The Role of Angiopoietin-2 in Signaling Through the Endothelial Receptor Tyrosine Kinase Tie1

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Pathology in the Graduate School
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2010

ABSTRACT

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Abstract

A functioning vasculature is critical for the supply of nutrients to all organ systems as well as for a host of physiologic and pathologic processes. Vascular development and maintenance are tightly regulated by a number of signaling processes, among which the Tie proteins are two functioning receptors. Although they have been shown to exhibit essential roles in endothelial cell sprouting and quiescence, the mechanistic details of Tie interactions and the effects of their associations with the Angiopoietins have not been elucidated. Studies in this thesis investigated the effects of Ang2 on Tie1 activation, signaling, and cellular responses within the context of both primary and immortalized endothelial cells. Additionally, we investigated the role of Ang2 in the cellular reorganization and subsequent downregulation of Tie1. We observed that Ang2, but not Ang1, induces phosphorylation of Tie1 in endothelial cells and that the extracellular domain of Tie2 is required for Ang2-mediated activation of Tie1. Furthermore, we demonstrated that Tie1 activation leads to signaling through the Akt axis, and the consequent stimulation of anti-apoptotic and proliferative cellular effects. Additionally, we demonstrated that Ang2 induces a concentration- and time-dependent downregulation of Tie1, and that Tie2's role in the process appears to be recruitment of the ligand to the multimeric Tie complexes. Interestingly, although Ang2 stimulation is necessary, we demonstrated that Ang2-mediated activation of Tie1 receptor complexes is not required for ligand induced downregulation of the receptor. Finally, we characterized the modulatory role of Tie1 with regards to Angiopoietin signaling through Tie2, and observed that removal of Tie1 from the surface of endothelial cells enhances Ang2-mediated activation of Tie2, leading to increases in cell survival signaling

cascades. Taken together, these data shed new light on Angiopoietin signaling through the Tie receptors, further characterize the interactions between Tie1 and Tie2, suggest novel forms of endothelial cell regulation within developing and mature vasculature, and may have implications for signaling within a host of physiologic and pathologic states.

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List of Abbreviations

Abbreviation	Definition
ATP	adenosine tri-phosphate
DMEM	Dulbecco's modified Eagle media
EBM	endothelial basal medium
EBM-2	endothelial basal medium 2
EC	endothelial cell
ECM	extra cellular matrix
ECRF	EC-RF24; immortalized HUVECs
ECRF-Tie1	ECRF cell with overexpression of Tie1
ECRF^{Tie1Lo}	ECRF cell with reduced expression of Tie1
ECRF^{Tie2Lo}	ECRF cell with reduced expression of Tie2
ECRF^{Tie2Lo}-mTie1	ECRF cell with reduced expression of Tie2 and overexpression of murine Tie1
ECRF^{Tie2Lo}-mTie1/Tie2	ECRF cell with reduced expression of Tie2, overexpression of murine Tie1, and overexpression of murine Tie2
ECRF^{Tie2Lo}-mTie1/Tie2K854R	ECRF cell with reduced expression of Tie2, overexpression of murine Tie1, and overexpression of murine Lys854Arg Tie2
ECRF^{Tie2Lo}-mTie1/Tie2TM	ECRF cell with reduced expression of Tie2, overexpression of murine Tie1, and overexpression of murine Tie2TM construct
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGM	endothelial growth medium
EGM-2	endothelial growth medium 2

EPC	endothelial progenitor cell
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FNIII	fibronectin type III
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HIF-1α	hypoxia-inducible factor-1α
HUVEC	human umbilical vein endothelial cell
IL-1β	interleukin-1-beta
IL 6	interleukin-6
IP	immunoprecipitation
JM	juxtamembrane
LDL	low density lipoprotein
MMP	matrix metalloprotease
mTOR	mammalian target of rapamycin
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM	platelet endothelial cell adhesion molecule
PH	pleckstrin homology

PI	phosphoinositide
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
RTK	receptor tyrosine kinase
SH2	Src homology 2
shRNA	short hairpin RNA
SMC	smooth muscle cell
TGF-β	transforming growth factor beta
TM	transmembrane
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor A
VEGFC	vascular endothelial growth factor C
VEGFR1	vascular endothelial growth factor receptor-1
VEGFR2	vascular endothelial growth factor receptor-2
VSMC	vascular smooth muscle cell
WPB	Weibel-Palade bodies
WT	wild-type

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1. Background and Significance

Due to the inefficient diffusion of molecules over distances greater than 100 um, complex organisms have developed circulatory systems to accommodate the transport of oxygen and nutrients from sites of absorption to peripheral tissues as well as export of carbon dioxide and waste products from the tissues to sites of excretion. The importance of this process is demonstrated by the fact that failure to develop a functional vasculature results in death early in embryonic development. The lumen of the vasculature is lined with a monolayer of endothelial cells, which serve as the initial barrier between the components of the blood and the tissues. Endothelial cells lie on a basement membrane and are surrounded by a network of pericytes, fibroblasts and intravasated smooth muscle cells that together make up the tunica intima and the internal elastic lamina. Moving peripherally from the lumen of the vasculature, vessels may contain layers of vascular smooth muscle comprising the tunica media, the external elastic lamina, and the tunica adventitia. These various layers as well as the blood allow the endothelial cells to come in contact with a host of factors that influence the overall phenotype of the ECs. The dynamic interaction between the endothelium and interacting factors determines the accessibility of the components of the blood to the stromal tissues.

1.1 Mechanisms of vascular growth

Both within the embryo and the mature organism, the various vessels of the vasculature form by one of three general mechanisms: vasculogenesis, angiogenesis, or arteriogenesis. Vasculogenesis is defined as the development of a new vasculature from the *de novo* condensation and subsequent organization of endothelial progenitor cells that migrate to sites of forming blood plexuses. Angiogenesis is defined as the

budding or sprouting of capillary branches from existing blood vessels in cases of sprouting angiogenesis or the intussusception of new vessels by the formation of novel lumens within capillary walls in cases of intussusceptive angiogenesis. Arteriogenesis is defined as the remodeling of existing arteries in response to changes in blood flow within the lumen of the vessel. Due to their differences in both form and function, the three mechanisms of vessel growth will be discussed further, with special emphasis on vasculogenesis and angiogenesis. The mechanisms for the various routes of vascularization and receptor cascades therein refer to the mechanisms within the circulatory system. The differences in both form and function of these three mechanisms of vessel growth will be discussed further, with special emphasis on vasculogenesis and angiogenesis. Although many of the mechanisms of the various routes of vascularization and the receptor signaling cascades that regulate them are common to both the blood vasculature and the lymphatic vasculature, there is much more known about the development and maturation/propagation of the blood vasculature than the lymphatic vasculature. In this dissertation, these differences, when present or known, will be highlighted.

1.1.1 Vasculogenesis

Vasculogenesis within complex organisms begins before the development of most organ systems (E7.5 in the mouse and during the third week of development in humans). Mesenchymal cells from the lateral and posterior mesoderm migrate and condense into blood islands near the extraembryonic yolk sac endoderm. The mesenchymal cells at the outer periphery of the plexuses flatten to form ECs and quickly create the rudimentary vascular network that will expand to give rise to the circulation. Blood islands initially form the dorsal aorta and yolk sac capillaries and it is these

locations that produce both the hematopoietic precursors for ECs and the cellular components of the blood. The source of hematopoietic precursors in the embryo migrates initially from the dorsal aorta and yolk sac to the fetal liver, and finally to the spleen and bone marrow(1).

Although it was originally thought that vasculogenesis occurred only in the developing embryo, recent evidence has shown that EC precursors, which can contribute to revascularization, are also produced in the mature organism. Islets of new ECs can be observed at the center of vascular transplant grafts that do not originate from the angiogenic endothelium found on the border of the graft. Furthermore, the peripheral infusion of mouse mononuclear cells to an ischemic insult leads to the maturation of these cells into ECs that can contribute up to 25% of the newly formed mature vasculature(2). The circulating endothelial progenitor cells derived from bone marrow hematopoetic stem cells responsible for adult vasculogenesis have been observed to hone to various sites of revascularization. In addition, they can contribute to vessel remodeling as part of the physiological development of the placenta and the corpus luteum or during pathological processes such as tumor growth and wound healing(3). These circulating EPCs express a host of factors in common with mature endothelial cells including VEGFR-2(3) and Tie2(4) and exhibit some similar signaling pathways, the roles of which will be discussed later.

1.1.2 Angiogenesis

Once a rudimentary capillary plexus is formed in the embryo, the majority of vascular expansion during development occurs by way of angiogenesis. Vascular maintenance, growth, and repair in both physiologic and pathologic processes postnatally also arise by angiogenic methods. Starting from approximately E9.0-9.5 in

the mouse, the development of the complex vascular network is predominantly controlled by the expansion of existing ECs into poorly perfused regions. Angiogenesis is triggered by the sensation of hypoxia through factors such as HIF-1α in the tissues that surround the vessels, which then turn on expression of pro-angiogenic factors. The existing endothelial cells of the microvasculature then proliferate and sprout primarily from the post-capillary venules into the basement membrane and form new junctions and vessels. This propagation occurs not only in the developing embryo but also in situations of ischemia in the adult, in which new vessel growth must occur to sustain cellular homeostasis. Since diffusion is limited to short distances, the increased density of capillaries allows for efficient delivery of oxygen and nutrients [reviewed extensively in(5)]. However, growth of microvasculature must be tightly regulated, and once the tissues are well perfused, cellular signals direct the endothelial cells to cease proliferation and reform the tight junctions that are responsible for separating the intra- and extra-vascular spaces (Figure 1). These cellular signaling mechanisms, of which the Tie1 and Tie2 receptors are integral players, will be discussed later.

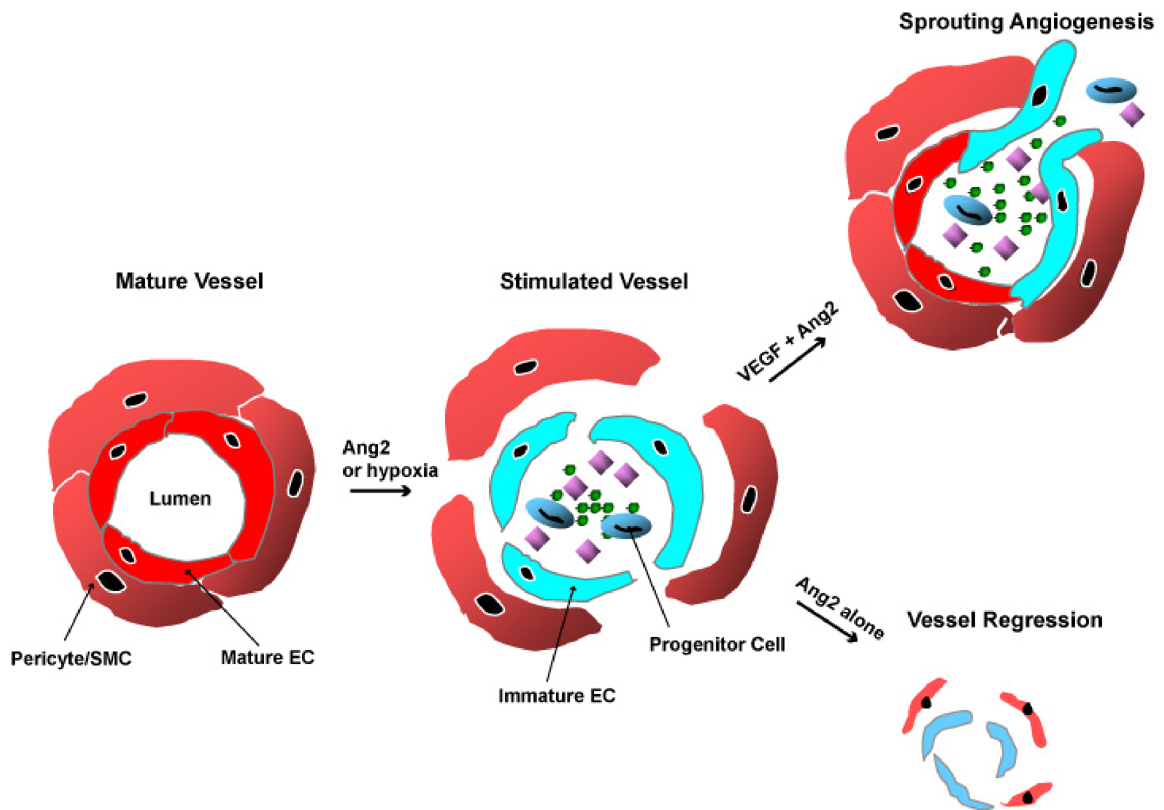

Figure 1: Mechanisms of Angiogenesis

During vessel quiescence, the lumen of the vasculature is encircled by a monolayer of endothelial cells layered on a basement membrane and its corresponding pericyte and Smooth Muscle Cell (SMC) constituents. The ECs are elongated, flattened, and form tight junctions between one another to prevent leakage of materials across the barrier. Stimulation of the system by either exogenous Ang2 or conditions of hypoxia leads to degradation of the basement membrane beneath the ECs and promotes looser associations between the endothelium and the underlying cells/matrix. Furthermore, a host of growth factors are released into both the lumen as well as the surrounding tissues. If VEGF stimulation occurs in conjunction with Ang2 addition, ECs begin to migrate and proliferate into the space underlying the old vessel and promote the formation of new vasculatures into the surrounding tissues. After Ang2 and VEGF stimulation subsides, the ECs reform the quiescent phenotype that is observed in mature vessels. However, if Ang2 stimulation occurs in the absence of VEGF, apoptosis cascades are initiated that lead to vessel regression.

1.1.3 Arteriogenesis

In contrast to angiogenesis, which is triggered by hypoxia, arteriogenesis is triggered by changes in shear stress. An increase in shear stress along the luminal surface of arterial ECs induces SMC proliferation, which then leads to increased endothelial proliferation at the sites of increased SMC growth, and consequently, the formation of larger collateral vessels(6). Furthermore, the changes in shear stress induce recruitment of monocytes and production of FGF2, which leads to formation of true collaterals that anastomose with occluded arteries(7). This is in contrast to the initial response of endothelial cells to increased blood pressure, in which simple dilatation is the predominant physiologic change.

1.1.4 Lymphangiogenesis

Although lymphangiogenesis occurs by way of previously mentioned growth processes, the resultant vessels are distinct from the blood vasculature. The functions of the lymphatic vasculature are to reabsorb fluids that are not taken up by the venules in the capillary beds, to redistribute the fluid into the venous circulation, to absorb chylomicrons from digestion, and to allow for the extravasation and movement of immune cells and molecules throughout the body. Lymphatic vessels develop after the formation of the cardiovascular system (E9.5-E10.0 in mice) by specific budding off of ECs from the cardinal veins. The budded ECs then migrate, form lymphatic plexuses, and develop into a separate vascular network that is connected to the blood vasculature only at the subclavian veins where the lymphatic fluid is emptied into the venous circulation(8). Lymphatic ECs are not adherent to an extensive basement membrane and are not ensheathed on the basolateral side by pericytes or SMCs. Rather, they are attached to the surrounding tissue by collagen filaments that can become taut in the

presence of fluid and allow for the enlargement of the lumen(9). Furthermore, the tight junctions that exist between lymphatic vascular ECs are less well developed than those between blood ECs due to the necessity for the extravasation of leukocytes into the parenchyma. Although differences exist in the signaling pathways between lymphatic and blood ECs, a number of different receptors, including Tie1 and Tie2, are expressed on both cell types, indicating partially overlapping mechanisms of regulation and control, which will be discussed later.

1.2 Pathological states involving angiogenesis

1.2.1 Pathophysiology of wound healing

Disruption of endothelial integrity elicits compensatory cellular responses in an attempt to restore tissue homeostasis. Wound healing is the result of a concerted response of circulating and mature ECs, inflammatory cells, SMCs, and fibroblasts. Injury of the vasculature causes leakage of luminal contents into the circumferential layers underlying the disrupted endothelium. Exposure of tissue factor results in the activation of platelets and consequently, the clotting cascade. The concomitant release of pro-inflammatory cytokines and growth factors leads to the recruitment of ECs, inflammatory cells, and fibroblasts to the site of injury. Leukocyte accumulation at the wound site is critical for the sustained secretion of chemoattractants as well as the phagocytosis of infectious agents and compromised tissue. After several days, macrophage infiltration at the site of injury leads to the phagocytosis of the previously recruited and dead neutrophils. Upon tissue infiltration, macrophages adopt a mature phenotype and secrete a large complement of growth factors such as VEGF, PDGF, TGF-β and FGF, all of which promote angiogenesis and the secretion of a new basement membrane overlying the site of injury(10). Complementary to EC-mediated

angiogenesis and vasculogenesis, recruited fibroblasts lay down ECM and differentiate into the myofibroblasts that are responsible for the production of granulation tissue that will initiate scar formation and remodeling. Angiogenesis occurs along a growth factor gradient along the ECM with the ECs degrading portions of the basement membrane and subsequently layering on top of the intimal tissue. This process is controlled by the secreted growth factor concentrations and basement membrane substituent fragments. At the time the wound site is filled with granulation tissue, the growth factor secretion by macrophages is greatly reduced. Consequently, the ECs stop proliferating, start regressing, and remodeling of the site is initiated, which eventually leads to scar formation.

1.2.2 Pathophysiology of atherosclerosis

Another pathophysiologic state that requires sustained signaling by the endothelium is atherosclerosis. Atherosclerotic plaque formation occurs as a combination of inflammatory, smooth muscle, and endothelial cell crosstalk and activation of compensatory mechanisms. Circulating low density lipoprotein (LDL) molecules are sequestered in the vessel intima and subsequently oxidized by reactive oxygen species. The oxidized LDL stimulates the ECs lining the lumen to express cell adhesion molecules that recruit monocytes to the vessel wall. In addition, LDL-mediated endothelial activation initiates chemoattractant molecule secretion, further homing circulating monocytes to sites of plaque formation. Monocytes extravasate into the intima, phagocytose oxidized LDL, and secrete cytokines as well as growth factors. The factors secreted by macrophages lead to SMC intravasation from the media to the intima as well as EC arteriogenesis. Macrophage and SMC phagocytosis of oxidized LDL leads to foam cell formation and continued secretion of cytokines and chemokines.

Eventually, explosion of the contents of the foam cells into the intima occurs, further exacerbating the inflammatory response. This leads to intimal thickening and protuberance of the plaque into the vessel lumen.

If the increased cellular mass within an atherosclerotic plaque leads to obstruction of blood flow to downstream tissues, the surrounding tissue experiences ischemia. Consequently, microvascular angiogenesis occurs from the vasa vasorum into the circumferential layers of the plaque.. However, the newly formed vessels lack the tight junction integrity of normal vasculature and therefore are leaky and less able to maintain the intraluminal pressures, and therefore are more prone to rupture. Rupture of the neovasculature within the intima leads to further inflammation, plaque progression, and the formation of hemorrhagic atheromas(11). In addition, the angiogenic ECs must break down some of the basement membrane during vessel expansion, and the matrix metallo-protease (MMP) secretion further destabilizes the plaque(12). Unstable plaque rupture initiates the thrombotic cascade which can either lead to complete vessel occlusion or to embolus formation and occlusion at sites distal to the atherosclerotic plaque.

1.3 Cellular signaling pathways in EC function

The interaction between the endothelium and the blood and surrounding tissues is very tightly regulated. One of the essential signaling pathways within the endothelium is receptor tyrosine kinase (RTK)-mediated activation of the Akt signaling cascade. Signaling by RTKs occurs following ligand binding, receptor clustering, and mutual transphosphorylation of the receptors' intracellular kinase domains. Upon ligand binding to the receptors' extracellular domains, the intracellular kinase domains undergo a conformational change involving the so-called activation loop, allowing binding of ATP to

an aspartate residue within the nucleotide binding loop, followed by phosphotransfer to specific tyrosine residues within the partner RTKs(13). Transfer of phosphate molecules leads to the recruitment of downstream effector molecules through the sequence-specific binding of SH2 domains to phosphotyrosine, as in the case of the p85 regulatory subunit of phosphoinositide 3-kinase(14). Binding and association of the p85 subunit of PI3K to RTKs leads to the activation of the p110 catalytic subunit(15), which phosphorlyates the D-3 hydroxyl residue of phosphoinositide 4,5-bisphosphate to form phosphoinositide 3,4,5-trisphosphate(16). PI(3,4,5)P_3 recruits the effector molecule Akt to the plasma membrane through its pleckstrin homology domain, and Akt is subsequently activated by phosphorylation of threonine 308 by PDK-1 and serine 473 by another kinase, likely ILK. Once activated, the serine/threonine kinase Akt phosphorylates a host of other downstream effectors that contain the consensus sequence R-X-R-X-X-S/T. Inhibition of proapoptotic molecules such as Bad and Procaspase-9 and activation of pro survival enzyme IκB, and subsequently NFκB, both lead to increased cell survival(17-18). Akt also promotes cell proliferation by activation of mTOR which leads to stabilization of the active Myc transcription factor as well as stabilization of the active E2F cell cycle progression transcription factor(19). Therefore, activation of the PI3K/Akt signaling axis by plasma membrane-bound RTKs can lead to a robust stimulation of both cell survival and proliferation pathways (Figure 2).

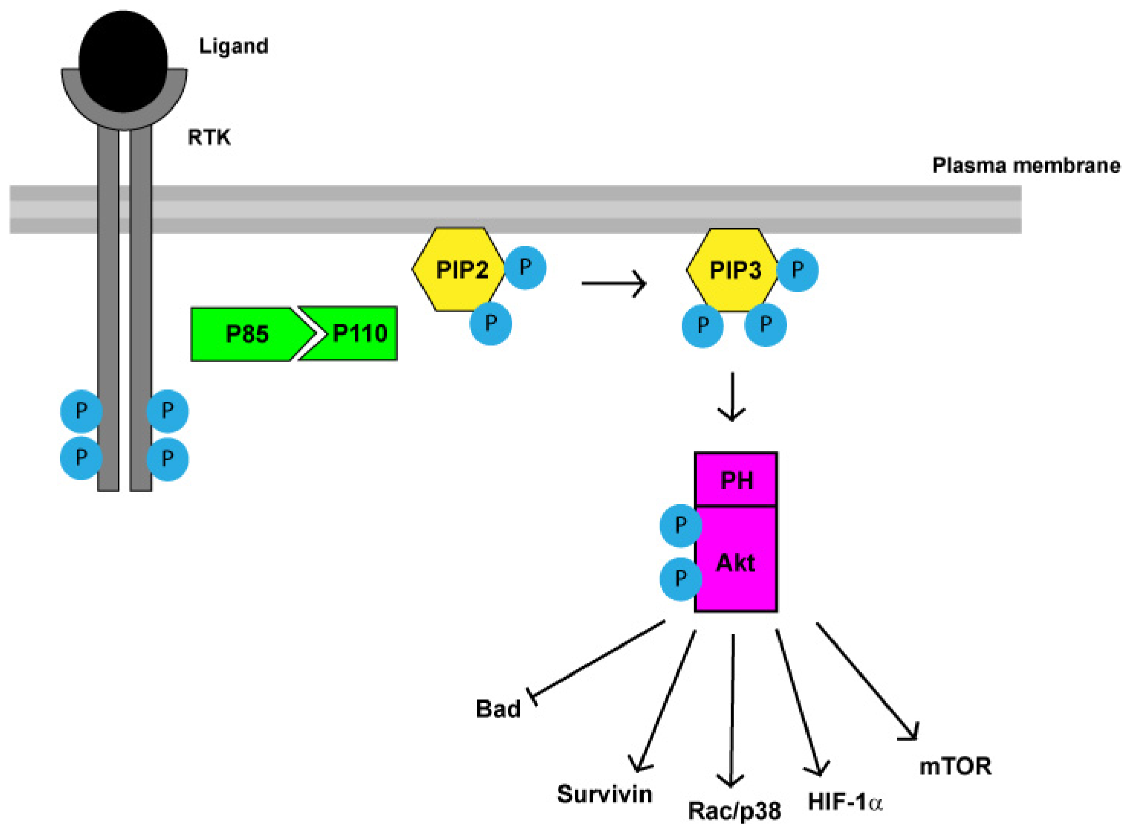

Figure 2: Mechanism of RTK signaling to activate Akt

Receptors multimerize and transphosphorylate intracellular Tyr residues upon ligand binding to RTK extracellular domains. The P85 subunit of PI3K recognizes and binds to specific phosphotyrosine residues, upon which the kinase is activated. The P110 catalytic subunit of active PI3K phosphorylates the substrate PIP2 to form PIP3. PIP3, as a second messenger, recruits Akt to the plasma membrane through its pleckstrin homology domain, whereupon it is activated by phosphorylation on specific Ser and Thr residues. Active Akt effects a number of distinct cellular effects through phosphorylation of a host of different targets, including BAD (inhibition of apoptosis), survivin (induction of pro-survival cascades), Rac/p38 (induction of both proliferation and migration), HIF-1α (hypoxia-mediated secretion of VEGFR-2 as well as Ang2), and mTOR (stimulation of protein synthesis).

1.3.1 VEGF and VEGFR-1, VEGFR-2 signaling

A critical RTK/ligand family involved in EC signaling is the vascular endothelial growth factor receptor family and its ligands, the VEGFs. The VEGF family of cytokines contains at least 5 isoforms, but for this discussion, only VEGF-A will be discussed, and will heretofore be referred to as VEGF, with a brief mention of VEGF-C and its role in lymphatic vasculature development. VEGF is critical in both the development of the embryo as well as the maintenance and growth of the postnatal vasculature. Mice lacking a single VEGF allele die in mid-gestation, between E11 and E12 due to impaired vasculogenesis with heterozygotes exhibiting a phenotype of severely impaired dorsal aorta formation. Homozygous knockout embryos had an even more severe phenotype with an almost complete failure of dorsal aortal formation(20). Mice lacking VEGF also had reduced numbers of nucleated red blood cells in the blood islands of the yolk sac, indicating that VEGF plays a role in early hematopoiesis as well as vasculogenesis. The role of VEGF-C is primarily in lymphangiogenesis, with transgenic mice overexpressing the ligand demonstrating hyperplasia of the lymphatic vasculature but no new vessel sprouting(21). Conversely, VEGF-C knockout mice die between E15.5 and E17.5 and exhibit edema from E12.5 with the presence of lymphatic endothelial cell lineages but no lymphatic vessel sprouting(22). However, blood vessels in VEGF-C knockout mice appear normal(22), highlighting the differences in EC signaling pathways regulating blood and lymphatic vascular development. Receptor knockout studies have demonstrated that the VEGF receptors are critical for vasculogenesis, and that other receptors cannot rescue aberrant VEGFR expressions. VEGFR-1 knockout mice die between E8.5 and E9.5 exhibiting a lack of endothelial cell organization into functional vessels, although ECs do form as evidenced by relatively normal dorsal aortas and yolk

sac blood islands. However, the ECs lining other future vessels appear to be greatly thickened and disorganized, with a lack of integrity(23). VEGFR-2 knockout mice die between E8.5 and E9.5 with a complete lack of yolk sac blood islands at E7.5. In addition, there is no presence of any blood vessel growth within the transgenic embryos or production of hematopoetic precursors(24).

The VEGFRs are primarily expressed on the surface of ECs and other hematopoietic stem cell lineages, although other cells, including monocytes and some tumor cells, have been documented to express the receptors. VEGF is alternatively spliced into at least three different active variants in the adult: $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, with the numbers referring to the number of amino acid residues in the mature human proteins (the corresponding murine proteins are all one residue shorter). Alternative splicing results in gradual loss of protein domains with affinity for heparin, thus the three VEGF variants range from entirely circulating ($VEGF_{121}$) to entirely extracellular matrix-bound ($VEGF_{189}$). The native $VEGF_{165}$ protein exists as a heparin-binding glycosylated homodimer of 45 kD that is able to bind and dimerize two receptor molecules to initiate signaling. VEGF binds VEGFR-1 and VEGFR-2 with K_ds of approximately 25 pM and 75-250 pM, respectively(25-26). Ligand binding induces dimerization of both receptors although VEGF-mediated VEGFR-1 phosphorylation is drastically less robust than agonist-induced VEGFR2 activation(27). Due to the high affinity of VEGF for the VEGFR-1 receptor but weak receptor phosphorylation, it has been proposed that the role of VEGFR-1 is to act as a ligand sink and prevent uncontrolled activation of VEGFR-2-mediated signaling pathways(28). This finding is supported by the previously noted results of VEGFR-1 knockout studies as well as by a follow-up study in which knock-in mice expressing a truncated form of VEGFR-1 lacking

the cytoplasmic kinase domain developed normally, demonstrating that VEGFR-1 signaling is not required for normal embryonic vascular development.

Upregulation of VEGF expression occurs as a response to tissue hypoxia through the activation of HIF-1α or as a response to the effects of other cytokines such as EGF, IL-1β, and TGF-β(29-31). Active VEGF receptors recruit PI3K and subsequently activate Akt, leading to increased endothelial cell mitogenesis and increased cellular survival through the expression of antiapoptotic proteins and the sequestration of Bad(32-33). However, in addition to survival and proliferation, activation of VEGF cascades leads to increased EC permeability and leakage, leading to increased inflammation at sites of ligand stimulation(34). Thus, although VEGF signaling is critical for initiation of angiogenesis, other ligand/receptor interactions play greater roles in the maturation and maintenance of the vasculature and the regulation of specific EC phenotypes.

1.3.2 Promotion of angiogenesis by various signaling cascades

A host of other soluble factors can stimulate EC growth and migration either directly or indirectly. Fibroblast growth factors bind to RTK FGF receptors and stimulate EC proliferation, migration and basement membrane degradation through recruitment of Shc and Crk(35). FGF stimulation also induces endothelial-derived matrix metalloproteinase expression which leads to tissue plasminogen activator-independent fibrinolysis and tubulogenesis both *in vitro* and *in vivo*(36-37). Platelet derived growth factor stimulation induces angiogenesis and vascular stabilization by the recruitment of mesenchymal progenitors such as pericytes and SMCs to the newly formed ECs. Insufficient recruitment of mural cells to newly formed capillaries by lack of signaling with PDGF leads to vessel enlargement, edema, impaired perfusion, and consequent

hypoxia(38). TNFα, Il-6, MCP-1, and TGFβ secretions, in low doses, lead to the recruitment of leukocytes that are then activated and secrete growth factors and proteinases both of which lead to endothelial cell activation(39).

Several receptor classes also influence vascular development and integrity. Ephrin (Eph) receptors are critical for the definition of arterial versus venous circulation as well as angiogenesis of endothelial and endocardial cells. Mice lacking the receptors die in mid gestation at E11.5 due to defects in arteriovenous junctions and vessel sprouting(40-41). The integrins, in addition to promoting angiogenesis independently, are able to affect EC adhesion and migration in conjunction with other cell surface receptors. Stimulation of blood ECs with VEGF leads to the direct association of VEGFR2 with αvβ3 integrin; an interaction that elicits the full potentiation of the VEGF induced signal through VEGFR2(42). In parallel, stimulation of lymphatic EC's with VEGFC leads to the association of α5β1 with VEGFR3 potentiating the VEGFC induced signal(43). α5β1 integrins have been shown to associate with other EC surface receptors such as Tie2 and potentiate their cellular responses of vascular maturation and stability(44), processes which will be discussed later.

Although the aforementioned cell signaling pathways contribute greatly to EC proliferation and migration, they do not fully explain the ability of endothelial cells to adopt a mature, quiescent phenotype. Neither do they explain the processes that control the maturation of newly formed vessels from a phenotype of leaky proliferating cells to one comprised of the tight junctions. However, the discovery of Tie1 and Tie2 and their respective expression profiles, signaling partners, and regulation have elucidated novel mechanisms of endothelial maturation and survival.

