ENDOTHELIAL BONE MORPHOGENIC PROTEIN 4 AND BONE MORPHOGENIC PROTEIN RECEPTOR II EXPRESSION IN INFLAMMATION AND ATHEROSCLEROSIS

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Engineering

Georgia Institute of Technology
April, 2008

ENDOTHELIAL BONE MORPHOGENIC PROTEIN 4 AND BONE

MORPHOGENIC PROTEIN RECEPTOR II EXPRESSION IN

INFLAMMATION AND ATHEROSCLEROSIS

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To My Parents.

ACKNOWLEDGEMENTS

I would like to thank Dr. Hanjoong Jo and all the members of the Jo Lab that have come and gone during my tenure.

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LIST OF SYMBOLS AND ABBREVIATIONS

ActRII	Activin receptor II
ALK	Activin like kinase
ApoE	Apolipoprotein E
AVF	Arteriovenous fistula
BAEC	Bovine aortic endotelial cell
BAMBI	BMP and activin membrane-bound inhibitor
BEC	Bovine endothelial cell
BMP4	Bone Morphogenic Protein-4
BMPR	Bone Morphogenic Protein Receptor
BMPRII	Bone Morphogenic Protein Receptor Type II
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
EC	Endothelial Cell
ECGS	Endothelial cell growth supplement
ECM	Extracellular Matrix
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular-Signal Regulated Kinase-1/2
FBS	Fetal Bovine Serum
FITC	fluorescein isothiocyanate
H_2O_2	Hydrogen peroxide
HAEC	Human aortic endothelial cell
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule – 1

IEL	Internal elastic lamina
IFN-γ	Interferon gamma
IHC	immunohistochemistry
IL-1β	Interleukin – 1 beta
JNK	c-jun N-Terminal Kinase
LDL	Low-Density Lipoprotein
LDLR	Low density lipoprotein receptor
LS	Unidirectional laminar shear stress
MAP Kinase	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein-1
mRNA	messenger ribonucleic acid
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NFκB	Nuclear factor κB
NO	Nitric oxide
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
OS	oscillatory shear stress
OxLDL	oxidized low-density lipoprotein
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI3K	Phosphatidyl inositol-3 kinase
PKC-ε	Protein kinase C-ε
PPH	Primary pulmonary hypertension
PVDF	polyvinyldifluoride
ROS	Reactive oxygen species

RPA	RNase protection assay
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	small interfering ribonucleic acid
Smad	`Sma and mothers against decaptaplegic
SMC	Smooth muscle cell
SSRE	Shear stress responsive elements
ST	static
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule – 1

SUMMARY

Atherosclerosis is an inflammatory disease, occurring preferentially in arterial regions with disturbed flow. We have shown that disturbed flow induces inflammation in endothelial cells (ECs) by producing bone morphogenic protein-4 (BMP4). Moreover, chronic BMP4 infusion induces endothelial dysfunction and systemic hypertension in mice. Here, we examined which BMP receptors (BMPR) mediate BMP4 action in ECs. Western blot, immunostaining and RT-PCR studies using human and bovine ECs, mouse aortas and human coronary arteries (HCA) showed that BMPRI (ALK2 and 6) and BMP-RII were expressed in ECs. As a functional test, ECs were treated with a BMPRII siRNA to knockdown expression. BMPRII knockdown blocked a well-known BMP4 response - smad1/5/8 phosphorylation, as expected. Unexpectedly, BMPRII knockdown itself significantly stimulated ICAM-1 and VCAM-1 expression and monocyte adhesion in a BMP4-independent manner. Inflammatory responses caused by BMPRII knockdown were blocked by inhibitors of NADPH oxidase and NFκB. From these results, we hypothesized that BMPRII knockdown in ECs would cause inflammation, which is a critical event in atherosclerosis initiation and progression. Genetic mutations of BMPRII have been linked to primary pulmonary hypertension. However, it is not known whether BMP-RII is regulated by atherosclerotic conditions and plays a role in non-pulmonary vessels causing inflammation and atherosclerosis. We examined BMPRII levels in HCA by immunostaining. While non-diseased arteries showed intense staining of BMPRII, the expression decreased as lesions became more advanced. BMPRII was virtually undetectable in the most advanced lesions. These findings suggested a potential link

between pro-atherosclerotic conditions and BMP-RII levels. We tested this hypothesis by treating ECs with pro-inflammatory cytokines found in atheromas: TNFα decreased BMPRII by 2-fold. In contrast, statins increased BMPRII by 4-fold. In summary, we demonstrate for the first time that BMPRII can be down- or up-regulated by pro- or anti-atherogenic conditions, respectively, and it is dramatically decreased in HCA with advanced plaques. Moreover, BMPRII knockdown in ECs induces inflammation, a critical atherogenic step. We propose that focal inflammation initiated by disturbed flow, together with circulating pro-atherogenic risk factors, may lead to a vicious cycle of BMPRII down-regulation causing secondary inflammation and atheroma progression.

CHAPTER 1

INTRODUCTION

Cardiovascular disease has been a leading cause of death in recent years, and atherosclerosis has been implicated in 75% of these deaths. To develop treatments for this widespread pathology, research has focused on finding the causes and mechanisms of this disease. It has been well established that hemodynamic factors affect atherosclerosis. Our work has shown that bone morphogenetic protein 4 (BMP4) may be an important protein in shear stress-induced endothelial dysfunction and atherogenesis. The following review will focus on this emerging field of shear stress, BMP4, BMP receptors (BMPR), inflammation and atherosclerosis.

Atherosclerosis

Atherosclerosis is a major cause of cardiovascular disease, including coronary artery disease, chronic heart failure, peripheral artery disease, chronic renal failure and stroke. It is characterized by a long process of plaque development that begins decades before clinical manifestation [1,2]. There is no doubt that the complex pathogenesis of atherosclerosis cannot be ascribed to a single factor. Environmental, genetic, and hemodynamic factors all play a part in its development. At the cellular level, a large number of growth factors, cytokines and vaso-regulatory molecules participate in this process [3].

While there are several well-known systemic risk factors causing atherosclerosis, such as smoking, diabetes, high cholesterol, oxidative stress, and obesity, one of its key

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features is the focal developmental pattern of plaques. Both systemic factors and local hemodynamic factors are responsible for endothelial dysfunction, which is characterized by a shift in the actions of the endothelium toward reduced vasodilation, a proinflammatory state, and prothrombotic properties[4]. Atherosclerotic plaques typically occur in branched or curved arterial regions where endothelial cells (ECs) are exposed to disturbed flow conditions such as low and oscillatory shear stress [5,6]. In contrast, the straight arterial regions exposed to laminar shear stress are well-protected from atherosclerosis development [5,6].

Atherosclerotic Plaque Formation

Atherosclerosis is now known as an inflammatory disease. Endothelial dysfunction is caused by both systemic and local factors that promote inflammation, and it is an important early event in the pathogenesis of atherosclerosis, contributing to plaque initiation and progression. The development of lesions includes a series of highly specific cellular and molecular responses that can best be described as chronic inflammation. The process begins with immune cells (monocytes and T lymphocytes) binding to activated endothelium and secreting cytokines [7,8]. Monocytes bind via the cell adhesion molecules, ICAM-1 and VCAM-1, and enter the blood vessel wall. Following transmigration, they differentiate into macrophages, take up lipoproteins, transform into foam cells, and eventually rupture and release their contents back into the sub-intimal space [7]. Cytokines, such as IL-1β and IFN-γ, released from macrophages and foam cells accelerate inflammation. They also reach smooth muscle cells in the medial layer and promote proliferation. Positive feedback continues as they have internalized too many

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lipoproteins and released all of their contents into the advancing plaque region until eventual plaque rupture. Plaque rupture is a dangerous condition that may result in a heart attack or stroke. After the rupture, the site of the rupture could seal over with an overlying clot, causing a potentially larger blockage in the artery. Also, the fatty particles may become accumulated further downstream in the artery.

Atherosclerosis and Hemodynamics

Cardiovascular disease and hemodynamics have been linked for the past two decades. Endothelial cells lining the blood vessel wall serve several functions. They inhibit platelet aggregation and coagulation, selectively transport macromolecules from the blood into the vessel wall, and sense blood flow. As described, blood flow profiles determine the site specificity of atherosclerotic plaque development. In 1969, Caro et al. identified the important correlation between low shear stress and atherosclerosis[9]. Zarins and colleagues modeled the human carotid bifurcation and correlated the localization of plaques to areas of flow disruption [6,10]. Similar modeling was performed on the abdominal aorta. In this area, oscillating flow and low shear stress were observed along the posterior wall of the infrarenal aorta where atherosclerotic lesions develop [11]. It was hypothesized that this low and oscillatory shear stress causes endothelial dysfunction by increasing the relative residence time of lipoproteins and inflammatory cells. Endothelial cell responsiveness to flow is essential for normal vascular function and plays a role in the development of atherosclerosis. Much research has shown that ECs respond differently to different types of shear stress, in both structure and function. They change their cell morphology through changes in the cytoskeleton and also exhibit the different

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gene expression profiles of the endothelial cell itself [12]. Via en face tissue staining from mouse aorta, Hajra et al. showed that EC morphology varies in athero prone versus athero protected regions of the vasculature[13]. Orientation of endothelial cells was more random and cell shape was more variable in athero prone regions with NFκ-B, ICAM-1, and VCAM-1 expression, whereas ECs aligned and were elongated with the direction of the blood flow in athero protected regions without adhesion molecule expression. All of these indicate that ECs sense shear stress and actively respond to it.

Shear Stress and Endothelial Cell Biology

Shear stress is defined as a tangential force over a surface area. In the vasculature, this corresponds to the dragging force of blood as it flows over the blood vessel wall. Mathematically, shear stress can be described as that the shear stress at the wall is proportional to the viscosity of the blood and the velocity gradient:

$$\tau = \mu \, du/dr$$

and can be described by

$$\tau_{wall} = \frac{32\mu Q}{\pi D^3}$$

for steady laminar flow in a straight tube (τ: shear stress, μ: viscosity, u: velocity, r: radius, Q: volume, D: diameter) [14] .

To investigate the effects of hemodynamic flow on endothelial cells in a controlled manner, several *in vitro* devices, such as the parallel plate [15], vertical-step flow chamber [16], cone- and-plate [17], and modified cone-and-plate [18], were developed [19]. ECs grown *in vitro* under static conditions have a "cobble stone" cell shape with a random

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orientation. When these cells are subject to uni-directional fluid flow, they elongate and align in the direction of flow [20,21] . However, under oscillatory flow with a net zero shear stress, they do not align and retain the morphology of cells under static conditions [15]. These *in vitro* studies using cell culture systems correspond well to the *in vivo* observations made by Hajra et al. as described above[13].

Several mechanotransduction pathways have been suggested, including integrins, ion channels, caveolae, G protein-linked receptors, mitogen-activated protein (MAP) kinase signaling, and the cytoskeleton [22,23]. In addition to changes in cell morphology, shear stress activates ion channels instantly, and in a more delayed manner, changes gene expression profiles[19]. Many groups, including ours, have performed microarray analyses and have identified gene expression profiles that are activated under various types of shear stress [24-27]. In addition, efforts to explain gene regulation by flow have led to the identification of promoter sequences termed SSRE (shear stress responsive elements). However, little is understood about the crosstalk between these elements and their bound transcription factors for each shear stress-regulated gene. Nitric oxide (NO), a vasodilator, intracelleular calcium and PKC-ε are activated with unidirectional laminar flow [28-30]. MAP kinase ERK and JNK are phosphorylated in a shear dependent manner [31-34].

In the case of oscillatory shear stress, many studies have focused on inflammatory responses including adhesion molecule expression and ROS stimulation [35-39]. Oxidative stress has been implicated in the development of numerous diseases including premature aging, cancer, neurodegenerative diseases, and atherosclerosis [40]. The reactive oxygen species (ROS) superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$)

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contribute significantly to such stress and have been intimately linked to atherogenesis via inflammatory responses that result from disturbed flow conditions [41-43]. We have previously shown that OS increases ROS levels in endothelial cells, while LS reduces ROS compared to static controls. OS stimulation of ROS leads to ICAM-1 expression and monocyte adhesion, an early and critical atherogenic event [44,45]. Furthermore, we have discovered one mechanosensitive gene, BMP4, that is produced by OS stimulation and acts as a potent inflammatory cytokine [17]. BMP4 protein is easily detected in endothelial cells cultured under static conditions. Expression is increased further by OS (1 day) and inhibited by LS exposure (1 day) [17]. The significance of this shear sensitive BMP4 expression was also supported by the finding that BMP4 protein is expressed in endothelial cells overlying foam cell lesions, but not in the normal "minimally diseased" areas of the human coronary arteries [17]. Furthermore, ICAM-1 staining, but not VCAM-1, is selectively increased in the similar endothelial patches expressing BMP4. While the immunohistochemical detection of BMP4 protein needs additional studies such as *in-situ* mRNA hybridization, to examine whether BMP4 is indeed produced by endothelial cells, these results are consistent with previous findings reported in another study using human carotid arteries with atherosclerosis [46].

Bone Morphogenetic Protein (BMP)

BMP was originally discovered as a bone-inducing protein [47]. Its critical and diverse roles now include embryonic development, patterning, cartilage formation, and cell differentiation [48,49]. BMPs are members of the transforming growth factor-β (TGF-β) superfamily. This superfamily consists of TGF-βs, inhibins, bone morphogenetic

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proteins, growth differentiation factors, anti-mullerian hormone, activins, and myostatin [48]. More than 30 BMP proteins have been identified, and BMP2/4 and BMP5/6/7 classes are the best characterized members.
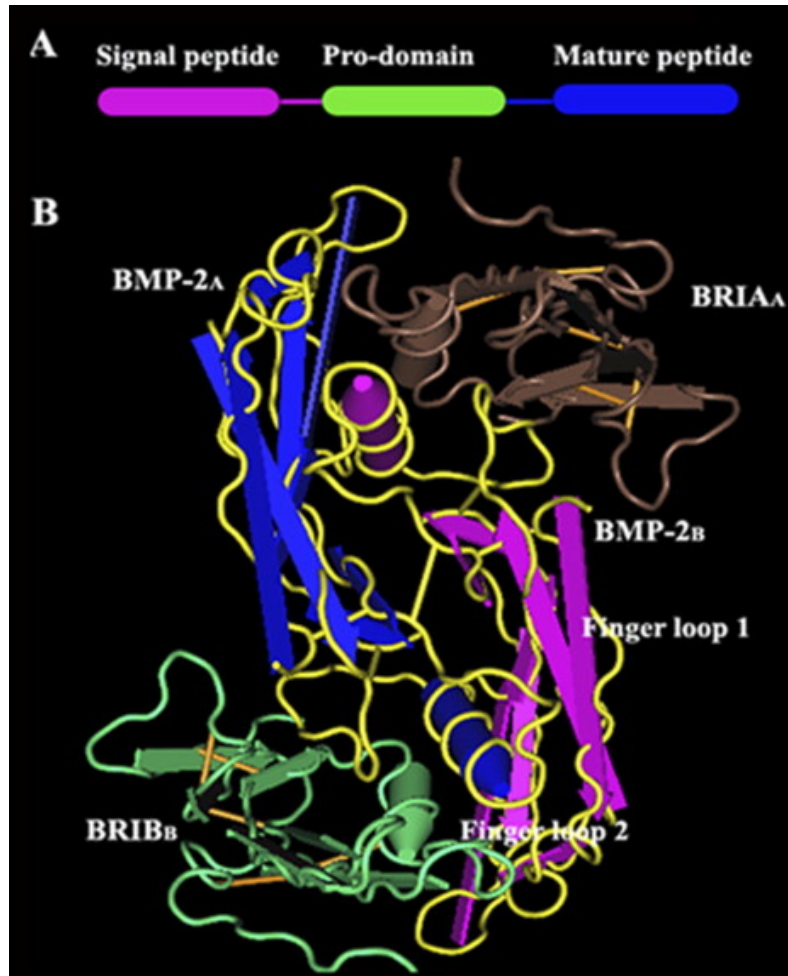
Figure 1.1. The structure of BMPs. (A) Schematic diagram of BMPs. (B) Three dimensional structure of BMP2 and BMP receptor IA and IB. This figure is taken from the review paper of Xiao et al[50] .

In particular, BMP4 has been identified as a protein that induces ectopic bone and cartilage formation when implanted in rats [48]. It is synthesized as a 408 amino acid precursor (pre-pro-precursor) that is proteolytically cleaved in the Golgi apparatus by pre-proconvertases such as furin that recognize the motif RRXR [51]. This leaves a C-

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terminal mature protein (116 amino acids) that has seven conserved cysteine residues. Figure 1.1 describes that BMPs synthesized as three domains and leaves mature peptide after the cleavage[50]. BMP4 is a secreted glycoprotein that can also dimerize. BMP4 is secreted as a 36 kDa pre-form, an 18 kDa monomer when glycosylated, and a 23 kDa dimer in a reduced condition [52]. BMP4 forms a homodimer when binding to its receptors, but there is a report that recombinant heterodimer between BMP4 and BMP7 is more potent in bioassays than BMP4 or BMP7 homodimers [53].

Unlike TGF-β, BMPs are secreted as active proteins and their activities are counterbalanced by secreted antagonists such as noggin and chordin [54]. BMP4 directly binds with high affinity to chordin (K_d= 300 pM) or noggin (K_d= 20 pM) [55]. Binding of BMP4 to noggin or chordin prevents it from binding to its cognate receptor. Although BMP7 can also interact with noggin, it does so with very low affinity [56]. Noggin and chordin do not bind TGF-β1 or activin [57]. Due to its relative specificity, noggin has been used as a valuable tool to dissect BMP4 function in cells and tissues [37].

The role of BMPs in vascular endothelial cells is an area of active research. BMP expression was shown to be increased in atherosclerotic plaques, although these authors did not address the involvement of ECs [58]. In ECs, BMP6 was reported to induce angiogenesis [59,60]. In addition, hypoxia and VEGF up-regulate BMP2 mRNA and protein expression in microvascular endothelial cells [61]. TNF-α increased BMP2 mRNA expression, and administration of H_2O_2 and high intravascular pressure also increased NFκB activation and BMP2 expression [62]. BMP4 mediates apoptosis of capillary endothelial cells [63]. Shin et al. [64] showed BMP4 produced by endothelial cells modulates osteogenesis in calcifying vascular cells.

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Through microarray studies and additional functional studies, our lab has found that oscillatory shear stress induces BMP4 expression in endothelial cells compared to the laminar shear stress. Under the static condition, BMP4 level was highly expressed, and OS increased BMP4 expression about 10% compared to static. BMP4 triggers a cascade of inflammatory responses including monocyte adhesion to endothelial cells. In addition, we have found that BMP4 expression is indeed increased in the endothelial cells overlying early atherosclerotic lesions in human coronary arteries [17,58].

BMP Receptors

BMPs bind to their receptors (BMPRs) to relay intracellular signaling. There are two types of signaling receptors specific for BMPs: BMP type I receptors (BMPRI) and type II receptors (BMPRII). Both are required for BMP signaling [56]. Three BMP type I receptors, BMPRIA (also known as ALK3, Activin-Like Kinase-3), BMPRIB (ALK6), and ALK2, as well as three type II receptors (BMPRII, Activin Receptor II (ActRII), and ActRIIB) have been identified [65]. Although somewhat variable depending upon species and vascular bed-origins, endothelial cells from mouse arteries as well as cultured murine and bovine aortic endothelial cells have been shown to express both type I (ALK2, 3, and 6) and type II BMPRs [66]. Unlike their well-known effects in bone formation and embryonic development, the functional importance of BMPRs in the vascular wall is an area of increasing attention in research. In endothelial cells, transfection with constitutively active mutants of ALK2, ALK3, and ALK6 has been shown to stimulate expression of the *id* gene and angiogenic responses [66]. One notable exception is in vascular smooth muscle cells where the loss-of-function mutations of BMPRII have been

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linked to familial primary pulmonary hypertension and sporadic primary pulmonary hypertension in humans [67]. In addition, it has been reported that BMPRII expression is reduced in the lungs of the primary pulmonary hypertension patients despite the lack of detectable mutations of the receptor [68]. Primary pulmonary hypertension (PPH) is characterized by obliteration of pre-capillary pulmonary arteries, leading to sustained elevation of pulmonary arterial pressure and subsequent right ventricular hypertrophy. PPH is typically associated with abnormal proliferation of endothelial and smooth muscle cells within the small pulmonary arteries [69].

BMP pseudoreceptor and co-receptor have also been identified. BMP and activin membrane-bound inhibitor (BAMBI) was cloned as a BMP pseudoreceptor that lacks an intracellular kinase domain, preventing the formation of a receptor complex [70]. In human colorectal tumors, activation and accumulation of β-catenin increased BAMBI expression. It seems that overexpressed BAMBI inhibits TGF-β-mediated growth arrest, and this may contribute to colorectal tumorigenesis [71]. Also, BMP co-receptor DRAGON (the glycosylphosphatidylinositol (GPI)-anchored protein) was recently identified [72,73]. DRAGON has the ability to bind to BMP2, BMPRIA and BMPRIB.

Intracellular Signaling of BMPs.

BMPs bind to BMPRII inducing homo-dimerization and auto-phosphorylation [74]. The dimerized type II receptor phosphorylates BMPRI, activating its kinase activity. Then, type I receptors phosphorylate SMAD proteins. In response to BMP4, SMAD-1, 5, and 8 proteins (also known as receptor-smad or R-smad) are phosphorylated, while TGFβ phosphorylates SMAD-2 and −3. Upon phosphorylation, SMAD-1 associates with

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SMAD-4 (co-smad) and the complex translocates to the nucleus where it can regulate transcription factors or bind directly to DNA [75] to regulate gene expression. SMAD-6 and −7 are inhibitory proteins, competing with SMAD-1, -5 and -8 and blocking BMP signaling. Interestingly, it has been shown that LS induces the expression of two inhibitory signaling molecules, SMAD-6 and -7 [76] , providing an additional mechanism by which LS prevents BMP4-dependent responses.
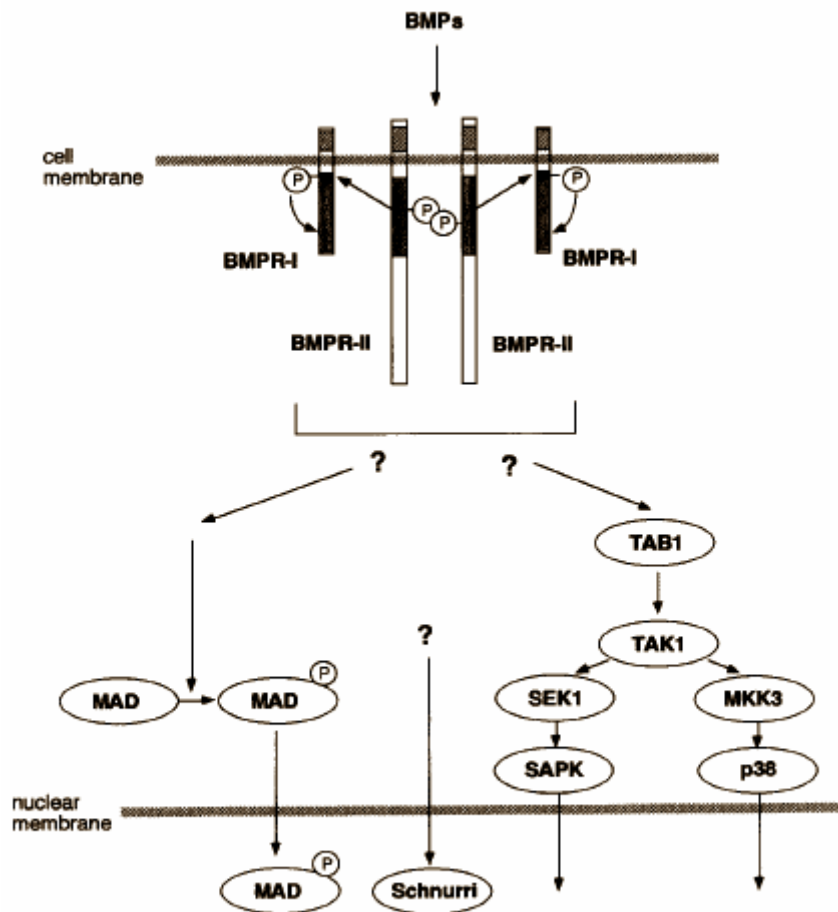
Figure 1.2. BMP receptors and signalling cascades. BMPs bind to BMP receptor type I and type II, then initiate intracellular signaling pathways. This figure is taken from the paper of Yamashita et al [77].

