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Buttons and Zippers: Endothelial Junctions in Lymphatic Vessels

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Abstract

Button-like junctions are discontinuous contacts at the border of oak-leaf-shaped endothelial cells of initial lymphatic vessels. These junctions are distinctively different from continuous zipper-like junctions that create the endothelial barrier in collecting lymphatics and blood vessels. Button junctions are point contacts, spaced about 3 μm apart, that border valve-like openings where fluid and immune cells enter lymphatics. In intestinal villi, openings between button junctions in lacteals also serve as entry routes for chylomicrons. Like zipper junctions that join endothelial cells, buttons consist of adherens junction proteins (VE-cadherin) and tight junction proteins (claudin-5, occludin, and others). Buttons in lymphatics form from zipper junctions during embryonic development, can convert into zippers in disease or after experimental genetic or pharmacological manipulation, and can revert back to buttons with treatment. Multiple signaling pathways and local microenvironmental factors have been found to contribute to button junction plasticity and could serve as therapeutic targets in pathological conditions ranging from pulmonary edema to obesity.

Button-like junctions, also called button junctions, are discontinuous intercellular contacts between endothelial cells of the initial region of lymphatic vessels in most organs (Baluk et al. 2007). Button junctions border valve-like openings that serve as routes for interstitial fluid and cells to enter initial lymphatics (lymphatic capillaries). The openings between buttons are positioned upstream to intraluminal valves in collecting lymphatics that prevent retrograde lymph flow. In the intestine, initial lymphatics are the site of entry of chylomicrons that carry dietary lipids to the venous bloodstream. Uniquely located in initial lymphatics, button junctions, and the openings between them, differ from continuous zipper-like junctions (zipper junctions) that seal the endothelium of collecting lymphatics, larger lymphatics, and blood vessels and form the transendothelial permeability barrier (Zhang et al. 2020; Norden and Kume 2021). Button junctions have not been described in other regions of lymphatics or in blood vessels.

This review develops a historical context for what has been learned about the formation, plasticity, and function of button junctions in lymphatics to build on and complement

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excellent recent reviews on junctions of lymphatic endothelial cells (Zhang et al. 2020; Norden and Kume 2021). The topic has a foundation of solid historical evidence for fluid entering lymphatics through a process coupled to mechanical forces in surrounding tissues. However, the nature of the path taken from interstitium into the lymphatic lumen has been debated over many years.

There is no dispute that lymph flows along a hydrostatic pressure gradient from initial lymphatics to collecting lymphatics, where it is propelled forward by smooth muscle contraction and mechanical forces in surrounding tissues and is prevented from flowing backward by intraluminal valves. The understanding of lymphatic valve development, function, and maintenance has benefitted from advancements described in excellent reviews (Schmid-Schonbein 2003; Muthuchamy and Zawieja 2008; Bazigou et al. 2014; Sabine and Petrova 2014; Vittet 2014; Bernier-Latmani and Petrova 2017; Iyer et al. 2020; Norden and Kume 2020; Petrova and Koh 2020; Francois et al. 2021; González-Loyola and Petrova 2021). By comparison, the process of fluid and cell entry into lymphatics has a more complicated history, where mechanisms have been proposed and adopted, only to be reinterpreted or invalidated by further evidence. The purpose of this review is to consider the evolution of understanding of cellular mechanisms underlying fluid and cell entry into lymphatics and how the discovery of button junctions in initial lymphatics has helped reconcile earlier concepts.

EVOLUTION OF UNDERSTANDING OF LYMPHATIC JUNCTIONS AND VALVES

We begin with historical developments that led to the current understanding of lymphatic junctions and valves. This history is often overlooked in modern texts that lose sight of original observations and concepts that are rediscovered. This history also provides a reminder that discoveries and interpretations reflect the techniques and knowledge of the time and are reinterpreted with continued advances. Throughout the process, scientific controversies and personality conflicts were no less heated historically than today.

Advances in the Seventeenth Century

The discovery of buttons and zipper junctions reflects an evolution built on the work of many. The story of the rediscovery of intestinal lymphatics by Gaspare Aselli in his experiments in 1622 on fed or fasting dogs is well documented (Mayerson 1969; Barrowman and Tso 1989; Suy et al. 2016, 2017). Although it is unclear who first recognized the specialization of initial lymphatics for fluid entry, the discovery of the intraluminal valves is attributed to at least four candidates. Aselli may well have observed lymphatic valves but did not describe them in his famous posthumously published treatise (Aselli 1627). In 1653 in Uppsala, Sweden, Olof Rudbeck clearly described valves (*valvula* in Latin) in lymphatics of the liver in his treatise entitled *Nova Excercitatio Anatomica*, translated into English (Nielsen et al. 1942), but Rudbeck depicted lymphatic vessels in his drawings as smooth tubes and did not illustrate valves.

The first accurate drawings of lymphatic valves were made a decade later by two colleagues at the University of Leiden in the Dutch Republic, which was at that time a hothouse of enlightened scientific experimentation. Jan Swammerdam was a 24-year-old medical student when he used fine tubes and ligatures to inflate lymphatics on June 22, 1664, to make the valves visible (Fig. 1A). He described and illustrated the results in a letter to friends, but they were not published until he completed his PhD thesis (Blasius 1666; Suy et al. 2017). Meanwhile, his friend Frederik Ruysch described very similar results in a short treatise in 1665 (Fig. 1B; Ruysch 1665). Multiple fascinating and sometimes conflicting accounts of this early history have been reported (Mayerson 1969; Barrowman and Tso 1989; Ijpma and van Gulik 2013; Suy et al. 2016, 2017; Irschick et al. 2019). The understanding reflected in these early reports has been extensively updated since then through careful study of the development, structure, and function of intraluminal valves (Bazigou et al. 2014; Petrova and Koh 2020; Francois et al. 2021; González-Loyola and Petrova 2021).

Advances in the Eighteenth and Nineteenth Centuries

In the eighteenth century, William Hunter, using tracer substances to visualize the lymphatics, developed the concept that lacteals and other lymphatics absorb fluid and return it to the blood system (Barrowman and Tso 1989). At the end of the nineteenth century, Ernest Starling and William Bayliss formulated equations of fluid transport from blood capillaries to the interstitial space and uptake by lymphatics, based on transendothelial hydrostatic pressure, osmotic pressure, and permeability (Starling 1894). This was in opposition to the view prevalent at that time that lymph formation involved active secretion (Fine 2014).

The concept of endothelial permeability invokes the barrier function of endothelial cells. Although the concept of intercellular junctions did not become formalized until the advent of transmission electron microscopy (TEM) in the 1950s and 1960s, the size and shape of individual lymphatic endothelial cells was well known in the mid-nineteenth century. Friedrich von Recklinghausen developed an empirical method using silver nitrate to stain endothelial cell borders, designated cement lines (von Recklinghausen 1862; Stadtmüller 1920). This method worked extraordinarily well for lymphatics and for blood vessels and gave results uncannily prescient of modern immunohistochemistry for junctional proteins, which was not developed until a century later. Von Recklinghausen correctly demonstrated that lymphatic vessels are composed of individual endothelial cells—in opposition to the concept of cellular syncytia prevailing at the time. However, he incorrectly concluded that the tips of initial lymphatics are open to the interstitial space. Nonetheless, the peculiar oak-leaf shape of endothelial cells was recognized in initial lymphatics, which were considered specialized for uptake of fluid and cells (Fig. 1C; von Recklinghausen 1862; Casparis 1918), but the significance of the distinctive oak-leaf shape was not linked to focal openings for fluid entry until more than 300 years after the discovery of intraluminal valves.

Advances in the Twentieth Century to Present

In the 1930s, Stephen Hudack and Philip McMaster explored the permeability of lymphatics and introduced the mouse ear and other models still in use today (Hudack and McMaster 1932; McMaster and Hudack 1932). They used vital dyes and particulate tracers to show

that, under baseline conditions, initial lymphatics are permeable to diffusible dyes, but not to particulates such as colloidal carbon (India ink) and concluded that the wall of lymphatics acts as a semipermeable membrane. However, they found that lymphatic permeability increased in inflammation resulting from mechanical, thermal, or chemical injury, and that this increase preceded the appearance of edema (McMaster and Hudack 1932). They also reported that lymphatics proliferated in sustained inflammation through a process now called lymphangiogenesis, and that lymphatics were spontaneously contractile in some tissues but not in others (Pullinger and Florey 1937).