1.4 Tie receptor discovery

Tie1 is a receptor tyrosine kinase that was first discovered by PCR cloning from an endothelial cell cDNA library based on sequence homology with other RTKs. The identification of several putative domains led to the naming of the molecule, Tyrosine kinase with Immunoglobulin-like domains and Epidermal growth factor repeats. The human tie1 gene is located on chromosome 1 at the locus 1p33-1p34 and encodes a single transmembrane spanning protein with an extracellular amino terminal sequence and an intracellular putative kinase domain. The Tie protein contains an approximately 15 amino acid residue signal sequence that directs it to the plasma membrane followed by two N-terminal Ig domains. The initial N-terminal Ig domains bear a loose but significant homology to the N-CAM Ig domain, indicating a potential function of association with immune molecules. After the two initial Ig domains, residues 214-344 comprise 3 Epidermal Growth Factor-like repeats, which resemble one another more closely than any other EGF repeats, although they have the greatest similarity to the laminin EGF domain. Distal to the EGF repeats, approximately 70 amino acid residues comprise another putative Ig domain followed by 3 Fibronectin Type III (FNIII) domains. Residues 761-786 comprise a region of hydrophobic residues that correspond to the transmembrane region of the protein followed by a 50 amino acid juxtamembrane (JM) region that may have regulatory roles. After the JM region, an intracellular bilobar kinase domain is present with the initial domain starting at residue 837 and containing a 14 residue kinase insert sequence between the two lobes of the kinase domain. The kinase domain bears the greatest homology to cKit, FGFR, and PDGFR. The C-terminal 31 residues bear no homology to other RTKs and may also have roles in regulation of receptor signaling(45-46).

Tie1, if isolated from cells, migrates at a molecular weight 10 kD larger than would be expected, indicating that the mature protein is heavily glycosylated at numerous putative N-glycosylation sites. Furthermore, Tie1 was found to be weakly phosphorylated when isolated from 3T3 cells, indicating that the kinase domain of Tie1 can be functional(45). Upon initial observations, *Tie1* is present beginning around E9.5 in developing mouse embryos and is ubiquitously expressed in both large and small vessels including the endocardium and the dorsal aorta. However, Tie1 expression is not noted in hepatic sinusoids(45-47).

Tie2 (the kinase initially called Tek) was first isolated as a cDNA sequence containing the C-terminal catalytic fragment of a kinase that bore a 42% sequence homology to the kinase domain of FGF. The Tie2 gene is located on mouse chromosome 4 between the *brown* and *pmv* loci within the mouse genome. Due to the expression of the transcript within the embryonic mouse heart at the location of the endocardium as well as the internal layer of most blood vessels at E12.5, the original name for the kinase was Tunica intima Endothelial cell Kinase, or TEK(48). By *in situ* hybridization, *tie2* expression was detected as early as E7.5-E8.5 in the yolk sac blood islands of the developing mouse embryo. Upon maturation, *tie2* expression is detectable in all of the major vessels, the endocardium, as well as arteries and veins, albeit at lower levels than in the developing embryo(48).

The full length Tie2 mRNA consists of a 4.2 kD transcript that encodes a single transmembrane spanning 1122 amino acid polypeptide. Similar to Tie1, the initial 18 residues constitute a signal peptide sequence followed by two approximately 70 residue Ig-like domains that have the most sequence homology to the C2 type of Ig domain. Residues 211-340 correspond to three EGF repeats that are most similar in sequence to

the laminin B1 and B2 motifs. The EGF repeats are followed by another Ig domain and further 3' along the receptor, residues 440-773 contain three FNIII repeats. Residues 746-772 comprise a region of hydrophobic residues corresponding to the transmembrane spanning region of the receptor. After a juxtamembrane region perhaps involved in kinase regulation, the bilobar kinase domain is initiated at residue 829 with a 21 amino acid interruption at residue 913. As with Tie1, the final C-terminal 32 residues of Tie2 do not have homologies with other RTK kinase domains. Mature Tek is N-glycosylated at 9 sites during processing. Due to the post translational modifications, the mature receptor migrates at 140 kDa, 15 kDa larger than the initially translated receptor. The mature protein, as with Tie1, is observed to be phosphorylated on tyrosine residues indicating active receptor function(47-50). The mature protein is found to be abundantly expressed in placenta and lungs in the highest levels, followed by the kidney and heart, although all vessels express the receptor(50).

When compared to one another, it was determined that Tie1 and Tek are in fact separate receptors belonging to a novel family of receptor tyrosine kinases and not orthologs of one another. Since the two receptors are rather similar to one another based on sequence homologies, the receptor Tek was renamed Tie2 to reflect the common family to which the receptors belong. The overall sequence similarity between the two receptors is around 70% with various regions having even greater similarities. Extracellularly, the two receptors share 50% sequence identity between the EGF repeats and 40% identity within the first FNIII domain, although the second and third FNIII domains have much less identity. The Ig domains also are highly homologous. There is 40% sequence identity throughout the transmembrane region and the kinase domains are even more similar, with an 80% sequence identity between the entire cytoplasmic

domains of Tie1 and Tie2(47). Based on sequence similarities and initial expression profiles, it was predicted that the two receptors would share similar functions within endothelial cells.

1.5 Tie proteins in development

1.5.1 Tie expression in development

Understanding the expression profiles of *Vegfr-2, Tie1, and Tie2* during murine development has shed light on the functions of the receptors within the context of vessel growth and maturation. *Flk-1* (VEGFR-2) is initially expressed at E7.0 of mouse development in the extraembryonic mesoderm which will become the embryonic yolk sac as well as the cardiogenic mesoderm that will form into the heart folds. In contrast, *Tie2* expression begins at E7.5 in the extraembryonic mesoderm of the developing yolk sac and *Tie1* expression does not commence until E8.0 at the extreme end of the yolk sac extraembryonic mesoderm. By E7.5, the expression profiles of *Flk-1* and *Tie2* are similar within the developing extraembryonic mesodermal derivatives, although *Tie2* expression is much more pronounced in the mesodermal layer of the amnion. By E8.5, the expression profiles of *Flk-1, Tie1* and *Tie2* are all highly homologous in the embryo proper at the sites of vascular and endocardial development including the dorsal aortas, endocardium, and intersegmental vessels(51). Although ubiquitously expressed in the vasculature, the expression of the receptors is localized only to the developing endothelium. *Tie2* by E8.5 is enriched in the brain capillary fraction of developing mouse embryos while no *tie2* mRNA is detectable in the brain tissues. Furthermore, *Tie2* expression corresponds with PECAM expression in both the invading brain capillaries, maturing endothelial tissues, and yolk sac endothelium further demonstrating the endothelial cell lineage specificity of tie family expression(52). At E12.5, expression of

Flk-1, Tie1, and *Tie2* are vastly downregulated within the extraembryonic yolk sac endothelium. However, the mRNA of all three is uniformly expressed around the embryonic vessels and vascular structures including the small vessels that permeate the organs such as the kidneys and lungs(51, 53).

Analysis of the *Tie1* and *Tie2* promoters also helps cement the potential functions of the tie receptors during development. The promoter of *Tie1*, when attached to a LacZ reporter, demonstrates expression of the reporter construct beginning at E8.0 at regions of the dorsal aorta and E8.5 of the developing endocardium and yolk sac blood islands. Promoter expression is evident at E9.5 in the carotid arteries and the intersomitic arteries of the developing mouse embryo. As seen in conjunction with *Tie1* mRNA, *Tie1* promoter activity is greatly reduced in the yolk sac by E12.5. *Tie1* promoter activity is ubiquitous in the developing vasculature including the lungs, kidneys, and hepatic arteries by E11.5 and continuing through until birth, at which point activity drops in the larger hepatic vessels. However, *Tie1* promoter activity is notably absent in hepatic sinusoidal capillaries at later developmental time points. This indicates that hepatic sinusoidal endothelial cells may develop by utilization of a distinct mechanism or that the *Tie1* mRNA that is expressed earlier in development is stabilized to allow for continued *Tie1* translation and processing(53). *Tie2* promoter activity also follows similar patterns as *Tie1* and *Tie2* mRNA expression in the developing embryo. By E10.5, *Tie2* promoter-induced LacZ expression is present in the developing vasculature as well as the hepatic sinusoids, but by E12.5, promoter activity is more restricted to the developing vasculature(54). Postnally, the Tie2 promoter also shows strong expression, indicating that Tie2 functions are necessary for vascular maintenance(55). Due to the exclusive localization of *Tie1* and *Tie2* expression, both tie promoters have been used to

drive endothelial cell-specific expression of target genes. Taken together, the Tie family of receptors comprises a group of proteins expressed at most stages of endothelial cell development and maintenance.

1.5.2 Tie2 requirement in development

The requirement for the Tie receptors in development has been demonstrated by observation of the phenotypes of organisms lacking one or both of the receptors. Embryos with deficient Tie2 receptors exhibit defects in the vasculature as well as within endocardial development. Homozygous mice with a dominant negative form of Tie2 (Lys853Ala) die *in utero* around E9.5 with gross deformities observed at E8.5. At E8.5, the yolk sacs of the embryos exhibit a cobblestone-like morphology with reduced numbers of endothelial cells lining the blood islands and consequent hemorrhage. At E9.5, the endocardium is thinner and functionally, the hearts of the embryos are unable to beat. In addition, hemorrhaging of the major vessels is noted at E9.5 as is collection of blood within the yolk sac blood islands due to a lack of the developing vasculature needed to distribute the formed blood(56). Tie2 knockout embryos die at E10.5 with indistinguishable large and small vessels, massive dilation of all vessels present , and hemorrhage around the malformed vasculature(57). In both instances (Tie2 dominant negative embryos and Tie2 knockouts), initial endothelial cell development occurs, indicating that vasculogenesis is not impaired, but there is a complete lack of functional angiogenesis. Tie2 expression under the control of a tetracycline promoter (Tet) permits observation of the receptor in later stages of development, when vascular maintenance is required. Tet-promoted Tie2 knockouts survive longer than E9.5 but exhibit massive hemorrhaging at E13.5 and an underdeveloped liver. By E14.5, the embryos demonstrate necrosis of the vasculature. However, the defects are not due to a failure

to grow since Tet-promoted knockouts are the same total body mass as day-matched wild type controls. Demonstrated by the knockouts, the absence of functional Tie2, once vessels initially form, leads to apoptotic destruction of the vasculature and consequent hemorrhaging. Interestingly, the heart appears normal, indicating that the effect of Tie2 on the endocardium is only necessary during the initial adherence to the myocardium and is dispensable afterward(58).

Much like VEGFR-2, the activity of Tie2 must be tightly regulated during development and maturation. Consequently, overexpression of active Tie2 also leads to malfunctioning vasculature. VE-PTP, a phosphotyrosine phosphatase found associated to Tie2 but not VEGFR-2, has been shown to be expressed at E9.5 in the dorsal aortas with expression spreading throughout most of the functional vasculature by E15.5. VE-PTP expression is highest along the endothelium throughout the smooth muscle cell lined vessels but noticeably absent within the microvasculature of the developing embryo. Co-immunopreciptation of Tie2 with VE-PTP demonstrates reduced phospho-Tie2 when associated with VE-PTP. Phenotypically, VE-PTP mutants have greater endothelial cell density throughout both large and small vessels, although the difference is much greater within the smooth muscle cell lined elastic arteries(59-60). A familial mutation leading to an overactive Tie2 (Arg to Trp mutation at 849) manifests as venous malformations throughout the entire vasculature with a uniform distribution of actin throughout the vessels. This may indicate that the overactive Tie2 prevents proper endothelial cell polarity leading to malformation of the vessel walls. Since the R849W mutant exhibits greater autophosphorylation which indicates greater activity, the aberrant phenotype is most likely due to aberrant signaling through activated Tie2 and not another cascade(61). Overall, Tie2 is dispensable in vasculogenesis but is required and

must be tightly regulated during subsequent angiogenic vessel sprouting and endothelial maintenance.

1.5.3 Tie1 requirement in development

Expression of Tie1 is also required for proper vascular development, but its role is distinct from the role of Tie2. Puri *et al* observed that *Tie1* is initially expressed in the yolk sac blood islands at E8.5 with ubiquitous expression in the vasculature by E10.5. At E13.5, embryos lacking functional Tie1 exhibited diffuse hemorrhaging. The major vessels of the mouse embryos appeared normal but there was loss of vessel integrity at the smaller capillary beds. The knockouts died at E14.5 due to diffuse hemorrhaging and a lack of vascular integrity observed by abdominal and pulmonary edema(62). Sato et al observed slightly different results in Tie1 knockout mice, mostly due to differences in the time of death and not in the phenotypic presentation. They observed that Tie1 knockout mice died immediately after birth due to lack of vascular integrity but were still viable at E14.5. At E13.5, diffuse edema of the neck of the embryos was noted. During development as well as in the immediate postnatal period, the embryos had smaller hearts and had massive hemorrhaging at the distal appendages as well as the internal organs. The deformities in the various compartments were ascribed to a lack of vessel integrity and endothelial cell death(57). Although Tie1 seems to be involved in vessel maintenance and the later stages of angiogenesis in development, it doesn't seem to be necessary for vasculogenesis. Embryos containing both Tie1-wild type and Tie1-deficient cells appear normal at E10.5 with Tie1 knockout cells contributing equally to the various vascular beds. By E15.5, Tie1 knockout cells persist in the dorsal aortic regions but are reduced in the capillary plexuses and are completely absent in the mature capillary beds. Taken together, the reduction in Tie1 knockout populations indicates a

role of the receptor in the later stages of sprouting angiogenesis. However, Tie1-knockout cells are still present in the megakaryocytes in late development indicating that not all hematopoetic cell derived lineages require the presence of the protein for proper function(63).

Recently, the necessity of Tie1 in the development of lymphatic vasculature has been demonstrated. Tie1 knockout embryos exhibit abnormally patterned lymph sacs at E13.0 without the development of mature lymphatic vessels and consequently, display generalized edema. The pulmonary leakage noted in the initial Tie1 knockout characterizations can now partially be attributed to the failure of the development of functional lymphatic vessels in the lungs (64). A hypomorphic floxed Tie1 embryo that exhibits a less severe phenotype (no hemorrhaging and lessened generalized edema) has elucidated several of the details with regard to the role of Tie1 in lymphatic development. Co-staining of Tie1 with lymphatic markers indicates that Tie1 is expressed on lymphatic precursors as well as mature lymphatic vasculature throughout the later stages of development, from E10.5 to E17.5. At E12.5, hypomorphic mutants demonstrate dilated lymph sacs and increased proliferation but subsequent apoptosis of endothelial cells within the lymph sacs. Some lymphatic vessels such as the diaphragmatic lymphatics completely regress from E16.5-17.5 while the lymphatic vasculature that remains is unable to transport fluid. The nonfunctional lymph leads to edema accumulation at sites of fluid introduction measured by FITC-dextran diffusion(65). Taken together, Tie1 expression must be tightly regulated in the development of lymphatic vasculature with reductions in levels of expression correlating with severity of phenotype.

Tie1/Tie2 dual knockout embryos exhibit an even more severe phenotype than either knockout individually. Dual knockout embryos die at E9.5 with a complete lack of yolk sac vasculature, complete lack of vessel segmentation, and a lack of vascular branching, although the initial dorsal aortas do form. The endocardial tissue in both the atria and ventricles is also absent(66). These results indicate that although Tie1 knockouts have a relatively normal phenotype at an initial stage of angiogenesis, Tie1 contributes to the process. Tie1/Tie2 double knockout hematopoetic stem cell lineages are present in all tissues in equivalent proportions to wild type cells at E13.5 with equal numbers of megakaryocytes. Cells lacking the receptors contribute equally to vascularizing the dorsal aorta as well as the ventral endothelium further demonstrating the dispensability of Tie for vasculogenesis(67-69). However, dual knockout hematopoetic stem cell lineages become underrepresented in the developing bone marrow and consequently the spleen, thymus, and peripheral blood in postnatal development. Still, cells deficient in the receptors are able to differentiate into mature lymphoid cells indicating a competitive disadvantage but not an inability to develop(69). Mouse chimeras containing Tie1/Tie2 dual knockout cells become grossly underrepresented in the vasculature at the later stages of embryogenesis indicating a selective pressure against knockout cells. Even dual heterozygous cells are absent in mature vasculature, although some heterozygous cells are present in a few hepatic sinusoids(66).

1.5.4 Angiopoietin associations to Tie2 in development

Demonstration of the importance of the Tie family of receptors in endothelial angiogenesis and maintenance led to the identification of ligands that induce receptor activation. Conditioned media from both SHEP-1 and C2C12 cells contain a factor,

Angiopoietin 1 (Ang1), that binds to Tie2 extracellular domains presented on BIACore chips and can be competed off by addition of soluble extracellular Tie2 domains(70-71). *In vivo*, embryonic *Ang1* expression localizes initially to the endocardium and later, spreads throughout the entire vasculature(70). The presence of Ang1 is required for embryonic development, since Ang1 knockout mice die at E12.5 from a phenotype that is reminiscent of Tie2 knockout mice. Ang1 deficient embryos have growth retardation of the developing heart and a lack of functional vasculature due to the presence of primitive vessels with no branching or intricate vascular networks. Furthermore, the developed endothelial cells of the embryos are much more rounded and unable to properly lay on the basement membrane. Inefficient layering prevents the formation of an effective barrier or recruitment of collagen fibers for the development of a mature interface(72).

When Tie1/Ang1 double transgenic embryos are observed, the phenotype is more drastic than either single knockout. Ang1 is expressed in large amounts at the sinus venouses, where both Tie1 and Tie2 are also expressed in the developing embryo. Double knockout mice, at E9.5, exhibit a normal left hand sinus but a lack of the right hand sinus coupled with a complete lack of anterior and posterior cardinal veins from the right hand sinus. In contrast, the cardinal veins on the right side are present in either of the single knockouts or wild type embryos at this stage of development. The dual knockout embryos exhibit normal development of the sinus venouses through E8.5 when Ang1 expression is uniform between the two sides of the neonate. By E9.0 however, the dual knockout embryo cardinal vein lumens of the right hand sinus are collapsed and disjointed leading to apoptosis of the vasculature(73). These results highlight the complex interdependence of the Tie receptors and their potential activating ligands.

Ang1 overexpression, perhaps predictably, leads to the opposite effect of knockouts in maturing systems. Transgenic mice induced to overexpress Ang1 around the skin have increased numbers of large vessels within the dermal tissues. In addition to the large vessels, the mice also have increased numbers of venules, capillaries and a more developed, intricate branched network of their capillary vasculature. The vessels' sizes are uniformly increased and electron microscope analysis reveals that the newly formed vasculature is not permeable, simply thicker(74). When compared to Ang1 overexpression in the skin, VEGF upregulation leads to greater numbers of tortuous, leaky vessels. VEGF/Ang1 dual overexpressing mice exhibit more vessel growth than either alone indicating that the EC responses to the two ligands may be additive. In addition, upregulation of both Ang1 and VEGF leads to the mature, non-leaky EC phenotype of wild type animals indicating that Ang1 can compensate for the cell permeability induced by VEGF. The vessels formed are also more resistant to inflammation-induced permeability than either the wild type or VEGF single overexpressing transgenic mice(75). However, increased Ang1 monomer expression in cardiac hypertrophy leads to cardiac monocyte recruitment by activation of integrins(76). Comparison of the last two studies indicates that the Tie receptors are not necessarily the only targets of Ang1 activation and that there exists a complex interplay between inflammatory stimuli and endothelial signaling. Adenovirus expressing Ang1 around the heart in diabetic mouse models leads to upregulated Akt, eNOS, and proangiogenic factors such as VEGF through the stabilization of HIF-1α. Due to the increases in vessel growth signals, Ad-Ang1 stimulates increased microvessel growth around atherosclerotic infarcts. Furthermore, Ang1 promotes recruitment of SMCs, decreases interstitial

fibrosis, and reduces overall cardiac hypertrophy(77). Overall, these results indicate that Ang1 is a powerful EC maturation and potential growth signal.

Although Ang1 was the first ligand identified for the Tie receptors, other Angiopoietins have been isolated with effects on the Tie signaling axis. Angiopoetin-2 (Ang2) binds to Tie2Fc domains as observed by BIACore but, unlike Ang1, initially did not induce Tie2 phosphorylation. Interestingly, Ang2 is unable to associate with the highly homologous Tie1 extracellular domains as measured by BIACore. With regards to Tie2 signaling, the differing expression profiles of Ang1 and Ang2 may explain some of the functional distinctions between the ligands. While Ang1 is present in the developing heart and the dorsal aortas, Ang2 is only present in the dorsal aortas. Furthermore, Ang1 is expressed after VEGF stimulation within angiogenic, sprouting vessels to promote vessel maturation while Ang2 seems to be expressed with VEGF at the forefront of vessel invasion, at sites of immature, dividing endothelium. Consistent with the observation that Ang2 is able to bind but not induce Tie2 phosphorylation, overexpression of Ang2 within developing mouse embryos leads to a phenotype highly homologous to either the Tie2 knockout or Ang1 knockout mouse(71).

Although Ang2 knockouts are not embryonic lethal, lack of Ang2 expression leads to certain specific phenotypic abnormalities in the maturing mouse. Ang2 knockout transgenics exhibit defects in their lymphatic vasculature, and as a result, exhibit both chylous ascities and pedal edema. The mice have a deficiency in the collecting ducts within the lymphatics and a defect in the maturation of the lymphatics observed in dermal tissues, although the physical positioning of the lymphatics is normal. The lymphatic vessels appear lacy, leaky, with a general dilated appearance, and a lack of fluid motion throughout the vasculature. The chylous ascities that were

previously mentioned are due to the poor uptake of chyle by the lymphatics after feeding of the mice(78-80). In addition to the abnormal lymphatic vasculature, Ang2 knockout transgenic mice have malformed retinas. During wild type retinal maturation, hyaloid vasculature from terminal branches of retinal artery that was developed during embryogenesis regresses after birth. Concurrent to the end of hyaloid regression, new vessels sprout laterally from central retinal artery to vascularize the superficial layer on the retinal surface. Eventually, the growing network of new arteries vascularizes the superficial surface of the retina. VEGF plays a critical role in this process, with expression levels being very high initially in the avascular retina to promote retinal artery endothelial sprouting into the avascular regions and then regressing once the retina is vascularized. The hyaloid vasculature is retained in Ang2 knockout mice which then leads to the lack of synthesis of the necessary collaterals that would penetrate and vascularize the retina. The remnant hyaloid vasculature is unable to provide the necessary blood to the inner surfaces of the retina, and the mice become blind as a result of degenerating retinas(78). Interestingly, Ang1 substitution for Ang2 in the Ang2 transgenic knockout mice rescues normal lymphatic phenotypes(78-79) but is unable to rescue the defect in retinal development(78). These experiments highlight two distinct roles of Ang2 in maturing vasculature. First, although Ang2 was initially shown to bind to but not induce Tie2 phosphorylation, there are conditions where Ang2 acts as an agonist (e.g. Ang1's agonistic effects are able to rescue normal lymphatic phenotypes in Ang2 deficient mice) along the Tie signaling axis. Second, Ang2's role in development is more complicated than as a simple agonist and may be present in part to curb the pro-maturation phenotype that would exist if only Ang1 was allowed to signal. (e.g. hyaloid regression does not occur even in Ang1-rescued Ang2 deficient mice)

1.6 Angiopoietin-2 in pathological states

Ang2 is expressed following perturbations in endothelial integrity, but its precise role in mediating recovery is still under debate. According to one study by Reiss et al, endothelial overexpression of Ang2 post-femoral artery occlusion in mice leads to reduced perfusion of the ischemic tissue and increased time of oxygen saturation of the tissues. Ang2 levels in the endothelium of the occluded limbs correlate inversely with levels of phospho-Tie2 and overexpression of Ang2 reduces collateral artery sizes. Furthermore, Ang2 expression leads to decreased recruitment of SMCs to new vessels leading to lower numbers of functional vessels as well as increased destruction of perivascular cells. A combination of both of these effects leads to a reduced thickness of the forming collateral arteries. In addition, Ang2 expression leads to increased inflammation of the occluded limbs and consequently, increased levels of necrosis of various cell types(81). However, according to Tressel et al, the inhibition of Ang2 expression after femoral artery ligation leads to impaired arteriogenesis. Ang2, as previously stated, is upregulated after ligation, but serves as a pro-healing ligand. Inhibition of Ang2 impairs recruitment of immune modulators to the wound site and reduces the amount of infiltration around the site of ligation due to decreased levels of the monocyte adhesion molecules ICAM and VCAM. Furthermore, inhibition of Ang2 prevents adequate smooth muscle coverage of the newly formed vasculature. Finally, prevention of Ang2 expression inhibits production of circulating CD11b-positive cells whose presence would further promote wound healing(82). Both studies highlight the recruitment of immune cells to sites of damage, but point to conflicting roles for Ang2 in mediating or prolonging recovery. Within another pathologic state, namely, atherosclerotic plaques, Ang2 plays a protective role but seems to have an opposite

effect on immune cell recruitment. According to Ahmed et al, Ang2 overexpression by adenovirus infection of ApoE knockout mice leads to less atherosclerosis with fewer monocytes contained within the plaques. The protective effect is mediated by way of Tie2-dependent activation of nitric oxide synthase and subsequent NO release. Ang2 expression reduces levels of oxidized LDL within the intima of the vessels and therefore prevents cytokine release and monocyte extravasation into sites of plaque formation(83). Clearly, due to the conflicting results, the intricacies of Ang2 signaling still need to be elucidated.

Expression levels of Ang2, in addition to sites of vascular injury, are increased in a host of solid tumors and the tumor vasculature. Increased translation of Ang2 in Tie2 null breast cancer cell lines correlates with increased metastatic potential of the tumor lines. Ang2 overexpression stimulates the increased activation of both tumor Akt and GSKβ leading to increased cell survival as well as loss of E-cadherin from the cellular surface increasing the propensity for tumor migration. Interestingly, these effects seem to be mediated through an αvβ1 integrin pathway, further highlighting the importance between integrin and RTK signaling(84). Development of an aptamer with a 200 fold specificity for Ang2 compared to Ang1 has led to the reduction of tumor size in mouse tumor flank models coupled with a decrease in the perfusion of the tumors treated with the anti-Ang2 aptamer(85-86). The aptamer prevents Tie2 phosphorylation *in vitro*, and, in addition to the anti-tumor effects, prevents angiogenesis in a rat corneal model even in the presence of EC growth factor bFGF(85). In colorectal cancers, stimulation of tumors with Ang2 inhibitors prevents tumor expansion due to lack of perfusion within the tumor bed but does not lead to tumor regression. Although prolonged anti-Ang2 therapies do not seem to confer resistance, stopping inhibitor treatments causes

renewed tumor growth. Therefore, in certain cancers, Ang2 presence induces greater perfusion due to effects on the endothelium(87). However, this is not a universal result. In mice with hepatocellular carcinomas, adenovirus-induced systemic overexpression of Ang2 destabilizes the tumor vasculature. Capillary dilatation occurs due to the loss of interactions between the pericytes along the basement membrane and the endothelial cells that adopt a more rounded phenotype. Transiently, overexpression of and stimulation by Ang2 leads to hypoxia, but sustained, chronic expression increases the overall perfusion to the tumor beds and eventually increases apoptosis of the hepatocellular carcinomas(88). Clearly, more research must be done to elucidate the role of Ang2 within the development and progression of cancer.

1.7 Angiopoietin-2 expression and release

The regulation of Ang2 expression and subsequent release by EC's may explain some of the differences that have been observed in the different physiologic states. Both cell growth as well as immune stimuli can induce Ang2 expression and secretion. *In vitro*, VEGF stimulation of bovine retinal and bovine microvascular arterial ECs increases *Ang2* mRNA transcript levels by increasing the rate of transcription with no effect on either *Ang2* mRNA stability or expression of Ang1 or Tie2. Furthermore, tyrsosine kinase inhibitors, a protein kinase C inhibitors, and MAPK inhibitors to the VEGF/VEGFR2 signaling axis prevent the VEGF-induced upregulation of transcription of *Ang2*. Interestingly, hypoxia also induces similar *Ang2* mRNA increases although this upregulation is independent of the VEGF axis. A VEGF neutralizing Ab does not inhibit the hypoxia-induced stimulation of transcription of *Ang2*. *In vivo*, mouse models of ischemia induce retinal neovascularization with an increase in the expression levels of *Ang2* mRNA within the angiogenic endothelium of the eye(89). The cytokine TNFα

induces Ang2 but not Ang1 transcription and subsequent protein translation in HUVECs. Inhibition of downstream targets of TNFα such as NF-κB reduces the cytokine induced Ang2 translation(90).

In addition to ligands, levels of shear stress also influence Ang2 synthesis. In HUVECs, sustained low shear stress (1 dyne/cm) leads to increases in mRNA transcription, protein translation, and subsequent secretion of Ang2. Conversely, sustained high shear stress (30 dynes/cm) stimulates the downregulation of mRNA transcripts and subsequently reduces protein levels and secretion of Ang2 after 24 hrs(91). Mechanistically, increases in shear stress lead to increased levels of pSer473 Akt and pThr172 AMPK in ECs with elevated levels of phosphorylated signaling molecules present as long as the shear stress is applied. Reductions in shear stress do not increase protein levels of Foxo1 but stabilize the transcription factor within the nuclear compartment of cells and thereby increase the binding of Foxo1 to its promoter sequences. Since the promoter sequence of Ang2 contains Foxo1 binding sites, low shear stress induces Ang2 transcription(91-92). Consequently, the lower levels of shear stress found in venous circulation lead to increased Ang2 production in veins compared to healthy arteries(91). Dominant negative AMPK introduction into ECs increases cell permeability due both to increases in Ang2 as well as cytokine production, signifying that low shear stress is an indication for both Ang2 production as well as monocyte attraction(92). It is important to note that only laminar stress reduces Ang2 production. HUVECs and capillary ECs exposed to oscillatory stresses increase Ang2 production, secretion, and consequent tubule formation and migration while those exposed laminar stresses do not upregulate or secrete Ang2. This effect is further demonstrated in the aortic arch where expression levels of Ang2 along the endothelium of the major curve

are significantly reduced compared to levels along the endothelium of the minor curve, due not to differences in overall stress but due to laminar versus disturbed flow(93).

Contrary to Ang1 which is stored in the basement membrane and activates endothelial cells through their basolateral surface, Ang2 is secreted by EC's into vessel lumens and is able to signal through the apical surface by autocrine or paracrine methods. Ang2, once glycosylated, is stored in Weibel Palade Bodies (WPB) exhibiting a granular pattern of expression in unstimulated EC's. Cytokines such as TNFα stimulate sphingosine-1-phosphate mediated activation of Phospho-Lipase C (PLC) and subsequent calcium release into the cytoplasm of activated endothelium. The increased calcium binds to calmodulin and releases stored granules of Ang2 by way of exocytosis in both lymphatic and blood vasculature(94). Maximal Ang2 release occurs 20 minutes after stimulation and continues as long as the stimulus to secrete ligand is present. Upon removal of the stimulus, complete repletion of the WPB stores of Ang2 is completed within 6 hours(95). This mechanism allows for the rapid induction of Ang2-mediated signaling cascades in manners quicker than would occur had translation of new protein had have to be initiated.