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While the Smad pathway is the well-characterized canonical signaling mechanism for BMPs, other pathways involving MAPK and NFκb [17,78] have also been shown to be BMP-inducible. Several groups have found that BMP4 treatment increased phosphorylation of MAPK such as ERK 1/2, p38 and JNK in various cell types [79,80]. The specific pathway activated by BMP4 may depend on the BMP receptor oligomerization pattern. Knaus' group suggested that preformed type I/ type II heterodimers activate the Smad pathway by ligand, whereas nascent formation of hetero-dimers of type I and type II complex upon ligand binding stimulates the p38 pathway [81,82]. Also, MAPK activation by BMP may act as a negative feedback mechanism for the Smad pathway by acting as an inhibitor of Smad1 nuclear translocation. It has been shown that phosphorylated ERK 1/2 binds to the linker domain of Smad1, which inhibits its binding to Smad4, thereby inhibiting its translocation into the nucleus [83].

Overall Hypothesis

Based on studies from literature and our lab's previous work, we constructed several specific aims over several years during PhD training.

In the beginning of this work, we have studied the mechanisms by which OS stimulates inflammatory responses and atherosclerosis. Using a cone-and-plate shear apparatus, we have shown that OS increases monocyte adhesion *in vitro* in a BMP4-dependent manner. OS induces BMP4 production, which leads an inflammatory response in endothelial cells [17]. The expression of adhesion molecules on the endothelial cell surface then allows monocytes to adhere to the endothelium [17]. The detailed mechanisms

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mediating OS and BMP4-induced monocyte adhesion at the BMP receptor level is the subject of this dissertation project.
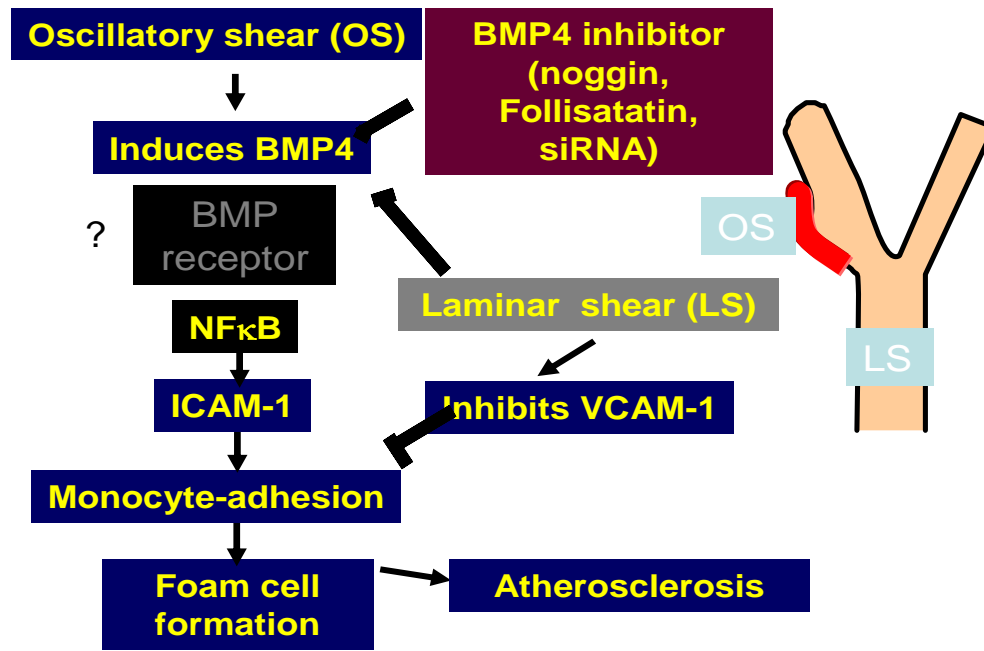
Figure 1.3 Summary of previous study. BMP4 produced by OS induces monocyte adhesion. BMP4 antagonists, noggin and follistatin or BMP4 siRNA can block this pathway. For this dissertation, we investigated a role of BMP receptors to induce monocyte adhesion.

Hypothesis: Oscillatory shear stress increases BMP4, which promotes monocyte adhesion and leads to atherosclerosis in branched arteries. This action is mediated by BMP receptors expressed in endothelial cells, with each receptor playing a unique role. Therefore, we set out to investigate a role for BMP receptors and downstream signaling of BMP4 in inflammation in endothelial cells. Unexpectedly, we found that knockdown of BMPRII increases inflammation independent of BMP4. Therefore, we hypothesize that BMPRII is a critical regulator of inflammation in endothelial cells and atherosclerosis.

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Specific Aim 1: Characterize endothelial BMP receptor expression in endothelial cells.

Hypothesis: It has been shown that different cell types and origins express different BMP receptor subtypes. Therefore, we hypothesized that endothelial cells express certain BMP receptors and the localization of each BMP receptor is important to its function and mediation of inflammation in response to OS-produced BMP4. This aim will be discussed in chapter 2.

Specific Aim 2: Identify a function for BMPRII and investigate downstream pathways mediating inflammation in endothelial cells.

Hypothesis: We first hypothesized that BMPRII mediates BMP4-induced inflammatory responses. Using a BMP receptors siRNA knockdown approach, we investigated functions of BMP receptors inducing BMP4-dependent and independent inflammation. Unexpectedly, we discovered that BMPRII knockdown increased monocyte adhesion in dependent of BMP4. Therefore, we modified our hypothesis which BMPRII depletion increases inflammatory responses. Also we investigated the downstream signaling pathways including ROS and NFκB pathways. This aim will be discussed in chapter 3.

Specific Aim 3: Investigate the regulation of BMPRII expression in endothelial cells.

Hypothesis: Recently, mutations of BMPRII have been associated with primary pulmonary hypertension. Also it has been shown that familial and sporadic cases of pulmonary hypertension patients decreased BMPRII expression in lungs. However, there has been no study to investigate BMPRII expression in atherosclerosis. Therefore, we

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hypothesized that endothelial BMPRII expression varies according to the state of atherosclerotic plaque development. In addition, pro-atherogenic factors such as cytokines, hyperlipidimea, hypertension, and diabetes, or anti-atherogenic factors, such as lipid lowering and anti-inflammatory drugs, regulate BMPRII expression conversely in endothelial cells. This aim will be discussed in chapter 4 and 5.

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Chapter 2

Characterization of BMP receptors in endothelial cells.

Introduction

BMPs bind to two types of BMP-specific receptors (Type I and Type II); there are BMPRI (ALK2, ALK3, ALK6) and BMPRII. When BMPs bind to BMPRII, BMPRII forms a hetero-dimer with BMPRI, phosphorylating it. Then, BMPRI phosphorylates Smad 1/5/8 proteins, which bind with Smad 4. This Smad complex translocates to the nucleus and binds to DNA to regulate gene expression (Figure 2.1). Inhibitory Smad 6 and 7 proteins can block this pathway. Smad 6 has been shown to preferentially inhibiting the BMP pathways by inhibiting phosphorylation of Smad 1 and preventing Smad 4 binding. In contrast, Smad 7 is a more general inhibitor of the TGF-β family. It binds to type I receptors to inhibit phosphorylation of Smad 1/5/8 [1,2].
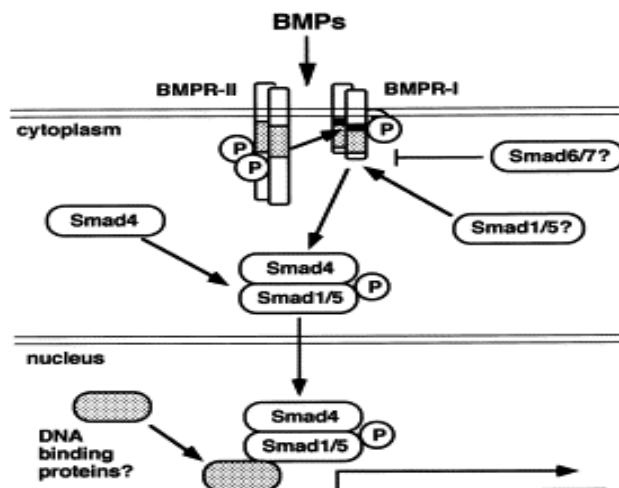
Figure 2.1. BMPs bind to BMP receptors to activate Smad signaling pathways [1]. BMPs bind to BMP receptors: BMPRII phosphorylates BMPRI, which phosphorylates Smad 1/5. Smad 1/5 phosphorylation can be inhibited by Smad 6/7, or phosphorylated Smad 1/5 can bind to Smad4 and translocate to nucleus. Smad proteins are transcription factors that involves in regulating several genes in response to the stimulus.

Each BMP family member binds to a different combination of BMP receptors. Table 1 summarizes BMP receptor oligomerization patterns by BMP2, BMP4 and BMP7 from literature. There are reports that BMP2 and BMP7 can bind to ALK2. In contrast, BMP4 has a weak binding affinity for ALK2 (personal communication with Peter ten Dijke), but has shown to bind to BMP type I receptors, ALK3 and ALK6. In contrast to TGF-β receptors, which were found to be fully homo-dimeric in the absence of ligand, BMP receptors can make homo-dimers and hetero-dimers in the presence and absence of ligand. It also seems that each cell types express and form different types of BMP receptor dimers.

Table 2.1. Each type of BMP ligand binds different combinations of receptors in different cell types. (COS-1: monkey kidney fibroblast, P19: mouse teratocarcinoma cells, MC3T3: mouse embryonic fibroblast, AG1518: human foreskin fibroblast, NIH3T3: mouse fibroblast cell line, MvILu: mink lung cells)

Ligand	Type I	Type II	Cell Type	Reference
BMP2	ALK2, ALK3, ALK6	BMPRII	COS	3
	ALK3, ALK6	BMPRII ActRII a, b	P19	4
BMP4	ALK 3, ALK6	BMPRII	COS, MC3T3, AG1518	5
	Not binding to ALK2	BMPRII	NIH 3T3	6
BMP7	ALK2, ALK3, ALK6	BMPRII	COS, MC3T3, MvILu,	4,5

Aortic endothelial cells have been shown to express both BMP receptor type I and II [7]. However, there is evidence that vascular cells of different origin express different types of receptors. For example, human pulmonary arterial endothelial cells express ALK6, not ALK3, while pulmonary smooth muscle cells express both ALK3 and ALK6 [8]. Table 2 summarizes the differential expression of BMP receptors in various cell types. This indicates that the specific combination of BMPRs may play an important role in each cell types.

Table 2.2. Different cell types express different combinations of BMP receptors. (MEEC: Mouse Embryonic Endothelial Cells, HPAEC: Human Pulmonary Aortic Endothelial cells, MPASM: Mouse Pulmonary Aortic Smooth Muscle cells, HASM: Human Aortic Smooth Muscle cells, RASM: Rat Aortic Smooth Muscle cells, HPASM: Human Pulmonary Aortic Smooth Muscle cells, MEF: Mouse Embryonic Fibroblasts, KS483: mouse pre osteoblast, O: expressed, ×: not expressed, -: not examined)

	Mouse aorta (EC)	MEEC	BAEC	HPAEC	MPASM	HASM	RASM	HPASM	MEF	KS483
ALK2	×	O	O	O	O	×	O	O	O	O
ALK3	O	O	×	×	O	O	O	O	O	O
ALK6	O	×	O	O	-	O	O	O	×	O
BMPRII	O	O	O	O	O	×	-	O	O	O
Method	IHC	PCR	PCR	PCR	PCR	PCR	PCR	PCR	PCR	PCR
Ref.	7	7	7	5	6	8	12	5	7	13

To elucidate the importance of BMPRs in cardiovascular pathology, we screened BMP receptor expression in various endothelial cells, including cultured BAEC, HUVEC, and HAEC, as well as mouse aortas, and human coronary arteries. To evaluate receptor expression, we used RT-PCR, Western blot, and immunostating to evaluate mRNA and protein expression of BMPR type I (ALK2, ALK3, and ALK6) and BMPRII.

Methods

Cell culture: HUVECs, purchased from the Department of Dermatology, Emory University, were cultured in M-199 medium supplemented with endothelial growth factor supplement (isolated by us), heparin (American Pharmaceutical Partners), and 20% heat inactivated fetal bovine serum (FBS, Atlanta Biolicals), and used between passages 3-6. BAECs, obtained from Cell Applications Inc., were maintained in a standard humidified incubator (37°C, 5% CO_2) in Dulbecco's minimum Eagle's medium (Gibco) supplemented with 10% FBS, heparin, endothelial cell growth supplement and minimum nonessential amino acids (Gibco). BAEC from passage 8-11 were used. HAECs purchased from Clonetics were cultured using the EGM-2 bullet kit (Clonetics) and used at passages 4–6.

RT-PCR: RT-PCR for ALK2, ALK3, ALK6 and BMPRII mRNA was performed using a BioRad MyCycler (Bio-Rad). Briefly, total RNA was reverse transcribed using random primers and a SuperscriptII kit (Invitrogen) to synthesize first-strand cDNA. The cDNA was purified using a MicroBiospin 30 column (Bio-Rad) in Tris buffer and stored at -20°C until used. Aliquots of the cDNA pool obtained were subjected to PCR and amplified in a 50 µl reaction mixture using *Taq* polymerase (Invitrogen) for 45 cycles. Reaction products were separated on a 1% agarose gel, and the amplified DNA fragments were visualized by ethidium bromide staining under UV light. PCR products were sequenced to confirm the intended product. The following primers were used to amplify human ALK2 (forward, 5-gcggtaatgaggaccactgt -3, reverse, 5-agaccagagccacttcctga -3), bovine ALK2 (forward, 5- ccccgaagttcttgatgaaa -3, reverse, 5-ctgtgagtctggcagatgga-3), human ALK3 (forward, 5-actttagcaccagaggatacc-3, reverse, 5-ttttcaccacgccatttaccc-3),

bovine ALK3 (forward, 5-ctacactgccccctgttgtt actttagcaccagaggatacc-3, reverse, 5-cgcattagcgcagtttgata-3), human ALK6 (forward, 5-caccctacactgcctccatt -3, reverse, 5-acttccccatagcgaccttt -3), bovine ALK6 (forward, 5-tatagctgacctgggcttgg-3, reverse, 5-ccgcagcttcttgatacaca -3),human BMPRII (forward, 5- gacaacattgcccgctttat -3, reverse, 5-atctcgatgggaaattgcag -3), and bovine BMPRII (forward, 5- gaagactgttgggaccagga -3, reverse, 5- ctggacatcgaatgctcaga -3).

Western blot: Following experimental treatments, endothelial cell lysates were prepared and analyzed by Western blot analysis as previously described[9,10]. Briefly, cells were washed in ice-cold phosphate-buffered saline and lysed in RIPA buffer, and protein concentrations were determined with a Bio-Rad DC assay. Aliquots of cell lysates were resolved via SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were probed with antibodies to ALK2 (1:1000, R&D), ALK3 (1:500, Santa Cruz), ALK6 (1:500, Santa Cruz), BMPRII (1:500, BD), ICAM-1 (1:1000, Santa Cruz), VCAM-1 (1:1000, Santa Cruz), or β-actin (1:1000, Santa Cruz), and with appropriate secondary antibodies conjugated to alkaline phosphatase. Protein bands were detected by a chemiluminescence method. To confirm the specificity of the antibody, we also used blocking peptide for ALK3 and ALK6 by incubating with primary antibodies, respectively.

Immunostaining: For immunohistochemical staining, frozen sections of human coronary arteries (10 μm) and mouse aortas (7 μm) were fixed in ice-cold acetone for 5 min, blocked for 1 hour with 10% donkey or goat serum and incubated with primary antibodies overnight at 4°C. Samples were then incubated with rhodamine-conjugated

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secondary antibodies (anti-goat IgG or anti-rat IgG) for 2 hours at room temperature, as described [10]. Nuclei were counter-stained with Hoechst 33258 (Sigma). Primary antibodies used were specific for ALK3 (1:50, Santa Cruz), ALK6 (1:50, Santa Cruz), BMPRII (1:50, Santa Cruz) or PECAM-1 (1:50, BD). Twenty different human coronary arteries were examined. Samples ranged from minimally diseased to those with advanced atheromas. Microscopic images were collected using a Zeiss epi-fluorescent microscope. For immunocytochemistry, confluent BEC were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked for 1hr with 3% bovine serum albumin. Cells were incubated with primary and secondary antibodies and observed via confocal microscopy (Axiovert, Zeiss), as we previously described [11].

Statistical Analysis: Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance was assessed by Student's t-test. $P<0.05$ was considered as statistically significant.

HAEC, HUVEC, and BAEC express ALK2, ALK3, ALK6, and BMPRII mRNA, but only ALK2, ALK6, and BMPRII proteins.
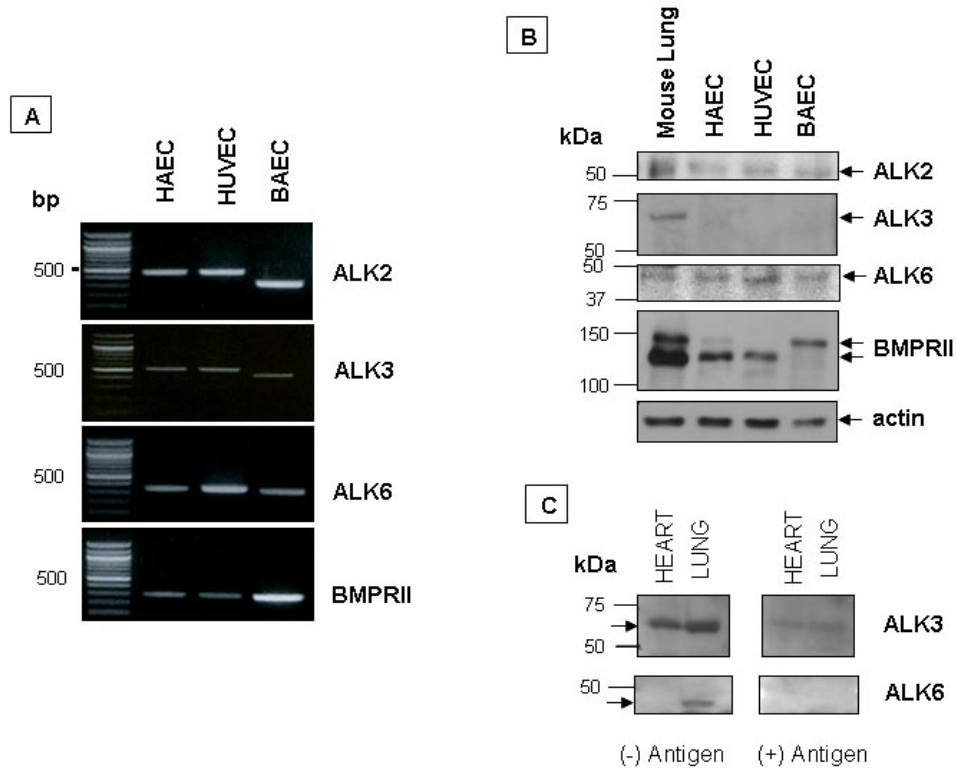
Figure 2.2. HAEC, HUVEC, and BAEC express ALK2, ALK3, ALK6, and BMPRII mRNA, but only ALK2, ALK6, and BMPRII proteins. The expression of BMP receptors was determined by RT-PCR (**A**), Western blot (**B**) (**A**) RT-PCR reactions for ALK2, 3, 6 and BMPRII were carried out using HAEC, HUVEC and BAEC. Each product was resolved by 1% agarose gel electrophoresis. (**B**) Lysates of mouse lung, HAEC, HUVEC, and BAEC were analyzed by immunoblot with antibodies to ALK2, 3 and 6, and BMPRII. β-actin was used as a loading control. (**C**) Lysates of mouse heart and lung were analyzed by immunoblot using antibodies to ALK3, and ALK6. For (+) antigen cases, antibodies were incubated with blocking peptides prior to incubation with blots.

HAEC, HUVEC, and BAEC expressed ALK2, ALK3, ALK6, and BMPRII mRNA as shown in Figure 2.2 (A). However, Western blots showed that HAEC,

HUVEC, and BAEC expressed only ALK2, ALK6, and BMPRII at the protein level. ALK3 was not detected. In comparison, mouse lung expressed ALK2, ALK3, ALK6 and BMPRII proteins (Fig. 2.2 (B)), while mouse heart expressed ALK2 and ALK3, but not ALK6 and BMPRII proteins (Fig.2.2 (C)). The specificity of antibodies for ALK3 and ALK6 was confirmed by competing with excess amounts of blocking peptide (Fig. 2.2 (D)). In these studies, we observed that the molecular weight of BMPRII varied between cell types. Mouse lung and HAEC expressed protein both at 140 and 120kDa, while HUVEC expressed only a 120kDa protein and BAEC only a140kDa protein. Cultured endothelial cells expressed all three isoforms of BMPRI (ALK2, ALK3, and ALK6) mRNA; however, ALK3 protein was undetectable.

Mouse endothelium expresses ALK6 and BMPRII.

Using immunohistochemical staining, we next examined BMP receptor expression in ECs from mouse aortas and human coronary arteries by. As shown in Fig. 2.3, BMPRII, but not ALK3 were easily detectable in ECs. In addition, a low level of staining for ALK6 was detected at the basal layer of ECs, as indicated by the faint pink staining just above the internal elastic lamina (marked with arrows in Fig. 2.3 (C)). PECAM-1 antibody (Fig. 2.3 (D)) confirmed the presence of an intact endothelial layer in all sections. Unlike endothelium, medial smooth muscle cells robustly expressed ALK3, ALK6 and BMPRII (Fig. 2.3). We were not able to find antibodies that were adequate for ALK2 staining with mouse aortas (data not shown). These data confirms that *in situ*

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investigation of endothelial cells as well as cultured endothelial cells express ALK6 and

BMPRII not ALK3.
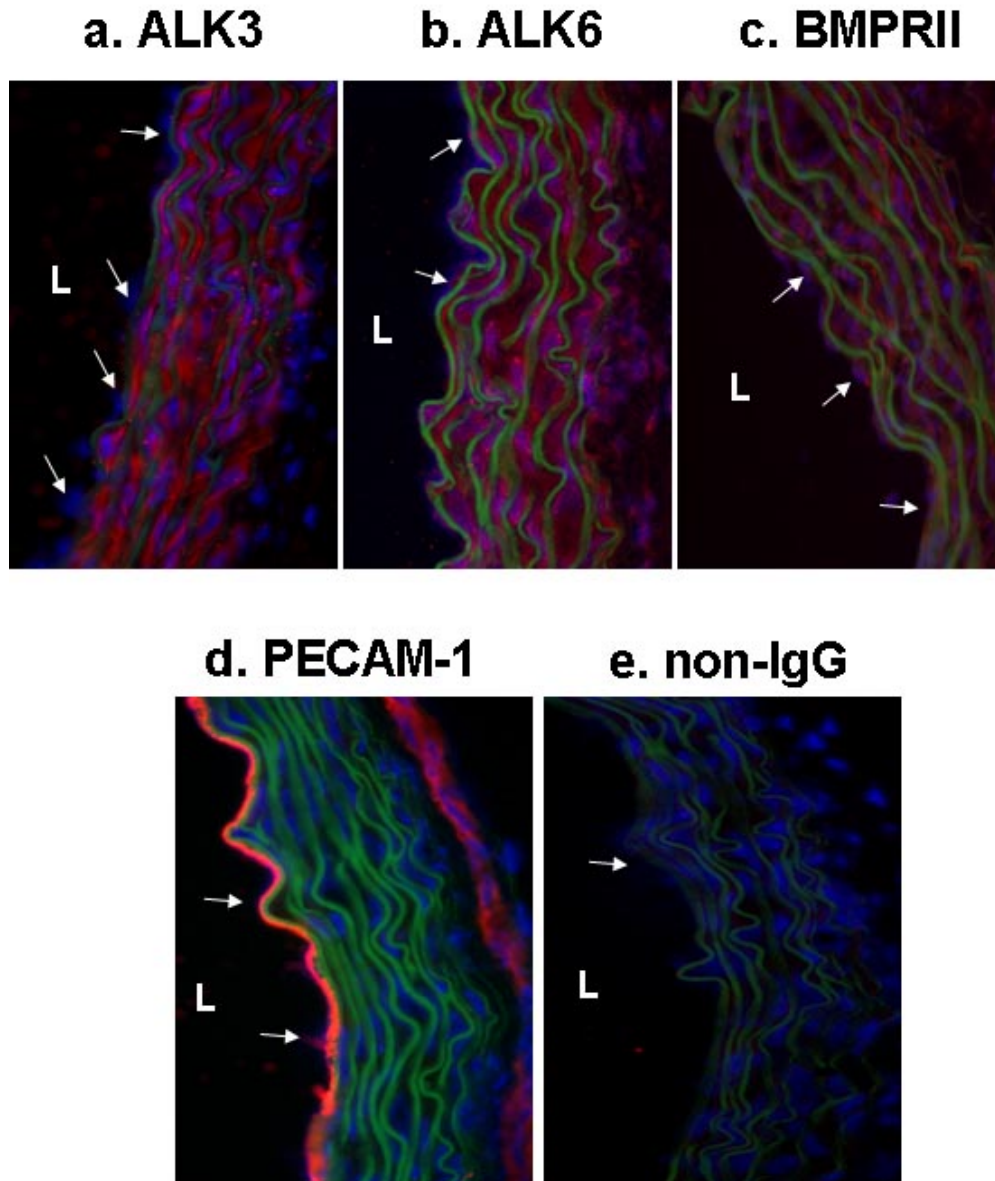
Figure 2.3. Mouse endothelium expresses ALK6 and BMPRII. Frozen sections of mouse thoracic aortas were stained with specific antibodies: ALK3, ALK6, BMPRII, and PECAM-1. Non-immune IgG control (non-IgG) was used as a control. Each antibody was detected with rhodamine-conjugated secondary antibodies (red signal) and observed by fluorescence microscopy (original magnification is 400x). Blue indicates nuclei stained with Hoechst while green signal indicates elastic lamina detected by auto fluorescence. L indicates the vessel lumen and arrows point to ECs.

