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Mucin-type O-glycans (O-glycans) are highly expressed in vascular ECs. However, it is not known whether they are important for vascular development. To investigate the roles of EC O-glycans, we generated mice lacking T-synthase, a glycosyltransferase encoded by the gene C1galt1 that is critical for the biosynthesis of core 1–derived O-glycans, in ECs and hematopoietic cells (termed here EHC T-syn+/− mice). EHC T-syn+/− mice exhibited embryonic and neonatal lethality associated with disorganized and blood-filled lymphatic vessels. Bone marrow transplantation and EC C1galt1 transgene rescue demonstrated that lymphangiogenesis specifically requires EC O-glycans, and intestinal lymphatic microvessels in EHC T-syn+/− mice expressed a mosaic of blood and lymphatic EC markers. The level of O-glycoprotein podoplanin was significantly reduced in EHC T-syn+/− lymphatics, and podoplanin-deficient mice developed blood-filled lymphatics resembling EHC T-syn+/− defects. In addition, postnatal inactivation of C1galt1 caused blood/lymphatic vessel misconnections that were similar to the vascular defects in the EHC T-syn+/− mice. One consequence of eliminating T-synthase in ECs and hematopoietic cells was that the EHC T-syn+/− pups developed fatty liver disease, because of direct chylomicron deposition via disconnected portal vein and intestinal lymphatic systems. Our studies therefore demonstrate that EC O-glycans control the separation of blood and lymphatic vessels during embryonic and postnatal development, in part by regulating podoplanin expression.

Introduction

During embryonic development, blood vessels initially arise from endothelial precursors. These progenitors first develop into a primary capillary plexus, a process known as vasculogenesis (1, 2). The vascular plexus then expands by means of endothelial cell sprouting (angiogenesis) into a vascular network (1–3). Through recruitment of mural cells and vascular specialization, the vascular network matures into a highly organized blood vascular system comprising arteries, veins, and capillaries (2, 4). This multistep process is governed by distinct factors (1, 2, 5). Among them, VEGFs and their receptors control endothelial cell sprouting (2, 6, 7). The specialization of arteries and veins is primarily regulated by the Notch family and the orphan nuclear receptor COUP-TFI, respectively (4, 8, 9). Later during vascular development, another important vascular system, the lymphatic system, also arises (10–12). Lymphatic endothelial cells differentiate from venous endothelial cells under control of the homeobox gene prospero-related homeobox 1 (PROX1) (11, 13). Thereafter, lymphatic endothelial cells form a lymphatic vascular network, for which VEGF-C is a key regulator (10). Although derived from veins, lymphatic vessels develop into an independent system (11, 12). Blood and lymphatic systems remain distinct despite the fact that they are in close proximity and that both angiogenesis and lymphangiogenesis are active during development and tissue remodeling (3, 11, 14). Therefore, there must be mechanisms that control the establishment and subsequently the independent integrity of these two systems. The hematopoietic signaling proteins Syk and SLP-76 regulate the separation between the blood and lymphatic vasculature during embryonic development (15). Beyond these two factors, however, little is known about the molecular mechanisms that regulate the blood and lymphatic vasculature as distinct systems.

The lymphatic system is essential for the transport of immune cells, interstitial fluids, and dietary lipids (2, 11). Dysfunction in the lymphatic system contributes to many pathological conditions such as edema and tumor development (10, 16), yet the clinical consequences of abnormal segregation of blood and lymphatic systems remain unknown.

The development of the blood and lymphatic systems is regulated by numerous glycoproteins (1, 2, 17, 18). Core 1–derived mucin-type O-glycans (O-glycans), which are present in most tissues/cells, modify many membrane and secreted proteins (19–21). Our previous study of mice with a global deficiency of T-synthase (referred to herein as T-syn−/− mice), a critical glycosyltransferase for biosynthesis of O-glycans encoded by the gene C1galt1, revealed an essential role of O-glycans during embryonic vascular development (22). However, whether O-glycosylation of endothelium,
which expresses high levels of O-glycans, is specifically required for this process remains unknown. Moreover, the molecular targets of O-glycosylation during vascular development are not known. Here we show that mice with endothelial cell–specific deletion of C1galt1 exhibit impaired expression/function of podoplanin, an O-glycoprotein also known as T1α, and develop lymphatic vascular defects and abnormal lymphatic functions. This phenotype was not observed in our previous study (22), as T-syn–/– embryos die before the lymphatic system becomes functional.