1.8 Angiopoietin structure

Due to the requirement for RTK's such as the Tie receptors to multimerize in order to establish signaling, the structure of the Angs and their multimerization are critical for activation. Ang1 is a 497 amino acid soluble polypeptide containing superclustering, coiled coil oligomerization, and fibrinogen-like binding domains with a monomeric molecular weight of ~70 kD due to post translational glycosylation. The first 50 aa's of Ang1 share no similarity with other molecules while residues 100-280 are similar to myosin and contain multiple Cys residues and sequences that can promote

oligomerization through coiled-coil domains(70, 96). Residues 280-497 form a domain similar to fibrinogen and are responsible for binding to the Tie receptors with an IC50 of 1.5 nM(70, 96). N terminal truncation mutants of Ang1 are unable to bind or activate the Tie receptor nearly as well as full length proteins with greater truncations leading to less efficient binding. A truncation mutant of Ang1 lacking the N-terminal 50 residues bound Tie2 with an IC50 of 94 nM while a truncation mutant containing only the necessary C-terminal fibrinogen like domain associated to Tie2 with an IC50 of 435 nM(96). Furthermore, the truncation mutants of Ang1 that are unable to form native-like quatrenary structures but instead form dimers and trimers are unable to activate and consequently phosphorylate Tie2(96-97).

According to stoichiometric calculations, the Angs bind to the Tie receptors in a 1:1 ratio through their fibrinogen-like domains(96, 98). Ang1 and Ang2 share 60% sequence homology and bind to Tie2 with very similar affinities (1.5-2.5 nM IC50's). The Angs form mushroom-like structures in solution, with the fibrinogen-like domains constituting the caps and the various N-terminal clustering domains forming the stalk in a coiled coil motif. In solution, both Ang1 and Ang2 form trimers, quatramers, and pentamers with a few larger order multimers. Although not proven, the proportion of higher order Ang1 multimers may be higher than higher order Ang2 multimers(97). Various Cys residues along the Ang structures are responsible for maintenance of the quatranary structure of the ligands. Cys 265 of Ang1 is engaged in stabilizing the coiled coil dimer while Cys 41 and Cys 54 form intermolecular disulfide bridges leading to tetrameric or larger multimeric structures. The Cys 265 residue is absent in Ang2 which initially was thought to be part of the reason for the differential signaling profiles of the two ligands. However, mutation of the equivalent Ser in Ang2 to a Cys does not induce

larger multimer formation or increased activity. Furthermore, mutation of Cys 265 to Ser in Ang1 does not lead to lower proportions of higher order multimers but does reduce Ang1 activity. Most likely, Cys 265 is involved in stabilization of the Ang1 molecule and not a critical residue for the Angs to adopt a proper multimeric status for activation of the Tie receptors(97). Taken together, due to similarities between Ang structures, the differences in activity of the two ligands cannot be attributable to theoretical quatrenary structure differences(96). The Ang2 fibrinogen-like domain consists of an alpha helix layered on 4 beta sheets with a calcium binding site very similar to gamma fibrinogen. Mutation of residues that are conserved within Angs and fibrinogens prevents Tie2 binding while mutation of residues only conserved within the Ang family but not conserved throughout fibrinogen retains the binding affinities of the ligands for the receptor(98) (Figure 3).

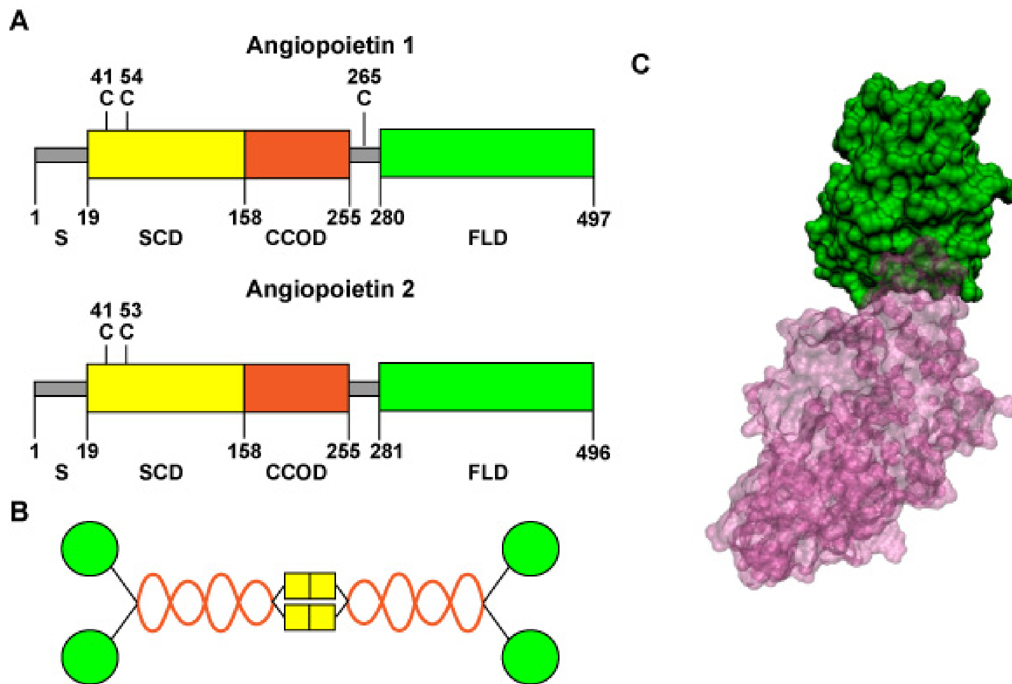

Figure 3: Structure and binding domains of Angiopoietins

(A) A schematic box representation of Ang1 and Ang2 including pertinent domains and residues. The sequences begin with a short signal sequence (labeled S) followed by a superclustering domain (colored yellow, labeled SCD), a coiled-coil oligomerization domain (colored orange, labeled CCOD), a linker, and end with the fibrinogen-like Tie binding domain (colored green, labeled FLD). The two Cys contained within the superclustering domain are believed to promote higher order multimerization of the ligands. Cys 265 of Ang1, absent in Ang2, promotes the stabilization of Ang1, but is not involved in mulimerization. (B) A cartoon representation of the association by four monomers of Ang to form the tetramer that can act as an agonist to the Tie receptors. The domain colors correspond to the scheme used in A. (C) A three dimensional, surface representation of the binding surface between Ang2 and Tie2. Residues 281-495 of Ang2 corresponding to the fibrinogen like domain and residues 23-445 of Tie2 corresponding to the Ig domains and EGF repeats were used to form the crystal structure. Ang2 is represented in green while Tie2 is represented in magenta. Ang1 is believed to bind to Tie2 in an analogous fashion.

1.9 Tie receptor structure

Analysis of the structure of the Tie receptors has begun to explain both the mechanisms of ligand activation as well as the initiation of transmission of downstream signal cascades. Due to the ability of the Angs to bind and activate Tie2, most of the analysis has been performed on Tie2, with comparisons to Tie1 structure based on sequence similarities. Human Tie1 is an 1138 amino acid single transmembrane-spanning RTK with a mouse homolog of 1134 aa's. In comparison, human Tie2 is comprised of 1134 aas with a mouse homolog of 1122 aa. The extracellular domain of Tie2 starting with the N terminal signal sequence contains 2 Ig domains followed by 3 EGF-like repeats, another Ig domain, and 3 fibronectin III-like domains, with the first two Ig loops being sufficient to bind Ang(99). A soluble receptor comprised of residues 1-360, corresponding to the first two Ig domains and the 3 EGF repeats, is necessary and sufficient to prevent binding of Ang to the full length receptor. Smaller truncation mutants comprising only the first two Ig domains are not able to competitively inhibit Ang binding indicating that the EGF repeats are involved in the stabilization of the three dimensional structure of the binding site(100). Three dimensionally, the extracellular domain structure looks like an arrowhead with FNIII domains comprising the shaft and the Ig domains coupled with the EGF repeats forming the arrowhead structure. The third Ig domain occupies the region at the base of the arrowhead and comprises the attachment point between the head and the stalk. The second Ig domain is at the tip of the arrowhead with the surface loop of the domain being the most exposed and is the region where Ang binding occurs(99, 101). Although Tie1's crystal structure has not been determined, sequence homologies predict a very similar structure(99). Within Tie2, 14 disulfide binds stabilize the arrowhead structure and another 20% of the

residues participate in either H-bonding or van der Waals interactions all contributing to a very rigid extracellular domain structure. Interestingly, based on crystal structures of the extracellular domain of Tie2 bound to Ang2, there are no major ligand-induced conformational changes to extracellular Tie2. The binding surface of the receptor with the Angs is dominated by hydrophobic residue interactions. For example, the phenyl ring of Phe 161 of Tie2 stacks with the phenyl ring of Phe 469 of Ang2 and interacts with the backbone of Asn 467 of Ang2 although there are several hydrophilic interactions that also contribute (e.g. Ser 164 of Tie2 is involved in hydrogen bonding with Ser 480 of Ang2). Although they have not been co-crystalized, Ang1 is predicted to bind in an identical manner to Ang2 with 6 out of 13 putative contact residues being completely conserved and another 2 residues having conservative mutations between the two ligands. Furthermore, mutation of Tie2 residues along the interface that abrogate binding to Ang2 also prevent Ang1 binding to the receptor(101). The similarities between Ang1 and Ang2 multimerization states as well as their similarities in binding to Tie2 indicate that the difference in effect between the two receptors is more nuanced than simple affinity.

Intracellularly, the Tie2 kinase domain folds into two lobar domains, with catalysis occurring in a cleft between the two regions. The kinase domain is comprised of a glycine-rich nucleotide binding loop (residues 831-836), a catalytic loop (residues 962-968), and an activation loop (residues 982-1008). The activation loop, or hinge region, in the unstimulated receptor forms a salt bridge with the C helix of the catalytic loop and prevents substrate access to the active sites(102). The activation loop also contains a Tyr (Tyr 992 in Tie2 and Tyr 1008 in Tie1) that, upon phosphorylation, destabilizes the association of the activation loop with the ATP and substrate binding sites and forms the

open conformation of the receptor. The phosphorylated activation loop releases the binding sites, associates with the disordered C-terminal residues, and leads to phosphorylation of Tyr residues that are able to recruit effector molecules and initiate signal cascades. The open conformation of the Tie2 receptor exposes Tyr 1101 and Tyr 1112 (Tyr 1113 and Tyr 1125 in Tie1) and allows for transphosphorylation of the exposed Tyr residues. Phosphorylation of the various C-terminal residues allows association of the receptor to a host of molecules through both SH2 and PTB domains. (Figure 4) Overall, the kinase domains of Tie1 and Tie2 most closely resemble the catalytic domain of FGFR with 45% sequence homology between the two receptor families and contain homologous sequences to other RTKs for SH2 and SH3 domain binding(103).

Elucidation of the mechanistic details of signal transduction along the Tie axis has led to greater understanding of the physiologic responses of endothelial cells to stimuli in various physiologic and pathologic situations. However, due to the fact that Angs are unable to bind to Tie1 extracellular domains when presented on BIACore, Tie1 was considered to be an orphan ligand for several years and the details concerning signaling were determined through the activation of Tie2. More recently, however, the Angs have been shown to be able to activate Tie1 signaling in mature vasculature, and this topic will be discussed in detail later. First, however, the role of Tie2 in signaling through the mature vasculature will be discussed.

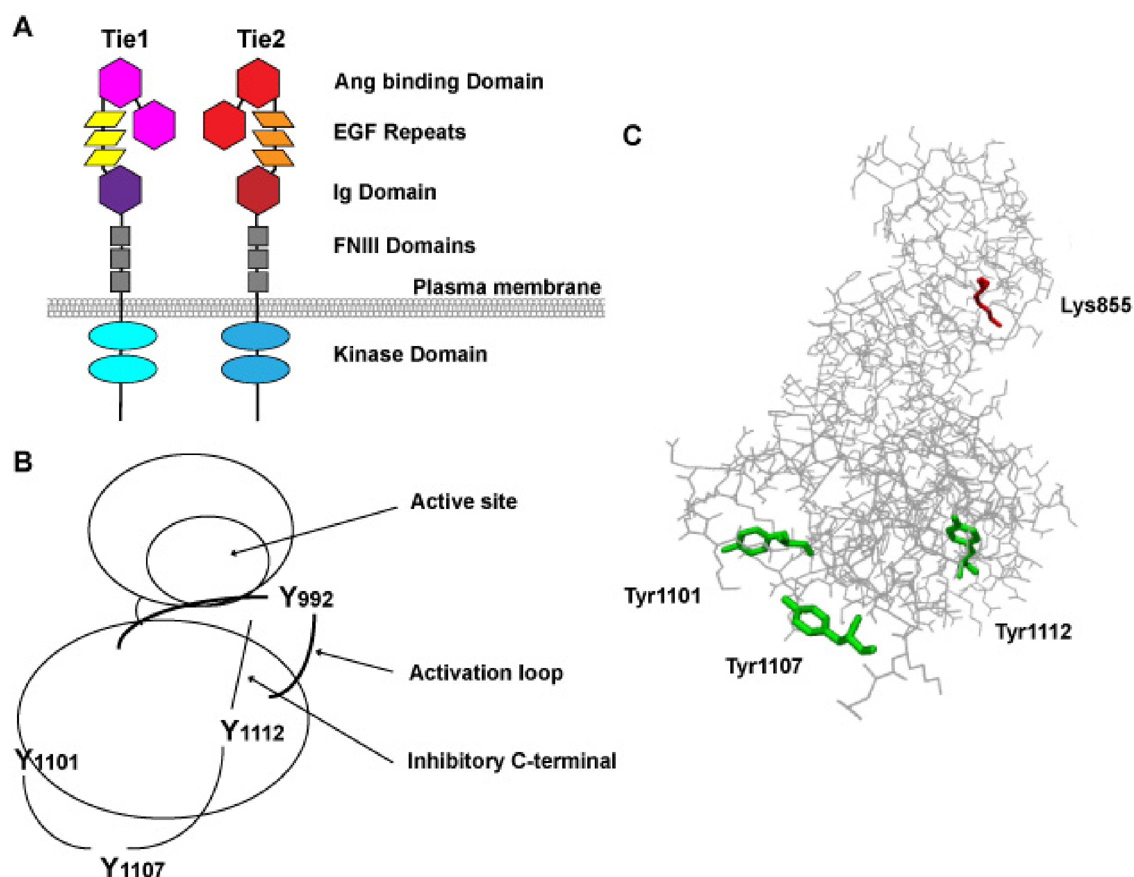

Figure 4: Structure and pertinent residues of Tie2

(A) A schematic representation of Tie1 and Tie2 highlighting both the intracellular and extracellular domains. Extracellularly, the receptors form an arrowhead structure with the ligand binding second Ig domain at the tip of the arrowhead. Intracellularly, the receptors have a juxtamembrane region followed by a bilobar kinase domain and an unordered C-terminal tail. Tie1 is predicted to have a similar structure to Tie2. (B) A cartoon representation of the kinase domain of Tie2. The N-terminal lobe contains the active site of the enzyme. Phosphorylation occurs along the loops between the two kinase domains. Phosphorylation of Tyr992 leads to removal of the inhibitory activation loop and permits binding of the C-terminal Tyrs into the active site. The disordered C-terminal folds back toward the active site of the receptor and sterically inhibits catalytic activity. (C) A three dimensional representation of the Tie2 kinase domain with important residues highlighted. Lys855 is critical for stabilization of the active site, and mutation of the residue leads to a dominant negative, kinase dead receptor. Tyr1101 and 1106 are phosphorylated upon receptor activation and transmit the signal to downstream second messengers. Tyr1112 is involved in sterically hindering association of other Tyr's within active sites; phosphorylation or mutation of the residue leads to greater Tie2 transmission of signaling cascades.

1.10 Molecular associations to Tie2

1.10.1 Binding partners to Tie2

Identification of the intracellular signaling molecules that can potentiate Tie2 signaling has elucidated several mechanistic details concering the Tie axis. The kinase domain of wild type Tie2 can bind the SH2 domain of the p85α subunit of PI3K while a kinase-dead mutant, Lys854Arg Tie2 is unable to associate with the downstream effector. Although the modification of Lys854 prevents binding, the association occurs at Tyr1101, a residue closer to the C-terminal of Tie2. When stimulated with colony stimulating factor (CSF), chimeric receptors with the extracellular domain of CSF receptor fused with the wild type intracellular domain of Tie2 cause the translocation of Akt to the plasma membrane. This translocation is inhibited by wortmannin, indicating that it is due to signaling through the PI3K axis(104). In addition to p85, a host of other signaling molecules have been observed to associate to the same location on Tie2 by utilization of SH2 domains. The SH2 domains of Grb2, Grb7, Grb14, and Shp2 are all identified as potential binding partners, although the p85 SH2 domain has the greatest binding affinity as determined by BIACore. The associations of Grb2, Grb7, and p85 are specific to Tyr1101 while Grb14 binds to Tyr1106 and Shp2 binds to both sites on the receptor(105).

In addition to SH2 domains binding to the C-terminal of Tie2, when analyzed by yeast two hybrid screens, the PTB domain of Dok-R also binds, albeit at Tyr1106 of Tie2. Dok-R is related to the rasGAP binding protein p62Dok with significant sequence homologies with the PTB domain of insulin receptor substrate 3. Furthermore, due to its PTB domain structure, Dok-R, upon binding, can act as a scaffold protein with the phosphorylated Tyr residues recruiting rasGAP, Crk, Abl, Src, Shp-2, or Nck(106). The

recruited effectors can then transmit signals through a host of molecules such as recruited Pak1 and Sos.

1.10.2 Ang1 activation of Tie2

Through ligand binding and subsequent recruitment of discrete binding partners, Tie2 induces the distinct physiologic effects of endothelial cell survival, motility, and maturation. Mechanistically, microvascular EC stimulation by Ang1 induces p85 recruitment at the phospho-Tyr1101 site of Tie2, and translocation of Akt to the receptor complex. Phosphorylation of Akt at Thr308 and Ser473 leads to upregulation of cell survival cascades along the previously mentioned canonical pathways. Ang1 stimulation also upregulates survivin mRNA levels with no increases in bcl-2 expression, which further stimulates cell survival. Addition of a dominant negative p85, wortmannin, or a dominant negative form of survivin all inhibit the Ang1-mediated pro-surivival effect indicating that the signaling is through the Tie2/PI3K axis(107). Physiologically, Ang1 pretreatment of EC's prevents starvation-, irradiation-, or mannitol-induced apoptosis in a dose-dependent manner. The anti-apoptotic effect is inhibited by the addition of soluble Tie2 which serves to sequester the ligand, or the addition of PI3K or Akt inhibitors that inhibit the signaling axis. Due the absence of Ang1 production by ECs, the Ang1 signaling most likely occurs by way of a paracrine mechanism. This is further supported by smooth muscle cell layers of the intima producing the ligand in the vicinity of the layered ECs(108-109).

To promote cell motility, Ang1 stimulation leads to phosphorylation of Tyr1106 on Tie2 and recruitment of Dok-R. Phosphorylation of Dok-R at Tyr351 allows for the association and co-IP of pNck as well as activated PAK. Activated Nck binding to Dok-R due to Ang1 stimulation induces actin cytoskeleton rearrangement to promote migration.

Pak recruitment to Tie2/Dok-R complexes at the plasma membrane potentiates the Ang1-mediated signaling through Dok-R thereby allowing lower levels of Ang1 to induce migratory signals(110-111). In addition to Dok-R recruitment, Ang1 activation of Tie2 stimulates p52 ShcA at Tie2 pTyr1101. This binding is specific to the p52 form of Shc; p46 and p66 Shc are not able to associate directly with Tie2 under known circumstances. Recruitment of Shc induces Tie2-mediated motility, an effect that can be completely blocked by overexpression of the SH2 binding domain of Shc in the stimulated cells. Interestingly, although Shc binding is completely inhibited, PI3K activation is only partly attenuated indicating that there may be other methods by which Ang's can signal through the Tie axis(112).

Since an aspect of EC migration is the requirement for removal of basement membrane structures in order to sprout neovessels, it would make intuitive sense that Ang1 would induce expression of proteases involved in basement membrane degradation. In pulmonary arterial endothelial cells, stimulation of Tie2 leads to PI3K-mediated activation of p125FAK and paxillin, two effectors whose activation leads to the increased transcription of matrix metalloproteases MMP-2 and MMP-9(113). On a more cellular level, activation of Tie2 leads to EC membrane ruffling, lamellipodia extensions, and F-actin cable appearances, all factors that assist in motility and chemotaxis by modification of the cytoskeletal structure of the endothelial cell(114). Tie2 associates with the trailing edge of migrating ECs but does not localize with aVb1 integrins, cellular fibronectin, or vinculin. Rather, Tie2 localizes to ends of actin fibers(115). These phenotypic changes are at least in part modulated by distinct roles of Rac1GTP and RhoGTP, demonstrated by the colocalization of Tie2 with the effector molecules. Rac1GTP, upon stimulation, localizes to the lamellipodia and the F-actin surrounding

leading edge while RhoGTP localizes to perinuclear space as well as all sites of F-actin accumulation. Further confirming that the signaling is through Tie2, inhibition of either Sos or PI3K prevents Rac/Rho induced motility(114).

In addition to other effects, Ang1 has been shown to promote EC maturation by reducing endothelial permeability along several potentially distinct routes. Gavard et al demonstrated that Ang1 activates mDia through the activation of RhoA along the Tie2/Sos axis. Activated mDia is able to associate with Src and act as a steric inhibitor, preventing VEGF mediated activation of Src. Sequestered Src, unable to be activated, is unable to induce the internalization of VE-cadherin, leaving the endothelium in a mature, non-permeable state(116). However, somewhat distinctly, Mammoto et al observed that Ang1 stimulation of Tie2 leads to activation of Rac1 and a suppression of RhoA. Through the mentioned pathway, p190RhoGAP coordinates the Ang1-mediated phosphorylation of myosin light chain kinase and stabilization of VE-cadherin(117). *In vivo*, endotoxin stimulation leads to disruption of endothelial junction integrity, an effect that is prevented by Ang1 co-stimulation. A p190RhoGAP siRNA, inhibiting the action of RhoGAP, blocks Ang1's ability to prevent endotoxin-mediated leakage resulting in interstitial edema(117). To further promote endothelial integrity through the formation of tight junctions between ECs, the subcellular localization of Tie2 changes upon Ang1 stimulation. Unstimulated ECs express Tie2 diffusely over the entire surface of the cell. Upon Ang1 stimulation, Tie2 localizes to the EC tight junctions with the tight junction-associated Tie2 being phosphorylated. pTie2 at tight junctions further stimulates phospho-eNOS through interaction of effectors through pY1101. However, to prevent unrestricted signaling, vascular endothelial protein tyrosine phosphatase also associates with Tie2 at cell-cell contacts and reduces Tie2 phosphorylation(115). The recruitment

of VE-PTP to tight junctions indicates that ECs need a constant stimulation by Ang1 to maintain a mature, non-permeable phenotype. Interestingly, stimulation with Ang2 leads to a similar localization of Tie1 and Tie2, but minimal phosphorylation of Tie2, highlighting the complexity of the signaling through the two receptors(115).

Complementing the agonist activity of Ang1, structural features inherent within the Tie2 receptor promote ligand-induced multimerization and activation. Cleavage of the C-terminal after the Tyr1107 phosphorylation site, although this removes one of the highly phosphorylated Tyr's (Tyr1112), leads to an increased phosphorylation of the receptor, both at baseline and upon stimulation. The inhibitory effect of the C-terminal residues of the receptor is mediated by the random coil peptide angling back toward the active site and sterically preventing binding to the kinase domain(118). Therefore, it appears that the phosphorylation of the Tyr1112 during receptor activation by Ang1 serves to repulse the inhibitory C-terminal tail from the active site. Tyr1112Asp and Tyr1112Glu Tie2 mutants, which mimic the phosphorylated state of Tie2, increase Dok-R and p85 phosphorylation upon Ang1 stimulation compared to wild type Tie2(119). In frame deletion of Leu914 of Tie2 leads to increased sequestration of the receptor in the endoplasmic reticulum and thereby prevents complete Ang1 activation of the receptor. Further mutations within the catalytic domain at locations Tyr897 and Arg915 increase sequestration in an additive fashion. *In vivo* mutations corresponding to these in-frame deletions cause sporadic venous malformations with uniformly enlarged venous channels surrounded by a patchy smooth muscle layer(120). By way of a distinct mechanism, EC layering on fibronectin potentiates Ang1 activation of Tie2. Heteromultimerization between α5β1 integrins and Tie2 can occur during fibronectin-mediated stimulation of ECs with Ang1 stimulation recruiting both p85 and FAK to the

heteromultimeric complex. The overall effect is the activation of downstream cascades at lower Ang1 concentrations than in the absence of integrin/Tie2 multimers. *In vivo*, inhibition of α5β1 partially prevents Ang1-mediated angiogenesis(44).

1.10.3 Ang2 activation of Tie2

Although initial experiments demonstrated that Ang1 was a requisite agonist for the Tie2 receptor and Ang2 bound but served as a competitive antagonist, it has since been shown that Ang2 can have context-based agonist properties toward Tie2. Ang2 stimulation prevents apoptosis in ECs by activating the same PI3K mediated signaling cascades as Ang1 stimulation. Furthermore, the anti-apoptotic effect can be inhibited by the addition of Akt inhibitors or soluble Tie2, demonstrating that the Ang2 can bind Tie2(121). Ang2 stimulation can also activate the ERK1/2 signaling cascade via p38 in the same manner as Ang1. However, contrary to Ang1 stimulation, VEGF stimulation concurrently with Ang2 inhibits ERK phosphorylation. Phenotypically, Ang2 is able to activate the PI3K or MAPK mediated anti-apoptotic signaling cascades but does not induce endothelial cell migration(122). In microvascular ECs, nucleus-sequestered Foxo1 induces the transpcription, translation, and secretion of Ang2. Subsequently, Ang2 binds to and activates Tie2 to activate PI3K-mediated survival cascades and subsequent downregulation of Foxo1. Complementing the mechanism, serum starvation of ECs in the presence of Ang2 sequestering antibodies induces greater apoptosis than starvation alone(123). This regulation of Ang2 feedback to inhibit cell growth signaling manifests in cancer cells with Ang2 expression preventing HIF-1α induced VEGF secretion in gliomas(124).

Using analogous signaling cascades to Ang1, Ang 2 is able to promote EC maturation as well as survival in animal models. Bovine mesenteric lymphatic

endothelial cells are activated by Ang2 more than venous or arterial endothelial cells with a greater accumulation of pERK. As a result, survival and proliferation, but not migration, are enhanced with Ang2 stimulation of lymphatic ECs(125). EC maturity is also enhanced; the exongenous addition of either Ang1 or Ang2 prevents the vascular leak of dye in a mouse ear extravasation model(123).

1.10.4 Synthetic ligand activation of Tie2

Due to the necessity for Angs to oligomerize to activate Tie2, modification of the Ang structures has led to a greater understanding of the requirements for Tie activation as well as the development of more effective agonists to the Tie receptors. The fibrinogen-like binding domains of Ang1 have been attached to the multimerization domains of other proteins to form Ang oligomers that are more stable than native Ang1 in specific multimeric states. Obligate dimers (GCN4-Ang1), a combination of dimeric and tetrameric molecules (MAT-Ang1), and obligate tetramers (COMP-Ang1) were synthesized and assayed for activity. When tested, the obligate tetramer COMP-Ang1 activates Tie2 to greater than extent than the other synthesized variants and even greater than native Ang1. COMP-Ang1 consists of the cartilage oligomeric matrix protein clustering domain instead of superclustering and coiled-coil oligomerization domains of Ang1 coupled to the fibrinogen-like Tie2 binding domain and exists solely as a tetrameric structure. The increased activation of Tie2 by COMP-Ang1 indicates that tetramers are the minimum required multimeric structures necessary to activate the Tie receptors(126). COMP-Ang1 stimluation increases Kruppel Like Factor 2 (KLF-2) expression and Monocyte Enhancement Factor 2 (MEF-2) in a PI3K/Akt dependent manner in ECs. As a result, stimulation of KLF-2 by Ang1 partially blocks VEGF-induced V-CAM expression and thereby maintains the mature EC phenotype of Ang-stimulated

ECs(127). Phenotypically, COMP-Ang1 leads to decreased apoptosis, increased sprouting, and increased migration in analogous pathways to native Ang1. *In vivo*, COMP-Ang1 induces a mature phenotype by promoting nonleaky vessel growth(126).

Within animal models, COMP-Ang1 stimulates endothelial survival, migration, and maturity. COMP-Ang1 localizes to predominantly lung and small intestine microvasculature, with its location mirroring the expression patterns of Tie2 in the mature organism. Subsequently the ligand induces Tie2 phosphorylation and Akt activation in lung vasculature. If animals are subjected to whole body irradiation, stimulation with COMP-Ang1 leads to decreases of apoptotic endothelial cells from 7-20 per field to 1-6 per field and increases average survival time from 217 hrs to 260 hrs. However, this effect is completely endothelial cell-specific; COMP-Ang1 has no effect on nonendothelial cell damage post radiation in the thymus, spleen, salivary glands, rectal cryptic cells, or lymphoid tissue(128). In models of wound healing, COMP-Ang1 promotes revascularization at sites of injury after stimulation. Increases in blood flow coupled with decreases in wound size are observed post stimulation with increases in cells staining positive for PECAM and LYVE(129). Post vessel injury, stimulation with COMP-Ang1 decreases vessel necrosis and increases survivin expression. In addition, skin grafts revascularize faster than controls with increased microvessel densities(130). Taken together, COMP-Ang1 activates specifically endothelial cell cascades to promote the production and maintenance of mature vasculature.

In addition to COMP-Ang1, COMP-Ang2 is able to stimulate Tie2 in an analogous fashion. An obligate pentamer, COMP-Ang2 binds to Tie2 and is four-fold more active in stimulation of Tie2 than native Ang2. COMP-Ang2 stimulated cells exhibit increased pSer473 Akt and increased vessel growth in an *in vivo* mouse ear injury

model(131). Together, the obligate multimers COMP-Ang1 and COMP-Ang2 are able to stimulate the Tie2 axis more effectively than the native ligands, and demonstrate the necessity of proper oligomerization of the ligands to induce Tie signaling. However, in addition to activation, downregulation of a receptor is also integral in producing desired or stemming unintended phenotypic effects. Otherwise, unregulated activation, as in the case of cancer, can occur. Therefore, a discussion on the mechanisms of downregulation of RTKs, and specifically, the downregulation of the Tie receptors is warranted.

1.11 Downregulation of RTKs

1.11.1 General mechanism of RTK downregulation

The downregulation and processing of RTKs was initially demonstrated with the EGFR and this mechanism is still used as the basis for discussions concerning downregulation. Binding of EGF to EGFR induces receptor dimerization and trans-phosphorylation of the receptor complex after which the receptors are endocytosed and recycled or processed. Kinase-inactive receptors or receptors exposed to small molecule kinase inhibitors are internalized at much lower rates than native receptors, and by different mechanisms due to the inefficient recruitment of the receptors to the endocytosed vesicles(132-133). Also, residues in the juxtamembrane region of EGFR such as Thr654 modulate the effectiveness of receptor endocytosis, with mutants being unable to be internalized as effectively as natives(134). Mechanistically, activation of EGFR induces Grb2 association by way of an SH2 domain(135). Grb2 is then able to act as a scaffold to recruit E3 ubiquitin ligases such a Cbl to the activated receptor complex(136). Recruited Cbl family members promote the ubiquitination of the EGFR receptor and its internalization into clathrin-coated endosomes. Upon internalization,

early endosomes are either recycled back to the surface or fuse together forming multivesicular bodies that accumulate in the perinuclear areas of the cell(137). The multivesicular bodies then fuse with early lysosomes that carry a host of proteolytic enzymes which rapidly degrade the activated receptor complexes(137). The essential nature of lysosomal degradation of EGFR is demonstrated by the complete inhibition of receptor degradation by lysosomal inhibitors(138). It is important to note that receptor-mediated signaling still occurs while the receptor is contained along the outer membranes of the vesicles since the receptor complex does not dissociate until it is degraded by the lysosomal proteases. Proteosomal inhibitors can also partially block the degradation of EGFR, but their roles are more indirect, involving the transport of the EGFR complexes from the outer to the inner membranes of the multivescular bodies after which they are acted upon by the lysosomal proteases(139) (Figure 5).

