THE ADHESION MOLECULE PECAM-1 DIRECTS ENDOTHELIAL NITRIC OXIDE SYNTHASE ACTIVITY AND CARDIAC FUNCTION TO REGULATE CARDIOVASCULAR HOMEOSTASIS

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ABSTRACT

MARGARET ELIZABETH MCCORMICK: The adhesion molecule PECAM-1 directs endothelial nitric oxide synthase activity and cardiac function to regulate cardiovascular homeostasis
(Under the direction of Ellie Tzima)

The mammalian vascular system is integral to the regulation of homeostasis within an organism. This system is composed of a vast network of blood vessels that transport oxygen and nutrients throughout the body. Blood vessels are lined by endothelial cells (ECs) that provide an interface between circulating blood and the underlying tissue. Based on their unique location, it is unsurprising that ECs are master regulators of numerous physiological processes. ECs are equipped with receptors that interact with their surrounding environment. Among these molecules is Platelet Endothelial Cell Adhesion Molecule-1, or PECAM-1. Since its identification in the early 1990s, PECAM-1 has gained increasing attention for its multi-faceted roles in vascular biology. In this dissertation, I describe two novel roles for PECAM-1 which advance our understanding for how this receptor regulates EC signaling and function.

One of the most important cellular signaling molecules in the vasculature is nitric oxide (NO) generated by the enzyme endothelial nitric oxide synthase (eNOS). NO is required for vasodilation and maintenance of the endothelium. Reduced NO production can lead to endothelial dysfunction, the first step in a host of diseases, including atherosclerosis (hardening of the arteries). Previous studies have suggested a role for PECAM-1 in regulating eNOS activity and NO production. Here, I define a pathway by which PECAM-1

regulates the expression of a trafficking protein that influences eNOS localization and activity.

In the second half of my dissertation, I focus *in vivo* using wild type and PECAM-1$^{-/-}$ mice. Our lab has shown that PECAM-1-mediated signaling facilitates activation and expression of molecules that participate in cellular crosstalk. Furthermore, it is well documented that cell crosstalk is central to proper cardiac function. Therefore, we hypothesized that genetic deletion of PECAM-1 may have deleterious effects on heart function. In agreement with this hypothesis, we are the first group to report that PECAM-1$^{-/-}$ mice display dilated cardiomyopathy. Work described in this dissertation defines the mechanism leading to these defects in function. Taken together, the work presented in this thesis further expands the evolving roles of PECAM-1 in cardiovascular biology and provides novel insight to its role in regulating organ function.

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First and foremost I would like to thank my mentor, Ellie Tzima, for all of her guidance and support over the last 5 years. I came to UNC knowing that I wanted to work in her lab and never once have I regretted this decision. I immensely respect her opinion and scientific foresight, her pursuit of cutting edge science and ideas, and her fearless endeavor into new areas of research. She has pushed me to have confidence in my ideas and myself. When my thesis work took us into the cardiac field, she never once shied away from pursuing it. I hope to take what I've learned from Ellie and expand on it as I continue to mature as a scientist.

I would also like to thank everyone in the Tzima lab, past and present: Dan Sweet, Caitlin Collins, Chris Givens, Zhongming Chen, Young Greenberg, Meghan Childs, Sruthi Cherkur, Meredith Brown and Yunhao Liu. Their unwavering support over the last 5 years has meant the world; most of this would not have been possible without them. To Dan, Caitlin and Chris, I look forward to being colleagues and friends for many years to come. Dan, for loving Pennsylvania as much as I do, and challenging me daily in the best way possible to make my projects more complete. Caitlin for reading everything I've ever written and for hearing every thought I've had and still being my colleague and friend (she knows how hard that is…). Zhongming Chen, AKA the mouse man, TGIF. And Chris, you've got some big shoes to fill (insert ha!), but I'm confident you can and will make us all proud.

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without his flexibility and positive attitude, my work would have been far less successful and significantly less enjoyable. A big thanks to the physiology department. I joined this department in 2007 because it felt like a supportive home to spend 5 years of school and it has been. Adriana Tavernise, Vicki Morgan and the rest of the staff have made my life, both scientifically and otherwise, run incredibly smoothly.

It is hard to put into words what my friends at UNC and in Chapel Hill have meant to me. They have all contributed in ways big and small to my well-being as a graduate student. Gray Camp is my role model for a thoughtful, kind, brilliant person. I truly cannot imagine what life here would have been like without him. Lauren Strickland for being such a positive influence in my life and for showing me what North Carolina is all about – hollerin'. Katy Liu for her non-judgmental friendship and support and a fantastic roommate (Juj included!). And thank you to Anne-Marie Neiser for being up for anything and always having a smile on your face. Also, a special thank you to my F32 roommates (Morgan, Tricia, and Jill) for keeping me social when science threatened to overwhelm me.

And of course I want to thank my parents, Bob and Melinda. My parents brought my brothers and I up to observe, question and think about the world around us. They exposed us to new places and experiences and instilled in us the curiosity they had for the world and all things nature. I believe all of that combined to put me where I am today and for that I am eternally grateful. I would also like to thank my brothers, Rob and Brian, for their love and support and being above all else, my friends.

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PREFACE

The work presented in Chapter II work was previously published in *Arteriosclerosis, Thrombosis and Vascular Biology* in March 2011. My roles in this project included helping design and perform the experiments, data analysis and writing the manuscript. Reema Goel and Debra Newman performed the knockdown studies and performed the corresponding data analysis (Figure S2.1). David Fulton performed the *in vivo* NO assays (Figure 2.1). Ellie Tzima was the principal investigator and helped design experiments, analyze data and write the manuscript.

The citation for the manuscript is as follows: **McCormick ME,** Goel R, Fulton D, Newman D, Tzima E. Platelet-Endothelial Cell Adhesion Molecule-1 Regulates Endothelial NO Synthase Activity and Localization Through Signal Transducers and Activators of Transcription 3-dependent NOSTRIN Expression. *Arterioscler Throm Vasc Biol*.(2011); 3:643-649.

Work described in Chapter III has currently been submitted for publication. My roles in this project included helping design and perform experiments as well as data analysis and writing the manuscript. Gianluigi Pironti and Howard Rockman performed the isolated cardiomyocyte experiments (Table 3.1). Mauricio Rojas performed the conscious echos, pressure-volume loops and TAC surgeries. Monte Willis performed conscious echocardiography. Ellie Tzima was the principal investigator and helped design experiments, analyze data and write the manuscript.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANP	Atrial Natriuretic Peptide
αSka	α-skeletal actin
BAEC	Bovine Aortic Endothelial Cell
βMHC	β-Myosin Heavy Chain
BMP	Bone Morphogenic Protein
Ca^{2+}	Calcium
Cav1	Caveolin-1
cGMP	cyclic Guanosine Monophosphate
ChIP	Chromatin Immunoprecipitation
DCM	Dilated Cardiomyopathy
EC	Endothelial Cell
EF	Ejection Fraction
eNOS	endothelial Nitric Oxide Synthase
Erk1/2	Extracellular related kinase 1/2
FS	Fractional Shortening
FSS	Fluid Shear Stress
HUVEC	Human Umbilical Vein Endothelial Cell
ITAM	Immunoreceptor Tyrosine Activation Motif
ITIM	Immunoreceptor Tyrosine Inhibitory Motif
JAK	Janus Kinase
L-NAME	L-NG-Nitroarginine Methyl Ester
LV	Left Ventricle
MAPK	Mitogen Activated Protein Kinase
NF-κB	Nuclear Factor Kappa B

NRG1	Neuregulin-1
NO	Nitric Oxide
NOSIP	eNOS Interacting Protein
NOSTRIN	eNOS Traffic Inducer
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PKA	Protein Kinase A
PLN	Phospholamban
PV	Pressure-Volume
qPCR	quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SERCA2	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
STAT3/5	Signal Transducer and Activator of Transcription 3/5
TAC	Transverse Aortic Constriction
TEM	Transmission Electron Microscopy
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VSMC	Vascular Smooth Muscle Cell
WT	Wild Type

CHAPTER I.

Introduction

The vascular endothelium

The vascular system plays a vital role in regulating homeostasis. Defects in vascular function lead to the development of diseases such as atherosclerosis and coronary artery disease. In addition to transporting oxygen and nutrients, the vasculature is also fundamental for the delivery of important hormonal/neurohormonal signals that control organ system function. An illustration of this is the hormone vasopressin, which is released by the posterior pituitary and travels through the vasculature to the renal tubules to increased blood volume and ultimately blood pressure.

William Harvey first described the presence of circulating blood in the early 1600s. Shortly after this observation, it was recognized that blood is separated from tissue by a series of vessels [1]. It took over a century to realize that a thin layer of cells, termed endothelial cells (ECs), line the blood vessel wall. Despite this observation, as recently as the 1960s, ECs were thought of simply as "the cellophane wrapper" of the vasculature [2]. A tremendous amount of work done in the following decades revealed that ECs play a dynamic role in both physiology and pathology. ECs are found in every organ throughout the body and are specialized for their unique locations and varied function. For instance, ECs in

the brain have tight junctions and a thick basement membrane to prevent the mixing of blood with cerebrospinal fluid. Conversely, ECs of the glomerulus have specialized filtration slits to permit filtration of fluids and small molecules into the renal tubules.

The endothelium is lined with receptors and adhesion molecules that provide a key interface between ECs and the extracellular environment. These proteins also mediate intracellular signaling and facilitate complex interactions with neighboring cell types. EC input comes in many varieties, including circulating growth factors (vascular endothelial growth factor, VEGF), hormones (endothelin-1), metabolites (nitric oxide, NO) and mechanical forces (blood flow). Due to their integral role, it is important to have a thorough understanding of how ECs are able to integrate information provided by their environment.

PECAM-1 Structure and Function

One important EC adhesion molecule is platelet endothelial cell adhesion molecule-1 (PECAM-1). In addition to being expressed on the surface of ECs, it is also found on platelets and leukocytes. PECAM-1 was initially identified for its role in leukocyte transendothelial migration, the process by which immune cells cross the vessel wall to underlying sites of inflammation [3]. More recently, it has been recognized as a multifaceted molecule in both endothelial and vascular biology.

PECAM-1 is a 130-kD single-pass transmembrane protein and is concentrated at endothelial cell-cell junctions (Figure 1.1A). The extracellular domain contains 6 Ig-like domains (Figure 1.1B). Ig-like domains 1 and 2 facilitate homophilic interactions with PECAM-1 molecules on neighboring ECs [4, 5]. The protein has a relatively short 118-amino acid cytoplasmic tail. This region consists of 8 exons that can be alternatively spliced to generate various isoforms; however, the full-length protein is the most abundant [6]. Within the cytoplasmic domain are 2 immunoreceptor tyrosine-based inhibitory motifs domains (ITIMs).

When tyrosine residues Y_{663} and Y_{686} within these domains are phosphorylated, PECAM-1 recruits Src-homology 2 (SH2) domain-containing proteins [6]. This is critical for PECAM-1 biology, as it allows the protein to participate in a number of important signaling pathways despite the lack of intrinsic kinase activity. PECAM-1 becomes tyrosine phosphorylated in response to a number of signals, including homophilic binding and shear stress. It has been previously shown that fluid shear stress (FSS) is a potent stimulus for PECAM-1 phosphorylation by the Src-family kinase Fyn [7, 8]. PECAM-1 mediates a number of shear stress-responsive pathways, including the activation of phosphoinositide-3 kinase and Akt. Importantly, PECAM-1 null ECs display impaired responses to shear stress [9].

The PECAM-$1^{-/-}$ mouse was generated, in part, to address its role in leukocyte transendothelial migration (TEM) [10]. Somewhat surprisingly, these animals were viable and had normal vasculogenesis despite previous studies implicating a role for PECAM-1 in angiogenesis [11, 12]. Initially, only minor defects in leukocyte TEM were identified in the PECAM-$1^{-/-}$ mouse. However, subsequent studies using other stress models, such as endotoxic induced-LPS shock, identified further defects in immunologic and angiogenic responses [13-15]. Additionally, our lab has demonstrated that PECAM-1 deficiency results in impaired flow-mediated vascular remodeling [16]. Furthermore, PECAM-1 null mice exhibit blunted arteriogenesis and reduced collateral remodeling due to deficits in shear stress-induced endothelial nitric oxide synthase (eNOS) and nuclear factor kappa B (NF-κB) signaling [17].

eNOS and NO Signaling in endothelial cells

Nitric Oxide (NO) is an important signaling molecule in the vascular system and regulates physiological activities including vascular tone, permeability of the endothelium, inflammation, cell proliferation, angiogenesis, and vascular remodeling [18]. Within the

endothelium, eNOS catalyzes the formation of NO and L-citrulline through the oxidation of L-arginine. NO regulates vascular tone through activation of guanylyl cyclase and subsequent cGMP production in vascular smooth muscle cells (VSMCs). In this cell type, cGMP acts a second messenger to mediate relaxation and vasodilation. Numerous stimuli activate eNOS (via phosphorylation) to generate NO, including humoral factors, such as VEGF, bradykinin and estrogen, as well as mechanical forces such as shear stress, which is one of the most potent activators of eNOS [19, 20].

The responsiveness of eNOS to diverse types of stimuli requires dynamic regulation of the enzyme. eNOS activity is regulated by several different mechanisms including protein-protein interactions (Ca^{2+}/Calmodulin (CaM), caveolin-1, and Hsp90); post-translational regulation (phosphorylation, acylation); cofactors and substrates (BH_4); and subcellular localization (plasma membrane caveolae, Golgi, and cytosolic compartments) [21, 22]. Basally, eNOS is kept inactive via association with the inhibitory protein caveolin-1 (Cav1), which typically occurs at the plasma membrane [18]. Following stimulation, either through growth factors such as VEGF or shear stress, Ca^{2+}/CaM is able to interact with eNOS. This helps displace eNOS from binding Cav1 and subsequently activates the enzyme. Additionally, FSS enhances the eNOS-Hsp90 interaction, thereby facilitating phosphorylation of Ser1179 by Akt [23, 24]. Phosphorylation of this serine residue plays a critical role in the early NO production in response to shear stress [19].

Subcellular localization is another important aspect of eNOS regulation. There are three pools of eNOS located within the cell: at the perinuclear Golgi complex; at the plasma membrane; and a cytosolic compartment [22]. eNOS is localized to the plasma membrane primarily in caveolae through acylation, including myristoylation and palmitoylation [18]. The plasma membrane pool of eNOS is highly responsive to increases in intracellular Ca^{2+} levels. eNOS in the *cis*-Golgi is less responsive to Ca^{2+} and highly responsive to Akt-

mediated activation [25]. The proteins NOSIP (eNOS interacting protein) [26] and NOSTRIN (eNOS traffic inducer) [27] mediate trafficking of eNOS between the plasma membrane and Golgi complex. Interestingly, the exact mechanisms that regulate this trafficking are not clearly established.

Misregulation of eNOS signaling has profound consequences on the health of the blood vessel. Endothelial dysfunction, present in a variety of pathologic conditions including atherosclerosis, is generally characterized by impaired NO production. It is therefore critical to characterize the mechanisms governing eNOS signaling to further understand how these processes contribute to vessel health and disease. $eNOS^{-/-}$ mice have been generated to address these important questions. Not surprisingly, these animals suffer from hypertension (through misregulated NO production), increased atherosclerotic lesions, and a heightened injury response [28, 29].

In Chapter II, I describe a novel mechanism of basal eNOS regulation. Previous studies have demonstrated a role for PECAM-1 in shear-induced eNOS activation. Here I show that PECAM-1 mediates basal eNOS activity through a pathway involving the eNOS trafficking protein NOSTRIN.

NOSTRIN regulates eNOS trafficking and activity

One of the more recently described regulators of eNOS activity and localization is the cytoplasmic protein NOSTRIN. This protein was discovered a decade ago in a yeast two-hybrid screen using the oxygenase domain of human eNOS as bait [27]. NOSTRIN is a 58-kD protein containing an N-terminal FCH region, a coiled-coil domain and C-terminal SH3 domain (Figure 1.2). The FCH region is followed by a coiled-coil stretch that composes the F-BAR domain, making it a member of the F-BAR (Fes/CIP4 homology and Bin/amphiphysin/Rvs) protein family. These proteins are regulators of membrane and

cytoskeletal interactions [30] and serve as adaptor proteins. The SH3 domain is required for binding eNOS [27] and the second coiled-coil domain facilitates oligomerization. NOSTRIN is expressed in highly vascularized tissues such as heart, lung and kidney [27]. Much of the focus on NOSTRIN has been its role as a member of the complex eNOS trafficking machinery [27]. It is able to independently bind both caveolin-1 and eNOS, forming a ternary complex important for eNOS localization [31]. Changes in NOSTRIN expression can alter eNOS activity and localization; when NOSTRIN is overexpressed, eNOS activity is significantly decreased [31, 32].

More recent studies have revealed additional functions for NOSTRIN in endothelial cells. *In vivo* studies using zebrafish have identified a novel role for NOSTRIN in mediating angiogenesis during development and in the adult [33]. The authors demonstrate that NOSTRIN forms a complex with Rac1 and Sos1, important for FGFR1-mediated Rac1 activation [33]. In addition to the full-length protein a shortened isoform, NOSTRINβ, was recently described [34]. Unlike NOSTRIN, this variant is primarily expressed in the nucleus and thought to regulate expression of the NOSTRIN gene. The importance of NOSTRIN in EC physiology and pathophysiology is currently the topic of ongoing investigation.

Cell-cell crosstalk and implications in cell and organ function

Cell-cell crosstalk, defined as communication through paracrine/autocrine factors released from one cell that affect the behavior and function of another cell, is an integral biological concept. This phenomenon is essential both during development and is critical for maintaining homeostasis within the adult. Crosstalk occurs through a number of mechanisms, including release of soluble factors, differential expression of receptors and ligands, as well of direct cell contact [35]. Importantly, cell crosstalk occurs in all tissues.

However, the research presented in this dissertation specifically focuses on the crosstalk between ECs and underlying cells.

During development tissues release signaling molecules that influence migration, proliferation, and differentiation of constituent cells. For example, most tissues release the signaling protein VEGF that signals to ECs to induce the growth of new blood vessels. This provides oxygen and nutrients to the newly forming tissue. VEGF-mediated signaling is also necessary for the patterning and maintenance of the nascent blood vessels [36]. Other examples of cytokines important in crosstalk during development include platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and bone morphogenic protein (BMP) [37]. Studies using genetic ablation of PDGF and its receptor PDGFR have demonstrated an important role for this signaling pathway in neural and vascular development [38]. In vascular development, PDGF is primarily secreted by ECs of the emerging vessel sprouts, while the receptor is expressed by pericytes and VSMCs. Here, EC-derived PDGF signaling stimulates both proliferation and migration of these neighboring cells to surround and stabilize developing blood vessels [38].

In the adult, cell crosstalk is essential for homeostasis. For instance, NO released from ECs signals to underlying VSMCs to regulate vessel tone. Misregulation of this signaling can lead to EC dysfunction and as mentioned previously, hypertension. Additionally, signaling from ECs to VSMCs can affect the growth and proliferation of VSMCs and their contractile properties. Cell crosstalk can also be facilitated through direct interaction of cells. For example, gap junctions, present in all cell types, mediate electrical and chemical communication. More specifically, within the heart, gap junctions are required for coordination of muscle contraction. Targeted deletion of any number of connexins leads to cardiac phenotypes ranging from embryonic lethality to a tendency for arrythymias [39].

