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−/− 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−/− 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.

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

The lymphatic system is essential for fluid homeostasis in the body. Lymphatic vessels collect extravascular 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 (LEC), 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 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). PIEZO2 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 vasorestriction 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

Significance

PIEZO2s 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 PIEZO2s beyond acute signaling molecules (e.g., touch sensation) and highlights the importance of these ion channels in controlling morphological/structural specification during development.
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 Piezo1fl/fox mice to generate Tie2Cre;Piezo1fl/fox mice (29, 33). Tie2Cre;Piezo1fl/fox 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;Piezo1fl/fox mice died within 2 wk after birth (Fig. 1A). 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; Piezo1fl/fox 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 ofloxP 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-tdTmato 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. S24). These results were identical to what was observed for tdTomato signal in Tie2Cre; Ai9 mesentery (SI Appendix, Fig. S1A). Therefore, Lyve1Cre; Piezo1flox/fl and Tie2Cre;Piezo1flox/fl both appear to delete PIEZO1 from LECs as well as BECs, although the efficiency of recombination in BECs might be less in Lyve1Cre;Piezo1flox/fl mice (35). Regardless, we used the Lyve1Cre;Piezo1flox/fl mice to confirm our results obtained from Tie2Cre;Piezo1flox/fl mice.

Lyve1Cre;Piezo1flox/fl mice showed postnatal lethality around 1–2 wk after birth and pleural effusion 1 wk after birth (Fig. 1D–F), resembling Tie2Cre;Piezo1flox/fl 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;Piezo1flox/fl 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;Piezo1flox/fl and Lyve1Cre;Piezo1flox/fl Mice. Next, we checked the structure of the lymphatic vasculature of Tie2Cre;Piezo1flox/fl and Lyve1Cre;Piezo1flox/fl 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 Prox1high nuclei and strong signal of integrin α9, were drastically reduced in Tie2Cre;Piezo1flox/fl mice compared with WT littermates (Fig. 2 A and B). Reduced numbers of lymphatic valves were also observed in P4 Lyve1Cre;Piezo1flox/fl 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

![Image](https://www.pnas.org/content/115/2/6093.f1)

**Fig. 1.** Postnatal lethality, pleural effusion in endothelial-specific Piezo1cko mice. (A) Survival rate of WT (n = 14), Het (Piezo1+− or Tie2Cre; Piezo1fl/fl) (n = 13), or Tie2Cre;Piezo1flox/fl (n = 14) newborn mice. (B) Chylous pleural effusion (red arrow) observed in 1-wk-old Tie2Cre;Piezo1flox/fl but not in WT littermate. (Scale bar: 5 mm.) (C) Amount of liquid in the chest cavity at P9. Mean ± SEM (n = 6 for WT, n = 5 for Tie2Cre;Piezo1flox/fl mice). *P < 0.05, unpaired t test. (D) Survival rate of WT (n = 13) or Lyve1Cre;Piezo1flox/fl (n = 15) newborn mice. (E) Chylous pleural effusion (red arrows) observed in 1-wk-old Lyve1Cre;Piezo1flox/fl mice, but not in WT littermate. (Scale bar: 5 mm.) (F) Amount of liquid in the chest cavity in 1-wk-old mice. Mean ± SEM (n = 16 for WT, n = 8 for Lyve1Cre;Piezo1flox/fl mice). *P < 0.05, unpaired t test.

Lyve1Cre, we performed the rest of the analysis with Tie2Cre; Piezo1flox/fl mice.

