# Soluble Tie 2: Mechanisms of Regulation and Role in Modulating Angiogenesis

by

Clarence Maurice Findley

Department of Pharmacology and Cancer Biology
Duke University

Date:_____

Approved:

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Christopher D. Kontos, MD, Supervisor

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Salvatore V. Pizzo, MD PhD

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Donald P. McDonnell, PhD

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Ann Marie Pendergast, PhD

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Daniel Kenan, MD PhD

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School
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ABSTRACT

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# Abstract

Angiogenesis, the production of new vessels from pre-existing vasculature, is a complex biological process that is dependent on a series of regulated events, including endothelial cell (EC) proliferation, migration, survival, and capillary morphogenesis (tube formation). These events are required for angiogenesis to occur properly and the steps are regulated by a variety of vascular growth factors and their receptors. Tie2, an endothelial receptor tyrosine kinase (RTK), is required for embryonic and postnatal angiogenesis. Studies have demonstrated that Tie2 is proteolytically cleaved, producing a 75 kDa soluble receptor fragment (sTie2). However, the mechanisms and function of sTie2 have not been elucidated. Here, we investigated signaling pathways and effector molecule(s) responsible for Tie2 cleavage. Additionally, we investigated the role of other growth factors and conditions on the degree of Tie2 cleavage. Finally, we examined sTie2 levels in peripheral artery disease, a human model of ischemic disease. We demonstrated that Tie2 cleavage is VEGF- and PI3K/Akt-dependent and sTie2 can bind Ang1 and Ang2 and prevent ligand-mediated Tie2 activation and downstream cellular responses. Also, ADAM15 cleaves Tie2 in a hypoxia-dependent manner and this response was also observed to be VEGF-mediated. With respect to peripheral artery disease, sTie2 levels were only significantly elevated in the most angiogenically compromised group (critical limb ischemia) of patients. These data shed light on the mechanism and function of Tie2 cleavage and suggest a role for sTie2 in mediating the angiogenic process.

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# List of Abbreviations

| | |
|---|---|
| 293-hTie2 | human epithelial kidney cell line stably expressing human Tie2 |
| 3T3-mTie2 | mouse fibroblast cell line stably expressing mouse Tie2 |
| ADAM | a disintegrin and metalloprotease protein |
| Ang | Angiopoietin |
| ATP | adenosine triphosphate |
| CLI | Critical Limb Ischemia |
| CSF | Colony stimulating factor |
| C-tail | Cytoplasmic tail |
| CVD | Cardiovascular Disease |
| DMEM | Dulbecco's Modified Eagle Medium |
| EBM | Endothelial Basal Medium |
| EGF | Epidermal growth factor |
| EPC | Endothelial Progenitor Cell |
| EC | Endothelial Cell |
| ECD | Extracellular domain |
| Eph | Ephrin |
| ExTie2 | Engineered Protein encompassing extracellular domain of Tie2 |
| FBS | Fetal Bovine Serum |
| FGF | Fibroblast growth factor |
| HEK293 | human epithelial kidney cell line |
| HUVEC | Human umbilical vein endothelial cell |
| IC | Intermittent Claudication |
| kDa | Kilodalton |

| | |
|---|---|
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix Metalloprotease |
| NGF | Nerve growth factor |
| NIH-3T3 | mouse fibroblast cell line |
| NRP | Neuropilin |
| PAD | Peripheral Artery Disease |
| PI3K | phosphoinositide-3-kinase |
| RTK | Receptor Tyrosine Kinase |
| sFlt | soluble VEGF receptor 1 |
| sTie2 | soluble Tie2 |
| TACE | TNF-alpha converting enzyme |
| Tie | Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains |
| VEGF | Vascular endothelial growth factor |

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First, I would like to thank my mother for making all of this possible. Without her love, guidance, sacrifice and financial support, none of this would have been possible. Her willingness and desire for both of her kids to achieve whatever it is they wanted has manifested itself in countless ways and for that we are both grateful. To my father, who challenged me to set goals for myself and stop at nothing to achieve them…"The squeaky wheel gets the grease." To my sister who has been nothing but supportive throughout this entire process…your time is coming soon. To all other family members, grandmother, aunts and uncles, thanks for your support…the doctor is in! To my girlfriend, Hewan, who has served as a constant emotional support, even when I questioned whether or not this process was worth it. Your encouraging words helped to bolster me as I navigated the PhD and I can only hope that I am half as supportive as you were for you and your aspirations.

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# 1. Introduction

Cardiovascular disease (CVD), including coronary, renal, cerebrovascular, and peripheral arterial disease, accounted for over 860,000 deaths in 2004 in the United States (1). The development of CVD is multi-factorial, with major predisposition conferred via genetic and environmental factors, including family history, tobacco use, hypercholesterolemia, and diabetes mellitus. Collectively, these factors contribute to the development of atherosclerosis of the arterial vasculature, which leads to progressive narrowing of the arteries and eventual production of ischemic tissue environments distal to stenotic lesions. One of the body's compensatory physiologic responses to ischemia is the growth of new blood vessels from pre-existing vascular structures, or angiogenesis, which occurs in an attempt to re-establish blood supply to nutrient-deficient, ischemic areas. Typically, however, the angiogenic response is insufficient to fully compensate for the degree of tissue ischemia. Hence, efforts to enhance the angiogenic response would have a dramatic impact on a large number of CVD patients.

## *1.1 Angiogenesis*

Angiogenesis is a complex process comprised of numerous steps, including endothelial cell (EC) proliferation, migration, survival, and capillary morphogenesis (tube formation). In the atherosclerotic setting, hypoxia induces the expression of angiogenic growth factors from stromal tissues as well as from ECs themselves, which ultimately results in the production of a variety of proteases (ie, MMP2 and MMP9) (2-5). These proteases attack the basement membrane and produce "channels" through which endothelial cells migrate to produce nascent vascular sprouts. These vascular sprouts

undergo a process of capillary morphogenesis, during which the sprout continues to migrate, elongate, proliferate and form capillary tubes. Pericyte and vascular smooth muscle cell recruitment aid in the maturation and ultimate stabilization of the new blood vessel. The angiogenic response to some stimuli can be extremely robust and produces an excessive number of vascular structures. However, a period characterized by the retraction of existing vascular structures, or vascular regression, follows the angiogenic response to regulate vessel over-production (6).

Numerous events are required for the angiogenic process to occur properly and these steps are regulated by a variety of vascular growth factors and their receptors (7). Among the most important regulators of angiogenesis are a group of receptor tyrosine kinases (RTK) and their cognate ligands, which have effects that are largely specific for endothelial cells. These factors include the vascular endothelial growth factors (VEGF) A-D and their receptors, VEGF receptor-1 (VEGFR-1), VEGFR-2, and neuropilin (NRP)-1 and -2; Angiopoietins (Ang) 1-4 and the Tie receptors (Tie1 and Tie2); the fibroblast growth factors (FGF) and their receptors; and the ephrin ligands and the Eph receptors, among others (8-12). VEGF and its receptors regulate the majority of steps in angiogenesis, including protease production, endothelial cell migration and proliferation and capillary morphogenesis. The Tie2-Ang system is known to regulate EC survival as well as the complex interplay between ECs and supporting perivascular cells, i.e. pericytes and vascular smooth muscle cells. FGF and its receptors regulate EC proliferation, migration, protease production, and integrin/cadherin receptor expression (13). Additionally, the ephrin ligands and Eph receptors mediate endothelial cell migration, vascular morphogenesis, and arterio-venous specification. The function of these receptor systems overlaps, however, receptor systems with similar functions

cannot fully compensate for perturbations in other receptor systems, as observed in a number of genetic knock-out studies (14-17). Each receptor system activates a unique complement of signaling pathways and investigating the complex relationship between these receptor systems will provide useful insight into angiogenesis.

The complexity of angiogenesis is highlighted by many failed attempts to promote therapeutic angiogenesis in patients with ischemic cardiovascular diseases (18-20). Given this complexity, it is not surprising that these studies have not promoted clinically relevant angiogenesis. In-depth knowledge of different receptor systems, their regulation and interplay will further current understanding of EC biology, and ultimately, lead to successful modulation of the angiogenic process for therapeutic gain.

## *1.2 Tie2 in Angiogenesis and Vascular Remodeling*

Hypoxia induces the expression of vascular endothelial growth factor (VEGF), which can be considered the prototypical angiogenic factor, along with other regulators of angiogenesis, including Ang2. As mentioned previously, VEGF expression is partly responsible for a number of early steps in angiogenesis, including basement membrane breakdown, vascular permeability, endothelial cell proliferation and migration, and capillary sprouting (17). However, VEGF does not mediate the initial vascular destabilization necessary to initiate angiogenesis or the ultimate maturation of newly formed blood vessels. Both of these steps of angiogenesis are regulated, in part, by the RTK, Tie2, and its ligands, the Angiopoietins.

The essential nature of Tie2 in embryonic vascular development is evident from the results of genetic studies from several investigators (16, 21-23). Tie2 -/- mice die at embryonic day 9.5 to 10.5 with severe vascular abnormalities characterized by dilated blood vessels with diminished vascular branching, especially in the head and heart; alterations in cardiac development; ultra-structural vascular changes consistent with disorganized subendothelial matrix; altered recruitment of periendothelial supporting cells; and decreased endothelial cell survival (22). Postnatally, the importance of Tie2 has been shown through a number of studies in which inhibition of the Tie2-Ang system resulted in decreased angiogenesis. For example, inhibition of Tie2 signaling with a soluble receptor (24-27), inhibition of Ang2 with an aptamer (28) or "peptibody" (29) all resulted in the effective inhibition of angiogenesis in tumor and corneal angiogenesis models. Moreover, mice lacking Ang2 displayed deficient retinal angiogenesis, a postnatal form of vascular growth that is VEGF-dependent, suggesting that Ang2 facilitates the early VEGF-mediated steps in angiogenesis. These findings demonstrate an important role for Tie2 in regulating vascular morphogenesis and remodeling in both the embryo and adult.

## *1.3 The Angiopoietins Have Distinct and Opposing Functions*

Tie2 is unique among RTKs in that its ligands, Angs 1-4, have been demonstrated to have opposing actions in both genetic and biochemical studies. The best characterized of these ligands are Ang1 and Ang2 (30, 31), which are highly homologous (~60% amino acid identity) and bind Tie2 with similar affinity ($K_D$ ~3 nM for both) (30-33). Thus, competition for Tie2 binding and subsequent effects on the

vasculature are likely dictated by the relative temporal and spatial expression of Ang1 and Ang2 within a given tissue. Interestingly, the phenotypes of both Ang1 and Tie2 knockout mice resemble that of transgenic mice overexpressing Ang2 in the endothelium, suggesting that Ang1 and Ang2 perform opposite roles during embryonic vascular development (14, 34, 35).

Biochemically, Ang1 induces Tie2 phosphorylation in endothelial cells whereas Ang2 does not, supporting the notion that Ang2 can act as a Tie2 inhibitor in certain contexts. Furthermore, while Ang2 has been shown to be upregulated in states of vascular regression, Ang1 has been shown to inhibit VEGF-mediated increases in vascular permeability and adhesion molecule expression (36). Based on these results, it is believed that Ang1 is constitutively expressed and activates Tie2 to promote vascular maturation and stabilization, rendering the endothelium resistant to many factors that disrupt normal vascular homeostasis. Vascular perturbations, such as wound healing, tumor angiogenesis and ischemia, increase Ang2 expression and ultimately destabilize the endothelium by inhibiting Ang1-mediated Tie2 activation. In the presence of endothelial mitogens like VEGF, vascular destabilization results in angiogenesis, whereas in the absence of VEGF, Ang2 appears to promote endothelial cell apoptosis and vascular regression (Fig. 1). Based on these observations, the Angiopoietins and Tie2 have become critically important targets for the therapeutic modulation of angiogenesis and other forms of pathological vascular remodeling.

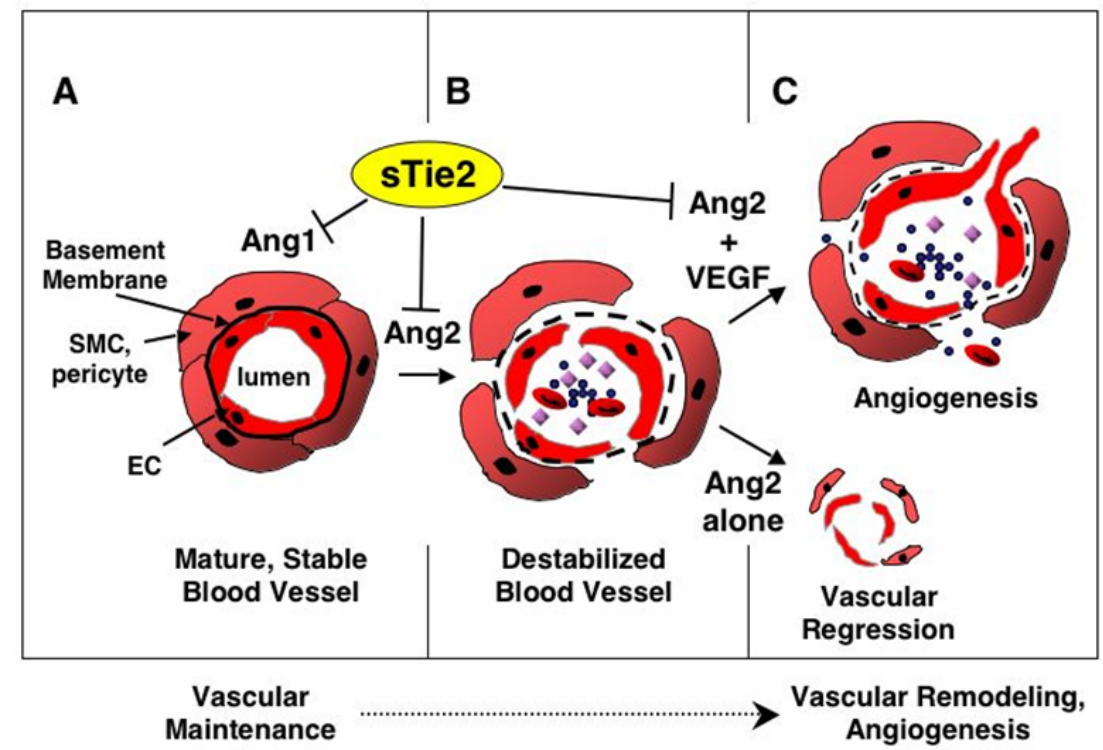


**Figure 1: Proposed model of the Angiopoietins and soluble Tie2 (sTie2) in the regulation of vascular maintenance and remodeling.**

Ang1 and Ang2, together with other endothelium-specific factors like VEGF and sTie2, regulate the transition from mature, stable blood vessels to angiogenic or remodeling vessels, as described in the text. Soluble Tie2 can negatively regulate the effects of the Angiopoietins.

## *1.4 Tie2/Angiopoietin-mediated Signaling Pathways*

Despite the abundance of information on the Angiopoeitin/Tie2 system derived from genetic studies, much remains unknown about the molecular mechanisms through which Tie2 regulates the transition between stable and remodeling blood vessels. The ligand binding domains for Ang1 and Ang2 appear to be the same, however the downstream responses are opposite, suggesting that Ang1 and Ang2 may active distinct sets of signaling pathways (37, 38). Ang1 has been shown to activate endothelial cell survival via the PI3-kinase/Akt pathway (39-41). Also, Ang1 has been demonstrated to regulate endothelial cell migration via activation of MEK (42). Recently, Ang1 was shown to activate RhoA, thereby leading to Src sequestration via mDia to mediate its EC blood vessel stabilizing function (i.e., inhibition of VEGF-induced permeability). The majority of studies investigating Tie2 signaling have focused on Ang1-mediated pathways. Less is known about the signals transduced by Ang2, in part due to the fact that Ang2 typically fails to activate Tie2 phosphorylation in endothelial cells, making the study of Ang2-mediated signaling pathways difficult. Ang2-induced activation of Tie2 has been observed in non-endothelial cells exogenously expressing Tie2, suggesting that in endothelial cells, Ang2 may induce the recruitment of a tyrosine phosphatase to Tie2, such as vascular endothelial protein tyrosine phosphatase (VE-PTP), thereby resulting in diminished tyrosine phosphorylation (43). Surprisingly, recent studies have demonstrated Ang2-mediated Tie2 phosphorylation and downstream signaling in endothelial cells (44). Ang2 elicited a transient increase in the phosphorylation of Tie2, protein kinase B (Akt), ERK1/2, and p38 mitogen-activated protein kinases. A potential explanation for these discrepant results comes from recent studies demonstrating that concomitant expression of Tie1 may dictate the functional effects of Ang2 on Tie2. In

endothelial progenitors cells (EPCs), Ang2 was shown to induce Tie2 phosphorylation, and this effect was due to the lack of Tie1 expression. In HUVECs that expressed Tie1, Ang2 failed to induce Tie2 activation, an effect that was reversed by silencing Tie1. Taken together, these findings support the notion that the effects of Ang2 are context-dependent and are influenced by co-expression of other proteins, including the related Tie1 receptor.

## *1.5 Regulation of Tie2 Expression, Activity and Function*

Tie2 is expressed exclusively in ECs and their precursors, and endothelial expression is regulated by the well-defined Tie2 promoter and enhancer elements. An autonomous endothelial-specific enhancer was identified within the first intron of the mouse Tie2 gene (45) and was demonstrated to regulate Tie2 expression uniformly in vascular structures. An upstream inhibitory region and two positive regulatory regions were identified within the promoter, and these regions are thought to contribute to the developmental and post-natal regulation of Tie2 expression (46). The transcription factor Ets-1 has been shown to transactivate the Tie2 gene promoter, resulting in a 15- to 20-fold induction in Tie2 transcript levels. Another Ets transcription factor family member, NERF2, was shown to bind to both the Tie2 gene promoter and enhancer regions. It is believed that the Ets factors synergistically act through both of these regions to regulate appropriate vascular expression of the Tie2 gene. Also, mutational analysis implicated a role for Ets factors in the regulation of Tie2 promoter basal activity in ECs (47).

In addition to expression, several studies have evaluated intrinsic Tie2 receptor activity. The crystal structure of the Tie2 kinase domain has been determined and several unique characteristics set this molecule apart from other receptor tyrosine kinase systems (48). Generally, the kinase domain is comprised of a catalytic core, kinase insert domain and a carboxy-terminal tail (C-tail). Unlike other RTKs in which the activation loop prohibits substrate or ATP binding in the inactive state, Tie2's unphosphorylated activation loop adopts an active conformation. Additionally, the nucleotide binding loop is maintained in an inhibitory position and the C-tail blocks the substrate binding site. To activate the receptor, conformational changes in the nucleotide binding loop, activation loop and C-tail are required for ATP and substrate binding. The C-tail contains tyrosines 1101, 1107 and 1112 (murine residues), which have been demonstrated to serve as binding sites for PI3K, Grb family proteins and Dok-R(40-42, 49). In the unphosphorylated state, the hydroxyl groups of Y1101 and Y1112 are hydrogen bonded to surrounding residues, likely stabilizing the C-tail in its inhibitory conformation. Studies from our lab demonstrated that deletion of the C-tail significantly enhanced Tie2 autophosphorylation, kinase activity and activation of Tie2- dependent signaling pathways both in the absence and presence of ligand-dependent stimulation (50). These findings confirmed that the C-tail performs a negative regulatory function and serves to modulate Tie2 activity.

Despite the wealth of knowledge surrounding Tie2 expression, activation and signaling, very little is known about post-translational mechanisms modulating Tie2 receptor expression, specifically at the cellular membrane. An important aspect of RTK regulation is the process of internalization and subsequent degradation. Typically, ligand-mediated activation of RTK signaling results in receptor dimerization or clustering

with subsequent autophosphorylation, substrate recruitment, and activation of downstream signaling cascades (51, 52). Ultimately, the receptor is internalized and either degraded or recycled back to the plasma membrane. In response to either Ang1- or Ang2-mediated stimulation, Tie2 internalization was observed, thereby demonstrating a role for receptor internalization in the regulation of Tie2 (53). To date, however, little is known about the mechanisms of Tie2 internalization or degradation. In addition to Tie2 receptor internalization, another mechanism of Tie2 regulation has been identified. Evidence from Reusch et al. demonstrated the presence of a truncated, soluble form of Tie2 (sTie2) in vivo in the serum of healthy volunteers, and in vitro in conditioned media from human umbilical vein endothelial cells (HUVECs) (54). A number of soluble receptors whose function is not well understood appear to act as negative regulators of angiogenesis by binding secreted growth factors, thereby prohibiting ligand interaction with their respective full-length, membrane-bound receptors. Evidence suggests that soluble receptors can regulate receptor signaling and function by decreasing receptor density at the plasma membrane and/or by producing soluble receptors that function as ligand traps to decrease ligand-dependent receptor activation (55).

## *1.7 Receptor Shedding, the ADAM Proteins, and Soluble Tie2 (sTie2)*

The recent identification of endogenously produced sTie2 provides an additional level of regulation for this receptor-ligand system, since sTie2 could act at multiple levels to regulate Tie2 signaling and function (Fig. 1). Unlike the soluble form of VEGFR-1 (also called sFlt), which is a splice variant of the VEGFR-1 transcript (56), sTie2 appears to result from metalloprotease-mediated cleavage of the Tie2 extracellular domain (ECD)

(Fig. 2B). Cleavage of RTKs and ectodomain shedding has most often been linked to the ADAM proteins, so-called because of their presence of a disintegrin and metalloprotease domain (57). A number of RTKs are regulated by protease-mediated shedding of their ECDs, including epidermal growth factor (EGF) receptors, colony-stimulating factor (CSF) receptor, nerve growth factor (NGF) receptor, and c-kit, among others (58-61). In all of these cases, ADAMs have been implicated as the major sheddases responsible for ECD cleavage (Fig. 2B), and the protein most commonly implicated is ADAM17, also known as tumor necrosis factor-$\alpha$ converting enzyme (TACE). To date, 23 ADAMs have been identified in humans (62). ADAM proteases cleave a variety of proteins such as receptors, adhesion molecules and membrane anchored growth factors, and as a result, serve an important role in regulating countless biological processes. Interestingly, several ADAMs have been implicated in cardio- and angiogenesis. Knockout mice for these ADAMs, specifically ADAM10, ADAM15, ADAM17 and ADAM19, display aberrations in cardiovascular (heart and blood vessel) development (57). Because of these ADAMs' essential role in cardiovascular biology and ability to cleave membrane spanning/anchored proteins, these are likely candidates for the effector molecules regulating Tie2 proteolysis.