Endothelial cells in human coronary arteries express ALK2, ALK6 and BMPRII.
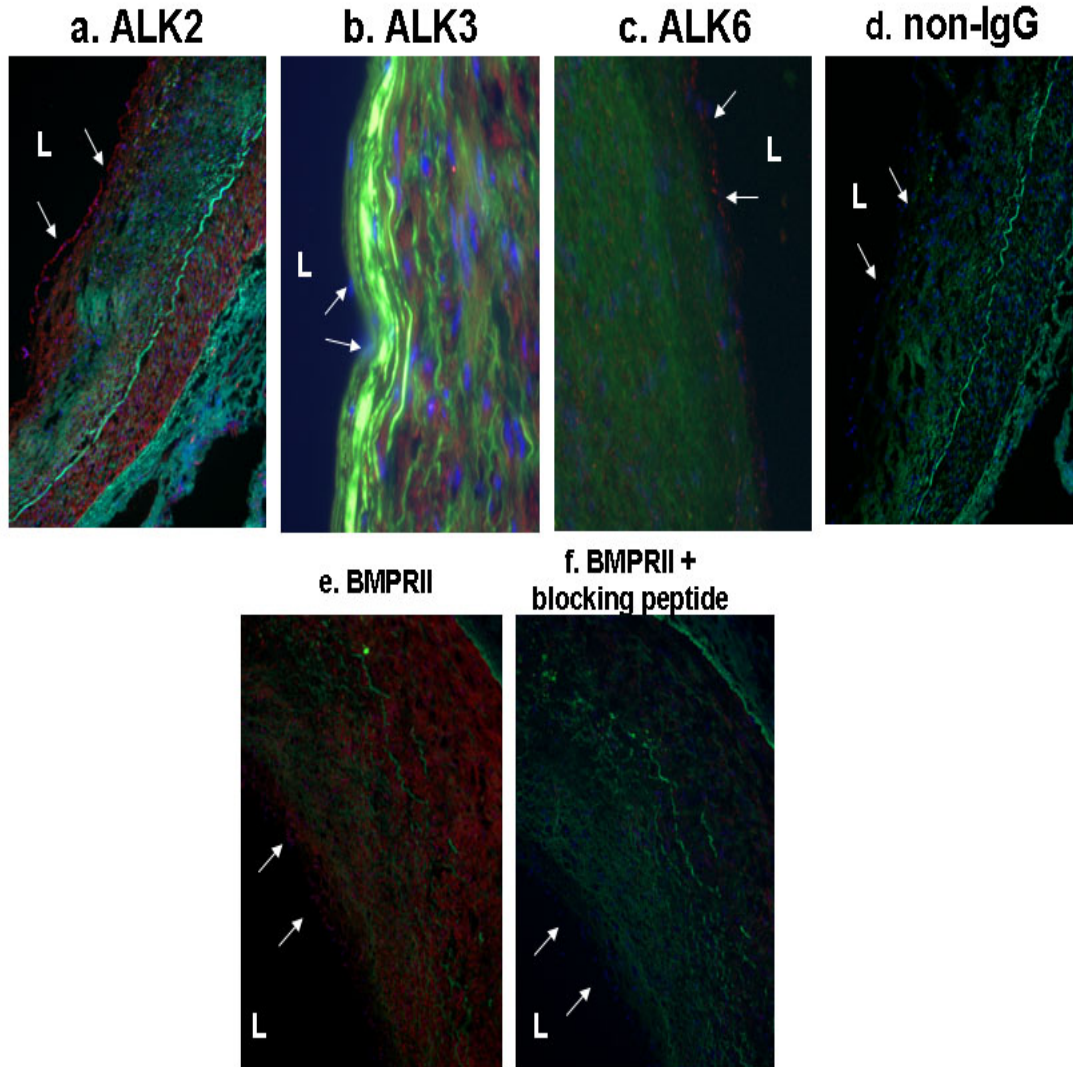
Figure 2.4. Endothelial cells in human coronary arteries express ALK2, ALK6 and BMPRII. Frozen sections of human coronary arteries were stained with specific antibodies: ALK2, ALK3, ALK6, BMPRII. Non-immune IgG control (non-IgG) was used as a control. For F) BMPRII antibody was pre-incubated with BMPRII blocking peptide prior to antibody incubation. Each antibody was detected with rhodamine-conjugated secondary antibodies (red signal) and observed by fluorescence microscopy (original magnification 100×). Blue indicates nuclei stained with Hoechst while green signal indicates elastic laminae detected by autofluorescence. L indicates the vessel lumen and arrows point to ECs.

To assess expression patterns in human samples, we also stained human coronary arteries for ALK2, ALK3, ALK6, and BMPRII. In human coronary arteries, we were able to detect ALK2 (Fig. 2.4 (A)) in both endothelium and smooth muscle cells. In addition, ECs from human coronary arteries expressed ALK6, but not ALK3 (Figure 2.4 (B)) and (C)). Specificity of BMPRII staining was confirmed with incubating antibody by competition staining (Figure 2.4 (E) and (F)). The secondary antibody alone (Fig. 2.4 (D)) was used as a staining control.

Together, these results suggest that ALK2, ALK6, and BMPRII are expressed in both cultured ECs and in intact mouse and human aortas. In the case of ALK3, although we were able to detect mRNA in cultured endothelial cells, it is possible that protein expression maybe very low and beneath the detection level of the antibody used. Since we were able to detect stable expression of BMPRII in both cultured endothelial cells and in situ mouse aortas and human coronary arteries, we have decided to focus on BMPRII as our the first target of the further studies.

BMPRII is expressed at the cell-cell junction in endothelial cells.

Our findings indicate that BMPRII expression is the most pronounced and consistent of BMPRs in both cultured endothelial cells and mouse aorta staining studies. As a result, we decided to further investigate BMPRII expression. We first carried out immunostaining studies to determine BMPRII expression in cultured BEC. This work

indicated that BMPRII was mainly expressed in cell-cell junctions (Fig. 2.5 (A)-b). To verify these finding, we co-stained confluent BEC for BMPRII and VE-cadherin, a marker of the cell-cell junction, together. As shown in confocal images (Fig. 2.5 (A) (b-d)), BMPRII co-localized with VE-cadherin in areas where cell-cell contact was maintained (marked with arrowheads), but not in those where the junctions were disrupted (marked with the arrows). To assess this pattern, we compared the BMPRII staining pattern in confluent BEC to sub-confluent BEC without formed junctions. Sub-confluent BEC did not exhibit the junctional staining pattern for BMPRII (Fig. 2.5 (A) a). In addition, when confluent BEC were scratched (marked as "sc") to remove junctions on one side of the cell monolayer and fixed two hours later, BECs lost the junctional BMPRII staining pattern on the scratched side (arrows in Fig. 2.5 (B) e-g). In contrast, unscratched areas maintained junctional staining (arrowheads in Fig. 2.5 (B) e-g). The junctional staining pattern of the BMPRII antibody was significantly and specifically reduced by peptide competition staining (compare e to h in Fig. 2.5). In the absence of the blocking peptide, the merged image (Fig. 2.5.g) of BMPRII and VE-cadherin staining showed yellow staining at the junction, indicative of co-localization of the two. On the other hand, in the presence of the blocking peptide, the merged image (Fig. 2.5.j) showed only red VE-cadherin staining at the cell-cell junction, indicating specific loss of the BMPRII signal. These results demonstrate that BMPRII is expressed in the cell-cell junction in EC. In HUVECs, we have not been able to show the immunostaining because of the technical problems that HUVECs were very hard to keep an undisrupted cell monolayer during the staining procedure.

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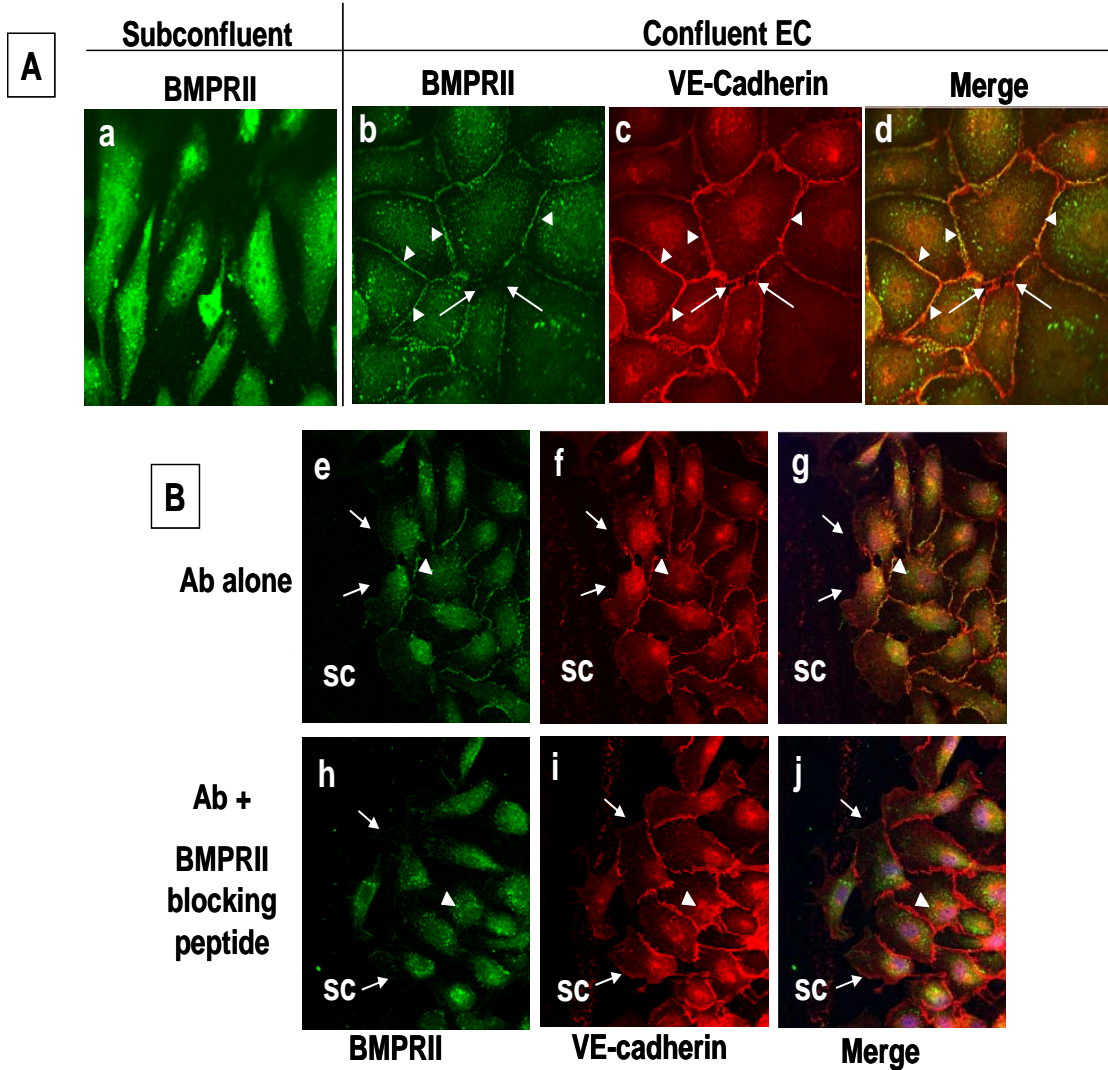
Figure 2.5. BMPRII is expressed at the cell-cell junction in endothelial cells. (A) Sub-confluent (a) or confluent (b-d) BEC were co-stained with antibodies to BMPRII (green stain) and VE-cadherin (red stain). In (**B**), confluent EC were scratched to remove the junction (as indicated by sc), then stained with BMPRII and VE-cadherin antibodies in the absence (-) or presence (+) of the antigenic peptides, demonstrating the specificity of the BMPRII antibody staining. Yellow staining in the merged images (d, g, j) indicates the co-localization of BMPRII and VE-cadherin. Confocal images (400x) are representatives of three independent studies. Arrowheads and arrows indicate intact and disrupted junctions, respectively.

BMPRII expression is not affected by shear stress.
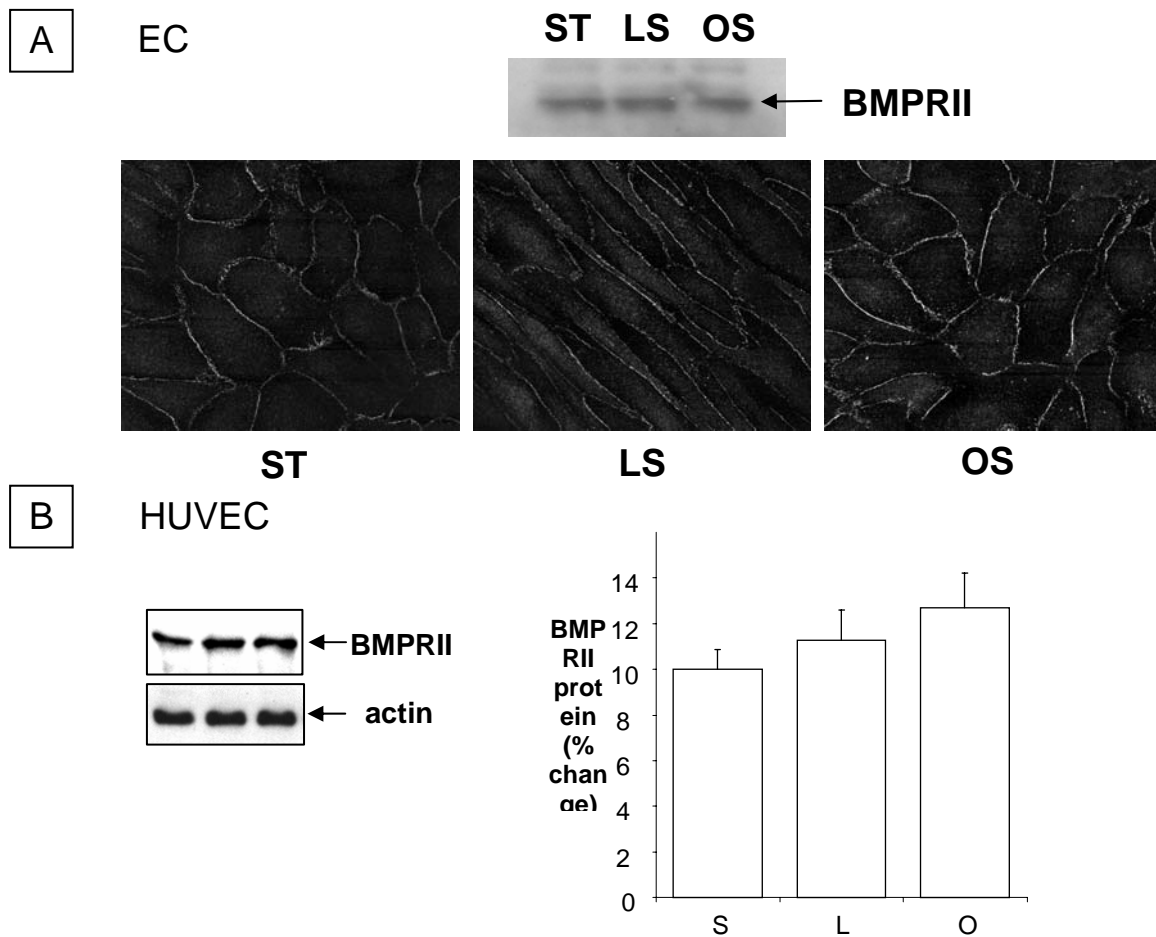
Figure 2.6. BMPRII expression is not affected by shear stress. (A) and (B) Confluent BEC and HUVECs were exposed to OS, LS, or static conditions for 24 hours. Cell lysates were collected, and equal amount of protein were assessed by Western blot. Blots were probed with BMPRIII antibodies. β-actin was used as an internal control. Shown are mean ± SEM (n=3, * p<0.05) **A**) Confluent EC were fixed with 4% paraformaldehyde after shear exposure, and stained with BMPRII antibody. Confocal images (400x) are representative of three independent studies.

It has been shown that several junctional proteins, such as PECAM-1, VE-Cadherin, or VEGF-R, suggested as a mechonasensor in endothelial cells. Since BMPRII exhibited the junctional staining pattern, we investigated whether BMPRII was a mechnosensitive protein or now. Therefore, we first examined whether BMPRII expression was regulated by shear stress. EC and HUVEC were exposed to ST, LS, and

OS for 24 hours. BMPRII expression did not appear to be shear-sensitive with 24 hour shear exposure. Both EC and HUVECs showed no change in protein level as analyzed by Western blot (Fig. 2.6 (A) and (B)). Also, there was no difference in the staining intensity of BMPRII after 24 hr shear exposure to LS versus OS (Fig 2.6 (A)). These data indicate that 24 hour shear exposure to both LS and OS does not affect either BMPRII protein expression or localization; however, it is still possible that different types of shear stress would change other junctional proteins which interact with BMPRII at the cell-cell junction.

Discussion

The major findings reported in this study are that a) HAEC, HUVEC, and BAEC express ALK2, ALK3, ALK6, and BMPRII mRNA. b) ALK2, ALK3, and BMPRII proteins were detected in HAEC, HUVEC, BAEC, mouse aortic ECs, and human coronary arterial ECs. c) BMPRII is expressed at the cell-cell junction in EC; and d) Shear stress does not affect the expression of BMPRII in EC or HUVECs. Together, these findings suggest that endothelial cells express both type I and type II receptors of BMPs which indicate that these cells can be stimulated by BMP signals.

There have been studies on BMP receptors in both biochemical studies and pathophysiological studies. BMP receptors have been identified and sequenced to determine sizes of proteins: For BMP type I recpetors, ALK2 is a 509 amino acid protein, ALK3 is a 532 amino acid protein, and ALK6 is a 502 amino acid protein. BMPRII is a 1038 amino acid protein with a long cytosolic tail (866 amino acid) with poorly defined

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function. Also, in terms of pathophysiological studies, mutations in ALK6 are known to cause brachydactyly type A2, which results in shortness of the fingers and toes [21]. ALK2 conditional knock out mice in neural crest cells display a cardiac outflow tract defect [22]. Recently, heterozygous germline BMPRII mutations have been found to underlie many cases of familial and sporadic primary pulmonary hypertension [23]. The disease is characterized by vascular cell proliferation and obliteration of the small pulmonary arteries.

We detected mRNA of all types of BMPRs (ALK2, ALK3, ALK6 and BMPR2) that we investigated in endothelial cells. We also detected proteins of ALK2, ALK6 and BMPRII; however, ALK3 protein level was under our detection limits in endothelial cells. Interestingly, we detected two different sizes of BMPRII using Western blot. In mouse lung and HAEC, both 120kDa and 140kDa band were detected. In HUVEC, only 120kDa band was detected, and in BAEC, only a 140kDa band was detected. This suggests that aortic cells express a different isoform of BMPRII than venous cells. Further studies will be necessary to confirm these findings. Using other cell types such as human pulmonary arterial and venous endothelial cells, we may be able to determine whether origin or species dictates BMPRII size. In addition, we may also study whether different splice variants exist, and what would affect the transcription of each variant. It is possible that there is a difference in glycosylation. Finally, functional and localizational studies of each variant would need to be performed in endothelial cells.

Our immunostaining study showed for the first time that BMPRII is expressed in the cell-cell junction of EC (Fig.2.4). To confirm its location and the antibody's specificity, several independent approaches were used: 1) comparison between confluent vs. sub-confluent or scratched ECs, 2) co-localization of VE-cadherin and BMPRII, 3) an antigenic peptide competition study with the BMPRII antibody, and 4) the specific loss of BMPRII signal by BMPRII siRNA but not by non-silencing siRNA (data not shown). Previously, Ramos et al. showed that some BMPRII (less than 6% of total) are located in lipid rafts, including caveolae, in human pulmonary arterial ECs (HPAEC) [9]. It is possible that some BMPRII may be located in caveolae in our cells, but this was not addressed here. Given the importance of the cell-cell junction in mechanosensing and mechanosignaling in ECs [25-27], BMPRII localization in the junction is interesting. A recent study reported that BMP induces nuclear localization of β-catenin mediated by adherence junction formation in chondrogenesis [28]. This finding and our finding that BMPRII is primarily located in the cell-cell junction raises the possibility that BMP action may be mediated through the cell-cell junction complex. Also junctional proteins such as VE-cadherin and PECAM-1 are known to be mechanically sensitive to fluid shear stress. For PECAM-1, onset of steady shear stress result in a rapid c-src dependent tyrosine phosphorylation of PECAM-1 and recruitment of SHP2 (a tyrosine phosphatase); also, ERK 1/2 activation is dependent on this PECAM-1/SHP2 activation [25,29]. As for VE-cadherin, the onset of steady flow triggers an immediate and transient increase in β-catenin and flk-1 (VEGFR2) association with VE-cadherin that decreases after 5 min of exposure and results in the activation of Akt [26]. Even though we have not seen any differences in BMPRII expression by shear stress, these findings indicate that

shear stress may change the composition of junctional complexes with BMPRII as a modulator.

Intercellular junction proteins regulate cell layer permeability, retard cell migration, maintain cellular integrity, and transfer intracellular signals. These proteins are recognized as possible mechanosensors because of their specific location in the cell. They can transfer information intracellularly by interacting with the cytoskeleton via several anchoring molecules [10]. Vascular Endothelial cadherin (VE-cadherin) and PECAM-1 are the two most well known junction proteins. VE-cadherin forms a junctional complex with armadillo family members including α, β, and γ-catenin, p120, Plakoglobin, and actin filament [11-14], and PECAM-1 with β and γ-catenin. Each of these proteins is involved in maintaining integrity and stabilization of the junction, or signaling via transcriptional activity [15,16]. Furthermore, Luscinskas' group has reported that the VE-cadherin complex may play an important role in the migration of circulating leukocytes under flow conditions [17]. This information suggested to us that BMPRII could also be involved in monocyte migration through the intercellular junction, in addition to BMP4-induced monocyte adhesion.

In summary, endothelial cells express both BMP type I receptors and type II receptors, and BMPRII could potentially be important in inflammation and monocyte infiltration mediated by endothelial cells.

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Chapter 3

BMPRII is a critical regulator of inflammation in endothelial cells.

Introduction

Atherosclerosis is known as an inflammatory disease [1,2], occurring preferentially in branched or curved arteries exposed to unstable flow conditions including low and oscillatory shear conditions (OS) [3-6]. In contrast, the straight regions of the vasculature, which are exposed to relatively high levels of laminar shear stress (LS), are resistant to atherogenesis. Despite the well-known importance of these two different flow conditions, the underlying mechanisms for shear-induced atherogenesis are still unclear.   In an attempt to identify mechanosensitive genes that are differentially regulated by OS and LS, several groups including us have carried out transcript profiling studies [6-12]. Some of these mechanosensitive genes identified by this work are likely to play critical roles in mediating site-specific inflammation and atherosclerosis development.

Through these microarray studies, we identified bone morphogenic protein 4 (BMP4) as a critical regulator of an inflammatory response. We have discovered that BMP4 is produced in ECs in response to OS, but is blunted by LS [8,13]. BMP4 stimulates inflammation via cell-surface expression of intercellular adhesion molecule-1 (ICAM-1) and monocyte adhesion to ECs, in an NF-κB- and NADPH oxidase-dependent manner [13-15], as discussed in chapter 2. More recently, we have shown that chronic infusion of BMP4 causes systemic hypertension by mechanisms involving activation of arterial NADPH oxidases and endothelial dysfunction in wild-type (C57BL/6) and ApoE-

deficient mice, but not in those mice lacking the p47phox component of the NADPH oxidase [16]. However, the detailed signaling mechanisms mediating the BMP4 effects are still not known. The aim of the study is to determine the underlying mechanisms.

BMP actions are mediated by two different types of the BMP receptors (BMPR): Type I receptors (ALK2, ALK3, and ALK6) and Type II receptors (BMPRII, ActRII and Act-RIIb) [17]. Upon binding to BMPs, Type I BMPRs are activated by BMPRII and phosphorylate smad-1/5/8 proteins which form a complex with co-smad (Smad 4). The smad complexes then translocate to the nucleus and regulate BMP-specific gene expression such as *inhibitor of diffentiation-1 (id-1)*[18,19]. Recently, loss-of-function mutations of BMPRII have been linked to primary pulmonary hypertension (PPH) in humans[20], demonstrating its importance in pulmonary arterial biology and pathophysiology. However, it is not known whether BMPRs play a critical role in pathophysiology of the non-pulmonary vasculature. Especially the conduit of vascular endothelial cells in regulation of the inflammation and atherosclerosis will be the focus of this study.

