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Segregated Foxc2, NFATc1 and Connexin expression at normal developing venous valves, and Connexin-specific differences in the valve phenotypes of Cx37, Cx43, and Cx47 knockout mice

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Abstract

Venous valves (VVs) are critical for unidirectional blood flow from superficial and deep veins towards the heart. Congenital valve aplasia or agenesis may, in some cases, be a direct cause of vascular disease, motivating an understanding of the molecular mechanisms underlying the development and maintenance of VVs. Three gap junction proteins (Connexins), Cx37, Cx43, and Cx47, are specifically expressed at VVs in a highly polarized fashion. VVs are absent from adult mice lacking Cx37; however it is not known if Cx37 is required for the initial formation of valves. In addition, the requirement of Cx43 and Cx47 for VV development has not been studied. Here, we provide a detailed description of Cx37, Cx43, and Cx47 expression during mouse vein development and show by gene knockout that each Cx is necessary for normal valve development. The valve phenotypes in the knockout lines exhibit Cx-specific differences, however, including whether peripheral or central VVs are affected by gene inactivation. In addition, we show that a *Cx47* null mutation impairs peripheral VV development but does not affect lymphatic valve formation, a finding of significance for understanding how some *CX47* mutations cause inherited lymphedema in humans. Finally, we demonstrate a striking segregation of Foxc2 and NFATc1 transcription factor expression between the downstream and upstream faces, respectively, of developing VV leaflets and show that this segregation is closely associated with the highly polarized expression of Cx37, Cx43, and Cx47. The partition of Foxc2 and NFATc1 expression at VV leaflets makes it unlikely that these factors directly cooperate during the leaflet elongation stage of VV development.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.02.033.

Keywords

Connexin; NFATc1; Foxc2; Valve development; Chronic venous disease; Lymphedema

1. Introduction

Venous valves (VVs), typically consisting of two cusps or leaflets extending into the lumen of a vein, are critical for unidirectional blood movement, act as flow modulators, and prevent sudden spikes in pressure in capillaries and venules during skeletal muscle contraction. The association of valvular incompetence with disorders including venous insufficiency, varicose veins, and venous hypertension highlights the importance of functional valves for vascular health (Eberhardt and Raffetto, 2014; Segiet et al., 2014). Incompetence of valves can be a sequela of venous disease, but primary valve failure or valve agenesis may in some cases be a direct cause of vascular disease. Mutations in the gene encoding the FOXC2 transcription factor, for example, cause lymphedema-distichiasis, a syndrome characterized by both lymphatic and venous dysfunction and most likely involves defective valve formation (Fang et al., 2000; Mellor et al., 2007; Ng et al., 2005).

VV leaflet formation requires the coordinated organization, growth, and migration of valve-forming cells from the endothelium as well as the formation of an organized extracellular matrix core. Kampmeier and La Fleur Birch, in 1927, provided the first histological description of the morphogenic events involved in VV formation (Kampmeier and La Fleur Birch, 1927). More recently, scanning electron microscopy, whole-mount immunostaining, and a β-gal reporter gene were used to visualize distinct stages of VV development (Bazigou et al., 2011). An early event is endothelial cell (EC) reorientation in which valve-forming cells align themselves perpendicular to the longitudinal axis of the vein. Ingrowth of these ECs forms a circumferential shelf and causes local luminal constriction. Although valve initiation occurs during embryogenesis, in peripheral veins valve leaflet elongation begins postnatally with the formation of the first commissure as the two leaflets come together at their insertion into the vessel wall, followed later by the formation of a second commissure.

At the molecular level, the development of VVs has only recently been studied. Several genes are highly expressed at emerging VVs, including *Prox1, Foxc2, Vegr3, Itga9, and Efnb2* (Bazigou et al., 2011; Norrmén et al., 2011). The induction of Efnb2 expression in venous ECs (VECs) is an early event in valve formation, and gene knockout studies have shown that Ephrin-B2 and integrin-α9 are both required for the development and maintenance of VVs (Bazigou et al., 2011). The transcription factor Prox1 is also important for valve formation, as a heterozygous mutation in the Prox1 gene results in the absence of VVs and lymphovenous valves (LVVs) (Srinivasan and Oliver, 2011). The genes highlighted above are also necessary for lymphatic vessel development and/or lymphatic valve (LV) formation, suggesting that there are common pathways controlling valve development in veins and lymphatic vessels (Bazigou et al., 2011, 2014).

Also highly concentrated at VVs and LVs are Connexin (Cx) proteins (Kanady and Simon, 2011; Kanady et al., 2011; Munger et al., 2013). Members of this family of structurally related proteins (21 Cxs in humans), typically form gap junction intercellular channels,

structures allowing for the direct cell-to-cell transfer of small molecules, including important signaling molecules (Goodenough and Paul, 2009). Gap junction channels exhibit selectivity and can be dynamically regulated in response to specific physiological stimuli (Ek-Vitorin and Burt, 2013; Solan and Lampe, 2014). In addition to their more commonly known role as mediators of direct intercellular communication, Cxs can also contribute to extracellular signaling by assembling undocked hemichannels that open transiently to act as release sites for signaling agents (Kar et al., 2012; Sáez and Leybaert, 2014; Stout et al., 2004). Cxs can also partner with other proteins and thereby contribute to diverse signaling pathways, in some cases independent of their channel function (Dbouk et al., 2009; Laird, 2010; Zhou and Jiang, 2014).

VV leaflets are comprised of two EC layers, one on the upstream side and one on the downstream side, separated by a connective tissue core. In the adult mouse, three Cxs (Cx37, Cx43, Cx47) are specifically expressed in the VV endothelium (Munger et al., 2013). Expression of Cxs at VV leaflets is highly polarized, similar to what is observed at LVs: Cx43 is present in the EC layer lining the upstream side of the leaflet whereas Cx37 is located on the downstream side. Venous Cx47 expression in the adult mouse is restricted to a small subset of cells in the VV, where it often localizes with Cx43. VVs are completely absent in peripheral veins from adult mice lacking Cx37, highlighting the importance of this family member for VVs (Munger et al., 2013). However, it is not presently known whether Cx37 is required for the initial formation of VVs or for the maintenance of valves once formed. In addition, the requirement of Cx43 and Cx47 for VV development has not yet been explored.

In this study, we provide a detailed description of Cx37, Cx43, and Cx47 expression during mouse vein development and use knockout mice to test whether each Cx is necessary for VV formation. In addition, LV development and lymphatic function is assessed in Cx47 null mice, to better understand the possible lymphatic effects of *CX47* mutations in human families with inherited lymphedema. We also document the surprisingly segregated expression of Foxc2 and NFATc1 transcription factors at developing venous valves, a segregation that is tightly associated with the polarized expression of Cxs, and we discuss how these findings relate to current models of Foxc2 and NFATc1 cooperation during valve development. Finally, we investigate the effect on VV formation of inactivating *Foxc2*, a likely regulator of Cx37 gene expression.

2. Material and methods

2.1. Mice

Cx37−/−(*Gja4*−/−) (Simon et al., 1997), *Cx43*+/− (*Gja1*+/−) (Reaume et al., 1995), *Cx47*−/− (*Gjc2*−/−) (Menichella et al., 2003) and *Foxc2*+/− (Iida et al., 1997) mice were described previously. *Cx47*−/− and *Cx43*+/− mice were interbred, as were *Cx37*−/− and *Foxc2*+/− mice, to generate doubly deficient mice. Mice were maintained on a C57BL/6 background and genotyped by PCR protocols for *Cx37*−/− (Kanady et al., 2011), *Cx43*+/− (Bobbie et al., 2010), *Cx47*−/− (Menichella et al., 2003) and *Foxc2*−/− (Kriederman et al., 2003) lines. The University of Arizona IACUC Committee approved all animal protocols.

2.2. Antibodies

Primary antibodies for immunostaining were: rabbit polyclonal antibodies to collagen IV (ColIV) (ab19808, Abcam), Cx32 (71-0600, Invitrogen), Cx37 (18264) (Simon et al., 2006), Cx40 (Gabriels and Paul, 1998), Cx43 (C6219, Sigma), Cx43 (71-0700, Invitrogen), Cx47 (364700, Invitrogen), laminin α5 (Lam α5) (405, a gift from Lydia Sorokin) (Sixt et al., 2001), laminin (Lam) (L9393, Sigma), Prox1 (11-002, AngioBio), Prox1 (ab11941, Abcam), von Willebrand Factor, vWF (A0082, Dako); mouse monoclonal antibody to NFATc1 (sc-7294, Santa Cruz), smooth muscle cell actin (SMA), Cy3 conjugated; rat monoclonal antibody to CD31 (HM1013, Hycult Biotech), LYVE-1 (14-0443, eBioscience); goat polyclonal antibodies to EphB4 (AF446, R&D Systems), Foxc2 (ab5060, Abcam), integrin α9 (ITA9) (AF3827, R&D Systems), VEGFR3 (AF743, R&D Systems); chicken polyclonal antibodies to laminin (ab14055, Abcam); Syrian hamster monoclonal antibody to podoplanin (127402, Biolegend). AffiniPure minimal cross reactivity secondary antibodies (conjugated to Alexa 488, Alex 555, Alexa 647, Cy3, Cy5, or Dylight 649) and Alexa 647 Streptavidin were from Jackson Immunoresearch or Invitrogen. Cx37 antibodies (18264) were directly labeled using the Alexa Fluor 555 Protein Labeling Kit (A20174, Invitrogen).

2.3. Section immunostaining

Veins were frozen unfixed in Tissue-tek OCT and sectioned at 10 μm. E16.5 embryos were frozen unfixed in OCT or fixed in 4% PFA, soaked in 30% sucrose and then frozen in OCT, before sectioning at 10–14 μm. Sections were fixed in acetone or methanol at −20 °C for 10 min, blocked in PBS containing 4% fish skin gelatin, 1% donkey serum, 0.25% Triton X-100 (or 0.1% Tween20), and incubated with primary antibodies for 1.5–3 h at room temperature or overnight at 4 °C. Sections were washed with PBS containing 0.25% Triton X-100 (or 0.1% Tween20) and then incubated with secondary antibodies for 30–40 min. When directly conjugated Alexa 555-Cx37 antibodies were used in conjunction with other rabbit primary antibodies, the unlabeled antibodies were incubated on the sections first, followed by anti-rabbit secondary antibodies and a 5% normal rabbit serum blocking step, before the Alexa 555-Cx37 antibodies were applied. For detection of NFATc1, a Mouse on Mouse detection system (BMK-2202, Vector Laboratories) was used to reduce non-specific staining and enhance signal. Nuclei were stained with a solution of 0.1 mg/ml DAPI for 3 min before the final wash. Sections were mounted either in Mowiol 40–88 (Aldrich) containing DABCO or in Prolong Gold (Life Technologies) and viewed with an Olympus BX51 microscope and Photometrics CoolSnap ES2 camera or with a Zeiss LSM 510 confocal microscope.

2.4. Whole-mount immunostaining

Mesentery was fixed in 1% PFA overnight at 4 °C, washed in PBS, permeabilized with PBS containing 0.3% Triton X-100, and then blocked overnight in PBS containing 3% donkey serum and 0.3% Triton X-100. Primary and secondary antibodies, diluted in PBS containing 0.3% Triton X-100, were sequentially applied to the tissue overnight at 4 °C. Mesenteries were mounted on slides in Citifluor mountant (Electron Microscopy Sciences). Ear tissue was treated similarly except fixation was for 1 h at room temperature.

2.5. In situ imaging of vein valves

Anesthetized mice (5–20 mice per genotype) were exsanguinated by clipping the right atrium. Residual blood in the veins provided contrast for visualizing the VVs. Several bilateral locations were examined for VVs: brachial vein (BV), proximal caudal femoral vein (PFV) and superficial caudal epigastric vein (SEV) (where the PFV and SEV enter the femoral vein), and proximal saphenous vein (PSV) (at the saphenofemoral junction). Hindlimb vein nomenclature was adapted from a study of mouse hindlimb arterial anatomy (Kochi et al., 2013).

2.6. Quantification of LVs

Mesenteries (3–7 mice per genotype) from E18.5 embryos, P4 pups, or P7 pups were cut into 3–4 segments and whole-mount co-immunostained in 24-well dishes for markers which highlight LVs: Prox1, CD31, Collagen IV (ColIV), laminin α5 (Lam α5), VEGFR3, Integrin α9 (ITA9), Cx37. The number of LVs per mesentery was determined by counting the valves in each segment. In some cases, the total number of immature valves present in the primary radial lymphatics was also quantified.

Ears from three adult mice per genotype were separated into dorsal and ventral halves and whole-mount co-immunostained for laminin α5 (a marker for LVs), LYVE-1 (expressed in initial lymphatics), and VEGFR3 (a marker for lymphatic ECs and LVs). LVs were counted in eight fields (10 × objective) spanning each ear sample and the average number of LVs per field was recorded.

