



Mechanically activated ion channel PIEZO1 is required for lymphatic valve formation

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PIEZO1 is a cation channel that is activated by mechanical forces such as fluid shear stress or membrane stretch. *PIEZO1* loss-of-function mutations in patients are associated with congenital lymphedema with pleural effusion. However, the mechanistic link between *PIEZO1* function and the development or function of the lymphatic system is currently unknown. Here, we analyzed two mouse lines lacking *PIEZO1* in endothelial cells (via *Tie2Cre* or *Lyve1Cre*) and found that they exhibited pleural effusion and died postnatally. Strikingly, the number of lymphatic valves was dramatically reduced in these mice. Lymphatic valves are essential for ensuring proper circulation of lymph. Mechanical forces have been implicated in the development of lymphatic vasculature and valve formation, but the identity of mechanosensors involved is unknown. Expression of *FOXC2* and *NFATc1*, transcription factors known to be required for lymphatic valve development, appeared normal in *Tie2Cre;Piezo1^{CKO}* mice. However, the process of protrusion in the valve leaflets, which is associated with collective cell migration, actin polymerization, and remodeling of cell–cell junctions, was impaired in *Tie2Cre;Piezo1^{CKO}* mice. Consistent with these genetic findings, activation of *PIEZO1* by *Yoda1* in cultured lymphatic endothelial cells induced active remodeling of actomyosin and VE-cadherin⁺ cell–cell adhesion sites. Our analysis provides evidence that mechanically activated ion channel *PIEZO1* is a key regulator of lymphatic valve formation.

and *PIEZO2* are nonselective cation channels activated by mechanical forces applied to the cell membrane, such as fluid shear stress and cell stretching (12–16). *PIEZOs* are large proteins containing more than 2,000 amino acids with 38 transmembrane domains per protomer. *PIEZO1* trimeric channels form a three-bladed propeller shape with a curved transmembrane region as revealed by cryoelectron microscopy. It has been proposed that a change in membrane tension induces conformational change of these propellers and leads to channel pore opening (17–20). *PIEZO2* functions as the key mechanotransducer for light touch sensation, proprioception, and control of respiration (21–24). *PIEZO1*, on the other hand, is expressed in endothelial cells, including LECs, and plays essential roles in developing blood vessels in mouse embryos and in flow-mediated vasoconstriction in adult mice (8, 15, 25–28). In addition, *PIEZO1* in red blood cells controls cell volume regulation, and gain-of-function mutations in *PIEZO1* cause dehydrated RBCs and some protection from malaria infection in mice and human (29–32). In this study, we analyzed mouse lines lacking *PIEZO1* in endothelial cells and found that *PIEZO1* is required for formation of lymphatic valves, highlighting the physiological importance of *PIEZO1* in the lymphatic system.

Results

Postnatal Pleural Effusion and Lethality of Endothelial-Specific *Piezo1* Conditional Knockout Mice. We previously reported that, when *PIEZO1* is constitutively and globally ablated (*Piezo1^{-/-}*), mice

PIEZO1 | mechanotransduction | ion channel | lymphatic system | valve formation

The lymphatic system is essential for fluid homeostasis in the body. Lymphatic vessels collect extracellular fluid from the interstitial space and drain it back to the venous circulation (1, 2). The lymphatic system is also involved in absorption of fat from the gut and is important for coordinating adaptive immune responses. Its dysfunction often results in peripheral lymphedema as well as pleural effusions and ascites. Lymphatic valves are required to ensure unidirectional flow of lymph in collecting vessels that are composed of contractile units known as lymphangions. The valves are formed from lymphatic endothelial cells (LECs), the main component of the lymphatic vasculature. Mouse genetic studies have identified transcription factors, adhesion proteins, and mediators of intercellular signaling as essential components for the development and maintenance of lymphatic vasculature and valves (1, 3–5). In addition, the contribution of mechanical forces to the development and maintenance of the lymphatic vasculature has been described (6). Accumulating evidence suggests that genes involved in valve formation are up-regulated when LECs are exposed to shear stress (7, 8). However, it still remains unclear how mechanical stimuli are converted into intracellular signals in the lymphatic endothelium.