Here, we examined the hypothesis that BMP4 induces inflammatory responses in ECs in a BMP receptor-dependent manner. To test this hypothesis, we first characterized BMP receptor mRNA and protein expression in cultured ECs and in mouse aortas, as discussed in chapter 3. Based on those results, we then examined the effect of BMPRII depletion on monocyte adhesion in control and BMP4-treated ECs. These results showed that BMPRII gene silencing inhibited BMP4-induced inflammation. Surprisingly, the

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knockdown of BMPRII itself induced inflammatory responses in the absence of BMP ligands. These findings uncovered BMPRII's constitutive role as a potential repressor of inflammation in ECs. Therefore, we hypothesized that down-regulation of BMPRII increases inflammation in endothelial cells. Subsequent studies focused on the identification of down-stream signaling pathways including ROS, mitogen activated protein kinase (MAPK), NFκB, and ICAM-1.

Methods

Cell culture and siRNA transfection: HUVEC purchased from Emory University were cultured in M-199 supplemented with endothelial growth factor supplement, heparin, and 20% fetal bovine serum (FBS), and used between passages 3-6. HUVEC were transfected with human BMPRII siRNA (5'-UGAACGCAACCUGUCACAUAAUAGGCG-3' from MWG Biotech) duplexes with 100 nM concentrations using Oligofectamine (Invitrogen) in serum-free media. After 6 hrs, the media were supplemented to raise FBS concentration to 10%. Two days post-transfection, the cells were exposed to rh-BMP4 (R&D) for 24 hrs in serum-free medium. BMPRII knockdown was confirmed by Western blot analysis using cell lysates.

Western blot: Following treatments, endothelial cell lysates were prepared and analyzed by Western blot analysis as described by us [13,21]. Briefly, cells were washed in ice-cold phosphate-buffered saline, lysed in RIPA buffer, and protein concentration determined with a Bio-Rad DC assay. Aliquots of cell lysates were resolved on a SDS-PAGE gel and

transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were probed with antibodies to BMPRII (1:1000, BD), ICAM-1 (1:1000, Santa Cruz), VCAM-1 (1:1000, Santa Cruz), phospho-Smad 1, 5, 8 (1:1000, cell signaling), total Smad 1,5,8 (1:1000, Santa Cruz), phospho and total p38 (1:1000, cell signaling), phospho and total IκBα (1:1000, cell signaling) or β-actin (1:1000, Santa Cruz), and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method.

Monocyte binding: Following BMPRII siRNA transfection for two days and BMP4 treatment for either 6hrs or 24hrs in HUVECs, the number of fluorescently labeled THP-1 monocytes bound to endothelial cells after a 45 min incubation period under static condition was determined microscopically as described previously [13,22,23]. In some studies of HUVECs, apocynin or an NFκB inhibitor, BAY 11-7082 were treated for two days after 6hr of transfection of BMPRII or non-silencing siRNA.

Amplex-Red assay: Using a horseradish peroxidase-linked Amplex Red fluorescence assay, extracellular H_2O_2 was measured. Briefly, after cells were transfected with non-silencing or BMPRII siRNA, media were washed twice with Krebs Ringer Phosphate (KRP) buffer and incubated with 5 µM Amplex UltraRed (Molecular Probes) and 0.1 U/ml horseradish peroxidase type II (Sigma-Aldrich) in KRP for 40 minutes. Triplicate reading were taken in a 96-well plate using 100 µl samples of media, and fluorescence was detected via plate reader at excitation and emission of 530 nm and 580 nm,

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respectively. Hydrogen peroxide levels were normalized to cellular protein as measured by the Bio-Rad DC assay.

Statistical Analysis: Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance was assessed by Student's t-test. *P*<0.05 was considered as statistically significant.

Results

We have shown previously that treatment of ECs with BMP4 stimulates monocyte adhesion to ECs [14], but it was not clear which BMP receptors mediated the inflammatory response. To examine whether BMPRII mediates BMP4-dependent monocyte adhesion in ECs, the cells were treated with siRNA to knockdown BMPRII expression, and then monocyte adhesion was determined. Prior to monocyte adhesion experiments, we investigated Smad 1/5/8 phosphorylation by BMP4 treatment in the presence of BMPRII siRNA (Fig 3.1.A)

BMPRII knockdown blocks BMP4 induced Smad 1/5/8 phosphorylation.

Transfection of HUVEC with 100nM BMPRII significantly reduced BMPRII proteins in Figure 4.1.b. We then tested the functional significance of the BMPRII knockdown by examining a canonical BMP4 signaling pathway, smad1/5/8 phosphorylation. As expected, BMP4 acutely (30 min and 60 min) stimulated smad1/5/8 phosphorylation in HUVEC treated with the non-silencing siRNA control (Fig. 3.1 (B)). In contrast, BMPRII knockdown prevented smad 1/5/8 phosphorylation in response to

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BMP4 (Fig. 3.1 (B)), supporting the expected notion that BMP4 stimulates smad1/5/8

phosphorylation after binding to BMP receptors.
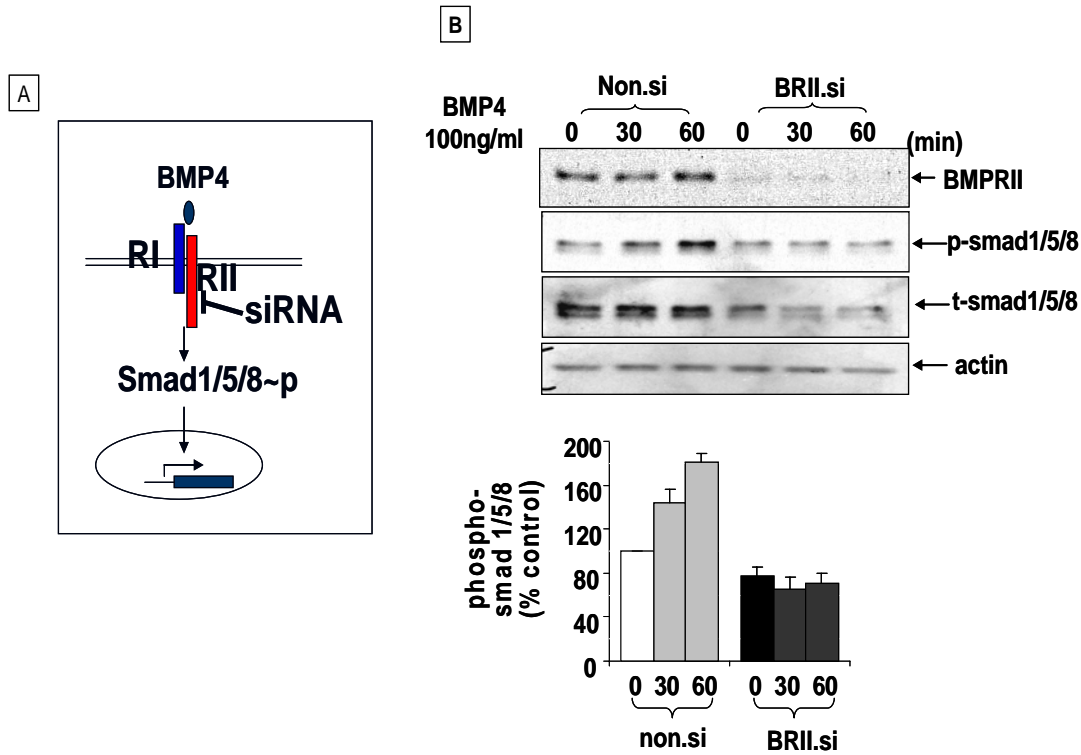
Figure 3.1. BMPRII knockdown blocks BMP4 induced Smad 1/5/8 phosphorylation.
(A) A diagram of BMP4 signaling pathway through BMP receptors (RI: BMP type I receptor, RII: BMP type II receptor). **(B)** Sub-confluent HUVECs were transfected with BMPRII siRNA (BRII.si, 100 nM) or non-silencing RNA (Non.si, 100nM) for 48 hours. Transfected cells were then treated with BMP4 (100 ng/ml) upto 60 minutes. BMPRII protein knockdown by siRNA was confirmed by Western blot. Cell lysates were collected and probed with antibodies to BMPRII, phospho-Smad 1/5/8, and total Smad 1/5/8a, β actin antibody as an internal control. Shown are mean ± SEM, and phospho-smad 1/5/8 signal was normalized to actin (*p<.05, n=3).

BMPRII knockdown stimulates inflammatory responses in basal cells.

We then carried out monocyte adhesion studies under the same conditions using the BMPRII siRNA and found a surprising result. First, as expected, BMP4 increased monocyte adhesion by 200% above the vehicle control in non-silencing siRNA treated cells. Unexpectedly, however, the BMPRII siRNA treatment of HUVEC significantly increased the monocyte adhesion response by 400% above the non-silencing vehicle control (Fig. 3.2 (B)). Moreover, BMP4 could no longer increase the monocyte adhesion response above that of the vehicle control in HUVEC treated with the BMPRII siRNA (Fig. 3.2 (B)). However, in the TNFα treatment, BMPRII siRNA transfection increased monocyte adhesion above the non-silencing TNFα treatment suggesting that BMPRII siRNA transfection alone did not saturate inflammation inducing monocyte binding. We also investigated adhesion molecule expression by BMPRII knock down. As shown in Figure 3.2 (C), BMPRII knock down increased ICAM-1 protein level by 6-fold, and VCAM-1 level by 3-fold above that of non-silencing control (Fig 3.2 (C)). On the other hand, BMPRII knock down did not affect BMP4 protein level. These results suggest that BMPRII knock down itself is sufficient to stimulate inflammatory response including ICAM-1 and VCAM-1 induction and monocyte adhesion.

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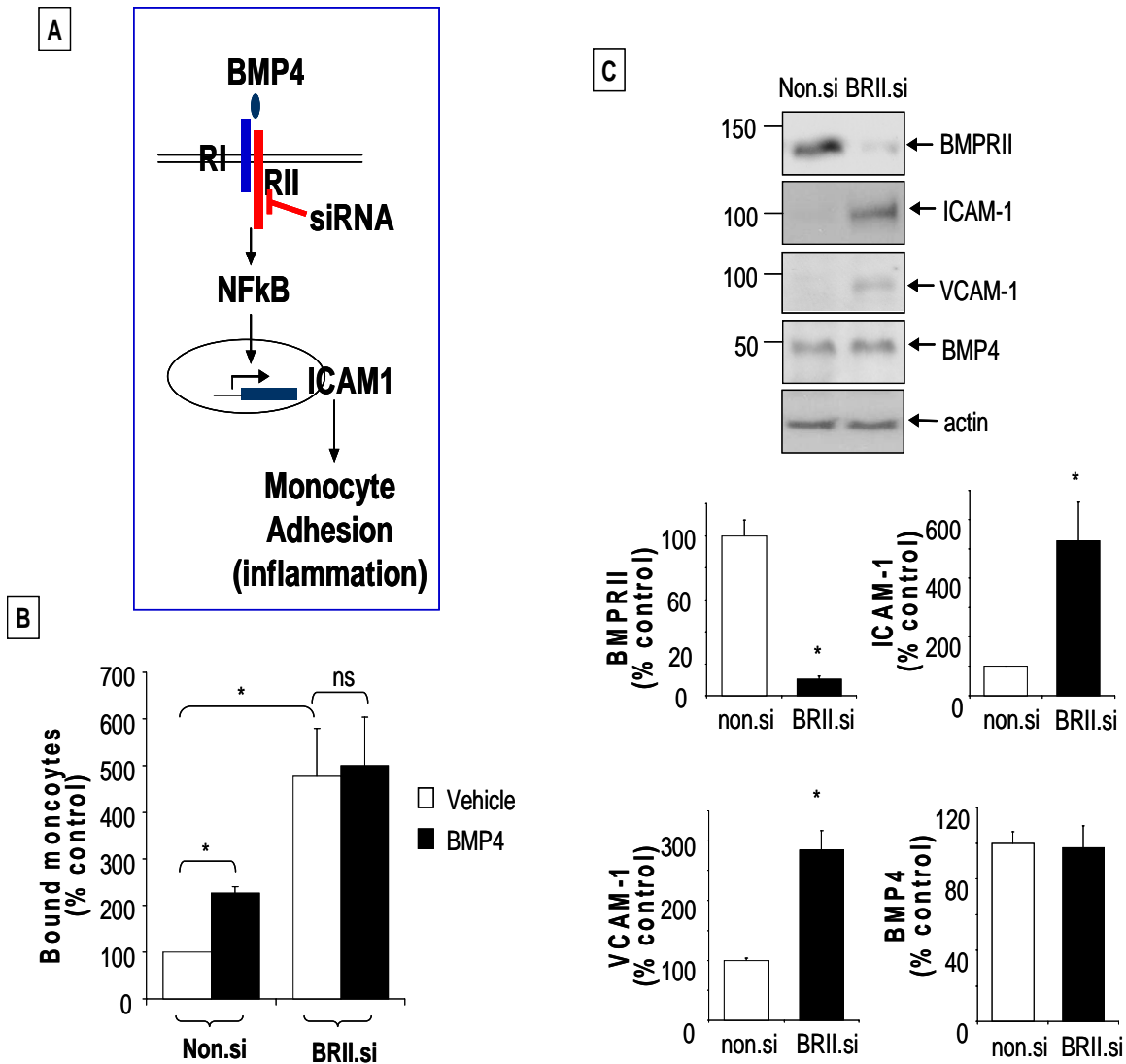
Figure 3.2. BMPRII knockdown stimulates inflammatory responses in basal cells.
(**A**) A diagram of BMP4 signaling pathway through BMP receptors (RI: BMP type I receptor, RII: BMP type II receptor). (**B** and **C**) HUVECs were treated with 100 nM BMPRII siRNA (BRII.si) or non-silencing siRNA (Non.si) for 2 days. (**B**) siRNA treated HUVECs were exposed to BMP4 (100ng/ml) for 4 hrs followed by monocyte binding assay using THP-1 cells. Data shown is expressed as mean ± SEM (n=6, *p < 0.05). (**C**) siRNA transfeced cells were lysed for immunoblot with antibodies to BMPRII, ICAM-1, and VCAM-1, using β-actin as loading controls. Data are expressed as mean ± SEM (n=4, *p < 0.05).

Knockdown of BMPRII increases inflammation through ROS dependent mechanism.

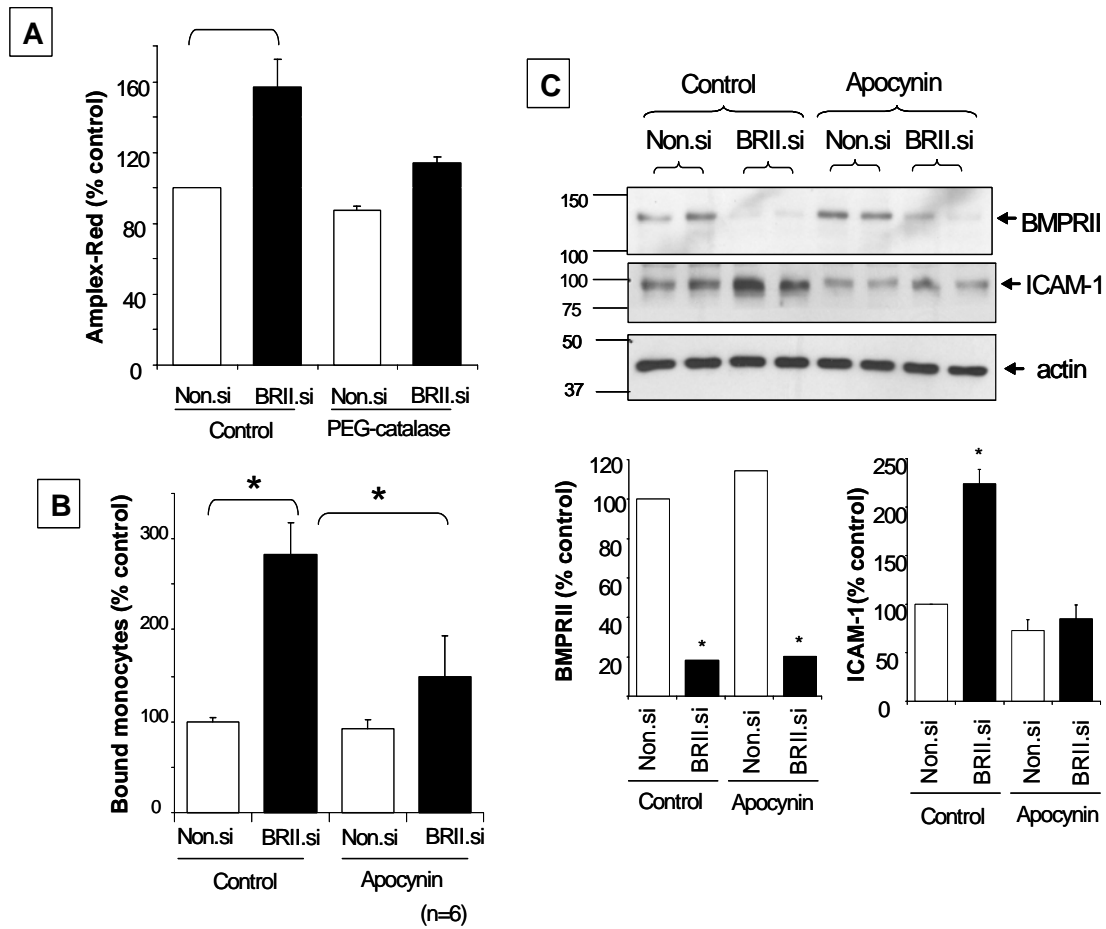**Figure 3.3. Knockdown of BMPRII increases inflammation through ROS dependent mechanism. A** to **C**) HUVECs were treated with 100 nM BMPRII siRNA (BRII.si) or non-silencing siRNA (Non.si) for 2 days. **B** and **C**) Apocynin (60 μM) were treated with siRNA for 2 days. **A**) siRNA ± PEG-catalase treated HUVECs were analyzed using Amplex-Red assay to measure hydrogen peroxide. (*, p<0.05, n=4) **B**) siRNA ± Apocynin treated HUVECs were followed by monocyte binding assay using THP-1 cells. Data shown is expressed as mean ± SEM (n=6, *p < 0.05). **C**) siRNA ± Apocynin transfeced cells were lysed for immunoblot with antibodies to BMPRII, and ICAM-1, using β-actin as loading controls. Data are expressed as mean ± SEM (n=4, *p < 0.05).

Next, we hypothesized that BMPRII knock down induced inflammation is due to increase in ROS production. Therefore, we measured extracellular hydrogen peroxide (H₂O₂) level using Amplex-Red Assay. As shown in Fig. 3.3 (A), BMPRII knockdown

extracellular H_2O_2 by 50%. Cell permeable catalase (PEG-catalase) was used as a control. When HUVECs were pre-treated with PEG-catalase after non-silencing and BMPRII siRNA transfection, H_2O_2 production was blocked with BMPRII knockdown. To block ROS production, HUVECs were treated with apocynin, an NADPH inhibitor. Apocynin treatment blocked BMPRII knock down induced inflammation measured by monocyte binding (Fig. 3.3 (B)), and also ICAM-1 expression (Fig. 3.3 (C)).

An NFκB inhibitor, BAY 11-7082, inhibits BMPRII knockdown induced inflammation.

To look at involvement of NFκB signaling pathway, we used a pharmacological NFκB inhibitor, BAY 11-7082. It has been shown that BAY 11-7082 inhibits IκB phosphorylation, down-regulates IκB expression, and blocks NFκB translocation into nucleus. Also it blocks TNFα induced - ICAM-1, VCAM-1, and E-selectin expression in endothelial cells[24]. After transfection with non-silencing and BMPRII siRNA, HUVECs were treated with BAY 11-7082. Monocyte binding induced by BMPRII siRNA transfection was completely blocked (Fig 3.4 (A)) Also BAY 11-7082 treatment blocked ICAM-1 expression (Fig 3.4 (B)). We also looked IκB phosphorylation and IκB expression. As shown in Fig 3.4 B, BMPRII knock down increased IκB phosphorylation, and BAY 11-7082 blocked the phosphorylation. Expression of IκB was decreased by BMPRII siRNA transfection, however, BAY 11-7082 treatment did not increase IκB expression. Even though IκB expression was not increased by BAY 11-7082 treatment, the basal level of IκB was lower in BAY 11-7082 treatment compared to control, and

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also IκB expression did not decrease further with BMPRII siRNA transfection in BAY 11-7082 treatment.
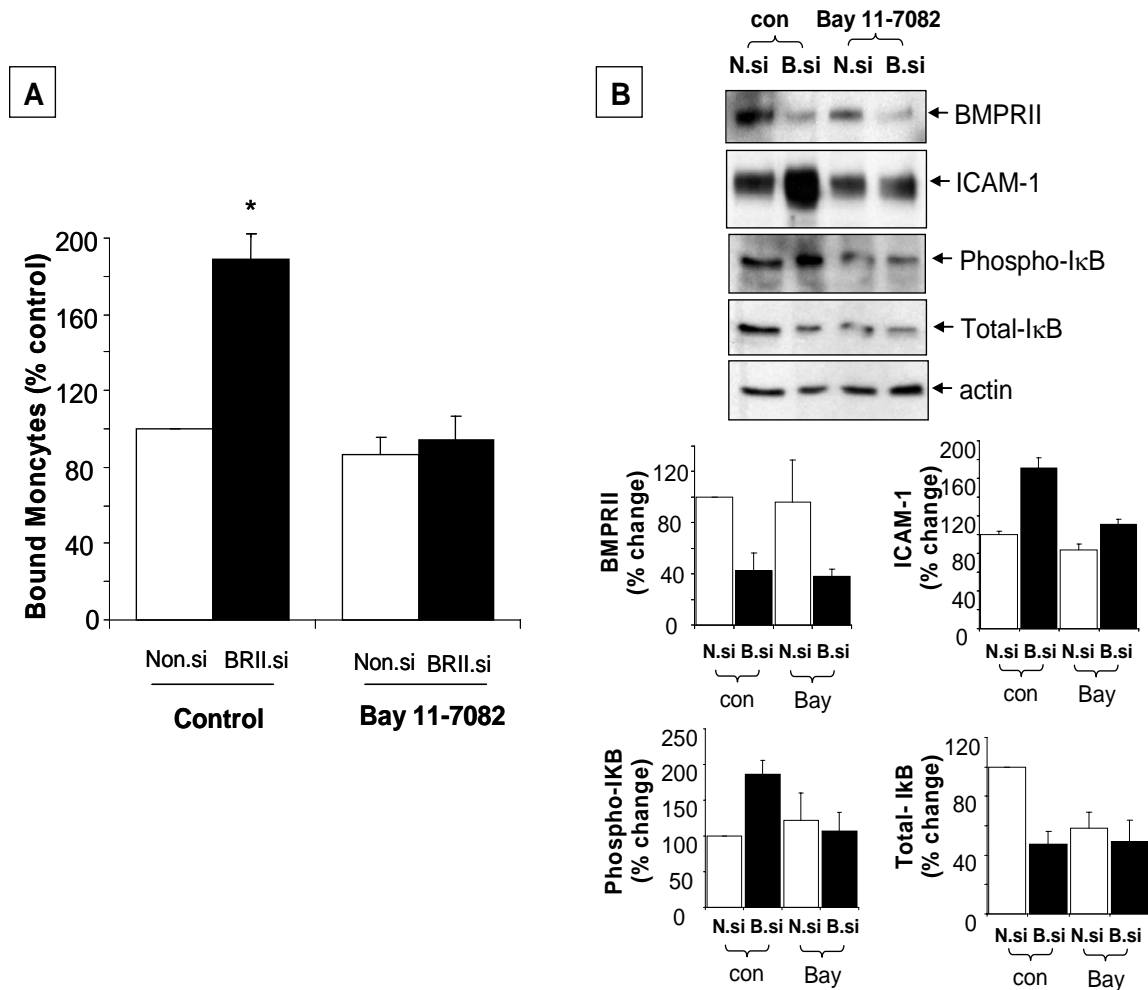
Figure 3.4 An NFκB inhibitor, BAY 11-7082, inhibits BMPRII knockdown induced inflammation. **(A)** Pre-confluent HUVECs were transfeced with 100 nM of non-silencing siRNA (Non.si) or BMPRII siRNA (BRII.si) for 2 days with or without Bay 11-7082. monocyte binding assay was performed using THP-1 cells. Data shown is expressed as mean ± SEM (n=4, *p < 0.05). **(B)** siRNA ± Bay 11-7082 treated cells were lysed for immunoblot with antibodies to BMPRII, ICAM-1, phosphor-IκB, and total IκB, using β-actin as loading controls. Data are expressed as mean ± SEM (n=4, *p < 0.05).