2.7. Lymphangiography with Evans blue dye

5–10 mice per genotype were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg)/xylazine (20 mg/kg) and placed on a warming pad. Evans blue dye (EBD) (1% w/v) was injected intradermally into both hindpaws and a dissecting microscope was used to trace EBD transport into the iliac lymph nodes and efferent lymphatics (Kriederman et al., 2003). Hindlimb skin and mesenteric lymph nodes were examined for abnormal dye reflux. The thoracic cavity was opened and transport of EBD into and along the thoracic duct was verified. Intercostal lymphatic vessels adjacent to the thoracic duct were checked for dye reflux. EBD was also injected into the forepaws, snout, or ear to examine EBD transport in axillary, jugular, and ear lymphatics.

3. Results

3.1. Cx37, Cx43, and Cx47 are dynamically expressed in VECs during development

Cryosections of embryonic (E16.5) and postnatal mouse veins (P0, P4, P11, P21) were immunostained using Cx antibodies. Three Cxs (Cx37, Cx43, and Cx47) were detected in the ECs of developing veins, exhibiting distinct expression patterns. In contrast, Cx32 and Cx40 were not detected in the VECs. Previously, we showed that Cx expression in peripheral veins of adult mice is restricted to the valves (Munger et al., 2013). We now find that Cx expression in developing veins is initially much more widespread than in the adult and becomes progressively more restricted to VVs as postnatal development proceeds. In addition, throughout vein maturation Cx37, Cx43, and Cx47 are differentially expressed

both at VVs and in the non-valve VECs. Immunostaining results for central veins are presented first, followed by peripheral veins.

3.2. E16.5 central veins

Centrally located veins form valves earlier during development than peripheral veins, so we examined the cervical region during embryogenesis. Frontal orientation sections collected at E16.5 in the area where the jugular and subclavian veins empty into the superior vena cava (see Fig. 1A for a schematic diagram) reliably reveal VVs where these central veins converge and also show the nearby LVVs where portions of the jugular lymph sac connect with the abutting vein (Srinivasan and Oliver, 2011). Immunostaining revealed that Cx37 was present on the downstream face of the E16.5 VV leaflets, whereas Cx43 was on the upstream face (Fig. 1B, B′). Cx47 tended to be present at the base of VV leaflets or extend only partly up the leaflet on the upstream side (Fig. 1C, C′). At LVVs, Cx37 was also found mainly on the downstream side of the valve leaflets and Cx43 was again on the upstream side (Fig. 1D–E). Cx47 was not detected in the LVV endothelium per se but was present in the immediately adjacent venous endothelium (not shown). A schematic summarizing polarized Cx expression in E16. 5 central VVs and LVVs is shown in Fig. 1A.

Cx37 was expressed more highly in the luminal (non-valve) VECs of E16.5 central veins than in the nearby jugular lymph sac endothelium (Figs. 1B, C and 6I, J). In more dorsally located sections (adjacent to the valve region), Cx37 expression was particularly high in the VECs that were pressed tightly against the jugular lymph sac (Fig. 6I, J). In contrast, Cx43 levels were higher in the lymphatic endothelium than in the neighboring VECs (Figs. 1B, 6I). Cx43 and Cx47 were expressed more prominently in the VECs of smaller veins (i.e. external jugular vein and subclavian vein) than in the larger superior vena cava (Fig. 1B, C).

3.3. P0–P11 peripheral veins

Cx expression in peripheral veins was studied postnatally from P0–P11. At P0, when peripheral valve leaflets are not yet evident, Cx expression was generally widespread in the VECs, however there was already a pronounced segregation of Cx expression within different domains of the endothelium (Fig. 2). Cx47 expression was generally high and was typically found in different areas of the endothelium than Cx37 (Fig. 2). Cx43 expression was often patchy at P0 and was frequently separated from adjacent domains of Cx37 expression (Fig. 2). Some areas of the venous endothelium exhibited Cx43 but little or no Cx37. Overall, there was significant sample-to-sample variability in the Cx expression patterns at P0, perhaps indicating ongoing dynamic changes at this timepoint.

From P4–P11, further dynamic changes in Cx expression occurred in the venous endothelium (Figs. 3, 4, S1, S2). Surprisingly, given its highly restricted expression pattern in adult veins (i.e. a small subset of cells within valves), Cx47 was found to be a major early VEC Cx, with widespread expression in many veins. At P4, for example, Cx47 expression was high in the VECs, particularly in smaller veins of the hindlimb like the distal portion of the saphenous vein (DSV) (Fig. 3C) or the superficial caudal epigastric vein (SEV) (Fig. 3H, S1). From P7–P11, however, non-valve expression of Cx47 started to decline (Figs. 4, S2). This was also generally true for Cx37 and Cx43; they were more broadly expressed in the

VECs at early stages and then progressively became more restricted to valves (Figs. 2–4, S1, S2). Eventually, in adults, Cx expression in peripheral veins is found only at valves (Munger et al., 2013).

Depending on anatomical location, there were significant differences in Cx expression in non-valve VECs from P4–P11. Cx47 expression, for example, was much higher in the DSV (Fig. 3C) than in the PSV (Fig. 3E) or FV (Fig. 3G) and, in non-valve regions of the brachial vein (BV), Cx47 levels were very low (Figs. 3K, 4G). A similar dependence on anatomical location was observed for Cx37 and Cx43 (Figs. 3, 4).

Valves, at different stages of maturity, were detected in peripheral veins at P4 (Figs. 3, S1), and more readily at P7 and P11 (Figs. 4, S2). The percentage of veins in which valves were detected in serial cryosections increased with developmental stage. By P11, valves (most of them mature) were consistently present at specific locations in the vasculature (Table 1). The pattern of Cx expression at developing VVs during these postnatal stages already closely resembled the adult pattern, with Cx37 expressed in the VECs forming the downstream face of the valve leaflet, and Cx43 and Cx47 co-expressed in VECs on the upstream face of the valve leaflet (Figs. 3, 4, S1, S2). A schematic summary of Cx expression at peripheral VVs is presented in Fig. 3B. The separation of Cx immunosignals in the P4–P11 valve sections was particularly striking and more easily imaged than in the adult, likely because valve morphology changes considerably during postnatal development from thicker leaflets early on to thinner leaflets at later stages with less separation between endothelial layers in the adult. At P4 (Figs. 3, S1) and P7 (Fig. 4), Cx47 expression at valves was typically uniform throughout the upstream face of the leaflet, but by P7–P11 (Figs. 4, S2), Cx47 was in some samples already restricted to a subset of cells within the upstream leaflet face (Fig. 4B, B′, K, K′, P). At P4–P11, in hindlimb veins in which there was still some Cx expression in the VECs immediately adjacent to valves, Cx43 and Cx47 tended to be present in the VECs upstream of the valve, whereas Cx37 was often expressed in VECs downstream of the valve (Figs. 3, 4, S1, S2). In contrast, in the forelimb, the BV at P4–P11 exhibited less Cx expression in the VECs immediately adjacent to valves compared to hindlimb veins. Thus, Cx expression in the BV is restricted to valve leaflets earlier compared to hindlimb veins (Figs. 3K–N, 4G–H, O–P).

3.4. Cx40 and Cx37 in vascular smooth muscle cells (SMCs) of developing peripheral veins

Section immunostaining also revealed the surprising presence of Cx40 and Cx37, but not Cx43 or Cx47, in the SMCs of developing hindlimb postnatal veins (P4–P11) (Fig. S3). While Cx37 was expressed in both SMCs and VECs, Cx40 was detected only in the SMCs (Fig. S3H–J, S5). Cx37 and Cx40 colocalized to the same gap junction plaques within venous SMCs (Fig. S3H–J). Depending on anatomical location, there were pronounced differences in the frequency of SMCs expressing Cx40 and Cx37. For example, in the DSV (P4–P11), Cx40 was prominently expressed in a high percentage of the SMCs (Fig. S3A, E), but in the FV a much lower fraction of the SMCs expressed Cx40, and they were intermittently spaced amongst the Cx40-negative SMCs (Fig. S3B, F). Furthermore, little or no Cx40 was detected in the SMCs of the BV (Fig. S3C, G). In the SEV, SMC Cx40 expression varied along its length; Cx40 was intermittent in the region near to where the

SEV enters the FV but was much more frequent in its upstream portion (except near a valve) (Fig. S4). Cx40 expression in venous SMCs declined during postnatal development, beginning around P11, and by P21, Cx40 expression was mostly gone (Fig. S3K–N). In the adult, Cx40 and Cx37 are not detected in venous SMCs (Munger et al., 2013).

3.5. Segregated Foxc2 and NFATc1 expression at VVs correlates closely with polarized Cx expression

Several developmentally important transcription factors are enriched at valves, including Foxc2 and Prox1, which are expressed at VVs and LVs (Bazigou et al., 2011; Norrmén et al., 2009, 2011; Petrova et al., 2004). NFATc1 is a calcium/calcineurin regulated transcription factor detected at LVs (Sabine et al., 2012), but its expression at VVs has not been studied. To investigate the relationship between Foxc2, NFATc1, Prox1 and Cx expression specifically at VVs, different combinations of triple label immunostaining was performed on sections of developing VVs (P7 and P11) (Figs. 5, S5). We found a remarkable correspondence between the segregated expression of Cxs at VVs (on the upstream and downstream sides of valve leaflets) and the expression of Foxc2 and NFATc1, which were surprisingly also segregated on opposite sides of the valve leaflets. Thus, in developing VV leaflets, Foxc2 and NFATc1 were not coexpressed in the same ECs. Foxc2 expression was very tightly associated only with cells expressing Cx37 on the downstream side of the valve leaflet (Figs. 5A, F–H, L, S5), whereas NFATc1 (localized to the nucleus and thus in an activated state) was closely associated only with Cx43 and Cx47 expression on the upstream side of the leaflet (Figs. 5D, E, I, S5). In contrast, Prox1 was expressed by ECs on both sides of the valve leaflet (Fig. 5J–M). A schematic summary of Cx37, Cx43, Cx47, Foxc2, NFATc1, and Prox1 expression at peripheral VVs is shown in Fig. 5N. Finally, in P11 valve samples that already exhibited some spatial restriction of Cx47 expression (to a subset of valve ECs), the remaining Cx47-positive cells were still associated with NFATc1 expression, however not all NFATc1-positive cells were associated with high Cx47 expression.

3.6. Cx37 is required for the development of central and peripheral VVs

To test whether Cx37 is required for the initial development of VVs, we probed vein sections from *Cx37*−/− mice collected at various stages (E16.5–P11) (Fig. 6). At E16.5 and E17.5, triple labeling (Prox1, VEGFR3, CD31) was used to identify valves in WT and *Cx37*+/− sections where the jugular and subclavian veins enter into the superior vena cava (Fig. 6A–D). No VVs were detected in the central veins in *Cx37*−/− samples, although there were typically some Prox1-positive cells in the venous endothelium where valves would normally have developed (Fig. 6B, D). LVVs were also absent in the *Cx37*−/− E16.5 embryos (not shown), in agreement with the recent results of Geng et al. (Geng et al., 2016). To assess peripheral veins, serial sections from the hindlimb and forelimb were analyzed for VVs at P4, P7, and P11 (Fig. 6E, F and Table 1). A combination of Cx43, Prox1, and EphB4 (or laminin) labeling showed a complete absence of peripheral VVs at all timepoints (Fig. 6E, F and Table 1). There were no obvious changes in the expression of Cx43 or Cx47 in non-valve regions of *Cx37*−/− veins during postnatal development. These results indicate that the absence of VVs in adult *Cx37*−/− mice can be explained by a defect in valve development rather than a failure to maintain valves.

3.7. Cx43 is necessary for normal development of central VVs

Cx43−/− E16.5 embryos were similarly analyzed for central VVs by immunostaining sections for Prox1, Podoplanin (a lymphatic marker), and CD31. A variable VV phenotype emerged (Fig. 7). Out of 3 *Cx43*−/− embryos analyzed, one had no central VVs and the other two had abnormal valves or partially absent valves, with shorter or discontinuous valve leaflets (Fig. 7B, C, E, F) compared to WT controls (Fig. 7A, D). *Cx43*−/− mice are not viable postnatally because of heart defects (Reaume et al., 1995), so it was not possible to look at peripheral vein valve formation.

