Tie1/Tie2-mediated PI3K/Akt signaling in lymphatic vessel quiescence and remodeling

Anni Lantta

Master's Thesis

University of Oulu

Degree Programme in Biology

June 2023

Abstract

The lymphatic vascular system is a network of blind-ended lymphatic capillaries which collect extracellular fluid, and larger collecting lymphatic vessels which transport the fluid back to blood circulation. The major functions of the lymphatic system are the maintenance of tissue fluid homeostasis, intestinal fat absorption and immune surveillance via importing antigens and antigen-presenting cells to lymph nodes. The maintenance and function of the lymphatic system is controlled by growth factor receptors expressed by lymphatic endothelial cells (LECs). Two major signaling pathways that regulate the quiescence and remodeling of the lymphatic vasculature are the angiopoietin/tyrosine kinase with Ig-like and EGF-like domains (Ang/Tie) signaling pathway and the vascular endothelial growth factor C/vascular endothelial growth factor receptor 3 (VEGF-C/VEGFR-3) signaling pathway. The phosphoinositide 3 kinase/protein kinase B (PI3K/Akt) pathway downstream regulates a variety of cellular functions including cell metabolism, growth, proliferation, and survival. One of the downstream effectors of PI3K/Akt signaling pathway is the transcription factor forkhead box protein O1 (FoxO1), which is inhibited via the activation of PI3K/Akt pathway. FoxO1 has a crucial role in inhibiting the formation of new lymphatic vessels by repressing genes involved in the process under normal conditions. During stress and inflammation, PI3K/Akt pathway is deactivated leading to FoxO1 translocation into the nucleus and where it activates the genes involved in vascular remodeling and destabilization. Understanding the pathways and mechanisms related to lymphatic vessel quiescence and remodeling has therapeutic value in manipulation of lymphangiogenesis e.g. in lymphedema and other pathologies in which lymphatic function is reduced.

Table of Contents

Abbreviations

Akt	Protein kinase B
Ang	Angiopoietin
BEC	Blood endothelial cell
EC	Endothelial cell
ECM	Extracellular matrix
FoxO1	Forkhead box protein O1
LEC	Lymphatic endothelial cell
LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
Nrp	Neuropilin
PECAM1	Platelet And Endothelial Cell Adhesion Molecule 1
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol (3,4) bisphosphate
PIP_3	Phoshatidylinositol (3,4,5) triphosphate
Prox1	Prospero-related homeodomain transcription factor
RTK	Receptor tyrosine kinase
Tie	Tyrosine kinase with Ig-like and EGF-like domains
TNFα	Tumor necrosis factor α
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1. Introduction

There are two parallel vascular systems in the human body: the blood and the lymphatic vascular system. The blood vascular system is a closed, treelike, hierarchical system of vessels whose main function is to deliver oxygen and nutrients to tissues and remove carbon dioxide and other waste products. The lymphatic vascular system is a network of blind-ended lymphatic capillaries which collect extracellular fluid, and larger collecting lymphatic vessels which transport the fluid back to blood circulation. The major functions of the lymphatic system are the maintenance of tissue fluid homeostasis, intestinal fat absorption and immune surveillance via importing antigens and antigen-presenting cells to lymph nodes (Kärkkäinen et al., 2003; Alitalo, 2011; Mortimer & Rockson, 2014). Both the blood and the lymphatic vascular systems are involved in the pathophysiology of a variety of diseases (Saharinen et al., 2017). Defects in the lymphatic vasculature lead to primary and secondary lymphedema, which are characterized by swelling of tissues due to fluid accumulation. The lymphatic system has also been considered as a route for cancer metastasis (Oliver et al., 2020).

Our understanding of different functions of the lymphatic vasculature has expanded during the last few years. The lymphatic system has been shown to have a role in conditions such as obesity, cardiovascular disease, atherosclerosis, and some neurological disorders (Brakenhielm & Alitalo, 2019). The maintenance and function of lymphatic vasculature is controlled by growth factor receptors expressed by lymphatic endothelial cells (LECs) that form the inner lining of the lymphatic vessels. Two major signaling pathways that regulate the quiescence and remodeling of the lymphatic system are the angiopoietin/tyrosine kinase with Ig-like and EGF-like domains (Ang/Tie) signaling pathway and the vascular endothelial growth factor C/vascular endothelial growth factor receptor 3 (VEGF-C/VEGFR-3) signaling pathway (Saharinen et al., 2017; Korhonen et al., 2022). The phosphoinositide 3 kinase/protein kinase B (PI3K/Akt) pathway downstream of Ang/Tie signaling pathway regulates a variety of cellular functions including cell metabolism, growth, proliferation, and survival (Hemmings & Restuccia, 2012). One of the downstream effectors of PI3K/Akt signaling pathway is the forkhead family of transcription factors (FoxOs), which are inhibited via the activation of PI3K/Akt pathway. FoxOs have a crucial role in many important cellular functions including cell cycle control, cell metabolism, differentiation, stress responses and apoptosis (Obsil & Obsilova, 2008). PI3K/Akt-mediated

phosphorylation of FoxOs leads to their exclusion from the nucleus and to their degradation in the cytoplasm and thus to changes in expression of FoxO-regulated genes. FoxO1 plays a role in inhibiting the formation of new lymphatic vessels by repressing genes involved in the process. Under normal conditions, the PI3K/Akt pathway-mediated inhibition of FoxO1 has been shown to maintain vascular stability. During stress and inflammation, PI3K/Akt pathway is deactivated leading to FoxO1 translocation into the nucleus and activation of genes involved in vascular remodeling and destabilization (Calissi et al., 2020). In the future, targeting of PI3K/Akt signaling and FoxO1 could provide a method to manipulate lymphatic vessel stability and growth under pathological conditions (Scallan et al., 2021; Korhonen et al., 2022).

1.1. The endothelium

The inner lining of the blood and lymphatic vessels consists of a monolayer of endothelial cells (ECs). This monolayer is called endothelium and it has a major role in controlling the functions of blood and lymphatic vessels, such as blood fluidity, platelet aggregation and vascular resistance. Endothelium also regulates immune responses and inflammation as well as angiogenesis and lymphangiogenesis. Endothelial cells are surrounded by a basement membrane (BM) and supported by smooth muscle cells and pericytes. The endothelium produces all proteins that form the BM and enzymes required for its degradation and remodeling. The ability to degrade and remodel the BM is crucial for vessel plasticity (Félétou, 2011).

Endothelial cells are generally thin and have an elongated shape. They are about 50–70 μm long, 10–30 μm wide and 0.1–10 μm thick (Félétou, 2011). Endothelial cells sense shear stress from the fluid flow through mechanotransduction pathway. The mechanotransduction pathway includes the adhesion molecule platelet and endothelial cell adhesion molecule 1 (PECAM1), which senses shear stress and initiates phosphorylation of vascular endothelial growth receptor 2 (VEGFR-2) and vascular endothelial growth receptor 3 (VEGFR-3). These proteins are held together by vascular endothelial cadherin (VE-cadherin), which has been proposed to regulate endothelial cell alignment according to the fluid flow (Yang et al. 2019).

Endothelial cells are aligned dynamically and their cell-cell junctions maintain endothelial barrier function (Hakanpää et al., 2017). Angiopoietin-1 (Ang1) strengthens endothelial cell adhesions and improves the barrier function (Fukuhara et al., 2010). Endothelial permeability is induced by

proinflammatory cytokines, some microbial components, and vascular endothelial growth factors (VEGFs), which bind their receptors on endothelial cells. The binding of these agents to their receptors activates downstream pathways, which can disturb VE-cadherin from endothelial cell-cell junctions and thus increase permeability. Remodeling of actin cytoskeleton within endothelial cells is stimulated by small GTPases of the Rho family and myosin light chain kinases (MLCK). The remodeling of actin cytoskeleton into actin stress fibers causes cellular tension during inflammation. Angiopoietin-2 (Ang2) is an endothelial cell-derived growth factor, which is up-regulated in pathological conditions that are associated with inflammation and vascular leakage. Ang2 acts together with various inflammatory agents and also promotes endothelial cell permeability (Hakanpää et al., 2017).

Endothelial cells are coupled to extracellular matrix (ECM) via integrin molecules. Integrins are transmembrane receptors which bind components of ECM and they are crucial for vascular development together with their fibronectin ligand (Aavramides et al., 2008; Rupp & Little, 2017). Integrins are divalent, cation-dependent heterodimeric membrane glycoproteins that consist of non-covalently associated α- and β-subunits (Aavramides et al., 2008). Integrin ectodomains attach ECs to ECM while the intracellular domain is engaged to the actin cytoskeleton via actin-binding proteins. Integrins are connected to actin cytoskeleton also in focal adhesions and they are linked to signaling pathways via linker and adapter proteins such as α-actinin and vinculin (Hakanpää et al., 2017). Integrin-mediated adhesion of endothelial cells mediates vascular stability, angiogenesis, and lymphangiogenesis. The major integrin heterodimer in endothelial cells is α5β1-integrin (Rupp & Little, 2001). β1-integrin regulates embryonic vascular sprouting and vessel lumen formation. It also has an important role in controlling postnatal vessel integrity by stabilizing VE-cadherin in developing endothelial cell junctions. It has been reported that Ang2 activates α5β1-integrins which leads to destabilization of the endothelial cell monolayer and further affects vascular stability and remodeling (Hakanpää et al., 2017).

Both the blood and the lymphatic vasculature are involved in the pathophysiology of a variety diseases. Angiogenesis, the formation of new blood vessels, is crucial for tissue regeneration and wound healing (Lugano et al., 2020). Angiogenesis is also involved in conditions such as neovascular eye diseases and cancer, and vascular leakage is increased during inflammation. In the eye, continued vascular leakage may lead to macular oedema, which is a common complication

in patients with diabetes. Vascular leakage may also cause tissue hypoperfusion in severe cases of infections. Insufficient function of lymphatic vasculature causes lymphedema and in cancer, the growth of new lymphatic vessels may increase tumor metastasis. Endothelial cells in blood and lymphatic vessels express growth factor receptors which control the development and functions of these structures (Saharinen et al., 2017).

1.1.1. Blood vasculature

The blood vascular system consists of the heart and a network of arteries, capillaries and veins. The main function of the blood vascular system is to supply oxygen and nutrients to tissues and to remove carbon dioxide and other waste products. The arterial system transfers blood from the heart into smaller arteries and capillaries, and the venous system carries blood back to the heart and lungs. The vessels of the blood vasculature consist of different cell types. The inner lining (tunica intima) is composed of endothelial cells that surround the lumen of the vessel. In smaller vessels and capillaries, the inner lining is supported by smooth muscle cells and pericytes, which are also known as mural cells. Larger vessels have a more complex structure, and their tunica intima is surrounded by a thicker layer of smooth muscle cells (tunica media) and an outer layer (tunica adventitia) that consist of connective tissue, elastic fibers and collagen. Contractile mural cells allow the vessels to respond to changes in blood flow by altering vessel diameter (Udan et al., 2012). New blood vessels may develop through vasculogenesis, which is the *de novo* formation of new blood vessels during embryogenesis, and through angiogenesis, which is the formation of new vessels from pre-existing ones (Vailhé et al., 2001).

Angiogenesis is a highly regulated process. During embryonic development, the primary vascular plexus forms from the endothelial precursors of the blood islands of the embryo. Remodeling of the primary vascular plexus leads to formation of a vascular network system (Vailhé et al., 2001). Development of the blood vasculature occurs before that of the lymphatic vasculature during embryogenesis. VEGF-A is the major growth factor in the formation blood vasculature via its receptor VEGFR-2. VEGFR-2-mediated signaling regulates EC proliferation, migration and survival as well as arterial-venous specification of the forming blood vasculature (Zhang et al., 2010). Angiogenesis is required for embryonic development and tissue repair. In pathologic conditions, it promotes chronic inflammation, tumor growth and metastasis. In tumors, new blood

vessels can sprout from the existing vessels or in minor cases, via recruiting circulating bone marrow-derived endothelial progenitor cells (Avraamides et al., 2008). Formation of new blood vessels promotes tumor growth by providing nutrients and removing waste products efficiently. Tumor cells secrete growth factors and chemokines such as VEGF-A, basic fibroblast growth factor (bFGF) and tumor necrosis factor α (TNFα), which activate quiescent vascular structures to form new vessels (Lugano et al., 2020).