Impaired Lymphatic Valve Protrusion in Tie2Cre;Piezo1flox/fl 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;Piezo1flox/fl embryos. The development of lymphatic valves is divided into several stages (7, 39). Up-regulation of Prox1 expression (Prox1high) is a marker for cells which will form valve leaflets (7, 39). First, a few Prox1high LECs appear in the vessel (stage 1). Next, the number of Prox1high cells increases and they align in the shape of a ring in the vessel (stage 2). The rings of Prox1high cells are often formed around branching points within vessels. Then, clusters of Prox1high cells further develop into valves. During this process, Prox1high cells align perpendicular to the vessel’s axis (reorientation) (stage 2.5). Reoriented Prox1high cells migrate toward the lumen (valve protrusion), resulting in disk-like structures (39) and constriction
of the vessel (stage 3). The valves continue to elongate until they form V-like shapes with leaflets in association with a thick extracellular matrix core (stage 4). Valve development is known to commence earlier in larger diameter, more proximal lymphatic vessels. Therefore, at embryonic day 18.5 (E18.5), valves at different stages of development can normally be identified in different segments of mouse mesentery. We first compared the number of valve-forming regions (VFRs), containing ≥10 Prox1\textsuperscript{high} nuclei, in E18.5 mesentery between WT and Tie2Cre;Piezo1\textsuperscript{KO} embryos. In the mesentery of E18.5 Tie2Cre;Piezo1\textsuperscript{KO} embryos, there was an overrepresentation of valves at stage 2 at the expense of stages 3 and 4, compared with WT littermates. The percentage of valves at intermediate phase (stage 2.5) was not different between Tie2Cre;Piezo1\textsuperscript{KO} and WT mesenteries (Fig. 3D). Next, we examined the structure of VFRs of Tie2Cre;Piezo1\textsuperscript{KO} embryos in more detail. Previous studies demonstrated that valve protrusion results from collective cell migration of Prox1\textsuperscript{high} cells, which is associated with actin polymerization and formation of cell–cell junctions (39, 40). In the optical cross-sections at VFRs of WT embryos, Prox1\textsuperscript{high} nuclei were observed not only on the wall of the vessel, but also in the more central regions, a sign of cell migration, which results in valve protrusion (Fig. 3 E and F). These Prox1\textsuperscript{high} nuclei were indeed associated with VE-cadherin and F-actin signals (Fig. 3 E, G, and H). In the cross-sections at VFRs of Tie2Cre;Piezo1\textsuperscript{KO} embryos, Prox1\textsuperscript{high} nuclei were lined as a single layered ring, suggesting a defect in cell migration (Fig. 3 E and F). In addition, in these sections, signals of F-actin or VE-cadherin were rarely observed in the more central regions than Prox1\textsuperscript{high} nuclei on the vessel wall, suggesting that structures such as cell extension toward lumen or cell–cell junction between valve-forming cells (that might contribute to valve protrusion) were not formed in VFRs of Tie2Cre;Piezo1\textsuperscript{KO} embryos (Fig. 3 E, G, and H). These data suggest that the protrusion process of lymphatic valves is impaired in Tie2Cre;Piezo1\textsuperscript{KO} lymphatic vasculature.

We also examined valves in the veins and the heart in Tie2Cre;Piezo1\textsuperscript{KO} mice. In P3 iliac veins, valves marked with Prox1\textsuperscript{high} nuclei were observed in WT mice but not in Tie2Cre;Piezo1\textsuperscript{KO} mice, suggesting that PIEZO1 is also required for venous valve formation (SI Appendix, Fig. S3A). In contrast, no obvious defects were detected in the heart valves of P6 Tie2Cre;Piezo1\textsuperscript{KO} mice (SI Appendix, Fig. S3B). Whether the deficits in venous valve formation contribute to the pathology or lethality of Tie2Cre;Piezo1\textsuperscript{KO} mice is not known.