Howard Florey, who received the Nobel Prize in Physiology or Medicine in 1945 for his work on penicillin, used TEM to examine cement lines visible after silver nitrate staining and found that the lines were up to 50 times wider than gaps between endothelial cells (Florey et al. 1959). Florey concluded that the lines represent more likely the overlap of adjacent endothelial cells than gaps between cells (Florey et al. 1959). Florey also suggested that the borders of endothelial cells have special cytochemical properties because similar lines were evident after highly negatively charged sodium indigotetrasulfonate, heparin, indigo-carmine, dextran sulfate, or other highly sulfonated dyes, followed by Azure II or other basic dye (Florey et al. 1959). Some years later, using TEM, Leak (1986) found lymphatic endothelial cell borders to have a high density of anionic sites that bind cationized ferritin particles. Subsequently, silver staining was found to be unusually wide at the tip of the scalloped borders of oak-leaf-shaped lymphatic endothelial cells and clearly different from the continuous borders of endothelial cells in collecting lymphatics (Zöltzer 2003).

In the second half of the twentieth century, openings between endothelial cells were described in initial lymphatics examined by TEM (Palay and Karlin 1959a; Casley-Smith 1965; Leak 1971). At that time, the nature of the route into lymphatics was hotly debated, but the striking TEM findings fit the view that intercellular openings were routes for entry of fluid, tracer particles, chylomicrons, and cells.

However, John Casley-Smith presciently considered four separate routes across the endothelial barrier, supported by evidence from TEM studies of ferritin, colloidal carbon, and thorium dioxide used as electron-dense tracers (Casley-Smith 1965). The first route was intercellular, where substances could pass through open junctions between endothelial cells, which were considered the main transit route in inflammation (Casley-Smith 1965). The other three routes were transendothelial pores, transcytosis of cellular organelles, and transporters that actively move substances into and across cells. Each route had its evidence and advocates, then and now, and was considered to subserve different functions (Leak and Burke 1968a; Azzali 2007; Triacca et al. 2017).

Fluid, cells, and chylomicrons were thought to enter lymphatics through openings between endothelial cells, while cholesterol and insulin entered by receptor-mediated transport (Lim et al. 2013; Yazdani et al. 2019). Caveolae and vesicles visible by TEM could participate in transport across lymphatic endothelial cells, but unlike openings at intercellular junctions, the distribution of tracers in vesicles did not change in inflammation, arguing against the involvement of transcytosis (French et al. 1960; Leak 1971; Azzali 2007). TEM studies also revealed that initial lymphatics had a thin or discontinuous basement membrane and lacked

smooth muscle cells that could interfere with entry of substances (Palay and Karlin 1959b; French et al. 1960; Casley-Smith and Florey 1961; Leak 1970).

TEM studies by Lee Leak supported the presence of open intercellular junctions in initial lymphatics and led to three-dimensional renderings of lymphatic endothelial cells under baseline conditions and when lymphatic endothelial cells were pulled apart by anchoring filaments tensioned by tissue swelling, enabling fluid entry (Fig. 2A,B; Leak and Burke 1968b; Leak 1970; Majno and Joris 1996). While the diagrams are visually attractive, they raised questions of mechanisms and feasibility of endothelial cells separating completely from one another and then reattaching to restore barrier function.

By using scanning electron microscopy (SEM) to observe the luminal surface of lymphatics, Anton Castenholz found that initial lymphatic endothelial cells had open junctions and overlapping endothelial cell borders that could function as valves (Fig. 2C; Castenholz 1984, 1987; Zöltzer 2003; Baluk et al. 2007). Complementary SEM views of the abluminal surface of lymphatics were obtained using a method introduced by Ushiki (1990) for removing extracellular connective tissue by collagenase digestion and alkali hydrolysis, but questions remained about the mechanism of fluid entry.

ANCHORING FILAMENTS OF LYMPHATICS

Anchoring filaments are thought to couple interstitial tension to the lymphatic lumen opening. These filaments were first described in 1935 by Beatrice Pullinger and Howard Florey who reported that lymphatics of the mouse ear were surrounded by a network of collagen and reticular fibers thought to promote lymphatic opening in the presence of edema (Pullinger and Florey 1935). Their work revealed anchoring filaments that attached the abluminal edges of lymphatic endothelial cells to the connective tissue matrix. They concluded that anchoring filaments open lymphatics as interstitial tension increases. Decades later, Lee Leak and John Burke described anchoring filaments viewed by TEM as 10-nm elastin-like filaments that connect regions near lymphatic endothelial cell junctions to the surrounding connective tissue (Fig. 2A,B; Leak and Burke 1968b).

Although further progress has been limited, the main protein component of anchoring filaments was reported to be fibrillin (Gerli et al. 2000). Fibrillin, which is secreted by lymphatic endothelial cells in culture (Weber et al. 2002), attaches to endothelial cells by integrins a2b1, a3b1, and avb3 and is linked to focal adhesion kinase (FAK) (Gerli et al. 2000; Vainionpää et al. 2007). Consistent with other unresolved issues, based on SEM observations, Castenholz questioned the nature of anchoring filaments described by Lee Leak, and reported instead a broader, more diffuse network of connective tissue filaments around initial lymphatics (Castenholz 1987). Casley-Smith maintained that anchoring filaments did not pull the wall of lymphatics open during normal fluid filling but did so in edema (Casley-Smith 1980).

BASEMENT MEMBRANE OF LYMPHATICS

Based on early TEM observations, endothelial cells of initial lymphatics were reported to have little or no basement membrane (basal lamina) (Palay and Karlin 1959b; French et

al. 1960; Casley-Smith and Florey 1961; Leak and Burke 1968a). However, this view has been revised in light of immunohistochemical evidence of a thin, discontinuous basement membrane, which—like that of blood vessels—contains type IV collagen, laminins, perlecan, nidogen, among other proteins (Vainionpää et al. 2007; Pflicke and Sixt 2009). The basement membrane is thought to contribute to binding and entry of immune cells into initial lymphatics without limiting fluid entry (Vainionpää et al. 2007; Pflicke and Sixt 2009).

CONCEPT OF PRIMARY AND SECONDARY VALVES

Ultrastructural observations together with functional and bioengineering considerations led Geert Schmid-Schönbein's group to advance the concept of primary and secondary valves in lymphatics (Trzewik et al. 2001; Mendoza and Schmid-Schönbein 2003; Lynch et al. 2007; Murfee et al. 2007). Accordingly, lymph entering through flap-like primary valves in initial lymphatics is propelled forward by smooth muscle contraction and mechanical forces in surrounding tissues and is prevented from flowing backward by secondary valves in collecting lymphatics (Fig. 2D). Microspheres up to 0.8 μm in diameter could enter lymphatics in their experiments (Lynch et al. 2007). Still unresolved, however, was the cellular mechanism underlying primary valves that enabled entry between lymphatic endothelial cells.

The mystery of primary valve openings began to be solved by findings of loosely overlapping flaps at scalloped endothelial cell borders visible when the abluminal surface of initial lymphatics was viewed in three dimensions by high-resolution field-emission SEM (Fig. 3A,B; Baluk et al. 2007). This finding fits with earlier evidence of oak-leaf-shaped endothelial cells and the presumptive entry route between endothelial cells based on TEM observations. Yet, still missing was a mechanism that reconciled the primary valve concept with the known structure of junctions between lymphatic endothelial cells.

JUNCTIONS BETWEEN LYMPHATIC ENDOTHELIAL CELLS

Endothelial cell junctions were initially identified and characterized by TEM (Brightman and Reese 1969; Simionescu et al. 1975; Pinto da Silva and Kachar 1982), but have acquired much greater functional significance through step-by-step characterization of the proteins of tight junctions and adherens junctions (Tsukita and Tsukita 1989; Lampugnani et al. 1993). Although the identification process began with proteins of desmosomes and then adherens junctions and tight junctions between epithelial cells (Tsukita and Tsukita 1985, 1989; Franke 2009), similar junctional proteins were subsequently found in endothelial cells of blood vessels and then lymphatics (Dejana 2004; Pfeiffer et al. 2008; Dejana et al. 2009; Duong and Vestweber 2020).

In endothelial cells, VE-cadherin is the principal adherens junction protein, and claudin-5, occludin, endothelial selective adhesion molecule (ESAM), and junctional adhesion molecule 1 (JAM1, JAM-A) are the best-studied tight junction proteins, but other claudins and JAMs have also been found (Claesson-Welsh et al. 2021). Gap junction proteins—connexins 37, 40, and 43—mediate electrical and chemical coupling of endothelial cells

but do not directly contribute to barrier function (Meens et al. 2017; Okamoto et al. 2019). Endothelial barrier function is governed by the interaction of proteins of tight junctions and adherens junctions with vascular endothelial tyrosine phosphatase (VE-PTP), zonula occludens-1 (ZO1), other cytoplasmic signaling molecules, and links to the actomyosin cytoskeleton (Orsenigo et al. 2012; Arif et al. 2021; Claesson-Welsh et al. 2021; Vestweber 2021).

