RESEARCH ARTICLE

Syndecan 4 controls lymphatic vasculature remodeling during mouse embryonic development

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ABSTRACT

The role of fluid shear stress in vasculature development and remodeling is well appreciated. However, the mechanisms regulating these effects remain elusive. We show that abnormal flow sensing in lymphatic endothelial cells (LECs) caused by Sdc4 or Pecam1 deletion in mice results in impaired lymphatic vessel remodeling, including abnormal valve morphogenesis. Ablation of either gene leads to the formation of irregular, enlarged and excessively branched lymphatic vessels. In both cases, lymphatic valve-forming endothelial cells are randomly oriented, resulting in the formation of abnormal valves. These abnormalities are much more pronounced in Sdc4−/−, Pecam1−/− double-knockout mice, which develop severe edema. In vitro, SDC4 knockdown human LECs fail to align under flow and exhibit high expression of the planar cell polarity protein VANGL2. Reducing VANGL2 levels in SDC4 knockdown LECs restores their alignment under flow, while VANGL2 overexpression in wild-type LECs mimics the flow alignment abnormalities seen in SDC4 knockdown LECs. SDC4 thus controls flow-induced LEC polarization via regulation of VANGL2 expression.

KEY WORDS: Syndecan 4, Lymphatic remodeling, Embryonic development

INTRODUCTION

Tissue remodeling is an important process during organogenesis. In the vascular system, fluid flow plays a crucial role in the morphogenesis of both blood and lymphatic vasculature in a manner that is still poorly understood. Lymphatic vessels constitute a distinct vascular network that takes up interstitial fluid (lymph) and returns it to systemic circulation. The effective unidirectional transport of the lymphatic fluid requires the formation of lymphatic intraluminal valves (hereafter referred to as lymphatic valves) that prevent backflow of lymph. A previous study has established that initiation of lymphatic fluid flow is unidirectional transport of the lymphatic fluid requires the receptor (Astudillo et al., 2014). Syndecan 4 also regulates the Wnt/β-catenin pathway in cardiomyocytes (Finsen et al., 2011), and in modulation of the Wnt/β-catenin pathway in Xenopus via interaction with the LR6 receptor (Astdullo et al., 2014). Syndecan 4 also regulates the Wnt/planar cell polarity (PCP) pathway during convergent extension (Muñoz et al., 2006), and induces clathrin-mediated endocytosis in PCP signaling (Ohkawara et al., 2011). In mouse, syndecan 4 (Sdc4) has been shown to interact genetically with the core PCP protein vang-like 2 (Vangl2), through which it regulates neural tube closure, wound healing and stereocilia orientation in sensory hair cells (Escobedo et al., 2013).

Given this involvement of SDC4 and PECAM1 in mechanotransduction, we set out to study the role of these proteins in lymphatic vasculature development. We find that deletion of either Sdc4 or Pecam1 in mice results in abnormal lymphatic vessel remodeling and, in particular, abnormal lymphatic valve formation due to the failure of lymphatic valve-forming ECs to properly align under flow. Deletion of both genes resulted in more profound abnormalities than caused by deletion of either gene alone. Finally, in the case of Sdc4 deletion, these effects were mediated by an abnormal flow-induced increase in Vangl2 levels.

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RESULTS

Lymphatic vessels show remodeling defects in Sdc4−/− mice

To study the function of Sdc4 in lymphatic vasculature formation, we used the development of mesenteric vessels as a model. In control mice, small-diameter, cord-like lymphatic endothelial structures (Prox1+, Vegfr3+, VE-cadherin+) are present by embryonic day (E) 14.5 (Fig. S1A, white arrow). The lymphatic endothelial cells (LECs) in these vessels extend fine cellular processes in order to connect to each other (Fig. S1A, yellow arrow). By E15.5, larger diameter lymphatic tubular structures are formed (Fig. S1A, arrow) and a complex, honeycombed primary lymphatic plexus (Prox1+, Vegfr3+) has developed by E16.5 (Fig. S1B). This primary lymphatic plexus is then remodeled into a complete lymphatic vascular network including capillaries and collecting lymphatic vessels (Fig. 1A,B).

In Sdc4−/− mice, the development of mesenteric lymphatic vessels is similar to that of the control from E14.5 through E16.5 (Fig. S1A,B). However, abnormal lymphatic vessels are observed in the mutants at later embryonic stages (Fig. 1C,D). At E18.5, instead of forming a hierarchal vascular network (Fig. 1A,B), mesenteric lymphatic vessels in Sdc4 nulls tend to form a vascular plexus with multiple branches (Fig. 1C). Compared with littermate controls, cell-cell junctions in Sdc4−/− mice are less linear and show numerous protrusions, exhibiting a wavy appearance (Fig. S1C). Moreover, enlarged, irregular lymphatic vessels are frequently seen in Sdc4 nulls (Fig. 1D).

To determine the cause of the increased lymphatic vessel diameter, we examined the cell cycle distribution of LECs and BECs in the mutants using fluorescence-activated cell sorting (FACS) (Fig. S2A,B). The number of LECs in the S/G2/M phases of the cell cycle was increased in Sdc4−/− mice compared with controls (Fig. S2A), suggesting increased proliferation. Importantly, this was not seen in BECs in Sdc4 nulls (Fig. S2B). These data are consistent with increased lymphatic but not blood vessel diameter in Sdc4−/− mice.