The heart is an organ whose function relies heavily on the crosstalk between cell types; primarily the crosstalk between ECs and cardiomyocytes (CMs, heart muscle cells). ECs exist in close proximity to CMs, providing a microenvironment that facilitates EC-CM communication (Figure 1.3A-D). Cardiac ECs are thought to release a number of paracrine factors, including neuregulin-1 (NRG1), VEGF, and NO that influence CM function. Interestingly, crosstalk is reciprocated, as CMs release VEGF and angiopoietin to affect EC growth and function [40]. Characterizing the regulation of these signaling pathways is physiologically relevant, as abnormal signaling can result in cardiac pathologies, such as cardiac hypertrophy.

Cardiac function and dilated cardiomyopathy

The heart is a fascinating organ; it beats continuously throughout our lifetime and is able to continuously adapt to changes in demand. In order to function properly, it requires coordination of signaling pathways and electrical signals. Imbalance of either of these components can lead to dramatic and deleterious changes in heart structure or function. Diseases that are characterized by disruptions to structure or function are termed cardiomyopathies. Dilated cardiomyopathy is the most common cardiomyopathy worldwide and represents one of the most common causes of heart failure. In this disorder, dilatation and impaired contraction of the ventricles develops and can lead to heart failure and sudden death. Although myocardial injury, as well as abnormalities in the function and integrity of CMs, can lead to dilated cardiomyopathy, cardiac endothelial dysfunction has also been implicated in progression of the disease [41]. In Chapter III, I describe a mechanism by which PECAM-1, expressed in ECs, is able to regulate signaling pathways in CMs that are important for proper coordination of contractility.

RESEARCH PRESENTED IN THIS DISSERTATION

As described in the following chapters, the goals of this thesis are as follows (Figure 1.4):

Chapter II. Determine the role of PECAM-1 in regulating basal eNOS activity

eNOS plays an important role in vascular biology. Misregulation can lead to endothelial dysfunction and cardiovascular disease. Previous studies have demonstrated a role for PECAM-1 in shear stress-induced eNOS activation. However, my preliminary data showed that PECAM-1 knockout ECs have higher basal pSer1179eNOS compared to PECAM-1 expressing cells. This observation suggests that PECAM-1 is not only required for shear-induced eNOS activation but also regulates basal eNOS activity. In this chapter I will *i) characterize the influence of PECAM-1 expression in basal eNOS activity and ii) determine the mechanism by which PECAM-1 regulates basal eNOS activity.* I show that PECAM-1 mediates basal eNOS activation, in part, by regulating the expression of the eNOS trafficking protein NOSTRIN.

Chapter III. Determine the role of PECAM-1 in regulating cardiac function

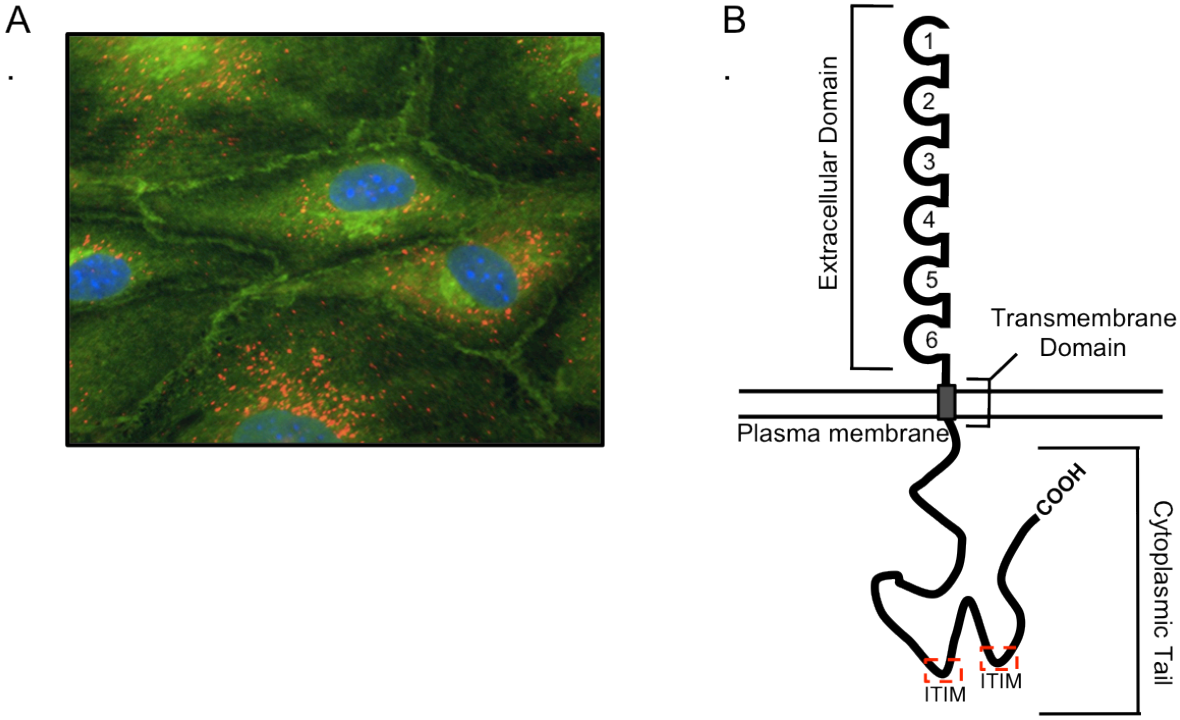
Previous studies have shown a requirement for PECAM-1 in regulating vascular function and remodeling. Because the vascular system is important for cardiac function, I hypothesized that cardiac function would be impaired in PECAM-1$^{-/-}$ animals. To address this possibility, we first performed echocardiography and found that the PECAM-1$^{-/-}$ hearts had impaired function relative to wild-type hearts. Using biochemical and *ex vivo* approaches, I show that this is due to misregulated signaling between ECs and CMs. In addition, by manipulating the hemodynamic environment of the heart, we implicate a role for PECAM-1 mechanosensing in regulation of cardiac function.

Chapter IV. Conclusions and Perspectives

In this chapter, I discuss the significance of this work for the cardiovascular biology field and tie Chapter II and III together through similar themes and questions raised.

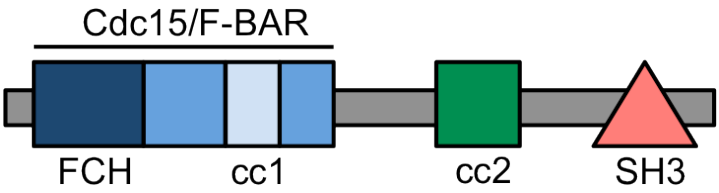
Figures

Figure 1.1. Structure and localization of PECAM-1.



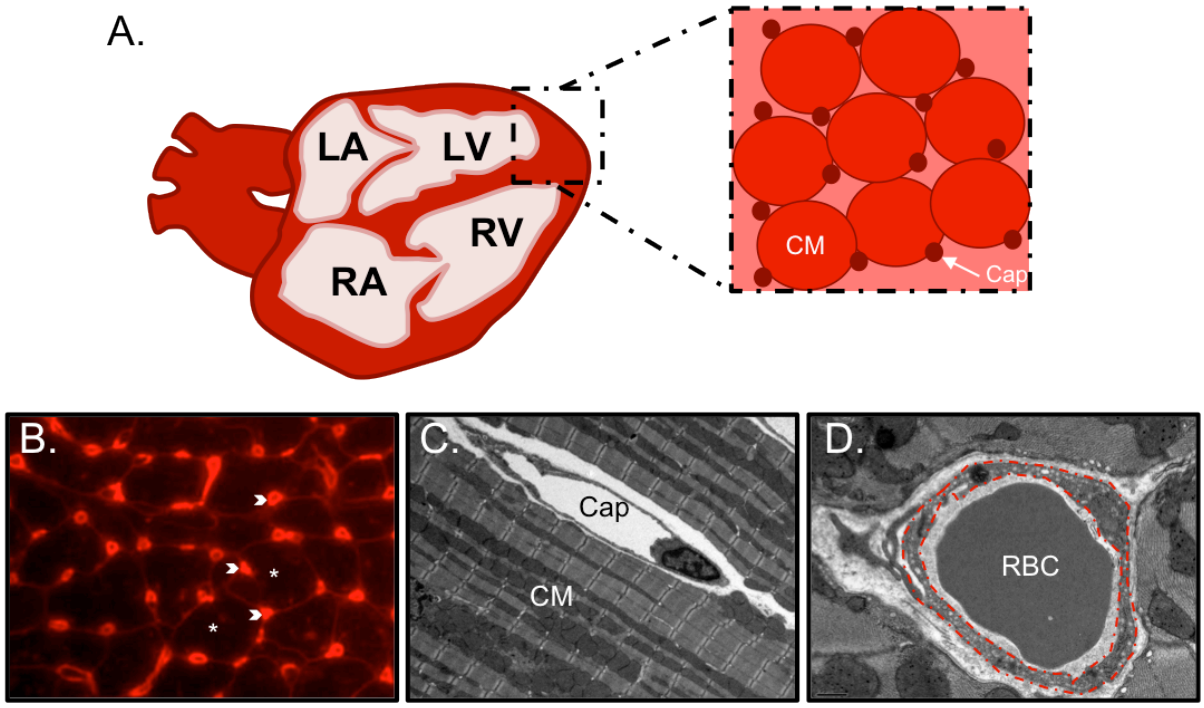
A. Mouse endothelial cells stained for PECAM-1 (green), acetylated LDL (red) and nucleus (DAPI). PECAM-1 staining is concentrated as cell-cell junctions. The accumulation of acetylated LDL within these cells is a marker of ECs. B. Representative structure of PECAM-1. The protein has 6 extracellular Ig-like domains, a single pass transmembrane domain and a relatively short cytoplasmic tail which contains ITIM residues Y_{663} and Y_{686} important for phosphorylation.

Figure 1.2. Multi-domain structure of NOSTRIN



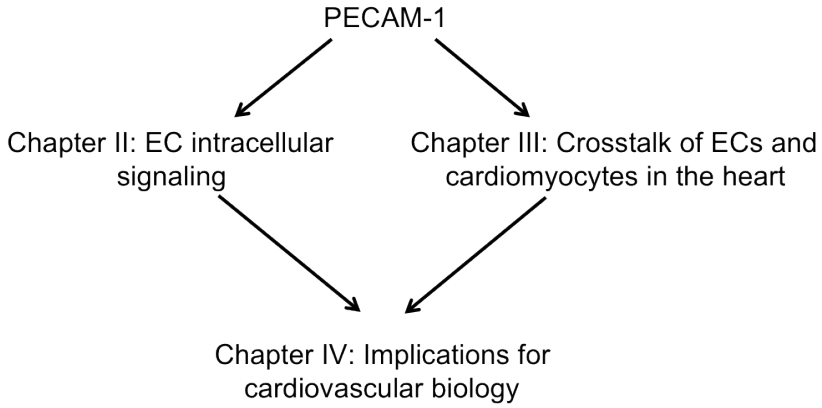
The cdc15/F-BAR domain, composed of an FCH and coiled-coil (cc1) domain, is important for directing the subcellular localization of NOSTRIN and particularly for membran binding. The C-terminal coiled-coil (cc2) domain is important for oligomerization of the protein and t he SH3 domain mediates interactions with binding partners such as eNOS and N-WASP. Image adapted from Ref. 32.

Figure 1.3. Intimate relationship between cardiac endothelial cells and cardiomyocytes.



A. Schematic representation of the heart and arrangement of capillaries and cardiomyocytes in the heart. Cardiomyocytes (CMs) are surrounded by approximately 4 capillaries (Cap). RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. B. TRITC-lectin staining in the LV. Capillaries are labeled in red (arrowhead). Faint staining shows the outline of CMs (asterisk). C-D. TEM images of mouse heart. C. The capillary (Cap) is situated just next to the CM, and more importantly, the contractile apparatus of the CM. D. A magnified view of a Cap lined by EC (outlined) with a red blood cell (RBC) in the center of the capillary.

Figure 1.4. Overview of research presented in this dissertation.



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CHAPTER II.

PECAM-1 regulates eNOS activity and localization through STAT3-dependent NOSTRIN expression.

Overview

Objective: Nitric oxide (NO) produced by the endothelial NO synthase (eNOS) is an important regulator of cardiovascular physiology and pathology. eNOS is activated by numerous stimuli and its activity is tightly regulated. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) has been implicated in regulating eNOS activity in response to shear stress. The goal of the current study is to determine the role of PECAM-1 in the regulation of basal eNOS activity.

Methods and Results: We demonstrate that PECAM-1 knockout ECs have increased basal eNOS activity and NO production. Mechanistically, increased eNOS activity is associated with a decrease in the inhibitory interaction of eNOS with caveolin-1; impaired subcellular localization of eNOS; and decreased NOSTRIN expression in the absence of PECAM-1. Furthermore, we demonstrate that blunted STAT3 activation in the absence of PECAM-1 results in decreased NOSTRIN expression via direct binding of STAT3 to the NOSTRIN promoter.

Conclusions: Taken together, our results reveal an elegant mechanism of eNOS regulation by PECAM-1 through STAT3-mediated transcriptional control of NOSTRIN.

Introduction

The production of NO is critical for cardiovascular homeostasis as NO regulates many fundamental cellular processes, including regulation of vessel tone, cell proliferation, and angiogenesis [1]. In the vascular endothelium, NO is synthesized from L-arginine by the constitutively expressed endothelial nitric oxide synthase (eNOS or NOS3) enzyme. eNOS function is critical, as genetic deletion of eNOS results in increased blood pressure [2, 3], impaired angiogenesis, abnormal vascular remodeling, and accelerated atherosclerosis [4]. Numerous stimuli promote the activation of eNOS through phosphorylation of serine residue 1179, leading to NO production. In this regard, it has been shown that the cell adhesion molecule PECAM-1 regulates eNOS activation *in vitro* and *in vivo*, possibly via a direct interaction between PECAM-1 and eNOS [5, 6]. However, the specifics of this interaction are not known.

eNOS is regulated by multiple interdependent control mechanisms; including post-translational lipid modifications, phosphorylation, localization and protein-protein interactions [7-9]. Together, these regulatory mechanisms ensure proper responses to diverse stimuli. eNOS is basally repressed through its interaction with an integral membrane protein, caveolin-1, which inhibits NO production [10]. In addition, studies show that trafficking and proper subcellular localization of eNOS are also critical for regulation of its activity [10, 11]. There are three pools of eNOS located within the cell: (1) the perinuclear Golgi complex; (2) the plasma membrane (primarily in caveolae); and (3) a cytosolic compartment. It is increasingly appreciated that eNOS can traffic between these compartments and that subcellular targeting can affect NO production in response to various stimuli [12, 13]. Recent studies have identified several eNOS trafficking proteins including NOSIP (eNOS interacting protein) [14] and NOSTRIN (eNOS traffic inducer) [12]. NOSTRIN is expressed in ECs both *in vitro* and *in vivo* [12, 15] and regulates eNOS trafficking and localization [12, 15, 16]. Although

proteins that influence localization have been identified, the mechanisms that control eNOS trafficking and the (patho)physiologic consequences are not fully understood.

Here, we investigate the role of PECAM-1 in the regulation of basal eNOS activity. We reveal an elegant mechanism of eNOS regulation through transcriptional control of NOSTRIN expression.

Materials and Methods

Animals

PECAM-1$^{-/-}$ C57BL/6 mice were kindly provided by Dr P. Newman (Blood Research Institute, BloodCenter of Wisconsin, Milwaukee) and eNOS-GFP transgenic mice were kindly provided by Rini de Crom (Erasmus University Medical Center, Rotterdam, The Netherlands). All mice were bred in house and used in accordance with the guidelines of the National Institute of Health and the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Mice aged 12-16 weeks were used for all experiments.

Cell culture and Reagents

PECAM-1 knockout (PE-KO) cells and cells reconstituted with murine full-length PECAM-1 (PE-RC) we prepared as described [17]. HUVECs (Lonza) were grown in M199 media supplemented with EGM2 bullet kit. Cucurbitacin I was purchased from Tocris Bioscience (Ellsville, Missouri). Antibodies to phospho-Ser1179 eNOS and phospho-Tyr STAT3 were from Cell Signaling (Danvers, MA). Anti-GM130 was from BD Biosciences (Transduction, San Diego) and total STAT3 (Santa Cruz, CA) and total GAPDH from Millipore (Temecula, CA).

Immunoprecipitation and Western Blotting

Cells were harvested in lysis buffer as previously described [18]. Lysates were assayed by Western blot. For immunoprecipitation, cells were harvested in OG buffer plus protease inhibitors [19]. Equivalent volumes of lysate were pre-cleared with Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology, Inc) for 1 hour at 4°C. Supernatants were then incubated with Protein A/G beads previously conjugated with anti-rabbit caveolin-1 (BD Transduction). Complexes were washed three times OG buffer and then assayed by Western blot.

Immunofluorescence

PE-RC and PE-KO cells were fixed and permeabilized in 3.7% formaldehyde with 0.2% Triton X-100 for 30 minutes at 37°C then blocked in 1% BSA in PBS for 30 minutes at 37°C. Cells were stained for total eNOS (BD Transduction) and Alexa488-conjugated Giantin (Covance). Images were taken using either the Zeiss LSM5 Pascal Confocal microscope using a 63X oil lens. Co-localization was determined using ImageJ and the Mander's colocalization coefficient [20]. Aorta *en face* preparations we prepared as previously described [21]. *En face* preparations were evaluated with a Zeiss LSM5 Pascal microscope.

Cellular Fractionation

Confluent PE-RC and PE-KO cells were fractionated as previously described [22]. The samples (25ul) were run on 4-12% SDS-PAGE gels then transferred to nitrocellulose membrane for Western blot analysis. Membranes were probed with total eNOS, GM130 as Golgi marker and caveolin-1 as the plasma membrane marker.

In vitro and *in vivo* NO Measurements

Cells were serum-starved for 24 hours then treated with either 100mmol/L L-Name, 1μmol/L ionomycin or L-Name plus ionomycin. Media NO levels were evaluated using the Griess reagent (NO Assay kit, R&D Systems). PECAM-1 $^{+/+}$ and $^{-/-}$ animals were anesthetized using intraperitoneal (IP) injections of ketamine (100mg/kg) and xylazine (15mg/kg) and injected with 100U heparin. Plasma was isolated for NO measurements, which were performed using a NO analyzer (Sievers Instruments, Boulder, CO) as previously described [23].

RNA Isolation and Quantitative PCR

Total RNA was isolated from a confluent cell monolayer using the TRIzol reagent (Invitrogen) and first-strand cDNA was transcribed using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using ABsolute SYBR Green ROX mix (Thermo Scientific). Relative levels of gene expression were normalized to mouse 18s expression using the comparative Ct method.

Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation was performed using the fast ChIP method described by Nelson et al [24] using a rabbit anti-STAT3 K15 antibody (Santa Cruz Biotechnology, Inc). Quantitative PCR was performed using primers specific for the NOSTRIN promoter.

Quantification and Statistical Analysis

Band intensity of immunoblots was quantified using the ImageJ program. Each experimental group was analyzed using single factor analysis of variance. P-values were obtained by performing two-tailed Student's *t* test using Excel (Microsoft). Statistical significance was defined as $P<0.05$.

