Distinct roles of VE-cadherin for development and maintenance of specific lymph vessel beds

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Abstract

Endothelial cells line blood and lymphatic vessels and form intercellular junctions, which preserve vessel structure and integrity. The vascular endothelial cadherin, VE-cadherin, mediates endothelial adhesion and is indispensable for blood vessel development and permeability regulation. However, its requirement for lymphatic vessels has not been addressed. During development, VE-cadherin deletion in lymphatic endothelial cells resulted in abortive lymphangiogenesis, edema, and prenatal death. Unexpectedly, inducible postnatal or adult deletion elicited vessel bed-specific responses. Mature dermal lymph vessels resisted VE-cadherin loss and maintained button junctions, which was associated with an upregulation of junctional molecules. Very different, mesenteric lymphatic collectors deteriorated and formed a strongly hyperplastic layer of lymphatic endothelial cells on the mesothelium. This massive hyperproliferation may have been favored by high mesenteric VEGF-C expression and was associated with VEGFR-3 phosphorylation and upregulation of the transcriptional activator TAZ. Finally, intestinal lacteals fragmented into cysts or became highly distended possibly as a consequence of the mesenteric defects. Taken together, we demonstrate here the importance of VE-cadherin for lymphatic vessel development and maintenance, which is however remarkably vessel bed-specific.

Keywords lymph vessels; lymphatic valves; vascular heterogeneity; VE-cadherin; YAP/TAZ

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Development & Differentiation; Vascular Biology & Angiogenesis

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Introduction

Endothelial cells form the inner lining of blood and lymphatic vessels, the body's conduits for fluids, solutes, and cells. Fast blood flow and high pressure require different properties of the circulatory system compared to lymph transport at low pressure through the dendriform lymphatic vessels (Potente & Makinen, 2017). Still, interendothelial adherence and tight junctions of very similar molecular composition link endothelial cells in both vascular systems and are essential for their formation, maintenance, and function (Dejana & Vestweber, 2013). Endothelial cell junctions mediate cell–cell adhesion, restrain migration and proliferation, establish and maintain cell polarity, provide essential survival signals, and regulate permeability. The central adhesive component of adherence junctions is the transmembrane protein vascular endothelial (VE)-cadherin, which mediates its function through extracellular homophilic trans-interactions and orchestrates an intracellular adhesion complex, containing β -catenin, plakoglobin, and p120 catenin (Kemler, 1992; Gumbiner, 1996). In a force-dependent manner, α -catenin conditionally together with vinculin and α -actinin links the VE-cadherin adhesion complex to the cortical actin cytoskeleton (Nelson & Weis, 2016). Direct fusion of VE-cadherin to α -catenin enhances this interaction, leading to reduced leukocyte extravasation and vascular dermal permeability (Schulte *et al*, 2011). Despite enforced interendothelial junctions, blood vessel development and morphogenesis are not discernibly affected in VE-cadherin- α -catenin mice. In contrast, on a C57BL/6 background covalent VE-cadherin- α -catenin linkage caused fetal edema and death due to the inability of primordial lymphatic vessels to remodel and subsequent fragmentation of the forming dermal lymphatic plexus (Dartsch *et al*, 2014). Reflecting their properties as a low-pressure, easy-entry conductance system, lymphatic vessels show a highly regulated spatial organization of adherence junction molecules. Lymphatic endothelial cells (LECs) in initial lymphatics or lymphatic capillaries display a discontinuous, button-like VE-cadherin distribution, which alternates with cell contact regions rich in the adhesion molecule

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PECAM1 and the hyaluronan receptor LYVE1 (Baluk *et al*, 2007). These are believed to serve as initial uptake sites for immune cells and tissue fluid (Pflücke & Sixt, 2009).

VE-cadherin provides a central membrane signaling hub that associates with a multitude of dynamically changing intracellular partners depending on vessel maturity and physiological activation state. Tyrosine residues in the cytoplasmic tail of VE-cadherin regulate endothelial junctions and several kinases and phosphatases directly interact with VE-cadherin (Nottebaum *et al*, 2008; Orsenigo *et al*, 2012; Vestweber *et al*, 2014; Wessel *et al*, 2014). Direct interaction partners of VE-cadherin also include p85/PI3-kinase, which is believed to provide essential survival signals (Carmeliet *et al*, 1999) and the transcriptional co-activator YAP (Giampietro *et al*, 2015). YAP competes in a cell density-dependent fashion with the actin-binding protein Eps8 for α -catenin binding (Giampietro *et al*, 2015) and controls epidermal proliferation downstream of α -catenin (Schlegelmilch *et al*, 2011). YAP and its paralog TAZ are major mechanosensitive regulators of transcription (Dupont *et al*, 2011; Halder *et al*, 2012; Finch-Edmondson & Sudol, 2016). In epithelial and endothelial cells, mechanical strain results in their nuclear translocation and subsequent cell cycle entry (Benham-Pyle *et al*, 2015; Wang *et al*, 2017).

Functional impairment of VE-cadherin by antibody injection destabilizes blood vascular junctions, resulting in increased permeability and neutrophil extravasation (Gotsch *et al*, 1997; Corada *et al*, 1999). Genetic deletion of VE-cadherin results in loss of endothelial morphogenetic competence following angioblast differentiation. Consequently, remodeling of the primary vascular plexus and sprouting angiogenesis fail, leading to developmental arrest at day E9.5 (Carmeliet *et al*, 1999; Gory-Faure *et al*, 1999). Inducible postnatal, pan-endothelial deletion of VE-cadherin resulted within days in increased vascular permeability in heart and lung, but not in skin and brain, despite near complete deletion in all organs (Frye *et al*, 2015). Surprisingly, even in lung where permeability was massively increased, loss of VE-cadherin did not result in an altered junctional ultrastructure, suggesting that once formed, VE-cadherin is dispensable for the maintenance of blood endothelial junctions. Specific requirements for VE-cadherin on lymphatic vessels have so far not been addressed.

Here, we analyzed the function of VE-cadherin in lymphatic vessel formation and maintenance applying inducible, LEC-specific deletion in mice. During development, LEC-specific loss of VE-cadherin caused fetal lethality due to a failure of the primary lymphatic plexus to remodel into functional lymph vessels. However, postnatal deletion of VE-cadherin revealed remarkably distinct and unexpected differences in the response of the dermal, mesenteric, and intestinal lymph vessel beds. Dermal lymph vessels fragmented upon early deletion in neonates, but became largely resistant to VE-cadherin loss following maturation. After puberty, maintenance of interendothelial junctions appeared to have become independent of VE-cadherin. In contrast, irrespective of the age, at which VE-cadherin was deleted, mesenteric lymphatics deteriorated, became hyperplastic, and ultimately grew as a layer of LECs on the mesothelium. This proliferative response, selectively observed in mesenteric LECs, was associated with constitutive phosphorylation of the vascular endothelial growth factor receptor-3 (VEGFR-3) and upregulation of the transcriptional co-activator TAZ. Finally, intestinal lacteals fragmented into cysts or highly distended vessels in a

process that was age-independent and may have been in part a secondary reaction to the mesenteric defects. In primary VE-cadherin-deficient LEC cultures, we detected reduced focal adhesion formation and increased motility. Taken together, we have for the first time demonstrated the importance of VE-cadherin for the maintenance of distinct lymphatic vessel beds. We suggest that specific differences in VEGF-C signaling and transcriptional regulation of junctional proteins in combination with altered migratory behavior of LECs may explain the distinct responses of dermal, intestinal, and mesenteric lymph vessels after VE-cadherin loss.

Results

VE-cadherin is indispensable for the development of functional fetal lymphatic vessels

We specifically probed for the requirement of VE-cadherin during lymphatic vessel development by crossing mice bearing a floxed *Cdh5* allele with the *Prox1-CreERT²* driver line. Deletion of VE-cadherin in lymphatic endothelial cells was induced by application of two or three optimal doses of 4-hydroxytamoxifen (4-OHT) at days E10.5 and E11.5 (analysis at E12.5) or E10.5, E11.5, and E12.5 (analysis at E14.5) of development. When pregnancies were terminated at day E12.5, 17% of the embryos analyzed showed signs of edema (Fig 1A). At developmental stage E14.5, all fetuses homozygous for the floxed *Cdh5* allele and positive for the *Prox1-CreERT²* driver displayed a pronounced dermal edema (Fig 1B, black arrowheads). Also, all VE-cadherin-deleted fetuses recovered at E15.5 and E18.5 displayed massive edema and we observed no viable *Cdh5^{lox/lox}; Prox1-CreERT²* pups, while viability of other genotypes was not noticeably affected (Fig 1A).

