2016

Analysis of brown adipocyte-derived VEGF-A

BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

ANALYSIS OF BROWN ADIPOCYTE-DERIVED VEGF-A

by

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B.S., Lehigh University, 2014

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science
2016

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ACKNOWLEDGMENTS

I would like to thank my friends and family for their love and support during my graduate career. You guys are the best.

I would also like to thank my mentor Dr. Tamar Aprahamian for allowing me to join her lab, training me in a variety of laboratory techniques, and improving my scientific writing. Similarly, I would like to thank Shi Su for also teaching me lab skills and being an excellent lab friend.

Lastly, I would like to thank Dr. Kiana Mahdaviani and the Shirihai lab for teaching me how to culture cells and allowing me to use their facilities and reagents at all hours of the day.

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ABSTRACT

Objective. While it has long been known that vascular endothelial growth factor A (VEGF-A) plays a role in vascular homeostasis, only recently have its effects been explored in adipose tissue. As perivascular adipose tissue (PVAT) is in close proximity with the aorta and coronary arteries and is known to contribute to vasodilation, it may influence vascular function via secretion of VEGF-A. The objective of this study is to analyze the effects of brown-adipocyte deletion of VEGF-A on circulating VEGF-A levels and distribution of VEGF-A isoforms. We hypothesize that ablation of VEGF-A in brown adipocytes will affect perivascular adipocyte and vascular function.

Materials/Methods. Mice harboring a brown adipose-specific VEGF deficiency, UCP1cre.VEGF$^{flox/flox}$ mice, were maintained on a chow diet. Primary adipocytes were isolated from brown adipose tissue (BAT) and thoracic PVAT by collagenase digestion and culturing. Gene expression was measured by RT-PCR from RNA extracted from tissues of UCP1cre.VEGF$^{flox/flox}$ mice. Circulating and tissue VEGF-A levels were quantified by ELISA.

Results. While VEGF-A ablation using the UCP1 promoter decreases VEGF- protein A levels in BAT and PVAT, it does not affect VEGF-A levels in the circulation.

Conclusion. This study confirms the functional utility of the UCP1cre.VEGF$^{flox/flox}$ mouse model, as it selectively reduces VEGF-A levels in BAT and PVAT without affecting

other tissues or circulating levels. As previous studies using VEGF ablation in all adipose tissues demonstrate an impaired thermogenic response and brown-adipocyte dysfunction, further study of the brown adipose-specific mouse model is warranted. Because PVAT provides protection against vascular stiffness, modulation of VEGF-A in PVAT may be a viable treatment for obesity-associated vascular complications.

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LIST OF ABBREVIATIONS

BAT.....Brown Adipose Tissue

CVD.....Cardiovascular Disease

DMEM.....Dulbecco's Modified Eagle Medium

FA.....Focal Adhesion

FABP4.....Fatty Acid Binding Protein 4

GAPDH.....Glyceraldehyde 3-Phosphate Dehydrogenase

HIF-1α.....Hypoxia Inducible Factor 1-Alpha

PE.....Phenylephrine

PGC-1α.....Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha

PI3K.....Phosphatidylinositol 3-Kinase

PVAT.....Perivascular Adipose Tissue

PWV.....Pulse Wave Velocity

SH.....Src Homology

UCP1.....Uncoupling Protein 1

VEGF.....Vascular Endothelial Growth Factor

VSMC.....Vascular Smooth Muscle Cell

WAT.....White Adipose Tissue

INTRODUCTION

Overview

Despite recent advances in the development of cardiovascular medications and surgical techniques, cardiovascular disease (CVD) remains the leading cause of death worldwide. In the United States alone, CVD affects an estimated 26.6 million people and accounts for one in four deaths each year (CDC, 2015). Furthermore, approximately half of all Americans possess one of three main risk factors for CVD: smoking, high blood pressure, and high LDL cholesterol. The emergence of the obesity epidemic has only increased the prevalence of hypertension and dyslipidemia and further contributed to heart disease in this susceptible population.

The Vascular Endothelial Growth Factor Pathway

Vascular Endothelial Growth Factor (VEGF) is a potent mitogen that can bind to heparin sulfate and the tyrosine kinase VEGF receptors, VEGFR1 (Flt-1), VEGFR2 (Flk-1), and the co-receptor neuropilin-1. Within the VEGF family, the VEGF-A protein has the greatest effects promoting angiogenesis, which is the outgrowth of new blood vessels from pre-existing vessels, and vasculogenesis, which is the de novo formation of new blood vessels. These vascular changes occur primarily due to interactions with VEGFR2, which is expressed on vascular endothelial cells and smooth muscle cells, as well as other cell types including neurons and Schwann cells (Sondell et al., 1999). Signaling through VEGFR2 occurs via ligand-binding and receptor dimerization, followed by transphosphorylation of tyrosine residues in the cytoplasmic kinase domain.

Subsequently, Src homology (SH) 2 domain proteins and adaptor proteins like Grb2 mediate downstream responses, which can range from stimulation of nitric oxide production to Ca^{2+} mobilization, migration, and proliferation. VEGFR2 activity has also been tied to activation of PLC-γ, ERK, c-Src, and the phosphatidylinositol 3-kinase (PI3k)/Akt pathway that is involved in cell survival (Zachary et al., 2001). Many of these pathways and thus the overall function of VEGF-A have been analyzed in endothelial cells, which leaves other adjacent tissue types wholly understudied despite the fact that they may secrete or respond to VEGF-A and possess the ability to modulate endothelial cell or arterial signaling properties.

Multiple isoforms of VEGF-A arise from alternative splicing of the pre-mRNA transcripts. The major isoforms possess either 121, 165, or 189 amino acids in humans and one less amino acid in mice (Arcondeguy et al., 2013; **Fig. 1A-B**). In mice, VEGF-A_{164} appears to be the most widely expressed, but VEGF-A_{120} is also prevalent in the kidney while VEGF-A_{188} is highest in the lung (Ng et al., 2001). Of these isoforms, VEGF-A_{121} and VEGF-A_{165} are secreted and both are able to act in a paracrine or autocrine fashion. For example, VEGF-A_{165} plays a critical role in angiogenesis at cartilage growth plates and promotes endochondral ossification via an autocrine signaling loop in mammalian chondrocytes (Carlevaro et al., 2000). In the kidney, paracrine signaling of VEGF-A through VEGFR2 maintains glomerular filtration barrier integrity, demonstrated by ablation of VEGFR2 from glomerular endothelial cells as well as podocyte-specific overexpression of VEGF-$A_{164,}$ resulting in proteinuria and endotheliosis in the glomerulus (Sison et al., 2010). Taken together, the secretory VEGF

isoforms appear to be important for homeostatic control and development across a variety of tissues.