Technical developments in creating reporter and mutant mice and dissecting the regulation of junctional proteins are advancing the understanding of endothelial junctions and barrier function in lymphatics (Zhang et al. 2020; Geng et al. 2021; Norden and Kume 2021; Stritt et al. 2021). Gene expression assessments of lymphatic endothelial cells by single-cell RNA sequencing have revealed clear differences among initial lymphatics, collecting lymphatics, and other lymphatic phenotypes in the mesentery (González-Loyola et al. 2021), skin (Hernández Vásquez et al. 2021), and lymph nodes (Takeda et al. 2019; Fujimoto et al. 2020; Xiang et al. 2020; Zhang et al. 2020; Norden and Kume 2021; Sibler et al. 2021), but links between gene-expression profiles and formation of regionally specific intercellular junctions remain to be identified.

BUTTON JUNCTIONS OF INITIAL LYMPHATICS

Structure and Composition

The realization that endothelial cells of initial lymphatics have distinctive intercellular junctions came initially from the discontinuous pattern of VE-cadherin staining in lymphatics of the mouse trachea (Fig. 4A,B; Baluk et al. 2007). The trachea proved well suited for this type of study because blood vessels and lymphatics in tissue whole mounts could be examined in three dimensions by confocal microscopy, which circumvented the limitation of interpreting thin histological sections (Baluk et al. 2005, 2007). Similar discontinuous VE-cadherin staining was found also in initial lymphatics of skin, diaphragm, urinary bladder, and intestinal villi (Baluk et al. 2007; Zheng et al. 2014). Tight junction proteins claudin-5, ESAM, and JAM1 had the same discontinuous distribution as VE-cadherin at the sides of overlapping flaps along the scalloped border of endothelial cells (Fig. 4C). In contrast, PECAM1 (platelet/endothelial cell-adhesion molecule 1, CD31) and LYVE1 were located at the tip of the flaps (Fig. 4C,D; Baluk et al. 2007).

The discontinuous pattern of junctional proteins in initial lymphatics was conspicuously different from continuous junctions of collecting lymphatics (Fig. 5A,B; Baluk et al. 2007). Unlike continuous junctions, the junctions in initial lymphatics consisted of roughly parallel linear segments of junctional proteins, about 3 μm in length and 3 μm apart at the sides of flaps along the border of oak-leaf-shaped endothelial cells (Fig. 5C,D). As initial lymphatics joined collecting lymphatics, the junctions transitioned from discontinuous to continuous (Fig. 5E; Baluk et al. 2007).

Discontinuous junctions in initial lymphatics were named “button junctions” because the focal contacts border openings between adjacent cells, analogous to buttons on a shirt. Consistent with the concept of primary valves, the region between buttons had no junctions to retard entry of fluid, cells, or particles the size of chylomicrons (Fig. 4D; Baluk et al.

2007). The presence of button junctions at the border of intercellular openings into initial lymphatics enabled flow to change with local physiological conditions without junction detachment and resealing.

Cell Entry between Button Junctions

Evidence of cell entry into lymphatics came initially from a remarkable series of real-time observations performed during the early twentieth century on living amphibian tail and rabbit ear preparations. Eliot and Eleanor Clark, who described many features of living blood vessels and lymphatics, reported that blood cells could enter or leave through holes in lymphatics (Clark and Clark 1932, 1933, 1935).

Later immunohistochemical studies of lymphatics in mouse tracheas 24 h after lipopolysaccharide exposure revealed MHC II–positive dendritic cells and macrophages near the tip of initial lymphatics with button junctions (Fig. 5F,G; Baluk et al. 2007). Leukocyte entry through transendothelial routes was not excluded (Azzali 2006) until Holger Pflicke and Michael Sixt (2009) used real-time confocal microscopic imaging of fluorescently labeled dendritic cells and lymphatics. Their studies documented dendritic cells entering initial lymphatics in mouse ear through what appeared to be preformed openings in the basement membrane within minutes of a stimulus. The observations have been confirmed and expanded by studies of leukocyte chemotaxis into initial lymphatics in lymphatic-reporter mice with labeled immune cells and other approaches to elucidate mechanisms of chemokine signaling, passage through the endothelium, crawling along the luminal surface, and transit to collecting lymphatics (Tal et al. 2011; Jackson 2019; Arasa et al. 2021a,b; Eichin et al. 2021; Johnson 2021).

While leukocyte entry into initial lymphatics receives greater attention, erythrocytes also can enter lymphatics (Clark and Clark 1926). Howard Florey described the clinical success of blood infusion into the abdomen of newborn infants being due to erythrocyte entry into lymphatics of the diaphragm en route to the bloodstream (Florey and Witts 1928). The process was promoted by increased intra-abdominal pressure, but the route oferythrocyte ingress into lymphatics was not determined (Florey and Witts 1928; Nagy 1992).

BUTTON JUNCTION PLASTICITY

The mechanistic understanding of button junctions has advanced through genetic and pharmacological approaches used to examine how junctions develop and change in disease and with experimental manipulation (Table 1; Zhang et al. 2020; Norden and Kume 2021). Junctional plasticity in initial lymphatics is manifested by transformation of button junctions into zippers or vice versa during development, genetic manipulation, disease, or therapeutic intervention. Button junctions have not been found in collecting lymphatics under normal or pathological conditions.

Developmental Changes

During embryonic development, endothelial cells of lymph sacs at E12.5 and tracheal lymphatics at E16.5 are interconnected by zipper junctions, not buttons (Fig. 6A; Yao et al. 2012). Just before birth, the proportion of buttons in tracheal lymphatics increases from 6%

at E17.5 to 12% at E18.5,35% at birth, 50% at postnatal day P7, 90% at P28, and 100% at 10 wk of age (Fig. 6B–D; Yao et al. 2012). This transformation also occurs in lymphatics in skin and diaphragm but at different times (Zheng et al. 2014).

Maturation of zippers into buttons can be promoted by the corticosteroid dexamethasone (Yao et al. 2012; Zheng et al. 2014). In the airways of neonates at P4, most junctions in initial lymphatics are intermediate between zippers and buttons (Fig. 6E). But all are buttons after treatment with dexamethasone from P0 to P4 (Fig. 6F; Yao et al. 2012). The junctional change in skin lymphatics can be advanced in utero by treatment of pregnant mice (Zheng et al. 2014). After dexamethasone, lymphatic endothelial cells become oak-leaf in shape, and the distribution of LYVE1 becomes complementary to VE-cadherin, as in the adult (Yao et al. 2012). Consistent with these changes after dexamethasone, nuclear glucocorticoid receptors in lymphatic endothelial cells are phosphorylated at Ser211, unlike in untreated mice (Fig. 6E,F; Yao et al. 2012).

Plasticity Accompanying Sprouting Lymphangiogenesis

Further evidence of button junction plasticity comes from observations that buttons in initial lymphatics are replaced by zippers during sustained inflammation after airway infection by the mouse pathogen *Mycoplasma pulmonis* (Fig. 6G; Baluk et al. 2007; Yao et al. 2012). Sprouting lymphangiogenesis is widespread in this condition (Baluk et al. 2005), and the sprouts have zippers instead of buttons (Baluk et al. 2007). Conversion of buttons to zippers after infection can be reversed by administration of dexamethasone (Yao et al. 2012).

Altered Button Junction Protein Expression

The adherens junction protein VE-cadherin (cadherin 5, CDH5) is essential for normal lymphatic development and function. Inhibition of VE-cadherin by blocking antibody results in rapid dissolution of button junctions in tracheal lymphatics of adult mice (Baluk et al. 2007). Genetic deletion of *Cdh5* during embryonic development impairs lymphatic growth and leads to edema and prenatal death (Hägerling et al. 2018). Deletion of *Cdh5* during the neonatal period results in structural abnormalities in lymphatics. Although the distribution of tight junction components of button junctions has not been examined under these conditions, genes encoding claudin-5 (*Cldn5)*, JAM1, and ZO1 are up-regulated in endothelial cells of dermal initial lymphatics after *Cdh5* deletion (Hägerling et al. 2018). Deletion of *Cldn5* does not abolish the barrier function of lymphatic endothelial cells in vitro, and postnatal conditional deletion of *Cldn5* (from P4 to P11) does not eliminate VE-cadherin from lymphatic endothelial cell junctions in vivo (Frye et al. 2020). Although a more complete understanding of the regulation of lymphatic endothelial junctions is clearly needed, depletion of *Cldn5* is not singularly essential for maintenance of the endothelial barrier or the integrity of VE-cadherin at adherens junctions in lymphatics (Frye et al. 2020).

EphrinB2/EphB4 signaling regulates claudin-5 at endothelial cell junctions through the Rac1/Rho pathway and is required for stabilization of cell junctions in collecting lymphatics (Frye et al. 2020). Deletion of EphrinB2 disrupts button junctions in initial lymphatics, but the alteration is considered secondary to effects on collecting lymphatics (Frye et al. 2020).