For further analysis, we immunostained the developing dermal lymphatic plexus in wholemount preparations from E14.5 fetuses for PROX1, PECAM1, and VEGFR-3. Compared to non-deleted control samples lacking the driver allele (*Cdh5^{lox/lox}*), deletion of VE-cadherin in lymphatic endothelial cells resulted in an irregularly shaped lymphatic plexus that was less ramified and displayed sheet-like vascular lacunae (Fig 1C, bottom row). Enumeration of the PROX1-expressing (PROX1⁺) nuclei and the area covered by lymphatic vessels revealed a roughly doubled number of LECs following VE-cadherin deletion (Fig 1D), associated with a significantly increased lymphatic vascular area (Fig 1E).

During heart development, PROX1 is expressed throughout the myocardium, the interventricular septum, the atrioventricular endocardium, and the mitral valve leaflets (Risebro *et al*, 2009). To exclude that the observed edema was due to cardiac defects, we analyzed sections and wholemount preparations of hearts from E12.5 and E14.5 fetuses that were stained for the cardiac isoform of Troponin T, Desmin, PECAM1, and VEGFR-3. At both developmental stages, heart development appeared unaffected by VE-cadherin deletion (Appendix Figs S1 and S2, and Videos S1 and S2).

Taken together, these results suggested that deletion of VE-cadherin in LECs during development resulted in disturbed lymph vessel development causing LEC hyperplasia and edema, which precluded survival to term.

Figure 1.

Figure 1. Genetic deletion of VE-cadherin in lymphatic endothelial cells results in edema and hyperplasia.

Pregnant dams were injected with 4-OHT at days E10.5 and E11.5 for analysis at day E12.5 or E10.5, E11.5, and E12.5 of development for later times of analysis.

- A Developmental loss of VE-cadherin leads to severe edema. Fetuses of the genotypes listed in the top row were analyzed at the developmental stages in the first column. Numbers in brackets denote embryos with signs of edema. Last column lists the percentage of viable fetuses in the VE-cadherin-deleted cohort.
- B Fetuses were explanted at day E14.5. Arrowheads indicate prominent edema along the back. The images are representative for 42 (*Cdh5^{lox/lox}*) and 25 (*Cdh5^{lox/lox}; Prox1-CreER^{T2}*) analyzed animals.
- C Skin wholemount preparations stained for PROX1, PECAM1, and VEGFR-3. Maximum intensity projections (MIPs) of tiled confocal stacks (*Cdh5^{lox/lox}* 2,075 × 2,075 μm, z = 50 μm; *Cdh5^{lox/lox}; Prox1-CreER^{T2}* 3,765 × 3,765 μm, z = 61 μm). Scale bars = 100 μm. The data are representative for six (*Cdh5^{lox/lox}*) and six (*Cdh5^{lox/lox}; Prox1-CreER^{T2}*) analyzed animals from three litters.
- D Enumeration of the PROX1-expressing (PROX1⁺) nuclei in three different VE-cadherin-deleted wholemount preparations analogous to Fig 1B. Nuclei were counted in a MIP corresponding to an area of 1.5 mm² and normalized to the number of nuclei in *Cdh5^{lox/lox}* control preparations.
- E The area covered by lymphatic vessels was determined in the samples evaluated in (D) and is depicted as relative area compared to *Cdh5^{lox/lox}* controls. The calculations and measurements were obtained from three animals and three confocal stacks per biological group. PROX1-positive nuclei were counted using the particle analysis tool of Fiji.

Data information: The data in (D and E) represent mean ± SD. ****P* < 0.001. Two-tailed unpaired Student's *t*-test.

Lymphatic-specific distribution of PECAM1 in button-type junctions is maintained in postnatal dermal lymph vessels after VE-cadherin deletion

The prenatal lethality caused by developmental deletion of VE-cadherin from lymphatic vessels precluded further analysis of VE-cadherin function in adult lymph vessel beds and of its role for maintenance of specialized button junctions. We therefore deleted VE-cadherin in LECs of neonates, by two optimal doses of 4-OHT at days P2 and P4 and analyzed the vasculature of wholemount ear skin preparations 6 weeks later. At this point, compared to control tissues (Fig 2A–D) VE-cadherin immunostaining was no longer detectable on the surface of LECs (Fig 2E and F), which were identified by surface PECAM1 and nuclear PROX1 expression. Despite the absence of VE-cadherin, dermal lymphatic vessels remained clearly identifiable; however, we frequently noted an irregular appearance of fragmentation and widened, initial lymphatics (Fig 2G, yellow arrows, H). When we zoomed into these structures, despite absence of VE-cadherin, the typical discontinuous junctional distribution of PECAM1 was retained (Fig 2C and G insets, yellow arrow heads).

Taken together, despite efficient postnatal deletion of VE-cadherin, dermal lymphatic vessels in the mouse ear maintained their gross appearance and typical structural features.

Age-dependent stabilization protects dermal lymphatic vessels against VE-cadherin loss

The observed irregularities after neonatal induction of VE-cadherin deletion prompted us to systematically analyze the consequences of LEC-specific VE-cadherin loss at different ages. Therefore, we induced deletion either in neonates (Fig 3A and B) or at 11 weeks (Fig 3C) or 42 weeks (Fig 3D) of age and analyzed the consequences 6 weeks (neonatal) or 5 weeks (11 and 42 weeks) later.

Independent of the time of induction, VE-cadherin was efficiently deleted from dermal lymphatic vessels (Appendix Fig S3). Compared to control samples (Fig 3A and E) in mice induced as neonates, loss of VE-cadherin led to the already described widening and fragmentation of lymph vessels (Fig 3B and F; white arrows), which was associated with the appearance of pointed vessel tips reminiscent of vascular sprouts (Fig 3F; white arrowheads) and an apparent deterioration of lymphatic valves. Comparable structural alterations were no longer detectable if VE-cadherin deletion was induced at 11 weeks (Fig 3C and G) or 42 weeks (Fig 3D and H). In

particular, valves appeared not affected upon later deletion and we readily detected their characteristic structure of two semilunar leaflets, as shown for induction at 11 weeks and analysis 5 weeks later in Fig 3I–L (yellow arrows).

These results suggested an age-related stabilization, which protected mature dermal lymph vessels against VE-cadherin loss.

Deletion of VE-cadherin causes mesenteric lymph vessel disintegration, independent of the age at the time of induction

While analyzing the consequences of VE-cadherin deletion on dermal lymph vessels at different ages, we noted development of edema and chylous ascites (Appendix Fig S4A and B). This prompted us to investigate mesenteric lymph vessels. Neonatal loss of VE-cadherin in dermal lymphatic vessels had provoked a mild expansion and occasional fragmentation of initial lymphatics (Fig 3B and F), while later deletion remained without apparent effect (Fig 3C, D, G and H). In pronounced contrast, mesenteric lymph vessels, which are mainly comprised of collectors (Fig 4A), were massively affected irrespective of the age of deletion. Following induction of neonates, mesenteric lymphatics became distended and dysmorphic within 6 weeks (Fig 4B). While PROX1⁺ cells still outlined the original vessel and valve positions (Fig 4B, white arrows), valves had largely deteriorated. A strong expansion of the LECs resulted in the formation of flat cell sheets comprised of a layer of PROX1⁺, VEGFR-3⁺, PECAM1⁺ LECs (Fig 4B yellow arrowheads). These sheets preferentially formed in the proximity of the intestine. One such area is outlined by the box in Fig 4B and shown enlarged in the adjacent inset and in Fig EV1A and B. Higher magnification (Fig EV1A and B) revealed heterogeneity in VEGFR-3 expression, which was frequently no longer detected on the cell surface, but found intracellularly next to the nucleus. Adjacent adipose tissue was rich in PECAM1⁺ blood capillaries (Fig 4B white arrowheads, Fig EV1C).

Clearly different from dermal lymph vessels, mesenteric lymphatics massively deteriorated, even when deletion had been induced as late as 6 month or 1 year of age (Fig 4C–F). Following induction at 6 month and analysis 11 weeks later (Fig 4C–E), we found a significant number of extremely thinned vessels (Fig 4C, white arrowheads) and again we noted the presence of large sheets or monolayers of LECs expressing PROX1, PECAM1, and VEGFR-3 (Fig 4C, white arrows and boxed area). A higher magnification of these sheet-like structures (Fig 4D) revealed, besides heterogeneous

Figure 2. Despite efficient postnatal deletion of VE-cadherin, the discontinuous distribution of PECAM1 is retained in dermal lymphatic vessels.

Deletion of VE-cadherin in lymphatic endothelial cells of newborn pups was induced by two optimal doses of 4-OHT at days P2 and P4. For analysis 6 weeks later, ear punches were subjected to wholemount immunostaining of the dermal vasculature.