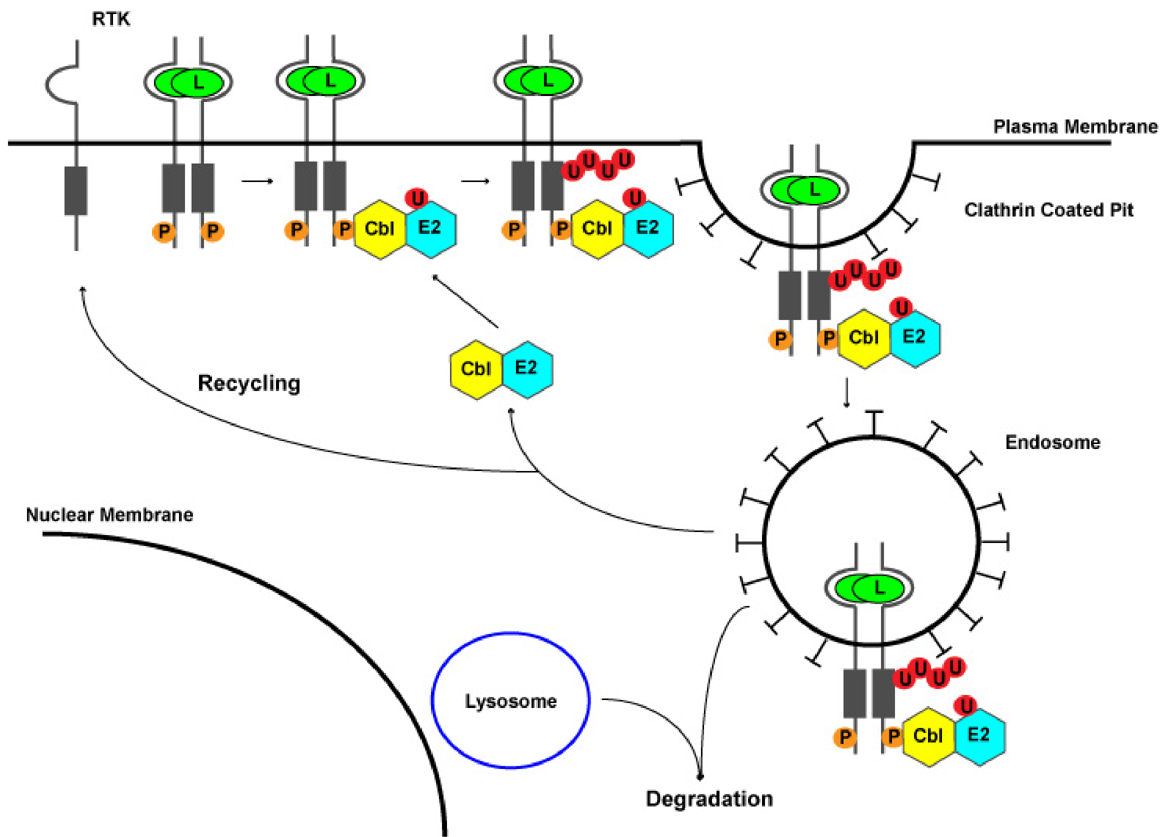

Figure 5: Mechanisms of RTK internalization from the plasma membrane

Ligand induced multimerization of RTK's leads to transphosphorylation of the receptors and subsequent transmission of signals. In addition to recruitment of second messengers that will stimulate downstream cascades, E3 ubiquitin ligases (eg, Cbl) coupled with E2 ubiquitin ligases associate with activated receptors. Ligase association leads to the polyubiquitination of activated receptors on Lys residues and internalization by the endocytosis of plasma membrane pieces within clathrin-coated pits. The newly formed endosomes either break apart, inactivate the receptors, and recycle them back to the plasma membrane or travel to the perinuclear region where they fuse with lysosomes. Fusion induces receptor degradation within the lysosomal compartments.

1.11.2 Internalization and downregulation of Tie2

The internalization of Tie2 occurs in a very similar manner to EGFR. Bogdanovic et al demonstrated that the surface half life of the unstimulated receptor in HUVECs is around 9 hrs, and is reduced to 3 hrs upon sustained Ang1 stimulation and 7 hrs upon sustained Ang2 stimulation. The difference in half life reduction between Ang1 and Ang2 is due to the increased phosphorylation of the receptor upon Ang1 stimulation. The reduction in the half life is at least partially due to receptor internalization and not simply cleavage, measured by the disappearance of biotinylated surface receptor upon Ang stimulation. Interestingly, the Angs dissociate from Tie receptor surfaces before internalization, with increased activation leading to increased receptor multimerization and subsequent release of ligand(140). Ang1 stimulation of HUVECs leads to ubiquitination by way of the E3 ubiquitin ligase c-Cbl. This is confirmed by the expression of the Tie2/Grb2 binding domain of c-Cbl (termed v-Cbl) in Ang1-stimulated HUVECs preventing c-Cbl binding to Tie2(141). Normally, Tie2 is localized in clusters on the surface of HUVEC cells with uniform distribution between the apical and basolateral surfaces. *In vivo*, Ang1 is present in clusters around the microvilli of HUVECs, and acts upon Tie2 at these junctions. Upon Ang1 stimulation, internalized Tie2 localizes with clathrin-coated pits near both the apical and basolateral surfaces, indicating that internalization occurs from both sides(142). Once internalized, Tie2 is trafficked to lysosomal compartments where it is degraded by proteases.

In addition to the canonical degradation pathway, Tie2, much like VEGFR-1 and VEGFR-2, can be cleaved from the cell surface. This cleavage can act as both a way to downregulate signaling through the receptor as well as a mechanism to sequester the circulating Angs. Although a 80-85 kDa extracellular portion of Tie2 termed sTie2 is

constitutively cleaved and present in ng/ml quantities in the sera of healthy individuals, PMA, FGF, or VEGF stimulation of HUVECs increases the level of cleaved sTie2 from the cell surface(143-144). The cleaved Tie2 prevents future Ang-mediated activation of Tie2 and thereby, the PI3K-mediated anti-apoptotic phenotypic effects of Ang stimulation. The cleavage itself is dependent on MAPK/p38 as well as MMP secretions(144), indicating that the accumulation of sTie2 in response to stimuli most likely involves several different endothelial signaling axis, and may have roles as feedback mechanism for Tie2 activation.

1.12 Expression and activation of Tie1 in mature vasculature

Due to the initial lack of ligands for Tie1, it was originally thought that the receptor only had roles in development and that Tie1 was dispensible for normal mature endothelial function. However, observation of Tie1 expression within developed vasculature under several different physiologic conditions indicated a role for the receptor postnatally. Hypoxia can induce tie1 expression in a time dependent manner in aortic endothelial cells through the activation of VEGF and VEGFR-2(145). Tie1 expression within mature vasculature has also been shown to be influenced by the movement of fluid across the endothelium, although the precise role of Tie1 or Ang2 has not been fully elucidated in different states of flow. Increases in shear stress or the maintenance of high levels of shear stress lead to decreases in tie1 mRNA and protein expression while they have no effect on Tie2 expression. Furthermore, shear stress induces rapid Tie1 cleavage from the surface of ECs(146). Chang et al demonstrated that high levels of non-random cyclic stress on the surface of cultured endothelial cells that leads to over 20% elongation of HUVECs induces Tie2 as well as Ang2 expression on ECs while transcription of tie1 or ang1 is unaffected(147). However, Chlench et al

observed that although Tie2 expression is increased, Ang2 expression is decreased upon cyclic stress induction of HUVECs. The increases in Tie2 expression occur by way of Foxo1 transcription repression and exclusion of Foxo1 from the nucleus. Foxo1 exclusion from the nucleus also leads to the inhibition of transcription and translation of Ang2(148). The reduction or absence of flow can also induce tie1 expression. The tie1 promoter is activated at locations of disturbed flow such as in aortic and retinal artery branch points as well as the semilunar valves of the heart. Furthermore, tie1 mRNA is expressed on the shoulders of atherosclerotic plaques where there is obstructed flow and an increased propensity to rupture. Finally, tie1 expression is also induced at arteriovenous junctions of grafts, where the flow is not uniform(149). However, not all cases of increases in shear stress lead to Tie1 downregulation or vice versa. In rat coronary microvascular endothelial cells but not HUVECs, Tie1 as well as VEGFR-2 expression is induced by cyclic stress and stretch of the ECs. In cases of cyclic stress, Ang production is also induced in SMCs(150). These results together indicate that Tie1 and Ang2 are not only involved in development, but also play important but complex roles in the integrity of mature endothelium. Furthermore, they show that in most endothelial cell lines that experience lessened shear stress, Tie1 expression is increased. However, physiologic effects of this upregulation are still unknown.

In addition to the demonstration that Tie1 is present on mature endothelial cells, progress has been made toward elucidating the mechanistic details of Tie1 activation. Chimeric receptors consisting of the extracellular domain of colony stimulating factor receptor fused to the intracellular kinase domain of Tie1 can multimerize and phosphorylate one another in 293 cells upon CSF stimulation. Furthermore, phosphorylated Tie1 activates the PI3K/Akt anti-apoptosis pathway and protects cells

from UV-induced apoptosis in an analogous fashion to Tie2, with mutations in the Tie1 Tyr1113 (corresponding to the Tyr1101 of Tie2) preventing p85 recruitment and Akt-mediated prosurvival pathways(151). These results indicate that the Tie1 kinase domain is functional, provided that proper multimerization and activation can be attained. Recently, several reports demonstrated that Ang1 can activate Tie1 on ECs. Ang1 and COMP-Ang1 but not Ang2 or Ang3 are able to modestly activate Tie1 in immortalized endothelial cells as well as native ECs, with the addition of soluble Tie2 preventing Ang activation(152-153). Interestingly, COMP-Ang1 is also able to activate the full length Tie1 receptor on the surface of 293T cells(152), which, due to the lack of binding of Angs to the extracellular domain of Tie1 by BIACore, may indicate that other receptors distinct from the Tie receptors are involved in the activation of Tie1.

1.13 Tie1/Tie2 Interactions

Due to the lack of ligand binding to Tie1 in the absence of other cofactors, the interactions of Tie1 with other receptors and the resultant signaling cascades in endothelial cells have begun to be investigated. Due perhaps to sequence similarities between the receptors or the possibility of transmitting signals to similar downstream effector molecules, it was hypothesized that Tie1 and Tie2 associate and interact on the endothelium. In fact, all regions of the two receptors are able to interact and promote heteromultimerization. Chimeric Tie1 and Tie2 associate on the surface of EC's, and upon stimulation with NGF, the ligand to the extracellular domains of the chimeric receptors, Tie2 but not Tie1 undergoes phosphorylation. With regards to the association, only the kinase domain of the Tie1 receptor endodomain is necessary for interaction; both the juxtamembrane region as well as the unordered C-terminal domains are dispensible for interaction(154). The transmembrane domains of both Tie1 and Tie2

mediate positive potential interactions with one another, indicating a positive propensity for multimer formations. Tie1's TM has a greater propensity for multimer formation than Tie2, but both receptors' TM domains contribute substantially to stabilized oligomer formations(155). Fluorescence-based resonance energy transfer (FRET) studies of the two receptors demonstrate that Tie1 and Tie2 associate on the surface of cells, and that the Angs have differential effects on the stabilization of the heteromultimeric complexes. Ang2 stabilizes the Tie1/Tie2 complex while Ang1 destabilizes the complex, allowing Tie2 homomultimeric complexes to predominate. The extracellular interaction surface between the receptors is complementary and is important for receptor interaction. A collection of positive charges along the Tie1 surface allows for association with a collection of negative charges along Tie2. Replacement of the positive surface charges of Tie1 with Asp's and Glu's abrogates binding of the two receptors unless complementary positive charges are reintroduced onto the Tie2 surface(156). Taken together, all gross domains of the two receptors have implications in mediating a heteromultimeric complex.

As a result of heteromultimerization of the Tie receptors, Ang signaling through Tie1 is most likely due to Ang binding to Tie2, and subsequent activation of Tie1 within the heteromultimer complexes. Ang1 activation of Tie1 in ECs(152-153) is prevented by knockdown of Tie2 receptors with no downregulation of Tie1 expression indicating that the initial association of Ang1 is to Tie2(153). Furthermore, IPs of Ang2 can contain both Tie1 and Tie2, and Tie1 cannot be IP'd associated to Angiopoietins without the presence of Tie2(157). Therefore, signaling through Tie1 can be attributed to the presence of Tie1/Tie2 heteromultimers whereby the active kinase domains of the receptors transphosphorylate one another.

One of the potential implications of Tie1/Tie2 heteromultimer formation is the modulation of Tie2 activity by the presence of Tie1. Consequently, Tie1's role may be to prevent Ang1 signaling through Tie2 by preventing full activation of Tie2 while Tie1 is present. In endothelial progenitor cells (EPCs), Tie1 and Tie2 are not associated while heteromultimers exist in HUVECs. Consequently, Ang2 is able to activate Tie2 along the PI3K/Akt axis in EPCs but not HUVECs, in an analogous fashion to Ang1 activation of Tie2 in ECs. If Tie1 expression is inhibited in HUVECs, Ang2 is then able to induce Tie2 phosphorylation in a similar fashion as in the case of native EPCs(158). Furthermore, reduction of Tie1 expression leads to greater potential activation of Tie2 in endothelial cells. ECs without functional heteromulimers have greater background activation of Tie2(156) and Tie1 knockdowns lead to greater activation of Tie2 by Ang1. Greater levels of pAkt as well as p42-44 MAPK occur with Ang1 stimulation of Tie1 knockdown HUVECs. Conversely, higher levels of activated caspases are present in Tie1-expressing HUVECs(153). Physiologically, a more extensive network of capillaries is visible in Tie1 knockout embryos coupled with increased levels of pAkt in the vasculature(153). Ang1 stimulation also leads to decreases in endothelial cell permeability and increases in survival cascades, and these results are completely abrogated by blocking Tie2 expression but aren't altered by reducing Tie1 expression(159).

A mechanism that can lead to the temporary reduction in Tie1 from the surface of the endothelium is stimulation-induced cleavage of the receptor. A host of different ligands, among them Il-1β, TNFα, VEGF, and PMA, can induce cleavage and subsequent shedding of the Tie1 extracellular domain at residue 749 from the EC surface in a PKC/calcium-dependent manner. Perhaps unsurprisingly, inhibition of the

MAPK pathway does not influence VEGF- or TNF-mediated Tie1 cleavage(160). Stimulation of ECs with either VEGF or PMA induces the accumulation of a 45 kD fragment of Tie1 still associated with Tie2(161) that is subsequently cleaved by a γ-secretase into a soluble 42 kD fragment that can be proteolytically degraded. Interestingly, reducing Tie2 expression prevents the 45 kD endodomain formation indicating that Tie2's presence is necessary for modulation of Tie1 presence on the surface of ECs(162). Phenotypically, activation of cleavage pathways of Tie1 and subsequent stimulation of endothelial cells with COMP-Ang1 does not alter Ang binding to Tie2 but rather induces greater Tie2 phosphorylation due to the lack of inhibitory extracellular Tie1/Tie2 heteromultimers(162). VEGF stimulation of ECs provokes Tie2 phosphorylation that is not abrogated by the addition of soluble Tie2 nor is it dependent on the association of VEGFR-2 with Tie2. Rather, the cleavage of Tie1 extracellular domains allow for better association of the Tie1/Tie2 endodomains, and VEGF-mediated phosphorylated Tie1 endodomains are able to phosphorylate full length Tie2 receptors to transmit downstream signals(163).

Although there is a battery of evidence to suggest that Tie1 modulates Ang1 signaling through Tie2, the role of Ang2 signaling through the Tie receptors is much less known and early evidence indicates that Tie1's role in the process may be distinct. Tie1 does not antagonize the partial agonist activity of Ang2 on Tie2(157), and, as previously stated, Ang2 stabilizes the Tie1/Tie2 hetermultimeric complex(156). Furthermore, adenovirus-mediated Ang2 overexpression in mouse dermal tissues leads to greater neovascularization(158). Therefore, the role of Tie1 in Ang2 signaling may be more of an agonist than a negative regulator, and other factors determine whether Ang2 association with ECs is stimulatory or inhibitory.

1.14 Tie1's role in inflammation

Although Tie1 has been shown to be upregulated in sites of occluded flow such as atherosclerotic lesions(149), the mechanistic implications for the upregulation are as yet unknown. One reason behind the observed findings may be that Tie1 stimulates inflammatory responses in endothelial cells that lead to the progression of immune responses. Both Tie1 and Ang2 are upregulated in the synovial joints of rheumatoid arthritis and osteoarthritis patients. Although the upregulation is contained primarily within the endothelium, the joint-infiltrated macrophages and SMCs also express higher than normal levels of Tie1 implicating the receptor in the inflammatory nature of the disease(164). Furthermore, a splice variant of Tie1 consisting of the extracellular domain coupled with the juxtamembrane region and several intron residues (121 kD receptor) associates with Tie1 and Tie2 but leads to decreased levels of arthritis in the joints of mice measured both by inflammation and paw thickness(165). The decrease in immune responses in the presence of the Tie1 variant is most likely due to the inability of a truncated Tie1 without cofactor binding domains to activate downstream signaling cascades and thereby activate immune responses. In non-arthritis models, Tie 1 overexpression leading to the ligand-independent autophosphorylation induces the expression of ICAM, VCAM, and E-selectin by way of a p38 mechanism. The induced chemokines then stimulate greater monocyte attachment to the ECs(166). Conversely, shRNA knockdown of Tie1 in HUVECs leads to the downregulation of a host of immune mediators including complement 3, duffy blood group chemokine receptor, TNF receptor superfamily 9, TLR2, granulocyte macrophage colony stimulating factor, Il-1β, and CXCL5 while transcription of tie2, eNOS, and TGFβ is not affected. Overall, the reduction in Tie1 expression prevents the endothelial cells from stimulating monocytes

by way of preventing the actions of monocyte chemoattractant protein 1 (MCP-1) (167). Although these results indicate that Tie1 has a role in eliciting immune responses in mature endothelium, more work needs to be done to understand the precise role of Tie1 within the context of inflammation.

1.15 Tie1 and cancer

Regarding the necessity of the Tie receptors in vascular maturity and maintenance coupled with Tie1's implications in inflammatory cascades, it is perhaps not surprising that the expression of Tie1 has been noted in a host of different cancers and the receptor can be used as a prognostic indicator in several different tumors. Hypoxia-driven pathways in cancer upregulate Tie1 expression and signaling, although the functional significance of these effects are not currently known. Norepinephrine and epinephrine-producing pheochromocytomas upregulate both Tie1 and Ang2 although overexpression of the two are not necessarily indicative of a worse prognosis(168). A host of angiogenic genes are upregulated in glioblastomas including, but not limited to, Tie1. However, increased levels of Tie1 correlate with worse clinical outcomes(169). Within gastrointestinal (GI) tumors, Tie1 overexpression leads to tumor progression. In gastric adenocarcinomas, the expression of Tie1 correlates inversely with survival and positively with increased potential for tumor invasion, adhesion, and angiogenesis into the tumor bed(170). Tie1, Tie2, and the Angs are all upregulated in GI stromal tumors as well as leiomyomas and schwannomas and the expression of these factors allows for the progression of tumors, but their presence does not seem to correlate with survival(171). Interestingly, there are cases where Tie1 expression indicates more positive outcomes. Specifically in thyroid papillary carcinomas, Tie1 is preferentially

expressed on the surface of tumors that are smaller sized and are better differentiated indicating that loss of Tie1 expression is indicative of tumor progression(172).

Within breast cancer, perhaps the most studied cancer system with regards to Tie1 expression, Tie1 is a definitive indicator of disease type and severity. The expression of Tie1 on breast cancer biopsies indicates a worse 5 year disease free survival rate, with the result being additive with the presence of lymph node metastases(173). Furthermore, Tie1 is expressed not only in the primary tumor, but also in the pectoral metastases of lung and ovarian as well as breast cancers(174). With regards to signaling, 43 kDa Tie1 endodomain fragments in addition to the full length Tie1 receptor are expressed on the surface of the tumor cells. Both the full length receptor and the truncation fragment are constitutively active, although Ang1 and Ang2 stimulation modestly increase signaling through the full length receptor(175-176). Interestingly, the signaling pathways activated by Tie1 in the case of breast cancer lines constitute both angiogenic and inflammatory cascades. Upregulation of tie1, tie2, ang1, and ang2 occur in inflammatory but not non-inflammatory breast cancers. Consequently, inflammatory breast cancers contain increased levels of circulating lymphatic cells(177). Taken together, Tie1 has an important role in mediating both angiogenic and inflammatory responses in host of tumors, and further understanding of the mechanistic details of Tie1 activation will help elucidate the role of Tie1 in tumor progression.

1.16 Conclusions

Tie1 has been demonstrated to have important roles in both developing and mature vasculatures, and has been implicated in a host of pathologic states. Although ligands can induce modest activation of the receptor, the signaling cascade is somewhat

unique due to the fact that activation is dependent on the association and presentation of ligand by/multimerization with the highly homologous Tie2. Progress has been made to understand the mechanistic details and physiologic effects of Tie1 signaling within endothelial cells but the role of Tie1 separate from Tie2 has not been elucidated. This project aimed to understand the distinct roles that Tie1 activation and downregulation play in Ang signaling in mature vasculatures. Furthermore, studies in this dissertation sought to distinguish the physiologic effects of Tie1 activation separate from Tie2 in the endothelium. As discussed in the subsequent chapters, Ang2 is able to activate Tie1 and transmit signals to induce prosurvival and proproliferative effects along the PI3K/Akt axis within ECs. In addition, this stimulatory effect is only dependent on Tie1/Tie2 associations along the extracellular and transmembrane surfaces. Furthermore, Ang2 induces a rapid and efficient downregulation of Tie1 from the EC surface that leads to the potential for hyperactivation of Tie2 by future Ang stimulations of the vasculature. Taken together, these results demonstrate a novel agonist for Tie1 signaling, may help explain differences in signaling through the lymphatic vasculature versus the blood vasculature, and help further the understanding of the role of Tie1 in the maintenance of vessels and the development of pathologies.

2. Angiopoietin-2 activates Tie1 signaling to promote endothelial cell survival through a Tie2 kinase-independent pathway

2.1 Introduction

Tie1 and Tie2 are receptor tyrosine kinases (RTKs) that are expressed primarily on endothelial cells and that are required for both embryonic vascular development and the maintenance of mature blood vessels(45, 48, 178). Substantial data now indicate that the Tie receptors and their ligands, Angiopoietins 1-4 (Ang1-4), regulate the transition between a mature, quiescent vasculature and angiogenic or remodeling blood vessels. Whereas Ang1 and Ang4 act as receptor agonists, Ang2 (and possibly Ang3) acts as a context-dependent agonist or antagonist for the Tie2 receptor(70-72, 74-75, 179). Structurally, the Angiopoietins consist of an N-terminal superclustering domain, a coiled-coil oligomerization domain, and a C-terminal fibrinogen-like receptor-binding domain. In their native conformations the Angiopoietins exist as higher order multimers, which are able to bind and cluster multiple Tie receptors to induce receptor activation and autophosphorylation(96-97, 180).

Activation of Tie2 has been linked to several important signaling pathways, most notably the phosphoinositol 3-kinase (PI3K)/Akt pathway, which promotes endothelial cell survival, sprouting, and migration(104, 181-182). Although less is known about signaling by Tie1, data from our laboratory have shown that Tie1 is also capable of activating the PI3K/Akt pathway to prevent apoptosis(151). *In vitro* studies have failed to demonstrate binding of the Tie1 extracellular domain to any of the Angiopoietins(70-71, 179), raising questions about the mechanisms of Tie1 activation *in vivo* and its role in Ang/Tie receptor signaling and function.

Tie1 is required for normal embryonic vascular development, as mice lacking Tie1 die late in gestation or in the immediate perinatal period with abnormalities of vascular integrity(57, 62), and recent data also demonstrate a requirement for Tie1 in lymphatic vessel development. Understanding Tie1's function and mechanisms of action has been complicated by its apparent lack of a ligand. This problem was initially circumvented by using a chimeric receptor approach to demonstrate activation of PI3K/Akt through a mechanism similar to that of Tie2(104, 151). In addition, proteolytic cleavage of the Tie1 extracellular domain has been shown to result in a cytoplasmic endodomain that can interact with and be phosphorylated by Tie2, although the functional consequences of this interaction are unknown(162, 183). Recently, Saharinen et al found that tyrosine phosphorylation of Tie1 could be induced by Ang1 or Ang4 in cells co-expressing Tie2, but that Tie2 kinase activity was not required for this effect. These findings suggest that Tie1 is unable to bind the Angiopoietins by itself, but that Ang-Tie2 binding facilitates ligand presentation to Tie1 followed by receptor activation(152). Notably however, effects of the Angiopoietins on Tie1-mediated signaling pathways and cellular responses remain unknown.

A number of recent studies have demonstrated effects of Tie1 expression on Tie2 signaling, and several reports have suggested that the differential effects of Angiopoietin signaling may be attributable to Tie1 expression status and its ability to heteromultimerize with Tie2. Reduced expression of Tie1, either endogenously in endothelial progenitor cells (EPCs) or using siRNA in human umbilical vein endothelial cells (HUVECs), was shown to enhance effects of Ang2, suggesting that Tie1 inhibits Ang2/Tie2 signaling(158). Furthermore, expression of Tie1 was found to attenuate Ang1/Tie2-mediated Akt activation, endothelial cell survival, and vascular density(162).

Taken together, these results demonstrate an important role for Tie1 in the modulation of Angiopoietin-induced endothelial cellular responses. To date, however, no studies have investigated signaling in endothelial cells mediated specifically by Tie1.

To further understand Ang2/Tie1 signaling, in this dissertation, we investigated the role of Ang2 in Tie1 activation and downstream signaling in endothelial cells. To distinguish effects of Tie1 from those mediated by Tie2 homo- or heteromultimerization, we developed an endothelial cell line in which Tie2 expression was silenced and rescued with a Tie2 mutant lacking the cytoplasmic kinase domain, thereby allowing Tie2 extracellular domain (ECD)-mediated ligand binding and presentation to Tie1. Ang2 was found to activate Tie1 and the downstream PI3K/Akt pathway, which required the presence of at least the Tie2 extracellular and transmembrane domains. Furthermore, Ang2-mediated activation of Tie1 led to increased endothelial cell survival and proliferation. Interestingly, we also observed autocrine activation of Tie1 by Ang2 in starved endothelial cells, suggesting a role for Tie1 in maintaining endothelial cell viability under stress conditions. Finally, similar to a previous report(158), silencing of Tie1 led to increased Ang2-mediated Tie2 activation. consistent with a role for Tie1 in the modulation of Ang2/Tie2 signaling. The findings in this chapter are the first to demonstrate a physiological role for Angiopoietin signaling in endothelial cells mediated specifically by Tie1, and they have broad implications for understanding the role of Tie1 in endothelial cell biology.

2.2 Methods

2.2.1 Reagents and cell culture

Mouse monoclonal Tie1 antibodies (clones 5D2, 21G6) were generously provided by Dr. Harvey Yamane (Amgen). Rabbit polyclonal anti-Tie1 (C-18) and

mouse monoclonal anti-phosphotyrosine (clone PY99) antibodies were from Santa Cruz Biotechnology. The Tie2 monoclonal antibody (clone 33) has been described previously(184). Rat monoclonal anti-α-tubulin (clone YL1/2) was from Serotec. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Recombinant human angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) were purchased from R&D Systems. The Tie kinase inhibitor (4-(6-Methoxy-2-napthyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidizole), cat. # 612085) and PI3K inhibitor LY294002 were purchased from Calbiochem.

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords essentially as described by Marin et al(185). Briefly, cords were flushed twice with RPMI medium (Invitrogen) and treated with collagenase (Sigma) to dislodge endothelial cells. Isolated cells were cultured in Endothelial Basal Medium-2 (EBM-2, Clonetics) containing growth factors and 5% fetal bovine serum (FBS; EGM-2-MV, Clonetics), according to the manufacturer's instructions. Endothelial cell populations were confirmed by immunostaining for CD31 (PECAM-1), cultured in EGM-2, and used between passages 1 and 4. EC-RF24 cells (ECRF) were generously provided by Dr. Ruud Fontijn (VU University Medical Center, Amsterdam) and cultured in EGM-MV (Clonetics) containing 10% FBS (Gibco). Human embryonic kidney (HEK)-293 cells and GP2-293 retroviral packaging cells (Clontech) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS, penicillin, and streptomycin. HEK-293 cells stably overexpressing Tie1 (293-Tie1) were cultured in DMEM supplemented with G418 (600 μg/mL, Invitrogen).

2.2.2 Retroviral vector generation

Plasmids pQCXIP, pQCXIH, and pQCXIX were from Clontech. To generate a vector with bleomycin resistance (pQCXIB), the cDNA encoding the bleomycin/zeocin resistance gene was amplified by PCR from the plasmid pBABE-Bleo(186) with the following primers: forward primer, 5'-GACGTAACGCGTTCACCATGGCCAAGTT-3'; reverse primer, 5'-AAGCTTCTCGAGTCAGTCCTGCTCCTC-3', which generated new *Mlu* I and *Xho* I restriction sites (underlined), respectively. The bleomycin resistance gene was cloned into the *Mlu* I and *Xho* I sites in pQCXIX to generate pQCXIB, and the murine Tie1 (mTie1) cDNA was then subcloned into the *Eco*R I site of pQCXIB. A Tie2 mutant that was truncated after the transmembrane (TM) domain and which lacked the entire cytosolic domain (designated Tie2TM) was generated by PCR with the following primers, which incorporated a stop codon immediately after the last residue of the transmembrane domain (isoleucine 782) and generated new *Not* I and *BsiW* I restriction sites: forward primer, 5'-TAGCGGCCGCGTATGGACTCTTTAGC-3'; reverse primer, 5'-GGACGTACGATCTAGGTACCTACGCT-3'. The Tie2TM construct was cloned into pQCXIH. A human (h)Tie1-specific shRNA in the plasmid pSM2 and corresponding to bp 3252-3272 (CCTGTGCCGAGCTCTATGAAA) of hTie1 (NCBI RefSeq# NM_005424) was obtained from OpenBiosystems (cat # RHS1764-9689489, ID# V2HS_69970) through the Duke RNAi core facility. This shRNA plasmid was used to generate recombinant retrovirus as described below.

2.2.3 Plasmid transfections, retrovirus generation, and retroviral infection

Transient transfection of 293-Tie1 cells was performed with pQCXIH-Tie2TM using Lipofectamine-PLUS (Invitrogen). Cells were allowed to grow for 24 hrs following transfection, after which they were stimulated as described below.

To generate recombinant retroviruses, GP2-293 cells were co-transfected with a given retroviral vector and a plasmid encoding the pantropic envelope protein, pVSV-G (Clontech), using Lipofectamine-PLUS. Following transfection, virus-containing supernatants were collected and passed through a 0.4 μm filter (Corning). For retroviral infections, ECRF cells were incubated with a 1:1 mixture of full growth medium and retrovirus-containing supernatant supplemented with polybrene (8 μg/mL, Sigma). Cells were cultured for 24 hours, after which the medium was replaced with fresh medium and virus-containing supernatant for a second round of infection. After two rounds of viral infection, the cells were changed into full growth medium and allowed to grow until confluent. When confluent, the cells were passaged and cultured in EGM-MV containing the appropriate selection antibiotic (puromycin, 5 μg/mL; hygromycin, 100 μg/mL; Zeocin, 400 μg/mL, all from Invitrogen) to obtain polyclonal cell populations. Cells were then screened for transgene expression by Western blotting.