Button junctions are not dependent on PECAM1. Although *Pecam1* expression is increased in lymphatic endothelial cells after *Cdh5* deletion (Hägerling et al. 2018), initial lymphatics in adult *Pecam1*-deficient mice have normal buttons and zippers (Baluk et al. 2007). After deletion of *Cdh5*, PECAM1 staining is discontinuous and located at the tip of endothelial flaps in initial lymphatics but can be broader than normal (Hägerling et al. 2018). Embryos of *Pecam1* knockout mice have structurally abnormal mesenteric lymphatics with defective intraluminal valves (Wang et al. 2016), but this occurs at a stage before button junctions form in initial lymphatics.

As a caveat, discontinuous staining for VE-cadherin at lymphatic endothelial cell borders after genetic manipulations can be deceptive and unrelated to button junctions when it instead results from altered junction formation or stability. Discontinuous lymphatic junctions are among many abnormalities in mice with deletion of the zinc-finger transcription factor gene Gata2 or its target *miRNA-126*, because GATA2 regulates expression of VE-cadherin and claudin-5 in lymphatic endothelial cells (Mahamud et al. 2019). Lymphatic junctions in these mice are jagged, disorganized, irregularly spaced (Mahamud et al. 2019), and unlike button junctions at the border of flaps in initial lymphatics.

RAS-interacting protein 1 (RASIP1), an endothelial-specific regulator of GTPase signaling in endothelial cells, is another factor essential for junction formation. Deletion of *Rasip1* in lymphatic endothelial cells results in disorganized junctions in embryos when zippers are normally present (Liu et al. 2018). Although discontinuous, the junctions in these mice lack the organized pattern of button junctions. Similarly disorganized junctions unlike buttons occur in collecting lymphatics after deletion of small GTPase Rasrelated protein genes *Rap1a* and *Rap1b* (Xu et al. 2018) and have also been described in lymphatic endothelial cells in culture, where the disorganization is amplified by TNF-α exposure (Kakei et al. 2014). Convincing evidence has not yet been obtained for button junction formation in cultured lymphatic endothelial cells despite concerted effort.

Angiopoietin-2 and VEGF Family Members

Unlike the disorganization of junctions that results from disruption of junctional proteins, zippers can form normally but fail to convert into buttons. Angiopoietin-2 (ANGPT2), a partial agonist of Tie2 receptors with potent effects on lymphatic development (Gale et al. 2007; Dellinger et al. 2008), regulates the conversion of zippers to buttons in initial lymphatics during development (Zheng et al. 2014). *Angpt2* deletion or inhibition by function-blocking antibody during gestation suppresses the formation of buttons; zippers persist postnatally (Zheng et al. 2014). However, once they have formed, button junctions are not altered by Angpt2 inhibition or overexpression (Zheng et al. 2014).

Manipulation of VEGF family growth factors and receptors has strong but complex effects on the development and maintenance of button junctions in intestinal lacteals (Zhang et al. 2018; Suh et al. 2019). If buttons change into zippers, lacteal uptake of chylomicrons made by intestinal epithelial cells is impaired and absorption of dietary lipid is disturbed (McDonald 2018; Zhang et al. 2018).

An elegant study by Anne Eichmann's group revealed that VEGF-A activation of VEGFR2 signaling induces button to zipper conversion in lacteals and reduces chylomicron uptake (Zhang et al. 2018). VEGFR2 inhibition has the opposite effect. Mechanistic experiments revealed that VEGFR1 expressed on endothelial cells of blood vessels in intestinal villi acts as a decoy receptor that limits the action of VEGF-A on lacteals (Zhang et al. 2018). Neuropilin-1 (NRP1) is a coreceptor for VEGF-A that acts similarly to VEGFR1. Deletion of *Nrp1* and *Vegfr1* genes together in endothelial cells increases VEGF-A availability, which promotes button conversion to zippers in lacteals and reduces dietary lipid uptake (Zhang et al. 2018). Similarly, inhibition of ROCK/Rho signaling by Y27632 promotes button to zipper conversion in lacteals and reduces uptake of dietary lipids (Zhang et al. 2018).

VEGF-C/VEGFR3 signaling in lacteals is another contributor to button junction maintenance and uptake of dietary lipid (Nurmi et al. 2015; Hong et al. 2020) and is influenced by the gut microbiota (Suh et al. 2019). Deletion of *Vegfr3* in lymphatic endothelial cells reduces button junctions in lacteals (Suh et al. 2019). Of potential clinical significance, depletion of gut organisms by antibiotics has a similar effect by reducing macrophage VEGF-C expression, which decreases buttons, increases zippers, and impairs dietary lipid absorption (Suh et al. 2019).

Delta-like4/Notch and YAP/TAZ Signaling

Among other pathways that influence button junctions, Delta-like4 (Dll4)/Notch signaling, which is downstream to VEGFR2 and VEGFR3, promotes continuous, slow regeneration of lacteals and is necessary for button junction formation (Bernier-Latmani et al. 2015). Inactivation of *Dll4* expression in lymphatic endothelial cells results in lacteal shortening, conversion of lacteal buttons to zippers, and impaired dietary lipid uptake, although button junctions are still present at the border of oak-leaf-shaped endothelial cells in lymphatics of the intestinal submucosa (Bernier-Latmani et al. 2015).

Activation of YAP/TAZ signaling promotes conversion of buttons to zippers in lacteals, with accompanying reduction of dietary lipid absorption (Hong et al. 2020). YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are activated by genetic deletion of the upstream inhibitors, *Lats1/2* (large tumor suppressor 1 and 2) (Hong et al. 2020). YAP and TAZ are the final transcriptional regulators in the canonical Hippo-YAP/TAZ pathway responsible for many aspects of cell differentiation and are negative regulators of *Prox1* expression in lymphatics (Cho et al. 2019). Expression of *Yap/Taz* in lymphatic endothelial cells is increased by depletion of VE-cadherin (Hägerling et al. 2018). *Yap/Taz* deletion or hyperactivation can result in abnormal lymphangiogenesis (Cho et al. 2019).

Adrenomedullin, S1P, and CD36

Tamoxifen-induced genetic deletion in lymphatic endothelial cells of calcitonin receptor–like receptor (*Calcrl*), the receptor for adrenomedullin (ADM), results in intestinal lymphangiectasia and more linear VE-cadherin staining of junctions in intestinal lacteals, with accompanying reduction in dietary lipid uptake (Davis et al. 2017, 2019; Xu et al. 2021), but changes in button junctions have not specifically been examined.

Sphingosine-1-phosphate (S1P), which is essential for lymphocyte egress from lymph nodes, is reduced in lymph—but not in plasma—of mice lacking the S1P synthesis genes sphingosine kinase 1 (*Sphk1*) and *Sphk2* in lymphatic endothelial cells (Pham et al. 2010). Tracheal lymphatics in these mice have fewer and more diffuse button junctions, implicating S1P signaling in the organization of these junctions (Pham et al. 2010). S1P signaling through S1P receptor 1 (S1PR1) regulates claudin-5 expression, promotes intercellular junction formation, and stabilizes lymphatic endothelial cells (Geng et al. 2020). These actions involve Delta-like4, ANGPT2, ADM, and other factors that have effects on lymphatic development and stability (Geng et al. 2020). ADM is expressed by lymphatic endothelial cells and has autocrine effects that stabilize intercellular junctions and the barrier function of the lymphatic endothelium (Klein and Caron 2015).

CD36, also known as scavenger receptor class B member 3, fatty acid translocase, and platelet glycoprotein 4, is a transmembrane protein that binds thrombospondin and long-chain fatty acids, among other ligands, and imports fatty acids into cells. The intensity of CD36 staining in lymphatic endothelial cells increases from intestinal lacteals to collecting lymphatics (Cifarelli et al. 2021). Inducible deletion of *Cd36* in lymphatic endothelial cells in adult mice impacts the structure and function of intestinal lacteals: VEGF-C signaling is reduced, intercellular junctions become disorganized, and lacteals are shortened (Cifarelli et al. 2021). Although button and zipper junctions have not been examined in detail in these mice, discontinuous junctions in lacteals have greater separation and are less numerous (Cifarelli et al. 2021).

CONCLUDING REMARKS

The understanding of the process of fluid and cell entry into lymphatics has evolved over many years. Evidence for the involvement of openings between button junctions in initial lymphatics is relatively new but fits with many earlier views and has been documented by diverse contemporary experiments. Developmental, pathophysiologic, and mechanistic studies have revealed the plasticity of button junctions, where buttons can convert to continuous zipper junctions and revert back to buttons, given the appropriate conditions.

Changes in lymphatic junction function can have important functional consequences. Reduction in lipid absorption by intestinal lacteals after button conversion to zippers highlights how junctional alterations in lymphatics of one organ can have systemic effects (Fig. 6H). Elucidation of the effects of button junction plasticity will provide insight into the contributions of lymphatic function to disease pathophysiology of each organ.