Results
Mice with targeted deletion of the C1galt1 gene in endothelial and hematopoietic cells do not express endothelial O-glycans (Figure 1A) are highly expressed in endothelial cells (22). To investigate their contributions to vascular development, we crossed mice in which the C1galt1 gene was flanked by loxP sites (C1galt1<sup>f/f</sup>) (Figure 1B) with Tie2Cre Tg mice (24). The resultant C1galt1<sup>f/f</sup>Tie2Cre<sup>+</sup> mice are deficient for T-synthase specifically in endothelial and hematopoietic cells (we refer to the mice as EHC T-syn<sup>−/−</sup> mice).

Lack of T-synthase activity in EHC T-syn<sup>−/−</sup> lung primary vascular endothelial cells indicated that the Cre-mediated in vivo deletion was efficient (Figure 1C). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) revealed that O-glycans were abundant in cultured WT (T-syn<sup>+</sup>) but not T-syn<sup>−/−</sup> endothelial cells (Figure 1D and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI36077DS1), indicating that T-synthase is a critical glycosyltransferase for O-glycan biosynthesis in endothelial cells. To determine the specificity of the deletion, we probed EHC T-syn<sup>−/−</sup> intestinal tissue sections with a mAb against Tn antigen, a truncated O-glycan structure expressed in the absence of T-synthase activity (Figure 1E, Supplemental Figure 1B, and data not shown). Enzymatic desialylation of tissue sections did not

Figure 1
Generation of EHC T-syn<sup>−/−</sup> mice. (A) Scheme for mucin-type O-glycan biosynthesis. Arrowheads indicate possible further branching, elongation, fucosylation, sialylation, and sulfation. (B) Diagram of WT (T-syn<sup>+</sup>), loxP site–flanked (T-syn<sup>f</sup>), and null (T-syn<sup>−/−</sup>) alleles of C1galt1. (C) T-synthase activity of primary endothelial cells isolated from T-syn<sup>−/−</sup> and EHC T-syn<sup>−/−</sup> lungs. The data represent the mean ± SEM of 2 independent experiments. (D) Annotated spectra of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analyses of 2-aminobenzamide–labeled (2-AB–labeled) O-glycans from T-syn<sup>−/−</sup> and T-syn<sup>+</sup> endothelial cells. The O-glycans with mass number in green contain a sodium instead of a hydrogen. MALDI-TOF-MS did not detect Tn antigen, which is exposed in the absence of T-synthase activity, because its size is below the detection limit. (E) Immunohistochemical staining of serial intestinal sections with antibodies against Tn antigen or Lyve-1. Tn is positive in endothelial cells of EHC T-syn<sup>−/−</sup> arteriole, blood capillaries, and Lyve-1–positive lymphatic vessels. A, arteriole; L, lymphatic vessels; V, vein; E, epithelium; BC, blood capillary. Scale bar: 50 μm.
expose more Tn antigen in EHC T-syn\(^{-/-}\) tissues (data not shown), indicating little expression of the sialylated Tn antigen (Figure 1A) in EHC T-syn\(^{-/-}\) tissues. These results indicate that the Cre-mediated recombination is efficient and specific in both blood and lymphatic endothelial cells (Figure 1E, right panel).

