# REGULATORY MECHANISMS OF LYMPHATIC DEVELOPMENT

A Dissertation

by

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# ABSTRACT

Cdc42 is a member of the Rho family of small GTPases and functions as a molecular switch regulating multiple cellular processes. We determined whether Cdc42 is involved in lymphatic development via generating two Cdc42 knockout mouse models. Our results demonstrated that endothelial cell (EC)-specific deletion of *Cdc42* caused embryonic lethality with severe edema. Furthermore, both EC-specific and lymphatic EC (LEC)-specific knockout mice exhibited impaired lymphatic vessel sprouting while the lumen size of lymphatic vessels significantly increased. Deletion of Cdc42 impaired the maturation of mesenteric collecting lymphatic vessels and interfered with the maturation of lymphatic valves (LVs). Furthermore, Cdc42 knockout led to disorganized lymphatic endothelial junctions, in which the distribution of ZO-1 (encoded by the *Tjp1* gene and also known as tight junction protein-1) was disorganized and discontinuous. We then generated a novel EC-specific *Tjp1* knockout mouse model in which vascular and lymphatic development was compromised. The defective lymphatic sprouting displayed in *TJP1*-deficient animals was similar to defects noted in the Cdc42 knockout mouse, indicating that ZO-1 may function in the Cdc42 signaling axis to regulate lymphatic development.

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, is a central point of convergence to regulate multiple signaling pathways. We generated both EC- and LEC-specific knockout mice to determine the functions of FAK in lymphangiogenesis. Deletion of FAK in ECs led to aberrantly formed lymph sacs. Also, EC-specific FAK

knockout in ECs compromised the sprouting lymphangiogenesis in multiple organs such as the skin and the diaphragm. LEC-specific knockout of FAK induced defective sprouting lymphangiogenesis in the dermal lymphatic vasculature. Meanwhile, the inactivation of *FAK* interfered with the development of mesenteric lymphatic vasculature, indicated by aberrant enlargement and less mature LVs. Deletion of FAK led to blood-filled lymphatic vessels, indicating the misconnection between the blood and lymphatic vasculatures. *In vitro* studies revealed that knockdown of FAK in cultured human dermal lymphatic endothelial cells (HDLECs) decreased phosphorylation of Erk and Akt.

In summary, our findings highlight the importance of multiple molecules, including Cdc42, ZO-1, and FAK, in lymphatic development.

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Dr. Peng, Dr. Zawieja, Dr. Meininger, and Dr. Tharakan contributed to the experimental design, data collection and processing, scientific discussions, and manuscript writing. Dr. Yixin Jin and Dr. Yang Liu contributed to experimental design and data collection.

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NOMENCLATURE

| | |
|---|---|
| CCBE1 | Collagen- and calcium-binding EGF domains 1 |
| Cdc42 | Cell division control protein 42 homolog |
| CLEC-2 | C-type lectin receptor 2 |
| COUP-TFII | Chicken ovalbumin upstream promoter-transcription factor II |
| CV | Cardinal vein |
| Cx | Connexin |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| ESAM | Endothelial cell-selective adhesion molecule |
| FAK | Focal adhesion kinase |
| FOXC2 | Forkhead box protein C2 |
| HDLEC | Human dermal lymphatic endothelial cell |
| ISV | Intersomitic blood vessel |
| JAM-A | Junctional adhesion molecule-A |
| LEC | Lymphatic endothelial cell |
| LFKO | Lyve1-Cre FAK knockout mouse |
| LRC | lymphatic ring complex |
| LS | Lymph sac |
| LV | Lymphatic valve |
| LVV | Lymphovenous valve |
| Lyve1 | Lymphatic vessel endothelial hyaluronan receptor 1 |

| | |
|---|---|
| NRP1 | Neuropilin-1 |
| NRP2 | Neuropilin-2 |
| PCP | Planar cell polarity |
| PDPN | Podoplanin |
| Prox1 | Prospero homeobox protein 1 |
| Sox18 | SRY-Box Transcription Factor 18 |
| Tie1 | Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 |
| Tie2 | Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 |
| TFKO | Tie2-Cre FAK knockout mouse |
| VE-Cadherin | Vascular endothelial cadherin |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| VFKO | VE-Cadherin-Cre FAK knockout mouse |
| ZO-1 | Zonula occludens-1 |
| β-gal | β-galactosidase |

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

Vertebrates develop two highly branched tubular vascular networks, the vascular and lymphatic systems. The lymphatic system is an extensive network consisting of lymph, lymphatic vessels, and lymphatic tissues and organs. Like the vascular system consisting of hierarchically arranged arteries, arterioles, capillary beds, venules, and veins, the lymphatic vasculature comprises three major components: lymphatic capillaries, lymphatic pre-collectors, and collecting/transport lymphatic vessels. A layer of LECs lines the interior surface of the vessel. The lymphatic vasculature was considered secondary to the circulatory system, and its importance has not been fully appreciated [1]. As a matter of fact, the lymphatic vasculature plays a critical role in maintaining body fluid homeostasis, regulating immune cell tracking, and absorbing dietary fat [2]. Furthermore, lymphangiogenesis, the growth of new lymphatic vessels, is actively implicated in the pathogenesis of multiple human diseases, such as inflammation, hypertension, and tumor metastasis. Therefore, uncovering the functions of lymphatic vasculature in physiological and pathologic conditions provides new therapeutic possibilities.

Impaired lymphatic formation or function can cause lymphedema where lymphatic vessel function is inadequate to drain protein-rich interstitial fluid. Consequently, patients could suffer from physical and psychological morbidity due to chronic tissue swelling, induration of affected areas, and impaired immune responses [3]. Unfortunately, effective cures are not currently available. Therefore, a better

understanding of the underlying mechanisms associated with lymphangiogenesis is essential for developing therapeutic strategies for lymphedema. Unlike angiogenesis, the growth of blood vessels, understanding of lymphatic development has lagged far behind. Since the late 20$^{th}$ century, considerable progress has been made to uncover the regulatory signaling pathways behind lymphatic development owing to discoveries of several lymphatic markers and the employment of various genetically modified mouse models. Yet, many questions are still elusive. This chapter begins with an introduction to lymphatic biology regarding history, structure, and function, followed by a summary of the major lymphangiogenic regulatory molecules. Next, we discuss essential events for lymphangiogenesis, including lymphatic valve (LV) formation and blood and lymphatic vascular separation. Finally, the molecules of interest in this dissertation work -- cell division control protein 42 homolog (Cdc42), focal adhesion kinase (FAK), and zonula occuldens-1 (ZO-1) are examined.

## Lymphatic System

### *The Historical Discoveries of the Lymphatic System*

The human body is vascularized by two tubular networks, blood vascular and lymphatic systems. Unlike the vascular system, which has received extensive scientific and clinical attention, the lymphatic system has long been considered secondary to the vascular system [1]. The lymphatic system was initially documented by Hippocrates (460 – 377 B.C.), a Greek physician regarded as the father of modern medicine. However, the studies and insights regarding the lymphatic system have lagged due to its

elusive morphological features and complex pathophysiology. One fact accountable for the lack of lymphatic research during early times is that the lymphatic network is mainly transparent and is very difficult to visualize [4].

**Early discoveries**

The first description of lymphoid tissue in terms of its anatomy and physiopathology can be traced back to ancient Greece. The Hippocratic treatise "On glands" documented the existence of lymph nodes and vessels and their association with diseases [5]. Lymph nodes were stated as "whitish and phlegmy" structures distributed over the whole body to "attract and receive" fluid [5].

The Alexandrian School also made significant contributions to the understanding of lymphatic systems. Unfortunately, most of the original documentation of their works no longer exists and can only be accessed through references by other authors. As reported by Galen of Pergamon (A.D. 129–199), Herophilos (300 B.C.) claimed the existence of "veins" ending in certain glandular bodies, and Erasistratus (310–250 B.C.) showed that mesenteric "arteries" are full of milky contents [6]. Whether or not these vessels were lymphatic vessels is still controversial. These descriptions were likely to be the first misinterpreted documentation for mesenteric lacteals. In addition to reiterating the work of Herophilos and Erasistratus, Galen provided unique insights obtained from the dissections of apes and pigs. He described turbid vessels passing from intestines to "spongy flesh bodies," which were mesenteric lymph nodes and lacteals containing chyle [6, 7].

**Renaissance of lymphatic discoveries**

Even though the first lymphatic discovery can be ascribed to the ancient Greek Hippocratic School, lymphatic research became quiescent during the following centuries. The lack of new insights for lymphatic findings was presumably due to difficulties in visualizing the invisible lymphatic structures.

In the early 17$^{th}$ century, Gaspare Aselli (1581-1626), an Italian physician, documented the difference between lymph vessels and veins, and he also, for the first time, discovered the chyliferous lacteals, "venae albae et lacteae" (lacteals), in well-fed dogs [8]. However, Aselli mistakenly believed that lacteals transported the chyle to the liver [9]. Aselli was not able to publish his work before his death. Two of his colleagues in Milan, Alexander Tadinus and Senator Septalius, published his work titled *De lactibus, sive lacteis venis* [10].

The following significant historical finding in lymphatic discoveries was made by Jean Pecquet (1624-1674). Pecquet observed the white liquid and blood mixture in a heart removed from a live dog. He believed that white liquid was chyle and traced its destination to the subclavian vein through a duct [7, 9]. The duct is now known as the thoracic duct. He also described valves of the thoracic duct. Pecquet further identified the connection between the duct and "receptaculum" into which the lacteals drained. This finding refuted Aselli's belief that the liver was the lacteals' destination [7]. The *cisterna chyli* is also referred to as Pecquet's receptacle in memory of his significant discovery [4]. A contemporary Swedish anatomist, Olaus Rudbeck (1630-1702),

independently discovered the thoracic duct. In 1653, Rudbeck published his observations on the lymphatic vasculature with the title *Nova exercitatio anatomica* [4, 11]. He described characteristics of lymphatic vessels, such as the direction of flow.

Paolo Mascagni (1755-1815), an anatomy professor at the University of Siena, published *vasorum lymphaticorum corporis humani historia et ichnographia* in 1787. This work has been considered a landmark in anatomical illustrations of the lymphatic system due to the accuracy and details of the human lymphatic system conveyed in his life-size illustrations of the entire human body. Mascagni mainly used mercury injection to visualize the lymphatic networks, which enabled him to name and depict almost all lymph vessels and glands in the human body. Around half of the lymphatic vessels currently known were discovered by Mascagni.

**Controversies on the lymphatic origin**

The origin of the lymphatic vasculature is a century-long debate. In the early 20th century, the research to uncover the origin of the lymphatic vasculature was primarily based on detailed anatomical analysis. Despite the lack of modern tools, researchers provided insightful hypotheses based on their observations. In 1902, India ink injection experiments conducted by Florence Sabin in the pig embryos argued that the lymphatic vasculature originated from the venous system. Concretely, venous ECs in the jugular and perimesonephric areas bud and give rise to the lymphatic vasculature [12-14]. Based on Sabin's view, these primary peripheral lymphatic vessels are derived from the centrifugal growth of veins at multiple primary centers. Shortly after Sabin's

hypothesis, George S. Huntington and Charles F. W. McClure provided an alternative model [15]. They conducted histological examinations on domestic cats and claimed that mesenchymal precursor cells are the source of the lymphatic vasculature. Those precursor cells from isolated spaces in the mesenchyme transform into LECs and form primitive lymphatic vessels. Therefore, lymphatic vasculature is of non-venous origin and connects to the venous system secondarily. Subsequent studies supported one view or the other. However, the origin of the lymphatic vasculature has remained elusive.

**Molecular era**

Towards the end of the 20th century, genetically modified mouse models in conjunction with lymphatic-specific markers such as VEGFR3 and Prox1 have significantly advanced our molecular understanding of lymphatic development mechanisms.

VEGFR3 is one of the first identified lymphatic markers. VEGFR3 is initially detected in angioblasts of head mesenchyme and the CVs of mouse embryos at E8.5 [16]. *VEGFR3* expression can be found in developing veins and lymphatic vessels at E12.5 and eventually becomes restricted to lymphatic vasculature [16]. The expression pattern of *VEGFR3* in the CV and lymphatic vessels provides supportive evidence for Sabin's model.

Recent evidence revealed by linage tracing analysis is also in favor of the venous origin of lymphatic vasculature. In mouse embryos, lymphatic vasculature starts developing as early as E 9.5 -10.5 [17, 18]. A subgroup of venous ECs in the dorsal

lateral side of CVs are committed to a lymphatic identity and differentiate into LEC progenitors. Subsequently, those cells migrate away and form the primordial lymphatic structures LSs, the dorsal peripheral longitudinal lymphatic vessel and the ventral primordial thoracic duct. These primordial structures give rise to peripheral lymphatic vasculature through centrifugal sprouting during later and postnatal gestation stages [19, 20]. During lymphangiogenesis, lymphatic capillary networks coalesce to form primitive lymphatic capillary plexus, which further remodel and develop different lymphatic vasculature components, capillaries, pre-collecting, and collecting lymphatic vessels. Each type of lymphatic vessel develops distinct structures to fulfill its specialized functions.

Although accumulating evidence supports Sabin's venous model, many findings suggest alternative sources for lymphatic endothelium. To determine the origins of deep and superficial lymphatics in avian species, grafting experiments in which quail paraxial mesoderm or dermatome tissues were transplanted into chick embryos were performed. The results suggested that deep parts of the jugular LSs originated from the paraxial mesoderm [21]. In contrast, superficial lymphatic structures were integrated from non-venous dermatomes [21]. In mice, a lineage-tracing study utilizing the Tie2 (endothelial marker) revealed that non–Tie2-lineage cells assemble into the lymphatic network of lumbar and dorsal midline skin [22]. We should note the potential issue of using $GFP^{-}$ LEC clusters from *Tie2-Cre*;*R26-mTmG* and *Prox1-CreER$^{T2}$;R26-mTmG* as indicators for cells with non-venous origin. The absence of labeling could be due to insufficient Cre-mediated recombination rather than differences in lineage. Further experiments

using different mouse models are required to determine whether non-venous-derived cells can contribute to lymphatic vessels. Recent findings also suggest heterogeneity in the origin of cardiac lymphatic vessels in which *Isl1*-expressing progenitors were integrated into cardiac lymphatic vessels [23]. Those *Isl1*-expressing progenitors cooperate with venous-derived LECs to form cardiac lymphatics with regional preferences [23]. In summary, current findings support multiple origins of the lymphatic vasculature. More investigations are required to uncover the origin of lymphatic vasculature in both physiological and pathological conditions.

### *Structure and Function of the Lymphatic System*

The lymphatic system, composed of lymphoid tissues and lymphatic vessels, plays a critical role in maintaining body fluid homeostasis. Although the venous system can recollect some fluid extravasated from the capillary bed (arterial side), the lymphatic system still needs to absorb significant amounts of the fluid [2]. Therefore, the primary function of the lymphatic vasculature is to collect the protein-rich tissue fluid and macromolecules extravasated from the arterial side of the capillary bed and transport it back to the venous circulation. Additionally, lacteal, lymphatic vessels in villi of the intestine, are required for dietary lipid absorption [24]. Moreover, lymphatic vessels also contribute to immune surveillance by transporting antigens and antigen-presenting cells (APCs) to interact with immune cells in the lymph nodes. As previously mentioned, the lymphatic vasculature mainly consists of three types of vessels: lymphatic capillaries, pre-collector lymphatic vessels, and collecting/transport lymphatic vessels

[25]. Unlike the circulatory blood vessels, the lymphatic network transport lymph in a unidirectional conduit system. Lymphatic capillaries are blind-ended and highly permeable, serving as entry sites for fluid extravasated from the blood capillary bed. The collected fluid, macromolecules, and associated cells are collectively referred to as lymph once they enter lymphatic capillaries. Lymph is then transported through pre-collector lymphatic vessels, followed by collecting/transporting lymphatic vessels. The contents are filtered through lymph nodes, subsequently joining lymph flow through the right lymphatic duct and thoracic duct, and eventually enter the blood circulation through the LVV in the jugular region.

**Lymphatic vessels**

Different types of lymphatic vessels assemble a unidirectional tubular network hierarchically. In addition, different types of lymphatic vessels develop specialized morphological and molecular features to better cope with functional demands.

*Lymphatic capillaries*

Lymphatic capillaries are thin-walled vessels with a wide range of sizes dependent on the tissue of origin, averaging approximately 20-70 μm in size [26]. This type of vessel is highly permeable due to specialized junctions between LECs. The intercellular junctions of initial lymphatic vessels are “button-like”, which is different from tighter “zipper-like” junctions present in the collecting lymphatic and blood vessels. Both “button-like” and “zipper-like” junctions are formed by VE-Cadherin and tight junction-associated proteins, such as ZO-), occludin, claudin-5, junctional adhesion

molecule–A (JAM-A), and ESAM [27]. However, intercellular junctions in initial lymphatics exhibit gaps between adjacent ECs that are only anchored on the sides, leaving overlapping flaps at the LEC tip free of junctional molecules. These overlapping cell-cell contacts in initial lymphatics are also referred to as primary valves [28]. Like other valves, these primary valves can prevent the backflow from vessels to the interstitial spaces. Also, the lymphatic capillaries are generally devoid of lymphatic muscle cells or pericytes and are sheathed by minimal or no basement membrane. Lymphatic capillaries contain no LVs. Moreover, lymphatic capillaries maintain a close relationship with the extracellular matrix (ECM) through unique bundles of microfibrils that radiate from endothelial cells to the ECM. The bundles of microfibrils are called anchoring filaments and provide mechanical support to prevent lymphatic capillaries from collapsing during interstitial pressure increases [25, 29]. The major component of an anchoring filament is fibrillin. Immunofluorescence staining analysis on initial lymphatics of human skin showed that fibrillin and integrin $\alpha 3\beta 1$ or FAK were contiguous [30], indicating the potential functions of integrin and FAK in the molecular and mechanical interaction between ECs and ECM. When tissue swells, the LECs of initial lymphatic vessels are pulled outward by anchoring filaments, allowing the opening of "button-like" junctions and, consequently, capturing the interstitial fluid.

*Pre-collecting lymphatic vessels*

The pre-collecting lymphatic vessels (pre-collectors) are transitional vessels between lymphatic capillaries and collecting lymphatic vessels. Pre-collectors are

between 70 and 150 μm in diameter [26]. Unlike initial lymphatic vessels where valvular structures are absent, pre-collecting lymphatic vessels develop LVs with single or double leaflets that ensure the unidirectional net flow of lymph. However, the LVs of pre-collectors are not as evenly distributed as those of collecting lymphatic vessels [26]. Detailed histological examination of superficial lymphatic pre-collectors from the human thigh revealed that this lymphatic vessel is rarely or discontinuously covered by smooth muscle cells. The location of muscular coverage does not rely on the site of intraluminal valves [31]. These smooth muscle cells allow the vessels to constrict and contract spontaneously. Pre-collector lymphatic vessels also contain portions with absorbing structures where thin-walled vessels have discontinuous coverage of basal lamina and anchoring filaments attaching to surrounding connective tissue. Therefore, the alternation of portions similar to initial lymphatics and portions covered by a muscular coat is a unique characteristic of pre-collector lymphatic vessels. These morphological features support the dual role of pre-collector lymphatic vessels: absorption and propulsion.

*Collecting lymphatic vessels*

Pumping the collecting lymphatic vessels is the primary driving force for lymph movement. The structure of collecting lymphatic vessels is generally similar to that of blood vessels. The wall of collecting lymphatic vessels contains an intima medial layer as well as an adventitia. The intima consists of ECs. Muscle, collagen, and elastic fibers are major media components, which are surrounded by adventitia. The adventitia

includes connective tissues, fibroblasts as well as nerve terminals. The collecting lymphatic vessel possesses a complete basal membrane, and junctions between LECs are continuous and "zipper-like" [27, 32]. Unlike primary valves in initial lymphatics, collecting lymphatic vessels have evenly distributed intraluminal LVs, also known as the secondary valves. The region between two adjacent valves is called a lymphangion. The rhythmical and intrinsic contraction of lymphatic smooth muscle is the primary mechanism to overcome the prevailing pressure gradients to pump the lymph throughout the lymphatic vasculature [33, 34]. Also, the surrounding contractions from skeletal muscle and arterial pulsations and other phasic tissue contractions can serve as extrinsic forces to aid in lymph movement.

### *Development of the Lymphatic Vasculature*

The mature lymphatic vasculature is functionally and structurally separated from the blood circulation with a few exceptions, such as unique junctions with the great veins in the neck. This vascular and lymphatic separation is essential for both vascular networks to function properly. Lymphatic vasculature development is an intricate process that needs spatial and temporal coordination of multiple signaling pathways. Most of the lymphatic vasculature is of venous origin. The ECs in embryonic CVs commit to a lymphatic endothelial fate and then migrate away from the CVs to assemble LSs, the precursors of lymphatic vessels. Subsequently, LECs sprout from these primitive lymphatic structures and undergo further branching, remodeling, and maturation.

As previously mentioned, pre-collecting and collecting lymphatic vessels develop LVs to ensure the unidirectional net flow of the lymph towards the venous system. The LV formation is also complex and tightly regulated by various molecules. The LV development can be generally classified into four stages: initiation, condensation, elongation, and maturation [35].

**Fate commitment of lymphatic endothelial cells**

One of the earliest expressed LEC-signature markers is Lyve1. The Lyve1 expression in ECs located at the dorsolateral side of the anterior CVs is considered a molecular indicator for the competence of ECs to respond to lymphatic-inducing signals [36]. Lyve1 was initially identified as a lymphatic-specific receptor for the glycosaminoglycan hyaluronan on the luminal side of the lymphatic vessel wall, and it is a homologue of CD44 [37]. Lyve1 is absent from most blood vessels with a few exceptions [38]. Expression of Lyve1 is downregulated to low levels in collecting lymphatic vessels as lymphatic vessels mature while initial lymphatic vessels maintain a high level of expression [39]. Nonetheless, knockout of Lyve1 or even double knockout of Lyve1 and CD44 did not lead to any apparent changes in lymphatic structure and function [40, 41]. Thus, although Lyve1 is a reliable lymphatic maker, it is likely not the key player in the differentiation of ECs towards LECs.

The most crucial regulatory molecule for lymphatic development is Prox1, the master regulator of murine lymphatic development [42]. The homeobox gene *Prox1* was initially cloned by Oliver et al. by homology to the Drosophila melanogaster gene

*prospero* [43]. Expression of *Prox1* can be found in various tissues, such as the central nervous system, eye lens, pancreas, liver, heart, and transiently in the skeletal muscle [43]. Functional inactivation of *Prox1* gene led to embryonic lethality at the mid-gestation stage with multiple developmental defects, such as lack of lymphatic system and failure of lens-fiber elongation [17, 44]. The inactivation of *Prox1* also led to many abnormalities in the eye, including aberrant apoptosis and proliferation, decreased expression levels of the cell-cycle inhibitors Cdkn1b and Cdkn1c, and non-polarized distribution of E-cadherin [17]. Many studies have corroborated the indispensable and specialized role of *Prox1* during lymphatic development [18, 19, 44-47]. Wigle et al. initially generated a genetically modified mouse model where *lacZ* gene (encoding β-gal) was inserted into *Prox1* gene to silence the gene function, which allowed the researchers to trace the expression of *Prox1* by β-gal detection throughout the mouse body [44]. From E10.0, β-gal-positive ECs were found at the dorsal lateral side of the CVs, and those β-gal-positive cells further budded away from the CVs in a polarized manner [44]. Later, Wigle et al. further revealed that *Prox1* activity is required for EC budding and differentiation towards a lymphatic identity. Budding of the lymphatic vasculature was prevented, and the lymphatic vasculature could not form in *Prox1*-null embryos [18]. Hong et al. were able to induce blood vascular endothelial cells to acquire a lymphatic identity through adenoviral gene transfer of *Prox1* cDNA [47], which provided further evidence to support the indispensable role of *Prox1* in lymphatic development. Later, Johnson et al. employed inducible knockout mice where Prox1 was downregulated during embryonic, postnatal, and adult stages, showing that loss of *Prox1*

was able to dedifferentiate LECs to ECs [45]. They also showed that *Prox1* gene knockdown in LECs led to a similar phenomenon, the reprogramming of LECs into BECs [45]. All the evidence supports the conclusion that Prox1 is the master regulator of the lymphatic vasculature. Constant Prox1 activity plays an indispensable role in the development of lymphatic vasculature and in maintaining the phenotypic identity of LECs.

What is the underlying mechanism of regulating *Prox1* gene expression? *Sox18*, encoding an SRY-related transcription factor, is located at distal mouse chromosome 2 [48]. *Sox18* is expressed in the cardiovascular system, vibrissae follicles, and pelage follicles, and its mutation likely underlies defective coat and vasculature development seen in the Ragged (Ra) mutant [49, 50]. Later, the study of patients with hereditary lymphedema revealed that mutations in *Sox18* also underlie the recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia [51]. To further determine the function of *Sox18* in the development of lymphatic vasculature, a Sox18-deficient mouse was generated and analyzed. The findings showed that functional inactivation of the *Sox18* gene caused edema and complete blockade of EC differentiation in CVs towards LECs [52]. Moreover, the results also revealed that Sox18 could directly activate the expression of Prox1 via its binding to the proximal promoter of Prox1 and expressing Sox18 in ECs induced expression of Prox1 and other lymphatic markers as well [52]. All evidence suggests that Sox18 serves as an upstream regulator of *Prox1*. However, other vessels like arteries do not express *Prox1* in the presence of *Sox18,* indicating that other

critical lymphatic-specific players in veins are also involved in the transition of ECs to LECs.

COUP-TFII belongs to the steroid/thyroid hormone receptor superfamily [53]. *COUP-TFII* is expressed in the cardiovascular system, and silencing *COUP-TFII* disrupted heart and blood vessel development [54, 55]. COUP-TFII also physically interacts with Prox1 in LECs, and it functions as a Prox1 regulator to control the differentiation of ECs to LECs [56, 57].

**Lymphatic sprouting and migration**

The unique dorsolateral arrangement of LEC progenitors in CVs and their directional migration implies the existence of external and lymphatic-inducing signals that polarly drive the differentiation and further migration of these progenitor cells. The VEGF-C/ VEGFR3 signaling pathway is the central player in regulating the budding of LECs from CVs [58]. VEGF-C belongs to the family of the PDGF/VEGF. VEGF-C was first identified as a ligand for VEGFR3 and VEGFR2 [59]. *Vegfc* gene is highly expressed in the region where lymphangiogenesis is active or lymphatic vasculature is rich, such as developing mesenteric, perimetanephric, axillary, and jugular regions [60]. VEGF-C is a specific lymphangiogenic factor that can induce LEC proliferation and the development of new lymphatic vessels [61]. ECs of CVs could commit to a lymphatic fate but could not sprout and further form lymphatic vessels in VEGF-C-deficient mice [62]. VEGF-C and VEGF-D could rescue the lymphatic defects shown in *Vegfc*-null mice could be rescued by VEGF-C and VEGF-D, whereas VEGF-A failed to do so [62].

This difference indicates that VEGF-C is a crucial paracrine regulator required for lymphangiogenesis. Like VEGF-C, VEGF-D is another important ligand for VEGFR2 and VEGR3 [63].

VEGFR3 functions as the primary receptor for VEGF-C. VEGFR3 (also known as FLT-4, encoded by the *FLT4* gene) is expressed in ECs during the early embryonic stage and is restricted to lymphatic vasculature at later developmental stage [16]. Inactivation of *FLT4* resulted in an early embryonic lethal phenotype with defectively formed blood vessels [64]. The expression of VEGFR3 is higher in angiogenic sprouts and is required for sprouting angiogenesis [65]. VEGFR3 contains an extracellular domain, a short transmembrane domain, and intracellular conserved kinase domains [66]. The extracellular domain of VEGFRs consists of seven immunoglobulin (lg)-like homology domains in which domains 1-3 (D1-3) provide the structural basis for ligand binding and conformational changes of domains 4-7 (D4-7) are required for receptor dimerization and activation [67]. Both the ligand-binding and kinase activities of VEGFR3 are critical during the development of lymphatic vasculature. Lack of kinase activity, but not the ligand-binding activity, blocked the sprouting of LECs from embryonic CVs [68]. *FLT4* gene mutation in humans underlies Milroy disease, a disease characterized by primary lymphedema [69].

VEGF-C can also induce lymphangiogenic responses via NRP2, a coreceptor of VEGFR3 [70]. NRP2 was initially discovered as a receptor for semaphorins (Sema) E and Sema IV, and it also functions as a crucial coordinator for axon guidance [71-74]. The *Nrp2* expression was found in blood vessels, including CVs of E10.0 mouse

embryos, and its expression remained strong in lymphatic vessels but not blood vessels from E13.0 onwards [75]. NRP2 is particularly essential for lymphatic sprouting through cooperation with VEGFR3 in response to VEGF-C [75, 76]. Blocking the binding between VEGF-C and NRP2 impaired the sprouting of lymphatic tip cells, as demonstrated by *in vitro* sprouting assays where NRP2 inhibition inhibited lymphatic sprouting and increased stalling and retraction [76].

CCBE1 also plays an essential role in lymphangiogenesis. Genetic screens identified *Ccbe1* gene in the zebrafish, and the zebrafish with mutant *Ccbe1* alleles developed no trunk lymphatic vessels [77]. Further evidence revealed that CCBE1, as a secreted protein, functions in the same developmental stage as VEGF-C [77]. Moreover, Prox1-positive LECs in CCBE1 knockout mice failed to bud [78]. Intriguingly, CCBE1 could significantly enhance lymphangiogenesis in the presence of VEGF-C, whereas it only showed a moderate impact on its own [78]. *Ccbe1* mutation underlies Hennekam syndrome, an autosomal recessive disease characterized by mental retardation, aberrant facial characteristics, lymphangiectasias, and lymphedema [79].

An interesting question is how LEC progenitor cells leave the CVs without compromising the integrity of these vessels. Yang et al. proposed a “budding” model where the LEC progenitors in CVs are interlinked by VE-Cadherin–expressing junctions and undergo collective cell migration [80]. As a result, ECs in CVs get close enough to establish new junctions and seal the gap as the collective LEC progenitors migrate away [80].

As the LEC progenitors exit the CVs, these budded LEC progenitor cells are positive for PDPN, a mucin-type transmembrane protein. PDPN is only present in LEC progenitors that have already exited the endothelium of the embryonic CVs [80, 81]. PDPN was initially identified on LECs as E11 antigen [82]. Approximately 40% increase in embryonic and fetal fatality rates was induced by PDPN knockout, and half of the neonatal *Pdpn*$^{-/-}$ mice died within a couple of days after birth [83]. Deletion of *Pdpn* also induced a "nonseparation" phenotype, where blood was present in both blood and lymphatic vasculature [84]. PDPN was able to induce platelet aggregates *in vitro*. Platelet thrombi could be detected at the junction between LSs and CVs of littermate controls, but not in PDPN-deficient mice [84]. Later, *PDPN* was postnatally inactivated in a LEC-specific manner. Knockout of PDPN in LECs recapitulated the blood-filled lymphatic phenotype and deletion of *Pdpn* impaired migration of dendritic cells towards lymph nodes [85]. A current model proposes that PDPN mediates the circulating platelet aggregation, which prevents the blood backflow at junctions between LSs and CVs, which eventually aids in the separation. However, the signals that underlie the activation of PDPN remain elusive.

**Lymphatic remodeling and maturation**

Mouse embryos develop initial lymphatic plexuses by approximately E14.5, which undergo further remodeling and maturation into different components, such as lymphatic capillaries, pre-collecting lymphatic vessels, and collecting lymphatic vessels. *Foxc2* mutation underlies lymphedema-distichiasis syndrome, a rare multisystem

disorder characterized by tissue swelling and extra eyelashes (distichiasis) [86]. *Foxc2* mutant mice show abnormal patterning of the lymphatic vasculature, lack of LVs, and increased coverage by pericytes [87]. Mice deficient in FOXC2 cannot develop a proper mature lymphatic vasculature. Defective vessels remain immature and capillary-like and lack valves [39]. The maturation defects shown in mice with inhibited NFAT signaling are reminiscent of defects caused by inactivation of *Foxc2* gene, and NFATc1 and FOXC2 cooperate during the maturation of lymphatic vessels [39]. Interestingly, we recently found that knockout of Cdc42 in ECs produced similar maturation defects where markers that should be downregulated in mature collecting vessels, such as Lyve1 and VEGFR3, remain high. Also, mature LVs could barely be found in the later developmental and postnatal stages.

The family of Ephrin ligands and corresponding Eph receptors coordinate the development of almost every tissue and organ through both "forward" and "reverse" signaling pathways. The conventional interaction between ligands and their receptor tyrosine kinase (RTK) counterparts is often referred to as "forward signaling." At the same time, Ephrin ligands also possess a receptor-like ability to transduce signals into host cells, which is known as "reverse signaling" [88]. Ephrins and their Eph receptors play critical and diverse roles during vascular development. Ephrin-B2, an Eph family transmembrane ligand, was explicitly detected in ECs of arteries, but not veins, whereas Eph-B4, a receptor for ephrin-B2, labels venous but not arterial ECs [89]. Ephrin-B2 mutants showed defective remodeling in arterial and venous capillaries [89]. Other Eph receptors or Ephrin ligands were also present in vascular systems, such as EphrinB1

detected in arteries and veins, and EphB3 expressed in veins and some arteries [90]. During lymphatic development, mice expressing ephrinB2 without the C-terminal PDZ interaction site displayed a remodeling defect where the primary lymphatic capillary plexus failed to form a network with hierarchical compartments, and the lymphatic vessels in mutants had abnormal smooth muscle cell coverage [91]. Additionally, EphrinB2 was primarily detected in the LECs of collecting lymphatic vessels, but not lymphatic capillaries. Nonetheless, EphB4 was present in both types of lymphatic vessels [91], indicating the potential function of EphrinB2-EphB4 interactions in lymphatic maturation.