2.2.4 Immunoprecipitations and Western blotting

Protein concentrations of cell lysates were determined using a BCA protein assay (Pierce Biotechnology). For immunoprecipitations (IPs), antibodies against Tie1 (clone 5D2) or Tie2 (Ab33) were added to lysates and allowed to bind at 4℃ for 16 hrs followed by addition of Protein G sepharose (GE Biosciences) for 1 hr at 4℃. Antibodies and bound proteins were eluted by boiling into Laemmli sample buffer,

separated by SDS-PAGE, transferred to nitrocellulose membranes, and Western blotted with the indicated antibodies.

2.2.5 Cell assays

To measure Tie receptor signaling and cellular responses, endothelial cells were seeded onto dishes coated with gelatin (0.2%, Sigma). Cells were cultured in growth medium until 90% confluent, washed once with EBM, and the medium was replaced with EBM containing 0.5% FBS. The cells were then starved for 4 hrs followed by treatment with or without Ang1 or Ang2 (500-1000 ng/mL in PBS + 0.1% BSA; R&D Systems) and sodium orthovanadate (VO_3, 1mM, Sigma) for 15 min. The cells were lysed with RIPA lysis buffer (100 mM KCl, 25 mM EDTA, 5 mM $MgCl_2$, 10 mM HEPES pH 7.0, 0.5% IGEPAL CA-630, 1% Triton X-100, 0.1% SDS, 10% glycerol) supplemented with VO_3 (1 mM) and protease inhibitors (Complete, Roche).

To measure cell viability, cells were grown until 70% confluent, washed with EBM, and starved for 4 hrs in EBM containing the indicated growth factor. Apoptosis was induced by addition of staurosporine (100 μM in DMSO, Invitrogen) for 90 min at 37℃. Cells were then either dislodged with a rubber cell scraper or trypsinized (0.25%, Gibco), then they were washed once with PBS, and trypan blue (0.4%, Sigma) was added to the cells. Total and viable cells were counted using a hemacytometer under light microscopy (Olympus CK2). Alternatively, cell viability was assessed following serum starvation. Cells were grown to 95% confluence, washed once with EBM, and incubated 48 hrs in serum-free EBM supplemented with the indicated growth factor. Cells were visualized by phase contrast microscopy (Olympus IX70, Optronics DEI-750 digital camera). Apoptosis was assessed with a caspase-3 and -7 activity assay (Caspase-Glo 3/7 Assay, Promega).

To measure cell proliferation, cells were seeded on 6 well plates and grown in full growth medium until ~40% confluent. Cells were washed with serum-free EBM and cultured in EBM for 24 hrs to synchronize them in the cell cycle. PBS or Ang2 (1000 ng/mL) was added to cells in EBM without FBS for 16 hrs then the cells were washed with EBM, and Click-It EdU Reagent (10 μM) was added for 4 hrs. Cells were visualized by phase contrast microscopy before and after addition of Ang2, and digital images were obtained (Olympus IX70, Optronics DEI-750 digital camera). The cells were removed by trypsinization, washed with PBS/1% BSA, and fixed with 4% formalin. The cells were permeabilized with saponin, stained with Alexafluor 488 azide (Invitrogen) to measure EdU incorporation, then washed and stained with CellCycle 488-red (a propidium iodide analog) to measure DNA content. The various populations of cells were counted on a flow cytometer (Guava) and analyzed by flow cytometry software (FloJo).

2.2.6 Statistical analysis

All blots shown are representative of at least three separate experiments. All results were expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the one-tailed Student's *t*-test (two sample, unequal variance), and $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Ang2, but not Ang1, induces Tie1 activation in endothelial cells

To investigate the mechanisms governing Tie1 activation in endothelial cells, we used both primary human umbilical vein endothelial cells (HUVECs) and an immortalized endothelial cell line derived from HUVECs, EC-RF24 (ECRFs)(187). Both cell types expressed similar amounts of full-length Tie1 and Tie2 proteins (Figure 6A). To

compare effects of the Angiopoietins on activation of Tie1 or Tie2, cells were briefly serum-starved and stimulated with either Angiopoietin-1 (Ang1) or Angiopoietin-2 (Ang2) for 15 min. Consistent with previous results(70-71) , stimulation with Ang1, but not Ang2, led to a robust activation of the Tie2 receptor in both HUVECs and ECRFs (Figure 6B). Somewhat surprisingly, the opposite effect was observed for Tie1, with Ang2, but not Ang1, inducing detectable Tie1 tyrosine phosphorylation in both endothelial cell lines (Figure 6C).

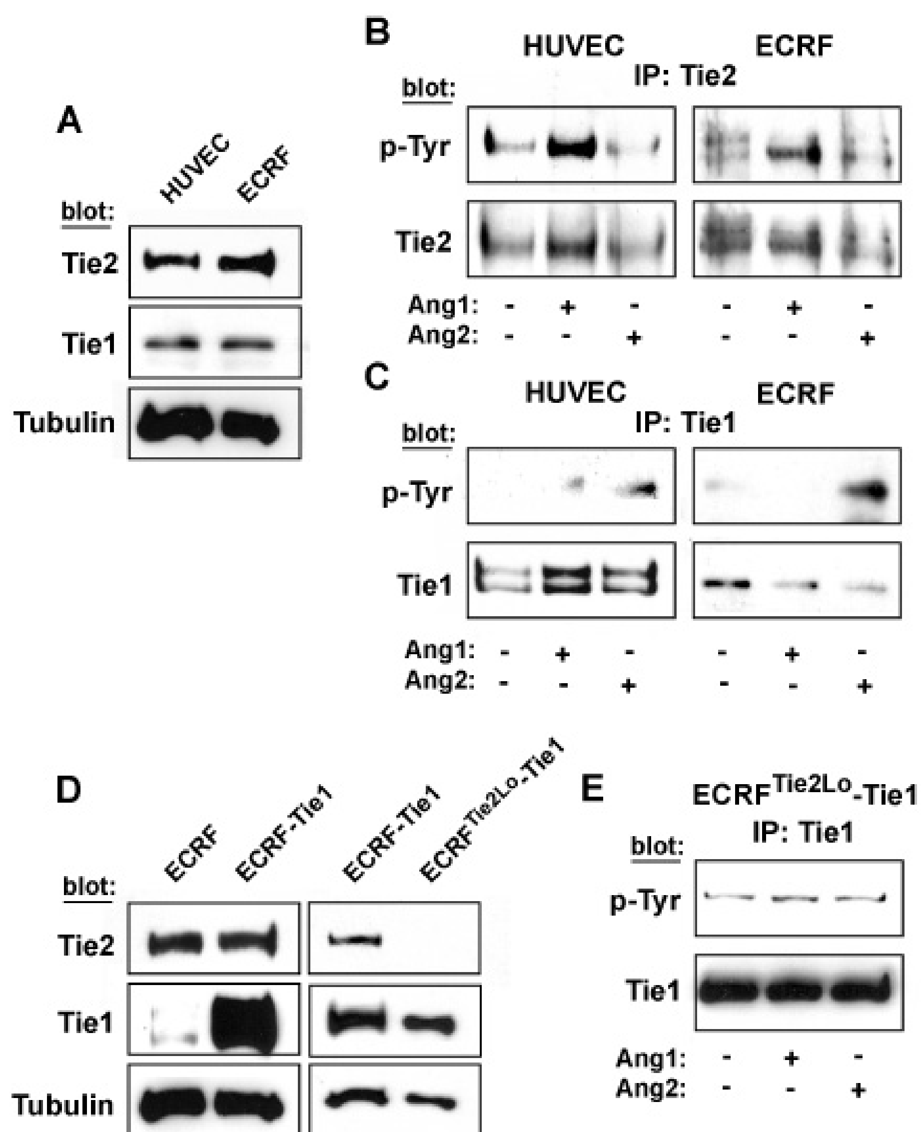

Figure 6: Ang2 induces Tie1 phosphorylation in a Tie2-dependent manner

(A) Whole cell lysates of HUVECs and ECRFs express comparable levels of Tie proteins. Proteins were detected by Western blotting with the indicated antibodies. (B, C) HUVEC and ECRF-Native cells were treated with or without Ang1 or Ang2 (500 ng/mL) for 15 minutes. Tie1 and Tie2 were immunoprecipitated (IP'd) from cell lysates, and proteins were Western blotted sequentially with anti-phosphotyrosine and anti-Tie1 or anti-Tie2. (D) Whole cell lysates of various stable ECRF cell lines either overexpress Tie1 (left panel) or overexpress Tie1 while have knocked down expression of Tie2 (right panel). Proteins were detected by Western blotting with the indicated antibodies. (E) ECRF-Tie2Lo/Tie1 cells were treated with or without Ang1 or Ang2 (500 ng/mL) for 15 minutes. Tie1 was IP'd from cell lysates, and proteins were Western blotted with the indicated antibodies.

2.3.2 Tie2 is required for Ang2-mediated Tie1 activation

Previous studies have shown that Ang1-mediated activation of Tie1 is dependent on co-expression of Tie2(153, 158), therefore we sought to investigate the requirement for Tie2 in Ang2-mediated Tie1 activation. Because patterns of Tie receptor expression and activation are retained in ECRF cells following derivation from HUVECs (Figure 6A), we used ECRFs to stably manipulate Tie receptor expression. To facilitate investigation of Tie1 activation and signaling, we first made an ECRF cell line stably overexpressing Tie1 above endogenous levels (Figure 6D, *left panel*). Compared to parental ECRF cells, ECRF-Tie1 cells express similar levels of Tie2 (Figure 6D), and ECRF-Tie1 cells are activated by Ang1 and Ang2 in a manner identical to that of HUVECs and parental ECRFs (data not shown), demonstrating that stable overexpression of Tie1 does not interfere with Angiopoietin signaling. To investigate the role of Tie2 in Ang2-mediated Tie1 activation, we used a retrovirally-expressed shRNA to silence Tie2 expression in ECRF-Tie1 cells. This construct resulted in silencing of over 95% of endogenous Tie2 in the resultant $ECRF^{Tie2Lo}$-Tie1 cells without altering Tie1 expression (Figure 6D, *right panel*). When $ECRF^{Tie2Lo}$-Tie1 cells were stimulated with high concentrations of either Ang1 or Ang2, neither ligand was able to induce Tie1 phosphorylation (Figure 6E). These findings demonstrate that Tie2 is required for Ang2-mediated Tie1 activation in ECRF cells.

2.3.3 The Tie2 extracellular domain is sufficient for Ang2-mediated Tie1 activation

Previous studies have found that Tie1 and Tie2 can heteromultimerize, raising the possibility that Ang2-induced phosphorylation of Tie1 was mediated by Tie2 kinase activity within a heteromultimeric complex, although Tie2 kinase activity has been found

to be dispensable for Ang1-induced Tie1 phosphorylation(152). To investigate this question, we stably expressed non-targeted Tie2 constructs in $ECRF^{Tie2Lo}$-Tie1 cells encoding either full-length murine Tie2 ($ECRF^{Tie2Lo}$-Tie1/Tie2 cells) or mutant murine Tie2 lacking the cytoplasmic domains (i.e., containing only the extracellular and transmembrane domains; $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells). Tie1 expression in both of these cell lines was unaffected (Figure 7A). Using an antibody against the extracellular domain (ECD) of Tie2, the re-expression of truncated Tie2TM was detectable at ~100 kDa compared to 150 kDa for full-length Tie2 (Figure 7A). Interestingly, stimulation of $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells with Ang2 led to activation of Tie1 similar to that observed in HUVECs and parental ECRFs (Figure 7B), demonstrating that Ang2-mediated Tie1 activation is independent of Tie2 kinase activity as well as any Tie2-associated cytoplasmic proteins. This finding suggests that Tie2 binds and stabilizes Ang2, thereby facilitating heteromultimerization with Tie1 receptors, which are then able to undergo mutual trans-phosphorylation.

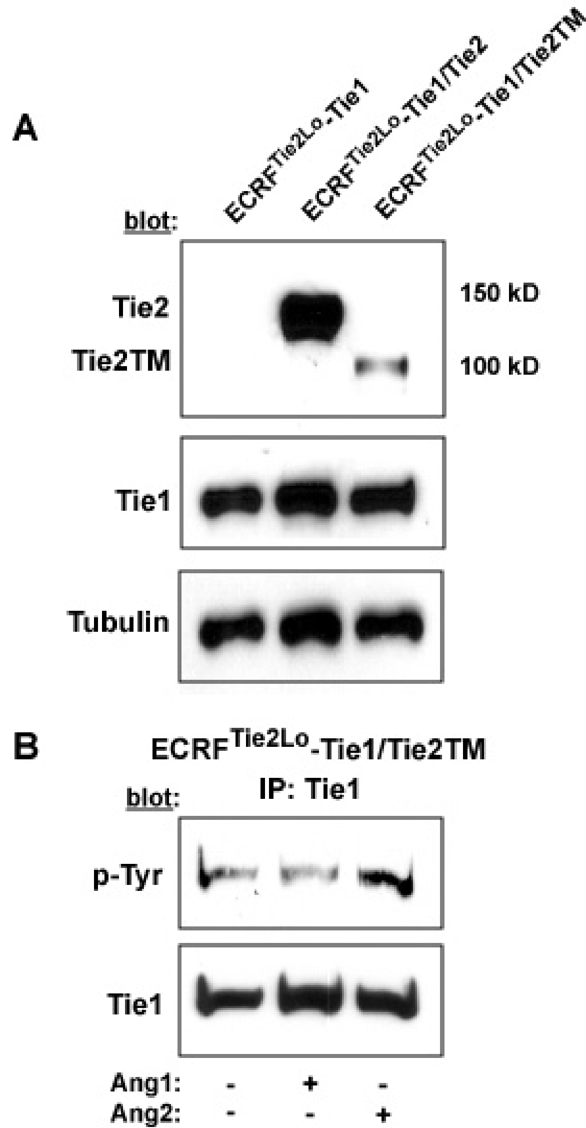

Figure 7: Tie2Tm is sufficient to induce Ang2-mediated Tie1 phosphorylation

(A) Whole cell lysates of various ECRF cell lines express various mutant Tie2 proteins. Proteins were detected by Western blotting with the indicated antibodies. (B) ECRF-Tie2Lo/Tie1/Tie2TM cells were treated with or without Ang1 or Ang2 (1000 ng/mL) for 15 minutes. Tie1 was IP'd from cell lysates, and proteins were Western blotted with the indicated antibodies.

2.3.4 Ang2 induces Tie1 kinase activity

To verify that Ang2-mediated Tie1 phosphorylation was due to activation of the Tie1 kinase, we tested a commercially available small molecule Tie2 kinase inhibitor for its effects on Tie1. Based on the high degree of homology between the Tie1 and Tie2 kinase domains (>80% amino acid identity)(47), we speculated that this Tie2 kinase inhibitor would also inhibit Tie1 kinase activity. This compound (hereafter referred to as "Tie inhibitor") is highly selective for Tie2 compared to serine-threonine kinases or other RTKs(188). Pre-incubation of $ECRF^{Tie2Lo}$-Tie1/Tie2 cells with the Tie inhibitor led to complete inhibition of Angiopoietin-induced Tie2 phosphorylation, including reduction of background Tie2 phosphorylation to undetectable levels (Figure 8A). As described, Tie1 is unable to bind the Angiopoietins in the absence of the Tie2 ECD. Therefore, to determine the inhibitory effects of the Tie inhibitor on Tie1 in the absence of a functional Tie2 kinase, HEK-293 cells stably overexpressing Tie1 were transiently transfected with a construct encoding Tie2TM, which led to robust expression of Tie2TM in addition to Tie1 (Figure 8B). 293-Tie1 cells transfected in this manner were stimulated with Ang2 in the absence or presence of the Tie inhibitor. As we observed in ECRF cells, Ang2 stimulation of Tie1 in the presence of Tie2TM resulted in a detectable increase in Tie1 tyrosine phosphorylation, however this effect was blocked by the Tie kinase inhibitor (Figure 8C). These findings demonstrate that the Tie kinase inhibitor is an effective tool for inhibition of Tie1 in the absence of a functional Tie2 kinase. In addition, they support the observation that Ang2 can induce Tie1 autophosphorylation that is independent of Tie2 kinase activity.

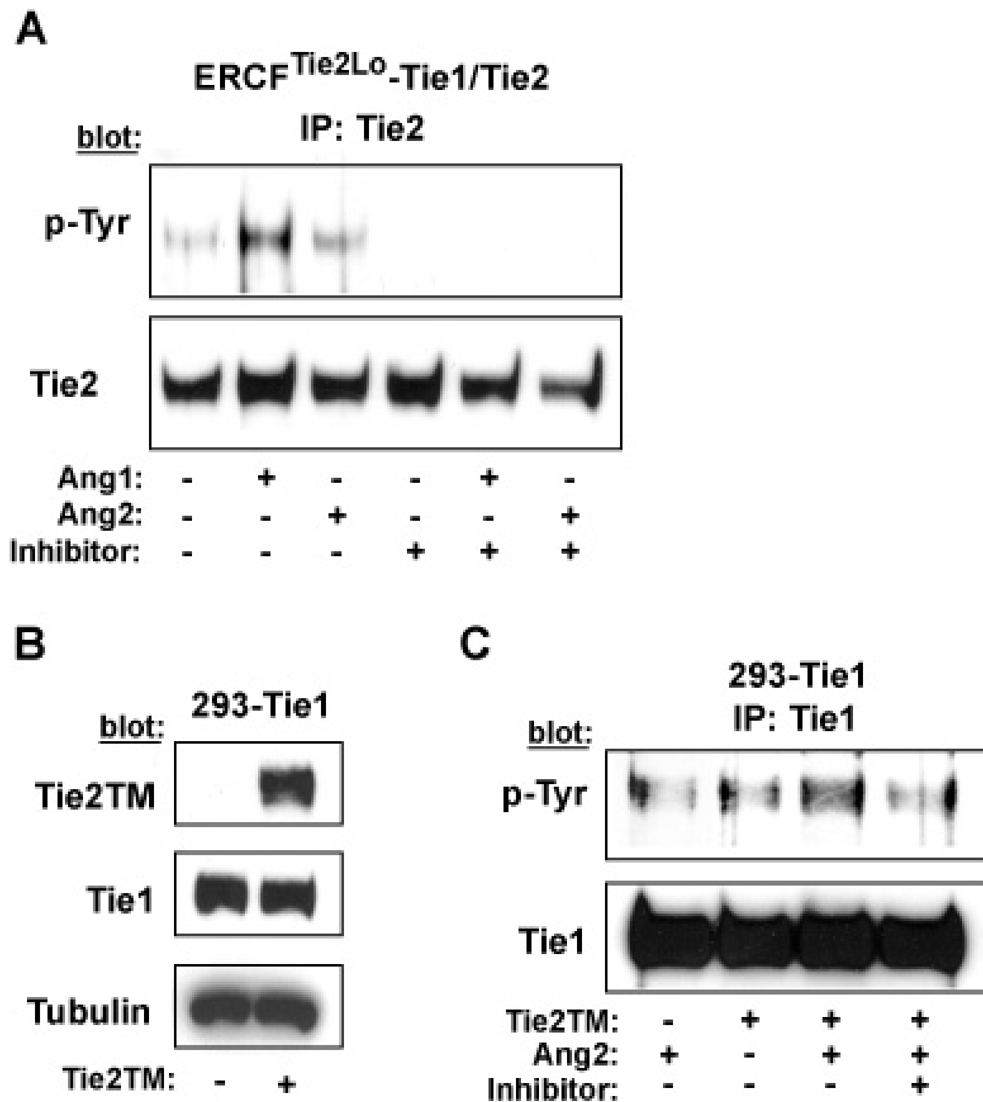

Figure 8: Tie inhibitor inhibits receptor phosphorylation in the presence of Angs

(A) ECRF-Tie2Lo/Tie1/Tie2 cells were treated with or without Ang1 or Ang2 (1000 ng/mL) for 15 minutes with or without Tie inhibitor (1.25 uM). Tie2 was IP'd from cell lysates, and proteins were Western blotted with the indicated antibodies. (B, C) 293-Tie1 cells were transiently transfected with plasmids encoding Tie2Tm. 293-Tie1 cells were treated with or without Ang1 or Ang2 (1000 ng/mL) for 15 minutes with or without Tie inhibitor (1.25 uM). Tie1 was IP'd from cell lysates, and proteins were Western blotted with the indicated antibodies.

2.3.5 Ang2-Tie1 signaling activates Akt to promote endothelial cell survival

We previously showed using a chimeric c-fms-Tie1 receptor that activation of the Tie1 kinase in fibroblasts could lead to signaling through PI3K/Akt and inhibition of apoptosis(151). We therefore sought to determine whether Ang2-mediated Tie1 activation in endothelial cells would elicit similar responses independently of Tie2. Stimulation of $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells with Ang2 led to a robust increase in Akt phosphorylation, which was abolished by the addition of the Tie inhibitor (Figure 9A), demonstrating that Ang2-Tie1 signaling proceeds, at least in part, through Akt. To determine whether Tie1-mediated Akt activation translated into an increase in cell survival, $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells were serum-starved in the absence or presence of Ang1 or Ang2, and apoptosis was induced by the addition of staurosporine. Ang2, but not Ang1, led to a significant improvement in cell viability, as measured by trypan blue exclusion (Figure 9B). To confirm the requirement for Tie2 in Ang2 presentation to Tie1, a similar cell viability study was performed to compare the effects of Ang2 on $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells and $ECRF^{Tie2Lo}$-Tie1 cells. Staurosporine-induced cell death was again significantly decreased by Ang2 in $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells, whereas no beneficial effect was observed in $ECRF^{Tie2Lo}$-Tie1 cells (Figure 9C). These results demonstrate that Ang2-Tie1 signaling promotes cell survival. Consistent with our previous observations on Tie1 receptor phosphorylation, this effect requires only the Tie2 extracellular and transmembrane domains, apparently for Ang2 presentation to Tie1.

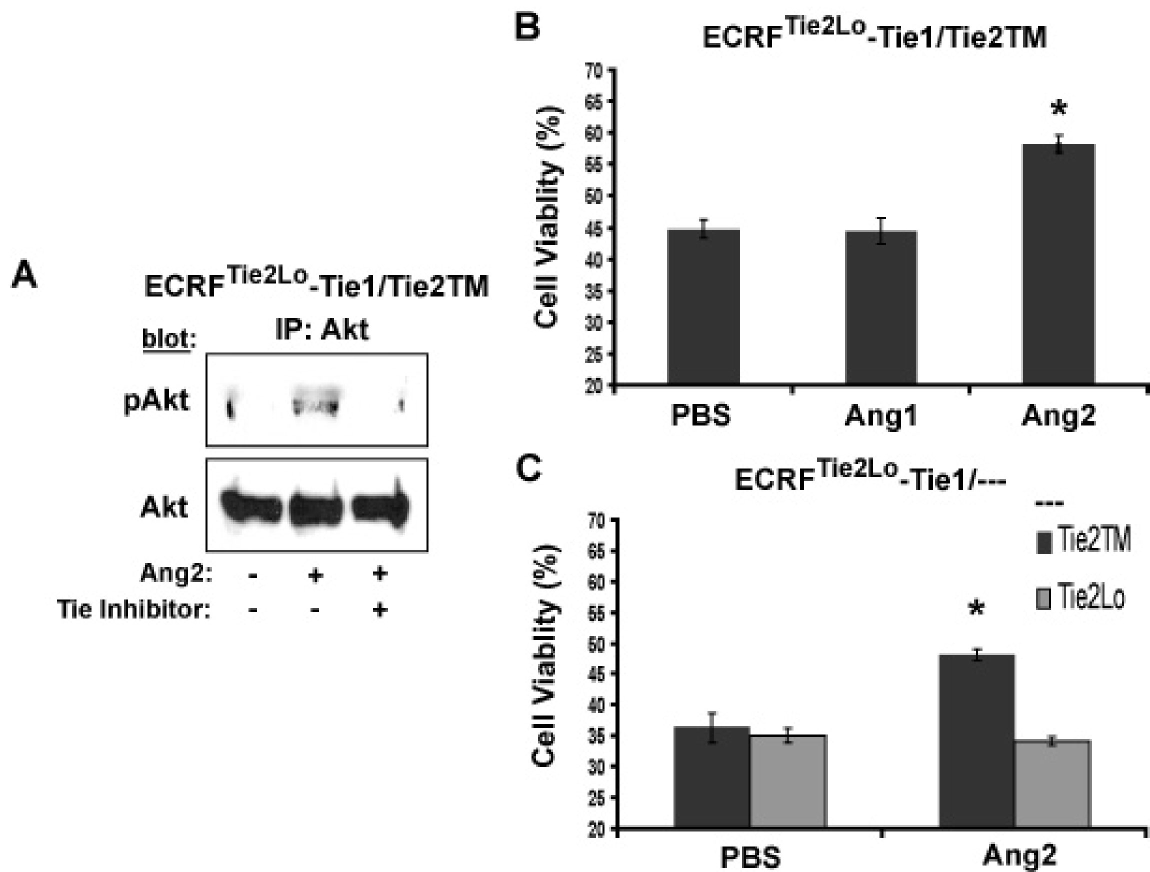

Figure 9: Ang 2 activation of Tie1 induces Akt-mediated cell survival cascades (1)

(A) ECRF-Tie2Lo/Tie1/Tie2 cells were treated with or without Ang2 (1000 ng/mL) and Tie inhibitor (1.25 uM) for 15 minutes. Akt was IP'd from cell lysates and proteins were Western blotted with the indicated antibodies. (B) Serum-starved ECRF-Tie2Lo/Tie1/Tie2TM cells were treated with or without Ang1 or Ang2 (1000 ng/mL). Effects on staurosporine-induced Trypan Blue cell viability were quantified. *P<0.005 by ANOVA. (C) Serum-starved ECRF-Tie2Lo/Tie1/Tie2TM or ECRF-Tie2Lo/Tie1 cells were treated and viability assessed as described in B. *P<0.005 by ANOVA.

To confirm the effects of Ang2-Tie1 on endothelial cell viability, we tested effects of this pathway on serum starvation-induced apoptosis. $ECRF^{Tie2Lo}$-Tie1/Tie2TM or $ECRF^{Tie2Lo}$-Tie1 cells were grown to sub-confluence and then incubated 48 hours in serum-free medium in the absence or presence of Ang2, either with or without the Tie kinase inhibitor. $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells displayed little observable cell death, either in the absence or presence of Ang2, whereas the Tie inhibitor significantly inhibited cell viability (by trypan blue exclusion) and enhanced apoptosis (by caspase-3 and -7 cleavage), both at baseline and in the presence of Ang2 (Figure 10, 11). Interestingly, serum starvation of $ECRF^{Tie2Lo}$-Tie1 cells resulted in overt cell death regardless of the presence of Ang2 or the Tie kinase inhibitor (Figure 10). Stressed endothelial cells secrete high concentrations of Ang2 (Figure 12)(95, 123), and these results suggested that autocrine Ang2 signaling could be responsible for the observed effects on cell survival in the absence of exogenous Ang2.

Next, we further investigated the mechanisms of Ang2-Tie1-mediated cell survival, including that mediated by autocrine Ang2. Since PI3K is upstream of Akt in the cell survival pathway, inhibition of either PI3K or Tie1 would be predicted to block Ang2-induced $ECRF^{Tie2Lo}$-Tie1/Tie2TM cell survival. Indeed, addition of LY294002, a PI3K inhibitor, or the Tie inhibitor significantly inhibited both basal and Ang2-induced viability of $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells following treatment with staurosporine, as measured by trypan blue exclusion (Figure 11A). To investigate the role of autocrine or intracrine Ang2 signaling on staurosporine-induced endothelial cell apoptosis, $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells were pretreated with either a recombinant soluble Tie2-Fc protein or the Tie inhibitor, and apoptosis was subsequently induced by the addition of staurosporine. Treatment with either Tie2-Fc or the Tie inhibitor reduced cell viability

(Figure 11B). Furthermore, there was no significant difference between treatment with Tie2-Fc or the Tie inhibitor, indicating that the Ang2-mediated protective effects are due to autocrine (i.e., secreted) Ang2 and not intracrine signaling, which was shown previously for VEGF-mediated endothelial cell survival(189). Finally, Ang2-Tie1 signaling resulted in significant inhibition of staurosporine-induced apoptosis as measured by effects on levels of activated caspase-3 and -7 (Figure 11C). Taken together, these results demonstrate that Ang2-Tie1 signaling inhibits endothelial cell apoptosis through a PI3K/Akt pathway that is Tie2 kinase-independent and is activated by autocrine secretion of Ang2.

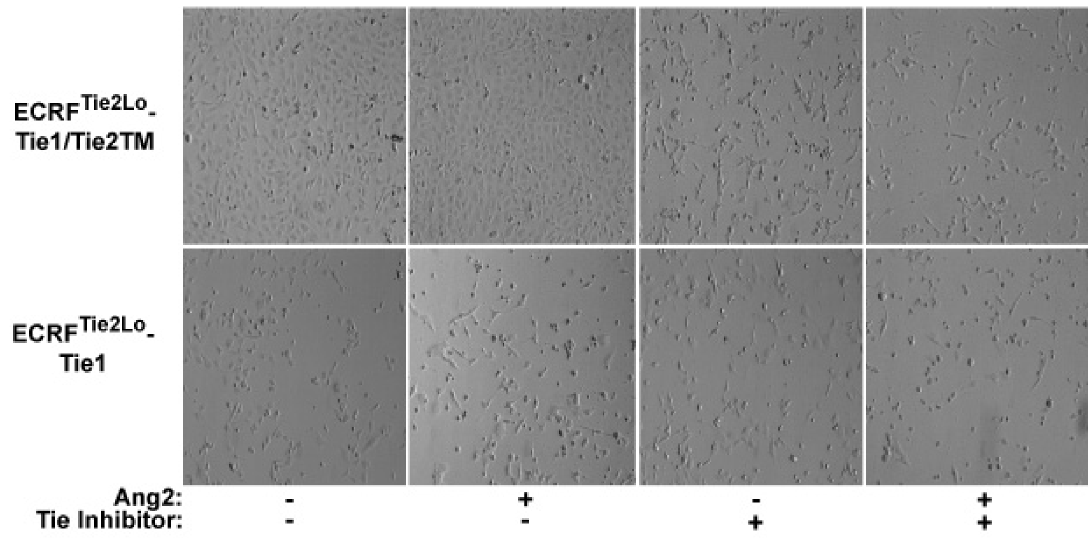

Figure 10: Ang 2 activation of Tie1 prevents starvation induced apoptosis

Serum-starved ECRF-Tie2Lo/Tie1/Tie2TM or ECRF-Tie2Lo/Tie1 cells were treated with or without Ang 2 (1000 ng/mL) and Tie inhibitor (1.25 uM) for 48 hours.