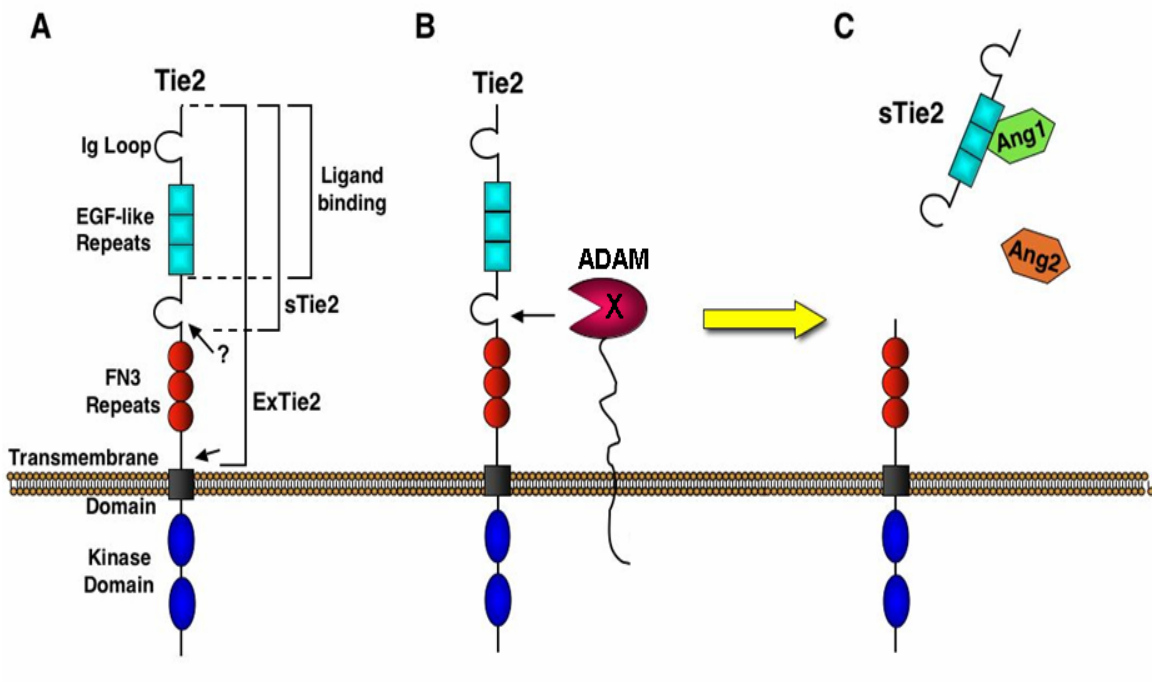


**Figure 2: Schematic diagram of soluble Tie2 proteins and putative shedding mechanism.**

(A) Domains within Tie2 are shown; the ligand-binding domain contains the first Ig loop and the EGF-like repeats. Soluble Tie2 proteins include the engineered ExTie2, terminating at the membrane (lower arrow), and endogenous sTie2. The approximate cleavage site of sTie2 is depicted by the arrow and "?". (B) sTie2 is likely shed by an ADAM protein (the "sheddase"), which can be activated by various factors inside and outside the cell. (C) Tie2 cleavage results in disruption of signaling and shedding of sTie2, which can act as a ligand trap, further inhibiting signaling.

## *1.8 Preclinical Studies of sTie2*

As for other RTKs, the function of Tie2 receptor shedding is unclear but may serve to modulate receptor expression at the plasma membrane or to bind ligand locally or systemically to modulate ligand-mediated Tie2 activation. As discussed below, preclinical studies suggest that sTie2 plays an inhibitory role in Tie2 signaling, possibly by thwarting the angiogenic process through inhibition of the early stages of angiogenesis (vascular destabilization) and/or inhibiting the later stages of angiogenesis (vessel maturation). However, the functional role of sTie2 may be more complex, and it is important to consider the physiological roles and expression of all components of the Tie2-Ang system. Ang1, which is secreted and incorporated into the extracellular matrix via its linker peptide region and is primarily extracellular matrix-bound, mediates vascular stabilization (63). Ang2, which is secreted and circulates systemically, mediates vascular destabilization. As demonstrated in a number of studies, Ang1 (~ 5-15 ng/ml) and Ang2 (~ 5-15 ng/mL) can both be readily identified in serum (64-66). Because these ligands have opposing actions on the vasculature, regulation of their expression and interaction with Tie2 is essential to the maintenance of vascular homeostasis. sTie2 circulates systemically and may possess the abilty to bind both Ang1 and Ang2 and inhibit ligand-mediated Tie2 interaction (54). As a result, sTie2 may function physiologically to maintain vascular homeostasis by preventing excessive Ang1- and/or Ang2-mediated Tie2 stimulation. However, as with any system, altering the tightly regulated expression levels of different proteins may have deleterious effects.

To date, the Tie2 cleavage site has not been identified, hence engineering a sTie2 construct similar to endogenous protein is not possible. The most relevant studies analyzing the effect of a Tie2 ligand trap on vascular biology have utilized an engineered

soluble Tie2 receptor that comprises the entire extracellular domain of full-length Tie2 (referred to as ExTie2). Engineered soluble receptors have been used in a number of studies as ligand traps to inhibit RTK signaling (67), and such an approach is also being used clinically to target tumor angiogenesis. ExTie2 has been shown to bind Ang1 and Ang2 and inhibit ligand-mediated Tie2 activation. ExTie2 is larger than the endogenous form of sTie2 (100kDa vs 75kDa), as it comprises the entire extracellular domain. Although the additional 25kDa of protein may alter protein activity, the data gleaned from ExTie2 and its effects on the vasculature are the most representative data available to date. Specifically, ExTie2 has been used by our laboratory and others to block Tie2 signaling and demonstrate that Tie2 is required for different forms of angiogenesis(24, 25, 68). For example, when mammary gland tumors were implanted subcutaneously in rats (24), the intravenous administration of an adenovirus encoding ExTie2 resulted in a 75% reduction in tumor volume as well as a 40% reduction in tumor vessel length density in established tumors. Additionally, these sets of studies also demonstrated decreased tumor metastasis in the rats overexpressing ExTie2, and these findings have been confirmed by other investigators (26). More recently, we demonstrated that adenovirus-mediated ExTie2 expression blocked both exercise-induced angiogenesis (69, 70). These experiments suggest that overexpression of ExTie2 inhibits angiogenesis in a variety of conditions. Based on these findings, it has been hypothesized that a similar phenomenon may occur in pathological states in humans (e.g. CVD or cancer), in which enhanced sTie2 concentrations result in an insufficient and/or aberrant angiogenic response.

## *1.9 Clinical Studies of sTie2*

Receptor shedding has been implicated in a number of pathological processes, such as inflammation, cell degeneration, apoptosis and oncogenesis. Since the Tie2-Angiopoeitin system has been implicated in several processes that disrupt vascular homeostasis, it is possible that Tie2 shedding may play a role in these processes. As mentioned previously, sTie2 was initially observed in vitro in the conditioned media of human umbilical vein endothelial cells (HUVECs) as well as in vivo in the serum of normal human volunteers (54). In addition to its presence in healthy individuals, reports have demonstrated that serum levels of sTie2 are elevated in patients with cancer. In a study of renal cell cancer patients compared to healthy control subjects, plasma sTie2 levels correlated with the presence of disease (71). In response to chemotherapy, decreases in sTie2 levels correlated with prolonged patient survival. Also, in patients with invasive breast and prostate cancers, sTie2 levels were significantly elevated compared to controls (72). In the breast cancer population, patients undergoing either mastectomy or wide local excision for biopsy-proven breast cancer, followed by standard best medical care (i.e., radiotherapy and/or chemotherapy with tamoxifen), experienced significant reductions in sTie2 levels three months post-procedure (73). These observations suggest a physiologically important and clinically relevant role for sTie2 in tumor angiogenesis.

In addition to its upregulation in cancer, sTie2 has also been shown to increase in patients with congestive heart failure, hypertension, and acute coronary syndromes, all of which are characterized by an important vascular remodeling component (pro- or anti- angiogenic) and EC dysfunction. In these disease states, sTie2 expression generally correlated positively with the expression of VEGF and Ang2, consistent with an

increased angiogenic state. Intriguingly, however, hypertension (66) and congestive heart failure (64) are associated with vascular rarefaction, therefore it is not clear whether the ultimate effect of sTie2 in these states is to promote or inhibit vascular growth. Elevated levels of soluble receptors in cardiovascular diseases likely result from aberrant signaling and cellular dysfunction, thus it is possible that the enhanced production of sTie2 is a marker of EC dysfunction. Moreover, sTie2 may play an inhibitory role in Tie2 signaling, thwarting the angiogenic process by inhibiting both the initial stages of angiogenesis (Ang2-mediated vascular destabilization) and the later stages of angiogenesis (Ang1-mediated vascular maturation). However, whether elevated levels of sTie2 and other soluble receptors are simply a marker of cardiovascular disease (including tumor angiogenesis) or are responsible for the development and progression of disease remains unknown. Work in this thesis will begin to address these issues.

## *1.10 Conclusions*

CVD is the leading cause of morbidity and mortality in the United States (1). In the setting of ischemia, the compensatory physiological response is collateral vessel growth and/or vascular remodeling. As a result, understanding the basic mechanisms of vascular growth and remodeling has clear implications for the treatment of CVD. The observations discussed here provide important insights into our understanding of Tie2's role in regulating angiogenesis and vascular remodeling. In the quiescent vasculature, sTie2 may regulate vascular stability by affecting Tie2 density at the plasma membrane and preventing excessive ligand-mediated effects of Tie2 activation on the vasculature.

However, in pathophysiological states, like cancer or CVD, cellular dysfunction arises and cell signaling becomes aberrant. In the case of sTie2, pathologically activated signaling pathways may lead to high levels of sTie2, which serve as a marker of endothelial cell dysfunction. Ang1 and Ang2 bind full-length Tie2 with similar affinity, therefore the temporal and spatial regulation of Ang concentrations likely plays a role in the transition from stable to remodeling blood vessels. Because sTie2 may bind the angiopoietins and prevent ligand-mediated stimulation of the receptor, sTie2 may serve a dual role. In the pathologic state, elevated levels of sTie2 may prevent initiation of both angiogenesis and vascular maturation by preventing Ang1- or Ang2-mediated Tie2 activation and, hence, compromise these essential physiological responses. Studies described in Chapters 2 and 3 of this thesis provide key insights into the mechanisms regulating sTie2 shedding as well as its functional effect on EC biology and the angiogenic process. Studies described in Chapter 4 provide clinically relevant insights into the role of sTie2 in peripheral artery disease (PAD), a poorly studied manifestation of CVD. It is hoped that these insights will lead to novel approaches to diagnose and treat a variety of vascular diseases, including heart disease, cancer and diabetes mellitus.

# 2. VEGF Induces Tie2 Shedding via a Phosphoinositide-3-Kinase/Akt-dependent Pathway to Modulate Tie2 Signaling

## *2.1* Introduction

Tie2, a RTK expressed predominantly on ECs and their embryonic precursors (74), is required for both embryonic and postnatal angiogenesis (22). Substantial data now indicate that Tie2 and its ligands, the angiopoietins, regulate the transition between mature, stable vasculature and angiogenic or remodeling blood vessels. Angiopoietin-1 (Ang1) acts primarily as a Tie2 agonist to promote vessel maturation by inducing interactions between endothelial and peri-endothelial support cells, including pericytes and vascular smooth muscle cells (16, 34, 35). In contrast, Angiopoieitin-2 (Ang2) is a context-dependent Tie2 antagonist, promoting vascular destabilization in part by opposing the effects of Ang1 but also through the activation of distinct endothelial signaling pathways (14, 30, 75). Despite advances in understanding the effects of angiopoietins on angiogenesis and vascular remodeling, little is known about mechanisms regulating Tie2 expression and downregulation.

Previous reports have demonstrated that the extracellular domain of Tie2 is proteolytically cleaved, resulting in the release of a 75 kDa soluble Tie2 (sTie2) protein. Shedding of sTie2 from endothelial cells can be stimulated by phorbol myristate acetate (PMA), but otherwise nothing is known about the mechanisms regulating this process (54). Soluble Tie2 is detectable in the serum of healthy individuals and is increased in a number of cardiovascular diseases, including congestive heart failure, coronary artery

disease, and cancer (64, 71, 76). Previous reports have demonstrated that Tie2 is expressed constitutively in the adult vasculature (77), and its expression increases in vascular remodeling states, such as breast cancer (78). Shedding of sTie2 in such conditions might regulate Tie2-Ang binding to facilitate angiogenesis. Alternatively, sTie2 shedding might simply be an indicator of normal receptor turnover. In either case, the function of sTie2 in endothelial cell biology remains unknown.

In this report, we investigated the role of soluble Tie2 in modulating Tie2 activity and cellular responses in vitro. sTie2 was found to bind both Ang1 and Ang2 and inhibit ligand-mediated Tie2 phosphorylation and endothelial cell apoptosis. Interestingly, Tie2 shedding was both constitutive and induced by VEGF via a PI3K/Akt-dependent mechanism, which had not been described previously. This suggests a novel mechanism by which VEGF may control Tie2-Ang1 activity to downregulate the stabilizing effects of Ang1 and promote a pro-angiogenic state. These findings have implications for understanding sTie2's role in the regulation of vascular growth and remodeling.

## *2.2 Materials and Methods*

### 2.2.1 Cell lines

HUVECs were freshly isolated from umbilical cords by standard techniques (79). Results obtained with HUVECs were validated with at least two different donor preparations of cells. NIH 3T3 and HEK-293 cells were from the American Type Culture Collection (ATCC). In all studies, HUVECs were used between passages 2 and 6 and were grown in endothelial growth medium containing microvascular endothelial cell supplement (EGM-MV, Clonetics Corp.), 10% fetal bovine serum (FBS), and 5%

penicillin/streptomycin and maintained in a 37°C, 5% $CO_2$ incubator. NIH 3T3 and HEK-293 cells were grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS and 5% penicillin/streptomycin/amphotericin (Invitrogen). Phoenix-Eco retroviral packaging cells were provided by Dr. Garry Nolan (Stanford University) and were grown in DMEM, 10% FBS plus pen/strep.

### 2.2.2 Antibodies and Reagents

Mouse monoclonal anti-Tie2 (clone 33), recognizing the extracellular domain of human and murine Tie2, has been described previously (78). Mouse monoclonal anti-phosphotyrosine (clone PY99) was from Santa Cruz Biotechnology. Mouse monoclonal anti-Ang1 and -Ang2 antibodies were from R&D Systems. Rat monoclonal anti-tubulin (clone YL1/2) was from Serotec. Rabbit polyclonal anti-Akt, anti-phosphoAkt (S473), and anti-cleaved caspase-3 antibodies were from Cell Signaling Technology. $Ni^{2+}$-NTA agarose was from Qiagen. Rabbit polyclonal antibody against phospho-Tie2 (pY1102/pY1108; Ab-1) was from Calbiochem. Phorbol-12-myristate 13-acetate (PMA), GM6001 (GM), bisindolylmaleimide I (BIS-I), PD98059 (PD), LY294002 (LY), SB203580 (SB), Isozyme-selective-Akt1/2 inhibitor VIII (AKTVIIIi) and Akt inhibitor X (AKTXi) were all from Calbiochem. Recombinant human Ang1, Ang2, and VEGF-$A_{165}$ were purchased from R&D Systems. The Cell Death Detection ELISA PLUS Assay was from Roche Applied Science.

### 2.2.3 Generation of Cell Lines Expressing Full-Length Tie2

HEK-293 cells stably expressing full-length human Tie2 (293-hTie2) have been

described previously (28). NIH 3T3 cells stably expressing full-length wild-type murine Tie2 (mTie2-WT) or the kinase-inactive K854R mutant of murine Tie2 (mTie2-KR) (49) were generated by retroviral infection. Briefly, the full-length murine Tie2 cDNA (49, 80) was cloned into the pLNCX retroviral expression vector (Clontech). Recombinant ecotrophic retrovirus was generated by transfection of LNCX-mTie2 or LNCX-mTie2-KR into Phoenix-Eco packaging cells (81). Retrovirus-containing supernatants were harvested and used to infect NIH 3T3 cells with polybrene (8 μg/ml), as described previously (40). Polyclonal populations of cells expressing mTie2 (3T3-mTie2 and 3T3-mTie2-KR) were selected with genetecin, G418 (600 μg/ml, Invitrogen), and analyzed for expression of mTie2 by Western blotting.

### 2.2.4 ELISA, Western Blotting, and Immunoprecipitation

HUVECs, 3T3-mTie2, or 293-hTie2 cells were grown to confluence in 6-well plates and stimulated in serum-free DMEM in the presence of ligands (Ang1, Ang2, or VEGF) or inhibitors (PMA, Bis-I, PD98059, SB203580, or LY294002, AKTi) for the indicated times at 37°C. Based on the relatively short half-life of Bis-I, in some cases cells were retreated 12h after the onset of the experiment. All other compounds were administered once. Where indicated, cells were preincubated with AKTi for 60 minutes prior to the addition of ligand. At the indicated times, conditioned media (CM) were collected, treated with 1mM sodium orthovanadate and Roche Complete Protease Inhibitor Cocktail tablets (according to the manufacturer's instructions), centrifuged at 14,000 $\times g$ for 10 min, and used for Western blotting or quantification of Tie2 or sTie2 by ELISA. For Western blotting from cell lysates, cells were lysed in Triton lysis buffer (137

mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0) supplemented with 1 mM sodium orthovanadate and Complete Protease Inhibitor Cocktail tablets (Roche). Soluble Tie2 in conditioned media and cell lysates was separated by SDS-PAGE and analyzed by Western blotting with anti-Tie2. Where indicated, sTie2 concentration in the CM was quantified by ELISA using a commercially available kit (R&D Systems) according to the manufacturer's instructions.

### 2.2.5 Analysis of Serum sTie2

Analysis of human serum samples was approved by the Duke University Institutional Review Board. Blood was collected from volunteers by venipuncture and placed into EDTA-containing tubes for serum sTie2 analysis. Briefly, whole blood samples were placed on ice and immediately centrifuged at 4000 ×g for 5 min. The plasma was then removed, aliquotted, and used immediately for analysis or stored at –80°C. Serum sTie2 concentrations were quantified by an enzyme-linked immunosorbent assay specific for Tie2 (R&D Systems), according to the manufacturer's instructions. For Western blotting analysis, blood was processed as described above, the plasma was diluted 20-fold in phosphate buffered saline, and samples were immunoprecipitated overnight with anti-Tie2 (clone 33) and Western blotted as described above.

### 2.2.6 Concentration of Conditioned Media

For the angiopoietin binding assays, CM from 3T3-mTie2 cells was concentrated approximately ten-fold using Centriprep Centrifugal Filter Units with an Ultracel YM-50 membrane (Millipore) according to the manufacturer's instructions. Protein

concentration in each sample was verified by Western blotting.

### 2.2.7 Angiopoietin Binding Assay

3T3-mTie2 cells were grown to confluence, the cells were rinsed once with Dulbecco's PBS (Invitrogen), and the medium was changed to serum-free DMEM. Following a 24-hour incubation at 37°C, the CM was harvested, centrifuged, and either stored at –80°C or used immediately for binding assays. Recombinant, 6-His-tagged Ang1 or Ang2 proteins (500 μg, R&D Systems) were diluted in 250 μl Triton lysis buffer plus protease inhibitors and purified on $Ni^{2+}$-NTA agarose beads (Qiagen) by overnight incubation at 4°C. As a negative control, $Ni^{2+}$-agarose beads were incubated with PBS without growth factors. CM from parental 3T3 or 3T3-mTie2-WT cells were concentrated 10-fold and added to tubes containing purified Ang1 or Ang2 or $Ni^{2+}$ beads alone and incubated overnight at 4°C. After overnight incubation, the agarose beads and bound proteins were washed five times with lysis buffer, and the proteins were eluted by boiling into Laemmli sample buffer and analyzed by Western blotting.

### 2.2.8 Analysis of Effects of sTie2 on Tie2 Phosphorylation and Apoptosis

To investigate effects of sTie2 on Angiopoietin-mediated Tie2 phosphorylation and endothelial cell survival, conditioned medium from parental NIH 3T3 cells or 3T3-mTie2 cells was collected and concentrated approximately 10-fold. For analysis of effects on Tie2 phosphorylation, HUVECs were serum starved for 3 hours in endothelial basal medium (EBM) without serum. Cells were then either left untreated or were

stimulated with Ang1 or Ang2 (300 ng/ml) in either control CM or sTie2-containing CM for 10 minutes. Cell lysates were collected and analyzed by Western blotting with anti-phospho-Tie2. To analyze effects of sTie2 on cell survival, HUVECs were serum-starved overnight in the absence or presence of Ang1 (300 ng/mL) in concentrated conditioned media from parental NIH 3T3 cells or 3T3-mTie2 cells. Apoptosis was induced by treatment with staurosporine (100 nM) for 90 min and then analyzed for either DNA fragmentation or caspase-3 cleavage. For DNA fragmentation, cell lysates were collected and analyzed using the Cell Death Detection ELISA-PLUS assay according to the manufacturer's instructions. For caspase-3 cleavage, cells were lysed with Triton lysis buffer and proteins were analyzed by Western blotting with anti-cleaved caspase-3. Data from three separate experiments were quantified from scanned images using ImageJ (v. 1.38x) and normalized to tubulin expression.