The Ang/Tie signaling pathway is essential for vascular and lymphatic development. Angs were identified as ligands for Ties, a family of RTK predominately expressed in ECs [92]. All known Angs, including Ang-1, Ang-2, Ang-3, and Ang-4 primarily bind to Tie2 (also known as Tek), which shares a high degree of homology with Tie1 [93]. Tie2 and Tie1 can form heterodimers [94]. Transgenic mice expressing dominant-negative alleles of the Tek receptor or homozygous null allele of the *tek* locus underwent embryonic lethality with vascular defects [95, 96]. Functional deletion of *Tie1* caused embryonic lethality during E13.5, and mice null for *Tie1* displayed severe edema with ruptured microvessels [97]. *Tie1* is expressed in LECs both embryonically and postnatally. Also, deletion of *Tie1* disrupted the formation of LSs and led to aberrant patterning of the lymphatic vasculature [98, 99]. Mouse embryos expressing Tie1 lacking intracellular domain developed edema and failed to remodel from primary lymphatic plexus to collecting lymphatic vessels. In contrast, inducible deletion of *Tie2*

postnatally did not induce any obvious lymphatic defects [100]. Mouse lacking Ang-1 died by E12.5 and showed cardiac and angiogenic defects, whereas ECs appeared rounded and detached from other types of cells and matrix [101]. Conversely, overexpression of Ang-1 in the skin increased the number, sprouting, and size of blood vessels [102]. Ang-2 functions as an antagonist for Tie2 during vascular development. Overexpression of Ang-2 induced blood vessel defects similar to those shown Ang-1- or Tie2- deficient mice [103]. Further studies revealed that Ang-2 regulates blood vessel formation in a context-dependent manner.

During lymphatic development, Ang-2 only functions as an agonist for Tie2. Mice lacking Ang-2 showed angiogenic defects in the postnatal retina vasculature and defective lymphatic development, including abnormal patterning, decreased sprouting, and lack of proper coverage by smooth muscle cells [104]. Interestingly, Ang-1 could rescue the lymphangiogenic but not angiogenic defects shown in mice lacking Ang-2 [104], indicating that Ang-1 can substitute for Ang-2 during lymphatic development [103]. Interestingly, Ang-2 may also function as a regulator of LEC junctions. Inhibition of Ang-2 in embryos disrupted the transformation of "button-like" junctions in initial lymphatics and integrity of "zipper-like" junctions in collecting lymphatic vessels [105].

**Lymphatic valve formation**

The lymphatic system's primary function is to regulate body fluid homeostasis by returning interstitial fluid to the venous system through unidirectional connections in the jugular area. Lymph initially taken up by lymphatic capillaries in the interstitium

flows through lymphatic pre-collectors and collecting lymphatic vessels towards the nodes. Postnodal lymphatic vessels then transport the lymph into the right lymphatic duct and thoracic duct, and these ducts connect to the venous system through the subclavian vein. The lymphatic system develops specialized structures, namely primary and secondary valves, to minimize lymph backflow. Dysfunction of LVs can cause reflux of lymph and contributes to the pathogenesis of lymphedema. Clinically, defective LVs may underlie the lymphatic dysfunction and edema in patients with lymphatic diseases such as lymphedema distichiasis and Emberger syndrome [87, 106].

*Primary valves*

As discussed earlier, intercellular junctions in initial lymphatic vessels are "button-like" where valve-like gaps are present due to the discontinuous arrangement of junctional proteins. In contrast, the junctions in collecting lymphatic vessels are tighter and appear to be "zipper-like." The proportion of "button-like" junctions increases as lymphatic vessels mature during physiological conditions [107]. Inflammation can convert "zipper-like" junctions into junctions with more "button-like" appearances [107].

The abluminal side of LECs is attached to the ECM via anchoring filaments, fibrillin-rich fibrils. Loss of EMILIN1, an ECM glycoprotein associated with elastic fibers, significantly reduced anchoring filaments in mice where lymphatic vessels are hyperplasic and deficient in function [108]. When interstitial fluid pressure surpasses the fluid pressure inside the initial lymphatic vessels, anchoring filaments attached in the

initial lymphatic vessels stretch the overlapping junctions apart and allow the water, ions, solutes, larger molecules, and even cells to enter into lymphatic vessels [109, 110]. On the contrary, if the pressure inside lymphatic vessels exceeds interstitial fluid pressure, the valve-like gaps are sealed via LEC contacts. Overall, these specialized valve-like gaps ensure the net unidirectional movement of the lymph into the initial lymphatic vessels without compromising the vessel integrity.

*Intraluminal secondary valves*

Unlike initial lymphatic capillaries, collecting lymphatic vessels develop intraluminal valves to prevent the backflow of lymph. Each mature LV consists of two semilunar leaflets, and each leaflet contains a connective tissue core wrapped by a single layer of LECs. Studies regarding LVs are primarily conducted through high-resolution imaging on mesenteric lymphatic vessels owing to the lack of reliable *in vitro* models. Mouse embryos initialize the formation of LVs at approximately E15.5. The valve formation can be classified into four stages: initiation, condensation, elongation, and maturation [35]. The LVs uniformly distribute along the collecting vessels. The initial trigger for the onset of formation is not well understood. Since LVs are often found at vessel bifurcations [111], alternation of flow dynamics within lymphatic vessels appears to be an essential driving force.

LV formation in the mouse initiates at E15.0-E16.0. The first indication of LV formation is the elevated expression of some transcription factors, such as Prox1, GATA2, and Foxc2, in a LECs cluster in the initial lymphatic plexus. GATA2, a zinc

finger transcription factor, regulates many critical genes for tissue development and maintenance. Mutation of *Gata2* underlies Emberger syndrome, a disorder characterized by lymphedema and predisposition to myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) [112, 113]. *Gata2* is abundantly expressed in lymphatic vessels, but not blood vessels [114]. Furthermore, GATA2 acts upstream and can regulate the expression of some genes that are indispensable for LV formation, such as *Prox1, Foxc2, Nfatc1, Itga9, and Angpt2* [106, 114]. The formation of the LV and LVV was disrupted in mouse embryos lacking GATA2 in LECs, and inducible deletion of *Gata2* caused degeneration of LVs [106]. These results suggest that *Gata2* is not only required for the formation but also maintenance of LVs. In addition to GATA2, Prox1 controls the formation of LVV. The formation of LVV is dependent on the dosage of Prox1 since LVVs were missing from *Prox1* heterozygous mice [46]. FOXC2-deficient mice caused agenesis of LVs [39].

During E16.0-E17.0, LV-forming endothelial cells will then demarcate LV territory. Connexins are transmembrane proteins that can exist in the forms of hemi and gap junction channels. Hemi-channels, also known as connexons, allow the exchange of ions and small molecules (< 1 kDa) between cytosol and extracellular spaces by diffusion. On the other hand, gap junction channels permit the diffusion between the cytosol of two adjacent cells [115]. Gap junction proteins Cx37 and Cx43 play an essential role in demarcation. Cx37, Cx43, and Cx47 are present in developing lymphatic vessels and are differentially distributed in the LV-forming zone [116]. Loss of Cx37 and Cx43 alone or in combination impaired LV formation [116]. Valve

assembly and territory delimitation are also accompanied by activation of calcineurin (CNB1)/NFATc1 signaling and deposition of ECM components such as collagen IV and laminin α5 [39, 117].

Mechanotransduction has also been regarded as a critical force for lymphatic morphogenesis. For example, PCP Celsr1 is required for directed cell rearrangements during lymphangiogenesis. LECs deficient in Celsr1 failed to rearrange and adopt a perpendicular direction during LV formation [118]. Recently, a study showed that the atypical cadherin FAT4 is essential for LECs to respond to flow. The deletion of FAT4 in LECs led to the failure of LECs to align with the direction of laminar shear stress *in vitro*. LEC polarity was impaired in FAT4-deficient mouse embryos [119]. Also, syndecan-4 acts as an essential regulator in flow-induced LEC polarization by regulating Vangl2 expression [120]. All evidence points out the significance of the polarization of LECs throughout lymphatic development.

A proposed model links several elements together. Mechanotransduction, Prox1, and FOXC2 cooperatively control LV morphogenesis via regulating Cx37 and calcineurin [35]. Cx37 and calcineurin are indispensable for assembly and demarcation of LV territory as well as the postnatal maintenance of established valves [35].

During E17.0-E18.0, valve leaflet assembly is a critical step for LV development. Integrin-α9 (encoded by *Itga9*) is present in both developing and mature lymphatic vessels, and its pattern of expression strongly correlates with the onset of LV leaflet formation [117]. Functional inactivation of *Itga9* led to the formation of rudimentary valve leaflets where fibronectin was disorganized, cusps were shorter, and loss of the

EIIIA domain of fibronectin recapitulated the defects caused by Integrin-α9 deficiency [117]. Integrin-α9 and EIIIA interaction can directly regulate the assembly of a fibronectin fibril in primary human LECs [117]. These observations uncover the significant role of integrin-α9 signaling during LV morphogenesis.

Mice deficient in *Sema3a* exhibited abnormally small LVs, and mice carrying mutant *Nrp1* alleles lacking the Sema3A binding site exhibited similar defects with valve morphogenesis [121]. Also, defective valves in mice deficient for Plxna1 resemble Sema3a mutants [121]. These *in vivo* observations, in conjunction with *in vitro* physical interaction among these molecules, support the conclusion that Sema3A-Nrp1-PlexinA1 signaling is required for LV morphogenesis.

As discussed above, Eph-ephrin signaling regulates the remodeling of primary lymphatic capillaries into hierarchical components of the lymphatic network. As a bidirectional signaling pathway, the function of Eph-ephrin signaling in LV formation has been examined. By generating different agonistic and antagonistic antibodies against EphB4 and ephrinB2, studies have shown that EphB4-forward signaling, but not ephrinB2-dependent reverse signaling, is required for lymphatic vessel formation [122]. Agonistic anti-EphB4 could rescue function-blocking ephrinB2-impaired formation of LVs, and loss of the cytoplasmic region of ephrinB2 did not decrease the number of valves [122].

Genes involved in LV formation are summarized in Table 1. LV formation is an intricate process that requires the proper cooperation and coordination of multiple signaling pathways and mechanotransduction. Conceptually, LV formation typically

follows the following steps. Initially, LECs demarcate valve-forming territory. Then, LECs reorientate and migrate at ridges of lymphatic vessels. Subsequently, the ECM reorganizes and forms a ring-like constriction. Then, valve leaflets elongate and form mature valves. The molecular mechanisms associated with the LV formation remain to be elucidated. In addition, more studies are required to uncover the importance of mechanical stimulation in the development and maintenance of LVs. Moreover, the role of LVs in pathological conditions requires more investigations as well.

**Table 1: Genes involved in lymphatic valve formation**

| Molecule | Function | Phenotype of Mutants | Reference |
|---|---|---|---|
| AKT1 | Serine/ threonine kinase | Valve agenesis in small collecting lymphatics of the superficial dermal layer of ear skin (adult) | Zhou et al., 2010. |
| PIK3R1 | PI3K subunits | Valve agenesis in mesenteric lymphatics (postnatal stage) | Mouta-Bellum et al., 2009. |
| Angiopoietin-2 | Growth factor | Decreased valve number in ear skin (adult) | Dellinger et al., 2008. |
| Integrin-α9 | Cell-matrix adhesion receptor | Rudimentary valve with disorganized fibronectin matrix, short cusps in the mesentery and skin (embryonic and postnatal stage). | Bazigou et al., 2009. |

**Table 1: Continued**

| Molecule | Function | Phenotype of Mutants | Reference |
|---|---|---|---|
| Fibronectin-EIIIA | ECM component | Lack of leaflets in the mesentery and skin (postnatal stage) | Bazigou et al., 2009. |
| Celsr1 | PCP protein | Failure of EC reorientation (embryonic stage) | Tatin et al., 2013. |
| Vangl2 | PCP protein | Failure of EC reorientation (embryonic stage) | Tatin et al., 2013. |
| Connexin 37 | Gap junction protein | Decreased valve number in the mesentery (embryonic stage) | Kanady et al., 2011 |
| Connexin 43 | Gap junction protein | Valve agenesis in the mesentery (embryonic stage) | Kanady et al., 2011 |
| BMP9 | Growth factor | Reduction of total valve number and mature valves (embryonic and postnatal stage) | Levet et al., 2013. |
| Integrin $\alpha5\beta1$ | Cell-matrix adhesion receptor | Defective lymphovenous valve formation | Turner et al., 2014. |

**Table 1: Continued**

| Molecule | Molecule | Molecule | Molecule |
|---|---|---|---|
| FOXC2 | Transcription factor | Valve agenesis in the mesentery (embryonic stage) | Norrmén et al., 2009. |
| EphrinB2 | Ephrin family protein | Valve agenesis in the mesentery and skin | Mäkinen et al., 2005. |
| Semaphorin3A-Neuropilin-1 signaling | | Defective valve formation in the mesentery | Jurisic et al., 2012. |
| EphB4 forward signaling | | Defective valve formation in the mesentery (postnatal stage) | Zhang et al., 2015. |
| Semaphorin3A-Neurophilin-1-PlexinA1 | | Reduction in valve number (postnatal stage) | Bouvrée et al., 2012. |

**Blood and lymphatic vascular separation**

The blood and lymphatic systems cooperate in many aspects, such as fluid homeostasis, to ensure proper functions of the human body. However, the two systems are functionally and structurally distinct. With a few exceptions, such as lymphovenous connections in the jugular region, mature blood circulatory, and lymphatic systems should remain separated to function properly [25, 123]. The LVV, a bicuspid valve,

serves as a guard to prevent backflow from the blood circulation with higher pressure to the lymphatic system with lower pressure. LVVs are where the thoracic duct or right lymphatic duct empty lymph into the venous system [124]. The misconnection between these two systems can lead to a blood-filled lymphatic phenotype where lymphatic vessels are abnormally filled with blood.

Platelets appear to be essential for blood and lymphatic vascular separation. Blood-filled LSs could be detected in mice deficient in Myeloid ecotropic viral integration site 1 (encoded by *Meis1*), a gene encoding a homeodomain transcription factor required to develop a definitive hematopoietic stem cell and megakaryocyte lineage. The specific lymphatic separation defect could also be found in megakaryocyte/platelet-specific deficient mice and mice treated with antibodies targeting circulating platelets [125].

The recognition of the significance of hematopoietic signaling in blood and lymphatic vascular separation stems from the blood-filled lymphatic vessels shown in mice deficient in SLP-76 or Syk, hematopoietic signaling proteins [126]. The expression of Syk and SLP76 is nearly restricted to hematopoietic cells, and blood-filled lymphatic vessels were present in wild-type animals reconstituted with SLP-76-deficient but not wild-type bone marrow [126]. GATA1-GFP-SLP-76 transgenic animals that expressed GFP-SLP-76 in a subset of hematopoietic lineages could rescue the lymphatic separation defects in SLP-76-deficient mice [127, 128]. Chimeric animals further revealed that SLP-76 and Syk contribute to vascular development in a cell-autonomous manner [127]. Later, a knockout mouse model in which *Syk* was conditionally silenced in

megakaryocytes showed the failure of lymphatic separation [129]. In summary, all the evidence corroborates that hematopoietic SLP-76/Syk signaling is essential for blood and lymphatic vascular separation.

Syk and SLP-76 act as downstream effectors of CLEC-2 (encoded by *Clec1b*) in response to rhodocytin [130]. Mice lacking CLEC-2 displayed a blood-filled lymphatic phenotype as well [131]. Knockout of CLEC-2 in megakaryocytes/platelets by *PF4*-Cre resulted in similar blood-filled lymphatic vessels as *Clec1b* null mice, indicating that CLEC-2 in megakaryocytes and platelets is essential for maintaining blood and lymphatic vascular separation [129]. However, how do platelets contribute to the lymphatic separation from the blood vascular system? CLEC-2 could induce thrombus stabilization [131]. A subsequent finding revealed that a fibrin-containing platelet thrombus was not detected in CLEC-2-deficient mice [124]. Src-Syk-phospholipase Cγ2 (PLCγ2) plays a critical role in megakaryocytes migration and thrombopoiesis [132]. Positional candidate cloning also identified *Plcg2* encoding PLCγ2, as a mediator for separating lymphatic vasculature [133]. PLCγ2 null mice exhibited blood-filled lymphatic vessels, which was also shown in lethally irradiated wild-type animals with reconstitution of PLCγ2-null bone marrow cells [133].

In addition to hematopoietic components, LEC is another crucial player during blood and lymphatic vascular separation. LEC PDPN has been shown to be essential for lymphatic vascular separation. PDPN on LECs can activate CLEC-2 of platelets [134]. Mice lacking T-synthase (encoded by *C1GALT1* gene), a glycosyltransferase required for the biosynthesis of core 1–derived O-glycans in endothelial and hematopoietic cells,

underwent embryonic and neonatal lethality accompanied by blood-filled and disorganized lymphatic vessels [135]. PDPN is also a type of mucin-type O-glycoprotein that was significantly decreased in T-synthase-deficient mice, and inactivation of *Pdpn* phenocopied the lymphatic defects caused by C1GALT1 deficiency [135]. PDPN is not only required for preventing misconnection between blood and lymphatic vasculature during development but also plays an indispensable role in maintaining the separation at the postnatal stage. Inducible deletion of *Pdpn* in LECs caused blood-filled lymph nodes and lymphatic vessels [85]. Interestingly, deficiency of PDPN *in* LECs also interfered with the efficient migration of dendritic cells to the lymph nodes [85].

Other signaling pathways are also involved in the separation between blood and lymphatic vasculature. LEC-specific deletion of these primary receptors of the transforming growth factor β signaling pathway led to blood-filled lymphatics [136]. Deletion of *Itga5* (encoding the integrin-α5 subunit) by Pdgfrb-Cre, also induced edema, hemorrhage, and blood-filled lymphatic vessels [137]. Interestingly, these defects were due to malformation of LVVs but were not directly induced by α5 deficiency in Pdgfrb-Cre-expressing mural cells or by defective hearts and great vessel formation [137]. Functional inactivation of *Rac1*, a gene encoding a small Rho GTPase, is also involved in separating the lymphatic vasculature from the blood circulation. Inactivation of *Rac1* in ECs by *Tie1*-Cre led to blood-filled LSs because the directed LEC migration induced by lymphatic-inductive signals from CVs were interfered [138]. Mice lacking Spred-1 and Spred-2, two Sprouty/Spred family proteins, also displayed blood-filled lymphatic vasculature [139]. Fasting-induced adipose factor (Fiaf), also known as angiopoietin-like

protein 4 (Angptl4)), is another molecule involved in blood and lymphatic separation. *Fiaf*$^{-/-}$ mutants showed defective partitioning of lymphatics from blood circulation.

Given the similar blood-filled defects and evidence of interaction among these molecules, the PDPN-CLEC-2-Syk-SLP76 signaling axis appears to be a central pathway in mediating the lymphatic separation from blood vasculature. However, further investigations are needed to evaluate whether thrombi formed by platelets can sufficiently prevent the backflow or signaling events induced by platelet-LEC interaction are more important for separating these two systems. Blood-filled lymphatic vessels may be a defect ascribed to multiple causes, such as failure to form thrombi, defective LVV formation, and impaired LEC functions. Further studies are required to dissect different reasons for blood-filled lymphatic vessels.

## Cdc42

Cdc42, a small GTPase, a member of the Rho GTPase family, is a convergence point for multiple intracellular signaling networks, for example signaling pathways initiated by G-protein coupled receptors, tyrosine kinase receptors, cytokine receptors, and integrins [140, 141]. Cdc42 functions as a molecular switch, playing a critical role in a wide array of cellular processes, such as filopodia formation, cell polarity establishment, vesicle trafficking, cell survival, and others [142-145]. Cdc42 cycles between a GTP-bound active state and an inactive state bound with GDP. Concretely, the activity of Cdc42 is regulated by three groups of proteins: GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide

exchange inhibitors (GDIs). GEFs mediate the activation of Cdc42 by converting GDP-bound inactive Cdc42 to GTP-bound active Cdc42. GAPs serve as inactivators, leading to hydrolysis of GTP and switching off Cdc42. GDIs can also regulate Cdc42 since it can maintain GTPase in an inactive state by preventing GEF-mediated nucleotide exchange and also prevent nucleotide hydrolysis when binding the GTPase in a GTP-bound state [146]. Patients with missense variants in Cdc42 show heterogeneous phenotypes, such as dysmorphic facial features, hematological anomalies, neurodevelopmental delay, cardiovascular defects, immunodeficiencies, and others [147]. A frequent mutation in Cdc42 (p.Tyr64Cys) underlies Takenouchi-Kosaki Syndrome. The syndrome is characterized by significant developmental delay, camptodactyly, facial dysmorphism, macrothrombocytopenia, as well as lymphedema [148-150]. The lymphatic defects caused by Cdc42 mutation indicate the indispensable role of Cdc42 during lymphatic development.

### *Functions of Cdc42 in vitro*

Cdc42 was initially identified as a crucial gene in *Saccharomyces cerevisiae*, in which Cdc42 was associated with the cell polarity establishment [151]. The human homolog Cdc42 was discovered in the same year [152]. Studies in *S. cerevisiae* further uncovered the essential function of Cdc42 in establishing cell polarity, especially the patterning of budding [153]. Microinjection experiments in mammalian cells such as Swiss 3T3 cells showed that the active form of Cdc42 triggered the filopodia formation

[145], thin and actin-rich plasma membrane protrusions [154]. Meanwhile, RhoA and Rac1, two other small GTP-binding proteins in the same family as Cdc42, induced actin stress fibers and lamellipodia formation in mammalian cells, respectively [155, 156]. These initial observations led to a belief that the Rho family of GTPases, particularly Cdc42, RhoA, and Rac1, are central regulators of actin dynamics. Moreover, Cdc42 is also a critical regulator in intracellular trafficking and cell growth. What follows are some key findings that shape our understanding of Cdc42 from the perspective of its cellular functions.

**Cdc42 as a key player in filopodia formation**

Filopodia consist of tight parallelly arranged bundles of filamentous (F)-actin and actin-associated proteins. Filopodia present as thin and finger-like cell protrusions. Filopodia are 0.1-0.2 μm in size and are regarded as "antennae" or "tentacles" for cells to sense the surrounding microenvironment when protruding [154]. Filopodia are required for many fundamental pathophysiological processes, such as wound healing and development. One of the best-studied mechanisms for Cdc42-mediated filopodia formation is the activation of Arp2/3 through direct interaction with proteins of the Wiskott-Aldrich syndrome protein (WASP) subfamily including WASP and neural (N)-WASP [157]. N-WASP was proposed as a direct downstream effector of Cdc42 in filopodia formation, and its verprolin homology, cofilin homology, and acidic region (VCA) actin-depolymerizing region might get exposed due to Cdc42 binding [158]. Active N-WASP might trigger actin-depolymerization and leave free barbed ends for

actin-polymerizing activity [158]. Findings showed that the N-WASP and the Arp2/3 complex interaction mediated Cdc42-dependent actin polymerization [159]. Binding of phosphatidylinositol (4,5)-bisphosphate (PIP2) to N-WASP's PIP2-binding domain acts in conjunction with Cdc42 binding to regulate N-WASP activation [160]. However, we should note that fibroblasts deficient in N-WASP and WASP could still form filopodia in response to Cdc42 [161, 162]. These results suggest that various signaling pathways may be involved in the process of filopodia formation. Meanwhile, mechanisms involved in filopodia formation may depend on cell types.

Cdc42 can regulate filopodia formation by an alternative mechanism via insulin-receptor substrate p53 (IRSp53). As a scaffolding protein, IRSp53 interacts with Cdc42 through its Cdc42 and Rac interactive binding motif (CRIB) domain and with actin-binding proteins such as Mena (Ena/VASP family member) through its src homology 3 (SH3) domain [163]. Cdc42 relieved the autoinhibitory effect exerted by its N terminus, which consequently led to the recruitment of Mena to initiate actin assembly [164]. Also, WAVE2, a sub-member of the WASP family, colocalized with IRSp53 at tips of protrusive lamellipodia and filopodia [165], and it serves as an important partner for IRSp53 in filopodia formation [166].

### *Cdc42 as a vital regulator for the cardiovascular system*

The signaling functions of Cdc42 in the development of various organs have been thoroughly reviewed [144]. Cdc42 plays an indispensable role for multiple organs, such as the heart, the nervous system, eye, pancreas, skin, blood, bone, immune system,

and liver [144]. Here, we will focus on the cardiovascular system. Heart-specific deletion of Cdc42 led to defective ventricular walls and ventricular septum, accompanied by impaired cardiomyocyte proliferation and cell-cell adhesion during embryonic development [167]. Heart-specific deletion of Cdc42 induced more significant cardiac hypertrophy following physiological and pathological stimuli, which was associated with increased cardiac nuclear factor of activated T cells (NFAT) activity due to inhibited c-Jun N-terminal kinase (JNK) activation [168]. Reestablishing JNK signaling rescued cardiac defects in Cdc42-deficient mice [168].

Cdc42 plays an essential role during blood vessel formation. Deletion of Cdc42 in ECs caused embryonic lethality with defective blood vessels [169, 170]. Loss of Cdc42 in ECs led to polarity establishment failure as well as disorganized cell-cell junctions and actin organization [169]. Postnatal EC-specific deletion of Cdc42 induced malformation of cerebral blood vessels, indicated by impaired sprouting, axial polarity, and abnormal distribution. These cerebral vascular defects caused by Cdc42 deletion were linked to enhanced MEKK3-dependent KLF4 expression [171]. Cdc42 also functions as an essential downstream effector for multiple signaling pathways in vascular development. For example, Hippo effectors YAP and TAZ control vascular network formation by regulating Cdc42 activity [172].

## FAK

FAK is a nonreceptor tyrosine kinase that plays a critical role in a wide spectrum of developmental processes and pathogenesis of many human diseases, such as

cardiovascular diseases and cancer. FAK functions as a multifunctional signaling molecule playing a significant role in integrin-mediated signaling pathways and pathways initiated by other cell surface receptors such as G-protein-coupled, cytokine, and growth factor receptors. Mechanistic forces can also induce conformational changes of FAK and trigger activation of FAK to mediate focal adhesion signaling [173]. FAK regulates fundamental cellular behaviors via both its kinase activity and scaffolding functions. FAK is expressed in most tissues.

FAK was initially identified by Schaller et al. in untransformed chicken embryo cells [174]. Immunofluorescence localization assays also revealed that FAK was localized to focal adhesions [174]. Hanks, et al. isolated cDNA encoding mouse FAK and reported FAK localization to focal adhesions [175]. They also showed that FAK could be phosphorylated in response to fibronectin [175]. Other independent findings indicated that FAK is an essential player in integrin-mediated signaling pathways [176, 177].

### *Structure and Function of FAK*

FAK is one of the important mediators in integrin signaling pathways. FAK also acts as a downstream effector of transmembrane receptors such as G-protein-coupled, cytokine, and growth factor receptors. FAK regulates essential cellular behaviors, such as cell migration, adhesion proliferation, polarity, and survival.

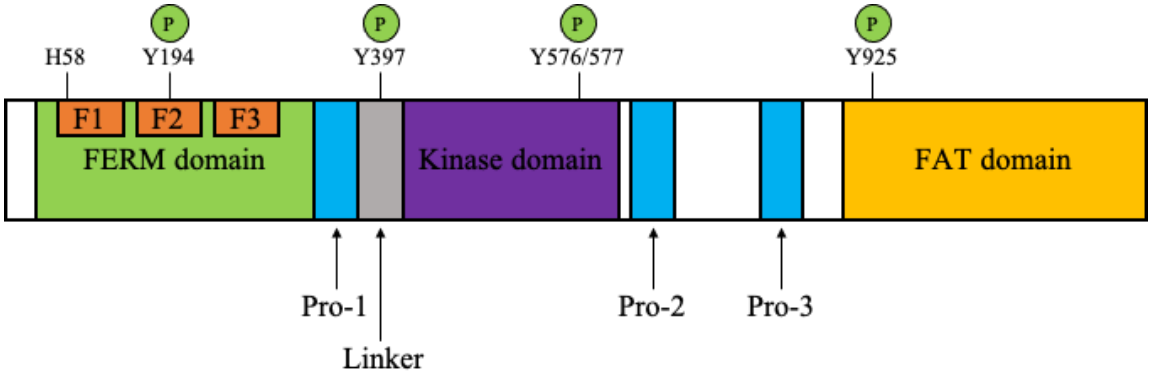



**Figure I-1 Schematic representation of domain organization of FAK.**
FAK contains an N-terminal FERM domain, a central kinase domain and a C-terminal focal adhesion-targeting (FAT) domain. The FERM domain consists of three lobes including F1, F2 and F3. H58 is histidine (H) residue 58. Y194, Y397, Y576/577 and Y925 are tyrosine (Y) residues. Pro-1, Pro-2 and Pro-3 are proline-rich domains. Adapted from Lawson and Schlaepfer [178].

FAK has multiple domains, including a central kinase domain flanked by a C-terminal focal-adhesion targeting (FAT) domain and an N-terminal band 4.1, ezrin, radixin, moesin homology (FERM) domain [179]. FAK has inactive and active states. In the inactive state, FAK is in an autoinhibited conformation in which the active site and several other regulatory phosphorylation sites are blocked by its FERM domain [180]. Upon activation of upstream signaling pathways, such as integrin- and growth factor receptor-initiated signaling pathways, FAK undergoes conformational changes. As a result, the FERM domain is displaced by other activating proteins. FAK is autophosphorylated at Y397, allowing Src family kinases to bind. Src family kinases in turn phosphorylate additional sites located in the activation loop of FAK and lead to FAK activation with full catalytic activity. The resulting FAK/Src complex can regulate

multiple cellular functions by phosphorylating downstream signaling pathways molecules such as the mitogen-activated kinases paxillin and p130Cas.

### The FERM domain

The FERM domain contains three subdomains: F1, F2, and F3, which compactly form a structure resembling the cloverleaf in shape [181]. In human FAK, there is an intervening 60-residue linker segment located between the FERM domain that terminates at residue 352 and the kinase domain that starts at approximately residue 415 [181]. This linker segment is essential for regulating FAK function, and it encompasses the $Tyr^{397}$ autophosphorylation site as well as the binding sites for the Src SH3 domain [181].

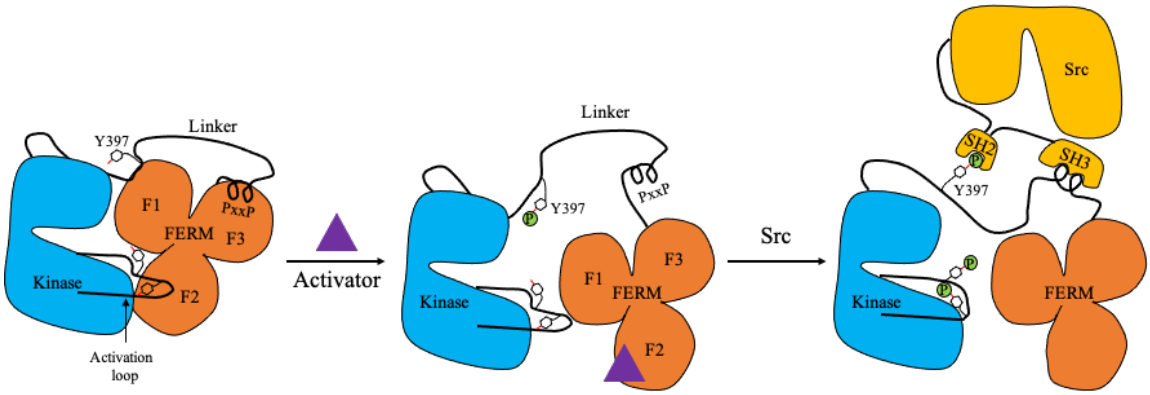



**Figure I-2 Schematic representation of FAK activation model.**
FAK switches between inactive and active states to regulate downstream signaling pathways. In its inactive state (first panel), kinase active sites and phosphorylation sites are blocked by the FERM domain containing F1, F2 and F3 lobes. Upon binding of activators such as cytoplasmic regions of integrin or growth factor receptors, the FERM domain is released, leading to quick autophosphorylation of the Tyr 397 residue in the linker region. Meanwhile, the docking sites for the SH2 and SH3 domains of Src kinase are revealed (second panel). In the following step, Src binds to pTyr 397 and PxxP sequence through its SH2 and SH3 domains respectively (third panel). Src then

phosphorylates Tyr 576 and Tyr 577 residues in the activation loop of FAK. Consequently, FAK gains full catalytic activity. Adapted from Lietha et al. [180].

The FERM domain is involved in the auto-inhibition of FAK. Recognition of the autoinhibitory effect of the FERM domain stemmed from the experimental observations in which the FERM domain could directly bind to its kinase domain, and deletion of the FERM domain increased FAK's catalytic activities [182, 183]. The crystal structure of FAK further revealed the structural basis for the mechanism of FAK's activation. The FERM domain was proposed to sequester the FAK's autophosphorylation and Src recruitment sites in the linker domain and limit the catalytic cleft's access [180]. The binding of activators, such as cytoplasmic regions of integrin or growth factor receptors, can result in the displacement of the FERM domain. The displacement causes rapid autophosphorylation of FAK at Tyr 397. Src also binds to the PxxP sequence in the linker domain through its SH3 domain. Src further phosphorylates Tyr 576 and Tyr 577 residues in the activation loop. FAK then reaches a state of full catalytic activation. The sequence of events in FAK activation may differ due to different activators [184].