Recently, studies of patients with familial lymphatic dysfunction suggested that mechanically activated ion channel *PIEZO1* is required for lymphatic system function. Specifically, homozygous and compound heterozygous mutations in *PIEZO1* were identified among patients suffering from persistent lymphedema, plural effusions, and ascites associated with congenital lymphatic dysplasia (9–11). *PIEZO1*

Significance

PIEZOs are mechanically activated cation channels. Recently, loss-of-function mutations of human *PIEZO1* were found among patients with familial lymphedema, suggesting a requirement of *PIEZO1* in the lymphatic system. In this paper, utilizing mouse models lacking *PIEZO1* in endothelial cells, we show that this ion channel is required for the formation of lymphatic valves, a key structure for proper circulation of lymph in the body. The requirement of *PIEZO1* in valve formation provides mechanistic insight on how *PIEZO1* variants cause lymphatic dysfunction in patients. This study also extends the relevance of *PIEZOs* beyond acute signaling molecules (e.g., touch sensation) and highlights the importance of these ion channels in controlling morphological/structural specification during development.

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die during embryonic midgestation, with growth retardation and defects in vascular remodeling in the yolk sac (15). To investigate the role of PIEZO1 in the lymphatic system in detail, we generated two mouse lines in which PIEZO1 was deleted specifically in endothelial cells.

The *Tie2Cre* mouse line, in which Cre recombinase is expressed in vascular endothelial cells, including both blood (BECs) and lymphatic endothelial cells (*SI Appendix, Fig. S1A*), was crossed with *Piezo1^{fllox/fllox}* mice to generate *Tie2Cre;Piezo1^{CKO}* mice (29, 33). *Tie2Cre;Piezo1^{CKO}* mice were born at the expected ratio [actual: 23.9% (28/117), expected: 25%], suggesting that they survive past embryonic development. Previously, endothelial-specific deletion of PIEZO1 in mice was reported to cause embryonic lethality (25). The variation in phenotype between the two studies could potentially be due to differences in genetic background (25, 29, 34). In our colony, most *Tie2Cre;Piezo1^{CKO}* mice died within 2 wk after birth (Fig. 1*A*). The body weight of the *Piezo1*-deficient mice was slightly less than control littermates during postnatal days (*SI Appendix, Fig. S1B*). Among human patients with *PIEZO1* loss-of-function variants, pleural effusion, a sign of defect in the lymphatic system, especially damage of lymphatic vessels close to the chest cavity, has been reported. Consistently, pleural effusion was evident in 1-wk-old *Tie2Cre;Piezo1^{CKO}* mice (Fig. 1*B* and *C*).

The second mouse line we utilized was initially aimed to more specifically ablate PIEZO1 in lymphatic endothelial cells. Here, Cre recombinase was expressed from the locus of *lymphatic vessel endothelial hyaluronan receptor 1* (*Lyve1*), a widely used marker for LECs (5, 35–37). High efficiency of Cre-mediated recombination of *loxP* sites in LECs was reported with this line; however, recombination in a subset of BECs was also reported (35). We examined the specificity of Cre activity in various endothelial cells by crossing the *Lyve1Cre* line to the *Ai9* line (*loxP-stop-loxP-tdTomato* reporter in the *ROSA26* locus) (38). In the mesentery of *Lyve1Cre;Ai9* mice, tdTomato signal was detected in arteries and veins, as well as lymphatic vessels (*SI Appendix, Fig. S2A*). These results were identical to what was observed for tdTomato signal in *Tie2Cre;Ai9* mesentery (*SI Appendix, Fig. S1A*). Therefore, *Lyve1Cre;Piezo1^{CKO}* and *Tie2Cre;Piezo1^{CKO}* both appear to delete PIEZO1 from LECs as well as BECs, although the efficiency of recombination in BECs might be less in *Lyve1Cre;Piezo1^{CKO}* mice (35). Regardless, we used the *Lyve1Cre;Piezo1^{CKO}* mice to confirm our results obtained from *Tie2Cre;Piezo1^{CKO}* mice.