3.8. Cx47 is necessary for the development of most peripheral VVs but not for the development of central VVs

Sections of *Cx47*−/− E16.5 embryos were immunostained for Prox1 and vWF to probe for valves (Fig. 7H, H′, J). VVs were clearly present and looked normal, indicating that Cx47 is not required for the formation of central vein valves. The development of LVVs was also not affected by the absence of Cx47 (Fig. 7H, J). We next looked for the presence of valves in peripheral veins of adult *Cx47*−/− mice by in situ vein valve imaging (Fig. 8L and Table 2). By directly imaging exposed veins, VVs were consistently detected at several locations in WT mice including in the BV, PFV, SEV, and PSV. In contrast, *Cx47*−/− mice, but not *Cx47*+/− mice, almost always lacked valves in peripheral veins (Fig. 8L and Table 2). Interestingly, one peripheral VV, in the SEV, consistently still formed normally in the absence of Cx47. By comparison, *Cx37*−/− mice, but not *Cx37*+/− mice, lacked this SEV valve, along with all other peripheral VVs examined, without exception. Immunostaining of sections from *Cx47*−/− mice collected at P4, P7, P11, and P21 showed that peripheral VVs failed to initially form in the *Cx47* null pups (Fig. 8A–F and Table 1), again with the exception of the SEV (Fig. 8G–K and Table 1). At *Cx47*−/− SEV valves, the normal segregation of Cx37 and Cx43 expression on opposite sides of the valve leaflets occurred (Fig. Fig. 8G, I–K), but Cx47 staining was absent as expected (Fig. 8H). No change in Cx37 or Cx43 expression was noted in non-valve areas of postnatal veins from *Cx47*−/− mice, compared to controls.

3.9. Cx47 is not required for the formation of LVs

Cx47 is also expressed in LVs and, importantly, Cx47 mutations have been identified in some families with dominantly inherited lymphedema (Ferrell et al., 2010; Kanady et al., 2011). We therefore assessed lymphatic function in *Cx47*−/− mice using Evans blue dye lymphangiography and examined LVs by immunostaining (Figs. 9, S6). In all 10 *Cx47*−/− mice tested, dye transport through the lymphatic vessels looked normal (Fig. 9A–C). To assess LVs, adult ears were whole-mount triple immunostained for laminin α5, VEGFR3, and LYVE-1. *Cx47*−/− ears had similar numbers of LVs compared to WT controls and LV morphology was normal (Fig. 9D–F). LVs were also assessed in P4 and P7 whole-mount mesentery by immunostaining for several lymphatic vessel and LV markers (Figs. 9G–J, S6). Mesenteric LV numbers in *Cx47*−/− mice were similar to WT controls, and valve maturity was also not different from WT (Figs. 9K, S6). SMC actin staining showed normal SMC coverage of the lymphatics (Figs. 9J, S6), and in adult *Cx47*−/− mesentery sections, LVs exhibited conventional morphology (not shown). Thus, a null mutation in *Cx47* in mice does

not affect LV formation and, at least to the level of resolution of the EBD assay, lymphatic function is not significantly affected by the absence of Cx47.

3.10. Cx47−/−Cx43+/− and Cx47−/−Cx43−/− mice

Cx47 and Cx43 are coexpressed at valves. To address the possibility that they functionally overlap, we interbred the two knockout lines. *Cx47*−/−*Cx43*−/− E16.5 embryos were found to lack both central VVs and LVVs (Fig. 7I, I′). Thus, mice deficient in both Cx47 and Cx43 have a more severe VV phenotype in central veins at E16.5 compared to *Cx43*−/− embryos. *Cx47*−/−*Cx43*−/− mice were not viable beyond birth so peripheral VVs could not be examined; however *Cx47*−/−*Cx43*+/− littermates were viable, and in those mice peripheral VVs were absent, again with the exception of the SEV valve (Fig. 8L and Table 2).

Regarding lymphatic vessels, we hypothesized that *Cx47*−/−*Cx43*+/− mice might mimic the situation of a dominant negative mutation in Cx47. In families with dominantly inherited lymphedema caused by mutations in Cx47, it has been suggested that dominant negative mutations in Cx47 may trigger the disease (Ferrell et al., 2010). Thus, we wanted to determine if *Cx47*−/−*Cx43*+/− mice, with no Cx47 present and presumably reduced Cx43 levels, would exhibit lymphatic defects. At E18.5, mesenteries collected from *Cx47*−/−*Cx43*+/− embryos showed normal numbers of LVs compared to *Cx47*−/− littermates, however there was an increase in immature LVs (Fig. 10D). However, by P4, mesenteric LVs in *Cx47*−/−*Cx43*+/− mice now appeared fully mature and there was still no difference in the total number of valves (Fig. 10E–G). Thus, although there was an initial delay in LV maturation, this delay resolved by P4. Moreover, adult *Cx47*−/−*Cx43*+/− mice had normal lymphatic function as determined by the EBD assay ($N = 5$), showed no signs of lymphedema, and displayed no effusions. In contrast, *Cx47*−/−*Cx43*−/− embryos collected at E18.5 completely lacked mesenteric LVs (Fig. 10C, D). This was expected, however, since we have previously shown that *Cx43*−/− embryos at this stage also lack mesenteric LVs (Kanady et al., 2011). Significantly, out of nine *Cx47*−/−*Cx43*−/− embryos collected at E18.5, four embryos showed signs of mild edema in the neck region (Fig. 10I), and some of these also exhibited blood within mesenteric lymphatics (Fig. 10K). Thus, although adult *Cx47*−/−*Cx43*+/− mice did not exhibit lymphedema, the phenotype of *Cx47*−/−*Cx43*−/− embryos does provide support for the idea that a combined deficiency in Cx47 and Cx43 can produce symptoms of lymphedema.

3.11. VV defects in Foxc2−/−, Foxc2+/−, and Foxc2+/−Cx37+/− mice

The close association between Foxc2 and Cx37 expression at VVs suggested that Foxc2 might induce the expression of Cx37 at valves. We therefore examined *Foxc2*−/− embryos for the presence of VVs in E16.5 central veins (Fig. 6H). Like *Cx37*−/− embryos, *Foxc2*−/− embryos at this stage lacked both VVs (Fig. 6H) and LVVs (not shown), consistent with the results of a recent study (Geng et al., 2016). Out of the three *Foxc2*−/− embryos examined, two had substantial edema and the third had very mild edema. Significantly, E16.5 *Foxc2*−/− embryos showed greatly reduced Cx37 expression, compared to controls, in the VECs adjacent to where VVs would normally be found (Fig. 6I–L).

Heterozygous (*Foxc2*+/−) embryos exhibited a less severe and more variable valve phenotype than *Foxc2*−/− embryos. Out of three *Foxc2*+/− E16.5 embryos examined, one lacked valves in the central veins, but two others had VVs in which the leaflets looked normal in some places (Fig. 6G) or appeared only slightly shorter than normal. Adult *Foxc2*+/− mice also showed a statistically significant increase in the occurrence of a common venous trunk formed by the merging of the SEV and PFV before they enter the FV (50% in *Foxc2*+/− mice versus 20% in WT), consistent with a role for Foxc2 in vascular patterning (Fig. S7) (Kanzaki-Kato et al., 2005; Xue et al., 2008).

Lastly, we examined doubly heterozygous *Foxc2*+/−*Cx37*+/− mice for evidence of a genetic interaction. Adult *Foxc2*+/−*Cx37*+/− mice exhibited a statistically significant 21% reduction in BV valve presence compared to WT controls (Table 2). This decrease was also significantly different from either *Cx37*+/− or *Foxc2*+/− mice, where VV numbers were similar to WT controls (Table 2). These results suggest a synergistic effect on BV valve formation when heterozygous mutations of Foxc2 and Cx37 are combined. However, the effect was not observed in other veins, indicating that the BV is more sensitive to the precise levels of these two proteins.

4. Discussion

4.1. Cx-specific differences in valve phenotypes

When Cx37, Cx43, and Cx47 are individually ablated, there are Cx-specific differences in the valve phenotype, indicating that their roles during valve development are not identical. The deficiencies vary in penetrance, severity, and whether central or peripheral valves are affected. The effect of Cx37 ablation is the most severe: with 100% penetrance, valves of both central and peripheral veins fail to form. In contrast, Cx47 is required for the development of peripheral VVs (with the exception of the SEV valve), but is not required for the formation of central VVs. Inactivation of Cx43 produces a central VV defect that is variable in severity. There are also differences in the requirement of Cxs for the development of VVs versus LVs. In the absence of Cx37, no VVs develop, yet some LVs are present, although they are considerably reduced in number (Kanady et al., 2011). On the other hand, Cx47 is required for most peripheral VVs to form, but is not required for LV development. The specific valve defect arising from Cx inactivation may depend on a number of factors, including where the Cx is expressed (e.g. upstream versus downstream), the timing of Cx expression, whether or not the Cx is coexpressed with another Cx family member, and Cx-specific differences in channel selectivity or regulation. Cx-specific interactions with other cellular proteins may also play a role.

4.2. Superficial caudal epigastric vein: an exception to the rule

The persistence of the SEV valve where it enters the FV in *Cx47*−/− mice was a consistent exception to the general requirement of Cx47 for the development of peripheral VVs. At the postnatal stages examined, Cx expression in the SEV looked qualitatively similar to other veins, however, it may be necessary to look at earlier time-points in more detail for subtle differences in the time-course of valve development or gene expression. Interestingly, unlike the other veins examined, the SEV is not embedded within skeletal muscle. It runs loosely

through the inguinal fat tissue and therefore it is likely to experience very different hemodynamic conditions compared to veins that are continuously exposed to the effects of skeletal muscle contraction. These factors could also potentially explain why Cx47 is required for VV formation in peripheral veins but not central veins.

4.3. Dynamic changes in Cx expression in postnatal veins

Cx37, Cx43, and Cx47 expression in veins is initially much more widespread throughout the luminal endothelium and only later becomes highly restricted to valves. Cx47, for example, is surprisingly widespread in the ECs of the early postnatal DSV, but in the adult, it is highly restricted to a small subset of valve ECs. A similar dynamic change in Cx expression occurs during the development of lymphatic collecting vessels. One possible role for widespread early expression is that Cxs could function to locally communicate second messengers at sites where an initial signal for valve formation is received or generated by the endothelium (Kumai et al., 2000). Thus, early prevalent Cx expression may provide initial flexibility, rather than a predetermined program, for the initiation of valve formation sites. Interestingly, the postnatal down-regulation of non-valve VEC Cx expression is not irreversible, as surgical wounding of the skin overlying adult superficial veins strongly induces transient Cx43 expression (Munger et al., 2013).

4.4. Differential expression of Cxs in postnatal veins

Differential expression of Cxs in peripheral veins is an early feature of their postnatal maturation. Segregated domains of Cx expression were observed at P0, even before valve leaflets are present. Similarly distinct domains of Cx expression have been observed in the developing jugular lymph sacs during embryogenesis (Kanady et al., 2011). Cx expression domains in the venous endothelium could be related to variations in shear stress along the maturing veins or correlate with potential valve initiation sites. Cx compartments could also help establish valve polarity. Postnatally, the highly polarized expression of Cxs at valve leaflets already closely mirrors what is observed in the adult, except that Cx47 is initially more uniformly expressed throughout the upstream face of valve leaflets. Moreover, the segregation of Cx expression in developing VVs between the upstream face of leaflets (Cx43, Cx47) and the downstream face (Cx37) is the same as in LVs (Kanady et al., 2011). The functional significance of segregated Cx expression at valves is not known but likely results from unequal mechanical stress experienced by the two faces of the valve, which are from an early stage exposed to distinct flow conditions and shear stress. Expression of Cx37 and Cx43 in the cardiovascular system is differentially regulated by mechanical forces, such as high laminar, low laminar, and oscillatory shear stress (Meens et al., 2013). Interestingly, however, the signaling pathways controlling Cx expression at LVs versus arterial endothelium depend on different shear stress patterns and involve distinct transcription factors (Meens et al., 2013). A comparison of Cx expression at VVs, LVs, LVVs, and cardiac valves is presented in Table S1.

4.5. Transient Cx expression in venous SMCs

Transient Cx40 expression in venous SMCs of postnatal veins was surprising given the exclusive EC location of Cx40 in arteries and veins of the adult mouse (Munger et al., 2013; Simon and McWhorter, 2003). Cx40 has been detected at some level in the SMCs of certain

arteries and arterioles in other species (Li and Simard, 1999; Little et al., 1995; van Kempen and Jongsma, 1999). Moreover, A7r5 cells, which are derived from embryonic rat aorta smooth muscle, are known to express high levels of Cx40 (Beyer et al., 1992; Kimes and Brandt, 1976), suggesting that the Cx profile in the embryonic and early postnatal vasculature may differ from the adult. Expression of Cx40 and Cx37 in venous SMCs was largely gone by P21. What Cx, if any, replaces Cx40 and Cx37 in the venous SMCs beyond P21 is unclear at present. A mouse model using an EGFP reporter gene showed Cx45 expression in venular SMCs, however activity was not readily detected in the SMCs of larger veins (Schmidt et al., 2012).