Results

PECAM-1 regulates basal eNOS activity

Previous studies suggest a requirement for PECAM-1 in shear-induced eNOS activation [10, 25, 26]. Our own results are in agreement with these observations (data not shown). Unexpectedly, our data revealed increased levels of phosphorylated eNOS in PECAM-1 knockout (PE-KO) cells compared to PECAM-1 expressing cells (PE-RC) (Figure 2.1A). PECAM-1 deletion, however, does not affect total eNOS expression [25]. To evaluate the functional consequences of increased basal eNOS phosphorylation in the PE-KO cells, we measured NO production. As shown in Figure 2.1B, there is a 3-fold increase in basal NO production in PE-KO cells compared to PE-RC cells. Stimulation with ionomycin increased NO production in both cell types, whereas L-NAME inhibited the production of NO. To determine if these observations are corroborated in whole animals, we measured NO levels from plasma. Indeed, plasma NO levels were higher in PECAM-$1^{-/-}$ compared to PECAM-$1^{+/+}$ animals (Figure 2.1C).

To further investigate the role of PECAM-1 in regulation of basal eNOS phosphorylation, we used siRNA to knockdown PECAM-1 expression in human umbilical vein endothelial cells (HUVECs). The degree of knockdown was assessed by Western blot (Supplemental Figure 2.1A). Interestingly, we observed an increase in both basal eNOS phosphorylation (Supplemental Figure 2.1B) and NO production (Supplemental Figure 2.1C) in PECAM-1-siRNA infected ECs compared to control siRNA cells. Together, these data suggest that absence of PECAM-1 results in higher basal eNOS activity and NO production.

Differential association of eNOS and caveolin-1 in the absence of PECAM-1

We next wanted to address the mechanism(s) responsible for the increased basal eNOS activity in the absence of PECAM-1. To determine if PECAM-1 affects the interaction

between eNOS and known regulatory proteins we performed co-immunoprecipitation assays. The caveolar scaffolding protein, caveolin-1, has been shown to regulate eNOS activity, primarily as an inhibitor of basal enzyme function [10, 19, 27]. Caveolin-1 is able to bind eNOS and block the calmodulin-binding site important for enzyme activation [28]. To determine if PECAM-1 affects the association of eNOS with caveolin-1, we performed co-immunoprecipitation assays. As shown in Figure 2.2, there was a decrease in basal eNOS-caveolin-1 association in the PE-KO cells compared to the PE-RC cells. Additionally, we see a decrease in the colocalization of eNOS and caveolin-1 by confocal microscopy, supporting our immunoprecipitation data (data not shown). These data suggest that PECAM-1 may regulate basal eNOS activity by promoting the association of eNOS with caveolin-1.

Role of PECAM-1 in eNOS localization/trafficking

Subcellular localization of eNOS influences its activation [11, 13, 23, 29]. To determine if PECAM-1 influences eNOS localization, we performed double-labeling immunofluorescence confocal microscopy in PE-RC and PE-KO cells. Cells were stained for total eNOS and giantin, a membrane-inserted component of the *cis*- and *medial*-Golgi complex. In PECAM-1 expressing cells, eNOS is found at both the perinuclear/Golgi complex and the plasma membrane (PM) (Figure 2.3A), consistent with the localization described in blood vessels *in vivo* [113]. In contrast, in cells that lack PECAM-1, eNOS is redistributed away from the perinuclear region (Figure 2.3A). Indeed, quantitative colocalization analysis (Mander's colocalization coefficient) showed decreased colocalization of eNOS with giantin in the absence of PECAM-1 (Figure 2.3A). Together, these data suggest that PECAM-1 influences the subcellular localization of eNOS.

To further support our *in vitro* findings, we isolated aortas from PECAM-1$^{+/+}$ and $^{-/-}$ mice expressing an eNOS-GFP fusion protein. The aortas were stained *en face* for giantin and visualized using confocal microscopy (Figure 2.3B). Similar to the PE-KO cells, quantitative colocalization analysis revealed a significant redistribution of eNOS away from the Golgi complex in the PECAM-1$^{-/-}$ aortas.

As a complementary approach, we utilized sodium carbonate extraction of cells followed by a discontinuous sucrose gradient. In this procedure, cholesterol-rich microdomains, including lipid rafts and caveolae, float as buoyant membranes at the 5–30% sucrose interface (fractions 3-4), whereas soluble proteins and heavy membranes remain at the bottom of the gradient (fractions 9–11). In all gradients, the distribution of caveolin-1 and GM130, a Golgi marker, were examined to confirm adequate separation of the fractions (Figure 2.3C). In PECAM-1-expressing ECs, eNOS distributed primarily into two distinct pools: light membranes highly enriched in caveolin-1 and heavy membranes enriched in GM130. In PE-KO cells, eNOS is also distributed into two pools. However, we noticed a significant redistribution of eNOS out of the Golgi-enriched fractions (fractions 10-11). These observations are consistent with the immunofluorescence data described above. Notably, PE-KO cells have increased eNOS activity and NO levels despite increased expression levels of the negative regulator caveolin-1 (not shown).

PECAM-1 mediates STAT3-dependent NOSTRIN expression in ECs

eNOS localization is a dynamic and well-coordinated process, mediated by a number of players including dynamin-2 and NOSTRIN[9]. To determine if PECAM-1 mediates eNOS localization through NOSTRIN, we measured NOSTRIN protein expression in PE-RC and PE-KO cells. Unexpectedly, we observed a significant decrease in NOSTRIN protein expression in PE-KO cells (Figure 2.4A). Reduced NOSTRIN expression in PE-KO cells could be due to either increased protein degradation or a reduction in mRNA levels. To

distinguish between these possible mechanisms, we first measured ubiquitination of NOSTRIN but did not observe a difference between the PE-RC and PE-KO cells (data not shown). Next, we used quantitative real-time PCR to determine the effect of PECAM-1 deletion on NOSTRIN mRNA levels. Our data demonstrate that NOSTRIN mRNA is significantly decreased in the absence of PECAM-1 (Figure 2.4B).

We next addressed the mechanism by which PECAM-1 affects NOSTRIN protein expression. It has previously been shown that the cytoplasmic tail of PECAM-1 is able to function as a scaffold for numerous signaling pathways, including signal transducers and activators of transcription (STAT) protein family members STAT5 and STAT3 [30, 31]. Additionally, ECs isolated from PECAM-1$^{-/-}$ mice have reduced STAT3 phosphorylation [31]. We asked whether the reduced levels of active STAT3 in the PECAM-1 knockout might result in decreased NOSTRIN expression. To determine if STAT3 phosphorylation is reduced in cells that lack PECAM-1, we probed PE-KO cell extracts for total STAT3 and phospho-STAT3. Importantly, our data are in agreement with previous reports showing that phospho-STAT3 levels are lower in the absence of PECAM-1 (Figure 2.5A). To determine if STAT3 activity affects NOSTRIN expression, we treated the PECAM-1 expressing ECs with cucurbitacin, a selective inhibitor of STAT3/JAK signaling, and assayed for changes in NOSTRIN expression by Western blot and quantitative real-time PCR. Cucurbitacin treatment results in a dose-dependent decrease in both NOSTRIN protein expression (Figure 2.5B) and mRNA levels (Figure 2.5C). These results suggest that PECAM-1 mediates STAT3-induced NOSTRIN expression, which, in turn, regulates eNOS localization.

Binding of STAT3 to the NOSTRIN promoter in endothelial cells

Analysis of the NOSTRIN promoter revealed several putative STAT3 binding sites (Supplemental Table 2.1). STAT family members bind to a conserved sequence of TT and

AA duplicates typically separated by 5 bases. In addition to binding TT(N5)AA nanomers, STAT3 is also able to bind octomers and decamers [32, 33]. We next investigated the possibility that STAT3 directly binds to the NOSTRIN promoter to modulate mRNA expression by chromatin immunoprecipitation (ChIP) assays with STAT3 antibodies. Immunoprecipitation of the chromatin lysates was followed by PCR with NOSTRIN promoter primers; NOSTRIN exon 10 primers served as a control. ChIP assays show that STAT3 binds to the NOSTRIN promoter region, but not the NOSTRIN coding region (Figure 2.5D). Together, these data suggest that STAT3 may regulate NOSTRIN mRNA levels by specifically binding to the NOSTRIN promoter.

Discussion

Understanding the mechanisms that regulate NO production in the endothelium can provide important insight into processes that initiate endothelial dysfunction and lead to the development of atherosclerosis. NO production is dynamically regulated by a number of humoral and mechanical factors. In this study, we investigated PECAM-1 regulation of basal eNOS activity. An unexpected finding of this study was the increased basal eNOS activity and NO production in PE-KO cells as well as PECAM-1$^{-/-}$ mice. An attractive hypothesis to explain the basal regulation of eNOS by PECAM-1 is their reported physical association. However, reports of this interaction have been highly conflicting. One group showed that shear stress induces a transient increase in the association of PECAM-1 and eNOS [6], while other studies have shown just the opposite [5]. Our own results suggest only a weak basal association of eNOS and PECAM-1 in static ECs (data not shown). In addition, ultra-structural and biochemical analyses suggest that eNOS resides within caveolae [10, 34], whereas PECAM-1 is found at a membrane network just below the plasmalemma at the cell borders that is distinct from caveolae [35]. Furthermore, studies in HUVECs have shown no

colocalization between PECAM-1 and caveolin-1 and that these two proteins do not co-migrate on sucrose gels [35]. The lack of physical association between eNOS and PECAM-1 led us to investigate differences in eNOS protein interactions and localization as possible mechanisms of regulation.

It is well recognized that correct subcellular targeting of eNOS is critical for proper regulation of its activity and NO bioavailability; thus, tight control of eNOS targeting to different compartments appears to be essential. In this regard, our data point towards a requirement for tightly regulated levels of NOSTRIN expression within ECs. Overexpression of NOSTRIN can promote the translocation of eNOS from the plasma membrane to intracellular vesicles, with a concomitant reduction in eNOS enzyme activity [12]. Conversely, decreased NOSTRIN expression also influences eNOS subcellular localization and may contribute to the increased NO levels observed in the PECAM-1 knockout. Interestingly, overexpression of the eNOS-binding partner, caveolin-1, leads to accelerated atherosclerosis formation in mice, partially through reduced NO production [36]; while persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension [37]. Thus, tight regulation of eNOS regulatory protein levels, including NOSTRIN and caveolin-1 expression, is required for proper eNOS function.

Here, we present exciting data to support a novel mechanism of eNOS regulation by PECAM-1. Our current working model is summarized in Figure 2.6. The cytoplasmic tail of PECAM-1 acts as a scaffold for STAT3 and mediates its activation. A possible candidate for the activation of STAT3 are the Src-family kinases, as Src-mediated STAT activation has been previously reported [38, 39]. Following activation, STAT3 dimerizes and translocates into the nucleus where it modulates expression of gene targets, including NOSTRIN. Once expressed, NOSTRIN facilitates eNOS trafficking and its correct subcellular localization. It is possible that the reduced NOSTRIN levels account for the difference in eNOS-caveolin-1

association, however this could be due to other undetermined mechanisms. Of note, it has been reported that eNOS, caveolin-1 and NOSTRIN form a ternary complex to facilitate eNOS translocation [16]. Additionally, our data are consistent with the hypothesis that NOSTRIN might serve to stabilize the inhibitory effect of caveolin-1 on eNOS [16].

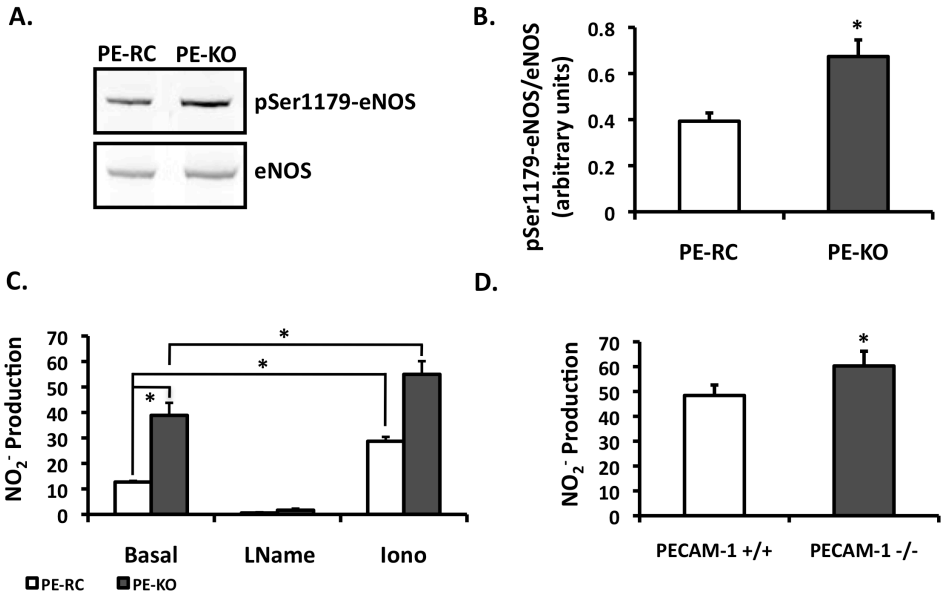
The signaling pathway identified here relates to the regulation of basal eNOS activity via PECAM-1. However, PECAM-1 is also known to regulate eNOS activation in response to the physiologic stimulus of shear stress [5, 6, 25, 40]. PE-KO cells are unable to activate eNOS in response to shear stress, yet they activate eNOS in response to ionomycin, indicating a specific requirement for PECAM-1 in flow-induced eNOS activation (Figure 2.1B and Supplemental Figure 2.2). It is worth noting here that PECAM-1 is also required for flow-induced activation of Akt and Src, two important upstream mediators of eNOS activity [25, 40]. The role of shear stress in NOSTRIN-mediated eNOS regulation is currently under investigation.

Blood flow and the NO signaling pathway are both known modulators of cardiovascular development and physiology. Two recent studies have provided compelling evidence for an evolutionarily conserved, shear stress- and NO-mediated pathway that also regulates hematopoiesis [41-43]. PECAM-1 is thought to be involved in flow mechanosensing, based on *in vitro* and *in vivo* experiments showing PECAM-1-dependent activation of flow-mediated intracellular signaling pathways and vascular remodeling [6, 44, 45]. Interestingly, PECAM-1 is required for NO-mediated dilation in response to shear stress in isolated skeletal muscle arterioles [40] as well as in the mouse coronary circulation [46], thus underscoring the importance of both PECAM-1 and NO in flow-mediated remodeling. Previous studies have also identified the importance of eNOS in flow-mediated remodeling [47]. We now reveal a sophisticated dual mode of eNOS regulation by PECAM-1 *in vitro*; while PECAM-1$^{-/-}$ ECs are unable to activate eNOS in response to shear stress, their basal eNOS

activity and NO levels are, paradoxically, increased through STAT3-mediated transcriptional control of NOSTRIN. Our findings underscore the dynamic regulation of proteins integral to the regulation of vascular homeostasis.

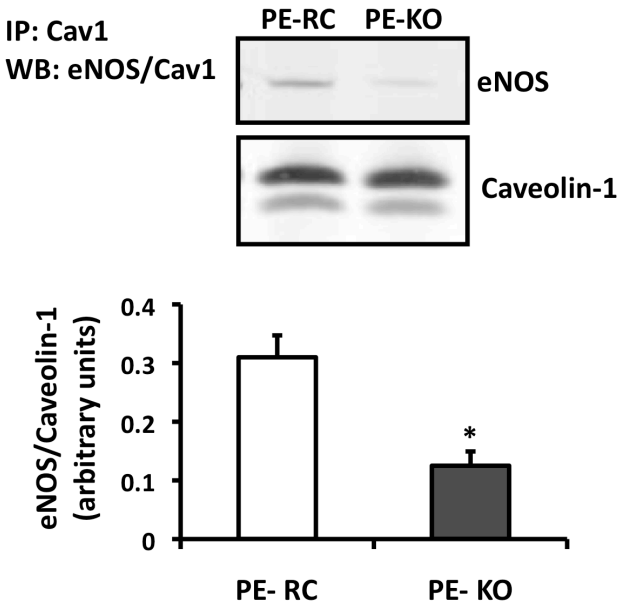
Figures

Figure 2.1. Basal eNOS phosphorylation and NO production in the PECAM-1 KO.



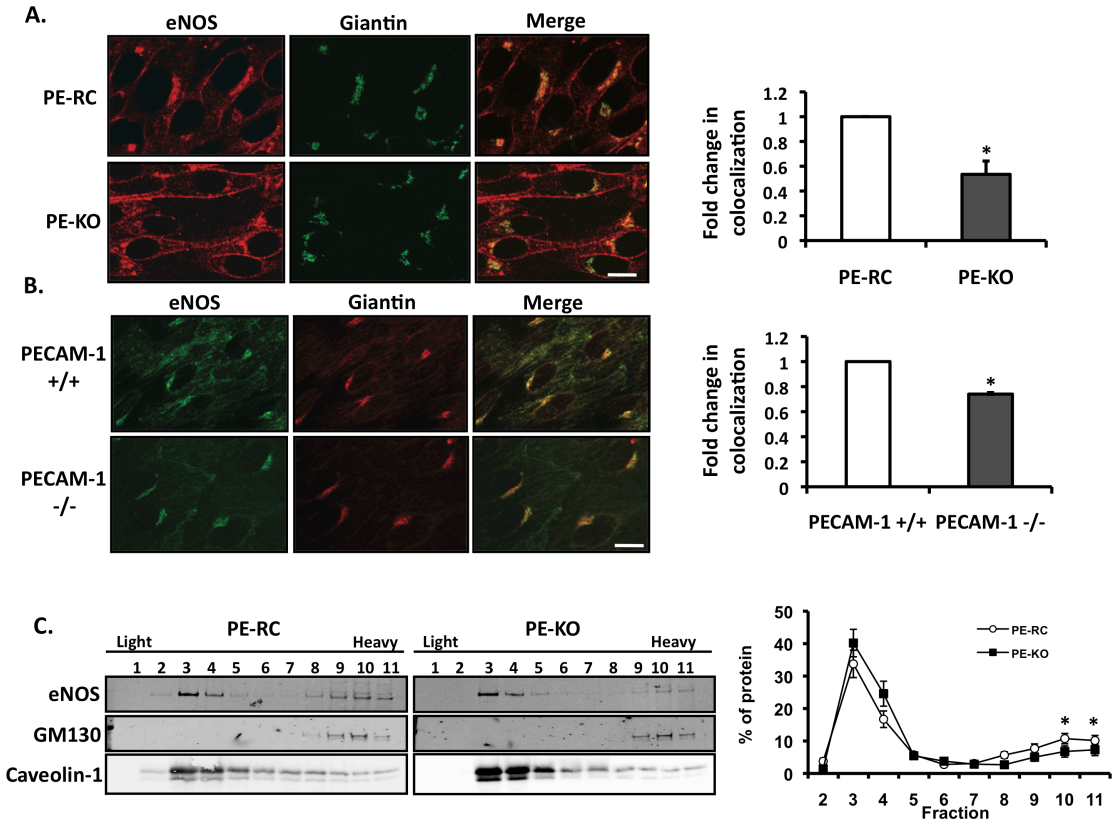
A, PE-KO cells have increased basal peNOS compared to PE-RC (n=6, *P<0.005). B, PE-RC and PE-KO cells were treated with 1mmol/L L-Name or 1μmol/L ionomycin and NO levels measured (n=3, *P<0.01 vs PE-RC basal). C, Plasma levels of NO from PECAM-$1^{+/+}$ and $^{-/-}$ mice were assayed as described. (n=7 mice;*P<0.05 vs PECAM-$1^{+/+}$).