![Image](image-url)

Fig. 1. Lymphatic vessels show remodeling defects in Sdc4−/− mice during late embryonic development. (A-D) Whole-mount immunofluorescence staining for Vegfr3, showing a hierarchal lymphatic vascular network in the mesentery of control mice at E18.5 (A,B); yellow arrow, capillaries; white arrows, lymphatic collecting vessels. In Sdc4 nulls, by contrast, lymphatic vessels are abnormally branched and tend to form a vascular plexus (C, arrows). Enlarged lymphatic vessels were also seen in Sdc4−/− mice (D, arrows). (E) Prox1high lymphatic valve-forming ECs reorient perpendicular to flow direction at E17.5 in control animals. The nuclei of valve-forming cells are highly elongated and are tightly packed (a, arrowheads). At E18.5, valve-forming cells reorganize again and form mature lymphatic valves containing two leaflets (b, arrowheads). Prox1high valve-forming cells are concentrated in valve areas in the control (c, arrows). (F) The nuclei of Prox1high valve-forming cells are rounded (a, arrowheads), randomly oriented and loosely arranged in Sdc4−/− mice at E17.5. Nuclei of similar morphology and organization were also seen in the mutants at E18.5 (b). Some of the Prox1high valve-forming cells in Sdc4−/− embryos do not reorient, and remain parallel to the longitudinal axis of lymphatic vessels (c, arrow). (G) Nuclear orientation of Prox1high cells in E17.5 mesenteric lymphatic vessels of control and Sdc4−/− mice (n=5). The percentage of nuclei reoriented >45° relative to flow direction is indicated. Mann–Whitney test. (H) Quantification of nuclear shape (roundness) of Prox1high cells in E17.5 mesenteric lymphatic vessels (n=5). Mann–Whitney test. Scale bars: 200 μm in A–D; 30 μm in Ea,b,Fa,b; 100 μm in Ec,Fc.
**Prox1\(^{\text{high}}\) lymphatic valve-forming ECs in Sdc4 nulls are abnormally oriented**

Given these remodeling abnormalities, we set out to investigate the development of lymphatic valves, an important event during lymphatic vasculature remodeling. In control mice, lymphatic valves start to develop in mesenteric lymphatic vessels at E16.5 (Sabine et al., 2012; Tatin et al., 2013). This is also the time when flow begins (Sabine et al., 2012). In agreement with previous reports (Sabine et al., 2012; Tatin et al., 2013), the development of lymphatic valves in control mice begins by the formation of clusters of Prox1\(^{\text{high}}\) lymphatic valve-forming ECs that are initially aligned along the longitudinal axis of lymphatic vessels (Fig. S1D). Valve-forming cells subsequently reorient 90° and become perpendicular to the direction of flow (Fig. 1Ea). During this reorientation process, the nuclei of Prox1\(^{\text{high}}\) valve-forming cells show morphological and organizational changes, becoming highly elongated and tightly packed (Fig. 1Ea). Furthermore, nuclear morphology and orientation are highly correlated with cell morphology and cell orientation (Fig. S3). By contrast, in Sdc4\(^{-/-}\) mice, the nuclei of Prox1\(^{\text{high}}\) cells are rounded, randomly oriented and loosely organized (Fig. 1Fa).

Since it is more accurate to measure nuclear rather than cell orientation, we assessed valve-forming cell orientation by measuring the angle of nuclei relative to the flow direction (Fig. 1G). In control mice, the majority of valve-forming cells (87%) are oriented ≥45° relative to flow direction at E17.5 (Fig. 1G). In Sdc4 nulls, even though the formation of lymphatic valves begins normally (Fig. S1D), only 57% of the valve-forming cells are oriented ≥45° (Fig. 1G). Nearly half of the valve-forming cells (43%) in Sdc4 nulls are arranged within 45° relative to flow direction (Fig. 1G).

The morphology of Prox1\(^{\text{high}}\) LECs was examined by measuring the roundness of the nuclei (Fig. 1H). In agreement with immunostaining results at E17.5 (Fig. 1Ea,Fa), Prox1\(^{\text{high}}\) valve-forming cells in Sdc4 nulls are less elongated than in controls (Fig. 1H). Moreover, Prox1\(^{\text{high}}\) valve-forming cells in Sdc4 nulls are loosely organized and form a wide band of cells (Fig. 1Fa,b), whereas they are tightly packed in control embryos (Fig. 1Ea,b). At E18.5, valve-forming cells in control animals reorient again to form mature lymphatic valves that have two leaflets (Fig. 1Eb), whereas in Sdc4 nulls the Prox1\(^{\text{high}}\) LECs exhibit a similar organization as at E17.5 (Fig. 1Fb). Also, some of the Prox1\(^{\text{high}}\) cells in Sdc4 nulls do not reorient and remain parallel to the flow direction along the lymphatic vessel wall (Fig. 1Fc). By contrast, Prox1\(^{\text{high}}\) LECs in control mice are concentrated in valve-forming areas (Fig. 1Ec).

We further investigated cell alignment by examining actin filament remodeling and alignment in lymphatic vessels. Phalloidin labeling showed that in control lymphatics the actin filaments are remodeled into long, fine fibers that are aligned in the direction of flow (Fig. 2A). By contrast, in Sdc4\(^{-/-}\) mice the actin fibers of LECs have a short, thick appearance and are randomly organized (Fig. 2B).