4.6. Cx expression in developing veins varies with anatomical location

Depending on vein location and size, we found considerable differences in Cx expression during postnatal vein development. Between the BV and the DSV, for example, Cx expression in the non-valve VECs differed greatly. It has become increasingly clear that there is considerable EC heterogeneity, not only between arteries and veins, but also amongst different arteries (Aird, 2012; Atkins et al., 2011; Chi et al., 2003; Simmons et al., 2012). Our results suggest that EC heterogeneity also extends to veins, at least during the period of postnatal maturation. VV formation, however, seems to be a more conserved element, since Cx expression at the developing VVs was similar in different anatomical locations.

4.7. Foxc2 and NFATc1 during VV development

Common signaling pathways and morphogenetic processes are thought to control valve formation in veins and lymphatic vessels (Bazigou et al., 2011; Munger et al., 2013). Our finding that Foxc2 and NFATc1 are separated on opposite sides of VV leaflets is therefore surprising, since it has been put forward that Foxc2 regulates the formation and maturation of lymphatic collecting vessels, including the formation of LVs, by directly cooperating with NFATc1 (Norrmén et al., 2009; Sabine et al., 2012). Several approaches, including reporter gene analysis with transfected cells, have provided evidence that cooperation between Foxc2 and NFATc1 can occur (Norrmén et al., 2009), however it is difficult to determine if direct cooperation between these transcription factors actually happens at developing valves. During the initiation phase of LV development, it was proposed that the valve forming cells co-express high levels of Foxc2 and activated NFATc1, in addition to Prox1 and Cx37 (Sabine et al., 2012). However, direct evidence for co-expression of Foxc2 and NFATc1 specifically in the initial valve-forming cells is lacking. Similarly, at later stages, co-localization of Foxc2 and NFATc1 within LV leaflet ECs has not been reported. Our data indicate that, in developing VVs, Foxc2 and NFATc1 are in fact on opposite sides of the valve leaflets. Thus, during VV leaflet elongation and maintenance, Foxc2 and NFATc1 are not likely to directly cooperate. These results suggest either that 1) cooperation between Foxc2 and NFATc1 happens only at an earlier stage of valve formation (i.e. initiation phase), before valve leaflet elongation occurs, or 2) that cooperation occurs during LV development but not during VV development. If the latter is true, it represents a significant difference in the signaling pathways operating during LV and VV development.

4.8. Cx37 and NFATc1 during valve development

The signals that initiate valve formation at specific sites in vessels are not well understood, but both extrinsic factors, such as fluid shear stress, and intrinsic genetic programs are likely to be involved (Kazenwadel et al., 2015; Sabine and Petrova, 2014; Sweet et al., 2015). Sabine et al. have proposed a mechanotransduction model for LV development in which Cx37 acts downstream of Prox1 and Foxc2 to allow a coordinated field of calcineurin/NFATc1 signaling, which must occur for valves to form (Sabine et al., 2012). A recent analysis of *Foxc2*+/−*Cx37*−/− mice showed that lymphatic network architecture and valve formation rely on the concurrent embryonic expression of Foxc2 and Cx37 (Kanady et al., 2015). Moreover, in a cultured lymphatic EC model, oscillatory fluid shear stress induced Cx37 expression and nuclear translocation of NFATc1 in a process requiring Foxc2 and Prox1. Extrapolating these and other data to LV-forming cells in vivo, it was proposed that Cx37 is required to locally synchronize Ca2+/calcineurin activity necessary for NFATc1 activation in the valve-forming cells (Sabine et al., 2012). Furthermore, it was anticipated that LV leaflets would coexpress Cx37 and NFATc1 on the downstream face (Sabine et al., 2012). In the case of developing VV leaflets, however, Cx37 and NFATc1 are surprisingly not expressed in the same valve leaflet ECs. Instead, Foxc2 is tightly correlated with Cx37 on the downstream side of VV leaflets whereas NFATc1 is closely associated with Cx43 and Cx47 on the upstream side. Thus, at the VV leaflet stage, Cx37 expression does not appear likely to be required for the activation of NFATc1. However, it remains possible that Cx37 is necessary earlier, during valve initiation, for coordinated activation of NFATc1 in the original valve-forming cells. It will be important to determine definitively if Cx37 and NFATc1 are coexpressed in the same valve-forming cells during the initiation stage in vivo, something that has been proposed for LVs but not directly demonstrated.

4.9. Foxc2 and Cx37 expression at valves

Cx37 expression was very tightly associated with Foxc2 expression on the downstream face of VV leaflets. In addition, *Foxc2*−/− embryos exhibited greatly reduced Cx37 expression in areas that show high Cx37 expression in WT embryos. Previous studies showed that *Foxc2*−/− embryos exhibit a sharp drop in Cx37 expression in the lymphatic endothelium of the jugular lymph sac and mesenteric lymphatics (Kanady et al., 2011; Sabine et al., 2012). In lymphatic EC cultures, oscillating shear stress-dependent induction of Cx37 expression required the presence of Foxc2 (Sabine et al., 2012). Moreover, it was recently demonstrated that conditional ablation of Cdk5, an activator of Foxc2 activity, greatly decreased Cx37 expression in lymphatic vessels (Liebl et al., 2015), and that knockdown of GATA2, which is upstream of Foxc2, blocked the flow-induced expression of Cx37 (Sweet et al., 2015). Furthermore, in the *Foxc2*+/−*Cx37*+/− mice generated in this study, there was significant inhibition of BV valve formation, consistent with a genetic interaction between *Foxc2* and *Cx37*. *Foxc2*+/−*Cx37*+/− embryos also show a reduction in the proportion of mature LVs during development (Sabine et al., 2012). Finally, *Foxc2*−/− and *Cx37*−/− mice have similar valve phenotypes. Taken together, these data strongly suggest that the Cx37 gene (Gja4) is a downstream target, possibly a direct target, of regulation by Foxc2.

4.10. NFATc1, Cx43 and Cx47 at valves

NFATc1 expression and activation was closely associated with Cx43 and Cx47 expression in developing VVs, raising the possibility that NFATc1 is an upstream factor regulating Cx expression at valves. Cx47 eventually is restricted to a subset of valve leaflet cells, however, and not all NFATc1-positive cells are associated with Cx47 expression. In contrast, the tight association between Cx43 expression and activated NFATc1 remained constant. NFATc1 is part of the NFAT family, whose members belong to the extended NF-κB/Rel family, and in some cases they can bind to NF-κB-like sites (Hogan et al., 2003). Interestingly, NF-κB was shown to bind to a site in the Cx43 gene promoter during AngII induction of Cx43 expression (Alonso et al., 2010). Thus, the NF-κB binding site present in the Cx43 upstream regulatory region may be a candidate site for NFATc1 binding during VV development.

4.11. Cx47 mutations and dominantly inherited lymphedema

Missense mutations in Cx47 have been found to cause dominantly inherited lymphedema in humans (Ferrell et al., 2010), but the role of Cx47 in lymphatic development and function has not been studied. The *CX47* mutations identified in affected families were suggested to be dominant negative; however this remains to be established (Ferrell et al., 2010). Our analysis of *Cx47* null mice revealed that Cx47 is required for the development of most peripheral VVs but is not required for LV development or lymphatic function. In humans, loss-of-function *CX47* mutations are associated with Pelizaeus-Merzbacher-like disease 1, a hypomyelinating disease resulting from loss of Cx47 from oligodendrocytes; however, lymphedema has not been reported in these patients (Henneke et al., 2008; Orthmann-Murphy et al., 2007). We propose that a lymphatic phenotype in families with dominant *CX47* mutations results from the combined inhibition of Cx47 and Cx43, perhaps because these Cxs may form heteromeric channels, and that the inhibition of Cx43 in particular might be a critical feature. In support of this model, Cx47 and Cx43 colocalize at lymphatic and VVs during development (Kanady et al., 2011; and this study). Moreover, *Cx43*−/− embryos fail to initially form LVs (Kanady et al., 2011). Thus, a mutation in Cx47 that dominantly inhibits Cx43 expression or function would be expected to impair LV formation. The absence of a lymphatic phenotype in *Cx47*−/−*Cx43*+/− mice besides an initial transient delay in valve maturation, however, suggests that dominant negative mutations in Cx47 may need to be quite strongly inhibitory towards Cx43 to manifest lymphatic disease symptoms. Nevertheless, some of the double null (*Cx47*−/−*Cx43*−/−) embryos in this study exhibited signs of edema and lacked both LVs and VVs, providing some support for the notion that a combined Cx deficiency may contribute to the development of lymphedema.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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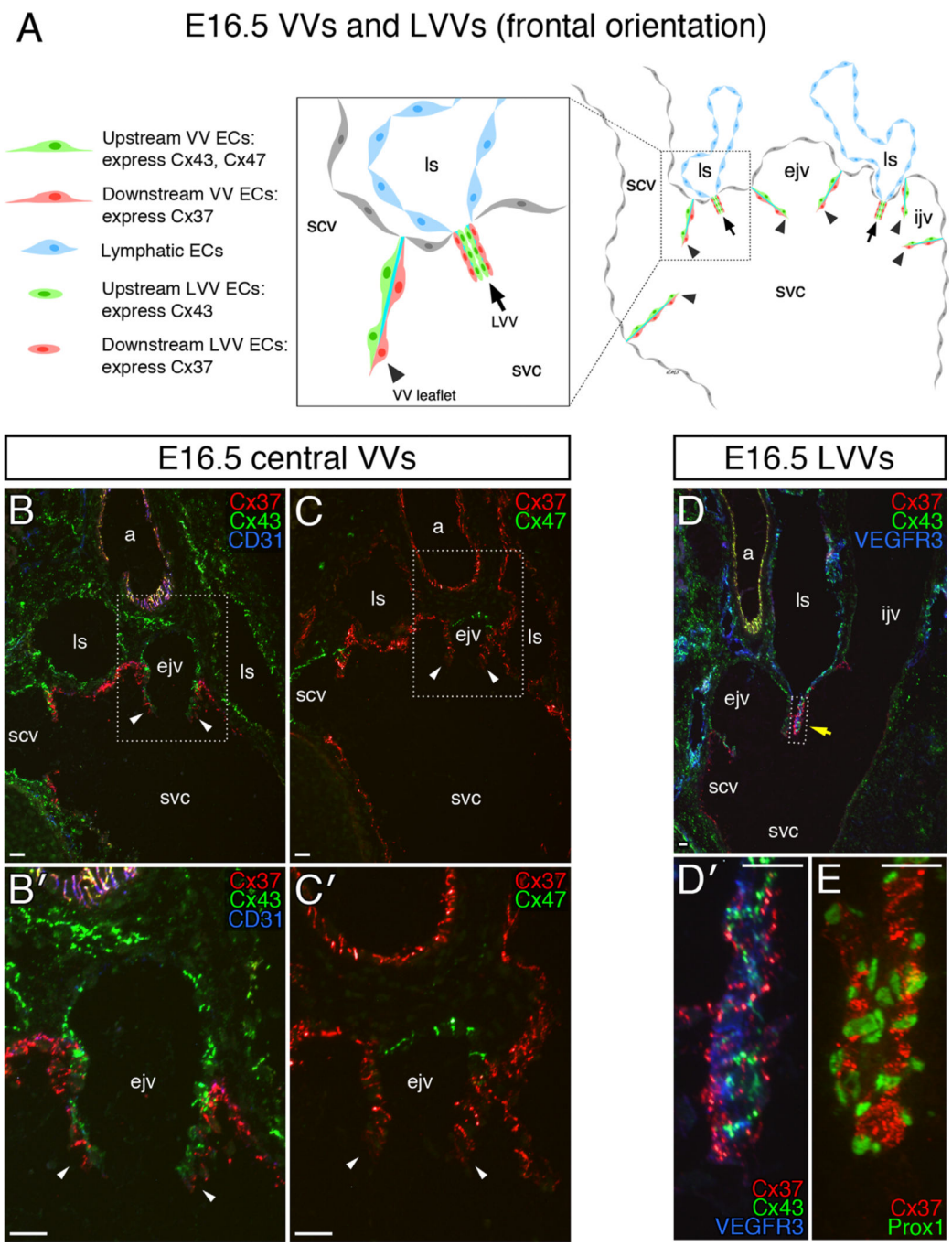

Fig. 1.

