# Cell Adhesion Mediated by VCAM–ITGα9 Interactions Enables Lymphatic Development

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***Objective***—Adhesive ligand–receptor interactions play key roles in blood vessel angiogenesis but remain poorly characterized during lymphatic vessel growth. In this study, we use genetic approaches in both fish and mice to address the roles of cell surface integrin ligand vascular cell adhesion molecule (VCAM) and its 2 receptors, integrins α9 and α4, during lymphatic vascular development.

***Approach and Results***—Conditional deletion of the *Vcam* gene was used to test VCAM function in lymphatic growth in midgestation mice. Morpholino knockdown and cRNA rescue of the 2 zebrafish vcam alleles, as well as integrins α9 and 4, were used to test the role of these ligands and receptors during lymphatic growth in the developing fish. We show that VCAM is essential for lymphatic development in the zebrafish embryo and that integrin α9 (Itgα9) rather than Itgα4 is the required VCAM receptor in the developing fish. VCAM is expressed along lines of lymphatic migration in the mouse intestine, but its loss only retards lymphatic growth.

***Conclusions***—These studies reveal an unexpected role for cell–cell adhesion mediated by Itgα9–VCAM interactions during lymphatic development in the fish but not in the mouse. We propose that the relative importance of cellular adhesive ligands is magnified under conditions of rapid tissue growth when the cell number increases faster than cell matrix, such as in the early zebrafish embryo. **(*Arterioscler Thromb Vasc Biol*. 2015;35:1179-1189. DOI: 10.1161/ATVBAHA.114.304997.)**

**Key Words:** cell adhesion molecules ■ growth and development ■ lymphatic vessels



## Introduction

Lymphatic vessels develop in all vertebrates where they function to drain fluid and cells that leak from the closed blood vasculature into the interstitium.[1] Lymphatic development has been studied in detail using genetic models in the mouse and fish.[2] These studies reveal that lymphatic endothelial cells (LECs) arise from venous blood endothelial cells and then migrate away from blood vessels to form primary lymphatic structures that sprout to create a network of lymphatic vessels. In the mouse, this primary structure is the lymph sac,[3] whereas in the fish, it is a line of parachordal lymphangioblasts.[4] In both fish and mice, lymphatic vessels arise in response to conserved molecular cues, such as the secreted factors VEGF-C and CCBE1.[5–10] However, the molecular mechanisms by which LECs rapidly migrate to generate this network remain incompletely understood.

## See cover image

The development of the lymphatic vascular network requires endothelial cell proliferation and migration, for example, in response to chemotactic factors that drive directional cell movement.[11] The physical movement of endothelial cells requires the engagement and release of adhesion receptors, especially integrins that are dynamically affinity modulated by extracellular signals, such as those chemokines. Integrin-dependent adhesion and cell anchoring are also necessary for endothelial cell survival.[12] Blood vessels form in matrix-rich tissues and endothelial cell adhesions, and migration studies performed ex vivo use conditions that favor matrix production; thus, the adhesive ligands that bind endothelial cell integrins in vivo are thought to be matrix proteins, such as collagen and fibronectin.[13,14] Consistent with this model of endothelial adhesion, genetic studies in mice have demonstrated endothelial cell requirements for integrin receptors during vascular development and postnatal angiogenesis.[15] In contrast, the role of cellular integrin ligands, such as vascular cell adhesion molecule (VCAM), remains restricted to leukocyte extravasation from the blood[16] and specific requirements for cell–cell adhesion during cardiac and placental development.[17,18]

Less is known about the adhesion receptors and ligands that drive lymphatic vessel development and growth. The Itgα9 interaction with the EIIIA form of fibronectin is required for the formation of lymphatic valve leaflets,[19–21] and the broadly used fibronectin receptor Itga5 is required for lymphovenous valve development.[22] Whether integrins play a more generally significant role during LEC migration and, if so, what ligands they use are not known. Blockade of integrin function is a

Received on: November 17, 2014; final version accepted on: February 22, 2015.

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**The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.304997/-/DC1.**

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| Nonstandard Abbreviations and Acronyms | |
|---|---|
| LEC | lymphatic endothelial cells |
| VCAM | vascular cell adhesion molecule |

promising antiangiogenic strategy for treatment of cancer,[15] thus identifying the integrin receptor–ligand interactions that participate in LEC migration might also prove valuable for antilymphangiogenic therapies.

To address the role of integrin receptors and ligands during lymphatic development, we have examined integrins α4 and α9 that have been implicated in postnatal lymphatic growth[23] and lymphatic valve development,[20] respectively, and their ligands fibronectin and VCAM1. We find that both VCAM and fibronectin are expressed along the path of migrating LEC in the developing mouse intestine but that loss of VCAM only slightly delays lymphatic network formation in that context. In contrast, loss of VCAM completely blocks lymphatic development in the zebrafish embryo. Endothelial Itgα4 is not required for blood or lymphatic vessel development in the mouse,[24] and we find that it is also dispensable in the developing fish. Instead, loss of Itgα9 blocks lymphatic development in a manner identical to that observed with loss of VCAM, and genetic studies reveal synergistic phenotypes with partial loss of both VCAM and Itgα9 consistent with function as a critical receptor–ligand interaction. These studies reveal an unexpected role for integrin-mediated cell–cell adhesion during lymphatic development that is essential in the rapidly developing fish embryo but dispensable in the more slowly growing mouse embryo. There may also exist postnatal or other developmental contexts in which VCAM and cell–cell adhesion play key roles during lymphatic growth.

## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### VCAM and Fibronectin Are Expressed Along the Path of Lymphatic Endothelial Growth in the Developing Intestine

During lymphatic development in the mouse, LECs reach the intestine from the mesentery at E15.5 (Figure 1A, 1B, 1G, and 1H) and subsequently migrate through the submucosa of