Figure 2.2. Differential association of the negative eNOS-binding partner, caveolin-1, in the absence of PECAM-1.



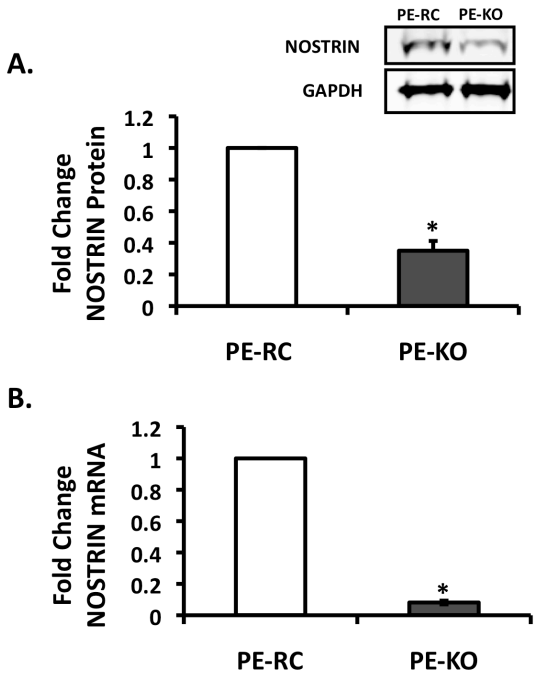
PE-RC and PE-KO cells were immunoprecipitated using an antibody against Cav1. Western blots were performed for total eNOS and Cav1. Quantitation is shown on the right (n=6; *P<0.001 vs PE-RC).

Figure 2.3. PECAM-1 regulates eNOS subcellular localization.



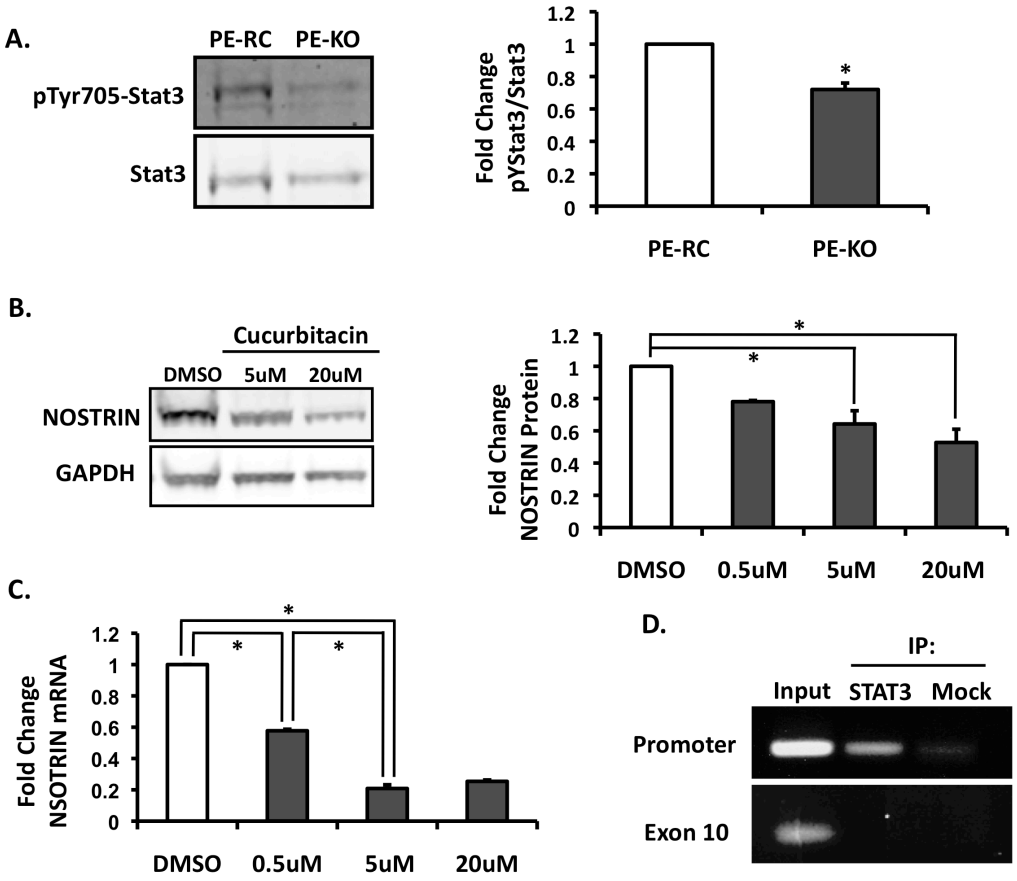
A, PE-RC and PE-KO cells stained for total eNOS (*red*) and Giantin (*green*). Colocalization was determined using the thresholded Mander's coefficient (n=4, 25 cells/experiment; *P<0.05). B, *En face* aortas from eNOS-GFP/PECAM-1 +/+ or -/- mice stained for Giantin (*red*) (50 cells/genotype; *P<0.05). C, Subcellular fractionation of PE-RC and PE-KO cells. Fractions were immunoblotted for total eNOS, GM130 and Cav-1 (n=3, *P<0.05). Bar = 5μm.

Figure 2.4. Reduced NOSTRIN expression in ECs lacking PECAM-1.



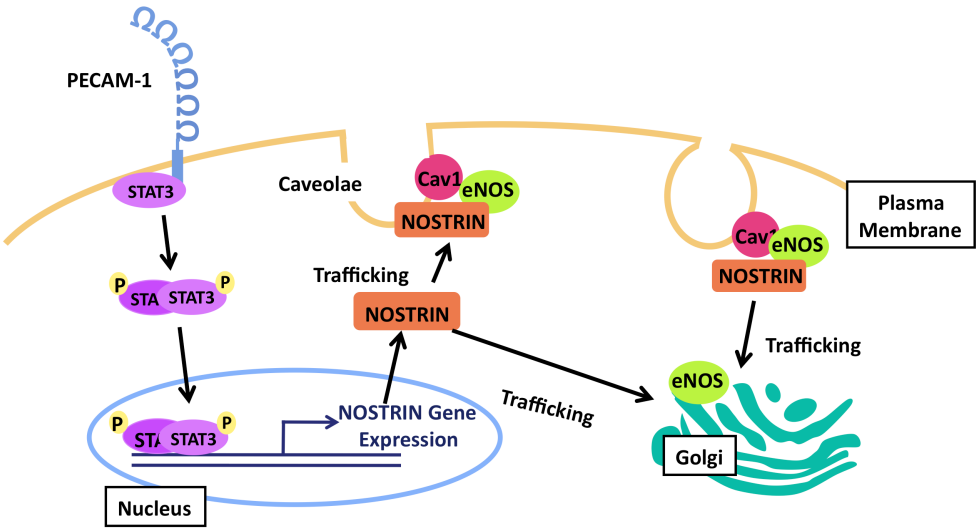
A, Levels of NOSTRIN protein were measured in PE-RC and PE-KO cells by Western Blot. GAPDH was measured for loading (n=4, *P<0.001 vs PE-RC). B, Real-time PCR analysis revealed decreased NOSTRIN mRNA levels in PE-KO cells (n=5, *P<0.001 vs

Figure 2.5. PECAM-1 regulates NOSTRIN in a STAT3-dependent manner.



A, Western blot for phosphorylated STAT3 in PE-RC and PE-KO cells. Quantitation shown on right (n=3, $P<0.01$ vs PE-RC). B, PE-RC and PE-KO cells treated for 24 hours with 0.5, 5 and 20μmol/L cucurbitacin then lysed and NOSTRIN protein expression determined by Western Blot. Values normalized to GAPDH (n=3, *$P<0.05$). C, Real-time PCR analysis of NOSTRIN mRNA expression in PE-RC cells treated for 12hrs with cucurbitacin. (n=3, *$P<0.001$). D. ChIP on PE-RC cells for STAT3 binding to NOSTRIN promoter region.

Figure 2.6. Model of PECAM-1-mediated NOSTRIN expression and eNOS trafficking.



The cytoplasmic tail of PECAM-1 acts as a scaffold for STAT3 binding. Following activation, STAT3 translocates to the nucleus where it regulates NOSTRIN mRNA expression. NOSTRIN protein is then able to bind eNOS and regulate its trafficking and localization with the cell.

Supplemental Material

Cellular Fractionation

Cells were washed twice with cold PBS then scraped into 2 ml of PBS and centrifuged at 1000 rpm for 3 minutes to pellet cells. The PBS was removed and the cells were resuspended in 750 μl of 500mmol/L sodium carbonate, pH 11. The cells were dounce-homogenized (25 strokes) on ice and sonicated at 50% power with 5 10-second bursts. The homogenate was adjusted to 42.5% sucrose using a 58% sucrose solution prepared in MBS (25mmol/L MES, pH 6.5, 0.15mol/L NaCl). 500 μl of homogenate was placed at the bottom of an ultracentrifuge tube then 1 ml of 30% sucrose was added followed by 600ul of 5% sucrose in MBS. The samples were centrifuged at 48,000 rpm for 3 hours at 4°C in using the SW50 Rotor (Beckman). Aliquots of 200ul were taken starting from the top (for a total of 12 samples) and added to 50ul 10X LSB. The samples (25ul) were run on 4-12% SDS-PAGE gels then transferred to nitrocellulose membrane for Western blot analysis. Membranes were probed with total eNOS, GM130 as Golgi marker and caveolin-1 as the plasma membrane marker.

***En Face* Staining**

Aortas were perfusion-fixed and dissected out under a dissection microscope. The aorta was trimmed of fat and excess tissue then cut longitudinally and permeabilized for 3 hours in 0.2% Tx100. Tissue was blocked in PBT (1% BSA, 0.1% Tween-20 in PBS) overnight at 4°C. Tissues were then placed on slides with the endothelium facing up, covered with Vectashield plus DAPI and mounted. Samples were incubated for 120 minutes at room temperature in Giantin antibody (1:50, Covance), then incubated for 60 minutes in secondary antibody at room temperature.

RNA Isolation and Quantitative PCR

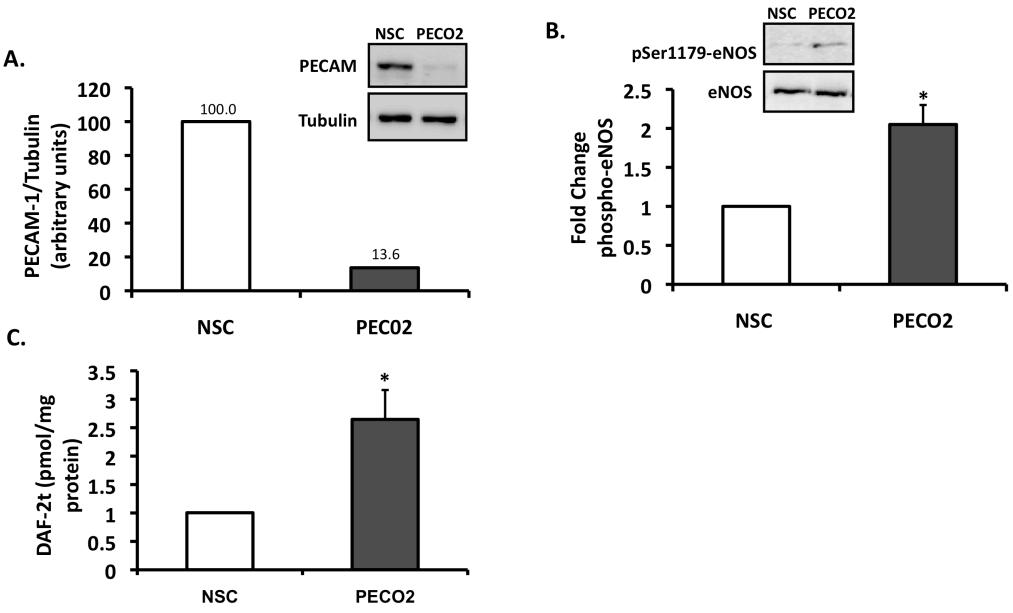
Total RNA was isolated from a confluent cell monolayer using the TRIzol reagent (Invitrogen) and DNAse treatment was performed using DNA-free (Ambion). First-strand cDNA was transcribed using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using ABsolute SYBR Green ROX mix (Thermo Scientific). Relative levels of gene expression were normalized to mouse 18s expression using the comparative Ct method. Primer sequences are listed in supplemental Table 1.

Chromatin Immunoprecipitation

Chromatin-containing lysates were incubated with rabbit anti-STAT3 K15 (Santa Cruz Biotechnology, Inc) for 30 minutes at 4°C in an ultrasonicator bath. DNA-protein complexes were precipitated using Protein A/G agarose beads and treated with proteinase K. DNA samples were extracted using 10% wt/vol Chelex (Bio-Rad) and PCR amplified using primers specific for the NOSTRIN promoter (sequences listed in Table 1).

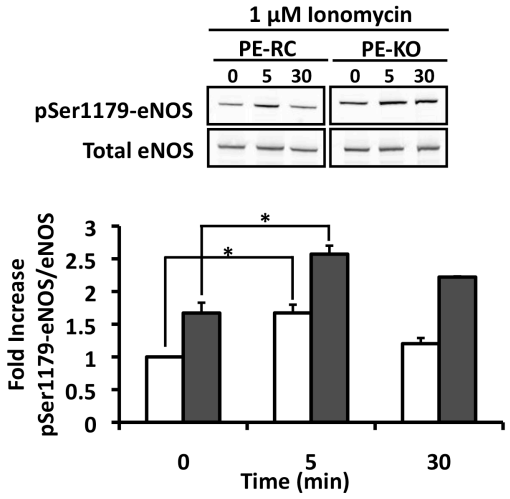
Supplemental Figures

Supplementary Figure 2.1. PECAM-1 knockdown in HUVECs.



A, HUVECs were treated with lentivirus expressing PECAM-1 siRNA (PECO2) or a non-specific control (NSC). Levels of knockdown were assayed by Western blot for PECAM-1 and tubulin. B, Lentivirus-treated HUVECs were assayed for pSer1179eNOS by Western blot (n=3; $P<0.05$ vs HUVEC NSC). C, NO levels in PECAM-1 knockdown HUVECS was assessed by DAF-2t levels.

Supplementary Figure 2.2. PECAM-1 is not required for ionomycin-induced eNOS activation.



PE-RC and PE-KO cells were stimulated with 1uM ionomycin and eNOS activation was assayed by Western blot. A Representative blot is shown (n=3, *P<0.01 vs PE-RC 0 min).

Supplemental Table 2.1. PCR and ChIP Primers

Primers (Mus musculus)	Sequence
18s Fwd	5'-CATTCGAACGTCTGCCCTATC-3'
18s Rev	5'-CCTGCTGCCTTCCTTGGA-3'
NOSTRIN Exon 10 Fwd	5'-CCAGGCTCTAATGGAAGAAGAACTG-3'
NOSTRIN Exon 10 Rev	5'-AGACTTCCGTCGCTCTTTGTCC-3'
NOSTRIN Promoter Fwd	5'-GCAGAAGGAAAGGGTTTATTGTGGCCT-3'
NOSTRIN Promoter Rev	5'-GCAATAGGACCAGCTGCCCTCAA-3'

Supplemental Table 2.2. Predicted STAT3 binding sites within the NOSTRIN promoter

From JASPAR database	
308-317	TGACAGGA
768-777	TTCCAGGCAT
906-915	TTTCAGGAAA
From Genomatix	
764-782	atcaaTTCCAGGCATccaga
900-918	tcatTTCCTGAAAaagcac

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CHAPTER III

A mechanosensor mediates crosstalk between endothelial cells and cardiomyocytes to regulate cardiac function

Overview

Background – Idiopathic dilated cardiomyopathy (IDCM) represents one of the most common causes of heart failure and death in the United States. Recent work has identified a role for vascularization and hemodynamics in regulating cardiac function. In the present study, we investigate the role of the junctional adhesion molecule Platelet Endothelial Cell Adhesion Molecule (PECAM-1) in the regulation of cardiac function.

Methods and Results –Conscious echocardiography revealed left ventricular (LV) chamber dilation and systolic dysfunction in PECAM-$1^{-/-}$ mice, which is further exacerbated in response to hemodynamic stress. Interestingly, despite deficits in cardiac function, cardiomyocytes isolated from PECAM-$1^{-/-}$ hearts displayed normal sarcomere structure and contractility. In the absence of PECAM-1, release of neuregulin (NRG-1) from endothelial cells (ECs) is deregulated, resulting in augmented phosphorylation of the NRG-1 receptor ErbB2. Furthermore, we observed aberrant activation of protein kinase A (PKA) and phospholamban, two proteins that are essential for the correct regulation of contractility in the cardiomyocyte, in the PECAM-$1^{-/-}$ mice.

Conclusions – Here, we identify a novel role for PECAM-1 in regulating cardiac function both at baseline and in response to hemodynamic stress. Based on our findings, we

propose that PECAM-1 is required for regulation of the NRG1 pathway in ECs, which ultimately acts on cardiomyocytes to affect cardiac contractility. These data highlight the importance of tightly regulated cellular communication for proper cardiac function.

Introduction

Endothelial cells (ECs) intimately associate with cardiomyocytes as each cardiomyocyte is surrounded by a dense network of capillary vessels comprised of ECs. The endothelium releases factors such as neuregulin (NRG-1), and nitric oxide (NO), that can affect numerous parameters of cardiomyocyte function, including their contractility [1]; cardiomyocytes, in turn, secrete factors that direct EC function [2]. Recent data provide a compelling link between EC dysfunction and the progression of dilated cardiomyopathy (DCM), a condition characterized by dilatation and impaired contraction of the heart that can lead to heart failure and sudden death [3]. Thus, to fully understand the biology and pathology of the heart, mechanisms of cellular crosstalk and their role in DCM must be investigated.

Our current understanding of the pathways that regulate EC-cardiomyocyte crosstalk is lacking. Importantly, we know that impaired release and/or activity of the molecules implicated in crosstalk can result in heart failure. For example, the knockout mouse of apelin, a protein released by ECs and important for a number of physiological functions, develops impaired contractility with age [4, 5]. The NRG1-ErbB signaling pathway represents another pertinent example. Interest in this pathway was generated following the observation that women treated with herceptin, a monoclonal antibody against the NRG-1 receptor ErbB2, have an increased risk for the development of heart failure [6]. Numerous studies have supported a role for NRG1 and ErbB2 in heart failure; the ventricular-specific knockout of ErbB2 develops DCM with age [7] and treatment with NRG-1 improves cardiac function in models of cardiomyopathy [8]. Finally, studies have suggested a role for hemodynamic stress,

hypoxia as well as mechanical load on cardiomyocytes and the extracellular matrix (ECM) in the development of DCM [9].

Mechanotransduction, or conversion of mechanical forces into biochemical or electrical signals, occurs in both cardiomyocytes and ECs [10-15]. Our own work has shown that the junctional cell adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM-1) functions as a mechanosensory protein in ECs and confers the ability to sense and respond to the hemodynamic force of flowing blood [10]. Although PECAM-1 initiates mechanosignaling specifically in ECs, its presence (or absence) can have profound consequences in the signals transmitted to other cell types, including vascular smooth muscle cells (VSMCs), and thus affect the physiology and pathology of the vessel as a whole. The PECAM-1$^{-/-}$ mouse is viable, but displays vascular defects in response to changes in hemodynamic forces. Specifically, PECAM-1$^{-/-}$ mice display impaired flow-mediated dilation due to mis-regulated NO production [16] and reduced VSMC relaxation [17, 18]. In addition, our lab has shown that PECAM-1 deficiency results in impaired flow-mediated vascular remodeling that results in reduced VSMC activation and signaling [19].