Further advances are needed for a complete understanding of button junction formation, maintenance, and plasticity. Single-cell RNA sequencing of gene expression in lymphatic endothelial cells has already revealed heterogeneity not previously appreciated but needs to be extended to address junction formation and the contributions of organ-specific microenvironments in health and disease. Additional studies are also needed to integrate knowledge of growth factors and signaling pathways known to influence button junction plasticity. As in the past, progress will continue step-by-step to uncover new features of

lymphatics; and as in the past, interpretations will accompany new findings but will often lag behind the eventual understanding.

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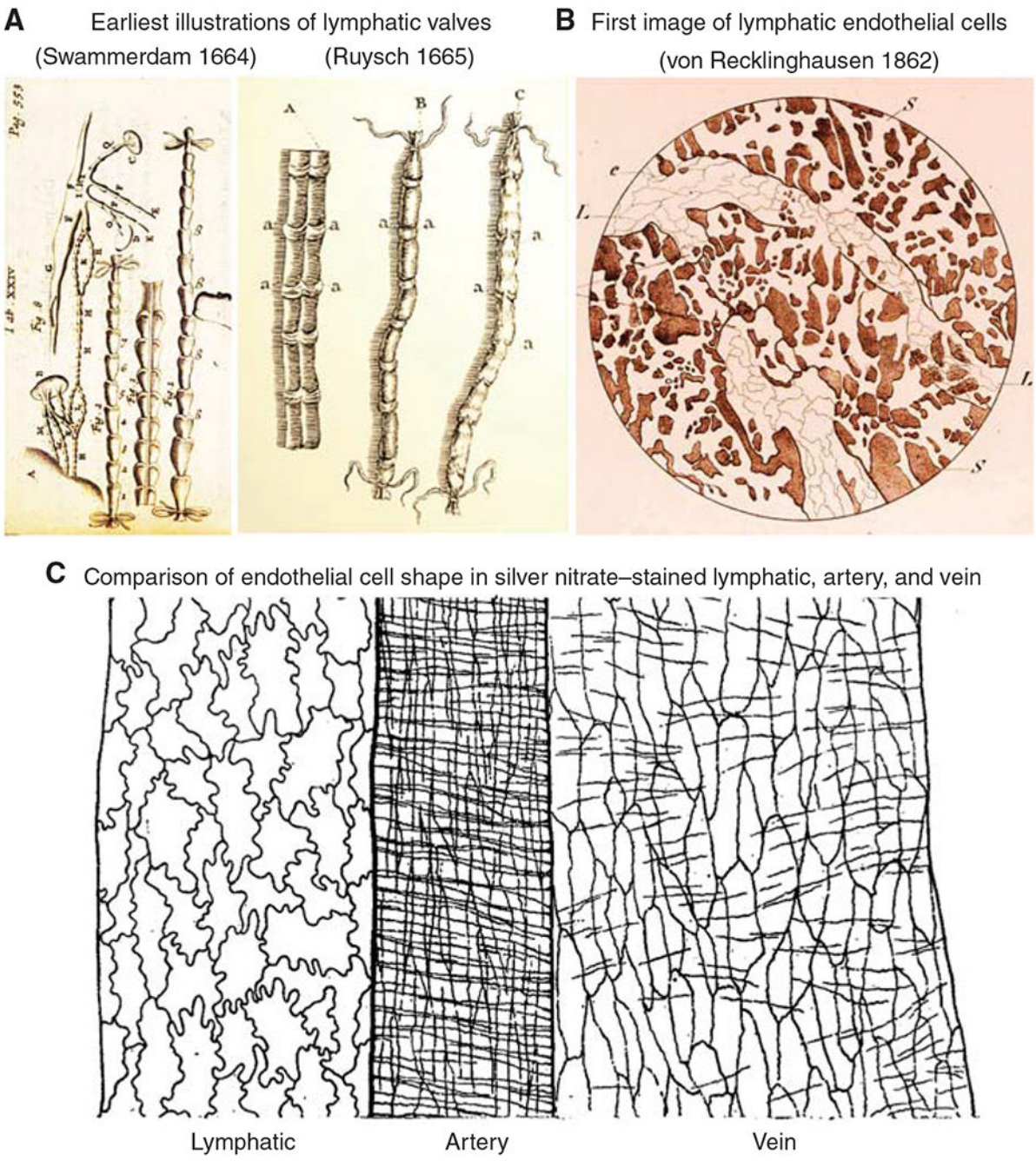

Figure 1.

Early studies of lymphatics. (*A*) The first illustrations of intraluminal (secondary) valves in lymphatics. (*Left* panel) Dog lymphatics drawn by Jan Swammerdam in June 1664 but only published in 1666 in a commentary on a contemporary textbook of anatomy. (Panel *A* reprinted from Table 24 in Blasius 1666 without restriction because figure is in the public domain.) (*Right* panel) Drawings by Frederik Ruysch published 1 year earlier (1665). The lymphatic labeled A has been dissected longitudinally to show the valves (labeled a). (Lymphatic labeled A, *right* side reprinted from Figure 1 in Ruysch 1665 without restriction

because figure is in the public domain.) (*B*,*C*) Early drawings of the shape of lymphatic endothelial cells stained by silver nitrate. (*B*) Faint endothelial cell borders in lymphatics of guinea-pig diaphragm drawn in 1862. (Panel *B* reprinted from Table 2, Figure 2 in von Recklinghausen (1862) and reprinted without restriction because table and figure are in the public domain.) (*C*) Endothelial cells in lymphatic, where the cells are oak-leaf shaped, and in artery and vein of cat omentum drawn in 1918. The transverse lines over the artery and veins are outlines of smooth muscle cells. (Panel *C* is reprinted from Figure 1 in Casparis 1918 without restriction because figure is in the public domain.)

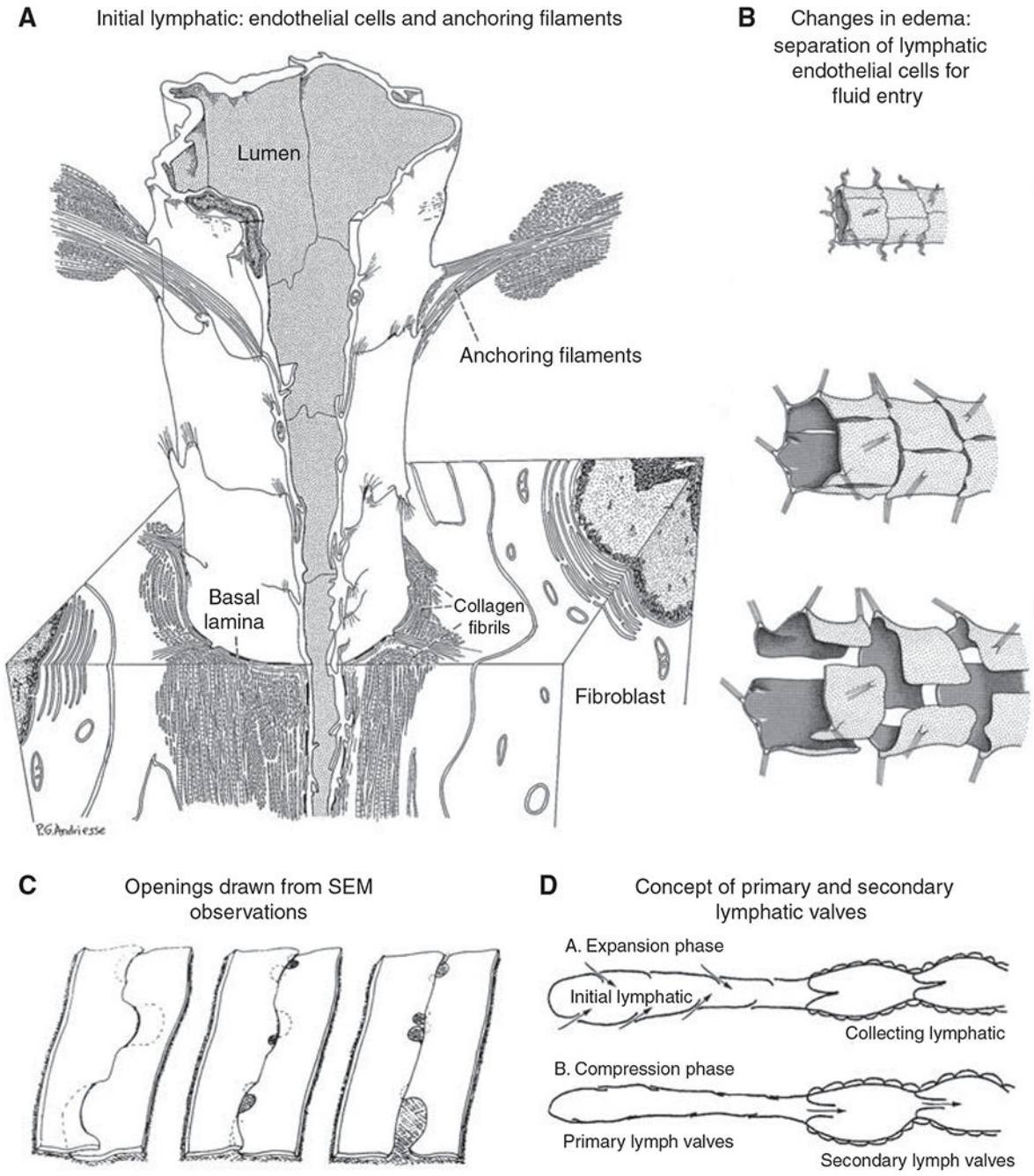

Figure 2.