Cx expression at central VVs and LVVs in E16.5 embryos. (A) Schematic depicting the arrangement of central veins, lymph sacs, VVs, and LVVs in frontal orientation. Polarized Cx expression in the VVs and LVVs is summarized and color-coded. (B)–(C′) Cx immunostaining of frontal orientation sections in the region where the EJV and SCV enter the SVC. Cx37 was detected on the downstream face of the VV leaflets (arrowheads) whereas Cx43 was on the upstream side. Cx47 was typically at the base of VV leaflets or only partly up the leaflets on the upstream side. Boxed regions in (B), (C), and (D) are

shown at higher magnification in (B′), (C′), and (D′), respectively. (D)–(E) show the lymph sac entering into the adjacent vein and the presence of a LVV (arrow). Note that at the tip of the LVV, shown at higher magnification in (D′) and (E), the two LVV leaflets are pressed together, bringing in close proximity four closely spaced EC layers. Cx37 was predominately located on the downstream side of the LVV leaflets and Cx43 was on the upstream side. VEGFR3 staining in this and subsequent figures is a marker for the lymphatic endothelium. (E) Prox1 was expressed at LVVs (and VVs, not shown), as expected. Nearby sections are shown in (B) and (C); (B′) and (C′); and (D′) and (E). Flow is from top to bottom. a, artery; ejv, external jugular vein; internal jugular vein; ls, lymph sac; scv, subclavian vein; svc, superior vena cava; Scale bars: 20 μm.

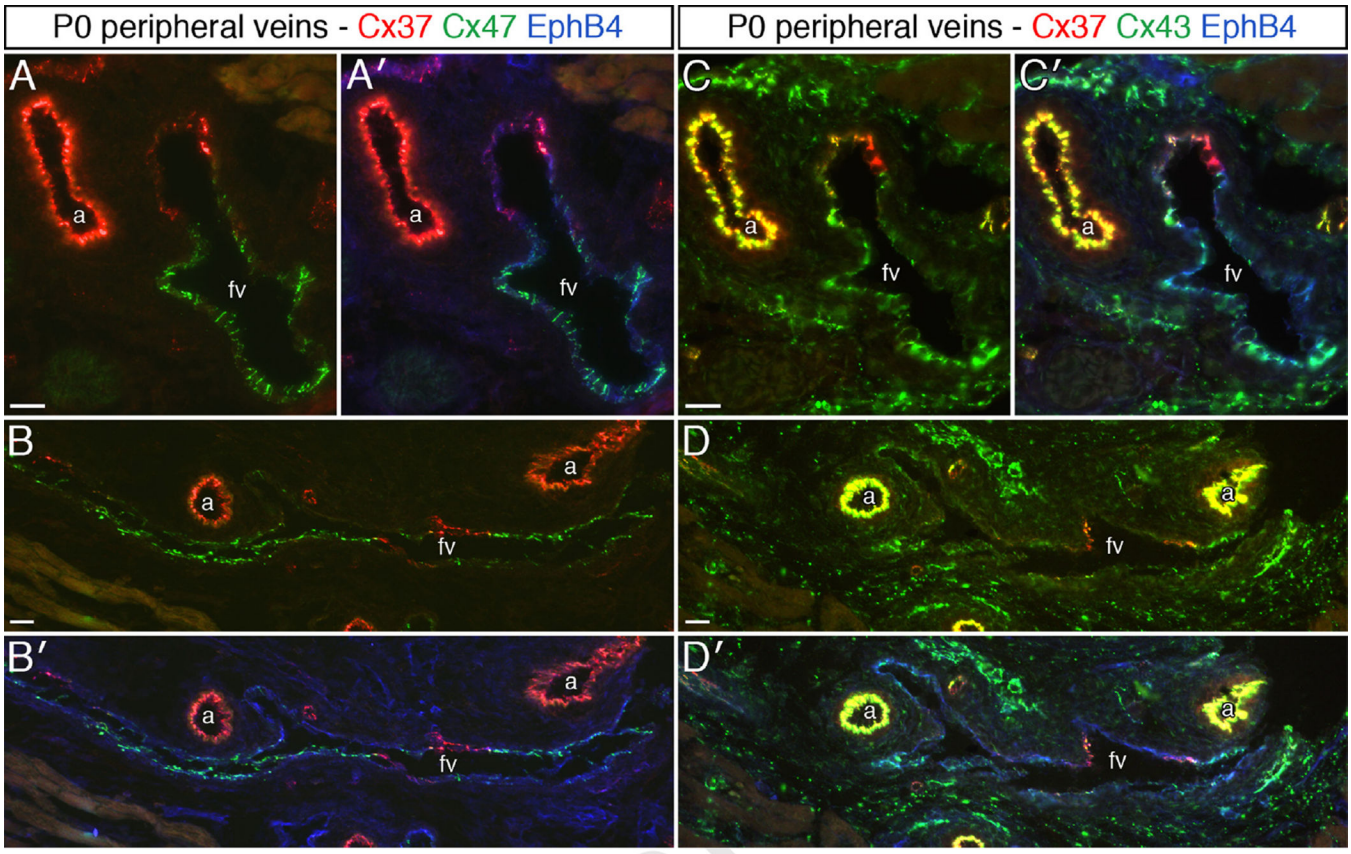

Fig. 2.

Widespread and segregated Cx expression in peripheral mouse veins at P0. Shown is Cx37, Cx43, and Cx47 immunostaining of sections from a P0 femoral vein (FV). Cx expression was widespread in the VECs, but there was often segregation of Cx expression into discreet domains. Valve leaflets were not yet present in peripheral veins at P0. EphB4 immunostaining in this and subsequent figures aids in the identification of veins. (A)–(B′) show Cx37 and Cx47 double labeling, and (C)-(D′) show Cx37 and Cx43 double labeling. (A′), (B′), (C′), (D′) include the EphB4 signal added to the Cx signals in (A), (B), (C), (D), respectively. (A) and (C) are nearby sections, as are (B) and (D). The FV was sectioned longitudinally in (B) and (D). Note that the EC signal in arteries obtained with the polyclonal Cx43 antibody (C6219, Sigma) in (C), (D), (G), (H) and in subsequent figures is due to cross-reactivity with abundant Cx40 in the arterial ECs. a, artery; fv, femoral vein. Scale bars: 20 μm.

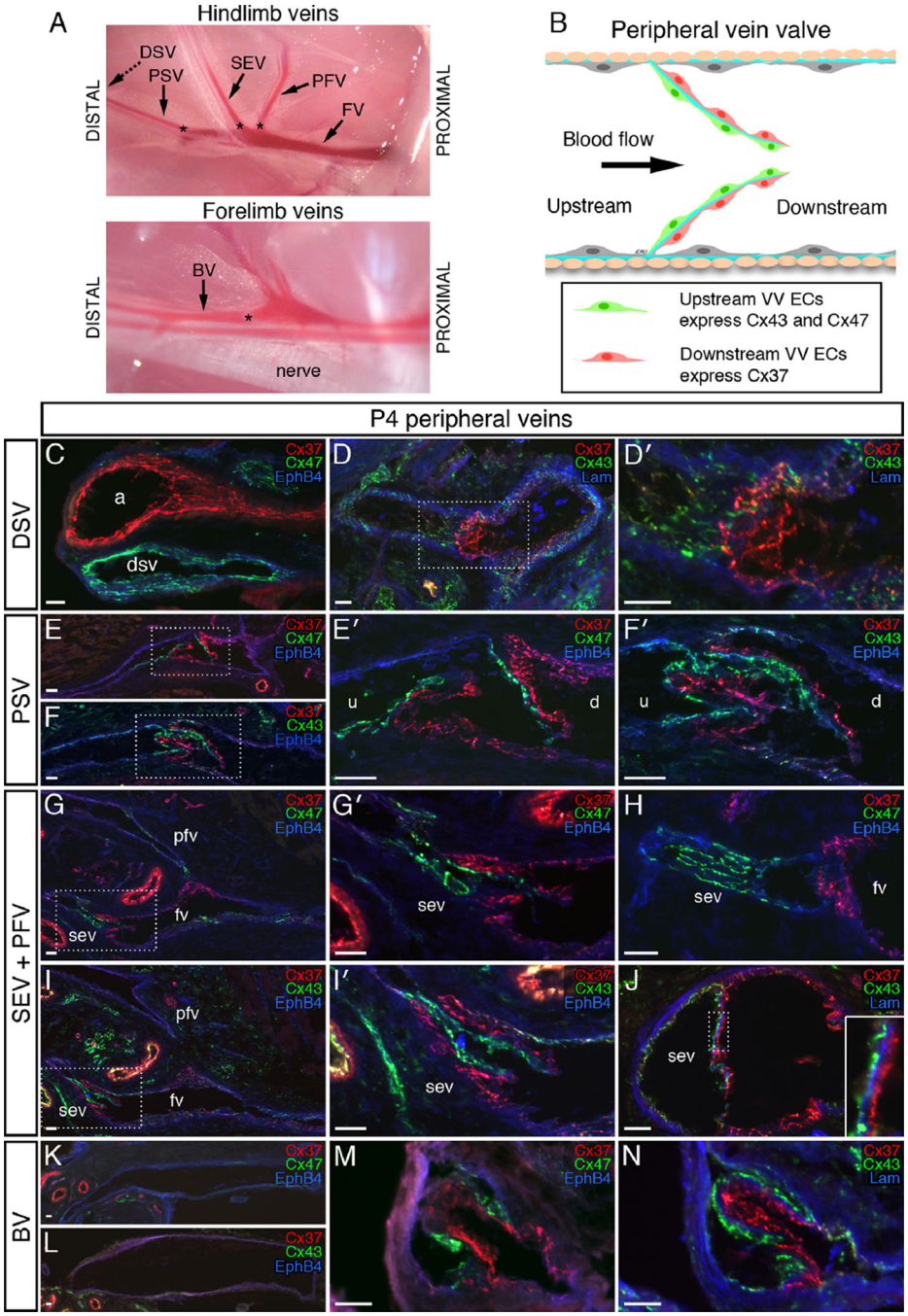

Fig. 3.

Cx37, Cx43, and Cx47 expression in peripheral mouse veins at P4, during VV formation. (A) Shown is the ventral aspect of an adult WT mouse hindlimb and forelimb, illustrating peripheral veins used in the study. Vein abbreviations are as follows: femoral vein (FV), proximal caudal femoral vein (PFV), superficial caudal epigastric vein (SEV), proximal saphenous vein (PSV), distal saphenous vein (DSV), brachial vein (BV). Note the DSV is located out of the field of view in (A), below the knee. VVs were typically located at the positions marked by asterisks. (B) Schematic depicting a peripheral VV, summarizing the

polarized Cx expression at VV leaflets. At P4, widespread Cx47 expression was observed in the ECs of some veins, like the DSV (C) and SEV (H). Cx37 and Cx43 were also more broadly expressed in the VECs of many veins at P4 compared with later postnatal stages; see (G) and (I) for example. VVs in different phases of development were detected, from early stage (D), (D′) to nearly mature (I), (I′). The pattern of Cx expression at valves already resembled what it will look like in the adult: Cx37 was found in the ECs forming the downstream face of the valve leaflet, whereas Cx43 and Cx47 were co-expressed in ECs on the upstream face. In the longitudinal sections shown, blood flow is from left to right. In the BV, Cx expression was very low in non-valve regions of the vein (K), (L), and Cx immunosignals were quite restricted to the valve (M), (N). Laminin (Lam) staining in (D), (D′), (J), and (N) outlines veins and highlights the extracellular matrix separating the Cx immunosignals on opposite sides of the valve leaflets (J) and (N). Nearby sections are shown in (E) and (F); (G) and (I); (M) and (N). Boxed regions in (D), (E), (F), (G), (I) are shown at higher magnification in (D′), (E′), (F′), (G′), (I′), respectively. The inset panel in (J) shows a higher magnification view of a portion of the valve leaflet (boxed region), revealing the clear separation of Cx37 and Cx43 signals, with laminin staining in between. a, artery; d, downstream; dsv, distal saphenous vein; fv, femoral vein; pfv, proximal caudal vein; sev, superficial caudal epigastric vein; u, upstream. Scale bars: 20 μm.