To examine whether the abnormal reorientation of lymphatic valve-forming cells in Sdc4\(^{-/-}\) mice compromises lymphatic valve development, we quantified the formation of valves. In control E18.5 animals, the majority (88.5%) of lymphatic valves are mature V-shaped valves (Fig. 2C), although there is a small minority (11.5%) of immature valves with a ring-shaped morphology (Fig. 2F). By contrast, in Sdc4 nulls, mature lymphatic valves are rarely seen (9.3%) at E18.5 (Fig. 2F). The majority of lymphatic valves in the mutants retain the ring-shaped appearance of immature valves (26%) (Fig. 2D,F) or appear highly abnormal (64.7%) with Prox1\(^{\text{high}}\) valve-forming cells either randomly oriented or aligned parallel to the flow direction (Fig. 2E,F).

Together, these data show that Sdc4 is important in regulating Prox1\(^{\text{high}}\) lymphatic valve-forming EC reorientation and lymphatic valve formation during embryonic development. Although Sdc4 null mice can survive to adulthood, abnormal lymphatic valves were also observed in dermal lymphatic vessels of Sdc4\(^{-/-}\) adults (Fig. S4C,D).

### Abnormal lymphatic vessel development in Pecam1 null mice

To establish whether abnormalities in lymphatic development noted in Sdc4\(^{-/-}\) mice were due to abnormal shear stress sensing or other features of Sdc4 biology, we examined lymphatic vessel morphogenesis in Pecam1 null mice that have a flow sensing defect. As in Sdc4\(^{-/-}\), the Pecam1\(^{-/-}\) mice did not display any lymphatic abnormalities during early stages of embryonic development (E14.5 to E16.5, Fig. S5A-F). However, by E18.5, unlike littermate controls (Fig. 3A), these mutants develop irregular (Fig. 3B, Fig. S6B), enlarged (Fig. 3C, Fig. S6C,G,H) and abnormally branched (Fig. 3D, Fig. S6D) mesenteric lymphatic vessels.

FACS analysis of LECs and BECs isolated from mesenteries of E18.5 embryos demonstrated a decrease in the number of cells in the S/G2/M phases of the cell cycle in Pecam1\(^{-/-}\) mice and increased numbers of cells at G1 phase (Fig. S2C,D). Thus, increased cell proliferation does not account for the increased vessel diameter seen in these animals.

Prox1\(^{\text{high}}\) valve-forming cells in Pecam1 nulls were less elongated, randomly oriented and loosely organized (Fig. 3Fa,b,G, Fig. S6I) compared with controls (Fig. 3Ea,b,G). Moreover, some of the valve-forming cells in Pecam1 nulls fail to reorient and are aligned along the lymphatic vessel wall (Fig. S6J). Prox1\(^{\text{high}}\) LECs that are not concentrated in valve-forming areas are often seen in Pecam1 nulls (Fig. 3Fc).

Unlike in control mice, where the majority of Prox1\(^{\text{high}}\) valve-forming cells are oriented perpendicular to the flow direction at E17.5 (Fig. 3Ea,c), with the majority (85%) of nuclei aligning at ≥45° relative to the flow direction (Fig. 3H), in Pecam1\(^{-/-}\) mice a much smaller proportion of valve-forming cells (52%) exhibited this degree of nuclear orientation (Fig. 3H). In agreement with these findings, actin filaments of LECs were not aligned with flow in Pecam1\(^{-/-}\) mice (Fig. 4A,B).

The abnormal reorientation of valve-forming cells in Pecam1 nulls results in significantly reduced formation of mature lymphatic valves and the development of immature and abnormal valves at E18.5 (Fig. 4C-E, Fig. S6K-N).

### Sdc4\(^{-/-}\); Pecam1\(^{-/-}\) double-knockout mice develop a severe lymphatic phenotype

To test a genetic interaction between Sdc4 and Pecam1 with regard to lymphatic vasculature development, we generated Sdc4\(^{-/-}\); Pecam1\(^{-/-}\) double-knockout mice. Examination of the mesenteric lymphatic vessels revealed much more extensive abnormalities, including irregular morphology, increased diameter and abnormal branching (Fig. 5A), in Sdc4\(^{-/-}\); Pecam1\(^{-/-}\) animals than in mice with a single gene deletion. Blood-filled jugular lymph sac was observed in Sdc4\(^{-/-}\); Pecam1\(^{-/-}\) embryos at E15.5 (Fig. S7). At E15.5, 8% of the Sdc4\(^{-/-}\); Pecam1\(^{-/-}\) embryos were lethal, 77% exhibited blood-filled lymphatic structures and/or edema, while 15% appeared normal. Those Sdc4\(^{-/-}\); Pecam1\(^{-/-}\) mice that survived to adulthood appeared normal and fertile.

Mural cell coverage in lymphatic vessels was somewhat increased in both Sdc4\(^{-/-}\) and Pecam1\(^{-/-}\) embryos compared with controls.
However, the increase was much more extensive in Sdc4−/−; Pecam1−/− double knockouts (Fig. 5B,C). Moreover, Sdc4−/−; Pecam1−/− mice developed abnormal lymphatic valves (Fig. 5D-F).