Evolving concepts of open junctions between lymphatic endothelial cells. (*A*) Three-dimensional rendering of initial lymphatic reconstructed from transmission electron micrographs. Anchoring filaments link endothelial cells to the surrounding connective tissue. (Panel *A* from Figure 25 in Leak and Burke 1968b; reprinted, with permission, from Rockefeller University Press © 1968.) (*B*) Concept of changes in endothelial cell junctions in initial lymphatic. (*Top*) Normal condition, junctions are closed. (*Middle*) Moderate edema, slits form between endothelial cells. (*Bottom*) Severe edema, endothelial

cells are fully detached and widely separated from one another. (Panel *B* from Figure 12.14 in Majno and Joris 1996; reprinted, with permission, from John Wiley © 1996.) (*C*) Three-dimensional renderings of lymphatic endothelial cell borders based on scanning electron microscopy (SEM) images of initial lymphatics with interstitial pressure at normal level (*left*), moderately increased (*middle*), and greatly increased (*right*). Focal regions of intercellular junction detachment are shown as shaded openings that enlarge as lymphatics dilate. (Panel *C* from Figure 16 in Castenholz 1987; reprinted, with permission, from the International Society of Lymphology © 1987.) (*D*) Concept of "expansion" phase, when primary valves are open, lymph enters the initial lymphatic along the hydrostatic pressure gradient and secondary valves are closed to prevent backflow, and "compression" phase, when primary valves are closed, secondary valves are open, and lymph is pushed through the collecting lymphatic. (Panel *D* is from Figure 7 in Mendoza and Schmid-Schonbein 2003; reprinted, with permission, from the American Society of Mechanical Engineers © 2003.)

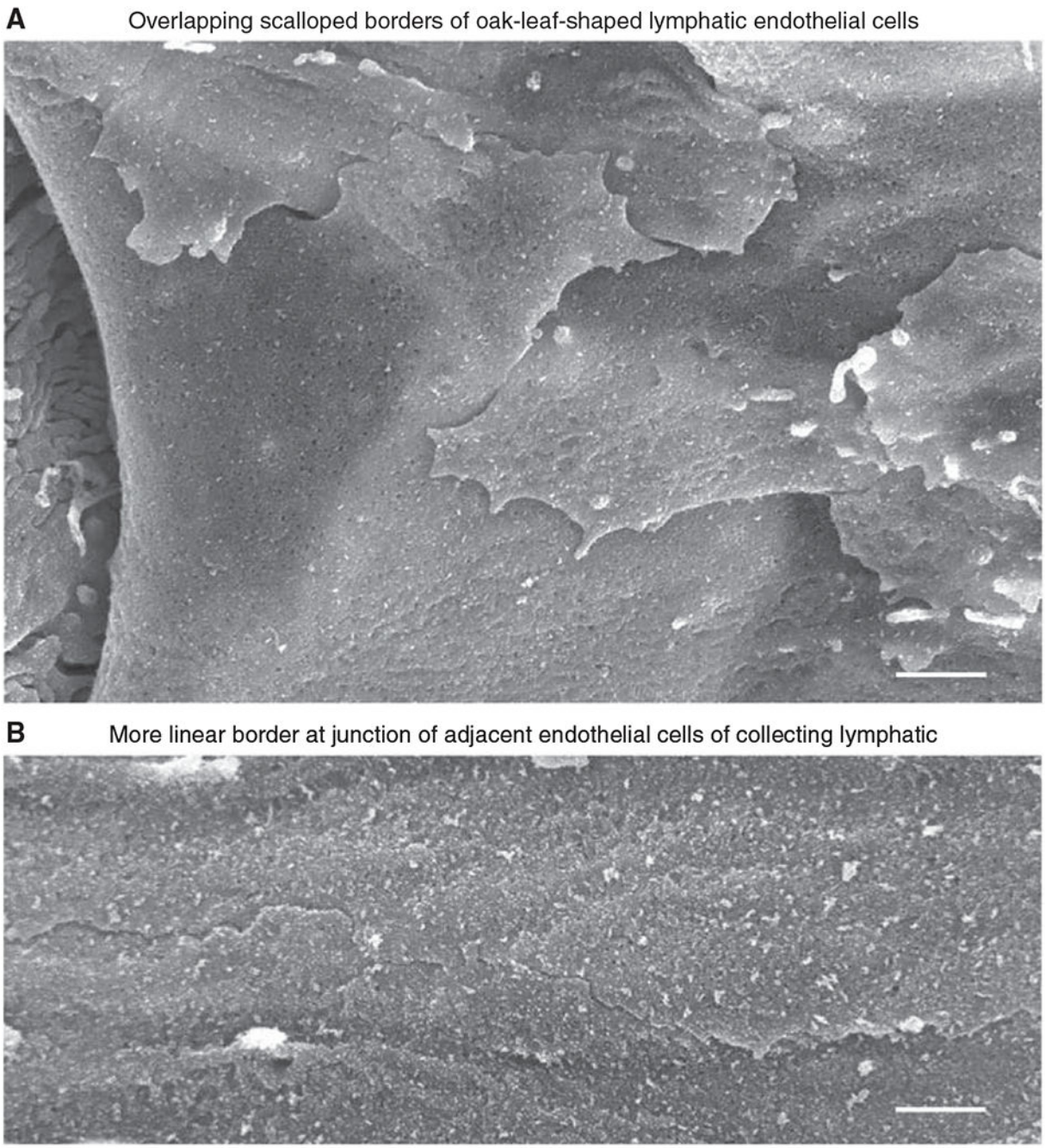

Figure 3.

Abluminal surface of lymphatic endothelial cell borders. Scanning electron microscopy view of abluminal surface of lymphatics after removal of surrounding connective tissue. (*A*) Initial lymphatic: Loosely apposed, overlapping, scallop-shaped cell borders of endothelial cells. (*B*) Collecting lymphatic: Tightly apposed linear borders of adjacent endothelial cells. Scale bars, 1 μm. (Panels *A* and *B* from Figure 1 and Supplemental Figure 1 in Baluk et al. 2007; reprinted courtesy of Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 Unported license).)