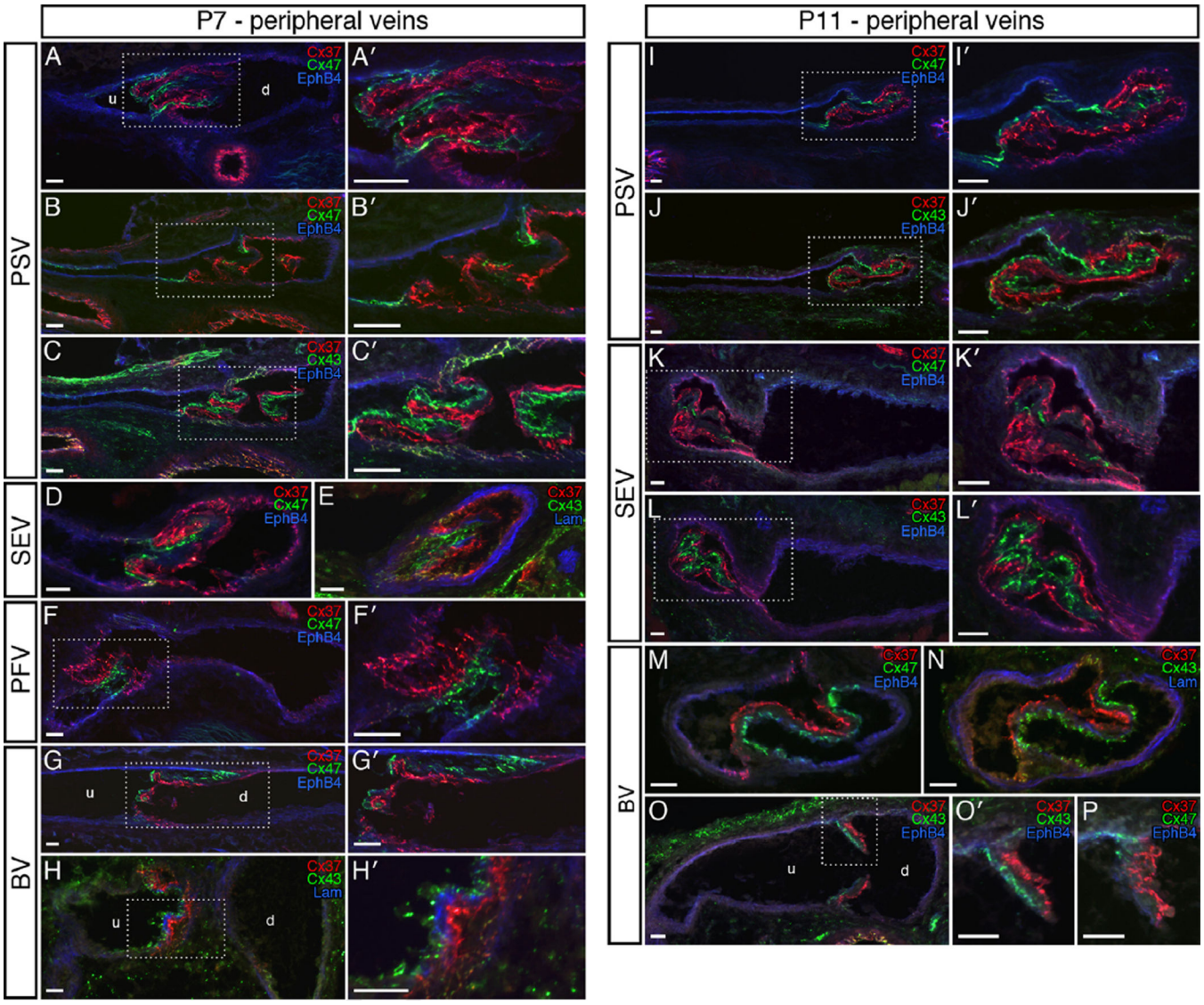

Fig. 4.

Cx expression in peripheral mouse veins and VVs at P7 and P11. Sections of PSV, SEV, PFV, BV were immunostained for Cx37, Cx43, and Cx47. At P7, there was less non-valve Cx expression than at earlier stages, although some expression outside of VVs persisted; see (B) and (F) for example. The BV in particular showed very little non-valve Cx expression (G). VVs were more frequently found at P7 than at P4 and more of the VVs were mature, although some immature VVs were still present, (F) for example. Cx37 was again found in the ECs on the downstream (d) face of the valve leaflet, whereas Cx43 and Cx47 were on the upstream (u) face. In longitudinal sections, blood flow is from left to right. Note that the morphology of some VVs appears complex because of the way the leaflets fold within the small luminal area. Cx47 expression at VVs was generally uniform throughout the upstream leaflet face (A), (D), (G), however in some cases it was restricted to a subset of cells on the upstream face (B), (B′). By P11, mature VVs were more prevalent. Cx37, Cx43, and Cx47 expression was further restricted to VVs and was highly polarized on the upstream and

downstream faces of valve leaflets. Cx47 expression was more frequently restricted to a subset of cells on the upstream face of VV leaflets, (K) and (P) for example. Nearby sections are shown in (B) and (C); (D) and (E); (I) and (J); (K) and (L); (M) and (N); (O′) and (P). Boxed regions in (A)–(C); (F)–(L) and (O) are shown at higher magnification in (A′)–(C′); (F′)–(L′) and (O′), respectively. d, downstream; u, upstream. Scale bars: 20 μm.

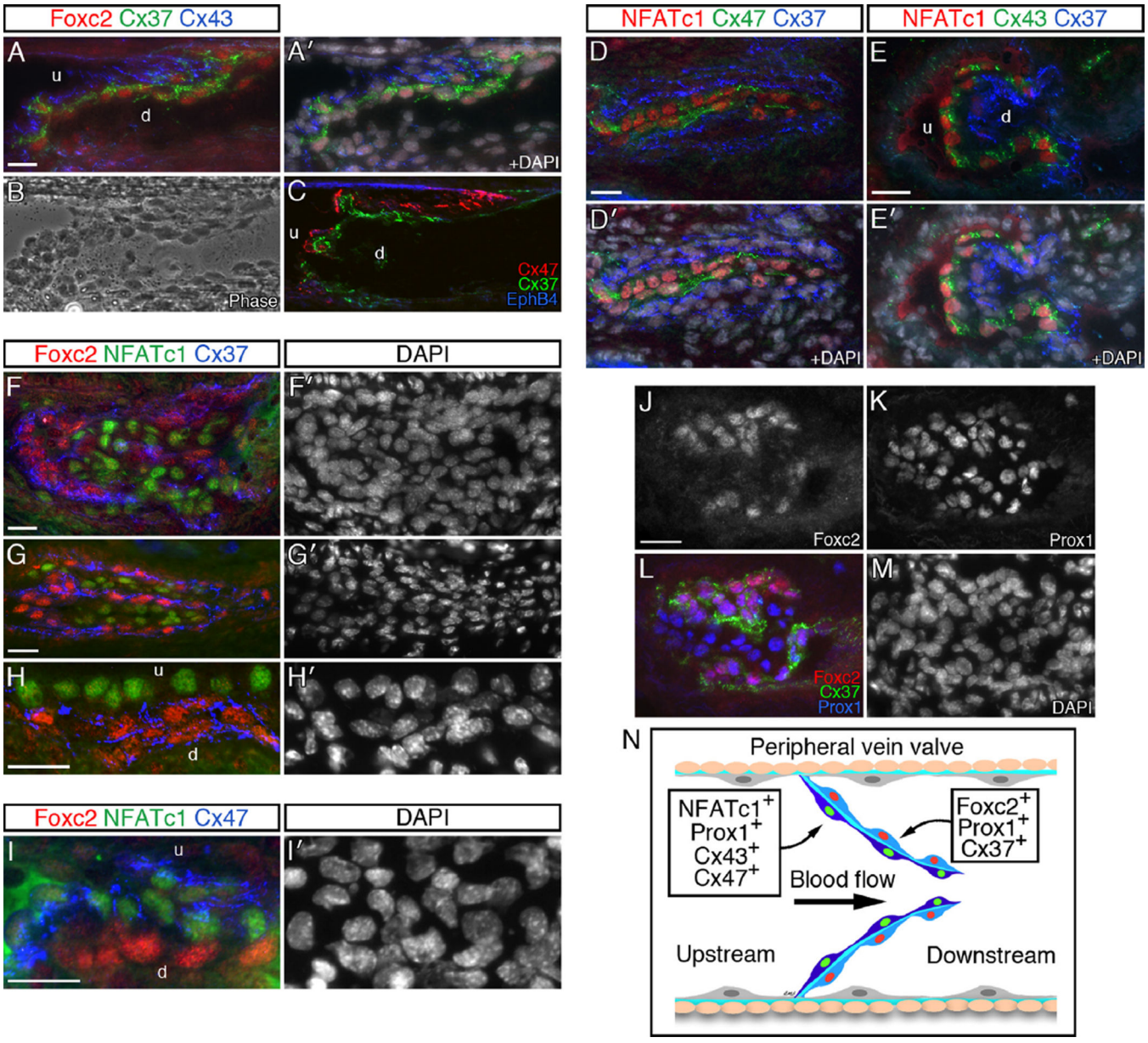

Fig. 5.

Segregated Foxc2 and NFATc1 expression in developing peripheral VVs correlates closely with polarized Cx expression. Sections of P7-P11 VVs were triple immunostained for Foxc2/Cx37/Cx43 (A); NFATc1/Cx47/Cx37 (D); NFATc1/Cx43/Cx37 (E); Foxc2/NFATc1/Cx37 (F)–(H); Foxc2/NFATc1/Cx47 (I); or Foxc2/Cx37/Prox1 (J)-(M). DAPI staining (white) of nuclei is shown superimposed in (A′), (D′), (E′) or as a separate signal in (F′)–(H′) and (M). (C) shows an adjacent section to (A) immunostained for Cx47/Cx37/EphB4 for comparison. Foxc2 expression was very tightly associated only with cells expressing Cx37 on the downstream (d) side of the valve leaflet (A), (F)–(H), (L) whereas NFATc1 expression was closely associated only with Cx47 (D), (I) and Cx43 (E) expression on the upstream (u) side of the leaflet. Co-immunostaining for both transcription factors directly confirmed that expression of Foxc2 and NFATc1 was segregated on opposite sides of the

valve leaflets (F)–(I). In contrast, Prox1 was expressed by VECs on both sides of the valve leaflet (J)–(M) (compare Prox1 distribution to that of Foxc2). (N) Schematic summary of Cx37, Cx43, Cx47, Foxc2, NFATc1, and Prox1 expression at peripheral VVs. d, downstream; u, upstream. Scale bars: 20 μm.

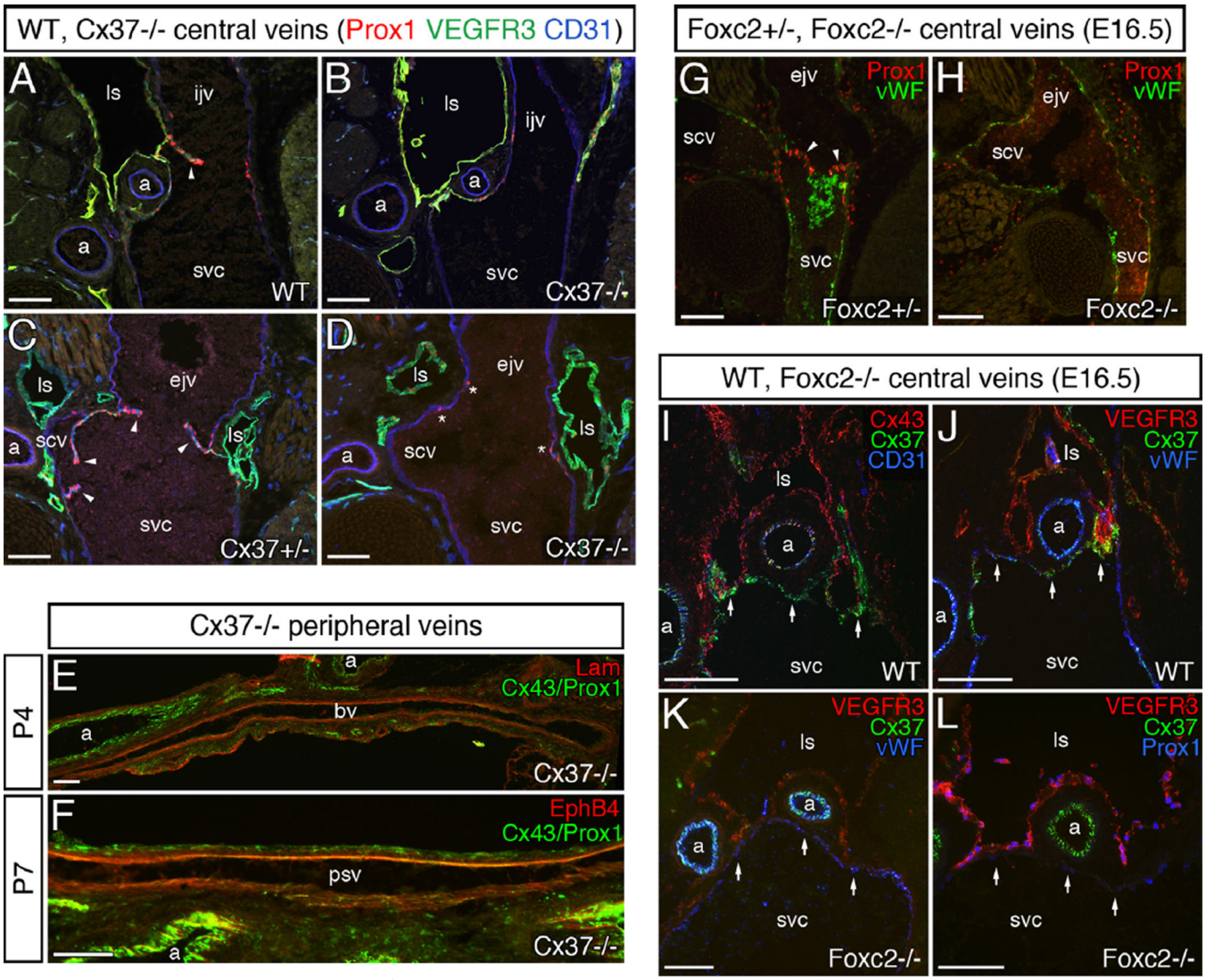

Fig. 6.