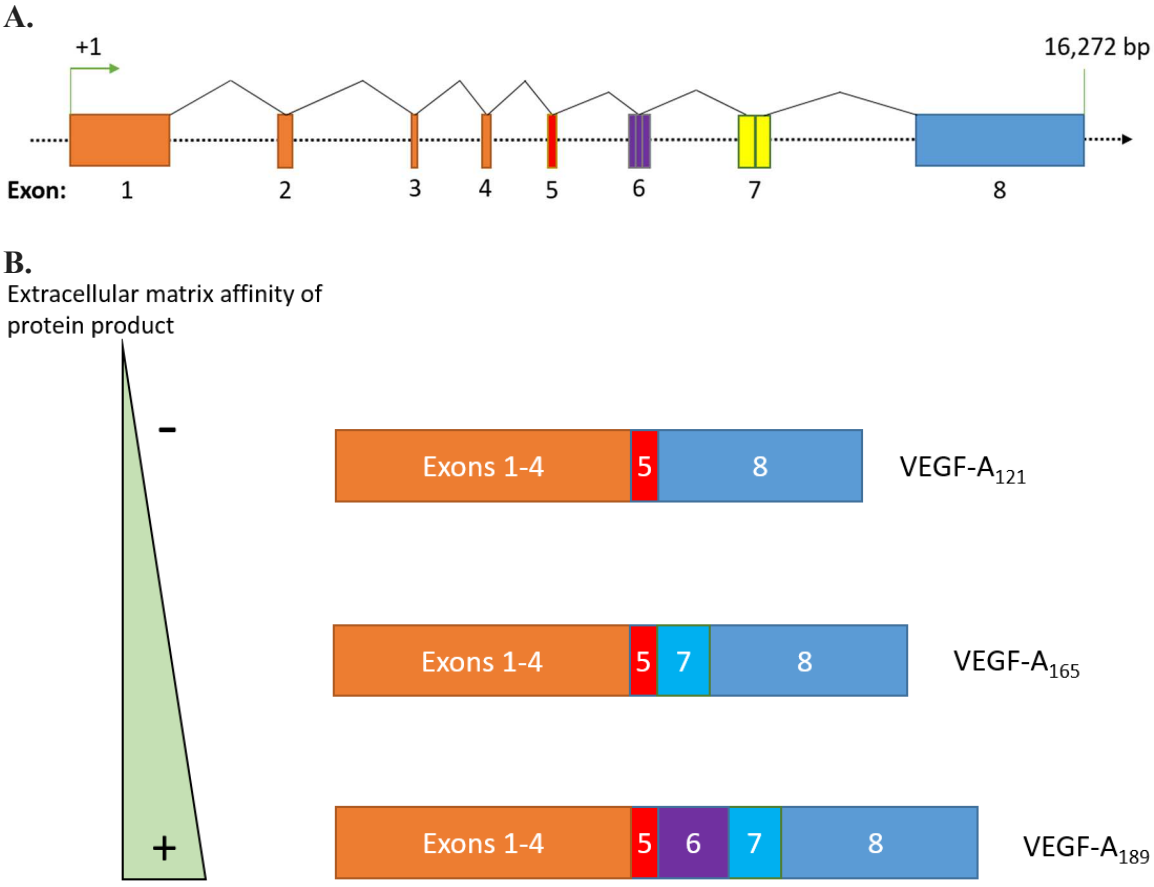


Figure 1: Gene structure of human VEGF-A isoforms. A) Exon map and VEGF-A gene length on chromosome 6p12. B) Exon composition of VEGF-A isoforms and their corresponding extracellular matrix affinity. (adapted from Arcondeguy et al., 2013)

In contrast to the secretory VEGF isoforms, VEGF-A_{189} is largely cell-associated due to tight binding with heparin proteoglycans in the extracellular matrix (Ferrara et al., 1991). As shown in **Fig. 1B**, extracellular matrix affinity is directly correlated with

VEGF-A isoform length, which prevents longer isoforms such as VEGF-A_{189} from acting in a paracrine manner on adjacent tissues. As a result, VEGF-A_{189} was thought to play a lesser role in VEGF-induced angiogenesis than the other isoforms, however recent studies have shown that VEGF-A_{189} is capable of causing endothelial cell migration and, to some extent, proliferation (Herve et al., 2005; Park et al., 1993). In fact, one study even suggests that the VEGF-A_{189} isoform, likely through interactions with heparin sulfate proteoglycans and VEGFR2, is the driving force behind tumor angiogenesis in non-small cell lung cancer and may also play a role in the development of nascent glioblastomas and adenocarcinomas (Yuan et al., 2001).

As a growth factor heavily involved in vascular processes, VEGF-A is critical for the proper development of vascular organs. The ability of VEGF-A to produce organized vascular beds is not only due to its mitogenic effects on endothelial cells, but also due to the differential distribution of VEGF-A isoforms in developing tissues. In mice, VEGF-A_{188} is highest in lung, heart, and liver because these are mesodermal-endodermal tissues whose early development require vasculogenesis, whereas VEGF-A_{120} and VEGF-A_{164} are highest in the mesodermal-ectodermal tissues of the brain, eye, and kidney that utilize angiogenesis stemming from the budding vascular network (Ng et al., 2001). A global knockout of VEGF in a mouse model is embryonic lethal, suggesting VEGF is necessary for embryonic development. Interestingly, chimeric mice that only express VEGF-A_{120} display defects in alveolar development due to decreased angiogenesis and increased vessel permeability. This suggests a need for the heparin-binding isoforms VEGF-A_{164} and VEGF-A_{188} for capillary remodeling and maturation, as VEGF-A_{120} cannot produce

the extensive branching necessary for proper alveolarization beyond initial vessel growth (Carmeliet et al., 1996)(Ng et al., 2001). Taken together, these results indicate that the VEGF-A isoforms control discrete as well as overlapping functions. Part of this difference may also be due to the fact that, while VEGF-A_{120} and VEGF-A_{165} have similar affinity for isolated VEGFR2, the natural heterodimerization of the VEGF-A_{165}-binding neuropilin-1 with VEGFR2 potentiates the phosphorylating effect of VEGF-A_{165} versus that of VEGF-A_{120} (Whitaker et al., 2001). While this required role of VEGF-A in embryonic development is well-understood, the manner in which VEGF contributes to vascular homeostasis in adult tissues remains to be fully elucidated. Furthermore, dysregulation of VEGF signaling can lead to significant vascular complications, particularly in the renal and cardiovascular systems.

VEGF in Human Disease

Major insights into the role that VEGF plays in maintaining a healthy vasculature come from attempts to modify VEGF in disease states, such as diabetes and cancer. Following its discovery in the 1980s and characterization in the 1990s, VEGF was thought to be one of many elusive factors that modulated tumor blood supply. A monoclonal antibody against VEGF was developed in 1993 that greatly reduced tumorigenesis *in vivo*, and in 2004 became the first FDA-approved, humanized angiogenesis inhibitor: bevacizumab (Avastin; Genentech) (Ferrara et al., 2004). Bevacizumab is still used to treat a variety of tumors, including lung, renal, brain, and metastatic colon cancers, demonstrating the broad tissue distribution and clinical

importance of VEGF.

While bevacizumab is effective at reducing cancer proliferation, off-target reductions in VEGF levels can damage otherwise healthy organs and result in significant side effects. In the kidney, for example, the glomerular microvasculature that filters the blood can be damaged and cause thrombotic microangiopathy and systemic hypertension in patients. This finding has been replicated by local VEGF ablation in mice (Eremina et al., 2008). These hypertensive side effects seen with use of VEGF inhibitors in cancer patients parallels the proteinuria and hypertension observed in the five percent of pregnant women who develop preeclampsia; interestingly, placental release of soluble, truncated VEGFR1 occurs in these women, such that VEGF is bound and sequestered without its downstream vasomodulating effects (Maynard et al., 2008). In some cases, such as dilative cardiomyopathy, VEGF-A_{165} and VEGF-A_{189} are downregulated, but it is not known whether this VEGF decrease is the cause or a result of the disease. Interestingly, in ischemic cardiomyopathy, VEGF and VEGF isoform expression is upregulated, however as ischemic hypoxia can induce VEGF transcription this is more likely an adaptive, protective response to injury rather than an instigator of dysfunction (Abraham et al., 2000). Observations from a VEGF deletion model that blocks the hypoxia response element, which results in motor neuron degeneration similar to amyotrophic lateral sclerosis (ALS), and use of VEGF to prevent cell death in a mouse model of Parkinson's disease, support the notion that maintenance of VEGF expression is important for both neurons and the renal system (Storkebaum et al., 2004). Thus, adequate VEGF levels are critical to prevent capillary rarefaction and renal defects that

can result in vascular pathologies across organ systems.

In contrast, glomerular VEGF is elevated in diabetes, which is the number one cause of end-stage renal failure in North America. It is not known whether the increase seen in patients is isoform-specific, however there is *in vitro* and *in vivo* evidence using VEGF-A_{165} overexpression that replicates the diabetes-induced renal damage. It is thought this occurs because VEGF-A_{165} is the dominant isoform expressed by both glomerular and tubular epithelial cells. Interestingly, renal podocytes exclusively express VEGFR2 in their cell body and foot processes, which also suggests that changes in VEGF-A_{165} levels could be most critical for renal homeostasis. While previous studies have demonstrated that global VEGF-A overexpression in mice results in proteinuria and other symptoms of diabetic nephropathy, a recent study that genetically deleted VEGF-A in mouse podocytes in an *in vivo* model of type 1 diabetes showed a concomitant increase in glomerular cell scarring, apoptosis, and overall mortality (Sivaskandarajah et al., 2012). These results suggest that moderate physiologic elevations of VEGF-A in the diabetic glomerulus may be a protective adaptation, which helps explain both the renal side-effects observed with anti-VEGF cancer therapies as well as VEGF overexpression. **Table 1** summarizes diseases and defects associated with VEGF deletion or overexpression:

Table 1: Phenotypic effects of VEGF-A Overexpression and Deletion in Mouse Models

VEGF-A Mouse Modification	Area of Interest	Physiologic Effects	Citation
Germline tetraploid embryonic stem cells, $VEGF^{+/-}$	VEGF heterozygosity	Embryonic lethal	Carmeliet et al. 1996
Exon deletion of $VEGF_{164,188}$ $VEGF^{120/120}$ chimeric mice	$VEGF_{120}$ & lung development	Decreased angiogenesis, retarded alveolarization	Ng et al. 2001
Bevacizumab, mAb for VEGF	Off-target effects	Thrombotic microangiopathy, systemic hypertension	Eremina et al. 2008
Podocyte-specific DOX-inducible Podocin-rtTa.tetO-cre.VEGF-A	Bevacizumab similarity	Glomerular lesions, hypertension, proteinuria	Eremina et al. 2008
Whole-body DOX-inducible tetO-cre.rosa-rtTa $VEGFR2^{flox/flox}$	VEGFR2 importance	Glomerular endothelial cell injury, heart/liver damage	Sison et al. 2010
Podocyte-specific DOX-inducible Nephrin-Cre.rosa-rtTa.tetO-$VEGF_{164}$	Renal failure	Proteinuria, endotheliosis	Sison et al. 2010
Podocyte-specific WT or STZ-treated Nphs2-rtTa.tetO-cre.$VEGF^{flox/flox}$	Elevated VEGF in diabetes	STZ-mice: glomerular scarring, apoptosis, proteinuria	Sivaskandarajah et al. 2012
Adipose-specific Adpn-rtTa.TRE-VEGF and mcr84 mAb inhibition	VEGF role in adipocytes	Improves angiogenesis and glucose tolerance against HFD during adipose expansion	Sun et al. 2012