As EC-cardiomyocyte crosstalk is important in regulating cardiac function and cellular communication in the vessel wall is perturbed in the absence of PECAM-1, we hypothesized that cardiac function is impaired in PECAM-1$^{-/-}$ mice. Here, we show that the absence of PECAM-1 results in impaired cardiac function associated with misregulated NRG-1β signaling in ECs and downstream defects in pathways that regulate cardiomyocyte contractility.

Materials and Methods

Animals

PECAM-1$^{-/-}$ C57BL/6 mice were kindly provided by Dr P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee), bred in house and used in accordance with the guideline of the National Institute of Health and for the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Male PECAM-1$^{-/-}$ and age-matched wild-type (WT) littermates.

Cell Culture and Reagents

PECAM-1-knockout (PE-KO) cells and cells reconstituted with murine full-length PECAM-1 (PE-RC) were prepared as previously described [16, 20].

Echocardiography Measurements

Conscious echocardiography was performed as previously described using a Vevo 2100 ultrasound biomicroscopy system [21, 22]. All LV dimension data are presented as the average of at least 3 independent waveforms.

Transverse Aortic Constriction

8-week old male WT and PECAM$^{-/-}$ mice were used for either sham operation or pressure-overload induced by transverse aortic constriction as previously described [22, 23]. Heart function was measured by echocardiography at 1 and 4 weeks after surgery.

RNA isolation and Quantitative PCR

Total RNA was isolated from mouse left ventricles using the Qiagen AllPrep Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. First-strand cDNA was transcribed

using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using ABsolute SYBR Green ROX mix (Thermo Scientific). Primers used are found in Supplemental Table 3.3.

Preparation of Lysates and Immunoblot Analysis

Hearts were homogenized and protein was extracted in a RIPA lysis buffer (50mM HEPES, 150mM NaCl, 2mM EDTA, 0.5% Triton X-100, 0.5% Na-Deoxycholate, 1% NP-40, 25mM β-glycerophosphate, 10% glycerol, 1mM sodium orthovanadate, 1mM phenylsulphfonyl fluoride, 10μg/ml leupeptin, 10μg/ml aprotinin, 10mM sodium fluoride, 1mM sodium pyrophosphate, pH 7.2). Protein extracts (30μg) were subjected to Western blot analysis with antibodies against JNK, phospho-JNK, pTyr877ErbB2, pTyr1284ErbB4, PKA-C, and pThr197PKA, (Cell Signaling); pSer16PLN, PLN Clone 1 and GAPDH (Millipore). Vinculin was from Sigma. Immunocomplexes were detected using the Licor Odyssey secondary detection system. NRG-1β was a gift from D. Sawyer and used as previously described. Media was concentrated as previously described [24] using Amicon Ultra-15 with Ultracel-3 membrane (Millipore, Co, Bedford, MA).

Histological analysis and immunohistochemistry

Hearts were perfused with 4% paraformaldehyde for 24 hours and then switched to 70% ethanol. The hearts were then paraffin embedded and sectioned into 5μm sections and stained with hematoxylin and eosin to assess overall morphology [22]. For cross-sectional analysis of cardiomyocytes and determination of capillary density, heart sections were stained with TRITC-conjugated lectin (*Triticum vulgaris*) and examined by fluorescence microscopy as previously described [21, 22].

Cardiomyocyte Isolation/Functional Assays

Adult mouse cardiomyocyte isolation, experimentation and analysis from WT and PECAM-1$^{-/-}$ mice were performed as previously described [25, 26].

Transmission Electron Microscopy

For electron microscopy, animals were euthanized and perfused with freshly made fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH 7.4. After perfusion, the hearts were removed and cut into 1-2 mm^3 cubes and stored from several hours to overnight in the fixative before processing for electron microscopy. Sections were observed using a LEO EM910 transmission electron microscope operating at 80kV (Carl Zeiss SMT, Inc., Peabody, MA) and photographed using a Gatan Orius SC1000 Digital Camera and Digital Micrograph 3.11.0 (Gatan, Inc., Pleasanton, CA).

Quantitation and Statistical Analysis

The band intensity of immunoblots was quantitated using computer software (ImageJ). Each experimental group was analyzed using single factor analysis of variance (Excel; Microsoft). Probability values were obtained by performing a 2-tailed Student *t*-test using the same program. Statistical significance was defined as $P<0.05$.

Results

PECAM-1$^{-/-}$ mice exhibit both systolic and diastolic dysfunction indicative of dilated cardiomyopathy

To first assess the cardiac performance of PECAM-1$^{-/-}$ hearts, we performed transthoracic echocardiography in conscious 14-16 week old male mice (Figure 3.1). In the PECAM-1$^{-/-}$ mice, we observed a significant enlargement of the left ventricular (LV) chamber at end diastole (LVID;d) compared to WT mice (Figure 3.1A,B). Echocardiography also revealed

impairment of LV function in PECAM-1$^{-/-}$ hearts, as evidenced by decreased ejection fraction (EF) and fractional shortening (FS) (Figure 3.1C). Interestingly, there was no significant difference in wall thickness at end diastole (Supplemental Table 3.1). We did, however, observe an increase in left ventricular mass (Figure 3.1D). This phenotype was present at every post-natal time point evaluated (Figure 3.1E, F and Supplemental Table 3.2). In summary, the echocardiographic data suggest that PECAM-1$^{-/-}$ mice have LV chamber dilation and systolic dysfunction, suggestive of dilated cardiomyopathy (DCM).

Studies have shown that increased blood pressure (hypertension) can lead to heart failure [27, 28]. To exclude the possibility that differences in blood pressure account for the impaired cardiac function in the PECAM-1$^{-/-}$ mouse, we measured mean arterial pressures (MAP) in conscious 12-14 week old mice. There was no significance difference in pressures between genotypes (Supplemental Figure 3.1), suggesting that cardiac dysfunction in the PECAM-1$^{-/-}$ animals is not associated with hypertension.

Phenotypic characterization of PECAM-1$^{-/-}$ mice

Reactivation of the fetal gene program is a common marker for various cardiac pathologies, including hypertrophy and DCM [29]. We therefore assessed fetal gene expression in adult WT and PECAM-1$^{-/-}$ hearts by quantitative PCR. The expression of β-myosin heavy chain (βMHC), atrial natriuretic peptide (ANP) and α-skeletal actin (αSka) were significantly increased in PECAM-1$^{-/-}$ hearts compared with WT littermates (Figure 3.2A). Interestingly, the increase in βMHC levels was not accompanied by a corresponding decrease in αMHC expression; this switch in isoform expression is frequently observed in mouse models of DCM [30].

We next investigated baseline cardiomyocyte area and capillary density in WT and PECAM-1$^{-/-}$ hearts. Cardiomyocyte cross-sectional area measurements revealed that

cardiomyocytes in PECAM-1$^{-/-}$ hearts were slightly larger than WT cardiomyocytes (174.8 μm^2 vs. 162.8 μm^2), although this did not reach statistical significance (Figure 3.2B). Recent studies have suggested a role for defective vascularization in the pathogenesis of idiopathic DCM (IDCM)[3]. Because PECAM-1 is expressed in ECs, and ECs line blood vessel walls, we hypothesized that differences in cardiac vascularity might account for differences in cardiac function of PECAM-1$^{-/-}$ animals. There were no obvious defects in coronary artery structure or number detected in the PECAM-1$^{-/-}$ hearts (data not shown). We also measured capillary density by TRITC-lectin staining and did not observe any differences between genotypes (Figure 3.2B). Despite having normal vascular architecture, the possibility exists that there is impaired tissue perfusion, which could compromise cardiac function. To address this possibility, we performed staining for tissue hypoxia and did not find any indication of hypoxia in PECAM-1$^{-/-}$ heart tissue (Supplemental Figure 3.2).

The fundamental contractile unit of cardiac muscle is the sarcomere. Previous studies have shown that defects in sarcomere protein expression and structure can lead to DCM[31]. In order to determine if alterations in sarcomere structure could explain the impaired contractility of the PECAM-1$^{-/-}$ hearts, we performed transmission electron microscopy (TEM). We did not detect any differences sarcomere organization or size between the WT and PECAM-1$^{-/-}$ mice (Figure 3.2C). When taken together with the vascular data, our studies suggest that the impaired cardiac function in the PECAM-1 null animals is not due to structural or architectural defects in the heart. This leads us to hypothesize that impaired EC-cardiomyocyte communication may be responsible for the observed phenotype.

Increased systolic dysfunction in PECAM-1$^{-/-}$ mice after biomechanical stress

To further explore the role of PECAM-1 in regulating cardiac function, we subjected PECAM-1$^{-/-}$ mice to transverse aortic constriction (TAC), a well-established model of

pressure overload. Following partial ligation of the aorta, there is an increase in biomechanical and hemodynamic stress on the heart as well as increased demand on the cardiomyocytes. This increased stress induces the formation of concentric hypertrophy as a mechanism of compensation [23]. In addition to effects on cardiomyocytes, the hemodynamic environment in the coronary vessels is also perturbed [32]. Previous studies have shown that PECAM-1$^{-/-}$ mice have impaired blood flow-mediated responses including impaired vascular remodeling and flow-mediated dilation [17, 19, 33]. Based on these studies, we hypothesized that PECAM-1$^{-/-}$ mice would have impaired remodeling after TAC. As expected, WT mice subjected to TAC displayed increased thickness of both the intraventricular septum and posterior wall (Figure 3.3A, B; Supplemental Table 1), accompanied by preservation of FS and EF (Figure 3.3C, D). In contrast, 4 weeks after TAC, PECAM-1$^{-/-}$ mice did not exhibit any significant increase in LV wall thickness. Additionally, these animals had a further increase in LV chamber size (Supplemental Table 3.1) and a significant deterioration in cardiac function (Figure 3.3C).

To assess morphological changes to the heart tissue after TAC, we analyzed the cross-sectional area of cardiomyocytes from WT and PECAM-1$^{-/-}$ hearts (Figure 3.4A). After 4 weeks of TAC, WT mice showed a significant increase in cardiomyocyte area, whereas the hypertrophic response was impaired in the PECAM-1$^{-/-}$ hearts (316.0 μm^2 vs. 247.5 μm^2) (Figure 3.4B). Similarly, we observed an increase in capillary density in WT mice after TAC, but this response was blunted in PECAM-1$^{-/-}$ mice (Figure 3.4B). There was no difference in fibrosis observed between genotypes (Supplemental Figure 3.4).

Another common feature of pathological hypertrophy is upregulation of fetal gene expression [29, 34]. We measured changes in gene expression after 4 weeks of TAC in the hearts (Figure 3.4C). Expression of βMHC, ANP, and αSka were increased in both WT and PECAM-1$^{-/-}$ hearts 7 days after TAC, although this increase was significantly reduced in

PECAM-1$^{-/-}$ animals (14-fold in WT vs. 4-fold in PECAM-1$^{-/-}$ for βMHC). This impaired activation is in agreement with the reduced hypertrophic response seen in the PECAM-1$^{-/-}$ mice (Figure 3.3). To gain insights into signaling pathways that might be affected in the absence of PECAM-1, we examined the MAPK pathway, which is implicated in mediating the development of cardiac hypertrophy [35, 36]. Immunoblots of heart lysates revealed blunted JNK phosphorylation in the PECAM-1$^{-/-}$ hearts after TAC compared to WT hearts (Figure 3.4D). There was no difference in activation of other hypertrophic regulators between genotypes.

Cardiomyocytes isolated from PECAM-1$^{-/-}$ hearts display normal contractility

Endothelial cell-cardiomyocyte crosstalk is thought to play an important role in regulating cardiac function [2]. As PECAM-1 is not expressed in cardiomyocytes (Supplemental Figure 3.3) [37, 38], we reasoned that the absence of PECAM-1 in ECs might lead to impaired EC-cardiomyocyte communication. Accordingly, we hypothesized that cardiomyocytes isolated from PECAM-1$^{-/-}$ hearts should behave like cardiomyocytes isolated from WT hearts. Basal rates of contraction and relaxation of cardiomyocytes isolated from PECAM-1$^{-/-}$ hearts were indistinguishable from those isolated from WT hearts. Additionally, we observed no differences in the maximum and minimum cardiomyocyte length (Table 3.1). These findings seem to be at odds with those made by echocardiography, which revealed impaired systolic and diastolic function in PECAM-1$^{-/-}$ hearts. They suggest that cardiomyocytes, when isolated and in the absence of EC input, are capable of contracting normally, whether they are isolated from PECAM-1$^{-/-}$ or WT hearts. Interestingly, the PECAM-1$^{-/-}$ cardiomyocytes have an enhanced response to isoproterenol treatment. The significance of this observation is currently being investigated.

Impaired Neuregulin-ErbB signaling in the PECAM-1$^{-/-}$ mice

A complex signaling network exists between cell types within the heart. Crosstalk between ECs and cardiomyocytes is mediated, in part, by secreted factors such as VEGF, NO, and NRG1 [2]. Interestingly, NRG1 and ErbB knockout mice display defects in cardiac function, including DCM [39, 40]. NRG-1 is a member of the EGF family of proteins and is able to bind and activate ErbB receptors within the heart. Upon activation, ErbB receptors signal through phosphoinositide-3 kinase and Akt to modulate cardiomyocyte contractility [41, 42]. It has previously been shown that release of the NRG1β isoform from ECs is mediated by reactive oxygen species (ROS) [43]. Furthermore, coronary arteries from PECAM-1 null mice have increased ROS production [17]. We therefore investigated if the impaired contractility in PECAM-1$^{-/-}$ mice is associated with defects in the NRG1 pathway. We first assayed ROS production *in vitro* using PECAM-1 knockout (PE-KO) and PECAM-1 expressing (PE-RC) ECs. There was a significant increase in ROS production from the PE-KO cells correlating with observations *in vivo* (Figure 3.5A). To determine levels of NRG1 release from ECs, we measured NRG-1β release by immunoblot analysis of conditioned cell media from PECAM-1 knockout (PE-KO) and PECAM-1 expressing (PE-RC) ECs. Interestingly, we observed increased NRG-1β in the media of PE-KO cells compared to PE-RC cells (Figure 3.5B). To determine if these observations translated *in vivo*, heart lysates from WT and PECAM-1$^{-/-}$ animals were assessed for NRG-1β release. Similar to our observations *in vitro,* we found higher levels of NRG-1β in PECAM-1$^{-/-}$ hearts (Figure 3.5C).

We next wanted to determine if the increased NRG-1β levels correlated with increased activity of its receptor. Importantly, we observed increased phosphorylation of ErbB2 in PECAM-1$^{-/-}$ hearts (Figure 3.5D). These data suggest that misregulated NRG-1β release in the PECAM-1$^{-/-}$ hearts could impair cardiac contractility via overactivation of ErbB receptor signaling in cardiomyocytes. Importantly, we did not observe any difference

between genotypes in expression of either total NRG1 or ErbB2 levels by both Western blot and quantitative PCR (data not shown).

NRG1/ErbB signaling affects the activity of proteins that mediate calcium handling from the sarcoplasmic reticulum of cardiomyocytes and proper regulation of these proteins is required for normal cardiac contractility. Protein kinase A (PKA) and phospholamban (PLN), a regulator of the Ca^{2+}-ATPase (SERCA2), are two important proteins in this process. Previous studies have shown that treatment of cardiomyocytes with NRG1 induces an increase in PLN phosphorylation [44]. Additionally PKA is known to mediate PLN phosphorylation on serine residue 16 [45]. We performed Westerns blots on heart tissue to assess PLN phosphorylation status and found a significant increase in pSer16PLN in PECAM-$1^{-/-}$ compared to WT hearts (Figure 3.5E,F). Similar to PLN, we found significant increases in pThr147PKA in the PECAM-$1^{-/-}$ hearts (Figure 3.5E and 3.5G). Importantly, levels of phosphorylation of threonine residue 17, a Ca^{2+}-calmodulin-dependent protein kinase II (CAMKII)-dependent phosphorylation site, were unaffected in the PECAM-$1^{-/-}$ (data not shown). Overall, these data suggest that impaired NRG-1 signaling in PECAM-$1^{-/-}$ hearts may impair cardiac contractility through mis-regulation of PKA and PLN.

Discussion

The findings presented here support a model in which PECAM-1, expressed in ECs and not cardiomyocytes, is able to modulate cardiomyocyte signaling and function, both under baseline conditions and in response to hemodynamic stress (Figure 3.5H). In the absence of PECAM-1, we observed increased NRG-1β release and concomitant increased phosphorylation of ErbB receptors. It is well established that signaling downstream of the ErbB receptors influences calcium handling and cardiomyocyte function [44]. Therefore, we propose that the absence of PECAM-1 facilitates increased NRG1β release from ECs, which

binds to the ErbB family of receptors on cardiomyocytes, leading to misregulated calcium handling and, ultimately, defects in cardiac contractility. It should be noted that mutations to PLN can lead to DCM in both human and mouse models [46, 47]. Additionally, constitutive activation of PKA can lead to impaired contractility and DCM [48].

The role of cardiomyocytes in regulating adult cardiac function has been well documented. This regulation is clinically relevant as mutations in sarcomere protein genes account for 10 percent of familial DCM [49]. Additionally, mouse models with mutations in sarcomere protein often develop severe forms of DCM (reviewed in [50]). Because the PECAM-1$^{-/-}$ animals have normal sarcomere structure, it is not surprising that they exhibit a mild form of DCM. In addition to normal sarcomere structure, the vasculature appears unperturbed, which is another potential risk factor for DCM development [51-53]. The fact that there are no obvious structural disruptions to either network implies that misregulated cellular communication results in impaired cardiac function. It is also possible that the PECAM-1$^{-/-}$ phenotype is mild due to compensation for the increased NRG-1/ErbB signaling. One result that points to this possibility is the isoproterenol data. Although both genotypes had a positive inotropic response of cardiomyocytes to isoproterenol (Table 3.1) this response was significantly enhanced in cardiomyocytes isolated from PECAM-1$^{-/-}$ hearts, suggestive of upregulation of the β-adrenergic pathway. This would not be surprising, as increased NRG1 is associated with decreased contractility through the muscarinic pathway [54], and proper coordination of these two pathways is required for contractility and efficient heart function.

Although we cannot exclude the possibility that other PECAM-1-expressing cell types may contribute to the impaired systolic function in the PECAM-1$^{-/-}$ mice, several pieces of data argue against this idea. First, previous studies have shown that there are no differences in either leukocyte or platelet counts in PECAM-1$^{-/-}$ mice [55]. Second, on a

C57Bl/6 background, no differences in platelet activation [56] or leukocyte emigration have been observed in PECAM-1$^{-/-}$ animals [55]. In addition, each cardiomyocyte is intimately associated with the surrounding EC-derived vasculature facilitating a unique interface for cellular crosstalk.