BMPRII knockdown increases p38 phosphorylation in a basal level, but BMP4 treatment decreases it.
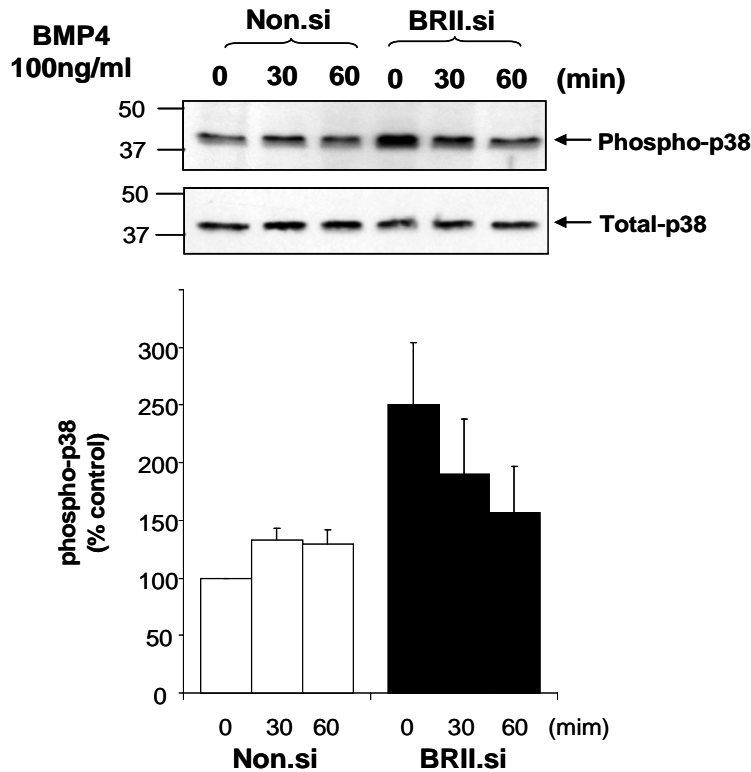
Figure 3.5. BMPRII knockdown increases p38 phosphorylation in a basal level, but BMP4 treatment decreases it. Sub-confluent HUVECs were transfected with BMPRII siRNA (BRII.si, 100 nM) or non-silencing RNA (Non.si, 100nM) for 48 hours. Transfected cells were then treated with BMP4 (100 ng/ml) upto 60 minutes. BMPRII protein knockdown by siRNA was confirmed by Western blot. Cell lysates were collected and probed with antibodies to phospho-p38, and total-p38, β actin antibody as an internal control. Shown are mean ± SEM, (*p<.05, n=3).

As we discussed in introduction, it has been shown that cells that overexpressed with BMPRII mutant cDNA showed up-regulation of p38 phosphorylation[25]. Therefore, we also investigated p38 signaling pathway. BMPRII knockdown increased p38 phosphorylation in the vehicle compared to non-silencing control, and also BMP4 treatment decreased p38 phosphorylation in the BMPRII siRNA transfected HUVECs. Even though Smad 1/5/8 pathway was blocked by BMPRII knock down, p38 pathway

seems to be an extra signaling pathway when BMPRII expression is affected. This data suggests that activation of p38 pathway may be due to releasing upstream signaling molecues of the pathway by downregulating BMPRII expression.

Discussion

The major findings reported in this study are that a) knockdown of BMPRII blocks BMP4-dependent smad1/5/8 phosphorylation and monocyte adhesion. Unexpectedly, we found that b) BMPRII knockdown in HUVEC unleashes inflammatory responses in the absence of BMP4. Collectively, these findings show for the first time that a decrease in BMPRII protein level in aortic endothelium is linked to inflammation. In subsequent studies, we showed that c) BMPRII knockdown increases ICAM-1 and monocyte adhesion through ROS, and NFκB dependent pathways.

Recently, heterozygous BMPRII mutations have been linked to familial and sporadic primary pulmonary hypertension (PPH) [20,26]. In addition, it has been reported that BMPRII expression is reduced in the lungs of the primary pulmonary hypertension patients even when they do not have any detectable mutations of the receptor [27]. PPH is characterized by obliteration of pre-capillary pulmonary arteries, leading to sustained elevation of pulmonary arterial pressure and subsequent right ventricular hypertrophy. PPH is typically associated with abnormal proliferation of endothelial and smooth muscle cells within the small pulmonary arteries [28]. Interestingly, PPH is also strongly associated with inflammation -as evidenced by accumulation of inflammatory cells infiltrating into the plexiform lesions in the lungs and increased inflammatory cytokines in the circulation

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of the patients [29]. Homozygotic deletion of BMPRII in mice causes an embryonic lethal phenotype [30], while heterozygotic BMPRII$^{+/-}$ mice show increased susceptibility to pulmonary hypertension in response to an inflammatory stress [31]. These results provide a link between BMPRII mutation or haplo-insufficiency and PPH as well as an association between inflammation and PPH. However, there is no published literature showing that BMPRII mutation or decreased BMPRII expression causes inflammation and atherosclerosis.

As far as we are aware, this is the first report demonstrating that a decrease in BMPRII expression in ECs induces inflammation. Several lines of evidence support our conclusions: 1) We have shown that BMPRII siRNA increased monocyte adhesion, while non-silencing siRNA did not (Fig.3.2); 2) BMPRII knockdown increased ICAM-1 and VCMA-1 expression in ECs (Fig.3.2); 3) BMPRII knock down increased ROS production, and BMPRII knock down induced-inflammation was abolished with apocynin treatment (Fig. 3.3); and 4) Monocyte adhesion induced by BMPRII knockdown was blocked by an NFκB inhibitor and an ICAM-1 neutralizing antibody (YN-1) (Fig. 3. and Fig. 3.5).

The effects of BMPRII and BMPs seem to be cell type- and vascular bed- specific. Recently, BMPRII knockdown by siRNA was shown to increase apoptosis in human pulmonary aortic ECs [32]. On the other hand, BMPRII knockdown in human pulmonary aortic smooth muscle cells (PASMC) inhibited apoptosis induced by BMP4/7 by mechanisms dependent on activation of caspases-3, 8 and 9 [33]. However, these studies

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did not examine whether BMPRII knockdown induces inflammation. Whether BMPRII knockdown causes apoptosis has not been examined in the current study, but BMP4 itself showed no significant effect on cell proliferation or apoptosis in "MAEC" and human aortic ECs (data not shown) previously in our lab. Even within the same pulmonary arterial bed, smooth muscle cells from peripheral and proximal arteries respond differently. BMP4 treatment increased proliferation of peripheral smooth muscle cells, but it inhibited that in proximal smooth muscle cells of pulmonary arteries [34].

The mechanism by which BMPRII knockdown stimulates ICAM-1 induction and subsequent monocyte adhesion in ECs is not known. However, we propose that the loss of BMPRII may unleash the signaling proteins that normally bind to the receptor, resulting in uncontrolled activation of inflammatory pathways. BMPRII is a protein (1,038 amino acids) with an extracellular BMP binding domain, a single transmembrane domain and a long cytoplasmic domain. The cytoplasmic domain (866 amino acids) contains a serine/threonine protein kinase domain followed by a C-terminal tail. Interestingly, this long cytoplasmic domain is unique to BMPRII, and not found in other members of the TGFβ receptor superfamily. Moreover, the cytoplasmic domain has been shown to bind various signaling proteins, cytoskeletal components and metabolic enzymes [35]. Among notable ones are p50b NFκB protein, MAP3K8, LIM kinase-1 and c-Src protein tyrosine kinase [35-37]. For example, the C-terminal tail interacts with c-Src, and the association has been shown to downregulate the Src tyrosine kinase activity [37]. More interestingly, BMPRII mutations truncating the C-terminus, as found in PPH patients, increase c-Src activity and subsequent proliferation of smooth muscle cells [37]. Perhaps,

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BMPRII, much like caveolins [38], serves as a docking protein in normal unstimulated conditions, holding the signaling proteins in an inactive state. When BMPRII level is decreased or its C-terminal is mutated or truncated as in the cases of PPH patients, the normally sequestered signaling proteins may be released and activated. Similarly, when BMPRII is knocked down by siRNA, the signaling molecules that are normally sequestered by the receptor may be released and activated, leading to a vicious cycle of ICAM-1 induction, monocyte adhesion, and eventual atherosclerosis development.

Also as shown in figure 3.6, BMPRII knock down by siRNA increased basal level of p38 phosphorylation, and BMP4 treatment decreased it. Previously, Rudarakanchana et al showed that transfection of mutant, but not wild-type, constructs into a mouse epithelial cell line (NMuMG cells) led to activation of p38 (MAPK) and increased serum-induced proliferation[25]. As we discussed earlier, it is very possible that BMPRII knock down or mutation releases MAP3K8, mitogen-activated protein kinase kinase kinase 8, from binding of BMPRII into cytosol, which activates p38 pathway. We need to pursue further study on this subject whether this speculation would be right using immunoprecipitation and inhibitor studies for MAP3K8. Also it shows that there might be other receptors would be involved in activating MAPK pathway by BMP4 treatment.

The major finding was that BMPRII knockdown inhibited monocyte adhesion and smad1/5/8 phosphorylation induced by BMP4, suggesting that BMPRII mediates the BMP4 effect (Fig. 3.1). These findings indicate that arterial inflammation could begin with BMP4 production in ECs exposed to disturbed flow conditions, priming the area for

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development of the early stages of atherosclerosis [13]. In the continued presence of disturbed flow and other risk factors for atherosclerosis such as hypercholesterolemia, hypertension, diabetes and smoking, atherosclerotic lesions may progress and BMPRII expression level decreases, which can further exacerbate inflammation and atherosclerosis.

In summary, we show that BMPRII mediates BMP4-dependent inflammation. More interestingly, BMPRII knockdown in ECs induces inflammation in ROS, NFκB, and ICAM-1 dependent way. For the future studies, we will pursue the rescue experiments using bovine siRNA to knock down BMPRII in BAEC, and then infect with human BMPRII adenovirus to rescue BMPRII expression without any effect from the siRNA because of the species specificity.

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Chapter 4

Atherogenic Conditions Downregulate BMPRII expression.

Introduction

Heterozygous mutations of BMPRII have been linked to familial and sporadic form of primary pulmonary hypertension (PPH) by both decreasing BMPRII expression as well as function[1,2]. In addition, it has been reported that BMPRII expression is reduced in the lungs of the primary pulmonary hypertension patients even when they do not have any detectable mutations of the receptor [3]. It seems that the condition of PPH may be correlated to regulation of BMPRII expression. In an animal model for pulmonary hypertension, Takahashi et al. showed that hypoxic condition in rats decreased BMPRII protein levels significantly[4]. There have been several evidences that showed decreased expression of BMPRII in both patients and animal models of PPH.

PPH is characterized by obliteration of pre-capillary pulmonary arteries, leading to sustained elevation of pulmonary arterial pressure and subsequent right ventricular hypertrophy. PPH is typically associated with abnormal proliferation of endothelial and smooth muscle cells within the small pulmonary arteries [5]. Interestingly, PPH is also strongly associated with inflammation, as evidenced by accumulation of inflammatory cells infiltrating into the plexiform lesions in the lungs and increased inflammatory cytokines in the circulation of PPH patients [6]. Homozygotic deletion of BMPRII in mice causes an embryonic lethal phenotype [7], while heterozygotic BMPRII^{+/-} mice show increased susceptibility to pulmonary hypertension in response to an inflammatory stress

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[8]. These results provide a link between BMPRII mutation or haplo-insufficiency and PPH as well as an association between inflammation and PPH. However, there is no published literature showing that BMPRII mutation or decreased BMPRII expression causes inflammation and atherosclerosis.

There have been studies on BMPRII expression in several research areas including cancer studies, HIV studies, and PPH. Down-regulation of BMPRII expression has also been seen in cancer patients. Several types of cancer cells such as renal carcinoma cells, bladder transitional carcinoma cells, and human prostate cancer cells showed decreased BMPRII expression [9-11]. Also restoration of BMPRII using the demethylating agent 5-aza-2'-deoxycytidine led to a decreased rate in tumor growth[10]. In addition, there have been reports of down-regulation of BMPRII from human immunodeficiency virus (HIV) infection. Cadlwell et al. showed that HIV-Tat overexpression decreases BMPR2 promoter activity in U937 monocytes[12]. Conversely, Hu et al. showed that whereas Simvastatin treatment decreased BMPRII reporter gene transcription, it increased mRNA and protein expression in human lung microvascular endothelial cells[13].

Based on these previous studies of pulmonary hypertension and cancer, we hypothesized that atherosclerotic conditions may be correlated to BMPRII expression in endothelial cells. We obtained human coronary artery sections with various stages of atherosclerosis to investigate BMPRII expression with progression of the disease. Interestingly, BMPRII expression decreased as atherosclerotic plaques advanced. This

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result led us to form a new hypothesis that pro-atherogenic cytokines may be involved in regulating BMPRII expression in endothelial cells. Therefore, we challenged HUVECs using a potent cytokine, TNFα. On the other hand, anti-atherogenic drugs such as a lipid lowering drugs like statins, which have been shown to increase BMPRII expression, may have protective effects through BMPRII.

Methods

Cell culture: Human umbilical vein ECs (HUVEC) purchased from the Department of Dermatology, Emory University were cultured in M-199 supplemented with endothelial growth factor supplement, heparin, and 20% fetal bovine serum (FBS), and used between passages 3-6.

Immunohistochemistry: Frozen sections of human coronary arteries (10 μm) were fixed in ice-cold acetone for 5 min, blocked for 1 hour with 10% donkey or goat serum, and incubated with primary antibodies overnight at 4°C, followed by rhodamine-conjugated secondary antibodies (anti-goat IgG, anti-mouse IgG or anti-rat IgG) for 2 hours at room temperature as described [14]. Nuclei were counter-stained with Hoechst 33258 (Sigma). Primary antibodies used were specific for ALK2 (1:50, R&D systems), BMPRII (1:50, Santa Cruz) or PECAM-1 (1:50, BD). Twenty different human coronary artery sections, containing various stages of atherosclerosis from minimally diseased to advanced atheroma stages, from nine different patients, were examined. Microscopic images were taken using a Zeiss epi-fluorescence microscope. The semi-quantification method was used based on the blind grading for atheroma intensity and BMPRII staining intensity in

endothelium using one to five scale (1: minimally diseased, low staining intensity, 5: advanced atheroma, high staining intensity).

Western blot: Following experimental treatments, endothelial cell lysates were prepared and analyzed by Western blot analysis, as described by us [14,15]. Briefly, cells were washed in ice-cold phosphate-buffered saline, and lysed in RIPA buffer, and protein concentration determined with a Bio-Rad DC assay. Aliquots of cell lysates were resolved on a SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were probed with antibodies to BMPRII (1:1000, BD), phospho-eNOS (1:1000, Cell signaling), total-eNOS (1:1000, Cell signaling), or β-actin (1:1000, Santa Cruz), and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by chemiluminescence method.

BMPRII levels are lower in endothelium of advanced atherosclerotic lesions than that in minimally diseased human coronary arteries.

To determine the pathophysiological significance of the *in vitro* finding that BMPRII knockdown induced inflammation, we examined whether BMPRII expression level changes in ECs in various stages of human atherosclerotic lesions. Serial frozen sections were obtained from human coronary arteries containing a spectrum of atherosclerotic lesion complexities, and examined by immunohistochemical staining with the BMPRII and PECAM-1 antibodies. In minimally diseased human coronary arteries, BMPRII staining was easily detected in luminal endothelium (arrows) and medial smooth muscle cells (Fig. 4.1 (B)-a). BMPRII staining intensity in the luminal endothelium appeared to be less in Type II to III atheromatous lesions when compared to that of minimally diseased arteries (compare Fig 4.1 (A) a, d, g and Fig. 4.1. (B) a, d, g). Furthermore, BMPRII staining in the luminal endothelium was nearly undetectable in advanced atheromatous lesions, (Fig. 4.1 (B)-g). Quantification of the BMPRII staining intensities in the luminal endothelium in various sections (n=17) showed a negative correlation (r^2=0.68) with respect to the atheroma lesion stages (Fig. 4.1 (C) Graph). These results suggest that BMPRII expression decreases as atherosclerotic lesion progresses, reaching an undetectable level in the advanced lesion stage.
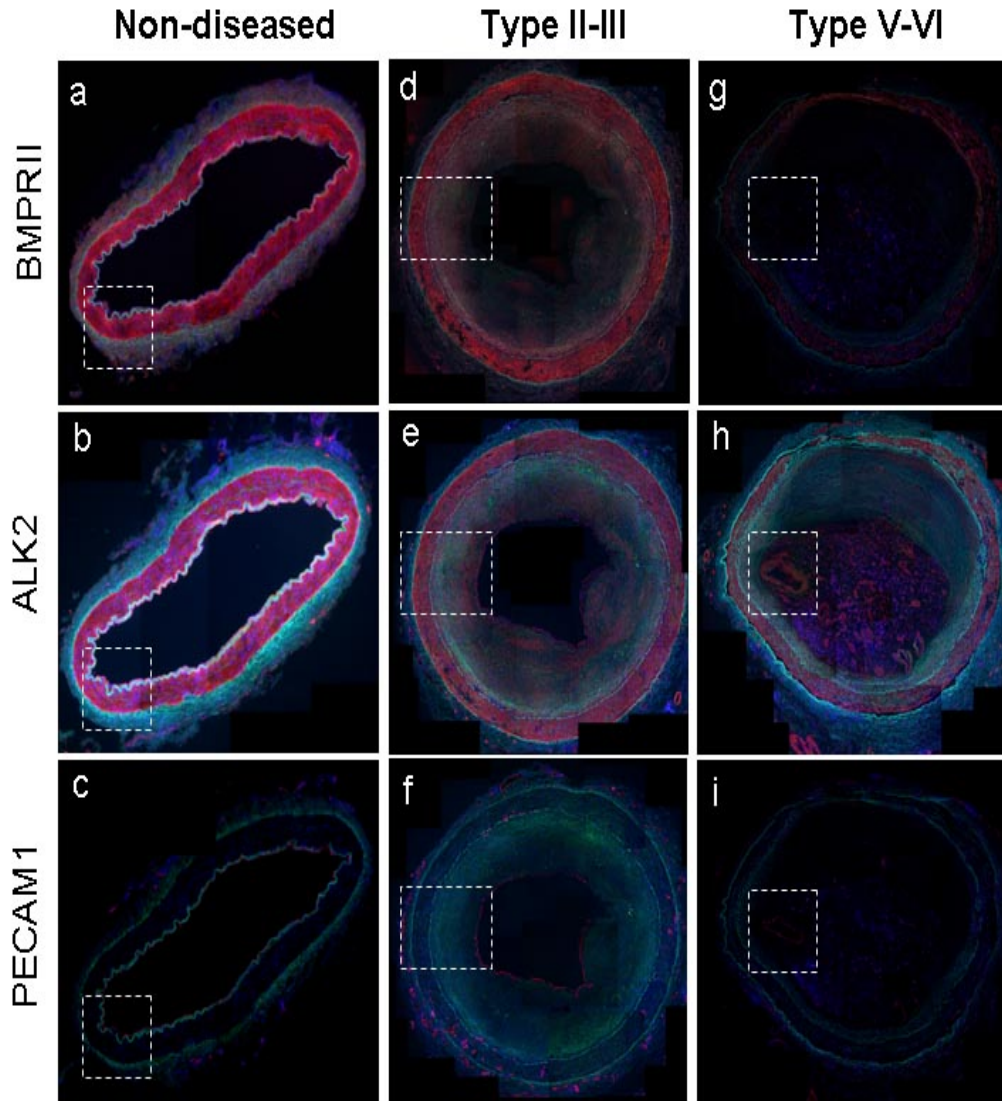
A

Figure 4.1. BMPRII levels are lower in endothelium in advanced atherosclerotic lesions than that in minimally diseased human coronary arteries. **(A)** Human coronary arteries with various stages of atherosclerotic plaques were stained with BMPRII (1:50, scbt), ALK2 (1:50, R&D systems), and PECAM-1 (1:50, BD Pharmingen) antibodies. These are composite figures from multiple 10x pictures taken by Zeiss epi-fluorescence microscope. Close-up images from the boxed areas are shown in Fig. 4.1 **(B)**

Figure 4.1. (Continued) BMPRII levels are lower in endothelium in advanced atherosclerotic lesions than that in minimally diseased human coronary arteries. (B) Close-up images from the boxed areas are shown (10x), Arrows indicate endothelium and L stands for the vessel lumen.
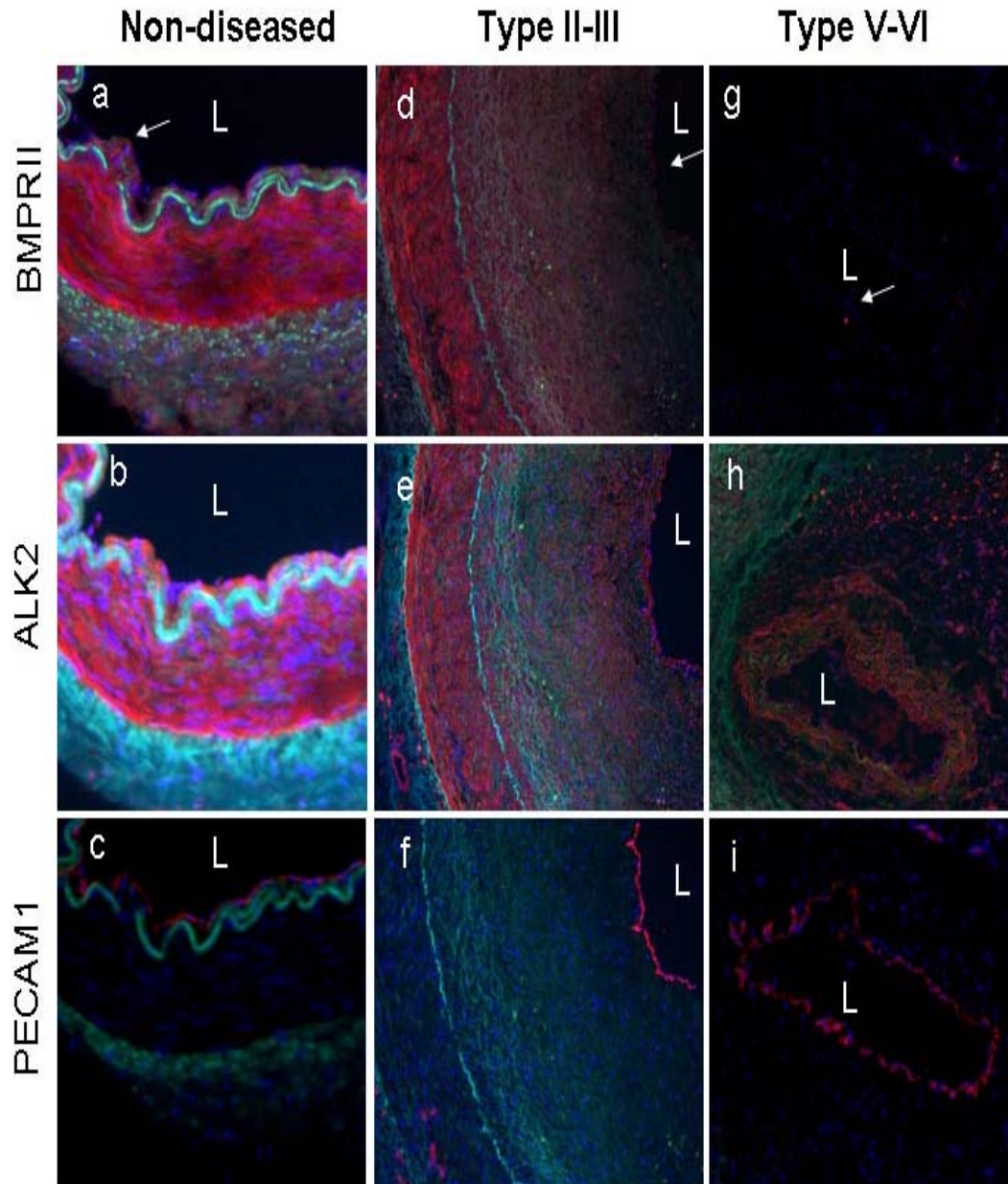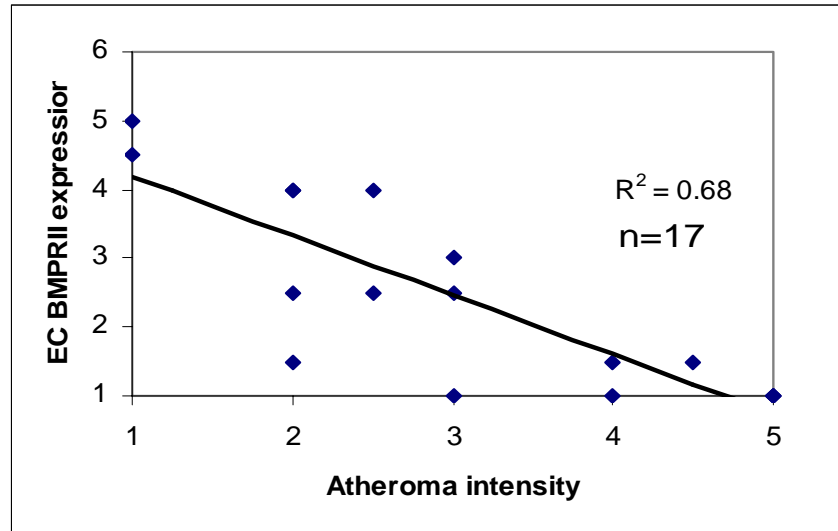
Figure 4.1. (Continued) BMPRII levels are lower in endothelium in advanced atherosclerotic lesions than that in minimally diseased human coronary arteries. (C) In total, 21 sections were stained, but 17 sections were blindly graded. Atheroma intensity: 1-minimally diseased to 5-advanced plaque, BMPRII expression: 1- low to 5-high.