Adipose-specific aP2.VEGF	Elevated VEGF in diabetes	Anti-inflammatory, insulin sensitive, anti-obesogenic	Elias et al. 2012
VEGF neutralization by adenoviral expression of soluble VEGFR1	VEGF role in brown adipocytes	Increased BAT apoptosis, mitochondrial degeneration	Bagchi et al. 2013
Adipose-specific ap2-cre.VEGF$^{flox/flox}$ and aP2-cre.rosa-rtTa.tetO-VEGF$_{164}$	VEGF role in adipocytes	Reduced vascularization & inflammation with deletion, rescued by overexpression	Sung et al. 2013
Adipose-specific ap2-cre.VEGF$^{flox/flox}$ and adenoviral-VEGF-A rescue	VEGF role in adipocytes	BAT whitening and capillary rarefaction, rescued by local VEGF-A injection	Shimizu et al. 2014
Brown adipocyte-specific UCP1-rtTa.TRE-VEGF-A	VEGF role in brown adipocytes	Improved thermogenesis, glucose metabolism, protects against HFD	Sun et al. 2014
Adipose-specific Fabp4cre.VEGF$^{flox/flox}$	VEGF role in adipocytes	Impaired mitochondrial oxidation/structure/vascularization	Mahdaviani et al. 2016

VEGF Signaling in Multiple Tissue Types

Though the role of adipocytes as secretory regulators of tissue function is becoming more appreciated, their role in cardiovascular function is not well understood. There is a need to study the interactions between adipocytes and other cell types, such as vascular smooth muscle cells. In the last decade, the existence of VEGF receptors in smooth muscle cells has been established, where before VEGF was thought to only act on vascular endothelial cells. While VEGF does not induce smooth muscle cell proliferation, it does appear to promote migration and act as a chemoattractant, which may be relevant for pathologic vascular processes, such as atherosclerosis and intimal invasion or scarring (Grosskreutz et al., 1999). As for adipose tissue, it is known that it expands following high caloric intake and requires increased vascularization to maintain normoxia and deliver nutrients within the fat. Obese mice display a deleterious reduction in VEGF-A and subsequent increase in adipose tissue hypoxia that promotes inflammation and insulin insensitivity. Adipocyte-localized doxycycline-inducible overexpression of VEGF-A during, but not after, adipose tissue expansion is able to ameliorate this negative metabolic shift that occurs as a result of VEGF-A dysregulation in obese *ob/ob* mice (Sun et al., 2012). Similarly, transgenic mice overexpressing VEGF-A are protected against insulin resistance due to a decrease in proinflammatory macrophage infiltration and improvements in glucose tolerance (Elias et al., 2012).

Perivascular adipose tissue (PVAT) surrounds vessels including the coronary artery and the aorta. Previous studies have demonstrated that PVAT impacts cardiovascular function via secretory interactions with the tunica externa and with the

aortic smooth muscle cells of the tunica media. Healthy PVAT has anti-contractile, vasodilating, and anti-inflammatory properties that can be attributed to its paracrine release of a host of vasoactive factors, such as adiponectin, a protein which enhances glucose metabolism and vasodilation of small arteries. In addition, PVAT also is a storage site of mesenchymal stem cells that can develop into adipocytes and vascular smooth muscle cells (Eringa et al., 2012). In obesity, however, aberrant PVAT expansion can induce macrophage infiltration, TNF-α release, reductions in adiponectin, and local hypoxia. Together, these changes can cause PVAT to promote a vasoconstrictive arterial state, which has also been implicated in the development of metabolic defects and atherosclerosis (Szasz et al., 2013).

It has recently been shown that PVAT surrounding the thoracic aorta is similar to interscapular brown adipose tissue (BAT), which is a highly oxidative tissue, rich in mitochondria, in both morphology and gene expression (Fitzgibbons et al., 2011). One study has suggested that VEGF-A can induce BAT proliferation and promote cell survival via interactions with VEGFR1, VEGFR2, and heparin-sulfate binding by the VEGF-A_{164} and VEGF-A_{188} isoforms (Bagchi et al., 2013). These results may also apply to PVAT and thus PVAT regulation of human aortic function. Interestingly, studies of adipose-specific VEGF-A overexpression demonstrate significant upregulation of uncoupling protein 1 (UCP1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) genes (Sun et al., 2012). These definitive molecular markers for BAT promote increased energy expenditure and may account for some of the metabolic improvements observed with VEGF-A overexpression in the context of obesity

and a high-fat diet.

Published studies from our laboratory have shown that VEGF-A secretion from fat regulates BAT to a greater extent than white adipose tissue (WAT), as VEGF-A expression levels are greater in adipocyte precursors than hematopoietic stem cells or endothelial cells and are highest in BAT over WAT. Using a cre-lox system to ablate VEGF specifically in adipose tissues by targeting the fatty acid binding protein 4 (Fabp4) promoter, our lab generated Fabp4cre.VEGF$^{flox/flox}$ mice to analyze the functional consequences of VEGF-A deletion in adipocytes. Fabp4cre^{+}.VEGF$^{flox/flox}$ mice displayed significant impairments in mitochondrial oxidation *in vivo* and of mitochondrial respiration *in vitro*, results which may be explained by the subsequent distortions in mitochondrial structure and decreased vascularization of the adipose tissue. This work identified that endogenous VEGF-A, acting in an autocrine manner, is necessary for regulation of BAT vascularization and thermogenic function (Mahdaviani et al., 2016).

While this autocrine mechanism within BAT has thus been established, it remains unknown if paracrine VEGF-A signaling from BAT-like tissues, such as PVAT, occurs or affects vascular function. Preliminary data from our lab have demonstrated that Fabp4cre^{+}.VEGF$^{flox/flox}$ mice exhibit increased collagen deposition in the thoracic aorta **(Fig. 2A-B)** consistent with the gradual pathogenesis of aortic remodeling. Fabp4cre^{+}.VEGF$^{flox/flox}$ mice also demonstrate a diminished aortic relaxation response to acetylcholine **(Fig. 2C)**, which is a functional measure of vascular tone that may be influenced by the observed increase in collagen deposition. These mice also display detrimental reductions in adiponectin expression in adipose tissue **(Fig. 2D)**, which is a

crucial regulator of tissue vascularity and inflammation and which may predispose these mice to CVD.