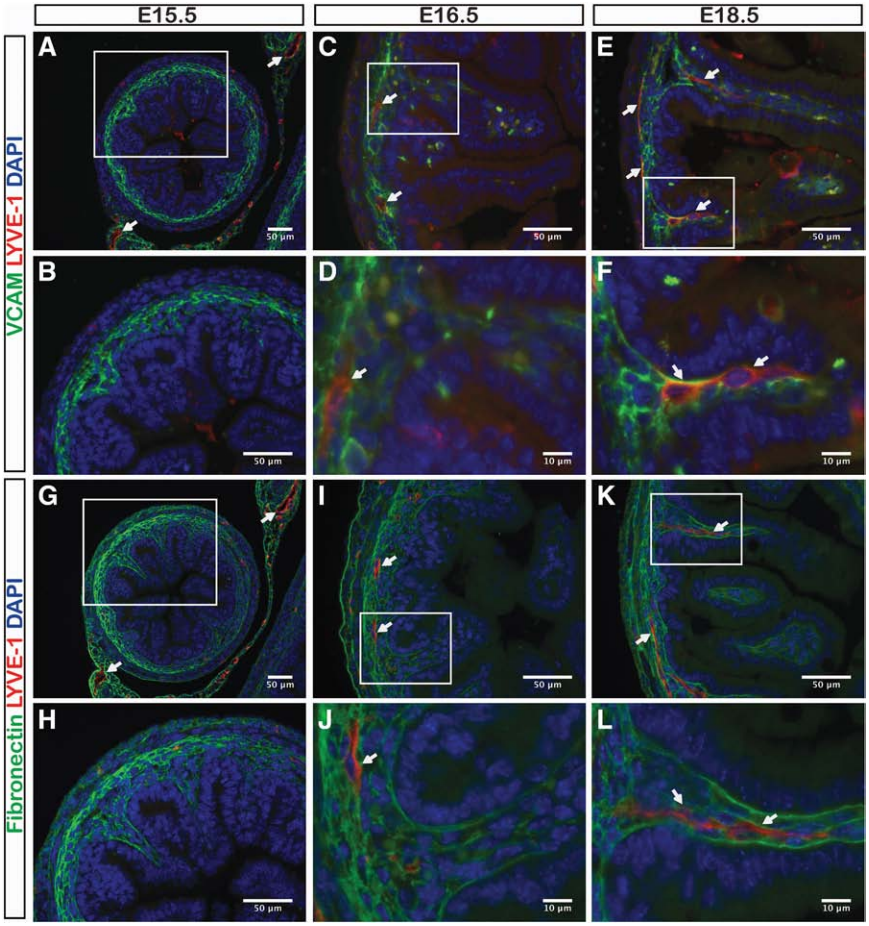


**Figure 1.** VCAM and fibronectin are expressed along the path of lymphatic endothelial cells (LECs) migration in the developing mouse intestine. **A** and **B**, VCAM$^+$ cells are detected in the submucosa before the arrival of LYVE1$^+$ LEC in the mouse intestine at E15.5. **C** and **D**, VCAM$^+$ cells are detected in the submucosa and villi at E16.5, a timepoint at which LYVE1$^+$ LEC are found in the submucosa. **E** and **F**, LYVE1$^+$ LECs are surrounded by VCAM$^+$ cells in both the submucosa and villi of mouse intestine at E18.5. **G**–**L**, Fibronectin is abundant throughout the submucosa and villi at E15.5 (**G** and **H**), E16.5 (**I** and **J**), and E18.5 (**K** and **L**). Boxed regions are shown at higher magnification in the below. Data show 1 representative of 3 experiments performed.