### 2.2.9 Adenovirus Infection and Use of Pharmacological Inhibitors

A recombinant, replication-defective adenovirus encoding myristoylated Akt (Ad-myrAkt) was generously provided by Dr. Ken Walsh (Boston University) and has been described previously (82). Recombinant adenoviruses encoding wild-type (WT) PTEN and the dominant negative C124S mutant (C/S) of PTEN have been described previously (83). An empty adenovirus without a cDNA insert (empty virus, AdEV) was used as a control for virus infection (83). To analyze the effects of these viruses on sTie2 production, HUVECs were plated in 6-well plates and grown until just subconfluent then infected overnight (approximately 16 h) with the indicated multiplicity of infection (moi) of each adenovirus in endothelial basal medium (EBM) containing 2% FBS. The

cells were allowed to recover for 24h in EGM-MV, then the medium was replaced with serum-free EBM for 24h, which was used for analysis of sTie2 concentration by ELISA. CM and cell lysates were collected and processed for sTie2 analysis as described above. To investigate effects of pharmacological inhibitors on sTie2 shedding, HUVECs were pre-treated for 30 minutes with the indicated inhibitors or vehicle then the medium was changed to serum-free EBM. The cells were incubated 24 hours at 37°C then CM were collected and sTie2 concentrations were analyzed by ELISA.

### 2.2.10 Statistical Analysis

All results are expressed as the mean ± SEM. All experiments were performed at least in triplicate to ensure that similar results were obtained in multiple experiments. Statistical comparisons between individual groups were performed using Student's *t*-test (two sample, unequal variance) and among multiple groups using ANOVA with Fisher's post-hoc test. $P<0.05$ was considered statistically significant.

## *2.3 Results*

### 2.3.1 Tie2 Is Constitutively Cleaved in Fibroblasts and HUVECs

Previous reports have demonstrated the presence of a soluble form of Tie2 (sTie2) in the cell culture medium of HUVECs and in human serum. To determine the mechanisms regulating sTie2 shedding, we first investigated whether sTie2 is produced in non-endothelial cells exogenously expressing Tie2. For the purposes of the present studies, cell culture conditioned medium (CM) is defined as serum-free medium in which cells are incubated for varying times and which contains secreted or shed proteins, such

as sTie2. Cell lysates and CM from NIH 3T3 fibroblasts stably expressing murine Tie2 (3T3-mTie2) and HEK-293 cells stably expressing human Tie2 (293-hTie2) were analyzed along with lysates and media from HUVECs. Western blotting with an antibody against the Tie2 extracellular domain detected a 75 kDa protein in CM but not lysates from 3T3-mTie2 cells and HUVECs (Figure 3A). The relative molecular weight of sTie2 from 3T3-mTie2 cells and HUVECs is similar to that described previously (54). Soluble Tie2 was undetectable in CM or lysates from 293-hTie2 cells by Western blotting (Figure 3A) or ELISA (data not shown), suggesting that the mechanisms of sTie2 shedding are conserved in cells of mesothelial origin. As expected, sTie2 was not detected from parental HEK-293 or NIH 3T3 cells (Figure 3B). To determine the time course of sTie2 shedding, conditioned media from HUVECs and 3T3-mTie2 cells were collected at varying times and sTie2 concentrations were quantified by ELISA. Tie2 shedding occurred rapidly in 3T3-mTie2 cells and peaked within 6 hours, whereas sTie2 concentrations in HUVEC conditioned media increased gradually over 48 hours (Figure 3C). In both cell types, peak sTie2 concentration was approximately 8 ng/ml at this time point.

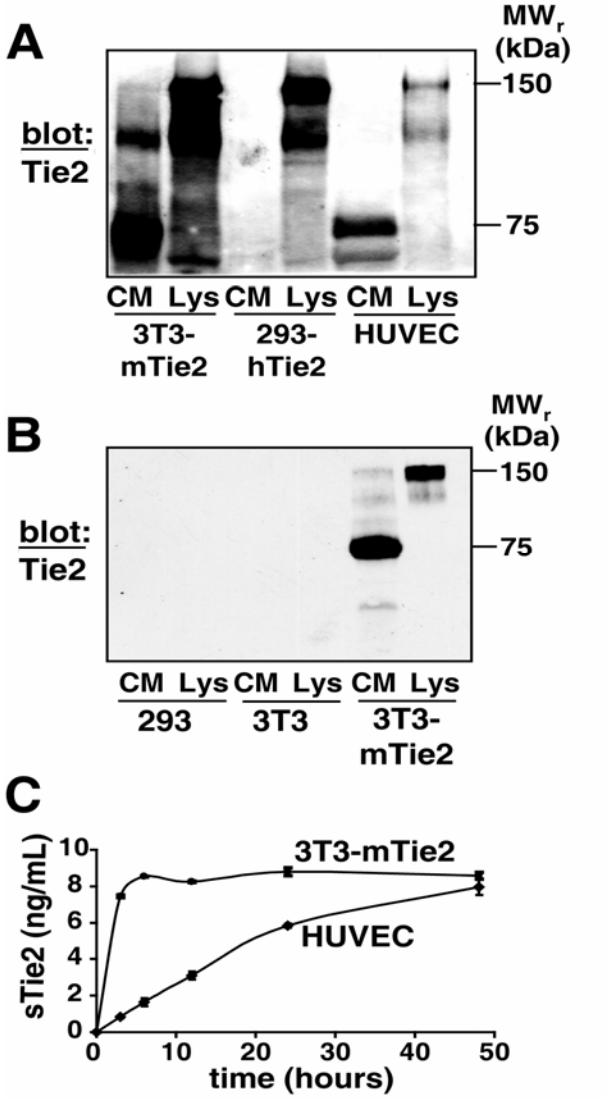


**Figure 3: sTie2 is shed from endothelial cells and fibroblasts *in vitro*.**

Conditioned media (CM) and cell lysates (Lys) from (A) 3T3-mTie2, 293-hTie2, and HUVECs or (B) parental 293 and 3T3 cells were obtained 24 hours after incubation in serum-free medium and Western blotted with anti-Tie2. Full-length Tie2 (150 kDa) was detectable in all three cell types, while sTie2 (75 kDa) was detectable in CM only from endothelial cells and fibroblasts. C, HUVECs and 3T3-mTie2 cells were grown in serum-free media for the indicated times, and sTie2 concentration in CM was quantified by ELISA.

### 2.3.2 sTie2 Is Detectable In Vivo

To investigate Tie2 shedding in human serum, blood was collected from 22 healthy human volunteers, and serum was analyzed by ELISA to quantify the sTie2 concentration. sTie2 was detectable in human serum by both ELISA and immunoprecipitation (Figure 4). The mean concentration of sTie2 detectable by ELISA was 20.6 ng/ml and ranged from 5.2 to 37.0 ng/ml (Figure 4), which is consistent with the serum sTie2 concentrations observed by other investigators (54, 66). The variability in sTie2 concentration was also evident by immunoprecipitation and Western blotting (Figure 2).

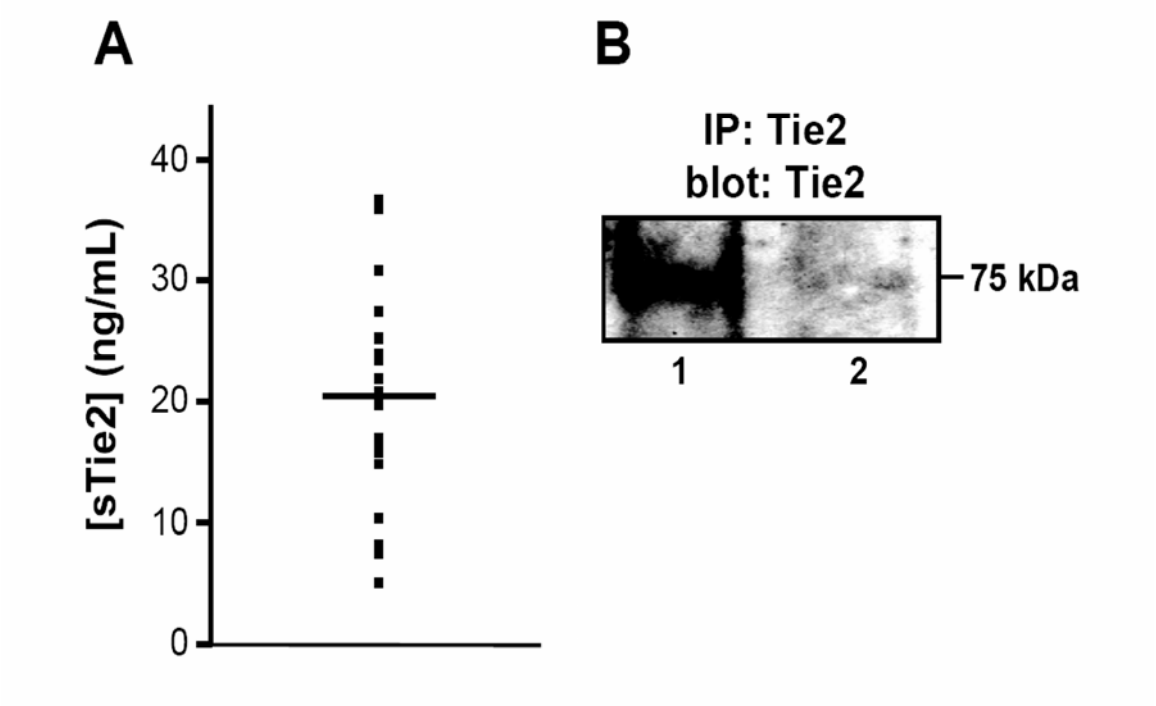


**Figure 4: sTie2 is detectable in human serum in vivo.**

A, Serum was collected from 22 healthy human subjects and sTie2 concentration was analyzed by ELISA. Mean sTie2 concentration was 20.6 ng/ml and ranged from 5.2 to 37.0 ng/ml. B, Serum from two healthy subjects was analyzed by immunoprecipitation and Western blotting with anti-Tie2. sTie2 expression was detectable in both subjects by this approach.

### 2.3.3 sTie2 Binds Recombinant Ang1 and Ang2

Tie2 shedding both *in vitro* and *in vivo* suggested that it might have functional effects on Tie2 signaling via angiopoietin binding. To investigate this possibility, concentrated sTie2-containing CM from 3T3-mTie2 cells or control CM from parental NIH 3T3 cells was mixed with recombinant, 6-His-tagged Ang1 or Ang2. Ang1 or Ang2 was precipitated on nickel agarose, and sTie2 binding was analyzed by Western blotting. Binding of sTie2 was undetectable in the absence of either angiopoietin protein (Figure 5A, *lane 6*), but binding of sTie2 was readily detectable in the presence of either Ang1 or Ang2 (Figure 5A, *lanes 7 and 8*). As expected, no sTie2 binding was detectable when the angiopoietins were incubated with parental 3T3 conditioned media, which lack sTie2 (Figure 5A, *lanes 2 and 3*). These findings demonstrate that constitutively shed sTie2 can bind both Ang1 and Ang2 *in vitro*.

### 2.3.4 sTie2 Inhibits Ang1- and Ang2-mediated Tie2 Phosphorylation

To investigate the effects of sTie2 on ligand-mediated Tie2 activation, 3T3-mTie2 conditioned media was evaluated for its ability to inhibit Ang1- or Ang2-induced tyrosine phosphorylation of Tie2. In endothelial cells, Ang1 induces Tie2 phosphorylation. Previously, Ang2 had not been shown to effect significant Tie2 activation in endothelial cells (30), although this has recently been described (53). However, in non-endothelial cells both Ang1 and Ang2 reproducibly induce significant Tie2 activation (30). Therefore, we tested whether sTie2 in 3T3-mTie2 conditioned media could inhibit angiopoietin-induced Tie2 phosphorylation in 293-hTie2 cells (28). As expected, both Ang1 and Ang2 induced Tie2 phosphorylation in the presence of parental 3T3

conditioned media, which lacks sTie2 (Figure 5B and C). However, in the presence of sTie2-containing conditioned media, the ligand-induced increase in Tie2 phosphorylation was markedly attenuated (Figure 5B and C).

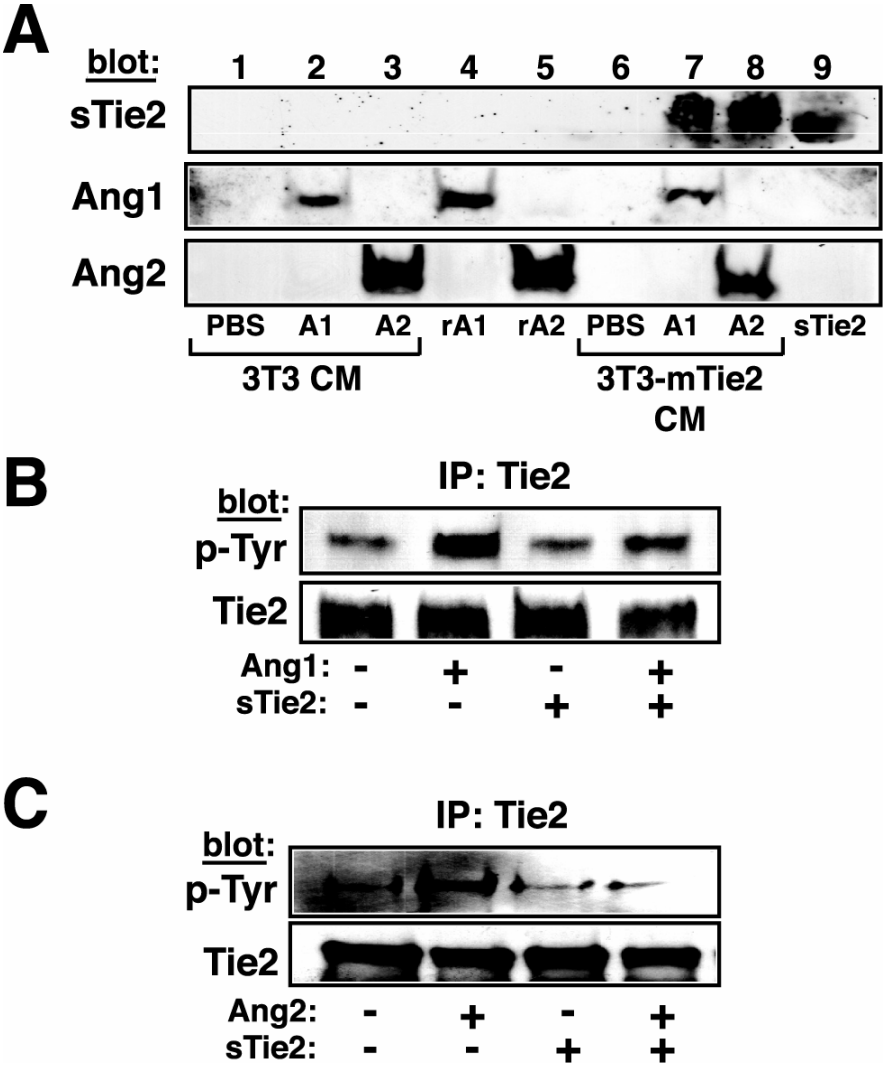


**Figure 5: sTie2 binds Ang1 and Ang2 and inhibits ligand-mediated Tie2 phosphorylation.**

A, sTie2 binds recombinant Ang1 and Ang2 proteins. Recombinant 6-His-tagged Ang1 (A1) and Ang2 (A2) were precipitated with nickel agarose and mixed with conditioned media (CM) from 3T3-mTie2 cells or parental 3T3 cells. PBS without recombinant Ang protein was used as a negative control. Bound proteins were detected by Western blotting with the indicated antibodies. Recombinant Ang proteins (rA1, rA2) and 3T3-mTie2 CM (sTie2) served as controls for the migration of each protein. B and C, 293-hTie2 cells were treated with or without Ang1 (B) or Ang2 (C) (300 ng/ml) for 10 min in the presence of concentrated conditioned media from parental NIH 3T3 cells (–sTie2) or 3T3-mTie2 cells (+sTie2). Tie2 was immunoprecipitated (IP) from cell lysates, and proteins were Western blotted sequentially with anti-phosphotyrosine and anti-Tie2.

### 2.3.5 sTie2 Inhibits Ang1-mediated Anti-apoptotic Effects in HUVECs

To determine whether the inhibitory effects of sTie2 on Tie2 activation translate into effects on Tie2-mediated cellular responses, HUVECs were treated with Ang1 in the absence or presence of sTie2-containing conditioned media from 3T3-mTie2 cells, and effects on apoptosis were examined. Apoptosis was induced in HUVECs with staurosporine, and effects of sTie2-containing CM from parental 3T3 cells or from 3T3 cells expressing mTie2 were tested. Treatment of serum-starved HUVECs with staurosporine for 90 minutes resulted in DNA fragmentation (Figure 6A) and caspase-3 cleavage (Figure 6B and C), consistent with induction of apoptosis, and these effects were inhibited by Ang1 in the presence of parental 3T3 conditioned media (Figure 6A-C). However, Ang1's anti-apoptotic effects were blocked in the presence of sTie2-containing conditioned media from 3T3-mTie2 cells (Figure 6A-C). Notably, essentially identical results were observed when we used conditioned media from 3T3 cells expressing a kinase inactive mutant of Tie2, indicating that overexpression of active Tie2 in 3T3-mTie2 cells did not result in the production of proteins that would adversely affect endothelial cell survival (data not shown). Taken together, these findings demonstrate that constitutively shed sTie2 binds both Ang1 and Ang2 and inhibits their effects on Tie2 activation and endothelial cell survival.

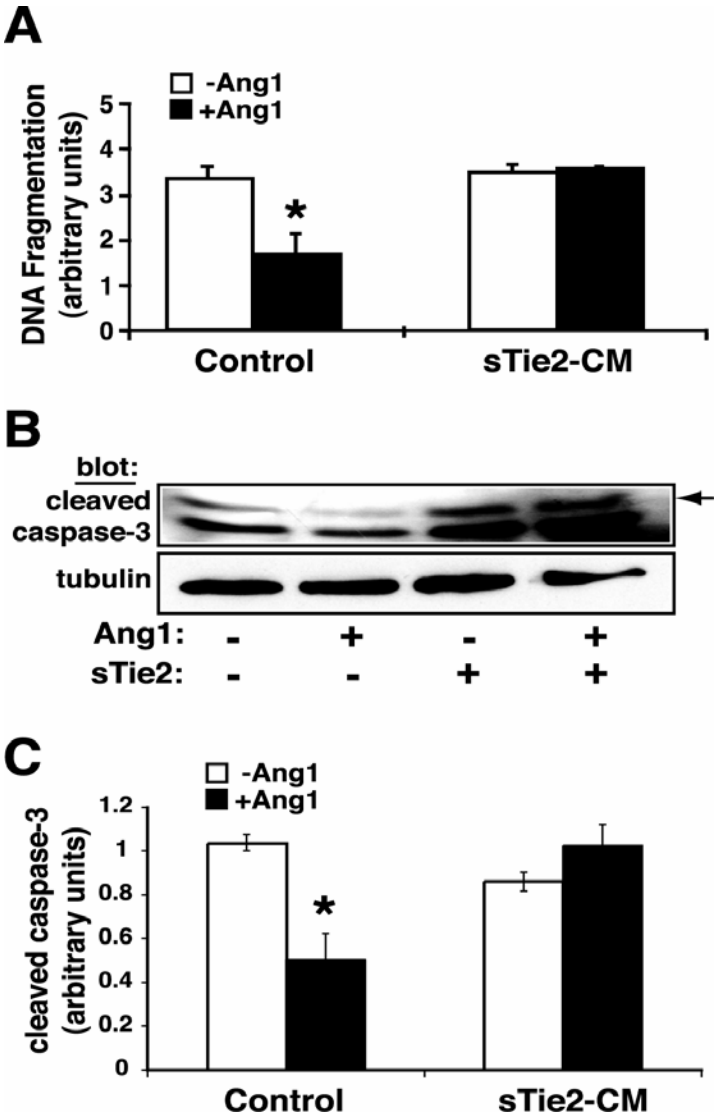


**Figure 6: sTie2-containing conditioned media inhibits Ang1-mediated endothelial cell survival.**

A, HUVECs were serum-starved overnight in the absence or presence of Ang1 (300 ng/mL) in concentrated conditioned media from parental 3T3 cells (Control) or 3T3-mTie2 cells (sTie2). Apoptosis was induced by treatment with staurosporine (100 nM), and cell lysates were analyzed for histone-associated DNA fragmentation using an ELISA-based approach. *, *P*<0.05 by ANOVA. B, HUVECs were treated as described in panel (A) and apoptosis was analyzed by Western blotting cell lysates with anti-cleaved caspase-3 (arrow). Blots were probed sequentially with anti-tubulin as a loading control. C, Cleaved caspase-3 was quantified from Western blots from three separate experiments as in panel (B) and normalized to expression of tubulin. *, *P*<0.05 by ANOVA.

### 2.3.6 sTie2 Shedding is PMA- and VEGF-Inducible

Phorbol myristate acetate (PMA) has been shown to induce shedding of other RTKs, including Tie2 (54), and VEGF has been shown to induce shedding of the related Tie1 receptor in HUVECs (84). To test the effect of VEGF on Tie2 shedding, HUVECs were stimulated with VEGF or with PMA as a positive control. Because primary endothelial cells undergo apoptosis after prolonged serum starvation, sTie2 shedding was examined after PMA significantly increased the amount of Tie2 shedding in HUVECs, as demonstrated by both ELISA and Western blotting (Figure 7A). Similarly, treatment of HUVECs with VEGF induced a significant increase in sTie2 concentration (Figure 7B).