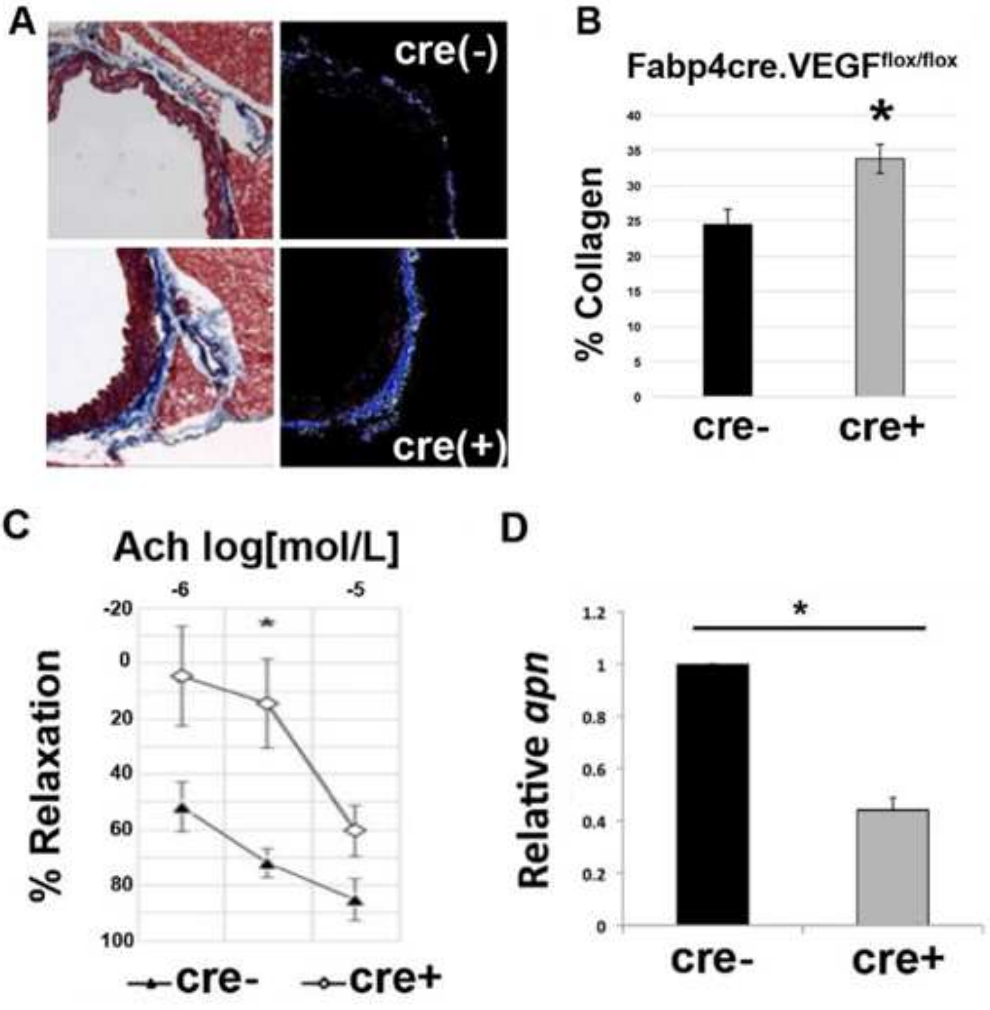


Figure 2: Impaired vascular tone in Fabp4cre.VEGF$^{flox/flox}$ mice. A) Representative aortic section stained with Masson's Trichrome (left) and area of analysis (right). B) Collagen deposition quantification. C) Impaired relaxation in Fabp4cre^{+}.VEGF$^{flox/flox}$ mice. D) Fabp4cre^{+}.VEGF$^{flox/flox}$ mice display reductions in PVAT, BAT adiponectin (n=4) *P < 0.05

Despite the presence of this dysfunctional phenotype with adipose-specific loss of VEGF-A, we do not know the exact mechanism by which this occurs. We hypothesize that VEGF-A plays a central role in PVAT function and that brown adipocyte-specific ablation, accomplished by generation of UCP1cre.VEGF$^{flox/flox}$ mice of VEGF-A will result in impaired oxidative and vascular function in these tissues. UCP1cre.VEGF$^{flox/flox}$ mice were generated by cross-breeding VEGF homozygous floxed mice with and UCP1cre+ mice, followed by crossing the F1 progeny–heterozygous for VEGF^{flox} and one cre^{+} and one cre^{-} , to obtain UCP1cre^{-}.VEGF$^{flox/flox}$ control and UCP1cre^{+}.VEGF$^{flox/flox}$ experimental mice **(Fig. 3)**. By using ELISA to analyze protein levels of VEGF-A across multiple tissue types, we were able to demonstrate that VEGF levels in lung and WAT were comparable between UCP1cre^{+}.VEGF$^{flox/flox}$ and UCP1cre^{-}.VEGF$^{flox/flox}$ mice **(Fig. 4A)**, but were reduced in BAT and PVAT of the UCP1cre^{+}.VEGF$^{flox/flox}$ mice; therefore, our model for VEGF-A ablation is both organ- and genotype-specific. Though we observe a selective and targeted decrease in VEGF gene expression in BAT and PVAT, we also wanted to study UCP1 expression. UCP1cre+.VEGF$^{flox/flox}$ mice demonstrate decreased UCP1 expression in BAT and PVAT suggesting that VEGF does indeed regulate UCP1 transcription, likely through PGC-1α as has been previously described **(Fig. 4B)**.

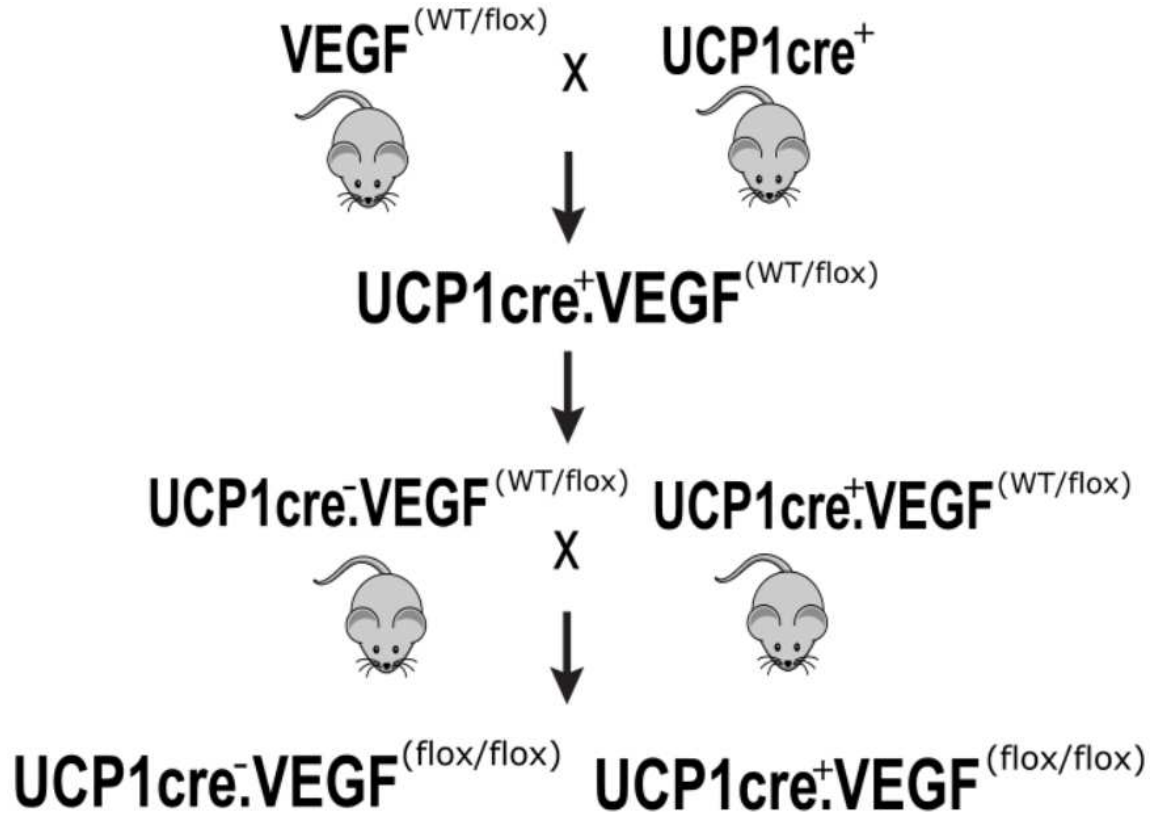


Figure 3: Generation of UCP1cre.VEGF$^{flox/flox}$ mice. Cross-breeding of multiple generations to select for UCP1cre.VEGF$^{flox/flox}$ mice.