EHC T-syn\(^{-/-}\) mice exhibit high embryonic and neonatal mortality, disorganized vasculature, and impaired lymphatic function. EHC T-syn\(^{-/-}\) embryos developed normally until E14.5, after which they exhibited disorganized blood-filled vasculature with blind ends primarily in the skin and small intestine but also in other organs such as heart (Figure 2, A and C, and data not shown). Of EHC T-syn\(^{-/-}\) embryos, 48% died after E14.5, and the surviving ones died during neonatal development or after weaning (Figure 2B). Of neonatal EHC T-syn\(^{-/-}\) mice, 60% had chylous ascites (Figure 2D), but none developed bloody ascites. More than 90% of those with chylous ascites died within a week after birth. Subcutaneous edema was common in most EHC T-syn\(^{-/-}\) embryos and surviving mice (Figure 2, A and G, and Supplemental Figure 2A). Widespread internal bleeding in small intestines was observed in approximately 75% of EHC T-syn\(^{-/-}\) mice at autopsy (Figure 2E and Supplemental Figure 2B), which might be a cause of the EHC T-syn\(^{-/-}\) postnatal lethality. Many EHC T-syn\(^{-/-}\) villi had a highly dilated vascular structure (Figure 2, F and H). Taken together, phenotypes such as chylous ascites, subcutaneous edema, and

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**Figure 2**

EHC T-syn\(^{-/-}\) mice exhibit high embryonic and neonatal mortality, disorganized vasculature, and impaired lymphatic function. (A) Representative E15.5 embryos showing dilated superficial vessels with scattered hemorrhages in an EHC T-syn\(^{+/+}\) mouse compared with its T-syn\(^{+/+}\) littermate. Arrows indicate abnormal vessels and subcutaneous bleeding. (B) Postnatal survival curves for T-syn\(^{+/+}\) mice and EHC T-syn\(^{-/-}\) mice. (C) Gross images of intestines of E17.5 T-syn\(^{+/+}\) and EHC T-syn\(^{-/-}\) embryos. Arrows indicate vessels. (D) Gross images of P7 T-syn\(^{+/+}\) and EHC T-syn\(^{-/-}\) jejunum. Arrows indicate lymphatic vessels. Asterisks indicate chylous ascites. (E) Four-week-old T-syn\(^{+/+}\) and EHC T-syn\(^{-/-}\) ileum. Arrow indicates blood vessels. Widespread bleeding (overall pink color) is evident in the EHC T-syn\(^{-/-}\) ileum. (F) Luminal surfaces of T-syn\(^{+/+}\) and EHC T-syn\(^{-/-}\) ileum. Arrows indicate dilated, blood-filled lacteals of EHC T-syn\(^{-/-}\) intestinal villi. (G) Histological sections of dorsal skin of E15.5 T-syn\(^{+/+}\) and EHC T-syn\(^{-/-}\) embryos. The asterisk marks enlarged subcutaneous fascia indicating edema. (H) Histological imaging of small intestine villi from 4-week-old T-syn\(^{+/+}\) and EHC T-syn\(^{-/-}\) mice. Arrows indicate potential blood-filled lymphatic vessels. Scale bars: 1 mm (A, C, D, and E); 50 μm (F–H).
abnormal blind-ended vessels in EHC T-syn−/− mice are suggestive of defective lymphatic vessel development/function.

EHC T-syn−/− mice form abnormal connections between blood and lymphatic vessels. To characterize the EHC T-syn−/− vascular abnormalities, whole-mount small intestines were stained with antibodies against CD31, a pan-endothelial marker (25), and Lyve-1, a lymphatic endothelial marker (11, 26). Starting from E15.5, EHC T-syn−/− mesenteric and intestinal submucosal lymphatic capillaries were disorganized compared with those of T-syn+/+ mice (Figure 3A and Supplemental Figure 3A). Intriguingly, EHC T-syn−/− lymphatic vessels were filled with blood (Figure 3B), indicating potential defects in the separation of blood and lymphatic vessels. Indeed, dextran-FITC (150 kDa) and fluorescent microbeads (1 µm) injected through the carotid artery went immediately into lymphatic vessels, such as intestinal lacteals, of EHC T-syn−/− mice but not T-syn+/+ mice (Figure 3, C and D). This confirms functional misconnections of blood and lymphatic vessels in EHC T-syn−/− mice. There was no visible extravascular dextran-FITC in EHC T-syn−/− mice, indicating that EHC T-syn−/− vessels were not leaky.

