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Integrin- $\alpha 5\beta 1$ is not required for mural cell functions during development of blood vessels but is required for lymphatic-blood vessel separation and lymphovenous valve formation

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Summary

Integrin $\alpha 5\beta 1$ is essential for vascular development but it remains unclear precisely where and how it functions. Here, we report that deletion of the gene encoding the integrin- $\alpha 5$ subunit [*Itga5*] using the *Pdgfrb-Cre* transgenic mouse line, leads to oedema, haemorrhage and increased levels of embryonic lethality. Unexpectedly, these defects were not caused by loss of $\alpha 5$ from *Pdgfrb-Cre* expressing mural cells (pericytes and vascular smooth muscle cells), which wrap around the endothelium and stabilise blood vessels, nor by defects in the heart or great vessels, but were due to abnormal development of the lymphatic vasculature. Reminiscent of the pathologies seen in the human lymphatic malformation, fetal cystic hygroma, $\alpha 5$ mutants display defects both in the separation of their blood and lymphatic vasculature and in the formation of the lymphovenous valves. As a consequence, $\alpha 5$ -deficient mice develop dilated, blood-filled lymphatic vessels and lymphatic capillaries that are ectopically covered with smooth muscle cells. Analysis of the expression of *Pdgfrb* during lymphatic development suggests that these defects probably arise from loss of $\alpha 5\beta 1$ integrin in subsets of specialised *Prox1*⁺ *Pdgfr*⁺ venous endothelial cells that are essential for the separation of the jugular lymph sac from the cardinal vein and formation of the lymphovenous valve leaflets.

Keywords

Integrin; mural cells; lymphatic vessels; PDGFR β

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Author Contributions

Experiments were conceived, designed and interpreted by CJT, KB-N and ROH.

Experiments were performed by CJT and KB-N; DC provided sections and AF generated *Itga5 flox/KO^{Tie2cre}* mice. The manuscript was written by CJT and ROH.

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Introduction

The integrin family of extracellular matrix receptors are key regulators of both blood and lymphatic vessel development (Astrof and Hynes, 2009; Avraamides et al., 2008; Chen et al., 2012; Hynes, 2007; Tammela and Alitalo, 2010). Multiple integrins, including the fibronectin receptors ($\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha 4\beta 1$), have been implicated to one degree or another in angiogenesis and a number of integrin inhibitors have entered clinical trials to test their ability to prevent tumour angiogenesis (Goodman and Picard, 2012). Integrin $\alpha 5\beta 1$ binds the Arg-Gly-Asp (RGD) motif in fibronectin and is the predominant fibronectin-binding integrin. $\alpha 5\beta 1$ is poorly expressed on quiescent endothelium, but is upregulated during developmental and tumour angiogenesis (Kim et al., 2000; Xie et al., 2011). Genetic ablation of $\alpha 5$, or its major ligand, fibronectin, results in embryonic lethality at ~E10.5 due to severe vascular defects (Francis et al., 2002; George et al., 1997; George et al., 1993; Yang et al., 1993). Furthermore, inhibition of $\alpha 5\beta 1$ leads to decreases in angiogenesis and tumour growth in both chicken and mouse models (Kim et al., 2000; Muether et al., 2007; Umeda et al., 2006).

Some studies have also implicated $\alpha 5\beta 1$ integrin in development of the lymphatic vasculature (Chen et al., 2012). The main function of the lymphatic system is to maintain tissue fluid balance (Földi et al., 2005). Blind-ended lymphatic capillaries collect excess interstitial fluid extravasated from the blood and drain it back via collecting lymphatic vessels, lymph nodes and finally the lymphatic duct to the venous circulation (Tammela and Alitalo, 2010). Within collecting lymphatic vessels, intra-luminal valves (herein referred to as lymphatic valves) ensure unidirectional lymphatic flow (Bazigou and Makinen, 2013), while at the junctions of the lymphatic ducts with the venous circulation, lymphovenous valves allow fluid to drain into the veins and prevent blood from entering into the lymphatic circulation. In vitro, $\alpha 5\beta 1$ is required for activation of vascular endothelial growth factor receptor-3 (VEGFR-3) (Zhang et al., 2005), which plays an essential role in lymphangiogenesis (Alitalo, 2011). Proliferation and growth of lymphatic endothelial cells (ECs) has also been blocked by small-molecule antagonists of $\alpha 5\beta 1$ in corneal and airway inflammation models in vivo (Dietrich et al., 2007; Okazaki et al., 2009).

The role of endothelial $\alpha 5\beta 1$ during development however remains unclear due to compensation by other integrins and the influence of strain-dependent genetic modifiers (van der Flier et al., 2010). Ablation of endothelial $\alpha 5\beta 1$ fails to produce the major defects seen with global knockouts of $\alpha 5$ (van der Flier et al., 2010). On a 129S4 background, *Tie2-Cre*-mediated deletion of *Itga5* leads to late embryonic lethality apparently due to patent ductus arteriosus (van der Flier et al., 2010). Ablation of both $\alpha 5$ and αv integrin subunits in endothelial cells produced remodelling defects in the major vessels but these were still much less severe than observed in the global $\alpha 5$ knockout mice. These results suggest that $\alpha 5\beta 1$ integrin functions in other cell types to contribute to the observed vascular defects in $\alpha 5$ -null embryos.

Much less information exists about the roles of integrins on mural cells, which wrap around both blood and collecting lymphatic vessels. The physiological importance of mural cells in blood vessel development can be seen in mice that lack platelet-derived-growth-factor-B

(PDGF-B), or its receptor, PDGF receptor- β (PDGFR β). PDGF-B is secreted from ECs and promotes the proliferation and migration of PDGFR β -positive mural cell precursors to the vessel wall (Hirschi et al., 1999). Loss of either PDGF-B or PDGFR β results in blood vessels that lack, or are incompletely covered by, mural cells (Leveen et al., 1994; Soriano, 1994). As a consequence, ECs hyperproliferate, form abnormal junctions and give rise to dilated, leaky vessels (Hellstrom et al., 1999; Lindahl et al., 1997).

Recently, two papers have shown that integrin- β 1 is essential for mural cell function in vivo. In both studies, mural-cell-specific deletion of β 1 led to the formation of aneurysms and defects in the assembly of ECM proteins within the vessel wall (Abraham et al., 2008; Turlo et al., 2012). In mice where *Itgb1* was deleted using the *Pdgfrb*-Cre transgene, mural cells also appeared round, poorly spread, and only loosely attached to the subendothelial basement membrane (Abraham et al., 2008). Electron microscopic studies have shown that dense fibronectin-rich plaques form at the pericyte-endothelial interface suggesting an important role for fibronectin-binding integrins (Courtoy and Boyles, 1983). Mural cells express several β 1 heterodimers that bind to fibronectin, including α 5 β 1 (Davenpeck et al., 2001), which is upregulated during differentiation of mesenchymal stem cells to pericytes (Kale et al., 2005). Integrin α 5 β 1 has also been shown to promote proliferation, migration, and switching of vascular smooth muscle cells (vSMCs) from a contractile to synthetic phenotype (Barillari et al., 2001; Davenpeck et al., 2001; Hedin and Thyberg, 1987), and to regulate signalling through PDGFR β (Veevers-Lowe et al., 2011). To date, however, no one has investigated the role of α 5 β 1 on either pericytes or vSMCs in vivo.

To address the function of α 5 β 1 on mural cells in vivo, we used a conditional gene targeting approach to disrupt expression of *Itga5* selectively in both pericytes and vSMCs. We found, again unexpectedly, that deletion of α 5 β 1 integrin from mural cells failed to produce major defects in blood vessel development. We did however observe defects in the lymphatics. Accordingly, we report here on the development of both blood vessels and lymphatics in mice lacking α 5 β 1 integrin in cell types that express *Pdgfr*-*Cre*—these include pericytes, vSMCs and, as we report here, a subset of endothelial cells that appear to be involved in separations between the two vascular systems.

Materials and Methods

Mouse lines

All mouse strains used were on a 129S4:C57BL/6 mixed genetic background. *Itga5* floxed mice (van der Flier et al., 2010), *Itgav* floxed mice (Lacy-Hulbert et al., 2007), transgenic *Pdgfrb*-*Cre* (Foo et al., 2006), *Tie2*-*Cre* (Kisanuki et al., 2001), mTmG (Muzumdar et al., 2007), *VE-Cadherin*-*CreERT*² (Benedito et al., 2009) and *Pf4*-*Cre* (Tiedt et al., 2007) mouse lines have all been described previously. Genotyping was performed on DNA isolated from tail snips either in-house or by Transnetyx. For experiments involving the *VE-Cadherin*-*CreERT*² line, pregnant females were given intraperitoneal injections of 2 mg Tamoxifen (dissolved in peanut oil) at E8.5, E9.5, and E10.5.

Histology and immunofluorescence staining

Freshly isolated embryos were embedded in Tissue-Tek OCT and sectioned (20 μ m) on a Cryostat; or fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight or in zinc fixative (BD) at room temperature (RT) for 48 hours, followed by embedding and sectioning (5 μ m) in paraffin wax. Selected paraffin sections were stained with hematoxylin and eosin (H&E) using standard protocols. For immunofluorescence stainings, deparaffinized tissue sections were subjected to heat-induced epitope retrieval (2 \times 5 min 800 W microwave) in 10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0, blocked in PBS containing 0.5% Tween and 2% goat or donkey serum (PBS-Tx), and incubated overnight at 4°C with primary antibodies diluted in 1:1 PBS:PBS-Tx. After washes in PBS-T (PBS 0.1% Tween), tissues were incubated either at RT for 2 hours, or overnight at 4°C, with fluorophore-conjugated secondary antibodies diluted in 1:1 PBS-Tx. Samples were then washed in PBS-T, mounted onto coverslips in Fluoromount (SouthernBiotech) and imaged using Zeiss LSM 510 or Nikon A1R scanning laser confocal microscopes. Images were processed using Volocity (Perkin Elmer) or Nikon elements software. Whole-mount staining of PFA-fixed embryonic back skin followed methods previously described (Foo et al., 2006).