Lyve1Cre;Piezo1^{CKO} mice showed postnatal lethality around 1–2 wk after birth and pleural effusion 1 wk after birth (Fig. 1*D–F*), resembling *Tie2Cre;Piezo1^{CKO}* mice. In human *PIEZO1*-deficient patients, respiratory defects resulting from severe pleural effusion were also reported (9). In line with this, three out of four *Lyve1Cre;Piezo1^{CKO}* mice exhibited collapsed alveoli at postnatal day 7 (P7), while this was never observed in WT mice (*SI Appendix, Fig. S2B*). Taken together, these mouse models exhibit similar defects in patients with *PIEZO1* loss-of-function mutations, suggesting that a role of PIEZO1 in the lymphatic system is likely conserved between human and mouse.

Reduced Number of Lymphatic Valves in *Tie2Cre;Piezo1^{CKO}* and *Lyve1Cre;Piezo1^{CKO}* Mice. Next, we checked the structure of the lymphatic vasculature of *Tie2Cre;Piezo1^{CKO}* and *Lyve1Cre;Piezo1^{CKO}* mice in the mesentery, where the development of lymphatic vasculature is well studied (7). At P4, it was evident that lymphatic valves, which are marked by clusters of Prox1^{high} nuclei and strong signal of integrin $\alpha 9$, were drastically reduced in *Tie2Cre;Piezo1^{CKO}* mice compared with WT littermates (Fig. 2*A* and *B*). Reduced numbers of lymphatic valves were also observed in P4 *Lyve1Cre;Piezo1^{CKO}* mice, compared with WT littermates (Fig. 2*C* and *D*). Therefore, endothelial PIEZO1 is required for the formation and/or maintenance of lymphatic valves. Given the similar phenotypes observed with *Tie2Cre* and