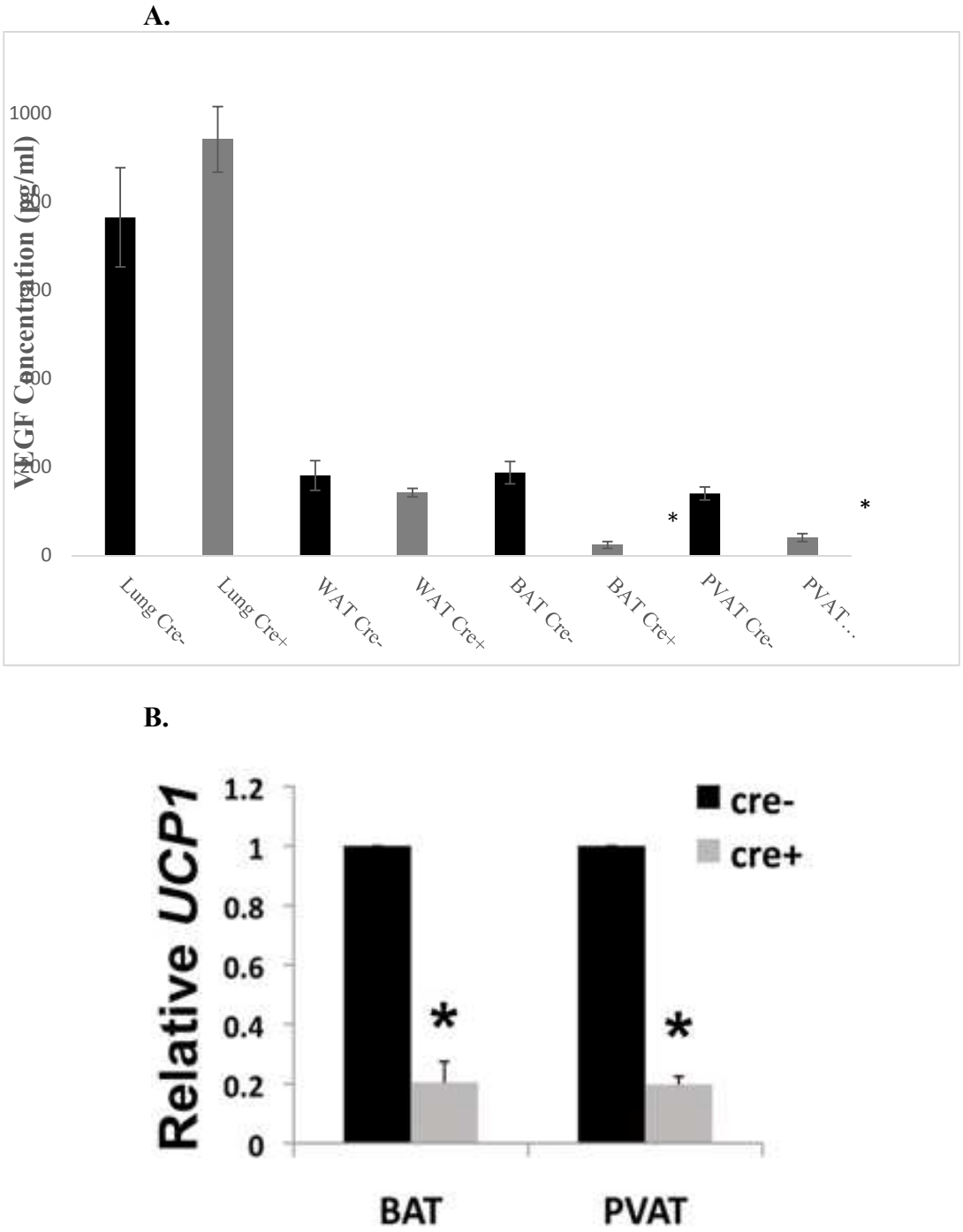


Figure 4: Tissue VEGF and UCP1 Concentrations in UCP1cre.VEGF$^{flox/flox}$ mice. A) Concentration of VEGF across tissue types including lung, WAT, BAT, and PVAT as measured by ELISA. B) UCP1 gene expression decreases in the BAT and PVAT of UCP1cre^{+}.VEGF$^{flox/flox}$ mice as expected and as measured by RT-PCR (cre^{-}, n=7-9; cre^{+}, n=6-9) *P < 0.01

In this thesis we will explore the presence and potential role of VEGF-A in thoracic PVAT and how this factor may affect overall aortic function in mice. We hypothesize that VEGF-A gene expression in PVAT may be similar to BAT and that brown adipocyte-specific ablation of VEGF-A in mice will alter PVAT molecular characteristics.

MATERIALS AND METHODS

Animals

For this study, mice were generated with brown adipose-specific VEGF-A ablation by crossing uncoupling protein-1 (UCP-1)-cre transgenic mice (obtained from Dr. Evan Rosen) and $VEGF^{flox/flox}$ mice (obtained from Genentech) on C57BL/6 genetic backgrounds to create experimental $UCP1cre^{+}.VEGF^{flox/flox}$ mice. $UCP1cre^{-}.VEGF^{flox/flox}$ mice were used as controls.

Tissue Isolation Protocol

Five different tissues were isolated from $UCP1cre^{+}.VEGF^{flox/flox}$ and $UCP1cre^{-}.VEGF^{flox/flox}$ mice, including the interscapular brown adipose tissue (BAT), subcutaneous white adipose tissue (WAT), heart, lung, and thoracic perivascular adipose tissue (PVAT) using a dissecting microscope. Tissues were immediately snap frozen in liquid nitrogen for direct RNA extraction.

Primary Cell Isolation and Culture Protocol

Interscapular BAT and thoracic PVAT were also extracted for use in primary cell culture prior to RNA extraction. Briefly, freshly isolated tissues were placed in Dulbecco's Modified Eagle Medium (DMEM) to maintain hydration. In a sterile hood, the cells were removed from the DMEM, shred with sterile scissors, and scraped into a falcon tube mixture of 5-6mg type 2 collagenase (Worthington) in 5ml HEPES buffer. Each cell-collagenase mixture was incubated in a shaking water bath for 20min at 37°C

and lightly vortexed every 5min. Using a syringe and 18.5G needle, the digested mixture was aspirated and filtered through a 100µm filter into a new falcon tube. After placing on ice for 10-20min to slow collagenase activity, this process was repeated with a 40µm filter and a minimum of 25mL DMEM was added through the filter to dilute the collagenase and further slow its activity. Mixtures were centrifuged at 1000RPM at 4°C for 10min, the supernatant was aspirated, and the remaining pellet was resuspended in 300µl to 1.5ml BAT differentiation media. Note: the DMEM wash and centrifugation step can be repeated, but was often skipped due to low cell yields. Resuspended cells were then plated in 48- or 96-well plates at 8% CO_2 and 37°C overnight, and washed with fresh media every other day thereafter for 7-8 days.

RNA Extraction

For cell cultures, RNA was extracted by direct lysis in culture plate wells with buffer RLT after removal of the BAT differentiation media and scraping with a sterile pipette tip. As cell cultures were not confluent, 350µl of buffer RLT was used in all cases. For tissues, RNA was extracted from the maximum amount of tissue available, wherein 600µl buffer RLT was used for ≤30mg tissue and 350µl buffer RLT was used for <20mg tissue; solid samples were disrupted and homogenized in a TissueLyser II with 5mm stainless steel beads and then centrifuged for 3 min at 14,000 RPM. After lysis of each sample type, RNA extraction was followed according to the manufacturer's instructions (Qiagen).

Real-Time Quantitative PCR

RNA was transcribed to cDNA using a Qiagen QuantiTect® reverse transcription kit and BioRad T100™ Thermal Cycler according to manufacturer instructions. Gene expression in PVAT, BAT, and heart from UCPcre^{+}.VEGF$^{flox/flox}$ and UCPcre^{-}.VEGF$^{flox/flox}$ mice was measured with the ViiA™ 7 Real-Time PCR system. The reference gene for all samples was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Taqman and Qiagen expression assays were used for UCP1, VEGF-A, and VEGF isoform gene expression. Relative mRNA was quantitated using the ΔΔCT method, and expressed as fold change relative to UCPcre(-).VEGF$^{flox/flox}$ mice. Primer sequences were designed and validated by Zhang et al. (2015):

Mouse VEGF common forward: 5'—GCCAGCACATAGAGAGAATGAGC—3'

Mouse VEGF$_{120}$ reverse: 5'—CGGCTTGTCACATTTTTCTGG—3'

Mouse VEGF$_{164}$ reverse: 5'—CAAGGCTCACAGTGATTTTCTTGG—3'

Mouse VEGF$_{188}$ reverse: 5'—AACAAGGCTCACAGTGAACGCT—3'

ELISA

Lung, WAT, BAT, and PVAT tissue and cell samples from UCP1cre.VEGF$^{flox/flox}$ mice were homogenized in a TissueLyser II (Qiagen) and proteins were extracted. VEGF-A protein was quantified using a mouse VEGF-A ELISA kit according to manufacturer's instructions (R&D Systems).

RESULTS

Circulating and Tissue-specific VEGF Concentrations

Since we observed increased aortic collagen deposition and decreased adiponectin in Fabp4cre^{+}.VEGF$^{flox/flox}$ mice **(Fig. 2)** with the most striking effects occurring in BAT, we used the brown-adipocyte specific UCP1cre.VEGF$^{flox/flox}$ mouse model to directly study the prevalence and importance of VEGF within BAT and PVAT to maintain their oxidative function and vascular homeostasis (**Fig. 3)**. Furthermore, as there was a phenotypic difference between Fabp4cre^{+}.VEGF$^{flox/flox}$ and Fabp4cre^{-}.VEGF$^{flox/flox}$ mice, there was a need to exclude the possibility that our gene modifications were affecting circulating VEGF levels in addition to the expected local VEGF deletion, as that could contribute to the observed vascular impairments. We confirmed that any observed effect was due to our localized deletion of VEGF and not a result of a global change in VEGF levels by measuring circulating VEGF-A concentrations in UCP1cre^{+}.VEGF$^{flox/flox}$ and UCP1cre^{-}.VEGF$^{flox/flox}$ mice. As shown in **Figure 5**, there is no significant difference in circulating VEGF levels.