Antibodies

Rat anti-mouse PECAM-1, MEC13.3 (BD Pharmingen), rabbit anti-mouse PECAM-1 (Abcam), mouse anti-human α -SMA, Clone 1A4-Cy3 (Sigma), rabbit anti-Desmin (Abcam), rat anti-mouse Ter-119 (BD Pharmingen), rabbit anti-mouse LYVE-1 (Abcam), hamster-anti mouse Podoplanin (Developmental Studies Hybridoma Bank), goat anti-mouse Integrin- α 9 (R&D systems), goat anti-human Prox1 (R&D systems), rabbit anti-Prox1 (Angiobio), rat anti-mouse integrin- α 5 (BD Pharmingen), goat anti-GFP (Abcam), rabbit anti-GFP (Cell Signalling), rabbit anti-mouse PDGFR β 28E1 (Cell Signaling), rabbit anti- α SMA (Abcam). Secondary antibodies were Alexa488, Alexa594, and Alexa647 conjugated antibodies (Invitrogen).

Micro-CT scans

PFA (4%)-fixed embryos were stained with a proprietary contrast agent and scanned with a high-resolution volumetric micro-CT scanner (μ CT40 ScanCo Medical, Zurich, CH) using the following parameters: 6 μ m isometric voxel resolution at 200 ms exposure time, 2000 views and 5 frames per view (Numira Biosciences (Salt Lake City, UT). The micro-CT generated DICOM files were analyzed using OsiriX and Volocity software.

Results

Loss of integrin- α 5 from PDGFR β -positive cells results in oedema

During development of the vascular system, α 5 β 1 is expressed on both endothelial and mural cells within the blood vessel wall (Fig. S1A). To study the role of α 5 β 1 on pericytes and vSMCs, female homozygous *Itga5*-floxed mice (van der Flier et al., 2010) were crossed to male *Pdgfrb-Cre*-positive (Foo et al., 2006) heterozygous *Itga5*-floxed mice. Isolation of the resulting mutant embryos, herein referred to as *Itga5^{Pdgfrb-cre}* mice, revealed efficient loss of α 5 protein within the vSMC layers of the aorta by embryonic day 13.5 (E13.5) (Fig.

S1B), and an increased incidence of intrauterine death from E13.5 (Fig. S2A). By E15.5, ~80% of *Itga5^{Pdgfrb-cre}* mice displayed oedema and, in 35% of mutants, widespread accumulation of blood within the skin (Fig 1A–C, S2B). *Itga5^{Pdgfrb-cre}* mice that survived to E17.5 (~65%) however appeared to have resolved these defects and survived to birth (Fig. S2A, S2B). Interestingly, *Itga5^{Pdgfrb-cre}* mice also developed blood-filled jugular lymphatic sacs (Fig. 1C–E). However in the majority of the mice, this was observed only on the left side of the embryos suggesting a left-side predilection (Fig. S2B). To rule out cardiac dysfunction as the cause of these defects, we completed micro-CT scans through the thoracic region of control and *Itga5^{Pdgfrb-cre}* embryos at E15.5. However, no obvious defects in either the development of the heart or remodelling of the outflow tract were detected in the absence of $\alpha 5\beta 1$ (Fig. 1F, G, Movie S1, S2).

Normal blood vessel development in *Itga5^{Pdgfrb-cre}* mice

Previous studies have shown that defective mural cell coverage leads to haemorrhage, oedema and embryonic lethality due to instability of the vessel wall (Hellstrom et al., 1999; Kogata et al., 2009). Analysis of the embryonic dermal vasculature by whole-mount immunostaining surprisingly revealed no obvious defects in vSMC or pericyte morphology (Fig. 2), despite efficient deletion of mural cell $\alpha 5$ protein (Fig. S1B). In contrast to the rounded morphology seen in mice lacking mural cell expression of all $\beta 1$ integrins (Abraham et al., 2008), *Itga5*-deficient vSMCs formed organised concentric rings around arteries and continuously covered large veins (Fig. 2A–D). Loss of *Itga5* did however cause abnormal recruitment of α SMA-positive cells to lymphatic capillaries of the skin (Fig. 2C–F), which in contrast to the collecting lymphatic vessels, are usually devoid of smooth muscle cells in order to allow efficient absorption of interstitial fluid (Petrova et al., 2004). Desmin-positive pericytes also appeared unaffected by the loss of integrin- $\alpha 5$. Pericytes in *Itga5^{Pdgfrb-cre}* mice were elongated, in close association with the capillary endothelium (Fig. 2G, H), and in similar numbers to those seen in control mice (Fig. S3).

Itga5^{Pdgfrb-cre} mice display blood-filled, hyperplastic lymphatic vessels

Since the haemorrhage and oedema in *Itga5^{Pdgfrb-cre}* mice could not be attributed to obvious defects in formation of the vascular wall and ectopic vSMC coverage was seen around lymphatic vessels (Fig. 2C–F), samples of embryonic skin from E15.5 embryos were stained with the pan-endothelial marker PECAM-1, the lymphatic endothelial marker LYVE-1, and the erythroid cell marker Ter-119 (Fig. 3A–F). In control mice, erythrocytes were contained within the blood vasculature (Fig. 3A, B), and blind-ended lymphatic capillaries appeared 2–3 times the size of the largest blood vessels. In contrast, in *Itga5^{Pdgfrb-cre}* embryos, lymphatic vessels appeared hyperplastic, tortuous and were often filled with blood (Fig. 3C, D). The distribution of LYVE-1 was also different in mutant embryos. Instead of the uniform LYVE-1 expression seen in control mice, endothelial cells in lymphatic capillaries of *Itga5^{Pdgfrb-cre}* embryos had increased levels of LYVE-1 at their cell junctions (Fig. 3D). Despite these lymphatic defects, blood vessels in *Itga5^{Pdgfrb-cre}* mutants appeared indistinguishable from those in control mice (Fig. 3E), and even in the most severely affected mutants, no aneurysms or dilation of blood vessels were ever observed (Fig. 3F).

***Pdgfrb*-Cre is expressed in a population of cells within the jugular lymph sac and lymphovenous valves**

Previous results have shown that *Pdgfrb* is expressed on newly formed lymphatic vessels in the corneas of mice treated with PDGF-B (Cao et al., 2004) and in endothelial cells in vitro (Heldin and Westermark, 1999). To address whether the defects observed in *Itga5^{Pdgfrb-cre}* mutants could be due to deletion of $\alpha 5$ throughout the lymphatic endothelium we analysed the expression of *Pdgfrb*, by crossing the *Pdgfrb-Cre* line with the mTmG reporter mouse (*Pdgfrb^{mtmg}*), in which Cre-mediated excision results in the expression of membrane-bound GFP (Muzumdar et al., 2007). As expected, *Pdgfrb* is highly expressed in both pericytes and vSMCs, but is absent from the vascular endothelium and lymphatic capillaries within the skin (Fig. S4A–D).

GFP⁺ cells were however detected in a subset of LYVE-1⁺ lymphatic ECs in the jugular lymph sacs (JLSs) (Fig. 4A, B), and in a population of Prox1⁺ cells in the internal jugular (IJV) and subclavian veins (SCV), adjacent to where the lymphatic sac (LS) merges with the venous endothelium to form the two lymphovenous valves and drain fluid back into the blood circulation (Fig. 4C–E). As described by Srinivasan and Oliver (2011), the lymphovenous valves form through the fusion of the LSs (Fig. 4A) with the veins in a region containing a subpopulation of Prox1⁺ FoxC2⁺ venous ECs within the IJV and SCV (Fig. 4A). Lymphovenous valves consist of two leaflets, each containing two layers of Prox1⁺ cells (Fig. 4A, S5A). The inner layer of cells is derived from the LSs and expresses podoplanin (Fig. 4A, S5B), while the outer layer is derived from the specialised Prox1⁺ FoxC2⁺ podoplanin⁻ cells found in the walls of the veins (Fig. 4A, S5B) (Srinivasan and Oliver, 2011). Interestingly, analysis of the *Pdgfrb^{mtmg}* mice revealed that *Pdgfrb-Cre* is also expressed within these specialised venous ECs in the outer layer of the lymphovenous valve leaflets or their progenitors (Fig. 4F). Furthermore, a subpopulation of PDGFR β -positive cells was still detectable in the outer layer of the valves at E13.5 (Fig. S5C). These cells did not appear to be pericytes or vSMCs, since they lacked the mural cell markers desmin and SMA (Fig. S5B). In addition to *Pdgfrb*, the lymphovenous valves also expressed high levels of integrin- $\alpha 5$ (Fig. 4G), along with the lymphatic valve marker (and fibronectin receptor) integrin- $\alpha 9$ (Fig. 4H) (Bazigou et al., 2009). This is in contrast to the valves within the collecting lymphatic vessels, where only low levels of $\alpha 5$ have been reported (Bazigou et al., 2009).