A–H (A, B, E, F) Overview MIPs of ear skin wholemount preparations immunostained for the indicated antigens. VEC, VE-cadherin; PRX, PROX1. (C, D, G, H) Magnified views of the areas in the red boxes in (A, E). Areas delimited by the stippled yellow boxes in (C) and (G) are enlarged in the insets (yellow solid boxes). Yellow arrows indicate widened lumen and yellow arrowheads denote oak leaf-shaped LECs with discontinuous junctions. Scale bars in (A) and (E) correspond to 100 μ m and in (C) and (G) to 50 μ m. The data are representative for six (*Cdh5^{lox/lox}*) and five (*Cdh5^{lox/lox}; Prox1-CreER^{T2}*) analyzed animals from two litters.

VEGFR-3 surface expression, continuously PECAM1-lined LEC contacts. Further, we noted vessel sprouts that appeared to emerge from deteriorated valve areas (Fig 4C, yellow arrowheads and E). Even upon induction at 1 year of age, mesenteric lymphatics had disintegrated when analyzed 8 weeks later and abundant sprouts emanated from the fragmented lymphatic vessels (Fig 4F).

For a detailed description, we analyzed the mesenteric LECs in a preparation derived from a mouse 11 weeks after VE-cadherin deletion, which was induced at 4 month (Fig EV2). Obvious and again striking were the layers of LECs that flanked original lymphatic vessels (vessels indicated by a string of asterisks in Fig EV2A). At higher resolution, we confirmed the previously observed heterogeneity of VEGFR-3 expression. LECs with low or absent surface VEGFR-3 were mostly located at the distal border of LEC sheets (yellow arrowheads in Fig EV2B and C). Within the LEC sheets, PECAM1 was uniformly expressed at the cell–cell contacts. Frequent intercellular gaps between LECs suggested open cell contacts due to insufficient junctional adhesion (Fig EV2D white arrows). Isolated PROX1[−] VEGFR-3[−] cells that displayed a high level of intracellular PECAM1 staining (white arrowheads in Fig EV2B) often flanked the LEC sheets and likely were phagocytes.

In summary, mesenteric lymph vessels were susceptible to the loss of VE-cadherin at all ages and reacted with a breakdown of the lumenized vessel structure leading to the formation of LEC sheets and broken LEC cell–cell contacts.

VE-cadherin deletion in LECs results in fragmentation or distension of intestinal lacteals

Development of chylous ascites in VE-cadherin-deleted mice suggested that also lacteals of intestinal villi were affected. In whole-mount preparations of the intestinal wall, triple staining for ESAM1, α SMA, and LYVE1 allowed simultaneous visualization of the centrally located lymphatic lacteal as well as the surrounding blood capillary network and smooth muscle cells (Fig 5A–E). Like PECAM1, the junctional molecule ESAM1 is expressed more strongly on blood endothelium compared to LECs and therefore readily identifies blood vessels (Hagerling *et al*, 2017). In wild-type mice, the central lacteal spans 70–90% of the villus length (Figs 5A and B, and EV3A), while α SMA-positive fibers extend along the entire length of the villus (Fig 5A and B, and EV3A and B; Bernier-Latmani *et al*, 2015).

Deletion of VE-cadherin in mice at various ages, even as old as 1 year, resulted in fragmentation of the central villi, giving rise to multiple cysts comprised of LYVE1-expressing LECs that extended an unusual number of filopodia (Figs 5C–E and 3C–E, and Appendix Fig S5 white arrows). In addition, we observed villi harboring exceptionally large, distended LYVE1⁺ lacteals (Fig 5C and D, Appendix Fig S5C yellow arrowhead, E). While we readily detected oak leaf-shaped LECs in the lacteals of control mice (Appendix Fig S6A–C arrowheads and inset), cell–cell contacts were ragged and individual cells extremely difficult to discern after

Figure 3. Dermal lymphatic vessels respond differentially to VE-cadherin loss, depending on the age at the time of induction.

A–L VE-cadherin deletion was initiated either in newborn pups (A, B, E, F) by two applications of 4-OHT at days P2 and P4 or in mice at 11 weeks (C, G) and 42 weeks (D, H) of age (three applications of Tamoxifen at 2-day intervals via oral gavage). Ear skins were prepared 6 weeks (A, B, E, F) or 5 weeks (C, D, G, H) after induction and immunostained for the indicated proteins. Shown are MIPs of confocal tile scans (approximately $750 \times 750 \mu\text{m}$) covering $40 \mu\text{m}$ in depth. White arrows in (F) highlight distorted and partially fragmented lymphatic vessels and white arrowheads denote aberrantly pointed vessels. (I–L) Lymphatic valves in the dermis of the ear were maintained for 5 weeks despite deletion of VE-cadherin at 11 weeks of age (yellow arrows in K, L). PEC1, PECAM1; PRX1, PROX1; VR3, VEGFR-3. Scale bars correspond to $100 \mu\text{m}$. The data represent wholemount stainings from six (A, E), five (B, F), six (C, G, K, L), three (D, H), and six (I, J) analyzed animals.

deletion of VE-cadherin (Appendix Fig S6D–F). These degenerative changes were observed in 100% of the lacteals of VE-cadherin-deleted mice.

Given the massive deterioration of the mesenteric lymphatic vessels, we wondered if the lacteal lymphatics deteriorated as a consequence of the demise of mesenteric collecting vessels. We therefore induced the deletion of VE-cadherin in neonates by two doses of 4-OHT and monitored in parallel intestinal and mesenteric lymphatics 5 days, 2, 4, and 6 weeks later (Figs EV3A–E and

EV4A–E). Five days after Tamoxifen treatment, intestinal lymphatics appeared unaffected (Fig EV3B and Appendix Fig S7A–D), while mesenteric collectors already showed clear changes including valve deterioration, vessel constriction, and the outgrowth of atypical sprouts (Fig EV4B). Mesenteric lymph vessels deterioration was progressive (Fig EV4C and D) and culminated 6 weeks after induction in the appearance of the already described sheets or islands of LECs (Fig EV4E, yellow asterisks). Lacteal fragmentation was the first detectable defect in intestinal lymphatics 2 weeks after

Figure 4. VE-cadherin is indispensable for the maintenance of mesenteric lymphatic vessels at all ages.

A–F Deletion of VE-cadherin was induced in mice at various age [4 weeks (A), neonate (B), 6 month (C, D, E), or 1 year (F)] by either two applications of 4-OHT at days P2 and P4 (neonates) or three applications of Tamoxifen at 2-day intervals via oral gavage (all other ages). The mesentery was prepared as indicated 4, 6, 8, or 11 weeks later and the wholemount preparations were immunostained for the proteins specified in color (left). (B) Inset in top panel shows a magnification of the area outlined by the white dashed line (for further magnification see Fig EV1A and B). Arrows indicate the previous position of lymphatic valves; white arrowheads indicate PECAM1⁺ capillaries supplying the intestinal fat tissue lining the vessels (magnified in Fig EV1C); yellow arrowheads denote island of LECs that display intracellular VEGFR-3 staining. (C) Arrows denote LECs that have converted from growth as tubes to sheets; white arrowheads, thinning of lymphatic vessels; yellow arrowheads, aberrant sprouting of LECs. The area outlined by a white box is depicted magnified in (D) and provides details on LECs growing as a sheet of cells. (E) Formation of ectopic sprouts from the valve area of degenerating lymphatic vessels. (F) Fully deteriorated lymphatic vessel with emanating sprouts that initiate sheet-like growth. PEC1, PECAM1; PRX1, PROX1; VR3, VEGFR-3. Scale bars = 100 μ m. The data represent $n = 5$ (A), $n = 5$ (B), $n = 3$ (C–E), and $n = 6$ (F) animals.

Tamoxifen application (Fig EV3C and Appendix Fig S7A) and hence lagged behind the mesenteric defects. At 4 weeks, we observed the first appearance of large bloated villi, leading to the fully fragmented and distended villi at 6 weeks (Fig EV3E, and Appendix Fig S7B and D). During the first six postnatal weeks, intestinal villi grow in length. Surprisingly, despite their defective structure, VE-cadherin-deficient lacteals still underwent this growth (Fig EV3 and Appendix Fig S7C).

Taken together, intestinal lacteals react with fragmentation and bloating in a distinct stereotypic way to the loss of VE-cadherin, which judged by the time of its appearance may be secondary to defects of the mesenteric collecting lymphatics.

LECs from different vessel beds react with unexpectedly heterogeneous changes in the expression of junctional proteins to VE-cadherin deletion

To further analyze the surprisingly different response of dermal, intestinal, and mesenteric lymphatic vessels to VE-cadherin deletion, we isolated LECs by FACS 5 weeks after induction of deletion (see Appendix Fig S8 and Materials and Methods section) and tested for a potential compensation of VE-cadherin loss by qRT–PCR using specific TaqMan probes for junctional proteins. Due to the limited

yields obtained, we pooled LECs from lymph capillaries and collecting vessels of each vessel bed for qRT–PCR analysis.

