability to adhere to collagen and increased apoptosis. *Lab Invest* 80, 1603-1615 (2000).

Weiss, D., Kools, J. J. & Taylor, W. R. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation* 103, 448-454 (2001).

Weiss, D., Sorescu, D. & Taylor, W. R. Angiotensin II and atherosclerosis. *Am J Cardiol* 87, 25C-32C (2001).

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. & Mizuno, K. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393, 809-812 (1998).

Yue, T. L., McKenna, P. J., Ohlstein, E. H., Farach-Carson, M. C., Butler, W. T., Johanson, K., McDevitt, P., Feuerstein, G. Z. & Stadel, J. M. Osteopontin-stimulated vascular smooth muscle cell migration is mediated by beta 3 integrin. *Exp Cell Res* 214, 459-464 (1994).

Zhu, N., Zhang, D., Chen, S., Liu, X., Lin, L., Huang, X., Guo, Z., Liu, J., Wang, Y., Yuan, W. & Qin, Y. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis* 215, 286-293.

Zhu, N., Zhang, D., Chen, S., Liu, X., Lin, L., Huang, X., Guo, Z., Liu, J., Wang, Y., Yuan, W. & Qin, Y. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis* 215, 286-293, doi:10.1016/j.atherosclerosis.2010.12.024 (2011).