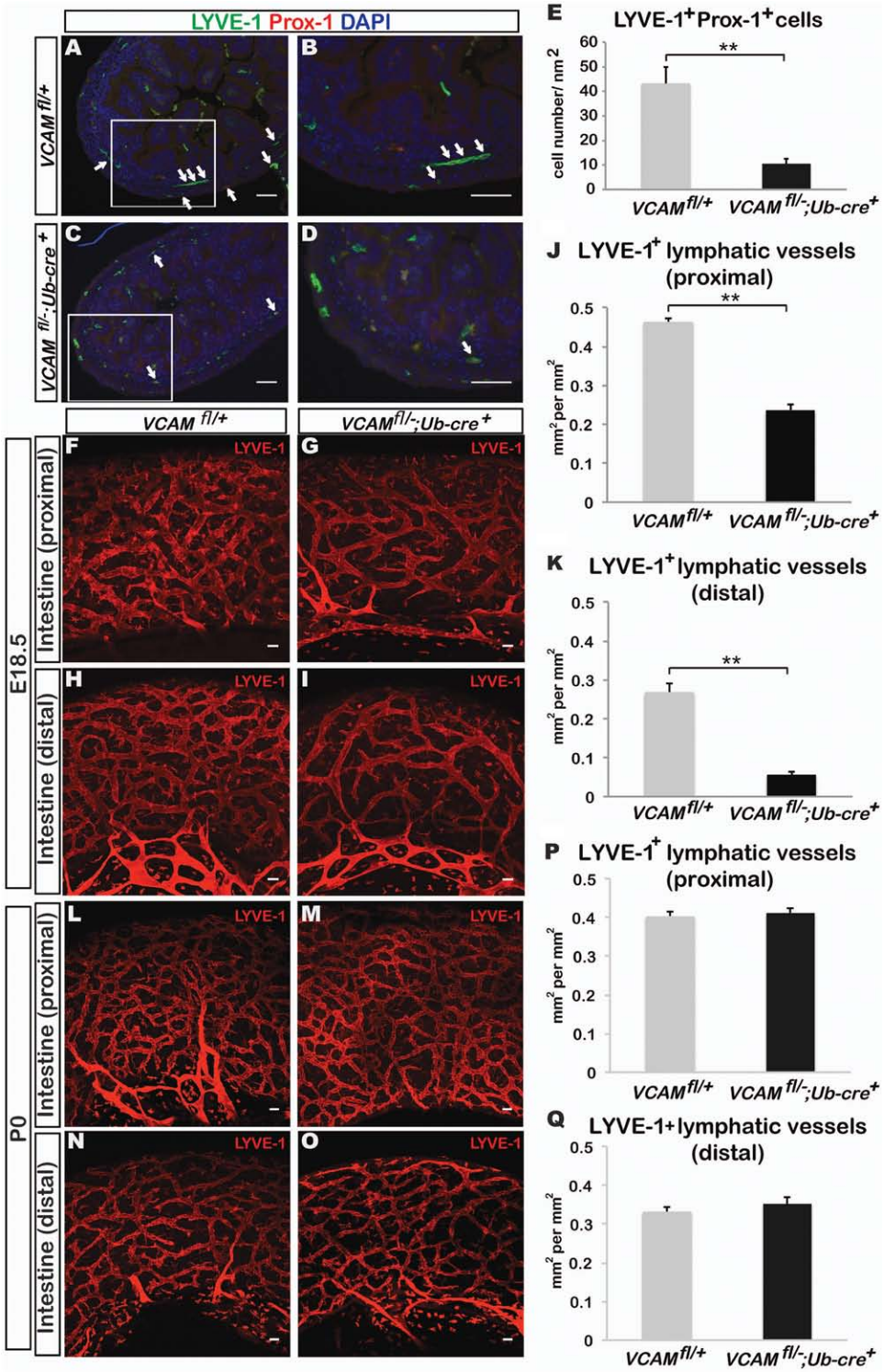


**Figure 2.** Inducible loss of VCAM in midgestation retards lymphatic growth into the mouse intestine. **A**–**D**, Staining for LYVE1⁺;PROX1⁺ lymphatic endothelial cells (LECs) reveals fewer lymphatic endothelial cells and vessels in the E16.5 Ub-CreERT2;*Vcam1*$^{fl/-}$ intestine. Arrows indicate individual PROX1⁺ nuclei in LECs. Boxes indicate regions in **A** and **C** that are shown at higher magnification in (**B**) and (**D**). Images were obtained with a Nikon Eclipse 80i microscope using a ×20/0.50 or ×40/0.75 numeric aperture (NA) dry objective. **E**, Quantification of LEC number in Ub-CreERT2;*Vcam1*$^{fl/-}$ and *Vcam1*$^{fl/+}$ control littermate intestines. n=5 for *Vcam1*$^{fl/+}$ and n=3 for Ub-CreERT2;*Vcam1*$^{fl/-}$. ** indicates $P<0.01$. **F**–**I**, Wholemount staining for LYVE1⁺ lymphatic vessels in Ub-CreERT2;*Vcam1*$^{fl/-}$ and control animals at E18.5 in the proximal (**F** and **G**) and distal (**H** and **I**) intestine. Images were acquired using a Leica TCS SP8 inverted microscope (Leica Microsystems, Wetzlar, Germany) with ×10 dry objective and assembled and analyzed using ImageJ (National Institutes of Health). **J** and **K**, Quantification of lymphatic vessel number in Ub-CreERT2;*Vcam1*$^{fl/-}$ and *Vcam1*$^{fl/+}$ control littermates in the E18.5 proximal and distal intestine. n=4; ** indicates $P<0.01$. **L**–**O**, Wholemount staining for LYVE1⁺ lymphatic vessels in Ub-CreERT2;*Vcam1*$^{fl/-}$ and control animals at P0 in the proximal (**L** and **M**) and distal (**N** and **O**) intestine. Images were acquired using a Leica TCS SP8 inverted microscope (Leica Microsystems, Wetzlar, Germany) with ×10 dry objective and assembled and analyzed using ImageJ (National Institutes of Health). **P** and **Q**, Quantification of lymphatic vessel number in Ub-CreERT2;*Vcam1*$^{fl/-}$ and *Vcam1*$^{fl/+}$ control littermates in the P0 proximal and distal intestine; n=5.

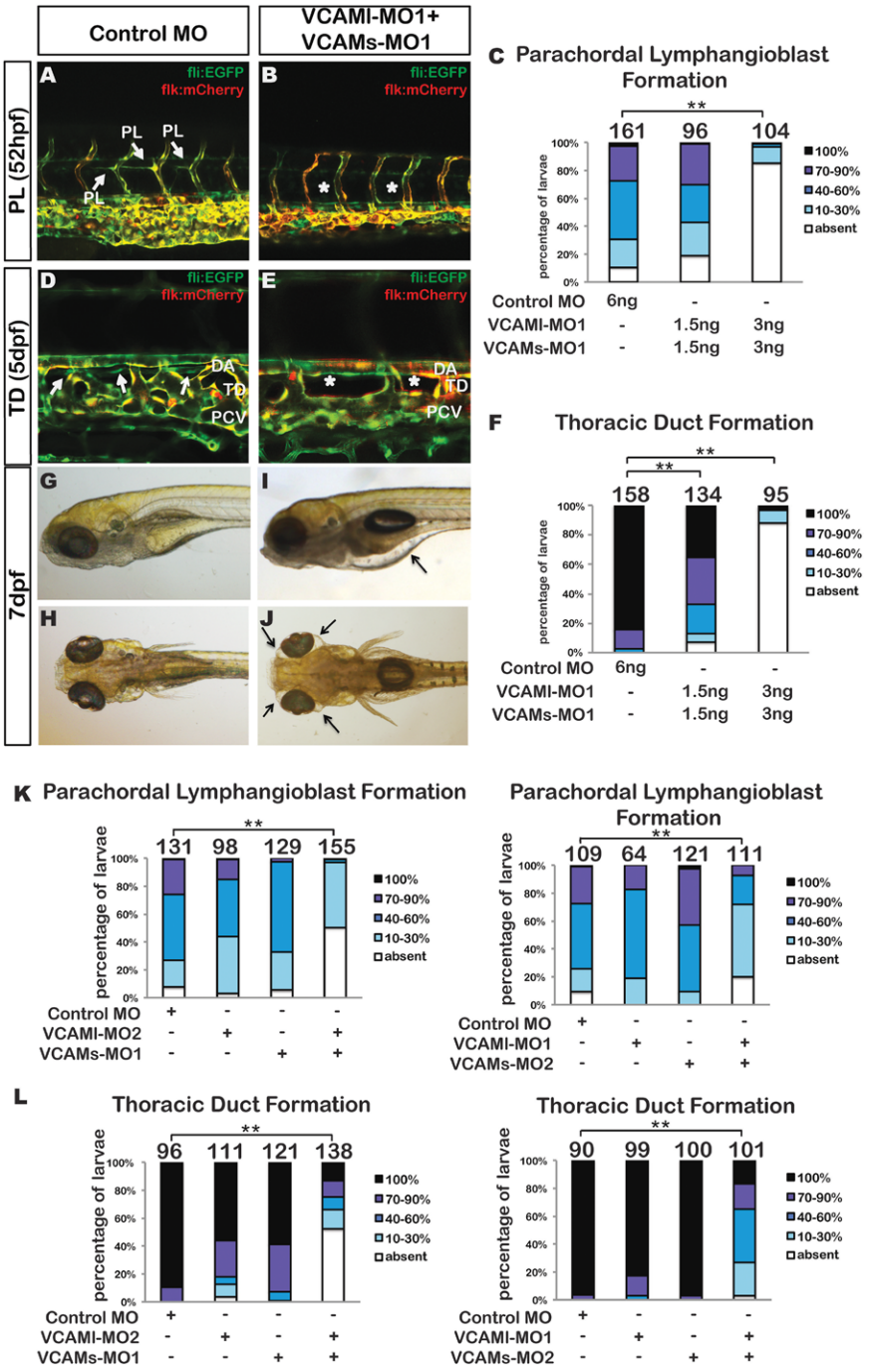


**Figure 3.** VCAM is required for lymphatic development in zebrafish. **A** and **B**, Detection of parachordal lymphangioblasts (PL, indicated by arrows) in 52-hpf zebrafish embryos treated with control or VCAMI+VCAMs morpholinos (3 ng+3 ng). **C**, Quantification of PL formation after injection of VCAMs and VCAMI morpholinos individually and together. ** indicates $P<0.001$. **D** and **E**, Detection of the thoracic duct (TD) between the dorsal aorta (DA) and posterior cardinal vein (PCV) in 5 dpf embryos treated with control or VCAMI+VCAMs morpholinos (3 ng+3 ng). Images were acquired using a Leica TCS SP8 inverted microscope (Leica Microsystems, Wetzlar, Germany) with 20x dry objective and assembled and analyzed using ImageJ (NIH). **F**, Quantification of TD formation after injection of VCAMs and VCAMI morpholinos individually and together. ** indicates $P<0.001$. **G**–**J**, Fish injected with VCAMI+VCAMs morpholinos (**I** and **J**) exhibit abdominal and periorbital edema compared with control morpholino injected fish (**G** and **H**). Images were acquired using an Olympus MVX10 microscope. **K**, PL formation after injection with alternate morpholinos targeting *vcaml* and *vcams*. **L**, TD after injection with alternate morpholinos targeting *vcaml* and *vcams*. Numbers above each column indicate the number of embryos analyzed in each group. MO indicates morpholino.