Sprouting angiogenesis occurs when new vessels start to sprout from existing vessels (Vailhé et al., 2001). Some endothelial cells become tip cells which are the most distal cells of the newly forming, temporarily blind-ended vessel. The neighboring ECs form the stalk cells whose main function is to proliferate and produce more cells to extend the new vessel (Lugano et al., 2020). Sprouting angiogenesis occurs in 5 stages. The first stage is to start the signaling to induce the formation of new vessels. The secretion of VEGF-A activates VEGFR-2 in nearby vessels. Endothelial cells that are exposed to the higher VEGF-A concentration become tip cells. The increased VEGF-A concentration and the proliferation of the stalk cells and tip cells lead to outgrowth of the vessels towards the VEGF-A gradient (Udan et al., 2012).

1.1.2. Lymphatic vasculature

The lymphatic vascular system is found in most organs (Zhang et al., 2020). It is a network of blind-ended lymphatic capillaries, collecting vessels and special secondary immune organs, such as lymph nodes, the spleen and tonsils (Avraamides et al., 2008). The lymphatic vascular system is an open system where interstitial fluid, proteins and extravasated fluid from blood capillaries are absorbed to lymphatic capillaries and transported back to the blood circulation (Mäkinen et al., 2007). The lymphatic vasculature has an essential role in regulation of fluid homeostasis in the body. The capillaries of the lymphatic vasculature absorb interstitial fluid and the collecting lymphatic vessels return lymph to the blood circulation at the thoracic duct. Lymphatic vessels also act as a route for antigen-presenting cells, which are transported to lymph nodes where they can activate B- and T-cell-mediated immune responses (Aacramides et al., 2008; Alitalo, 2011). Lymphatic vessels also have an essential role also in absorption of dietary fat. Lymphatic capillaries inside the villi of the small intestine are called lacteals. Dietary fat is taken up by

intestinal enterocytes and transported into lymph via lacteals as lipid particles or chylomicrons (Mäkinen et al., 2007).

Lymphatic endothelial cells (LECs) in lymphatic capillaries have discontinuous, button-like cell-cell junctions, little or no basal membrane and they lack mural cell coverage which enables the entry of lymph into the capillaries. Lymphatic endothelial cells are coupled to the extracellular matrix by thin fibril structures called anchoring filaments. The anchoring filaments prevent the lymphatic capillaries from collapsing under high interstitial pressure. Collecting lymphatic vessels, also known as collectors, have continuous zipper-like cell-cell junctions, a surrounding basal membrane and a layer of smooth muscle cells (SMCs) which prevents leakage of fluid and enables fluid transportation (Mäkinen et al., 2007).

Unlike the blood vascular system, the lymphatic vasculature doesn't have a pump to maintain the flow of fluid. Lymph is transported in the lymphatic system by contractions of collecting lymphatic vessel SMCs and surrounding skeletal muscles (Mäkinen et al., 2016). Lymphatic collecting vessels also contain intraluminal valves that prevent backflow of the fluid. Lymphatic valves have two leaflets, which both have a layer of LECs. The leaflets are opened and closed by changes in pressure which allows lymph to move forward (Scallan et al., 2021).

Lymphangiogenesis, the formation of new lymphatic vessels, comprises of different cellular processes including proliferation, migration, sprouting, and tube formation. During embryogenesis, lymphatic vascular system development begins around mid-gestation when the blood vascular system has already developed (González-Loyola & Petrova, 2021). Lymphatic vessels form when blood endothelial cells (BECs) in the cardinal vein differentiate into lymphatic endothelial cells that express Prospero homeobox protein 1 (Prox1) and SRY-Box Transcription Factor 18 (Sox18). These polarized endothelial cells bud from the cardinal vein and begin to migrate out to form the first lymphatic structures in the jugular region of the embryo (Udan et al., 2012). During embryogenesis, the primary lymphatic plexus is remodeled to form capillary and collecting vessels (González-Loyola & Petrova, 2021).

Prox1 regulates cellular differentiation and organogenesis in many tissues including lymphatic vessels. Prox1 is required for the formation of lymphatic vessels during embryogenesis, but it is also necessary for postnatal development and maintenance of the lymphatic vasculature (Mäkinen

et al., 2007). *Prox1* expression has been detected to start around embryonic day (E) 9.75 in mice. It is expressed in a subpopulation of endothelial cells that will develop into LECs (Srinivasan et al., 2010). Prox1 also sustains high expression of VEGFR-3 in LECs while the VEGFR-3 expression is downregulated in developing blood vessels during later stages of development (Kaipainen et al 1995; González-Loyola & Petrova, 2021).

The survival, migration, and proliferation of LECs is controlled through VEGFR-2 and VEGFR-3 and their ligands VEGF-C and VEGF-D. Activation of these receptors stimulates protein kinase C-dependent activation of extracellular signal-regulated kinase ERK1 and ERK2 signaling pathways and phosphorylation of Akt through PI3K signaling pathway (Stacker et al., 2014). VEGFR-3 together with its ligand VEGF-C is required for the proliferation, migration, and survival of LECs. VEGF-C is secreted by the vascular smooth muscle cells and the mesenchymal cells when lymphatic vessels start to develop from blood vascular system during embryogenesis, and it is absolutely required for the development of the lymphatic system (Kärkkäinen et al., 2004; Mäkinen et al., 2007).

1.2. Angiogenic and lymphangiogenic factors

1.2.1. Receptor-tyrosine kinases

Receptor tyrosine kinases (RTKs) are highly conserved key regulators of many cellular processes including proliferation, differentiation, cell survival, migration, and cell cycle control. RTKs are required for cell signaling pathways during both embryogenesis and adult homeostasis. The molecular structure is similar in all RTKs: they consist of an extracellular domain containing ligand-binding regions, a transmembrane helix, and an intracellular region containing the protein tyrosine kinase domain, an additional carboxy terminal, and regulatory regions. Mutations in RTKs and their altered activation are linked to different diseases and conditions such as inflammation, cancer, and atherosclerosis. RTKs are activated by growth factors. Binding of the ligands induces dimerization of the receptors which leads to auto- or transactivation of the receptor tyrosine residues and activation of downstream signaling effectors (Hubbard & Miller, 2007; Lemmon & Schlessinger, 2010).

1.2.2. Vascular endothelial growth factors and their receptors

Vascular endothelial growth factors (VEGFs) are a family of growth factors which includes five proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and a placenta growth factor (PlGF). VEGFs have different isoforms that are formed through alternative splicing or proteolytic processing. After secretion, some isoforms may diffuse quite freely in tissues while some of the isoforms bind to heparan sulfate proteoglycans on the cell membrane (Tammela et al., 2005). VEGFs bind three tyrosine kinases, VEGFR-1–3 and to two non-kinase receptors: neuropilin 1 (Nrp1) and neuropilin 2 (Nrp2), which are co-receptors of VEGFRs (Zhang et al., 2010). Nrp-1 and Nrp-2 have roles in immunology and neuronal development, and they are also involved in angiogenesis and lymphangiogenesis. They bind class 3 semaphorins, which are molecules that mediate repulsive signals during neuronal axon development. Nrp-1 also binds VEGF-A, VEGF-B and PlGF and Nrp-2 binds VEGF-A, VEGF-C and PlGF. Nrp-1 acts as a co-receptor to enhance the interactions of VEGF-A and VEGFR-2 and it may also form complexes with VEGFR-1 (Tammela et al., 2005; Roy et al., 2006).

VEGF receptors consist of three domains: an extracellular domain for ligand binding, a transmembrane domain, and an intracellular domain with the tyrosine kinase activity. Binding of VEGFs to their receptors leads to activation of the tyrosine kinase enzyme in the intracellular domain, which phosphorylates the tyrosine residues and activates the downstream pathways (Melincovici et al., 2018). VEGFR-1 is expressed in a variety of cell types, including ECs, pericytes, osteoblasts and macrophages. VEGFR-1 binds VEGF-A, VEGF-B and PlGF and its deletion has been shown to lead to death at early embryogenesis in mice due to disorganized blood vasculature and overgrowth of ECs (Roy et al., 2006). VEGFR-1 forms heterodimers with VEGFR-2 and has strong signaling properties in the heterodimeric form. VEGFR-1 expression is upregulated during angiogenesis and in hypoxic conditions (Roy et al., 2006; Mäkinen et al., 2001).

VEGFR-2 binds VEGF-A, VEGF-C and VEGF-D and it is the primary receptor transmitting VEGF-mediated signals in ECs. VEGFR-2 signaling pathway regulates vasodilation, migration and proliferation of ECs. VEGFR-3 binds VEGF-C and VEGF-D. VEGFR-3 signaling is crucial for development of blood vessels during embryonic development, but the expression is reduced in

mature vessels (Roy et al., 2006, Mäkinen et al., 2001). However, VEGFR-3 is activated in BECs during angiogenesis (Tammela et al., 2008). VEGFR-3 is required for lymphangiogenesis and its expression is upregulated in ECs of vascular tumors (Roy et al., 2006). Mutations in VEGFR-3 have been linked to hereditary lymphedema, also known as primary lymphedema (Tammela et al., 2005).

1.2.2.1. VEGF-A, PlGF and VEGF-B

VEGF-A is a multifunctional cytokine whose expression is induced by hypoxic stress and other cytokines including platetet-derived growth factor and epidermal growth factor. VEGF-A promotes angiogenesis via VEGFR-1 and VEGFR-2 (Cudmore et al., 2006). VEGF-A also promotes EC survival by increasing the expression of different anti-apoptotic factors such as Bcl-2 and A1. Double deletion of VEGF-A leads to death at E8–E9 in mice, and embryos lacking even one VEGF-A allele die at E11–E12 (Roy et al., 2006).

PlGF and VEGF-B are ligands for VEGFR-1 and Nrp-1. PlGF is mostly expressed in the placenta, heart, and lungs and it can form heterodimers with VEGF-A. VEGF-B is also able to form heterodimers with VEGF-A and it is predominantly expressed in striated muscles, brown fat and myocardium (Tammela et al., 2005). The physiological role of VEGF-B is still being studied as it does not have a clear effect on angiogenesis or lymphangiogenesis. However, VEGF-B is highly expressed in the heart, and it has been shown that VEGF-B deficiency affects the blood vessel density in ischemic mice (Li et al., 2009). It has also been found that VEGF-B gene or protein transfer increases blood vessel density during infarcts and in ischemic mouse hearts suggesting that VEGF-B has angiogenic activity in the heart, specifically related to pathological conditions. VEGF-B deficiency has been shown to lead to poor blood vessel survival in the cornea and it seems to have a survival effect on different types of neurons, such as retinal neurons, brain cortex neurons and motor neurons in the spinal cord (Li et al., 2009; Räsänen et al., 2021).

1.2.2.2. VEGF-C and VEGF-D

VEGF-C is produced as a precursor protein and activated by intracellular secretory proprotein convertases. VEGF-C is further proteolyzed in the extracellular space by plasmin and some other proteases. This modification produces a 21 kD non-disulfide-bound homodimeric protein which

has affinity for both VEGFR-2 and VEGFR-3. VEGF-C induces mitogenesis, migration and survival of ECs. During embryonic development, VEGF-C and VEGFR-3 are expressed mostly in the sites of lymphangiogenesis. At adult state, the expression of VEGF-C decreases in most tissues but remains high in the lymph nodes, lungs and heart (Kukk et al., 1996). It has been shown that adenoviral VEGF-C transduction induces growth of new lymphatic vessels. Loss of single allele has been shown to result in lymphedema and hypoplasia of the cutaneous lymphatic vessels (Tammela et al., 2005). VEGF-C is the primary lymphangiogenic growth factor and it also mediates blood vascular permeability via VEGFR-2 (Roy et al., 2006).

VEGF-C/VEGFR-3 signaling is also regulated by mechanical forces, such as stretching of the cells due to increased hydrostatic pressure and flow shear stress. In vitro studies have shown that mechanical stretching of LECs causes interactions between integrin ß1and VEGFR-3 leading to VEGF-C-mediated phosphorylation of VEGFR-3 which is followed by LEC proliferation. Flow shear stress has shown to increase the proliferation and sprouting of LECs. These results indicate that the flow of lymph itself enhances lymphangiogenesis (González-Loyola & Petrova, 2021).

VEGF-D is expressed in a variety of adult tissues, including the vascular endothelium, heart, lung, and skeletal muscle. VEGF-D is a subject to proteolytic modifications in its N-terminal and C-terminal ends. The mature form binds both VEGFR-2 and VEGFR-3. VEGF-D is associated with endothelial cell proliferation, and it has been proposed to have a role in tumor angiogenesis and lymphangiogenesis (Roy et al., 2006).