Cx37 and Foxc2 are each required for the development of VVs. Prox1/VEGFR3/CD31 labeling of E16.5 (A), (B) and E17.5 (C), (D) embryo sections (frontal orientation) showed that VVs (arrowheads) were present in WT and *Cx37*+/− central veins, but not in *Cx37*−/− veins. There were typically a few Prox1-positive cells (asterisks) associated with the *Cx37*−/− venous endothelium in the area where valves would normally have formed. (E), (F) VVs were absent in sections of *Cx37*−/− peripheral veins (P4 or P7) immunostained for Cx43, Prox1, and laminin or EphB4. VVs were also absent in central veins of E16.5 *Foxc2*−/− embryos (H), but VV leaflets were detected (arrowheads) in two out of three *Foxc2*+/− littermates (G). In WT sections just dorsal to where valves are found, Cx37 expression (arrows) was normally high in the VECs immediately adjacent to the jugular lymph sac (ls) (I), (J). In contrast, E16.5 *Foxc2*−/− embryos showed very little Cx37 expression in the VECs in the corresponding region (K), (L). vWF staining in (G), (H), (J), (K) and subsequent figures labels the vein and arteries (a) but not lymph sacs. Nearby sections are

shown in (I), (J) and (K), (L). a, artery; bv, brachial vein; ejv, external jugular vein; ijv, internal jugular vein; svc, superior vena cava. Scale bars: 100 μm.

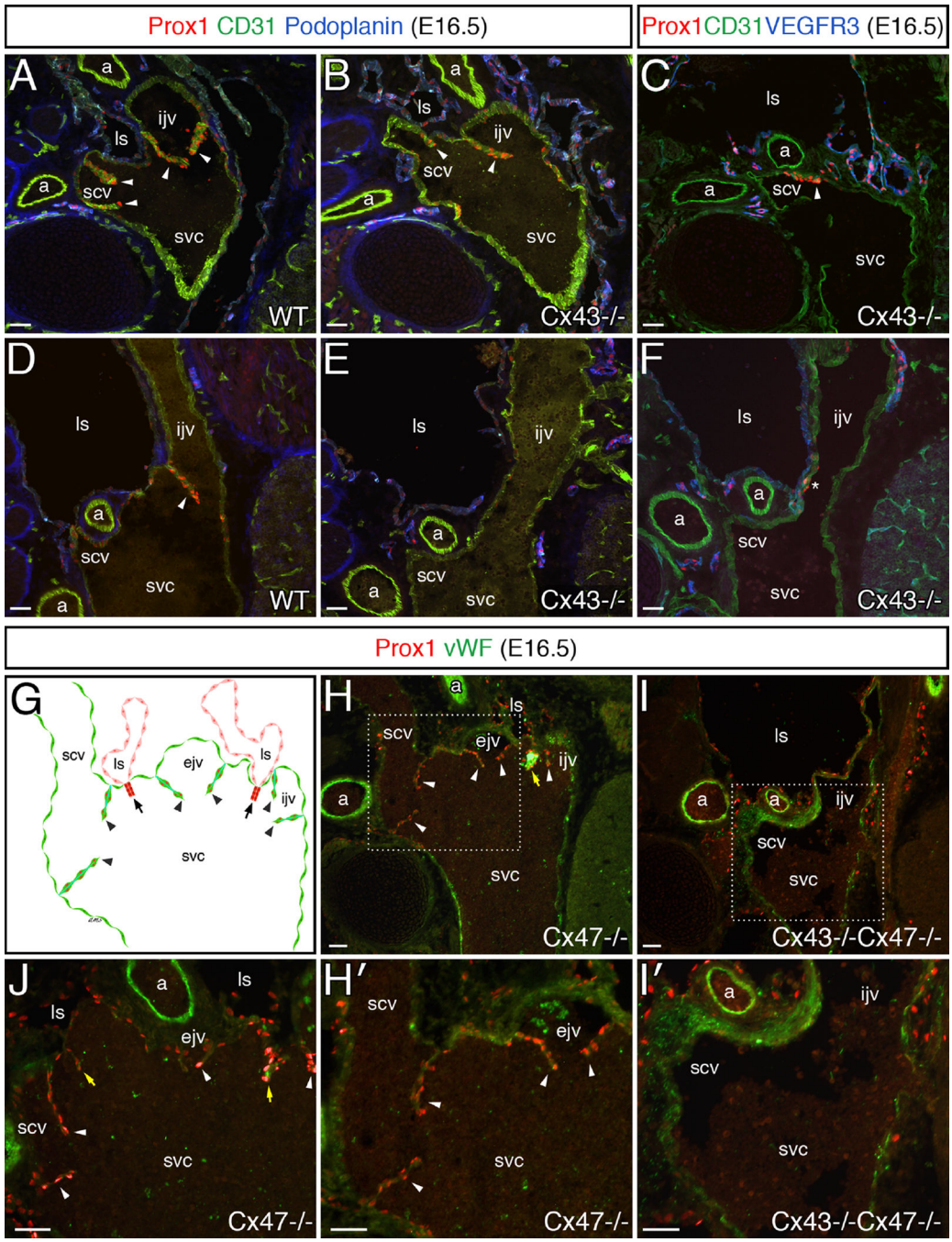

Fig. 7.

Cx43 is necessary for normal development of VVs in central veins; Cx47 is not required for the formation of central VVs or LVVs. WT or *Cx43*−/− E16.5 embryo sections (frontal orientation) were immunostained for Prox1/CD31/Podoplanin (A), (B), (D), (E) or Prox1/VEGFR3/CD31 (C), (F) to detect VVs (arrowheads). Podoplanin, like VEGFR3, is a marker for lymphatic ECs. Compared to WT embryos (A), (D), *Cx43*−/− embryos either lacked VVs in the central veins (E) and (F), or had abnormal VVs or partially absent VVs (B) and (C). In some cases, there were a few Prox1-positive cells (asterisks) associated with the

Cx43−/− VECs in the area where VVs would normally have formed (F). (G) Schematic guide to frontal orientation sections at E16.5. (H), (J) *Cx47*−/− E16.5 embryo sections were immunostained for Prox1 and vWF, and normal VVs (arrowheads) were detected in the central veins. Boxed regions in (H) and (I) are shown at higher magnification in (H′) and (I′), respectively. The development of LVVs (arrows) was also not affected by the absence of Cx47 (H), (J). (I), (I′) In contrast, *Cx47*−/−*Cx43*−/− E16.5 embryos lacked both VVs and LVVs. a, artery; ejv, external jugular vein; ijv, internal jugular vein; ls, lymph sac; scv, subclavian vein; svc, superior vena cava. Scale bars: 50 μm.

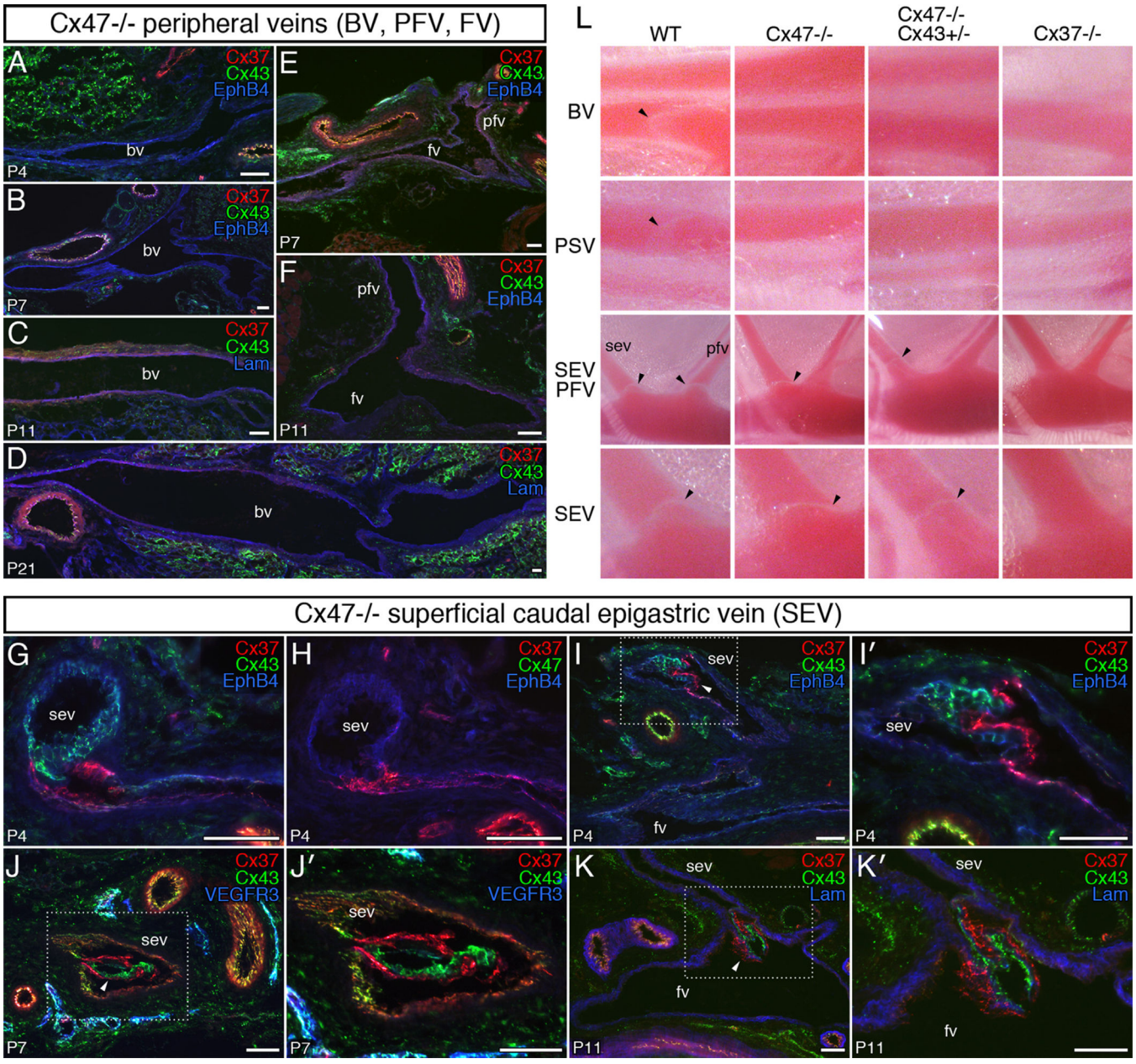

Fig. 8.

Cx47 is necessary for the development of most peripheral VVs; the SEV is an exception. (A)–(D) Peripheral vein sections from *Cx47*−/− mice collected at P4–P21 were immunostained for Cx37, Cx43, and either EphB4 or laminin. VVs failed to develop in most peripheral veins examined (BV, FV, PFV). (G)–(K) A consistent exception was the SEV, where the VV formed normally in *Cx47*−/− mice (P4–P11). Segregated Cx37 and Cx43 expression on opposite sides of the valve leaflets (arrowheads) was still observed at *Cx47*−/− SEV valves during development (G), (I)–(K), but Cx47 staining was absent as expected (H). (G) and (H) show adjacent sections. Boxed regions in (I), (J), (K) are shown at higher magnification in (I′), (J′), (K′), respectively. (L) In situ VV imaging of adult mice detected valves (arrowheads) in BV, PSV, SEV, and PFV of WT mice. However, *Cx47*−/− and

Cx47−/−*Cx43*+/− adult mice lacked these VVs, with the exception of the SEV, where valves (arrowheads) persisted. By comparison, *Cx37*−/− mice lacked the SEV valve, along with all other peripheral VVs examined. bv, brachial vein; fv, femoral vein; pfv, proximal caudal vein; sev, superficial caudal epigastric vein. Scale bars: 50 μm.