Due to its discovery as a central player of integrin signaling in focal adhesions, FAK was named after its typical distribution in fibronectin and was regarded as being limited to the cytosol and cytoplasmic membrane [185]. FERM domains typically mediate binding to cytoskeleton- or membrane-linked proteins as well as phospholipids [186]. Interestingly, findings also revealed that FAK's FERM domain contains the sequences for its transport between the nucleus and cytoplasm. Nuclear localization signal (NLS) within the F2 lobe of FERM mediates FAK's localization within the

nucleus [187]. FAK also possesses two nuclear export signal (NES) sequences, including NES1 in the F1 lobe of the FERM domain and NES2 in the kinase domain [188]. These sequences mediate the export of FAK from the nucleus to the cytoplasm. NLS and NES regulate FAK dynamics between the nucleus and the cytoplasm. Nuclear FAK also provides a different angle to evaluate its function.

**The C-terminal FAT domain and proline-rich regions**

The C-terminal FAT domain is indispensable and sufficient for FAK's linkage to integrin and focal adhesions [189], which is necessary for FAK to mediate integrin signaling. The FAT domain forms a four-helix bundle, involving direct binding with paxillin, a 68-kD focal adhesion phosphoprotein [190]. Structural analysis showed that leucine-rich 'LD' motifs of paxillin directly interacts with the helices of the FAT domain [191, 192]. The requirement for the integrity of its helical bundle to bind to other proteins is different [192]. For example, integrity of the helical bundle is required for biding to paxillin, but not talin, another focal adhesion protein [192]. The FAT domain is also required for direct interaction with p190RhoGEF, a RhoA-specific GDP/GTP exchange factor, and the FAK/p190RhoGEF axis regulates RhoA activation [193].

The C-terminal domain contains two proline-rich regions.The proline-rich regions serve as binding sites for some SH3-domain-containing proteins such as $p130^{Cas}$ [194]. The proline-rich p130Cas binding site is important for FAK-dependent cell mobility [195]. Graf belongs to the GAP family of GTPase regulators, and it can interact with a proline-containing sequence of FAK in the C-terminal region. As a GAP, Graf

can regulate the activities of Rho and Cdc42 [196]. The SH3-domain-containing protein ASAP1 (ADP ribosylation factor [ARF]- GAP can also interact with proline-rich motifs in the C-terminal region of FAK, and transient overexpression of ASAP1 inhibited cell migration [197].

**FAK in cell migration**

FAK is a critical regulator of cell migration in various cell types. Typically, cell migration requires three steps: determining the direction of movement, producing protrusive activity at the leading edge, and retracing the trailing edge to produce net migration [198]. FAK involves in all three phases of cell migration. With few exceptions [199], inhibition of FAK typically compromises cell migration, whereas increases in FAK promote cell migration.

Directional migration is a complicated cellular process that needs coordinated signals for cytoskeletal changes and the establishment of cell polarity. Knockdown of FAK impaired spontaneous polarization of cells, indicated by lack of broad leading edge and more disorganized distribution of Golgi structures [200]. FAK, p120RasGAP, and p190RhoGAP (p190A) form a mechanical complex at the leading edge of focal adhesions to control cell polarity via RhoA GTPase activity [201]. Shear stress can also induce directional migration of ECs, accompanied by polarized recruitment of FAK at nascent focal adhesions [202]. FAK is assembled in the protrusions of living migrating ECs [203]. This polarized distribution of FAK in migrating cells further corroborates the function of FAK in determining the direction of migration.

FAK also regulates protrusive behaviors. $FAK^{-/-}$ macrophages showed decreased colony-stimulating factor-1-dependent motility while displaying increased lamellipodial protrusive as well as retractive activities in responses to colony-stimulating factor-1 [204]. Live cell imaging also recorded movement towards the protrusion of the cell front in migrating ECs [203]. FAK is also an acritical factor for neuronal filopodia formation by regulating actin nucleation through N-WASP [205].

FAK controls the retraction of the trailing edge during cell movement. Lysophosphatidic acid was used in fibroblasts to mimic the dynamics of tail retraction in migrating cells. Downregulation of FAK impaired trailing edge retraction induced by Lysophosphatidic acid [206].

One possible mechanism for FAK to regulate cell migration is through focal adhesion turnover [207]. FAK-null fibroblasts could not transiently suppress the Rho activity induced by fibronectin, and activation or inhibition of Rho activities phenocopied impact of presence or absence of FAK on turnover of focal adhesions [208]. Furthermore, Rho activity inversely correlates with the turnover of focal adhesions [208]. These findings indicate that constitutive activation of Rho activity due to FAK deficiency may underlie the inhibitory turnover of focal adhesions and defective cell migration.

FAK regulates the recruitment of SH2 and SH3 domain-containing signaling proteins to affect cell migration [209]. Fibroblast-like cells from FAK-deficient embryos displayed impaired mobility *in vitro*, accompanied by focal adhesion increases [210].

These findings indicate that FAK involves cell migration by regulating focal adhesion turnover.

### FAK *in Development of Cardiovascular Systems*

FAK can be detected in most tissues, and it is highly conserved across different species from eukaryotic organisms to mammalian species. FAK is critical for diverse developmental processes, such as blood vessels and hearts.

The importance of FAK in angiogenesis has been revealed both *in vivo* and *in vitro*. In 1994, findings showed abundant expression of FAK in developing blood vessels, which indicated the potential involvement of FAK in angiogenesis [211]. FAK knockout mouse unsurprisingly led to early embryonic lethality while the animal displayed extensive cardiovascular defects [210, 212]. Later, several studies employing transgenic and conditional knockout mice further uncovered the significance of FAK in angiogenesis *in vivo.* Overexpression of FAK in vascular ECs caused increased both hindlimb ischemia and wounded-induced angiogenesis [213]. On the contrary, EC-specific knockout of FAK led to defective angiogenesis due to impairment of multiple EC functions, such as cell survival, proliferation, and migration [214, 215].

Proper heart development also requires FAK. Knockout of FAK in cardiomyocytes by light chain 2v (MLC2v) Cre promoted hypertrophy upon challenge by angiotensin II (Ang II) or pressure overload [216]. Also, the inactivation of *FAK* by MLC2v Cre induced fibrosis [216]. Deleting FAK in *nkx2-5*-expressing cells caused lethality shortly after birth with significant defects with subaortic ventricular septum and

outflow tract alignment [217]. Later, FAK was deleted in cardiomyocytes by myosin light chain-2a (MLC2a) Cre during development led to embryonic lethality associated with thin ventricular walls and ventricular septal defects [218]. Some knockout mice were viable but showed spontaneous eccentric right ventricle hypertrophy [218].

FAK has not been studied in lymphangiogenesis. Therefore, the studies proposed here are going to provide insight into the molecular mechanisms of how FAK is involved in lymphangiogenesis.

## ZO-1

Epithelia and endothelia require tight junctions (TJs) to set the boundaries for different tissue compartments and to isolate multicellular organisms from the external environment. The primary physiological function of tight junctions serves as a barrier to preventing solute and water leakage. Meanwhile, it can also function as selective gates for diffusion-based on size and charge [219]. In addition, tight junctions have also been linked to intracellular singalongs that regulate cell proliferation, polarity, and differentiation [220]. Tight junctions consist of transmembrane proteins, including occludins, claudins, junction adhesion molecule (JAM) proteins, and cytoplasmic proteins such as Zonula occluden-1 (ZO-1). These cytoplasmic proteins interact with cytoplasmic domains of junctional transmembrane proteins and link these junctional proteins with cytoskeleton actin components.

ZO-1, known as tight junction protein-1, is a 220-kD peripheral tight junction adaptor protein belonging to the family of multidomain proteins known as the

membrane-associated guanylate kinase homologs (MAGUKs). ZO-1 is the first tight junction component identified using a monoclonal antibody that could recognize a 225-kD tight junction-associated polypeptide, which was ZO-1 [221]. ZO family also consists of ZO-2 and ZO-3. ZO-1 includes three PSD95, DlgA, ZO1 homology (PDZ) domains, an SRC homology 3 (SH3) domain (PDZ-1, PDZ-2, and PDZ-3), a yeast guanylate kinase homology (GUK) domain, and a proline-rich region at the C-terminus. ZO-1 interacts with many tight junctional components such as occludins, claudins, junction adhesion molecule (JAM) proteins, coxsackievirus and adenovirus receptor (CAR), and tricellulin [222-227]. Concretely, ZO-1 interacts with the C-terminus of claudins through its PDZ-1 domain [223]. The interaction with JAM was mediated by its PDZ-2/3 domains [228]. A region at the SH3-hinge-GUK domain is essential for ZO-1's interaction with occludin [229]. ZO-1 can also interact with the cytoskeleton in both direct and indirect fashion. The direct interaction with actin filaments is through ZO-1's COOH terminal region [230]. The binding between ZO-1 and cytoskeletal elements is bridged by actin-binding proteins such as cortactin [231], alpha-catenin [232], cingulin [226], and Shroom [233]. ZO-1 also interacts and talks to signaling molecules such as serin protein kinase ZAK and G protein subunit (G 12) [234].

Although ZO-1 was initially regarded as a unique component of TJs, the following findings widened the understanding by showing that ZO-1 was also associated with adherens junctions (AJs) [235, 236] and gap junctions (GJs) [116, 227, 237]. Furthermore, in addition to structural barrier functions, ZO-1 also interacts with many

intracellular proteins, such as adaptors, signaling molecules, and transcriptional regulators [234].

# CHAPTER II

# CDC42 IS REQUIRED FOR SPROUTING LYMPHANGIOGENESIS AND LYMPHATIC VALVE FORMATION DURING EMBRYONIC DEVELOPMENT


## Abstract

Cdc42, a Ras-related small GTPase that regulates remodeling of the actin cytoskeleton, plays an essential role in vasculogenesis and angiogenesis. Here, we determined whether Cdc42 contributes to lymphatic development by generating two Cdc42 knockout mouse lines by crossing *Cdc42*/flox mice with *VE-Cadherin-Cre* or *Prox1-CreERT2* transgenic mice. Our findings showed that deletion of Cdc42 in ECs led to embryonic lethality with severe edema. Furthermore, both EC- and LEC-specific knockout animals displayed impaired lymphatic vessel sprouting while the lumen size of lymphatic vessels significantly increased. Additionally, the inactivation of *Cdc42* inhibited mesenteric collecting lymphatic vessel maturation and prevented mature LV formation. Our findings also suggested that the Cdc42 knockout caused disorganized intercellular LEC junctions where the distribution of ZO-1(also known as tight junction protein-1) was disrupted.

Furthermore, we employed the Cre-loxP system and generated a novel vascular EC-specific *Tjp1* (encoding ZO-1) knockout mouse model via crossing $Tjp1^{flox/flox}$ with *Tie2-Cre* transgenic mice. Our findings revealed that the inactivation of *Tjp1* in the ECs led to lethality along with severe edema and hemorrhage. Also, visualization of the entire vascular network revealed defective blood vessel sprouting in ZO-1-deficient

mice. Meanwhile, ZO-1 deficiency also led to both morphological defects with lymphatic system development. The dermal lymphatic vasculature displayed highly reduced network sprouting complexity, while the lymphatic vessels were overly enlarged. Collecting lymphatic vessels in the mesentery also showed significant enlargement. In summary, these data demonstrate that ZO-1 is crucial for both vascular and lymphatic development during embryogenesis. The defective lymphatic sprouting defects displayed in ZO-1-deficient animals were reminiscent of defects exhibited by Cdc42 knockout mice, indicating ZO-1 may function in the Cdc42 signaling axis to regulate lymphatic development.

## Introduction

The lymphatic vasculature is a hierarchical tubular network that is required for regulating body tissue fluid hemostasis, orchestrating immune protection, and absorbing and transporting dietary fats [58, 238, 239]. The lymphatic vasculature is composed of various functional components, including initial lymph capillaries, pre-collecting lymphatic vessels (known as lymphatic pre-collectors), muscularized pre-nodal and post-nodal collecting lymphatic vessels [240]. Growing new lymphatic vessels from pre-existing lymphatic structures is termed as lymphangiogenesis. At approximately embryonic day (E) 9.5 in the mouse, Sox 18 and COUP-TFII drive a subset of ECs in CVs to express *Prox1* gene and commit lymphatic fate [58, 241, 242]. These cells then sprout and migrate away from the CVs and ISVs at multiple sites, giving rise to the primitive lymphatic structures. Other than sprouting from veins, recent studies have

revealed that non-venous sources also contribute to lymphatic development in multiple organs [239], such as the heart [243, 244], the mesentery [245, 246], and the skin [22].

During lymphangiogenesis, LECs must undergo differentiation, migration, and proliferation. Meanwhile, they have to interact with other components like other types of cells and the ECM responding to various extracellular stimuli [1]. VEGF-C/VEGFR3 is the central signaling pathway that controls the sprouting and migration of lymphatic vessels [247]. Deletion of *Vegfc* gene impairs LEC sprouting from the CVs and disrupts LS formation in mice [62]. Similarly, loss of functional VEGF-C in humans underlies impaired lymphatic function and lymphedema [248, 249].

Functional LVs and lymphatic muscle cells can ensure net and unidirectional movement of lymph from peripheral locations back to the venous system [240]. Mature LVs are composed of two semilunar leaflets that direct lymph flows and prevent backflow. Extracellular stimuli drive valve-forming LECs to transform to an elongated structure and then condense as ring-like structures [35, 118]. Simultaneously, the cells express high levels of lymphatic molecules such as Prox1 and FOXC2 [35]. During the LV-forming process, PCP signaling also regulates the assembly/disassembly of EC adherens junctions [118]. However, the cellular mechanisms underlying the dynamic changes of cytoskeleton structure and cell-cell junctions during lymphatic valve formation are still largely unknown.

Cdc42 belongs to the Rho family of small GTPases. It acts as a molecular switch in multiple cellular processes, such as filopodia formation, cell polarity establishment, vesicle trafficking, and cell-cell adhesion dynamics [141, 250]. Cdc42 cycles between an

'on' state and an 'off' state. The active Cdc42 (GTP-bound) recognizes and binds to its downstream effector proteins to regulate downstream signal transduction. After GTP is hydrolyzed, inactive Cdc42 (GDP-bound) switches off the signal transduction. The activity of Cdc42 is mainly regulated by three groups of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP-dissociation inhibitors (GDIs) [142]. The cytosolic inactive Cdc42 is translocated to the plasma membrane and becomes activated upon extracellular stimuli.

Consistent with its crucial functions *in vitro*, various missense variants in *Cdc42* led to a broad spectrum of developmental defects in humans, such as brain malformations, facial dysmorphism, cardiac malformations, lymphatic anomalies, etc. [147-150, 251]. Tyrosine residue at Tyr64 site may modulate the interaction between Cdc42 and RhoGDI [252]. Importantly, Cdc42 (p.Tyr64Cys) is a frequent mutation that likely underlies a novel disease state, Takenouchi-Kosaki syndrome, in which lymphedema is a common feature [150, 253]. Cdc42 is critical for developing most tissues and organs, such as neurons, pancreas, lung, intestine, heart, etc.

Since Cdc42 EC-null mice perished before the onset of lymphatic development [169, 170], the role of Cdc42 in lymphangiogenesis remains elusive. Thus, we generated two new Cdc42 EC knockout mouse lines by crossing $Cdc42^{flox/flox}$ mice with either *VE-Cadherin-Cre* [254] or *Prox1-CreER*$^{T2}$ [19] mice to evaluate the function of Cdc42 in the lymphatic development. Our findings suggest that Cdc42 is essential for the remodeling of blood vessels and multiple aspects of lymphatic formation, such as maturation of collecting lymphatic vessels and LV formation. Furthermore, deletion of

Cdc42 also resulted in disorganized lymphatic endothelial junctions in which the distribution of ZO-1 was disorganized and discontinuous.

Intercellular tight junctions are important for controlling paracellular diffusion. ZO-1 is a member of a family of tight junction-associated proteins [220]. ZO-1 is an adapter protein that binds with junction transmembrane proteins, including claudin, occludin, and JAM family adhesion proteins [223, 226, 232]. In addition, ZO-1 regulates actomyosin cytoskeleton remodeling through F-actin [236]. The deficiency of ZO-1 caused a lethal phenotype at approximately E9.5 with angiogenic defects in the yolk sac [255]. However, it remains elusive whether the angiogenic defects were directly associated with loss of ZO-1 in vascular ECs or were merely secondary to non-endothelial defects. Recent studies have documented that ZO-1 is required for angiogenesis and barrier formation *in vitro* [256]. However, the role of ZO-1 in blood and lymphatic vessel formation in EC-specific knockout animals has not been examined. To address these critical questions, we created endothelium-specific ZO-1 knockout mice by crossing *Tie2-Cre*$^{-}$;*Tjp1*$^{flox/flox}$ with *Tie2-Cre*$^{+}$;*Tjp1*$^{flox/wt}$ transgenic mice. Our results showed that loss of ZO-1 in ECs caused neonatal lethality with dramatic edema starting from embryonic stages. Whole-mount staining results revealed that deletion of ZO-1 disrupted the sprouting angiogenesis at multiple organs during early embryonic stages. Meanwhile, development of the lymphatic system was also impaired, indicated by the aberrant formation of LSs and defective sprouting lymphangiogenesis in multiple organs. These findings indicated that ZO-1 is required for the blood circulatory and lymphatic system development.

## Materials and Methods

### *Generation of Cdc42 EC or LEC-specific knockout mice*

Cdc42 EC-specific knockout mice were generated by crossing *Cdc42*/flox mice with *VE-Cadherin-Cre* mice. Cdc42 LEC-specific knockout mice were generated by crossing *Cdc42*/flox mice with *Prox1-CreER$^{T2}$* mice. All study protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M Health Science Center.

### *Whole-mount staining*

The isolated embryos were fixed by 4% paraformaldehyde (PFA), followed by stepwise dehydration by methanol. After treatment with 30% $H_2O_2$ in methanol, the samples were rehydrated with methanol. The embryos were then treated in blocking buffer, followed by incubation with antibody against CD105 (1:50) (BD biosciences) at 4°C for two overnights. Subsequently, the samples were washed with phosphate buffered saline (PBS) containing 0.1% Triton X-100, followed by incubation with HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in the blocking buffer. The blood vasculature was then visualized by tetramethylbenzidine (Sigma-Aldrich) substrate.

For whole-mount immunofluorescence staining of mesenteries and skin, samples were fixed by PFA for 1 hour at room temperature. The samples were washed with PBS and then incubated overnight with different primary antibodies, including VEGFR3 (R&D systems), LYVE-1 (AngioBio), Prox-1 (AngioBio), smooth muscle actin

(Abcam), and CD31 (BD biosciences). After washes with PBS at room temperature, the samples were incubated with secondary antibodies. ProLong gold antifade reagent (Invitrogen) was then applied on the samples to diminish photobleaching. The images were acquired using Olympus confocal microscope (Fluoview FV3000) or Leica TCS SP2.

### *Tamoxifen administration*

Tamoxifen (Sigma) was dissolved in corn oil (Sigma-Aldrich) at a final concentration of 20 mg/mL. Tamoxifen (5 mg) was injected intraperitoneally into pregnant females for two days starting from E9.5.

### *Cell culture.*

Human dermal lymphatic endothelial cells (HDLECs, PromoCell) were cultured with endothelial cell growth medium MV2 kit (PromoCell) and used before passage 9. HDLECs (2 x $10^5$ cells/well) were plated in 6 well culture plates and incubated with 20 nM siRNAs (Qiagen) and HiPerFect transfection reagent (Qiagen) for 72 hours, according to the manufacturer's instructions. Subsequently, HDLECs were used for the following experiments. 100ng/ml VEGF-C was used throughout all experiments. For the human phospho-kinase array assay (R&D system), HDLECs were treated with VEGF-C for 15 minutes before the assay. The experiments were performed based on the manufacturer's instructions.

*Immunofluorescence staining*

Cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature, followed by PBS washes and permeabilization with 0.1% Triton X-100 in PBS for 15 minutes. The cells were then blocked with PBS containing 5% fetal bovine serum for 2 hours at room temperature, followed by incubation with primary antibodies against VE-Cadherin (Santa Cruz biotechnology), Prox1(AngioBio), and ZO-1(Invitrogen) overnight. After washes with PBS, the cells were then incubated with Alexa-488 or/and Alexa-594-conjugated secondary antibodies (Invitrogen) from various sources for 2 hours at room temperature to visualize the bound primary antibodies. After another four washes with PBS, the sample was mounted with ProLong gold antifade reagent (Invitrogen) with DAPI to minimize photobleaching.

## Analysis of Statistical Significance

Data sets were evaluated by Student's t-test. Data were considered as statistically significant at $P<0.05$. All data were presented as mean ± SD.

## Results

### *Deletion of Cdc42 in vascular ECs caused embryonic lethality with vascular remodeling.*

To determine the function of Cdc42 in vascular and lymphatic development, we generated an EC-specific Cdc42 knockout mouse line (VCKO) by crossing *Cdc42* $^{flox/flox}$ mice with *VE-Cadherin-Cre* mice, in which Cre recombinase driven by the *VE-Cadherin* promoter shows mosaic expression during early embryonic stage [257]. *Cdc42* $^{flox/flox}$ animals were used as littermate controls. The mosaicism leads to a higher rate of survival of Cdc42 knockout mice.

Screening 21 litters of 105 weaned pups showed the number of pups for each genotype: 39 (37%) of *Cdc42*$^{flox/flox}$ control pups, 38 (36%) of *Cdc42*$^{flow/+}$;*VE-Cadherin-Cre*$^{+}$ pups, and 28 (27%) of *Cdc42*$^{flox/+}$;*VE-Cadherin-Cre*$^{-}$ pups. The lack of viable VCKO mice indicated that the deletion of Cdc42 in an EC-specific manner caused embryonic and/or postnatal lethality. The ratio of viable VCKO embryos matched Mendel's law before E14.5. However, the ratio of VCKO embryos decreased after E14.5. Less than 30% of VCKO embryos survived beyond E16.5.

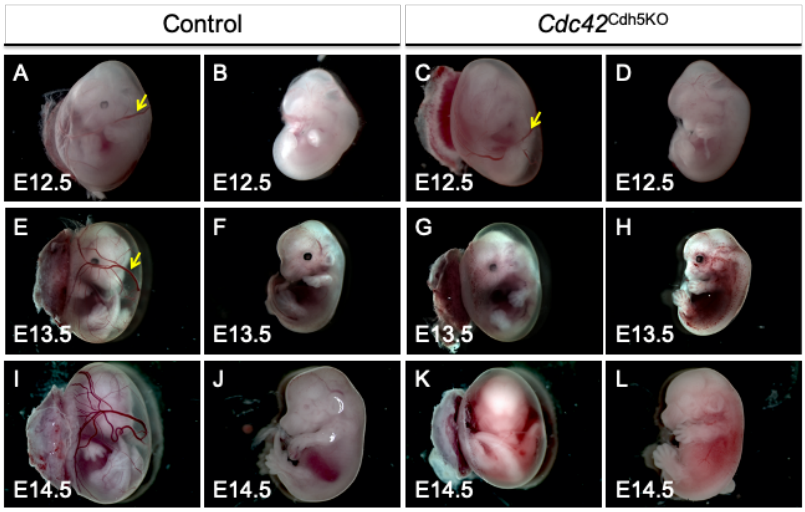



**Figure II-1 Deletion of Cdc42 in ECs disrupted vascular development.**
Gross examination of littermate controls (A, B, E, F, I, J) and VCKO (C, D, G, H, K, L) embryos at E12.5 (A-D), E13.5 (E-H), and E14.5 (I-L) with or without the yolk sac.

Control and VCKO embryos were harvested during the early developmental stage. E12.5 control and VCKO embryos were visually indistinguishable. Embryo bodies and yolk sacs are vascularized by normal blood vasculature (Fig. II-1, A-D). Later, compared with the controls (Fig. II-1, E-F), most (around 60%) VCKO embryos started to display vascular defects in both the yolk sacs and embryo bodies by E13.5 (Fig. II-1, G-H). These defects became even more evident as embryos further developed. (Fig. II-1, I-L).

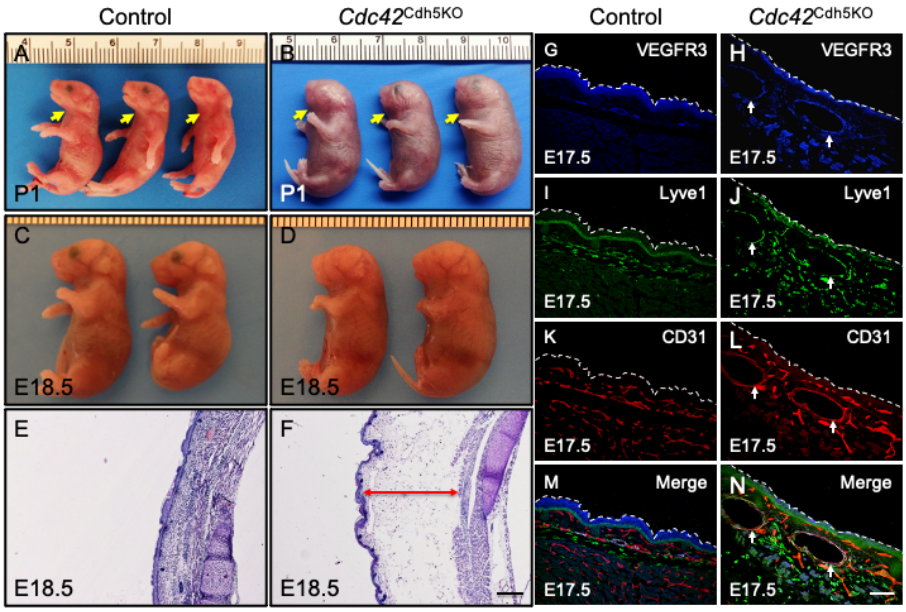



**Figure II-2 Cdc42 is required for lymphatic development during embryogenesis.** Gross examination of P1 pups (A, B) and E18.5 embryos (C, D). P1 VCKO pups showed severe edema with purple skin (B), but P1 control pups with pink appearances displayed no signs of edema (A). Side view of E18.5 VCKO embryos showed edema (D), but no edema was detected in control embryos (C). Histological analysis with H&E staining on the dorsal skin of E18.5 control (E) and VCKO (F) embryos. The subcutaneous tissues of E18.5 VCKO embryos exhibited a distinct extension (red line) (F). Immunofluorescence staining for VEGFR3 (G, H), Lyve1 (I, J), and CD31 (K, L) on control (G, I, K, M) and VCKO (H, J, L, N) embryos. The enlarged lymphatic vessels are indicated by white arrows. Bars: 125 μm (F), 100 μm (N).

*Knockout of Cdc42 in ECs caused severe edema.*

A small portion of the VCKO embryos was still viable at the early postnatal stage. However, these pups displayed severe edema with purple skins, and perished shortly after birth (Fig. II-2, B). Compared with control animals (Fig. II-2, A, C), the distances between the lower lip and the ventral neck of the VCKO pup was significantly narrower (Fig. II-2, B, D, yellow arrows), indicating subcutaneous edema caused by interstitial fluid accumulation.

Lymphatic vessels play an indispensable role in regulating body fluid homeostasis. The dramatic subcutaneous edema in the VCKO embryos prompted us to assess the impact of EC-specific Cdc42 knockout on dermal lymphatic development. To this end, we conducted immunofluorescence staining on dorsal skin sections dissected from E17.5 embryos using Lyve-1, a marker to label LECs. The results revealed significantly enlarged vessels in the dermal lymphatic networks from the E17.5 VCKO embryos (Fig. II-2, J, N), but not in the controls (Fig. II-2, I, M). VEGFR3, another lymphatic specific marker, showed similar lymphatic abnormalities as the Lyve1 staining (Fig. II-2, G, H).

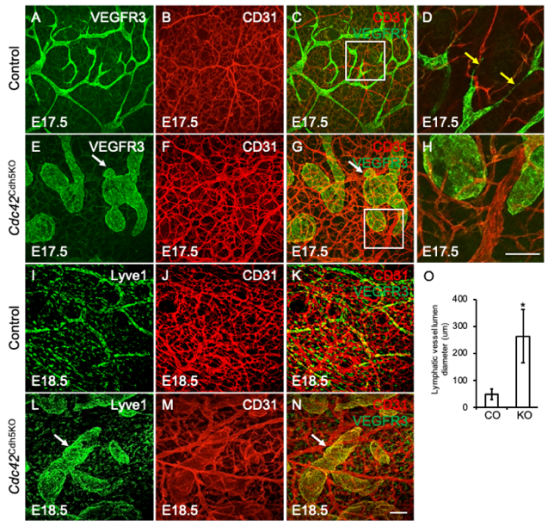



**Figure II-3 Loss of Cdc42 prevented LEC filopodia formation and increased the lumen size of lymphatic vessels in the skin.**
Immunostaining of E17.5 control (A-D) and VCKO embryonic skin (E-H) with VEGFR3 (A, E) and CD31 (B, F). D and H are higher magnification views of the boxed regions. It showed that the filopodia (yellow arrows) in the control embryo (D) are more abundant than the knockout embryos (H). Immunostaining of E18.5 control (I-K) and VCKO (L-N) embryonic skin with CD31 (J, M) and Lyve1 (I, L). The lymphatic lumen size was increased in the knockout animals (G, N), compared with the controls (C, K). Quantification of the diameter of lymphatic vessels from skin of E17.5 embryos (O). N = 4 per genotype. Data are presented as mean ± SD. *P<0.05. Bars: 100 μm.

To further dissect the impact of Cdc42 knockout on lymphangiogenesis, we utilized whole-mount immunofluorescence staining to visualize E17.5 control and VCKO dermal lymphatic vasculature with antibodies against CD31 and VEGFR3 (Fig. II-3, A-H). The size of the lymphatic lumen was significantly increased in VCKO (Fig. II-3, E, G, white arrows) when compared with the controls (Fig. II-3, A, C). Meanwhile, filopodium-like protrusions are abundantly present in control LECs (Fig. II-3, D, yellow arrows), whereas the number of filopodia was significantly decreased in the VCKO embryos (Fig. II-3, H). Similar aberrant enlargement could also be detected in E18.5 dorsal skin from VCKO embryos by Lyve1 and CD31 staining (Fig. II-3, L, N, white arrows).

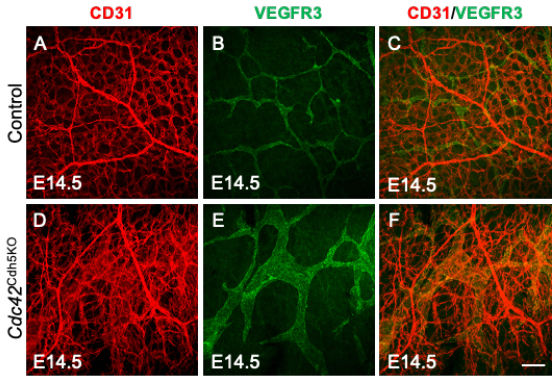



**Figure II-4 Deletion of Cdc42 resulted in lymphatic vessel enlargement.**
Whole-mount immunofluorescence staining was performed on E14.5 control (A, B, C) and VCKO (D, E, F) embryonic skins with CD31 (A, D) and VEGFR3 (B, E). Bar: 100 μm.

To determine the relationship between aberrant lymphatic enlargement and subcutaneous edema, we collected those VCKO embryos showing no apparent signs of edema at E14.5. Whole-mount staining results showed that the inactivation of *Cdc42* led

to aberrant lymphatic enlargement before the onset of dramatic subcutaneous edema (Fig. II-4, D-F) compared with the controls (Fig. II-4, A-C). These findings indicate that impaired lymphatic formation could contribute to the edema in VCKO embryos.

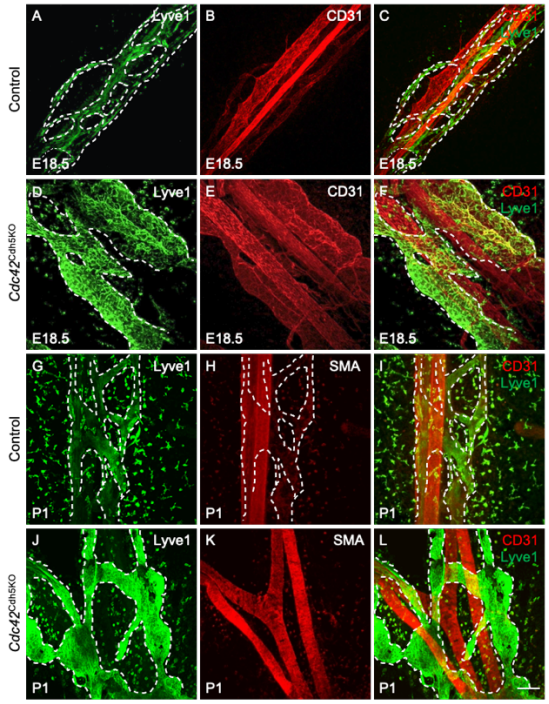



**Figure II-5 Cdc42 is essential for collecting lymphatic vessel maturation.** Immunofluorescence staining for Lyve1 (A, D) and CD31 (B, E) on the mesenteries of E18.5 control (A, B, C) and VCKO (D, E, F) embryos. The lymphatic lumen size of VCKO animals (F) was larger than that of control animals (C). Lyve1 (G, J) and smooth muscle actin (H, K) double staining on control (G, H, I) and VCKO (J, K, L) mesenteries. Smooth muscle actin positive cells were recruited in P1 control lymphatics (I), but not in VCKO (L) collecting lymphatic vessels. Bar: 100 μm.