1.2.3. The Tie receptor family

Tie1 and Tie2 are receptor tyrosine kinases expressed by endothelial cells. They have a unique extracellular structure containing epidermal growth factor, immunoglobulin, and fibronectin type III domains. Angiopoietin growth factors are ligands for Tie2 receptor, while Tie1 is an orphan receptor whose full function is still to be investigated (Saharinen et al., 2015). Tie1 and Tie2 can form heterodimers together. In the heterodimeric form Tie1 modulates the signaling of Tie2 by either inhibiting or up-regulating the activation of Tie2 (Zhang et al., 2021). Together with VEGFs and their receptors, the Tie receptors and angiopoietins form the second endothelial specific RTK signaling pathway (Saharinen et al., 2015). Tie1 and Tie2 are expressed in endothelial cells and in some hematopoietic stem cells and pericytes. The extracellular region of both Tie1 and Tie2

contains three EGF-like modules which are connected by two immunoglobulin (Ig)-like domains, and three fibronectin type III (FNIII) repeats. In Tie2, the first Ig-like domain and the EGF-like modules are required for binding of both Ang1 and Ang2. The cytoplasmic regions of both receptors consist of tyrosine kinase domains offering a site for phosphorylation and protein interactions (Macdonald et al., 2006). Growth factors Ang1, Ang2 and Ang4 are ligands for Tie2 but they do not bind Tie1 (Davis et al., 1996; Lee et al., 2004; Maisonpierre et al., 1997). However, Tie1 can be activated by angiopoietins via its interaction with Tie2. It has been shown that the complete activation of Tie2 by Ang1 and Ang2 requires the activation of Tie1 as well (Saharinen et al., 2017).

1.2.3.1. Angiopoietin-Tie pathway

The angiopoietin-Tie signaling pathway regulates vascular permeability and vascular remodeling in endothelial cells during inflammation, tumor angiogenesis and metastasis (Saharinen et al., 2017). Vascular remodeling may result from molecular, structural, or functional changes in endothelial cells. These changes can cause plasma leakage and leukocyte influx from microvasculature during inflammation. Remodeling is induced by cytokines such as VEGFs, tumor necrosis factors, Ang1 and Ang2 (Korhonen et al., 2016). Ang1 is the major ligand in quiescent vasculature and Ang2 is activated during inflammatory, septic, and other pathological conditions. Ang1 is an agonist for Tie2 receptor phosphorylation regulating vascular remodeling during embryonic development and maintenance of vascular stability after gestation (Kim et al., 2016). Dysregulation of the Angiopoietin-Tie signaling pathway can cause vascular impairment, ischemia, and reperfusion as well as promote the development of atherosclerotic plaques (Zhang et al., 2019). It has been shown that deletion of either Ang1, Tie1 or Tie2 results in embryonic lethality in mice (Korhonen et al., 2016).

Ang1 is an endogenous agonist for Tie2 and critical for Tie2 activation. Ang1 has an N-terminal super-clustering domain, a central coiled-coil domain, and a C-terminal fibrinogen-like domain which is responsible for Tie2 binding. Ang2 acts as a weaker Tie2 agonist or antagonist depending on the occasion (Jo et al., 2021). Ang1 is primarily produced by pericytes and vascular smooth muscle cells (Fukuhara et al., 2010). Ang2 is produced by endothelial cells and it is stored in Weibel-Palade bodies within the cells. Ang2 levels increase during vascular remodeling and in

response to TNF and VEGF stimulation. Ang2 expression is also increased in hypoxic conditions. During inflammation, Ang2 is released from Weibel-Palade bodies in response to inflammatory cytokines and Ang1/Tie2 signaling is reduced leading to increased downstream expression of Ang2 and transcription factors such as Forkhead box protein O1 (FoxO1). Ang2 destabilizes endothelial cells by activating the formation of actin stress fibers (Kim et al., 2016; Saharinen et al., 2017).

In vivo and in vitro experiments have shown that the Ang-Tie signaling pathway is modulated by endothelial integrins. Integrin α5β1 enhances Ang1-induced phosphorylation of Tie1 and Tie2, Akt phosphorylation, and the nuclear exclusion of FoxO1 (Saharinen et al., 2017). Microvascular dysfunction and the activation of endothelial cells are associated with increased endothelial permeability and inflammation. These conditions are a clinical problem in many pathological cases such as sepsis, vascular complications of diabetes, impaired wound healing, ischemia, reperfusion injuries, and atherosclerosis. Ang1 acts as a strong vascular stabilizer. In vivo and in vitro experiments have shown that Ang1 stimulation inhibits vascular leaking induced by inflammatory cytokines such as histamine, VEGF, and thrombin. Ang2 acts differently as it increases vascular leaking together with inflammatory cytokines while a high dose of recombinant Ang2 has been reported to reduce vascular permeability in mice (Saharinen et al., 2017).

1.2.4. Phosphoinositide 3-kinases

Phosphoinositide 3-kinases (PI3Ks) are enzymes that phosphorylate the 3-OH groups of inositol membrane lipids and thus control the activity of intracellular protein effectors and regulate cellular functions (Vanhaesebroeck et al., 2012). There are eight mammalian isoforms of PI3Ks which are divided into three groups based on their structure and lipid substrates (Graupera & Potente, 2013). The molecular structure consists of three different catalytic subunits (p110α, β, and δ) and five different isoforms of the regulatory subunit (p85α, p55α, p50α, p85β and p55γ). The role of these regulatory proteins is to bind, stabilize, and inhibit the catalytic subunits in basal conditions. Regulatory subunits also bring the catalytic subunits in contact with their lipid substrates at the membranes when they are activated. PI3Ks are divided into three main groups: I, II and III. Class I PI3Ks are further divided into two subclasses: IA and IB (Karar & Maity, 2011). The class I PI3Ks generate 3-phosphoinositide lipids, which are direct activators of signal transduction

pathways. The class II and III PI3Ks regulate membrane trafficking within the endocytic route and in autophagy and endosomal recycling. All PI3K classes consist of a PI3K core, which has a C2 domain, a helical domain, and a kinase domain (Bilanges et al., 2019).

1.2.4.1. Class I PI3Ks

Class I PI3Ks are heterodimers which have a p110 catalytic subunit and a regulatory unit. Class I PI3Ks are further divided into two subclasses: IA and IB. They consist of the PI3K catalytic core and N-terminal RAS-binding domain. Class IA PI3Ks also have a binding domain for a regulatory unit. Catalytic subunits in class IA are p110α, p110β, and p110δ, which are in complex with a p85 regulatory subunit. Class IB PI3Ks consist of a p110γ catalytic subunit and a p84 or p101 regulatory subunit. The recruitment of class IA PI3Ks to the plasma membrane occurs via binding of the SH2 domains of p85 regulatory subunit to tyrosine-phosphorylated proteins like RTKs or other membrane-bound receptors or proteins (Bilanges et al., 2019). Class I PI3Ks are activated by RTKs, G protein-coupled receptors, and some small GTPases. The interaction of regulatory subunits of PI3Ks and phosphotyrosine residues of the RTKs recruit p110 to the cell membrane, which leads to the activation of its catalytic activity (Vidal et al., 2021). Activated class I PI3Ks phosphorylate phosphatidylinositol 4,5-bisphoshate at the cell membrane. This phosphorylation further generates the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) which is metabolized to phosphatidylinositol 3,4-bisphoshpate (PIP_2). Beside the catalytic activity of class I PI3Ks, their catalytic subunits have also protein scaffolding activity, which allows them to act as adaptor proteins in assembling of protein-protein complexes and to regulate a variety of physiological functions. It has been proposed that there are at least 10–30 proteins in a typical mammalian cell that bind class I PI3Ks (Bilanges et al., 2019).

1.2.4.2. Class II PI3Ks

Class II PI3Ks are a less known class of PI3Ks. There are three different isoforms in vertebrates: PI3KC2α, PI3KC2β and PI3KC2γ. The first two are ubiquitous and the last one is expressed mainly in the liver. Class II PI3Ks don't have a classic signal transducing activity downstream of plasma membrane receptors. They have a role in regulating intracellular membrane dynamics and membrane trafficking and their functions seem to vary between different cell types. It has been proposed that class II PI3Ks have important roles in endothelial cells, as they are required for

angiogenic signaling in response to growth factors. They consist of a PI3K catalytic core with non-structured N-terminal and C-terminal phox homology domain as well as C2 domain. Class II PI3Ks have been linked to endocytosis and recycling endosomes together with late endosomes or lysosomes (Bilanges et al,. 2019).

PI3KC2α is encoded by *Pik3c2a* gene. Yoshioka et al. (2012) found that *Pik3c2a* knockout mice embryos showed retarded growth from E8.5 and the embryos died between E10.5–11.5 due to vascular defects. The knockout embryos showed highly disorganized or absent blood vessel pattern at the dorsal aorta, intersomitic vessels and branchial arches. PI3KC2α is required in sprouting angiogenesis and in maintaining vascular hyperpermeability in case of vascular damage. Class II PI3Kα is also essential for endothelial cell migration, proliferation, and survival, as well as assembling of VE-cadherin at cell-cell junctions (Yoshioka et al. 2012).

1.2.4.3. Class III PI3Ks

Class III PI3Ks, also known as VPS34, have critical functions in many physiological processes, such as vesicular trafficking, phagocytosis, macroautophagy, cytokinesis and nutrient sensing (Iershov et al. 2019). VPS34 has two main complexes that consist of VPS34, VPS15 and beclin1 including either ATG14 (complex I) or UVRAG (complex II). Complex I produces PIP_3 on autophasosome precursor membranes and is recruited to the initiation sites of phagophores, mainly at the endoplasmic reticulum. Sufficient amount of PIP_3 in the ER is crucial for the formation and elongation of the developing autophagosome. Complex II also controls the maturation of endosomes and promotes the fusion of late endosomes/lysosomes (Bilanges et al. 2019). As the class II PI3Ks, class III PI3Ks don't have a direct impact on signal transducing. However, through the regulation of endosomal trafficking, they regulate signaling indirectly via protein kinases such as Ser/Thr kinase SGK3 or LKB1. It has been found that double *Vps34* knockout is lethal and leads to death at early embryogenesis in mice (Bilanges et al., 2019).

1.2.5. PI3K-Akt signaling pathway

Protein kinase B, also known as Akt, belongs to the AGC group of protein kinases. Its main function is to mediate PI3K pathway (Fayard et al., 2010). Akt is activated by PIP_3 or PIP_2-driven recruitment to the plasma membrane and phosphorylation of the Thr308 and Ser473 residues.

Activated Akt phosphorylates a variety of substrates upon activation of PI3K pathway signaling. To date, over 100 Akt substrates have been identified. These substrates take part in regulating many physiological functions such as cell cycle progression, differentiation, survival, and apoptosis (Bilanges et al., 2019). There are three mammalian isoforms of Akt: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). Akt1 is a ubiquitous protein kinase found in all tissues. Akt2 is mainly found in insulin-sensitive tissues and Akt3 is mostly found in testis and brain. The structure of Akt comprises of three functional domains: an amino-terminal pleckstrin homology domain, a central catalytic domain, and a carboxyl-terminal regulatory domain that contains the hydrophobic motif FPQFSY (phenylalanine, proline, glutamine, serine and tyrosine, respectively) (Fayard et al., 2010).

Akt has many direct substrates including members of the forkhead family of transcription factors (FoxOs). FoxOs are important mediators of the cell cycle facilitating the G1/S transition via transcriptional regulation of the cyclin-dependent kinase inhibitors p21 and p27. P21 has also been shown to be directly phosphorylated by Akt. Akt increases cyclin D1 stability via phosphorylation and via inhibition of glycogen synthase kinase-3 (GSK3) which promotes cell cycle progression (Fayard et al., 2010). Akt affects cellular metabolism, growth, proliferation, and overall survival (Hemmings & Restuccia, 2021).

The PI3K/Akt signaling pathway is a highly conserved pathway and its activation is tightly controlled. The pathway is activated by growth factors including insulin, which bind the receptors on cell surface (Hemmings & Restuccia, 2021). Activated PI3Ks catalyze the conversion of PIP_2 to PIP_3, which recruits multiple effector proteins including Akt and 3-phosphoinositide-dependent kinase 1 (PDK1). Fully activated Akt inhibits for example p21, p27, and the FoxO family of transcription factors. Akt activates IκB kinase alpha, MDM2, and telomerase reverse transcriptase. The PI3K/Akt pathway is negatively regulated by the phosphatase and tensin homolog which catalyzes the conversion of PIP_3 back to PIP_2 (Vidal et al., 2021).