### 2.3.7 Constitutive sTie2 shedding is MMP-, p38 MAPK-, and PI3K-dependent

Our data indicated that Tie2 shedding occurs both constitutively and following ligand activation with VEGF. To investigate the mechanisms responsible for these two processes, we first used a panel of pharmacological inhibitors of various signaling pathways to explore the regulation of constitutive Tie2 shedding in HUVECs. Matrix metalloprotease (MMP) inhibition with the non-specific inhibitor GM6001 significantly decreased sTie2 shedding from HUVECs, demonstrating that Tie2 cleavage is metalloprotease-dependent (Figure 7C). Shedding of other RTKs has been shown to be mediated by PKC and ERK. Treatment of serum-starved HUVECs with either the non-selective PKC inhibitor bisindolylmaleimide I (Bis-I) or the MEK inhibitor PD98059 had no significant effect on constitutive Tie2 shedding (Figure 7C). Two other signaling

pathways important for vascular growth and remodeling are the p38 MAP kinase pathway and the PI3K/Akt pathway. Inhibition of either p38 with SB203580 or PI3K with LY294002 significantly blocked Tie2 shedding in HUVECs (Figure 7D). However, inhibition of both pathways simultaneously had no further effect on Tie2 shedding (Figure 5D), indicating that p38 and PI3K lie within a common pathway for the induction of Tie2 shedding. Taken together, these findings demonstrate that Tie2 shedding is MMP- and p38-dependent, as is the case for other RTKs. However, these data also demonstrate that Tie2 shedding is PI3K-dependent, indicating a novel role for the PI3K pathway in Tie2 shedding.

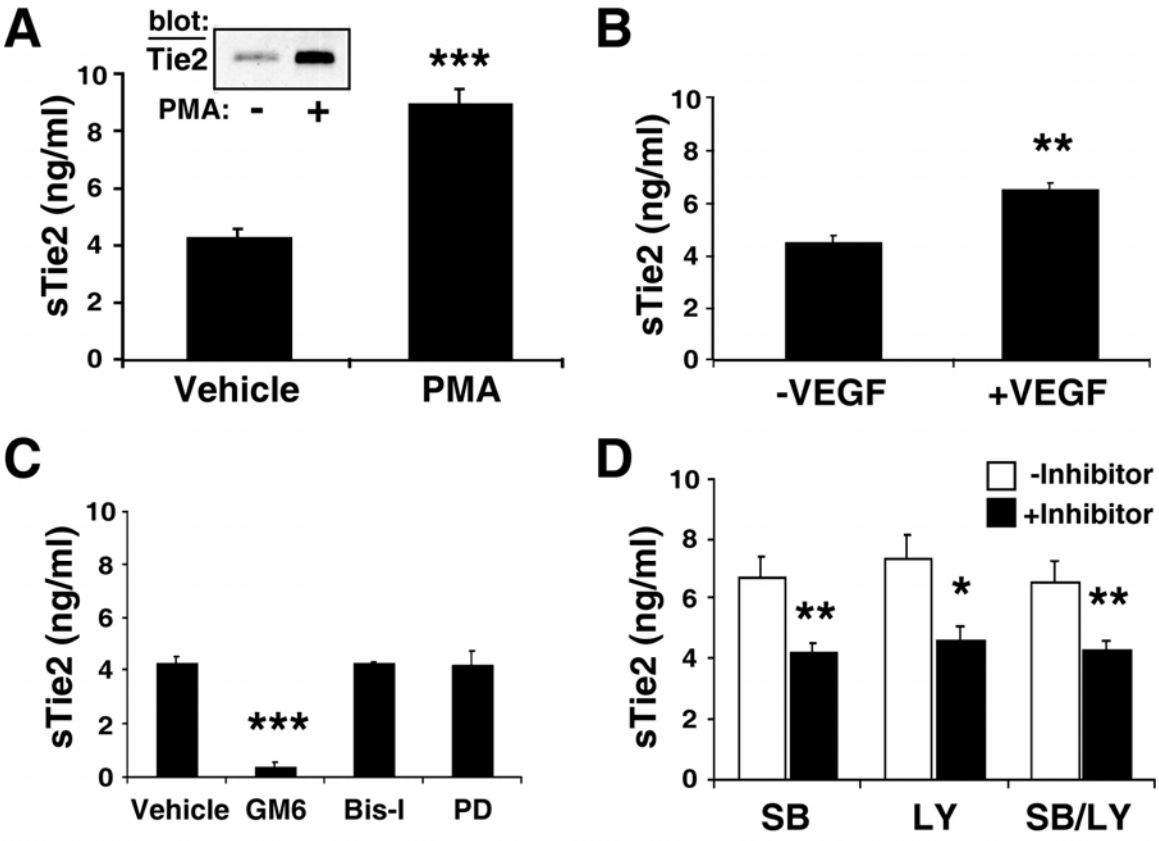


**Figure 7: sTie2 shedding is PMA- and VEGF-inducible, and constitutive sTie2 shedding is MMP-, p38 MAPK-, and PI3K/Akt-dependent.**

HUVECs were treated with or without PMA (100 nM) (A) or VEGF (20 ng/mL) (B), and sTie2 was analyzed by ELISA and Western blotting (A). Both PMA (***, *P*<0.005) and VEGF (**, *P*<0.01) significantly increased sTie2 shedding. C, HUVECs were treated with GM6001 (GM6, 100 nM), bisindolylmaleimide I (BisI, 100 nM), or PD98059 (PD, 50 μM), and sTie2 concentration in the conditioned media was analyzed by ELISA (***, *P*<0.005). D, HUVECs were treated with SB203580 (SB, 20 μM) or LY294002 (LY, 50 μM) alone or in combination, and sTie2 shedding was quantified (*, *P*<0.05; **, *P*<0.01). In all studies, conditioned media were collected 24 hours after treatment with inhibitors.

### 2.3.8 VEGF-inducible sTie2 Shedding Is PI3K/Akt-dependent

VEGF activates both p38 and PI3K, therefore we investigated whether VEGF-inducible Tie2 shedding was affected by inhibition of either of these pathways. HUVECs were treated with or without VEGF in the absence or presence of LY294002 or SB203580, and sTie2 release was measured by ELISA. Similar to our previous findings in unstimulated HUVECs, both the LY compound (Figure 8A) and the SB compound (Figure 8B) significantly inhibited VEGF-induced sTie2 release. As noted, p38 has been linked previously to RTK shedding but PI3K has not, therefore we focused our subsequent investigation on the role of the PI3K pathway in VEGF-mediated Tie2 shedding. To confirm the effects of PI3K on Tie2 shedding, we tested the effects of PTEN on this process, since PTEN is the major phosphatase in cells that hydrolyzes the lipid products of PI3K. HUVECs were infected with recombinant adenoviruses to overexpress either wild-type (WT) catalytically inactive PTEN (C/S). PTEN-C/S acts as a dominant negative inhibitor in endothelial cells (83), and it induced an increase in Akt phosphorylation (Figure 8C). The effects of PTEN were examined on both untreated and VEGF-treated cells. PTEN-WT significantly reduced Tie2 shedding in both untreated and VEGF-treated HUVECs compared to cells infected with control virus. Furthermore, dominant negative PTEN significantly increased Tie2 shedding both in the presence and absence of VEGF (Figure 8D). Taken together, these results demonstrate that both constitutive and VEGF-mediated Tie2 shedding are dependent on PI3K activity.

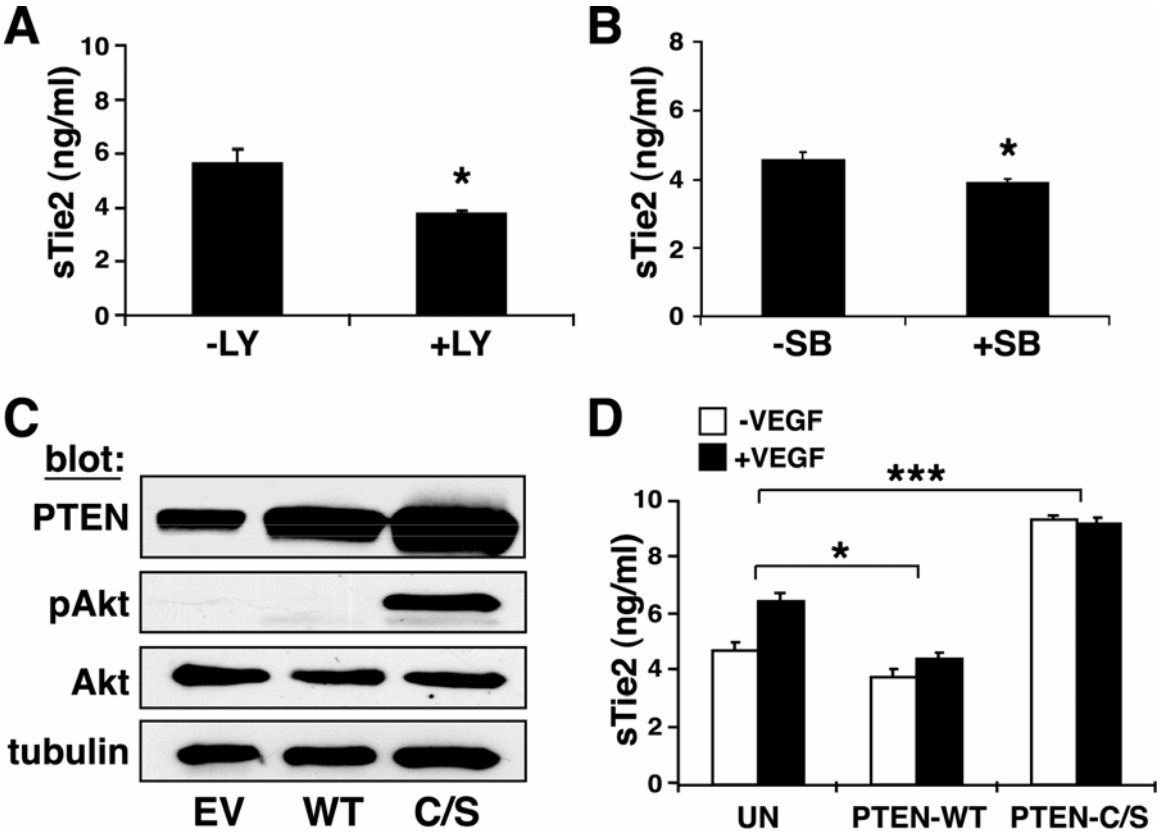


**Figure 8: VEGF-inducible sTie2 shedding is PI3K/Akt-dependent.**

A, HUVECs were treated with VEGF (20 ng/ml) in the absence or presence of LY294002 (LY, 50 μM), conditioned media were collected 24 hours later, and sTie2 concentration was quantified by ELISA (*, $P<0.05$). B, HUVECs were treated with VEGF (20 ng/ml) in the absence or presence of SB203580 (20 μM) and conditioned media were analyzed as described in panel (A) (*, $P<0.05$). C, HUVECs were infected with a control, empty adenovirus (EV) or with adenoviruses encoding wild-type (WT) or dominant-negative PTEN (C/S), all at a multiplicity of infection of 100. Proteins were detected by Western blotting with the indicated antibodies. D, HUVECs were uninfected (UN) or infected with the indicated adenoviruses, treated with or without VEGF (20 ng/ml) for 24 h, and sTie2 concentration was determined in conditioned media (*, $P<0.05$; ***, $P<0.005$).

PI3K activates multiple downstream effector molecules, including Akt, which is essential for VEGF-induced angiogenesis and endothelial cell survival (85). Therefore, we focused on the role of Akt in PI3K-dependent Tie2 receptor cleavage. HUVECs were first treated with or without VEGF in the presence or absence of a selective pharmacological inhibitor of Akt. The Akt inhibitor significantly decreased both basal and VEGF-induced sTie2 shedding (Figure 9A). Together with the results of PTEN inhibition, these findings suggested that Akt activation might be sufficient to induce sTie2 shedding. To investigate this possibility, HUVECs were infected with an adenovirus encoding constitutively active myristoylated Akt (myr-Akt) and sTie2 shedding was quantified. Compared to uninfected or control virus-infected cells, Admyr-Akt induced a dose-dependent increase in phospho-Akt (Figure 9B), which corresponded with significant increases in sTie2 shedding (Figure 9C). Additionally, the dominant-negative PTEN (PTEN C/S)-mediated increase in Tie2 cleavage was abrogated by pharmacological inhibition of Akt kinase activity (Figure 9D), confirming that PTEN-C/S-induced sTie2 shedding is mediated through Akt. Since Akt activation was sufficient to induce Tie2 shedding, we asked whether overexpression of myrAkt could induce this process in 293-hTie2 cells, in which sTie2 was undetectable (Figure 3A). Adenoviral infection of these replication-competent cells resulted in cell lysis and release of full-length Tie2 (Figure 10A and B), but AdmyrAkt failed to induce an increase in sTie2 shedding compared to control virus infection (Figure 10A and B). Taken together, these observations demonstrate that Akt activity is both necessary and sufficient for sTie2 shedding, but the necessary protease downstream of Akt is either not expressed in 293 cells or may not be activated by the same mechanisms in these cells.

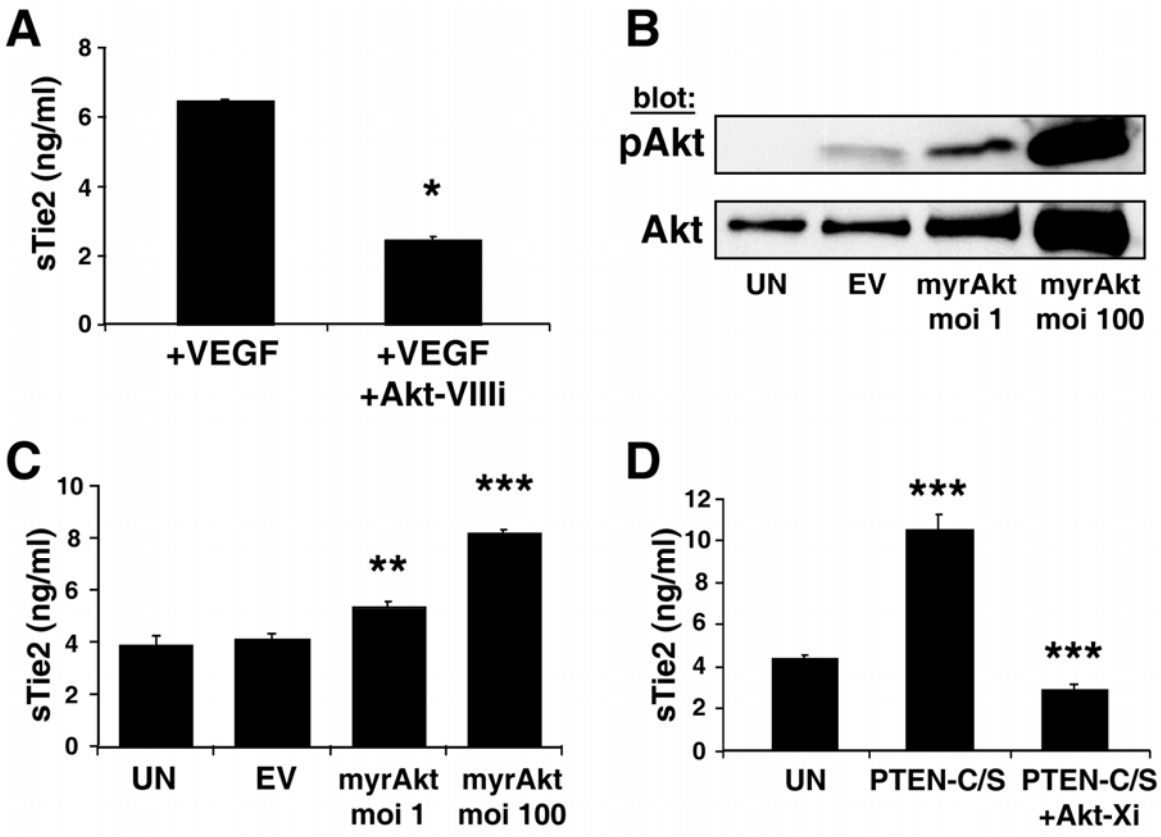


**Figure 9: Akt activation is necessary and sufficient for sTie2 shedding.**

A, HUVECs were pre-treated with vehicle or an Akt inhibitor (AKTVIIIi, 30 nM) for 60 minutes then grown in the absence or presence of VEGF (20 ng/mL) for 24 h and sTie2 shedding was quantified. B, HUVECs were uninfected (UN) or infected with a control, empty adenovirus (EV) or with the indicated multiplicity of infection (m.o.i.) of an adenovirus encoding myristoylated Akt (myrAkt). Proteins were detected by Western blotting sequentially with antibodies against phospho-Akt (pAKT) or total Akt. C, HUVECs were infected with the indicated adenoviruses, and sTie2 concentration was analyzed in conditioned media by ELISA 24 hours after changing cells to serum-free media. D, HUVECS were infected with adenovirus encoding dominant-negative PTEN (C/S) and treated with vehicle or an Akt kinase inhibitor (AKTXi, 25 μM) for 24 hours, and sTie2 concentration in CM was analyzed by ELISA (*, $P<0.05$; **, $P<0.01$; ***, $P<0.005$).

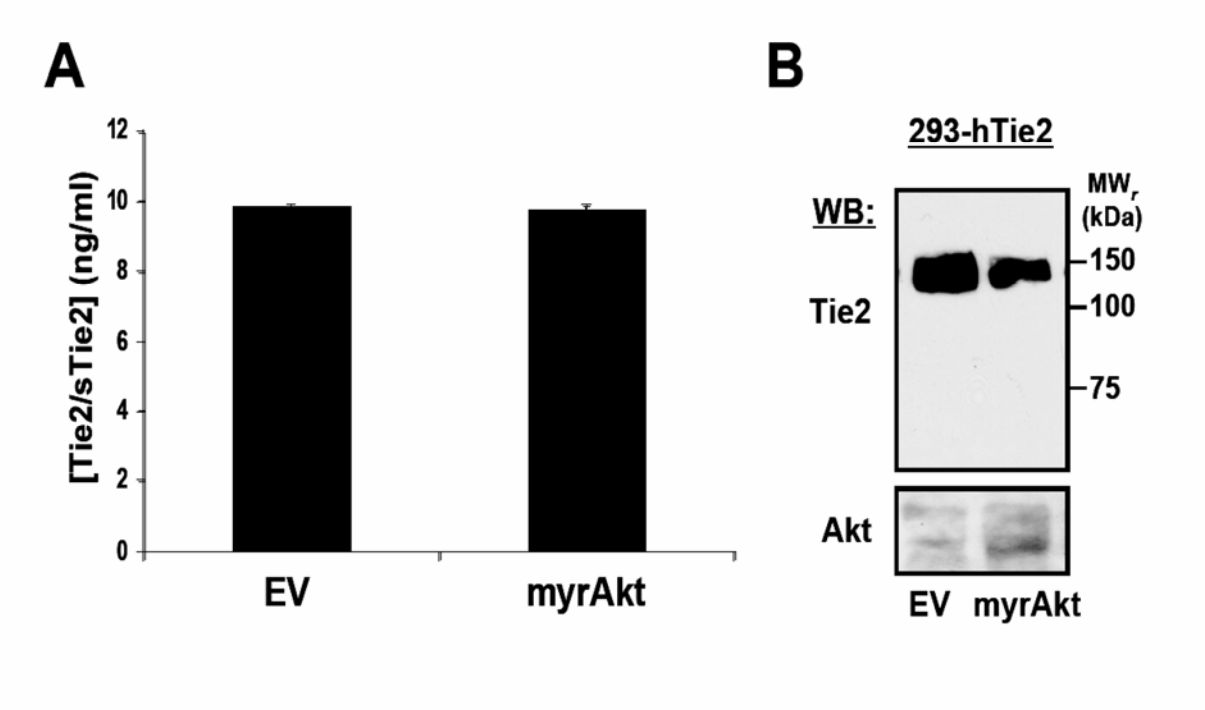


**Figure 10: Constitutively active Akt does not induce sTie2 shedding in 293-hTie2 cells.**

293-hTie2 cells were grown to confluence and infected with either empty adenovirus (EV) or adenovirus encoding constitutively active Akt (myrAkt). The medium was then changed to serum-free medium for 24 hours and CM was collected for analysis by ELISA (A) and Western blotting (B). No differences in Tie2/sTie2 concentration were observed between AdEV- and AdmyrAkt-infected cells. Western blotting revealed that the Tie2 detected by ELISA in panel (A) was exclusively full-length Tie2 (150 kDa), as no sTie2 (75 kDa) was detected. Additionally, the detection of Akt (a cytosolic protein) in the CM of both groups was consistent with marked cell lysis and release of cytosolic and membrane-bound proteins, including full-length Tie2.

## *2.4 Discussion*

Tie2 is an endothelial cell-specific RTK that is required for vascular growth and remodeling. Tie2 has been shown to be proteolytically cleaved (54), yet the mechanisms and biological significance of this process have not been examined previously. Here, we demonstrate that Tie2 shedding occurs in both a constitutive and VEGF-inducible manner and that sTie2 is functional, as it binds both Ang1 and Ang2 to inhibit ligand-mediated receptor activation and downstream cellular responses. Further, we show that Tie2 shedding is regulated by PI3K/Akt- and p38 MAPK-dependent pathways and that Akt activation is both necessary and sufficient to induce sTie2 shedding. Although a role for p38 MAPK in RTK cleavage has been demonstrated previously, this is the first study to demonstrate a requisite role for the PI3K/Akt pathway in RTK shedding. Moreover, this is the first report to demonstrate a direct effect of VEGF on the regulation of the Tie2-Angiopioetin system via receptor cleavage.