the intestine wall to the villi to form a complete lymphatic vascular network.[25] To assess whether both cell-associated and matrix integrin ligands might participate in LEC migration during this process, we first stained for fibronectin and VCAM1 and LECs in the developing mouse intestine. At E16.5, a timepoint at which LYVE1$^+$ LECs have entered the submucosa but not yet migrated through it to reach the villi, VCAM expression was detected in both the submucosa

and the villus (Figure 1C and 1D). By E18.5, LYVE1$^+$ LECs could be detected in the villi where they were surrounded by VCAM$^+$ cells (Figure 1E and 1F). Staining for fibronectin revealed abundant protein in the submucosa and villus at both E16.5 and E18.5 (Figure 1I–1L). Thus, both cell surface and matrix integrin ligands are present along the path of LEC growth in the developing intestine.

## Loss of VCAM Retards Lymphatic Growth in the Intestine But Not Skin of the Developing Mouse

Almost all VCAM-deficient animals die before E15 because of requirements for VCAM–Itgα4 adhesion in the heart and placenta, but rare surviving VCAM knockout animals have been described that live to adulthood without overt phenotypes.[17,18,26] The lack of edema in rare surviving VCAM-deficient animals could indicate that VCAM is not essential for lymphatic growth or merely reflect the ability of some animals to escape VCAM-deficient phenotypes because of a favorable genetic background. To more rigorously assess the requirement for VCAM during lymphatic development, we generated Ub-CreERT2;*Vcam1*$^{fl/-}$ animals and activated Cre activity in utero with maternal tamoxifen administration starting at E12.5 (Figure IA in the online-only Data Supplement). Analysis of E16.5 intestine from Ub-CreERT2;*Vcam1*$^{fl/-}$ animals revealed virtually complete loss of VCAM protein assayed both by immunostaining of the intestine wall (Figure IB and IC in the online-only Data Supplement) and immunoblot analysis of total intestine protein (Figure ID in the online-only Data Supplement). At E16.5, Ub-CreERT2;*Vcam1*$^{fl/-}$ animals exhibited 75% fewer LYVE1$^+$ Prox1$^+$ LECs in the submucosa of the intestine compared with *Vcam1*$^{fl/+}$ control littermates (Figure 2A–2E) and had not yet formed larger lymphatic vessels (Figure 2A–2D). At E18.5, the number of lymphatic vessels visualized by anti-LYVE1 whole-mount staining of the intestine wall was reduced by ≈50% in the proximal intestine and by >75% in the distal intestine in Ub-CreERT2;*Vcam1*$^{fl/-}$ animals compared with littermate controls (Figure 2F–2K). By P0, however, the number of lymphatic vessels in both the proximal and distal intestine did not differ significantly between Ub-CreERT2;*Vcam1*$^{fl/-}$ and control littermates (Figure 2L–2Q). The loss of VCAM therefore retards but does not completely block the migration of LECs into the intestine and the formation of the gut lymphatic network, an observation consistent with overlapping roles for cell-associated and matrix integrin ligands in this context. This possibility is supported by the observations that prenatal deletion of VCAM had no effect on lymphatic development in the matrix-rich dorsal skin (Figure IIA–IID in the online-only Data Supplement), and postnatal deletion of VCAM did not affect lymphatic growth in the ear (Figure IIE–IIH in the online-only Data Supplement), a matrix-rich organ that develops after birth.

## VCAM Is Required for Lymphatic Development in Zebrafish Embryos

The finding that VCAM contributes to lymphatic development in the intestine but not the ear in the mouse suggested that the role of cellular integrin ligands may be context-dependent and augmented when matrix ligands are less abundant. The lymphatic vasculature develops earlier in the zebrafish embryo than in the mouse embryo, suggesting that matrix may be less available and LECs may use cell–cell adhesion to a greater extent than in the mouse. Genome database analysis revealed 2 zebrafish genes that are syntenic with and exhibit similar levels of amino acid homology to mouse and chicken VCAM1 that were designated as VCAM short (VCAMs) and VCAM long (VCAMl; Figures III and IV in the online-only Data Supplement). VCAMs has fewer immunoglobulin domains and is similar to the single chicken VCAM orthologue, whereas VCAMl has a greater number of immunoglobulin domains and more closely resembles mammalian VCAM (Figure IV). Morpholino knockdown of either VCAMl or VCAMs alone had no effect on lymphatic development, measured either at the parachordal lymphangioblast formation stage (52 hpf) or at the thoracic duct formation stage (5 dpf) using *fli1a:eGFP;flk:mCherry* double transgenic embryos in which blood endothelial cells express both GFP and mCherry, whereas LECs express only GFP[27] (Figures V and VI in the online-only Data Supplement; Figure 3).

Injection of low dose (3 ng each) of morpholinos targeting both VCAMs and VCAMl resulted in a nearly complete loss of parachordal lymphangioblast formation at 52 hpf (Figure 3A–3C) and thoracic duct formation at 5 dpf (Figure 3D–3F). Consistent with the loss of lymphatic function, VCAMs+VCAMl double morphant fish exhibited marked abdominal and periorbital edema at 7 dpf (Figure 3G–3J). To test the specificity of these findings, we first assessed whether combinations of different morpholinos targeting VCAMs and VCAMl conferred similar defects in lymphatic vessel growth. Loss of parachordal lymphangioblast formation (Figure 3K) and thoracic duct formation (Figure 3L) was also observed with combined use of 2 distinct nonoverlapping morpholinos directed against VCAMs and VCAMl, providing strong evidence for the specificity of the morpholino phenotypes.