*Loss of Cdc42 in ECs compromised maturation of collecting lymphatic vessels in the mesentery.*

Mesenteric collecting lymphatic vessels are essential for moving absorbed dietary fat towards the venous system. To determine the function of Cdc42 in the mesenteric lymphatic development, we collected the mesenteries control and deficient

embryos at E18.5 (Fig. II-5, A-F). We then stained the mesenteric preparations with Lyve1 (Fig. II-5, A, D) and CD31 (Fig. II-5, B, E). The collecting lymphatic vessels with relatively even diameters in control mesenteries developed along the blood vessels (Fig. II-5. A, C). Nonetheless, Lyve-1-positive mesenteric lymphatic vessels in VCKO appeared to be enlarged and irregular in diameter (Fig. II-5, D, F). Meanwhile, the level of Lyve1 in lymphatic vessels from VCKO animals is significantly higher. To determine whether the lymphatic defects were still present at the postnatal stage, we assessed the structure of the mesenteric collecting lymphatic vessels at postnatal day (P) 1 (Fig. II-5, G-L). Strikingly, collecting lymphatic vessels from VCKO appeared to be highly enlarged while Lyve1 expression was still maintained at a high level (Fig. II-5, J, L) at P1. On the contrary, collecting lymphatic vessels in the control embryos were relatively even in diameter and exhibited a lower level of Lyve1 expression (Fig. II-5, G, I).

Another critical characteristic during the development of collecting lymphatic vessels is recruitment of muscle cells. As expected, mature collecting lymphatic vessels from P1 control animals were indeed sheathed by lymphatic muscle cells positive smooth muscle actin (Fig. II-5, H, I, white lines). Nonetheless, lymphatic vessels in the VCKO mesenteries were devoid of muscle cells positive for smooth muscle actin (Fig. II-5, K, L), indicating that the Cdc42 deficiency compromised the recruitment of lymphatic muscle cells. In summary, these results showed that maturation of collecting lymphatic vessels in the mesentery requires Cdc42.

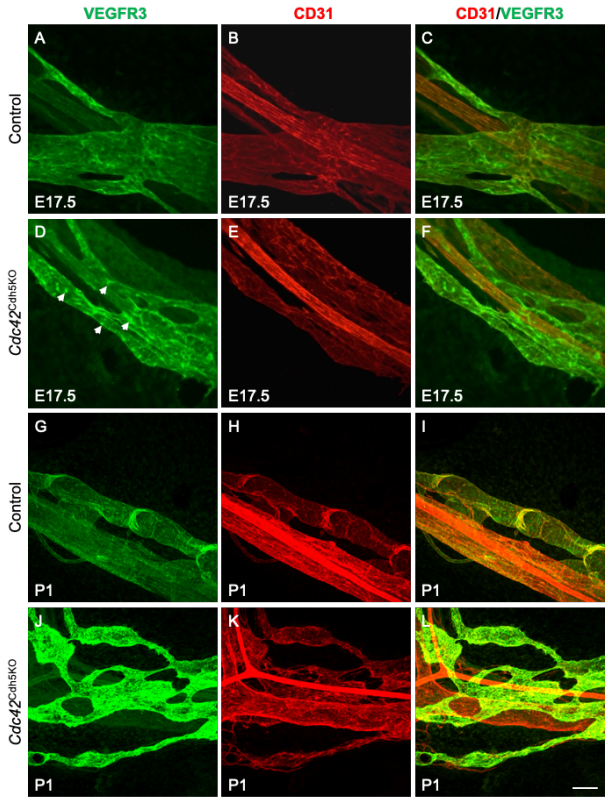



**Figure II-6 Inactivation of *Cdc42* in ECs compromised maturation of mesenteric collecting lymphatic vessels.**
Whole-mount immunofluorescence staining with antibodies against VEGFR3 (A, D, G, J) and CD31 (B, E, H, K) on E17.5 (A-F) and P1 (G-L) mesenteries. Bar: 100 μm.

Staining E17.5 control (Fig. II-6, A-C) and knockout (Fig. II-6, D-F) embryos with VEGFR3 showed that VEGFR3 was relatively distributed in the collecting lymphatic vessels in control animals (Fig. II-6, A). In contrast, puncta could often be detected in collecting lymphatic vessels from VCKO embryos (Fig. II-6, D, white arrows). Consistent with the lymphatic defects exhibited at E17.5, collecting lymphatic networks were disorganized in P1 VCKO embryos (Fig. II-6, J-L), compared with the control embryos (Fig. II-6, G-I). These disorganized lymphatic vessels may indicate compromised intussusceptive vascular pruning. These defects in the mesenteric

lymphatic vasculature are reminiscent of maturation defects displayed in *Foxc2*$^{-/-}$ mice [39], in which immature collecting vessels displayed high levels of Lyve1 and VEGFR3. In summary, these findings indicate that Cdc42 deficiency in ECs impairs the maturation of collecting lymphatic vessels in the mesentery.

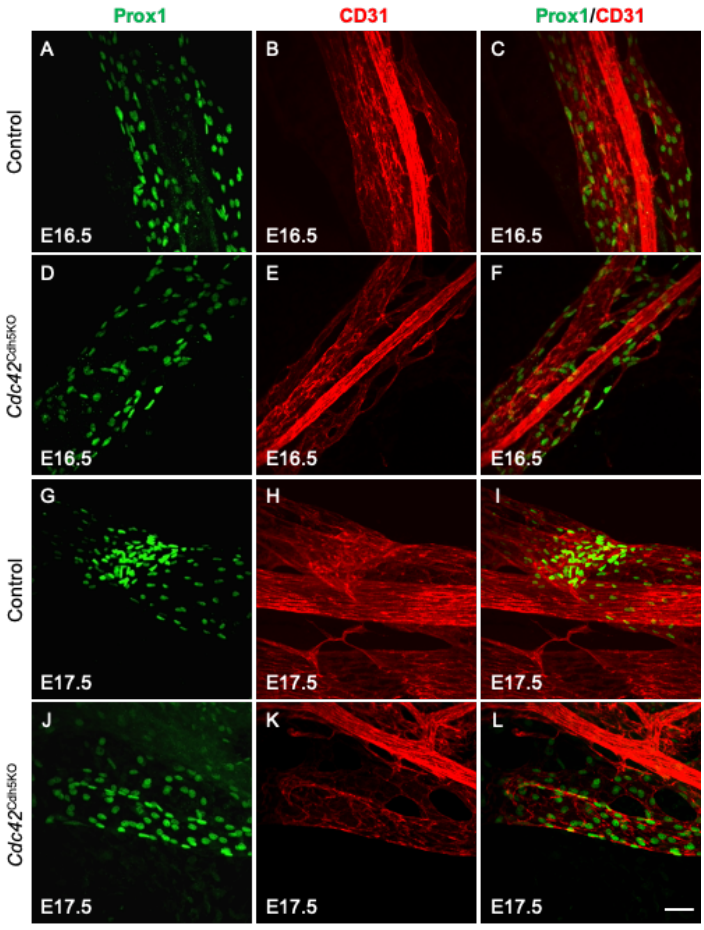



**Figure II-7 Inactivation of *Cdc42* disrupted the formation of LVs.**
Whole-mount immunofluorescence staining was performed on E16.5 (A-F) and E17.5 (G-L) mesenteries with antibodies against Prox1 (A, D, G, J) and CD31 (B, E, H, K). Bar: 100 μm.

*Inactivation of Cdc42 impaired LV maturation.*

LV formation is an intricate process that is sophisticatedly controlled by coordinated activities of various signaling molecules. To assess whether Cdc42 is involved in this process, we conducted co-staining with CD31 and Prox1 on mesenteries from embryos from E16.5 to P1. Prox1 is evenly distributed along the mesenteric lymphatic vessels at E16.5, and the LVs could not be visually detected in control (Fig. II-7, A-C) or VCKO (Fig. II-7, D-F) embryos. By E17.5, a cluster of LECs with high *Prox1* expression was detected along the longitudinal axis of vessels in both control (Fig. II-7, G-I) and VCKO (Fig. II-7, J-L) embryos. In addition, a subset of LECs with high *Prox1* expression turned 90 degrees and moved towards the vessel lumen in control animals (Fig. II-7, G) but these cells could not reorient in VCKO embryos (Fig. II-7, J).

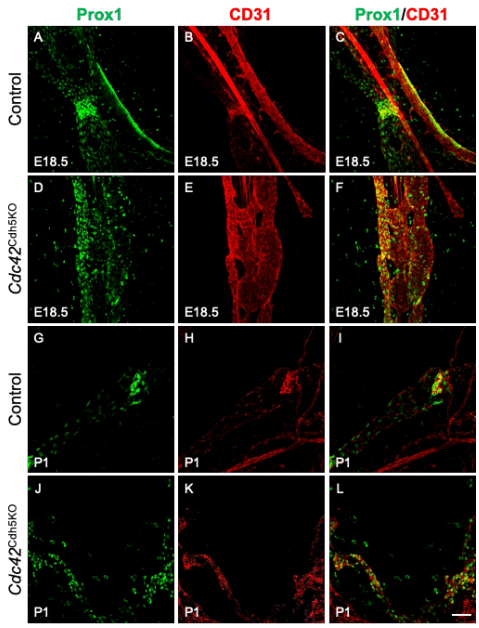



**Figure II-8 Cdc42 is required for lymphatic valve formation.**
Immunofluorescence staining for Prox1 (A, D) and CD31 (B, E) on E18.5 control (A, B, C) and VCKO mesentery (D, E, F). Prox1 level was increased in the LV-forming region but was expressed at a moderate level in the lymphangion regions (A, C). The

expression level of *Prox1* was relatively even in the knockout animals (D, F). P1 mesenteric vessels were stained with Prox1 (G, J) and CD31 (H, K). P1 LVs formed mature leaflets with high *Prox1* expression in the control animals (I), but no mature LVs were visualized in the VCKO embryos (L). Bar: 100 μm.

Moreover, *Prox1* expression from the lymphangion region is higher than the rest of the parts of the vessels in control animals (Fig. II-8, A). At E18.5, LV-forming cells clustered together and formed ring-like constrictions in control embryos (Fig. II-8, A). Nonetheless, obvious ring-like structures could hardly be detected in VCKO animals (Fig. II-8, D). At P1, LVs in controls were mature and characterized by well-formed leaflets (Fig. II-8, G-I). However, we barely detected any mature LVs with leaflets from VCKO (Fig. II-8, J-L). These abnormalities associated with lymphatic vessels suggested that Cdc42 is required for LV formation.

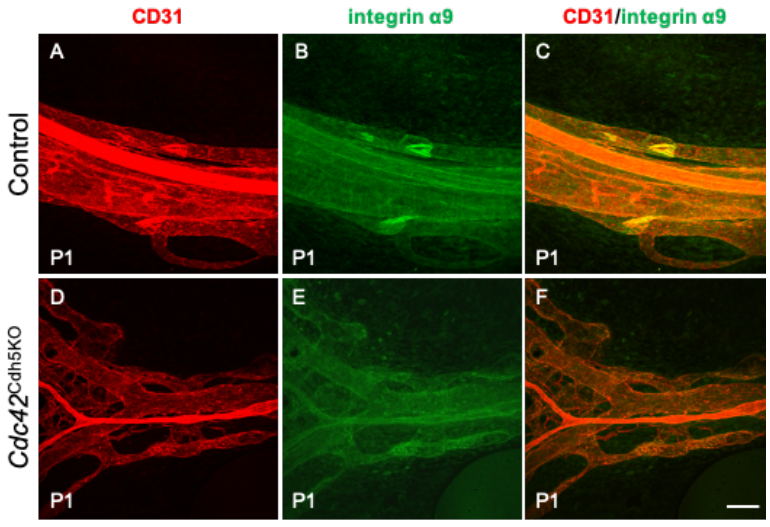



**Figure II-9 Deletion of Cdc42 prevented LV formation in the mesentery.**
Whole-mount immunofluorescence staining was performed on P1 control (A, B, C) and VCKO (D, E, F) mesenteries with antibodies against CD31 (A, D) and integrin α9 (B, E). Bar: 100 μm.

We also visualized the mesenteric lymphatic vessels with another marker for LVs, integrin α9 (Fig. II-9, B, E), and CD31 (Fig. II-9, A, D). Consistent with Prox1 staining results, the V-shaped valves were present in the control embryos (Fig. II-9, A-C), but no mature LVs formed in the VCKO embryos (Fig. II-9, D-F). In summary, Cdc42 is essential for LV formation.

*Cdc42 regulated lymphatic endothelial cell-cell junctions.*

Deletion of Cdc42 resulted in aberrant cell-cell adherens junctions in multiple types of cells, such as ECs. In the present study, the results suggested that EC-specific knockout of Cdc42 caused lymphatic enlargement and embryonic edema. Therefore, we speculated that Cdc42 is critical for maintaining lymphatic endothelial cell-cell junctions. To test this hypothesis, we collected mesenteries from E17.5 VCKO and littermate control embryos and performed whole-mount staining with antibodies against VE-Cadherin and Prox1. We detected robust and uniformly distributed VE-Cadherin at LEC-LEC junctions in control mesenteric lymphatic vessels (Fig. II-10, A-C). However, the distribution of VE-Cadherin was disrupted, and VE-Cadherin-labeled cell-cell junctions appeared to be thinner and aggregated in VCKO lymphatics (Fig. II-10, D-F, yellow arrows). Consistent with these results, the distribution of adaptor protein ZO-1 was irregular and discontinuous in VCKO animals and often displayed a serrated appearance (Fig. II-10, J-L), which was different from its uniform distribution in control embryos (Fig. II-10, G-I).

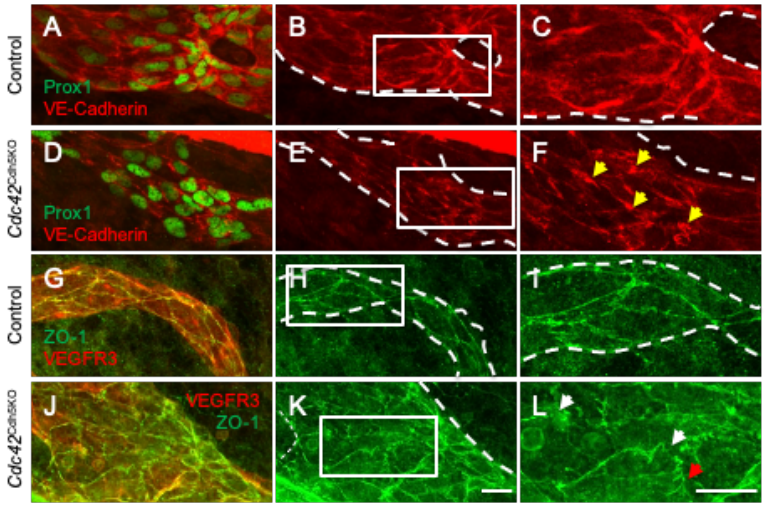



**Figure II-10 Knockout of Cdc42 disrupted LEC junctions *in vivo*.**
(A-F) Confocal images of E17.5 mesenteric lymphatic vessels from control (A-C) and VCKO (D-F) embryos stained for VE-Cadherin (red) and Prox1 (green). C, F are higher magnification views of boxed regions shown in B, E, respectively. VE-Cadherin was more evenly distributed in control animals (C), whereas the VE-Cadherin appeared to form condensed aggregates in VCKO embryos (yellow arrows, F). (G-L) E17.5 mesenteric lymphatic vessels stained with VEGFR3 (red) and ZO-1 (green) from control (G-I) and VCKO (J-L) embryos. ZO-1 was uniformly distributed at the intercellular regions between cells (H), whereas Cdc42 deficiency led to irregular localization of ZO-1 at LEC junctions (K). Images with higher magnifications revealed that ZO-1-positive junctions were frequently discontinuous in VCKO embryos (white arrows, L). Also, junctions appeared to be serrated (red arrow, L). Bars: 20 μm.

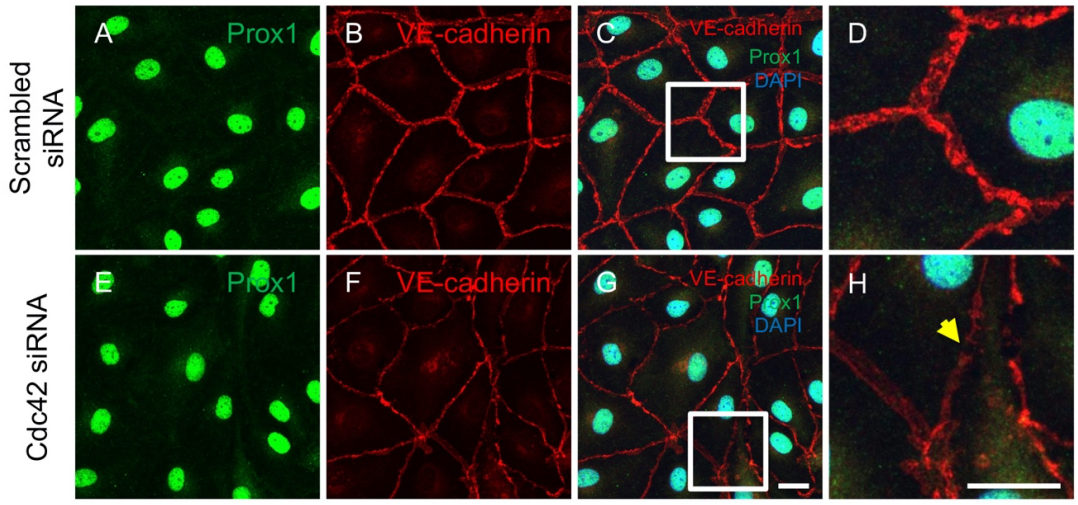



**Figure II-11 Knockdown of Cdc42 in HDLECs altered VE-Cadherin distribution.** (A-H) Confocal images of cultured HDLECs stained with Prox1 (green), VE-Cadherin (red), and DAPI (blue). D, H are the higher magnification views of boxed regions shown in C, G respectively. The siCdc42-treated HDLECs display discontinuous cell-cell junctions (H, yellow arrow), whereas the junctions in control HDLECs appear continuous and thicker. Bars: 20 μm.

To further investigate the role of Cdc42 in regulating the formation of cell-cell junctions, we performed Cdc42 siRNA knockdown in cultured HDLECs. Prox1-positive nuclei confirmed LEC identity (Fig. II-11, A, E). Consistent with the *in vivo* findings, Cdc42 knockdown led to discontinuous cell-cell boundaries labeled by ZO-1 and VE-Cadherin (Fig. II-12, E-H), whereas intercellular junctions in HDLECs treated with scrambled siRNA appeared continuous and thicker (Fig. II-12, A-D). Since the formation and maintenance of cell-cell junctions rely on cytoskeleton support, we investigated whether Cdc42 knockdown also affected cytoskeleton organization in cultured HDLECs. Immunostaining for ZO-1 and phalloidin revealed that the disruptions of cell-cell junctions often aligned with the aberrant organization of cytoskeleton where

discontinuous cell-cell junctions colocalized with disorganized phalloidin-positive filamentous (F)-actin (Fig. II-12, M-P), compared with scrambled siRNA-treated control cells (Fig. II-12. I-L). These results suggest that Cdc42 regulates lymphatic intercellular junction formation both *in vitro* and *in vivo*.

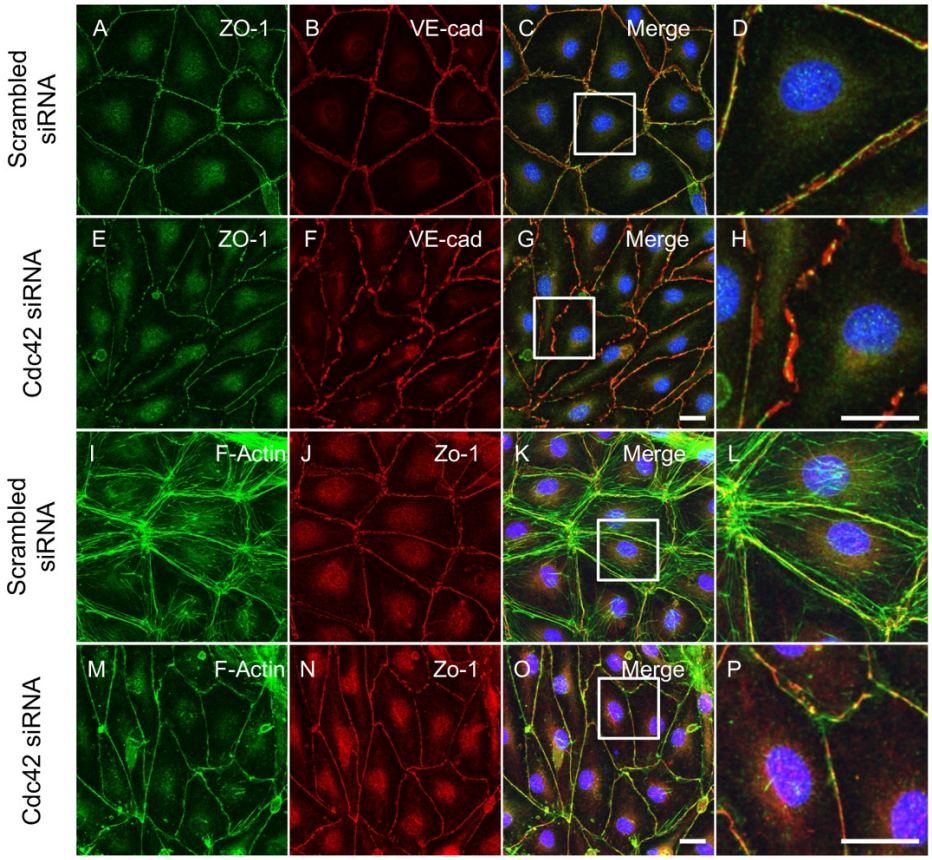



**Figure II-12 Knockdown of Cdc42 in cultured HDLECs disrupted intercellular junctions *in vitro*.**
(A-H) Confocal images of cultured HDLECs treated with scrambled siRNA (A-D) or siRNA targeting Cdc42 (siCdc42) (E-H) stained with ZO-1 (green), VE-Cadherin (red), and DAPI (blue). D, H are higher magnification views of the boxed region shown in C, G respectively. The siCdc42-treated HDLECs displayed discontinuous cell-cell junctions (H), whereas the junctions in cell treated with scrambled siRNA appeared continuous and thicker (D). Confocal images of cultured HDLECs treated with scrambled siRNA (I-L) or siRNA targeting Cdc42 (M-P) stained with phalloidin (green), ZO-1 (red), and DAPI (blue). L, P are higher magnification views of boxed regions shown in K, O.

Consistently, scrambled siRNA-treated cells showed abundant phalloidin-positive F-actin, and ZO-1-labeled junctions were well-formed (L). Nonetheless, siCdc42-treated HDLECs had disrupted cell-cell boundaries, and discontinuous ZO-1-positive junctions colocalized with the aberrant organization of phalloidin-positive F-actin. Bars: 20 μm.

*The inactivation of Cdc42 in LECs led to embryonic edema with lymphatic enlargement.*

We then generated a Cdc42 LEC-specific knockout mouse line by crossing *Cdc42*$^{flox/flox}$ mice with *Prox1-CreER*$^{T2}$ mice. Our results revealed that the deletion of Cdc42 in LECs led to severe edema in E15.5 PCKO (*Cdc42*$^{flox/flox}$;*Prox1-CreER*$^{T2+}$) embryos (Fig. II-13, E). In addition, whole-mount staining for VEGFR3 (Fig. II-13, B and F) and CD31 (Fig. II-13, C and G) on dorsal skin from these knockout embryos showed enlarged lymphatic vessels (Fig. II-13, F and H). In contrast, these aberrant lymphatic features were not present in the control embryos (Fig. II-13, B and D), indicating that lymphatic Cdc42 is indispensable for embryonic lymphatic development.

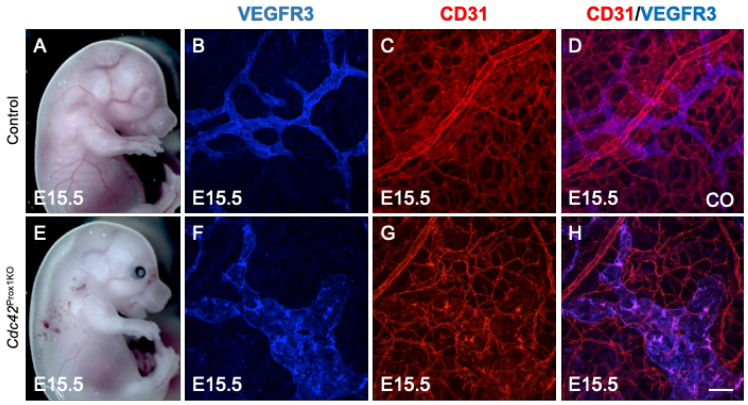
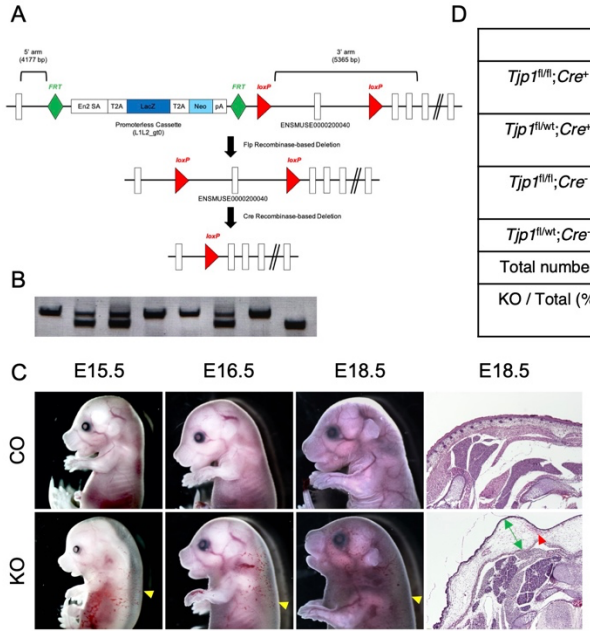



**Figure II-13 Deletion of Cdc42 in LECs resulted in embryonic edema with enlarged lymphatic vessels in the skin.**
Gross examination of E15.5 control (A) and PCKO (E) embryos. PCKO embryos displayed edema. Immunostaining of E15.5 control (B-D) and PCKO (F-H) embryonic skins for VEGFR3 (B, F) and CD31 (C, G) showed lymphatic enlargement due to LEC-specific Cdc42 knockout. Bar: 100 μm.

*Deletion of ZO-1 led to a lethal phenotype and edema.*

Since Cdc42 knockout altered ZO-1 distribution both *in vitro* and *in vivo,* we evaluated whether ZO-1 might function in the Cdc42 signaling axis during vascular and lymphatic development. To understand the function of ZO-1 in blood vessel formation, we used a gene-trap mutagenic strategy to introduce *loxP* sites flanking exon 2 of the *Tjp1* gene (Fig. II-14, A). We generated an EC-specific ZO-1 knockout mouse line by crossing *Tie2-Cre*$^{-}$;*Tjp1*$^{flox/flox}$ with *Tie2-Cre*$^{+}$;*Tjp1*$^{flox/wt}$ transgenic mice. Examining genotypes of embryos at E18.5 days revealed that ZO-1-deficient mice (*Tjp1*$^{flox/flox}$;*Tie2-Cre*$^{+}$) were born at nearly Mendelian frequency (23.7%). However, the ratio of ZO-1-deficient homozygotes decreased to 10.4% by P21, suggesting that knockout of *Tjp1* gene led to neonatal lethality. To investigate how deletion of ZO-1 in ECs caused the lethal phenotype, *Tjp1*$^{flox/flox}$;*Tie2-Cre*$^{+}$ (hereafter referred to as TZKO) and *Tjp1*$^{flox/flox}$;*Tie2-Cre*$^{-}$ or *Tjp1*$^{flox/wt}$;*Tie2-Cre*$^{+}$ (hereafter collectively referred to as control) embryos from various developmental stages were examined. At E15.5 days, TZKO embryos displayed severe edema, and the edema continued to later developmental stages, including E16.5 and E18.5 days (Fig. II-14, C). H&E staining showed obvious extension of subcutaneous tissues (Fig. II-14, C, green arrow), indicating the defective formation of vascular or/and lymphatic systems. Also, TZKO embryos displayed hemorrhage in subcutaneous areas (Fig. II-14, C, red arrowhead). In summary, ZO-1 deficiency resulted in neonatal lethality accompanied by severe subcutaneous edema.




| | E18.5 | P21 |
|---|---|---|
| $Tjp1^{fl/fl};Cre^{+}$ | 33 | 5 |
| $Tjp1^{fl/wt};Cre^{+}$ | 43 | 8 |
| $Tjp1^{fl/fl};Cre^{-}$ | 24 | 15 |
| $Tjp1^{fl/wt};Cre^{-}$ | 39 | 20 |
| Total number | 139 | 48 |
| KO / Total (%) | 23.7% | 10.4% |

**Figure II-14 Generation of EC-specific *Tjp1* knockout mice.**
(A) A gene-trap transposon vector-containing splice acceptor (SA) sequence, LacZ and neomycin resistant gene were used to create ZO-1 knockout mice. Flp recombinase was used to remove the LacZ and Neo sequence by recognizing and slicing at the FRT sites. Cre recombinase was used to delete the gene of interest, *Tjp1*, by recognizing and splicing at the *loxP* sites. Mice that were heterozygous for a *loxP*-flanked allele were intercrossed to generate mice with homozygous for *loxP*-flanked alleles. The resulting embryos were genotyped (B). (C) At E15.5, the deletion of ZO-1 in vascular ECs caused severe edema in the knockout when compared to the control embryos. At E18.5, ZO-1 deficiency resulted in severe edema in the knockout compared with the control embryos. H&E staining of the skin shows dilation and hemorrhaging in the knockout embryos.

*ZO-1-deficient mice displayed aberrant blood vessel formation.*

The subcutaneous edema displayed in TZKO embryos indicated defects associated with vascular development. Since the ratio of TZKO embryos still approximately matched the Mendelian frequency at E18.5 days, vasculogenesis, the differentiation of precursor cells (angioblasts) into ECs and subsequent *de novo* formation of primitive vascular networks, should not be severely affected. To evaluate

blood vessel formation, especially angiogenesis, the growth of new capillaries from pre-existing blood vessels, we performed whole-mount immunostaining on E11.5 embryos with endoglin (CD105) [258], a marker of neovascularization. At E11.5 days, control embryos developed well-formed hierarchical vascular trees in brain regions (Fig. II-15, A, B). Nonetheless, the complexity of vascular trees in TZKO embryos was significantly decreased (Fig. II-15, E, F). In addition, the larger vessels appeared enlarged while the capillaries failed to sprout from pre-existing vessels (Fig. II-15, F). Also, the length of ISVs in TZKO was significantly reduced, and vascular densities were decreased as well (Fig. II-15, G, H), compared with control embryos (Fig. II-15, C, D). To gain more insight into angiogenesis, we performed whole-mount fluorescent staining for CD31 on E15.5 embryonic anterior dorsal skin (Fig. II-15, K, green area). E15.5 dorsal skin from control embryos was completely vascularized by blood vessels (Fig. II-15, L). Although whole anterior dorsal skin from TZKO embryos was vascularized with blood vessels as well (Fig. II-15, M), the vascular densities appeared to decrease (Fig. II-15, N). Visualizing dermal blood vessels at later developmental stages, including E16.5 (Fig. II-15, P), E17.5 (Fig. II-15, Q), and E18.5 (Fig. II-15, R, S) revealed similar attenuation of vascular densities. These findings suggested that ZO-1 is required for blood vessel formation during embryogenesis.