Compared to Tamoxifen-treated, VE-cadherin-expressing control samples, we detected threefold or higher upregulation of the genes coding for *Pecam1*, *Jam1*, *Jam3*, *Zo1*, and *Claudin-5*, but not *Cdh2*, in VE-cadherin-deleted dermal LECs (Fig EV5A). In whole-mount immunostainings of the skin, we were able to confirm protein upregulation of Claudin-5, JAM-A, and ZO-1 in initial lymphatics (Appendix Fig S9, white boxed bottom insets in B, D, F and I, J, K). Only upregulation of Claudin-5 reached statistical significance (Appendix Fig S9I). Interestingly, while these tight junction-associated proteins were strictly confined to the button part of the junctions in control LECs, their distribution remained discontinuous in VE-cadherin-deleted LECs, but extended more into the interbutton regions (most pronounced for Claudin-5 and JAM-A, see yellow boxed top inset in Appendix Fig S9B, D and F). In contrast, we detected no expression differences in collecting vessels and valves that were however characterized by already very high expression of the respective proteins (Appendix Fig S9A–F).

In contrast, mesenteric LECs, which are mainly comprised of cells from collecting vessels, displayed distinctly different expression changes compared to dermal LECs after VE-cadherin loss. We

Figure 5. Deletion of VE-cadherin results in fragmentation and distension of the intestinal lacteals.

A–E Deletion of VE-cadherin was induced in mice at various ages [neonatal (C), 16 weeks (D) or 1 year (E)] by either two applications of 4-OHT at days P2 and P4 (neonates) or three applications of Tamoxifen at 2-day intervals via oral gavage (all other ages). Control mice of the same age were subjected to an identical regimen (A, B). Intestinal wholemount preparations of the jejunum and ileum were generated 5–8 weeks after Tamoxifen induction and immunostained for the indicated proteins, scale bars = 100 μm. The data represent wholemount stainings from three (D), five (B, E), and six (A, C) animals analyzed for each genotype.

found *Claudin-5* threefold and *Cdh2* more than tenfold upregulated. Expression of the other tested junctional molecules was not different from controls and *Occludin* expression was even reduced (Fig EV5B). While we never detected N-cadherin expression in LECs of control tissues nor in dermal and intestinal VE-cadherin-deleted LECs, it was weakly detectable in mesenteric LEC sheets. However, N-cadherin immunoreactivity remained largely cytoplasmic (Appendix Fig S9G and H). Immunostaining also revealed reexpression of Lyve1 (Appendix Fig S9G and H inset). Finally, in intestinal LECs, which are comprised of lacteals and collecting vessels, we found no upregulation of junctional protein expression. Surprisingly, PECAM1 mRNA was even downregulated (Fig EV5C).

Taken together, the relative insensitivity of dermal LECs to VE-cadherin deletion may at least in part be due to compensatory upregulation of junctional proteins in the initial lymphatic vessels, which was not observed in the lymph vessels of intestine and mesentery.

Mesenteric LECs selectively respond with hyperplasia to the loss of VE-cadherin

The massive expansion of mesenteric LECs after VE-cadherin deletion suggested strongly increased cell proliferation. We tested this assumption by determining EdU incorporation over 24 h in VE-cadherin-deleted mice. We neither detected EdU/PROX1 double-positive nuclei in intestine nor dermis of VE-cadherin-deleted or

control mice (not shown). Also mesenteric LECs of Tamoxifen-treated control mice had not incorporated EdU (Fig 6A and B), while approximately 13% of VE-cadherin-deficient mesenteric LECs had incorporated EdU (Fig 6C and D), demonstrating a strong selective hyperplasia of this vessel bed (Fig 6E). Despite the lack of EdU-positive lacteals after 24-h incorporation, Prox1-staining revealed an increased number of nuclei particularly in strongly distended lacteals after several weeks of VE-cadherin deletion (Appendix Fig S5B and E). This demonstrated a limited proliferative response, which was likely too mild to be detected by 24 h EdU incorporation.

To further explore the molecular basis of the mesenteric hyperplasia, we probed for phosphorylation of the major lymphatic growth factor VEGFR-3 on tyrosines 1230/1231, which are phosphorylated following ligand stimulation. Phospho-VEGFR-3 was only detectable in mesenteric LECs of VE-cadherin-deleted mice (Fig 6F–I), raising the possibility of increased VEGF-C signaling activity compared to, e.g., skin. We addressed this issue by testing lysates of dermis, intestine, and mesentery from wild-type mice for tissue-specific VEGF-C and VEGF-D expression using qRT-PCR. Indeed, we detect roughly twofold higher levels of both growth factors in mesentery compared to skin (Fig 6J). While intestinal VEGF-C levels were mildly increased, VEGF-D was virtually absent in this organ. Within the mesenteric LEC layer, cells that no longer showed surface expression of VEGFR-3 upregulated VEGFR-2, presumably as a compensatory mechanism (Fig 6K–L) exposing a second potentially proliferative receptor.

Figure 6. Deletion of VE-cadherin leads to selectively increased proliferation of mesenteric LECs.

- A–D Deletion of VE-cadherin was induced in adult mice (13 weeks) and EdU incorporation into PROX1-positive cell nuclei was determined 6 weeks after Tamoxifen administration. Shown are mesenteric wholemount stainings prepared from control (A, B; $n = 3$) or VE-cadherin-deleted mice (C, D; $n = 3$). (B) and (D) show a magnification of the area outlined by the white dashed line in (A) and (C). Antigens shown in addition to the EdU incorporation are depicted in color on the left side of the first panel. PEC, PECAM1; PRX1, PROX1. Scale bars = 100 μm .
- E Enumeration of EdU-positive LECs. The measurements were obtained from three animals and nine confocal stacks per biological group. PROX1-positive nuclei were counted using the particle analysis tool of Fiji. Co-localization of PROX1 and EdU was determined manually in each confocal plane, using the cell counting tool of Fiji. The data represent mean $\pm$ SD. **** $P \leq 0.0001$. Two-tailed unpaired Student's *t*-test.
- F–I Aberrantly enhanced VEGFR-3 phosphorylation in VE-cadherin-deficient mesenteric LECs of 6 weeks old mice that had received 4-OHT injections at P2 and P4. Shown are wholemount immunostainings for the antigens shown on the left in color. pVR3, pVEGFR-3, PRX1, PROX1; PEC1, PECAM1. (G) and (I) show a magnification of the area outlined by the white dashed box in (F) and (H). Scale bars = 50 μm . The data are representative for 6 (*Cdh5^{lox/lox}*) and 5 (*Cdh5^{lox/lox}; Prox1-CreER²*) analyzed animals from two litters.
- J Dermal, mesenteric and intestinal tissue pieces of 15-week-old control (*Cdh5^{lox/lox}*) mice were dissociated and total RNA was extracted immediately. For reverse transcription, 2.0 μg of total RNA was used for each tissue type. Expression of lymphangiogenic factors VEGF-C and VEGF-D was determined by quantitative real-time (qRT)-PCR using specific TaqMan probes. Samples were measured in triplicate and diagrams are representative for three independent experiments. Relative expression ($\Delta\Delta C_t$) of target genes was normalized to the control transcript UBC.
- K, L Loss of VEGFR-3 surface expression on mesenteric LECs growing in cell sheets was associated with concomitant upregulation of VEGFR-2, scale bars = 50 μm . The data are representative for four analyzed animals from two litters. VR2, VEGFR-2; VR3, VEGFR-3, PRX1, PROX1.
- M, N Expression of the transcriptional co-activators YAP and TAZ was analyzed in mesenteric wholemount preparations 8 weeks after induction of VE-cadherin deletion at 1 year of age by three doses of Tamoxifen. Respective antigens depicted are indicated on the top of the panels in color. The highly related molecules YAP and TAZ were both recognized by the antibody used and are therefore collectively abbreviated Y/T. Scale bars = 100 μm .
- O PROX1⁺ nuclei (red in panels M and N) were classified into YAP/TAZ low-, intermediate-, and high-expressing cells, according to their relative YAP/TAZ immunoreactivity in wild-type and VE-cadherin-deleted mice. The data were obtained from three animals per biological group. PROX1-positive and YAP/TAZ-positive nuclei were counted manually. The pie charts represent mean values.

The YAP/TAZ signaling pathway regulates organ size via cell proliferation and is under direct control of geometrical and mechanical strains that impact on the cytoskeleton. In our TaqMan qRT-PCR analysis, we noted differential expression of the transcriptional regulators YAP and TAZ by LECs. While dermal LECs responded to VE-cadherin deletion with upregulation of both YAP and TAZ, mesenteric LECs only upregulated TAZ. In intestinal LECs, both transcriptional regulators were downregulated, possibly caused by vessel deterioration due to downstream blockade of lymph flow (Fig EV5).