The PI3K/Akt pathway has an important role in many cellular functions such as proliferation, migration, adhesion, invasion, survival and metabolism, and the pathway is upregulated in many cancers. Activation of PI3K/Akt signaling pathway is required for angiogenesis and lymphangiogenesis as well as for vascular permeability. The PI3K/Akt pathway modulates the

expression of other angiogenic factors as well, for example nitric oxides and angiopoietins. The effects of VEGF in endothelial cells are partly mediated by the PI3K/Akt pathway as it has been shown that binding of VEGF to its receptor activates the PI3K/Akt pathway in endothelial cells (Karar & Maity, 2011).

1.2.6. FoxO1

Forkhead box protein O1 (FoxO1) is a transcription factor that acts as a repressor of gene expression in the endothelium (Scallan et al., 2021). Endothelial-specific deletion of *FoxO1* has shown to increase the number on lymphatic vessels while *FoxO1* overexpression leads to decreased amount of lymphatic vessels in mice (Niimi et al., 2021). The activation and nuclear localization of FoxO1 is regulated by Akt via phosphorylation. Phosphorylated FoxO1 is excluded from the nucleus which directly inhibits its activity (Scallan et al., 2021). FoxO1 is involved in many cellular processes such as oxidative stress resistance, DNA repair, cell cycle arrest and energy metabolism. The deletion of *FoxO1* results in embryonic lethality in mice due to severe malformations in cardiovasculature. Activation of PI3K/Akt signaling pathway by RTKs (figure 1) leads to nuclear exclusion of FoxO1 and repression of its target genes (Hemmings & Restuccia, 2021).

FoxO1 affects vascular stability and remodeling through up-regulation of genes associated with endothelial cell apoptosis and vascular destabilization. Ang1/Tie-mediated FoxO1 inhibition leads to improved endothelial cell survival and blood vessel stability together with PI3K/Akt pathway-induced up-regulation of survivin expression (Fukuhara et al., 2010). FoxO1 induces the secretion of Ang2 leading to Tie2 phosphorylation (Daly et al., 2006). During inflammation, loss of Tie1 due to ectodomain shedding promotes the antagonistic binding of Ang2 on Tie2 causing a positive feedback loop and leading to suppression of phosphorylated Tie2, activation of FoxO1, up-regulation of Ang2 expression and thus to vascular remodeling. Ang2 blockade during infection inhibits nuclear accumulation of FoxO1 and Ang2 expression, and leads to vascular enlargement. Under normal conditions, the transcriptional activity of FoxO1 and the expression Ang2 are low or absent. However, the increased amount of nuclear FoxO1 and Ang2 expression can be prevented by Ang2 blockade or by giving exogenous Ang1 to activate Tie2/PI3K/Akt signaling (Kim et al., 2016).

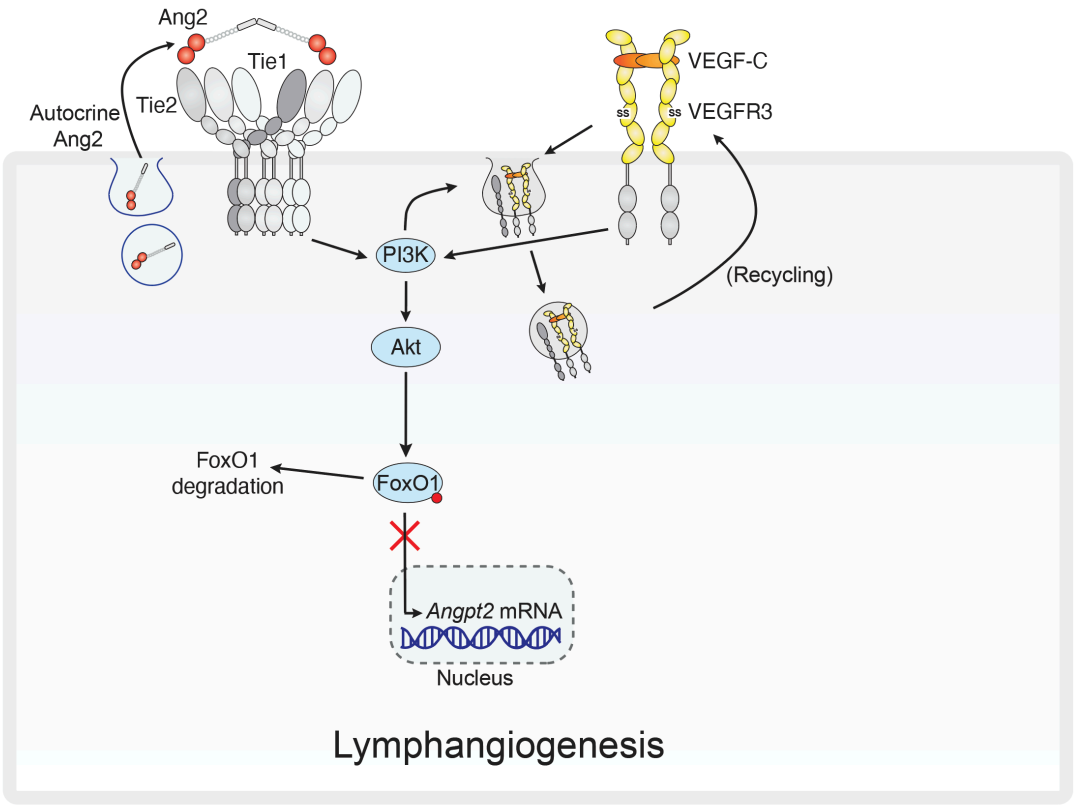


Figure 1. Schematic overview of Ang2/Tie/PI3K signaling pathway. Ang2/Tie and VEGF-C/VEGFR-3 signaling pathways both activate PI3K/Akt signaling leading to phosphorylation of transcription factor FoxO1. Phosphorylated FoxO1 is excluded from the nucleus leading to inhibition of its target genes including *Angpt2*. VEGF-C induces internalization of its receptor VEGFR-3. Internalized VEGFR-3 is trafficked for degradation or recycling. (Image modified from Korhonen et al., 2022.)

Oscillatory shear stress caused by lymph flow in lymphatic vasculature drives lymphatic vessel development. Oscillatory shear stress activates Akt in lymphatic endothelial cells and leads to phosphorylation of serine or threonine residues of FoxO1 resulting in its transportation into cytoplasm. When FoxO1 is transported into cytoplasm, it gets degraded via a ubiquitin-dependent pathway. It has been demonstrated that FoxO1 localizes in the cytoplasm especially in valve forming LECs. This is also characterized by high *Prox1* expression, whereas non-valve forming LECs show low *Prox1* expression together with nuclear FoxO1 (Niimi et al., 2021).

FoxO1 has a role in inhibiting the formation of new lymphatic vessels by repressing genes involved in the process (Scallan et al., 2021). It seems that the main target of FoxO1 is *FOXC2*, which is a shear-responsive gene involved in lymphatic vessel formation. (Niimi et al., 2021). Loss of *FoxO1* in a mouse model of lymphedema-distichiasis has shown to fully restore the number of lymphatic vessels and the functioning of the vessels back to normal level. These results indicate that controlling of FoxO1 is of clinical interest for the treatment of lymphedema characterized by lack of functional lymphatic vessels. Deletion of *FoxO1* has shown to lead to formation of new lymphatic vessels. These newly formed vessels have normal morphological and molecular structures and they can be used to restore lymphatic vessels in lymphedema mouse models. The clinical value of FoxO1 also holds the fact that it has been shown to selectively restore the defective lymphatic vessels without leading to uncontrolled growth in healthy vessels (Scallan et al., 2021).

1.3. Aims of the thesis

FoxO1 is an important regulator of lymphangiogenesis. It has been shown that in endothelial cells, FoxO1 is activated downstream of PI3K/Akt signaling which is activated by both Ang2/Tie and VEGF-C/VEGFR-3 signaling pathways. The aim of this thesis was to study the crosstalk of Ang2/Tie and VEGF-C/VEGFR-3 signaling pathways. The effects of these pathways were studied in vitro by examining the PI3K/Akt signaling-mediated FoxO1 inhibition in cultured LECs. The localization of Tie and VEGFR-3 receptors on the lymphatic endothelial cell membrane was studied with proximity ligation assay. To examine the crosstalk of these two pathways in vivo, we studied the effect of lymphatic-specific Tie1, Tie2 or Tie1;Tie2 double deletion on VEGF-C induced lymphangiogenesis in skeletal muscles of mice.

The hypothesis were:

1) Activation of VEGFR-3 via VEGF-C stimulation leads to nuclear exclusion of transcription factor FoxO1, and use of Ang2 blocking antibody (MEDI3617) may alter the localization.

2) Due to the crosstalk of these pathways, Tie1 and VEGFR-3 receptors are located in close proximity on the cell membrane.

3) Tie1, Tie2 or Tie1;Tie2 double deletion changes the rate of VEGF-C induced lymphangiogenesis in skeletal muscles of mice.

2. Materials and methods

This work is a part of the publication by Korhonen et al. *Lymphangiogenesis requires Ang2/Tie/PI3K signaling for VEGFR3 cell-surface expression* (2022). For the publication and background research, I carried out the in vitro experiments presented in this thesis, including cell culture, growth factor treatments, proximity ligation assay, immunofluorescence staining, imaging, and quantification. For the in vivo experiments, I prepared cryosections from previously collected skeletal muscles of mice and carried out immunofluorescence staining, imaging, and quantification.

2.1. Lymphatic endothelial cell culture

The activation of PI3K/Akt signaling pathway and the localization of FoxO1 were studied in cultured LECs. As it has been shown that Ang2 antibody reduces the expression of VEGFR-3 on LECs (Korhonen et al., 2022), we wanted to investigate whether stimulation with Ang2 blocking antibody (MEDI3617) would alter the localization of FoxO1 compared to treatment with VEGF-C or with MEDI3617 and VEGF-C together. The preceding activation of PI3K/Akt signaling was checked with an additional staining of phosphorylated Akt. Human dermal lymphatic endothelial cells (HDLEC, PromoCell, C-12216) were cultured in Endothelial Cell Growth Medium MV2 (PromoCell C-39221) on 10 cm plates coated with 0.1 % gelatin. For the experiments, the cells were transferred onto 0.12 cm diameter glass coverslips on 10 cm plates and grown on MV2 medium until confluent. When the cells had reached confluency, the medium was changed to Endothelial Cell Growth Medium MV (PromoCell C-39220) without VEGF-C and the cells were incubated overnight.

2.1.1. Cell stimulation and immunofluorescence staining

The cells were incubated in MV medium overnight, and the next day the coverslips were transferred onto 6-well plates for the experiment. The cells were treated for 2 hours with either anti-Ang2 antibody MEDI3617 (5 µg/ml) or human IgG (5 µg/ml) as a control. After the antibody treatment, the cells were stimulated with VEGF-C (100 ng/ml) for 0.5, 1, 3 or 6 hours. After stimulation, the cells were rinsed quickly with phosphate buffered saline (PBS) and fixed with 4 % paraformaldehyde (PFA) in PBS for 10 minutes. After fixation, the cells were washed 3 x 10

minutes with PBS. The cells were permeabilized with 0.3 % Triton-X (Tx) in PBS for 5 minutes. After permeabilization, the cells were washed with PBS and incubated in blocking solution containing 5 % normal donkey serum (NDS) and 0.1 % NaN_3 in PBS, for 1 hour at room temperature. The cells were incubated with primary antibodies diluted in antibody diluent (2.5 % NDS, 0.05 % NaN_3, 0.05 % Tween-20 in PBS) overnight at +4 °C. The cells were immunostained for phosphorylated Akt and FoxO1 in two different stainings. Antibodies are listed in table 1.

Table 1. Primary and secondary antibodies used in immunostaining of LECs.

Primary antibodies			Secondary antibodies		
Antibody	Manufacturer	Catalog No.	Antibody	Manufacturer	Catalog No.
FoxO1	Cell Signaling Technology	#28805	Donkey-a-rabbit, Alexa Fluor 488	Invitrogen	#A-32790
pAkt	Cell Signaling	#9271S			
Prox1	R&D Systems	#AF2727	Donkey-a-goat, Alexa Fluor 594	Invitrogen	#A-11058
VE-Cadh	BD Pharmingen	#555661	Donkey-a-mouse, Alexa Fluor 647	Invitrogen	#A-31571

After the overnight incubation with primary antibodies, the cells were washed 3 x 15 minutes with PBS. Secondary antibodies were diluted in antibody diluent and incubated for 1 hour at room temperature. After the incubation, the cells were washed 2 x 10 minutes with PBS. To stain the nuclei, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI, 1 ug/ml) in PBS for 10 minutes at room temperature and washed with PBS. The cells were post-fixed with 4 % PFA in PBS for 10 minutes and washed 3 x 5 minutes with PBS. After the final washes, the coverslips were dipped in sterile water to remove any salt residues, and air-dried on bench. Finally, the coverslips were mounted with Vectashield (Vector Laboratories) and sealed with Cytoseal 60 (Epredia™). The cells were imaged using Zeiss LSM880 confocal microscope with ZEN 2.3 SP1 black edition acquisition software.