Separation of the jugular lymph sacs from the cardinal vein is delayed in *Itga5^{Pdgfrb-cre}* mice

Development of the lymphatic system in mice starts at E9.75 when Prox1 turns on in a subpopulation of LYVE-1⁺ ECs in the lateral parts of the anterior cardinal vein (CV) (Srinivasan et al., 2007; Wigle and Oliver, 1999). Prox1⁺ lymphatic progenitor cells then bud, and migrate from the CV in response to VEGF-C from the mesoderm to form the JLSs and the superficial lymphatic vessels, respectively (Francois et al., 2012; Hagerling et al., 2013; Yang et al., 2012). During the budding of the JLSs, nascent lymph sacs become transiently filled with blood until complete separation from the CV occurs at E12.5, and the lymphovenous valves form and allow the lymph sacs to drain into the venous circulation (Francois et al., 2012; van der Putte, 1975). Failure to separate the JLSs from the CV leads

to prolonged bleeding into the lymph sacs, tortuous blood-filled cutaneous lymphatic vessels and eventually embryonic death (Abtahian et al., 2003; D'Amico et al., 2009; Uhrin et al., 2010).

Since *Pdgfrb-Cre* is expressed within Prox1⁺ ECs in the venous endothelium and in a subset of cells within the JLSs (Fig. 4), we examined whether the defects observed in *Itga5^{Pdgfrb-cre}* embryos might also be due to a failure of the JLS to separate from the CV. Consistent with previous reports, at E10.5 the anterior CV of *Itga5^{Pdgfrb-cre}* embryos expressed LYVE-1 (Wigle et al., 2002) and contained equivalent numbers of Prox1⁺ progenitor cells when compared with control mice (Fig. 5A, B). By E12.5, LYVE-1 expression was no longer present in the CV, and both control and mutant embryos had clearly visible lymph sacs (Fig. 5C, D). However, in contrast to control embryos (Fig. 5C), JLSs in E12.5 *Itga5^{Pdgfrb-cre}* embryos were often filled with blood (Fig. 5D, S2) and consisted of both LYVE-1⁺ lymphatic ECs and LYVE-1⁻ ECs in both haemorrhaging (Fig. 5D) and non-haemorrhaging mice (Fig. S6). The lack of LYVE-1⁺/Prox1⁺ lymphatic endothelial cells was particularly noticeable in the region directly adjacent to the CV (Fig. 5D, S6), suggesting that the absence of *a5* integrin in these cells compromised the separation of JLS from CV in *Itga5^{Pdgfrb-cre}* mice, which as a result were susceptible to excessive amounts of blood entering into the lymphatic circulation.

Previous studies have shown platelets are essential for separation of the JLS from the CV (Bertozzi et al., 2010). Mutant mice with defects in podoplanin-induced platelet activation fail to separate their lymphatic vessels from the blood circulation and, as a result, develop blood-filled lymphatics (Bertozzi et al., 2010). Platelets have been reported to express PDGFR β in humans (Yang et al., 1997), therefore, *Itga5* might have been deleted from platelets in our mutant mice and be the cause of the lymphatic defects observed. However, deletion of *Itga5* specifically within platelets, using the *Pf4-Cre* mouse line (Tiedt et al., 2007) failed to replicate the phenotype seen in *Itga5^{Pdgfrb-cre}* mice. *Itga5^{Pf4-cre}* mice developed to term and formed organised lymphatic capillaries within the skin (Fig. S7). Thus, the development of blood-filled lymphatic vessels in *Itga5^{Pdgfrb-cre}* mice is not due to platelet defects.

Integrin- $\alpha 5$ is required for formation of the lymphovenous valves

In the human lymphatic malformation, fetal cystic hygroma, failure to connect the LSs to the venous circulation results in large swollen lymphatic vessels, oedema and, unless resolved, death due to inability to drain interstitial fluid back into the venous circulation (Chervenak et al., 1983). To investigate whether the defects in *Itga5^{Pdgfrb-cre}* mice might also involve a failure to form the lymphovenous valves, we analysed sequential coronal sections through E15.5 control and mutant embryos. At E15.5, the lymphovenous valves should have been fully formed for at least 48 hours (Fig. 4C–E) (Srinivasan and Oliver, 2011). Indeed, in control mice (Fig. 6A–C) the first valve of the left JLS is present just below the vertebral artery, where the LS splits into two portions, and drains into the IJV (arrow, Fig. 6B), while the second valve is positioned more caudally, and drains into the junction of the IJV and SCV (arrow, Fig. 6C). In oedematous *Itga5^{Pdgfrb-cre}* mice however, no obvious connections between the LSs and the venous circulation were apparent, and despite careful examination

of ~30 oedematous embryos, no lymphovenous valves were detected (Fig. 6D–F). Instead, by E15.5, only *Prox1*⁺ lymphovenous valve rudiments were found on the walls of both the jugular and subclavian veins (Fig. 6E, F).

As a result, lymph fluid and blood that has entered the lymph sacs during the separation from the CV, is unable to drain into the venous circulation, accumulates in the lymphatic network (Fig. 6D, E), and leads to dilation of the lymph sac (Fig. 6D). Interestingly, lymphovenous valves were found in *Itga5*^{*Pdgfrb-cre*} embryos that lacked any obvious signs of oedema (Fig. S8A). Furthermore, in agreement with the predisposition of mutants to develop a blood-filled JLS only on the left side of the embryo, we also observed that in a large proportion of *Itga5*^{*Pdgfrb-cre*} embryos analysed, only the left lymphatic sac failed to connect to the venous endothelium, and only in rare cases were both lymphovenous valves absent (Fig. S8B). Lymphovenous valve defects have previously been reported in mice lacking a single copy of the *Prox1* transcription factor and as a result they develop oedema and occasionally blood-filled JLSs (Srinivasan and Oliver, 2011). In that study, the authors proposed that the development of the lymphovenous valves is dependent on expression of *Prox1* in the population of venous ECs that contribute to formation of the valve leaflets. Loss of *Prox1* expression did not seem to be the cause for the valve defects in *Itga5*^{*Pdgfrb-cre*} embryos however, since similar numbers of *Prox1*⁺ vascular ECs were visible at regions adjacent to the lymphatic sacs, in both control and *Itga5*^{*Pdgfrb-cre*} mice (arrowhead, Fig. 6A, D).

Endothelial expression of both $\alpha 5$ and αv integrins are required for proper lymphatic development

Since our results suggest that deletion of $\alpha 5$ in a subset of venous endothelial cells, rather than mural cells (Fig. S5B) or platelets (Fig. S7), is the cause of the lymphatic defects seen in *Itga5*^{*Pdgfrb-cre*} embryos, we re-examined embryos in which *Itga5* had been deleted using *Tie2-Cre*, which is expressed in both the lymphatic and blood vasculature. As previously reported by van der Flier et al. (2010), on a C57BL/6 background none of the *Itga5 flox/KO*^{*Tie2-cre*} embryos analysed developed oedema or blood-filled lymphatics (Fig. S9A, B), and only relatively mild dilation of lymphatics was observed (Fig. S9B). In contrast, on a 129S4 background, ~40% of the *Itga5 flox/flox*^{*Tie2-cre*} embryos developed oedema (4/9 analysed), and hyperplastic blood-filled lymphatic vessels (1/4 mutants) by E14.5 (Fig S9C, D), confirming that the lymphatic defects in *Itga5*^{*Pdgfrb-cre*} embryos are indeed endothelium-specific. The penetrance of this phenotype was enhanced further by the addition of a single integrin- αv KO allele (Fig. S9E), or by deleting both *Itga5* and *Itgav* specifically within the endothelium from E8.5 using the inducible *VE-Cadherin-Cre*^{*ert2*} mouse (6/6 mutants) (Fig. S9F). The phenotype of endothelium-specific $\alpha 5$, and possibly *Itga5*^{*Pdgfrb-cre*} embryos, appears therefore to be dependent on a strain-dependent genetic modifier and on the ability of αv integrins to compensate for the loss of $\alpha 5\beta 1$.

Discussion

We report here two unexpected findings about the role of $\alpha 5\beta 1$ integrin in vascular development. First, ablation of $\alpha 5$ integrin from mural cells fails to cause any notable

defects in the development of the blood vasculature. Second, deletion of $\alpha 5$ integrin in *Itga5^{Pdgfrb-cre}* mice produces lymphatic defects.

Integrin- $\alpha 5$ is dispensable for mural cell functions during vascular development

Despite efficient ablation of $\alpha 5\beta 1$ integrin from mural cells (Fig. S1B), blood vessels in *Itga5^{Pdgfrb-cre}* embryos lack obvious mural cell defects (Fig. 2). $\alpha 5$ -deficient pericytes covered capillaries and extended long cellular extensions (Fig. 2F), while vSMCs formed organised layers around the aorta (Fig. S1B) and aligned in concentric rings around arteries within the skin of mutant mice (Fig. 2B). This was particularly surprising since $\alpha 5\beta 1$ is highly expressed on mural cells (Fig. S1A) (Moiseeva, 2001). We have reported earlier that mice lacking endothelial expression of $\alpha 5\beta 1$ also fail to display the angiogenic defects observed in global $\alpha 5$ -KO mice (van der Flier et al., 2010). Thus, $\alpha 5\beta 1$ integrin seems to be largely dispensable in each cell type for formation of the blood vasculature. It is possible that $\alpha 5\beta 1$ integrin in *both* cell types contributes to assembly of a fibronectin-rich ECM between them and that either cell type can perform this function. Alternatively, other fibronectin-binding integrins could contribute overlapping or compensatory functions that cover for the absence of $\alpha 5\beta 1$ integrin. αv integrins can also assemble fibronectin but global knockouts of all αv integrins or ablation of $\alpha v\beta 3$ and $\alpha v\beta 5$ also fail to produce general vascular defects (Bader et al., 1998; Hoidalva-Dilke et al., 1999; Huang et al., 2000a; Reynolds et al., 2002), demonstrating that, if αv integrins are involved, they are less essential than $\alpha 5\beta 1$ integrin. We have shown that mice lacking endothelial expression of both $\alpha 5$ and αv integrin subunits, both of which are capable of fibronectin assembly in vitro, show no obvious defects in the deposition of fibronectin within their basement membranes in vivo (van der Flier et al., 2010).