To this end, we verified YAP/TAZ upregulation by immunostaining of the mesentery. We identified so far one antibody suitable for wholemount staining, which however only provided a specific signal in mesenteric wholemount preparations but not intestine or skin and did not distinguish between YAP and TAZ. In mesenteric control preparations (Fig 6M), we detected only low YAP/TAZ expression in PROX1⁺ LECs (90% of the cells). Due to the low expression level, nuclear localization of YAP/TAZ was negligible. Following VE-cadherin deletion, we noted a strong upregulation of YAP/TAZ immunoreactivity in the cytoplasm and nucleus of LECs growing in sheets atop the mesenteric mesothelium (Fig 6N) with 68% of Prox1⁺ cells expressing intermediate and 18.7% high YAP/TAZ levels (Fig 6O).

These results suggest that mesenteric LECs specifically develop into hyperplastic cell layers in response to VE-cadherin loss. Initial proliferation and activation of the VEGFR-3 pathway may be triggered by increased availability of the lymphangiogenic growth factor VEGF-C. The ensuing mechanical strain may contribute to upregulation of the YAP/TAZ transcriptional regulators that are likely supporting the proliferative response.

VE-cadherin-deficient LECs in culture show altered cytoskeletal architecture and reduced cell–cell adhesion

To obtain sufficient material for further cell biological and biochemical analyses, we isolated PECAM⁺, LYVE1⁺, PDPN⁺ LECs from

Tamoxifen-treated mice for *in vitro* culture (Appendix Fig S10A). To this end, we used primary dermal LEC cultures (pdLECs) for our analysis, which reproducibly developed into homogeneous, primary cultures. Mesenteric and intestinal LECs *in vitro* often lost lymphatic marker expression and adopted a heterogeneous morphological appearance.

Western blot analysis revealed that genetic deletion of VE-cadherin resulted in a significantly reduced expression of the entire adhesion complex including β -catenin, α -catenin, and plakoglobin (Appendix Fig S10B and C). In contrast to dermal LECs *in vivo*, cultured pdLECs upregulated N-cadherin, which localized to cell–cell contacts (Appendix Fig S10D and E). Also different from the intact vessel bed, pdLECs upregulated TAZ, but not YAP transiently at intermediate cell density. In this respect, VE-cadherin-deleted pdLECs shared properties of mesenteric VE-cadherin-deleted LECs. Enhanced TAZ expression coincided with elevated TAZ-mediated transcriptional activity as indicated by a robust upregulation of the target genes *Trp73*, *Cyr61*, and *Ctgf* (Appendix Fig S11A and B).

Culture of equivalent numbers of VE-cadherin-expressing and VE-cadherin-deleted pdLECs revealed distinct differences. Already 24 h after seeding, VE-cadherin-expressing pdLECs clustered in small islands, characteristic for endothelial cells, and had initiated PECAM1⁺ cell contacts (Appendix Fig S11C white arrowheads). In contrast, VE-cadherin-deficient pdLECs covered a larger surface and displayed reduced cell–cell contact formation (Appendix Fig S11D), which became more pronounced after 3 (Appendix Fig S11E and F) or 7 days in culture (Appendix Fig S11G and H). VE-cadherin-expressing cells grew to a dense confluent cell layer with robust cell contacts (Appendix Fig S11E and G), while VE-cadherin-deleted cells retained their significantly larger surface, often were binucleated and even at the highest cell density displayed broken cell–cell contacts (Appendix Fig S11F and H). In particular at intermediate and high density, VE-cadherin-deleted pdLECs displayed a pronounced ring of circumferential actin (Appendix Fig S11E–H). Hence, we wondered if

Figure 6.

the observed reduced junction formation was the consequence of reduced adhesive strength between cells, leading to cell retraction during permeabilization and staining. To test this possibility, we

stained cultures with directly labeled anti-PECAM1 antibodies, performing only brief wash steps in the presence of 1 mM Ca^{2+} . Under these conditions, overtly extensive gaps between cells were

no longer observed, albeit contact formation appeared still less tight with many junctional gaps compared to control cultures (Appendix Fig S12). Immunostaining of pdLECs confirmed higher YAP/TAZ expression levels after VE-cadherin deletion. In 3-day cultures, nuclear localization of YAP/TAZ was increased; however, similar to mesenteric LECs, the change in the ratio of nuclear to cytoplasmic localization was not significant (not shown).

Taken together, VE-cadherin-deficient pdLECs were characterized by reduced intracellular adhesion, cell density-independent enhanced circumferential actin, and upregulation of the YAP/TAZ transcriptional co-activators.

Reduction of focal adhesion formation and higher mobility of cultured VE-cadherin-deficient pdLECs

The altered cytoskeletal arrangements in VE-cadherin-deficient pdLECs appeared to be associated with differences in cell spreading. Cell spreading is largely driven by focal adhesions that form dynamic anchor points connecting the cytoskeleton to sites of integrin activation. We visualized focal adhesions by co-staining actin in 3-day cultures, with either vinculin or the focal adhesion kinase FAK (Fig 7A and B). At comparable cell density, cytoplasmic FAK and vinculin immunoreactivity were elevated in VE-cadherin-deficient pdLECs, yet, the number of focal adhesions per cell was approximately 35% reduced (Fig 7C and D). Because focal adhesions are also central elements regulating cells dynamics and movement, we wondered if the reduced number of focal adhesions in VE-cadherin-deleted pdLECs would impact on their motility and change their capacity to close the denuded zone in a scratch wound assay. Indeed, VE-cadherin-deficient pdLECs displayed an increased capacity to cover a scratch wound, indicative of increased motility. During the 24 h assay period, the effect was most pronounced from 16 h onwards (Fig 8A and B).

In summary, despite elevated cytoplasmic FAK and vinculin levels, VE-cadherin-deleted pdLECs displayed a reduced number of focal adhesions, which likely contributed to their increased motility.

Discussion

Here, we have investigated the requirement of the major endothelial adhesion molecule VE-cadherin for development, maintenance, and function of lymphatic vessels, by deleting VE-cadherin from LECs. While in the absence of VE-cadherin the primitive lymphatic vessel network still formed, we report a failure to remodel and mature, causing edema and fetal death (Fig 1). In contrast, postnatal deletion revealed unexpected differences in the requirement of specific lymphatic vessel beds for VE-cadherin. Dermal lymph vessels were only susceptible to VE-cadherin loss upon induction of deletion in neonates, but did not develop significant alterations when VE-cadherin was deleted at later ages (Fig 3). Very differently, mesenteric lymph vessels were exquisitely sensitive and selectively proliferated in response to VE-cadherin loss at all ages (Fig 4). Starting from the lymphatic valves, collecting mesenteric vessels deteriorated, giving rise to vessel fragments and ultimately planar sheets of LECs (Figs 4, EV1, EV2, and EV4). Similarly and possibly related, intestinal lacteals fragmented or became extremely dilated irrespective of the age at VE-cadherin deletion (Figs 5 and EV3).

Global deletion of VE-cadherin results in a developmental arrest around E9.5 due to massive vascular malformations (Carmeliet *et al*, 1999; Gory-Faure *et al*, 1999), precluding the analysis of the formation of the first lymph vessels. Inducible postnatal endothelial deletion caused rapidly appearing, dominant blood vessel permeability defects (Frye *et al*, 2015). Interestingly, blood vessels in different organs were differentially affected. Heart and lung permeability was increased already 7 days after induction, while blood vessels in skin and brain remained unaffected. Their independence of VE-cadherin expression suggests an intrinsically higher stability of the dermal and brain vascular beds, possibly due to stronger matrix and mural cell stabilization. Despite increased permeability in lung and heart, VE-cadherin-deleted blood vessels remained ultrastructurally intact (Frye *et al*, 2015). Formation of inconspicuous interendothelial junctions had already been reported in VE-cadherin null embryos (Carmeliet *et al*, 1999; Gory-Faure *et al*, 1999). The absence of notable edema within the first 2 weeks after endothelial VE-cadherin deletion indicated that the lymphatic system was still functional and able to balance the increased plasma leakage. Of note, 3–4 weeks after induction of VE-cadherin deletion, Frye *et al* (2015) also noted signs of dermal edema. This was interpreted as a possible consequence of progressive heart insufficiency; however, it may also have been indicative of the onset of lymph vessel defects.

Our developmental analysis was dominated by appearance of massive edema in the last third of gestation. Because PROX1 is also expressed in the developing heart, we tested if PROX1-driven VE-cadherin deletion would result in cardiac defects. In sections, but also wholemount staining, heart development appeared comparable between VE-cadherin-deleted and control animals (Appendix Figs S1 and S2 and Videos S1 and S2), suggesting that indeed edema formation was due to malfunctioning lymphatic vessels.