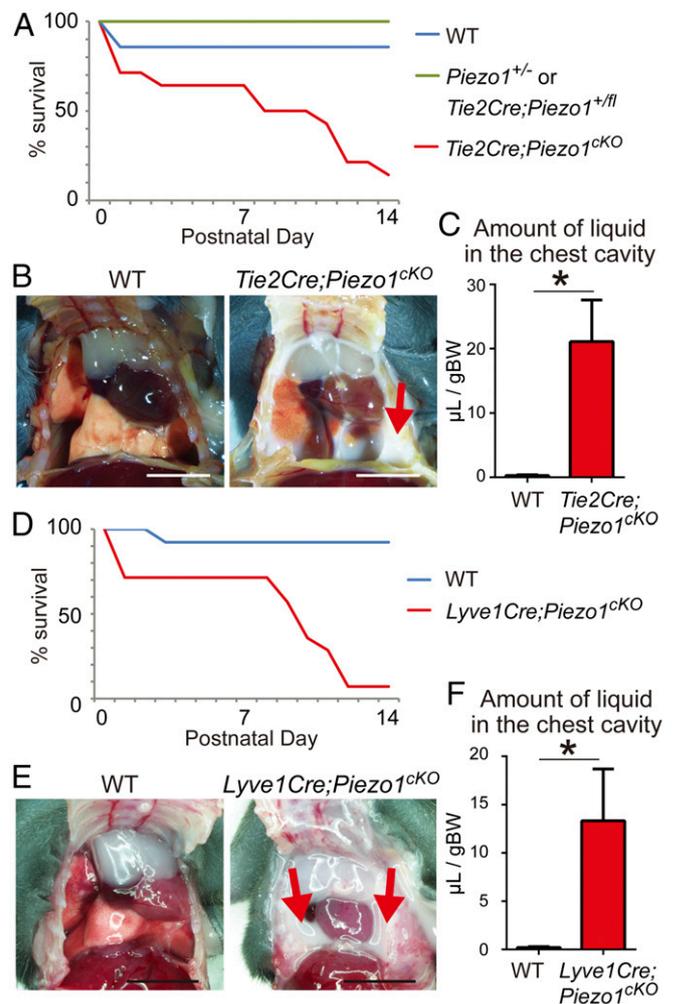


Fig. 1. Postnatal lethality, pleural effusion in endothelial-specific *Piezo1* cKO mice. (*A*) Survival rate of WT ($n = 14$), Het (*Piezo1^{+/-}* or *Tie2Cre;Piezo1^{+fl}*) ($n = 13$), or *Tie2Cre;Piezo1^{CKO}* ($n = 14$) newborn mice. (*B*) Chylous pleural effusion (red arrow) observed in 1-wk-old *Tie2Cre;Piezo1^{CKO}*, but not in WT littermate. (Scale bar: 5 mm.) (*C*) Amount of liquid in the chest cavity at P9. Mean \pm SEM ($n = 6$ for WT, $n = 5$ for *Tie2Cre;Piezo1^{CKO}* mice). * $P < 0.05$, unpaired *t* test. (*D*) Survival rate of WT ($n = 13$) or *Lyve1Cre;Piezo1^{CKO}* ($n = 15$) newborn mice. (*E*) Chylous pleural effusion (red arrows) observed in 1-wk-old *Lyve1Cre;Piezo1^{CKO}* mice, but not in WT littermate. (Scale bar: 5 mm.) (*F*) Amount of liquid in the chest cavity in 1-wk-old mice. Mean \pm SEM ($n = 16$ for WT, $n = 8$ for *Lyve1Cre;Piezo1^{CKO}* mice). * $P < 0.05$, unpaired *t* test.

Lyve1Cre, we performed the rest of the analysis with *Tie2Cre;Piezo1^{CKO}* mice.

Impaired Lymphatic Valve Protrusion in *Tie2Cre;Piezo1^{CKO}* Mice. Our results suggested an almost complete absence of lymphatic valves at postnatal day 4. Next, we checked the development of lymphatic valves in *Tie2Cre;Piezo1^{CKO}* embryos. The development of lymphatic valves is divided into several stages (7, 39). Up-regulation of Prox1 expression (Prox1^{high}) is a marker for cells which will form valve leaflets (7, 39). First, a few Prox1^{high} LECs appear in the vessel (stage 1). Next, the number of Prox1^{high} cells increases and they align in the shape of a ring in the vessel (stage 2). The rings of Prox1^{high} cells are often formed around branching points within vessels. Then, clusters of Prox1^{high} cells further develop into valves. During this process, Prox1^{high} cells align perpendicular to the vessel's axis (reorientation) (stage 2.5). Reoriented Prox1^{high} cells migrate toward the lumen (valve protrusion), resulting in disk-like structures (39) and constriction