Mural cell deletion of *Itgb1*, the common beta subunit of a dozen different integrins, including $\alpha 5$, as well as other potential minor fibronectin receptors ($\alpha 4$, $\alpha 8$, $\alpha 9$) allows development until late in gestation, although skin vessels do show dilations. Some pups are born but, postnatally, aneurysms form and the mural cells show defects in their adherence to vessels and in the assembly of fibronectin and other ECM proteins around vessels within the skin (Abraham et al., 2008). None of those other alpha subunits, when individually deleted globally, has much if any effect on vascular development and, in the absence of $\alpha 5\beta 1$ on mural cells, αv integrins are presumably still expressed by the mural cells. In light of these results, there are two possible explanations for the absence of detectable blood vessel defects in *Itga5^{Pdgfrb-cre}* embryos. First, the role of $\alpha 5$ may be adequately replaced (through overlapping functions or by compensation) by other integrins expressed on the mural cells, or by $\alpha 5$ expressed on ECs during angiogenesis. Mural cells express several fibronectin receptors ($\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha 4\beta 1$) (Moiseeva, 2001), and defects in pericyte and vSMCs distribution have been reported in $\alpha 4$ -KO mice (Garmy-Susini et al., 2005; Grazioli et al., 2006). To address these possibilities, tissue-specific mutants lacking multiple fibronectin integrins and mice lacking $\alpha 5$ in both endothelial and mural cell compartments will need to be studied. Second, mural cell function and vessel stability may in fact, be more reliant on the collagen and laminin receptors included among the $\beta 1$ integrins. vSMCs reportedly express high levels of $\alpha 3\beta 1$ (a laminin receptor) and $\alpha 1\beta 1$ (a collagen receptor) in

vivo; however to date, only the $\alpha 7 \beta 1$ integrin (another laminin receptor) has been shown to have a role in mural cell function during development (Flintoff-Dye et al., 2005).

Selective deletion of $\alpha 5 \beta 1$ integrin causes lymphatic defects

Lymphatic malformations occur in 1 in 1750 live births and 1 in 200 spontaneous abortions. The exact genes and cells involved in these malformations remain unclear. Here we report that $\alpha 5 \beta 1$, the major fibronectin receptor, is essential for proper lymphatic development. Reminiscent of the lymphatic malformation, fetal cystic hygroma (Chervenak et al., 1983), *Itga5^{Pdgfrb-cre}* embryos fail to form lymphovenous valves on their left side (Fig. 6D–F). Consequently, $\alpha 5$ mutants fail to drain their left jugular lymph sacs into the venous circulation, accumulate interstitial fluid in their lymphatic circulation and develop swollen hyperplastic lymphatic vessels and oedema (Fig. 1B, 3C). *Itga5^{Pdgfrb-cre}* mice that manage to connect their JLSs before intrauterine death appear to resolve their oedema and survive to birth (Fig. S2, S8). Nevertheless, even without obvious persistent oedema, these mice still displayed enlarged lymphatic vessels and ectopic smooth muscle cell coverage around their capillary lymphatic vessels (Fig. 2D, E), just as observed in patients with lymphedema distichiasis, and in mice lacking the transcription factor *Foxc2* (Petrova et al., 2004). Interestingly, lymphatic hyperplasia and abnormal recruitment of vSMCs to the lymphatic capillaries, without blood vessel defects, are also seen in mice lacking the PDZ-binding domain of ephrin-B2, which like *FoxC2* mutants have defective lymphatic valves and abnormal lymphatic circulation (Makinen et al., 2005; Petrova et al., 2004). Intriguingly, deletion of ephrin-B2 using the same *Pdgfrb-Cre* line used in this study, also leads to ectopic vSMC cell coverage on the dermal lymphatic capillaries, in addition to causing mural cell defects (Foo et al., 2006).

Since the majority of *Itga5^{Pdgfrb-cre}* embryos developed oedema in the absence of obvious haemorrhaging, and blood-filled lymph sacs were observed before the formation of the lymphovenous valves at E12.5 (Fig. 5D, S2), we believe that the appearance of a blood-filled lymphatic network in $\alpha 5$ mutants requires both a delay in the separation of the JLS from the CV (Fig. 5D) and defective lymphovenous valve formation (Fig. 6). Previous studies have shown that, even in wild-type mice, blood can enter the lymphatic circulation as the nascent lymph sacs bud from the CV (Francois et al., 2012; van der Putte, 1975). Therefore, prolonging this process would result in excess blood entering the lymphatic system. Indeed, numerous reports have shown that failure or delay in separating the JLS from the CV results in blood-filled lymphatic vasculature (Abtahian et al., 2003; D'Amico et al., 2009; Uhrin et al., 2010). In wild-type mice, blood that enters during development is, most likely, drained back into the venous circulation via the lymphovenous valves. However, since no such connection is made in the majority of *Itga5^{Pdgfrb-cre}* embryos by E15.5, this blood is prevented from leaving the lymphatics and leads to the blood-filled phenotype observed. Interestingly, blood-filled lymphatics have also been reported to occur in *Prox1* heterozygous mutants, which also lack lymphovenous valves (Srinivasan and Oliver, 2011). In that study, the authors suggest that blood could enter the lymphatic circulation through the formation of abrupt fusions between the lymph sac and the IJV close to the valves (Srinivasan and Oliver, 2011). However, since <10% of *Prox1* heterozygous embryos develop blood-filled lymphatic vessels (Srinivasan and Oliver, 2011) and an abrupt

fusion between the LS and the IJV was only seen in one *Itga5^{Pdgfrb-cre}* embryo (data not shown), it is unlikely that this connection is the primary site where blood enters the lymphatic circulation in our mutant embryos.

Expression of PDGFR β and integrin α 5 β 1 during lymphatic development

Our analysis of *Pdgfrb-Cre* expression during embryonic development suggests that the lymphatic defects observed in *Itga5^{Pdgfrb-cre}* embryos are caused by loss of α 5 β 1 in a specialised population of *Prox1⁺ Pdgfr⁺* venous endothelial cells that contribute to the outer layer of the lymphovenous valve leaflets (Fig. 4C–G). Elegant work from Srinivasan and Oliver (2011) has shown that these cells also express the transcription factor *Foxc2*, in contrast to the lymphatic ECs that form the inner layer of the valve leaflets which are *Prox1⁺* but *Foxc2⁻* (Fig. 4A). Interestingly, *Foxc2* is a direct transcriptional activator of PDGFR β expression and promotes mesenchymal transition (Hollier et al., 2013). This suggests that expression of PDGFR β within the outer layer of the valves and in the JLS is downstream of *Foxc2*, and therefore of *Prox1* (Harada et al., 2009), and may indicate an important role for PDGF-B signalling in formation of both the JLS and the lymphovenous valves. Consistent with this model, *Pdgfrb* KO mice, in addition to displaying severe blood vasculature defects, also display dilated, tortuous, blood-filled lymphatic vessels, analogous to those seen in *Itga5^{Pdgfrb-cre}* embryos (Haiko, 2008). Furthermore, mice lacking a single copy of *Prox1* lose expression of *Prox1* and *Foxc2* in the cells within the IJV and SCV that contribute to the outer layer of the valve and fail to develop lymphovenous valves (Srinivasan and Oliver, 2011).

Possible roles of integrin- α 5 in lymphatic development

In this study, we have found that α 5 is highly expressed in the lymphovenous valves (Fig. 4G), and is required by the specialised population of *Prox1⁺ Pdgfrb⁺*-expressing cells for the proper formation of the JLS and the lymphovenous valves. The importance of α 5 within these cells could be attributable to several factors. First, α 5 may be mediating growth factor signalling. Integrin- α 5 is required for optimal activation of the lymphatic endothelial growth factor receptor, VEGFR-3 (Zhang et al., 2005), and proliferation of lymphatic ECs is blocked by small-molecule antagonists of α 5 β 1 in vitro (Dietrich et al., 2007). α 5 β 1 has also been implicated in the activation of PDGFR β , in the absence of growth factor stimulation, and anti- α 5 blocking antibodies significantly reduce PDGFR β phosphorylation (Veevers-Lowe et al., 2011). Second, loss of α 5 may reduce cell migration or adhesion of cells to fibronectin and fibrillin, the main component of the anchoring filaments securing lymphatic vessels to the interstitial matrix. Finally, α 5 may be required for deposition of fibronectin. Integrin- α 5 plays a major role in assembly of fibronectin (Wierzbicka-Patynowski and Schwarzbauer, 2003) and loss of α 5 in either vascular or lymphatic ECs leads to a reduction in fibronectin fibrillogenesis (Bazigou et al., 2009; van der Flier et al., 2010). Interestingly, assembly of fibronectin containing the extra type III domain (EIIIA) has been shown to be essential for the elongation of the intra-lymphatic valve leaflets (Bazigou et al., 2009). Mice lacking endothelial integrin- α 9, fail to organise EIIIA⁺ fibronectin into fibrils and as a result develop ring-like valve structures that are unable to prevent the backflow of lymph fluid within the body (Bazigou et al., 2009). It is conceivable therefore, that a similar process regulates the development of lymphovenous valve leaflets.