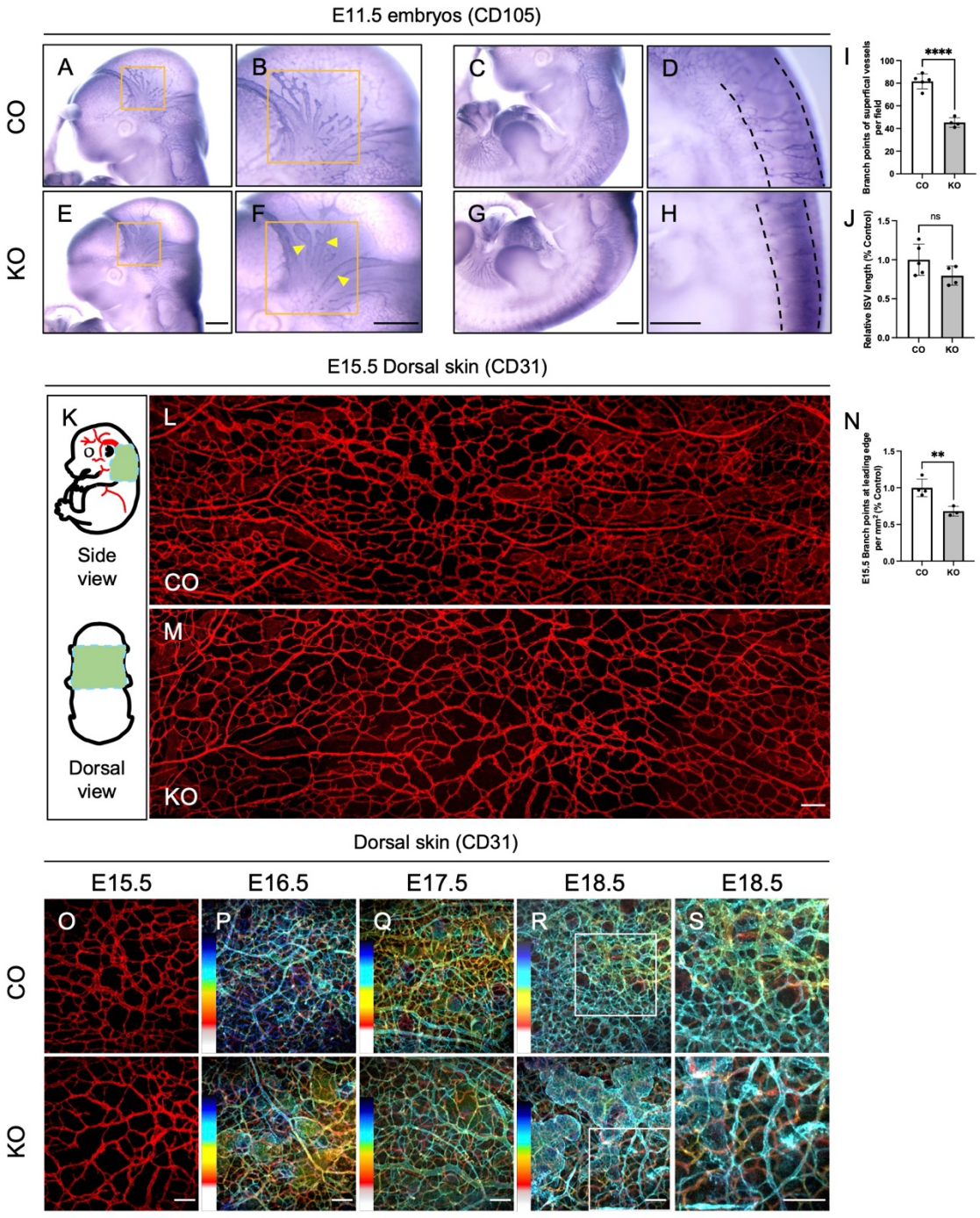



**Figure II-15 Loss of ZO-1 compromised vascular development.**
(A-H) Whole-mount immunofluorescence staining for CD105 on E11.5 control (A, B, C, D) and ZO-1-deficient embryos (E, F, G, H). In the ZO-1-deficient embryos, vascular patterning and morphology were disorganized and defective. The blood vasculature in the head region did not properly remodel as shown in the yellow box. B and F are

images with higher magnification. D and H are higher magnification images for representative ISVs. (I) Quantification for branch points of boxed regions (CO, n=5 embryos; KO, n=4 embryos). (J) Quantification for relative ISV length (CO, n=5 embryos; KO, n=4 embryos). (D and H) Area demarcated by dotted lines showed the ISV area used in quantification. (K) Schematic of embryonic anterior dorsal skin used to visualize the vascular development. (L and M) Immunofluorescent staining for CD31 (red) on the dorsal skin from E15.5 embryos. Loss of ZO-1 caused decreased blood vessel density (M), compared with the littermate control (L). (N) Quantification for branch points per $mm^2$. Higher magnification images are displayed in (O) for CO and KO respectively. (M) Quantification analysis of number of branch points in dorsal skin per $mm^2$ (CO, n=5 embryos; KO, n=4 embryos). (P, Q, R) Depth projection of representative images of skin immunostaining for CD31 at different developmental stages. Inactivation of *Tjp1* in ECs leads to decreased vascular density at different time points including E16.5 (P), E17.5 (Q) as well as E18.5 (R). (S) Higher magnification images for the boxed regions shown in (R). The data represents the means ± SD. $^{**}P<0.01$; $^{****}P<0.0001$. Bars, 500 μm (E, F, G, H), 100 μm (M, O, P, Q, R, S).

*ZO-1-deficient mice exhibited aberrant lymphatic development.*

Since most lymphatic vasculature is of venous origin, the deletion of *Tjp1* gene by Tie2-Cre might interfere with the formation of the lymphatic vasculature as well. To examine whether lymphatic development was affected, LSs, the primitive lymphatic structures from which the lymphatic vessels sprout, were examined by immunofluorescence staining for PDPN and CD31. PDPN is a marker to label LEC progenitors that fully exit the CVs [58]. Transverse sections were collected at the position indicated by the orange plate from E13.5 embryos (Fig. II-16, A). PDPN-positive LSs could be detected in control embryos. PDPN-positive LSs were present in TZKO embryos as well, indicating deletion of ZO-1 did not block the migration of LECs away from embryonic CVs (Fig. II-16, D, F). However, the LSs in TZKO embryos appeared enlarged (Fig. II-16, E, G). The aberrantly formed LSs indicated that ZO-1 was involved in the formation of the LS.

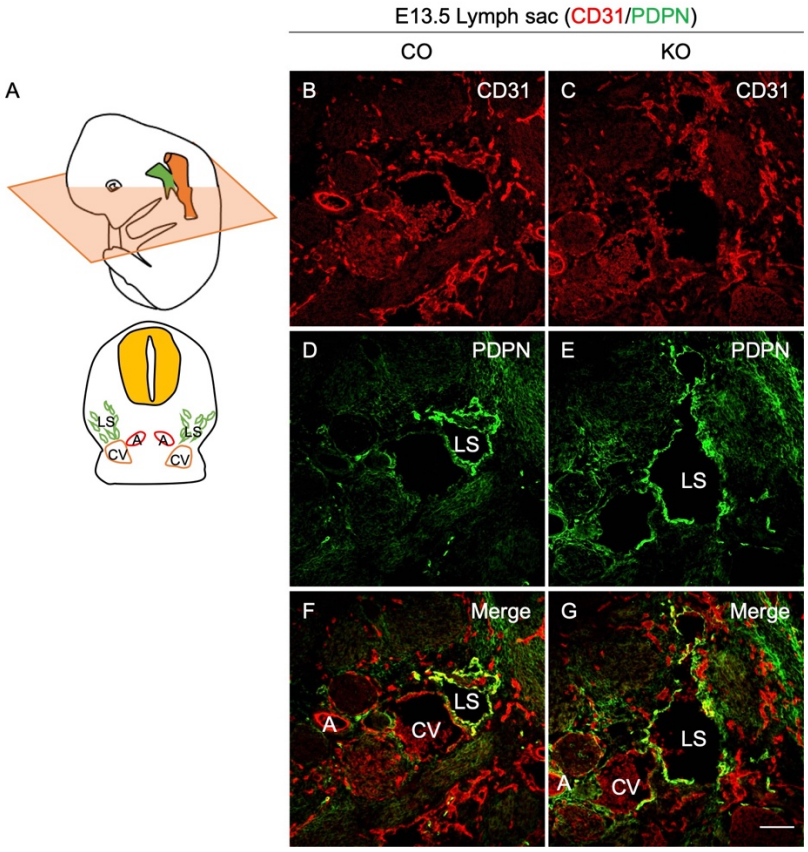



**Figure II-16 Loss of ZO-1 led to aberrantly formed lymph sac.**
(A and B) Schematic representation displaying anatomy of a developing CV and LS in a transverse plane. (C-F) Immunostaining for CD31 (red) and PDPN (green) on the sections collected at the position shown in (A). Loss of ZO-1 resulted in enlarged LSs in knockout (KO) (C, E, G), but not in control (CO) (B, D, F) embryos. LS, lymph sac; CV, cardinal vein; A, artery. Bar, 100 μm.

The dermal lymphatic vasculature plays an important role in fluid homeostasis. Therefore, malformation of the dermal lymphatic vasculature could contribute to edema. LECs start invading the anterior dorsal skin at approximately E12.5 [136]. Then, the progressive ingrowth of dermal lymphatic vessels moves towards the midline of the anterior dorsal skin (Fig. II-17, A). By E17.5, most skin is vascularized by the lymphatic

vasculature, and the leading edge of the lymphatic network should reach the midline of the skin (Fig. II-17, A). To evaluate the function of ZO-1 in the development of dermal lymphatics, the embryonic skins were dissected and stained with the endothelial marker CD31 and lymphatic endothelial marker VEGFR3 at E16.5. VEGFR3-positive lymphatic vessels formed a meshwork in control embryos (Fig. II-17, B), while dermal lymphatic vessels E16.5 TZKO embryos were striking enlarged (Fig. II-17, C). We also quantified the branch points (Fig. II-17, G) and distance to closure (Fig. II-17, H) at E15.5 when lymphatic vessels were actively sprouting and found that ZO-1 deficiency significantly reduced the network complexity of the lymphatic vasculature and increased the distance to closure. Immunofluorescence staining with another lymphatic endothelial maker Lyve1 and CD31 on traverse sections taken from E16.5 embryos showed that Lyve1-positive lymphatic vessels displayed enlarged lumen size in TZKO embryos (Fig. II-17, E), compared with control embryos (Fig. II-17, D). A small portion of TZKO embryos did not show severe edema (Fig. II-17, K) compared with littermate controls (Fig. II-17, J). We used these embryos to evaluate whether ZO-1 deficiency impaired lymphatic vasculature development without apparent signs of edema. The E17.5 embryonic skin was dissected from knockout embryos with no apparent edema and littermate controls and then was stained for CD31 and VEGFR3. In E17.5 control embryos, the entire anterior dorsal skin was vascularized by VEGFR3-positive lymphatic vessels (Fig. II-17, L). VEGFR3-positive lymphatic vessels reached the midline as expected (Fig. II-17, L). However, the anterior dorsal skin from TZKO embryos appeared to be less vascularized by VEGFR3-positive dermal lymphatic

vessels, particularly in the area towards the midline (Fig. II-17, M). Also, the complexity of the lymphatic network was significantly reduced as well (Fig. II-17, M). Higher magnification images showed that control lymphatic vessels displayed normal morphological features, such as regular diameters and sprouting (Fig. II-17, N). On the contrary, the TZKO lymphatic vessels showed enlarged diameter (Fig. II-17, O). In addition, some lymphatic sprouts appeared blunt (Fig. II-17, O), indicating that ZO-1 is required for lymphatic sprouting.

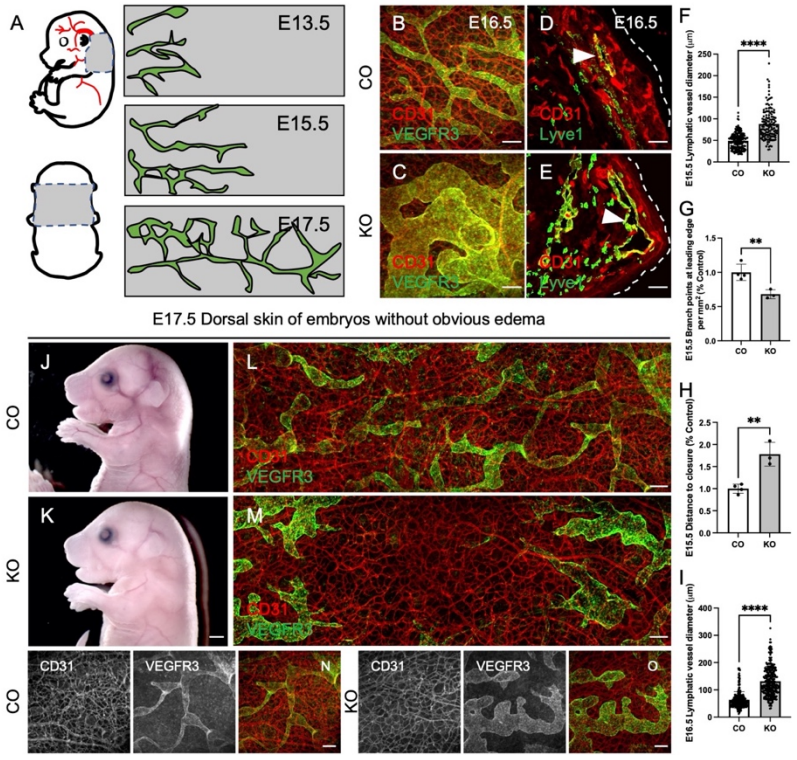



**Figure II-17 EC-specific deletion of ZO-1 interfered with lymphatic development in skin.**
(A) Schematic of embryonic anterior dorsal skin used to evaluate lymphatic development. The cartoon shows development of the dermal lymphatic vasculature at different time points. Lymphatic vessels start migrating towards the midline at approximately E12.5, further develop, and eventually reach the midline at approximate E17.5. (B and C) Representative images of immunofluorescence staining on anterior dorsal skin for CD31(red) and VEGFR3 (green) from E16.5 embryos. Lymphatic vessels

in the knockout (KO) embryos (C) were highly dilated, compared with the control (CO) embryos (B). (D and E) Representative images of immunofluorescence staining on transverse section of E16.5 embryos for CD31(red) and Lyve1(green). (F, G, H, I) Quantification of E15.5 lymphatic vessel diameter (CO, n=4 embryos; KO, n=4 embryos), E15.5 number of branch points per $mm^2$ (CO, n=4 embryos; KO, n=3 embryos), E15.5 distance to closure (CO, n=4 embryos; KO, n=3 embryos), and E16.5 lymphatic vessel diameter (CO, n=4 embryos; KO, n=4 embryos). (J and K) Gross examination of CO and KO at E17.5 days. A portion of ZO-1 embryos did not display severe edema (K), compared with littermate controls (J). Immunostaining for CD31 (red) and VEGFR3 (green) to label the blood and lymphatic vasculature in the dorsal skin, respectively, revealing lymphatic vessels are actively remodeling and have reached the midline in CO (L). However, lymphatic vasculature of KO is less branched, migrating towards, but has not yet fully covered the skin at E17.5 (M). (N) individual channels and merged image display the well-branched blood and lymphatic vasculature. KO, on the contrary, shows dilated lymphatic vasculature (O). The data represents the means ± SD. **P*<0.05; ***P*<0.01; *****P*<0.0001. Scale bar, 1 mm (K), 100 μm (B, C, D, E, L, M, N, O).

*ZO-1 is required for lymphatic development in multiple organs.*

The lymphatic vasculature develops in an organ-specific manner [259]. Therefore, we evaluated whether ZO-1 was involved in the development of lymphatic vasculature in other organs as well. For example, cardiac lymphatic vessels are required for maintaining fluid balance, which is indispensable for producing normal cardiac output [260]. Also, recent findings show that the lymphatic vasculature may be a therapeutic target for optimal healing following cardiac diseases, such as myocardial ischemia and infarction.

To investigate whether ZO-1 is involved in cardiac lymphatic development, hearts were isolated from E18.5 control and TZKO embryos. The cardiac lymphatic vessels were visualized by immunochemistry staining for VEGFR3. In control hearts, lymphatic vessels formed a meshwork on the pericardial surface, VEGFR3-positive lymphatic vessels reached the apex of the heart (Fig. II-18, A, C). However, the density

of cardiac lymphatic vessels from TZKO hearts was significantly reduced, and vessels failed to extend to the apex of hearts (Fig. II-18, B, D). The branch points, vascular density and lymphatic vessel length were significantly decreased due to ZO-1 deficiency (Fig. II-18, E-G).

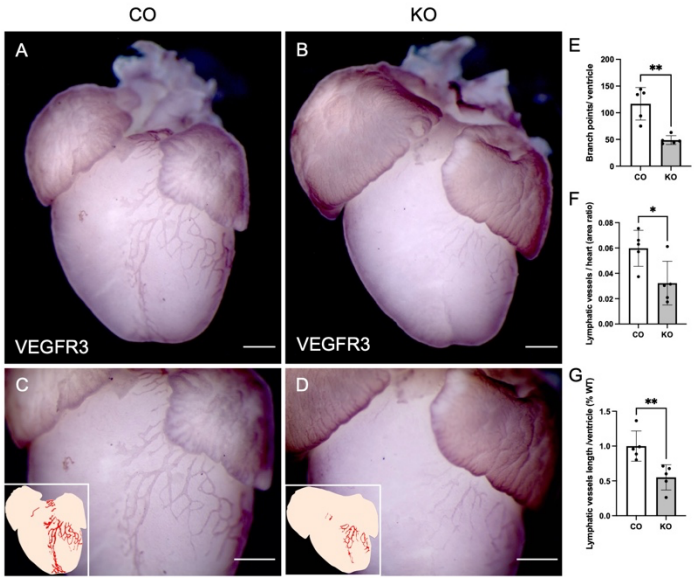



**Figure II-18 ZO-1 deficiency impaired cardiac lymphatic development.**
(A, B, C and D) Immunochemical staining for VEGFR3 on the embryonic heart from E18.5 embryos. Deficiency of ZO-1 in an EC-specific manner results in defective cardiac lymphatic development, suggested by fewer branch points in the knockout (KO) mice (C), compared with that of control (CO) mice as shown in (A). (B and D) are the higher magnification images of the red box regions in (A and C). (E) Quantification of the number of branch points of whole heart (anterior side), suggesting a significant downregulation of the number of branch points caused by ZO-1 knockout (CO, n=5 embryos; KO, n=5 embryos). The data represents the means ± SD. **P*<0.05; ***P*<0.01. Scale bar, 500 μm (A, B, C, D).

Mesenteric lymphatic vessels are required for the absorption of dietary fats. Therefore, we investigated the impact of ZO-1 deficiency on the development of mesenteric lymphatic vessels. The embryonic mesenteries were examined at E18.5 with whole-mount immunofluorescence staining for CD31, VEGFR3, and Lyve1. VEGFR3-

and Lyve1-positive mesenteric lymphatic vessels from control embryos appeared to develop normally (Fig. II-19, A-D). Mesenteric lymphatic vessels were also present in TZKO embryos (Fig. II-19, E-H). However, the morphological characteristics were aberrant, such as abnormal enlargement (Fig. II-19, E-H).

Interestingly, the development of the lymphatic vasculature in other organs such as the diaphragm was not apparently affected by ZO-1 deficiency (data not shown). Therefore, ZO-1 is crucial for embryonic development in an organ-specific manner.

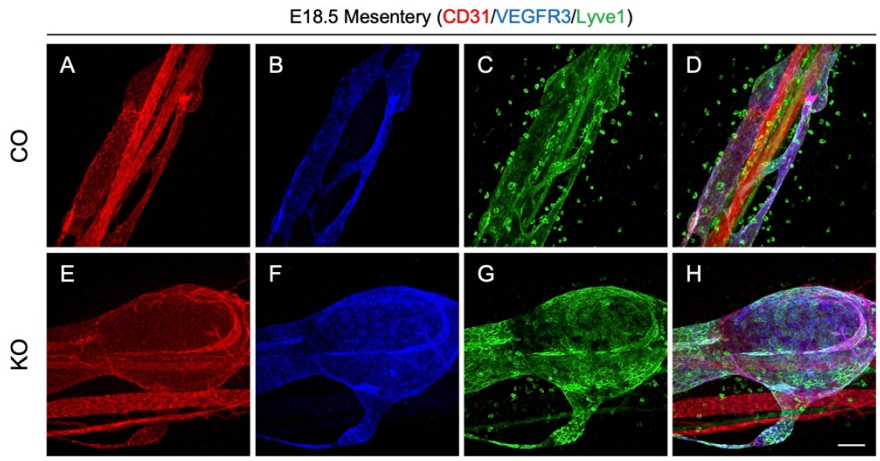



**Figure II-19 Knockout of *Tjp1* gene caused mesenteric lymphatic vessel enlargement.**
(A to H) Immunochemical staining for CD31 (red), VEGFR3 (blue) and Lyve1 (green) on the mesentery from E18.5 embryos. Deficiency of ZO-1 results in lymphatic vessel dilation in knockout (KO) (E, F, G and H), compared with normal mesenteric lymphatic vessels in control (CO) embryos (A, B, C and D). Scale bar, 100 μm (H).

## Discussion

During lymphangiogenesis, LECs need to assemble and disassemble cell-cell and cell-matrix adhesions [261]. This process is dependent on properly coordinated

cytoskeletal remodeling. Cdc42 is an essential regulator of actin polymerization and plays a vital role in controlling cell differentiation, migration, proliferation, and survival [142]. However, the role of Cdc42 in lymphatic vessel formation and maturation was unknown. Here, we have demonstrated that the deletion of Cdc42 in ECs led to severe edema with dilated lymphatic vessels. Furthermore, immunofluorescence staining results provided evidence that Cdc42 was required for filopodia formation in the skin. Strikingly, we found that the removal of Cdc42 in ECs prevented LV formation, lymphatic muscle cell recruitment, and lymphatic vessel remodeling.

Filopodia are finger-like membrane protrusions filled with tight F-actin bundles. Filopodia have growth factor receptors, integrins, cadherins, etc., and play an essential role in environmental probing, cell migration, and cell-cell adhesion formation [154, 262]. In response to extracellular stimuli, the active Cdc42 interacts with the GBD/CRIB motif of the neural Wiskott-Aldrich Syndrome protein (N-WASP) and exposes its C-terminal VCA domain. Subsequently, the active N-WASP induces conformational changes in Actin-Related Proteins (Arp) 2/3, which result in actin polymerization and filopodia formation [159, 160]. Alternatively, Cdc42 binds to scaffolding proteins insulin-receptor substrate p53 and interacts with WAVE2 and ENA/VASP to stimulate filopodia formation [163]. The first evidence suggesting the role of Cdc42 in filopodia formation stemmed from active Cdc42 protein injection-induced filopodia formation in Swiss 3T3 cells [145]. Further studies demonstrated that Cdc42 is essential in tumor necrosis factor α [263], transforming growth factor β [264], Nrp1, and VE-Cadherin-induced filopodia formation [265]. However, the deletion of Cdc42 in mouse embryonic

fibroblasts did not affect filopodia formation [266], suggesting that the role of Cdc42 in filopodia formation is cell-type-dependent. Our data provided solid evidence in favor of a Cdc42 requirement for LEC filopodia formation. It is well documented that Cdc42 activates atypical protein kinase C (aPKC) and regulates cell polarity establishment and filopodia formation via the aPKC-Par6-Par3 complex in various cell types [267]. Moreover, aPKC has been demonstrated to play a role in inhibiting stalk EC proliferation during angiogenesis in the retina [268]. If lymphatic development shares similar regulatory mechanisms with retinal angiogenesis, the deletion of Cdc42 may interfere with aPKC activation and impair tip lymphatic EC filopodia formation. Also, the loss of Cdc42 in the stalk LECs may cause aPKC to lose its inhibitory effect on LEC proliferation, consequently causing the dilated lymphatic vessels in the Cdc42 knockout embryos.

The second profound phenotype in Cdc42 EC-specific knockout mice is defective maturation of collecting lymphatic vessels. We have also shown that inactivating Cdc42 prevented LV formation. Interestingly, Cdc42 is not required for the initiation stage of LV formation but is essential for LV-forming cell condensation to form a ring-like constriction. The underlying molecular mechanisms for LV-forming cell condensation remain unclear. The differential adhesion hypothesis may explain cell sorting and boundary establishment during embryo development [269]. The high-affinity cells tend to aggregate in the center to decrease tissue surface tension, while relatively lower-affinity cells are located in the periphery. It was reported that LECs with high Prox1 expression tended to aggregate while the expression level of VE-Cadherin in these

LV-forming cells increased. Also, Cdc42 through IQGAP1 enhances cell-cell adhesions. Previous studies have documented that LVs frequently form at lymphatic vessel bifurcations, where LECs are exposed to disturbed shear stresses. Therefore, it is possible that the disturbed shear stress activates Cdc42 and then enhances VE-Cadherin-based cell-cell adhesions during the condensation of LV-forming cells. This hypothesis may align with defects displayed in Cdc42 knockout mice where the LV-forming cells could not establish strong cell-cell adhesions and LV-forming cells failed to condense. We showed that the inactivation of *Cdc42* in LECs impaired VE-Cadherin-mediated adherens junctions, indicating Cdc42-regulated LV formation may act through VE-Cadherin. Recent studies provide direct evidence for the importance of VE-Cadherin in LV formation and maintenance [270]. We also observed that the inactivation of *Cdc42* impaired lymphatic muscle cell recruitment. Previous studies have documented that ECs and vascular smooth muscle cells interact through N-cadherin [271, 272]. Our previous studies have demonstrated that Cdc42 is required for N-cadherin-mediated cardiomyocyte adhesions [167]. Therefore, deleting Cdc42 potentially affects LEC N-cadherin function and results in failed recruitment of lymphatic muscle cells in the mesenteric collecting lymphatic vessels.

As a critical junctional adaptor protein, ZO-1 interacts with many other junctional molecules, such as the transmembrane proteins of the claudin, occludin and, JAM families [223, 226, 227]. ZO-1 knockout led to an early embryonic lethal phenotype approximately at E9.5 – E10.5 and no mutant mice could survive beyond E11.5 [255]. ZO-1 deficiency caused impaired vascular development, such as defective

formation of vascular trees [255]. Aligned with a global ZO-1 knockout mouse model, further research revealed that ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation [256]. However, the function of ZO-1 in angiogenesis has not been evaluated *in vivo*. Also, whether ZO-1 is involved in lymphatic development remains elusive. To determine the role of ZO-1 in angiogenesis and lymphangiogenesis *in vivo*, we generated ZO-1 EC-specific knockout mice. EC-specific deletion of ZO-1 led to a lethal phenotype that mostly occurred during the postnatal stage. Deletion of ZO-1 induced edema during embryogenesis. Whole-mount staining results showed that ZO-1 deletion impaired vascular development, such as branching in various regions. Inactivation of *Tjp1* also interfered with lymphatic development indicated by the aberrant formation of the LS. Further histological analysis revealed that ZO-1 played a critical role in the lymphatic development of various organs, including skin, heart as well as mesentery. Functional studies using florescent tracers showed that ZO-1 is required for lymph uptake. Taken together, endothelial ZO-1 is required for blood angiogenesis and lymphangiogenesis during embryonic development.

Both ZO-1- and Cdc42-deficient mice displayed sprouting lymphangiogenesis defects. The similarity of phenotypes indicated potential connections between these two molecules during lymphangiogenesis. It has been shown that ZO-1 directly interacts with a Cdc42 downstream effector MRCKβ to regulate cell migration and protrusion at the migrating leading edge [273]. The formation of a ZO-1/MRCKβ complex requires the opening of close conformation of MRCKβ by Cdc42 [273]. Therefore, both ZO-1 and Cdc42 deficiency in LECs might disrupt the formation of the ZO-1/ MRCKβ

complex and consequently causes defective migration and protrusion. The relationship between ZO-1 and Cdc42 during lymphatic development requires further studies.

# CHAPTER III

# FAK IS REQUIRED FOR SPROUTING LYMPHANGIOGENESIS


## Abstract

The lymphatic vasculature is lined by a single layer of overlapping LECs that forms an intricate hierarchical tubular network. The lymphatic vasculature regulates body fluid homeostasis by returning blood components extravasated from the arterial side of the capillary bed back to the blood circulation. Lymphatic vessels also play a significant role in dietary fat absorption and immune surveillance. FAK, a non-receptor tyrosine kinase, plays an essential role in establishing blood vasculature through regulating cell migration, proliferation, and survival. However, it remains unclear whether FAK participates in the development of the lymphatic vasculature. In the present study, we employed EC- and LEC-specific knockout mice models and found that FAK was involved in lymphatic vasculature formation. FAK deficiency in ECs led to defective sprouting lymphangiogenesis in multiple organs during embryogenesis, such as the skin and the diaphragm. Deletion of FAK in ECs also resulted in aberrant enlargement of mesenteric lymphatic vessels. Lymphatic-specific deletion of FAK caused similar sprouting and patterning defects during embryogenesis. Examination of EC-specific knockout mice showed that sprouting defects continued to the postnatal stage at which the complexity of the lymphatic network in the mouse tail was significantly reduced, and the lymphatic vasculature also displayed patterning defects at P10. In addition, the inactivation of *FAK* led to delayed LV maturation. Moreover,

deletion of FAK induced blood-filled lymphatics during embryogenesis. Knockdown of FAK in cultured HDLECs revealed that phosphorylation of Erk and Akt were downregulated. In summary, our findings suggest that FAK functions as a regulator for lymphangiogenesis and is also involved in the separation between the blood and lymphatic vasculatures.

## Introduction

The lymphatic vasculature, a complex tubular network, plays an essential role in fluid homeostasis [238, 274]. Concretely, the lymphatic system can collect the protein-rich tissue fluid and macromolecules extravasated from blood capillaries and return the contents to the blood circulation. The lymphatic vasculature is also essential for immune surveillance and lipid absorption. Impaired formation or function of the lymphatic vasculature leads to lymphedema, in which patients suffer from limb swelling due to interstitial fluid build-up [275, 276]. Unlike the blood circulation, the lymphatic vasculature is a unidirectional network of conduits. The main components of the lymphatic vasculature are lymphatic capillaries and collecting lymphatic vessels. Lymphatic capillaries are highly permeable since they lack pericytes, are sheathed with a discontinuous basement membrane and develop specialized “button-like” junctions between LECs [27]. The collecting lymphatic vessels form tighter “zipper-like” junctions and are covered by smooth muscle cells. The collecting lymphatic vessels move the contents (lymph) collected by lymphatic capillaries through the lymph nodes towards the venous bloodstream. Transported lymph flows through two main outflow

paths, the right lymphatic duct and the thoracic duct, and enters the blood circulation through the LVVs in the jugular region. The lymphatic system is associated with the pathogenesis of lymphedema and implicated in cardiovascular diseases, hypertension, tumor metastasis, etc. [12].

Developmentally, most of the lymphatic vasculature is of venous origin [22, 23, 277]. Around E9.5, a subset of venous ECs in the CVs starts to express the homeobox gene *Prox1*, the master control gene of the lymphatic vasculature [18, 19, 278]. Those *Prox1*-expressing cells migrate towards the dorsolateral side and give rise to the LSs [44]. An environmental cue, VEGF-C, and its corresponding receptor, VEGFR3, expressed in *Prox1*-expressing cells are indispensable for the sprouting of *Prox1*-expressing cells from CVs [62, 68]. Those *Prox1*-expressing cells form initial lymphatic plexuses, which further develop through sprouting, remodeling, and maturation. The mature lymphatic network hierarchically consists of lymphatic capillaries and collecting lymphatic vessels. Once flow begins within the lymphatic vasculature, the specialized development of collecting lymphatic vessels is initiated [279]. LVs ensure the unidirectional net flow of lymph. LECs with upregulated levels of transcriptional factors, such as Prox1, FOXC2, and GATA2, delimit the territories of valve-forming areas [241]. Those valve-forming cells undergo polarization, reorientation, and invagination. A mature valve consists of two leaflets composed of a thick ECM core covered by a layer of LECs on each side [117]. Impaired function or formation of LVs can cause lymph reflux and lead to lymphedema. Clinically, defective development of LVs may underlie

the lymphedema in patients with lymphedema-distichiasis syndrome and Emberger syndrome [87, 106].

FAK, a non-receptor tyrosine kinase, plays a critical role in embryogenesis and the pathogenesis of many human diseases, such as cancer and cardiovascular diseases [280]. FAK contains three major domains, including a central kinase domain, an N-terminal FERM domain at the N-terminal, and a C-terminal focal adhesion targeting (FAT) domain [179, 198]. A linker region that contains proline-rich regions separates both terminal regions from the central kinase domain [281]. Thus, FAK is a prominent player in multiple signaling pathways, such as integrin- and growth factor-mediated signal transduction pathways [282, 283]. Integrin-mediated cell adhesion or growth factors cause rapid FAK activation, indicated by the elevation in phosphorylation at Tyr397 and other sites in FAK in multiple cell types. Then, active FAK leads to the recruitment of multiple SH2-domain- and SH3-domain-containing proteins, such as Src family kinase and phospholipase C-γ, to switch on corresponding downstream signaling pathways.

The binding of Src leads to the phosphorylation of additional sites on FAK. FAK consequently gains full catalytic activities. The FAK/Src complex can regulate many other substrates such as paxillin. FAK-mediated activation of these signaling pathways is further involved in a broad spectrum of cellular functions, including cell migration, cell cycle progression, and cell survival.

FAK regulates diverse developmental processes by controlling cell migration, proliferation, and survival. FAK is indispensable for angiogenesis. EC-specific knockout

of FAK led to hemorrhage and a lethal phenotype during embryogenesis [214, 215]. The blood vessels in the FAK mutant appeared to be disorganized and enlarged, while *in vitro* studies with isolated ECs further revealed that FAK is required for tubulogenesis, cell proliferation, migration, and survival [214]. Kinase-defective FAK could rescue EC apoptosis, but not defective angiogenesis in FAK knockout mice [284]. Since ECs and LECs share many similarities in cellular functions and underlying molecular mechanisms, FAK may regulate the development of the lymphatic vasculature by regulating the cellular behaviors of LECs. The potential role of FAK in lymphatic development was also indicated by our earlier finding where VEGF-C stimulation led to increased phosphorylation of FAK at Y397. However, as a multifunctional molecule, the role of FAK in lymphatic development has not been determined.

We employed different FAK knockout mouse models and characterized the lymphatic development from the embryonic to the postnatal stage. Our findings support that FAK participates in multiple aspects of lymphatic development. Deletion of FAK led to aberrant LSs. Also, the lymphatic sprouting was defective in FAK mutants both embryonically and postnatally. Inactivation of *FAK* also induced a blood-filled lymphatic phenotype in which blood is present inside of the lymphatic vasculature. *In vitro* studies also suggest that the downstream effectors were affected due to FAK knockdown in responses to VEGF-C.