After cessation of growth in adult mice, dermal lymph vessels became resistant to VE-cadherin loss (Fig 3), suggesting a stabilizing vessel maturation effect. The highly specialized button junctions in mature, dermal lymphatic capillaries were retained in the absence of VE-cadherin (Fig 2), raising the question if other adhesion molecules compensated the loss of VE-cadherin. Indeed, we found several tight junction proteins including JAM-A, ZO-1, and Claudin-5 upregulated specifically in dermal initial lymphatics (Fig EV5 and Appendix Fig S9, insets). These proteins codistribute with VE-cadherin at cell–cell contacts (Baluk *et al*, 2007), and interestingly, while their discontinuous distribution was largely retained, a tendency to spread more into the interbutton space was observed in the absence of VE-cadherin (Appendix Fig S9, yellow boxed insets). We did not detect altered expression in collector vessels and lymphatic valves, which already express very high levels of junctional proteins.

In agreement with studies where VE-cadherin had been deleted in blood endothelial cells *in vivo* (Carmeliet *et al*, 1999; Gory-Faure *et al*, 1999; Frye *et al*, 2015), we never found N-cadherin expressed in dermal or intestinal LECs. As reported for VE-cadherin-deficient cultured primary endothelial cells (Frye *et al*, 2015) and cell lines (Giampietro *et al*, 2012), we observed N-cadherin expression in pdLECs *in vitro* (Appendix Fig S10D and E). In our model, N-cadherin upregulation *in vivo* was exclusively confined to atypical sheet-like cell layers of mesenteric LECs,

Figure 7. Loss of VE-cadherin from lymphatic endothelial cells results in increased circumferential actin and a reduced number of focal adhesions.

A, B Primary dermal LECs were isolated as described in Appendix Fig S8A. PdLECs were used for up to four passages and control and VE-cadherin-deleted pdLECs had been cultured for the same number of passages. Cells were seeded at a density of 2.5×10^4 cells on gelatine-coated 15 mm glass coverslips and co-stained after 3 days of culture for the actin cytoskeleton (Phalloidin) and either vinculin (A) or FAK (B) for the visualization of focal adhesions. PROX1 staining in (A) identifies LECs, and nuclei in (B) were counterstained with Hoechst. Scale bars correspond to 50 μ m.

C, D Bar diagrams show the number of focal adhesions/cell, which were enumerated using Image J from the vinculin and FAK stainings shown in (A and B). Data are mean values + SEM derived from three cell lines and nine viewfields of $256 \times 256 \mu$ m per biological group. *** $P \leq 0.001$. Two-tailed unpaired Student's *t*-test.

Figure 8. Enhanced collective cell migration in VE-cadherin-deficient lymphatic endothelial cells.

A Collective cell migration of VE-cadherin-deficient and control LECs was assessed in wound scratch assays. LECs were grown up to four passages and seeded at a density of 5×10^4 cells on fibronectin-coated 8-well μ -chamber slides (growth area 0.22 cm^2); scale bars = $500 \mu\text{m}$.
 B The area of the zone denuded from cells is depicted in the curve diagram showing mean values $\pm$ SD of three individual experiments conducted in quadruplets.
 * $P \leq 0.01$. Two-tailed unpaired Student's t-test.

where N-cadherin was, however, not localized to junctions (Appendix Fig S9G and H). The aberrant proliferation (Fig 6) and morphogenetic behavior of these cells but also their loss of the endothelial marker VEGFR-3, in combination with its diffuse distribution, raise the possibility that N-cadherin upregulation reflects more a loss of endothelial identity than a process compensating VE-cadherin deficiency. While LYVE 1 became reexpressed, PECAM1 was homogeneously distributed at contacts, but gaps between LECs indicated insufficient adhesive force (Fig EV2D). In initial lymphatics, PECAM1 is restricted to the junctional regions between buttons, which form portals or primary valves for fluid, solutes, and leukocyte entry (Baluk *et al*, 2007; Pflücke & Sixt, 2009). This spatially separated distribution in combination with the low PECAM1 expression in lymphatic vessels compared to arteries and veins (Hägerling *et al*, 2013; Fig 2) may help LECs to limit adhesion and allow easy access of cells and fluids. The molecular mechanisms responsible for button formation and the discontinuous PECAM1 distribution in initial lymphatics remain largely unknown. Genetic deletion or inhibition of Ang2 prevents formation of button-type junctions, demonstrating involvement in a pathway that regulates lymphatic junctions (Zheng *et al*, 2014). Tie receptors had previously already been implicated in the maintenance of lymphatic junctions as suggested by the development of chylous ascites and peripheral lymphedema (Gale *et al*, 2002) and the failure to mature and develop a hierarchical lymph vessel system (Dellinger *et al*, 2008) in Ang2-deficient mice. Postnatal Ang2 inhibition results in chylous ascites due to junctional defects in mesenteric collector vessels, where Ang2 inhibition interferes with valve maturation and maintenance of the transcription factor FOXC2 in valve forming areas (Zheng *et al*, 2014).

After VE-cadherin deletion, lymphatic valves in dermal vessels remained intact for up to 11 weeks (Fig 3I and J and not shown), but rapidly deteriorated in mesenteric vessels (Figs 4 and EV4). Valves are of central importance for the function and preservation

of collecting lymph vessels. The transcription factor FOXC2 couples oscillatory flow to valve specific gene expression, making it indispensable for development and maintenance of lymphatic valves (Sabine *et al*, 2012). FOXC2 has also been shown to directly influence the distribution of VE-cadherin and the associated actin cytoskeleton in response to flow (Sabine *et al*, 2015) and is therefore an excellent candidate for an upstream regulator of VE-cadherin. Postnatal, LEC-specific FOXC2 deletion causes valve deterioration, chylous ascites, and chylothorax, resulting from loss of junctional integrity and formation of atypical sprouts from deteriorating valves (Sabine *et al*, 2015). We observed highly reminiscent changes in the mesentery of VE-cadherin-deleted mice, namely deterioration of and sprouting from lymphatic valves (Fig 4C yellow arrowheads). This supports the idea that FOXC2 maintains valve formation at least partially via maintenance of VE-cadherin junctional expression. Valves of mesenteric vessels may be particularly susceptible to VE-cadherin loss and the associated weakening of cell–cell adhesion, because of the high mechanical stress exerted on the valve area during contraction of collector lymphangions.

Besides mesenteric collecting vessels, intestinal lacteals were sensitive to VE-cadherin loss independent of the age at induction; however, the LEC response was distinctly different. Like the initial dermal lymphatics, lacteals are largely formed by oak leaf-shaped LECs (Appendix Fig S6A–C). After VE-cadherin deletion, lacteals either fragmented into spheres or became massively dilated. In both cases, the oak leaf shape of LECs was lost and junctions deteriorated. However, despite intense filopodia extension, LECs always remained integrated into lumenized structures. Highly distended lacteals reacted with a limited proliferative response (Appendix Fig S5), which may have been caused by increased mechanical stretch due to obstruction of the mesenteric collectors. Such mechano-stimulation of LEC proliferation has been shown to be VEGFR-3-mediated and independent of VEGF-C levels, but

dependent on YAP/TAZ nuclear localization (Planas-Paz *et al*, 2012; Neto *et al*, 2018). Supporting the assumption that intestinal defects were caused by the demise of mesenteric collectors, mesenteric defects temporally preceded fragmentation of lacteals (Figs EV3 and EV4).

Only mesenteric LECs reacted to VE-cadherin deletion with massive hyperplasia and a progressive loss of morphogenetic properties, ultimately leading to growth as cell sheets on the mesothelium. While mechano-stimulation alone was unlikely to have caused such massive proliferation, elevated VEGF-C and VEGF-D expression levels, compared to skin and intestine, likely contributed to the continued mesenteric hyperplastic response (Fig 6J) and would explain the high levels of active phosphorylated VEGFR-3 on VE-cadherin-deficient LECs. Mesenteric LECs that had lost VEGFR-3 surface expression, but upregulated VEGFR-2, which has previously been reported for retinal blood vessels (Zarkada *et al*, 2015). Proliferation of ECs in response to VEGF-A is independent of YAP/TAZ (Neto *et al*, 2018). Still YAP/TAZ can become activated downstream of VEGF-A or in response to mechanic signals and then provide the optimal transcriptional program for VEGF-driven endothelial proliferation (Wang *et al*, 2017; Neto *et al*, 2018).

Our results suggest that mechanical load peaks at the valves of mesenteric collecting vessels, leading to disruption of LEC contacts and to formation of aberrant sprouts in the absence of the adhesive force of VE-cadherin. The mechanic stress during this process would promote nuclear localization of YAP/TAZ, ensuring optimal conditions for subsequent proliferative signaling through the VEGFR-3 in response to the elevated mesenteric VEGF-C and VEGF-D levels. Remarkably, TAZ has previously been identified as required for the hyperproliferation following FOXC2 deletion in LECs (Sabine *et al*, 2015).