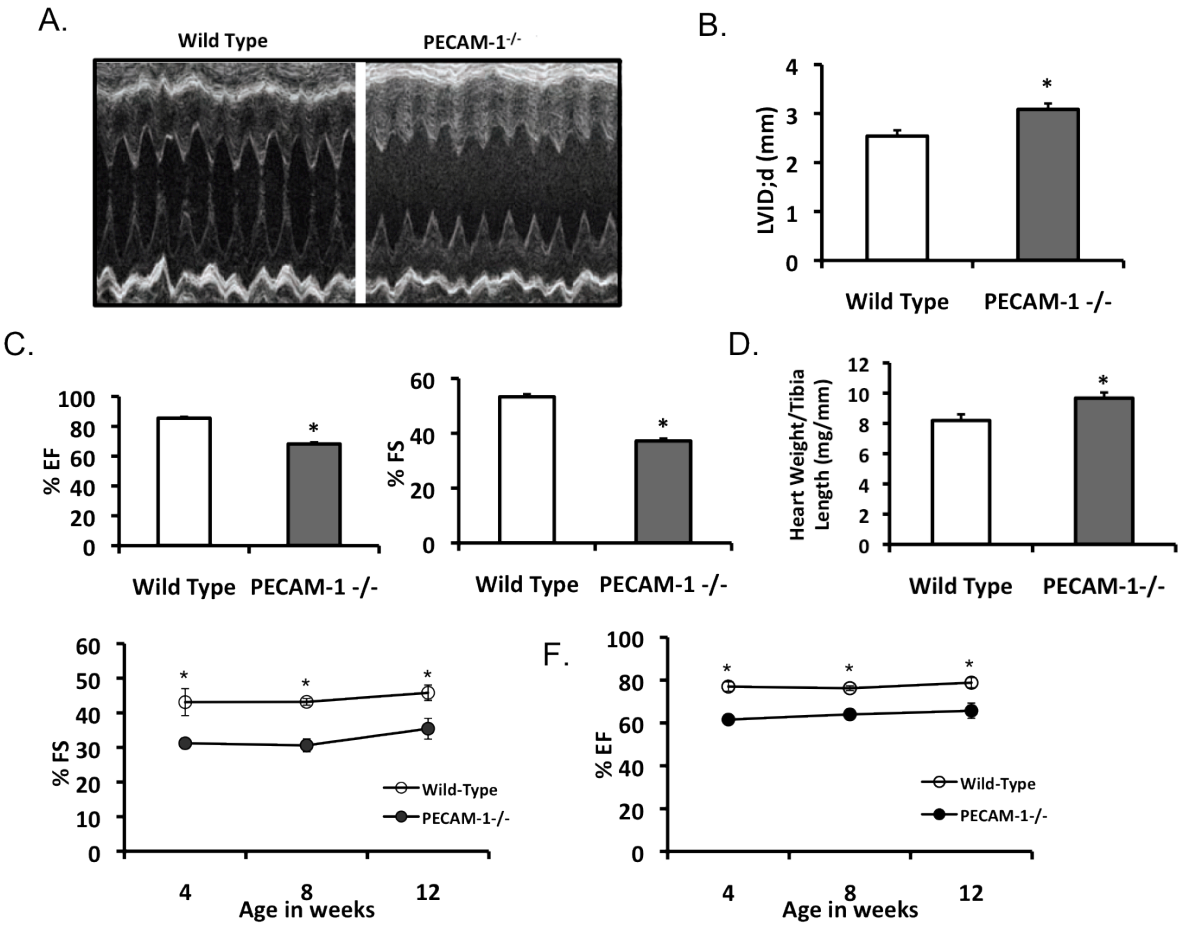
To date, the rather intuitive, consensus in the field is that EC signaling can affect global heart function. However, studies supporting this thinking are limited. There are only a handful of reports of EC-specific knockout animals demonstrating impaired cardiac contractility. A role for vascular density in regulating heart growth and function has recently been reported [57]. Importantly, not all EC knockout models display a basal cardiac phenotype. For example, the HIF2α EC-specific knockout has normal contractility, but once challenged by TAC, there is rapid decompensation to heart failure [58]. Previously, work in the caveolin-1$^{-/-}$ global KO mouse showed development of DCM at 5 months of age [59]. This was attributed to misregulation of eNOS/NO signaling in non-myocytes, a signaling pathway thought to play an important role in EC-cardiomyocyte crosstalk. However, additional studies suggest that Cav1 is required in fibroblasts [60]. In general, although it is accepted that ECs release molecules that can affect cardiomyocyte function, the mechanisms that regulate this release remain unclear.

Another important observation from our studies is the impaired remodeling in the PECAM-1$^{-/-}$ mice after TAC. This phenotype is likely due to a mechanism independent of the impaired NRG-1β signaling. We know from our previous studies that PECAM-1$^{-/-}$ animals have impaired remodeling in response to changes in hemodynamic stress in models of carotid artery ligation and impaired collateral remodeling. In these models, the absence of PECAM-1 prevents the detection of changes in blood flow and subsequent activation of signaling pathways. It is likely that a similar phenomenon is occurring in the heart, as TAC dramatically changes the hemodynamic environment.

Currently, our understanding of the role of shear stress and hemodynamic forces in regulating heart function is limited. Studies in zebrafish have shown that manipulating the flow environment, either genetically or mechanically, has profound effects on heart formation [61]. Additionally, loss of the primary cilium in mouse causes significant changes to heart formation [62]. Our data suggest that the PECAM-1$^{-/-}$ mouse is a relevant model to study the role of shear stress on adult heart function. When the hemodynamic environment in manipulated (via TAC), PECAM-1$^{-/-}$ animals show impaired remodeling compared to WT animals. Using this model may help elucidate signaling pathways that are required in shear stress signaling in both cardiac endothelial cells and the heart. Additionally, this study furthers our understanding of the potential causes of DCM.

Figures

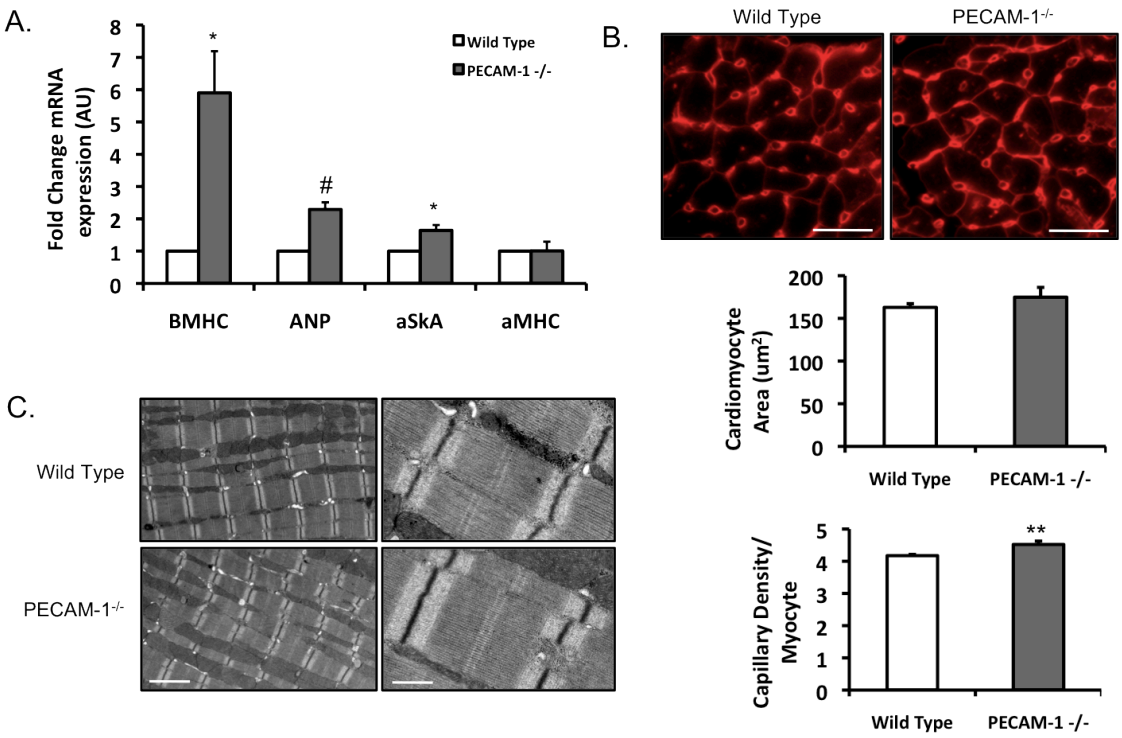
Figure 3.1. PECAM-1$^{-/-}$ mice have increased chamber size with systolic and diastolic dysfunction.



A. Representative image of M-Mode echocardiography for baseline heart function in age-matched adult WT and PECAM-1$^{-/-}$ mice. B-C. Echocardiographic measurement assessment of left ventricular dilation and cardiac function (ejection fraction, %EF, fractional shortening, %FS). Echocardiography results represent at least 10 mice per group ($^{*}P<0.01$). Measurements were collected at the level of the papillary muscle in M-mode. D. Analysis of left ventricle weight to tibia length to normalize left ventricular mass (n=10/genotype,

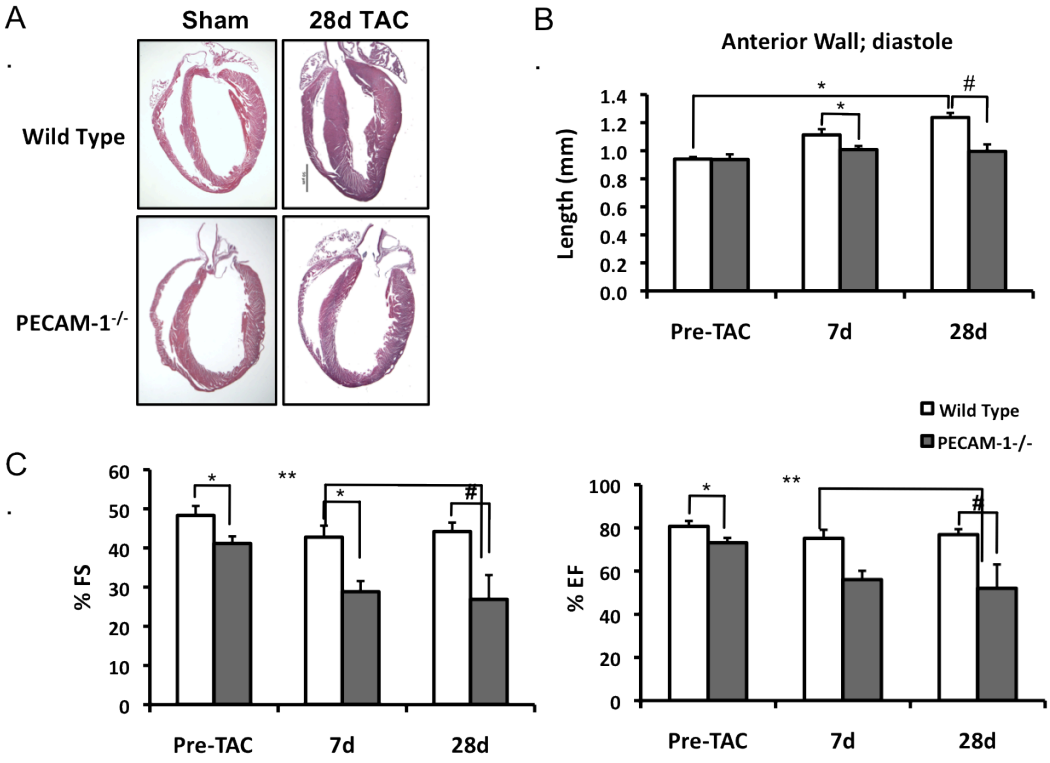
P<0.005). E-F. Echocardiographic measurement assessment of left ventricular function by fractional shortening (E) and ejection fraction (F). Echocardiography results represent at least 10 mice per group (P<0.01).

Figure 3.2. Normal cellular architecture in PECAM-1$^{-/-}$ hearts.



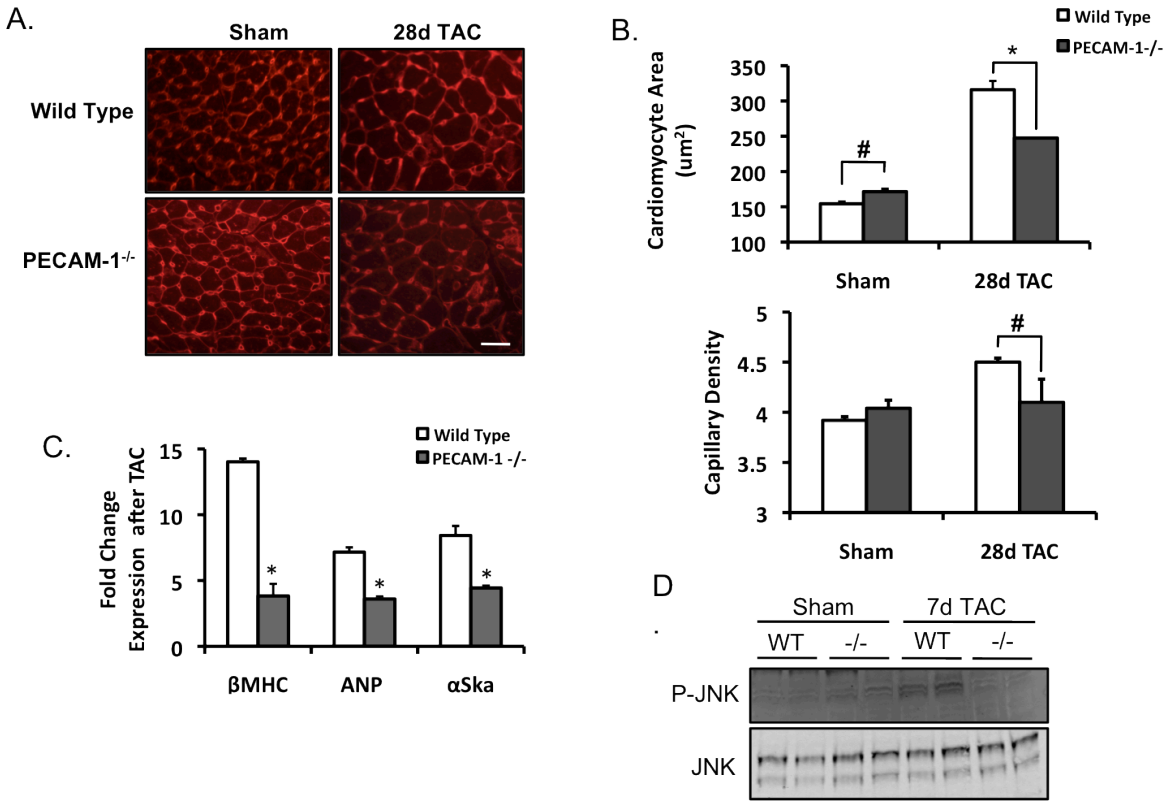
A. Quantitative real-time RT-PCR of cardiac fetal gene expression in 12-week-old male mice. All mRNA species were quantified relative to 18s housekeeping gene expression and presented as fold change (arbitrary units [AU]) relative to WT mice. (n=3/genotype, $^{*}P<0.01$ $^{\#}P<0.05$). βMHC indicates β-myosin heavy chain; ANP, atrial natriuretic peptide; αSka, α skeletal actin; αMHC, α myosin heavy chain. B. TRITC-lectin stained sections for baseline cardiomyocyte cross-sectional area and capillary density in WT and PECAM-1$^{-/-}$ mice. Scale bar 20μm (n=3-4/genotype, $^{**}P<0.01$). C. The ultrastructure of cardiac muscle from WT and PECAM-1$^{-/-}$ mice by transmission electron micrograph. 10000X and 25000X representative images shown, scale bars 2μm and 1μm, respectively.

Figure 3.3. PECAM-1$^{-/-}$ mice have impaired response to TAC.



A. 8-week-old WT and PECAM-1$^{-/-}$ mice underwent either sham or TAC surgeries. Representative H&E stained sections are shown. B. Left ventricular anterior wall dimensions in diastole for sham, 7 day and 28 day WT and PECAM-1$^{-/-}$ hearts (n= 6/genotype for sham, n=10/genotype for TAC, $^{*}P<0.001$ $^{\#}P<0.005$) C. Fractional shortening and ejection fraction for sham, 7 day and 28 day hearts (n= 6/genotype for sham, n=10/genotype for TAC, $^{*}P<0.005$ $^{**}P<0.02$, $^{\#}P<0.05$).

Figure 3.4. Impaired cellular activation and remodeling after TAC in PECAM-1$^{-/-}$ mice.



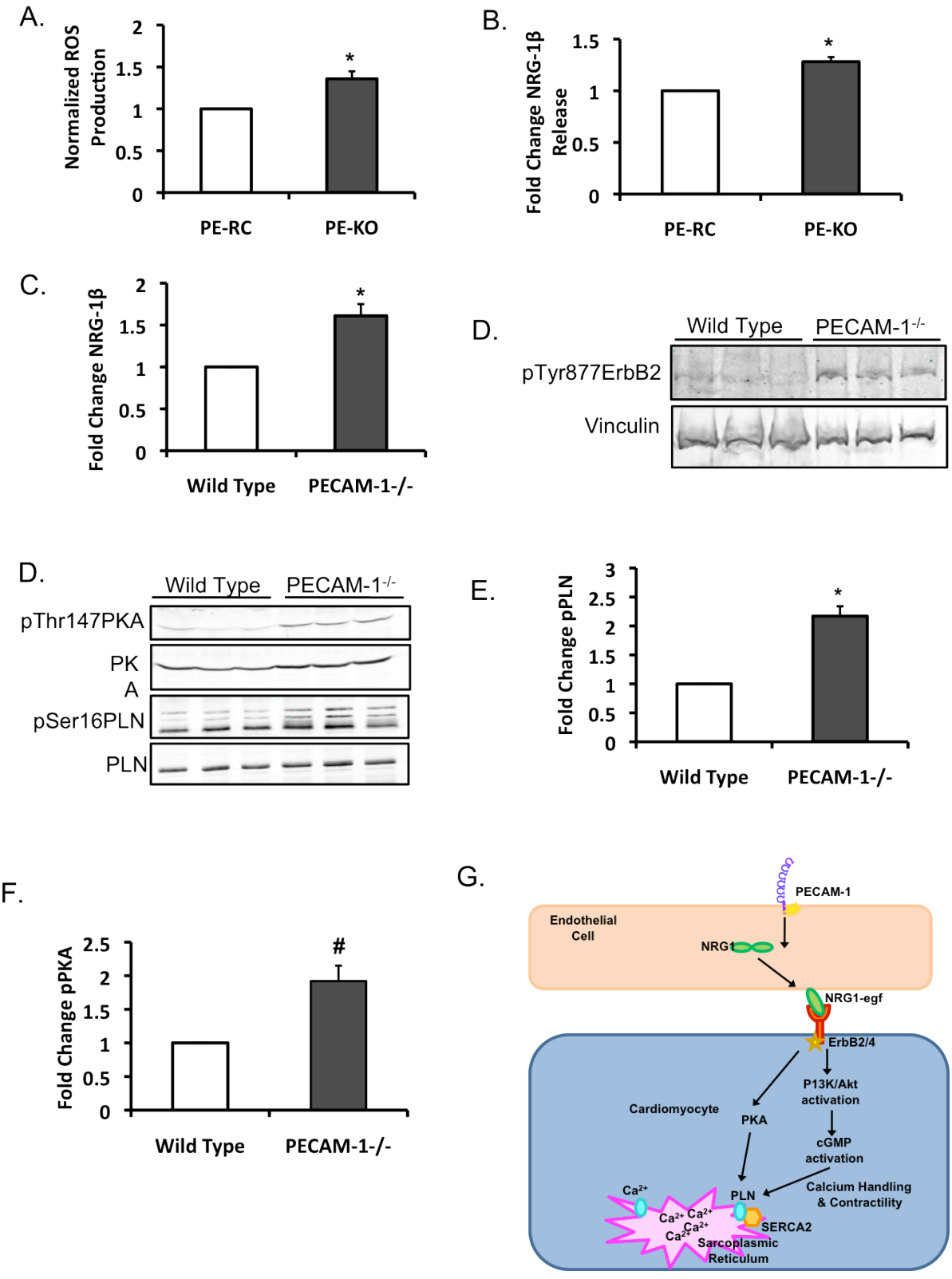
A. TRITC-lectin staining of sham and 4 week TAC WT and PECAM-1$^{-/-}$ hearts. B. Quantitation of staining in A for baseline and 28 days post-TAC cardiomyocyte cross-sectional area and capillary density in WT and PECAM-1$^{-/-}$ mice (n=3-4/genotype, $^{*}P<0.001$, $^{\#}P<0.05$). C. Quantitative real-time RT-PCR of cardiac fetal gene expression in 12-week-old male mice. All mRNA species were quantified relative to 18s housekeeping gene expression and presented as fold change (arbitrary units [AU]) relative to sham mice. (n=3/genotype, $^{*}P<0.01$). D. Representative Western blot for pThr183/pTyr185JNK in sham and 7d post-TAC WT and PECAM-1$^{-/-}$ hearts.