## Materials and Methods

### *Mice*

Homozygous ($FAK^{flox/flox}$) floxed mice [214] were intercrossed with *Tie2-Cre* or *VE-Cadherin*-Cre transgenic mice to generate EC-specific knockout mice. *Lyve1-Cre* transgenic mice were used in conjunction with $FAK^{flox/flox}$ mice to generate lymphatic endothelial cell (LEC)-specific knockout mice. *Tie2-Cre*$^{+}$; $FAK^{flox/flox}$, *VE-Cadherin-Cre*$^{+}$; $FAK^{flox/flox}$ and *Lyve1-Cre*$^{+}$; $FAK^{flox/flox}$ were used as knockout mice. $FAK^{flox/flox}$ or Heterozygous ($FAK^{flox/wt}$) with corresponding Cre deleter strain was used as littermate controls (no apparent difference between the two types of controls was observed). The day the vaginal plug was detected was considered as E0.5. For *Tie2-Cre* and *VE-Cadherin-Cre* genotyping, the forward primer was 5'-CGCAGAACCTGAAGATGTTCGCGATT-3' and the reverse primer was 5'-TCTCCCACCGTCAGTACGTGAGATAT-3'. For Lyve1Cre genotyping, the following primers were used: wild-type forward primer, 5'-TGCCACCTGAAGTCTCTCCT-3'; mutant forward primer, 5'- GAGGATGGGGACTGAAACTG-3'; and reverse primer, 5'-TGAGCCACAGAAGGGTTA GG-3'. The wild-type allele gave a 425-bp band, while the mutant allele showed a 210-bp band. Forward and reverse primers for FAK genotyping were 5'-GAATCATACACCAAGTTCAAGTG-3' and 5'-GGTTTAGAAATCCTTTGGGCAGG-3', respectively. All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. The procedures on mice were carried out in compliance with institutional guidelines.

*Whole-mount immunofluorescence staining*

For whole-mount embryonic mesentery preparation, the samples were dissected and fixed in 4% PFA in PBS for 1 hour at room temperature or overnight at 4°C. For whole-mount preparation of embryonic anterior dorsal skin, E15.5-17.5 embryos were fixed in 4% PFA overnight at 4°C. Extra layers of muscle and connective tissues were gently removed with microdissection forceps without destroying the dermal superficial lymphatic vasculature. The dissected mesentery or skin was washed with PBS three times for 30 min at room temperature to remove PFA. The sample was then treated with PBS containing 0.1% Triton X-100 for 30 minutes at room temperature, followed by treatment with blocking buffer (5% fetal bovine serum in PBS) for 2 hours at room temperature. Then, the sample was stained with primary antibodies diluted in blocking buffer overnight at 4°C. After four washes with PBS, the tissue was then incubated with Alexa-488 or/and Alexa-594-conjugated secondary antibodies from various sources for 2 hours at room temperature to visualize the primary antibodies. After another four washes with PBS, the sample was mounted with ProLong gold antifade reagent (Invitrogen) with DAPI to minimize photobleaching. Confocal images were acquired with Leica TCS SP2 or Olympus FV3000 confocal microscope.

For whole-mount preparation of postnatal tail, experiments were performed in accordance with the protocol described by previous literature [76] with minor modifications. Briefly, 1-2 centimeters of tail skin were dissected. The skin was cut open longitudinally on the dorsal side. Then, the tail bone was gently removed with microdissection forceps. The tail was then treated with 20 mM EDTA in PBS on an

incubator shaker at 37°C, followed by carefully removing the epidermis. The resulting sample was fixed in 4% PFA 3 hours at room temperature and subjected to similar whole-mount staining procedures described above.

For immunofluorescence staining of cryosections of embryos, mouse embryos were embedded in OCT compound (Sakura Finetek) overnight at -80°C. 8-μm sections were collected and fixed in PFA for 30 minutes at room temperature, followed by three washes with PBS for 30 minutes at room temperature to get rid of PFA. Then sections were permeabilized with PBS containing 0.1% Triton X-100 for 20 minutes at room temperature. After three washes with PBS for 30 minutes at room temperature, the sections were blocked with blocking buffer for 1 hour at room temperature, followed by incubation with primary antibodies overnight at room temperature. The sections were then washed in PBS and subsequently stained with Alexa-488 or/and Alexa-594-conjugated secondary antibodies. ProLong gold antifade reagent (Invitrogen) was eventually applied on the sections to diminish photobleaching. Then stained sections were subjected to examination with Leica TCS SP2 or Olympus FV3000 confocal microscope.

*Immunochemistry staining*

For diaphragm whole-mount preparation, the embryonic diaphragm was dissected from E18.5 embryos, followed by 4% PFA treatment overnight at 4°C. The diaphragm was stepwise dehydrated with 25%, 50%, 75%, and 100% methanol in PBS 15 min for each concentration at room temperature and then treated with

methanol/30%$H_2O_2$ (5:1) for 5 hours at room temperature to bleach endogenous peroxidase. Next, stepwise rehydration was performed by using 75%, 50%, 25%, and 0% methanol in PBS sequentially. The sample was blocked with blocking buffer (5% fetal bovine serum and 0.5% Triton X-100 in PBS) for two hours at room temperature. Then, the diaphragm was incubated with anti-mouse VEGFR3 (R&D system) diluted in PBS overnight at room temperature. Subsequently, the sample was washed five times with PBS containing 0.1% Triton X-100 for one hour, followed by HRP-conjugated secondary antibodies in blocking buffer. The lymphatic vasculature was then visualized using tetramethylbenzidine (Sigma-Aldrich) substrate.

### *Cell culture and siRNA transfection*

Human dermal lymphatic endothelial cells (HDLECs, PromoCell) were cultured with endothelial cell growth medium MV2 kit (PromoCell) and used prior to passage six. The cells were maintained in a humidified environment containing 5% $CO_2$. siRNAs for FAK (QIAGEN) were transfected with HiPerFect transfection reagent (QIAGEN) for 72 hours in accordance with the manufacturer's instruction.

### *Western blotting analysis*

HDLECs reaching ~100% confluency were lysed with RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail (Thermo Scientific) and phosphatase inhibitor (Thermo Scientific). Antibodies against FAK (Cell Signaling), Phospho-FAK (Tyr397) (Cell Signaling), Akt (Cell Signaling), Phospho-Akt (Ser473) (Cell Signaling),

Erk1/2(Cell Signaling), Phospho-Erk1/2 (Thr202/Tyr204) (Cell Signaling), VE-Cadherin (Santa Cruz Biotechnology) and β-Catenin (Santa Cruz Biotechnology) were utilized. The PVDF membrane was washed with stripping buffer (Thermo Scientific) according to manufacturer's instruction and re-probed with GAPDH (Proteintech) and vinculin (Sigma-Aldrich).

## Analysis of Statistical Significance

Datasets were evaluated by Student's t-test. Data were considered as statistically significant at $P<0.05$. All data were presented as mean ± SD.

## Results

*Endothelial cell-specific deletion of FAK caused aberrant enlargement of lymph sacs*.

To investigate the role of FAK in lymphangiogenesis *in vivo*, we initially examined the existing FAK EC-specific knockout mouse model, $FAK^{flox/flox}$;$Tie2\text{-}Cre^{+}$ mice (designed as Tie2 FAK knockout (TFKO) hereafter) [214]. TFKO embryos displayed severe edema and multifocal superficial hemorrhages at approximately E12.5. The defects became more evident at E13.5 (Fig. III-1, A), whereas littermate controls displayed normal gross morphology (Fig. III-1, A). The subcutaneous edema indicated malformation or malfunction associated with blood and/or lymphatic vasculature. Consistent with previous findings, deletion of FAK in ECs led to angiogenic defects. However, whether deletion of FAK impaired lymphatic vasculature development remained elusive.

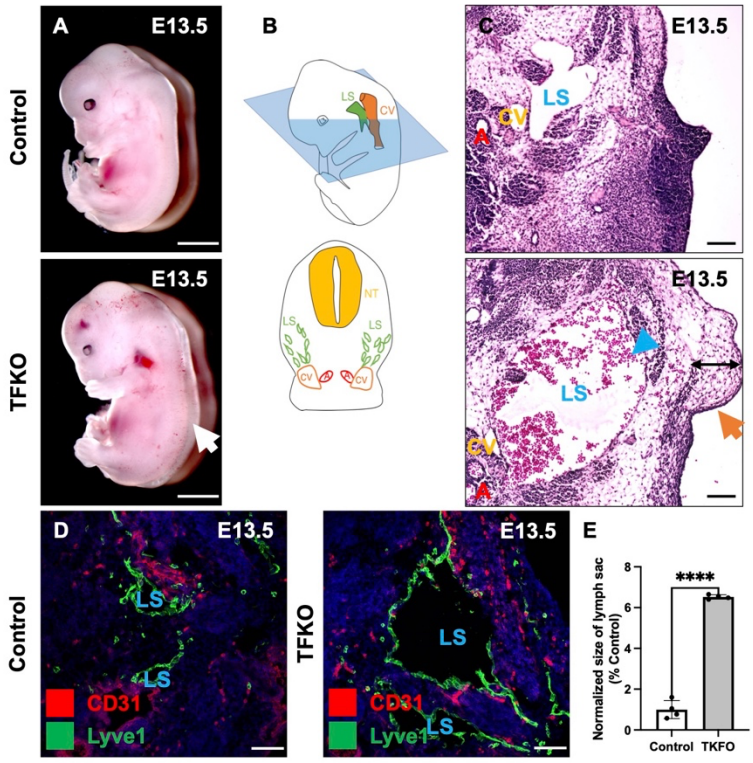



**Figure III-1 EC-specific knockout of FAK by Tie2-Cre caused enlargement of lymph sacs.**
(A) Gross examination of E13.5 control and TFKO embryos. Control embryos did not display phenotypic abnormalities, whereas deletion of FAK in ECs induced subcutaneous edema in TFKO embryos (white arrow). (B) Schematic representation depicting the anatomic location of cardinal veins (CVs) and lymph sacs (LSs) in a developing embryo (upper panel) and transverse section at the position indicated by the blue plane (button panel). (C) H&E staining of transverse sections in the jugular region of the E13.5 embryo. Control animals developed well-formed lymph sacs, whereas the LSs of FAK-deficient mice were significantly enlarged (blue arrowhead). TFKO displayed subcutaneous expansion due to edema (orange arrow). (D) Co-immunostaining for CD31 and Lyve1 in transverse sections of E13.5 control and TFKO embryos. Compared with the lymph sac from the control embryo, the Lyve1-positive lymph sac (green) in the TFKO animal was significantly increased in size. (E) Quantification of lymph sac areas normalized to the area of the whole transverse section. Increased size of jugular lymph sacs in TFKO compared with littermate controls. The data represents the means ± SD. ****$P$<0.0001. Scale bars: 2 mm (A, C); 100 μm (D).

To evaluate the function of FAK in lymphatic development, we examined the LSs, the primitive lymphatic structures. At E13.5 days, we performed transverse sections

at the jugular area, indicated by the blue plane (Fig. III-1, B, blue plane). We then examined the morphology of LSs by H&E staining (Fig. III-1, C). Consistent with the observation of gross morphology, the excess interstitial fluid accumulated in subcutaneous spaces of TFKO embryos (Fig. III-1, C, orange arrow). Also, the size of LSs was significantly enlarged in TFKO embryos (Fig. III-1, C). Meanwhile, a regular and well-formed LS was present in control embryos (Fig. III-1, C). To further confirm the lymphatic identity of the enlarged structure in FAK knockout mice, we performed immunofluorescence staining with a lymphatic marker (Lyve1) as well as a pan endothelial marker (CD31) on frozen sections of E13.5 embryos (Fig. III-1, D). Like H&E staining results, both control and TFKO embryos could develop LSs at E13.5 days. However, the size of LSs positive for Lyve1 significantly increased due to EC-specific FAK knockout (Fig. III-1, E). In summary, these results suggest that FAK is involved in the early development of the lymphatic vasculature.

*Deletion of FAK disrupted lymphatic sprouting during embryogenesis.*

Deletion of FAK by Tie2-Cre induced early embryonic lethal phenotype. The early embryonic lethality prevented us from further investigating the role of FAK in lymphatic development during the later embryonic stage. Therefore, we generated another EC-specific FAK conditional knockout mouse model to assess the functions of FAK in lymphangiogenesis. $FAK^{flox/flox}$ was crossed with $FAK^{flox/+}$; *VE-Cadherin-Cre*$^{+}$ to generate $FAK^{flox/flox}$; *VE-Cadherin-Cre*$^{+}$ mice (denoted as VE-Cadherin FAK knockout (VFKO) hereafter). The *VE-Cadherin* promoter driver induced mosaic expression of *Cre*

recombinase [257], and this mosaicism increased the survival rate of FAK-deficient mice. We could then utilize the VFKO embryos to study the role of FAK in lymphatic development. It turned out that compared with littermate controls, FAK knockout via VE-Cadherin-Cre did not induce severe edema during embryogenesis (Fig. III-2, A), and the ratio of VFKO still matched the expected Mendelian frequency at E18.5. This allowed us to investigate the function of FAK in lymphatic development. We evaluated the dermal lymphatic vascular network formation to examine whether FAK plays a role in lymphangiogenesis. A portion of anterior dorsal skin (Fig. III-2, B, the green region) was isolated and stained with a lymphatic endothelial marker (VEGFR3) as well as the pan endothelial marker CD31 (Fig. III-2, C-D). EC-specific deletion of FAK caused aberrant lymphatic development. At first glance, control animals' entire anterior dorsal skin was vascularized by lymphatic vessels at E17.5 days (Fig. III-2, C). Nonetheless, areas along the midline of the skin in VFKO animals were still devoid of lymphatic vessels (Fig. III-2, C). The lumen size of lymphatic vessels in VFKO animals was dramatically enlarged compared with control animals (Fig. III-2, C).

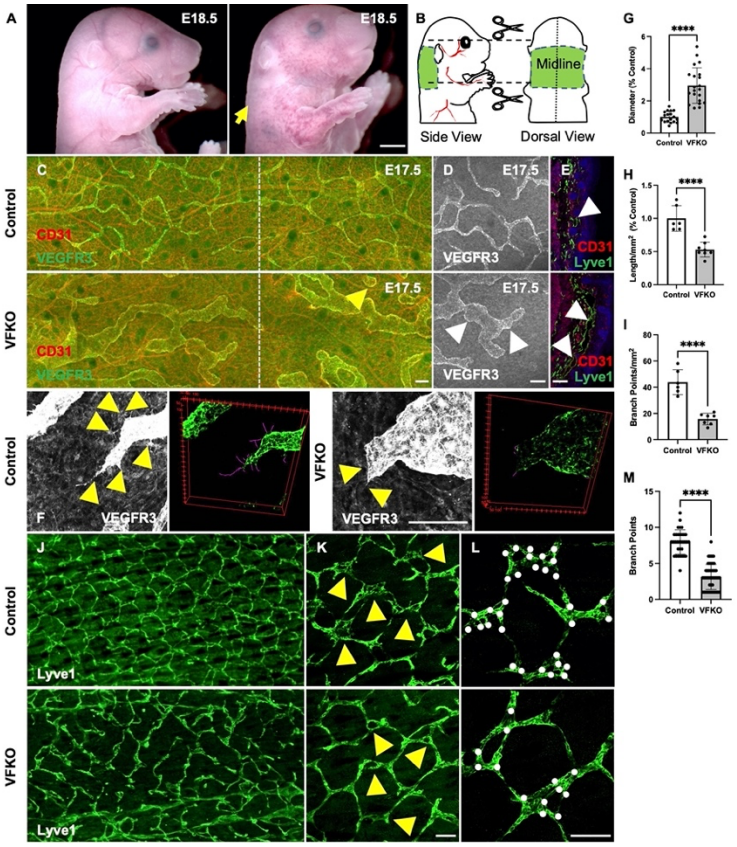



**Figure III-2 Inactivation of *FAK* in endothelial cells impaired sprouting lymphangiogenesis in the skin.**

(A) Macroscopic examination of E18.5 control and VFKO embryos. VFKO animals showed mild subcutaneous edema in contrast to normally developed control embryos. (B) Schematic representation showing the dermal region used for whole-mount staining. A part of anterior dorsal skin (green area) was dissected at E17.5, followed by whole-mount immunofluorescence staining for vascular endothelial growth factor receptor (VEGFR)-3 (green) and CD31 (red). (C-D) Whole-mount staining for VEGFR3 (green) and CD31 (red) in wild-type and VFKO embryos. VEGFR-3-positive lymphatic vessels formed meshwork in wild-type animals, and the network highly sprouted. In contrast, the sprouting lymphangiogenesis was impaired in VFKO. The network complexity was reduced, and some isolated lymphatic structures (C, yellow arrowhead) were detected within dermal lymphatic networks. The size of the lymphatic vessels significantly increased. Some lymphatic vessels failed to elongate and formed some bumps (D, white arrowheads). (E) Transverse sections stained with CD31 (red) and Lyve1(green). White arrowheads point out lymphatic vessels. (F) Representative images of sprouting lymphatic vessels from wild-type and VFKO embryos. VEGFR-3-positive lymphatic vessels from controls embryos displayed much more filopodia than VFKO animals. (G-I) Quantification of the relative diameter of lymphatic vessels (G), relative length of lymphatic vessels (H), and the number of branch points (I) in the dermal lymphatic

vasculature. The lymphatic vessels were dramatically enlarged due to the knockout of FAK (G). The total length of the lymphatic vessels in VFKO animals relative to littermate controls decreased (H). Also. the number of branch points was significantly reduced in mutants (I). (J to L) Whole-mount immunofluorescence staining for Lyve1 (Green) in P10 mouse tails of control and VFKO embryos. The mouse tail from the control animal was vascularized with Lyve1-positive lymphatic capillaries, which was finely arranged in the form of hexagon lattices at P10, whereas the complexity of the lymphatic network in VFKO was reduced (J). The LRCs were present at the junctions of matrixes (K, L), and each LRC is normally composed of 4 to 6 rings. In VFKO, the number of rings in LRC was decreased (K, L). (M) Quantification of the number of branch points per LRC. The data represents the means ± SD. ****$P<0.0001$. Scale bars: 2 mm (A); 100 μm (C, D, E, F, K, L).

Lymphatic sprouting was also impaired, indicated by the significantly decreased number of branch points in VFKO embryos compared with littermate controls (Fig. III-2, C). Some lymphatic structures failed to branch out and could not attach to the main lymphatic network (Fig. III-2, C, yellow arrowheads). Some lymphatic sprouting events in VFKO embryos appeared to initialize, but they failed to continue. Consequently, those lymphatic sprouts ended up forming rounded bumps (Fig. III-2, D, white arrowheads). We could observe much more rounded bumps in lymphatic vessels from VFKO embryos compared with controls. Those defects caused by FAK knockout indicated that FAK plays an essential role in lymphatic sprouting. Staining on transverse sections from E17.5 embryos also revealed enlarged lumen size due to FAK knockout (Fig. III-2, E). To further evaluate the function of FAK in lymphatic sprouting, we examined the structure of filopodia (Fig. III-2, F), the plasma membrane protrusions required for vessels sprouting [154, 157]. It turned out that FAK participated in filopodia formation since the deletion of FAK in ECs disrupted filopodia formation. Filopodia were

abundantly present in the lymphatic vessels of wild-type animals, whereas the number and length of filopodia were decreased in VFKO (Fig. III-2, F) animals.

*FAK-deficient mice displayed defective lymphatic sprouting postnatally.*

Accumulating evidence from embryonic lymphangiogenesis analysis indicates FAK plays a critical role in lymphatic sprouting. We took advantage of viable knockout mice to investigate whether FAK is involved in lymphatic sprouting during the postnatal stage. We used the dermal lymphatic network of the mouse tail as a model to study postnatal lymphangiogenesis. The lymphatic network of the mouse tail actively undergoes remodeling and branching during the first postnatal week and is fully established around P10 [76]. This dermal lymphatic vasculature initially sprouts from the major lateral lymphatic vessels embedded in the deeper dermis. The tail's mature superficial dermal lymphatic network is characterized by a honeycomb-like structure composed of finely arranged hexagon lattices of lymphatic capillaries [76]. The so-called LRCs are present at each matrix junction, connecting the superficial network to collecting ducts. The absence of FAK in ECs interfered with the sprouting of the lymphatic vessels since the Lyve1-positive lymphatic networks from VFKOs were less complex than lymphatic networks in littermate controls. The lymphatic networks in mutant animals were irregular and disorganized (Fig. III-2, J). The lymphatic hexagon lattices displayed in control animals were reduced to pentagon or rectangle in FAK knockout animals (Fig. III-2, K). Compared with control animals, the branch points of LRCs were significantly decreased in mutant mice (Fig. III-2, L-M). Combined with

observations from embryogenesis, our data suggest that FAK plays an indispensable role in sprouting lymphangiogenesis at both embryonic and postnatal stages.

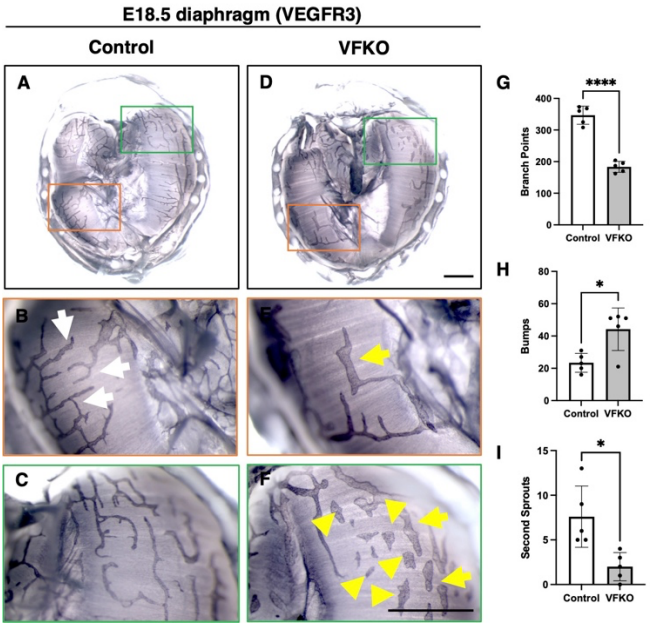



**Figure III-3 Deletion of endothelial FAK disrupted the lymphatic development in the diaphragm.**
(A, D) Whole-mount immunohistochemistry staining for VEGFR3 in diaphragms of E18.5 control and VFKO embryos. Control animals possessed lymphatic networks with normally patterned VEGFR3-positive lymphatic vessels (A). The lymphatic vasculature of diaphragms in VFKO was discontinuous and aberrant (D). (B, C, E, F) Representative images of E18.5 diaphragms labeled with VEGFR3 at higher magnifications. Lymphatic vessels positive for VEGFR3 showed rich sprouts (B, white arrows) in control animals. The sprouting lymphangiogenesis in VFKO was impaired, indicated by the reduced complexity of the lymphatic network (E). The defective lymphatic vessels were enlarged (E, yellow arrows). Some VEGFR3-positive structures could not connect (F, yellow arrowheads) and ended up forming isolated "islands". (G-I) Quantification of branch points (G), bumps (H), and second sprouts (I) in E18.5 diaphragms. Branch points and second sprouts were reduced in VFKO's lymphatic networks. Meanwhile, the number of bumps indicating failure of sprouting was increased in VFKO (H). The data represents the means ± SD. **P*<0.05; *****P*<0.0001. Scale bars: 1 mm (D, F).

*Deletion of FAK in ECs disrupted lymphatic development in the diaphragm.*

Since the lymphatic vasculatures in different organs fulfill different functions, we assessed the lymphatic development in other organs as well. Lymphatic vessels in the diaphragm are required for draining the fluid from the peritoneal cavity. The lymphatic vascular network in the diaphragm was examined at E18.5 by immunohistochemistry (IHC) staining (Fig. III-3, A-F). Compared with the regular and highly sprouted lymphatic network in control embryos (Fig. III-3, A), the diaphragmatic lymphatic network from mutants was less complex and discontinuous (Fig. III-3, D). Like lymphatic defects observed in embryonic dorsal skin, some diaphragmatic lymphatic vessels were enlarged, and the lymphatic vessels were heterogeneous in size (Fig. III-3, E-F, yellow arrows). In contrast, the lymphatic vessels in the diaphragms of littermate controls formed a uniformly sized vascular network (Fig. III-3, B-C). In addition, unlike the lymphatic network formed by connected and interwoven lymphatic vessels in control animals (Fig. III-3, C), some lymphatic vessels in mutant embryos failed to branch out and ended up being isolated “islands” in the diaphragm (Fig. III-3, F, yellow arrowheads). These defects were reminiscent of the defects displayed in dermal lymphatic networks from VFKOs. Those sprouting defects further reinforced the importance of FAK for lymphatic sprouting in various organs.

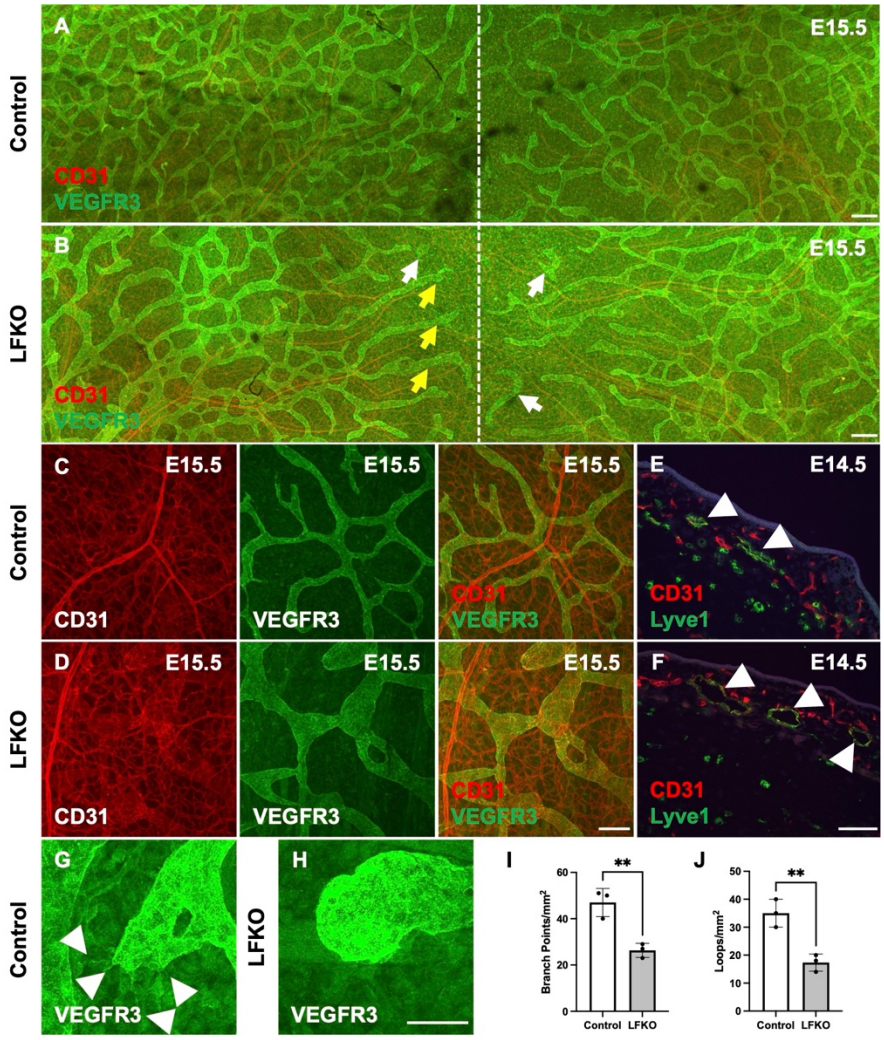



**Figure III-4 LEC-specific knockout of FAK resulted in defective sprouting lymphangiogenesis in the skin.**

(A-B) Whole-mount staining for VEGFR3 and CD31 in control and LFKO embryos at E15.5. Control animals developed VEGFR-3-positive dermal lymphatic vasculature, actively migrating towards the midline (A). Lymphatic vessels in LFKO were sprouting and migrating towards the midline as well. However, the complexity of the lymphatic network was significantly reduced, especially at leading fronts of migrating lymphatic vessels (yellow arrows, B). In addition, some lymphatic structures could not connect the main lymphatic network (white arrows, B) in LFKO embryos. (C-D) Representative images from whole-mount staining of anterior dorsal skin at E15.5. CD31-positive blood vessels (red) in control mice were comparable to LFKO. Control animals were vascularized with normal VEGFR3-positive lymphatic vessels (C). Dermis in LFKO displayed lymphatic hyperplasia and the reduced sprouting complexity of lymphatic vasculature (D). (E-F) Transverse sections were double-stained with CD31 (red) and Lyve1 (green). Dermis in wild-type animals appeared to show Lyve1-positive lymphatic vessels with normal lumen size (arrowheads, E). Lyve1-positive lymphatic vessels in

LFKO showed enlarged lumens (arrowheads, F). (G-H) Representative images showed growth of filopodia in both control and LFKO animals. The filopodia were abundantly present in control animals (arrowheads, G), whereas the number of filopodia was reduced in LFKO animals. (I-J) Quantification of branch points and the number of loops in E15.5 skin. The branching complexity of the lymphatic network was significantly decreased in LFKO, indicated by branch points (I) and loops formed by lymphatic vessels (J). The data represents the means ± SD. $^{**}P<0.01$. Scale bars: 100 μm.

*Inactivation of FAK in LECs impaired sprouting lymphangiogenesis.*

Since FAK was deleted in both ECs and LECs from VFKO, the defects shown in VFKO might have derived from both defective lymphatic and vascular network formation. To further dissect the function of lymphatic FAK in lymphangiogenesis, we employed *Lyve1-Cre* transgenic mice to generate a LEC-specific FAK conditional knockout mouse (LFKO) model where the deletion of FAK only occurred in LECs. The dorsal skin was isolated and stained with CD31 and VEGFR3 at E15.5 (Fig. III-4, A-B). Like the defects displayed in VFKO, the lymphatic vessels in LFKO underwent sprouting defects as well (Fig. III-4, B). Compared with littermate controls (Fig. III-4, A), the number of branch points and loops formed by dermal lymphatic vessels in LFKO significantly decreased due to FAK knockout (Fig. III-4, B). We noticed that the sprouting defects were more evident at the leading edge of the lymphatic network. Some lymphatic vessels at the leading edge could not sprout at E15.5 days and were displayed as a few parallel vessels (Fig. III-4, B, yellow arrows). More dramatically, some lymphatic structures from LFKO could not connect to the rest of the lymphatic network and ended up being the isolated “islands” near the midline of dermal skin (Fig. III-4, B, white arrows), which was barely observed in control animals (Fig. III-4, A).

Previous work has shown that certain dermal lymphatic vessels have a local origin where lymphatic vessels do not originate from LSs [245]. Therefore, deletion of FAK might have attenuated the ability of those isolated "lymphatic islands" to sprout out and connect to the main lymphatic network. Consequently, they could not connect with the developing lymphatic networks. Representative images showed that CD31-positive blood vessels were not affected due to FAK knockout in LECs (Fig. III-4, C-D). The lymphatic vessel from littermate controls displayed normal morphology and showed a regular lumen size (Fig. III-4, C). Nonetheless, we could see that the lymphatic vessels were less branched, and the lumen size of lymphatic vessels was highly enlarged as well in LFKO animals (Fig. III-4, D). We also performed the immunofluorescence staining on E14.5 transverse sections with antibodies specific for Lyve1 and CD31. The results showed that knockout of FAK in LECs led to enlarged lymphatic vessels but not blood vessels. Moreover, we could observe many rounded lymphatic protrusions from LFKO animals (Fig. III-4, G), whereas the ends of lymphatic vessels from control embryos were much sharper and more abundant in filopodia (Fig. III-4, H). These findings highlight that lymphatic endothelial FAK regulates lymphatic sprouting.

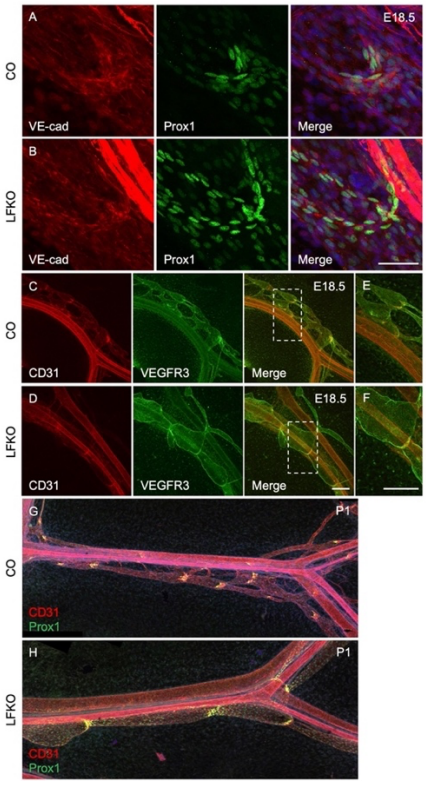



**Figure III-5 Attenuation of FAK in ECs interfered with lymphatic valve maturation.**
(A-B) Whole-mount immunofluorescence staining for VE-Cadherin (red) and Prox1 (green) with E18.5 control and VFKO embryos mesenteries. Expression levels of VE-Cadherin and Prox1 elevated in valve-forming zones in both control (A) and LFKO (B) animals. (C-D) Whole-mount immunofluorescence staining for CD31 (red) and VEGFR3 (green) with E18.5 control and VFKO embryos mesenteries. Compared with the control vessels with typical appearances (C), mesenteric lymphatic vessels from LFKO showed dramatic enlargement (D). (E-F) Higher magnification views of boxed areas delineating the valve-forming areas. (G-H) Whole-mount immunofluorescence staining to visualize the valves in the mesenteric lymphatic vessels from P1 control (G) and LFKO (H) animals. Scale bars: 50 μm (B), 100 μm (D, F).