Conclusions

Despite numerous *in vitro* and *in vivo* studies, the roles of $\alpha 5\beta 1$ in vascular development remain unclear. Both endothelial and mural cells express $\alpha 5\beta 1$ during development but it is not essential in either cell type alone – maybe it functions similarly and, to some degree redundantly, in both cell types, for example in correct assembly of extracellular matrix. Furthermore, other integrins appear to play more minor, but perhaps overlapping, functions in vascular development. Some such functions have been revealed in experiments where multiple integrins have been ablated (van der Flier et al., 2010) and additional experiments of that sort, although difficult to accomplish, may shed further light on the complex roles of integrins in development of the cardiovascular system. It is also becoming clear that integrins, in particular fibronectin-binding integrins, have prominent roles in controlling the development of the lymphatic system. Mice lacking integrin- $\alpha 9$ develop abnormal lymphatic valves and chylothorax (Bazigou et al., 2009; Huang et al., 2000b), while integrin- $\alpha 4$ is required for tumour lymphangiogenesis (Garmy-Susini et al., 2010). Our present study has shown that integrin $\alpha 5\beta 1$ is essential in a subset of *Pdgfrb*⁺ cells for the proper separation of the blood and lymphatic vasculature and the development of the lymphovenous valves. Thus, while the known roles served by integrins in vascular and lymphatic development continue to increase, there undoubtedly remain yet other contributions to be discovered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Abraham S, Kogata N, Fassler R, Adams RH. Integrin beta1 subunit controls mural cell adhesion, spreading, and blood vessel wall stability. *Circ Res*. 2008; 102:562–570. [PubMed: 18202311]
- Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, Myers EE, Huang B, Jackson DG, Ferrari VA, Tybulewicz V, Lowell CA, Lepore JJ, Koretzky GA, Kahn ML. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science*. 2003; 299:247–251. [PubMed: 12522250]
- Alitalo K. The lymphatic vasculature in disease. *Nat Med*. 2011; 17:1371–1380. [PubMed: 22064427]
- Astrof S, Hynes RO. Fibronectins in vascular morphogenesis. *Angiogenesis*. 2009; 12:165–175. [PubMed: 19219555]
- Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. *Nat Rev Cancer*. 2008; 8:604–617. [PubMed: 18497750]
- Bader BL, Rayburn H, Crowley D, Hynes RO. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell*. 1998; 95:507–519. [PubMed: 9827803]

- Barillari G, Albonici L, Incerpi S, Bogetto L, Pistrutto G, Volpi A, Ensoli B, Manzari V. Inflammatory cytokines stimulate vascular smooth muscle cells locomotion and growth by enhancing alpha5beta1 integrin expression and function. *Atherosclerosis*. 2001; 154:377–385. [PubMed: 11166770]
- Bazigou E, Makinen T. Flow control in our vessels: vascular valves make sure there is no way back. *Cell Mol Life Sci*. 2013; 70:1055–1066. [PubMed: 22922986]
- Bazigou E, Xie S, Chen C, Weston A, Miura N, Sorokin L, Adams R, Muro AF, Sheppard D, Makinen T. Integrin-alpha9 is required for fibronectin matrix assembly during lymphatic valve morphogenesis. *Dev Cell*. 2009; 17:175–186. [PubMed: 19686679]
- Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams RH. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell*. 2009; 137:1124–1135. [PubMed: 19524514]
- Bertozi CC, Hess PR, Kahn ML. Platelets: covert regulators of lymphatic development. *Arterioscler Thromb Vasc Biol*. 2010; 30:2368–2371. [PubMed: 21071706]
- Cao R, Bjorndahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsarlis K, Dissing S, Ohhashi T, Jackson DG, Cao Y. PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell*. 2004; 6:333–345. [PubMed: 15488757]
- Chen J, Alexander JS, Orr AW. Integrins and their extracellular matrix ligands in lymphangiogenesis and lymph node metastasis. *Int J Cell Biol*. 2012; 2012:853703. [PubMed: 22505936]
- Chervenak FA, Isaacson G, Blakemore KJ, Breg WR, Hobbins JC, Berkowitz RL, Tortora M, Mayden K, Mahoney MJ. Fetal cystic hygroma. Cause and natural history. *N Engl J Med*. 1983; 309:822–825. [PubMed: 6888468]
- Courtoy PJ, Boyles J. Fibronectin in the microvasculature: localization in the pericyte-endothelial interstitium. *J Ultrastruct Res*. 1983; 83:258–273. [PubMed: 6348302]
- D'Amico G, Jones DT, Nye E, Sapienza K, Ramjuan AR, Reynolds LE, Robinson SD, Kostourou V, Martinez D, Aubyn D, Grose R, Thomas GJ, Spencer-Dene B, Zicha D, Davies D, Tybulewicz V, Hodivala-Dilke KM. Regulation of lymphatic-blood vessel separation by endothelial Rac1. *Development*. 2009; 136:4043–4053. [PubMed: 19906871]
- Davenpeck KL, Marcinkiewicz C, Wang D, Niculescu R, Shi Y, Martin JL, Zalewski A. Regional differences in integrin expression: role of alpha(5)beta(1) in regulating smooth muscle cell functions. *Circ Res*. 2001; 88:352–358. [PubMed: 11179205]
- Dietrich T, Onderka J, Bock F, Kruse FE, Vossmeier D, Stragies R, Zahn G, Cursiefen C. Inhibition of inflammatory lymphangiogenesis by integrin alpha5 blockade. *Am J Pathol*. 2007; 171:361–372. [PubMed: 17591980]
- Flintoff-Dye NL, Welser J, Rooney J, Scowen P, Tamowski S, Hatton W, Burkin DJ. Role for the alpha7beta1 integrin in vascular development and integrity. *Dev Dyn*. 2005; 234:11–21. [PubMed: 16003770]
- Földi, M.; Strössenreuther, RHK. ebrary Inc. Foundations of manual lymph drainage. 3. Mosby; St. Louis: 2005.
- Foo SS, Turner CJ, Adams S, Compagni A, Aubyn D, Kogata N, Lindblom P, Shani M, Zicha D, Adams RH. Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. *Cell*. 2006; 124:161–173. [PubMed: 16413489]
- Francis SE, Goh KL, Hodivala-Dilke K, Bader BL, Stark M, Davidson D, Hynes RO. Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol*. 2002; 22:927–933.
- Francois M, Short K, Seeker GA, Combes A, Schwarz Q, Davidson TL, Smyth I, Hong YK, Harvey NL, Koopman P. Segmental territories along the cardinal veins generate lymph sacs via a ballooning mechanism during embryonic lymphangiogenesis in mice. *Dev Biol*. 2012; 364:89–98. [PubMed: 22230615]
- Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA. Integrin alpha4beta1 signaling is required for lymphangiogenesis and tumor metastasis. *Cancer Res*. 2010; 70:3042–3051. [PubMed: 20388801]

- Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J. Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. *J Clin Invest.* 2005; 115:1542–1551. [PubMed: 15902308]
- George EL, Baldwin HS, Hynes RO. Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells. *Blood.* 1997; 90:3073–3081. [PubMed: 9376588]
- George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development.* 1993; 119:1079–1091. [PubMed: 8306876]
- Goodman SL, Picard M. Integrins as therapeutic targets. *Trends Pharmacol Sci.* 2012; 33:405–412. [PubMed: 22633092]
- Grazioli A, Alves CS, Konstantopoulos K, Yang JT. Defective blood vessel development and pericyte/pvSMC distribution in alpha 4 integrin-deficient mouse embryos. *Dev Biol.* 2006; 293:165–177. [PubMed: 16529735]
- Hagerling R, Pollmann C, Andreas M, Schmidt C, Nurmi H, Adams RH, Alitalo K, Andresen V, Schulte-Merker S, Kiefer F. A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. *EMBO J.* 2013; 32:629–644. [PubMed: 23299940]
- Haiko, P. VEGF-C/VEGFR-3 and PDGF-B/PDGFR-beta pathways in embryonic lymphangiogenesis. Haartman Institute and Biomedicum Helsinki. University of Helsinki; 2008.
- Harada K, Yamazaki T, Iwata C, Yoshimatsu Y, Sase H, Mishima K, Morishita Y, Hirashima M, Oike Y, Suda T, Miura N, Watabe T, Miyazono K. Identification of targets of Prox1 during in vitro vascular differentiation from embryonic stem cells: functional roles of HoxD8 in lymphangiogenesis. *J Cell Sci.* 2009; 122:3923–3930. [PubMed: 19825936]
- Hedin U, Thyberg J. Plasma fibronectin promotes modulation of arterial smooth-muscle cells from contractile to synthetic phenotype. *Differentiation.* 1987; 33:239–246. [PubMed: 3596086]
- Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev.* 1999; 79:1283–1316. [PubMed: 10508235]
- Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development.* 1999; 126:3047–3055. [PubMed: 10375497]
- Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ Res.* 1999; 84:298–305. [PubMed: 10024303]
- Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S, Hynes RO. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest.* 1999; 103:229–238. [PubMed: 9916135]
- Hollier BG, Tinnirello AA, Werden SJ, Evans KW, Taube JH, Sarkar TR, Sphyris N, Shariati M, Kumar SV, Battula VL, Herschkowitz JI, Guerra R, Chang JT, Miura N, Rosen JM, Mani SA. FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. *Cancer Res.* 2013; 73:1981–1992. [PubMed: 23378344]
- Huang X, Griffiths M, Wu J, Farese RV Jr, Sheppard D. Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol Cell Biol.* 2000a; 20:755–759. [PubMed: 10629031]
- Huang XZ, Wu JF, Ferrando R, Lee JH, Wang YL, Farese RV Jr, Sheppard D. Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. *Mol Cell Biol.* 2000b; 20:5208–5215. [PubMed: 10866676]
- Hynes RO. Cell-matrix adhesion in vascular development. *J Thromb Haemost.* 2007; 5(Suppl 1):32–40. [PubMed: 17635706]
- Kale S, Hanai J, Chan B, Karihaloo A, Grotendorst G, Cantley L, Sukhatme VP. Microarray analysis of in vitro pericyte differentiation reveals an angiogenic program of gene expression. *FASEB.* 2005; J19:270–271.