To investigate whether there is any compensation between Sdc4 and Pecam1, we examined Pecam1 levels in lymphatic vessels of Sdc4−/− mice or Sdc4 levels in Pecam1−/− mice by qRT-PCR. No significant changes in expression were observed (Fig. S9A,B).

**Vanl2 expression is upregulated in Sdc4−/− mice**

The reorientation defect of Proxhigh valve-forming ECs observed in Sdc4−/− or Pecam1−/− mutants is reminiscent of mice deficient in the PCP protein Vanl2 (Tatin et al., 2013). We therefore measured VANGL2 expression in SDC4 knockdown (KD) or PECAM1 KD LECs. Exposure to laminar flow increased VANGL2 levels in controls as well as in SDC4 KD and PECAM1 KD LECs (Fig. 6A). However, the increase was much higher in SDC4 KD LECs compared with controls (7-fold versus 3-fold), while the increase was less marked in PECAM1 KD cells (Fig. 6A).

Similar results were observed in vivo. We isolated thoracic duct (TD) from wild-type (WT) and Sdc4−/− mice and examined the expression levels of Vanl2 by qRT-PCR. Since TD contains a mixed pool of cell types, we normalized our qRT-PCR results to Cdh5 (Fig. 6B). There was a significant (5.8-fold) increase in Vanl2 expression in Sdc4−/− compared with WT mice (Fig. 6B).
addition, there was a less profound (2.6-fold) increase in \textit{Celsr1}, which encodes a PCP protein, in \textit{Sdc4} nulls (Fig. 6B), whereas \textit{Celsr1} levels in \textit{Pecam1}−/− mice appeared similar to those in WT (Fig. S9C). To examine whether increased \textit{Vangl2} levels in \textit{Sdc4} nulls mediate increased \textit{Celsr1} expression, we overexpressed \textit{VANGL2} in LECs (Fig. S9D) and measured \textit{CELSR1} expression in these cells using qRT-PCR. VANGL2 overexpression resulted in an increase in \textit{CELSR1} levels (Fig. S9E) that was similar to that shown in vivo above.

**SDC4 controls LEC response to flow signals through VANGL2**

We postulated that an increase in \textit{Vangl2} levels was responsible for the mis-sensing of flow signals in \textit{Sdc4} null LECs, leading to lymphatic vessel remodeling defects. To test this hypothesis, we first examined whether increased \textit{Vangl2} levels in \textit{Sdc4} nulls mediate increased \textit{Celsr1} expression, we overexpressed VANGL2 in LECs (Fig. S9D) and measured \textit{CELSR1} expression in these cells using qRT-PCR. VANGL2 overexpression resulted in an increase in \textit{CELSR1} levels (Fig. S9E) that was similar to that shown in vivo above.

**Fig. 3. Pecam1−/− mice show lymphatic remodeling defects at E18.5.** (A–D) Irregular (B, arrow), enlarged (C, arrow) and abnormally branched (D, arrow) mesenteric lymphatic vessels developed in \textit{Pecam1}−/− mice but not in controls (A, arrow) at E18.5. (E,F) Compared with control (Ea,b, arrowheads), the nuclei of \textit{Prox1}high valve-forming cells in \textit{Pecam1}−/− are less elongated (Fa,b, arrowheads), randomly oriented and loosely organized. Nuclei of \textit{Prox1}high valve-forming cells aligned along the longitudinal axis of lymphatic vessels were also seen in \textit{Pecam1}−/− embryos (Fc, arrow). In control mice, by contrast, the nuclei of \textit{Prox1}high valve-forming cells are concentrated in valve areas (Ec, arrows). (G) Quantification of nuclear shape (roundness) of \textit{Prox1}high cells in E17.5 mesenteric lymphatic vessels (n=5). Mann–Whitney test. (H) Nuclear orientation of \textit{Prox1}high cells in E17.5 mesenteric lymphatic vessels (n=5). The percentage of nuclei reoriented >45° relative to flow direction is indicated. Mann–Whitney test. Scale bars: 200 μm in A–D; 30 μm in Ea,b,Fa,b; 100 μm in Ec,Fc.

Vessels before mature functional valves have developed, we subjected LECs to laminar flow in vitro. In the absence of flow (static condition), LECs are randomly oriented and thus display an average orientation of ∼45° to flow direction (perfect alignment of all cells with flow direction is 0°, and perpendicular alignment is 90°). LECs transduced with a control virus aligned normally to flow direction, with an average orientation of ∼43°, close to the value measured in cells that were not exposed to flow (Fig. 6C–E).

To relate the abnormal polarization of lymphatic valve-forming ECs in \textit{Sdc4}−/− mice to a flow sensing defect, we tested the ability of LECs with reduced \textit{Sdc4} expression to align under flow. Primary LECs were transfected with scrambled siRNA (siScrambled) or siRNA against \textit{SDC4} (siSDC4) (Fig. S10A,B) and subjected to laminar flow (8 dynes/cm²). After 16 h of flow exposure, control LECs (transfected with siScrambled) were elongated along the flow axis, with phalloidin labeling demonstrating remodeling of actin.
fibers into long, fine filaments (Fig. 7A,B). By contrast, LECs transfected with siSDC4 (SDC4 KD LECs) exhibited a cuboidal morphology, with the actin fibers in these cells retaining a thick and short appearance, and failed to align along the direction of flow (Fig. 7B).