Analyses of cryosections of small intestines demonstrated that T-syn+/+ blood endothelium expressed a high level of CD31 and no Lyve-1 and was surrounded by α-SMA–positive cells. T-syn−/− intestinal submucosal lymphatic microvascular endothelium did express Lyve-1, displayed a lower level of CD31, and had no α-SMA coating (Figure 3E). The high CD31 level profile appeared unaffected in EHC T-syn−/− arteries, veins, and blood capillaries (Figure 3, A and E). In contrast, most EHC T-syn−/− intestinal submucosal lymphatic capillaries were disorganized, dilated, and expressed abnormally high levels of CD31 (Figure 3E, Figure 4, A and B, and Supplemental Figure 3, A and B). All EHC T-syn−/− lymphatic capillaries expressed Prox1 (Figure 3F, Figure 4, D and E, and Supplemental Figure 3, C and D), a definitive lymphatic endothelial marker (11), yet approximately 65% of these vessels partially lost their expression of Lyve-1 (probed with an O-glycosylation–independent polyclonal antibody) (Figure 3E, Figure 4, A, D, and E, and Supplemental Figure 3, A and B). In contrast, Lyve-1 was expressed uniformly at a high level in T-syn+/+ intestinal lymphatic microvascular endothelium (Figure 3, A and C–E; Figure 4A; and Supplemental Figure 3, A and B). Prox1-positive lymphatic microvessels in EHC T-syn−/− intestines had ectopic staining of α-SMA (Figure 3, E and F), which is known not to be associated with intestinal lymphatic microvessels (27). Antibodies against CD105 and endomucin, which are blood vascular endothelial cell–specific markers (25, 28, 29), labeled only blood vascular endothelium in T-syn+/+ mice (Figure 4, E and F, and Supplemental Figure 3D). In contrast, anti-CD105 and anti-endomucin stained both EHC T-syn−/− blood and lymphatic endothelial cells, although the staining of anti-endomucin was patchy (Figure 4, E and F, and Supplemental Figure 3D). Thus, the observed functional misconnections correlated with hybrid blood and lymphatic vessel marker expression within...
individual vessel profiles. The number of EHC T-syn+/– Prox1-positive lymphatic endothelial cells appeared to be comparable with that of T-syn+/+ lymphatic endothelial cells, although some of the lymphatic endothelial cells were attenuated with cytoplasmic thinning (Figure 3F, Figure 4, D and E, and Supplemental Figure 3B). These experiments indicate that endothelial O-glycans perform an important function in the segregation of blood and lymphatic endothelial cells during embryonic development.

EHC T-syn+/– arteries were normal based on their morphology (Figure 3E). The smooth muscle cell coverage of venous endothelial cells appeared not to be as uniform in EHC T-syn–/– mice as in control T-syn+/+ mice (Figure 3E). Although skin and brain blood capillaries were comparable in T-syn+/+ and EHC T-syn–/– mice (Supplemental Figure 3C and Supplemental Figure 4), EHC T-syn–/– intestinal blood capillaries had a moderate degree of abnormal branching (Figure 4F and Supplemental Figure 3A). This phenotype is similar, albeit much less severe, to the abnormal angio genesis in global T-syn–/– mice, suggesting that endothelial O-glycans contribute to angiogenesis.

Endothelial O-glycans are specifically required for vascular development. To differentiate the contribution of endothelial cells and hematopoietic cells to abnormal vascular development in EHC T-syn–/– mice, we transplanted EHC T-syn–/– bone marrow cells into lethally irradiated WT B6.SJL recipients and vice versa to generate hematopoietic cell–specific T synthase–deficient mice (HC T-syn–/–) and endothelial cell–specific T synthase–deficient mice (EC T-syn–/–) (Supplemental Figure 5A). Nine months later, HC T-syn–/– mice did not develop vascular abnormalities, whereas the EC T-syn–/– mice retained abnormal vasculature (Supplemental Figure 5B). These data indicate that WT bone marrow is unable to restore the EHC T-syn–/– vascular defects and that loss of T-synthase in bone marrow cells of WT adult mice cannot induce a postnatal abnormal vascular phenotype.