## Lymphatic Defects Conferred by vcam Morpholinos Are Rescued by vcam cRNA Injection But Not p53 Knockdown

Lymphatic phenotypes were conferred by low-dose morpholinos designed to specifically knock down expression of the 2 zebrafish vcam alleles. The specificity of these findings is supported by the fact that phenotypes were observed only with simultaneous knockdown of both vcam alleles, but additional evidence that morpholino phenotypes are not because of off-target effects is required. A direct means of testing the specificity of morpholino-induced knockdown is to rescue the phenotype by injection of cRNA that encodes the target protein but is not blocked by the morpholino.[28] We coinjected VCAMl cRNA with morpholinos targeting VCAMl and VCAMs to test specific rescue of the lymphatic phenotype. Embryos injected with VCAMl cRNA alone did not exhibit developmental abnormalities or overgrowth of parachordal lymphangioblasts (Figure 4A–4D). However, VCAMl cRNA significantly rescued parachordal lymphangioblast formation at 52 hpf ($P$<0.001, Figure 4E–4I),

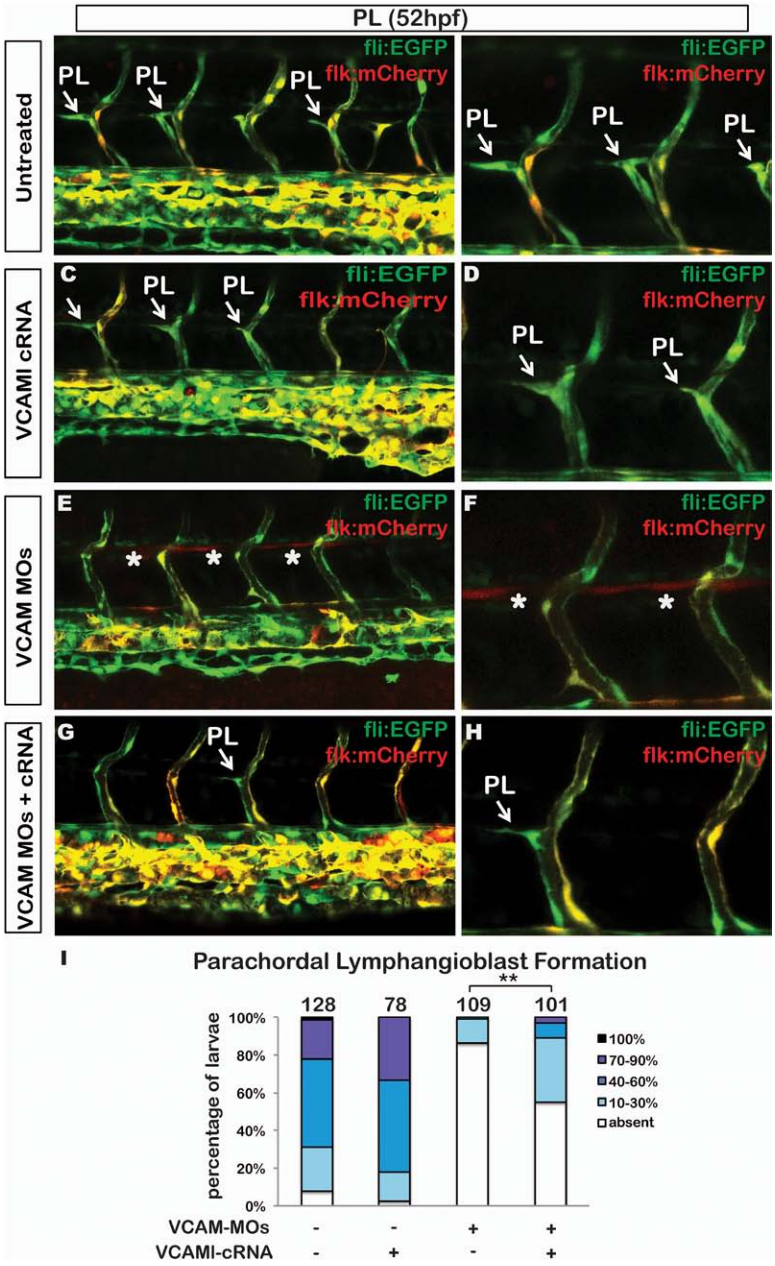


**Figure 4.** VCAM cRNA injection rescues lymphatic development in VCAM morphant fish embryos. **A**–**D**, Injection of 200 pg *vcaml* cRNA does not interfere with parachordal lymphangioblast (PL) formation at 52 hpf. Arrows indicate GFP⁺ PL. **E** and **F**, Injection of 3 ng of VCAMl MO1 and 3 ng of VCAMs MO1 blocks PL formation. **G** and **H**, Coinjection of 200 pg of vcaml cRNA rescues PL formation after injection of VCAM morpholinos. **I**, Quantification of the rescue effect of vcaml cRNA in VCAM morphants on PL formation at 52 hpf. Numbers indicate the number of embryos analyzed in each group. ** indicates $P<0.001$. MO indicates morpholino.

consistent with a specific requirement for VCAM during lymphatic development.

A common mechanism by which morpholinos confer off-target phenotypes is through p53 activation, and coinjection of morpholinos targeting p53 provides a second means of testing the specificity of morpholino phenotypes in zebrafish embryos.[29] To further address the specificity of the lymphatic phenotypes conferred by morpholino knockdown, we coinjected p53 morpholinos. In contrast to vcam cRNA injection, knockdown of p53 failed to rescue lymphatic growth in vcam morphant embryos (Figure VII in the online-only Data Supplement), suggesting that loss of lymphatic growth is because of loss of vcam expression and is not an off-target morpholino effect.

## VCAM Receptor Itgα9 But Not Itgα4 Is Required for Lymphatic Development in Zebrafish

The best characterized receptors for VCAM are integrin α4β1 that mediates leukocyte adhesion to endothelial cells[30] and integrin α4β7 that functions primarily in B cells.[31] VCAM–Itgα4 interactions also play important roles during development, as Itgα4-deficient mice exhibit cardiac and placental defects identical to those of VCAM-deficient mice.[18,26] Importantly, α4β1 integrins have also been demonstrated to play important roles in postnatal lymphatic growth in mammals.[23] To identify the VCAM receptor required for lymphatic development in fish, we therefore first tested the loss of zebrafish *itgα4*. Injection of 4 ng of a splice morpholino directed against the splice donor of exon 6 of itgα4 resulted in highly efficient knockdown of itgα4