RTKs and other cell surface receptors are cleaved by metalloproteases, and the primary candidates for Tie2 shedding are the ADAM (a disintegrin and metalloprotease) and ADAMTS (ADAM with thrombospondin motifs) families of metalloproteases (57, 58, 86). ADAMs 10, 15, 17, and 19 have been implicated in cardiac development and angiogenesis and are therefore potential candidates for the regulation of sTie2 shedding (87-89). The activation of RTK shedding by ADAMs has been linked to several signaling proteins, including ERK, p38 MAPK and PKC (57). In our studies, sTie2 shedding was mediated by p38 MAPK and PI3K/Akt, which regulated both basal (constitutive) and VEGF-inducible Tie2 shedding. Interestingly, the effect of simultaneous inhibition of p38 MAPK and PI3K on sTie2 shedding was not synergistic, suggesting that these proteins lie in the same pathway regulating Tie2 cleavage. Accordingly, evidence exists for

crosstalk between the PI3K/Akt and p38 MAPK pathways (90, 91). Although p38 MAPK signaling has been linked to shedding of a variety of proteins, to our knowledge this is the first study to demonstrate a role for PI3K/Akt in RTK shedding (92-94). Notably, VEGF-mediated activation of PI3K/Akt is required for endothelial cell survival and angiogenesis (85), and our findings suggest that VEGF-mediated angiogenesis might involve sTie2 shedding. Moreover, our results provide potential functional relevance for this process, as it may serve to regulate the balance between angiogenesis and vascular quiescence.

Although the role of Tie2-Ang signaling in vascular growth and remodeling is quite complex, it is generally accepted that Ang1-mediated activation of Tie2 promotes vascular stabilization and quiescence (35), whereas Ang2 acts in opposition to Ang1 to facilitate VEGF-mediated angiogenesis (14). Our data demonstrate that sTie2 can bind both Ang1 and Ang2 and inhibit ligand-mediated Tie2 signaling. Because Ang1 and Ang2 have distinct, context-dependent effects on the vasculature, the ultimate effect of sTie2 shedding on vascular growth and/or remodeling likely depends on relative expression of the different Angs within a given vascular bed. Tie2 has been shown to be activated by Ang1 in the quiescent adult vasculature (77), and Ang1 inhibits VEGF-mediated increases in endothelial permeability (34). In this context, VEGF-mediated Tie2 shedding would be predicted to result in vascular destabilization, thereby facilitating angiogenesis. Interestingly, the related Tie1 receptor, which has also been shown to play a role in vascular maturation and stabilization, is also proteolytically cleaved in both a VEGF- and PMA-inducible manner (84, 95). Taken together, these observations suggest that an important component of VEGF-mediated signaling is the activation of proteases that cleave and downregulate the Tie receptors, thereby shifting the

vasculature from a quiescent to an angiogenic state.

In vivo, sTie2 has been detected in the serum of healthy human subjects (54, 96), a finding confirmed by our studies. In addition, recent reports have demonstrated increased levels of sTie2 in a variety of disease states characterized by vascular remodeling, including congestive heart failure, hypertension, and acute coronary syndromes (64, 65, 76, 97). Although the role of sTie2 is not entirely clear, serum sTie2 concentrations in patients with renal cell carcinoma correlated with disease stage and mortality, suggesting that sTie2 correlates with increases in tumor angiogenesis (71).

Pathophysiologically, it is also possible that enhanced sTie2 production serves as a marker of endothelial dysfunction, as chronic hyperactivation of multiple signaling pathways may result in enhanced Tie2 cleavage. Interestingly, preparations of HUVECs from different donors display significantly different basal levels of Tie2 shedding (data not shown), consistent with the wide range of serum concentrations of sTie2 detected in vivo. However, additional studies will be required to determine whether elevated sTie2 is a marker of aberrant signaling and cellular dysfunction (i.e., a result of disease) or whether it contributes to the progression of vascular disease. The findings in this study provide key insights into a novel mechanism by which VEGF counteracts the vascular stabilizing effects of Tie2. Chapter 3 will begin to further investigate the role of VEGF in mediating Tie2 cleavage and identify the effector molecules responsible for Tie2 cleavage.

# 3. VEGF-dependent activation of ADAM15 is responsible for Tie2 cleavage under hypoxic conditions

## *3.1 Introduction*

Tie2 signaling functions to maintain vascular integrity by mediating the complex interaction between endothelial cells (EC) and vascular supporting cells, such as pericytes and vascular smooth muscle cells. Genetic and biochemical studies have demonstrated that the ligands for Tie2, the angiopoietins, can regulate opposing functions in the vasculature. Ang1 has been shown to induce Tie2 phosphorylation, resulting in PI3K-Akt activation, increased cell survival and enhanced vascular maturation. In contrast, Ang2 is expressed almost exclusively in the remodeling vasculature and has been linked to both vascular growth and regression. Treatment of endothelial cells with Ang2 has been shown to inhibit Tie2 phosphorylation, and Ang2 can promote endothelial cell apoptosis and enhance vascular destabilization. Because both Ang1 and Ang2 bind Tie2 with similar affinity ($K_D$ ~3 nM), precise spatial and temporal regulation of these ligands' tissue concentrations is likely essential for proper development, remodeling, and maintenance of the vasculature. However, mechanisms regulating the Tie2-Angiopoietin system remain incompletely understood.

Recent data from our laboratory and others have suggested a previously under-appreciated mechanism regulating Angiopoietin availability and Tie2 activity and function. Proteolytic cleavage of the extracellular domain of Tie2 results in shedding of a soluble form of the receptor that can bind the Angiopoietins and inhibit their ability to bind and activate full-length Tie2 (98). Receptor shedding may be a means of regulating full-length receptor expression at the plasma membrane or a mechanism for producing

an endogenous ligand trap to inhibit receptor signaling (58). Soluble Tie2 (sTie2) was originally demonstrated by Reusch et al both in vitro from cultured human umbilical vein endothelial cells (HUVEC) and in vivo in the serum of healthy human subjects (54). We recently demonstrated that Tie2 shedding from endothelial cells (ECs) is induced by vascular endothelial growth factor (VEGF)-mediated activation of the pro-survival phosphoinositide 3-kinase (PI3K)/Akt pathway. Although sTie2 released from ECs could prevent Angiopoietin-mediated Tie2 activation, the exact function of sTie2 in vascular remodeling remains unknown. Studies have demonstrated that serum or plasma concentrations of sTie2 are increased in a variety of disease states, including cancer (71, 72) and cardiovascular disease (65, 66, 76, 99), suggesting that sTie2 may be either pro- or anti-angiogenic depending on the context. However, the effector molecules responsible for sTie2 shedding have not been investigated.

Proteolytic cleavage and shedding of receptor tyrosine kinases (RTKs) is mediated largely by the ADAM (a disintegrin and metalloprotease) family of proteases. To date, over 23 human ADAM proteins have been identified, and they have been shown to regulate a variety of cellular processes, such as inflammation, fertilization, tumoriogenesis, cardiogenesis and neurogenesis (58). Whereas ADAM17 is the prototypical sheddase and has been implicated in cleavage of numerous RTKs, ADAMs 15 and 19 (57) have been implicated in cardiac development and angiogenesis and are therefore potential candidates for the regulation of Tie2 shedding. We hypothesize that Tie2 cleavage is mediated by either of these ADAMs. To investigate this, we explored the role of these ADAMs in Tie2 shedding. Moreover, because we have shown that sTie2 is increased in patients with ischemic vascular disease, we sought to determine the role of hypoxia on the regulation of Tie2 shedding.

## *3.2 Methods*

### 3.2.1 Cell lines

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords by standard techniques (79). In all studies, HUVECs were used between passages 2 and 6 and were grown in endothelial growth medium containing microvascular endothelial cell supplement (EGM-MV, Clonetics Corp.), 10% fetal bovine serum (FBS), and 5% penicillin/streptomycin and maintained in a 37°C, 5% $CO_2$ incubator.

### 3.2.2 Antibodies and Reagents

Monoclonal anti-Tie2 (clone 33), which recognizes the extracellular domain of Tie2, has been described previously (78). Rat monoclonal anti-tubulin (clone YL1/2) was from Serotec. Isozyme-selective-Akt1/2 inhibitor VIII (AKTVIIIi) was from Calbiochem. Neutralizing VEGF antibody (AF-293-NA), control goat IgG and human VEGF-$A_{165}$ were purchased from R&D Systems. siRNA pools (OnTarget PLUS Smartpool) were purchased from Dharmacon.

### 3.2.3 Mice

Male C57BL/6 mice (12- to 16-weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME) directly. The mice were provided water and food *ad libitum*. All studies were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

### 3.2.4 Hindlimb Ischemia and Laser Doppler Perfusion Imaging

Unilateral hindlimb ischemia was induced by femoral artery ligation and excision, as described previously (100, 101). Serum concentrations of sTie2 were quantified in all animals pre-operatively and two days post-operatively using venous blood collected from the tail vein. Serum was collected into heparinized tubes and centrifuged at 4000×*g* for 10 minutes at 4°C then analyzed immediately by ELISA or stored at -80°C for later analysis. Quantification of serum sTie2 was performed using a mouse Tie2 ELISA kit (Quantikine, R&D Systems). Perfusion in the ischemic and contralateral non-ischemic limbs was measured using the laser Doppler perfusion imaging system (Perimed, Stockholm, Sweden), as described previously (same references in previous two sentences). Perfusion was assessed immediately post-operatively, two days post-operatively and on post-operative day 21. Perfusion recovery was expressed as the ratio of perfusion in the left (ischemic) to the right (nonischemic) hind limb.

### 3.2.5 ELISA, Western Blotting and Immunoprecipitation

HUVECs were grown to confluence and treated with or without ligands (VEGF) in serum-free DMEM at 37°C for the indicated times. Where indicated, cells were preincubated with AKTi for 60 minutes prior to the addition of ligand. At the indicated times, conditioned media (CM) were collected, treated with 1mM sodium orthovanadate and Roche Complete Protease Inhibitor Cocktail tablets (according to the manufacturer's instructions), centrifuged at 14,000 ×*g* for 10 min, and used for Western blotting or quantification of Tie2 or sTie2 by ELISA. For Western blotting from cell lysates, cells

were lysed in Triton lysis buffer (137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0) supplemented with 1 mM sodium orthovanadate and Complete Protease Inhibitor Cocktail tablets (Roche). Soluble Tie2 in conditioned media and cell lysates was separated by SDS-PAGE and analyzed by Western blotting with anti-Tie2. Where indicated, sTie2 concentration in the CM or serum was quantified by ELISA using a commercially available kit (R&D Systems) according to the manufacturer's instructions.

### 3.2.6 siRNA-mediated Gene Silencing

Because of the long half-life of ADAM15 and ADAM19 (12-24 hours), we employed an extended siRNA transfection protocol. HUVECS were plated and grown to 50% confluence then transfected with siRNAs against human ADAM15 or ADAM19 or a scrambled control using Lipofectamine 2000, according to the manufacturer's instructions. After 6 hours, the media containing the transfection reagents was removed and replaced with normal endothelial cell growth medium. 48 hours after the first transfection, the cells were transfected again according to the same protocol. Twenty-four hours after the second transfection, the cells were placed in serum-free media in the presence or absence of VEGF (20 ng/ml) for analysis of effects on sTie2 shedding.

### 3.2.7 Adenovirus Infection and Use of Pharmacological Inhibitors

A recombinant, replication-defective adenovirus encoding myristoylated Akt (Ad-myrAkt) was generously provided by Dr. Ken Walsh (Boston University) and has been described previously (82). An empty adenovirus without a cDNA insert (empty virus, AdEV) was used as a control for virus infection (83). To

analyze the effects of these viruses on sTie2 production, HUVECs were grown to 90% confluence then infected overnight (approximately 16 h) with the indicated multiplicity of infection (moi) of each adenovirus in endothelial basal medium (EBM) containing 2% FBS. The cells were allowed to recover for 24h in EGM-MV, then the medium was replaced with serum-free EBM for 24h, which was collected for analysis of sTie2 concentration by ELISA. CM and cell lysates were collected and processed for sTie2 analysis as described above.

### 3.2.8 Semi-quantitative PCR

Total RNA was extracted using the Qiagen RNeasy Miniprep Kit, according to the manufacturer's instructions. Approximately 1 ug of harvested RNA was reverse-transcribed using RT-SuperScript III (Invitrogen), and the resulting cDNA was stored at -20°C until further analysis. For semi-quantitative PCR, cDNA samples were amplified with primers specific for human ADAM15 as previously described (102): forward primer: 5'-CAAATATAGGTGGCACTGAGGAG-3'; reverse primer: 5'-TAGCAGCAGTTCTCCAAAGTGTG-3', yielding a 285-bp product. A 234-bp fragment of β-actin was amplified to control for RNA template loading using the following primers: forward primer: 5'-ACCAACTGGGACGACATGGAG-3'; reverse primer: 5'-AGTCCATCACAATGCCTGTGG-3'. Semi-quantitative PCR was performed in a total volume of 30 ul (1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 40 ng forward and reverse primers, 1.5 U Taq polymerase) and added to 1 ug cDNA sample, 40 ng purified ADAM15 plasmid (positive control) or $dH_2O$ (negative control). All samples were amplified as follows: after an initial denaturation at 95°C for 4 mins, samples were denatured at 95°C for 30 secs, primers were annealed at 50°C for 30 secs, and

elongated at 72°C for 30 secs. The cycle was repeated 21 times. A final elongation at 72°C for 10 mins completed the PCR. Following amplification, amplicons were run on a 2% agarose gel.

### 3.2.9 Statistical Analysis

All experiments were performed at least in triplicate, and results are expressed as the mean ± SEM. Statistical comparisons between individual groups were performed using Student's *t*-test (two sample, unequal variance) and among multiple groups using ANOVA with Fisher's post-hoc test. $P<0.05$ was considered statistically significant.

## *3.3 Results*

### 3.3.1 sTie2 levels are increased acutely following hindlimb ischemia

Previous reports have demonstrated the presence of sTie2 in the cell culture medium of HUVECs and human serum, and we recently demonstrated that sTie2 is increased in critical limb ischemia in humans with peripheral arterial disease (98). To explore mechanisms regulating Tie2 shedding, we first investigated whether sTie2 is increased acutely as a result of ischemia *in vivo*. To do this, serum sTie2 concentrations were measured pre- and post-operatively in C57BL/6 male mice subjected mice to surgical femoral artery ligation. Hind limb ischemia was confirmed by laser Doppler perfusion imaging (Figure 11A). On post-operative day two, sTie2 concentrations were

significantly increased compared to pre-operative levels (Figure 11B). These data demonstrate that acute tissue ischemia induces an increase in Tie2 cleavage.

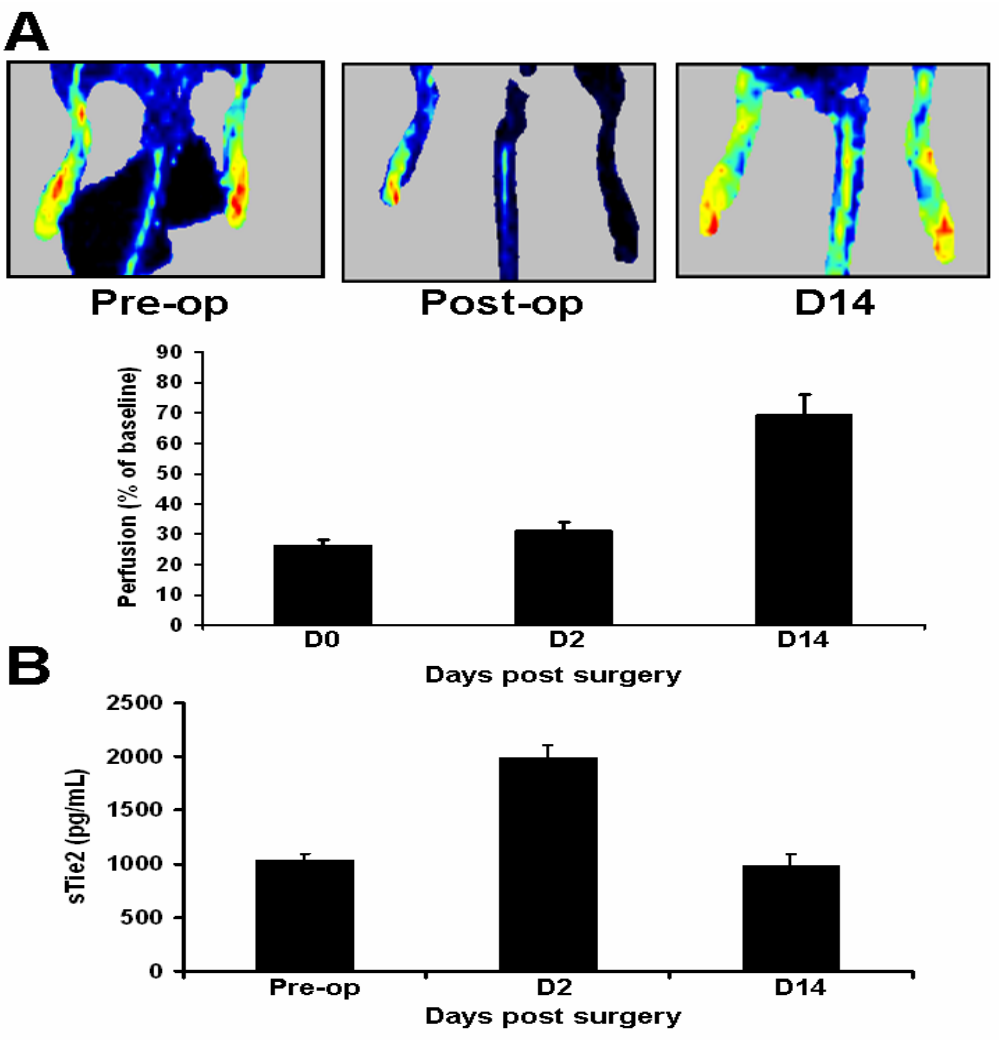


**Figure 11: sTie2 levels are elevated under hypoxic conditions.**

Hindlimb ischemia was induced and perfusion was measured by laser Doppler perfusion imaging in C57BL/6 mice. A) Representative laser Doppler perfusion images of mice before surgery (pre-op), immediately post-operatively (D0), and 14 days (D14) into recovery are displayed. Perfusion levels over a 14-day period are depicted graphically

and are represented as percentage of baseline. B) Levels of sTie2 post-operatively (D0) and two (D2) and fourteen (D14) days postoperatively are also depicted.

### 3.3.2 Hypoxia activates Tie2 cleavage in a VEGF-dependent manner

To determine whether increased Tie2 shedding following femoral artery ligation was induced by tissue hypoxia, we subjected HUVECs to chronic hypoxia for 48 hours. In addition, HUVECs were treated with $CoCl_2$, which mimics hypoxia via HIF-1$\alpha$ stabilization (103). In both cases, sTie2 shedding was significantly increased (Fig. 12A).

Hypoxia induces the expression of numerous genes, including VEGF and other angiogenic genes. Based on our recent observation that VEGF induces Tie2 cleavage (98), we next investigated whether the enhanced sTie2 production in response to hypoxia was VEGF-dependent. Cells were exposed to $CoCl_2$ for 18 hours in the presence or absence of a VEGF-A inhibitory antibody. The $CoCl_2$-induced increase in Tie2 shedding was abrogated in the setting of VEGF inhibition (Fig 12B).

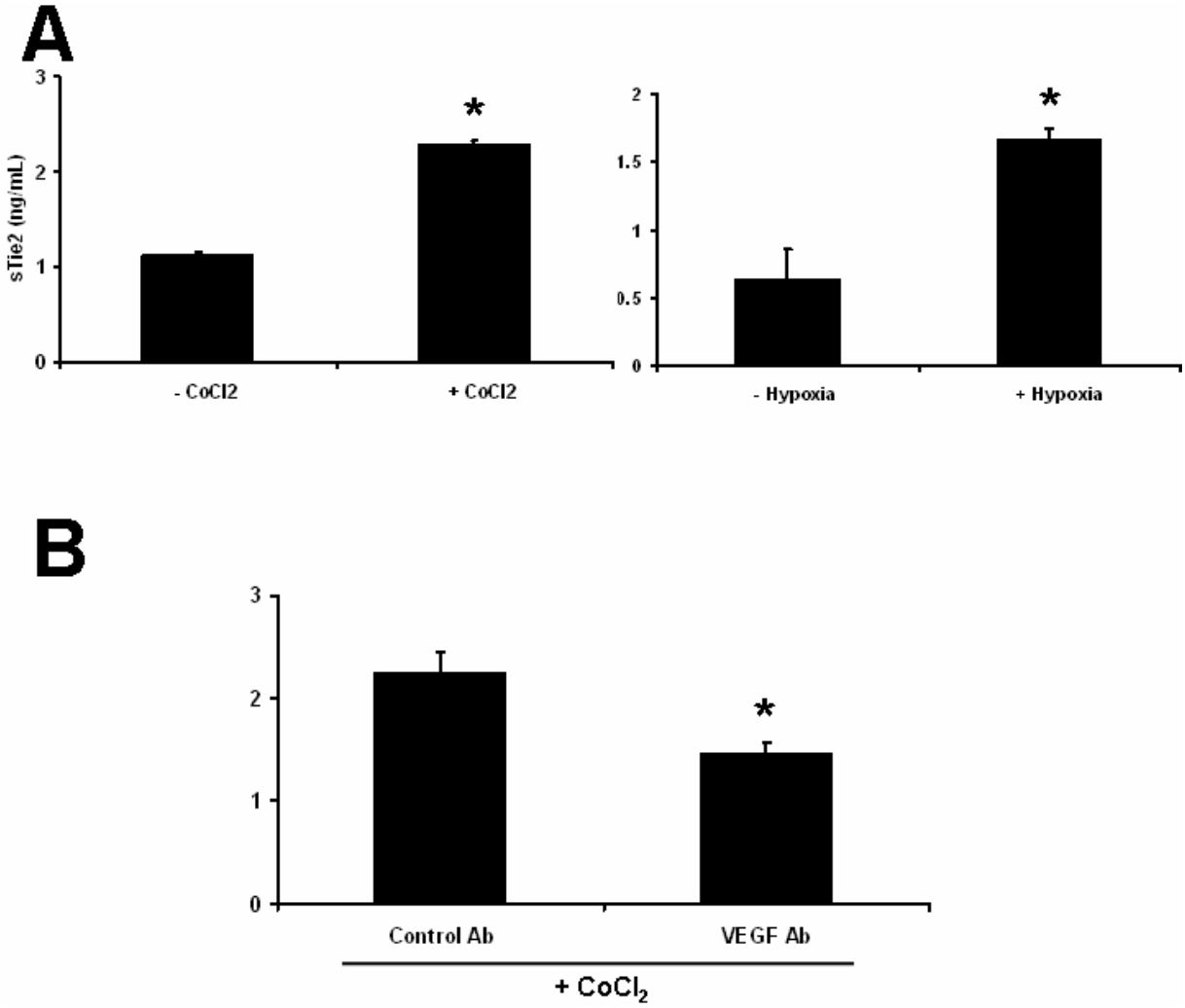


**Figure 12: Hypoxia induces Tie2 cleavage in a VEGF-dependent manner**

A, HUVECs were stimulated with CoCl2 (500 μM, 18 hours) or exposed to hypoxic conditions (48 hrs). CM was then analyzed by ELISA to examine the effect of hypoxia on Tie2 cleavage. B, HUVECs were stimulated with CoCl2 in the presence of either control antibody (goat IgG) or VEGF-A inhibitory Ab at 2.5 μg/ml. After 18 hours, the CM was then analyzed for sTie2 content.