- Kim S, Bell K, Mousa SA, Varner JA. Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. *Am J Pathol.* 2000; 156:1345–1362. [PubMed: 10751360]
- Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol.* 2001; 230:230–242. [PubMed: 11161575]
- Kogata N, Tribe RM, Fassler R, Way M, Adams RH. Integrin-linked kinase controls vascular wall formation by negatively regulating Rho/ROCK-mediated vascular smooth muscle cell contraction. *Genes Dev.* 2009; 23:2278–2283. [PubMed: 19797768]
- Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Branson RT, Roes JT, Savill JS, Hynes RO. Ulcerative colitis and autoimmunity induced by loss of myeloid alpha5 integrins. *Proc Natl Acad Sci U S A.* 2007; 104:15823–15828. [PubMed: 17895374]
- Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* 1994; 8:1875–1887. [PubMed: 7958863]
- Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science.* 1997; 277:242–245. [PubMed: 9211853]
- Makinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, Klein R, Wilkinson GA. PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. *Genes Dev.* 2005; 19:397–410. [PubMed: 15687262]
- Moiseeva EP. Adhesion receptors of vascular smooth muscle cells and their functions. *Cardiovasc Res.* 2001; 52:372–386. [PubMed: 11738054]
- Muether PS, Dell S, Kociok N, Zahn G, Stragies R, Vossmeier D, Jousen AM. The role of integrin alpha5beta1 in the regulation of corneal neovascularization. *Exp Eye Res.* 2007; 85:356–365. [PubMed: 17659277]
- Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis.* 2007; 45:593–605. [PubMed: 17868096]
- Okazaki T, Ni A, Ayeni OA, Baluk P, Yao LC, Vossmeier D, Zischinsky G, Zahn G, Knolle J, Christner C, McDonald DM. alpha5beta1 Integrin blockade inhibits lymphangiogenesis in airway inflammation. *Am J Pathol.* 2009; 174:2378–2387. [PubMed: 19443705]
- Petrova TV, Karpanen T, Norrmen C, Mellor R, Tamakoshi T, Finegold D, Ferrell R, Kerjaschki D, Mortimer P, Yla-Herttuala S, Miura N, Alitalo K. Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. *Nat Med.* 2004; 10:974–981. [PubMed: 15322537]
- Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, Hodivala-Dilke KM. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nat Med.* 2002; 8:27–34. [PubMed: 11786903]
- Soriano P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev.* 1994; 8:1888–1896. [PubMed: 7958864]
- Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM, Oliver G. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. *Genes Dev.* 2007; 21:2422–2432. [PubMed: 17908929]
- Srinivasan RS, Oliver G. Prox1 dosage controls the number of lymphatic endothelial cell progenitors and the formation of the lymphovenous valves. *Genes Dev.* 2011; 25:2187–2197. [PubMed: 22012621]
- Tammela T, Alitalo K. Lymphangiogenesis: Molecular mechanisms and future promise. *Cell.* 2010; 140:460–476. [PubMed: 20178740]
- Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood.* 2007; 109:1503–1506. [PubMed: 17032923]
- Turlo KA, Noel OD, Vora R, LaRussa M, Fassler R, Hall-Glenn F, Iruela-Arispe ML. An essential requirement for beta1 integrin in the assembly of extracellular matrix proteins within the vascular wall. *Dev Biol.* 2012; 365:23–35. [PubMed: 22331032]

- Uhrin P, Zaujec J, Breuss JM, Olcaydu D, Chrenek P, Stockinger H, Fuertbauer E, Moser M, Haiko P, Fassler R, Alitalo K, Binder BR, Kerjaschki D. Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. *Blood*. 2010; 115:3997–4005. [PubMed: 20110424]
- Umeda N, Kachi S, Akiyama H, Zahn G, Vossmeier D, Stragies R, Campochiaro PA. Suppression and regression of choroidal neovascularization by systemic administration of an alpha5beta1 integrin antagonist. *Mol Pharmacol*. 2006; 69:1820–1828. [PubMed: 16527907]
- van der Flier A, Badu-Nkansah K, Whittaker CA, Crowley D, Bronson RT, Lacy-Hulbert A, Hynes RO. Endothelial alpha5 and alphaV integrins cooperate in remodeling of the vasculature during development. *Development*. 2010; 137:2439–2449. [PubMed: 20570943]
- van der Putte SC. The development of the lymphatic system in man. *Adv Anat Embryol Cell Biol*. 1975; 51:3–60. [PubMed: 1136873]
- Veevers-Lowe J, Ball SG, Shuttleworth A, Kielty CM. Mesenchymal stem cell migration is regulated by fibronectin through alpha5beta1-integrin-mediated activation of PDGFR-beta and potentiation of growth factor signals. *J Cell Sci*. 2011; 124:1288–1300. [PubMed: 21429937]
- Wierzbicka-Patynowski I, Schwarzbauer JE. The ins and outs of fibronectin matrix assembly. *J Cell Sci*. 2003; 116:3269–3276. [PubMed: 12857786]
- Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J*. 2002; 21:1505–1513. [PubMed: 11927535]
- Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell*. 1999; 98:769–778. [PubMed: 10499794]
- Xie L, Duncan MB, Pahler J, Sugimoto H, Martino M, Lively J, Mundel T, Soubasakos M, Rubin K, Takeda T, Inoue M, Lawler J, Hynes RO, Hanahan D, Kalluri R. Counterbalancing angiogenic regulatory factors control the rate of cancer progression and survival in a stage-specific manner. *Proc Natl Acad Sci U S A*. 2011; 108:9939–9944. [PubMed: 21622854]
- Yang JT, Rayburn H, Hynes RO. Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development*. 1993; 119:1093–1105. [PubMed: 7508365]
- Yang M, Khachigian LM, Hicks C, Chesterman CN, Chong BH. Identification of PDGF receptors on human megakaryocytes and megakaryocytic cell lines. *Thromb Haemost*. 1997; 78:892–896. [PubMed: 9268191]
- Yang Y, Garcia-Verdugo JM, Soriano-Navarro M, Srinivasan RS, Scallan JP, Singh MK, Epstein JA, Oliver G. Lymphatic endothelial progenitors bud from the cardinal vein and intersomitic vessels in mammalian embryos. *Blood*. 2012; 120:2340–2348. [PubMed: 22859612]
- Zhang X, Groopman JE, Wang JF. Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin alpha5beta1. *J Cell Physiol*. 2005; 202:205–214. [PubMed: 15389531]

- $\alpha 5\beta 1$ integrin is expressed on mural cells but is dispensable for normal blood vessel development.
- $\alpha 5\beta 1$ integrin is also expressed on a subset of *Prox1*⁺ *Pdgfr*⁺ venous endothelial cells.
- Deletion of $\alpha 5\beta 1$ integrin from these ECs causes distended, blood-filled lymphatics.
- $\alpha 5\beta 1$ integrin is required for separation of jugular lymph sacs from cardinal veins.
- $\alpha 5\beta 1$ integrin is required for proper formation of lymphovenous valves.