*Inactivation of FAK interfered with lymphatic vessel structure and valve maturation.*

Since mesenteric lymphatic vessels play an indispensable role in dietary fat absorption, we continued to characterize the impact of FAK knockout on the development of mesenteric lymphatic vessels. The LV development is divided into four

stages: initiation, condensation, elongation, and maturation [35]. A subset of lymphatic endothelial cells shows increases in some essential molecules for LV formation during the initiation stage, such as Prox1 and VE-Cadherin. Then, a few rows of Prox1-high cells coalesce and compose a ring-like constriction. Subsequently, Prox1-high cells reorient and invaginate towards the lumen to form valve leaflets during elongation. Finally, the lymphatic valve-forming cells further develop with the maturation of LVs and deposition of ECM components during maturation.

The initiation of LV development was not noticeably affected by FAK deficiency, since elevation of the signature molecules such as Prox1 and VE-Cadherin could be detected in both control and LFKO animals (Fig. III-5, A-B). However, loss of endothelial FAK disrupted the normal development of the lymphatic vasculature, indicated by lymphatic vessel enlargement where characteristic features of lymphatic vessels such as lymphangions were also affected (Fig. III-5, C-D). We could observe VEGFR3-positive collecting lymphatic vessels displaying typical features in control animals, including sinus and valves (Fig. III-5, E). Meanwhile, some portions of lymphatic vessels were highly enlarged where we barely detected any mature valves at E18.5 days in FAK-deficient mice (Fig. III-5, F).

To further understand if FAK is involved in LV formation, LVs were visualized with CD31 and Prox1 immunofluorescence staining on P1 mesenteries (Fig. III-5, G-H). Instead of imaging a specific valve, we tried to profile multiple valves along the lymphatic vessels. The LVs that were positive for CD31 and Prox1 were evenly distributed along the lymphatic vessels in control animals (Fig. III-5, G), whereas mature

LVs decreased in the LFKO animals (Fig. III-5, H). These findings revealed that FAK knockout in LECs interfered with the LV formation in the mesentery. The reduction in mature LVs highlighted the importance of FAK in lymphatic valve formation.

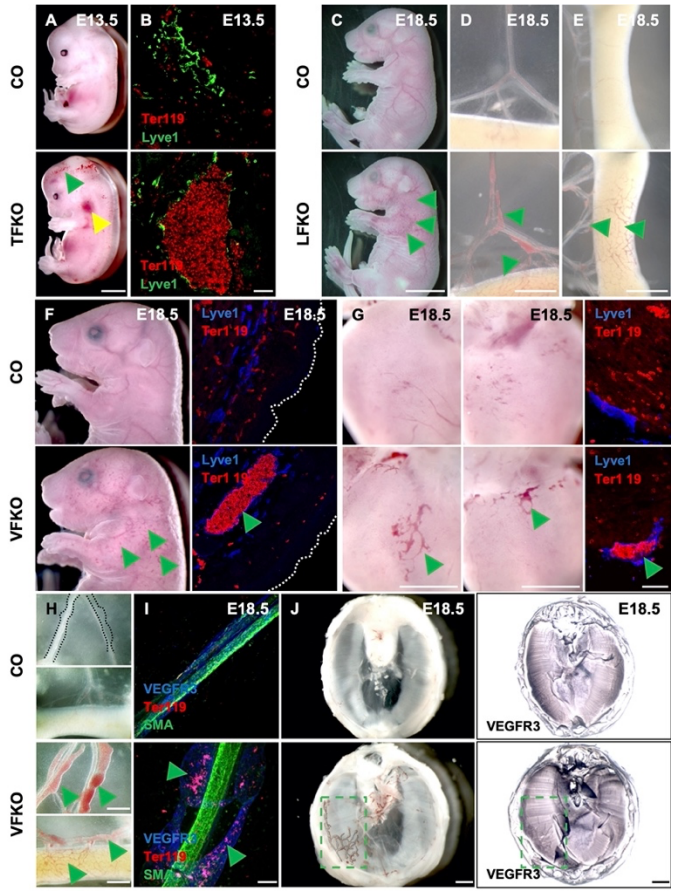



**Figure III-6 FAK deficiency induced blood-filled lymphatic vessels.**
(A) Gross examination of E13.5 control and TFKO embryos showed pooling of blood at the jugular region of TFKO embryos but not control animals. (B) Immunofluorescence staining with antibodies specific for Ter119 (red) and Lyve1 (green) in transverse sections collected from the jugular region confirmed the presence of erythrocytes inside of Lyve1-positive lymph sacs. (C) Gross examination of E18.5 control and LFKO embryos revealed that blood-filled lymphatic vessels were present in LFKO embryos (green arrowheads) but not in littermate controls. The lymphatic vasculature in the mesentery (D) and the intestine (E) of LFKO embryos contained blood (green arrowheads). (F) Gross examination of E18.5 VFKO embryos showed blood-filled lymphatic vessels in the skin of mutant embryos. (G) The lymphatic vessels in the

dissected hearts of VFKO were filled with erythrocytes. The cardiac lymphatic vessels in VFKO contain Ter119-positive red blood cells (green arrowheads). Immunofluorescence staining results demonstrated that Ter119-positive erythrocytes (red) were found in the lumen of Lyve1-positive lymphatic vessels (blue). (H) Mesenteries and intestines from VFKO embryos showed blood-filled lymphatic vessels. (I) Immunofluorescence staining with VEGFR3 (blue), Ter119 (red), and smooth muscle actin (green) confirmed that erythrocytes were detected in lymphatic vessels of VFKO embryos. (J) Dissected E18.5 diaphragms from control and VFKO embryos. Microscopic examination shows blood-filled diaphragmatic lymphatic vessels (green box). Immunohistochemistry staining with VEGFR3 confirmed the lymphatic identity of blood-filled vessels.

*FAK deficiency induced blood-filled lymphatic vessels.*

The blood and lymphatic vascular system are anatomically separated. Even though vascular and lymphatic systems are transiently connected during embryogenesis, they should remain separated to function properly as these two systems develop. Blood-filled lymphatic vessels indicate misconnections between lymphatic and blood vascular systems. In TFKOs, VFKOs, and LFKOs, FAK deficiency caused blood-filled lymphatic vessels. A blood-filled structure was present at the jugular region of FAK-deficient mice induced by Tie2-Cre (Fig. III-6, A). Erythrocytes stained by Ter119 were present in Lyve1-positive lymph sacs in TFKO embryos (Fig. III-6, B). LFKO embryos were also examined. The blood was present in lymphatic vessels of the skin (Fig. III-6, C), the mesentery (Fig. III-6, D), as well as the intestine (Fig. III-6, E). In VFKO embryos, the penetration of this blood-filled lymphatic phenotype was even higher (> 90%). Blood could be found in the skin (Fig. III-6, F), the heart (Fig. III-6, G), the intestine (Fig. III-6, H), the mesentery (Fig. III-6, H-I), and the diaphragm (Fig. III-6, J) in the VFKO animals. These findings suggest that FAK is involved in the process of blood and lymphatic vascular separation.

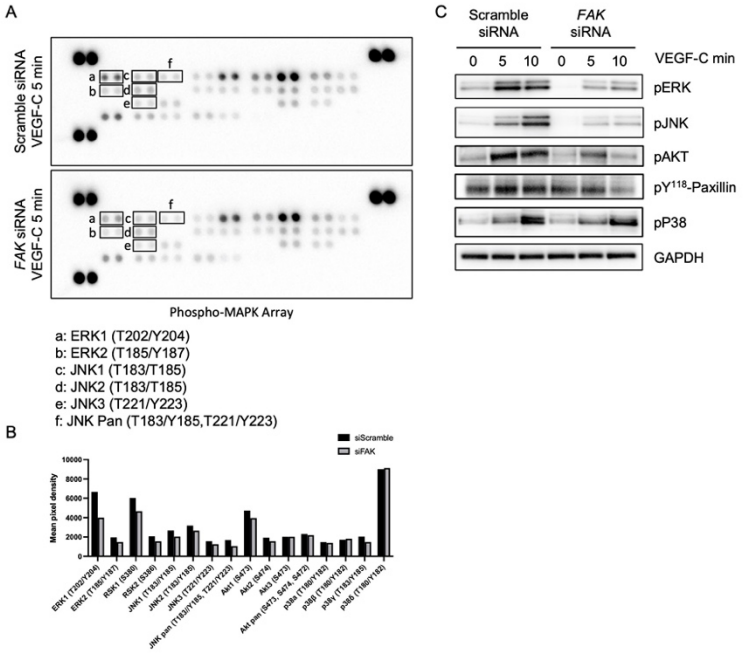



**Figure III-7 Knockdown of FAK led in defective responses of HDLECs to VEGF-C stimulation.**
(A) HDLECs were transfected with scrambled siRNA or FAK siRNA, followed by VEGF-C treatment for 5 minutes. Cell lysates were used for a Phospho-MAP kinase array assay. (B) Mean pixel density for selected targets from the Phospho-MAP kinase array assay. (C) HDLECs were transfected with scrambled siRNA or FAK siRNA, followed by VEGF-C treatment for 0, 5, or 10 minutes. The cell lysates were analyzed by western blotting with antibodies as indicated.

*Knockdown of FAK in HDLECs reduced MAP kinase signaling pathways.*

As a point of convergence of signal transductions, multiple downstream effectors and signaling pathways have been implicated in mediating FAK regulation on various cellular functions. Therefore, we evaluated the active states of multiple effectors of FAK in cultured HDLECs. MAP kinase signaling pathways have been shown to mediate FAK-dependent cell migration and proliferation. Therefore, we examined the effect of FAK knockdown on active states of MAP kinase signaling pathways by performing a Phospho-MAP kinase array assay (Fig. III-7, A). HDLECs were treated with either

scrambled siRNA or siRNA targeting FAK, followed by VEGF-C stimulation for 5 minutes. The resulting cell lysates were used to perform a Phospho-MAP kinase array assay. The results revealed that phosphorylation of ERK1/2 (Fig. III-7, a-b) decreased due to FAK knockdown. Phosphorylation of JNK (C-Jun NH2-terminal kinase) (Fig. III-7, c-f) also decreased in the HDLECs treated with FAK siRNA. We then performed western blotting to validate the Phospho-MAP kinase array assay results. HDLECs are transfected with either scrambled siRNA or FAK siRNA, followed by VEGF-C stimulation for 0, 5, or 10 minutes (Fig. III-7, C). Phosphorylation of ERK1/2 was enhanced as the treatment time increased (Fig. III-7, C). However, the level of ERK1/2 phosphorylation significantly decreased due to FAK knockdown. Phosphorylation of AKT also showed moderate reduction (Fig. III-7, C). Paxillin is a direct target of FAK, and phosphorylation of paxillin at Tyr118 is crucial for cell migration and spreading [285]. The phosphorylation of paxillin at Tyr118 was also reduced due to FAK knockdown (Fig. III-7, C). Together, these findings indicate that reduced phosphorylation of paxillin, AKT, and ERK signaling may be responsible for lymphatic defects exhibited in VFKO and LFKO.

## Discussion

As a nonreceptor tyrosine kinase that plays prominent roles in various signaling pathways, active FAK recruits many SH2-domain- and SH3-domain-containing proteins. Consequently, several downstream pathways are activated. These FAK-mediated downstream pathways are involved in a broad spectrum of cellular functions such as cell

migration, proliferation, and survival in various cell types. Many studies have revealed that FAK is required for angiogenesis by regulating behaviors of ECs in both physiological and pathological conditions. However, whether FAK plays a role in forming the lymphatic vasculature composed of LECs has not been examined. In this study, we evaluated the role of FAK in lymphatic development by utilizing multiple genetically modified mouse models. Through analyzing the development of lymphatic vasculature in an existing Tie2-Cre induced FAK knockout animal model, our data suggested that FAK was involved in the development of the lymphatic vasculature, indicated by aberrantly enlarged and blood-filled lymph sacs in the mutant embryos.

In addition, deletion of FAK in ECs by VE-Cadherin-Cre impaired the sprouting lymphangiogenesis in multiple organs during embryogenesis, such as the skin and the diaphragm. Further analysis on postnatal development of the lymphatic vasculature in mouse tails revealed that FAK was required for lymphatic sprouting postnatally, indicated by irregular patterning of lymphatic network and reduced lymphatic vessel sprouting in FAK knockout embryos. LEC-specific deletion of FAK led to a similar sprouting defect in the skin, which confirmed the specific role of lymphatic FAK in lymphatic development. Our findings also showed that deletion of FAK interfered with mesenteric lymphatic development, indicated by aberrant morphology and delayed LV formation. Moreover, FAK knockout also induced a novel blood-filled lymphatic phenotype, indicating that FAK was involved in blood and lymphatic vascular separation.

One striking defect caused by FAK knockout was impaired lymphatic sprouting at both embryonic and postnatal stages. Filopodia formation was also impaired due to FAK deficiency. The lymphatic defect displayed in the present study is reminiscent of impaired actin nucleation and neuronal filopodia formation of axonal growth from NEX-Cre-induced FAK conditional knockout mice [205]. Phosphorylation of the actin nucleation-promoting factor neural Wiskott-Aldrich syndrome protein (N-WASP) at Tyr256 was regarded as a critical mediator for neuronal filopodia formation [205]. Therefore, phosphorylation of N-WASP in the NEX-Cre-induced FAK knockout mice may account for impaired actin nucleation and deficient filopodia formation during axonal growth. In the present study, the EC-specific deletion of FAK resulted in a significant reduction of sprouting lymphangiogenesis accompanied by defective filopodia formation. This defect might also be due to impaired phosphorylation of N-WASP at Tyr256 by FAK.

Alternatively, FAK may regulate cytoskeletal dynamics by directly interacting with Actin Related Protein 2/3 (Arp2/3) complex. Arp2/3 promotes actin polymerization by generating new nucleation cores, the initial step of forming an action filament. Arp2/3 also mediates of binding of newly formed polymers to pre-existing ones. It has been shown that FAK regulates actin assembly through interacting with the Arp2/3 complex via its FERM domain [286]. FERM domain can bind Arp2/3 and enhance actin polymerization mediated by Arp2/3 [286]. Therefore, dysregulated Arp2/3 caused by FAK knockout may account for lack of filopodia formation and consequent lymphatic branching defects.

Another novel phenotype caused by FAK knockout was blood-filled lymphatics, where the blood was present in lymphatic vessels. The function of lymphatic vasculature is impaired if high-pressure blood vascular hemodynamic forces are introduced. Therefore, it is essential to maintain the separation between the blood and lymphatic vasculatures. Furthermore, valves, including LVs and LVVs, cooperate with platelet-mediated lymphovenous hemostasis to safeguard blood-lymphatic separation throughout life [124]. Concretely, PDPN present in LECs interacts with the CLEC-2 receptor in platelets, activating Syk and SLP76 axis and eventually leads to the formation of platelet thrombi. Thus, platelet aggregates may stabilize the thrombi and participate in blood and lymphatic vascular separation. In the present study, we did not observe apparent defects associated with LVVs in VFKO mice. However, we did find that deletion of FAK delayed the formation of LVs, which may account for temporal blood-filled lymphatic vessels shown in the embryonic stage. It may also explain why most of the blood-filled lymphatic vessels were resolved at the postnatal stage. Additionally, the multiple Cre transgenic mice used in the present study have been reported to express in specific hematopoietic stem cells. Therefore, FAK could be deleted in certain hematopoietic stem cells, which interfered with the development of platelets and contributed to the formation of blood-filled lymphatic vessels.

# DISCUSSION AND FURTHER DIRECTIONS

Lymphedema, including both primary and secondary lymphedema, affects millions of people globally. The causes for lymphedema are complicated. Primary lymphedema is caused by genetic conditions such as inheritable congenital lymphatic hyperplasia, hypoplasia or aplasia [287]. Secondary lymphedema is induced by damage or dysfunction of the existing lymphatic system. Common causes for secondary lymphedema include oncologic treatment and filariasis infection [287]. Although active management, such as compression therapy, can significantly alleviate the symptoms, effective cures are not currently available. The lack of cures can be largely ascribed to the complicated nature of lymphatic abnormalities in the pathogenesis of different diseases. In some circumstances, we may need to boost lymphangiogenesis by administering growth factors to compensate for the impaired functional demands caused by hypoplasia. In other circumstances, we may have to block hyperplasia to alleviate the side effects of lymphatic overgrowth. To therapeutically manipulate the lymphatic system, understanding the molecular mechanisms is a critical building block to facilitate understanding the regulatory mechanisms involved in the development of the lymphatic vasculature. In the present study, we identified and characterized multiple molecules involved in the development of the lymphatic system.

We started by investigating the role of Cdc42 in the development of lymphatic vasculature. We found that EC-specific deletion of Cdc42 caused embryonic lethality accompanied by severe edema. Detailed histological analyses revealed that loss of Cdc42 in ECs impaired sprouting lymphangiogenesis in the skin. EC-specific deletion of

Cdc42 also caused maturation defects in the mesenteric lymphatic vasculature. Moreover, mature LVs were barely detected in the Cdc42 mutants, suggesting the critical role of Cdc42 in LV formation. EC-specific deletion of Cdc42 also disrupted distribution of lymphatic endothelial cell-cell junctions in which ZO-1-positive junctions were discontinuous. In cultured HDLECs, knockdown of Cdc42 led to discontinuous cell-cell junctions. The altered distribution of ZO-1 indicates that ZO-1 may function in Cdc42 signaling axis in lymphatic development. We generated a novel EC-specific *Tjp1* knockout mouse model. ZO-1 deficiency in ECs led to severe edema and lethality. Histological findings revealed that ZO-1 deficiency resulted in defective blood vessel sprouting. Meanwhile, lymphatic development was impaired in multiple organs, such as skin, heart, and the mesentery. Sprouting lymphangiogenesis in embryonic skin was compromised. The dermal lymphatic vessels in the mutants displayed reduced complexity. The development of cardiac lymphatic vessels was also compromised. The hearts in mutants were significantly less vascularized by lymphatic vessels. Meanwhile, mesenteric lymphatic vessels were significantly enlarged due to ZO-1 deficiency. These findings signify that ZO-1 plays an important role in lymphatic development. ZO-1 may function as a downstream effector in the Cdc42 signaling axis during lymphatic development. ZO-1 directly interacts with a Cdc42 downstream effector, MRCKβ, to regulate cell migration and protrusion at the migrating leading edge [273]. Future work is needed to evaluate how Cdc42 interacts and regulates ZO-1 in LECs.

In the second part, we evaluated the role of FAK in lymphatic development. We showed that deletion of FAK in ECs interfered with formation of the LS. Moreover,

inactivation of *FAK* impaired lymphatic sprouting in multiple organs, such as the skin as well as diaphragm during embryonic development. FAK is also involved in postnatal lymphangiogenesis, indicated by defective formation of the lymphatic vasculature in the skin of mouse tails. Knockdown of FAK reduced the phosphorylation of multiple targets, including ERK1/2, JNK, AKT and paxillin. The reduced phosphorylation of paxillin, JNK, ERK signaling may be responsible for the lymphatic defects seen in FAK knockout animals. A novel defect caused by FAK knockout was blood-filled lymphatic vessels. Future work is required to understand how FAK is involved in regulation of lymphatic separation from the blood vasculature. Were blood-filled lymphatic vessels directly caused by FAK deficiency in ECs? Was endothelial FAK able to modulate the functions of platelets? Was FAK in platelets affected due to leakage of Cre expression? These are the questions that need to be addressed to dissect the causes for blood-filled lymphatic vessels.

REFERENCES


1. Choi, I., S. Lee, and Y.K. Hong, *The New Era of the Lymphatic System: No Longer Secondary to the Blood Vascular System.* Cold Spring Harbor Perspectives in Medicine, 2012. **2**(4).
2. Tammela, T. and K. Alitalo, *Lymphangiogenesis: Molecular mechanisms and future promise.* Cell, 2010. **140**(4): p. 460-76.
3. Warren, A.G., et al., *Lymphedema: a comprehensive review.* Ann Plast Surg, 2007. **59**(4): p. 464-72.
4. Natale, G., G. Bocci, and D. Ribatti, *Scholars and scientists in the history of the lymphatic system.* Journal of anatomy, 2017. **231**(3): p. 417-429.
5. Crivellato, E., L. Travan, and D. Ribatti, *The Hippocratic treatise 'On glands': the first document on lymphoid tissue and lymph nodes.* Leukemia, 2007. **21**(4): p. 591-2.
6. Irschick, R., C. Siemon, and E. Brenner, *The history of anatomical research of lymphatics - From the ancient times to the end of the European Renaissance.* Ann Anat, 2019. **223**: p. 49-69.
7. Loukas, M., et al., *The lymphatic system: a historical perspective.* Clin Anat, 2011. **24**(7): p. 807-16.
8. Asellius, G., *De lactibus sive lacteis venis.* JB Bidellium, Milan, 1968. **1627**.
9. Flourens, P., *A History of the Discovery of the Circulation of the Blood*. 1859: Rickey, Mallory.
10. Tretbar, L.L., et al., *Lymphedema*. 2008: Springer.

11. Fransson, S.-G., *Olof Rudbeck.* Clinical cardiology, 1997. **20**(11): p. 974-976.

12. Alitalo, K., T. Tammela, and T.V. Petrova, *Lymphangiogenesis in development and human disease.* Nature, 2005. **438**(7070): p. 946-53.

13. Sabin, F.R., *On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig.* American Journal of Anatomy, 1902. **1**(3): p. 367-389.

14. Sabin, F.R., *On the development of the superficial lymphatics in the skin of the pig.* American Journal of Anatomy, 1904. **3**(2): p. 183-195.

15. Huntington, G.S. and C.F. McClure, *The anatomy and development of the jugular lymph sacs in the domestic cat (Felis domestica).* American Journal of Anatomy, 1910. **10**(1): p. 177-312.

16. Kaipainen, A., et al., *Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development.* Proceedings of the National Academy of Sciences, 1995. **92**(8): p. 3566-3570.

17. Wigle, J.T., et al., *Prox1 function is crucial for mouse lens-fibre elongation.* Nature Genetics, 1999. **21**(3): p. 318-322.

18. Wigle, J.T., et al., *An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype.* EMBO J, 2002. **21**(7): p. 1505-13.

19. Srinivasan, R.S., et al., *Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature.* Genes Dev, 2007. **21**(19): p. 2422-32.

20. Hagerling, R., et al., *A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy.* EMBO J, 2013. **32**(5): p. 629-44.

21. Wilting, J., et al., *Dual origin of avian lymphatics.* Developmental Biology, 2006. **292**(1): p. 165-173.

22. Martinez-Corral, I., et al., *Nonvenous Origin of Dermal Lymphatic Vasculature.* Circulation Research, 2015. **116**(10): p. 1649-1654.

23. Maruyama, K., et al., *Isl1-expressing non-venous cell lineage contributes to cardiac lymphatic vessel development.* Developmental biology, 2019. **452**(2): p. 134-143.

24. Casley-Smith, J.R., *The identification of chylomicra and lipoproteins in tissue sections and their passage into jejunal lacteals.* J Cell Biol, 1962. **15**: p. 259-77.

25. Maby-El Hajjami, H. and T.V. Petrova, *Developmental and pathological lymphangiogenesis: from models to human disease.* Histochem Cell Biol, 2008. **130**(6): p. 1063-78.

26. Suami, H. and M.F. Scaglioni, *Anatomy of the Lymphatic System and the Lymphosome Concept with Reference to Lymphedema.* Seminars in Plastic Surgery, 2018. **32**(1): p. 5-11.

27. Baluk, P., et al., *Functionally specialized junctions between endothelial cells of lymphatic vessels.* J Exp Med, 2007. **204**(10): p. 2349-62.

28. Trzewik, J., et al., *Evidence for a second valve system in lymphatics: endothelial microvalves.* FASEB J, 2001. **15**(10): p. 1711-7.

29. Leak, L.V., *The structure of lymphatic capillaries in lymph formation.* Fed Proc, 1976. **35**(8): p. 1863-71.

30. Gerli, R., et al., *Specific adhesion molecules bind anchoring filaments and endothelial cells in human skin initial lymphatics.* Lymphology, 2000. **33**(4): p. 148-57.

31. Sacchi, G., et al., *The structure of superficial lymphatics in the human thigh: precollectors.* Anat Rec, 1997. **247**(1): p. 53-62.

32. Margaris, K.N. and R.A. Black, *Modelling the lymphatic system: challenges and opportunities.* J R Soc Interface, 2012. **9**(69): p. 601-12.

33. von der Weid, P.Y. and D.C. Zawieja, *Lymphatic smooth muscle: the motor unit of lymph drainage.* Int J Biochem Cell Biol, 2004. **36**(7): p. 1147-53.

34. Muthuchamy, M. and D. Zawieja, *Molecular regulation of lymphatic contractility.* Ann N Y Acad Sci, 2008. **1131**: p. 89-99.

35. Sabine, A., et al., *Mechanotransduction, PROX1, and FOXC2 cooperate to control connexin37 and calcineurin during lymphatic-valve formation.* Dev Cell, 2012. **22**(2): p. 430-45.

36. Oliver, G., *Lymphatic vasculature development.* Nature Reviews Immunology, 2004. **4**(1): p. 35-45.

37. Banerji, S., et al., *LYVE-1, a New Homologue of the CD44 Glycoprotein, Is a Lymph-specific Receptor for Hyaluronan.* Journal of Cell Biology, 1999. **144**(4): p. 789-801.

38. Gordon, E.J., N.W. Gale, and N.L. Harvey, *Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE-1 is also*

*expressed on embryonic blood vessels.* Developmental Dynamics, 2008. **237**(7): p. 1901-1909.

39. Norrmen, C., et al., *FOXC2 controls formation and maturation of lymphatic collecting vessels through cooperation with NFATc1.* J Cell Biol, 2009. **185**(3): p. 439-57.

40. Luong, M.X., et al., *Lack of lymphatic vessel phenotype in LYVE-1/CD44 double knockout mice.* Journal of Cellular Physiology, 2009. **219**(2): p. 430-437.

41. Gale, N.W., et al., *Normal Lymphatic Development and Function in Mice Deficient for the Lymphatic Hyaluronan Receptor LYVE-1.* Molecular and Cellular Biology, 2007. **27**(2): p. 595-604.

42. Hong, Y.K. and M. Detmar, *Prox1, master regulator of the lymphatic vasculature phenotype.* Cell and Tissue Research, 2003. **314**(1): p. 85-92.

43. Oliver, G., et al., *Prox-1, a Prospero-Related Homeobox Gene Expressed during Mouse Development.* Mechanisms of Development, 1993. **44**(1): p. 3-16.

44. Wigle, J.T. and G. Oliver, *Prox1 function is required for the development of the murine lymphatic system.* Cell, 1999. **98**(6): p. 769-78.

45. Johnson, N.C., et al., *Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity.* Genes Dev, 2008. **22**(23): p. 3282-91.

46. Srinivasan, R.S. and G. Oliver, *Prox1 dosage controls the number of lymphatic endothelial cell progenitors and the formation of the lymphovenous valves.* Genes Dev, 2011. **25**(20): p. 2187-97.

47. Hong, Y.K., et al., *Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate.* Dev Dyn, 2002. **225**(3): p. 351-7.

48. Greenfield, A., et al., *The Sry-related gene Sox18 maps to distal mouse chromosome 2.* Genomics, 1996. **36**(3): p. 558-9.

49. Pennisi, D., et al., *Mutations in Sox18 underlie cardiovascular and hair follicle defects in ragged mice.* Nat Genet, 2000. **24**(4): p. 434-7.

50. Carter, T.C. and R.J.S. Phillips, *Ragged, a Semidominant Coat Texture Mutant - in the House Mouse.* Journal of Heredity, 1954. **45**(4): p. 150-154.

51. Irrthum, A., et al., *Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia.* Am J Hum Genet, 2003. **72**(6): p. 1470-8.

52. Francois, M., et al., *Sox18 induces development of the lymphatic vasculature in mice.* Nature, 2008. **456**(7222): p. 643-7.

53. Tsai, S.Y. and M.J. Tsai, *Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age.* Endocr Rev, 1997. **18**(2): p. 229-40.

54. Pereira, F.A., et al., *The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development.* Genes & Development, 1999. **13**(8): p. 1037-1049.

55. Pereira, F.A., et al., *Chicken ovalbumin upstream promoter transcription factor (COUP-TF): expression during mouse embryogenesis.* J Steroid Biochem Mol Biol, 1995. **53**(1-6): p. 503-8.

56. Lee, S., et al., *Prox1 physically and functionally interacts with COUP-TFII to specify lymphatic endothelial cell fate.* Blood, 2009. **113**(8): p. 1856-9.

57. Srinivasan, R.S., et al., *The nuclear hormone receptor Coup-TFII is required for the initiation and early maintenance of Prox1 expression in lymphatic endothelial cells.* Genes Dev, 2010. **24**(7): p. 696-707.

58. Yang, Y. and G. Oliver, *Development of the mammalian lymphatic vasculature.* J Clin Invest, 2014. **124**(3): p. 888-97.

59. Joukov, V., et al., *A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases.* EMBO J, 1996. **15**(7): p. 1751.

60. Kukk, E., et al., *VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development.* Development, 1996. **122**(12): p. 3829-37.

61. Oh, S.-J., et al., *VEGF and VEGF-C: Specific Induction of Angiogenesis and Lymphangiogenesis in the Differentiated Avian Chorioallantoic Membrane.* Developmental Biology, 1997. **188**(1): p. 96-109.

62. Karkkainen, M.J., et al., *Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins.* Nature Immunology, 2004. **5**(1): p. 74-80.

63. Achen, M.G., et al., *Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4).* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 548-53.

64. Dumont, D.J., et al., *Cardiovascular failure in mouse embryos deficient in VEGF receptor-3.* Science, 1998. **282**(5390): p. 946-9.

65. Tammela, T., et al., *Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation.* Nature, 2008. **454**(7204): p. 656-60.

66. Secker, G.A. and N.L. Harvey, *VEGFR signaling during lymphatic vascular development: From progenitor cells to functional vessels.* Dev Dyn, 2015. **244**(3): p. 323-31.

67. Leppanen, V.M., et al., *Structural and mechanistic insights into VEGF receptor 3 ligand binding and activation.* Proc Natl Acad Sci U S A, 2013. **110**(32): p. 12960-5.

68. Zhang, L., et al., *VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis.* Cell Res, 2010. **20**(12): p. 1319-31.

69. Brice, G., *Milroy disease and the VEGFR-3 mutation phenotype.* Journal of Medical Genetics, 2005. **42**(2): p. 98-102.

70. Karpanen, T., et al., *Functional interaction of VEGF-C and VEGF-D with neuropilin receptors.* FASEB J, 2006. **20**(9): p. 1462-72.

71. Chen, H., et al., *Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III.* Neuron, 1997. **19**(3): p. 547-59.

72. Giger, R.J., et al., *Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity.* Neuron, 1998. **21**(5): p. 1079-92.

73. Giger, R.J., et al., *Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins.* Neuron, 2000. **25**(1): p. 29-41.

74. Chen, H., et al., *Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections.* Neuron, 2000. **25**(1): p. 43-56.

75. Yuan, L., et al., *Abnormal lymphatic vessel development in neuropilin 2 mutant mice.* Development, 2002. **129**(20): p. 4797-806.

76. Xu, Y., et al., *Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3.* J Cell Biol, 2010. **188**(1): p. 115-30.

77. Hogan, B.M., et al., *ccbe1 is required for embryonic lymphangiogenesis and venous sprouting.* Nature Genetics, 2009. **41**(4): p. 396-398.

78. Bos, F.L., et al., *CCBE1 Is Essential for Mammalian Lymphatic Vascular Development and Enhances the Lymphangiogenic Effect of Vascular Endothelial Growth Factor-C In Vivo.* Circulation Research, 2011. **109**(5): p. 486-491.

79. Alders, M., et al., *Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans.* Nat Genet, 2009. **41**(12): p. 1272-4.

80. Yang, Y., et al., *Lymphatic endothelial progenitors bud from the cardinal vein and intersomitic vessels in mammalian embryos.* Blood, 2012. **120**(11): p. 2340-8.

81. Breiteneder-Geleff, S., et al., *Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium.* Am J Pathol, 1999. **154**(2): p. 385-94.

82. Wetterwald, A., et al., *Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes.* Bone, 1996. **18**(2): p. 125-32.

83. Mahtab, E.A.F., et al., *Cardiac malformations and myocardial abnormalities inpodoplanin knockout mouse embryos: Correlation with abnormal epicardial development.* Developmental Dynamics, 2008. **237**(3): p. 847-857.

84. Uhrin, P., et al., *Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation.* Blood, 2010. **115**(19): p. 3997-4005.

85. Bianchi, R., et al., *Postnatal Deletion of Podoplanin in Lymphatic Endothelium Results in Blood Filling of the Lymphatic System and Impairs Dendritic Cell Migration to Lymph Nodes.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2017. **37**(1): p. 108-117.

86. Traboulsi, E.I., et al., *Lymphedema-distichiasis syndrome and FOXC2 gene mutation.* Am J Ophthalmol, 2002. **134**(4): p. 592-6.

87. Petrova, T.V., et al., *Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis.* Nat Med, 2004. **10**(9): p. 974-81.

88. Kuijper, S., C.J. Turner, and R.H. Adams, *Regulation of angiogenesis by Eph-ephrin interactions.* Trends Cardiovasc Med, 2007. **17**(5): p. 145-51.