A potent cytokine, TNFα, decreases BMPRII expression in HUVECs.

Based on the results from BMPRII staining of various stages of human coronary arteries, we speculated that BMPRII expression may be regulated by atherogenic conditions. Therefore, we decided to test the hypothesis that two potent cytokines, Tumor Necrosis Factor α (TNFα) and Lipopolysaccharide (LPS), which are known to increase inflammation, may be responsible for decreasing BMPRII expression.

As shown in Figure 4.2 (A), TNFα treatment decreased BMPRII expression in HUVEC. After 24 hour of treatment, BMPRII expression was decreased about 50% compared to control. LPS treatment, however, had a tendency to decrease BMPRII expression only slightly; while there was a trend, it was not statistically significant (Fig 4.2 (B)). These results support our hypothesis that atherosclerotic cytokines decrease BMPRII expression in endothelial cells.

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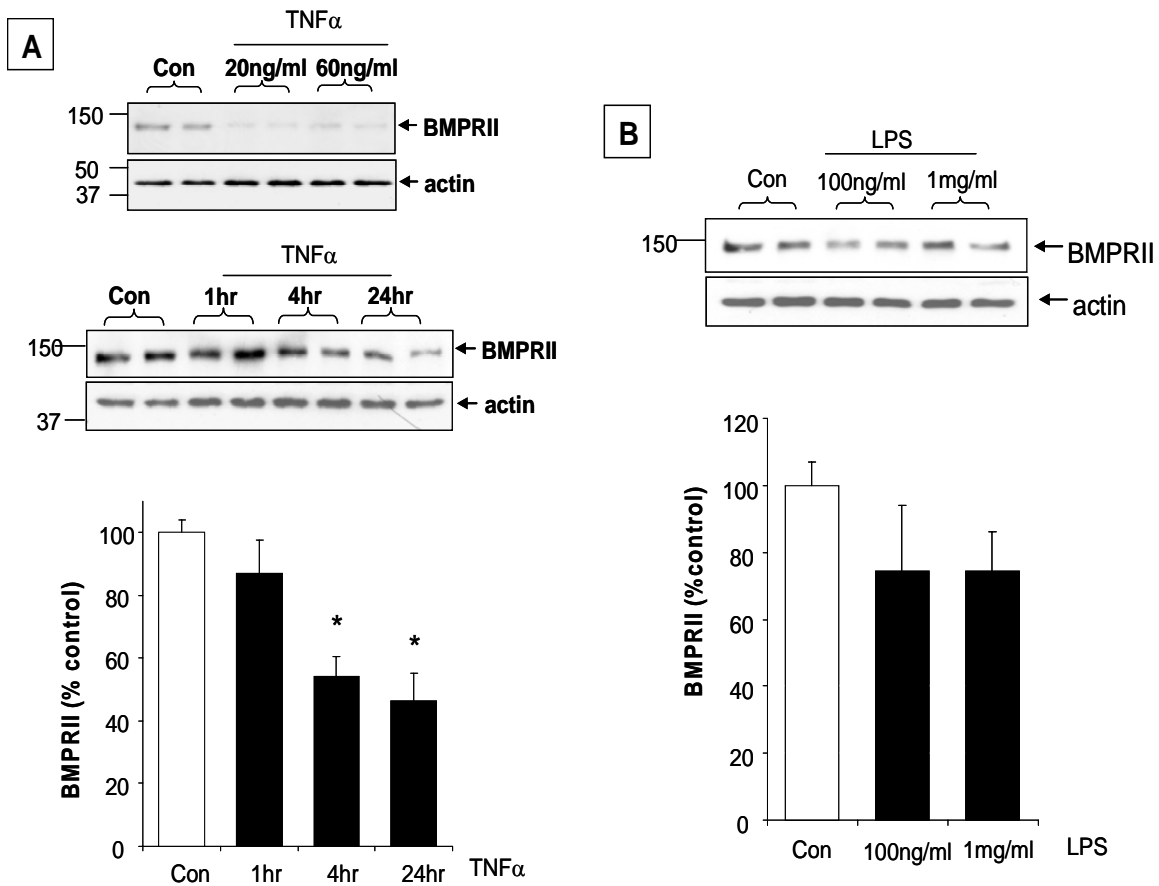
Figure 4.2. A potent cytokine, TNFα, decreases BMPRII expression in HUVECs. (A)
Confluent HUVECs were treated with TNFα (20 ng/ml and 60 ng/ml) for 1 hr, 4 hr, and
24 hrs. Cells were harvested in RIPA buffer and protein lysates were analyzed by
Western blot using BMPRII (1:1000, BD pharmigen) and actin (1:1000, scbt) antibodies.
Densitometry and statistical analysis were performed (n=6, *, p<0.05). (**B**) Confluent
HUVECs were treated with LPS (100ng/ml and 1mg/ml) for 24 hrs. Cell lysates were
processed and analyzed as described in **A**). (n=4, *, p<0.05)

Statins increase BMPRII expression in HUVECs.
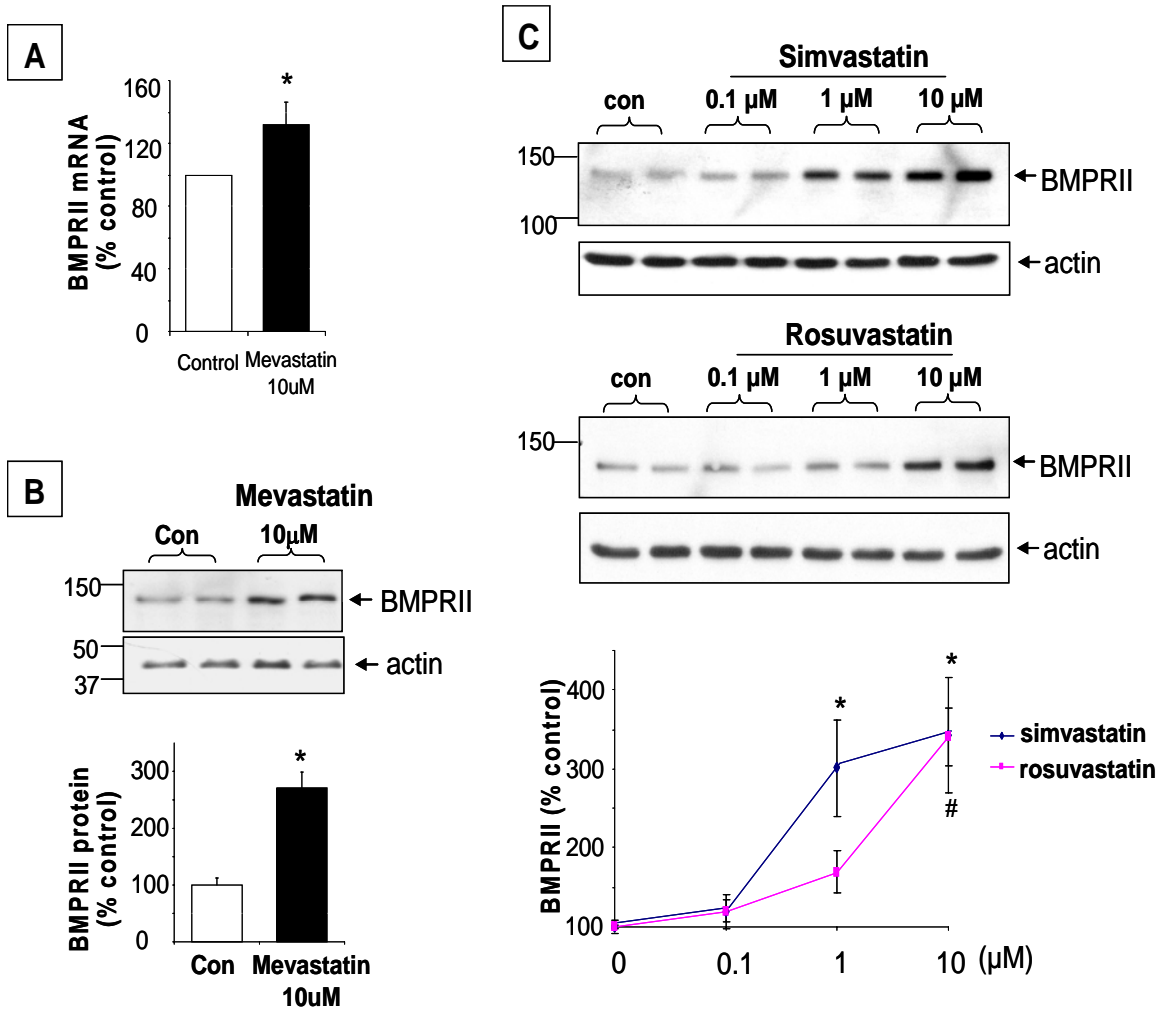
Figure 4.3. Statins increase BMPRII expression in HUVECs. (A) Confluent HUVECs were treated with 10μM mevastatin for 24 hrs. Total RNA was isolated and real-time PCR was performed (*, p<0.05, n=4). (**B**) Cells were treated the same way as in (**A**) and then immunoblot analysis was performed as in Fig 5.2 (*, p<0.05, n=4). **C**) Confluent HUVECs were treated with several concentrations of simvastatin (Calbiochem), and rosuvastatin (gift from Dr. Searles). (*, p<0.05, n=6).

Hu et al. previously showed that simvastatin increases BMPRII expression in human lung microvascular endothelial cells[13]. Figure 4.3 (A) shows that mevastatin (10μM) treatment for 24 hours in HUVECs increased BMPRII mRNA approximated

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40% as measured by quantitative PCR. BMPRII protein levels were also increased with mevastatin treatment by about 250% (Fig.4.3 (B)). We then tested two other statins, simvastatin and rosuvastatin. Simvastatin is categorized as hydrophobic; whereas, rosuvastatin is hydrophilic. As shown in Figure 4.3 (C), simvastatin increased BMPRII expression by about 350% at as low a concentration as $1\mu M$. Also, rosuvastatin increased BMPRII expression to the same level as simvastatin at a concentration of $10\mu M$. This suggests that simvastatin is 10-fold more potent in increasing BMPRII expression.

Mevalonate blocks simvastatin-induced BMPRII overexpression.

Statins are inhibitors of HMG-CoA reductase, which is the rate-limiting enzyme in the cholesterol synthesis pathway that converts HMG-CoA to mevalonate (figure 4.4 (A)). To show that statins' upregulation of BMPRII expression is due to inhibition of the HMG-CoA reductase pathway, we treated HUVECs with simvastatin along with mevalonate. As shown in Figure 4.4 (B), simvastatin treatment increased BMPRII expression by about 400%, and concurrent mevalonate treatment with simvastatin completely blocked simvastatin-induced BMPRII up regulation. This result suggests that upregulation of BMPRII by statins is mediated by the cholesterol synthesis pathway.
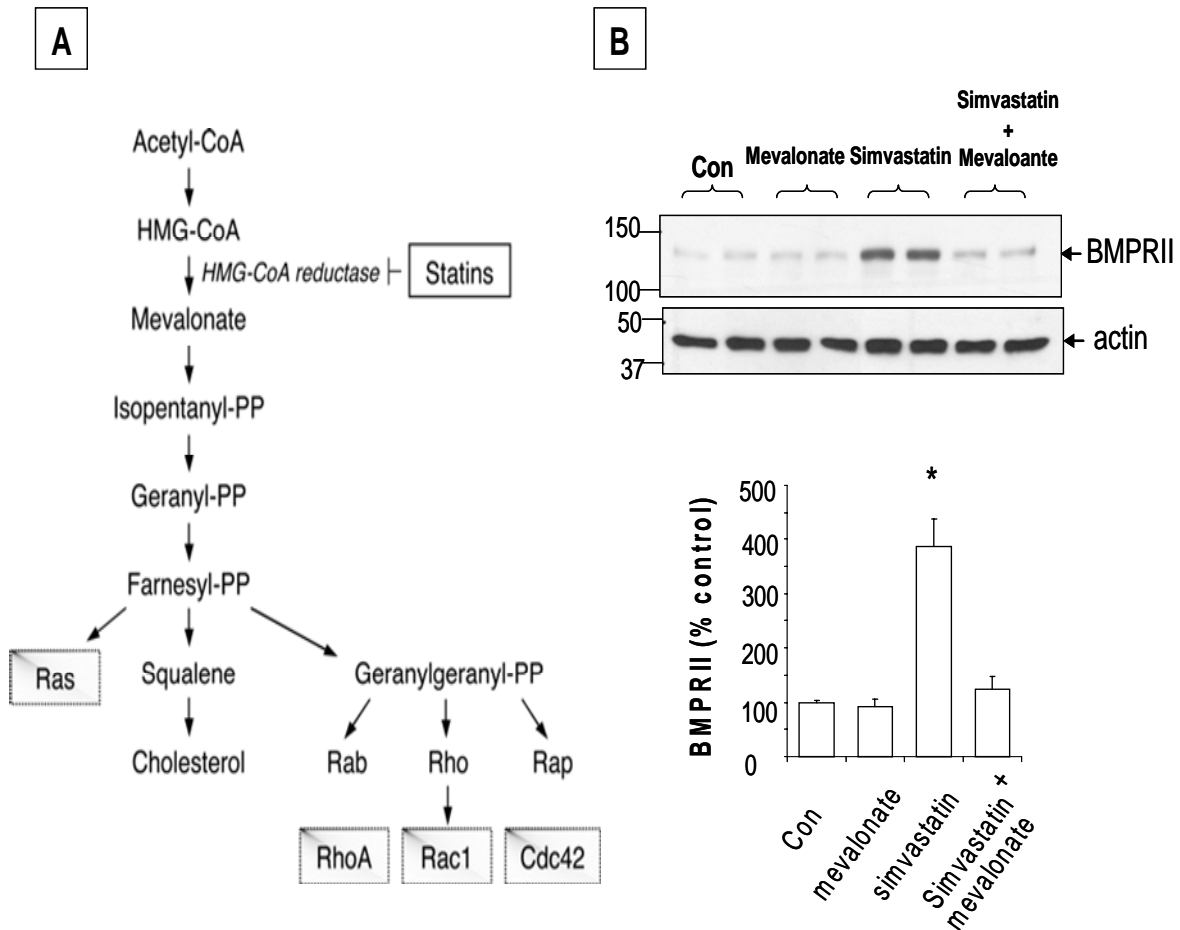
Figure 4.4. Mevalonate blocks simvastatin-induced BMPRII overexpression. (A) A diagram of cholesterol synthesis pathway. (**B**) Confluent HUVECs were treated with 10μM simvastatin with or without mevalonate for 24 hrs. Cell lyasates were collected and then immunoblot analysis was performed (*, p<0.05, n=4).

BMPRII knockdown attenuates LS-induced eNOS phosphorylation.
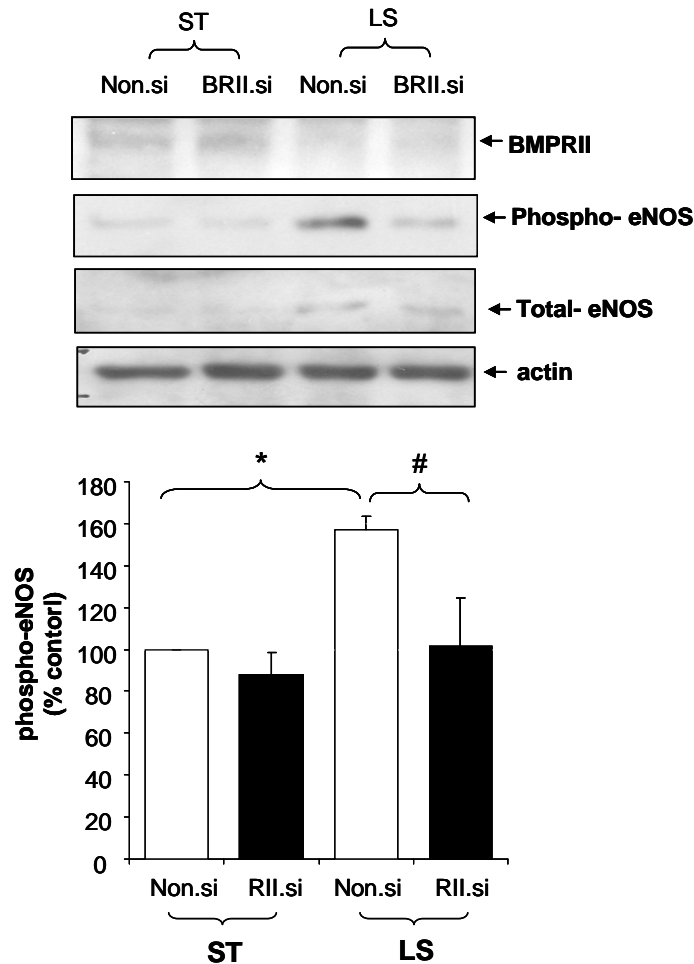
Figure 4.5. **BMPRII knockdown attenuates LS-induced eNOS phosphorylation.** HUVECs were transfected with non-silencing siRNA or BMPRII siRNA (50nM) for two days, and then exposed to uni-directional laminar shear for 24hrs. Cell lyasates were collected and then immunoblot analysis was performed (*, p<0.05, n=3).

Previously, it has been shown that statins increase eNOS expression and activity[16,17]. From our statin studies, we showed that statins increase BMPRII expression. Therefore, we speculated that BMPRII expression may affect eNOS expression. When we knocked down BMPRII, there was no effect on eNOS expression under basal

conditions. However, BMPRII siRNA-transfected cells exposed to LS showed less phosphorylated eNOS expression compared to Non-silencing controls (Fig 4.5). This data suggests that in PPH patients, vascular dysfunction might be due to decreased eNOS activity induced by decreased expression of BMPRII.

Discussion

The major findings reported in this study are that a) BMPRII expression is progressively lost as athermanous lesions advance in human coronary arteries, b) the cytokines TNFα decrease BMPRII expression in HUVECs, c) statins increase BMPRII expression through a HMG-coA reductase dependent pathway, and d) knockdown of BMPRII decreases eNOS phosphorylation by LS. Collectively, these findings show that BMPRII protein level is regulated in endothelium and may be negatively correlated with atherosclerotic lesion development.

Downregulation of BMPRII has been shown in familial and sporadic primary pulmonary hypertension as well as some types of cancers such as prostate and bladder cancers. However, it has not been shown that atherosclerotic plaques also lose BMPRII expression. It is highly possible that a proliferative cell state might be responsible for down regulation of BMPRII expression because proliferation is one of the phenotypes that is common among three areas of research-pulmonary hypertension, cancer and atherosclerosis. However, here, we are suggesting that inflammatory cytokines also decrease BMPRII expression in endothelial cells.

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We have shown here that pro-inflammatory cytokines, TNFα, decreased BMPRII expression in HUVECs (Fig. 5.2). Several reports of BMPRII downregulation have been reviewed, especially in pulmonary hypertension and cancer research[2,3,4, 9-11]. In this study, we also showed that atherosclerotic conditions downregulate BMPRII expression using human coronary arteries with various atheroma developments (Fig. 4.1).

Atherosclerosis is a long term disease process which involves risk factors such as hypertension, age, smoking, and hyperlipidemia. Also, other risk factors that could contribute to a further understanding of vascular pathology include markers of inflammation and growth factors. In this study, we showed the effects of only TNFα effects on BMPRII expression; however, for future studies, we may need to determine the effects of various cytokines such as interleukin family members (IL-2, IL4, IL-6, IL-8, IL-10, IL-1α, IL-1β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), interferon-γ (IFNγ), and monocyte chemotactic protein-1 (MCP-1). We also may need to ask how important other risk factors, such as hyperlipidimia and hypertension, are in regulating BMPRII expression as well as to what extent BMPRII contributes in a protective role during atherogenesis.

Interestingly, in our lab, we have shown that BMP4 levels increase with the progression of atherosclerosis in human coronary arteries[18]. In those studies, ECs in the lesser curvature exposed to disturbed flow, but not those in the greater curvature and straight arterial regions exposed to undisturbed flow, showed coexpression of BMP4 and BMP antagonists. Similarly, in human coronary arteries, expression of BMP4 and BMP

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antagonists in ECs positively correlated with the severity of atherosclerosis. San Martin et al. showed that in db/db mice (Type 2 diabetes model) BMP4 levels increased with the progression of diabetes, and after 8 week of treatment with the superoxide scavenger Tempol, 12-week old db/db mice had lower BMP4 protein expression when compared to non-treated mice[19].

On the other hand, Grainger et al. showed that TGF-ß, which is included in the same family member as BMP4, decreases with the development of atherosclerosis. They postulated that TGF-ß played an important role in maintaining normal vessel wall structure and that loss of this protective effect contributed to the development of atherosclerosis[20]. Kulkarni et al. generated TGF-β knock-out mice and showed a widespread inflammatory reaction in multiple organs causing cardiopulmonary death at 3-4 weeks of age due to profound pathology in heart and lungs[21]. However, Bobik et al. suggested contradictory findings, that TGF-ß is most active in fatty atherosclerotic lesions of the human aorta, showing properties of proatherogenic cytokine, promoting the retention of lipoprotein[22].

In this study, we have not shown that BMPRII has a protective effect on atherogenesis. However, we did show that statins increase expression of BMPRII in HUVECs (Fig. 4.3). For future studies, we will need to focus on the protective effect of BMPRII by using other drugs which may be regulate over expression of BMPRII as well as mouse models where BMPRII is overexpression in both endothelial cells and smooth

muscle cells. However, to our knowledge, there are no BMPRII overexpressing mice models.

The mechanisms of statins' effects on eNOS expression and activity have been studied. Because statins inhibit an early step in the cholesterol biosynthetic pathway, they also inhibit the synthesis of isoprenoids such as farnesylpyrophosphate and geranylgeranylpyrophosphate, which are important posttranslational lipid attachments for intracellular signaling molecules such as the Rho GTPases. Indeed, a decrease in Rho GTPase responses as a consequence of statin treatment increases the production and bioavailability of endothelium-derived NO. The mechanism involves, in part, Rho/Rho-kinase (ROCK)-mediated changes in the actin cytoskeleton, which leads to decreases in eNOS mRNA stability. The regulation of eNOS by Rho GTPases, therefore, may be an important mechanism underlying the cardiovascular protective effect of statins[23]. Laufs et al. showed that Rho negatively regulates eNOS expression and statins up-regulate eNOS expression by blocking Rho geranylgeranylation, which is necessary for its membrane-associated activity[24].

As such, the Rho/ROCK pathway has gained important prominence as a promising therapeutic target in cardiovascular diseases. Here, we showed that statins increase BMPRII expression and also BMPRII knockdown decreased, LS-induced eNOS phosphorylation compared to non-silencing siRNA controls. This indicates that BMPRII might have a protective effect through eNOS. Statin treatment may increase eNOS activity through a BMPRII/Rho/ROCK dependent pathway. However, our preliminary

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data shows that statins alone increase adhesion molecules, including ICAM-1 and VCAM-1. This area of study seems to be controversial because several groups have shown that statin treatment increases adhesion molecules and inflammatory responses[25-27], but many groups have also shown that statins actually protect from inflammation by cytokine treatment with TNFa or high glucose[28,29]. We will need more thorough studies to determine the mechanism of statin's effects on BMPRII.

In summary, we show that BMPRII levels decrease in atherosclerotic ECs and that pro-atherogenic cytokine, such as TNFα decrease BMPRII expression. On the other hand, statin treatment increased BMPRII expression, and BMPRII knockdown decreased eNOS phosphorylation by LS indicating that statin might have effects on eNOS through BMPRII. Therefore, BMPRII expression in EC's could be used as a novel biomarker of atherosclerosis and also BMPRII overexpression may have a protective role during atherogenesis, which is a subject that needs more thorough investigation.