Expression of Transcription Factors Involved in Valve Development in Tie2Cre;Piezo1\textsuperscript{KO} Mice. A number of proteins, including transcription factors, are specifically expressed or activated at VFRs and are involved in the initiation of valve development (3). Recent studies utilizing cultured LECs suggest that shear stress induced by lymph flow is upstream and induces these changes, implying that mechanotransduction is involved in this process (6–8, 41). Our previous in vitro data suggested that PIEZO1 is not required for flow-induced expression of FOXC2 and GATA2 in LECs (8). Nuclear localization of another transcription factor NFATc1 at VFRs is suggested to occur via Ca\textsuperscript{2+} signaling (7). To assess the involvement of PIEZO1 in expression of transcriptional factors at VFRs, we examined the distribution of FOXC2 and NFATc1 in Tie2Cre;Piezo1\textsuperscript{KO} lymphatic vasculature. Clusters of FOXC2\textsuperscript{high} or NFATc1\textsuperscript{high} nuclei were observed overlapping with Prox1\textsuperscript{high} signal in both WT and Tie2Cre;Piezo1\textsuperscript{KO} mesenteries (Fig. 4). Other transcription factors and proteins are involved in the initiation of valve development, and it remains an open question whether PIEZO1 is involved in such pathways. However, in vivo data suggest that up-regulated expression of FOXC2 and nuclear localization of NFATc1 at VFRs, which are currently proposed to occur through mechanotransduction, are not dependent on PIEZO1.

Piezo1 Activation Induced Cytoskeletal and Cell–Cell Adhesion Remodeling in Cultured LECs. Our in vivo data suggest that PIEZO1 is involved in the protrusion process of lymphatic valves, which was previously shown to be associated with remodeling of the cytoskeleton and
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
$^+\text{-cell – cell adhesion}$ appeared punctate. To activate PIEZO1, we used Yoda1 at 1.5 $\mu$M. This represents a moderate stimulus as the EC$_{50}$ of Yoda1 activation of PIEZO1 is $\sim$25 $\mu$M. Treatments with higher concentrations, such as 5 $\mu$M, resulted in detachment of LECs (SI Appendix, Fig. S4A and B). When LECs were exposed to 1.5 $\mu$M Yoda1 for 1 h, drastic changes were observed in the LEC morphology described above (Fig. S4A). 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
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 \( \text{PIEZO1} \) (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 \( \text{PIEZO1} \) in the lymphatic system is likely to be conserved between human and mouse.

The severity of phenotypes associated with loss-of-function \( \text{PIEZO1} \) 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 \( \text{PIEZO1} \) variants, there may be genetic modifiers or environmental effects, which might also explain the phenotypic difference between \( \text{Tie2Cre;Piezo1 cKO} \) 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 \( \text{PIEZO1} \) 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 \( \text{PIEZO1} \) is dispensable for early events in valve development, as the up-regulation of \( \text{FOXC2} \) and \( \text{NFATc1} \), key transcription factors in valve development that are up-regulated by oscillatory shear stress in cultured LECs, were not affected in \( \text{Tie2Cre;Piezo1 cKO} \) mice (7, 8). Our in vivo data are in line with previous findings showing that knockdown of \( \text{PIEZO1} \) and/or \( \text{PIEZO2} \) does not affect transcriptional up-regulation of \( \text{FOXC2} \), \( \text{Cx37} \), \( \text{Integrin} \alpha9 \), or \( \text{GATA2} \) 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 \( \text{PIEZO1} \) 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. 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 \( \mu \)m.)](https://www.pnas.org/content/early/2020/07/19/2004935117)

![Fig. 5. \( \text{PIEZO1} \) 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 \( \mu \)M Yoda1 for 0, 1, or 16 h. (Scale bar: 50 \( \mu \)m.) (B) and (C) Staining of F-actin, pMLC2, and VE-cadherin in LECs treated with control (B) or \( \text{PIEZO1} \) siRNA (C) before Yoda1 administration. \( n = 2 \) experiments.](https://www.pnas.org/content/early/2020/07/19/2004935117)
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 radially 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 α4β1 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, we data highlight the requirement of mechanosensor channel PIEZO1 in the formation of lymphatic valves, an essential structure for lymph circulation.

**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 α9 (10 μg/mL, AF3827; R&D Systems), FOXC2 (10 μg/mL, AF3827; R&D Systems), NFATc1-488 (1:50, 649604; BioLegend), mVE-cadherin (1:200, 555289; BD Biosciences), hVE-cadherin (3 μg/mL, AF938; R&D Systems), pMLC2 (1:300, 3675; Cell Signaling Technology), and Phalloidin-S419 (1:200, A12381; Thermo Fisher Scientific).

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

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