89. Wang, H.U., Z.F. Chen, and D.J. Anderson, *Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4.* Cell, 1998. **93**(5): p. 741-53.

90. Adams, R.H., et al., *Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis.* Genes Dev, 1999. **13**(3): p. 295-306.

91. Makinen, T., *PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature.* Genes & Development, 2005. **19**(3): p. 397-410.

92. Fiedler, U. and H.G. Augustin, *Angiopoietins: a link between angiogenesis and inflammation.* Trends Immunol, 2006. **27**(12): p. 552-8.

93. Schnurch, H. and W. Risau, *Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage.* Development, 1993. **119**(3): p. 957-68.

94. Yancopoulos, G.D., et al., *Vascular-specific growth factors and blood vessel formation.* Nature, 2000. **407**(6801): p. 242-248.

95. Dumont, D.J., et al., *Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo.* Genes Dev, 1994. **8**(16): p. 1897-909.

96. Sato, T.N., et al., *Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation.* Nature, 1995. **376**(6535): p. 70-4.

97. Puri, M.C., et al., *The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells.* EMBO J, 1995. **14**(23): p. 5884-91.

98. Qu, X., et al., *Abnormal embryonic lymphatic vessel development in Tie1 hypomorphic mice.* Development, 2010. **137**(8): p. 1285-95.

99. D'Amico, G., et al., *Loss of endothelial Tie1 receptor impairs lymphatic vessel development-brief report.* Arterioscler Thromb Vasc Biol, 2010. **30**(2): p. 207-9.

100. Shen, B., et al., *Genetic dissection of tie pathway in mouse lymphatic maturation and valve development.* Arterioscler Thromb Vasc Biol, 2014. **34**(6): p. 1221-30.

101. Suri, C., et al., *Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis.* Cell, 1996. **87**(7): p. 1171-80.

102. Suri, C., et al., *Increased vascularization in mice overexpressing angiopoietin-1.* Science, 1998. **282**(5388): p. 468-71.

103. Maisonpierre, P.C., et al., *Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis.* Science, 1997. **277**(5322): p. 55-60.

104. Gale, N.W., et al., *Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1.* Dev Cell, 2002. **3**(3): p. 411-23.

105. Zheng, W., et al., *Angiopoietin 2 regulates the transformation and integrity of lymphatic endothelial cell junctions.* Genes Dev, 2014. **28**(14): p. 1592-603.

106. Kazenwadel, J., et al., *GATA2 is required for lymphatic vessel valve development and maintenance.* J Clin Invest, 2015. **125**(8): p. 2979-94.

107. Yao, L.C., et al., *Plasticity of button-like junctions in the endothelium of airway lymphatics in development and inflammation.* Am J Pathol, 2012. **180**(6): p. 2561-75.

108. Danussi, C., et al., *EMILIN1/alpha9beta1 integrin interaction is crucial in lymphatic valve formation and maintenance.* Mol Cell Biol, 2013. **33**(22): p. 4381-94.

109. Bazigou, E., J.T. Wilson, and J.E. Moore, Jr., *Primary and secondary lymphatic valve development: molecular, functional and mechanical insights.* Microvasc Res, 2014. **96**: p. 38-45.

110. Pflicke, H. and M. Sixt, *Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels.* J Exp Med, 2009. **206**(13): p. 2925-35.

111. Kampmeier, O.F., *The genetic history of the valves in the lymphatic system of man.* American Journal of Anatomy, 1928. **40**(3): p. 413-457.

112. Mansour, S., et al., *Emberger syndrome-primary lymphedema with myelodysplasia: report of seven new cases.* Am J Med Genet A, 2010. **152A**(9): p. 2287-96.

113. Ostergaard, P., et al., *Mutations in GATA2 cause primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome).* Nat Genet, 2011. **43**(10): p. 929-31.

114. Kazenwadel, J., et al., *Loss-of-function germline GATA2 mutations in patients with MDS/AML or MonoMAC syndrome and primary lymphedema reveal a key role for GATA2 in the lymphatic vasculature.* Blood, 2012. **119**(5): p. 1283-91.

115. Meens, M.J., et al., *Connexins in lymphatic vessel physiology and disease.* FEBS Lett, 2014. **588**(8): p. 1271-7.

116. Kanady, J.D., et al., *Connexin37 and Connexin43 deficiencies in mice disrupt lymphatic valve development and result in lymphatic disorders including lymphedema and chylothorax.* Dev Biol, 2011. **354**(2): p. 253-66.

117. Bazigou, E., et al., *Integrin-alpha9 is required for fibronectin matrix assembly during lymphatic valve morphogenesis.* Dev Cell, 2009. **17**(2): p. 175-86.

118. Tatin, F., et al., *Planar cell polarity protein Celsr1 regulates endothelial adherens junctions and directed cell rearrangements during valve morphogenesis.* Dev Cell, 2013. **26**(1): p. 31-44.

119. Betterman, K.L., et al., *Atypical cadherin FAT4 orchestrates lymphatic endothelial cell polarity in response to flow.* Journal of Clinical Investigation, 2020. **130**(6): p. 3315-3328.

120. Wang, Y.D., et al., *Syndecan 4 controls lymphatic vasculature remodeling during mouse embryonic development.* Development, 2016. **143**(23): p. 4441-4451.

121. Bouvree, K., et al., *Semaphorin3A, Neuropilin-1, and PlexinA1 are required for lymphatic valve formation.* Circ Res, 2012. **111**(4): p. 437-45.

122. Zhang, G., et al., *EphB4 forward signalling regulates lymphatic valve development.* Nat Commun, 2015. **6**: p. 6625.

123. Kim, H. and G.Y. Koh, *Platelets take the lead in lymphatic separation.* Circ Res, 2010. **106**(7): p. 1184-6.

124. Hess, P.R., et al., *Platelets mediate lymphovenous hemostasis to maintain blood-lymphatic separation throughout life.* J Clin Invest, 2014. **124**(1): p. 273-84.

125. Carramolino, L., et al., *Platelets play an essential role in separating the blood and lymphatic vasculatures during embryonic angiogenesis.* Circ Res, 2010. **106**(7): p. 1197-201.

126. Abtahian, F., et al., *Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk.* Science, 2003. **299**(5604): p. 247-51.

127. Sebzda, E., et al., *Syk and Slp-76 mutant mice reveal a cell-autonomous hematopoietic cell contribution to vascular development.* Dev Cell, 2006. **11**(3): p. 349-61.

128. McDevitt, M.A., et al., *An upstream, DNase I hypersensitive region of the hematopoietic-expressed transcription factor GATA-1 gene confers developmental specificity in transgenic mice.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 7976-81.

129. Finney, B.A., et al., *CLEC-2 and Syk in the megakaryocytic/platelet lineage are essential for development.* Blood, 2012. **119**(7): p. 1747-56.

130. Suzuki-Inoue, K., et al., *A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2.* Blood, 2006. **107**(2): p. 542-9.

131. Suzuki-Inoue, K., et al., *Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets.* J Biol Chem, 2010. **285**(32): p. 24494-507.

132. Mazharian, A., et al., *Critical role of Src-Syk-PLC{gamma}2 signaling in megakaryocyte migration and thrombopoiesis.* Blood, 2010. **116**(5): p. 793-800.

133. Ichise, H., et al., *Phospholipase Cgamma2 is necessary for separation of blood and lymphatic vasculature in mice.* Development, 2009. **136**(2): p. 191-5.

134. Pan, Y. and L. Xia, *Emerging roles of podoplanin in vascular development and homeostasis.* Front Med, 2015. **9**(4): p. 421-30.

135. Fu, J., et al., *Endothelial cell O-glycan deficiency causes blood/lymphatic misconnections and consequent fatty liver disease in mice.* Journal of Clinical Investigation, 2008. **118**(11): p. 3725-3737.

136. James, J.M., A. Nalbandian, and Y.S. Mukouyama, *TGF  signaling is required for sprouting lymphangiogenesis during lymphatic network development in the skin.* Development, 2013. **140**(18): p. 3903-3914.

137. Turner, C.J., et al., *Integrin-alpha5beta1 is not required for mural cell functions during development of blood vessels but is required for lymphatic-blood vessel separation and lymphovenous valve formation.* Dev Biol, 2014. **392**(2): p. 381-92.

138. D'Amico, G., et al., *Regulation of lymphatic-blood vessel separation by endothelial Rac1.* Development, 2009. **136**(23): p. 4043-53.

139. Taniguchi, K., et al., *Spreds are essential for embryonic lymphangiogenesis by regulating vascular endothelial growth factor receptor 3 signaling.* Mol Cell Biol, 2007. **27**(12): p. 4541-50.

140. Sinha, S. and W. Yang, *Cellular signaling for activation of Rho GTPase Cdc42.* Cell Signal, 2008. **20**(11): p. 1927-34.

141. Cerione, R.A., *Cdc42: new roads to travel.* Trends Cell Biol, 2004. **14**(3): p. 127-32.

142. Etienne-Manneville, S. and A. Hall, *Rho GTPases in cell biology.* Nature, 2002. **420**(6916): p. 629-35.

143. Kozma, R., et al., *The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts.* Mol Cell Biol, 1995. **15**(4): p. 1942-52.

144. Melendez, J., M. Grogg, and Y. Zheng, *Signaling role of Cdc42 in regulating mammalian physiology.* J Biol Chem, 2011. **286**(4): p. 2375-81.

145. Nobes, C.D. and A. Hall, *Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.* Cell, 1995. **81**(1): p. 53-62.

146. Maldonado, M.D.M. and S. Dharmawardhane, *Targeting Rac and Cdc42 GTPases in Cancer.* Cancer Res, 2018. **78**(12): p. 3101-3111.

147. Martinelli, S., et al., *Functional Dysregulation of CDC42 Causes Diverse Developmental Phenotypes.* Am J Hum Genet, 2018. **102**(2): p. 309-320.

148. Motokawa, M., et al., *A hot-spot mutation in CDC42 (p.Tyr64Cys) and novel phenotypes in the third patient with Takenouchi-Kosaki syndrome.* J Hum Genet, 2018. **63**(3): p. 387-390.

149. Szczawinska-Poplonyk, A., et al., *A Novel CDC42 Mutation in an 11-Year Old Child Manifesting as Syndromic Immunodeficiency, Autoinflammation,*

*Hemophagocytic Lymphohistiocytosis, and Malignancy: A Case Report.* Front Immunol, 2020. **11**: p. 318.

150. Takenouchi, T., et al., *Further evidence of a mutation in CDC42 as a cause of a recognizable syndromic form of thrombocytopenia.* Am J Med Genet A, 2016. **170A**(4): p. 852-5.

151. Johnson, D.I. and J.R. Pringle, *Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity.* J Cell Biol, 1990. **111**(1): p. 143-52.

152. Shinjo, K., et al., *Molecular cloning of the gene for the human placental GTP-binding protein Gp (G25K): identification of this GTP-binding protein as the human homolog of the yeast cell-division-cycle protein CDC42.* Proc Natl Acad Sci U S A, 1990. **87**(24): p. 9853-7.

153. Drubin, D.G., *Development of cell polarity in budding yeast.* Cell, 1991. **65**(7): p. 1093-6.

154. Mattila, P.K. and P. Lappalainen, *Filopodia: molecular architecture and cellular functions.* Nat Rev Mol Cell Biol, 2008. **9**(6): p. 446-54.

155. Ridley, A.J. and A. Hall, *The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors.* Cell, 1992. **70**(3): p. 389-99.

156. Ridley, A.J., et al., *The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.* Cell, 1992. **70**(3): p. 401-10.

157. Faix, J. and K. Rottner, *The making of filopodia.* Curr Opin Cell Biol, 2006. **18**(1): p. 18-25.

158. Miki, H., et al., *Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP.* Nature, 1998. **391**(6662): p. 93-6.

159. Rohatgi, R., et al., *The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly.* Cell, 1999. **97**(2): p. 221-31.

160. Prehoda, K.E., et al., *Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex.* Science, 2000. **290**(5492): p. 801-6.

161. Lommel, S., et al., *Actin pedestal formation by enteropathogenic Escherichia coli and intracellular motility of Shigella flexneri are abolished in N-WASP-defective cells.* EMBO Rep, 2001. **2**(9): p. 850-7.

162. Snapper, S.B., et al., *N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility.* Nat Cell Biol, 2001. **3**(10): p. 897-904.

163. Scita, G., et al., *IRSp53: crossing the road of membrane and actin dynamics in the formation of membrane protrusions.* Trends Cell Biol, 2008. **18**(2): p. 52-60.

164. Krugmann, S., et al., *Cdc42 induces filopodia by promoting the formation of an IRSp53:Mena complex.* Curr Biol, 2001. **11**(21): p. 1645-55.

165. Nakagawa, H., et al., *IRSp53 is colocalised with WAVE2 at the tips of protruding lamellipodia and filopodia independently of Mena.* J Cell Sci, 2003. **116**(Pt 12): p. 2577-83.

166. Goh, W.I., et al., *mDia1 and WAVE2 proteins interact directly with IRSp53 in filopodia and are involved in filopodium formation.* J Biol Chem, 2012. **287**(7): p. 4702-14.

167. Li, J., et al., *Essential role of Cdc42 in cardiomyocyte proliferation and cell-cell adhesion during heart development.* Dev Biol, 2017. **421**(2): p. 271-283.

168. Maillet, M., et al., *Cdc42 is an antihypertrophic molecular switch in the mouse heart.* J Clin Invest, 2009. **119**(10): p. 3079-88.

169. Barry, D.M., et al., *Cdc42 is required for cytoskeletal support of endothelial cell adhesion during blood vessel formation in mice.* Development, 2015. **142**(17): p. 3058-70.

170. Jin, Y., et al., *Deletion of Cdc42 enhances ADAM17-mediated vascular endothelial growth factor receptor 2 shedding and impairs vascular endothelial cell survival and vasculogenesis.* Mol Cell Biol, 2013. **33**(21): p. 4181-97.

171. Castro, M., et al., *CDC42 Deletion Elicits Cerebral Vascular Malformations via Increased MEKK3-Dependent KLF4 Expression.* Circ Res, 2019. **124**(8): p. 1240-1252.

172. Sakabe, M., et al., *YAP/TAZ-CDC42 signaling regulates vascular tip cell migration.* Proc Natl Acad Sci U S A, 2017. **114**(41): p. 10918-10923.

173. Bauer, M.S., et al., *Structural and mechanistic insights into mechanoactivation of focal adhesion kinase.* Proc Natl Acad Sci U S A, 2019. **116**(14): p. 6766-6774.

174. Schaller, M.D., et al., *pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions.* Proc Natl Acad Sci U S A, 1992. **89**(11): p. 5192-6.

175. Hanks, S.K., et al., *Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin.* Proc Natl Acad Sci U S A, 1992. **89**(18): p. 8487-91.

176. Guan, J.L., J.E. Trevithick, and R.O. Hynes, *Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein.* Cell Regul, 1991. **2**(11): p. 951-64.

177. Kornberg, L., et al., *Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase.* J Biol Chem, 1992. **267**(33): p. 23439-42.

178. Lawson, C. and D.D. Schlaepfer, *pHocal adhesion kinase regulation is on a FERM foundation.* J Cell Biol, 2013. **202**(6): p. 833-6.

179. Zhao, X. and J.L. Guan, *Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis.* Adv Drug Deliv Rev, 2011. **63**(8): p. 610-5.

180. Lietha, D., et al., *Structural basis for the autoinhibition of focal adhesion kinase.* Cell, 2007. **129**(6): p. 1177-87.

181. Ceccarelli, D.F., et al., *Crystal structure of the FERM domain of focal adhesion kinase.* J Biol Chem, 2006. **281**(1): p. 252-9.

182. Cooper, L.A., T.L. Shen, and J.L. Guan, *Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction.* Mol Cell Biol, 2003. **23**(22): p. 8030-41.

183. Jacamo, R.O. and E. Rozengurt, *A truncated FAK lacking the FERM domain displays high catalytic activity but retains responsiveness to adhesion-mediated signals.* Biochem Biophys Res Commun, 2005. **334**(4): p. 1299-304.

184. Goni, G.M., et al., *Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes.* Proc Natl Acad Sci U S A, 2014. **111**(31): p. E3177-86.

185. Murphy, J.M., et al., *Targeting focal adhesion kinase in cancer cells and the tumor microenvironment.* Exp Mol Med, 2020. **52**(6): p. 877-886.

186. Frame, M.C., et al., *The FERM domain: organizing the structure and function of FAK.* Nat Rev Mol Cell Biol, 2010. **11**(11): p. 802-14.

187. Lim, S.T., et al., *Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation.* Mol Cell, 2008. **29**(1): p. 9-22.

188. Ossovskaya, V., et al., *FAK nuclear export signal sequences.* FEBS Lett, 2008. **582**(16): p. 2402-6.

189. Prutzman, K.C., et al., *The focal adhesion targeting domain of focal adhesion kinase contains a hinge region that modulates tyrosine 926 phosphorylation.* Structure, 2004. **12**(5): p. 881-91.

190. Tachibana, K., et al., *Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK.* J Exp Med, 1995. **182**(4): p. 1089-99.

191. Liu, G., C.D. Guibao, and J. Zheng, *Structural insight into the mechanisms of targeting and signaling of focal adhesion kinase.* Mol Cell Biol, 2002. **22**(8): p. 2751-60.

192. Hayashi, I., K. Vuori, and R.C. Liddington, *The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin.* Nat Struct Biol, 2002. **9**(2): p. 101-6.

193. Zhai, J., et al., *Direct interaction of focal adhesion kinase with p190RhoGEF.* J Biol Chem, 2003. **278**(27): p. 24865-73.

194. Polte, T.R. and S.K. Hanks, *Complexes of focal adhesion kinase (FAK) and Crk-associated substrate (p130(Cas)) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation. Requirements for Src kinase activity and FAK proline-rich motifs.* J Biol Chem, 1997. **272**(9): p. 5501-9.

195. Cary, L.A., et al., *Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration.* J Cell Biol, 1998. **140**(1): p. 211-21.

196. Hildebrand, J.D., J.M. Taylor, and J.T. Parsons, *An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase.* Mol Cell Biol, 1996. **16**(6): p. 3169-78.

197. Liu, Y., et al., *The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly.* Mol Biol Cell, 2002. **13**(6): p. 2147-56.

198. Schaller, M.D., *Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions.* J Cell Sci, 2010. **123**(Pt 7): p. 1007-13.

199. Yano, H., et al., *Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion.* J Cell Biol, 2004. **166**(2): p. 283-95.

200. Tilghman, R.W., et al., *Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells.* J Cell Sci, 2005. **118**(Pt 12): p. 2613-23.

201. Tomar, A., et al., *A FAK-p120RasGAP-p190RhoGAP complex regulates polarity in migrating cells (vol 122. pg 1852, 2009).* Journal of Cell Science, 2009. **122**(16): p. 3005-3005.

202. Li, S., et al., *The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3546-51.

203. Hu, Y.L., et al., *FAK and paxillin dynamics at focal adhesions in the protrusions of migrating cells.* Sci Rep, 2014. **4**: p. 6024.

204. Owen, K.A., et al., *Regulation of lamellipodial persistence, adhesion turnover, and motility in macrophages by focal adhesion kinase.* J Cell Biol, 2007. **179**(6): p. 1275-87.

205. Chacon, M.R., et al., *Focal adhesion kinase regulates actin nucleation and neuronal filopodia formation during axonal growth.* Development, 2012. **139**(17): p. 3200-10.

206. Iwanicki, M.P., et al., *FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts.* J Cell Sci, 2008. **121**(Pt 6): p. 895-905.

207. Webb, D.J., et al., *FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly.* Nat Cell Biol, 2004. **6**(2): p. 154-61.

208. Ren, X.D., et al., *Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover.* J Cell Sci, 2000. **113 ( Pt 20)**: p. 3673-8.

209. Sieg, D.J., C.R. Hauck, and D.D. Schlaepfer, *Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration.* J Cell Sci, 1999. **112 ( Pt 16)**: p. 2677-91.

210. Illc, D., et al., *Reduced Sell Motility and Enhanced Focal Adhesion Contact Formation in Cells from Fak-Deficient Mice.* Nature, 1995. **377**(6549): p. 539-544.

211. Polte, T.R., A.J. Naftilan, and S.K. Hanks, *Focal adhesion kinase is abundant in developing blood vessels and elevation of its phosphotyrosine content in vascular smooth muscle cells is a rapid response to angiotensin II.* J Cell Biochem, 1994. **55**(1): p. 106-19.

212. Ilic, D., et al., *Focal adhesion kinase is required for blood vessel morphogenesis.* Circ Res, 2003. **92**(3): p. 300-7.

213. Peng, X., et al., *Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice.* Cardiovasc Res, 2004. **64**(3): p. 421-30.

214. Shen, T.-L., et al., *Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis.* The Journal of Cell Biology, 2005. **169**(6): p. 941-952.

215. Braren, R., et al., *Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation.* J Cell Biol, 2006. **172**(1): p. 151-62.

216. Peng, X., et al., *Inactivation of focal adhesion kinase in cardiomyocytes promotes eccentric cardiac hypertrophy and fibrosis in mice.* J Clin Invest, 2006. **116**(1): p. 217-27.

217. Hakim, Z.S., et al., *Conditional deletion of focal adhesion kinase leads to defects in ventricular septation and outflow tract alignment.* Mol Cell Biol, 2007. **27**(15): p. 5352-64.

218. Peng, X., et al., *Cardiac developmental defects and eccentric right ventricular hypertrophy in cardiomyocyte focal adhesion kinase (FAK) conditional knockout mice.* Proc Natl Acad Sci U S A, 2008. **105**(18): p. 6638-43.

219. Zihni, C., et al., *Tight junctions: from simple barriers to multifunctional molecular gates.* Nat Rev Mol Cell Biol, 2016. **17**(9): p. 564-80.

220. Balda, M.S. and K. Matter, *Tight junctions at a glance.* Journal of cell science, 2008. **121**(22): p. 3677-3682.

221. Stevenson, B.R., et al., *Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia.* J Cell Biol, 1986. **103**(3): p. 755-66.

222. Schmidt, A., et al., *Occludin binds to the SH3-hinge-GuK unit of zonula occludens protein 1: potential mechanism of tight junction regulation.* Cell Mol Life Sci, 2004. **61**(11): p. 1354-65.

223. Itoh, M., et al., *Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins.* J Cell Biol, 1999. **147**(6): p. 1351-63.

224. Cohen, C.J., et al., *Multiple regions within the coxsackievirus and adenovirus receptor cytoplasmic domain are required for basolateral sorting.* J Biol Chem, 2001. **276**(27): p. 25392-8.

225. Riazuddin, S., et al., *Tricellulin is a tight-junction protein necessary for hearing.* Am J Hum Genet, 2006. **79**(6): p. 1040-51.

226. Bazzoni, G., et al., *Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin.* J Biol Chem, 2000. **275**(27): p. 20520-6.

227. Furuse, M., et al., *Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions.* J Cell Biol, 1994. **127**(6 Pt 1): p. 1617-26.

228. Ebnet, K., et al., *Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1.* J Biol Chem, 2000. **275**(36): p. 27979-88.

229. Fanning, A.S., et al., *The unique-5 and -6 motifs of ZO-1 regulate tight junction strand localization and scaffolding properties.* Mol Biol Cell, 2007. **18**(3): p. 721-31.

230. Fanning, A.S., T.Y. Ma, and J.M. Anderson, *Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1.* FASEB J, 2002. **16**(13): p. 1835-7.

231. Katsube, T., et al., *Cortactin associates with the cell-cell junction protein ZO-1 in both Drosophila and mouse.* J Biol Chem, 1998. **273**(45): p. 29672-7.

232. Muller, S.L., et al., *The tight junction protein occludin and the adherens junction protein alpha-catenin share a common interaction mechanism with ZO-1.* J Biol Chem, 2005. **280**(5): p. 3747-56.

233. Etournay, R., et al., *Shroom2, a myosin-VIIa- and actin-binding protein, directly interacts with ZO-1 at tight junctions.* J Cell Sci, 2007. **120**(Pt 16): p. 2838-50.

234. Chai, K., et al., *The epithelium-molecular landscaping for an interactive barrier.* J Biomed Biotechnol, 2010. **2010**: p. 870506.

235. Ikenouchi, J., et al., *Requirement of ZO-1 for the formation of belt-like adherens junctions during epithelial cell polarization.* J Cell Biol, 2007. **176**(6): p. 779-86.

236. Itoh, M., et al., *Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments.* J Cell Biol, 1997. **138**(1): p. 181-92.

237. Kausalya, P.J., M. Reichert, and W. Hunziker, *Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells.* FEBS Lett, 2001. **505**(1): p. 92-6.

238. Alitalo, K., *The lymphatic vasculature in disease.* Nat Med, 2011. **17**(11): p. 1371-80.

239. Semo, J., J. Nicenboim, and K. Yaniv, *Development of the lymphatic system: new questions and paradigms.* Development, 2016. **143**(6): p. 924-35.

240. Bridenbaugh, E.A., A.A. Gashev, and D.C. Zawieja, *Lymphatic muscle: a review of contractile function.* Lymphat Res Biol, 2003. **1**(2): p. 147-58.

241. Koltowska, K., et al., *Getting out and about: the emergence and morphogenesis of the vertebrate lymphatic vasculature.* Development, 2013. **140**(9): p. 1857-70.

242. Coso, S., E. Bovay, and T.V. Petrova, *Pressing the right buttons: signaling in lymphangiogenesis.* Blood, 2014. **123**(17): p. 2614-24.

243. Klotz, L., et al., *Cardiac lymphatics are heterogeneous in origin and respond to injury.* Nature, 2015. **522**(7554): p. 62-7.

244. Lioux, G., et al., *A Second Heart Field-Derived Vasculogenic Niche Contributes to Cardiac Lymphatics.* Dev Cell, 2020. **52**(3): p. 350-363 e6.

245. Stanczuk, L., et al., *cKit Lineage Hemogenic Endothelium-Derived Cells Contribute to Mesenteric Lymphatic Vessels.* Cell Rep, 2015. **10**(10): p. 1708-1721.

246. Mahadevan, A., et al., *The left-right Pitx2 pathway drives organ-specific arterial and lymphatic development in the intestine.* Dev Cell, 2014. **31**(6): p. 690-706.

247. Lohela, M., et al., *VEGFs and receptors involved in angiogenesis versus lymphangiogenesis.* Curr Opin Cell Biol, 2009. **21**(2): p. 154-65.

248. Gordon, K., et al., *Mutation in vascular endothelial growth factor-C, a ligand for vascular endothelial growth factor receptor-3, is associated with autosomal dominant milroy-like primary lymphedema.* Circ Res, 2013. **112**(6): p. 956-60.

249. Balboa-Beltran, E., et al., *A novel stop mutation in the vascular endothelial growth factor-C gene (VEGFC) results in Milroy-like disease.* J Med Genet, 2014. **51**(7): p. 475-8.

250. Ridley, A.J., *Rho GTPase signalling in cell migration.* Curr Opin Cell Biol, 2015. **36**: p. 103-12.

251. Takenouchi, T., et al., *Macrothrombocytopenia and developmental delay with a de novo CDC42 mutation: Yet another locus for thrombocytopenia and developmental delay.* Am J Med Genet A, 2015. **167A**(11): p. 2822-5.

252. Tu, S., et al., *Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase.* J Biol Chem, 2003. **278**(49): p. 49293-300.

253. Uehara, T., et al., *Pathogenetic basis of Takenouchi-Kosaki syndrome: Electron microscopy study using platelets in patients and functional studies in a Caenorhabditis elegans model.* Sci Rep, 2019. **9**(1): p. 4418.

254. Alva, J.A., et al., *VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells.* Dev Dyn, 2006. **235**(3): p. 759-67.

255. Katsuno, T., et al., *Deficiency of zonula occludens-1 causes embryonic lethal phenotype associated with defected yolk sac angiogenesis and apoptosis of embryonic cells.* Mol Biol Cell, 2008. **19**(6): p. 2465-75.

256. Tornavaca, O., et al., *ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation.* J Cell Biol, 2015. **208**(6): p. 821-38.

257. Grimsley-Myers, C.M., et al., *VE-cadherin endocytosis controls vascular integrity and patterning during development.* J Cell Biol, 2020. **219**(5).

258. Fonsatti, E., et al., *Emerging role of endoglin (CD105) as a marker of angiogenesis with clinical potential in human malignancies.* Curr Cancer Drug Targets, 2003. **3**(6): p. 427-32.

259. Mouta-Bellum, C., et al., *Organ-specific lymphangiectasia, arrested lymphatic sprouting, and maturation defects resulting from gene-targeting of the PI3K regulatory isoforms p85alpha, p55alpha, and p50alpha.* Dev Dyn, 2009. **238**(10): p. 2670-9.

260. Huang, L.H., K.J. Lavine, and G.J. Randolph, *Cardiac Lymphatic Vessels, Transport, and Healing of the Infarcted Heart.* JACC Basic Transl Sci, 2017. **2**(4): p. 477-483.

261. Paupert, J., N.E. Sounni, and A. Noel, *Lymphangiogenesis in post-natal tissue remodeling: lymphatic endothelial cell connection with its environment.* Mol Aspects Med, 2011. **32**(2): p. 146-58.

262. De Smet, F., et al., *Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way.* Arterioscler Thromb Vasc Biol, 2009. **29**(5): p. 639-49.

263. Gadea, G., et al., *TNFalpha induces sequential activation of Cdc42- and p38/p53-dependent pathways that antagonistically regulate filopodia formation.* J Cell Sci, 2004. **117**(Pt 26): p. 6355-64.

264. Oh, S.Y., et al., *The type III TGFbeta receptor regulates filopodia formation via a Cdc42-mediated IRSp53-N-WASP interaction in epithelial cells.* Biochem J, 2013. **454**(1): p. 79-89.

265. Kouklis, P., M. Konstantoulaki, and A.B. Malik, *VE-cadherin-induced Cdc42 signaling regulates formation of membrane protrusions in endothelial cells.* J Biol Chem, 2003. **278**(18): p. 16230-6.

266. Czuchra, A., et al., *Cdc42 is not essential for filopodium formation, directed migration, cell polarization, and mitosis in fibroblastoid cells.* Mol Biol Cell, 2005. **16**(10): p. 4473-84.

267. Lin, D., et al., *A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity.* Nat Cell Biol, 2000. **2**(8): p. 540-7.

268. Nakayama, M., et al., *Spatial regulation of VEGF receptor endocytosis in angiogenesis.* Nat Cell Biol, 2013. **15**(3): p. 249-60.

269. Amack, J.D. and M.L. Manning, *Knowing the boundaries: extending the differential adhesion hypothesis in embryonic cell sorting.* Science, 2012. **338**(6104): p. 212-5.

270. Yang, Y., et al., *VE-Cadherin Is Required for Lymphatic Valve Formation and Maintenance.* Cell Rep, 2019. **28**(9): p. 2397-2412 e4.

271. Dejana, E., *Endothelial cell-cell junctions: happy together.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 261-70.

272. Gerhardt, H. and C. Betsholtz, *Endothelial-pericyte interactions in angiogenesis.* Cell Tissue Res, 2003. **314**(1): p. 15-23.

273. Huo, L., et al., *Cdc42-dependent formation of the ZO-1/MRCKbeta complex at the leading edge controls cell migration.* EMBO J, 2011. **30**(4): p. 665-78.

274. Oliver, G., et al., *The Lymphatic Vasculature in the 21(st) Century: Novel Functional Roles in Homeostasis and Disease.* Cell, 2020. **182**(2): p. 270-296.

275. Jurisic, G. and M. Detmar, *Lymphatic endothelium in health and disease.* Cell Tissue Res, 2009. **335**(1): p. 97-108.

276. Schulte-Merker, S., A. Sabine, and T.V. Petrova, *Lymphatic vascular morphogenesis in development, physiology, and disease.* J Cell Biol, 2011. **193**(4): p. 607-18.

277. Yang, Y. and G. Oliver, *Development of the mammalian lymphatic vasculature.* Journal of Clinical Investigation, 2014. **124**(3): p. 888-897.

278. Wigle, J.T., et al., *Prox1 function is crucial for mouse lens-fibre elongation.* Nat Genet, 1999. **21**(3): p. 318-22.

279. Sweet, D.T., et al., *Lymph flow regulates collecting lymphatic vessel maturation in vivo.* J Clin Invest, 2015. **125**(8): p. 2995-3007.

280. Parsons, J.T., *Focal adhesion kinase: the first ten years.* J Cell Sci, 2003. **116**(Pt 8): p. 1409-16.

281. Sulzmaier, F.J., C. Jean, and D.D. Schlaepfer, *FAK in cancer: mechanistic findings and clinical applications.* Nat Rev Cancer, 2014. **14**(9): p. 598-610.

282. Guan, J.L., *Role of focal adhesion kinase in integrin signaling.* Int J Biochem Cell Biol, 1997. **29**(8-9): p. 1085-96.

283. Abedi, H. and I. Zachary, *Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells.* J Biol Chem, 1997. **272**(24): p. 15442-51.

284. Zhao, X., et al., *Role of kinase-independent and -dependent functions of FAK in endothelial cell survival and barrier function during embryonic development.* J Cell Biol, 2010. **189**(6): p. 955-65.

285. Petit, V., et al., *Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells.* J Cell Biol, 2000. **148**(5): p. 957-70.

286. Serrels, B., et al., *Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex.* Nat Cell Biol, 2007. **9**(9): p. 1046-56.

287. Sleigh, B.C. and B. Manna, *Lymphedema*, in *StatPearls*. 2021: Treasure Island (FL).