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Chapter 5

In vivo mouse model for BMPRII regulation and atherosclerosis

Introduction

Atherosclerotic cardiovascular disease is the main cause of death in the Western world. Atherosclerosis is a chronic inflammatory disease, which develops slowly, resulting in cumulative atheromatous plaques. These plaque formations usually begin in childhood, well before the age of 10, and progress over time. Autopsies of healthy young men who died during the Korean and Vietnam Wars already showed coronary disease[1]. Endothelial dysfunction causes leukocytes and monocytes to adhere and infiltrate into the intima, under the endothelium, to develop atherosclerotic lesions, filled with excess lipids, smooth muscle cells, and collagen deposition. As the plaques grow and narrow the lumen, blood flow in the arteries is hindered. If they rupture and travel to smaller arteries and block the blood flow, this can cause heart attack or stroke.

There have been studies investigating atherosclerotic cardiovascular disease in many animal models, including nonhuman primates, swine, dogs, rabbits, rats and mice. The ideal animal model of cardiovascular disease will mimic the human disease process metabolically and pathophysiologically, and will develop end-stage disease comparable to that in the human. Among these, pigs and monkeys are the best models resembling the human atherosclerotic process; however, the high cost and difficulties with handling the animals are hinderances for large amounts of research[2]. Therefore, the possibility of a murine (mouse) atherosclerosis model was suggested. Generally, mice are highly

resistant to atherosclerosis because they normally have a high percentage of high density lipoprotein (HDL), carrying most of the plasma cholesterol on HDL; in comparison, humans carry about 75% of their plasma cholesterol on low density lipoprotein (LDL). Early atherosclerotic mouse models were limited to C57BL/6 mice with special diets of 30% fat, 5% cholesterol, and 2% cholic acid in Wissler's laboratory. Later Paigen et al. modified this to the "Paigen diet", consisting of 15% fat, 1.25% cholesterol, and 0.5% cholic acid. Paigen et al. screened ten inbred strains of mice for their ability to form fatty streaks and C57BL/6 mice reached a fatty streak stage at the aortic root within 14 weeks to 9 months on this diet[3]. However, the lesions from these mice consist of macrophage foam cells without any proliferation of smooth muscle cells into the intima, also, and atherogenic diet is necessary to induce atherosclerosis. There have been several genetically engineered atherosclerotic mouse models developed, including ApoE knock out, LDL receptor knock out, and ApoE *3Leiden mice. In 1992, Piedrahita et al. and Plump et al. developed ApoE knock out mouse[4,5]. ApoE is a structural component of all lipoprotein particles which coats them, functioning as a ligand for receptor-mediated uptake of chylomicron and VLDL remnants, as well as apoE-rich HDL. Jawien et al. showed that in ApoE knock out mice, the lipid profile was shifted from high HDL and low LDL contents to mostly vLDL[6]. As a result, total plasma cholesterol level in ApoE knock out mice increase 5-fold compared to normal mice. Triglycerides increase by about 2-fold, as shown in table 5.1.

Table 5.1. Lipid profile from ApoE knock mice with chow diet. Taken from reference 6.

Animals	Total cholesterol in mg/dl ± SD	HDL cholesterol In mg/dl ± SD	Triglyceride In mg/dl ± SD
Normal	86 ± 20	73 ± 28	73 ± 36
Heterozygous	88 ± 22	75 ± 18	102 ± 40
Homozygous	434 ± 129	33 ± 15	123 ± 51

Later on, Dansky et al. showed that according to genetic background, even among ApoE knock out mice, mice respond differently to atherosclerosis development[7]. They compared FVB/NJ and C57BL/6J backgrounds of ApoE knock out mice. Although FVB/NJ background mice had higher cholesterol levels than C57BL/6J background mice, the atherosclerotic lesion areas were less in FVB/NJ mice compared to C57BL/6J at 16 weeks of age. The advantage of ApoE knock out mice is that they develop atherosclerosis without a high-cholesterol diet. As shown in Figure 5.1, at 3 months of age, ApoE knock out mice start to develop macrophage accumulation in the aortic sinus (Fig 5.1.b) compared to the ApoE +/+ control litter mates (Fig 5.2.c) which did not develop any signs of atherosclerosis at 6 months. At 10 months of age, ApoE -/- mice develop plaques in the ascending aorta and carotid arteries as well in the aortic sinus (Fig 5.2 d, e, and f).

a) Normal, aortic sinus, 6 month

b) Apo E KO, aortic sinus, 3 month

c) Apo E KO, aortic sinus, 5 month

d) Apo E KO, aortic sinus, 10 month

e) Apo E KO, ascending aorta, 10 month
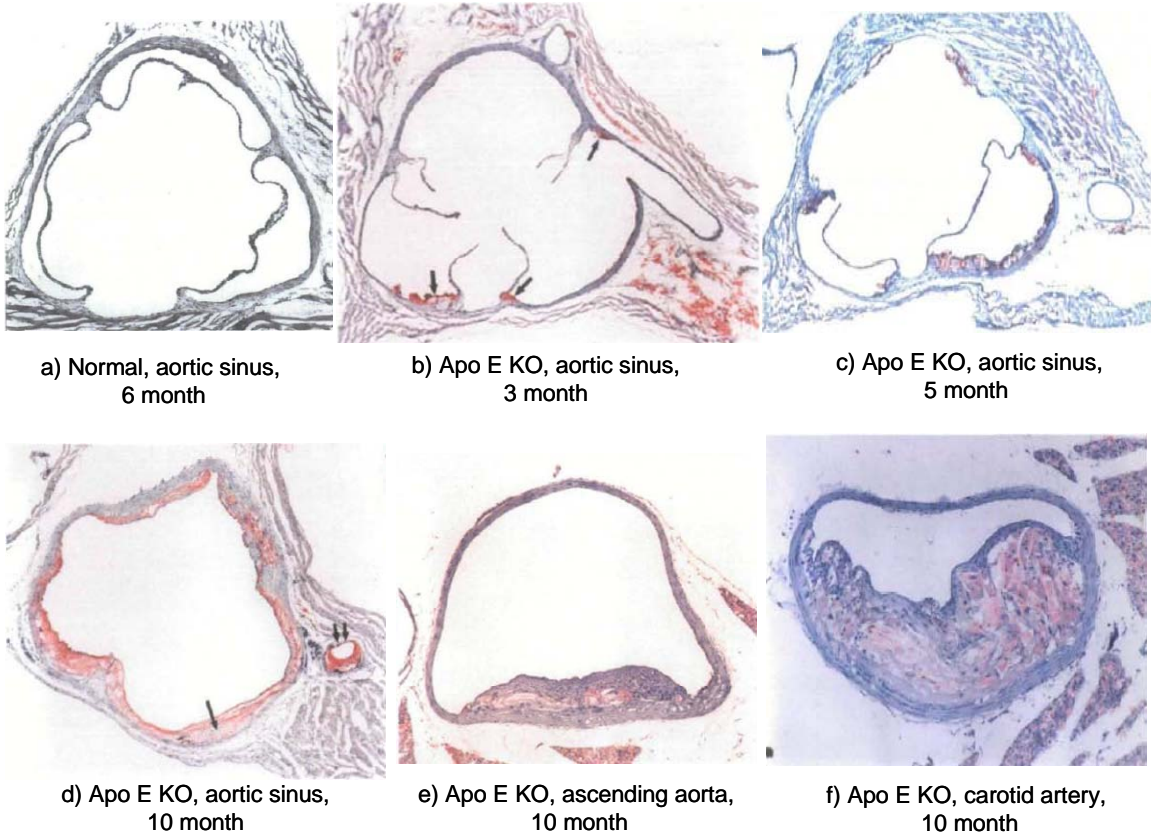
f) Apo E KO, carotid artery, 10 month

Figure 5.1. Lipid staining of aortic sinus, ascending aorta, and carotid artery sections from various ages of ApoE knock out mice on chow diet. This figure is reconstructed from Reddick et al.' paper[8].
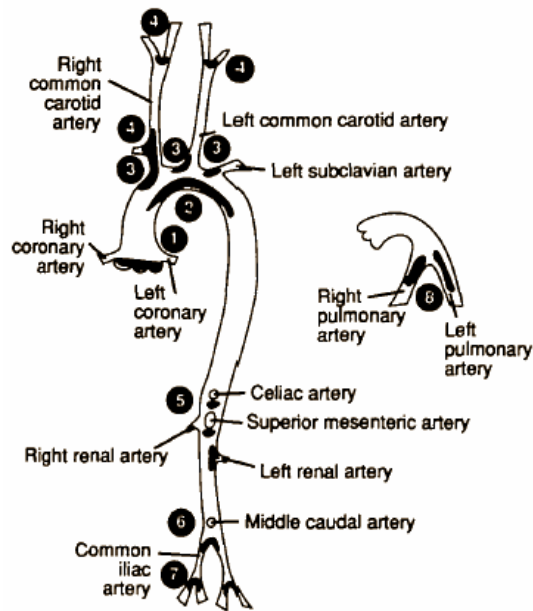
Figure 5.2. Sites of predilection for lesion development are indicated in black: 1) aortic root, at the base of the valves; 2) lesser curvature of the aortic arch; 3) principal branches of the thoracic aorta; 4) carotid artery; 5) principal branches of the abdominal aorta; 6) aortic bifurcation; 7) iliac artery; and 8) pulmonary arteries. This is taken from Nakashima et.al[9].

Figure 5.2 shows areas of atherosclerotic lesion development throughout the vasculature in mice. Similar to human atherosclerotic plaque development, mice develop lesions preferentially in disturbed flow areas such as the aortic sinus, curvature, and bifurcation. If ApoE knock out mice are fed a high-fat atherogenic diet, plaque development can be accelerated by about two to five weeks, as shown in Figure 5.3.
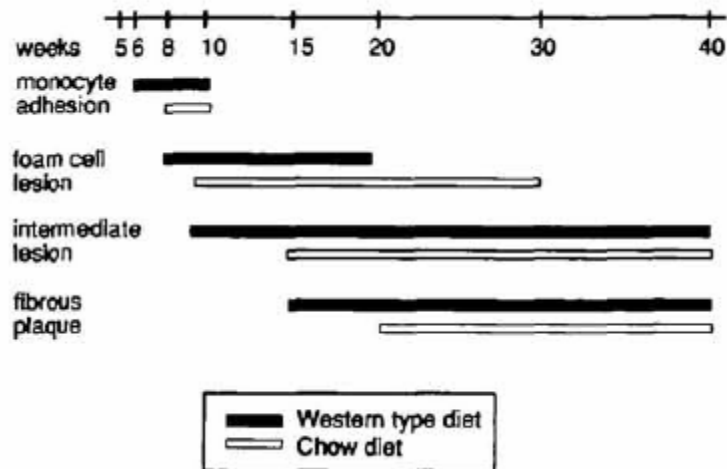
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Figure 5.3. Diagram showing how lesion formation in chow-fed mice is delayed in comparison with mice fed a Western-type diet. The diagram shows the time periods over which indicated lesion types were observed. This diagram is taken from Nakashima et.al[9].

The LDL receptor knock out mouse is a model of human familial hypercholesterolemia developed by Ishibashi et al. in 1993. LDL receptor knock out mice do not get atherosclerosis with chow diet; however, extensive hypercholesterolemia and lesion formation can be induced by high-fat diet whereas ApoE knock out mice are characterized by spontaneous hyperlipidemia and atherosclerosis.

Apo E *3Leiden mice carry mutant forms of human apoE. ApoE *3Leiden is a tandem duplication of codons 120 through 126 in the apoE gene, and the presence of a single allele for this mutation results in the expression of familial dyslipoproteinemia. Dysfunctional apoE Leiden has high affinity over wild-type apoE for triglyceride-rich lipoproteins. ApoE*3 Leiden mice are less susceptible to lesion development than ApoE KO mice, which develop lesions even on a normal chow diet. ApoE*3 Leiden mice have the ability to synthesize functional endogenous apoE.

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Even though these mice follow the human atherosclerotic disease process to some extent, mouse models do not develop the same end-stage ischemic heart lesions, including occlusive coronary artery disease, myocardial infarction, cardiac dysfunction, and premature death. Atherosclerotic lesions can be categorized in several types; Type I are initial lesions with isolated macrophage foam cells, Type II have fatty streaks with mainly intracellular lipid accumulation, Type III are intermediate lesions with type II changes and small extracellular lipid pools, Type IV are atheromas with Type II changes and a core of extracellular lipid, Type Va are fibroatheromata with lipid cores and fibrotic layers, Type Vb are mainly calcific Vc: fibrotic fibroatheromata, and Type VI have plaque rupture, thrombus formation and hemorrhage[10]. Also, it has been controversial as to whether mice develop plaque rupture of Type VI lesions. Schwartz et al. recently published a very extensive review on plaque rupture in mice[11].

To overcome these disadvantages in a diet-induced genetically engineered mouse model and to develop a model which mimics human atherosclerotic plaques, there have been several papers published using mechanical intervention in the arteries. Vascular remodeling, neointima formation, and atherosclerosis have been studied in mice whose arteries are injured from angioplasty, carotid artery ligation, or cuff placement. Leidenfrost et al. showed that temporary ligation yielded a significant luminal narrowing and the lesion was more advanced, with inclusion of foam cells, cholesterol clefts, necrotic cores, and fibrous caps, than the *de novo* plaques produced by diet alone[12]. In addition, this approach generated a significant plaque in 3 weeks compared to 6 to 12 months needed with high-fat diet alone. When the high fat diet was stopped after

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angioplasty, the vessels generated a densely cellular, hyperplastic lesion analogous to the neointimal thickening seen in human restenosis, but with high fat diet, intimal lesions resemble fatty streaks and not hypercellulear, fibrotic intimal lesions seen with restenosis in humans. Primary atherosclerotic intima shows less smooth muscle cells and more macrophage-derived foam cells. Also, Chen at al. showed that shear stress is responsible for composition of plaques[13]. They used a cast to change shear stress levels in the mouse carotid artery, and showed that oscillatory shear stress makes more lipids and macrophage-type plaques, while low shear stress makes more smooth muscle cells and collagen-type plaques.

These mechanically induced injury models also give us very useful tools to study the effects of atherogenic factors on disease progression and also to test candidate hypolipidemic and antiatherosclerotic drugs. Because of the intrinsic differences in anatomy and physiology between the human and mouse, it seems necessary to use several model systems to study the atherosclerotic disease process.

The importance of BMPRII in pulmonary hypertension has recently been supported by transgenic studies. BMPRII knock out mice are embryonic lethal; therefore, Beppu et al. developed a heterozygote BMPRII mice model [14,15]. Song et al. and Long et al. showed that these mice have increased susceptibility to pulmonary hypertension in response to either serotonin or inflammation induced by lipoxygenase adenovirus delivery, respectively [16,17]. Also, West el al. developed a tetracycline-responsive, smooth muscle-specific dominant-negative BMPRII mouse[18]. When these mice were fed doxycycline, they developed pulmonary hypertension. Later, from the same group, Tada

et al, performed a gene array analysis of whole lung mRNA from mice with the transgene activated for 1 or 8 weeks compared to non-activated mice [19]. Their major findings were that in these lungs, they lose markers of smooth muscle differentiation but increase inflammatory gene expression. In addition, Reynolds et al, performed BMPRII adenoviral gene therapy in hypoxia- induced pulmonary hypertension rat models, and showed that up-regulation of BMPRII could reduce pulmonary hypertension but could not completely reverse it to a normal state[20].

In this study, we have decided to expand our findings from *in vitro* cell culture and human atherosclerosis studies to *in vivo* mouse model studies. For the first part of the study, we hypothesized that hyperlipidimic conditions in Apo E knock out mice or vascular injury induced by carotid artery ligation might be responsible for decreased expression of BMPRII in endothelial cells. For the second part of the study, we obtained BMPRII heterzygote mice from Dr. Beppu at Harvard University to test whether decreased expression of BMPRII is responsible for inflammation and acceleration of atherosclerosis.

Method

Animal studies for diet induced atherosclerosis model: Male Apolipoprotein E–null (ApoE$^{-/-}$) mice from Jackson Laboratory (Bar Harbor, Me) were purchased at the age of 6 weeks. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Emory University. Mice were fed Paigen's atherogenic diet from

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Research Diets (New Brunswick, NJ) for 2 weeks, 4 weeks, 8 weeks and 6 months. The animals were humanely killed by CO_2 inhalation at the end of treatment.

Partial carotid ligation model: 6 weeks old female ApoE knock out mice were purchased from Jackson Laboratory (Bar Harbor, Me). On the day of surgery, anesthesia was induced by using a Ketamine/Xylazine mixture injected intraperitoneally. After hair removal from the neck of each mouse, one-inch mid-line incision was made from the level of the larynx down to the clavicle. A blunt dissection was done just left of the trachea, with resultant exposure of the left common carotid and its caudal divisions. The left internal carotid, external carotid and occipital arteries were ligated with 6-0 silk suture. For matching sham surgery controls, ligation was not done, but blunt dissection was performed as noted above. The skin was closed with 6-0 silk suture using simple, interrupted stitches. A Western diet was started on the day of surgery to accelerate atherosclerosis. Mouse carotid arteries in OCT blocks were then collected at different time points for analysis.

Immunohistochemistry: Frozen sections of mouse aortas and carotid arteries (10 μm) were fixed in ice-cold acetone for 5 min, blocked for 1 hour with 10% donkey or goat serum, and incubated with primary antibodies overnight at 4°C, followed by rhodamine-conjugated secondary antibodies (anti-goat IgG or anti-rat IgG) for 2 hours at room temperature as described [21]. Nuclei were counter-stained with Hoechst 33258 (Sigma). Primary antibodies used were specific for BMPRII (1:50, Santa Cruz) or PECAM-1 (1:50, BD). Microscopic images were taken using a Zeiss epi-fluorescence microscope. The

semi-quantification method was used to grade BMPRII staining intensity in endothelium using one to five scales (1: low staining intensity, 5: high staining intensity).

Results

High-fat diet increases total cholesterol, LDL, and VLDL, but decreases HDL and triglycerides in ApoE knock out mice.

From the high-fat diet induced atherosclerosis mouse studies using ApoE knock out mice, we collected blood samples at the time of sacrifice. The average serum cholesterol at baseline with chow diet was between 600 and 1000 mg/dL, with no significant differences between groups. As expected, high-fat diet had a dramatic increase in total serum cholesterol between 1400 and 2600 mg/ml. As shown in Figure 6.4, total cholesterol levels went down over time, peaking at 2 weeks in high-fat diet mice. Since we analyzed plasma samples on a different date, it is necessary to confirm this data. Interestingly, LDL and vLDL levels also decreased with time with high-fat diet, while HDL levels increased. Unexpectedly, triglyceride levels in chow-diet mice were higher than those in the high-fat diet group. We do not know whether this is a specific effect of the diet we used. However, this data clearly shows that high-fat diet induced hyperlipidemia in Apo E knock out mice.

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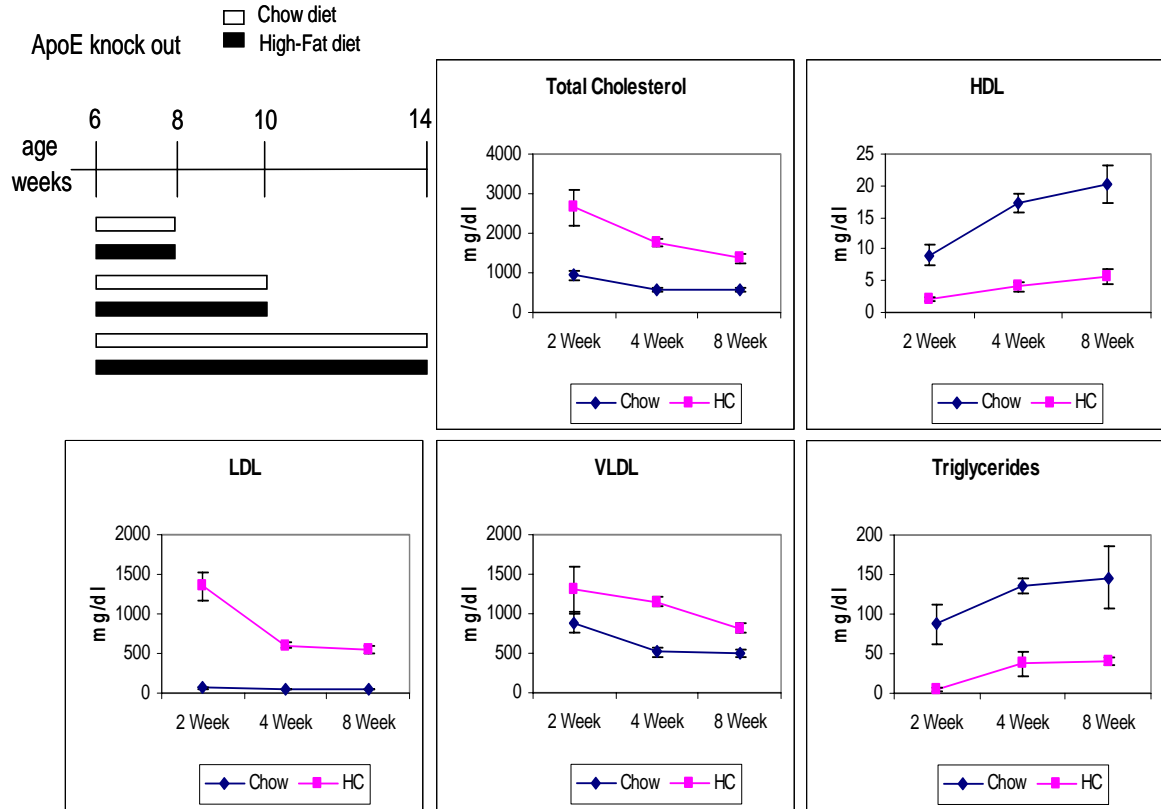
Figure 5.4. Total cholesterol, LDL, and VLDL levels increased in high fat diet ApoE knock out mice compared to chow diet, while HDL decreased. Serum lipid profiles, including total serum cholesterol, HDL, LDL, vLDL, and triglycerides, were measured at 2 weeks, 4 weeks and 8 weeks for chow-diet or high-fat diet ApoE knock out mice. Three to five representative samples were analyzed from each group (Chow: Chow diet, HC: High-fat diet)

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BMPRII expression decreases in aortas with aging of ApoE knock out mice.