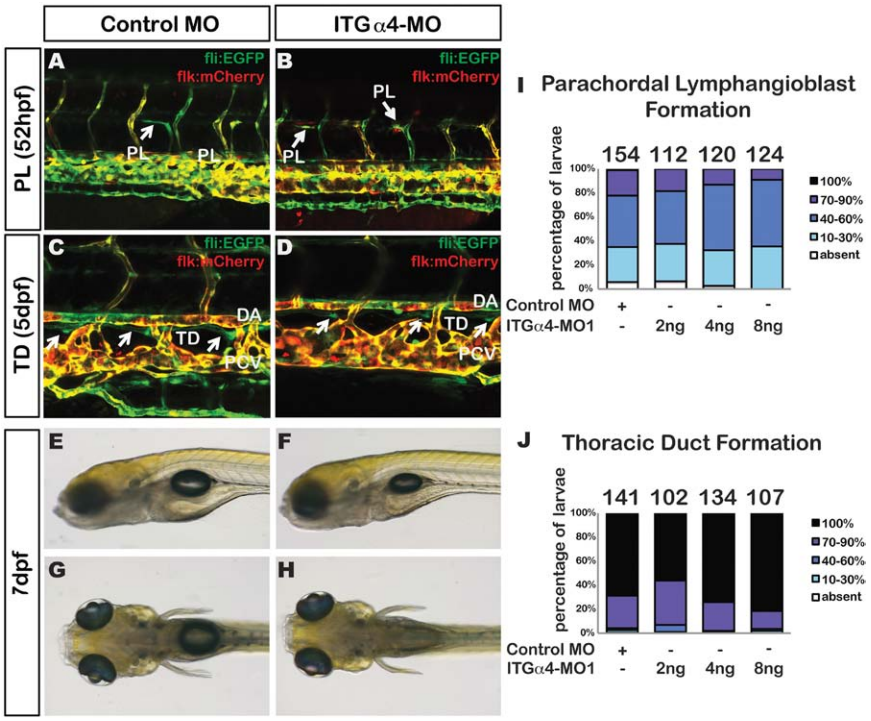


**Figure 5.** *itgα4* is not required for lymphatic development in zebrafish. **A** and **B**, Parachordal lymphangioblast (PL) formation at 52 hpf proceeds normally in zebrafish embryos treated with 4 ng of *itgα4* splice morpholino. **C** and **D**, Thoracic duct (TD) formation in 5 dpf embryos treated with control or itgα4 morpholino. **E–H**, *itgα4* morphant embryos do not exhibit abdominal or periorbital edema. **I** and **J**, Quantification of PL and TD formation in zebrafish embryos after injection of 6 ng control and escalating doses of *itga4* morpholinos. Numbers indicate the number of embryos analyzed in each group. MO indicates morpholino.



mRNA (Figure VIC in the online-only Data Supplement). However, itgα4 morphant embryos exhibited normal lymphatic growth at both the parachordal lymphangioblast (Figure 5A, 5B, and 5I) and thoracic duct (Figure 5C, 5D, and 5J)stages and were not edematous at 7 dpf (Figure 5E–5H). Embryos injected with a second splice morpholino targeting the splice donor of exon4 of itgα4 also failed to exhibit a defect in lymphangiogenesis (data not shown). As in VCAM morphants, we observed no defects in cardiac development or pericardial edema in itgα4 morphant embryos (Figure 5F). These studies suggest that α4 integrins are not the VCAM receptors required for lymphatic growth and development in fish.

A second, less characterized receptor for VCAM is the integrin α9β1. α9β1 has been reported to mediate neutrophil adhesion to VCAM on activated endothelial cells,[32] but affinity studies reveal a much lower affinity for VCAM compared with α4β1.[33] In mice, the in vivo role of Itgα9 is tightly linked to the EIIIA fibronectin ligand as loss of either results in defective lymphatic valve formation but otherwise normal lymphatic vascular growth and development.[20] Injection of 2 distinct morpholinos targeting the ATG and the exon 2 splice acceptor of zebrafish *itgα9* (Figure VID in the online-only Data Supplement) resulted in a severe loss of parachordal lymphangioblasts (Figure 6A, 6B, 6I, and 6K) and thoracic duct (Figure 6C, 6D, 6J, and 6L) formation similar to that seen with loss of VCAM. Consistent with these findings, itgα9 morphant fish also exhibited abdominal and periorbital edema at 7 dpf (Figure 6E–6H). These data reveal that itgα9 is required for lymphatic development in zebrafish.

## VCAM and Itgα9 Exhibit Genetic Interactions During Zebrafish Lymphatic Development

The studies described above suggested that VCAM and Itgα9 may function as a ligand–receptor pair during lymphatic network formation in the fish. To test this hypothesis, we first examined vcam and itgα9 gene expression in zebrafish embryos using in situ hybridization. At 52 hpf, vcaml is expressed in the brain, whereas itgα9 is expressed in the heart and fin bud (Figure 7A–7C). Strikingly, both vcaml and itgα9 are expressed in the midline of the embryo, where the parachordal lymphangioblast forms, and between the somites, where the intersegmental lymphatic vessels form (Figure 7B and 7D). Thus the spatial pattern of VCAM and Itgα9 expression parallels that of lymphatic development in the fish.

We next examined genetic interactions between the vcam and itgα9 genes in developing fish embryos using compound knockdown experiments with low doses of morpholinos targeting VCAMs, VCAMl, and itgα9. Low dose morpholino treatment against VCAMs and VCAMl genes or itgα9 alone did not impair parachordal lymphangioblast or thoracic duct formation (Figure 7E–7J, 7M, and 7N). Combined knockdown, however, resulted in significant loss of both lymphatic structures, consistent with a synergistic effect on lymphatic growth (Figure 7K–7N). These studies support genetic interactions between VCAM and Itgα9 during lymphatic development and suggest that this