2.2. Proximity ligation assay

Proximity ligation assay (PLA) is a method for detecting protein-protein interactions within cells. In the assay, target proteins are first stained with immunofluorescence-compatible primary antibodies and then incubated with PLA probes. The probes bind primary antibodies and form

circular DNA after hybridization. The formed circular DNA is amplified and visualized as spots of proximity with fluorescence microscopy (Alam, 2018).

In this experiment,. PLA was used to investigate whether Tie1+VEGFR-3 and Notch1+VEGFR-3 are located in close proximity to each other in permeabilized LECs. PLA was done according to the Duolink® PLA Protocol using Duolink® flowPLA Detection Kit - Red (Sigma-Aldrich DUO94001) with anti-mouse PLUS (DUO92001) and anti-human MINUS (DUO92021) PLA probes. LECs were cultured in MV2 medium on 0.1 % gelatin coated coverslips until about 90 % confluency. Then the cells were incubated overnight in MV medium, fixed with 4 % PFA in PBS for 10 minutes, permeabilized with 0.3 % PBS-Tx for 5 minutes and blocked with Duolink Blocking solution (DUO82007) for 1 hour. Primary antibodies (listed in table 3) were diluted in antibody diluent and incubated overnight at +4 °C. After primary antibody incubation, the cells were washed 2 x 5 minutes with Wash Buffer A at room temperature. Wash buffer wash replaced with PLA probe solution and the coverslips were incubated in a humidity chamber for 1 hour at +37 °C. Then the coverslips were washed 2 x 5 minutes with Wash Buffer A and incubated with ligation solution in a humidity chamber for 30 minutes at +37 °C. After ligation, the coverslips were washed 2 x 5 minutes with Wash Buffer A and incubated in a humidity chamber with amplification solution for 100 minutes at +37 °C. The coverslips were washed 2 x 10 minutes with 1x Wash Buffer B (with DAPI in the second wash) and 1 minute with 0.01x Wash Buffer B. After the washes, the coverslips were post-fixed with 4 % PFA in PBS, washed 3 x 10 minutes with PBS, dipped in sterile water, air-dried, and mounted with with Vectashield (Vector Laboratories) and sealed with Cytoseal 60 (Epredia™). The coverslips were imaged using Zeiss LSM880 confocal microscope using ZEN 2.3 SP1 black edition acquisition software.

Table 2. Primary antibodies used in PLA.

Antibody	Manufacturer	Catalog No.
Human anti-Tie1 Dx2240	Creative Biolabs	#AFC-586CL
Mouse anti-human Notch1 ab44986	Abcam	ab44986
Mouse anti-human VEGFR-3 9D9	ReliaTech	#101-M36
Human anti-human VEGFR-3 3C5	ThermoFischer	#14-5988-82
Human IgG		
Mouse IgG		

2.3. Mouse models

A tetracycline-regulated mouse model was used to study the interaction between Tie1 and Tie2 receptors and VEGF-C growth factor induced lymphangiogenesis. Lymphatic-specific Tie1, Tie2 and Tie1;Tie2 deletions were achieved by crossing mice carrying floxed Tie1 and/or Tie2 alleles to Prox1CreERT2 driver mice (Tie1-del, Tie2-del and Tie1;Tie2-del mice). Mice carrying only floxed alleles and no Cre were used as controls. Recombination was induced in adult mice by giving the mice 5 doses of Tamoxifen orally on 5 consecutive days. Adeno-associated virus (AAV)-mediated VEGF-C (or AAV-Empty as a control) was injected into tibialis anterior muscles ($1x10^{11}$ vp/muscle) of these mice to see whether the deletion of Tie receptors alters VEGF-C-induced lymphangiogenesis in skeletal muscles. Since skeletal muscle tissue doesn't normally contain lymphatic vessels, all lymphangiogenic growth seen in the muscles of these mice was considered as resulting from the AAV-injections. Lymphatic growth was detected by immunostaining the muscle sections with Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) antibody, which is a lymphatic specific marker.

For analysis of lymphangiogenic response in tibialis anterior muscles, the mice were perfused with 2 % PFA and the tibialis anterior muscles were collected and embedded in Surgipath FSC 22 Clear Frozen Section Compound (Leica Byosystems Richmond, Inc.), frozen and stored at -80 °C. Tissue blocks were sectioned with cryotome (Cryostar NX70, Thermo Fisher Scientific). 10 µm sections were cut onto Superfrost^{TM}Plus Adhesion Microscope Slides (Epredia, Gerhard Menzel GmbH). The sections were dried at room temperature for 30 minutes and fixed with 1 % PFA in PBS for 5 minutes, washed 3 x 7–10 minutes with PBS and 8 minutes with 0.3 % PBS-Tx. The sections were blocked with donkey immunomix (DIM; 5% donkey serum, 0.2% bovine serum albumin, 0.3% Triton-X, 0.05% NaN_3 in PBS) for one hour at room temperature. Primary antibodies were diluted in DIM and incubated overnight at +4 °C. After the primary antibody incubation, the sections were washed 3 x 5–10 minutes with 0.1 % PBS-Tx. Secondary antibodies were diluted in DIM and incubated one hour at room temperature. Then the sections were washed 3 x 5–10 minutes with 0.1 % PBS-Tx, incubated with DAPI in PBS for 5 minutes, and washed twice with 0.1 % PBS-Tx and once with PBS for 5–10 minutes. The sections were post-fixed with 1 % PFA in PBS for 5 minutes and washed 3 x 5–10 minutes with PBS. The sections were then dipped in sterile water

and mounted in ProLong Gold Antifade mounting media (Thermo Fisher Scientific). The primary and secondary antibodies used for all muscle sections are listed in table 3.

Table 3. Antibodies used for staining Tibialis anterior muscle sections.

Primary antibodies			Secondary antibodies		
Antibody	Manufacturer	Catalog No	Antibody	Manufacturer	Catalog No
LYVE1	”home-made”		Donkey-a-rabbit, Alexa Fluor 488	Invitrogen	#A-21208
CD31	Cell Signaling Technology	#77699	Donkey-a-rat, Alexa Fluor 594	Invitrogen	#A-18741
VEGFR-3	R&D Systems	#AF743	Donkey-a-goat, Alexa Fluor 647	Invitrogen	#A-21447

2.4. Quantification

Fiji/ImageJ (Schindelin et al., 2012) was used for quantification of the data. In the FoxO1 staining, the nuclear/cytoplasmic ratio was quantified by its localization (nuclear or cytoplasmic) within the cell by measuring optical density (OD). For quantification, 20 cells from each condition and timepoint were checked to be Prox1 positive. FoxO1 localization was determined by selecting the area of the whole cell according to VE-cadherin staining (OD_{cell}) and the area of the nucleus according to DAPI staining ($OD_{nucleus}$), and the integrated density of the areas was measured. The nuclear/cytoplasmic ratio (N/C ratio) was calculated by subtracting the optical density of the nucleus from the optical density of the whole cell ($OD_{cytoplasm}$ = Odc_{ell}-$OD_{nucleus}$). The proximity ligation assay was quantified by measuring the amount of PLA spots per nuclei from 5 fields of view from each condition.

AAV-VEGF-C-induced formation of lymphatic vessels in tibialis anterior muscles of Tie1-deleted, Tie2-deleted and Tie1;Tie2-deleted mice was quantified by measuring the LYVE1 area (%) from whole muscle sections (N=6 muscles for Tie1 control + AAV-Empty, Tie1 control + AAV-VEGF-C, Tie1-del + AAV-Empty, Tie1-del + AAV-VEGF-C, Tie2-del + AAV-VEGF-C, Tie1;Tie2 control + AAV-VEGF-C, Tie1;Tie2-del + AAV-Empty, and for Tie1;Tie2-del + AAV-VEGF-C. N=4 muscles for Tie2 control + AAV-Empty, Tie2 control + AAV-VEGF-C, Tie2-del + AAV-Empty and Tie1;Tie2 control + AAV-Empty).

For statistical analyses, one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons was performed using GraphPad Prism version 9.4.1 for Mac (GraphPad Software, San Diego, California USA, www.graphpad.com).

3. Results

3.1. VEGF-C effect on pAkt and FoxO1 localization in LECs

The in vitro study was performed to observe whether antibody-mediated blocking of Ang2 affects the VEGF-C-mediated localization of FoxO1 in LECs. In addition, phosphorylated Akt (pAkt) staining (figure 2) was performed to study the activation of PI3K/Akt signaling pathway upstream of FoxO1. The pAkt staining showed that the kinetics of pAkt were altered upon VEGF-C stimulation as expected. 30 minutes after VEGF-C stimulation, pAkt staining pattern was vesicle-like, while 60 minutes after stimulation the pattern was more diffuse which might possibly indicate the redistribution of Akt trafficking to the plasma membrane. Interestingly, when the cells were treated together with an Ang2-blocking antibody, MEDI3617, pAkt staining pattern remained vesicle-like 60 minutes after VEGF-C stimulation, possibly indicating a change in the signaling activity. Staining of the IgG-treated control showed pAkt in small vesicles diffusely in the cytoplasm while cells treated with Ang2-blocking antibody, MEDI3617, showed larger vesicles. The kinetics remained similar after the 1-hour timepoint (appendix 1).

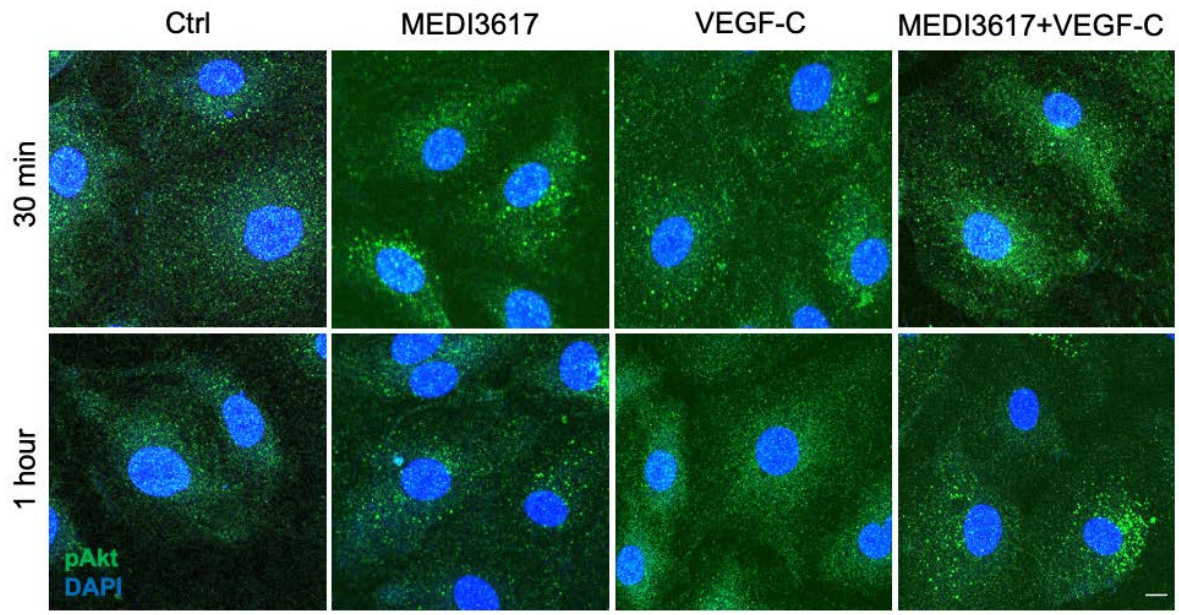


Figure 2. Effects of MEDI3617 and VEGF-C on the cellular localization of phosphorylated Akt in LECs. The cells were treated with IgG control, MEDI3617, VEGF-C or both MEDI3617 and VEGF-C. Permeabilized cells were stained with DAPI and pAkt antibody. Scalebar 10 µm.

The FoxO1 staining (figure 3) showed that in LECs treated with only IgG or MEDI3617, the localization of FoxO1 was more nuclear, and the amount of FoxO1 in the cytoplasm was quite small at all timepoints.