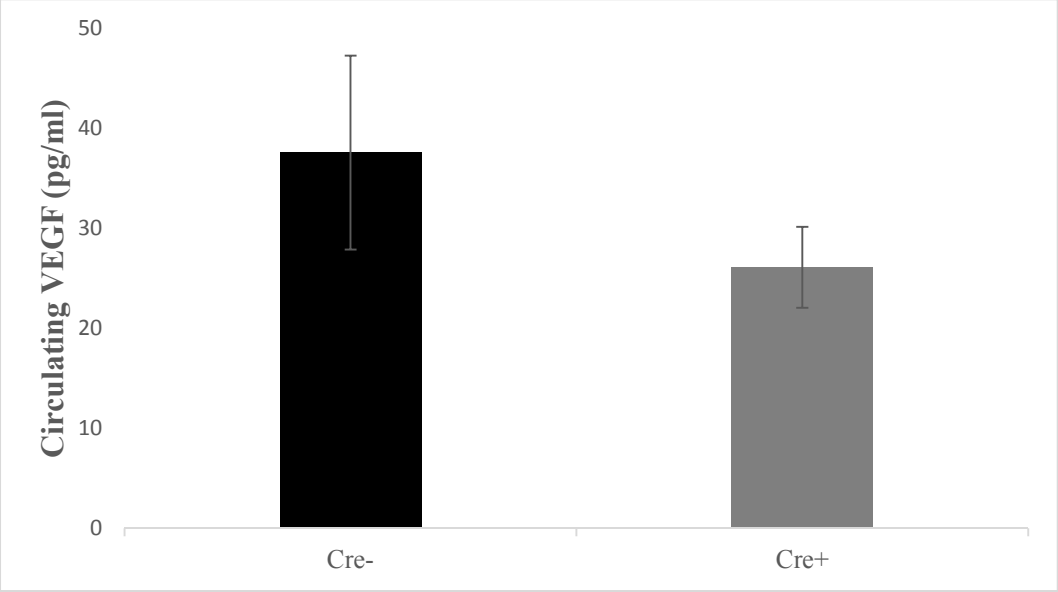


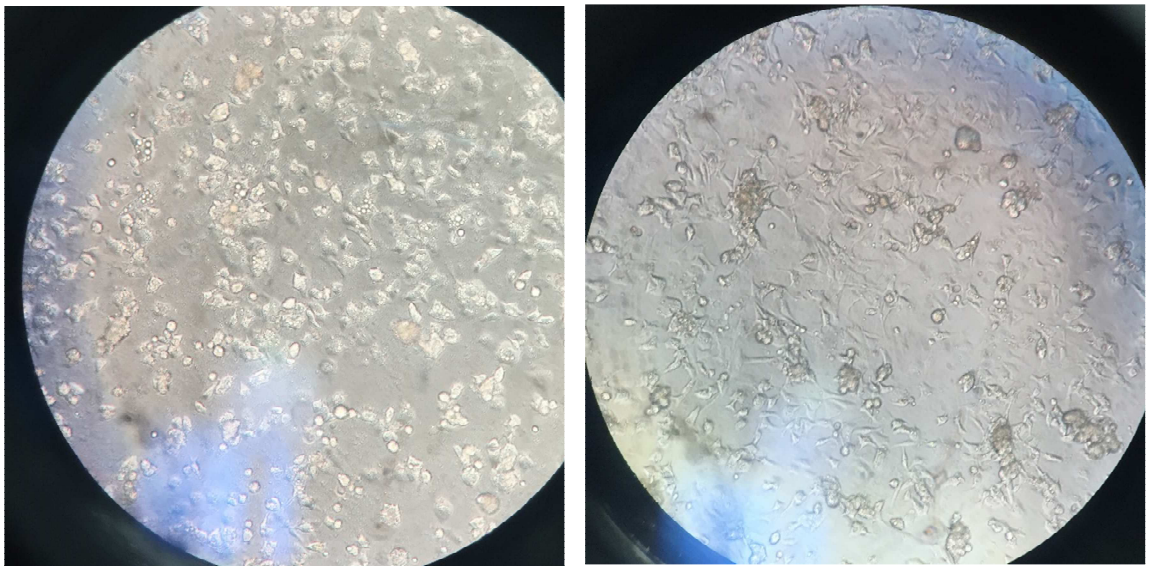
Figure 5: Circulating VEGF Concentrations in UCP1cre.VEGF$^{flox/flox}$ mice. Circulating concentration of VEGF in cre^{-} and cre^{+} mice as measured by ELISA. (cre^{-}, n=7-9; cre^{+}, n=6-9)

Primary Adipocyte Culturing and RNA Extraction

In order to analyze VEGF-A gene expression and distribution in UCP1cre^{+}.VEGF$^{flox/flox}$ and UCP1cre^{-}.VEGF$^{flox/flox}$ mice, we first needed to extract RNA from tissues or cell samples. Primary adipocyte cells, though difficult to obtain and to culture, would provide the greatest specificity for our research; isolated tissues, like BAT and PVAT, can be very heterogeneous as WAT, smooth muscle cells, and other extraneous cellular material can easily contaminate the samples. In contrast, primary brown or perivascular adipocytes, as detailed in the methods section, are more precisely isolated through collagenase digestion that breaks down extracellular matrices and filtration through cell strainers. Therefore, we initially cultured primary brown and perivascular adipocytes from UCP1cre^{-}.VEGF$^{flox/flox}$, UCP1cre^{+}.VEGF$^{flox/flox}$, and even

wild-type C57BL6/J mice. Photomicrographs of brown adipocyte cell culture are displayed in **Fig. 6A**. RNA was extracted from primary cell cultures, and whole tissue samples of BAT, PVAT, and heart. RNA concentrations and purity, as measured by 260/280nm absorbance ratio, were high in the isolated tissues and even in some of the UCP1cre^{-}.VEGF$^{flox/flox}$ samples, indicating the RNA extractions were a success (**Fig. 6B)**. At the same time, however, many of the cultured UCP1cre^{+}.VEGF$^{flox/flox}$ adipocyte samples, though pooled from multiple wells and mice (n=2 per group), still yielded low RNA concentrations that have hampered further analysis. Efforts will be made in the future to improve primary adipocyte culture by modifying factors such as mouse age and reagent freshness to obtain maximally viable and confluent cell cultures in the future, as detailed in the **Assessing Paracrine VEGF Signaling** section of the discussion.

A.



BAT Cre^{-} PVAT Cre^{-}

B.

Sample Source	RNA Concentration (ng/ul)	Absorbance (260nm/280nm)
Tissue		
Cre^- Heart	259.90	2.12
Cre^- Heart	113.70	2.11
Cre^- Heart	314.10	2.12
Cre^- Heart	356.50	2.12
Cre^- Heart	237.50	2.12
Cre^- Heart	268.30	2.14
Cre^+ Heart	383.20	2.13
Cre^+ Heart	314.80	2.12
Cre^+ Heart	327.10	2.14
Cre^+ Heart	271.90	2.13
Cre^+ Heart	346.50	2.12
Cre^- PVAT	263.00	2.12
Cre^- PVAT	143.10	2.10
Cre^- PVAT	205.90	2.10
Cre^- PVAT	235.90	2.12
Cre^- PVAT	245.00	2.1
Cre^- PVAT	254.30	2.12
Cre^- PVAT	292.90	2.11
Cre^+ PVAT	161.50	2.11
Cre^+ PVAT	215.10	2.11

Cre^+ PVAT	279.40	2.13
Cre^+ PVAT	164.00	2.11
Cre^+ PVAT	301.00	2.11
Cre^- BAT	400.80	2.12
Cre^- BAT	106.30	2.10
Cre^- BAT	453.10	2.11
Cre^- BAT	282.70	2.11
Cre^- BAT	175.40	2.11
Cre^- BAT	435.20	2.12
Cre^- BAT	379.70	2.11
Cre^+ BAT	246.60	2.14
Cre^+ BAT	459.00	2.11
Cre^+ BAT	297.60	2.12
Cre^+ BAT	248.50	2.10
Cre^+ BAT	208.00	2.11
Young PVAT	14.5	2.03
Young PVAT	120	2.09
Young PVAT	138.4	2.10
Young PVAT	182.7	2.10
Young PVAT	212.4	2.10
Young PVAT	220.9	2.10
Young PVAT	311.5	2.10
Young PVAT	205.3	2.09

Young PVAT	171.1	2.10
Aged PVAT	292.8	2.09
Aged PVAT	163.5	2.08
Aged PVAT	146.9	2.07
Aged PVAT	277.3	2.09
Cell Culture		
C57BL6/J BAT	47.8	2.03
C57BL6/J PVAT	82.9	2.05
Cre^{+} PVAT	12.30	1.98
Cre^{-} PVAT	42.60	2.11
Cre^{+} BAT	111.80	2.09
Cre^{-} BAT	132.20	1.91

Figure 6: PVAT primary cell culture and RNA extraction. A) Representative images, taken by phone through light microscope lens at 10X, of UCP1cre^{-}.$VEGF^{flox/flox}$ differentiated primary brown adipocyte (left) and perivascular adipocyte (right) cultures on day 7. B) RNA extraction data categorized by sample source, cre status (red is negative, blue is positive), RNA concentration, and purity as measured by the 260nm/280nm absorbance ratio (A: BAT and PVAT cre^{-}, n=2; B: C57BL6/J, n=3)

DISCUSSION & FUTURE DIRECTIONS

This study was designed to explore the basic characteristics of the VEGF pathway and analyze the expression of VEGF-A in PVAT as a primary regulator of vascular and oxidative function. Our UCP1cre.VEGF$^{flox/flox}$ mouse model, which specifically ablates VEGF in brown adipocytes, will allow us to more precisely determine the extent to which brown adipocyte-derived VEGF directly affects vascular function without possible confounding from global deletion of WAT-derived VEGF. With this model, we can analyze the VEGF angiogenic pathway from release of VEGF-A from thoracic PVAT to its interactions with vascular smooth muscle cells and endothelial cells that play a role in atherosclerosis of the aorta and other major arteries. Future study of obesity- and age-associated dysregulation of VEGF-A in PVAT may elucidate novel mechanisms that contribute to the pathogenesis of CVD and targets for next-generation vascular therapeutics.