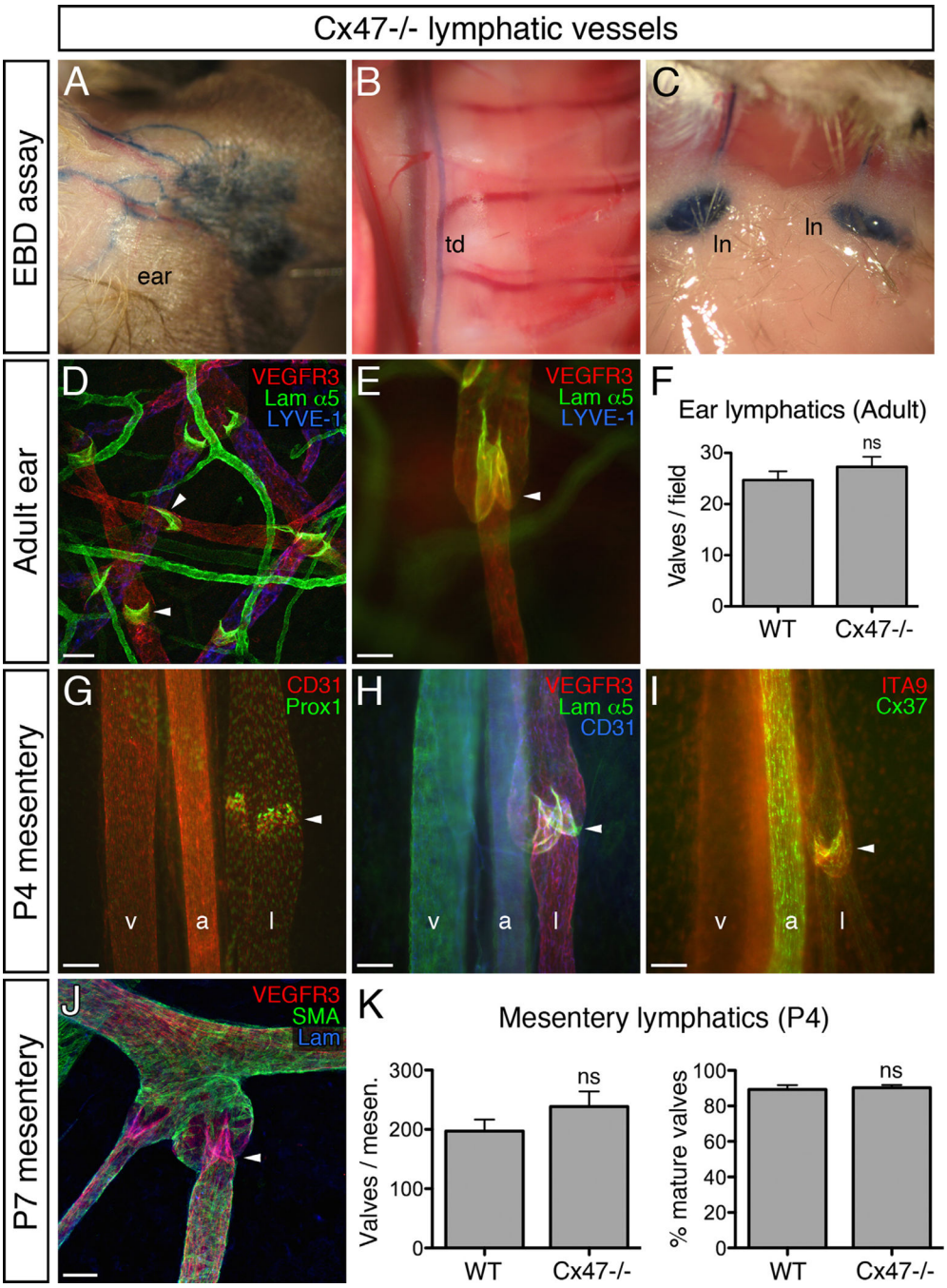

Fig. 9.

Cx47 is not required for the formation or maintenance of LVs. Evans blue dye lymphangiography of *Cx47*−/− adult mice showed that dye transport through ear lymphatics (A), thoracic duct (td) (B), and maxillary lymphatics (C) was normal. (D), (E) Whole-mount *Cx47*−/− adult ear immunostaining showed that LVs (arrowheads) were present in ear lymphatics and displayed normal morphology (E). (F) *Cx47*−/− adult ear LVs were present in normal numbers compared to WT controls. Whole-mount immunostaining of P4 (G)–(I) or P7 (J) *Cx47*−/− mesentery showed that these LVs (arrowheads) exhibit normal

morphology and gene expression during postnatal development. Prox1, Laminin α5, VEGFR3, Cx37, and Integrin α9 (ITA9), were all enriched in the normal fashion at the *Cx47*−/− LVs. (J) SMC actin staining revealed that coverage of lymphatics by SMCs was normal in *Cx47*−/− mesentery. (K) At P4, mesenteric LV numbers in *Cx47*−/− mice were similar to WT controls, and valve maturity was also not different from WT. a, artery; l, lymphatic vessel; ln, lymph node; ns, not significant; td, thoracic duct; v, vein. Scale bars: 50 μm.

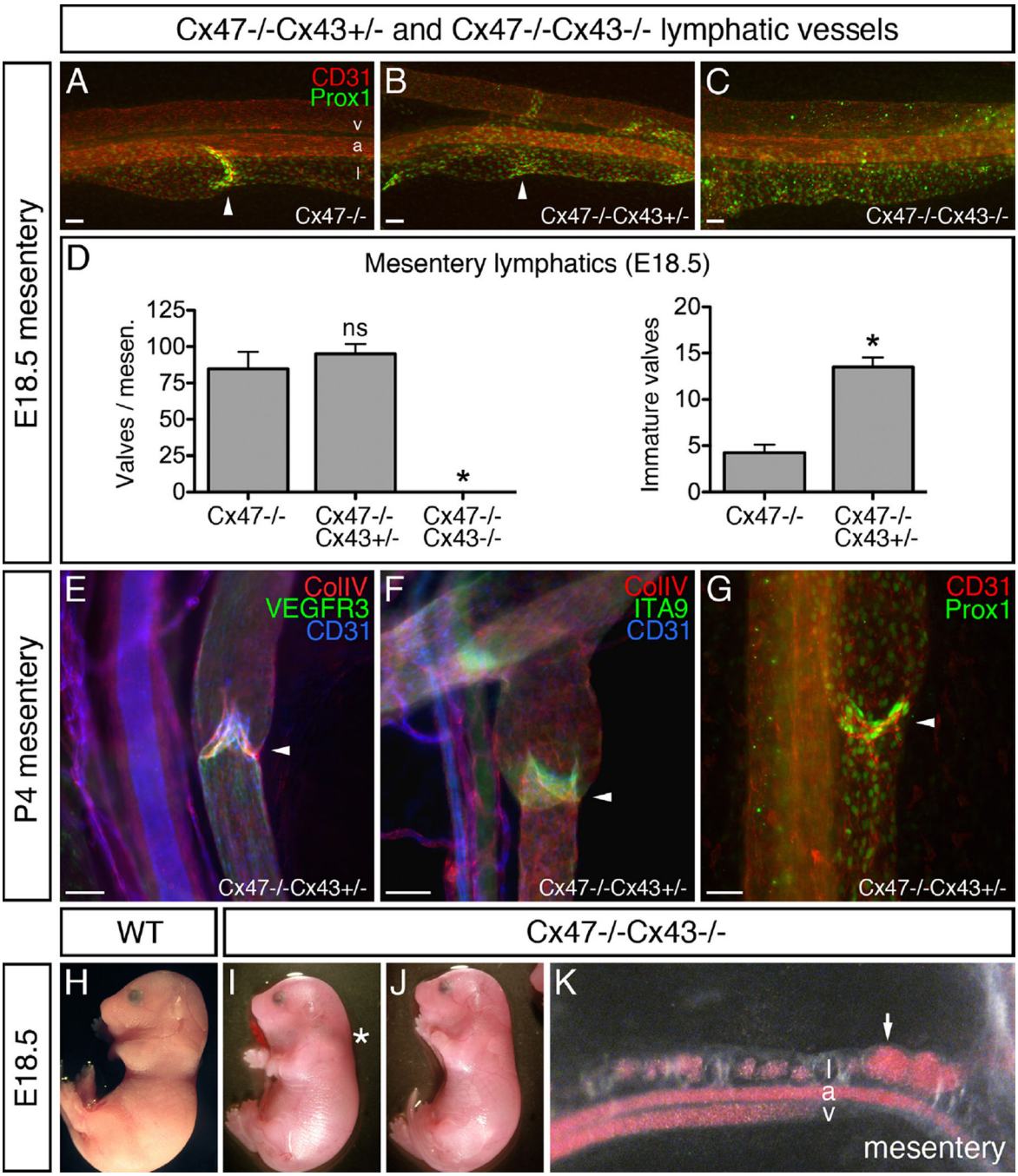

Fig. 10.

Lymphatic phenotypes of *Cx47*−/−*Cx43*+/− and *Cx47*−/−*Cx43*−/− mice. (A)–(D) At E18.5, mesenteries collected from *Cx47*−/−*Cx43*+/− embryos had normal numbers of LVs (arrowheads) compared to *Cx47*−/− littermates (D, left graph), however there was an increase in immature valves (B, arrowhead) and (D, right graph). In contrast, *Cx47*−/−*Cx43*−/− embryos collected at E18.5 completely lacked mesenteric LVs (C) and (D, left graph). Asterisks denote a statistically significant ($P < 0.05$) difference from *Cx47*−/− samples. (E)–(G) At P4, mesenteric LVs in *Cx47*−/−*Cx43*+/− mice now appeared fully

mature as evinced through whole-mount immunostaining for a number of valve markers. Out of 9 *Cx47*−/−*Cx43*−/− embryos collected at E18.5, four showed signs of mild edema in the cervical region. (I) shows an example of an E18.5 *Cx47*−/−*Cx43*−/− embryo with mild edema (asterisk), while (J) is an example of a *Cx47*−/−*Cx43*−/− embryo that looked similar to a WT control (H). (K) The *Cx47*−/−*Cx43*−/− embryo shown in (I) also exhibited blood in some of the mesenteric lymphatic vessels (arrow). a, artery; l, lymphatic; ns, not significant; v, vein. Scale bars: 50 μm.

Table 1

Frequency of valves in veins collected from WT, *Cx37*−/−, and *Cx47*−/− mice at different postnatal developmental stages.

Genotype	Developmental stage			
	P0	**P4**	**P7**	**P11**
WT				
SEV	0/8	4/4	11/11	9/9
Other veins	ND	12/18	6/9	10/12
Cx37−/−				
SEV	ND	ND	ND	ND
Other veins	ND	0/18*	0/18*	0/9*
Cx47−/−				
SEV	ND	6/8	2/2	4/4
Other veins	ND	0/3	0/2	0/14*

The number of veins with a valve present (determined by immunostaining serial cryosections) per total number of veins examined is shown in the table.

SEV, superficial caudal epigastric vein; Other veins: pooled data from brachial vein, proximal saphenous vein, or distal saphenous vein samples.

The number of animals used for each genotype was: WT (P0: 4; P4: 5; P7: 7; P11: 9), *Cx37*−/− (P4: 3; P7: 4; P11: 3), *Cx47*−/− (P4: 6; P7: 1; P11: 5). Statistical significance was tested using Fisher's exact test (two-tailed).

ND, not determined.

* $P < 0.05$ versus WT.

Table 2

Frequency of valve presence in veins of adult mice with different genotypes.

Genotype	Type of vein			
	BV	SEV	PFV	PSV
WT	23/24	35/35	19/22	14/14
Cx37+/−	25/26	32/32	25/32	18/22
Cx37−/−	0/16*	0/16*	0/16*	0/2*
Cx47+/−	10/10	10/10	10/10	9/9
Cx47−/−	0/31*	39/40	0/10*	1/20*
Cx47−/−*Cx43*+/−	0/4*	10/10	0/10*	0/4*
Foxc2+/−	18/18	18/18	13/18	17/18
Cx37+/−*Foxc2*+/−	30/40*#	39/39	26/40	15/15

The number of veins with a valve present per total number of veins examined by in situ imaging is presented.

BV, brachial vein; SEV, superficial caudal epigastric vein; PFV, proximal caudal femoral vein; PSV, proximal saphenous vein.

The number of animals used for each genotype was: WT (18), *Cx37*+/− (16), *Cx37*−/− (8), *Cx47*+/− (5), *Cx47*−/− (20), *Cx47*−/−*Cx43*+/− (5), *Foxc2*+/− (9), *Cx37*+/−*Foxc2*+/− (20).

Statistical significance was tested using Fisher's exact test (two-tailed).

*$P < 0.05$ versus WT.

#$P < 0.05$ versus *Cx37*+/− or *Foxc2*+/−.