Under physiological conditions, contact inhibition of growth is mediated by stable cell–cell junctions and hence VE-cadherin has been implicated in this process. One mechanism how VE-cadherin can directly impact on cell proliferation is via regulation of YAP/TAZ. In confluent cells, YAP/TAZ are prevented from nuclear entry through multiple mechanisms, e.g., complex formation with angiomotins (Zhao *et al*, 2011). Of particular relevance here is the reported competition between the actin-signaling adaptor EPS8 and YAP for binding the VE-cadherin–catenin complex via α -catenin (Giampietro *et al*, 2015). In this model, in cells with immature junctions, YAP freely enters the nucleus and activates transcription. At the same time, EPS8 binds the VE-cadherin– α -catenin complex and masks the YAP binding site. Upon confluence and development of stable junctions, VE-cadherin-mediated AKT-activation leads to YAP-phosphorylation, subsequent 14-3-3 binding and sequestration to the junctional VE-cadherin complex. However, complex formation between the YAP-related protein TAZ and the VE-cadherin–catenin complex has so far not directly been demonstrated (Giampietro *et al*, 2015).

For *in vitro* analysis, we used pdLECS, which had a number of commonalities with VE-cadherin-deficient mesenteric LECs, including continuous proliferation in a monolayer and the selective upregulation of N-cadherin and TAZ. The later may result from mechano-transduction caused by altered cell and cytoskeletal architecture enforced by growth as a cell layer.

As outlined above, properties observed in mesenteric LECs after loss of VE-cadherin were partially shared by pdLECs, which we used for biochemical and cell biological assays. Besides an increase in circumferential actin of VE-cadherin-deficient pdLECs (Fig 7 and Appendix Fig S11), we detected a decrease of approximately 30% in focal adhesions per cell compared to controls (Fig 7). Cell-ECM traction has been demonstrated to be directly correlated with cell–cell tension (Maruthamuthu *et al*, 2011; Sehgal *et al*, 2018). In addition, the reduced focal adhesions may indicate increased actin remodeling and the activation of TAZ contributed to the increased motility (Fig 8) of VE-cadherin-deleted pdLECs. Similar changes in the actin cytoskeleton have been described for the endothelial deletion of afadin, which remarkably affected only lymphatic LECs, causing a reduction of junctional VE-cadherin and junctional dissolution (Majima *et al*, 2013).

We show here remarkable similarities between blood and lymph vessels like the insensitivity of both vessel types in the dermis to VE-cadherin loss. But we also found unexpected differences, like the sensitivity of mesenteric lymph vessels to VE-cadherin deletion, which caused selective hyperplasia and the rapid loss of morphogenetic properties by mesenteric LECs. These results add to the growing evidence that despite the overall similar molecular makeup of intercellular junctions in blood and lymphatic endothelial cells, both endothelial cell types possess very different personalities.

A better understanding of this specific behavior will necessitate more insights into the interactions of LECs with matrix and mural cells, but also a better understanding of the different mechanical strains, impacting on the different vessel beds. The resulting insights will be of importance for the long-standing quest to modulate lymph vessel growth and function.

Materials and Methods

Antibodies and reagents

The following reagents and antibodies were used: Hoechst 33342 (Life Technologies), rabbit polyclonal anti-human Prox1 (ReliaTech, 102-PA30), goat polyclonal anti-human Prox1 (R&D Systems, AF2727), goat polyclonal anti-mouse VEGFR-3 detecting ligand stimulated phosphorylation of Tyr 1230 and 1231 (R&D Systems, AF743), rat monoclonal anti-mouse PECAM-1 [clone 5D2.6 and clone 1G5.1 (Wegmann *et al*, 2006)], goat polyclonal anti-mouse Lyve1 (R&D Systems, AF2125), rat monoclonal anti-mouse Lyve-1 (clone ALY7) coupled to Alexa 488 (eBioscience), sheep polyclonal anti-mouse N-Cadherin (R&D Systems, AF6426), rabbit polyclonal anti-mouse ESAM1 [VE-19, (Nasdala *et al*, 2002)], rabbit polyclonal anti-mouse VE-cadherin [VE-42, (Broermann *et al*, 2011)], rabbit polyclonal anti-mouse YAP/TAZ (CST, 8418), mouse monoclonal anti-human α -SMA coupled to Cy3 (Sigma), Phalloidin Alexa 488 (Life Technologies, A12379), rat monoclonal anti-mouse CD31 (BD Bioscience, clone MEC 13.3) coupled to APC (BD Bioscience), hamster monoclonal anti-mouse Podoplanin (clone 8.1.1) coupled to PE (BD Bioscience), mouse monoclonal anti-mouse vinculin (Abcam, ab18058), mouse monoclonal anti-mouse FAK (BD Biosciences, 610088), rabbit polyclonal anti-mouse Claudin-5 (Thermo

Fisher Scientific, 34-1600), rabbit polyclonal anti-mouse ZO-1 (Thermo Fisher Scientific, 40-2200), rabbit polyclonal anti-mouse JAM-A (VE 65, D. Vestweber), rabbit polyclonal anti-human pVEGFR-3 (Tyr1230/1231) (Cell Applications, CY1115), mouse monoclonal anti-mouse Troponin T cardiac isoform (Thermo Fisher Scientific, MA5-12960), rabbit monoclonal anti-mouse Desmin XP (CST, clone D93F5), mouse polyclonal anti-mouse alpha-Catenin (BD Bioscience, 610194), mouse monoclonal anti-mouse beta-Catenin (BD Bioscience, clone14), mouse monoclonal anti-mouse Plakoglobin (BD Bioscience, 610254), and rabbit polyclonal anti-mouse beta-tubulin (BD Bioscience, clone 9F3). Secondary Alexa-dye-labeled antibodies were obtained from Life Technologies and peroxidase-labeled secondary antibodies from Jackson ImmunoResearch.

Mouse strains

Wild-type, VE-cadherin flox/flox (Frye *et al*, 2015), and *Prox1-CreER^{T2}* (Bazigou *et al*, 2009) mouse embryos and adult mice of C57Bl/6 genetic background were analyzed at different ages (5 days–1 year) and developmental stages between E12.5 and E15.5. Control mice were matched by genetic background, sex, and age. Embryonic staging was determined by the day of the vaginal plug (E 0.5). All animal experiments were approved by the responsible animal committees at the sites of animal experimentation.

Induction of Cre-mediated deletion of conditional KO mice

For *in vivo* induction of Cre recombinase activity, adult mice were either injected intraperitoneally with 50 µg/g Tamoxifen at three consecutive days with additional injections at days 5 and 8 or administered three applications of Tamoxifen by oral gavage at 2-day intervals. Adult mice were analyzed 2–11 weeks after induction. Pregnant mice were treated with 50 µg/g Z-4-hydroxytamoxifen (4-OHT), starting at developmental day E10.5. For induction in newborn mice, 2.5 µg 4-OHT was injected subcutaneously into the neck at P2 and P4.

Isolation and culture of murine lymphatic endothelial cells

Primary dermal lymphatic endothelial cells (pdLECs) were isolated from the tail of wild-type and mutant mice and cultured as previously described (Frye *et al*, 2015). For cultivation of pdLECs, preselected batches of ECGS (BT-203, Alfa Aesar) that favor the expansion and maintenance of lymphatic endothelium were used.

Isolation of murine dermal, mesenteric, and intestinal LECs for direct gene expression analysis

Lymphatic endothelial cells were isolated from the tail, mesentery, and intestine of VE-cadherin-deleted and control mice. Tissues were mechanically dissociated using the GentleMACS™ Dissociator (Miltenyi) and further enzymatically digested in 1 mg/ml Collagenase A solution for 10–15 min at 37°C. Single cells were collected and stained for 15 min with dye-conjugated primary antibodies diluted in FACS buffer (3% fetal calf serum in PBS). After antibody staining, the cells were analyzed and sorted on a FACS Aria IIIu cell

sorter (BD Biosciences) using a 85 µm nozzle. Cells were gated based on FSC and SSC to exclude cellular debris and doublets, and on DAPI exclusion for viability. Due to the limited cell numbers obtained from the different vessel beds, we did not attempt to individually sort LECs from capillaries and collecting vessels for qRT-PCR. LECs were sorted on the basis of CD31⁺, Podoplanin⁺ double positivity. While this strategy should comprise both populations, we noted that a fraction of LYVE1⁺ and Podoplanin⁺ double positive LECs (likely from capillaries) were not included in the CD31⁺, PDPN⁺ gate. To optimize our yield, both populations were pooled. For total RNA isolation, the cells were directly sorted into phenol/guanidinium-isothiocyanate buffer (PEQLAB Biotechnologie GmbH). Data analysis was done using FlowJo software (LLC).

Preparation of whole tissue RNA lysates for determination of VEGF-C and VEGF-D expression levels

Tissue pieces were isolated from the tail, mesentery, and intestine of 15-week-old control mice and mechanically dissociated using the GentleMACS™ Dissociator (Miltenyi). For subsequent isolation of total RNA, the tissue was directly dissociated in phenol/guanidinium-isothiocyanate buffer (PEQLAB Biotechnologie GmbH).