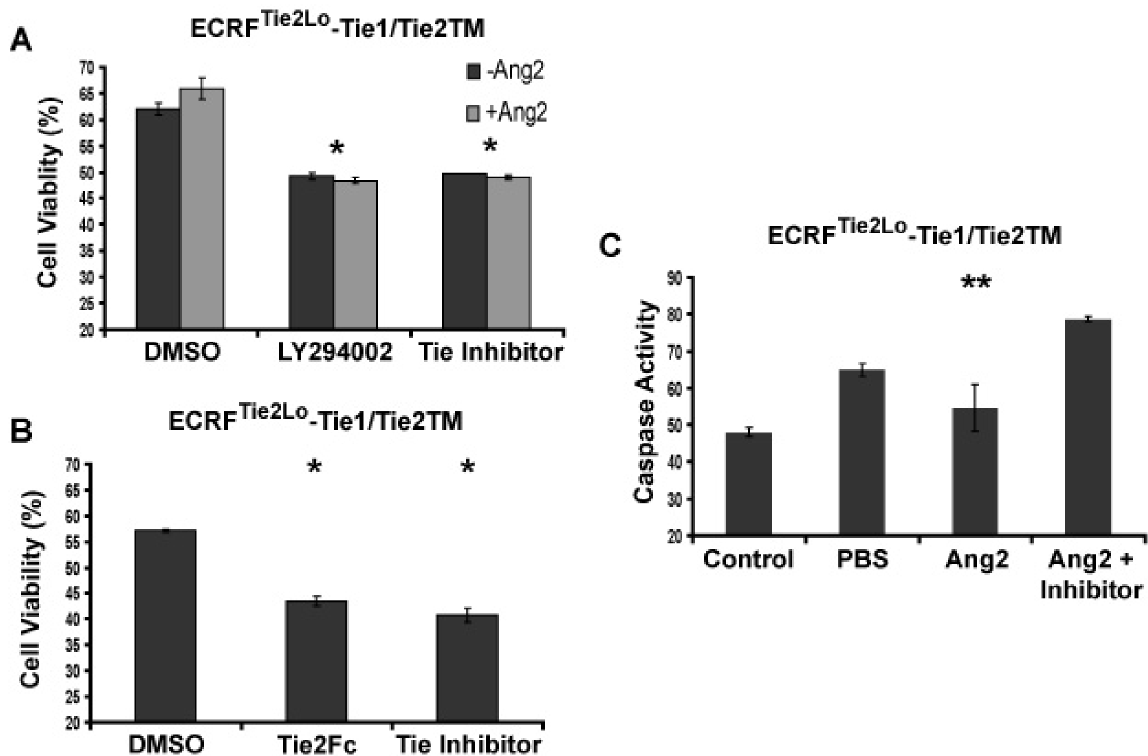

Figure 11: Ang 2 activation of Tie1 induces Akt-mediated cell survival cascades (2)

(A, B) Serum-starved ECRF-Tie2Lo/Tie1/Tie2TM cells were treated with Ang2 and the inhibitors indicated, and viability assessed as described in B. *P<0.005 by ANOVA. (C) Serum-starved ECRF-Tie2Lo/Tie1/Tie2TM cells were treated with or without Ang2 (1000 ng/mL) and Tie inhibitor (1.25 uM). Effects on activation of caspase 3/7 were quantified. **P<0.05 by ANOVA.

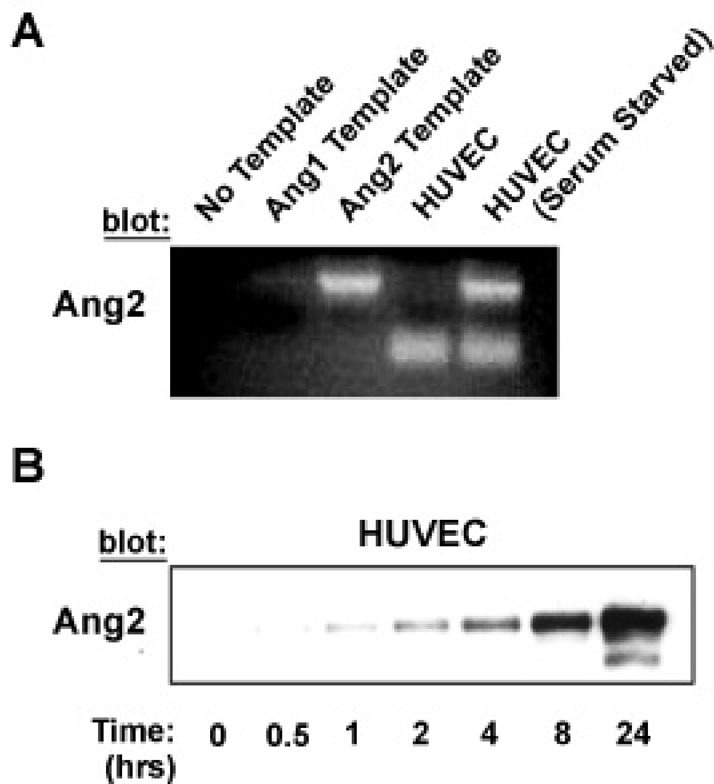

Figure 12: Starved ECs secrete Ang2 in a time dependent manner

(A) RT-PCR of Ang2 mRNA levels of unstarved and starved HUVECs. Templates comprised of scramble, hAng1, and hAng2 sequences were used to confirm specificity of the Ang2 primers. (B) Conditioned media from serum starved HUVECs from the indicated time points was Western blotted with anti-Ang2 antibodies.

Results obtained by Julie Roy

2.3.6 Ang2-Tie1 signaling induces endothelial cell proliferation

Activation of Akt can lead to increases in cell proliferation as well as cell survival, and we noted that $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells treated with Ang2 appeared to grow more densely than corresponding vehicle-treated cells (Figure 10, top row, compare panels 1 and 2). To investigate whether Ang2-induced activation of Tie1 would lead to mitogenesis, $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells were serum starved to synchronize them in the cell cycle, and then they were either left untreated or were treated with Ang2 in the presence or absence of the Tie kinase inhibitor, and proliferation was assessed. As observed previously, stimulation with Ang2 appeared to increase cell number compared to control-treated cells, and this effect was blunted by the Tie inhibitor (Figure 13A). To quantify the extent of cell proliferation, incorporation of ethynyl-deoxyuridine (EdU) was assessed by flow cytometry. EdU incorporation was significantly greater in Ang2-treated $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells than in control (PBS)-treated cells. Treatment with the Tie kinase inhibitor significantly inhibited Ang2-mediated proliferation (Figures 13B and C). Moreover, the Tie inhibitor significantly blunted the basal rate of DNA synthesis observed in PBS-treated cells, consistent with autocrine Ang2 inducing Tie1-mediated cell proliferation (Figures 13B and C). To confirm that EdU incorporation corresponded with DNA synthesis, cells were simultaneously labeled with propidium iodide. As expected, cells that had incorporated EdU were in the G2/M phases of the cell cycle, while those without EdU incorporation were those in the G0/G1 phases of the cell cycle (data not shown).

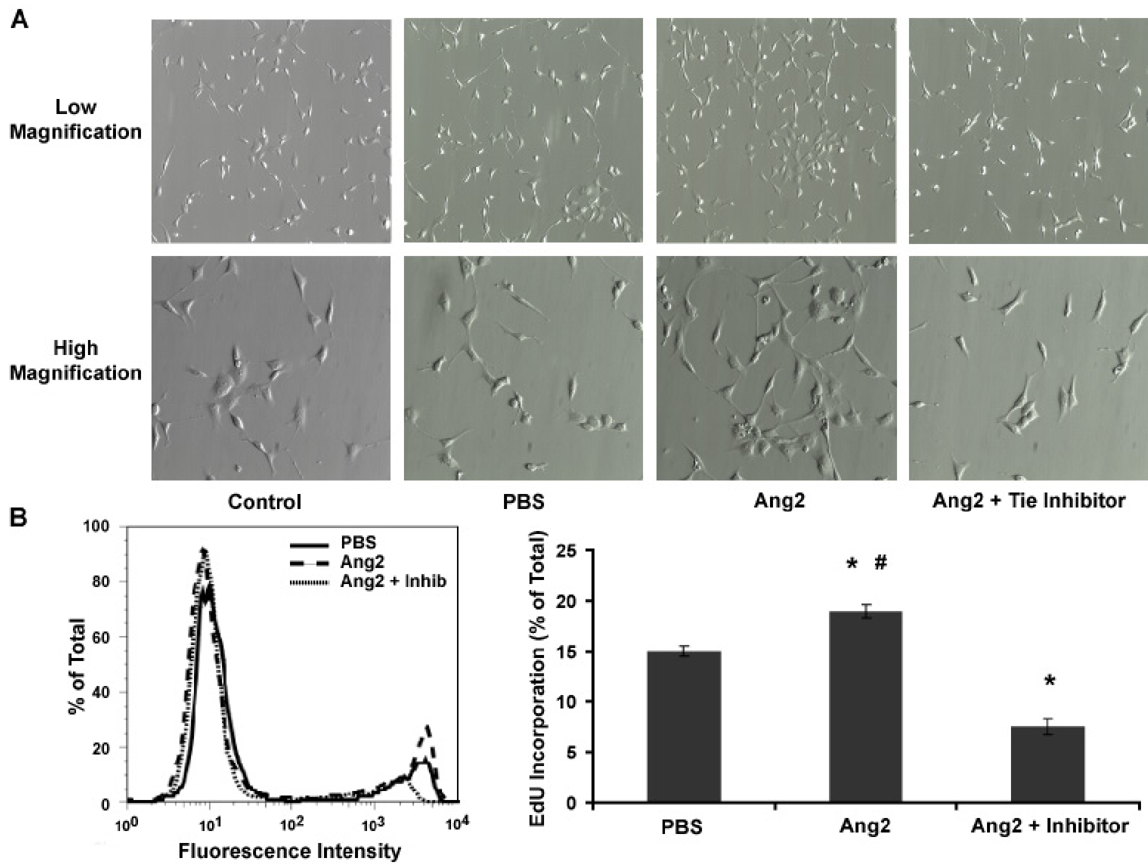

Figure 13: Ang2 activation of Tie1 induces cell proliferation

Serum-starved ECRF-Tie2Lo/Tie1/Tie2TM cells were treated with or without Ang2 (1000 ng/mL) and Tie inhibitor (1.25 uM). (A) Proliferation was visualized after 16 hrs stimulation. (B, C) DNA synthesis was quantified using EdU incorporation and flow cytometry of stimulated cells. Alexafluor 488 was conjugated to the EdU incorporated into the cells, and the fluorescence intensity of the 488 conjugate was determined as the measure of EdU incorporation. *,#P<0.005 by ANOVA.

2.3.7 Loss of Tie1 expression enhances Angiopoietin-Tie2 signaling

Previous studies of Ang2 have shown conflicting results with respect to its effects on Tie2 activation, signaling, and cellular responses, leading to its characterization as a context-dependent Tie2 ligand. Furthermore, recent studies have found that Ang-Tie2 signaling is reduced in the presence of Tie1 expression(153, 162). Therefore, we evaluated the effects of loss of Tie1 expression on Ang2-Tie2 signaling in our system. ECRF cells stably expressing a Tie1-specific shRNA were generated using recombinant retrovirus. Tie1 expression in the resultant $ECRF^{Tie1Lo}$ cells was reduced to less than 10% of parental levels, while Tie2 expression was unaffected (Figure 14A). $ECRF^{Tie1Lo}$ cells were treated with Ang1 or with Ang2 in the absence or presence of the Tie inhibitor. In contrast to the effects observed in parental ECRF cells (Figure 6B), in the absence of Tie1 both Ang1 and Ang2 induced robust tyrosine phosphorylation of Tie2, the latter of which was blocked by the Tie kinase inhibitor, consistent with Ang2-induced Tie2 autophosphorylation (Figure 14B). To investigate a physiological effect of Ang2 in this context, $ECRF^{Tie1Lo}$ cells were serum starved then treated as in Figure 14B, and effects on staurosporine-induced apoptosis were determined. Stimulation with either Ang1 or Ang2 led to a significant improvement in cell viability, as measured by trypan blue exclusion, while addition of the Tie inhibitor blunted the pro-survival effects of Ang2 (Figure 14C). These results demonstrate that Ang2's agonist effects are not exclusive to Tie1. Furthermore, these findings suggest that Tie1 plays an important role in modulation of Ang2-Tie2 signaling.

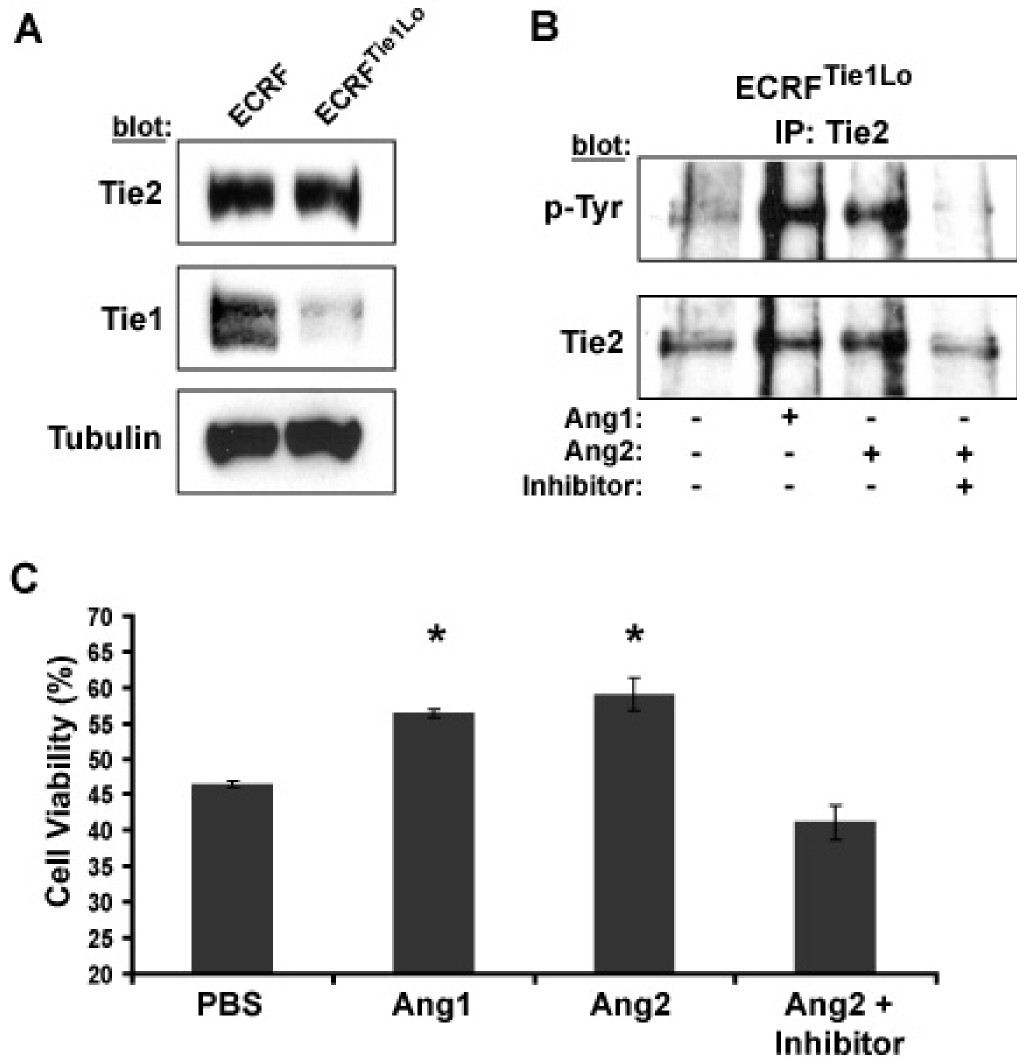

Figure 14: Angiopoietins activate Tie2 and lead to induction of cell survival cascades in the absence of Tie1

(A) Whole cell lysates of various ECRF cell lines express varying levels of Tie1. Proteins were detected by Western blotting with the indicated antibodies. (B, C) ECRF-Tie1Lo cells were treated with or without Ang1 or Ang2 (1000 ng/mL) and Tie inhibitor (1.25 uM) for 15 minutes. (B) Tie1 was IP'd from cell lysates, and proteins were Western blotted with the indicated antibodies. (C) Effects on staurosporine-induced Trypan Blue cell viability were quantified. *P<0.005 by ANOVA.

2.4 Discussion

The importance of Tie1 in vascular development has been demonstrated clearly by knockout studies, which demonstrated embryonic lethality due to defects in vascular integrity(57, 62). However, studies investigating the signaling pathways and corresponding functional effects of Tie1 have been limited by the lack of a known ligand. Previously, we circumvented this problem by using a chimeric receptor containing the kinase domain of Tie1 to demonstrate that Tie1 signaling can activate PI3K/Akt and promote cell survival(151). Importantly, these findings had not been validated with the full-length Tie1 receptor in endothelial cells. Recent reports have shown that Ang1 and Ang4 can activate Tie1 in the presence of Tie2(152) and that Tie1 and Tie2 can heteromultimerize(152, 158), but no physiological effects have been ascribed to these interactions. Results in this chapter extended our prior observations by demonstrating that Ang2 is able to activate Tie1 in endothelial cells. In addition, we developed a model system utilizing a truncated Tie2 receptor, which allowed us to eliminate any effects of the Tie2 kinase or Tie2-associated cytoplasmic molecules and, therefore, to demonstrate effects of Ang2 transduced specifically through Tie1. Using this system, we found that Ang2-Tie1 signaling activated PI3K/Akt, resulting in increased endothelial cell survival and mitogenesis. Furthermore, we found that the survival and proliferative effects of Tie1 were mediated in part by autocrine, endothelial cell-secreted Ang2. Finally, consistent with other recent reports(158, 162), expression of Tie1 resulted in modulation of Ang2-Tie2 signaling in endothelial cells.

An important finding in this chapter is that the Tie1 kinase is able to transduce Ang2-mediated signals in a manner that is independent of the Tie2 kinase. However,

Ang2 was unable to activate Tie1 unless at least the Tie2 ECD was present. Saharinen et al first demonstrated that Ang1 and Ang4 could induce phosphorylation of Tie1 when it was co-expressed with Tie2(152). Moreover, Ang1-mediated Tie1 phosphorylation occurred even in the presence of a kinase-inactive Tie2 mutant, consistent with Tie1 undergoing autophosphorylation. In that study, however, it was possible that a Tie2-associated kinase (e.g., a non-receptor tyrosine kinase) could mediate Tie1 phosphorylation, although our prior results with the chimeric Tie1 kinase suggest that this is unlikely. In our current chapter, use of the Tie2TM construct definitively establishes that Ang2-Tie1 signaling occurs independently of the Tie2 kinase. Our findings contrast somewhat with those of Saharinen et al, who were unable to demonstrate Ang2-mediated activation of Tie1(152). Furthermore, we did not observe robust Ang1-mediated activation of Tie1, as they reported. Although the reasons for these differences are unclear, we speculate that they are related to differences in the particular ligand preparations and cell systems used by each of our groups. Importantly, however, our results are consistent in the observation that Angiopoietin-mediated Tie1 activation requires contributions from Tie2.

In this chapter, the finding that Ang2 induces Tie1 *auto*phosphorylation was supported by the observed reduction in signaling and downstream responses in the presence of a pharmacological Tie kinase inhibitor. A limitation of this ATP mimetic is that it could have off-target effects (i.e., non-Tie kinases), however concentrations used were close to the IC_{50} for Tie2, which was not present in our assays, thereby increasing the likelihood that observed effects were due to inhibition of Tie1. It is important to note that the absence of the Tie2 kinase domain did not abolish Ang2-mediated activation of downstream signaling pathways, supporting the idea that these pathways are

transduced via the Tie1 kinase. Notably, these findings are novel, as no functional effects have previously been attributed to Angiopoietin-mediated Tie1 activation.

The observation that Ang2 induced Tie1 activation in the presence of the Tie2TM protein implies that Ang2 is able to cluster several Tie1 molecules, thus enabling Tie1 autophosphorylation (Figure 15). Based on previous reports, Ang2 has been thought to exist primarily as a dimer, although trimers and higher order multimers have been described in solution(96-97). In contrast, Ang1 activates Tie2 as an obligate tetramer, a conformation that would enable Tie1-Tie2 heteromultimererization and trans-phosphorylation, as suggested previously(152). We have found that a substantial population of Ang2 exists in a higher order multimeric state (e.g., tetrameric; Otvos, Balint; Roy, Julie; Kontos, Christopher, unpublished observations), which presumably would be necessary for Tie1 activation after binding at least one Tie2TM molecule. Our data, along with that of others, suggest that Tie2 binds Ang2 (or other Angiopoietins) and then is able to present it to Tie1, which can then be stabilized within a multimeric complex and undergo phosphorylation either by another Tie1 molecule or by Tie2 within the complex. Assuming one receptor-binding site per ligand molecule, activation of Tie1 in our model system would require at least one Tie2TM molecule and two Tie1 molecules, thus Ang2 would have to be at least trimeric (Figure 15). Importantly, these findings suggest that different oligomeric forms of Ang2 could differentially recruit Tie1 following Tie2 binding, thereby determining the activation state of the Tie receptors and the ultimate biological effect on the vasculature. The implications of these findings are particularly important for understanding the biology of Ang2, which is widely recognized to have context-dependent effects. Thus, it is likely that Tie1 plays an important role in modulation of Ang2-Tie2 signaling and function.

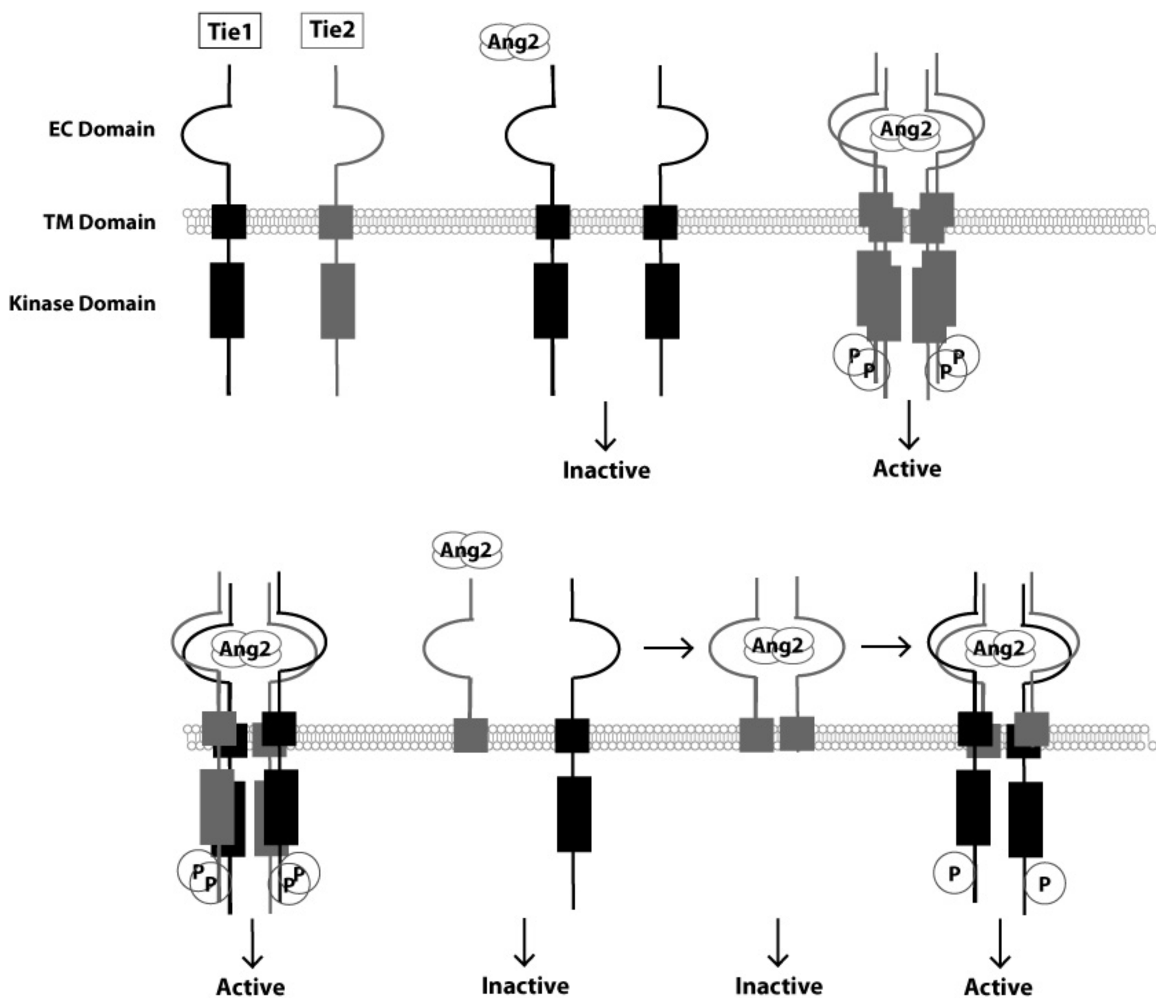

Figure 15: Proposed mechanism of action of Ang2-induced activation of Tie receptors

Tie1 is indicated by a black receptor; Tie2 is indicated by a gray receptor (top left). In absence of Tie2, Ang2 is unable to bind to or multimerize Tie1. In contrast, Tie1 is dispensable for Tie2-Ang2 binding, although phosphorylation is dependent on the cellular context (top center and top right). Ang2 induces Tie1/Tie2 heteromultimer formation and subsequent phosphorylation of receptors, although the degree to which Tie2 is required for this is unclear (or unable to be elucidated) (bottom left). In the presence of a truncated Tie2 mutant, Ang2 binds to Tie2Tm receptors, which are unable to signal but facilitate Tie1 recruitment and multimerization. Heteromultimerization leads to trans-phosphorylation of Tie1 kinase domains, consistent with the clustering of multiple Tie1 receptors in this complex (bottom center and right).

The biological effects of Ang2-Tie1 signaling observed here may also shed light on the results of several recent studies of both Ang2 and Tie1. Although knockout of Ang2 in mice does not cause embryonic lethality, it results in perinatal death due to abnormal lymphatic vessel remodeling(78). Interestingly, Ang2 was found to be highly expressed in arterial vascular smooth muscle, although no overt arterial phenotype was observed in Ang2$^{-/-}$ mice, indicating that Ang2 is not required for arterial specification or remodeling during development(78). However, we recently found that exogenous delivery of Ang2 significantly inhibited the development of aortic atherosclerosis in ApoE$^{-/-}$ mice, consistent with a role for Ang2 in arterial vascular maintenance(83). Although this result might seem inconsistent with previous descriptions of Ang2 as an antagonist or vascular destabilizing factor, our demonstration that Ang2-Tie1 signaling promotes endothelial cell survival suggests a possible mechanism for Ang2's beneficial effect on atherosclerosis. Porat et al demonstrated that Tie1 expression is increased at points of disturbed blood flow, such as arterial branch points and aortic valve leaflets, which are prone to atherosclerosis development (149). Upregulation of Tie1 and subsequent activation by Ang2 could therefore have an important vasculoprotective effect by promoting endothelial cell survival and enhancing barrier function, thereby limiting the development of atherosclerosis.

In addition to effects on blood vessels, Ang2-Tie1 signaling may also be physiologically relevant in the setting of lymphatic vessel development and maintenance. Two separate reports demonstrated that Tie1$^{-/-}$ mice died in mid- to late gestation due to abnormalities of vascular integrity(57, 62). Recently, however, D'Amico et al developed a mouse homozygous for a hypomorphic Tie1 allele, which survived longer than Tie1-null mice, allowing a more detailed developmental analysis. These mice were found to

have lymphatic vascular defects with onset of edema before the development of hemorrhage or other overt blood vascular abnormalities, indicating that Tie1 is required for both lymphatic and blood vessel development. Taken together with our findings and those in Ang2 knockout mice, it is possible that Ang2-Tie1 signaling plays an important role in proper lymphatic vessel development and maintenance.

Consistent with previous reports, findings in this chapter demonstrate that Tie1 can modulate Ang2's effects on Tie2 in endothelial cells. Silencing of Tie1 led to robust Ang2-mediated activation of Tie2 and increased cell viability. These findings have important implications in the context of angiogenesis and vascular remodeling. Since VEGF has been shown to induce Tie1 extracellular domain shedding(144, 190), upregulation of VEGF would be predicted to result in loss of Ang2-Tie1 signaling and a shift toward Ang2 signaling via homomultimeric Tie2 complexes. This could result in hyperactivation of Tie2 by Ang2, or, at a later stage of vessel remodeling, by Ang1. In this context, Tie1 may in fact play an important modulatory role by limiting excess Tie2 signaling, not unlike the role of VEGFR-1 in limiting excess VEGF signaling(23, 191). Activating mutations of Tie2 (R849W, Y897S) have been observed in the setting of inherited venous malformations, suggesting that dysregulated Tie2 activity leads to vascular dysmorphogenesis(61, 192-193). These findings suggest that vascular integrity is regulated by very precise control of Angiopoietin-Tie receptor signaling and that functional Tie1 on the surface of endothelial cells may act as a rheostat to modulate the vascular activation state.

Although much work remains to be done to define the precise role of Tie1 in vascular function, this chapter is the first to show that Ang2 can signal specifically through Tie1 and to demonstrate that this results in physiological responses in

endothelial cells. Moreover, our results define the role of Tie2 in this process by demonstrating that it does not require the cytoplasmic kinase domain, but that the Tie2 extracellular domain is necessary for Ang2 binding. Further studies elucidating the precise nature of the interactions among the Tie receptors and Angiopoietins may lead to an improved understanding of vascular growth and remodeling in both physiological and pathological states.

3. Angiopoietin-2 induces Tie1 internalization and downregulation by a lysosomal, activation independent pathway

3.1 Introduction

Since downregulation of receptors constitutes a critical mechanism for the regulation of signaling cascades and we observed ligand-mediated activation of Tie1, we hypothesized that Ang2 would also elicit Tie1 downregulation cascades. Following ligand binding, receptors are internalized and either recycled or degraded. Tie1 and Tie2 comprise a family of RTKs that are expressed primarily on endothelial cells and that have essential roles in both embryonic vascular development and the adult vasculature(45, 48, 56-57). Mice lacking Tie1 die in mid to late gestation due to a lack of vessel integrity leading to edema and hemorrhage while Tie2 knockout mice fail to produce branched, mature vessels(57, 62). Substantial data demonstrate that the Tie receptors and their ligands, the Angiopoietins, regulate the transition from mature, quiescent vessels to an angiogenic phenotype, with Ang1 acting as a requisite agonist and Ang2 acting as a context-dependent agonist or antagonist(66, 70-72). Structurally, the angiopoietins are comprised of an N-terminal superclustering domain, a coiled-coil oligomerization domain, and a C-terminal fibrinogen-like receptor binding domain. Although monomers of the fibrinogen like domains are able to bind Tie2 in solution, Angiopoietins form higher order multimeric structures that are able to bind to and appropriately cluster the Tie receptors to induce activation and downstream signaling cascades(96-97, 180).

Initially, signaling through the Tie receptor axis was thought to occur exclusively through Tie2 due to Tie1's inability to bind to the Angiopoietins in the absence of

Tie2(70-71). However, Tie1 has been found to heteromultimerize with Tie2 thereby resulting in ligand-mediated receptor phosphorylation(153, 158). Tie1/Tie2 heteromeric complexes have been shown to inhibit signaling through Tie2, and removal of the sequestering role of Tie1 leads to hyperactivation of Tie2 by Ang1(153, 159, 162, 183). Recently, Seegar et al, using FRET, demonstrated that Tie1 and Tie2 form pre-existing heteromers with Ang1 destabilizing the complexes and Ang2 actually stabilizing the heteromeric complexes(156). Taken together, the differences between signaling though the Tie receptors may be due to the different receptor associations induced by various Ang ligands.