We reasoned that if the absence of endothelial O-glycans causes the phenotypes in EHC T-syn–/– mice, endothelial cell–specific expression of T-synthase would rescue EHC T-syn–/– embryos and correct EHC T-syn–/– lymphatic defects. To test this hypothesis, we generated Tg lines that expressed T-synthase specifically in endothelial cells under control of the Tie2 promoter and enhancer (Tie2–T-syn Tg; Figure 5A) (30), which drives the expression of the transgene in all endothelial cells, only transiently in hematopoietic stem cells, but not in differentiated hematopoietic cells (30, 31). Crosses between two independent Tie2–T-syn Tg founders and EHC T-syn–/– mice produced rescued EHC T-syn–/– viable pups.
with a normal Mendelian distribution (Figure 5B). The rescued EHC T-syn+/ mice developed normally and had normal vasculature (Figure 5, C and D). As expected, the Tn mAb did not stain the rescued EHC T-syn+/ vascular endothelial cells but stained hematopoietic cells, whereas the mAb stained arterial, venous, and lymphatic endothelial cells and hematopoietic cells in non-rescued EHC T-syn–/– mice (Figure 5E and data not shown). This result confirms that the rescue is blood and lymphatic endothelial specific. Taken together, these experiments demonstrated that endothelial, but not hematopoietic, O-glycans are specifically required for lymphangiogenesis.

Silencing of C1galt1 in zebrafish and tadpole reveals existence of an additional C1galt1 gene. To verify the lymphatic abnormalities revealed by EHC T-syn+/ mice in different organisms, we silenced the known C1galt1 gene in zebrafish (Zebra danio) and tadpole (Xenopus laevis). The C1galt1 knockdown was efficient in both organisms (Supplemental Figure 6A and Supplemental Figure 7, A and B). However, the knockdown tissues were not positive for Tn, which should be exposed in the absence of T-synthase; instead, they expressed a similar level of core 1 O-glycans as control tissues (data not shown). Moreover, comprehensive analyses revealed no significant blood and lymphatic vascular abnormalities in these C1galt1-silenced organisms (Supplemental Figure 6 and 7). We found a new C1galt1 (Q08BL) in the June 2008 Zebra danio database (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NM_001077374) and a new EST that shares a high degree of homology with C1galt1 in Xenopus laevis (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucest&id=8884333; February 2006). The new Xenopus C1galt1 EST is not a “B” copy of the Xenopus laevis pseudotetraploid genome, because it is quite divergent. These results suggest that the lack of vascular phenotype after silencing of C1galt1 in both zebrafish and tadpole is due to the existence of another gene for T-synthase. Although only one functional C1galt1 gene has been identified in vertebrate and invertebrate animals (19), these findings suggest that there is a functionally redundant C1galt1 in these small aquatic vertebrate animal models.

Lack of O-glycans results in impaired expression of podoplanin, and mice without podoplanin phenotype EHC T-syn–/– mice. Lack of O-glycans may alter the expression and/or function of many glycoproteins that regulate lymphangiogenesis. To identify the molecular targets, we first screened molecules that are important in angiogenesis and lymphangiogenesis by RT-PCR and Western blotting of primary endothelial cells or tissue lysates from T-syn+/+ and EHC T-syn–/– mice. Levels of transcripts of genes that are associated with vascular development were similar in T-syn+/+ and EHC T-syn–/– mice (Supplemental 8A and data not shown), suggesting that abnormal O-glycosylation does not significantly affect gene expression. Among these candidate molecules, expression of podoplanin protein, a mucin-type O-glycoprotein (32), was substantially decreased in EHC T-syn–/– endothelial cells as measured by different glycosylation-independent antibodies (Figure 6A and Supplemental Figure 8B). EHC T-syn–/– endothelial podoplanin exhibited several smaller bands on a reducing SDS-PAGE gel (Figure 6A). Immunoblotting of immunoprecipitated podoplanin from primary endothelial cell and intestinal tissue lysates with the lectin Helix pomatia agglutinin (HPA), which recognizes Tn antigen, indicated that the smaller podoplanin bands were Tn positive (Figure 6, B and C), suggesting that they were underglycosylated and possibly degradation products of podoplanin. Podoplanin was markedly reduced in Lyve-1–positive intestinal submucosal