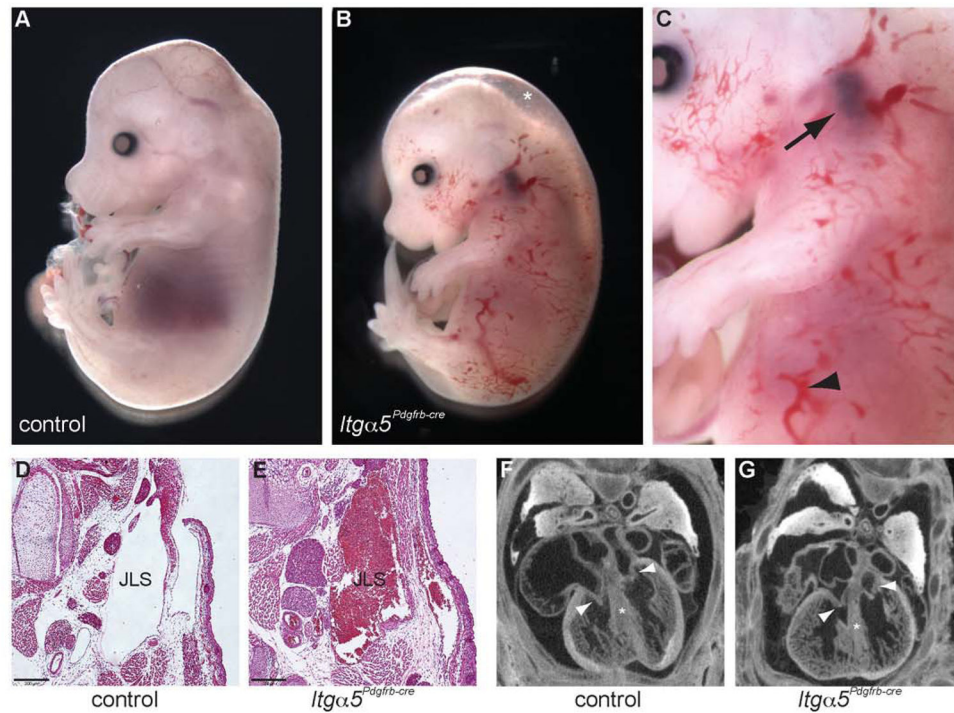


Figure 1. Phenotype of *Itga5*^{Pdgfrb-cre} mutant mice

Freshly isolated E15.5 control (A) and *Itga5*^{Pdgfrb-cre} embryos (B, enlarged in C). *Itga5*^{Pdgfrb-cre} mutants display oedema (asterisk, B), accumulation of blood within the skin (arrowhead in C), and blood-filled jugular lymph sacs (arrow, C). H&E sections showing control (D) and blood-filled jugular lymph sac (JLS) in E15.5 *Itga5*^{Pdgfrb-cre} mutants (E). Micro-CT section of E15.5 control (F) and *Itga5*^{Pdgfrb-cre} hearts showing development of the tricuspid and mitral valves (arrowheads) and the absence of ventricular septal defects (*) in mutant embryos (G). See also Movies 1 and 2 in Supplementary Material. Scale bar: 20 μ m.

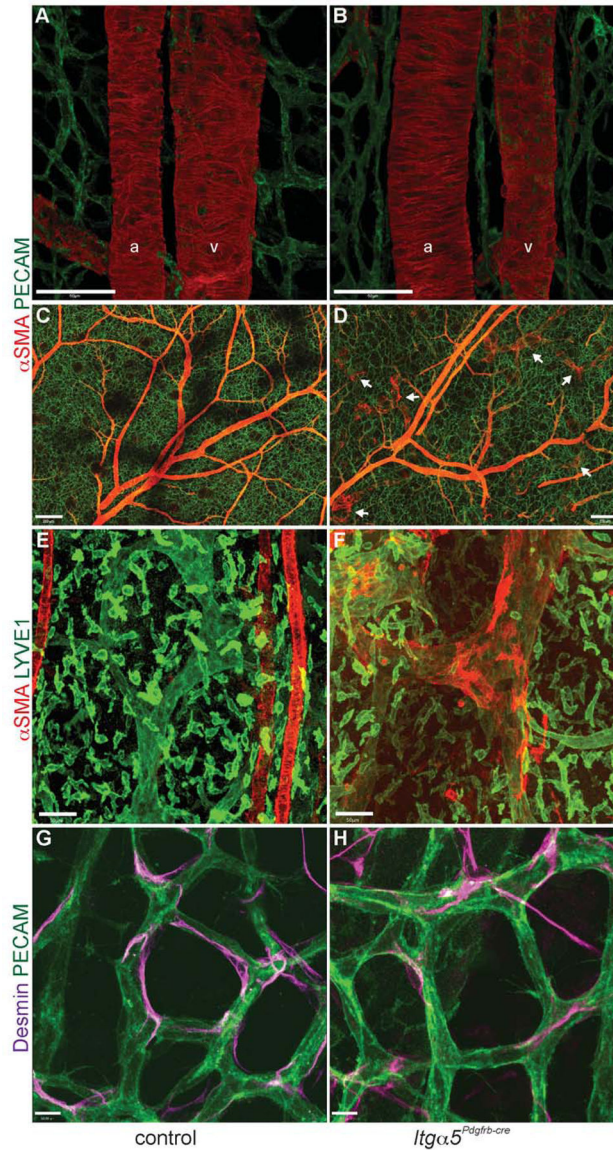


Figure 2. Normal blood vessel morphology in *Itga5^{Pdgfrb-cre}* mice

(A–H) Whole-mount immunofluorescence stainings of control and mutant embryonic skin at E17.5. (A, B) No obvious defects seen in vSMC (red) attachment or morphology around arteries (a) and veins (v) in *Itga5^{Pdgfrb-cre}* mice. (C, D) Low magnification images showing the extent of vSMC coverage throughout the vasculature in a control (C) and *Itga5^{Pdgfrb-cre}* embryo (D). Notice the ectopic vSMC coverage around lymphatic capillaries (white arrows) (D), which are absent in control embryos (C). Higher magnification image of a lymphatic capillary (green) in control skin (E) and ectopic α SMA coverage around a lymphatic capillary in an *Itga5^{Pdgfrb-cre}* embryo (F). Desmin-positive pericytes apposed to microvessels in control (G) and *Itga5^{Pdgfrb-cre}* mutants (H). Scale bars: 50 μ m (A, B), 200 μ m (C, D), 50 μ m (E, F), 10 μ m (G, H).

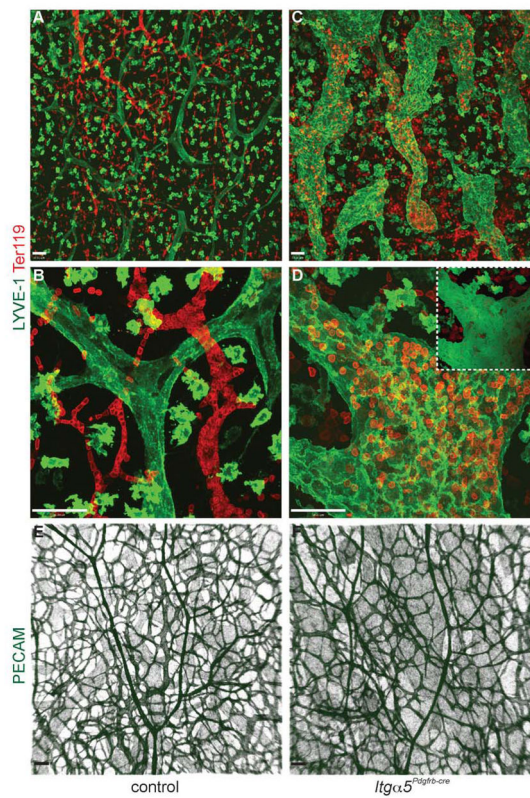


Figure 3. *Itg α 5^{Pdgfrb-cre}* mice display hyperplastic, blood-filled lymphatic vessels
 (A–D) Confocal images showing the lymphatic capillaries (LYVE-1, green; note that LYVE-1 is also expressed on macrophages) and erythrocytes (Ter119, red) in E15.5 control and *Itg α 5^{Pdgfrb-cre}* skin. In contrast to control mice (A and B), lymphatic vessels in *Itg α 5^{Pdgfrb-cre}* mutants are hyperplastic, tortuous and filled with red blood cells (C and D). Inset shows 3D rendering of image in (D) confirming presence of blood within the lymphatic vessel. Blood vessels in *Itg α 5^{Pdgfrb-cre}* mice (strong PECAM stain, green) however remain unaffected by the deletion of integrin- α 5 (E, F). Note lymphatic vessels (weak PECAM stain) are also visible in both E and F. Scale bars: 50 μ m.