A number of recent studies demonstrated a mechanism by which Tie1 shedding and cleavage modulates ligand responsiveness to Tie2. VEGF or PMA stimulation of endothelial cells induces Tie1 extracellular domain cleavage and subsequent proteolytic processing of the kinase domain. The processing is limited to Tie1, and consequently, upon Tie1 degradation, the cells are hyperactivatable along the Ang1/Tie2 axis(157, 159, 162, 183). Although this mechanism demonstrates one way to modulate the Ang/Tie signaling cascade, it doesn't address either the subsequent feedback signals or the differences with regards to multimer stability upon ligand binding. Other groups have observed modest Tie1 phosphorylation upon Ang1 binding(140, 153) and we have recently shown that Ang2 induces Tie1 activation after heteromultimer formation. Coupled with Seegar et al's results describing the increased stability of Tie1/Tie2 complexes upon Ang2 binding, more work must be done to elucidate the potential feedback mechanisms that are employed upon ligand associations.

In this chapter, we investigated the roles of the Angiopoietins in inducing the downstream internalization of the Tie1 receptor. Recently, it has been demonstrated

that ligand-mediated Tie2 processing occurs by way of ubiquitination and internalization into clathrin coated pits, in much the same mechanism as regulation of the EGFR(141-142). Therefore, based on structural and functional similarities between Tie1 and Tie2, we hypothesized that Ang2-mediated Tie1 processing would occur along analogous pathways. Ang2 but not Ang1 was found to lead to a concentration- and time-dependent downregulation of Tie1 from the extracellular surface of endothelial cells. Tie2's role in mediating this process was simply to allow for the association and presumed stabilization of Tie heteromultimeric complexes upon Ang2 binding. Furthermore, Tie1 processing was found to occur by way of receptor internalization and subsequent degradation by lysosomal compartments. Finally, receptor activation was found to be superfluous for downregulation of Tie1, raising the possibility of the association of other, as of yet unknown, proteins to the intracellular domains of Tie1 that mediate the process of internalization. These findings highlight a distinct mechanism of Tie1 downregulation that is a result of ligand binding to the receptor complexes, and have broad implications in understanding the role of Tie1 specifically with regards to the modulation of Tie2 signaling and broadly in the whole of endothelial cell biology.

3.2 Methods

3.2.1 Reagents and cell culture

Mouse monoclonal Tie1 antibodies (clones 5D2, 42G10) were generously provided by Dr. Harvey Yamane (Amgen). The rabbit polyclonal anti-Tie1 (C-18) antibody was from Santa Cruz Biotechnology. Rat monoclonal anti-α-tubulin (clone YL1/2) antibody was from Serotec. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Alexafluor 568 goat anti-mouse IgG secondary antibody and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. Recombinant

human angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) were purchased from R&D Systems. Ethidium bromide was purchased from Sigma. The Tie kinase inhibitor (4-(6-Methoxy-2-napthyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidizole), cat. # 612085) was purchased from Calbiochem.

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords essentially as described by Marin et al. Briefly, cords were flushed twice with RPMI medium (Invitrogen) and treated with collagenase (Sigma) to dislodge endothelial cells. Isolated cells were cultured in Endothelial Basal Medium-2 (EBM-2, Clonetics) containing growth factors and 5% fetal bovine serum (FBS; EGM-2-MV, Clonetics), according to the manufacturer's instructions. Endothelial cell populations were confirmed by immunostaining for CD31 (PECAM-1), cultured in EGM-2, and used between passages 1 and 4. EC-RF24 cells (ECRF) were generously provided by Dr. Ruud Fontijn (VU University Medical Center, Amsterdam) and cultured in EGM-MV (Clonetics) containing 10% FBS (Gibco). Modified ECRF cells were generated and cultured as described previously.

3.2.2 Immunoprecipitations and Western blotting

Protein concentrations of cell lysates were determined using a BCA protein assay (Pierce Biotechnology). For immunoprecipitations (IPs), antibodies against Tie1 (clone 5D2) were added to lysates and allowed to bind at 4℃ for 16 hrs followed by addition of Protein G sepharose (GE Biosciences) for 1 hr at 4℃. Antibodies and bound proteins were eluted by boiling into Laemmli sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and Western blotted with the indicated antibodies.

3.2.3 Cell assays

To measure Tie receptor downregulation, endothelial cells were seeded onto dishes coated with gelatin (0.2%, Sigma). Cells were cultured in growth medium until 90% confluent, washed once with EBM, and the medium was replaced with EBM containing 0.5% FBS. The cells were then starved for 3.5 hrs followed by treatment with EBM containing cycloheximide (5 μg/mL in DMSO, Sigma) for 30 min. The cells were treated with or without Ang1 or Ang2 (100-1000 ng/mL in PBS + 0.1% BSA; R&D Systems) and cycloheximide for the times indicated. The cells were lysed with RIPA lysis buffer (100 mM KCl, 25 mM EDTA, 5 mM $MgCl_2$, 10 mM HEPES pH 7.0, 0.5% IGEPAL CA-630, 1% Triton X-100, 0.1% SDS, 10% glycerol) supplemented with sodium orthovanadate (1 mM) and protease inhibitors (Complete, Roche).

To observe changes in cellular localization of Tie1, HUVECs were seeded on glass coverslips and grown until 90% confluent, washed with EBM, and starved for 4 hrs in EBM containing 0.5% FBS. The cells were treated with or without Ang1 or Ang2 (500 ng/mL in PBS + 0.1% BSA) for 30 min. The cells were washed 3 times with PBS followed by fixation with 4% paraformaldehyde (Sigma) at 4℃ for 15 min. Permeabilization of the cellular membranes was achieved by incubating the cells in ice-cold methanol (Sigma) at -20℃ for 5 min. Cells were washed 3 times with PBS, and nonspecific antibody binding was blocked by incubation of cells with 5% goat serum at room temperature (RT) for 1 hr. The cells were washed with PBS, incubated with anti-Tie1 antibodies (clone 42G10) in 5% goat serum at RT for 1 hr, washed again, and treated with Alexafluor 568-conjugated anti-mouse antibodies in 5% goat serum at RT for 1 hr. After subsequent washing with PBS, cells were treated with DAPI (1:20,000 dilution in PBS) at RT for 2 min. Cells were subsequently washed and allowed to adhere

onto the surface of glass microscope slides. Cells were visualized by fluorescence microscopy, and digital images were obtained with an Olympus IX70 microscope connected to a PaxCam ARC digital camera.

To determine lysosome- versus proteasome-mediated receptor downregulation, endothelial cells were seeded on dishes coated with gelatin and grown to confluence as described previously. Cells were starved for 2 hrs, after which they were treated with EBM containing 0.5% FBS supplemented with cycloheximide (5 μg/mL) and chloroquine (250 μM in EtOH, Sigma) or MG132 (21 μM in DMSO, Sigma) for 2 hrs. The cells were then stimulated with Ang1 or Ang2 in the continued presence of the inhibitors, then lysed and blotted as described.

To determine the requirement for Tie kinase activity for receptor internalization, endothelial cells were seeded on dishes coated with gelatin and grown to confluence, as described previously. Cells were starved for 3.5 hours, after which they were treated with EBM containing 0.5% FBS supplemented with the Tie inhibitor (1.25 μM in DMSO). The cells were then stimulated with Ang1 or Ang2 in the continued presence of the inhibitors, lysed, and blotted as described.

3.2.4 Semiquantitative PCR

To measure mRNA transcript levels, cells were seeded onto dishes coated with gelatin, grown to confluence, starved, and treated with Ang1 or Ang2 (500 ng/mL in PBS + 0.1% BSA) for 30 min as described. Total RNA was extracted from cells using TRIZol (Invitrogen), and cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) and oligo(dT) primers. Tie1 mRNA levels were assessed by semiquantitative PCR with the following primers derived from the kinase domain of Tie1: forward primer, 5'-CCCCAACATCATCAACCTCCTG-3'; reverse primer, 5'-

GGCTCACTATCTCCCAAAGAAGG-3'. These primers amplified a 455 bp PCR product, and band intensities were assessed after 24 and 27 cycles.

3.2.5 Statistical analysis

All blots shown are representative of at least three separate experiments. All graphs utilized the normalized averages of five separate experiments. All results were expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the one-tailed Student's *t*-test (two sample, unequal variance), and $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Ang2, but not Ang1 induces Tie1 downregulation in endothelial cells

To investigate the mechanisms governing Tie1 downregulation in endothelial cells, we used EC-RF24 cells (ECRFs), an immortalized endothelial cell line derived from primary human umbilical vein endothelial cells. This cell line allowed us to stably manipulate expression of Tie1 and Tie2 to evaluate effects of different receptor mutations on Tie1 internalization. We developed a cell line expressing endogenous levels of Tie2 in which expression of Tie1 has been increased dramatically by way of a stable retroviral infection of native ECRF cells, termed ECRF-Tie1. To compare the effects of the Angiopoietins on downregulation of Tie1, cells were treated with cycloheximide (CHX) to prevent new protein synthesis, and stimulated with either Angiopoietin 1 (Ang1) or Angiopoietin 2 (Ang2) in the presence of CHX. Stimulation with Ang2 for 30 min led to a robust dose-dependent downregulation of Tie1 in ECRF-Tie1 cells while Ang1 had no effect on Tie1 expression at concentrations up to 1000 ng/mL (Figure 16A). Futhermore, stimulation with an intermediate concentration (500 ng/mL) of

Ang2 led to a time-dependent downregulation of Tie1 in ECRF-Tie1 cells (Figure 16B). Stimulation with Ang1 induced minimal receptor downregulation compared to Ang2. Reduced Tie1 levels were observed after 60 min of stimulation in both Ang1 and Ang2 stimulated cells which likely reflect basal receptor half life (Figure 16B). To quantify the rate of receptor downregulation, the half-life of Tie1 in endothelial cells in response to Ang1 versus Ang2 was determined by stimulation with the Angiopoietins in the presence of CHX. Stimulation of ECs with Ang2 led to a Tie1 half life of 61 min (Figure 16C). Stimulation of ECs with Ang1 led to a half life of multiple hours which is consistent with prior reports that have quantified the basal half-life of Tie2 as being ~5 fold longer than stimulated receptor(140) suggesting that Ang1 has no added effect (Figure 16C).

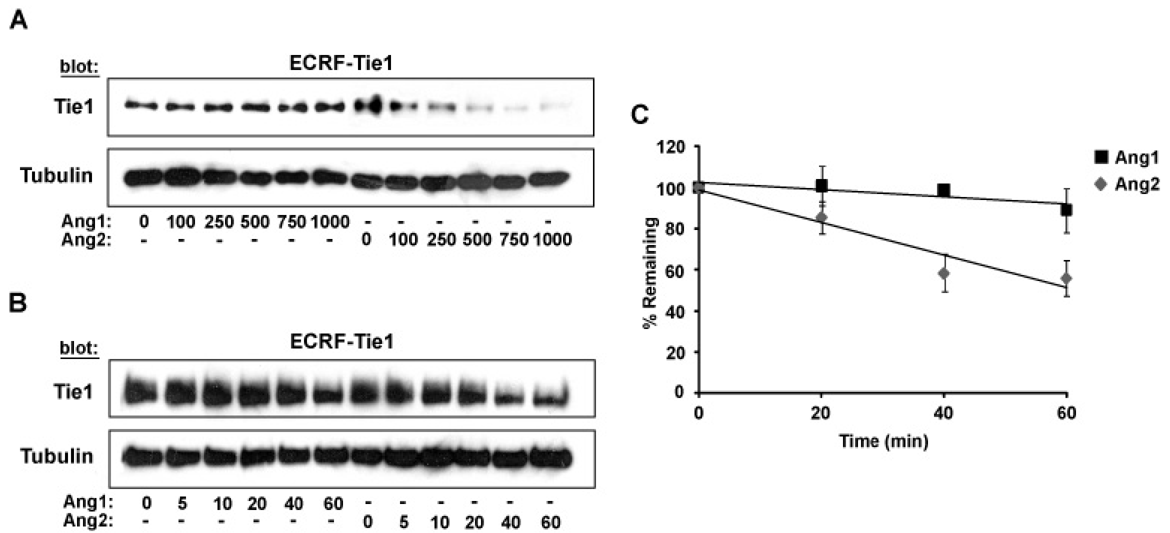

Figure 16: Concentration- and time-dependent Ang2 stimulation induces Tie1 downregulation in endothelial cells

(A) ECRF-Tie1 cells were starved and treated with or without Ang1 or Ang2 for 30 min at the concentrations indicated. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Western blots from whole cell lysates were treated with antibodies against Tie1 and Tubulin. (B) ECRF-Tie1 cells were starved and treated with Ang1 or Ang2 (500 ng/mL) for the times indicated. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Western blots from whole cell lysates were treated with antibodies against Tie1 and Tubulin. (C) ECRF-Tie1 cells were starved and treated with Ang1 or Ang2 (500 ng/mL) as described. Tie1 band intensities were measured by densitometry and normalized against tubulin loading controls. Relative intensities were calculated as a percent of the Tie1 band intensity at 0 min. Tie1 half lives after ligand stimulation were calculated as a linear regression of the average measured relative intensities, and calculated as 61 min following Ang2 stimulation.

3.3.2 Downregulation of Tie1 is not due to transcription repression

To confirm that Ang2-mediated effects on Tie1 were at the post-translational level and not transcriptional we evaluated ligand-induced effects on Tie1 mRNA. To compare Tie1 transcription levels, HUVECs were stimulated with Ang1 or Ang2, and Tie1 mRNA was quantified by semi-quantitative PCR. Stimulation with Ang1 or Ang2 (500 ng/mL) for 30 min led to no change in the total Tie1 mRNA levels indicating that Ang2-induced Tie1 downregulation is not due to alterations in the transcription of Tie1 (Figure 17).

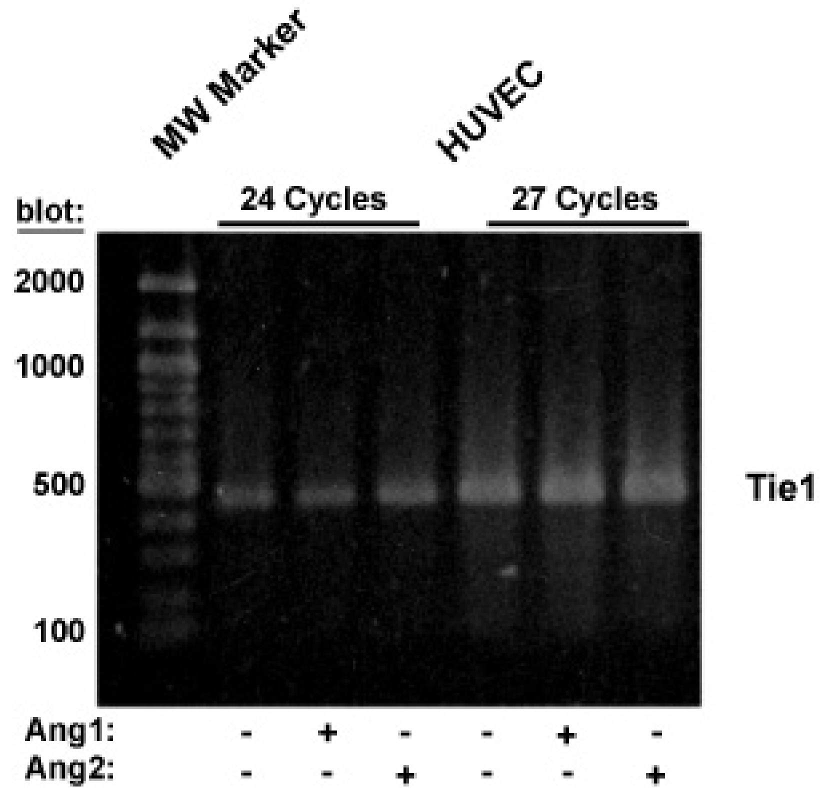

Figure 17: Tie1 expression is not affected by Ang1 or Ang2 stimulation

HUVEC cells were starved and stimulated with Ang1 or Ang2 (500 ng/mL) for 30 min as described. cDNA was generated from total isolated mRNA transcripts using oligo-dT primers. Transcript levels were measured by primers to Tie1 KD and intensities after 24 and 27 cycles were ascertained.

3.3.3 The extracellular and transmembrane domains of Tie2 are sufficient for Ang2-mediated Tie1 downregulation

We have previously shown that Ang2-induced activation of Tie1 is dependent only on the presence of Tie2's extracellular and transmembrane domains, presumably for Ang2 binding to induce proper Tie1/Tie2 heteromultimer formation. Having observed Tie1 downregulation in the presence of Ang2, we sought to determine the role of Tie2 in mediating the process, and whether, as in the case of Tie1 activation, the Tie2 kinase domain is dispensible for Tie1 downregulation. To determine whether Tie2 is required for Tie1 downregulation, we used $ECRF^{Tie2Lo}$ cells, in which Tie2 expression was silenced by ~95%. $ECRF^{Tie2Lo}$-Tie1 cells were stimulated with Ang1 or Ang2 in the presence of cycloheximide. Neither Ang1 nor Ang2 resulted in any downregulation of Tie1 in $ECRF^{Tie2Lo}$-Tie1 cells demonstrating the requirement for Tie2 in Tie1 downregulation (Figure 18A). To further characterize the requirement for the Tie2 cytoplasmic domain and/or kinase activity in mediating the Tie1 response, we used recombinant retrovirus to stably re-express either kinase inactive Tie2 ($ECRF^{Tie2Lo}$-Tie1/K854R cells) or a variant of Tie2 truncated after the transmembrane domain ($ECRF^{Tie2Lo}$-Tie1/Tie2TM cells) in $ECRF^{Tie2Lo}$ cells. Ang2 but not Ang1 stimulation led to a time-dependent downregulation of Tie1 in cells expressing either kinase-inactive Tie2 or the truncated Tie2-TM lacking the cytoplasmic kinase domain (Figure 18B, C). Together, these results indicate that Tie1 downregulation mirrors activation, as the Tie2 extracellular domain, but not its kinase domain, is required for Ang2-mediated downregulation.

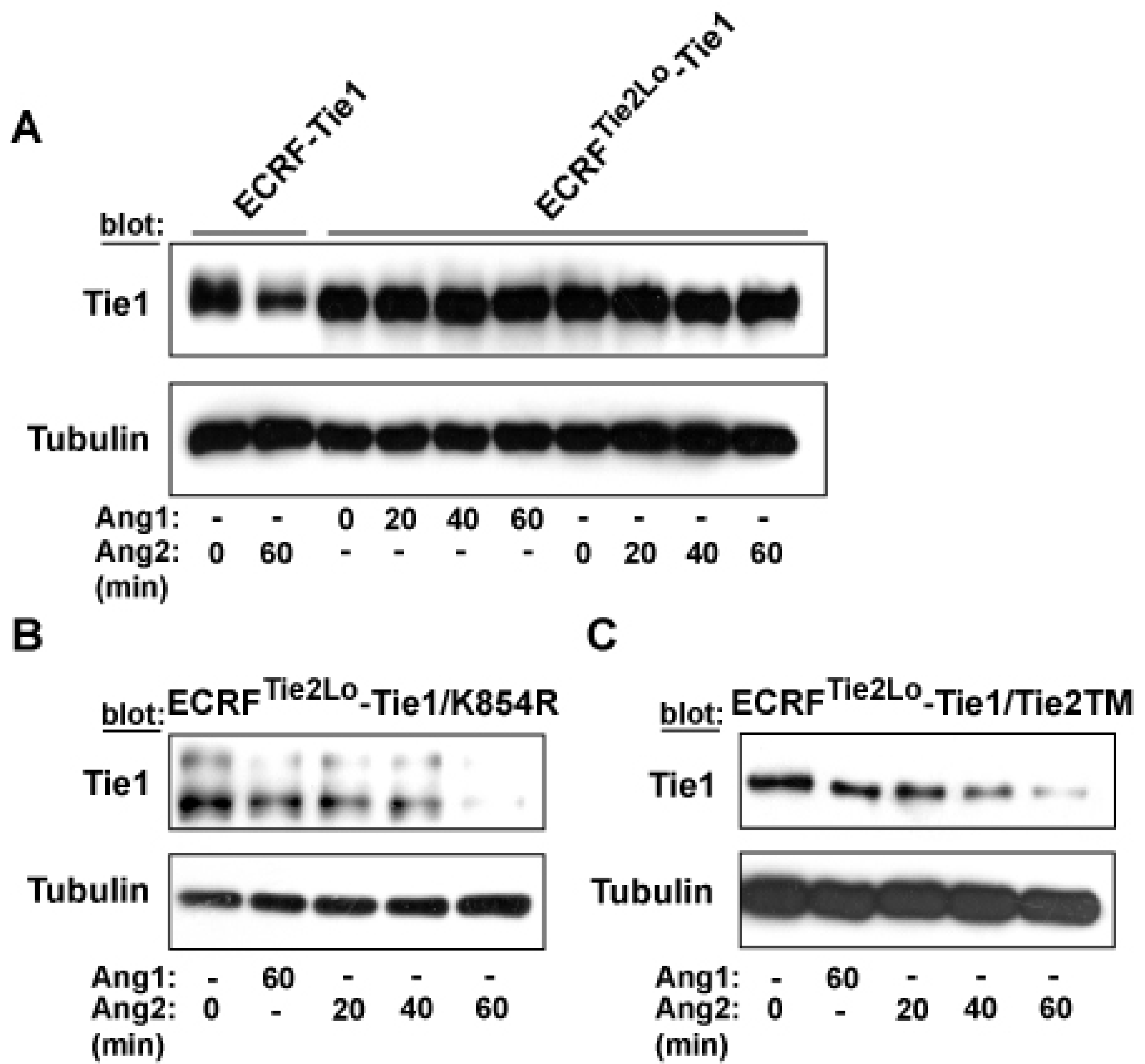

Figure 18: The Tie2 extracellular domain is required for Ang2-mediated downregulation

(A) $ECRF^{Tie2Lo}$-Tie1 cells were grown to confluence, starved, and treated with or without Ang1 or Ang2 (500 ng/mL) for the times indicated. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Western blots from whole cell lysates were analyzed with antibodies against Tie1 and Tubulin. (B) $ECRF^{Tie2Lo}$-Tie1/Tie2K854R cells were grown to confluence, starved, and treated with or without Ang1 or Ang2 (500 ng/mL) for the times indicated. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Western blots from whole cell lysates were treated with antibodies against Tie1 and Tubulin. (C) $ECRF^{Tie2Lo}$-Tie1/Tie2TM cells were grown to confluence, starved, and treated with or without Ang1 or Ang2 (500 ng/mL) for the times indicated. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Western blots from whole cell lysates were analyzed with antibodies against Tie1 and Tubulin.

3.3.4 Ang2 stimulation induces Tie1 translocation from plasma membrane

Upon ligand association, one of the mechanisms by which receptors are processed is the internalization of the ligand/receptor complex and subsequent recycling of the receptor or targeted degradation of the complex. Therefore, we sought to determine whether Ang2 stimulation of endothelial cells would lead to a shift in sub-cellular localization of Tie1. To observe changes in Tie1 localization, HUVECs were stimulated with Ang1 or Ang2 and stained with an antibody specific to the extracellular domain of Tie1. Stimulation of HUVECs by Ang2 but not Ang1 for 60 min led to the removal of Tie1 from the plasma membrane of endothelial cells, as observed by immunoflourescence microscopy (Figure 19).

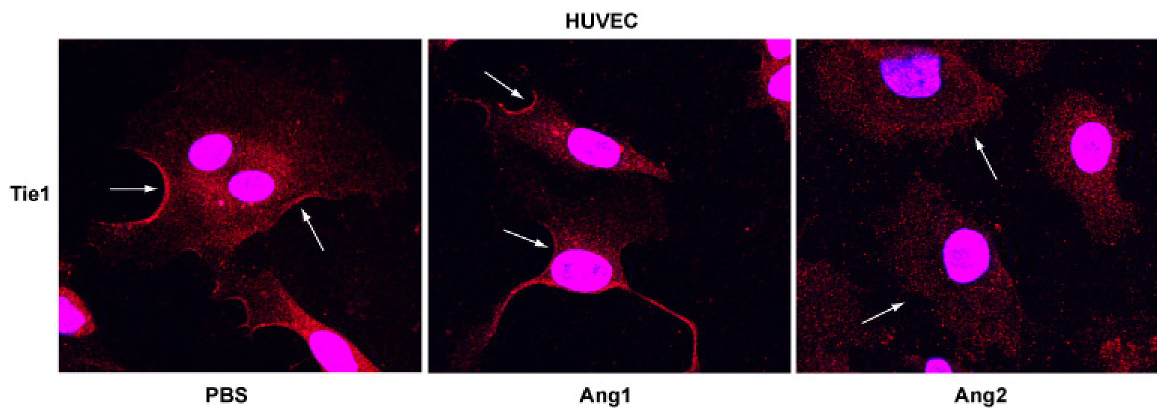

Figure 19: Ang2 induces Tie1 internalization from endothelial cell surfaces

HUVEC cells were seeded on glass coverslips, starved, and treated with or without Ang1 or Ang2 (500 ng/mL) for 60 min. Cells were fixed, permeabilized, and treated with Tie1 antibodies to the extracellular domain of the receptor. Tie1 was visualized by treatment of the cells with a fluorescent antibody, Alexafluor 568. Images were obtained by confocal microscopy, original magnification 400x. Arrows indicate the location of the HUVEC plasma membrane. Images are representative of 4 random distinct microscope fields.

3.3.5 Tie1 is degraded partly in lysosomes

Previous studies from our lab and others have demonstrated that Tie2 is internalized into clathrin-coated pits and degraded within lysosomes. Due to the similar structure and function of Tie1, we speculated that Tie1 follows a similar route of degradation. To determine the mechanism of Tie1 downregulation, we treated ECRF-Tie1 cells with pharmacological inhibitors of either the 26S proteosome (MG-132) or lysosomes (chloroquine), then stimulated cells with the Angiopoietins. Co-incubation with chloroquine, but not MG-132, led to a marked inhibition of the vast majority of Ang2-mediated Tie1 downregulation (Figure 20). These results indicate that Tie1 is downregulated upon Ang2 binding, primarily through lysosomes consistent with previous reports requiring ubiquitination and internalization of Tie2 into endosomes prior to degradation.

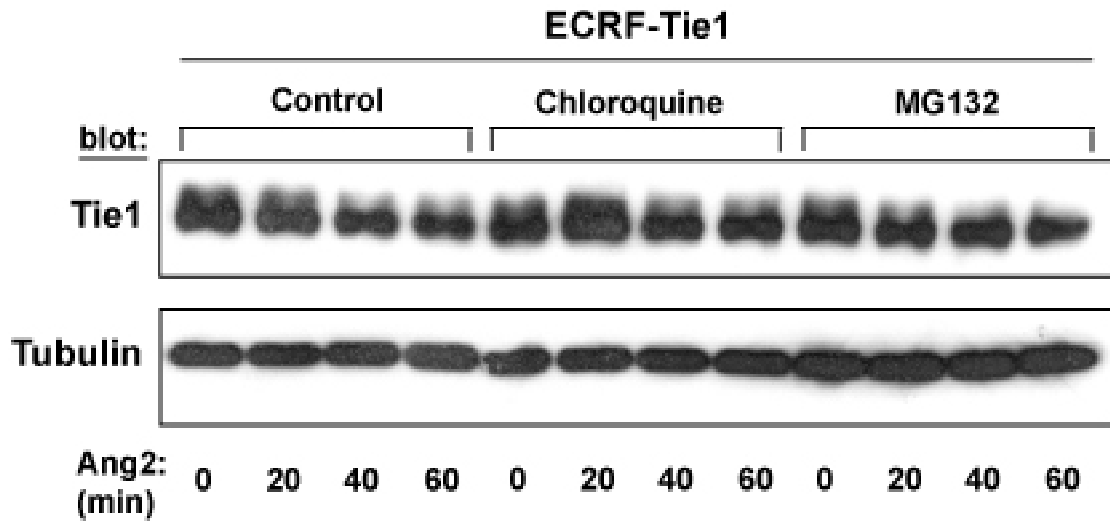

Figure 20: Tie1 downregulation occurs by lysosomal pathways

ECRF-Tie1 cells were grown to confluence, starved, and treated with or without Ang2 (500 ng/mL) for the times indicated. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Indicated cells were also pretreated with either chloroquine, a lysosomal inhibitor (250uM in EtOH) or MG132, a 26S proteosomal inhibitor (21uM in DMSO) for 2 hrs. Western blots from whole cell lysates were analyzed with antibodies against Tie1 and Tubulin.

3.3.6 Tie1 activity is not required for downregulation

Although treatment of endothelial cells with Ang2 leads to Tie1 downregulation, we next sought to determine whether Ang2 binding to the Tie1/Tie2 heteromeric complex is sufficient to induce Tie1 downregulation or whether Tie1 kinase activity is required for this process. Previously, we have found that a commercially available, ATP mimetic, small molecule Tie2 inhibitor has cross-reactivity for Tie1. Stimulation of ECRF-Tie1 cells with Ang2 and the Tie inhibitor had no effect on Tie1 downregulation, demonstrating that kinase activity of Tie1 or Tie2 is not required for ligand-mediated degradation (Figure 21). These findings suggest that the signals responsible for Tie1 internalization are transduced in a Tie kinase-independent manner.

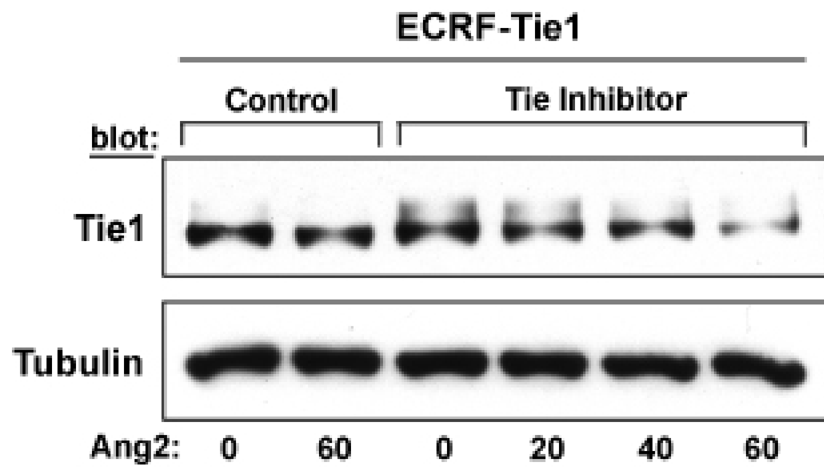

Figure 21: Tie1 activity is not required for Ang2-mediated downregulation

ECRF-Tie1 cells were grown to confluence, starved, and treated with or without Ang2 (500 ng/mL) for the times indicated. Cells were pretreated with an active site directed inhibitor to the Tie receptors (1.25 uM in DMSO) for 4 hrs prior to Ang2 stimulation. All cells were pretreated with cyclohexamide (5 ug/ml) for 30 min to prevent novel protein synthesis. Western blots from whole cell lysates were analyzed with antibodies against Tie1 and Tubulin.

3.4 Discussion

Genetic studies in mice have demonstrated Tie1's integral role in vascular development, as knockout studies have shown that absence of the receptor results in embryonic lethality due abnormal vascular integrity. However, mechanistic studies to elucidate Tie1's role in the endothelium have been hindered by the lack of known ligands and downstream signaling pathways. Previous studies have demonstrated that Tie1 and Tie2 are able to heteromultimerize and that Tie1 can modulate Ang signaling through Tie2. Furthermore, studies have shown removal of Tie1 from these complexes by proteolytic cleavage, and this can lead to increased activation of Tie2 by Ang1. Previously, we have demonstrated that Ang2 can activate Tie1 in the presence of the Tie2 ECD, and that Ang2 but not Ang1 transduces signals through Tie1. However, the effect of the Ang2-Tie1 interaction on receptor downregulation has not been examined. Results in this chapter demonstrated that Ang2 is able to induce rapid Tie1 downregulation in endothelial cells. This downregulation is not due to a repression of transcription but rather to mature receptor internalization and subsequent degradation that occurs at least partly in lysosomes. In addition, we found that the Ang2-mediated Tie1 downregulation requires Ang2 association with the extracellular domain of Tie2 but does not require Tie2 cytoplasmic domains or kinase activity. Similarly, Ang2-induced Tie1 downregulation was independent of Tie kinase activity, suggesting that Ang2 signals receptor internalization through pathways that are phosphotyrosine-independent.