Measurements of cell orientation in response to laminar flow confirmed these observations. When subjected to flow, control LECs aligned to flow with the average orientation between 35° and 40° relative to flow direction (Fig. 7C, siScrambled). By contrast, flow had no effect on the alignment of SDC4 KD cells, which showed an average orientation of ∼45°, similar to cells that were not subjected to flow (static) (Fig. 7C, siSDC4). Remarkably, reducing VANGL2 levels in SDC4 KD LECs restored their ability to align under flow (Fig. 7B,C, siSDC4; siVANGL2). In fact, these cells aligned as well as control cells (Fig. 7B,C).

We also examined flow-mediated activation of VEGFR3 signaling in control and SDC4 KD LECs (Fig. S10C). As expected, flow activated VEGFR3 signaling in control LECs (Fig. S10C). By contrast, flow-induced activation of VEGFR3 signaling was significantly reduced in SDC4 KD cells (Fig. S10C).

**DISCUSSION**

The data presented in this study identify two new regulators of FSS-mediated lymphatic vasculature remodeling: Sdc4 and Pecam1. A deletion of either gene in mice resulted in a similar lymphatic remodeling defect during embryonic development. Moreover, simultaneous deletion of both genes caused a more severe lymphatic phenotype that included the appearance of blood-filled jugular lymph sac at E15.5. This phenotype is unlikely to be secondary to blood vessel defects, as hemorrhage or other obvious blood vessel abnormalities were not seen in these animals. One potential explanation is the formation of abnormal lympho-venous valves, resulting in blood backflow into the lymphatic vascular network. Another phenotype observed in Sdc4−/− or Pecam1−/− embryos was an increase in mural cell coverage in lymphatic vessels and this was even more pronounced in Sdc4−/−; Pecam1−/− double nulls. These data support the existence of abnormal flow signaling in these mutants and are consistent with a previous report (Sweet et al., 2015). The enlarged lymphatic vessel diameter in Sdc4−/− embryos has been linked with increased LEC proliferation. This is not the case in Pecam1 nulls. Although Sdc4−/− and some Sdc4−/−;
Pecam1−/−/− double-knockout mice can survive to adulthood, the presence of abnormal lymphatic valves in the adult may affect the function of lymphatic vessels leading to increased morbidity (Alexander et al., 2010).

The more severe lymphatic phenotype that developed in Sdc4−/−/−; Pecam1−/−/− double nulls suggests that Sdc4 and Pecam1 function through independent flow signaling pathways. While both have been implicated in flow signaling in LECs, the molecular mechanisms involved are distinct: in the case of Sdc4, it involves regulation of flow-mediated Vangl2 expression, whereas PCP proteins do not seem to be involved in Pecam1-controlled flow signaling (Fig. S11).

Fig. 5. Sdc4−/−; Pecam1−/− double nulls show a severe lymphatic phenotype. (A) Mesenteric lymphatic vessels in Sdc4−/−; Pecam1−/− embryos show irregular morphology (middle, white arrow), increased diameter (middle, yellow arrow) and abnormal branching (bottom, arrows) at E18.5, which were not seen in controls (top, arrows). (B) Sparse mural cell (α-SMA+) coverage in mesenteric lymphatic vessels in control mice at E18.5. (C) Extensive mural cell recruitment in lymphatic vessels of Sdc4−/−; Pecam1−/− double nulls. A, artery; V, vein; L, lymphatic vessel. (D) Mature V-shaped valves in mesenteric lymphatic vessels of control mice at E18.5 (arrows). (E) Abnormal lymphatic valves containing randomly oriented Prox1high valve-forming ECs formed in Sdc4−/−; Pecam1−/− mice at E18.5 (arrows). (F) Quantification of lymphatic valves formed in the mesentery of control and Sdc4−/−; Pecam1−/− mice at E18.5 according to antibody staining for Prox1 and VE-cadherin. The proportion (%) of different types of valves relative to the total number of valves formed per mesentery is shown. Mature valves are V-shaped; immature valves are ring shaped; abnormal valves are those consisting of randomly organized Prox1high valve-forming cells or Prox1high cells aligned parallel to the longitudinal axis of lymphatic vessels. Student’s t-test (two-tailed). Data represent mean±s.e.m. (n=3). Scale bars: 200 μm in A; 100 μm in B,C; 15 μm in D,E.
PECAM1 involvement in mechanotransduction is well established in BECs, where it transduces shear forces to activate a complex with VE-cadherin and VEGF receptor to mediate shear stress signaling (Tzima et al., 2005). However, its involvement in flow sensing events in the lymphatic endothelium has not been reported previously. A recent study has demonstrated that the transmembrane domain of VE-cadherin can bind directly to the transmembrane domains of VEGFR2 and VEGFR3 (Coon et al., 2015), suggesting that VEGFR3, which is highly expressed in LECs, could be a component of a mechanotransduction complex. This is supported by the observation that changes in Vegfr3 levels in LECs, could be a component of a mechanotransduction complex with VE-cadherin and VEGF receptor to mediate shear sensing events in the lymphatic endothelium has not been established in BECs, where it transduces shear forces to activate a complex with VE-cadherin and VEGF receptor to mediate shear stress signaling (Tzima et al., 2005). However, its involvement in flow sensing events in the lymphatic endothelium has not been reported previously. A recent study has demonstrated that the transmembrane domain of VE-cadherin can bind directly to the transmembrane domains of VEGFR2 and VEGFR3 (Coon et al., 2015), suggesting that VEGFR3, which is highly expressed in LECs, could be a component of a mechanotransduction complex. This is supported by the observation that changes in Vegfr3 levels in LECs, could be a component of a mechanotransduction complex.