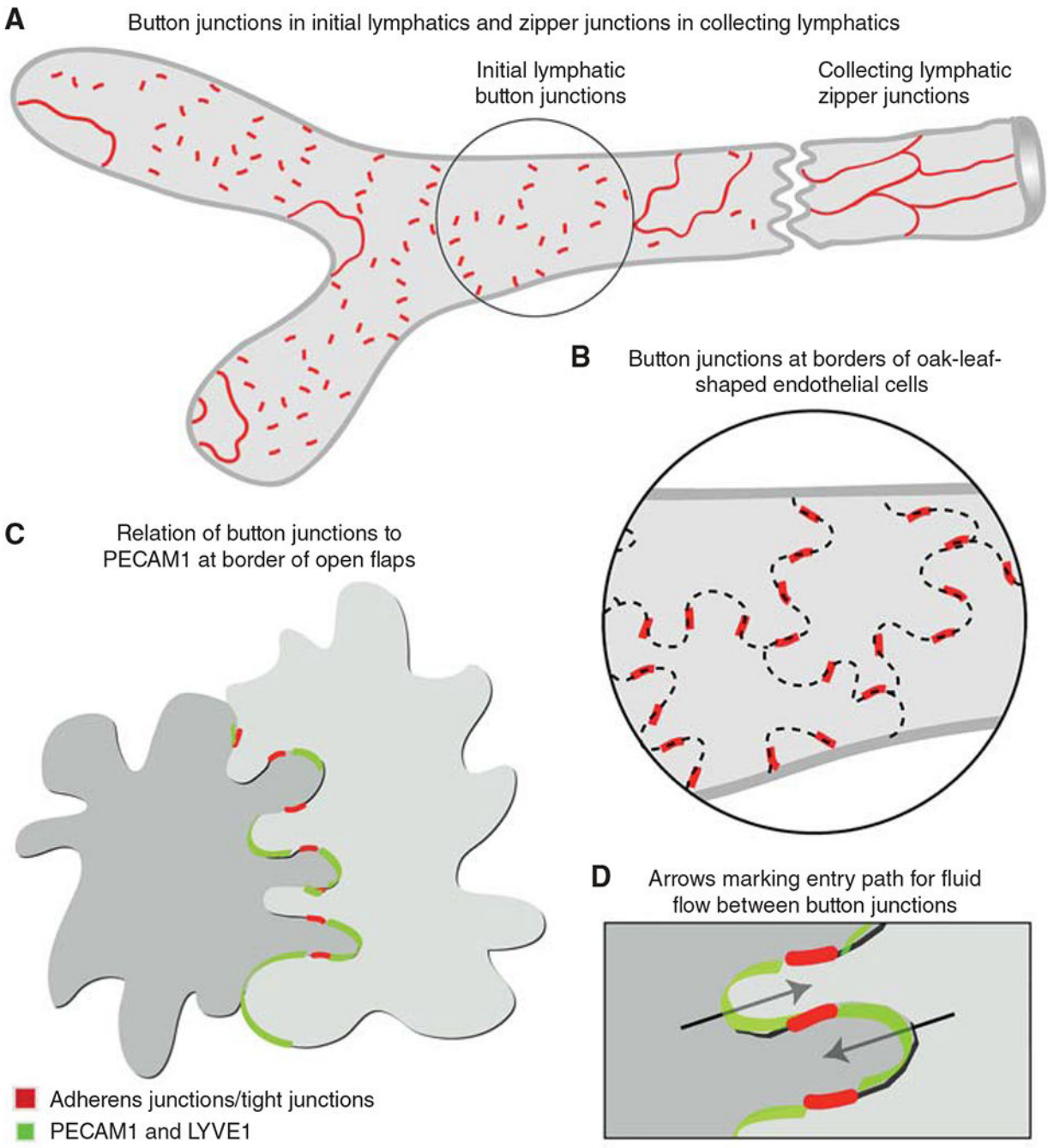

Figure 4.

Diagrams of button and zipper junctions in lymphatics. (*A*) Drawing showing discontinuous button junctions (red line segments) in endothelium of initial lymphatics and continuous zipper junctions (continuous red lines) in collecting lymphatics. Both types of junctions consist of proteins typical of adherens junctions and tight junctions. (*B*) More detailed view showing the oak-leaf shape of endothelial cells (dashed lines) of an initial lymphatic. Buttons (red) appear to be oriented perpendicular to the cell border but are in fact parallel to the sides of flaps. (*C,D*) Enlarged views of buttons along the sides of flaps of

adjacent oak-leaf-shaped endothelial cells with overlapping edges. PECAM1 and LYVE1 are located at the tip of flaps. Button-like adherens junctions and tight junctions at the sides of flaps direct fluid entry (arrows) through the junction-free region at the tip. Fluid traverses the basement membrane (not shown) and enters initial lymphatics through these openings without disruption of junctions. (Panels *A*–*D* from Figure 7 in Baluk et al. 2007; reprinted courtesy of Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 Unported license).)

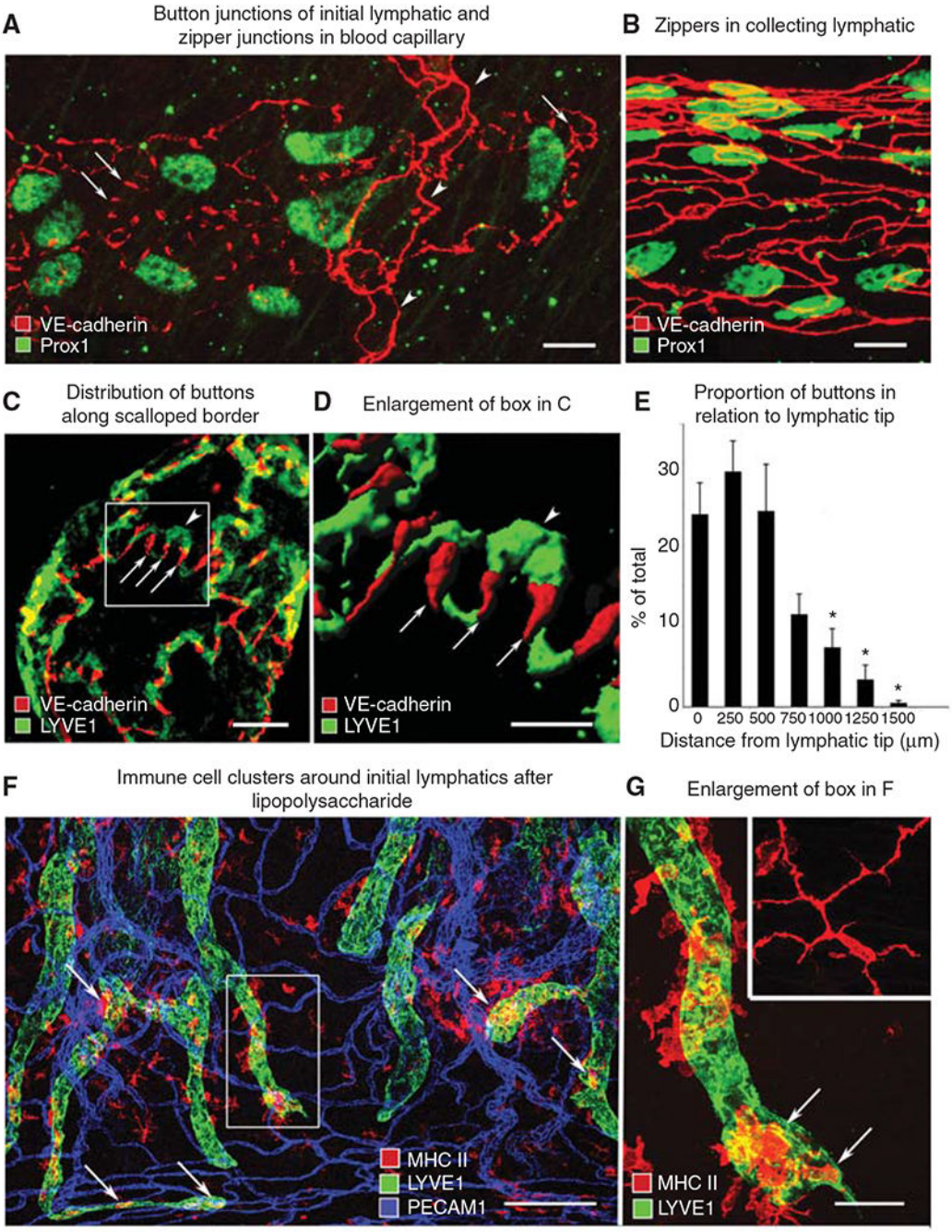

Figure 5.

Buttons and zippers and cell entry into initial lymphatics. Confocal microscopic images of mouse tracheal lymphatics. (*A*) Staining for VE-cadherin marks discontinuous buttons (arrows) in an initial lymphatic. Arrowheads point to zipper junctions in a blood capillary. (*B*) Continuous zipper junctions in a collecting lymphatic. (*C,D*) Confocal images showing VE-cadherin at buttons (arrows) and LYVE1 between buttons (arrowhead) at the border of oak-leaf-shaped endothelial cells of initial lymphatic. (*D*) Imaris isosurface rendering of confocal image stack of enlarged boxed region in panel *C*. (*E*) Gradient in abundance of

buttons shown as a function of distance from the tip of initial lymphatics. Mean ± SEM; $*P < 0.05$ compared to proportion at the tip. (Panels *A*–*E* from Figure 1 in Baluk et al. 2007; reprinted courtesy of Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 Unported license).) (*F*) Lymphatics (LYVE1, green) and MHC II–positive immune cells (arrows, red) in whole mount of mouse trachea 24 h after intratracheal instillation of lipopolysaccharide. (*G*) Enlargement of boxed region in F of an initial lymphatic containing MHC II–positive cells (arrows) that are rounded and have fewer processes than a corresponding cell in a pathogen-free mouse (*inset*). Scale bars, 10 μm (*A*–*C*); 5 μm (*D*); 200 μm (*F*); 50 μm (*G*, *inset*). (Panels *F* and *G* from Figure 6 in Baluk et al. 2007; reprinted courtesy of Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 Unported license).)