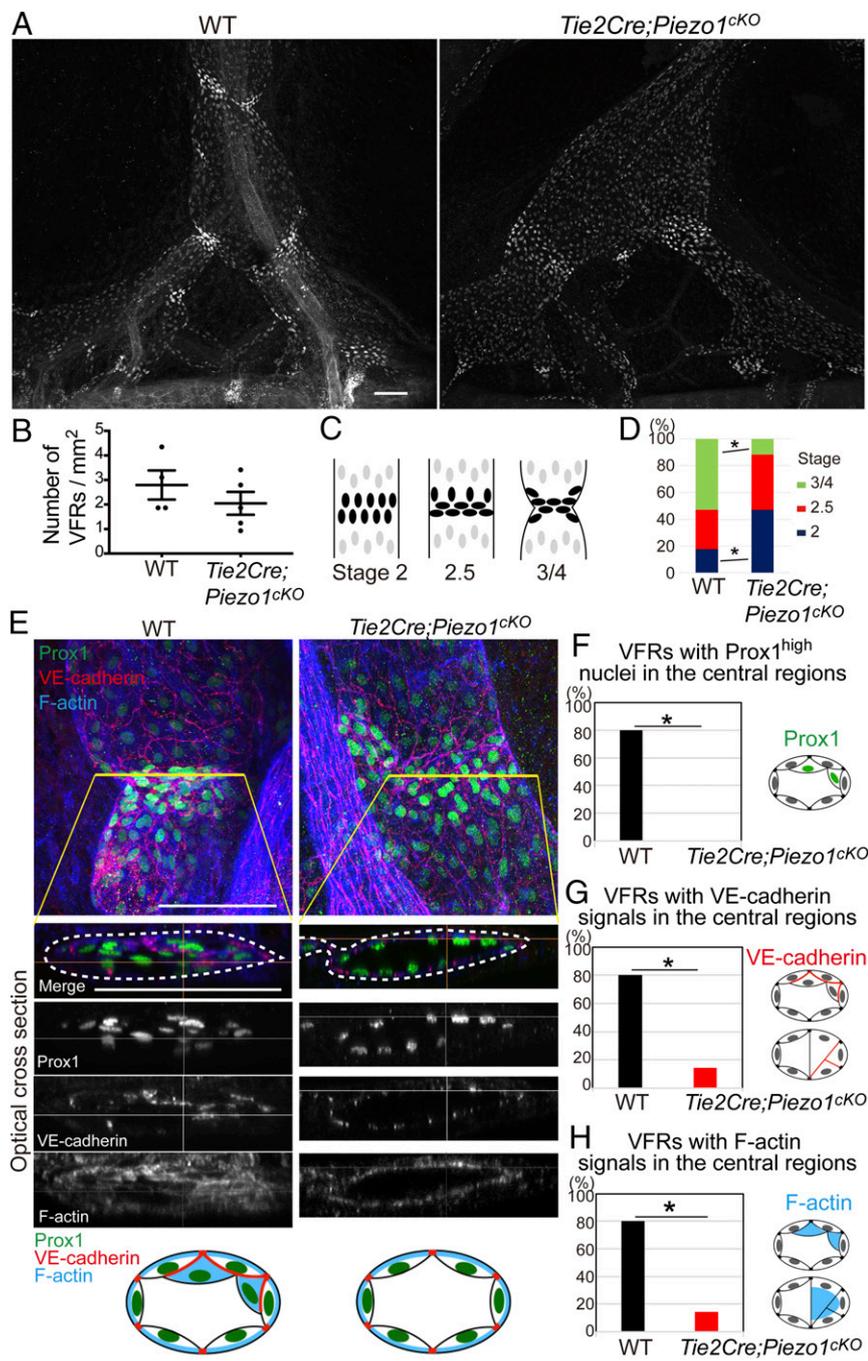


Fig. 3. Impaired lymphatic valve protrusion in *Tie2Cre;Piezo1^{cKO}* mice. (A) E18.5 mesentery with Prox1 staining. (Scale bar: 100 μ m.) (B) Number of VFRs (containing $n \geq 10$ Prox1^{high} nuclei) per area in E18.5 mesentery. Mean \pm SEM ($n = 4$ for WT, $n = 5$ for *Tie2Cre;Piezo1^{cKO}* embryos). $P = 0.35$, unpaired t test. (C) Stages of lymphatic valve formation. Stage 2: there are $n < 5$ reoriented Prox1^{high} nuclei and the vessel is not constricted. Stage 2.5: $n \geq 5$ Prox1^{high} nuclei are reoriented but the vessel is not constricted. Stages 3 and 4: $n \geq 5$ Prox1^{high} nuclei are reoriented and the vessel is constricted. (D) Percentage of each stage among VFRs in E18.5 mesentery. $n = 17$ VFRs from four embryos per genotype. $*P < 0.05$, χ^2 test. (E) Representative images of VFRs in E18.5 mesentery. Yellow lines (Top, side view) show where images were optically cross-sectioned (Middle) and schematic illustration of distribution of Prox1^{high} nucleus (green), VE-cadherin (red), and F-actin (blue) signals (Bottom). Dotted lines demarcate lymphatic vessels. (Scale bar: 100 μ m.) (F–H) Percentage of VFRs in E18.5 mesentery in which several Prox1^{high} nuclei (F), VE-cadherin (G), or F-actin (H) signal was located in the more central regions than Prox1^{high} nuclei on the vessel wall. Right shows examples of patterns of each signal. $n = 10$ images from four WT embryos, $n = 14$ images from four *Tie2Cre;Piezo1^{cKO}* embryos. $*P < 0.05$, χ^2 test.