The role of Pecam1 in flow sensing has been extensively investigated, whereas relatively little is known about Sdc4 involvement in this process. We recently demonstrated that aortic ECs of Sdc4 null mice are poorly aligned in the direction of flow and that SDC4 KD in human umbilical vein ECs inhibits flow-induced alignment, a defect rescued by re-expression of the gene (Baeyens et al., 2014).

Surprisingly, the PCP protein Vangl2 was identified as an important component of Sdc4-dependent shear stress signals in this study. Laminar flow upregulates VANGL2 expression in LECs in vitro, indicating that VANGL2 is a flow-responsive gene. Consistent with this observation, flow had only minimal effects on VANGL2 expression in PECAM1 KD LECs, which are defective in transducing mechanical forces. However, in the setting of reduced SDC4 expression, exposure to flow led to a much greater increase in VANGL2 expression than that seen in control LECs. Increased Vangl2 levels were also observed in lymphatic vessels in Sdc4 null mice. These results suggest that SDC4 controls the extent of flow-induced increase in VANGL2 expression.

The excessive increase in VANGL2 expression in response to flow in LECs with reduced SDC4 expression accounts for the misalignment of these cells. Indeed, VANGL2 overexpression in control (WT) LECs impaired their ability to align to flow, while the reduction of VANGL2 expression in SDC4 KD LECs restored their ability to align. Combined with observations of abnormal lymphatic valve morphogenesis in Vangl2-deficient mice (Tatin et al., 2013), these data suggest that there is an optimal level of Vangl2 expression in LECs. The concept of the optimal expression level of a particular protein with regard to the regulation of flow-mediated cell alignment is consistent with a recent description of an endothelial flow-sensing set-point study (Baeyens et al., 2015).

The involvement of PCP proteins in lymphatic valve morphogenesis has been reported previously. Ablation of the
core PCP proteins Celsr1 or Vangl2 in mice results in the formation of abnormal lymphatic valves due to a reorientation defect of lymphatic valve-forming cells (Tatin et al., 2013). Moreover, Vangl2 has been shown to be involved in flow-controlled ependymal cilia orientation (Guirao et al., 2010), suggesting that this PCP protein plays a role in the integration of FSS-mediated signals. Yet, how it achieves this effect remains unknown. Our results expand this concept by demonstrating that VANGL2 is a flow-responsive gene and its expression levels, as induced by flow, are critical for LECs to align to flow. We have further identified that SDC4 is an important regulator in this process.

The need for an optimal Vangl2 level controlling cell orientation is consistent with what is known about PCP protein involvement in cell polarization. In vertebrate inner ear hair cells, the core PCP proteins Frizzled (Fz) and Dishevelled (Dvl) form a complex on one side of the cell that is opposite to Van Gogh (Vangl) and Prickle (Pk) on the other. This asymmetric distribution of the PCP proteins is consistent with what is known about PCP protein involvement in cell polarization. In vertebrate inner ear hair cells, the core PCP proteins Frizzled (Fz) and Dishevelled (Dvl) form a complex on one side of the cell that is opposite to Van Gogh (Vangl) and Prickle (Pk) on the other. This asymmetric distribution of the PCP proteins is consistent with what is known about PCP protein involvement in cell polarization.

The appearance of ‘wavy’ endothelial cell-cell junctions in the lymphatic vessels of Sdc4−/− mice is consistent with a degree of destabilization. Indeed, the involvement of PCP proteins in controlling classical cadherins has been reported previously (Nagaoka et al., 2014a,b). In the nervous system, Vangl2 binds directly to the intracellular domain of N-cadherin to stabilize cell-cell junctions (Nagaoka et al., 2014b). Similarly, Vangl2 controls E-cadherin in epithelial cells (Nagaoka et al., 2014a). It is therefore likely that, in LECs, Vangl2 binds VE-cadherin to regulate the formation of endothelial adherens junctions.

In summary, our study has identified a new mechanism of FSS-mediated signaling that involves Sdc4-dependent upregulation of the PCP protein Vangl2 driven by flow.

MATERIALS AND METHODS

Mice

Sdc4−/− and Pecam1−/− strains were maintained in a C57BL/6J background. Sdc4−/−; Pecam1−/− mice were generated by breeding Sdc4−/−; Pecam1−/− with Sdc4−/−; Pecam1−/−. The morning that the vaginal plug was found is designated embryonic day (E) 0.5. All mouse experiments complied with protocols approved by the Yale University Institutional Animal Care and Use Committee.