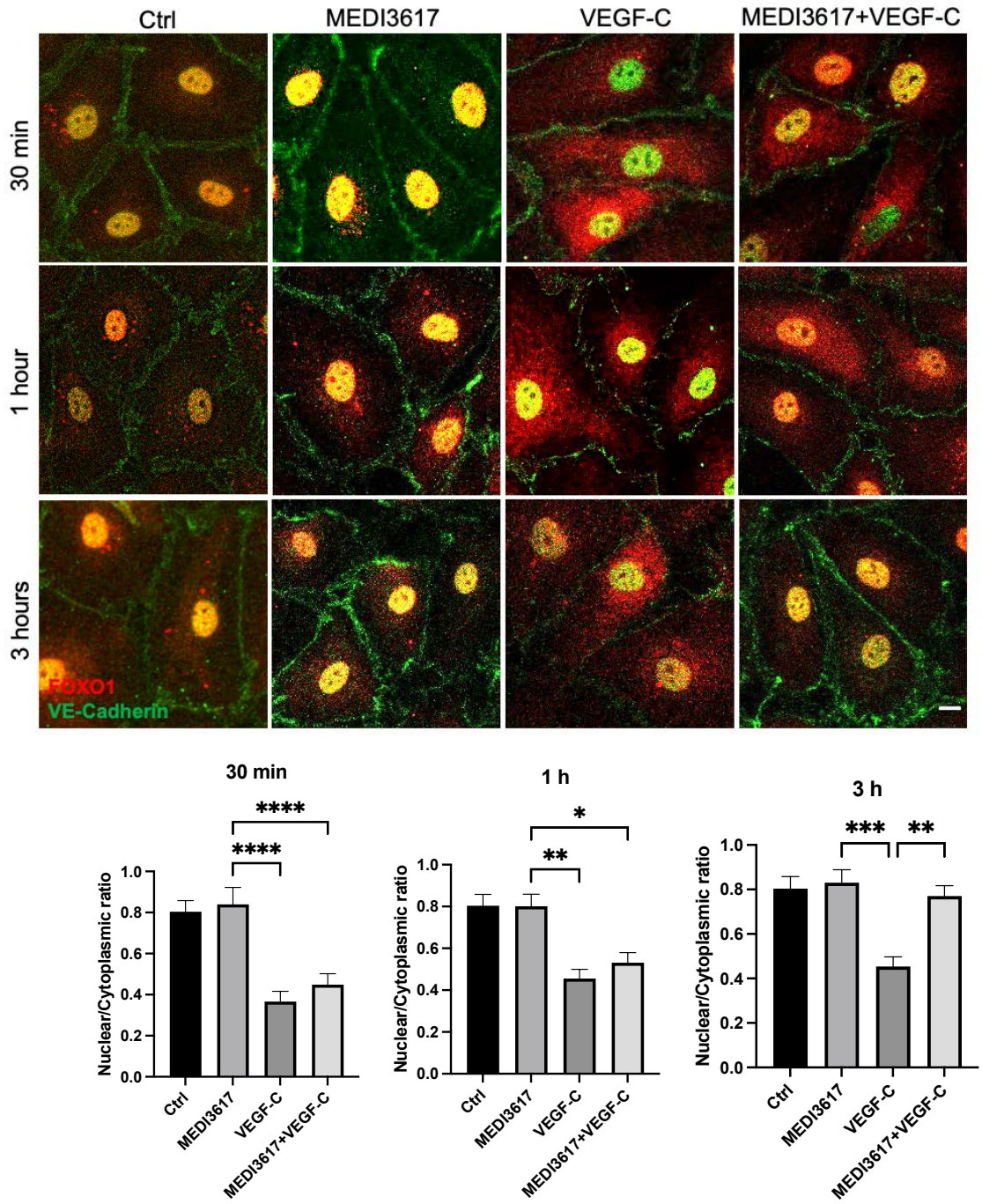


Figure 3. Effects of MEDI3617 and VEGF-C on the cellular localization of FoxO1 in LECs. The cells were stimulated with IgG control, MEDI3617, VEGF-C or both MEDI3617 and VEGF-C. Permeabilized cells were stained with FoxO1 and VE-Cadherin antibodies. Images were quantified by calculating the nuclear/cytoplasmic ratio of FoxO1. Scalebar 10 µm. Mean ± SEM, 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons. *p<0.05, **P<0.01, ***p<0.001, ****p<0.0001.

In VEGF-C-treated cells, the localization of FoxO1 was cytoplasmic at all timepoints indicating that the presence of VEGF-C in the growth medium activated both VEGFR-3 and PI3K/Akt signaling, and that the action remained for a rather long time. In cells treated with both MEDI3617 and VEGF-C, FoxO1 was cytoplasmic at 30 min and 1-hour timepoints, but nuclear at 3-hour timepoint compared to the VEGF-C treated cells ($F(3, 76) = 11.9$, $p<0.01$). The results were similar at the 6-hour timepoint (appendix 2). In control IgG or MEDI3617- treated cells, FoxO1 remained nuclear at all timepoints confirming that FoxO1 enters the nucleus when PI3K/Akt signaling is not activated.

3.2. Tie1 and VEGFR-3 are detected in close proximity in LECs

The PLA was carried out to determine whether VEGFR-3 and Tie1 are located in close proximity to each other in LECs. We also performed PLA for VEGFR-3 and Notch1 and VEGFR-3/Tie1 + control IgG to ensure that the assay does not produce non-specific staining. As expected, The PLA staining was strong with Tie1 and VEGFR-3 antibodies ($F(3, 16) = 201.5$, $p<0.0001$) while the amount of PLA events in Notch1+VEGFR-3 and IgG + VEGFR-3/Tie1 antibodies were weak (figure 4).

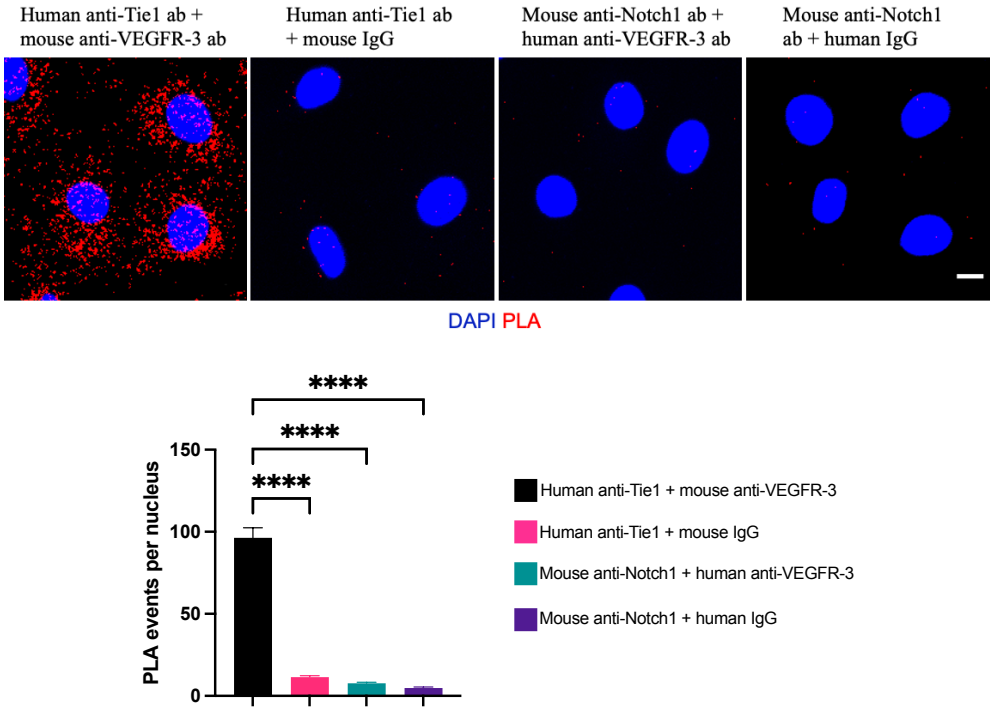


Figure 4. Proximity ligation assay of Tie1+VEGFR-3 and Notch1+VEGFR-3. Scalebar 10 µm. Mean ± SEM, 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons. ****P<0.0001.

3.3. Effect of Tie1, Tie2 or Tie1;Tie2-double deletion on VEGF-C-induced lymphangiogenesis in adult mice

To study the effects of Tie and Tie2 on VEGF-C-induced lymphangiogenesis we deleted Tie1, Tie2 or both from the lymphatic endothelium of adult mice and injected AAV vector encoding VEGF-C into tibialis anterior (TA) muscle of the mutant mice. Sections of TA muscles of Tie1-del, Tie2-del and Tie1;Tie2-del mice were stained with lymphatic marker LYVE1 to analyze the lymphangiogenic response in these mutant mice (figure 5). Both Tie1 and Tie1;Tie2-deletions significantly reduced lymphatic growth compared to controls with no deletion ($F(3, 20) = 11.5$, $p<0.01$ for Tie1 deletion and $F(3, 18) = 34.4$, $p<0.01$ for Tie1;Tie2 deletion). However, deletion of Tie2 ($F(3, 14) = 15.79$, $p = 0.498$) did not result in statistically significant difference compared to littermate controls.

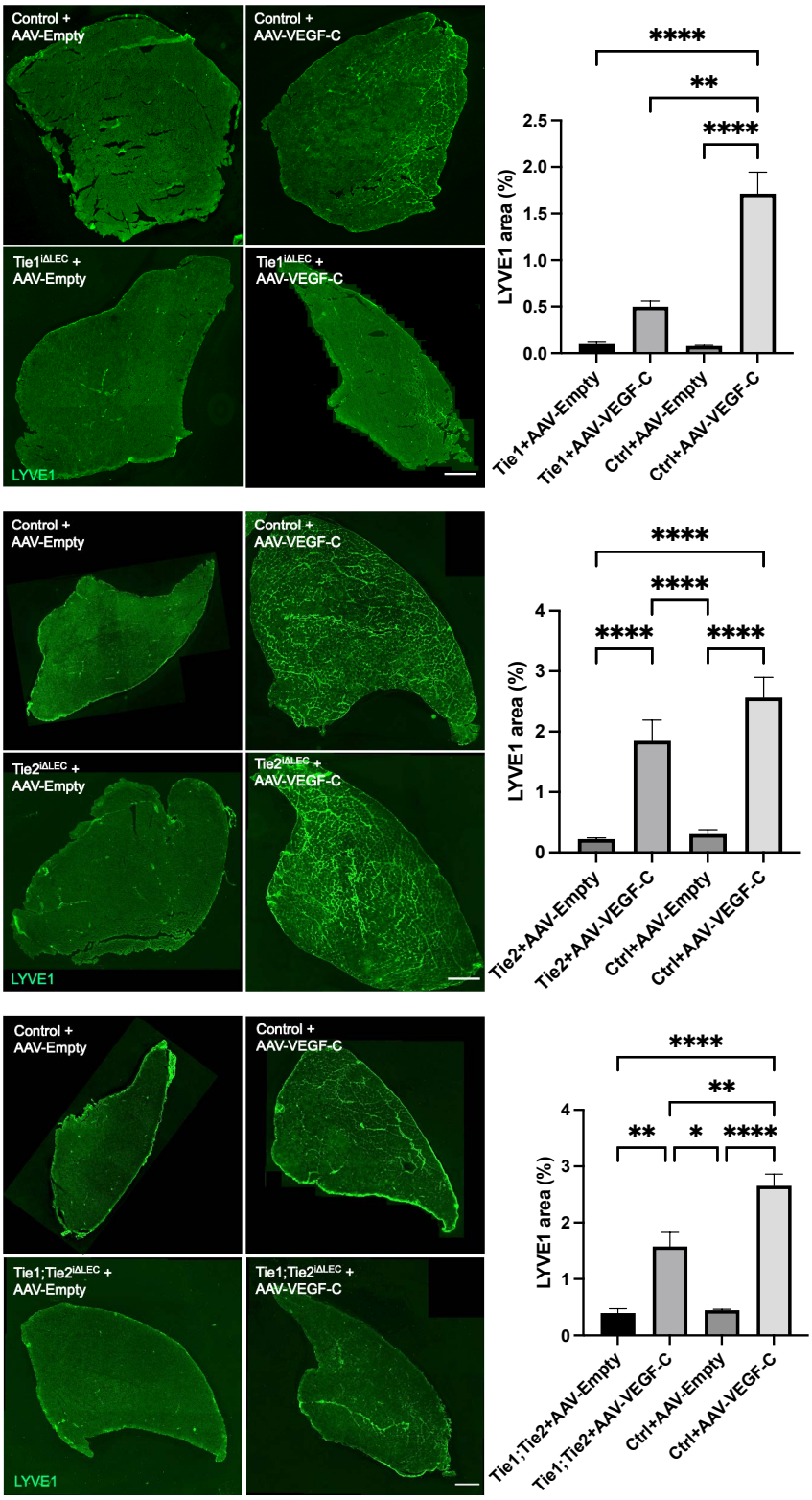


Figure 5. LYVE1 staining of tibialis anterior muscles of Tie1-deleted, Tie2-deleted and Tie1;Tie2-deleted mice treated with AAV-VEGF-C or AAV-Empty. Quantification of LYVE1 area (%). Scalebars 500 µm. Mean ± SEM, 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons.. Ns = P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4. Discussion

The aim of this thesis was to study the crosstalk between the two main signaling pathways in lymphatic vessel growth and maintenance; the Ang/Tie and the VEGF-C/VEGFR-3 pathways. The aim was to study how PI3K/Akt signaling pathway downstream of Ang2/Tie pathway affects the phosphorylation of FoxO1 and whether treatment with Ang2 blocking antibody would alter FoxO1 localization in LECs. Proximity ligation assay was performed to determine the proximity of Tie1 and VEGFR-3 receptors in cultured LECs. The role of the Tie receptors in VEGF-C-induced growth of new lymphatic vessels was studied with mouse models which had inducible lymphatic-specific deletion of Tie1, Tie2 or both Tie1 and Tie2 receptors. AAV-VEGF-C or AAV-Empty was injected into tibialis anterior muscles of these mice and control mice to study the role of Tie1 and Tie2 in VEGF-C-induced lymphangiogenesis.