### 3.3.3 ADAM15 mediates both VEGF-dependent and hypoxia-induced Tie2 shedding

To further examine the mechanisms involved in Tie2 shedding, we next investigated the effector molecule(s) responsible for sTie2 production. The ADAM proteins, a family of transmembrane metalloproteases, have been shown to regulate shedding of a number of other RTKs. We began by focusing on ADAMs that have been implicated in cardiovascular development, which would suggest a potential role in sTie2 shedding. Specifically, ADAM15 and ADAM19 knockout mice display decreased neovascularization and defective heart development, respectively (57, 88, 89). To evaluate the role of these ADAMs in Tie2 shedding, HUVECs were transfected with siRNAs specific for either human ADAM15 or ADAM19 or with a scrambled control siRNA and sTie2 levels were quantified following treatment with VEGF. These siRNAs yielded near complete silencing of both ADAM15 and ADAM19 in HUVECs (Fig 13A). Compared to cells treated with control siRNA, VEGF-induced sTie2 shedding was significantly inhibited in the presence of ADAM15 siRNA but not ADAM19 siRNA, demonstrating that Tie2 shedding is ADAM15-dependent, in both VEGF-induced and constitutive settings (Fig 13A). Additionally, we evaluated whether hypoxia-induced Tie2 shedding, which we have shown to be VEGF-dependent, was also mediated by ADAM15. As depicted in Figure 13B, ADAM15 knockdown resulted in significantly reduced shedding under hypoxic conditions.

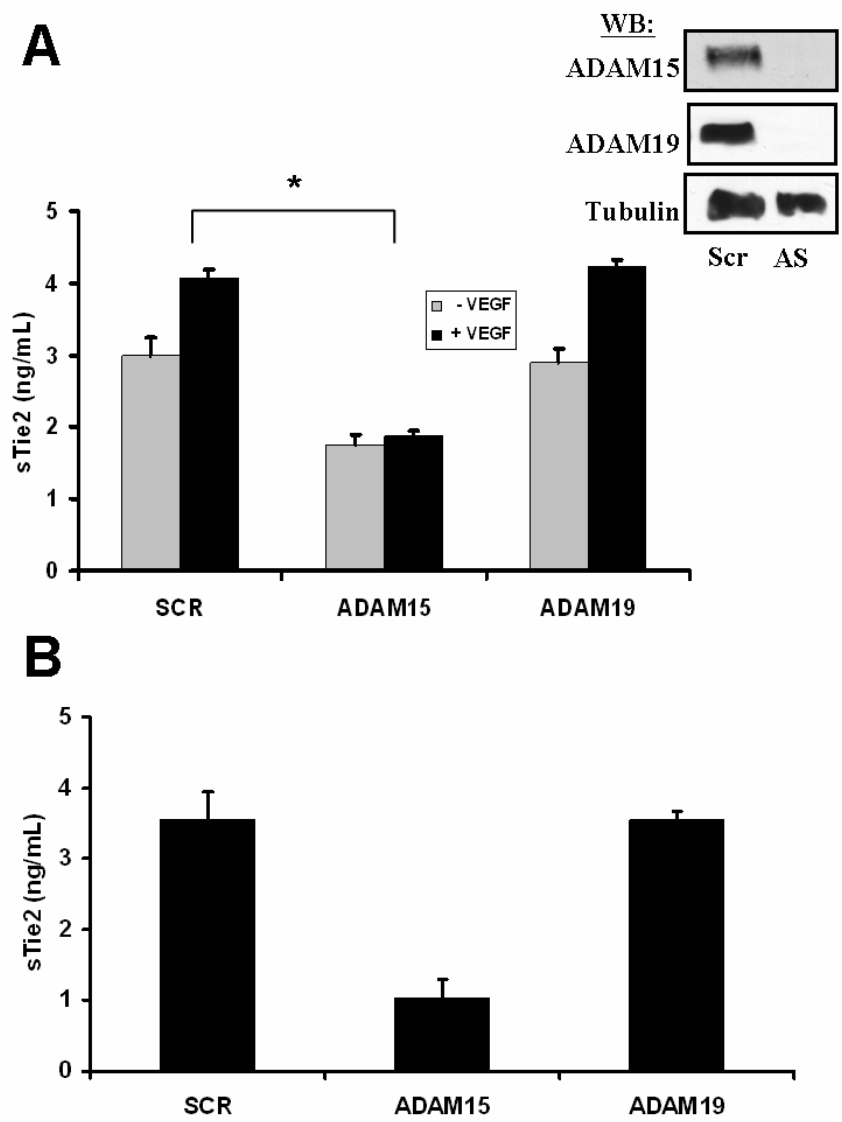


**Figure 13: ADAM 15 cleaves Tie2 in a VEGF-dependent manner**

A, HUVECS were transfected with siRNA targeting either ADAM15, ADAM19 or a non-targeting siRNA (scramble[SCR]) as described in the Methods section. The cells were then stimulated with either VEGF (20 ng/mL) or PBS, as indicated. Conditioned media was removed and analyzed via ELISA for sTie2 content. Additionally, lysates from the samples were analyzed by westernblot to determine presence of corresponding ADAM proteins. B, HUVECs were transfected with siRNAs, as described above, and exposed to hypoxia for 48 hours. The CM was then analyzed by ELISA to determine sTie2 concentration.

### 3.3.4 VEGF activates ADAM15 transcription

Having demonstratedshown that hypoxia-induced Tie2 shedding is VEGF- and Akt-dependent and that VEGF-mediated Tie2 shedding is ADAM15-dependent, we next investigated the mechanisms by which VEGF regulates ADAM15. To determine whether VEGF regulates ADAM15 transcription and/or translation, we stimulated HUVECs with VEGF for varying times and measured changes in ADAM15 RNA by semi-quantitative RT-PCR (Figure 14). VEGF treatment increased the transcription of ADAM15 by 6 hours and an increase in ADAM15 protein was observed by 6 hours. These studies suggest that prolonged stimulation of the VEGF receptor on the EC cell surface activates cellular pathways involved in the upregulation of ADAM15 transcription, leading to eventual increases in ADAM15 expression and activity on the cell surface.

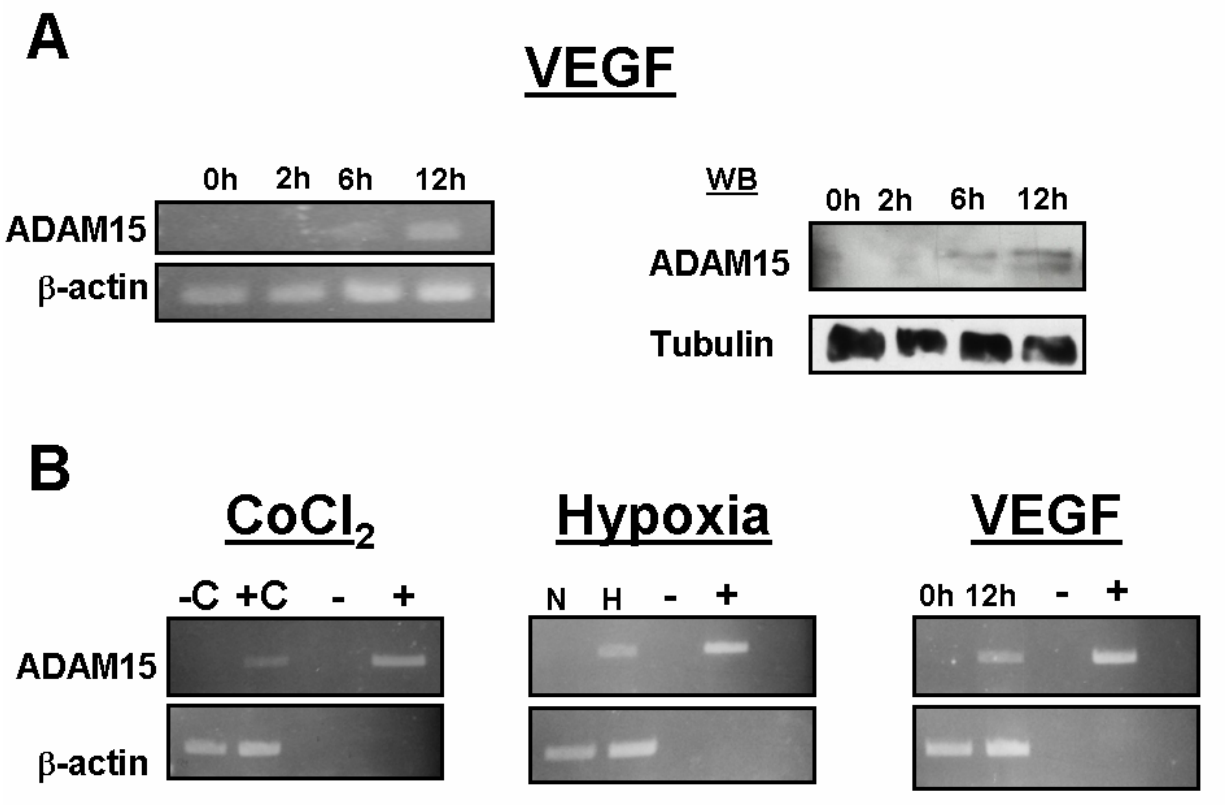


**Figure 14: VEGF upregulates ADAM15 transcription.**

A, HUVECs were grown to confluence and stimulated with VEGF (20ng/mL) for the indicated timepoints. Cells were lysed and RNA was harvested. Semi-quantitative RT-PCR was performed to examine ADAM15 transcripts (left). Also, cells were lysed and samples were analyzed by westernblot (right). B, To examine the effects of hypoxia on ADAM15 transcription, cells were stimulated in the presence (+C) or absence(-C) of CoCl2 (500μM) for 18 hours or grown in hypoxic (H) or normoxic (N) conditions for 48 hours or stimulated with VEGF for the indicated timepoints.
Negative control (-); Positive control (+)

## *3.4 Discussion*

Tie2 is known to be proteolytically cleaved resulting in shedding of sTie2 (54), but to date few studies have examined the mechanisms and/or biological significance of this process. We previously demonstrated that activation of ECs with VEGF, a potent angiogenic factor, induces sTie2 shedding via activation of the PI3K/Akt pathway (98). Moreover, sTie2 was found to be increased in the plasma of patients with critical limb ischemia (99). Here, we investigated the link between these two processes and demonstrated that Tie2 shedding is induced by hypoxia in a VEGF-dependent manner. Further, we show that VEGF-inducible Tie2 shedding is ADAM15-dependent. Interestingly, ADAM15 is transcriptionally regulated by VEGF. This is the first report to identify an effector molecule responsible for the proteolytic cleavage of Tie2 and to demonstrate a role for hypoxia in sTie2 shedding. These findings provide an important mechanistic link between sTie2 release and ischemic vascular disease.

The proteolytic cleavage of RTKs and shedding of soluble receptors are mediated largely by the ADAM (a disintegrin and metalloprotease) family of metalloproteases. Because ADAM15 and -19 have been implicated in cardiac development and angiogenesis, these proteins were the principal candidate mediators of Tie2 shedding analyzed in our study. RTK shedding by ADAMs had previously been linked to a number of signaling proteins, including ERK, p38 MAPK and PKC. However, recent work from our laboratory demonstrated that activation of the PI3K/Akt pathway is both necessary and sufficient for Tie2 cleavage, although the effector protease responsible for this effect had not been identified. Using siRNAs to silence different ADAM proteins, we demonstrated that ADAM15 is required for VEGF-mediated Tie2 shedding.

According to Fig 13a, we achieved significant knockdown (>95%) of ADAM15, however, we were unable to completely abrogate Tie2 cleavage. As a result, we cannot exclude the possibility for the role of other protease(s) in Tie2 cleavage. ADAM17, which was not studied in this report, has been shown to effect Tie2 cleavage in the PMA-induced setting (data not shown, personal communication from C. P. Blobel). Activation with PMA is artificial, as endogenous analogues of PMA do not exist, and it is likely that ADAM17 involvement in Tie2 cleavage in the physiologic setting may not occur or occur in pathological states. Despite this, the role for other metalloproteases in Tie2 cleavage cannot be negated.

ADAM15 has been shown to play a significant role in a variety of diseases with a significant vascular component. In tumors, ADAM15 protein was found to be significantly increased in multiple types of adenocarcinoma, specifically in prostate and breast cancer specimens (104). As will be discussed, significant elevations in serum sTie2 have also been observed in patients with breast or prostate cancer. In addition, ADAM15 upregulation has also been observed in a variety of cardiovascular diseases, such as dilated cardiomyopathy, atrial fibriillaiton and atherosclerosis (105-107). Because of ADAM15s ability to cleave Tie2, it is likely that ADAM15 upregulation contributes to the increased sTie2 production observed in these disease states.

Interestingly, the presence of sTie2 in normal patients (~20 ng/mL) suggests a normal role for this protein in modulating Tie2. Elevated sTie2 levels have been observed in a variety of disease processes with a significant angiogenic component. In a study of renal cell cancer, elevated sTie2 levels were associated with disease, as compared to control subjects (71). In response to chemotherapy, decreases in sTie2 levels correlated with prolonged patient survival. Also, in patients with invasive breast

and prostate cancers, sTie2 levels were significantly elevated. In the breast cancer population, patients undergoing either mastectomy or wide local excision for biopsy-proven breast cancer, followed by standard best medical care (i.e., radiotherapy and/or chemotherapy with tamoxifen), as dictated by clinical necessity, experienced significant reductions in sTie2 levels three months post-procedure (104). In another form of human disease, sTie2 levels were elevated in hypertension, congestive heart failure, acute coronary and peripheral artery disease patients (64-66). The role of sTie2 in disease is unknown, as sTie2 elevations could be a marker of disease or contribute to disease progression. However, concomitant elevations of sTie2 and ADAM15 in similar disease states suggest a correlation and potential contribution to disease progression.

VEGF, a hypoxia-responsive gene, is significantly upregulated in the setting of hypoxia. In this study, we demonstrated that hypoxia enhances Tie2 cleavage and does so in a VEGF-dependent manner. We have also implicated the role of ADAM15 in the cleavage process. Naturally, the role of VEGF in modulating ADAM15 transcription and/or expression would be called into question. Interestingly, Komiya et al have implicated a role for VEGF in modulating ADAM15 expression levels. $VEGF_{165}$ was shown to enhance ADAM15 RNA expression levels (transcript) in a dose-dependent manner over a 24-hour period (108). We were also able to demonstrate similar results, as ADAM15 was shown to be upregulated significantly at 12 and 24 hours. These results have enabled us to propose the following mechanism. The body's natural compensatory response to hypoxia is angiogenesis. Angiogenesis, the growth of new blood vessels from pre-existing vessels, contributes significantly to collateral blood vessel growth and enables the body to appropriately respond to ischemic environments. Results from this study and data from previous studies has led to the development of a model describing

the mechanism of Tie2 cleavage in hypoxia. In settings of hypoxia, the angiogenic cascade is initiated. One of the most potent inducers of angiogenesis, VEGF, is upregulated and secreted from cells to act in an autocrine and paracrine fashion. VEGF activates the VEGF receptor leading to activated signaling cascades, more specifically the PI3K-Akt pathway, which results in the upregulation of ADAM15 transcription in settings of chronic VEGF exposure (> 6 hours). ADAM15 is translated and trafficks to the membrane where it is activated and effects Tie2 cleavage (109). sTie2 can then bind and inhibit the ligands, Angiopoeitin 1 and Angiopoietin2 in the periphery, and potentially affect vascular homeostasis.

Additional studies are necessary to elucidate whether elevated sTie2 is a marker of aberrant signaling and cellular dysfunction (i.e., a result of disease) or whether it contributes to the progression of vascular disease. Findings in this study provide a mechanistic basis from which to investigate these possibilities, as they provide key insights into a mechanism by VEGF and ischemia function to enhance angiogenesis by counteracting the vessel stabilizing effects of Tie2 via ADAM15-depnedent Tie2 cleavage.

# 4. Plasma Levels of Soluble Tie2 and VEGF Distinguish Critical Limb Ischemia from Intermittent Claudication in Patients with Peripheral Arterial Disease

## *4.1 Introduction*

Peripheral arterial disease (PAD) is characterized by atherosclerosis of non-cardiac vascular beds and most commonly affects the lower extremities. Although widely under-recognized, PAD is a major health problem, affecting 8 to 12 million individuals in the United States (110). The two major clinical manifestations of PAD of the lower extremities are intermittent claudication (IC) and critical limb ischemia (CLI), which have markedly different clinical outcomes (111). IC is characterized by reproducible pain on exertion that is relieved with rest. CLI is the most severe form of PAD and is characterized by the inability of arterial blood flow to meet the metabolic demands of resting muscle or tissue, resulting in rest pain and/or tissue necrosis, which frequently necessitates amputation (112, 113). Whereas annual mortality in patients with IC is only 1-2%, the annual risk of death in patients with CLI is up to 20% (112, 113). Therefore, early identification would be expected to provide improved treatment options for patients with PAD.

Currently, the ankle-brachial index (ABI) is the gold-standard for PAD diagnosis, however the correlation between ABI and severity of PAD is poor (114). Aside from the ABI, there are no other reliable diagnostic tests for PAD. Furthermore, the diagnosis of IC vs. CLI is purely clinical. Patients with IC and CLI can present with virtually identical clinical risk factors (e.g., tobacco use, diabetes mellitus), peripheral hemodynamic

parameters, and degree of atherosclerotic burden (115). Thus, minimally invasive tests or biomarkers to diagnose PAD and potentially distinguish between IC and CLI are needed.

In PAD, atherosclerotic arterial occlusive disease results in tissue ischemia and varying degrees of collateral blood vessel growth (i.e., angiogenesis). The extent of collateral blood vessel formation has the potential to impact the clinical manifestations of PAD, thus insufficient angiogenesis may be responsible for the different clinical presentations of patients with IC and CLI. A number of angiogenic growth factors and endogenous inhibitors of angiogenesis have the potential to modulate vascular growth in PAD. Among these, vascular endothelial growth factor (VEGF)-A is perhaps the most important pro-angiogenic factor, as it is required for both physiological and pathological angiogenesis (9), and even minor changes in its expression can lead to dramatic alterations in vascular growth and remodeling (116). The related placenta growth factor (PlGF) binds a common receptor, VEGF receptor-1 (VEGFR-1), and although it is not required for normal embryonic vascular development, it plays a critical role in pathological angiogenesis(117). The angiopoietins are ligands for the endothelial receptor Tie2 (10), and angiopoieitin-2 (Ang2) is upregulated by VEGF (118) and in some cases has been shown to be required for VEGF-mediated angiogenesis(14). Interestingly, soluble forms of both VEGFR-1 and Tie2 are produced and secreted into the circulation, where they can bind their respective growth factors and inhibit angiogenesis(119). Whereas soluble VEGFR-1 (sVEGFR-1) is produced by alternative splicing of the VEGFR1 gene (120), soluble Tie2 (sTie2) results from proteolytic cleavage of the full-length, membrane-bound receptor (98), and both proteins have been implicated in the pathogenesis of cardiovascular disease(101, 121).

Here, we hypothesized that differences in expression of these angiogenic growth factors and/or their soluble receptors might provide markers of PAD and its different clinical manifestations. To investigate this possibility, plasma from control subjects and patients with IC and CLI was analyzed for changes in the pro-angiogenic growth factors Ang2, VEGF-A, and PlGF, along with their soluble receptors sVEGFR-1 and sTie2, which act as endogenous inhibitors of angiogenesis. We found that patients with PAD have significantly higher plasma concentrations of Ang2, VEGF, and sTie2 compared to control subjects, but only VEGF and sTie2 concentrations distinguish patients with CLI from those with IC.

## *4.2 Methods*

### 4.2.1 Subject recruitment

Subjects with PAD were recruited consecutively from the vascular medicine clinics at Duke University Medical Center, and control subjects were recruited from the vascular screening clinics if they showed no evidence of PAD, as demonstrated by an ankle-brachial index (ABI) >1.0 or other diagnostic testing. All patient studies were approved by the Duke University Institutional Review Board. All patients were over 18 years of age and able to give informed consent for a single 10 ml blood draw. The study population was comprised of 23 control subjects without PAD and 46 patients with PAD. PAD subjects included 23 patients with intermittent claudication (IC) and 23 patients with critical limb ischemia (CLI), based on the Rutherford criteria (122). IC patients had an ABI of 0.5-0.9 and exercise-limiting claudication, and CLI subjects were identified by an ABI <0.5 and the presence of either a non-healing lower extremity ulcer, rest pain, or gangrene. Control subjects were age- and gender-matched, and were without clinical

evidence of vascular, metabolic, or neoplastic disease based on history and physical examination and routine laboratory studies. Patients were excluded from all groups if they had evidence of acute coronary syndromes and/or acute decompensated heart failure in the previous 6 weeks or Child-Pugh class C liver disease. Sample size was determined based on 80% power to detect a significant difference in at least one measured factor between control and PAD subjects without distinction between IC and CLI subgroups. The subjects' demographic characteristics are shown in Table 1.