Whole-mount immunofluorescence staining and image acquisition

For whole-mount immunofluorescence staining of mesentery, tissue was fixed in 1% paraformaldehyde (PFA) for 1 h at room temperature, then washed with ice-cold PBS three times. Tissue was blocked in blocking buffer comprising 5% donkey serum, 0.2% BSA, 0.3% Triton X-100, goat anti-VE-cadherin (AF743, R&D Systems, 1:100), rabbit anti-laminin α5 (Ringelmann et al., 1999) (1:1000), rat anti-VE-cadherin (clone 11D4.1, 555289, BD Biosciences, 1:100), goat anti-Vegfr3 (AF743, R&D Systems, 1:100), hamster anti-podoplanin (Developmental Studies Hybridoma Bank, University of Iowa, 1:1000) and Cy3-conjugated oSMA (clone 1A4, C6198, Sigma-Aldrich, 1:400); Alexa Fluor 488-conjugated phalloidin was used to stain actin (A12379, Life Technologies, 1:200).

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MATERIALS AND METHODS

Mice

Sdc4−/− and Pecam1−/− strains were maintained in a C57BL/6J background. Sdc4−/−; Pecam1−/− mice were generated by breeding Sdc4−/−; Pecam1−/− with Sdc4−/−; Pecam1−/−. The morning that the vaginal plug was found is designated embryonic day (E) 0.5. All mouse experiments complied with protocols approved by the Yale University Institutional Animal Care and Use Committee.

Whole-mount immunofluorescence staining and image acquisition

For whole-mount immunofluorescence staining of mesentery, tissue was fixed in 1% paraformaldehyde (PFA) for 1 h at room temperature, then washed with ice-cold PBS three times. Tissue was blocked in blocking buffer comprising 5% donkey serum, 0.2% BSA, 0.3% Triton X-100 in PBS for 1 h at room temperature, then incubated with primary antibody diluted in blocking buffer overnight at room temperature. After washing with wash buffer comprising 0.3% Triton X-100 in PBS, tissue was incubated with secondary antibody diluted in blocking buffer at 4°C overnight. Tissue was then washed with wash buffer and flat mounted using fluorescent mounting medium (DAKO). Images were acquired using a Leica DM6000 CS confocal or Nikon Eclipse 80i microscope. Confocal images are maximum intensity projections of z-stacks. Nuclear orientation and shape (roundness) of Prox1high valve-forming ECs were quantified using ImageJ (NIH).

Primary antibodies were: rabbit anti-Prox1 (11,002, AngioBio, 1:200), rabbit anti-Lyve1 (11-034, AngioBio, 1:400), rabbit anti-laminin α5 (Ringelmann et al., 1999) (1:1000), rat anti-VE-cadherin (clone 11D4.1, 555289, BD Biosciences, 1:100), goat anti-Vegfr3 (AF743, R&D Systems, 1:100), hamster anti-podoplanin (Developmental Studies Hybridoma Bank, University of Iowa, 1:1000) and Cy3-conjugated oSMA (clone 1A4, C6198, Sigma-Aldrich, 1:400); Alexa Fluor 488-conjugated phalloidin was used to stain actin (A12379, Life Technologies, 1:200).

In summary, our study has identified a new mechanism of FSS-mediated signaling that involves Sdc4-dependent upregulation of the PCP protein Vangl2 driven by flow.

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Cell culture and siRNA transfection
Human dermal LECs (Lanza, CC-2812) were used in this study, and were cultured in EGM-2MV medium (Lanza, CC-3202). For gene KD in LECs, cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. siRNA was washed out 6-8 h after transfection. Transfected cells were harvested for experiments 72 h after transfection.

siRNAs used for gene KD were from OriGene Technologies: scrambled siRNA (NC1 Control SR30004) and siRNA against human SDC4 (SR304301), human VANGL2 (SR311428) or human PECAM1 (SR303439), each at 15 nM.

Lentiviral transduction
LEC were transduced with lentiviral particles expressing control (OHS5833, GE Healthcare Dharmacon) or VANGL2 (OHS8999-202624111, GE Healthcare Dharmacon). Protein expression was identified by immunoblot analysis.

qRT-PCR analysis
cDNA was synthesized from 1 µg total RNA using the Superscript III First-Strand Synthesis System (Invitrogen) following the manufacturer’s instructions. 3 µl cDNA was used for each qPCR reaction, which was performed in a 25 µl reaction volume in triplicate on a CFX96 Real-Time System C1000 thermal cycler (Bio-Rad) using iQ SYBB Green Supermix (Bio-Rad). Four independent experiments were carried out. The cycle employed an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. qPCR primers for human VANGL2 were purchased from Qiagen (PhP12377B-200). Other primers were as follows (5’-3’, forward and reverse): mouse Pecam1, CCACGCG-CCTTCAAAATGTC and TCTCCAGGATCACCAGTGC; mouse Celsr1, GGCACTGATGACCTTGGACTA and AGCTGATTCCCAATCTGCAC; mouse Vangl2, CATCTTTGAGAGAACTGAGGTCTTG and CCTTCTTTGTCATGCGGTACA; mouse Sdc4, CATCTTTGAGAGAACTGAGGTCTTG and CCTTCTTTGTCATGCGGTACA; mouse Pecam1, CGGTGTTCAGCGAGATCC and CGGCAATCATGGTCAGGGTACTA; mouse 18S rRNA (Rn18s), AGGAA-TTCCACTAAGAAGG and GCTCTCATAACATCCACAC; human ACTB, TCCACGACCCAGAGGAATGGGAAACAGTCA; human 18S rRNA (Rn18s), AGGAA-TTCCACTAAGAAGG and GCTCTCATAACATCCACAC; human SDC4, GGGAGAAATCTGAGCTTTG and GCGCATCAGGAGGCCTTC; human CELSR1, TGGATATCTCCAGGCGCTGA and AGGGCGATAGGTGCAATCTCT.