Table 3.1. Contractile parameters in adult myocytes isolated from WT and PECAM-1$^{-/-}$ mice

	Wild-Type (n = 6)		PECAM-1$^{-/-}$ (n = 7)	
	Basal	Iso (10^{-7} M)	Basal	Iso (10^{-7} M)
-dL/dt (μM/ms)	-0.283±0.02	-0.365±0.01	-0.299±0.02	-0.419±0.01[a]
+dL/dt (μM/ms)	0.259±0.02	0.303±0.01	0.280±0.01	0.357±0.01[b]
Maximum Length (μM)	126.4±2.8	126.9±3.7	128.2±3.6	134.4±3.6
Minimum Length (μM)	105.4±2.7	97.9±2.6	105.6±2.6	103.22±3.4

[a] $p<0.015$ PECAM-1-/- vs WT
[b] $p<0.005$ PECAM-1-/- vs WT

Figure 3.5. Misregulated NRG-1/ErbB signaling in PECAM-1$^{-/-}$ hearts.



A. Confluent monolayers of PE-RC and PE-KO cells were serum-starved for 24 hours then loaded with 2,7-H_2DCFDA (10μmol/L for 30 minutes at 37°C) then lysed. Fluorescence was measured using a plate reader. Fluorescence was normalized to total protein levels in the lysate (n=7, $^{*}P<0.01$). B. Quantitation of Western blot for NRG-1β release from PE-RC and PE-KO cells. Media was collected after 24hrs and concentrated. Concentrated media was then run on a polyacrylamide gel (n=5, $^{*}P<0.05$). C. Quantitation of Western blot for NRG-1β in heart tissue from WT and PECAM-1$^{-/-}$ hearts. (n=3/genotype, $^{*}P<0.05$). D. Representative Western blots for phospho-ErbB2 (n=6/genotype). E. Levels of pSer16PLN and pThr147PKA were assayed by Western blot. Samples were normalized to total PLN and total PKA respectively. Quantitation is shown for pSer16PLN in (F) and pThr147PKA in (G) (n=6/genotype, $^{*}P<0.03$ $^{\#}P<0.005$). H. Model of PECAM-1 regulation of cardiac contractility.

Supplemental Materials and Methods

Blood pressure measurements

Blood pressure was non-invasively measured by determining the tail blood volume with a volume pressure-recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT).

Tissue hypoxia staining

Tissue hypoxia was assessed using the Hypoxyprobe™-1 Kit (Hypoxyprobe, Inc. Burlington, MA) according to the manufacturer's instructions and as previously described [63]. Briefly, animals were injected i.p. with 60mg/kg hypoxyprobe for 60 minutes. Hearts were then harvested and fixed in 4% PFA, embedded in paraffin and sectioned at 8 μm. Tissue sections were stained with the Hypoxyprobe-1 antibody and visualized using DAB.

Supplemental Figure Legends

Supplemental Table 3.1. Echocardiographic data from WT and PECAM-1$^{-/-}$ mice pre- and post-TAC

	Wild Type (n=5-12)		PECAM-1$^{-/-}$ (n=5-12)	
	Pre-TAC	28d TAC	Pre-TAC	28d TAC
Heart Rate (BPM)	710 ± 15.45	680 ± 8.8	660 ± 12.5[b]	660 ± 10.6
IVS;d (mm)	1.01 ± 0.02	1.23 ± 0.3[e]	1.06 ± 0.02	0.99 ± 0.05[d]
IVS;s (mm)	1.70 ± 0.02	1.86 ± 0.04	1.58 ± 0.02	1.41 ± 0.16
LVPW;d (mm)	0.96 ± 0.01	1.20 ± 0.05[e]	0.99 ± 0.02	0.99 ± 0.05
LVPW;s (mm)	1.52 ± 0.02	1.65 ± 0.04	1.4 ± 0.03	1.25 ± 0.06
LVID;d (mm)	2.94 ± 0.17	2.79 ± 0.1	3.29 ± 0.12[b]	3.43 ± 0.31
LVID;s (mm)	1.38 ± 0.10	1.56 ± 0.11	2.06 ± 0.08	2.54 ± 0.4
LV Vol;d (µl)	34.9 ± 5.1	29.6 ± 2.7	44.6 ± 3.9	49.6 ± 11.2
LV Vol;s (µl)	5.33 ± 1.0	7.04 ± 1.3	14.2 ± 1.4	28.48 ± 12
EF%	85.5 ± 0.87	76.8 ± 2.5	68.2 ± 1.14[a]	52.0 ± 9.8[c]
FS%	48.3 ± 2.4	44.2 ± 2.2	37.2 ± 0.88[a]	26.8 ± 6.2[c]

HR, heart rate; IVS, interventricular septum; LVPW, left ventricular posterior wall; LVID, left ventricular internal dimension; LV vol, left ventricular volume; EF, ejection fraction; FS, fractional shortening.

[a] $P<0.005$ PECAM-1$^{-/-}$ vs WT pre-TAC
[b] $P<0.01$ PECAM-1$^{-/-}$ vs WT pre-TAC
[c] $P<0.05$ PECAM-1$^{-/-}$ vs WT 28d TAC
[d] $P<0.05$ PECAM-1$^{-/-}$ vs WT 28d TAC
[e] $P<0.05$ WT pre- vs 28d TAC

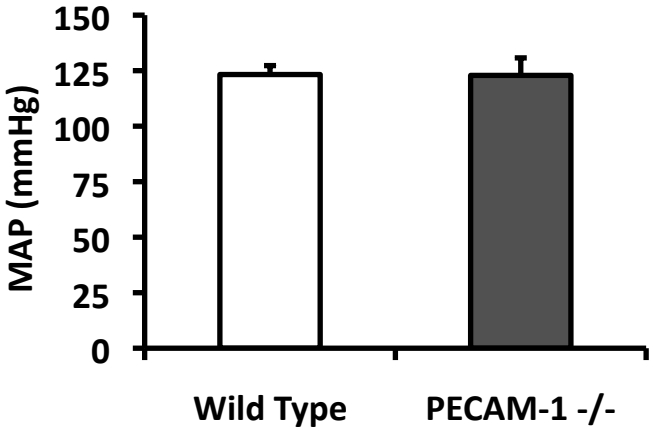
Supplemental Table 3.2. Echocardiographic data from aging WT and PECAM-1$^{-/-}$ mice

	Wild Type (n=10)			PECAM-1$^{-/-}$ (n=10)		
	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Heart Rate (BPM)	684 ± 24.4	737 ± 8.23	725 ± 9.3	612 ± 24.3	664 ± 22.1	678 ± 17.1
IVS;d (mm)	0.73.3 ± 0.01	0.88 ± 0.03	0.98 ± 0.03	0.70 ± 0.03	0.86 ± 0.02	0.99 ± 0.02
IVS;s (mm)	1.21 ± 0.03	1.46 ± 0.03	1.62 ± 0.02	1.07 ± 0.05[c]	1.35 ± 0.04[c]	1.57 ± 0.04
LVPW;d (mm)	0.72 ± 0.02	0.84 ± 0.03	0.91 ± 0.03	0.62 ± 0.03[a]	0.82 ± 0.04	0.92 ± 0.04
LVPW;s (mm)	1.15 ± 0.04	1.33 ± 0.04	1.49 ± 0.05	0.93 ± 0.02[a]	1.2 ± 0.06	1.34 ± 0.05
LVID;d (mm)	2.42 ± 0.1	2.69 ± 0.07	2.89 ± 0.05	2.77 ± 0.1[c]	3.33 ± 0.2[a]	3.19 ± 0.13[c]
LVID;s (mm)	1.36 ± 0.08	1.5 ± 0.05	1.53 ± 0.06	1.89 ± 0.09[a]	2.19 ± 0.2[a]	2.08 ± 0.1[a]
LV Vol;d (µl)	21.3 ± 1.87	27.1 ± 1.68	31.2 ± 1.25	29.5 ± 2.8[c]	46.8 ± 6.0[a]	42.5 ± 4.1[c]
LV Vol;s (µl)	4.9 ± 0.6	6.17 ± 0.5	6.52 ± 0.63	11.4 ± 1.4[a]	14.9 ± 3.2[a]	14.7 ± 2.5[b]
EF%	76.9 ± 2.2	77.3 ± 1.0	79.8 ± 1.5	61.6 ± 2.1[a]	64.9 ± 2.5[a]	65.2 ± 2.4[a]
FS%	44.1 ± 2.0	44.3 ± 0.9	47.2 ± 1.5	31.9 ± 1.5[a]	34.7 ± 1.7[a]	34.0 ± 1.8[a]

HR, heart rate; IVS, interventricular septum; LVPW, left ventricular posterior wall; LVID, left ventricular internal dimension; LV vol, left ventricular volume; EF, ejection fraction; FS, fractional shortening.

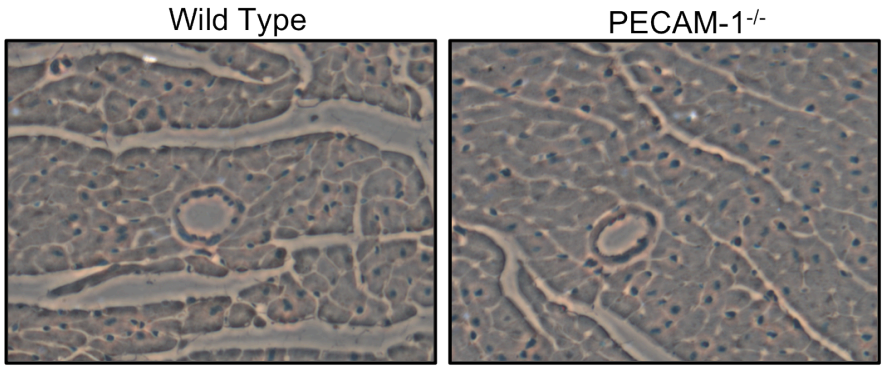
[a]$P<0.005$, [b]$P<0.01$, [c]$P<0.05$ All statistics were calculated PECAM-1$^{-/-}$ vs WT at corresponding age

Supplemental Figure 3.1. Mean arterial pressure (MAP) from PECAM-1$^{-/-}$ mice similar to WT mice.



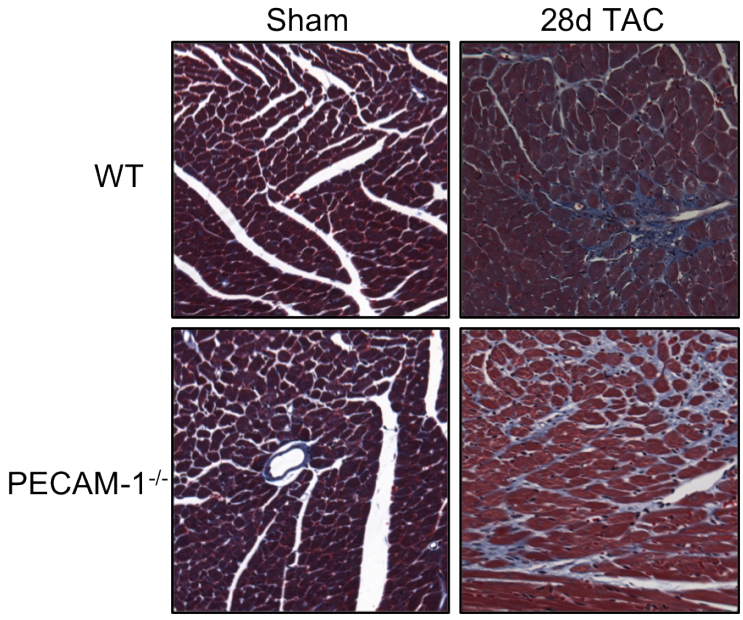
Blood pressures from WT and PECAM-1$^{-/-}$ mice were performed by tail-cuff measurement (n=9-10/genotype).

Supplemental Figure 3.2. No evidence for hypoxia in PECAM-1$^{-/-}$ hearts



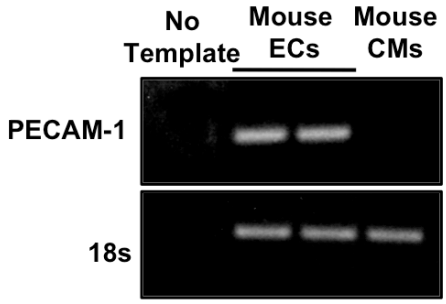
WT and PECAM-1$^{-/-}$ were injected with Hypoxyprobe-1^{TM} 60 minutes prior to sacrifice. The tissue was fixed, paraffin-embedded and sectioned. There was no apparent positive staining in either genotype.

Supplemental Figure 3.3. No difference in fibrosis between genotypes after TAC.



Masson's trichrome staining in either sham or 28 days after TAC in WT and PECAM-1$^{-/-}$ mice to look at fibrosis.

Supplemental Figure 3.4. Adult mouse cardiomyocytes do not express PECAM-1.



qPCR was performed on RNA isolated from two lines of mouse endothelial cells as well as mouse cardiomyocytes (HL1) cells. No expression was detected in the CMs compared to the robust signal from the endothelial cells.

Supplemental Table 3.3. ***Primers used for quantitative PCR***

Primers		Sequence
ANP	For	5’ TGGGACCCCTCCGATAGATC 3’
	Rev	5’ TCGTGATAGATGAAGGCAGGAA 3’
βMHC	For	5’ TTGAGAATCCAAGGCTCAGC 3’
	Rev	5’ CTTCTCAGACTTCCGCAGGA 3’
αMHC	For	5’ CTACGCGGCCTGGATGAT 3’
	Rev	5’ GCCACTTGTAGGGGTTGAC 3’
αSK-Actin	For	5’ CAGCTCTGGCTCCCAGCACC 3’
	Rev	5’ AATGGCTGGCTTTAATGCTTCA 3’
18s	For	5’ CATTCGAACGTCTGCCCTATC 3’
	Rev	5’ CCTGCTGCCTTCCTTGGA 3’
PECAM-1	For	5’ CACCTCGAAAAGCAGGTCTC 3’
	Rev	5’ CGTTATACACCATCGCATCG 3’

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CHAPTER IV.

Conclusions and Perspectives

Overview

In chapters II and III of this dissertation, I have identified two new aspects of PECAM-1 biology: 1. Regulation of eNOS activity via a novel mechanistic pathway and 2. A role for PECAM-1 in regulation of heart function via modulation of EC-CM crosstalk. PECAM-1 was originally identified in 1990 as a glycoprotein expressed on the surface of endothelial cells (ECs) [1]. Over the past 22 years, we have learned a tremendous amount about its complex biology, which ranges from junctional adhesion molecule to a more recently identified role as an endothelial mechanosensor. My work, described in Chapter II, identifies a novel pathway by which PECAM-1 modulates eNOS localization and activity in ECs. In Chapter III, I describe the mechanism through which PECAM-1 expression leads to normal cardiac function. This is a novel role for PECAM-1, as relatively little attention has been paid to its role in organ systems, due in part to the viability and phenotypic normality of the PECAM-1$^{-/-}$ mouse.

While both Chapter II and III provide novel insights into PECAM-1 biology, they also highlight important underlying concepts in the field of vascular biology. In this chapter I will address relevant topics and unanswered questions generated by my research, such as the

role of shear stress both *in vitro* and *in vivo*. I conclude with a discussion of how my work furthers our understanding of cell signaling in the cardiovascular system.

CHAPTER II: PECAM-1 REGULATES eNOS ACTIVTY AND LOCALIZATION THROUGH STAT3-DEPENDENT NOSTRIN EXPRESSION

In Chapter II, I set out to identify the mechanisms by which PECAM-1 regulates basal eNOS activity. Ultimately my research identified a novel-signaling pathway in ECs that regulates eNOS function. However, despite thorough characterization of this pathway, several important questions remain, including definition of a role for shear stress and the mechanism by which JAK/STAT signaling regulates NOSTRIN expression and subsequent eNOS activity.

The role of shear stress

One of the most significant inputs to the endothelium is the frictional shear stress of blood flow. Shear stress plays an integral role in regulating both normal physiology, as well as vascular pathologies including the development of atherosclerosis [2]. As early as the 1960s, researchers and physicians recognized the non-uniform distribution of atherosclerotic lesions; they preferentially formed in large arteries at branch points or areas of high curvature, such as the inner curve of the aortic arch [3]. It was hypothesized that hemodynamic force, or shear stress, was responsible for this focal distribution. In these regions of the vasculature, blood flow, and consequently shear stress, is low and disturbed. A considerable amount of research has been done in the last 50 years to identify how the vessel, and more specifically ECs, can detect differences in shear stress. We now know that ECs are equipped with mechanosensory molecules that detect mechanical forces such, as

shear stress, and trigger diverse EC signaling cascades in response to distinct mechanical inputs.

Of particular interest to our lab is a recently described mechanosensory complex consisting of PECAM-1, VE-Cadherin, and VEGFR2[4]. In this complex, PECAM-1 functions as the force transducer, converting shear stress into an intracellular response. In the absence of PECAM-1, ECs are unable to activate shear stress-responsive signaling pathways such as Akt and Erk1/2 [4]. Thus, both the PECAM-1$^{-/-}$ animals, as well as ECs isolated from these mice, are useful tools for identifying the role of shear stress in physiologically relevant pathways[5, 6]. For my dissertation research, I have utilized these tools to answer questions about PECAM-1 cardiovascular biology.

PECAM-1 is required for eNOS activation in response to shear stress; there is no increase eNOS phosphorylation in ECs lacking PECAM-1 (PE-KO). However, my research revealed that despite the requirement for PECAM-1 in response to shear, PE-KO cells have higher basal levels of eNOS phosphorylation, which correlates with increased NO production. This was an intriguing observation and led us to characterize a novel mechanism by which PECAM-1 regulates eNOS activity and localization through the eNOS trafficking protein NOSTRIN described in Chapter II. These observations, coupled with what we know about PECAM-1 biology, lead us to ask whether shear stress plays a role in mediating NOSTRIN expression.