### 4.2.2 Sample acquisition and analysis of angiogenesis-modulating factors

Blood was collected by venipuncture into EDTA-containing tubes. Whole blood samples were placed on ice and immediately centrifuged at 2000 $\times$g for 5 minutes at 4°C for plasma separation. Plasma was aliquoted and stored at –80°C. The concentrations of sTie2, VEGF-$A_{165}$, Ang2, placenta growth factor (PlGF), and soluble VEGF receptor-1 (sVEGFR-1) were quantified using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Assay sensitivities, quantified as the mean minimal detectable concentration for each assay, were: sTie2, 14 pg/ml; VEGF-A, 5.0-9.0 pg/ml; Ang2, 8.3 pg/ml; PlGF, 7 pg/ml; sVEGFR-1, 3.5 pg/ml. Coefficients of variation (intra-assay precision) were less than 4% for all assays.

### 4.2.3 Cell culture studies

Human umbilical vein endothelial cells (HUVECs) were harvested from fresh umbilical cords and phenotyped as described previously (79) and used between passages 2 and 6. Cells were grown in endothelial growth medium (EGM)-MV (Cambrex) until confluent. The cells were then changed to serum-free medium, and triplicate samples were treated with vehicle or with recombinant human $VEGF_{165}$ (25ng/mL; R&D Systems) for 24 hours. The conditioned media were then collected and sTie2 concentration was quantified by ELISA, as described above. ELISA data are presented as the means ± the standard deviation (SD) from triplicate samples. Experiments were repeated on at least three separate occasions. Statistical comparisons were performed using Student's t-test, and significance was set at $P<0.05$.

### 4.2.4 Statistical Analysis

Statistical analysis was performed using SPSS 16.0 for Windows. Patient demographic data were compared across all three groups by ANOVA for continuous variables followed by chi-squared analysis to compare IC and CLI patients. Categorical variables were compared between IC and CLI groups using a non-parametric chi-squared test. Categorical variables that were significantly different between the IC and CLI patients were controlled for using analysis of covariance (ANCOVA) to compare levels of VEGF and sTie2 within the PAD cohort. Similarly, ANCOVA was used to control for the effects of differences in ABI on VEGF and sTie2 concentrations in IC vs. CLI patients. For analysis of ELISA data, which were not normally distributed, the data

were log-transformed and analyzed by one-way ANOVA with Fisher's protected least significant difference post-hoc test or Student's t-test where appropriate. Graphical data are presented as the non-log-transformed values and are expressed as the mean ± SD. Significance was set at $P<0.05$.

## *4.3 Results*

### 4.3.1 Baseline patient characteristics

The baseline demographic and clinical characteristics of the study population are shown in Table 1. Comparing patients with IC and CLI, a significantly greater percentage with CLI were male, diabetic, hypertensive, and had a history of smoking and peripheral vascular intervention (Table 1). There were no significant differences in the use of cardiovascular medications (ACEI/ARB or statins) between patients with IC and CLI (Table 1).

**Table 1: Patient Demographics**

| | Control (n=23) | Intermittent Claudication (n=23) | Critical Limb Ischemia (n=23) | *P*(ANOVA) | *P* ($\chi^2$, IC vs. CLI) |
|---|---|---|---|---|---|
| Age, median (min-max), yrs | 51.7(36-72) | 56.1 (40-69) | 62.3 (29-90) | 0.02 | 0.25 |
| Male, n (%) | 14 (61.2) | 12 (52.2) | 15 (65.2) | | 0.001 |
| Caucasian, n (%) | 13 (56.5) | 15 (65.2) | 17 (74.1) | | 0.13 |
| ABI*, median (min-max) | 1.09 (0.91-1.36) (n=22) | 0.55 (0.48-0.86) (n=21) | 0.39(0.08-0.53) (n=17) | 0.001 | 0.001 |
| Diabetes mellitus | 0 (0.0) | 4 (17.4) | 9 (42.9) | | 0.003 |
| Smoking history, n (%) | 11 (47.8) | 16 (69.5) | 18 (78.2) | | 0.001 |
| Prior Coronary Artery Disease, n (%) | 3 (14.3) | 9 (39.1) | 17 (74.1) | | 0.37 |
| Prior Lower Extremity Vascular Intervention | 0 (0.0) | 9 (39.1) | 19 (82.6) | | 0.001 |
| Hyperlipidemia (LDL>130) | 8 (34.7) | 15 (65.2) | 11 (47.8) | | 0.37 |
| Statin use | 4 (17.3) | 19 (82.6) | 16 (69.5) | | 0.08 |
| Hypertension | 7 (30.4) | 14 (60.8) | 15 (65.2) | | 0.01 |
| ACEI/ARB** | 2 (8.6) | 8 (34.8) | 11 (47.8) | | 0.37 |

*ABI = ankle-brachial index; ABI data were not available for all patients

**ACEI/ARB = angiotensin converting enzyme inhibitor or angiotensin receptor blocker

### 4.3.2 Analysis of angiogenic growth factors and inhibitors in PAD and control subjects

Plasma concentrations of the five different factors were analyzed in controls vs. all PAD patients combined. Plasma concentrations of sVEGFR-1 and PlGF were not significantly different in PAD patients compared to controls (Fig. 1A and B). However, levels of Ang2, VEGF, and sTie2 were significantly higher in PAD patients (Fig. 1C-E).

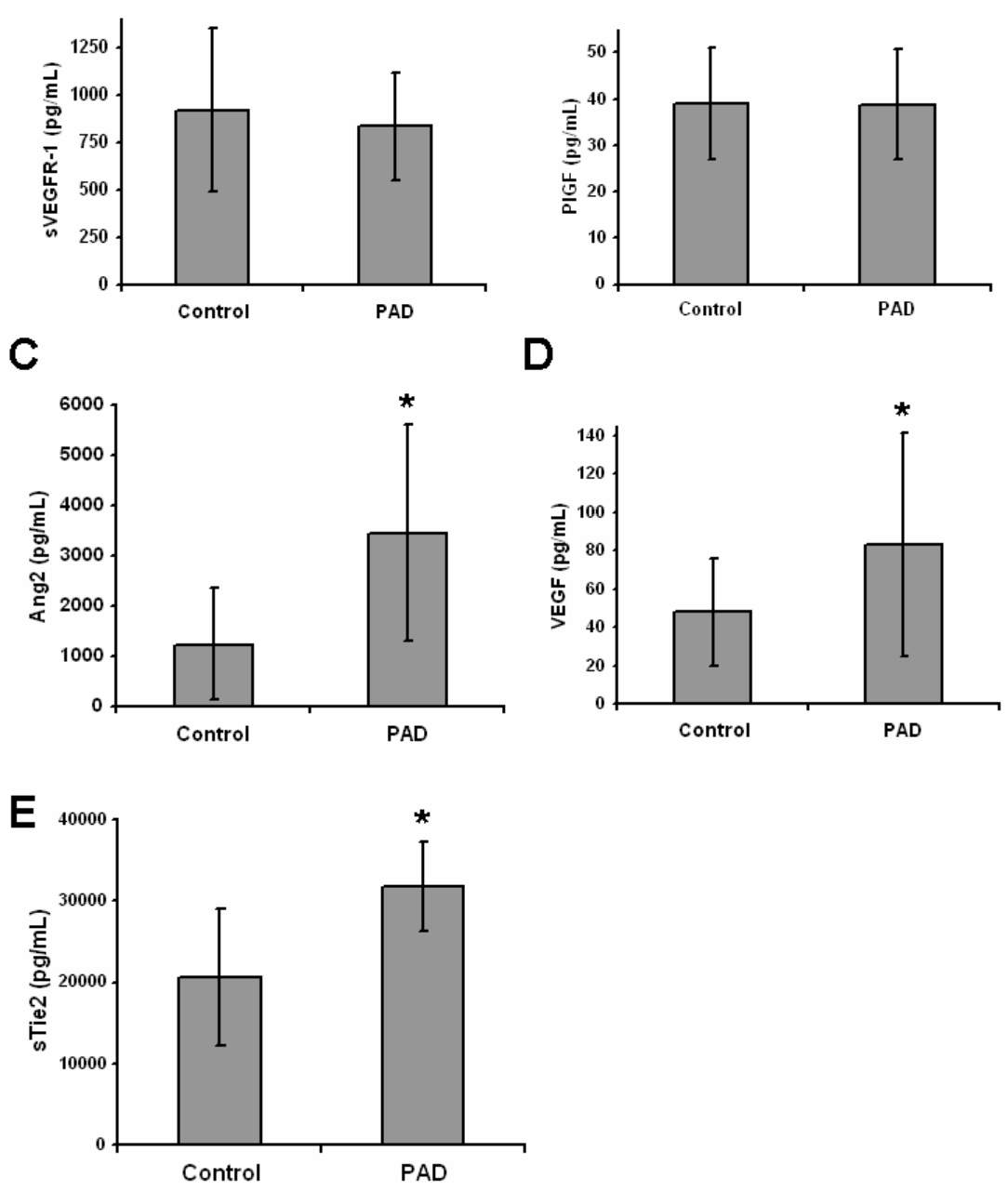


**Figure 15: Plasma concentrations of Ang2, VEGF, and sTie2 are increased in PAD.**

Plasma concentrations of sVEGFR-1 (A), PlGF (B), Ang2 (C), VEGF (D), and Tie2 (E) were measured in control subjects (n=23) and patients with PAD (n=46). Concentrations of sVEGFR-1 and PlGF were not significantly different between the two populations, however Ang2 (*, $P<0.0001$), VEGF (*, $P<0.01$), and sTie2 (*, $P<0.01$) were significantly greater in PAD patients.

### 4.3.3 Analysis of angiogenic growth factors and inhibitors within PAD groups (IC vs. CLI)

Visually, there was a stepwise increase in plasma Ang2 expression from control subjects to patients with IC to patients with CLI (Fig. 2A). Although Ang2 concentrations were significantly greater in both PAD groups compared to controls, Ang2 expression was not statistically different in patients with IC and CLI (Fig. 2A). In contrast, plasma VEGF expression was not significantly increased in IC patients compared to controls (Fig. 2B). However, plasma VEGF expression was significantly higher in patients with CLI compared to those with IC (Fig. 2B). Similarly, plasma sTie2 levels were not significantly different in patients with IC compared to controls, but they were significantly higher in CLI patients compared to either IC patients or controls. Mean sTie2 levels in the three groups were 20.6 ± 1.8, 22.8 ± 1.5, and 32.1 ± 1.4 ng/mL in control, IC, and CLI patients, respectively.

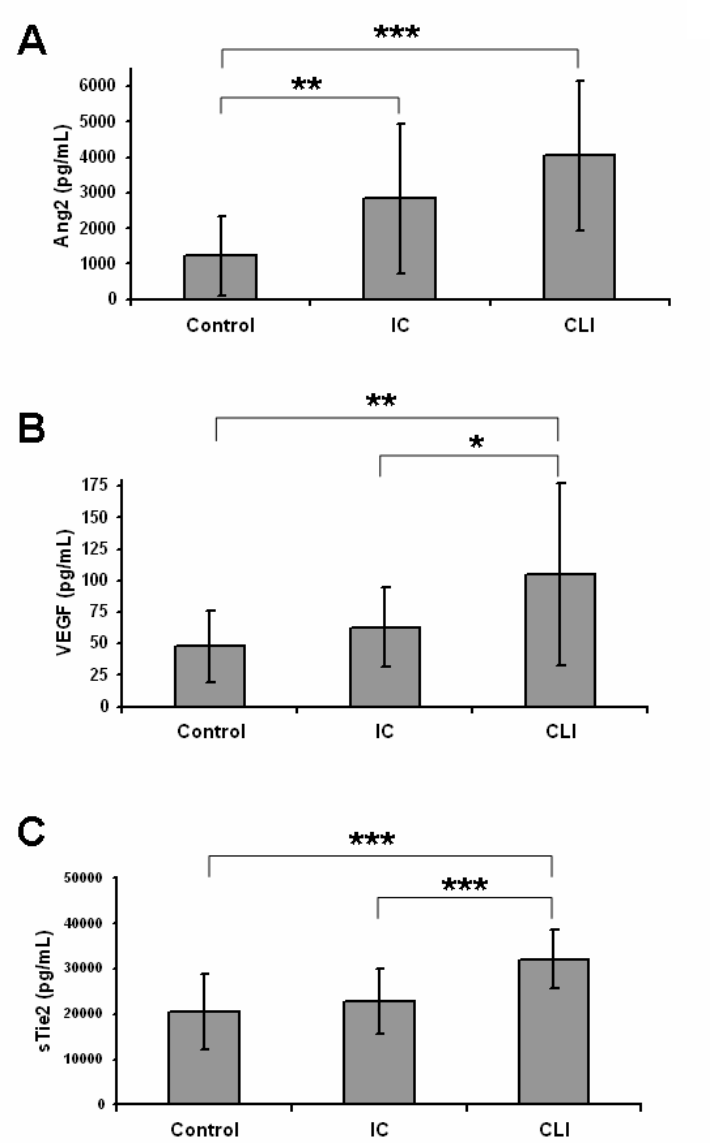


**Figure 16: Plasma VEGF and sTie2 levels distinguish patients with CLI from controls or patients with IC**

Plasma concentrations of Ang2, VEGF, and sTie2 were measured and compared in control subjects (n=23) and those with IC (n=23) or CLI (n=23). (A) Plasma concentrations of Ang2 were significantly increased in both IC (**, *P*<0.01) and CLI patients (***, *P*<0.0001) compared to controls, but there was no significant difference between IC and CLI. (B) VEGF levels were significantly increased in CLI patients compared to controls (**, P<0.01) and IC (*, P<0.05), but there was no difference between IC and controls. (C) sTie2 concentrations were significantly greater in CLI patients compared to controls (***, P<0.0001) and IC (***, P<0.0001), but there was no difference between IC and controls.

### 4.3.4 sTie2 and VEGF concentrations are independent of cardiovascular risk factors

Circulating concentrations of VEGF and sTie2 could be altered as a result of concomitant cardiovascular disease (CVD) and endothelial dysfunction. Analysis of the baseline demographic and clinical characteristics of patients with IC and CLI revealed that significantly more CLI patients were male, diabetic, hypertensive, and had a history of smoking (Table 1). Therefore, we performed analysis of covariance (ANCOVA) for each of these categorical variables with VEGF and sTie2 concentrations and found that differences in both VEGF and sTie2 between the IC and CLI groups remained significant ($P$<0.001) in our study population.

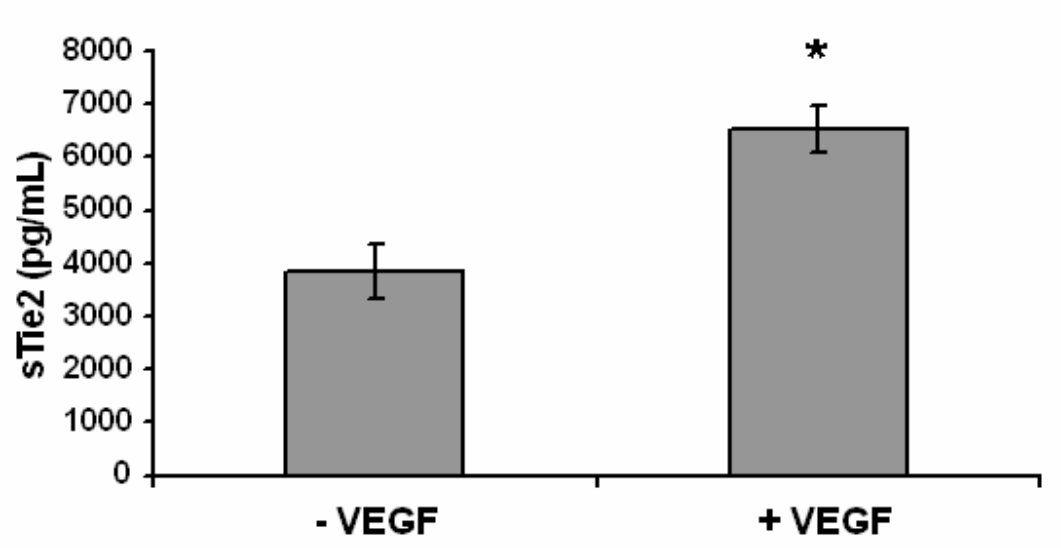


**Figure 17: sTie2 production is induced by VEGF *in vitro***

HUVECs were stimulated with VEGF-$A_{165}$ (25 ng/mL) for 24 hours. Concentration of sTie2 in the cell conditioned media was quantified by ELISA. VEGF treatment significantly increased release of sTie2 (*, $P<0.01$).

### 4.3.5 sTie2 and VEGF concentrations are independent of the ABI

ABI values were lower within our CLI patients than in those with IC (Table 1). However, when we controlled for differences in ABI, the increased concentrations of both sTie2 and VEGF in CLI vs. IC patients remained significantly different ($P$<0.001). These findings demonstrate that increased levels of sTie2 and VEGF correlate with disease state but not with the ABI, suggesting that these proteins are potential markers of CLI.

### 4.3.6 Interaction between VEGF and sTie2

The finding that sTie2 and VEGF concentrations were similarly elevated in subjects with CLI compared to controls or those with IC suggested a potential mechanistic link between VEGF and sTie2. VEGF is known to activate proteases upon stimulation of endothelial cells (9), and sTie2 is a proteolytic cleavage product of the full-length, membrane-bound receptor (54). Moreover, VEGF has been shown to induce proteolysis of the related Tie1 receptor(84). Therefore, we stimulated human umbilical vein endothelial cells (HUVECs) with recombinant human VEGF and analyzed cell conditioned media for the presence of cleaved sTie2 by ELISA. VEGF induced a significant increase in sTie2 *in vitro* (Fig. 3), suggesting that increased sTie2 levels in CLI are a result of increased VEGF expression.

## *4.4 Discussion*

Peripheral arterial disease is a major health care problem in the US, and the prevalence of PAD is increasing. Although the ABI is currently the gold standard for the diagnosis of PAD, there are a number of limitations to its use, and new diagnostic tests are needed. Because PAD is characterized by decreased tissue perfusion and tissue hypoxia, we hypothesized that concentrations of circulating angiogenic growth factors and/or inhibitors of angiogenesis would be altered in subjects with PAD, and these factors might provide important diagnostic and therapeutic targets in this disease. We found that plasma concentrations of Ang2, VEGF, and sTie2 were all significantly elevated in patients with PAD compared to control subjects. Furthermore, plasma concentrations of VEGF and sTie2, but not Ang2, were significantly increased in patients with critical limb ischemia compared to those with intermittent claudication. Increases in VEGF and sTie2 in CLI patients were independent of CVD risk factors and the ABI. Moreover, VEGF was found to induce shedding of sTie2 from endothelial cells *in vitro*, suggesting a potential mechanistic link between these two proteins. Taken together, these findings suggest that sTie2 and VEGF may provide novel biomarkers for PAD in general and CLI more specifically. These results have implications for understanding the pathophysiology and potentially improving the diagnosis and treatment of PAD.

Despite its increasing prevalence, PAD continues to be clinically under-recognized. Recent data indicate that a significant percentage of patients with PAD are asymptomatic or they present with atypical symptoms(123). Moreover, patients with PAD frequently have concomitant CAD, and their risk of cardiovascular events is significantly higher than in patients without PAD. As a result, the early identification of PAD is of paramount importance, as it would lead to earlier and more aggressive

cardiovascular risk factor modification. The identification of novel biomarkers of PAD would aid substantially in this effort. To this end, Wilson et al recently used an unbiased proteomic approach to demonstrate that β2-microglobulin (β2M) is upreglated in the serum of patients with PAD(124). β2M is a component of the class I major histocompatibility complex and is present on virtually all cell membranes, and although the mechanism of its increased release is not understood, its identification as a potential biomarker of PAD represents an important step forward in the diagnosis and management of this disease (125). Proteomic techniques such as the mass spectroscopy approach used by Wilson et al provide an attractive means to identify potential protein biomarkers. However, an important limitation of these approaches is that they can detect only a small subset of the total serum proteome. Many serum proteins are masked by albumin or other abundant proteins, and a number of proteins of interest, including most cytokines and growth factors, are present in serum at concentrations that are below the level of detection with these techniques. Therefore, the hypothesis-driven, targeted approach used in our study provides an important complement to investigate potential biomarkers for PAD.

PAD is characterized by tissue ischemia, and the resulting tissue hypoxia provides a potent angiogenic stimulus. Therefore, PAD should result in upregulation of angiogenic factors in order to induce a compensatory angiogenic response, and VEGF, Ang2, and sVEGFR-1 are all known to be upregulated by hypoxia *in vitro(126-128)*. A number of studies have examined circulating levels of angiogenesis-modulating proteins in the setting of cardiovascular disease or in patients with CVD risk factors, such as hypertension and diabetes mellitus. Similar to our results, plasma concentrations of VEGF, Ang2, and sTie2 have been found to be increased the setting of coronary artery

disease, acute coronary syndromes (65, 76), and hypertension (66). Increased VEGF and Ang2 have also been demonstrated in the setting of diabetes (97), although there appear to be no prior data on sTie2 levels in diabetics. In the context of PAD, only VEGF and sVEGFR-1 have been studied previously, and consistent with our findings, plasma VEGF concentrations were found to be higher in patients with PAD compared to controls (129-131). Also in agreement with our results, circulating levels of sVEGFR-1 were not increased in patients with PAD but were either unchanged(129, 131) or reduced (130) compared to controls. Importantly, after controlling for major demographic and cardiovascular risk factors in our study, the differences in circulating sTie2 and VEGF levels in CLI patients remained significant.