4.1. In vitro experiments

PLA using Tie1 and VEGFR-3 antibodies showed strong staining while the amount of PLA events in Notch1+VEGFR-3 and Tie1/VEGFR-3 + IgG controls was low. Based on the PLA, there is a pool of Tie1 and VEGFR-3 receptors in LECs that are in close proximity to each other and a possibility of interaction between these proteins as hypothesized. The pAkt staining showed that VEGF-C activates PI3K/Akt signaling pathway in cultured LECs and that this action can be altered with an Ang2 blocking antibody. However, more research is needed regarding the kinetics and vesicle transportation of Akt in the cells in order to fully understand the altered response.

To study the effects of the same treatments downstream of PI3K/Akt, cells were stained for FoxO1 and its cellular localization was quantified. Cells that were treated with VEGF-C alone showed a strong cytoplasmic localization of FoxO1 indicating that VEGF-C-induced activation of VEGFR-3/PI3K/Akt signaling leads to FoxO1 phosphorylation and exclusion from the nucleus. It has been suggested that the increase in Ang2 e.g. after infection is caused by the transcriptional activation of FoxO1 which results from the suppressed activation of Tie2/PI3K/Akt signaling in LECs (Kim et al., 2016). VEGF-C has also been shown to increase Ang2 release from stimulated LECs leading to activation of Tie2/PI3K/Akt signaling and thus theoretically promote nuclear localization of FoxO1 (Korhonen et al., 2022). However, in this experiment Ang2 blocking antibodies were able

to inhibit VEGF-C induced nuclear exclusion of FoxO1 at 3-hour timepoint, suggesting that the crosstalk of these two ligands is more complex than previously thought. The hypothesis regarding the altered localization of FoxO1 upon anti-Ang2 antibody followed by VEGF-C stimulation was partly confirmed, but the effect of Ang2 blockade was lesser than expected. In future studies, the actions of these ligands and their inhibition should be examined in a strictly controlled manner perhaps by measuring the concentration of Ang2 released by the cells. Also, the concentration of VEGF-C was quite high in these studies and concentrations more close to the physiological range would possibly provide interesting and more plausible results.

Possible sources of error in these experiments were technical issues including cell culture and handling of the cells as well as immunofluorescence staining protocols. The quantifications were carried out by hand, including choosing the cells for quantification and adjusting the intensities for the images, which made the quantification and statistical analysis more prone to errors.

4.2. In vivo experiment

The effect of Tie receptor deletions on VEGF-C-induced lymphangiogenesis in adult mice was studied by administering either VEGF-C-producing or empty control AAV into tibialis anterior muscles of mice with lymphatic specific deletion of Tie1, Tie2 or both Tie1 and Tie2, and quantifying lymphatic area in immunostained muscle sections. Quantification showed a significant decrease in AAV-VEGF-C induced lymphatic vessel growth in Tie1 and Tie1;Tie2 double deleted mice compared to control mice treated with AAV-VEGF-C indicating that Tie receptors are required for full lymphangiogenic response to VEGF-C. Interestingly, Tie1-deleted mice treated with AAV-VEGF-C showed very little lymphatic growth compared to Tie1-del mice treated with AAV-Empty. This could be due to technical issues or perhaps Tie1-deletion in these mice did indeed completely inhibit lymphangiogenesis. In contrast to Tie1 and Tie1;Tie2 double deletion, deletion of Tie2 did not significantly affect lymphangiogenesis. The primary hypothesis was that the deletion of both Tie1 and Tie2 would have an impact on the lymphangiogenic rate, but regarding these results, only Tie1 and Tie1;Tie2 double deletion resulted in statistically significant changes.

Previously it has been shown that Tie1 deletion leads to lymphatic abnormalities in mouse embryos, and that deletion of Ang1, Tie1 or Tie2 results in embryonic lethality in mice (Qu et al.,

2015; D'Amico et al., 2009; Korhonen et al., 2016). However, the results of this thesis suggest that the orphan receptor Tie1 has a major role also in adult lymphangiogenesis even though its role has not been fully revealed yet. It should be noted that the data of this thesis is preliminary and future experiments need more mice per group. In addition to the low amount of mice per group, other potential sources of error are the possible differences in AAV-injections, and the cryosections cut from the muscles may have been from different parts of the muscle. This could have been taken into account by cutting and staining more sections per muscle, and by having sections from different parts of the tissue to gain better understanding of the distribution of the new lymphatic growth. Also in this experiment the quantifications were done by hand, which might have affected the results.

5. Conclusions

There are two major signaling pathways responsible for lymphatic vessel growth and remodeling: the Ang2/Tie and VEGF-C/VEGFR-3 pathways. The crosstalk of these pathways includes many downstream effectors including PI3K/Akt signaling. The in vitro results of this thesis suggest that VEGF-C-mediated activation of PI3K/Akt signaling leads to FoxO1 exclusion from the nucleus and that this action is slightly shifted towards more cytoplasmic localization upon Ang2 blocking antibody treatment. Also, PLA showed that there is a pool of VEGFR-3 and Tie1 molecules that are in close proximity thus enabling their interaction. Furthermore, the preliminary in vivo results presented here show that Tie1 receptor has a role in VEGF-C-induced lymphangiogenesis in adult mice, while deletion of Tie2 alone does not impair lymphangiogenesis. The in vitro and in vivo data presented in this thesis support the idea of a crosstalk of Ang/Tie and VEGF-C/VEGFR-3 pathways in lymphangiogenesis, as was recently showed by Korhonen et al. (2022). Further knowledge on the crosstalk between these two signaling pathways has therapeutic value in manipulation of lymphangiogenesis e.g. in lymphedema and other settings in which lymphatic function is reduced.

6. Acknowledgements

I would like to thank my supervisors Aino Tedeton and Andrey Anisimov from the University of Helsinki, and Heikki Helanterä from the University of Oulu for all the guidance and support. I would also like to thank all members of the Alitalo lab for their kindness, help and patience throughout the time I spent there, I learned so much.

7. References

Alam, M. S. (2018). Proximity Ligation Assay (PLA). *Current Protocols in Immunology*, *123*(1), e58. https://doi.org/10.1002/cpim.58

Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis—PubMed. (n.d.). Retrieved 31 May 2023, from https://pubmed.ncbi.nlm.nih.gov/9204896/

Avraamides, C. J., Garmy-Susini, B., & Varner, J. A. (2008). Integrins in angiogenesis and lymphangiogenesis. *Nature Reviews. Cancer*, *8*(8), 604–617. https://doi.org/10.1038/nrc2353

Brakenhielm, E., & Alitalo, K. (2019). Cardiac lymphatics in health and disease. *Nature Reviews Cardiology*, *16*(1), Article 1. https://doi.org/10.1038/s41569-018-0087-8

Cell signaling by receptor-tyrosine kinases—PMC. (n.d.). Retrieved 31 May 2023, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2914105/

Cudmore, M. J., Hewett, P. W., Ahmad, S., Wang, K.-Q., Cai, M., Al-Ani, B., Fujisawa, T., Ma, B., Sissaoui, S., Ramma, W., Miller, M. R., Newby, D. E., Gu, Y., Barleon, B., Weich, H., & Ahmed, A. (2012). The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nature Communications*, *3*, 972. https://doi.org/10.1038/ncomms1977

D'Amico, G., Korhonen, E. A., Waltari, M., Saharinen, P., Laakkonen, P., & Alitalo, K. (2010). Loss of endothelial Tie1 receptor impairs lymphatic vessel development-brief report. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *30*(2), 207–209. https://doi.org/10.1161/ATVBAHA.109.196618

Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C., & Yancopoulos, G. D. (1996). Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell*, *87*(7), 1161–1169. https://doi.org/10.1016/s0092-8674(00)81812-7

Fayard, E., Xue, G., Parcellier, A., Bozulic, L., & Hemmings, B. A. (2010). Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway. *Current Topics in Microbiology and Immunology*, *346*, 31–56. https://doi.org/10.1007/82_2010_58

Félétou, M. (2011). *The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-Derived Vasoactive Mediators*. Morgan & Claypool Life Sciences. http://www.ncbi.nlm.nih.gov/books/NBK57149/

Fukuhara, S., Sako, K., Noda, K., Zhang, J., Minami, M., & Mochizuki, N. (2010). Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis. *Histology and Histopathology*, *25*(3), 387–396. https://doi.org/10.14670/HH-25.387

González-Loyola, A., & Petrova, T. V. (2021). Development and aging of the lymphatic vascular system. *Advanced Drug Delivery Reviews*, *169*, 63–78. https://doi.org/10.1016/j.addr.2020.12.005

Graupera, M., Guillermet-Guibert, J., Foukas, L. C., Phng, L.-K., Cain, R. J., Salpekar, A., Pearce, W., Meek, S., Millan, J., Cutillas, P. R., Smith, A. J. H., Ridley, A. J., Ruhrberg, C., Gerhardt, H., & Vanhaesebroeck, B. (2008). Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. *Nature*, *453*(7195), 662–666. https://doi.org/10.1038/nature06892

Graupera, M., & Potente, M. (2013). Regulation of angiogenesis by PI3K signaling networks. *Experimental Cell Research*, *319*(9), 1348–1355. https://doi.org/10.1016/j.yexcr.2013.02.021

Hemmings, B. A., & Restuccia, D. F. (2012). PI3K-PKB/Akt pathway. *Cold Spring Harbor Perspectives in Biology*, *4*(9), a011189. https://doi.org/10.1101/cshperspect.a011189

Hubbard, S. R., & Miller, W. T. (2007). Receptor tyrosine kinases: Mechanisms of activation and signaling. *Current Opinion in Cell Biology*, *19*(2), 117–123. https://doi.org/10.1016/j.ceb.2007.02.010

JCI - Foxo1 deletion promotes the growth of new lymphatic valves. (n.d.). Retrieved 31 May 2023, from https://www.jci.org/articles/view/142341

Karar, J., & Maity, A. (2011). PI3K/AKT/mTOR Pathway in Angiogenesis. *Frontiers in Molecular Neuroscience*, *4*, 51. https://doi.org/10.3389/fnmol.2011.00051

Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H., Betsholtz, C., & Alitalo, K. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nature Immunology*, *5*(1), 74–80. https://doi.org/10.1038/ni1013

Kim, M., Allen, B., Korhonen, E. A., Nitschké, M., Yang, H. W., Baluk, P., Saharinen, P., Alitalo, K., Daly, C., Thurston, G., & McDonald, D. M. (2016). Opposing actions of angiopoietin-2 on Tie2 signaling and FOXO1 activation. *The Journal of Clinical Investigation*, *126*(9), 3511–3525. https://doi.org/10.1172/JCI84871

Korhonen, E. A., Murtomäki, A., Jha, S. K., Anisimov, A., Pink, A., Zhang, Y., Stritt, S., Liaqat, I., Stanczuk, L., Alderfer, L., Sun, Z., Kapiainen, E., Singh, A., Sultan, I., Lantta, A., Leppänen, V.-M., Eklund, L., He, Y., Augustin, H. G., … Alitalo, K. (2022). Lymphangiogenesis requires Ang2/Tie/PI3K signaling for VEGFR3 cell-surface expression. *The Journal of Clinical Investigation*, *132*(15), e155478. https://doi.org/10.1172/JCI155478