NOSTRIN was first described a decade ago, and the majority of work done in the years since has focused on its role in directing eNOS localization and activity[7]. More recent work has identified a role for NOSTRIN in FGF-mediated signaling and vascular development independent of its association with eNOS[8]. Given that most studies have focused on the function of the protein, relatively little is known about how NOSTRIN expression is regulated. My work has shown that NOSTRIN expression is regulated, in part,

by the transcription factor STAT3, and through direct interaction of STAT3 with the NOSTRIN promoter. To address whether shear stress mediates NOSTRIN expression, I have done preliminary experiments in PECAM-1 expressing cells. After 4 hours of laminar shear stress, I observed an increase in NOSTRIN expression (as measured by qPCR). This suggests that NOSTRIN expression is shear stress-responsive. However, it remains unclear if this increase in expression occurs in a PECAM-1/STAT3-dependent manner.

In vivo, the distribution of eNOS is modified by different patterns of shear stress (laminar vs. disturbed)[9]. This is particularly relevant in regions prone to atherosclerosis where redistribution of eNOS correlates with decreased NO production[10]. Therefore, it may be possible that distinct shear stress patterns direct changes in NOSTRIN expression. This could account for changes in eNOS distribution. One might hypothesize that in atheroprotective regions, NOSTRIN expression levels would also be high. Conversely, in atheroprone areas where shear stress is disturbed, NOSTRIN expression would be low. Thus eNOS would be mislocalized and subsequently NO production would be reduced. Understanding the complex relationships between these signaling pathways might help further our understanding about the production of NO, a critical mediator of vascular health and disease.

PECAM-1 regulation of JAK/STAT activity

In Chapter II, we describe a role for JAK/STAT3 signaling in the regulation of NOSTRIN expression. PECAM-1 is required for this signaling, as in its absence, we observe reduced STAT3 phosphorylation and reduced NOSTRIN expression. Previous studies have shown that PECAM-1 can act as a scaffold for both STAT3 and STAT5, facilitating their activation[11], but it is unclear how PECAM-1 promotes STAT phosphorylation. STAT3 is activated by phosphorylation of either tyrosine residue 705

(Tyr705) or serine residue 727 (Ser727), both of which are important for influencing its transcriptional activity. It is thought that protein kinase C delta (PKCδ) phosphorylates Ser727, whereas the more canonical janus kinase (JAK) phosphorylates Tyr705 [12]. In agreement with previous studies, the PECAM-1 KO ECs have reduced Tyr705 phosphorylation. By treating the PECAM-1 expressing (PE-RC) ECs with the JAK/STAT3 inhibitor, cucurbitacin, we are able to phenocopy the PE-KO cells and reduce NOSTRIN expression. This suggests that although Ser727 is an important activation site, PKCδ is less likely to be important in our system. Other studies have implicated a role for Src in regulating STAT3 activation [13, 14]. Because PECAM-1 facilitates Src activation in response to specific stimuli, it is also possible that Src is important in controlling STAT3 activity.

We have yet to investigate the activity of PKCδ or JAK to address whether these upstream kinases are affected by PECAM-1 deletion. In addition, to our knowledge, there are no reports of PECAM-1-JAK association, although JAKs are known to bind to other transmembrane receptors. Specifically, JAKs are usually coupled to cytokine receptors such as the gp130 rector family and become activated in response to cytokine stimulation. It has been shown that STAT3 binds PECAM-1 in an ITIM-independent manner [15]. Thus, it is possible that the scaffolding role of PECAM-1 is important for facilitating the spatial localization of molecules important in activating STAT3. It would be feasible to address this hypothesis by using PECAM-1 truncation mutants and assaying STAT3 activity or assessing PECAM-1-STAT3 interaction via biochemical analysis. Alternatively, it is possible that JAKs bind to the cytoplasmic domain of PECAM-1 which would spatially restrict them to an area where shear stress-responsive kinases such as Src-family kinases are located. Interestingly, previous studies have shown interplay between JAK and the Src-family kinase Lyn, which act synergistically in mature red blood cells to promote maturation [16]. Understanding the relationship between JAK/STAT and PECAM-1 is a relevant

consideration, as it may help explain or identify novel transcriptional targets downstream of PECAM-1.

As mentioned in the previous section, my preliminary studies suggest that shear stress influences NOSTRIN expression. Currently, it is unclear whether the change in NOSTRIN expression by shear stress is STAT3-dependent. Interestingly, relatively little is known about STAT3 function in ECs, and in particular, its regulation/activation by shear stress. The majority of work investigating STAT3 activation and transcriptional regulation has been done in the context of cytokine signaling and cancer. Some studies have been done to address the relationship between shear stress and STAT3 signaling. However, differences in methodology make conclusions from these experiments unclear [12, 17]. One commonality is that shear stress appears to stimulate a transient increase in Ser727 and a decrease in Tyr705 phosphorylation, and phosphorylation of both residues is reduced after 8h of shear. My preliminary studies suggest an increase in NOSTRIN expression after 4hrs of shear. Therefore, a more stringent time course of STAT3 phosphorylation by shear should be conducted to gain insight into the relationship between these events. However, these data may also implicate a more direct role for Src in STAT3 phosphorylation, since it activated by shear stress and thought to play a role in STAT activation.

CHAPTER III: A MECHANOSENSOR MEDIATES CROSSTALK BETWEEN ENDOTHELIAL CELLS AND CARDIOMYOCYTES TO REGULATE CARDIAC FUNCTION

Previous work from our lab has suggested that PECAM-1 is important for mediating signaling between ECs and underlying cell types such as VSMCs [5]. Given that the heart is composed of a significant proportion of ECs, which outnumber CMs 3:1 [18], we hypothesized that the same might be true in the heart. In Chapter III I describe a novel role for PECAM-1

in regulating EC-CM crosstalk which affects cardiac function. Our results were very intriguing and led us to ask additional questions regarding the complexity of this crosstalk.

Shear stress signaling in the heart

Due to its role as a flow sensor, we hypothesized that the defects in function observed in the PECAM-1$^{-/-}$ animals were due to impaired flow signaling. Although a role for shear stress has been shown during cardiac development in zebrafish and other model systems [19, 20], it is unclear how shear stress influences cardiac function in adult animals. Flow patterns generated in the heart are dynamic and varied depending on location. Therefore, ECs within the heart are exposed to different types of shear stress. For instance, endocardial ECs experience the filling and emptying of the ventricular chambers with every cardiac cycle, whereas the myocardial ECs encounter highly pulsatile flow, as the myocytes squeeze these vessels during contraction.

To investigate the effect that shear stress has on the heart, one needs a model in which blood flow is manipulated with minimal effects on oxygen and nutrient delivery to the myocardium. One method is transverse aortic constriction (TAC). In this model, partial ligation of the aorta increases the hemodynamic (shear) stress on the heart, in the ventricles and the myocapillaries. The characteristic response to the increased stress is the development of cardiac hypertrophy. The contribution of the endothelium to the development of hypertrophy is not well understood. Using the PECAM-1$^{-/-}$ mice, we observed impaired remodeling and decreased activation of cardiac stress-responsive pathways. This suggests that PECAM-1 is capable of detecting changes in shear stress that are important for cardiac hypertrophy. This finding is particularly intriguing, as the pathway(s) that initiate compensatory remodeling are poorly characterized. It has previously been shown that CMs are able to detect changes in the hemodynamic environment which manifests as increased stress on the sarcomere. The muscle-specific protein muscle LIM

protein (MLP) is thought to function as a CM-specific mechanosensor[21, 22]. As changes in the hemodynamic environment also affect the CMs, it is possible that CM-specific pathways, such as MLP signaling may contribute to the minimal remodeling observed in the PECAM-1$^{-/-}$ mice after TAC (Figure 3.3).

I have shown that impaired NRG-1-ErbB signaling within the heart contributes to the cardiac dysfunction observed in the PECAM-1$^{-/-}$ animals. However, I have not shown whether this is due to an inability to regulate NRG-1 signaling in response to shear stress. Interestingly, previous studies have shown that one component of this pathway, the production of reactive oxygen species (ROS), is shear responsive. Therefore, if shear-induced ROS production is impaired in the PECAM-1$^{-/-}$ ECs, it would not be surprising that these cells have reduced NRG1 release. We can address this question by subjecting PECAM-1 expressing and knockout ECs to shear stress using our *in vitro* flow system and measure subsequent NRG1 release. An important consideration for these experiments is the type of flow that is experienced by the ECs in the complex environment of the heart. Our system allows manipulation of flow patterns allowing us to mimic the flow experienced by the myocapillary ECs, as well as the endocardial ECs.

Which endothelial cells are important for regulating cardiac function?

The work presented in Chapter III further strengthens the importance of EC-CM crosstalk in regulating cardiac function. However, it does not address which population of ECs within the heart is required to regulate crosstalk. There are three major populations of ECs within the heart: coronary, myocapillary, and endocardial. Coronary ECs line the coronary blood vessels, which are superficial vessels that run along the surface of the heart. These vessels supply the cardiac muscle with oxygen-rich blood. Despite this essential function, their contribution to EC-CM crosstalk is minimal. These cells are anatomically

remote from the cardiomyocytes, thereby preventing the diffusion of important signaling molecules to underlying CMs.

The myocapillary ECs, which surround each cardiomyocyte, and the endocardial ECs, which line the ventricular chambers, are in close proximity to the underlying cardiomyocytes. These distances range from ~1μm for myocapillary ECs to 10-50μm for endocardial ECs [18]. Myocapillary ECs and endocardial ECs differ in a number of ways, including cell morphology, type of cell junctions, and PECAM-1 distribution. Evidence for the latter comes from immunofluorescent localization of PECAM-1 in cardiac tissue sections. In endocardial ECs, PECAM-1 is found primarily at border zones where ECs overlap [23]. Conversely, PECAM-1 staining in myocapillary ECs is observed diffusely over the cell surface [18]. One could imagine that the differential localization of PECAM-1 in these cell populations could have profound effects on cell crosstalk and signaling. We know that PECAM-1 forms homophilic interactions with PECAM-1 on adjacent cells [24] and that this interface is important for mediating a subset of signaling events. However, it is unclear whether these interactions are important for regulating NRG1 release from ECs and more broadly, mediating signaling within the heart.

To address which cardiac EC population is important for NRG1 signaling and elevated ErbB activation, I have performed preliminary experiments using cardiac tissue sections. The sections were stained with antibodies against phosphorylated ErbB2/4 and imaged using confocal microscopy. Unfortunately, images taken of the tissues were difficult to interpret due to challenges with staining, which we attribute to poor quality reagents. It may be worthwhile to repeat this experiment using other available antibodies, as spatial differences in ErbB activation could be revealed. A second method to establish the relevant EC signaling population would be to remove one population of ECs from the heart and assay cardiac function *ex vivo*. It is extremely difficult to remove the myocapillary ECs, but

previous studies have denuded the endocardial ECs to assess their role in regulating cardiac function [25]. One might hypothesize that if NRG1/ErbB signaling were elevated in endocardial ECs, removal of this layer of cells would reverse the systolic and diastolic dysfunction present in the PECAM-1$^{-/-}$ mouse. If the phenotype persists, these data would suggest that the myocapillary ECs might be important for mediating NRG-1 release. However, it is likely that the endocardial and myocapillary ECs play distinct roles in cellular crosstalk, and therefore, both contribute to cardiac function.

INTEGRATING *IN VITRO* AND *IN VIVO* RESEARCH

Although Chapter II and III seem disparate at first glance, it has become obvious, as both have progressed that they are more related than initially thought. Several common themes are present in this dissertation, including the balance of signaling molecules and the interplay between pathways. The aim of this section is to integrate these ideas and discuss them in the larger context of cardiovascular biology.

Balance of signaling

In 1865, Claude Bernard first published the idea of *milieu interieur*, now referred to as homeostasis. This concept describes the adaptations in behavior or signaling that maintain a constant condition, for example temperature. Homeostasis is an incredibly dynamic process and requires the presence of compensatory mechanisms to maintain values within a normal range. The work presented in this thesis highlights the requirement for a balance of signaling molecules to maintain normal cardiovascular physiology. Central to both stories is the misregulaton of signaling molecules that are important for cellular crosstalk. In Chapter II, we observed increased NO production from the PE-KO cells, as well as in the plasma of PECAM-1$^{-/-}$ mice, and in Chapter III, we show increased NRG1-ErbB2

signaling in the PECAM-1$^{-/-}$ hearts. In both cases, homeostasis must be maintained by the activation of compensatory mechanisms, which currently remain unknown. However, we have identified several interesting possibilities, particularly *in vivo.*

Contribution of alternative signaling pathways

Studies in the 1980s by Furchgott and Zawadski demonstrated that ECs release compounds that affect vasomotricity (tone) of the surrounding VSMCs [26]. The compound, initially termed endothelium derived relaxing factor, was later identified as NO [27]. Their observations expanded the evolving role of ECs in vascular biology and revealed the possibility that ECs could play an important role in cell crosstalk. Accordingly, more than a decade after the discovery of NO, studies determined that ECs could also influence cardiac contractility [28, 29]. This occurs through signaling molecules such as VEGF, ET-1, prostaglandins, NO, and NRG-1 [30]. Although my work demonstrates that PECAM-1 is important for NO and NRG-1 release, it is highly likely that it also facilitates the production and/or release of additional signaling molecules.

Blood vessel tone is a dynamically regulated process, consisting of changes in vasodilation (widening of the blood vessel) and vasoconstriction (narrowing of the blood vessel). As discussed previously, NO participates in vasodilation, increasing perfusion of target tissues. We found that PECAM-1$^{-/-}$ mice have increased plasma NO that may contribute to a decrease in blood pressure. However, when we measure MAP in these animals, it is identical to wild-type controls. These results suggest upregulation of vasoconstrictor signaling, such as increased expression of endothelin-1 (ET-1), in these mice. It would be possible to test this hypothesis both *in vitro* and *in vivo* by measuring plasma ET-1 levels or ET-1 mRNA expression in ECs. One might also expect that components of the endothelin signaling pathway may also be upregulated such as

endothelin receptors, ET_A on VSMCs and ET_B on ECs. An interesting experiment would be to treat isolated arterioles with endothelin and measure the contractile response with the expectation that the PECAM-$1^{-/-}$ vessels are more responsive (exhibit increased contractility) to ET-1 treatment.

An interesting observation in the isolated cardiomyocyte studies led us to hypothesize a role for an alternative mechanism. We noticed that PECAM-$1^{-/-}$ CMs were more responsive to isoproterenol stimulation than WT CMs. Isoproterenol is a β-adrenergic agonist that is structurally similar to adrenaline. Therefore, the compound stimulates β-adrenergic receptors in the heart to increase heart rate and contractility. One hypothesis for the increased responsiveness to isoproterenol in the PECAM-$1^{-/-}$ CMs is increased expression/activity of the adrenergic signaling pathway to compensate for higher muscarinic activity. It is well accepted that muscarinic and adrenergic signaling function to maintain normal cardiac function. Thus NO and NRG1 signaling have been shown to converge at the level of muscarinic signaling [31], I hypothesize that increased input from the adrenergic pathway may be a compensatory mechanism for the depressed heart function.

Regulation of adrenergic signaling is a complex process, involving the activity of downstream kinases such as G protein-coupled receptor kinase-2 (GRK2) as well as G-proteins and β-arrestins. Ligand binding to β-adrenergic receptors (βAR) leads to a conformational change in the receptor, facilitating its phosphorylation by GRK2, leading to receptor desensitization and internalization [32]. Interestingly, adrenergic signaling is relevant in heart failure; decreased adrenergic signaling is observed in patients with congestive heart failure [33]References. Furthermore, either peptide-mediated inhibition or genetic ablation of GRK2 can prevent the progression to heart failure [34, 35]. Because we have increased responsiveness to adrenergic stimulation, it is possible that levels of GRK2 are reduced in the PECAM-$1^{-/-}$ hearts. Furthermore, there may be reduced levels of β-arrestins in CMs of

PECAM-1$^{-/-}$ mice. These questions can be answered by measuring changes in gene and/or protein expression of these components.

The signaling relationship between PECAM-1 and eNOS/NO in the heart

While ET-1 signaling, among other pathway, may contribute to the phenotype of the PECAM-1$^{-/-}$ hearts, there is compelling evidence that eNOS/NO signaling may predominate. In Chapter II I show that PECAM-1 regulates eNOS and NO release. Interestingly, there is a large body of literature implicating a role for NO in regulating cardiac contractility [36]. In fact, it has been demonstrated that high levels of NO reduce contractility (negative inotropy), whereas low levels of NO increase contractility (positive inotropy) [36]. We have measured cGMP from WT and PECAM-1$^{-/-}$ hearts as an indirect measure of NO production. Our preliminary data suggest that there is increased cGMP in the PECAM-1$^{-/-}$ hearts. This correlates with my observations from PECAM-1$^{-/-}$ plasma and may indicate that contractility may be affected on two levels: one, through elevated NRG1/ErbB signaling and two, through elevated NO production.

To address the possibility that PECAM-1-eNOS signaling is important in regulating cardiac function, we have generated a PECAM-1$^{-/-}$/eNOS$^{-/-}$ mouse. Preliminary characterization of this mouse revealed a markedly different phenotype from the PECAM-1$^{-/-}$ mouse. These mice have reduced litter sizes and birthrates compared to either the PECAM-1$^{-/-}$ or eNOS$^{-/-}$ animals. Interestingly, the mice that survive develop significant hypertrophic cardiomyopathy as early as 4 weeks, as determined by conscious echocardiography. The hypertrophy is likely not due to high blood pressure, as the mean arterial pressure (MAP) in the PECAM-1$^{-/-}$/eNOS$^{-/-}$ mice is only slightly increased above that of the PECAM-1$^{-/-}$ and significantly less than the eNOS$^{-/-}$. Further characterization of these animals is currently under investigation in the lab.

Implications for cardiovascular biology

The work presented in this thesis has broad implications for cardiovascular biology, both on the cellular and organ/animal level. Both PECAM-1 and eNOS are promiscuous proteins involved in many EC processes and signaling pathways. By further characterizing their functions, we will gain a better understanding of EC function as a whole. In addition to elucidating a role for PECAM-1 in the regulation of eNOS activity, the work in Chapter II also provides a clearer picture of eNOS regulation. Endothelial dysfunction is characterized by misregulated eNOS/NO production, and its role in the progression of diseases like atherosclerosis is well documented. Therefore, understanding the regulation of eNOS is central to the development of new targets for manipulating the health of the vessel.

In Chapter III, we demonstrate the importance of PECAM-1, an endothelial protein, in directing cellular crosstalk and subsequent cardiac function. Despite the appreciation that ECs modify heart function, the specific role of these cells remains poorly defined. The contribution of CMs to cardiac function is well established, as are the intracellular components that regulate their function. Genetic mutations in both cytoskeletal anchoring proteins and sarcolemma proteins can lead to the development of DCM. This includes the proteins titin [37], which accounts for 25% of familial and 18% of sporadic DCM, cardiac myosin binding protein (MYBPC3) [38], and laminin A/C [39] among others. Importantly, a large percent of DCM cases are idiopathic. This population of disease may represent defects in cell-cell communication whose signaling pathways are poorly characterized. More importantly, our work strengthens previous observations implicating the vasculature as a critical regulator of cardiac function.

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