In vivo EdU-based proliferation assays

EdU labeling of proliferative cells was performed using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific). In brief, mice were intraperitoneally administered 4 mg/kg (body weight) EdU 24 h prior to euthanasia and tissue preparation. Tissues were fixed for 4 h in 4% PFA/PBS, washed in PBS, permeabilized (0.5% Triton X/PBS), and blocked (3% BSA/PBS). Thereafter, tissues were stained for indicated antigens, and secondary antibodies were Alexa-dye conjugated (Life Technologies). Enumeration of EdU-positive LECs was obtained from confocal stacks using the particle analysis tool and the cell counting tool of Fiji.

Immunohistochemistry

Tissues were prefixed for 2–4 h in 4% PFA/PBS, washed in PBS, embedded, and snap-frozen in OCT compound. 10–20 µm cryosections were fixed in 4% PFA/PBS for 15 min, washed in PBS, and blocked in Blocking Solution (10% Chicken Serum, 0.3% Triton X-100 in PBS). Sections were incubated for 1 h with primary antibodies diluted in Dilution Solution (1% BSA, 1% Chicken Serum, 0.3% Triton X-100 in PBS), washed three times in PBS-T (0.1% Tween-20 in PBS), and finally incubated in Alexa-dye-conjugated secondary antibodies (Life Technologies). After mounting with Mowiol (Calbiochem, 475904), confocal images were captured using a Zeiss LSM 780 (20×, NA = 0.8; 40× water, NA = 1.2; 63× oil, NA = 1.4) confocal microscope.

Hematoxylin–Eosin staining and peroxidase staining

Peroxidase staining was performed using the EnVision⁺ System Kit (Dako) according to the manufacturer's protocol. Briefly, cryosections were fixed using 4% PFA, washed with PBS, and subsequently blocked with Peroxidase Block. Following this step, the tissue was incubated with primary antibodies for 30 min, followed

by three washing steps with PBS-T (0.1% Tween-20 in PBS). Subsequently, the substrate-chromogen solution was added and incubated for 10 min. Finally, samples were hematoxylin-counterstained and mounted in Mowiol.

Embryonic skin wholemount stainings

Wholemount staining of embryonic tissue was previously described (Hägerling *et al*, 2013). Briefly, embryos were dissected and fixed in 4% PFA at 4°C. Skin wholemounts were prepared, permeabilized (0.5% Triton X-100/PBS), and blocked in PermBlock (1% BSA, 0.5% Tween-20). Skins were stained with primary antibodies, and secondary antibodies were Alexa-dye conjugated (Life Technologies).

Wholemount staining of embryonic and adult tissues

After preparation, tissues were fixed in 4% PFA for 2–4 h and washed with PBS. Subsequently, samples were permeabilized in 0.5% Triton X-100 in PBS, blocked in PermBlock, and stained with primary antibodies at 4°C. Following three washing steps with PBS-T, tissues were incubated in secondary antibodies labeled with Alexa-dyes (Life Technologies). Analysis of wholemount staining was performed using an Ultramicroscope II (LaVision BioTec, Bielefeld, Germany) or a Zeiss LSM 780 (20×, NA = 0.8; 40× water, NA = 1.2; 63× oil, NA = 1.4) confocal microscope.

Optical clearing

Optical clearing was described previously (Hägerling *et al*, 2013). Briefly, for visualization, embryonic and adult wholemount stainings were embedded in 1% low-melting point agarose. After dehydration in methanol (50, 70, 95, 100, 100% methanol, each step 1 h), samples were optically cleared in a benzyl alcohol: benzyl benzoate solution (ratio 1:2) for 4 h. Cleared samples were subsequently imaged by ultramicroscopy.

Ultramicroscopy

After wholemount immunofluorescence staining, optically cleared samples were imaged using a LaVision Ultramicroscope II (LaVision BioTec, Bielefeld, Germany). Stacks were captured with a step size of 1–2 µm and at different magnifications. 3D reconstruction and analysis of ultramicroscopy stacks were performed by using the volume rendering software Voreen.

Immunofluorescence staining of cells

Primary dermal LECs were fixed in 4% PFA/PBS for 15 min at RT, washed twice in PBS, and permeabilized in 0.5% Triton X-100 in PBS for 30 min at RT. After blocking in PermBlock for 1 h at RT, the cells were incubated with primary antibodies diluted in Dilution Solution (1% BSA, 1% Chicken Serum, 0.3% Triton X-100 in PBS) over night at 4°C, washed three times in PBS-T (0.1% Tween-20 in PBS), and finally incubated in Alexa-dye-conjugated secondary antibodies (Life Technologies). After mounting with Mowiol (Calbiochem, 475904), confocal images were captured using a Zeiss LSM 780 (20×, NA = 0.8; 40× water, NA = 1.2; 63× oil, NA = 1.4) confocal microscope.

Scratch wound assay

For scratch wound assays, 1-µm slides 8-well fibronectin coated (ibidi 80823) were equipped with 2-well culture inserts (ibidi, 80209) to introduce a defined scratch of 500 µm. pdLECs were collected using Accutase® solution and 5×10^4 cells were seeded into each well. After formation of a confluent monolayer (approximately 5–6 h), the culture insert was removed and fresh medium was applied. Cell migration was visualized at 37°C and 10% CO₂ using the Axiovert 200 m microscope (Carl Zeiss). Images were captured every 10 min in a 24 h time frame and 10 images, covering the time frame in equal intervals, were used to evaluate the assay. The area uncovered from cells was measured in Fiji.

Western blotting

Total cell lysates, containing equal amounts of total protein, were separated by SDS-PAGE and transferred to nitrocellulose membranes using wet blotting technique. Blocking and antibody incubation was performed in TBS-T (0.1% Tween-20 in PBS) containing 5% BSA. After blocking, blots were incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 h at RT followed by three washing steps with TBS-T. Blots were quantified using the luminescent image analyzer LAS-4000 (Fuji Film) and the software MultiGauge V3.2 (Fuji Film). Quantification of the signals was standardized to β-tubulin signals.

RNA isolation and quantitative real-time PCR (qPCR) using TaqMan Assays

Total RNA was isolated from cell lysates in phenol/guanidinium-isothiocyanate buffer (PEQLAB Biotechnologie GmbH). First-strand cDNA was synthesized using SuperScript IV Reverse Transcriptase (Life Technologies), primed by random hexamers (Applied Biosystems). Subsequently, transcript levels were determined by quantitative real-time PCR (qPCR) using corresponding TaqMan gene expression assays (Applied Biosystems) on a 7300 RT-PCR System (Applied Biosystems).

Gene symbol	Reference
Angpt1	Mm01129232_m1
Angpt2	Mm00545822_m1
Cdh2	Mm01162497_m1
Cldn5	Mm00727012_s1
Ctgf	Mm01192933_g1
Cyr61	Mm00487498_m1
Esam	Mm00518378_m1
F11r (Jam1)	Mm00554113_m1
Jam2	Mm00470197_m1
Jam3	Mm00499214_m1
Ocln	Mm00500912_m1
Pecam1	Mm01242584_m1
Tek	Mm00443243_m1
Tjp1 (Zo-1)	Mm00493699_m1

Gene symbol	Reference
Trp73	Mm00660220_m1
Vegf-C	Mm01202430_m1
Vegf-D	Mm01131929_m1
Wwtr1 (Taz)	Mm01289583_m1
Yap1	Mm01143263_m1

FACS analysis of primary murine dermal lymphatic endothelial cells

For flow cytometry, pLECs were washed with PBS and incubated with Accutase® solution (Sigma) for approximately 10 min at 37°C. Detached cells were collected by centrifugation and resuspended in FACS buffer (3% fetal calf serum in PBS). Cells were stained for 20 min with dye-conjugated primary antibodies diluted in FACS buffer on ice. After washing, cells were measured using a BD FACS Canto Flow Cytometer.

Statistical analysis

Statistical significance was analyzed using two-tailed unpaired Student's *t*-tests with Welch's correction for assumption of unequal SD. For data analysis, SigmaPlot 11 and GraphPad Prism 7 software were used. The significance threshold was set at 0.05. *P*-values are indicated by asterisks: **P* ≤ 0.05; ***P* < 0.01; ****P* < 0.001, and *****P* < 0.0001. Results are shown as means ± SEM or mean ± SD. Western blot signals were quantified using the software MultiGauge V3.2 (Fuji) and Image J/Fiji.

Expanded View for this article is available online.

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Author contributions

FK conceived the project, designed experiments, analyzed data, and wrote the manuscript; RH, EH, and CD designed and performed experiments, analyzed and interpreted data, and contributed to the manuscript; MS performed experiments, analyzed and interpreted data; TM provided reagents, enabled experimentation, and contributed to the manuscript; SB and DV provided reagents and support and contributed to the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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