cell–cell junctions (39, 40). To test whether the activation of PIEZO1 can affect these components in LECs, we used Yoda1, a chemical agonist of PIEZO1, but not of PIEZO2 (42, 43). Before Yoda1 administration, actin fibers associated with phospho-myosin light chain 2 (pMLC2) were abundantly observed in cultured LECs, while VE-cadherin⁺ cell–cell adhesion appeared punctate. To activate PIEZO1, we used Yoda1 at 1.5 μ M. This represents a moderate stimulus as the EC₅₀ of Yoda1 activation of PIEZO1 is ~ 25 μ M. Treatments with higher concentrations, such as 5 μ M, resulted in detachment of LECs (SI Appendix, Fig. S4A and B). When LECs were exposed to 1.5 μ M Yoda1 for 1 h, drastic changes were observed in the LEC morphology described above (Fig. 5A). The abundant F-actin stress fibers reorganized into predominantly cortical actin structures. pMLC2 signal was greatly reduced, and VE-cadherin⁺ cell–cell boundaries were straightened.

Continued exposure to Yoda1 (16 h) led to further changes in cell morphology. LECs now became elongated with F-actin stress fibers and pMLC2 signals, while VE-cadherin⁺ cell–cell adhesion was poorly formed. These LECs often overlapped each other. These effects of Yoda1 were suppressed by knockdown of *PIEZO1* induced by siRNA treatment, confirming that these changes in F-actin, pMLC2, and VE-cadherin are dependent on PIEZO1 activation (Fig. 5B and C and SI Appendix, Fig. S4C). These data suggest that activation of PIEZO1 in LECs can induce remodeling of actomyosin and/or VE-cadherin⁺ cell–cell adhesion.

Discussion

Lymphatic valves are an essential structure for proper flow of the lymph throughout the body. In this study, by utilizing mouse lines lacking PIEZO1 in endothelial cells, we show that PIEZO1 is

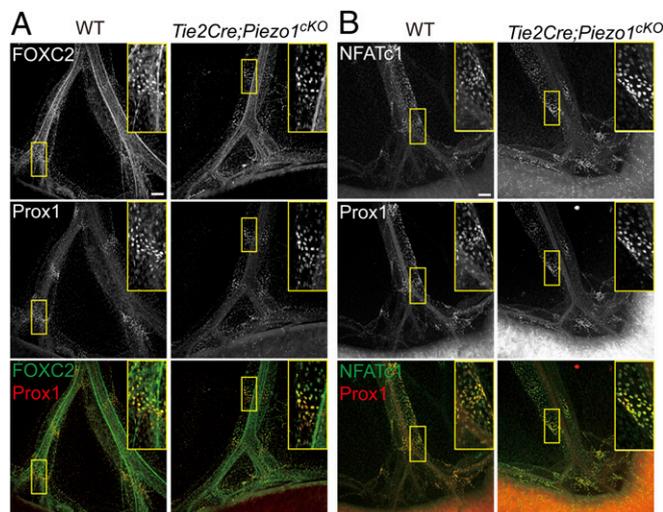


Fig. 4. Expression of transcription factors involved in initiation of valve development in *Tie2Cre;Piezo1^{CKO}* mice. (A and B) FOXC2 (A) or NFATc1 (B) with Prox1 staining in E17.5 mesentery of WT or *Tie2Cre;Piezo1^{CKO}* embryos. Insets show magnified images. FOXC2: $n = 8$ for WT, $n = 7$ for *Tie2Cre;Piezo1^{CKO}* embryos. NFATc1: $n = 7$ for WT, $n = 6$ for *Tie2Cre;Piezo1^{CKO}* embryos. (Scale bar: 100 μm .)

required for the formation of lymphatic valves. These knockout mice also exhibited pleural effusion, possibly leading to collapsed alveoli, and difficulty in breathing, which might cause the lethality of these mice. These phenotypes bear a strong resemblance to what was observed in human patients with familial lymphatic dysfunctions associated with loss-of-function variants of *PIEZO1* (9–11). It is worth noting that in those patients, deep rerouting of lymph was observed in the lower limbs, likely resulted from attenuated drainage by superficial lymphatic vessels (10). These data suggest that the function of *PIEZO1* in the lymphatic system is likely to be conserved between human and mouse.