Isolation of total RNA from thoracic duct Thoracic ducts were dissected out from ten adult WT and ten adult Sdc4−/− mice and were collected in RLT Plus Buffer (Qiagen). Tissue was homogenized using TissueLyser II (Qiagen) followed by centrifugation at 15,000 rpm (21,000 g) at 4°C. Supernatant was collected, from which total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen).

Fluorescence-activated cell sorting Mesenteric vessels of E18.5 control, Sdc4−/− or Pecam1−/− embryos were digested for 30 min at 37°C in 1 mg/ml collagenase type II with periodic gentle inversion to mix, followed by mechanical dissociation via repeated pipetting. Single cells were incubated in 10 µg/ml Hoechst 33342 (Sigma) for 30 min at 37°C, followed by an additional 15 min incubation with 0.5 µg/ml Pyronin Y (Sigma) and fluorescently conjugated antibodies Pecam1 (CD31)-FITC, VE-cadherin (CD144)-FITC, CD45-PECy7 and Lyve1-Alexa 488. ECs were identified as events negative for CD45 (Ppre) and positive for either the Pecam1 (Sdc4−/− experiments) or VE-cadherin (Pecam1−/− experiments) endothelial marker; LECs were subsequently discriminated as Lyve1−. Two-dimensional cell cycle FACS analysis was then performed on BEC and LEC events by comparing DNA (Hoechst) and RNA (Pyronin Y) content.

Western blotting analysis Cells were rapidly washed with ice-cold PBS twice and lysed with 100 µl RIPA lysis buffer containing complete mini EDTA-free protease inhibitors (1183617001, Roche) and phosphatase inhibitors (0490683701, Roche). Cell lysates were subjected to two cycles of snap-freezing in liquid nitrogen and then cleared by centrifugation at 15,000 rpm (21,000 g) for 10 min. Protein concentration was determined using BCA protein assay (Thermo Scientific) and equalized in each sample. Then, 40 µg total protein from each sample was loaded onto a 4-15% TGX gel (Bio-Rad) with Tris/glycine/SDS buffer (Bio-Rad) for SDS-PAGE and transferred to an Immobilon-P (PVDF) membrane (IPVH 00010, Millipore). Membranes were blocked with 5% non-fat dried milk (AB 10109-0100, AmericanBio) in Tris-buffered saline containing 0.5% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with primary antibodies at 4°C overnight. Membranes were washed with TBS-T and incubated with secondary antibodies for 2 h at room temperature. Protein bands were visualized using HRP-conjugated secondary antibodies by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, WBKLI0500, Millipore) recorded using a digital acquisition system (G-Box, Syngene) equipped with a CCD camera with ‘true’ 1.4 megapixel resolution.

Primary antibodies were: anti-syndecan 4 (Abcam, ab24511, 1:1000), anti-actin (Santa Cruz, sc-1615, 1:200), anti-VANGL2 (R&D Systems, AF4815, 1:100), anti-VEGFR3 (Cell Signaling Technology, 2638, 1:1000), anti-phospho-VEGFR3 (Cell Applications, cy1115, 1:1000) and anti-Jk-actin (Sigma, A1978, 1:10,000). Peroxidase-labeled secondary antibodies (all 1:2000) were: goat anti-rabbit IgG (Vector Laboratories, PI-1000), horse anti-goat IgG (Vector Laboratories, PI-9500), rabbit anti-sheep IgG (Thermo Scientific, 31480) and horse anti-mouse IgG (Vector Laboratories, PI-2000).

Shear stress LECs were seeded on fibronectin-coated (20 µg/ml) tissue culture plastic slides. On reaching confluence, cells were starved with EBM-2 medium (Lonza, CC-3156) containing 5% FBS, 100 UI/ml penicillin and 100 µg/ml streptomycin for 4 h. Shear stress with a calculated intensity of 8 dynes/cm² was applied in a parallel flow chamber with starvation medium. After 16 or 20 h of steady laminar flow, cell alignment was quantified by measuring nuclear orientation using a custom-made MATLAB (MathWorks) function (Baeyens et al., 2014). At least 50 images/condition/experiment taken with a 20× objective were used for quantification.

Statistical analysis GraphPad Prism 6 was used for statistical analysis. Student’s t-test (two-tailed), Mann-Whitney test, Kruskal–Wallis test, one-way ANOVA and two-way ANOVA were performed to determine statistical significance.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Y.W. carried out most of the experiments and prepared figures. N.B. conducted flow experiments and quantified the shape and the orientation of lymphatic valve-forming cells. F.C. performed western blotting. K.T. conducted flow experiments and western blotting. J.S.F. performed FACS. J.Z. isolated thoracic ducts from mice. Y.J. helped with data analysis, study design and manuscript preparation. Y.W. and M.S. helped with data analysis, study design and manuscript preparation. Y.W. and M.S. designed the project and wrote the manuscript. M.S. supervised all aspects of the project.

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Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.140129.supplemental

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