Assessing Paracrine VEGF Signaling

In the present study, we have explored the role that VEGF-A expression and secretion from adipose tissue plays in the coordination of angiogenesis and vascular homeostasis. Despite the success of other laboratory members to culture primary brown adipocytes isolated from UCP1cre.VEGF$^{flox/flox}$ mice, recent attempts have met with little success. Briefly, low cell yields, qualitatively defined as a lack of confluency in cultured primary brown adipocytes, were repeatedly observed upon collagenase digestion, adipocyte isolation, and immediate plating. Though experimenters varied through multiple run-throughs, improvements came from changes including: making fresh buffer

solutions, decreasing the time from dissection to plating, and eliminating one of the two wash steps. The greatest improvement was observed after using a syringe plunger to facilitate the movement of the mixture of cells in collagenase through the 100μm and 40μm filters, followed by enhanced collection of the filtrate from the filter underside using a pipette. Though experimental conditions remained essentially identical to prior successful iterations of the primary brown adipocyte culturing protocol, RNA extraction of these non-confluent adipocyte cultures yielded low RNA concentrations that prevented proper use in subsequent RT-PCR analysis. Furthermore, our RT-PCR experiments with UCP1cre.VEGF$^{flox/flox}$ mouse cDNA, collected from RNA extracted directly from isolated BAT, WAT, and heart tissues, were often inconclusive and error-prone, but we have yet to identify the cause(s). These experimental inconsistencies have prevented reliable analysis from our more recent experiments and, as such, much of this manuscript has focused on prior work and future directions.

Beyond enhancing the reliability of our primary culture and RT-PCR protocol, in the future we would like to explore other aspects of VEGF-A and VEGF isoform expression. Once successful, we could use RT-PCR to measure VEGF isoform distribution across tissue types, including heart, BAT, and PVAT. With confluent cultures of primary brown and perivascular adipocytes from UCP1cre^{+}.VEGF$^{flox/flox}$ and UCP1cre^{-}.VEGF$^{flox/flox}$ mice, we could also analyze protein levels of VEGF-A_{120}, VEGF-A_{164}, and VEGF-A_{188} as that would be a more direct method of analyzing the VEGF-A isoform distribution in brown-like adipocytes. It would be of interest to generate conditioned media from these primary cultures and treat vascular smooth muscle cells

(VSMCs) to analyze how VEGF ablation affects rates of proliferation and differentiation in culture as well as migration when measured by wound healing assay.

VEGF and Aging

During the course of this study, we attempted to analyze VEGF-A isoform expression in the PVAT of young mice, aged 3-4 months, and older mice, aged 24 months. Though experimental inconsistencies have hampered our ability to draw conclusions from our RT-PCR study of VEGF-A gene expression, addressing aging-associated changes in VEGF expression across tissue types is critical to better our understanding of vascular pathologies, such as arteriosclerosis and cerebral microangiopathy, which often develop later in life. In the past decade, studies have identified that VEGF as well as VEGFR1 and VEGFR2 expression is reduced as cells age (Baffert et al., 2004; Iemitsu et al., 2006; Villar-Cheda et al., 2009; Wang et al., 2004). Aortic smooth muscle cells isolated from aged rabbits display a lack of proper upregulation of VEGF in hypoxic conditions, a process which is normally stimulated by the recruitment of the transcription factor hypoxia inducible factor 1α (HIF-1α). Decreases in HIF-1α protein levels and binding activity thus constitute a deleterious state that occurs with progressive aging and can lead to VEGF reductions and subsequent impairments in angiogenesis and tissue oxygenation following ischemia (Rivard et al., 2000).

More recently it has also been shown that, compared to neonatal endothelial cells, aged human dermal microvascular endothelial cells exhibit reductions in VEGF

expression and angiogenesis. Expanding on previous work, the authors assert that this reduction is due to corresponding decreases in nuclear transport of the transcription factors P-STAT3 and P-CREB, which typically activate VEGF gene expression in concert with HIF-1α (Ahluwalia et al., 2014). Future therapeutic strategies will need to address this harmful decrease in HIF-1α activity. In fact, it has already been shown in aged mice that exercise can rescue HIF-1α activity and the ischemia-induced neovascularization needed for recovery via a phosphatidylinositol 3-kinase-dependent mechanism; pharmacologic or, indeed, physical activation of this upstream component of the VEGF pathway in elderly patients following ischemia may thus be a potent therapeutic tool to promote vascular regeneration (Cheng et al., 2010). Not all injuries are ischemic, however. It has also been shown that aged mice display defects in their ability to upregulate VEGF following mechanical injury to the sciatic nerve, illustrating the need to better understand all aspects of the VEGF angiogenic pathway in order to develop accurate and effective therapies (Pola et al., 2004).

In Vivo/Ex Vivo Arterial Stiffness and Function

Since aberrant VEGF expression is observed with aging and we observe changes in the vasculature when we ablate VEGF in brown-adipocytes, we hypothesize that VEGF deletion will increase stiffening of major arteries, such as the thoracic aorta, that are prone to vascular damage. In this regard, alternative analyses of vascular and arterial function from a mechanical as well as molecular perspective may shed further light on negative, age-associated cardiovascular changes. Aortic stiffness, an independent risk factor and pre-symptomatic biomarker for cardiovascular disease, has enjoyed recent

attention in scientific research. On a macroscopic level, a less distensible aorta reduces energy absorption of the pressure pulse and results in a corresponding increase in circulatory pressure. At the tissue level, heterogeneity of the arterial architecture influences blood vessel function; for example, endothelial cells in the intima control differentiated vascular smooth muscle cell (dVSMC) tone in the media. In large arteries such as the aorta, dVSMC contraction moderately reduces lumen size and significantly enhances stiffness. Cellular and molecular regulation of these actin-myosin contractions occurs in many ways, but recent work has highlighted the role of nonmuscle cytoskeletal actin and integrin-mediated focal adhesions (FAs) between the cell and extracellular matrix. Specifically, tyrosine phosphorylation on FA proteins is a signal of FA turnover following establishment of a FA. FAs take part in mechanotransduction of external physical forces to internal biological signals, which may play a role in the development of cardiovascular disease when aberrantly regulated (Saphirstein and Morgan, 2014). As PVAT surrounds coronary arteries and are known to signal to dVSMC through VEGF-A in a paracrine manner, ablation of endogenous VEGF-A in UCP1cre+.VEGF$^{flox/flox}$ mice may impair vascular tone and FA homeostasis.

These basic physiological insights must be paired with biomechanic analyses to fully describe the cellular regulation of aortic stiffness and focal adhesions. At low pressure, distensible elastin maintains wall pressure, whereas at high pressure collagen reorganizes to stiffen the vessel wall and bear more of the load. Mechanically, blood vessels are nonlinear, anisotropic, and viscoelastic with hysteresivity independent of strain rate; *in vivo* the vessel wall is pre-stretched and stressed, all of which suggests

dynamic and complex regulation of the vessel architecture at a cellular level. Contrary to previous theories which suggested that dVSMC transiently adjusts vessel tension distribution between elastin and collagen, recent studies have indicated that age and hypertension increase the inherent stiffness of aortic SMCs. Building on our basic mouse model, we could include age as a variable and analyze aortic SMC stiffness in young as well as old UCP1cre^{+}.VEGF$^{flox/flox}$ and UCP1cre^{-}.VEGF$^{flox/flox}$ mice. With this setup, we would expect aged control mice to display inherent impairments in vessel elasticity, while aged experimental mice would possess even stiffer arteries. Lastly, we could confirm the critical role of paracrine-derived VEGF-A in maintaining vessel homeostasis by performing a gain-of-function experiment; to accomplish this, a microinfusion catheter would deliver exogenous VEGF-A to the perivascular adipose tissue and/or thoracic aorta, as has recently been validated in swine and humans, in an attempt to rescue vessel elasticity (Karanian et al., 2010; Owens et al., 2014).