Our study subdivided PAD patients by their diagnosis of intermittent claudication or critical limb ischemia, which have markedly different outcomes. Currently, the diagnosis of IC vs. CLI is largely clinical and is based on a combination of signs, symptoms, and, to a certain degree, the ABI. Our finding that VEGF and sTie2 were significantly increased in patients with CLI compared to IC indicates that plasma concentrations of these proteins may distinguish between these two conditions. To our knowledge, only one study has attempted to correlate expression of angiogenic proteins with severity of PAD. Makin et al (129) examined VEGF levels in PAD patients as a function of the ABI and found that VEGF concentrations did not differ in patients with ABIs above or below 0.52. Although that study did not classify patients clinically as having CLI, no correlation was found between plasma VEGF concentration and the presence of rest pain (129). A critical difference in our study is that patients were given a clinical diagnosis of CLI that was not based on the ABI alone, since it correlates poorly with disease state. Importantly, our data demonstrate a significant association between

levels of sTie2 or VEGF and the clinical diagnosis of CLI that is independent of the ABI. These findings suggest that sTie2 and/or VEGF may provide novel biomarkers for this manifestation of PAD.

Whereas VEGF is likely upregulated as a compensatory response to ischemia in PAD, sTie2 appears more likely to play a role in the pathogenesis of the disease. Although IC and CLI have traditionally been thought of as early vs. late or minor vs. severe variants of the same disease, recent evidence suggests that these manifestations of PAD may be caused by distinct mechanisms. In this regard, the upregulation of an inhibitor of angiogenesis like sTie2 could worsen the clinical presentation despite having a similar extent of vascular occlusive disease. If so, sTie2 could serve as a target for therapeutic intervention in PAD. Furthermore, the increase in circulating levels of both VEGF and sTie2 in CLI patients suggests a mechanistic link between these two proteins. Accordingly, our data demonstrated that VEGF induced proteolytic cleavage and shedding of sTie2 *in vitro*, and recent concomitant work from our laboratory has demonstrated that this process is mediated through the phosphoinositide 3-kinase/Akt pathway (98). Thus, it is possible that ischemia-induced increases in VEGF expression result in dysregulated cleavage of sTie2, thereby limiting the angiogenic response to tissue hypoxia in those patients with the most severe ischemia, eventually resulting in CLI.

The regulation of Tie2 cleavage and subsequent shedding of sTie2 is poorly understood. Previous studies have demonstrated sTie2 in the serum of healthy individuals (54) as well as in patients with a variety of cardiovascular diseases, including congestive heart failure, hypertension, and acute coronary syndromes(64-66). Moreover, in renal cell carcinoma patients, sTie2 concentrations correlated with severity

of disease and decreased survival (71). Our study is the first to examine sTie2 levels in PAD, and the correlation of sTie2 with CLI provides important insights into this manifestation of PAD.

### 4.4.1 Study Limitations

Although a clear limitation of the current study is the modest sample size, a unique feature of the study is the analysis of subclassifications of patients with PAD (e.g., IC and CLI). Moreover, investigation of changes in factors involved in angiogenesis provides novel insights into the pathophysiology of this disease and its different manifestations. Although our results suggest that plasma concentrations of Ang2 are not significantly different between patients with IC and CLI, the current study was underpowered to rule out a type II error in this analysis (i.e., a false negative). Thus, assessment of larger populations will be necessary to confirm whether Ang2 might also serve as a marker of CLI as well as to validate our observations on sTie2 and VEGF in this population. Furthermore, investigation of temporal changes in these factors in PAD populations may be clinically relevant, and additional studies will be needed to establish quantitative values of sTie2 and/or VEGF that define CLI with relatively high sensitivity and specificity. The targeted, hypothesis-driven approach used here to investigate changes in angiogenesis modulatory proteins is limited in its analysis of only a small subset of potential biomarkers of PAD. Therefore, additional unbiased proteomic approaches, as described recently for the identification of β2M (23), might lead to the identification of additional PAD biomarkers.

### 4.4.2 Conclusion

This study demonstrates that plasma levels of sTie2 and VEGF are significantly increased in patients with PAD and that increases in these proteins distinguish patients with CLI from those with IC. Because the signs and symptoms of CLI can overlap with those of other disease states, including neuropathies and venous ulcers, these results could have important implications for the diagnosis of CLI. These results also demonstrate a potential mechanistic link between VEGF and sTie2 in PAD, suggesting that sTie2 may serve not only as a marker of CLI but also as a viable target for therapeutic intervention in this disease.

# 5. Conclusions and Future Directions

Angiogenesis is a complex biological process, and our ability to harness its potential for therapeutic purposes depends on a thorough understanding of its regulatory mechanisms. Elucidating the various components of angiogenesis and understanding their roles in the angiogenic process, from temporal, spatial, and functional perspectives, will likely promote significant advances in the treatment of vascular diseases. However, this will require that all components of the angiogenic cascade be considered critical to this process and explored fully to define their relative contributions. To date, the contribution of soluble receptors to angiogenesis has been poorly understood. However, mounting evidence from this body of work and others highlights the importance of sTie2 and other soluble receptors in this process and reinforces the need for further investigation into their mechanisms of action and regulation.

Tie2 and the angiopoietins modulate the transition from stable to remodeling blood vessels. As a result, this receptor-ligand system serves an integral function in both the initiation of angiogenesis via vessel destabilization and the promotion of vessel maturation and stabilization. Initiating angiogenesis necessitates disruption of the tight interactions between endothelial cells and supporting vascular cells (vascular smooth muscle cells and pericytes), while the formation of stable, functional conduits for blood flow requires stabilization of these interactions. Regulation of Tie2 ligand binding influences both of these processes, thus the identification of sTie2 and understanding its role in the modulation of angiogenesis and vascular remodeling is critically important.

As demonstrated by studies in this thesis, full-length Tie2 is expressed at the membrane but undergoes metalloprotease-mediated cleavage of the extracellular domain to form sTie2. sTie2 is functional, as it can bind Ang1 and Ang2 to prevent

ligand-dependent Tie2 receptor activation in the setting of either Ang1- or Ang2-mediated stimulation. Additionally, sTie2 inhibits the Ang1-induced pro-survival response. Importantly, these studies have demonstrated a role for the PI3K-Akt pathway in the Tie2 cleavage and shedding process. Prior to this observation, a role for PI3K or Akt in mediating RTK cleavage had not been described. Most importantly, we demonstrated a role for the prototypical angiogenic factor, VEGF, in activation of Tie2 cleavage in HUVECs and other endothelial cell types. Such a role for VEGF-mediated Tie2 regulation had not been previously appreciated, and these findings highlight the complex nature of angiogenesis regulation. Several questions arose from this set of observations: How does VEGF activate Tie2 cleavage? What protease(s) are responsible for Tie2 cleavage? What is the mechanism by which PI3K-Akt regulates this process?

To begin to answer these questions, we elected to investigate these phenomena in a mouse model of hindlimb ischemia. Ischemia is one of the most potent inducers of angiogenesis, as tissue hypoxia results in robust increases in VEGF expression (132). As a result, we investigated whether ischemia-induced VEGF would lead to altered sTie2 levels. Interestingly, we observed a near two-fold increase in sTie2 levels within two days after femoral artery ligation, and this elevation dissipated as perfusion was restored. These data suggest that the ischemia-induced tissue hypoxia was responsible for the elevation in sTie2, however, once normal oxygen tension was restored, sTie2 levels normalized. We investigated this response *in vitro* and observed induction of Tie2 cleavage in response to hypoxia. This hypoxia-dependent response was found to be VEGF-dependent, as demonstrated by VEGF inhibition studies. Importantly, VEGF upregulated the transcription of ADAM15, the protease responsible for Tie2 cleavage, in

an Akt-dependent manner. This set of experiments supports a role for VEGF in the regulation of sTie2 shedding in an Akt-dependent, ADAM15-mediated manner.

Integrating results from both sets of studies suggests a role for sTie2 shedding as a positive regulator of the angiogenic response. From a mechanistic standpoint, tissue hypoxia stimulates VEGF production from stromal tissues as well as ECs. VEGF acts in a paracrine/autocrine fashion, resulting in activation of VEGF receptor-mediated signaling pathways and cellular responses. Through the PI3K-Akt pathway, VEGF transcriptionally upregulates ADAM15 expression, which is likely in an enzymatically active form, thereby resulting in enhanced Tie2 cleavage. Insights from the hindlimb ischemia model begin to suggest a pro-angiogenic role for sTie2. Classically, VEGF and the PI3K/Akt pathway have been considered integral components of the EC pro-survival pathway, and it is likely that the Tie2 cleavage response, which is both VEGF- and PI3K-Akt dependent, serves to maintain endothelial cell viability and shift the angiogenic balance towards endothelial cell growth. Enhanced sTie2 production may be a normal and necessary component of the angiogenic response. However, these findings are in direct contrast to what has been described previously. In animal models of angiogenesis, overexpression of an engineered form of sTie2, ExTie2, resulted in a blunted angiogenic response (see Chapter 1). So, how does one rationalize an increase in endogenous sTie2 concentrations in settings such as ischemia if sTie2 is, in fact, an angiogenesic inhibitor?

To understand this apparent discrepancy between our results and prior studies, we began to explore the role of sTie2 in patients with PAD, clinically relevant ischemic vascular disease. PAD presents a unique opportunity to examine the body's response to ischemia since different manifestations of this disease (IC vs. CLI) represent populations

with different degrees of ischemia (IC vs. CLI). We examined several putative pro- and anti-angiogenic factors in this patient population and found that VEGF, Ang2 and sTie2 were significantly elevated across the spectrum of patients with PAD. Importantly, in the IC group, which represents the least angiogenically compromised of PAD patients, sTie2 levels were similar to controls. However, sTie2 levels were significantly elevated in patients with CLI. It is possible that the severity of ischemia in IC patients is not severe enough to induce Tie2 cleavage. However, these patients clearly have intermittent episodes of ischemia, which would be expected to induce at least transient elevations in VEGF and, therefore, sTie2. Such an intermittent ischemic response may make it difficult to demonstrate changes in sTie2 levels, and further studies are necessary to investigate this possibility.

Although it has been speculated that increased sTie2 levels in CLI could be causative by contributing to the reduced angiogenic response, increasing evidence suggests that a defect in CLI is the inability of the muscle tissue (the "substrate") to respond appropriately to growth and/or survival cues. Thus, elevated sTie2 levels would still correspond with an increased angiogenic program, but one in which the tissue is unable to respond appropriately by growing new blood vessels. This possibility is consistent with the fact that VEGF and Ang2 levels were also significantly elevated in CLI patients. In this case, the defect would not be the increase in sTie2, rather it is the inability to grow new blood vessels in response to increased VEGF, Ang2, and sTie2. Consistent with this possibility, sTie2 levels decreased in the mouse hind limb ischemia model over time as perfusion improved. These studies raise the important question of what sTie2's role is during ischemia-induced angiogenesis. Studies demonstrating the spatial and temporal changes in expression of the angiopoietins, sTie2 production, or

Tie2 activation in ischemia are limited. As discussed previously, the balance in expression of Ang1 and Ang2 dictates the ultimate Tie2 response. Therefore, elucidating the changes in these factors at the tissue level will be critical to understand how sTie2 regulates the observed phenotype in a given setting. If sTie2 is present in the extracellular matrix, it might preferentially bind Ang1, shifting the balance toward Ang2-mediated vessel destabilization. In contrast, high sTie2 levels in the blood might preferentially block Ang2's effects. In this respect, it is possible or even likely that markedly elevated sTie2 levels would abrogate all angiopoietin-mediated Tie2 activation and inhibition of angiogenesis. Prolonged ischemia, such as in CLI, could result in significantly elevated sTie2 concentrations that are anti-angiogenic, and this might explain why preclinical studies in which sTie2 was expressed at very high levels inhibited angiogenesis. Overall, these studies highlight the delicate balance necessary to maintain vascular homeostasis or promote a pro- or anti- angiogenic phenotype. Additional studies will be critical to clarify the precise role of sTie2 in these processes.

## 5.1 Clinical Implications

Vascular remodeling can counteract damage in ischemic tissues, and the combination of current modes of intervention (PCI or bypass surgery) with factors that promote collateral vessel development could prove to be the optimal mode of treatment in patients with vascular disease. On the other hand, poor candidates for PCI or bypass surgery may stand to benefit greatly from improved treatments that promote collateral vessel growth and stabilization. With modern day advances in medical technology, percu taneous coronary interventions (PCI) have become a mainstay of treatment for

symptomatic arterial occlusive disease, however the procedure is not always successful**.** In some patients, restenosis will inevitably lead to vascular reocclusion, return of symptoms, and the need for reintervention on an already diseased vessel.

Peripheral artery disease (PAD), a form of CVD, is characterized by atherosclerosis of the peripheral vasculature resulting in disease of the renal, carotid, iliac, femoral, and popliteal vessels and their respective tissues and organs of distribution. PAD has been recognized as a marker of systemic atherosclerosis and is associated with a 2-3 fold-risk in CVD morbidity and mortality. The increasing prevalence of PAD is of great concern and necessitates further investigation to understand and combat this looming “epidemic.”

Treatment of PAD can be divided into three major subgroups: (a) risk factor modification, (b) medical therapy, and (c) surgical or percutaneous interventions. Risk factor modification includes smoking cessation and the initiation of an exercise regimen with a low fat diet, which are designed to retard the progression of disease and reduce the incidence of complications associated with PAD. Current medical management includes anti-platelet agents, anti-hypertensive therapy, oral hypoglycemic agents, lipid lowering agents (statins) and pro-circulatory agents. More invasive modes of treatment include vessel bypass surgery, percutaneous vascular interventions (angioplasty and stent placement), and in cases of advanced disease, amputation. At present, no data support the use of primary pharmacologic therapy in patients with critical limb ischemia. The best options are revascularization with surgery or percutaneous interventions. However, for patients with CLI who are not suitable candidates for revascularization, amputation is the only recourse. This is associated with high morbidity/mortality rates, especially in the elderly, as these patients may be less able to function with prostheses. As a result, amputation in a previously ambulatory individual may mean the difference

between independence and good quality of life versus nursing home-dependence, poor quality of life, and markedly increased healthcare costs. If a method of identifying patients at risk for progression to CLI were available, more aggressive modes of treatment could be enacted in this patient population. Currently, the ankle-brachial index (ABI) is the gold standard for PAD diagnosis. However, the correlation between ABI values and the severity of PAD is poor. Soluble Tie2, which is upregulated in settings of hypoxia and significantly elevated in CLI patients, could serve as a serum biomarker for this specific disease subpopulation. Identifying elevated sTie2 levels in a PAD patient could facilitate more rapid risk stratification and aggressive therapeutic management that might prevent progression to skin ulceration, amputation, and/or physical debilitation.

## 5.2 Future Directions

The studies described in this thesis have established the role of VEGF and PI3K/Akt in Tie2 cleavage and sTie2 shedding. Additionally, the role of VEGF- and Akt-mediated transcriptional upregulation of ADAM15 was demonstrated. However, the link between Akt activation and ADAM15 transcription has not been explored. Likely candidates for mediating this response are downstream effectors of Akt, such as mTOR, MDM2, and GSK3β. Utilizing a targeted siRNA approach would help to evaluate the role of these candidates in upregulation of ADAM15 expression.

Additionally, investigating the Tie2 cleavage site would add significant information to the limited body of knowledge surrounding sTie2. This knowledge would facilitate the engineering of a construct to explore the binding properties of this molecule (i.e. does sTie2 bind Ang1 or Ang2 more tightly?), which would help to elucidate details of its role

in angiogenesis. Additionally, conducting in vivo studies with endogenous sTie2 will add greater credibility to existing studies that have demonstrated angiogenic inhibition in the setting of ExTie2 overexpression.

The role of ADAM 15 in Tie2 cleavage has been established by these studies. However, examining the angiogenic response in ADAM15 knockout mice would support the hypothesis that ADAM15 cleaves Tie2 in the setting of ischemia and that elevations in sTie2 post-ischemia are necessary for an appropriate angiogenic response. It appears that sTie2 levels must be tightly regulated to maintain vascular homeostasis, hence, investigating the effects of varying levels of ExTie2 or sTie2 on the post-ischemic angiogenic response would be beneficial.

In the human studies in this thesis, sTie2 was found to be significantly elevated only in the CLI patient population. Because of the morbidity and mortality associated with CLI, stratifying patients early and implementing an aggressive treatment strategy could be beneficial. To investigate the utility of serum sTie2 as a biomarker for CLI, determining the temporal sequence of elevations in sTie2 is absolutely necessary. Specifically, is sTie2 transiently increased in IC patients and is sTie2 significantly elevated over time in CLI patients? In either case, sTie2 could be employed as a potential biomarker in vascular medicine and surgery clinics and may enhance therapy and outcomes for these difficult to treat patients.

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# Biography

## Clarence M. Findley

**Born**: January 20, 1979 in Washington, DC, USA

### EDUCATION

**Duke University** — Durham, NC
PhD candidate in Pharmacology — August 2003-May 2008
MD candidate — August 2001-May 2009

**Princeton University** — Princeton, NJ
AB. Department of Molecular Biology — September 1996-May 2000

### ABSTRACTS AND PRESENTATIONS

**Findley CM** and Kontos CD. "Tie2 Cleavage is Mediated by ADAM15 in a VEGF- and PI3K/Akt-dependent Manner (oral)." 15th International Vascular Biology Meeting. Sydney, Australia. June 2008.

**Findley CM** and Kontos CD**.** "VEGF Induces MMP-dependent Tie2 Cleavage (poster)." American Heart Association- Scientific Sessions. Orlando, FL. October 2007.

**Findley CM** and Kontos CD. "ADAM proteins Mediate PMA- and VEGF-Inducible Tie2 Cleavage (oral)" National Medical Association- 2007 Annual Convention and Scientific Assembly. Honolulu, HI. August 2007.

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**Findley CM** and Kontos, CD. "Inducible Tie2 Cleavage Results in Shedding of Soluble Tie2 and Modulation of Tie2 Activity" (oral). 17th Annual Scientific Sessions- Society of Vascular Medicine and Biology. Philadelphia, PA. June 2007.

**Findley CM** and Kontos CD. "VEGF Induces ADAM-dependent cleavage via the PI3K-Akt Pathway (poster)" European Hematological Society-Angiogenesis. Albufeira, Portugal. May 2007.

**Findley CM** and Kontos CD**.** "Soluble Tie2 Modulates Tie2 Activity and Regulates Endothelial Cellular Responses (oral and poster)" Research Trainee Forum. Sunny Isles Beach, FL September 2006.

**Findley CM** and Kontos, CD. "Constitutive Tie2 Cleavage Results in Shedding of Soluble Tie2 and Modulation of Tie2 Activity" (poster). UNCF-Merck Fellows Day Conference. West Point, PA. June 2006.

Mitchell RG, **Findley CM**, Kontos CD, and Annex BH. "Soluble Tie2: A Biologic Inhibitor of Angiogenesis is Markedly Elevated in Patients with Critical Limb Ischemia" (poster). 17th Annual Scientific Sessions- Society of Vascular Medicine and Biology. Philadelphia, PA. June 2006.

**Findley CM** and Kontos, CD. "Metalloprotease-Dependent Production of Soluble Tie2 Modulates Tie2 Activity" (oral) American Heart Association-7th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology. Denver, CO. April 2006.

Xie D, Reed EA, Timberlake SA, **Findley CM**, Odronic SI, Pippen AM, Wu F, Li Y, Annex BH, Donatucci GF. "Vasoreactivity Changes Following VEGF Signaling Blockade in bFGF Treated Corporal Tissue of Hypercholesterolemic Rabbits." (poster) Sexual Medicine Society of North America 2005 Fall Meeting. New York, NY. November 2005.

## PUBLICATIONS

**Findley CM,** Hart JL, Dokun AR, and Kontos CD. VEGF-dependent activation of ADAM15 is responsible fo rTie2 cleavage under hypoxic conditions (manuscript in preparation), 2008.

**Findley CM**, Mitchell RG, Annex BA, and Kontos, CD. Plasma Levels of Soluble Tie2 and VEGF Distinguish Critical Limb Ischemia from Intermittent Claudication in Patients with Peripheral Arterial Disease (in press). *Journal of American College of Cardiology* 2008.

Xie D, **Findley CM,** Timberlake SH, Reed EA, Pippen AM., Kontos CD, Donatucci CF, and Annex BA. VEGF Mediates the Beneficial Effects of bFGF on Vasoreactivity in Corporal tissues of Hypercholesterolemic Rabbits (in press). *Journal of Sexual Medicine* 2008.

**Findley CM***,* Cudmore MJ, Ahmed A and Kontos CD. VEGF induces Tie2 shedding via a phosphoinositide 3-kinase/Akt dependent pathway to modulate Tie2 signaling. *Art Throm Vasc Biol. 2007;* ***27****: 2619-26.*

## HONORS AND AWARDS

2007 Jay D. Coffman Young Investigator Award (2nd place)- Society for Vascular Medicine
2007 European Commission Full Scholarship- European Hematologic Society
2006 Acres of Diamonds Award- Minority Trainee Research Forum
2006 Arteriosclerosis, Thrombosis, and Vascular Biology New Investigator Travel Award
2006 UNCF- Merck Graduate Fellow
2006 Biotechnology Institute- Amgen Minority and Indigenous Fellow
2004 American Heart Association- Council on Basic Cardiovascular Science Minority Travel Grant

## FUNDING

***1 R36 AG027584-01 (PI: Clarence M. Findley)***

NIH/NIA
Title: Tie2- Regulation and Role in Angiogenesis
The goals of this project are to investigate unknown aspects of Tie2 receptor regulation. Additionally, the project aims to examine the role of Tie2 in pathological angiogenesis using a mouse model of hindlimb ischemia.
Funded: 7/1/06-7/1/08

***Graduate Dissertation Research Fellowship Award (PI: Clarence M. Findley)***

Merck
Title: Soluble Tie2- Regulation and Role in Pathological Angiogenesis
This a pre-doctoral fellowship award used to support a graduate student's stipend, conference travel and equipment/supplies.
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