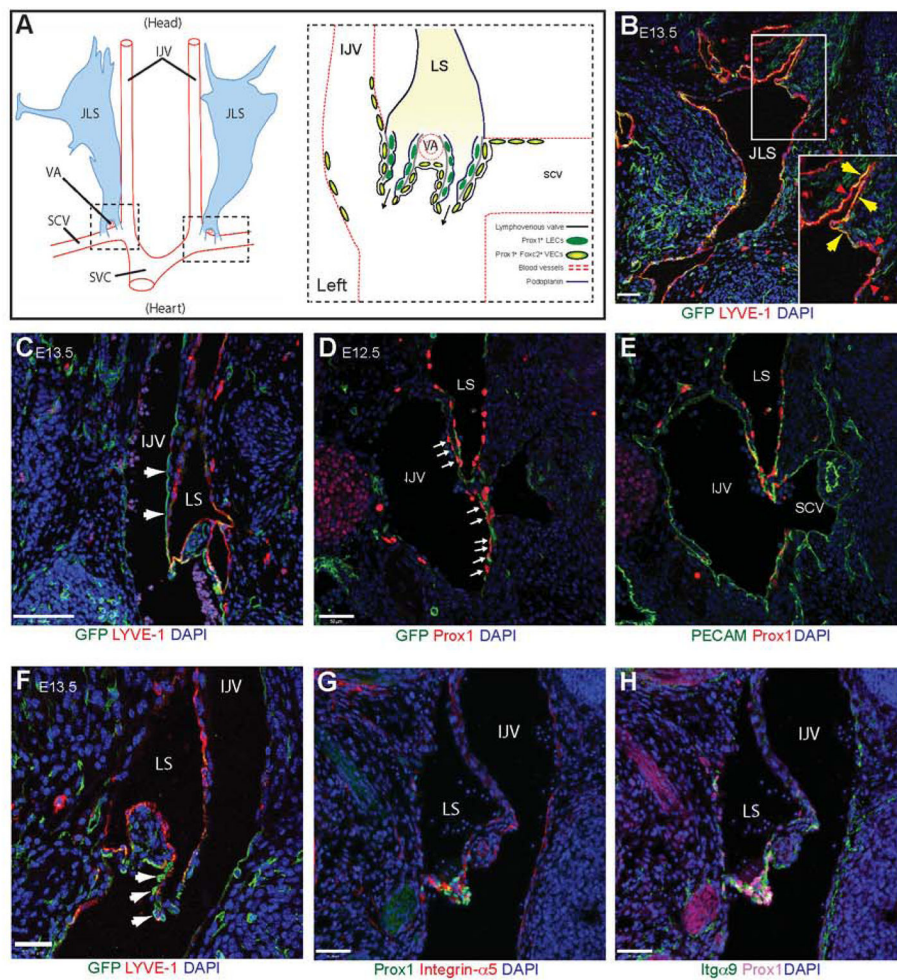


Figure 4. Lymphatic expression of *Pdgfrb-Cre*

(A) Schematic representation of the position (modified from Srinivasan and Oliver (2011)) and morphology of the jugular lymphatic sacs (JLS) and the lymphovenous valves in E13.5 mouse embryos. The head is orientated to the top and the heart towards the bottom of the figure. The JLS runs from the neck posteriorly to the level of the thymus, where it is split into two portions by the vertebral artery (VA). The lymphovenous valves (dashed boxes) form at the end of these two lymph sacs (LS) through the fusion of the posterior region of the LSs with the internal jugular (IJV) and subclavian (SCV) veins where they merge and drain into the superior vena cava (SVC). Specialised Prox1⁺ Foxc2⁺ podoplanin⁻ venous endothelial cells (VECs) are found in these regions. The lymphovenous valve leaflets consist of two layers of Prox1⁺ cells. The inner layer is composed of Prox1⁺ Foxc2⁻ podoplanin⁻ lymphatic endothelial cells (LEC) from the LSs, while the outer layer is derived from the Prox1⁺ Foxc2⁺ podoplanin⁻ VECs. (B) Immunofluorescence staining on a coronal section through the JLS of a *Pdgfrb^{mtmg}* mouse at E13.5 (*Pdgfrb^β*⁺ cells will be GFP⁺). Note that the JLS appears to contain both GFP⁺ (yellow, arrows) and GFP⁻ (red, arrowheads) lymphatic endothelial cells (B, enlarged in inset). (C) GFP⁺ cells are also present in a population of cells in the IJV and SCV (arrows), adjacent to the JLS where the LS merge with venous circulation to form the lymphovenous valves. (D, E) Sequential transverse sections stained

with antibodies against the transcription factor Prox1 and (D) GFP and (E) PECAM showing *Pdgfrb-Cre* expression in the LS and in a specialised population of venous endothelial cells within the IJV and SCV (arrows) in an E12.5 *Pdgfrb^{mtmg}* embryo. Note the lack of *Pdgfrb-Cre* expression in Prox1-negative venous endothelium. (F) *Pdgfrb⁺* cells (arrows) in the outer layer of the lymphovenous valve. (G, H) Immunofluorescence staining on a cryosection through the lymphovenous valve showing high expression of (G) integrin- α 5 and (H) integrin- α 9 within the Prox1⁺ valve leaflets. Scale bars: 50 μ m.

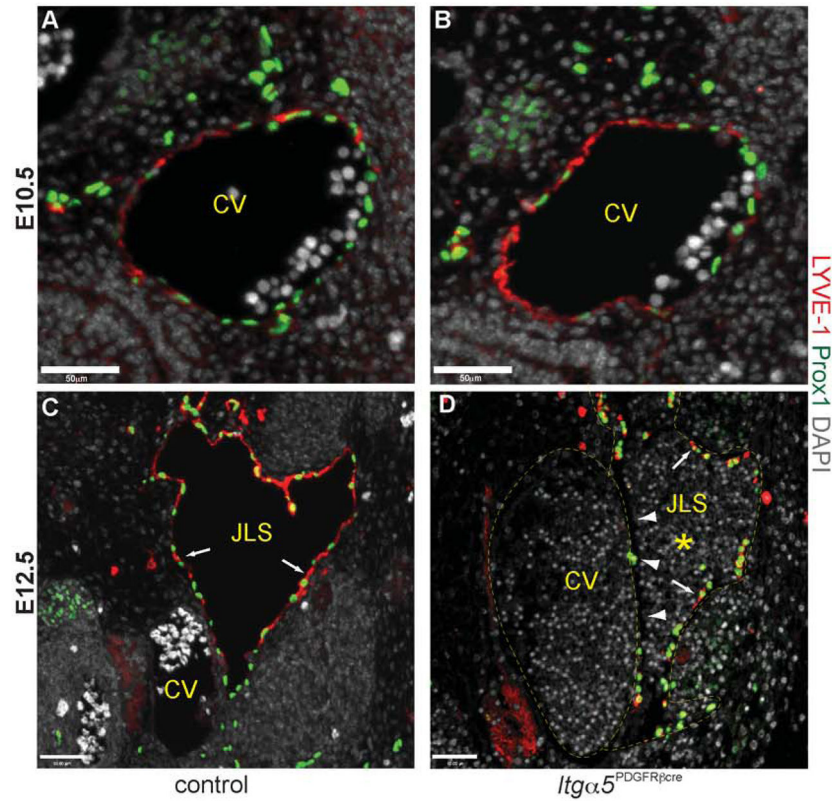


Figure 5. Separation of the jugular lymph sac from the cardinal vein is abnormal in *Itg* $\alpha 5^{PDGFR\beta-cre}$ embryos

Transverse sections through the cardinal vein (CV) showing expression of LYVE-1 (red) and Prox1 (green) in control and *Itg* $\alpha 5^{PDGFR\beta-cre}$ embryos. At E10.5, equivalent numbers of Prox1⁺ lymphatic endothelial progenitor cells are expressed on the dorsal lateral side of the anterior cardinal vein in control (A) and *Itg* $\alpha 5^{PDGFR\beta-cre}$ mice (B). Note that LYVE-1 is expressed throughout the CV at this stage.

These cells then bud, and migrate from the CV to form the jugular lymph sacs (JLS) by E12.5. In control embryos the JLSs now consist entirely of Prox1⁺ LYVE-1⁺ lymphatic endothelial cells (arrows, C) and contain no red blood cells. In contrast, JLSs in mutant embryos often contained blood (asterisk, D) and appeared to consist of both Prox1⁺ LYVE-1⁺ lymphatic endothelial cells (arrows, D), and Prox1⁺ Lyve1⁻ and Prox1⁻ Lyve1⁻ blood endothelial cells. This is most apparent directly adjacent to the CV (arrowheads, D). Scale bars: 50 μ m.

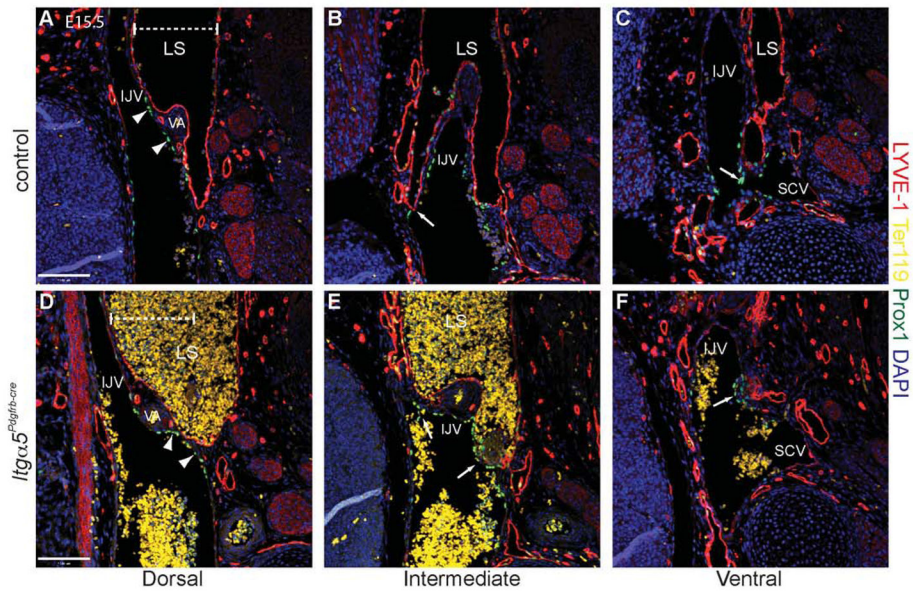


Figure 6. *Itg α5^{Pdgfrb-cre}* mice fail to form lymphovenous valves

Sequential coronal sections at the junction of the IJV and SCVs stained with LYVE-1 (red), Prox1 (green), and Ter119 (yellow), showing the morphology of the left lymphovenous valves in a control (A–C) and an *Itga5^{Pdgfrb-cre}* embryo (D–F) at E15.5. In control mice, the first valve leaflets open into the IJV (arrow, B), while the second valve drains into the SCV/IJV junction (arrow, C). Note the Prox1 expression in the venous endothelium adjacent to the LS (arrowheads, A), and the lack of red blood cells (yellow) within the LS in control mice (A–C). In contrast, no valve-like structures are observed in *Itga5^{Pdgfrb-cre}* mice (D–F) and, at the regions where the LS should connect at either the IJV (E) or the SCV (F), only Prox1⁺ rudiments are visible (arrows, E and F). Consequently, lymph fluid and sometimes blood (D and E), accumulates and leads to dilation of the lymphatic sac (D). Dashed line indicates the width of the LS in a control embryo (A) for comparison. Note, just as observed in control embryos, Prox1⁺ VECs are also clearly visible in mutant embryos (arrowheads, D). Scale bars: 50 μm.