The severity of phenotypes associated with loss-of-function *PIEZO1* mutations in patients covers a wide range. Some patients survive to adulthood, while others experience fetal or neonatal demise due to severe tissue edema sometimes characterized as fetal or neonatal hydrops (9–11). As varying severity was reported between siblings carrying the same *PIEZO1* variants, there may be genetic modifiers or environmental effects, which might also explain the phenotypic difference between *Tie2Cre;Piezo1^{CKO}* mice of our colony and one previously reported (11, 25).

Mechanotransduction plays an essential role in the development, maintenance, and function of the vasculature. Here, we show that *PIEZO1* is one of the mechanosensors required for lymphatic and venous valve formation, likely playing a role during the valve protrusion process. However, our data do not exclude the possibility that phenotypes other than impaired valve formation contribute to the phenotypes observed in the mutant mice. In contrast, we find that *PIEZO1* is dispensable for early events in valve development, as the up-regulation of FOXC2 and NFATc1, key transcription factors in valve development that are up-regulated by oscillatory shear stress in cultured LECs, were not affected in *Tie2Cre;Piezo1cKO* mice (7, 8). Our in vivo data are in line with previous findings showing that knockdown of *PIEZO1* and/or *PIEZO2* does not affect transcriptional up-regulation of FOXC2, *Cx37*, *Integrin $\alpha 9$* , or *GATA2* induced by oscillatory shear stress (8). These data suggest that there are at least two different mechanotransduction pathways involved in the formation of lymphatic

valves. The identity of the other mechanosensor required for initiation of valve formation remains unknown.

Our in vitro data suggest that activation of *PIEZO1* can induce dynamic remodeling of actomyosin and/or VE-cadherin⁺ cell–cell adhesion in LECs. It is important to note, however, that we do not present data showing whether mechanical forces relevant for LECs