Figure 5.5. **BMPRII expression decreases in aortas with age in ApoE knock out mice**. Aortas from ApoE knock out mice with chow or high fat diet at 2 weeks, 4 weeks, or 8 weeks were stained with BMPRII (1:50, Santa Cruz) antibody. These are 40x pictures taken with a Zeiss epi-fluorescence microscope. Arrows indicate endothelial cells, and L stands for the vessel lumen. Aortas are from ApoE knock out mice with (a) 2 weeks of chow diet, (b) 2 weeks of high fat diet, (c) 4 weeks of chow diet, (d) 4 weeks of high-fat diet, (e) 8 weeks of chow diet, and (f) 8 weeks of high-fat diet.
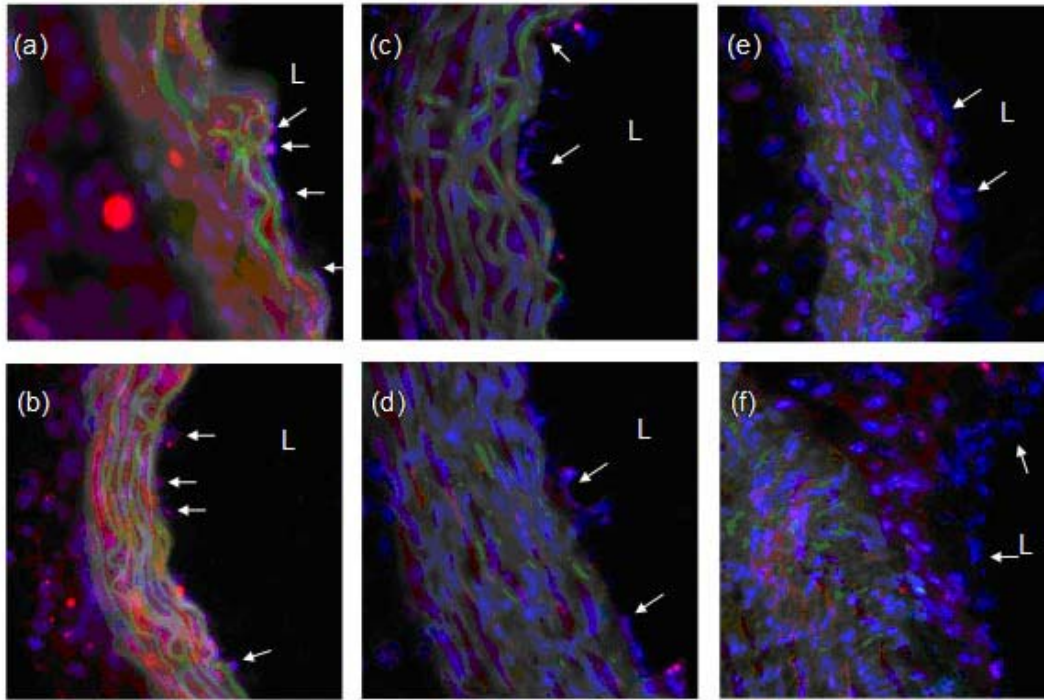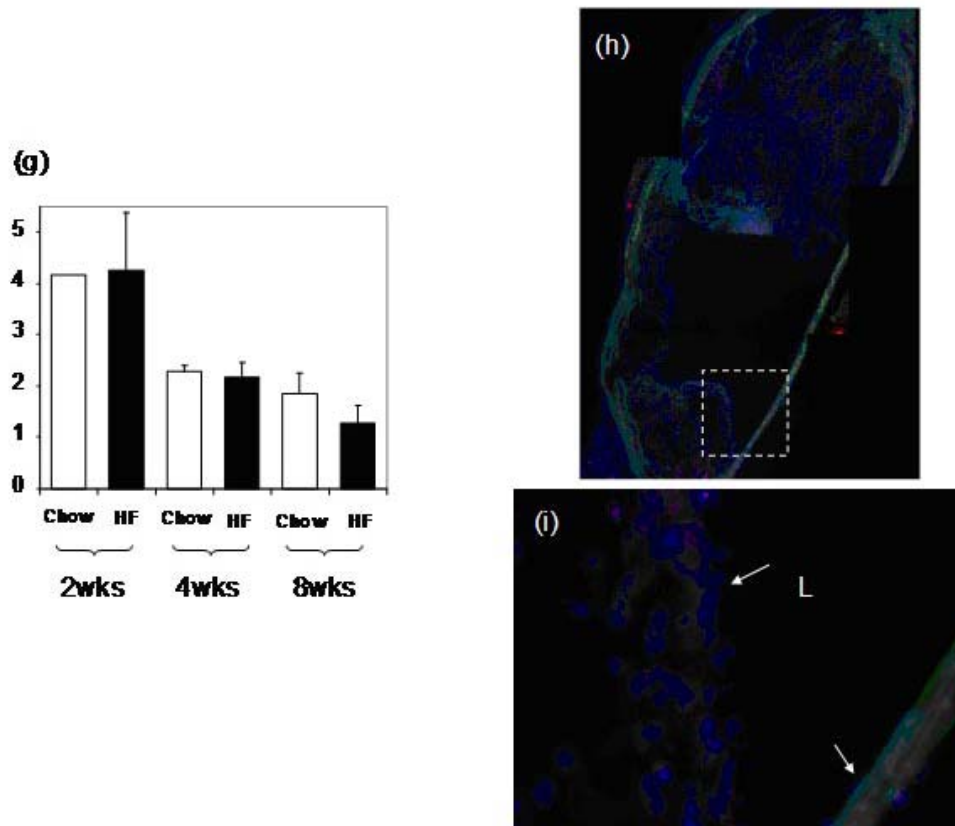
Figure 5.5. **(Continued) BMPRII expression decreases in aortas with age in ApoE knock out mice**. Aortas from ApoE knock out mice with chow or high fat diet at 2 weeks, 4 weeks, or 8 weeks were stained with BMPRII (1:50, Santa Cruz) antibody. These are 40x pictures taken with a Zeiss epi-fluorescence microscope. Arrows indicate endothelial cells, and L stands for the vessel lumen. (g) A total of 24 sections were stained and blindly graded. BMPRII expression: 1- low to 5- high. (h) Aortas from 1 year old ApoE knock out mice were stained with BMPRII. (i) Close up pictures from the box area in Figure 5.5 (h) taken at 40x magnification. Arrows indicate endothelium and L stands for vessel lumen.

Next, we examined BMPRII expression from the aortas of ApoE knock out mice on chow or high-fat diet. As shown in Figure 5.5, endothelial cells after 2 weeks of either chow or high fat diet expressed the highest levels of BMPRII, as indicated by pink staining (arrows in Figure 5.5 (a) and (b)). BMPRII expression decreased after 4 weeks or 8 weeks on chow or high fat diet, as shown in Figure 5.5 (c) through (f). A total of

twenty-four aorta sections for six conditions were graded blindly by three people. Quantification of BMPRII staining intensities in the luminal endothelium in various sections showed a decrease with respect to the age (Fig.5.5 (g) Graph). However, there was no difference between chow diet and high-fat diet. These results suggest that BMPRII expression decreases with age in ApoE knock out mice. As shown in Figure 5.5 (h) and (i), 1 year old ApoE knock out mice had an undetectable levels of BMPRII with advanced lesions. These data support our hypothesis that with atherosclerotic disease progression, BMPRII expression in endothelial cells is decreased.

Preliminary BMPRII staining from partial carotid artery ligation model

Carotid arteries from the partial ligating model were stained with BMPRII antibody to investigate protein expression levels. In this model, the left carotid artery was ligated, and the right artery was undamaged at the time of surgery. As shown in Figure 6.6, after 5 days of ligation, the right carotid artery was enlarged compared to the sham surgery. On the eighth day after surgery, the left carotid artery started to develop vascular remodeling and atherosclerosis. In addition, BMPRII expression was decreased in the left carotid artery compared to right side on the eighth day. These data are from one animal for each condition; therefore, we will need to confirm the results with more animals.
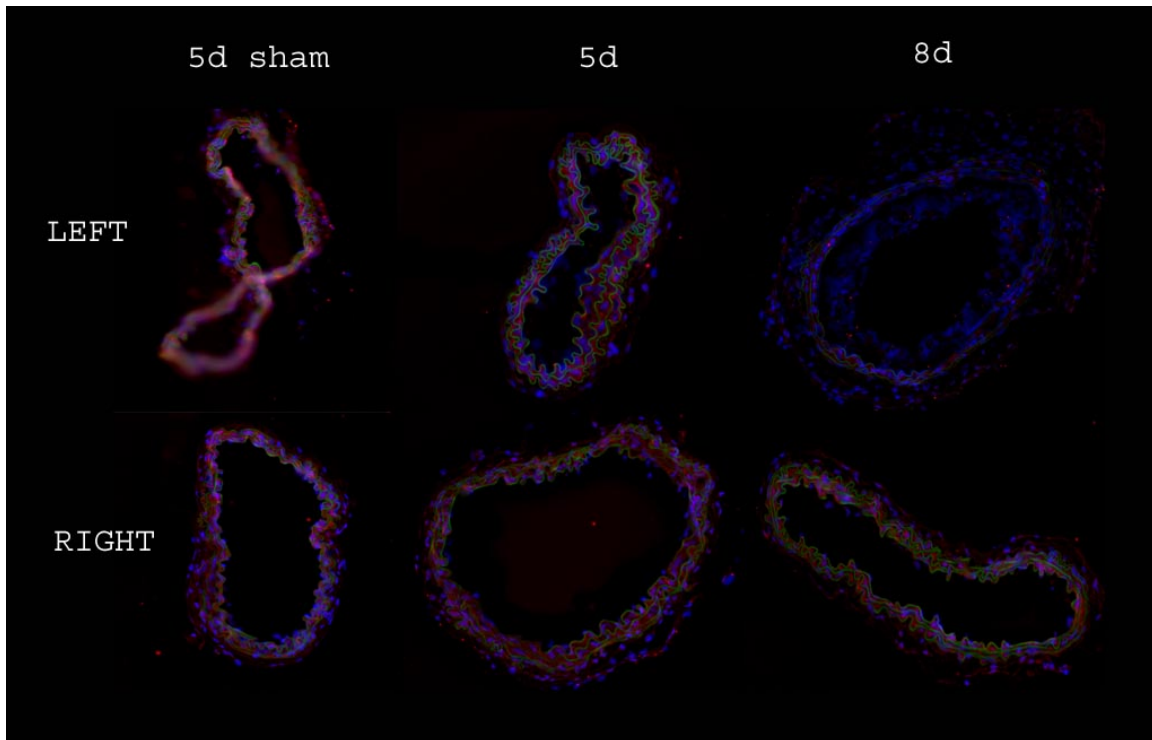
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Figure 5.6 Preliminary BMPRII stain from partial carotid artery ligation model. Carotid arteries 5 days and 8s day after sham and ligating surgery of ApoE knock out mice were stained with BMPRII (1:25, Santa Cruz) antibody. These are figures from 20x pictures taken with a Zeiss epi-fluorescence microscope.

Discussion

From these *in vivo* atherosclerotic mouse studies, we showed that a) ApoE knock out mice on high- fat diet develop exacerbated hyperlipidimic conditions after 2 weeks of diet compared to chow diet (Fig 5.1), b) foam cell development with age and high-fat diet appear to be responsible for decreased expression of BMPRII in endothelial cells in ApoE knock out mice (Fig 5.2), and c) a preliminary study from a carotid ligation model showed that with medial thickening and atherosclerosis development, BMPRII expression decreased in endothelial cells as well as in smooth muscle cells (Fig. 5.3).

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From our previous studies using human coronary arteries in chapter 4, we showed that BMPRII expression is lost in both smooth muscle cells and endothelial cells with the advanced atheroma development. However, our *in vivo* ApoE knock out mouse studies showed that decreased BMPRII expression is more evident and faster in endothelial cells than smooth muscle cells. Although this is a very preliminary study, our carotid ligation model seems to give us similar results to human coronary arteries. We think that any discrepancies may come from the differences inherent in the animal model.

These animal model approaches are trials to simulate the human atherosclerotic process. Though these studies give very important insights into how atherosclerosis develops, validation of certain findings in several animal models and human beings is necessary. Therefore, we have decided to also employ a partial carotid ligation model for atherosclerotic development in mice.

There are several *in vivo* mouse models that accelerate the atherosclerotic process through surgical intervention of blood flow.

Ligation of the carotid artery is used *in vivo* to block blood flow and, afterwards, to examine the response of vascular remodeling. In mice, sufficient blood flow still reaches the brain through other vessels so that the ligation is not fatal. This model of flow cessation causes the induction of proteases, intimal hyperplasia, and macrophage infiltration, all with an intact endothelium [22-25]. In addition, low flow models *in vivo* have been accomplished by partial ligation of the carotid arteries [26] or by ligation of internal carotid artery and three of the other branches of the external carotid artery [27]. Partial

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outflow occlusion models also lead to lower flow through the artery of interest [28]. Among these, we have started a partial carotid ligation model, and we have some preliminary data showing changes in BMPRII expression.

Moreover, for on going and future studies, we have begun to feed BMPR-II heterozygote mice and C57BL/6 control mice with high-fat diet for 8 weeks. We are planning to look at lipid profiles, en face Oil-Redo staining for overall evaluation of atherosclerosis, and macrophage infiltration and atherosclerosis in the aortic sinus. Also, we are planning to pursue partial carotid ligation surgeries to compare BMPR-II heterozygote mice and C57BL/6 control mice in terms of atherosclerotic progression. In addition, we are breeding BMPRII heterozygote mice and ApoE knock out mice to accelerate atherosclerosis in our model. With these mice, we will look at atherosclerosis as well as pulmonary hypertension in collaboration with Dr. Beppu at Harvard School of Medicine.

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Chapter 6

Discussion and Future Studies

The novel findings of this study are that, a) OS increases nox1 and nox2 mRNA expression while decreasing nox4, b) BMP4 increases only nox1 mRNA level, c) knocking down nox1 blocks OS-induced monocyte adhesion, d) knocking down BMP4 inhibits OS-induced ROS production and prevents monocyte adhesion, e) aortic ECs express ALK2, ALK6 and BMPRII, and f) knockdown of BMPRII blocks BMP4-dependent smad1/5/8 phosphorylation and monocyte adhesion. Unexpectedly, we found that g) BMPRII knockdown in MAEC and HUVEC unleashes an inflammatory response in the absence of BMP4 and that h) BMPRII expression is progressively lost as atheromatous lesions advanced in human coronary arteries. In addition, i) cytokines, TNFα and LPS decrease BMPRII expression in HUVECs, j) statins increase BMPRII expression through an HMG-CoA reductase-dependent pathway, and k) knockdown of BMPRII decreases eNOS phosphorylation by LS.

Collectively, these novel findings strongly suggest that BMP4, produced in endothelial cells in response to OS, acts as a pro-inflammatory cytokine by stimulating ROS production in a nox1-dependent manner, eventually leading to a monocyte adhesion response. Also, we show that BMPRII mediates BMP4-dependent inflammation. More interestingly, BMPRII knockdown in ECs induces inflammation. We show for the first time that a decrease in BMPRII protein level in aortic endothelium is linked to inflammation and atherosclerosis. Therefore, BMPRII expression in EC's could be used as a novel biomarker of atherosclerosis. Furthermore, BMPRII overexpression might

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have a protective role during atherogenesis, a speculation that warrants more thorough investigation.

Atherosclerosis is now well known to be an inflammatory disease preferentially occurring in lesion-prone areas associated with unstable shear stress in branched or curved arteries. In addition, increased levels of ROS have been strongly implicated in atherosclerotic plaque development[1]. In our previous study[2], we found that monocyte adhesion by endothelial cells in response to OS was observed only after a chronic exposure (18 hrs or longer), but not by a shorter exposure (4 hrs), suggesting a requirement for *de novo* synthesis of proteins such as BMP4. Consistent with this idea, we have recently provided evidence that BMP4 induction, requiring several hours of OS exposure, is responsible for triggering the monocyte adhesion response in endothelial cells[3]. Furthermore, our current data, showing a significant increase in ROS production following 20 hrs of BMP4 treatment, may be due to an increase in nox1 mRNA level. Taken together, these results demonstrate a critical role for BMP4 in mediating OS-dependent ROS production and inflammatory responses.

Recently, heterozygous BMPRII mutations have been linked to familial and sporadic primary pulmonary hypertension (PPH) [4,5]. In addition, it has been reported that BMPRII expression is reduced in the lungs of primary pulmonary hypertension patients even when they do not have any detectable mutations of the receptor [6]. PPH is characterized by obliteration of pre-capillary pulmonary arteries, leading to sustained elevation of pulmonary arterial pressure and subsequent right ventricular hypertrophy. PPH is typically associated with abnormal proliferation of endothelial and smooth muscle cells within the small pulmonary arteries [7]. Interestingly, PPH is also strongly associated with inflammation, as evidenced by accumulation of inflammatory cells infiltrating into

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plexiform lesions in the lungs and increased inflammatory cytokines in the circulation of PPH patients [8]. Homozygotic deletion of BMPRII in mice causes an embryonic lethal phenotype [9], while heterozygotic BMPRII$^{+/-}$ mice show increased susceptibility to pulmonary hypertension in response to an inflammatory stress [10]. These results provide a link between BMPRII mutation or haplo-insufficiency and PPH as well as an association between inflammation and PPH. However, there is no published literature showing that BMPRII mutation or decreased BMPRII expression causes inflammation and atherosclerosis.

The effects of BMPRII and BMPs seem to be cell type- and vascular bed- specific. Recently, BMPRII knockdown by siRNA was shown to increase apoptosis in human pulmonary aortic ECs [11]. On the other hand, BMPRII knockdown in human pulmonary aortic smooth muscle cells (PASMC) inhibited apoptosis induced by BMP4/7 by mechanisms dependent on activation of the caspases-3, 8 and 9 [12]. However, these studies did not examine whether BMPRII knockdown induces inflammation. Whether BMPRII knockdown causes apoptosis has not been examined in the current study, but BMP4 itself showed no significant effect on cell proliferation or apoptosis in ECs (data not shown). Even within the same pulmonary arterial bed, smooth muscle cells from peripheral and proximal arteries respond differently. BMP4 treatment increased proliferation of peripheral smooth muscle cells, but it inhibited it in proximal smooth muscle cells of pulmonary arteries [13].

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The mechanism by which BMPRII knockdown stimulates ICAM-1 induction and subsequent monocyte adhesion in ECs is not known. However, we propose that the loss of BMPRII may unleash the signaling proteins that normally bind to the receptor, resulting in uncontrolled activation of inflammatory pathways. BMPRII is a protein (1,038 amino acids) with an extracellular BMP binding domain, a single transmembrane domain and a long cytoplasmic domain. The cytoplasmic domain (866 amino acids) contains a serine/threonine protein kinase domain followed by a C-terminal tail. Interestingly, this long cytoplasmic domain is unique to BMPRII and not found in other members of the TGFβ receptor superfamily. Moreover, the cytoplasmic domain has been shown to bind various signaling proteins, cytoskeletal components and metabolic enzymes [14]. Among notable ones are p50b NFκB protein, MAP3K8, LIM kinase-1 and c-Src protein tyrosine kinase [14-16]. For example, the C-terminal tail interacts with c-Src, and the association has been shown to downregulate the Src tyrosine kinase activity [16]. More interestingly, BMPRII mutations truncating the C-terminus, as found in PPH patients, increase c-Src activity and subsequent proliferation of smooth muscle cells [16]. Perhaps, BMPRII, much like caveolins [17], serves as a docking protein in normal unstimulated conditions, holding the signaling proteins in an inactive state. When BMPRII level is decreased or its C-terminal is mutated or truncated as in the cases of PPH patients, the normally sequestered signaling proteins may be released and activated. Similarly, when BMPRII is knocked down by siRNA or its level is decreased in ECs in advanced atherosclerotic lesions, the signaling molecules that are normally sequestered by the receptor may be released and activated, leading to a vicious cycle of ICAM-1 induction, monocyte adhesion, and eventual atherosclerosis development. In the continued presence

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of disturbed flow and other risk factors for atherosclerosis such as hypercholesterolemia, hypertension, diabetes and smoking, atherosclerotic lesions may progress and BMPRII expression level decreases, which can further exacerbate inflammation and atherosclerosis.

Downregulation of BMPRII has been shown in familial and sporadic primary pulmonary hypertension as well as in some types of cancers such as prostate and bladder cancers. However, it has not been shown that atherosclerotic plaques also lose BMPRII expression. It is very possible that a proliferative cell state may be responsible for down regulation of BMPRII expression because proliferation is one of the phenotypes that are in common among these three areas of research: pulmonary hypertension, cancer and atherosclerosis. However, we are suggesting that inflammatory cytokines also decrease BMPRII expression in endothelial cells. Here, we have shown that pro-inflammatory cytokines, TNFα and LPS, decrease BMPRII expression in HUVECs. Several reports of BMPRII down regulation have been reviewed, particularly in pulmonary hypertension and cancer research[2,6, 18, 19-21]. In this study, we also showed, using human coronary arteries with various atheroma developments, that atherosclerotic conditions downregulate BMPRII expression.

Taken together, we are suggesting a new hypothesis that disturbed flow begins inflammation, mediated by BMP4, which initiates foam cell formation. This increases secretion of pro-atherogenic factors and cytokines, and this, along with other atherogenic risk factors, including hypercholesterolemia, hypertension, and diabetes, is responsible

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for decreasing BMPRII expression. As we showed with BMPRII knockdown studies, decreased BMPRII expression induces secondary inflammation, which accelerates atherosclerosis. Figure 7.1 illustrates our overall hypothesis.

Based on our hypothesis, we still need to show that pro-atherogenic cytokines other than TNFα and LPS or atherogenic risk factors including hypercholesterolemia, hypertension, or diabetes, may be responsible for downregulation of BMPRII expression and accelerated atherosclerosis. Atherosclerosis is a long term disease process which involves risk factors such as hypertension, age, smoking, and hyperlipidemia. Also, other risk factors that could contribute to further understanding of vascular pathology include markers of inflammation and growth factors. In this study, we showed only the effects of TNFα and LPS on BMPRII expression; however, in future studies, we will need to determine the effect of various kinds of cytokines such as interlukin family members (IL-2, IL4, IL-6, IL-8, IL-10, IL-1α, IL-1β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), interferon-γ (IFNγ), and monocyte chemotactic protein-1 (MCP-1). Secondly, we will need to address how important other risk factors such as hyperlipidemia and hypertension are in regulating BMPRII expression as well as to what extent BMPRII contributes in the protective role during atherogenesis.
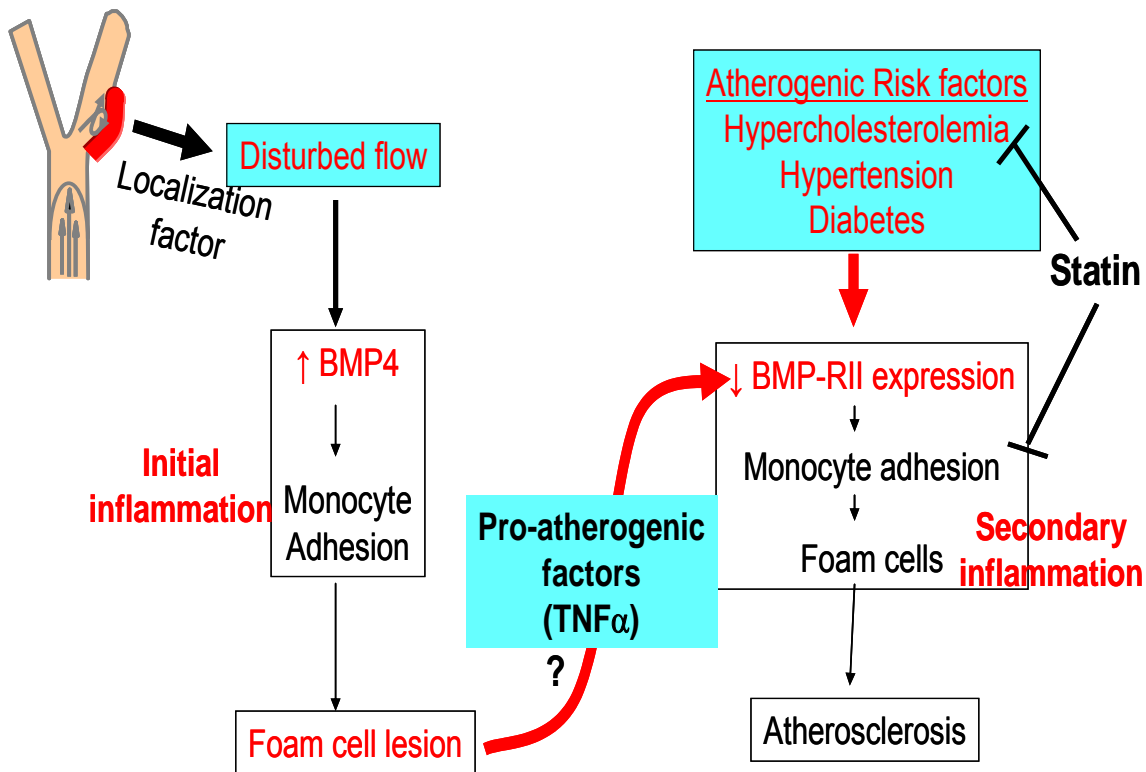
Figure 6.1. Overall hypothesis. Disturbed flow begins inflammation, mediated by BMP4, which initiates foam cell formation. This increases secretion of pro-atherogenic factors and cytokines, and this, along with other atherogenic risk factors, including hypercholesterolemia, hypertension, and diabetes, is responsible for decreasing BMPRII expression. As we showed with BMPRII knockdown studies, decreased BMPRII expression induces secondary inflammation, which accelerates atherosclerosis.

Furthermore, there may be a new mechanism involved in the downregulation of BMPRII. There has been increasing evidence that microRNAs play a crucial role in cell type development and chronic diseases. New knowledge about the functional role of microRNAs (miRNAs) is revolutionizing cell biology and will have a major impact on biomedical research and novel therapeutic opportunities. MiRNAs are a recently discovered class of endogenous, small, noncoding, single-stranded RNAs of 22 nucleotides that regulate about 30% of the encoding genes in the human genome. So far, about 400 miRNAs have been identified and as many as 1000 miRNAs are estimated.

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However, the role of miRNAs in vascular disease is currently completely unknown. To date, there has been only one paper regarding miRNA and atherosclerosis by Ji et. al[22]. They ran miRNA microarray analysis using rat miRNA array probes from a carotid artery balloon injury model. As we used siRNA to knock down BMPRII expression, it is possible that atherosclerotic conditions may increase miRNA and then downregulate BMPRII. We searched miRNA hits from human BMPRII sequence, and there were 12 miRNAs predicted to bind (http://microrna.sanger.ac.uk). Certainly, we need to accumulate more data on miRNA profiles in atherosclerosis, and we also need to verify which miRNA may be responsible for down regulation of BMPRII.

We are suggesting that one of the mechanisms by BMPRII knockdown induces inflammation is by releasing its binding proteins. Although there had been studies of the cytoplasmic domain of BMPRII that have shown it to bind various signaling proteins, cytoskeletal components and metabolic enzymes [14-16], there is no data on endogenous binding protein profiles specifically in endothelial cells. Therefore, we are planning to pursue proteomics studies after pulling down binding proteins of BMPRII.

Also, we have obtained global BMPRII heterozygote mice, and we are crossing these with ApoE knock out mice to study the development of atherosclerosis. We will use both a high-fat diet induced atherosclerosis model as well as a partial carotid artery ligation model for neointima formation. These animal models will give us more information about how BMPRII is protective for atherogenesis.

In conclusion, these studies support the hypothesis that oscillatory shear stress increases BMP4 expression which induces inflammation through a ROS-dependent pathway. Moreover, atherogenic conditions, such as pro-atherogenic cytokines and several risk factors, decrease BMPRII expression which further increases inflammation and atherosclerosis.

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