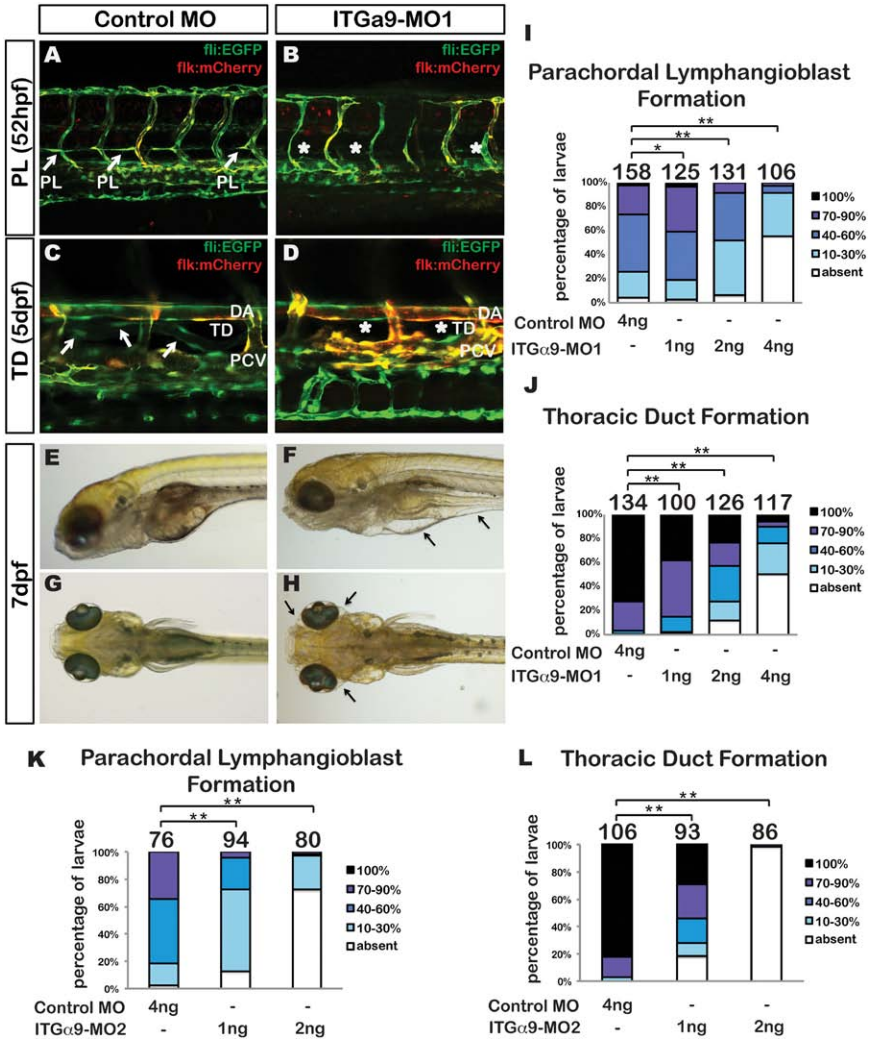


**Figure 6.** Loss of itgα9 blocks lymphatic development in zebrafish. **A** and **B**, Parachordal lymphangioblast (PL) formation at 52 hpf is blocked in zebrafish embryos treated with 4 ng of itgα9 splice morpholino. **C** and **D**, Thoracic duct (TD) formation in 5 dpf embryos treated with control or itgα9 morpholino. **E**–**H**, itgα9 morphant embryos exhibit abdominal or periorbital edema. **I** and **J**, Quantification of PL and TD formation in zebrafish embryos after injection of 4 ng control and escalating doses of itgα9 morpholino number 1. **K** and **L**, Quantification of PL and TD formation in zebrafish embryos after injection of 4 ng control and escalating doses of ATG morpholino against itgα9 (itgα9 morpholino number 2). Numbers indicate the number of embryos analyzed in each group. * indicates $P<0.05$ and ** indicates $P<0.001$. MO indicates morpholino. MO indicates morpholino.

is a functionally important receptor–ligand interaction during lymphatic development.

## Discussion

The lymphatic vascular network forms rapidly as LECs proliferate and migrate from their site of venous origin to tissues throughout the body.[34] Such rapid growth and invasion of preexisting tissues require factors that support LEC proliferation (eg, VEGFC[5,6] and CCBE1[7–10]), guide LEC migration (eg, CXCL12 and CXCR4[11]), and mediate the physical movement of LECs. The adhesive ligands and receptors that enable LEC movement during this process of rapid migration and vessel growth remain obscure. Our findings reveal a critical role for the cell surface ligand VCAM and the integrin α9 receptor during zebrafish lymphatic development. In contrast, in the developing mouse, our studies and published work reveal only supportive roles for VCAM and ITGα9 in lymphatic growth of the developing intestine and the formation of lymphatic valve leaflets, respectively. These studies therefore reveal an unexpected mechanism of cell–cell adhesion that is essential during fish but not mammalian lymphatic development.

A recent study has revealed that many morphant fish phenotypes are not reproduced by genetic mutants,[35] raising the question of whether and to what extent our findings accurately reflect roles for VCAM and ITGα9 in fish lymphatic development. Several lines of evidence suggest that the differences in the requirement for VCAM and ITGα9 observed in the fish and mouse reflect real species differences rather than artificial phenotypes in the fish conferred by morpholinos that act nonspecifically. First, *vcam* and *itgα9* are expressed in a coordinate manner along the path of lymphatic endothelial migration in the developing fish. In contrast, in the developing mouse intestine, VCAM and ITGα4 exhibited minimal coexpression at sites of lymphatic growth (Figure VIIIA–VIIID in the online-only Data Supplement), whereas ITGα9 expression was not spatially associated with lymphatics (Figure

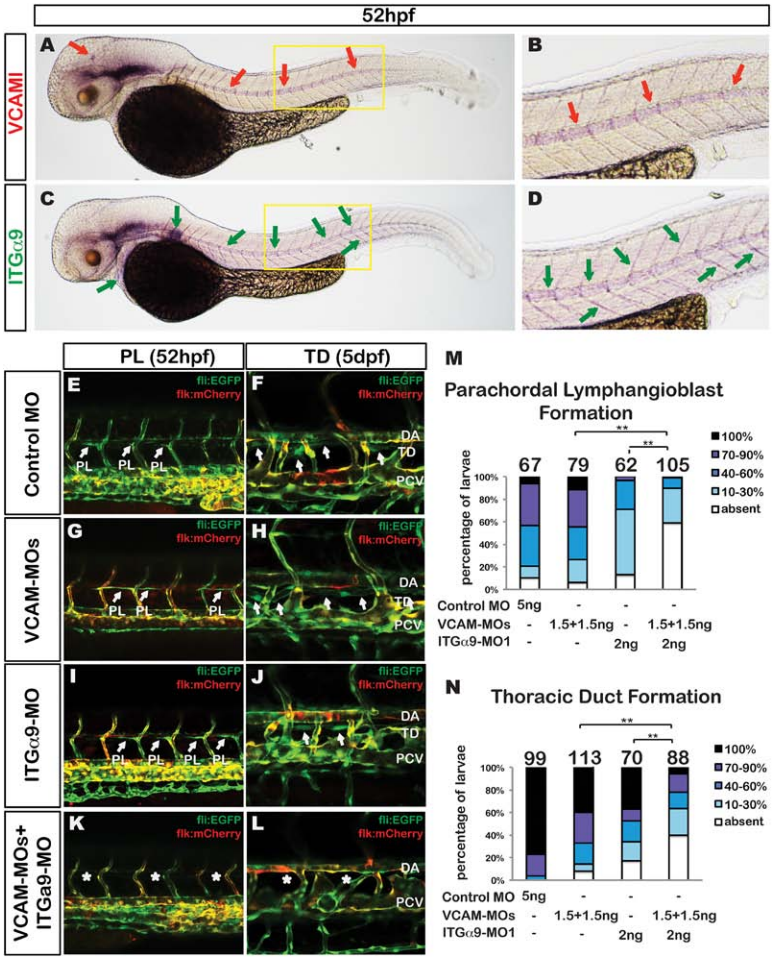


**Figure 7.** Synergistic lymphatic-deficient phenotypes with loss of both vcam and itgα9. **A** and **B**, In situ hybridization of *vcaml* in zebrafish embryos at 52 hpf. **C** and **D**, In situ hybridization of *itgα9* in zebrafish embryos at 52 hpf. Boxed regions are shown at higher magnification on the right. Shown is a single study that is representative of 5 separate experiments. **E**–**L**, Parachordal lymphangiobasts (PLs) and thoracic ducts (TDs) were visualized at 52 hpf and 5 dpf, respectively, after injection of 5 ng control (**E** and **F**), 1.5 ng vcams+1.5 ng vcaml (**G** and **H**), 2 ng itgα9 (**I** and **J**), or 1.5 ng vcams+1.5 ng vcaml+2 ng itgα9 (**K** and **L**) morpholinos. **M** and **N**, Quantification of PL and TD formation in zebrafish embryos after injection of the indicated morpholinos. Numbers indicate the number of embryos analyzed in each group. ** indicates $P<0.001$. MO indicates morpholino.