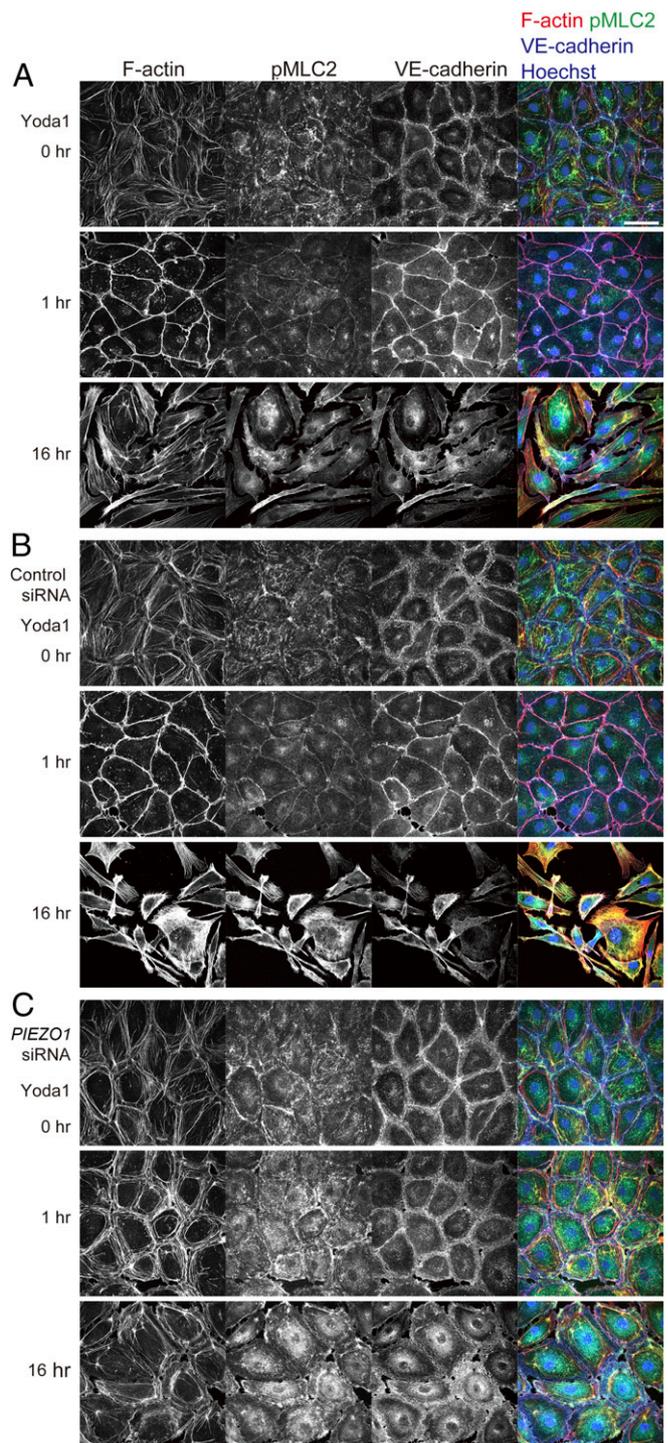


Fig. 5. *PIEZO1* activation-induced changes in actomyosin and VE-cadherin⁺ cell–cell adhesion in cultured LECs. (A) Staining of F-actin, pMLC2, and VE-cadherin in LECs treated with 1.5 μM Yoda1 for 0, 1, or 16 h. (Scale bar: 50 μm .) (B and C) Staining of F-actin, pMLC2, and VE-cadherin in LECs treated with control (B) or *PIEZO1* siRNA (C) before Yoda1 administration. $n = 2$ experiments.

during valve formation are sufficient to induce the changes we observe by Yoda1. Future experiments will address this issue.

Nevertheless, as Yoda1 was administered, VE-cadherin⁺ cell-cell junctions changed their form from punctate into straightened ones. These structures may have similarities to previously reported focal adherens junction (also called punctate or discontinuous) and linear adherens junction (also called continuous); the former is connected to radial actin bundles that pull on the cadherin–catenin complex, and the latter is supported by cortical parallel actin bundles (44, 45). As implied by the change in the amount of pMLC2, contracting force on actomyosin might be attenuated by Yoda1-induced PIEZO1 activation.

The effect of PIEZO1 on cellular shape or migration has been reported in BECs and other cell types (15, 25). In BECs, it is suggested that the effect of PIEZO1 on cell alignment against flow is through calpain-induced remodeling of focal adhesion (25). In CHO cells ectopically expressing $\alpha 4\beta 1$ integrin, PIEZO1-dependent intracellular calcium increase is suggested to negatively regulate protein kinase A, which in turn modulates proteins, including Rac1, a modulator of F-actin dynamics (46). While the molecular mechanisms through which PIEZO1 activation leads to the remodeling of cytoskeleton and/or cell–cell adhesion in LECs remain to be determined, regulation of these cellular components is likely the key for valve morphogenesis. Together, our data highlight the requirement of mechanosensor channel PIEZO1 in the formation of lymphatic valves, an essential structure for lymph circulation.

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Methods

All animal procedures were approved by The Scripps Research Institute, National Institute for Basic Biology, and University of Pennsylvania Institutional Animal Care and Use Committees. Detailed methods are provided in *SI Appendix, SI Materials and Methods*.

Antibodies and Dyes. The following antibodies and dyes were used: Prox1 (1:200, 76696; abcam), integrin $\alpha 9$ (10 μ g/mL, AF3827; R&D Systems), FOXC2 (10 μ g/mL, AF3827; R&D Systems), NFATc1-488 (1:50, 649604; BioLegend), mVE-cadherin (1:200, 555289; BD Biosciences), hVE-cadherin (3 μ g/mL, AF938; R&D Systems), pMLC2 (1:300, 3675; Cell Signaling Technology), and Phalloidin-594 (1:200, A12381; Thermo Fisher Scientific).

Chemically Induced Piezo1 Activation. Round-shaped cover glasses (Matsunami) were coated with 20 μ g/mL human plasma fibronectin (Millipore) in PBS, then LECs were seeded onto slides and grew to 80–100% confluence. Culture medium was replaced with one containing 1.5 μ M Yoda1 (Sigma). After 1 or 16 h, medium was washed out and fixed with 4% PFA for 15 min. Immunostaining was performed as previously described (15).

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