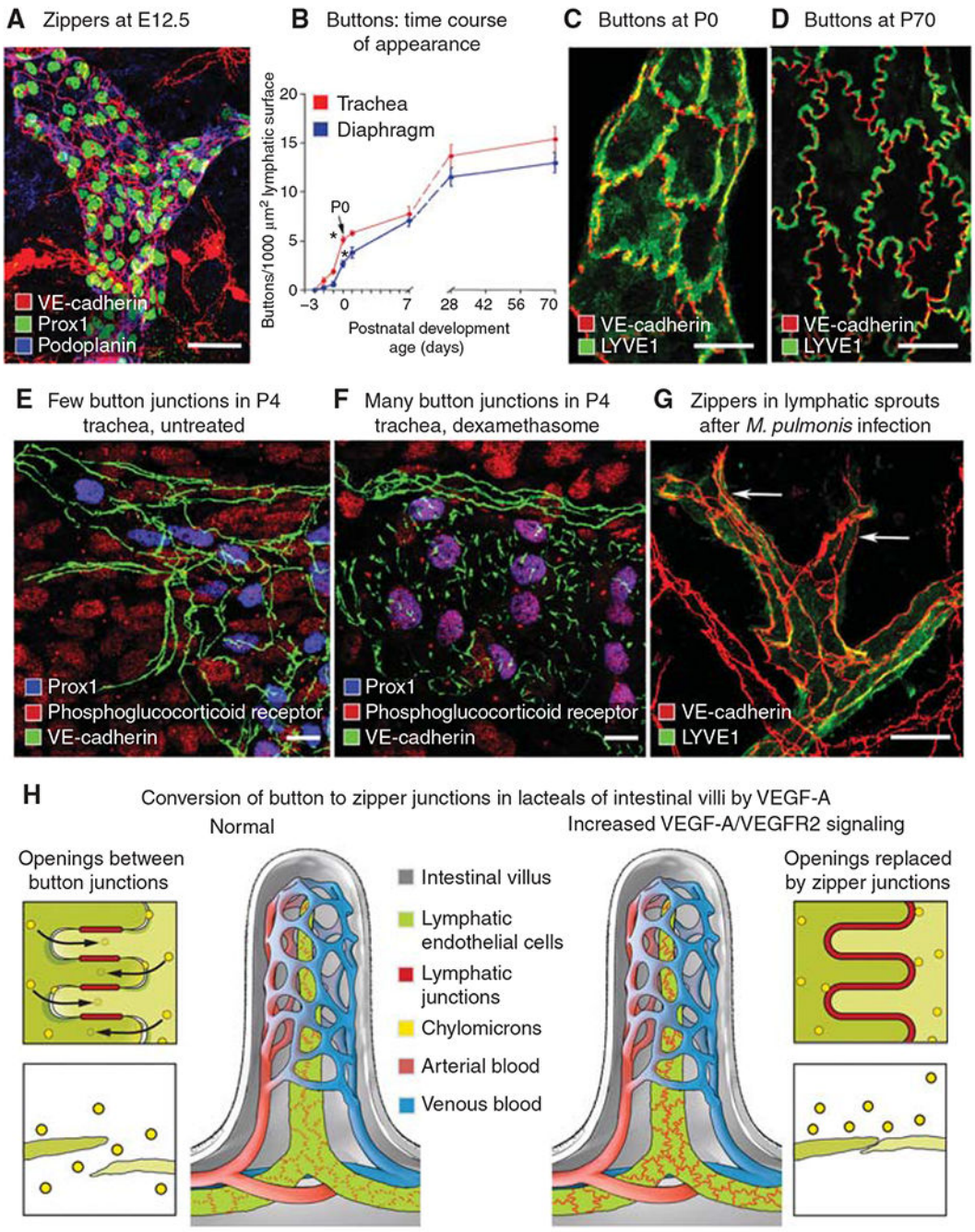

Figure 6.

Plasticity of button junctions in initial lymphatics. (*A*) Endothelial cells joined by zipper junctions (arrows) in embryonic lymphatics at E12.5. (*B*) Time course of development of button junctions in lymphatics of trachea (red) and diaphragm (blue) from E16.5 to P70. Buttons are expressed as number of VE-cadherin-stained junctional segments. Buttons that appear before birth represent only about 35% of the eventual number in adult initial lymphatics in the trachea and 20% in the diaphragm at P70. $*P < 0.05$, values at P0 (arrow) are significantly different from those at E18.5. (*C*,*D*) Confocal microscopic images

that illustrate the increasing proportion of buttons in initial lymphatics from P0 to P70. (*E,F*) Confocal images of tracheal lymphatics of P4 pups, either untreated (*E*) or treated with dexamethasone from P0 to P4 (*F*). Without treatment (*E*), lymphatic endothelial cells (Prox1, blue nuclei) have zipper junctions (VE-cadherin, green) and faint staining for phosphoglucocorticoid receptor (Ser211, red). After dexamethasone (*F*), lymphatics have button junctions and strong phosphoglucocorticoid receptor staining that colocalizes with Prox1 in lymphatic endothelial cell nuclei (purple, arrows). (*G*) Zippers in lymphatic endothelium after *Mycoplasma pulmonis* infection for 14 d. (Panels *A–G* from Figures 1, 2, 4, and 7 in Yao et al. 2012; reprinted, with permission, from the American Society for Investigative Pathology published by Elsevier © 2012.) (*H*) Entry of chylomicrons through openings between button junctions in normal intestinal lacteal (*left*) and lack of entry after conversion of buttons to zippers by activation of VEGF-A/VEGFR2 signaling in lacteal endothelial cells (*right*). Scale bars, 20 μm (*A*, *E–G*); 10 μm (*C,D*). (Panel *H* from McDonald 2018; adapted, with permission, from the author.)

Table 1.

Effects of mechanistic manipulations on button junctions in lymphatics

Mechanistic manipulation	Effect on buttons	References
Angiopoietin-2 (ANGPT2)	Necessary for button formation in initial lymphatics	Zheng et al. 2014
Calcitonin receptor–like receptor (CALCR1)	Adrenomedullin receptor upstream of DLL4; lymphatic endothelial cell-specific *Calcr1* mutations result in conversion of buttons to zippers, reduced lipid uptake, and intestinal lymphangiectasia	Davis et al. 2017, 2019
CD36	Lymphatic endothelial cell-specific *Cd36* deletion disrupts button junctions in lacteals and collecting lymphatics in intestine and mesentery and reduced uptake of chylomicrons	Cifarelli et al. 2021
Dexamethasone	Promotes conversion of zippers to buttons during development and in inflammation	Yao et al. 2010
Delta-like4 (DLL4) Notch signaling	Necessary for button formation in lacteals, signaling downstream of VEGFR2 and VEGFR3, supports lacteal regeneration	Bernier-Latmani et al. 2015
EphrinB2/EphB4 signaling	Necessary for stabilization of lymphatic junctions, regulates claudin-5 expression in lymphatic endothelial cells	Frye et al. 2020
Gut microbiome	Promotes VEGF-C production and button formation in lacteals, antibiotic depletion of gut bacteria results in conversion of buttons to zippers in lacteals	Suh et al. 2019
Inflammation	Promotes zipper formation in lymphatic sprouts in mouse airways after infection by *Mycoplasma pulmonis*	Baluk et al. 2007; Yao et al. 2012
LYVE1	*Lyve1* deletion has no apparent effect on lymphatic junctions or fluid drainage but reduces leukocyte transit and amplifies chronic inflammation	Gale et al. 2007; Vieira et al. 2018
Mechanical stretch	Promotes VEGF-C production and button formation in lacteals	Hong et al. 2020
Neuropilin1 (NRP1)	Deletion of *Vegfr1* and *Nrp1* together promotes VEGF-A/VEGFR2 signaling and conversion of buttons to zippers in lacteals, reduces chylomicron uptake	Zhang et al. 2018
PECAM1	Deletion of *Pecam1* has no apparent effect on button junctions or leukocyte entry into initial lymphatics	Baluk et al. 2007; Wang et al. 2016
RhoA/ROCK signaling	Necessary for junction formation in lacteals and for secondary valves in collecting lymphatics, ROCK inhibition by Y-27632 promotes conversion of buttons to zippers	Zhang et al. 2018; Norden and Kume 2021
S1P/S1PR1 signaling	S1PR1 regulates lymphatic quiescence by inhibiting VEGF-C signaling induced by shear stress, regulates claudin-5 expression	Pham et al. 2010; Geng et al. 2020
VE-cadherin (cadherin-5, CDH5)	Anti-VE-cadherin antibody promotes rapid dissolution of button junctions, postnatal *Cdh5* deletion has pronounced effects on lacteals and limited effect on skin lymphatics	Baluk et al. 2007; Hägerling et al. 2018
VEGF-A/VEGFR2 signaling	VEGF-A signaling through VEGFR2 in lacteals results in button conversion to zippers and reduces chylomicron uptake, inhibition of VEGFR2 by DC101 antibody promotes button formation	Zhang et al. 2018
VEGFR1	Combined deletion of *Vegfr1* and *Nrp1* promotes VEGF-A/VEGFR2 signaling and results in button conversion to zipper junctions in lacteals, reduces chylomicron uptake	Zhang et al. 2018
VEGF-C/VEGFR3 signaling	Promotes button formation in lacteals	Nurmi et al. 2015; Hong et al. 2020
YAP/TAZ signaling	Upstream of Vegfc in maintenance of buttons in lacteals, *Lats1/2* deletion activates YAP/TAZ signaling and reduces buttons, YAP/TAZ signaling balance needed to maintain button integrity	Cho et al. 2019; Hong et al. 2020