An important finding in this chapter is that Ang2, but not Ang1, leads to a rapid, concentration-dependent downregulation of Tie1 from the EC plasma membrane that is independent of the Tie2 kinase domain but dependent on the presence of the Tie2 ECD. Furthermore, this effect is not due to a change in transcription of the receptor or

shedding of the extracellular domain of the protein, but rather due to specific ligand-induced internalization of mature receptor complexes from the plasma membrane. Although previous studies have demonstrated that stimulation of endothelial cells with VEGF or PMA induces Tie1 cleavage, we observed no accumulation of 45 or 42 kD Tie1 fragments upon Ang2 stimulation, indicating that the mechanism of Ang2-mediated downregulation is distinct from that of Tie1 cleavage. Furthermore, immunofluorescence microscopy with antibodies directed against the extracellular domain of Tie1 (Figure 19) indicates that Ang2 induces Tie1 translocation from the plasma membrane to intracellular compartments. Receptor shedding, as in the case of VEGF stimulation, would lead to a reduction of signal from the cell surface but not a subsequent accumulation of receptors within the endothelial cell cytoplasm. Also, the finding that chloroquine, a lysosomal inhibitor, blunted Ang2-mediated Tie1 downregulation supports the possibility that the full length receptor is internalized and degraded after internalization and is not cleaved prior to endocytosis. Taken together, these results indicate that in addition to proteolytic shedding, Tie1 expression in endothelial cells can be modulated by Ang2-induced receptor internalization.

Surprisingly, we found that Ang2-induced downregulation of Tie1 is independent of Tie activity, a result that is distinct from the canonical mechanisms of RTK internalization. Methods responsible for receptor tyrosine kinase (RTK) downregulation have been elucidated largely by studying the mechanisms of Epidermal Growth Factor Receptor (EGFR) internalization, with ligand binding and activation inducing endocytosis of the receptor, and it is these mechanisms that have served as the basis for essentially all RTK downregulation cascades. EGF binding to EGFR induces receptor multimerization, activation, and subsequent ubiquitination and endocytosis of the

activated receptor into clathrin coated pits and early endosomes(132-133, 135-136, 194). The endosomes then migrate to the perinuclear regions of the cell, fuse with lysosomes, and the contents are then degraded by the lysosomal enzymes contained within, extinguishing the active receptor-induced signaling cascades(137-139). Based on our prior observation that Ang2 is able to activate Tie1, we assumed that active Tie1 was being degraded in a feedback loop analogous to the downregulation of activated EGFR to prevent uncontrolled signaling. However, we found that incubation with an ATP mimetic pharmacological Tie2 inhibitor did not prevent Ang2-mediated downregulation of Tie1. We have previously demonstrated that the Tie inhibitor cross-reacts with both Tie1 and Tie2 indicating that the inhibitor prevents all Tie kinase signaling. Since ligand-induced downregulation still occurred in the presence of the inhibitor, this indicates the presence of other factors that can associate with the Tie1/Tie2 heteromeric complex that then induce receptor internalization, albeit in a phosphotyrosine-independent manner. Currently, the only signaling pathway documented to be activated by Tie1 following ligand binding is the PI3K/Akt pathway, which requires Tie1 kinase activity and subsequent phosphorylation of Tyr1113 in the receptor carboxy-terminal. Although preliminary studies have identified proteins that bind Tie1 in a phosphotyrosine-independent manner, it remains unclear whether these proteins are regulated by ligand binding or if they are involved in receptor internalization. Recently, Ang2 was shown to stabilize Tie1/Tie2 complexes, while Ang1 destabilizes these heteromeric complexes. Our results support the notion that Ang2 stabilization of the Tie1/Tie2 complex could facilitate association of cytosolic proteins with Tie1, which then induce receptor downregulation even in the absence of kinase activation.

Previous reports have demonstrated that silencing Tie1 expression with siRNA or shedding of the receptor ECD leads to increased Tie2 activation by the Angiopoietins. We have also found Tie2 is more readily activated by either Ang1 or Ang2 in Tie1-deficient endothelial cells. Therefore, one role of Ang2 in signaling through the Tie receptors may be to downregulate Tie1 in a manner distinct from receptor cleavage and allow for Ang1 to subsequently activate the Tie2 signaling axis. This process could occur readily in the angiogenesis of mature vessels. Previous reports have indicated that Ang2 is expressed along the leading edge of endothelial cells during sprouting angiogenesis, whereas Ang1 expression occurs along the trailing edge, where vessel maturation is occurring. Therefore, during angiogenesis, one of the roles of Ang2 could be to downregulate Tie1 from the cell surface to allow for subsequent stimulation of Tie2 by Ang1 within the maturing endothelium once invasion is complete. This model would require a precise spatio-temporal relationship between Ang2 and Tie1 in modulating signaling through Tie2 during vessel maturation. In support of this possibility, hyperactivating mutations in (R849W, Y897S) have been shown to result in venous malformations, indicating that dysregulation of Tie2 leads to vascular dysmorphogenesis. Overall, these results suggest that vascular integrity is very tightly regulated by the Tie/Angiopoietin system, and that Tie1 may act as a rheostat to modulate the activity of Tie2 in a precise spatial and temporal manner.

In addition to its effects on modulating Tie2 activity during angiogenesis, Ang2-mediated downregulation of Tie1 may be an important component of the endothelial cell response to hypoxia. Ang2 and VEGF expression and secretion are both upregulated in endothelial cells in hypoxia, while Tie levels remain relatively unchanged. We have previously demonstrated that autocrine signaling by secreted Ang2 can induce activation

of the Tie/Angiopoietin signaling axis in stressed or starved ECs. Therefore, a role of Ang2 upregulation during hypoxia may be to enchance the VEGF-induced cleavage of the receptor by promoting the downregulation of Tie1, priming Tie2 for activation by Ang1. Once normoxia is attained and angiogenesis subsides, the reduction in Ang2 secretion allows for the reaccumulation of Tie1 at the plasma membrane, and continued modulation of the Tie2/Ang signaling axis by Tie1. Taken together, our findings suggest that Ang2 and Tie1 may have important roles in the regulation of the Tie axis during tissue ischemia and cellular hypoxia.

Although a significant work remains to be done to elucidate the role of Tie1 in the mature vasculature and to understand the mechanisms of differential signaling between Ang1 and Ang2 along the Tie receptor axis, results in this chapter are the first to demonstrate that Ang2 specifically induces downregulation of Tie1 from the plasma membrane in a manner distinct from receptor cleavage and shedding. Moreover, our results define the role of the Tie kinase domains in this process by demonstrating that Tie kinase activity is not required for receptor internalization, and they suggest the possibility of, as yet unknown, signaling molecules that are differentially recruited to Tie1/Tie2 heteromeric complexes in response to Ang2 versus Ang1. Further experiments elucidating the interactions between the Angiopoeitins and Tie receptors, as well as their downstream signaling partners may lead to a greater understanding of vascular growth and remodeling in both physiologic and pathologic states.

4. Conclusions and Future Directions

The endothelium interacts with both the contents of the blood and the underlying basement membrane in tightly regulated and coordinated processes. Understanding the precise components and signaling cascades within the quiescent, angiogenic, and thrombotic phenotypes as well as the differences between the various endothelial states should result in significant advances in the treatment of disease. To accomplish this task, the breadth of endothelial cell surface receptors must be examined and their relative contributions to the aforementioned states must be determined. Although numerous studies have demonstrated the importance of Tie2 signaling in the development and maintenance of a mature vasculature, the roles of the related Tie1 receptor have been, as of yet, poorly understood. Results from this body of work establish a novel method of Tie1 activation in mature endothelial cells and support the role of Tie1 in influencing signaling through Tie2. Furthermore, they reinforce the complexity of the Tie receptor axis, and the necessity for further studies to elucidate the physiologic effects of Tie1 receptor activation and regulation.

Tie receptors and their ligands, the Angiopoietins, mediate the transition between quiescent and angiogenic vessels. A battery of evidence has characterized Angiopoietin association with, and activation of, Tie2 and the subsequent phenotypic changes in ECs. However, the lack of a ligand for Tie1 had initially prevented elucidation of the role of this receptor beyond mouse genetic studies. Still, the increased expression of Tie1 in disturbed flow, lymphatic, and tumor vasculature suggests that Tie1 plays a role in altering the phenotype of the endothelium through mechanisms distinct from those of Tie2. Recently, the interaction between Tie1 and Tie2 on the surface of endothelial cells and Tie1's role in modulating ligand-induced signaling through Tie2 has been more

firmly established(154, 156). However, our data indicate that Tie1's role is more complex than that of a simple inhibitor of Ang/Tie2 signal transduction, as results from this body of work demonstrate that Ang2/Tie1 signaling results in multiple mechanistic and physiologic effects.

As demonstrated by studies in this dissertation, Ang2 is able to induce phosphorylation and activation of Tie1 in both primary and immortalized endothelial cells. These results are the first to reveal that a ligand for the Tie1 receptor can induce not only receptor phosphorylation but also physiologic pro-survival and pro-proliferative effects. Although previous studies from our lab have characterized the intracellular kinase domain of the Tie1 receptor and its ability to activate the PI3K/Akt signaling cascade(151), this is the first report of physiologic effects of Tie1 signaling observed in endothelial cells. Importantly, these findings reveal a novel ligand/receptor interaction and a mechanism by which Tie1 can prevent apoptosis and promote proliferation.

Previous studies showed that the Angiopoietins are unable to bind to Tie1 extracellular domain individually and that Tie1/Tie2 heteromers are present on the surface of ECs even in the absence of ligand(71, 156). Our data demonstrate a novel mechanism by which Tie2 directs the Ang2/Tie1 interaction. The requirement for Tie2 in Tie1 activation was characterized in this body of work by the generation and analysis of a host of different immortalized EC lines. Results from this thesis demonstrated that although the extracellular domain is necessary, the kinase domain of Tie2 is not required for Ang2-mediated Tie1 activation. These findings support the idea that an important role of Tie2 in Tie1 activation is to bind ligand and present it to Tie1, facilitating proper oligomerization. Moreover, they demonstrate that the Tie1 kinase domain is functional in primary endothelial cells.

One of the limitations of previous studies of Tie1 *in vitro* was the inability to observe the effects of Tie1 activation separate from Tie2. The use of ECRF-Tie1/Tie2TM cells has allowed for distinguishing Tie1's effects from those of Tie2. We have demonstrated that Tie1 is able to signal through the PI3K/Akt axis to promote the physiologic effects previously described. The mechanism of anti-apoptosis by Tie1 appears to be identical to that described for Tie2, a fact that is not entirely surprising given the high degree of homology between the Tie1 and Tie2 kinase domains. What remains unclear from our results, however, is the relative levels of contribution of the two receptors. Since Tie1 and Tie2 are both functional on the endothelial surface and Tie1 is unable to signal in the absence of Tie2, activation of heteromeric Tie1/Tie2 complexes appears likely to lead to signal transduction by both Tie1 and Tie2 kinase domains. Further studies will be necessary to characterize the proportional contributions of each receptor, which may be accomplished through the generation of cell lines with inducible expression of Tie1 and/or Tie2.

These results are in contrast with several reports demonstrating that Ang1, but not Ang2, can induce modest Tie1 phosphorylation(152-153). There are several potential explanations for the discrepancy between those studies and the results in this body of work. First, one of the ligands used in the report of Saharinen et al was COMP-Ang1, an Ang1 derivative engineered specifically to form an obligate tetramer and which induces hyperactivation of Tie2 in multiple model systems. Therefore, any heteromers that would be present on the surface of the cells at the time of stimulation would likely be activated, increasing the likelihood that these hyperactivated complexes could lead to Tie1 phosphorylation. Second, the immortalized endothelial cells typically used by other groups to examine Tie receptor phosphorylation, EA.hy926 cells, which are also derived

from HUVECs, are a distinct cell population and therefore may have distinct Tie signaling mechanisms. Third, the expression levels of Tie1 on the surface of distinct HUVEC populations can vary considerably. Although the mechanisms responsible for varying Tie1 expression are unknown, it can be hypothesized that a HUVEC population with greater expression of Tie1 would have a greater proportion of Tie1/Tie2 heteromers, and therefore a greater likelihood that either Ang1 or Ang2 could activate Tie1.

More difficult to explain is the fact that other groups have not observed Ang2-mediated Tie1 activation, particularly in light of recent structural observations regarding Tie1 and Tie2. In particular, Seegar et al demonstrated that the extra-cellular domains of Tie1 and Tie2 present complementary surfaces with respect to their electrostatic charge, thereby forming pre-existing heteromeric complexes(156). As measured by FRET, Ang2 was found to stabilize these heteromeric complexes while Ang1 destabilized Tie1/Tie2 interactions. Therefore, Ang1 stimulation of ECs would be predicted to lead to fewer Tie1/Tie2 complexes, possibly favoring Ang1-Tie2 interactions. The results of Seegar et al support our finding that Ang2 promotes association between Tie1 and Tie2 and subsequent activation of Tie1. Furthermore, studies presented here indicate that one role of Tie2 is to present Ang2 to Tie1, although Tie2 may not necessarily activate Tie1. It is important to note that the design of our studies, particularly through the use of the Tie2TM-expressing cells, did not address the contribution of the Tie2 kinase to Tie1 activation. Nonetheless, one would predict that Ang2-mediated stabilization of Tie1/Tie2 heteromers would allow greater Tie1 autoactivation as well as possibly Tie2-mediated transactivation.

In addition to receptor phosphorylation, results from this body of work characterized Ang2's role in Tie1 internalization and downregulation. As noted, previous

reports have shown that Tie1 can modulate Tie2 signaling, and that elimination of Tie1 enhances ligand-mediated Tie2 activation(153, 162, 183). However, the aforementioned studies analyzed effects of Tie1 silencing or proteolytic shedding following treatment with VEGF or PMA. Although these mechanisms lead to release of Tie1's inhibitory role in Tie2 signaling, they do not shed light on the effects of the Angiopoietins' interaction with Tie receptor heteromeric complexes. We were able to characterize a ligand (Ang2)-mediated downregulation of Tie1 receptors in endothelial cells that is dependent on the presence of Tie2, apparently for ligand binding. This is the first demonstration of ligand-induced Tie1 downregulation, and describes a potential feedback mechanism to prevent uncontrolled signaling through activated Tie1 receptors. Together, these studies, coupled with previous groups' data, demonstrate multiple mechanisms of Tie1 regulation that lead to removal from the endothelial surface.

As in the case of Tie1 activation, Tie2 is required for Ang2-mediated Tie1 internalization. However, our studies using the Tie2TM-expressing cells point to an important difference in Tie2's roles in these two processes. As noted previously, although Tie1 activation requires the Tie2 ECD, apparently for ligand presentation to Tie1, removal of the Tie2 kinase domain does not allow an assessment of Tie2's relative contribution to Tie1 activation. In the case of Tie1 internalization, this process clearly proceeds rapidly even in the absence of Tie2 kinase activity. Moreover, Tie1 kinase activity is not required for Tie1 processing, demonstrating that ligand binding is sufficient to induce downregulation, and demonstrating a novel, kinase-independent internalization pathway. Intriguingly, additional studies from our lab have shown that Ang2 does not induce Tie2 internalization (unpublished observations), consistent with a distinct Ang2/Tie1-mediated internalization signal. Although Ang2-mediated Tie1 degradation

occurs by internalization into lysosomes in a manner analogous to the turnover of other RTKs, the observation that inhibition of Tie kinase activity does not prevent processing demonstrates that the conformational changes occurring upon Ang2 binding to Tie1 is sufficient to initiate downregulation. This result highlights [an] two important point[s]. It demonstrates the necessity for other, currently unknown, binding partners to associate with the cytoplasmic domain of Tie1. In attempting to understand how Tie1 and Tie2 differ, it is important to examine their structural differences. Notably, the region of greatest difference between Tie1 and Tie2 is their juxtamembrane (JM) domains. Whereas Tie1 contains 13 serine and threonine residues in its JM region, Tie2 contains only 3. Many of these sites have the potential to be phosphorylated by Ser/Thr kinases, which might associate with Tie1 following Ang2 binding, thereby recruiting interacting proteins that promote receptor downregulation. More work must be performed to elucidate the binding partners and novel sites of Tie1 modification apart from its kinase activity to understand the precise mechanism of Ang2-mediated Tie1 internalization.

Differences in Ang2's quaternary structure may be responsible for the conflicting reports regarding Ang2's ability to activate the Tie receptor signaling axis. Previous reports have demonstrated that, at minimum, tetramers of Ang1 are necessary to induce Tie2 phosphorylation but lower order multimers can still associate with Tie2(96-97). Since both Ang1 and Ang2 appear to bind in analogous fashions to Tie2, it is presumed that similar multimeric Ang2 structures are necessary for activation but lower order oligomers will still interact with receptors(99, 101). Therefore, dimers or trimers of Ang2 may facilitate internalization by orienting Tie receptor complexes into proper conformational structures to allow cofactor binding to Tie1 while preventing Tyr phosphorylation of the receptor. *In vitro*, we have observed that recombinant Ang2

exists as a mixture of dimers and tetramers. Stimulation of endothelial cells with predominantly dimeric recombinant Ang2 may be one reason for previous groups' observations of the inability of Ang2 to activate Tie1. The characterization of Ang2's conformational requirements for Tie1 downregulation versus activation *in vitro* and comparison to Ang2's state in various physiologic conditions *in vivo* will more fully explain the regulation of vascular maturation along the Tie axis.

In addition to the multimeric state of Ang2 regulating signaling through the Tie axis, the ability of endothelial cells to synthesize and secrete Ang2, in contrast to the SMC requirement for Ang1 secretion, suggests another mechanism of regulation. Results from this body of work, as well as previous reports, have demonstrated that stressed or hypoxic endothelial cells rapidly exocytose Ang2 from Weibel-Palade bodies and increase Ang2 expression to allow for sustained secretion of ligand(89, 95). In addition, we also observed that secreted Ang2 activates Tie1 in an autocrine manner. This mechanism may afford ECs the ability to transiently prevent apoptosis when subjected to stresses *in vivo*, and it may represent another signaling cascade to stimulate chemotaxis in hypoxic states. Notably, we did not investigate whether Ang2-mediated Tie1 activation induces EC migration, although its ability to activate the PI3K/Akt pathway is consistent with this possibility. Furthermore, downregulation of Tie1 in an autocrine manner may facilitate activation of Tie2 by Ang1 once a mature endothelial cell phenotype is required.

4.1 Implications for normal physiology

Tie2, being expressed ubiquitously along the surface of both blood and lymphatic vessels, has been shown to regulate the transition between angiogenic and mature endothelium. Recently, Tie1 has been implicated in modulation of Tie2 signaling along

the endothelium in various physiologic states. Results from this dissertation may further explain the interactions between endothelial cells and their surrounding environment, and they highlight several novel considerations for the role of this receptor family in various vascular systems.

The ability of Ang2 to activate and downregulate Tie1 may shed light on signaling mechanisms at play during sprouting angiogenesis. Ang2 has been shown to be upregulated at the leading edge of invading endothelial cells while Ang1 is expressed primarily at the trailing edge, where a more mature phenotype is required(115). Therefore, circulating Ang2 may activate Tie1 and, through the downstream signaling cascades invoked, delay apoptosis/anoikis at sites of initiating angiogenesis, providing the sprouting ECs more time to develop into functional vessels and correct the hypoxia induced state. Subsequently, due to the inherent ability of Ang2 to induce internalization of Tie1, endothelial cell surfaces at sites distal to those undergoing active angiogenesis and where a quiescent phenotype is desired might exhibit a greater proportion of Tie2 homomultimers. There, Ang1 secretion by cells within the basement membrane activates Tie2 at the trailing end of the vessel sprout and thereby promotes the transition to a mature vasculature.

Although the Ang/Tie system catalyzes critical steps in sprouting angiogenesis, other ligand/receptor systems, most notably VEGF/VEGFR-2, contribute to the overall mechanism of blood vessel growth. Ang2 stimulation without concurrent VEGF appears to lead to vessel regression, indicating that the pro-survival signals induced by Ang2 binding to Tie1 may not be sufficient to prevent EC apoptosis after prolonged periods of time(71). However, since Ang2 is rapidly secreted upon stimulation and can act in an autocrine manner before VEGF/VEGFR-2 signaling, the role of Ang2/Tie1 activation in

vessel sprouting may be to delay, not prevent, apoptosis until additional growth factor signaling can occur(95). Alternatively, concurrent activation of ECs by VEGF and Ang2 may transduce different signals than Ang2 alone. In addition to stimulation of growth, VEGF treatment of endothelial cells has been shown induce Tie1 cleavage and processing(162-163). In this regard, VEGF-induced Tie1 shedding could lead to preferential Ang2-Tie2 signaling, which would be predicted to result in effects distinct from those of Ang2-Tie1, i.e., in the absence of VEGF. Although the function of circulating soluble Tie1 is not currently known, the cleaved receptor fragment on the angiogenic endothelial cells would be expected to increase the proportion of Tie2 homomultimers, and indeed has been shown to lead to enhanced Tie2 activation(162). Therefore, both VEGF and Ang2 induce Tie1 downregulation, albeit through different mechanisms, thereby potentiating Ang1-mediated Tie2 activation, possibly to promote vascular quiescence.

In addition to the roles played in blood vessel angiogenesis, Tie1 has recently been shown to have critical effects in both the development and maintenance of the lymphatic vasculature. Two recent reports have described lymphatic vascular defects in mouse embryos bearing hypomorphic Tie1 alleles, and they have observed that Tie1 knockout animals develop dilated, disorganized, and non-functional lymphatic vessels(64-65). During the initial stages of lymphangiogenesis, the lymphatic endothelium of hypomorphic Tie1 embryos is hyperproliferative compared to wild-type animals, but during later periods of vessel maturation it takes on a more apoptotic phenotype. Other studies have demonstrated that Ang2-deficient mice develop a dilated lymphatic vasculature similar to that seen in hypomorphic Tie1 embryos, and that Ang1 can rescue this lymphatic phenotype when knocked into the Ang2 locus. Taken

together, these studies are consistent with the results of this work, as they demonstrate that both Ang2 and Tie1 act as agonists during lymphatic vascular development, and they suggest that Ang2 signaling occurs through Tie1 in this context.

4.2 Implications for pathologic states

With the greater knowledge of Tie receptor expression profiles and signaling cascades, the respective roles of these receptors are beginning to be clarified in pathological conditions. Distinct from Tie2's activities, Tie1 has recently been linked to a role in inflammation(167). Silencing Tie1 expression in HUVECs resulted in a decrease in expression of a variety of chemokines and inflammatory cytokines, whereas Tie1 overexpression had the opposite effect. *In vivo,* Tie1 has been shown to be overexpressed on the surface of endothelial cells in the synovial joints of both rheumatoid (RA) and osteoarthritis (OA) and has been linked to the stimulation of mononuclear cells at sites of inflammatory disease(164-165). Furthermore, expression of alternative splice variants of Tie1 within RA joints reduces the levels of inflammatory molecules and has been implicated in better outcomes(165). The improved outcomes appear to be due to both increased soluble Tie1 shedding from endothelial cells and, consequently, less expression of full-length Tie1. Therefore, better outcomes may be due to the binding of an unknown circulating Tie1 ligand to sTie1 thereby preventing its activation of intact receptors or the inability for Tie1 to be activated by known ligands such as the Angiopoietins . However, the aforementioned physiologic effects are, at least in part, due to the reduced quantity of activated Tie1 promoting inflammatory cascades. . Taken together, these results indicate that activated Tie1 signaling stimulates endothelial pro-inflammatory responses and may contribute to a variety of inflammatory diseases, including rheumatoid arthritis.

Another important disease in which Tie1 activation may promote inflammatory cell recruitment and progression of disease is atherosclerosis. Tie1 mRNA is overexpressed in the endothelium at sites of disturbed blood flow, including adjacent to vascular occlusions and on the surface of atherosclerotic plaques(149). Increased transcription of the receptor in these instances suggests a compensatory response by the endothelium in an attempt to correct the pathologic state. Recent reports have demonstrated that endothelial cells lacking Tie1 are unable to stimulate monocyte recruitment as a result of decreased secretion of MCP-1(167). Therefore, Tie1's role in atherosclerotic plaques may be recruitment of inflammatory cells, possibly as a wound healing response, but which ultimately leads to progression of the disease. Ang2 is overexpressed in the endothelium and secreted at sites of decreased shear stress(91-93), such as surfaces of atherosclerotic plaques. Overexpression and secretion of Ang2, coupled with increased Tie1 expression, most likely induces activation of the receptor specifically at sites of plaque initiation and encourages monocyte recruitment and extravasation to promote atherosclerosis progression. This possibility is consistent with results from this body of work that demonstrate Ang2-mediated Tie1 activation and signaling through cascades distinct from Tie2, which could be at least partly responsible for the pathologic response.

Although autocrine Ang2/Tie1 signaling seems to support atherosclerotic plaque progression, understanding the distinction between Ang2-mediated Tie1 activation and internalization may facilitate the design of new therapeutic approaches for atherosclerosis. We have demonstrated that Tie1 is able to be downregulated even in the absence of activation, provided Tie2 binds and presents the ligand to Tie1. Furthermore, we have demonstrated that EC secretion of Ang2 provides functional

ligand that is able to activate Tie1 in an autocrine fashion. Finally, the binding of Ang2 to endothelial cells appears to occur along the luminal side of the cells, in contrast to Ang1 binding, indicating that soluble ligands in the blood have the opportunity to bind to receptors that are able to be activated. Therefore, the sustained use of Ang2 multimers that are unable to activate Tie1 (perhaps obligate dimers or trimers) would lead to Tie1 internalization without activation and inflammatory signaling. Since reduced Tie1 expression prevents monocyte stimulation, progression of disease should be slowed, if not halted. Consistent with this possibility, a recent report from our lab demonstrated that systemic Ang2 delivery in atherosclerosis-prone ApoE knockout mice resulted in inhibition of atherosclerosis progression.

In addition to their roles in inflammatory diseases, Ang2 and Tie1 have both been demonstrated to be upregulated in a host of cancers and perhaps more importantly have been implicated in tumor progression. In both breast cancer and gastrointestinal tumors, endothelial Tie1 expression indicates a greater propensity for tumor angiogenesis and an increased predisposition to tumor invasion and metastasis(170, 172-173). Since Tie1 is able to activate the PI3K/Akt signaling cascade, a role of Tie1 may be to stimulate stromal vessel proliferation to supply the growing tumor. Interestingly, Tie1 expression has also been demonstrated within breast tumor cells, that specifically also stimulate inflammatory responses(177). Therefore, Tie1 signaling in these tumors might result in induction of inflammatory signaling cascades that recruit tumor associated macrophages (TAMs). The presence of TAMs provokes local immune suppression and progression of disease. Clearly, more work must be performed to elucidate the precise role of Tie1 throughout the tumor microenvironment, including blood vessels, stromal tissues, and tumors, to promote cancer development. Interestingly, if similar mechanisms of Tie1

downregulation exist in tumor cells compared with ECs, modified Ang2 molecules that induce internalization but not activation of receptors may represent a novel therapeutic approach to treat cancer as well.

4.3 Future Directions

This dissertation has defined a novel Angiopoietin-2-mediated Tie1 signaling cascade within endothelial cells. Additionally, a previously unknown ligand-induced receptor internalization system has been elucidated, and the requirements for Tie1 phosphorylation and Tie2's involvement have been defined for each of these processes. Finally, a set of cell lines and reagents have been generated that have allowed important observations to be made, clarifying important distinctions between Tie1 and Tie2 signaling within endothelial cells. Although this work defines some of the basic mechanisms responsible for Tie1's physiological effects, more work must be done to characterize the precise nature of ligand-induced Tie1 signaling and its roles *in vivo*.

Although there has been some speculation regarding modulation of Tie2 signaling by Tie1, no reports have demonstrated that Ang2-induced downregulation of Tie1 leads to hyperactivation of Tie2 homomultimers. Comparison of the effects of Ang1 on Tie2 activation to the effects of Ang2 to induce internalization of Tie1 followed by addition of Ang1 to activate Tie2 would confirm the hypothesis that removal of the inhibitory role of Tie1 in this manner results in increased Tie2 activation. This result should be confirmed *in vivo* by observation of the cell surface expression levels of Tie1 at the invasive versus trailing edges of sprouting angiogenic vessels.

Since internalization of Tie1 is dependent on Tie2 only for ligand presentation and not receptor activation, other cofactors must be present either to modify Tie1 or recruit the machinery integral to receptor processing. Interestingly, the juxtamembrane

region of Tie1 contains a multitude of modifiable residues that may be acted upon to induce internalization. Employment of proteomic techniques to identify important associated proteins after Ang2 stimulation would characterize the players and may indicate, based on sequence analysis, the mechanisms of association that lead to Tie1 downregulation. Mass spectroscopic analysis of Ang2-stimulated Tie1 receptors would also allow identification of post-translationally modified residues involved in the fate of the protein. Furthermore, mutations of these amino acids may point to mechanisms of pathologic conditions in which Tie1 dysregulation is implicated (lymphatic malformations or cancer).

The reagents generated during the course of experimentation within this dissertation, as stated, allow for the distinction between Tie1 and Tie2 signaling. Therefore, the signals that are transduced through Tie1 specifically following Ang2 stimulation can be determined. Since Tie1 seems to exhibit an inflammatory function in endothelial cells, utilizing the engineered Tie2TM cell line will allow for the characterization of the inflammatory gene expression mediated solely by stimulation of Tie1. Thus, the contributions of the Tie receptors versus other endothelial plasma membrane proteins (e.g., integrins) can be assessed in inflammatory states.

Utilizing the Tie2TM-expressing cells may also help shed light on the mechanisms of lymphangiogenesis and maintenance of the lymphatic vasculature. Development of lymphatic EC-specific Tie2 knockdown and Tie2TM-rescue cell lines would allow for activation of Tie1 independent of Tie2. If the Tie2TM-expressing lympatic ECs exhibit a wild-type phenotype, then lymphatic development and maintenance could be inferred to be a Tie1-dependent process, and Tie2 is simply a conduit for ligand recruitment. However, if the mutant phenotype predominates, then the

role of Tie1 is at least partially to modulate signaling through Tie2. In either scenario, the experiment would characterize the precise roles of the various Tie receptors in the development of distinct vascular systems within the embryo.

In addition to describing the functions of Tie1/Tie2 complexes in EC signaling, the results in this dissertation implicate the varying effects of different Angiopoietin multimeric states. Therefore, the characterization of the quaternary structures of Ang2 necessary to elicit internalization versus activation of Tie1 should be investigated. Furthermore, once the precise oligomeric structure of Ang2 required for downregulation but not phosphorylation is determined, therapeutic approaches based on inhibiting Tie1's inflammatory function could be designed. Utilizing synthetic analogs to the superclustering and coiled coil oligomerization domains while retaining the native receptor-binding domain of Ang2 will allow for the synthesis of obligate multimers that mediate Tie1internalization. Once synthesized, these novel therapeutics can be tested with regards to prevention of pathologic states in model systems that have already been characterized (such as atherosclerosis in ApoE knockout mice). Therefore, extension of the results obtained within this dissertation may lead to improved understanding of and novel approaches to treat a host of distinct illnesses through modification of the Tie1 signaling axis.

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PUBLICATIONS

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