According to recent research, aortic stiffness can be classified as either functional or material. Material aortic stiffness is geometry-independent (e.g. Young's Modulus), whereas functional aortic stiffness (e.g. impedance) is geometry- and material composition-dependent. Functional aortic stiffness is best measured by pulse wave velocity (PWV), which is defined as transit distance divided by transit time between carotid and femoral pressure waveforms in the vessel walls; one can also measure aortic characteristic impedance (Z_c), which describes the ability of a vessel to limit flow caused by temporally varying gradients in pressure. The latter method is five-fold more sensitive to changes in diameter than the former, which is important when considering the vessel

context being studied. On the other hand, while material stiffness *in vivo* can be calculated from functional stiffness methods (using Bramwell-Hill or Moens-Korteweg equations), the results can be inaccurate due to a lack of control of geometric and loading factors. Thus, *in vitro* studies for material stiffness are considered superior, such as uniaxial ring stretching, biaxial tissue stretching, or pressurization of vessels, any of which could be performed on aortic rings or tissue isolated from UCP1cre^{+}.VEGF$^{flox/flox}$ and UCP1cre^{-}.VEGF$^{flox/flox}$ mice (Saphirstein and Morgan, 2014).

Increased functional aortic stiffness due to aging equilibrates impedance from the aorta and muscular arteries, which generates excessive pulse pressure and injures the microvasculature in high-flow, low-impedance organs such as the kidneys; this increased pressure itself can contribute to hypertrophy, promoting hypertension and further vascular insult. As mentioned previously, molecular mechanisms can also contribute to aortic stiffening. For example, elastin cannot be repaired and is thus highly susceptible to permanent crosslinking by advanced glycation end-products, calcification, and degradation by matrix metalloproteinases (Saphirstein and Morgan, 2014). One hypoxia-independent mechanism of VEGFR2-mediated angiogenesis involves phosphorylation of Src kinase via SH2 docking. Once activated, Src blocks apoptosis via Raf-1 stimulation and promotes focal adhesion development and cell migration due to recruitment of FAK and paxillin proteins that are a component of the FA system (Rahimi, 2006). Contractile stimulation of dVSMCs or aortic rings with phenylephrine (PE), an alpha adrenergic agonist and vasoconstrictor, results in tyrosine phosphorylation of FAK and paxillin proteins (Min et al., 2012).

Future work could assess if there is an impairment in activation of these proteins, as measured by phosphotyrosine immunoblotting, *in vitro* with dVSMCs isolated from UCP1cre^{+}.VEGF$^{flox/flox}$ mouse aortas compared to controls. Similarly, we could examine *ex vivo* the PE-induced isometric tension of aortic rings isolated from UCP1cre.VEGF$^{flox/flox}$ mice, which would be an improvement over previous work in our laboratory that only analyzed adipose-specific Fabp4cre.VEGF$^{flox/flox}$ mice. One interesting experimental variation would be to develop a VSMC-specific VEGFR2 knockout mouse model that would mimic the impairments in isometric tension we expect to see in UCP1cre.VEGF$^{flox/flox}$ aortic rings and support the necessity of paracrine VEGF-VEGFR2 signaling interactions for proper vascular tone.

Lastly, *in* vivo one could assess functional aortic stiffness of UCP1cre.VEGF$^{flox/flox}$ mice using invasive pressure catheterization (Millar Instruments) of the thoracic aorta, as prior work from our laboratory failed to find stiffness differences from typical PWV measurements, which only target the WAT-like abdominal aorta, in Fabp4cre.VEGF$^{flox/flox}$ mice. As our new model of UCP1-targeted VEGF ablation should only affect the BAT-like thoracic aorta, results from thoracic pressure catheterization could be compared not only across cre^{+} and cre^{-} mice, but also between thoracic and abdominal aortic stiffness. In any of these experiments, if a difference was identified it would shed light on a new role for BAT and PVAT-derived VEGF in the modulation of age-dependent aortic stiffness and SMC contractility from a mechanical as well as molecular standpoint. In the future, therapeutics may be developed that temporarily sequester substrates away from Src or that reduce vasoconstrictor induction of VSMC

contractile signaling and thereby lessen the tendency for aortic stiffening with age (Min et al. 2012).

Based on this study and our previous work, we continue to hypothesize that VEGF plays a critical role in maintaining vascular homeostasis and modulating arterial function. The development of novel therapies that target VEGF pathways may therefore enhance existing treatments for cancer and age- or obesity-associated vascular diseases.

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CURRICULUM VITAE

Adam Winslow Long

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Birth year: 1993

Education

Lehigh University – Bethlehem, PA
B.S., *magna cum laude* in Behavioral Neuroscience, 2014
GPA: 3.66

Relevant Courses: Atherosclerosis Research Proposal; Synapses, Plasticity, and Learning; Experimental Neuroscience Lab; Diseases of the Nervous System; Endocrinology of Behavior; Development and Disease; Biochemistry I/II with Lab, Biostatistics; Biomedical Externship at St. Luke's Hospital

Honors: National Society of Collegiate Scholars *2012*; AP Scholar with Distinction *2011*; Dean's List *Fall 2011, Spring 2013, Fall 2013, Spring 2014*

Work Experience

Ophthalmic Technician at Brighton Eye Associates – Brighton, MA
November 2014-March 2015

Optometry Practice specializing in family care

- Interviewed and recorded patient medical and optometric histories
- Performed non-contact tonometry, autorefraction keratometry, fundus photography, optical coherence tomography, and administered dilation drops
- Scheduled appointments and ordered prescription contact lens supplies

Summer Intern at Akamai Technologies – Cambridge, MA
Summer 2008-2011

Member of Network Support group, which maintains social and financial relationships with ISPs

- Updated database of network information and organized files of peering contracts
- Contacted Internet Service Providers (ISPs) to optimize content delivery
- Validated payment invoices (up to $200K)

Research Experience

Research Assistant - Lehigh University, 3 Semesters & 1 Summer
Fall 2012-Spring 2014

Lab of Dr. Marcos Pires, Department of Chemistry

- Wrote grant proposals for the synthesis of a molecular vehicle to monitor protein-protein interactions and received $1500 Lehigh College of Arts and Sciences

Undergraduate Research Grant and $4000 grant from the Lehigh Grants for Experiential Learning in Health program

- Designed and characterized an AsLOV2/Intein construct for photoinducible intein-mediated protein ligation
- Tested use of biologically inert D-amino acids for inhibition of *Staphylococcus aureus* biofilms

Summer Intern at Genetic Services, Inc. – Cambridge, MA
Summer 2012

- Prepared fly embryos for microinjection of client-specified DNA sequences
- Cross-bred transformed flies and isolated mutant offspring with conspicuous phenotypes to establish custom strains for clients

Researcher in PHAGES Research Program – Lehigh University
Spring 2012

Phage **H**unters **A**dvancing **G**enomics and **E**volutionary **S**cience, Science Education Alliance program

- Used Bacteriophage Recombineering of Electroporated DNA (BRED) and bioinformatics tools to analyze function of orphan genes in the mycobacteria bacteriophage *Butters*

Volunteer Experience

Nutrition Instructor for Cooking Matters Program - Massachusetts
September 2014-Present

- Taught low-income parents in Lawrence and Brighton, MA how to shop for and prepare healthy, affordable meals as part of Share Our Strength's No Kid Hungry campaign
- Introduced basic nutrition concepts such as reading food labels and meal planning

Volunteer at Massachusetts General Hospital – Boston, MA
July 2014-Present

- Transported Emergency Room patients to radiology for MRIs, CTs, Ultrasounds, and X-Rays
- Provided emotional support and hospitality services for radiation oncology patients

Nutrition Chair of Lehigh Fitness and Weightlifting Club – Lehigh University
Fall 2013-Spring 2014

- Founded organization to promote health and fitness using peer motivation and educational programs
- Set up a weightlifting mentoring program at a local middle school and nutrition panel discussions at Lehigh
- Researched nutrition topics and wrote articles for official blog: lehighfitnessandweightlifting.tumblr.com

Fundraising Chair of Engineers Without Borders – Lehigh University
Spring 2013-Summer 2014

Member since Spring 2012

- Researched non-governmental organizations for partnership in La Fragosa, Honduras

- Led a group of students to San Juan del Sur, Nicaragua to identify prospective water system projects and establish a working relationship with Nicaraguan communities and the local mayor's office
- Personally raised $40,000 from grants to support philanthropic projects in the United States, Honduras, and Nicaragua

Volunteer at St. Luke's Hospital – Bethlehem, PA
Summer 2013

- Assisted patients in the Emergency Department by setting up patients in rooms, providing hospitality services, and supporting families during emergencies

Research Skills

Spectrophotometry; Histology; RT-PCR; BRED; Restriction Mapping; Electrophoresis; Molecular Cloning; Light and Fluorescence Microscopy; SDS-PAGE; Bradford Assay; Western Blot; ELISA; Chromatography (Thin-layer, Column, Gel, Affinity, Ion-Exchange); Dissection; Cell culture

Other Activities

Boston Ski & Sports Club Soccer, Casa League Soccer
November 2014-Present
Alpha Chi Sigma Chemical Fraternity (AXS), Undergraduate and Professional Chapters
Winter 2011-Present
Lehigh United Ultimate Frisbee Team DIII
Fall 2012-Spring 2014
American Cancer Society Relay For Life
Spring 2007-Spring 2014
Leukemia and Lymphoma Society Walk For A Cure
Fall 1993-2011