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., & Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development (Cambridge, England)*, *122*(12), 3829–3837. https://doi.org/10.1242/dev.122.12.3829

Lee, H. J., Cho, C.-H., Hwang, S.-J., Choi, H.-H., Kim, K.-T., Ahn, S. Y., Kim, J.-H., Oh, J.-L., Lee, G. M., & Koh, G. Y. (2004). Biological characterization of angiopoietin-3 and angiopoietin-4. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *18*(11), 1200–1208. https://doi.org/10.1096/fj.03-1466com

Li, X., Lee, C., Tang, Z., Zhang, F., Arjunan, P., Li, Y., Hou, X., Kumar, A., & Dong, L. (2009). VEGF-B: A survival, or an angiogenic factor? *Cell Adhesion & Migration*, *3*(4), 322–327. https://doi.org/10.4161/cam.3.4.9459

Lugano, R., Ramachandran, M., & Dimberg, A. (2020). Tumor angiogenesis: Causes, consequences, challenges and opportunities. *Cellular and Molecular Life Sciences: CMLS*, *77*(9), 1745–1770. https://doi.org/10.1007/s00018-019-03351-7

Mäkinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M. I., Pulkkanen, K. J., Kauppinen, R., Jackson, D. G., Kubo, H., Nishikawa, S., Ylä-Herttuala, S., & Alitalo, K. (2001). Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nature Medicine*, *7*(2), 199–205. https://doi.org/10.1038/84651

Mäkinen, T., Norrmén, C., & Petrova, T. V. (2007). Molecular mechanisms of lymphatic vascular development. *Cellular and Molecular Life Sciences: CMLS*, *64*(15), 1915–1929. https://doi.org/10.1007/s00018-007-7040-z

Niimi, K., Nakae, J., Inagaki, S., & Furuyama, T. (2021). FOXO1 represses lymphatic valve formation and maintenance via PRDM1. *Cell Reports*, *37*(9), 110048. https://doi.org/10.1016/j.celrep.2021.110048

Obsil, T., & Obsilova, V. (2008). Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene*, *27*(16), 2263–2275. https://doi.org/10.1038/onc.2008.20

PI3K isoforms in cell signalling and vesicle trafficking | Nature Reviews Molecular Cell Biology. (n.d.). Retrieved 31 May 2023, from https://www.nature.com/articles/s41580-019-0129-z

Qu, X., Zhou, B., & Baldwin, H. S. (2015). Tie1 is required for lymphatic valve and collecting vessel development. *Developmental Biology*, *399*(1), 117–128. https://doi.org/10.1016/j.ydbio.2014.12.021

Räsänen, M., Sultan, I., Paech, J., Hemanthakumar, K. A., Yu, W., He, L., Tang, J., Sun, Y., Hlushchuk, R., Huan, X., Armstrong, E., Khoma, O.-Z., Mervaala, E., Djonov, V., Betsholtz, C., Zhou, B., Kivelä, R., & Alitalo, K. (2021). VEGF-B Promotes Endocardium-Derived Coronary Vessel Development and Cardiac Regeneration. *Circulation*, *143*(1), 65–77. https://doi.org/10.1161/CIRCULATIONAHA.120.050635

Roy, H., Bhardwaj, S., & Ylä-Herttuala, S. (2006). Biology of vascular endothelial growth factors. *FEBS Letters*, *580*(12), 2879–2887. https://doi.org/10.1016/j.febslet.2006.03.087

Rupp, P. A., & Little, C. D. (2001). Integrins in vascular development. *Circulation Research*, *89*(7), 566–572. https://doi.org/10.1161/hh1901.097747

Saharinen, P., Eklund, L., & Alitalo, K. (2017). Therapeutic targeting of the angiopoietin-TIE pathway. *Nature Reviews. Drug Discovery*, *16*(9), 635–661. https://doi.org/10.1038/nrd.2016.278

Saharinen, P., Jeltsch, M., Santoyo, M. M., Leppänen, V.-M., & Alitalo, K. (2015). The TIE Receptor Family. *Receptor Tyrosine Kinases: Family and Subfamilies*, 743–775. https://doi.org/10.1007/978-3-319-11888-8_16

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), Article 7. https://doi.org/10.1038/nmeth.2019

Srinivasan, R. S., Geng, X., Yang, Y., Wang, Y., Mukatira, S., Studer, M., Porto, M. P. R., Lagutin, O., & Oliver, G. (2010). The nuclear hormone receptor Coup-TFII is required for the initiation and early maintenance of Prox1 expression in lymphatic endothelial cells. *Genes & Development*, *24*(7), 696–707. https://doi.org/10.1101/gad.1859310

Stacker, S. A., Williams, S. P., Karnezis, T., Shayan, R., Fox, S. B., & Achen, M. G. (2014). Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature Reviews Cancer*, *14*(3), Article 3. https://doi.org/10.1038/nrc3677

Structure of the extracellular domain of Tie receptor tyrosine kinases and localization of the angiopoietin-binding epitope—PubMed. (n.d.). Retrieved 31 May 2023, from https://pubmed.ncbi.nlm.nih.gov/16849318/

Tammela, T., Enholm, B., Alitalo, K., & Paavonen, K. (2005). The biology of vascular endothelial growth factors. *Cardiovascular Research*, *65*(3), 550–563. https://doi.org/10.1016/j.cardiores.2004.12.002

The class 3 PI3K coordinates autophagy and mitochondrial lipid catabolism by controlling nuclear receptor PPARα | Nature Communications. (n.d.). Retrieved 31 May 2023, from https://www.nature.com/articles/s41467-019-09598-9

The lymphatic vasculature in disease | Nature Medicine. (n.d.). Retrieved 31 May 2023, from https://www.nature.com/articles/nm.2545

The Lymphatic Vasculature in the 21st Century: Novel Functional Roles in Homeostasis and Disease—PubMed. (n.d.). Retrieved 31 May 2023, from https://pubmed.ncbi.nlm.nih.gov/32707093/

Therapeutic strategies targeting FOXO transcription factors | Nature Reviews Drug Discovery. (n.d.). Retrieved 31 May 2023, from https://www.nature.com/articles/s41573-020-0088-2

Understanding vascular development—PubMed. (n.d.). Retrieved 31 May 2023, from https://pubmed.ncbi.nlm.nih.gov/23799579/

Vailhé, B., Vittet, D., & Feige, J.-J. (2001). In Vitro Models of Vasculogenesis and Angiogenesis. *Laboratory Investigation*, *81*(4), Article 4. https://doi.org/10.1038/labinvest.3780252

Vanhaesebroeck, B., Stephens, L., & Hawkins, P. (2012). PI3K signalling: The path to discovery and understanding. *Nature Reviews Molecular Cell Biology*, *13*(3), Article 3. https://doi.org/10.1038/nrm3290

Vascular endothelial growth factor (VEGF)—Key factor in normal and pathological angiogenesis—PubMed. (n.d.). Retrieved 31 May 2023, from https://pubmed.ncbi.nlm.nih.gov/30173249/

Yang, Y., Cha, B., Motawe, Z. Y., Srinivasan, R. S., & Scallan, J. P. (2019). VE-Cadherin Is Required for Lymphatic Valve Formation and Maintenance. *Cell Reports*, *28*(9), 2397-2412.e4. https://doi.org/10.1016/j.celrep.2019.07.072

Yoshioka, K., Yoshida, K., Cui, H., Wakayama, T., Takuwa, N., Okamoto, Y., Du, W., Qi, X., Asanuma, K., Sugihara, K., Aki, S., Miyazawa, H., Biswas, K., Nagakura, C., Ueno, M., Iseki, S., Schwartz, R. J., Okamoto, H., Sasaki, T., … Takuwa, Y. (2012). Endothelial PI3K-C2α, a class II PI3K, has an essential role in angiogenesis and vascular barrier function. *Nature Medicine*, *18*(10), Article 10. https://doi.org/10.1038/nm.2928

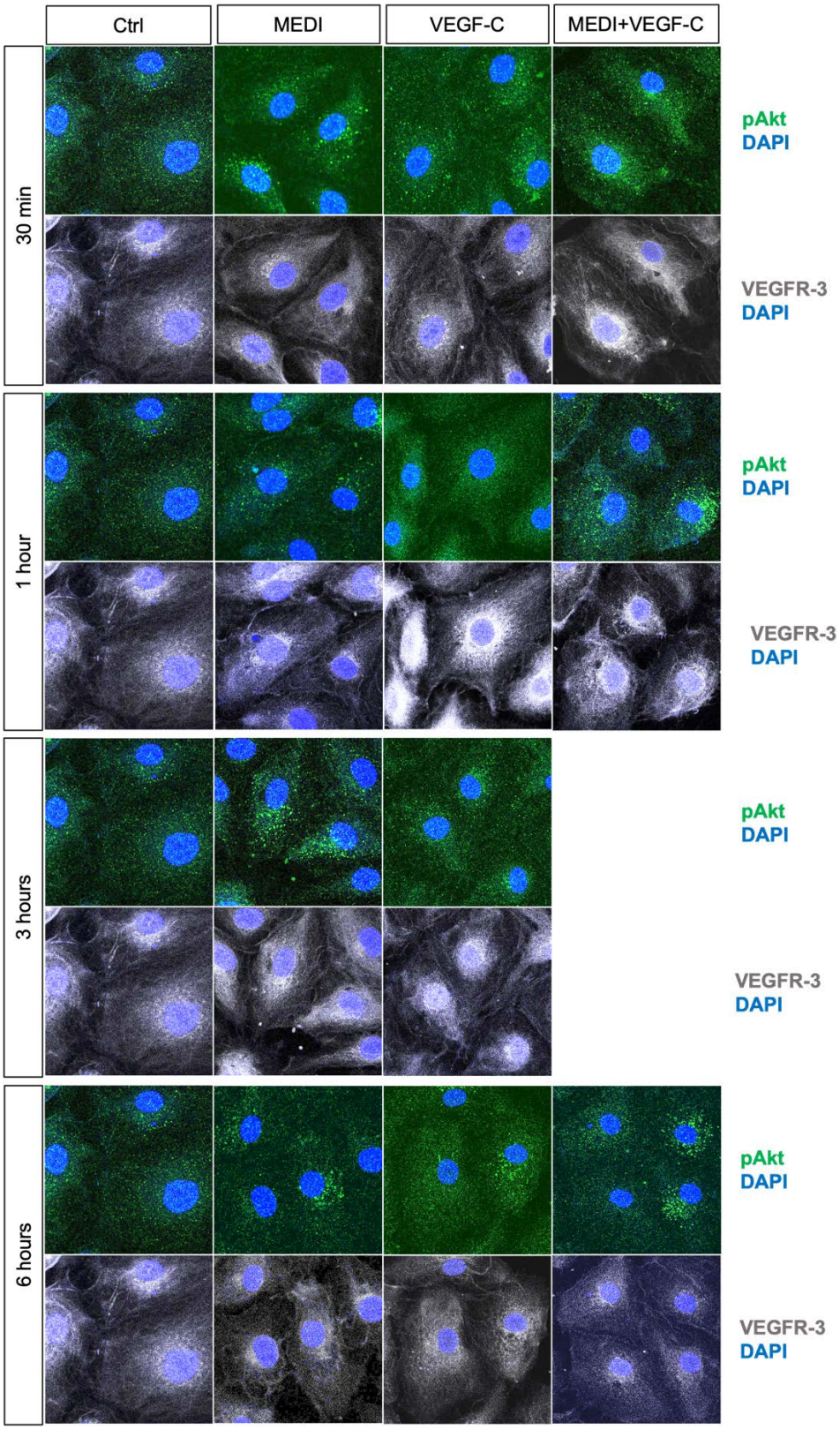
Zhang, F., Zarkada, G., Yi, S., & Eichmann, A. (2020). Lymphatic Endothelial Cell Junctions: Molecular Regulation in Physiology and Diseases. *Frontiers in Physiology*, *11*, 509. https://doi.org/10.3389/fphys.2020.00509

Zhang, L., Zhou, F., Han, W., Shen, B., Luo, J., Shibuya, M., & He, Y. (2010). VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis. *Cell Research*, *20*(12), Article 12. https://doi.org/10.1038/cr.2010.116

Zhang, Y., Kontos, C. D., Annex, B. H., & Popel, A. S. (2021). A systems biology model of junctional localization and downstream signaling of the Ang–Tie signaling pathway. *Npj Systems Biology and Applications*, *7*(1), Article 1. https://doi.org/10.1038/s41540-021-00194-6

8. Appendices

Appendix 1. pAkt staining experiment.



Appendix 2. FoxO1 staining experiment.