VIIIE and VIIIF in the online-only Data Supplement). Thus, expression and function in the 2 species differ in coordinate ways. Second, we used multiple morpholinos to target the *vcam* and *itgα9* genes in fish and observe identical phenotypes with distinct morpholinos injected at low dosages. Third, the lack of any phenotype in fish treated with only a single *vcam* morpholino speaks to specific functional redundancy of the 2 *vcam* genes rather than an off-target morpholino effect that is typically conferred in a dominant manner. Fourth, the lymphatic phenotypes conferred by the morpholinos used in this study are not rescued by reducing p53 levels (Figure VII in the online-only Data Supplement), indicating that p53 activation, a common mechanism of off-target morpholino effects,[29] does not play a role in the genesis of these phenotypes. Fifth, loss of *vcam* and loss of *itgα9* in the fish result in identical defects in lymphatic growth but not venous sprouting that are distinct from defects in both observed with loss of either *ccbe1* or *vegfc* (Figure IX in the online-only Data Supplement). Finally, loss of lymphatic growth conferred by *vcam* morpholinos is rescued by coinjection of morpholino-resistant vcam-encoding cRNA. Thus, although future studies using fish genetic models will be needed to fully explore this biology, our expression and functional studies support the conclusion that VCAM and ITGα9 play essential roles in early zebrafish lymphatic development and nonessential roles in mouse lymphatic development.

Endothelial cell adhesion and migration during angiogenesis are thought to be mediated by cell–matrix interactions that are dynamically regulated by integrin receptors in response to external signals, such as chemokines and VEGF.[36,37] Studies of the transmembrane protein VCAM1 and related proteins, such as MADCAM, have demonstrated that they function as cell surface ligands for integrin receptors (eg, α4β1 and α4β7) that enable hematopoietic cells to bind endothelial cells and exit the blood.[30,31] The possibility that VCAM–integrin interactions might also play important adhesive roles during development and in nonhematopoietic cells was raised by studies of VCAM-deficient and ITGα4-deficient mice that exhibited similar lethal defects in myocardial–epicardial adhesion and placental growth before E12.5.[17,18,26] Our studies of mouse embryos in which VCAM has been inducibly and efficiently deleted after E12.5 reveal a delay in lymphatic vascular development in the gut, a result consistent with the coexpression of fibronectin along the path of LEC migration in the developing intestine and the lack of an overt vascular phenotype in embryos in which Itgα4 has been deleted selectively in endothelial cells.[24] Thus, in the mouse endothelial cell–cell adhesion seems redundant with cell–matrix adhesion and is not required for lymphatic development. In contrast, loss of VCAM function in zebrafish embryos resulted in a complete block in lymphatic development that, in contrast to the defects conferred by loss of vegfc or ccbe1, has no detectable effect on

blood vessel development (Figure VII in the online-only Data Supplement).[6,8] The reason for this striking species difference in the use of VCAM is not yet clear, but one possibility is that lymphatic outgrowth from the blood vascular system occurs early in fish compared with mammals; thus, there may be less time for matrix protein synthesis and deposition before lymphatic outgrowth and a correspondingly increased reliance on the cellular adhesive ligand VCAM and its receptor ITGα9.

Although LECs in the developing fish may require VCAM for cell adhesion, a second possible explanation for the VCAM–ITGα9 requirement is endothelial survival and proliferation. Integrin–matrix interactions have been shown to be required during active angiogenesis both in vivo[38] and in vitro.[39] To address this possible mechanism, we examined lymphatic endothelial proliferation and apoptosis in the E18.5 intestine of VCAM-deficient and control mouse embryos. These studies failed to reveal significant changes with loss of VCAM (Figure X in the online-only Data Supplement), suggesting either that LEC adhesion and migration are more important than cell survival or that the VCAM-deficient lymphatic phenotype is too subtle in the mouse to detect important changes in cell survival. Whether for cell migration or cell survival, it seems most likely that the greater role of VCAM in the fish embryo is because of the rapid timecourse of its vascular development that provides less opportunity to synthesize and use matrix proteins.

Finally, if the dependence of lymphatic growth on VCAM–ITGα9 interactions in the fish is contextual, for example, because of a relative lack of matrix at that early timepoint in development, it is also possible that there exist mammalian contexts in which VCAM and ITGα9 play critical roles in lymphatic growth. Recent studies of lymphatic growth in adult mice during tumor growth or matrigel lymphangiogenesis have revealed a requirement for ITGα4, an integrin subunit that is expendable during lymphatic development.[23] Whether lymphatic growth in these more aggressive postnatal contexts may also require VCAM and ITGα9 remains to be investigated. Antiangiogenic agents directed against integrin avb3 show promise in the blood vascular system; thus, similar strategies directed against adhesive receptor–ligand interactions may also provide therapeutic approaches to blocking pathological lymphangiogenesis.



## Acknowledgments

We thank Zoltan Jakus, Dan Sweet, and other members of the Kahn laboratory for their thoughtful comments and suggestions during the course of this work. We thank Dr Brant Weinstein and Jie He for help with the zebrafish studies.

## Sources of Funding

This work was supported by the Lymphatic Education and Research Network (Y. Yang), National Institutes of Health R01 HL11553 (M. Kahn), and the Leducq Foundation (M. Kahn and Y. Yang).

## Disclosures

None.

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## Significance

Lymphatic vessels develop after the formation of the blood vascular network through rapid expansion of a small number of lymphatic endothelial progenitors that arise in veins. Understanding how lymphatic vessels develop in the vertebrate embryo is expected to provide insight that may be applied toward therapeutic lymphangiogenesis, for example, in individuals with chronic lymphedema. Using both mouse and zebrafish models, we establish a role for cell–cell adhesion during lymphatic growth that is mediated by the integrin $\alpha 9\beta 1$ and its cell-associated ligand VCAM1. These studies highlight the role of adhesive interactions during lymphatic growth and demonstrate important differences between mammals and fish in their use during this process.