Figure 5

Endothelial expression of C1galt1 rescues EHC T-syn–/– embryonic lethality and defective vascular development. (A) Diagram of the DNA construct used to generate Tie2–T-syn Tg mice and of T-syn–/–, T-syn1, and T-syn–/– alleles, with positions of primers used in genotyping. Tie2 P, Tie2 promoter; Tie2 E, Tie2 intronic enhancer. (B) PCR genotyping of tail genomic DNA from offspring of Tie2–T-syn Tg crossed with EHC T-syn–/– mice. Lane 3 represents the genotype of the rescued EHC T-syn–/– mice. (C) Intestinal vasculature of EHC T-syn–/– mice. Dark brown indicates positive staining. Arrows, vascular endothelial cells; arrowheads, blood cells. Data are representative of at least 3 experiments. Scale bars: 1 mm (C); 50 μm

(P and D).
lymphatic endothelium in EHC T-syn−/− mice (Figure 6D). The expression of surface podoplanin was reduced in permeabilized Prox1-positive primary EHC T-syn+/− skin endothelial cells, and there was no intracellular accumulation of underglycosylated podoplanin (Supplemental Figure 9). These data suggest that O-glycosylation of endothelial cells is essential for the cell surface expression of podoplanin.

The size of Lyve-1 was smaller in EHC T-syn−/− endothelial cells than in T-syn+/+ endothelial cells, indicating that Lyve-1 is O-glycosylated (Supplemental Figure 8B). Anti–Lyve-1 consistently stained Lyve-1 in T-syn+/+ and EHC T-syn−/− primary endothelial cell lysates (Supplemental Figure 8B), suggesting that changes in Lyve-1 O-glycosylation do not affect its stability and that the anti–Lyve-1 epitope is not dependent on O-glycosylation.

Pdpn−/− mice (Pdpn−/−, also known as T1α−/−) die shortly after birth due to respiratory failure (32, 33). Although Pdpn−/− mice have abnormal lymphatic vessels, they were not reported to have misconnections between blood and lymphatic vessels (32). We found that, Pdpn−/− mice like EHC T-syn−/− mice, had disorganized and blood-filled lymphatic vessels (Figure 6, E–H). These data suggest that O-glycosylated podoplanin is required for the separation of blood and lymphatic vessels during embryonic development.

Endothelial O-glycans are required for the maintenance of distinct blood and lymphatic vascular systems during postnatal development. To examine whether O-glycans are important for the continued segregation of distinct blood and lymphatic systems, we generated mice with postnatal deletion of C1galt1 (inducible T-syn−/− mice; Figure 7A). Two weeks after induction, inducible T-syn−/− endothelial and hematopoietic cells, which normally express high levels of O-glycans, started to be positive for anti-Tn mAb staining (Figure 7B and data not shown), indicating induced deletion of C1galt1. Six months later, inducible T-syn−/− mice developed misconnections between blood and lymphatic vessels that resembled the phenotype of EHC T-syn−/− mice (Figure 7B). Intravital microscopy revealed that arterially injected India ink flowed directly from intestinal veins into lymphatic vessels, demonstrating direct connections between T-syn−/− venous and lymphatic vessels (Figure 7C and Supplemental Videos 1 and 2). As in EHC T-syn−/− mice, some of the inducible T-syn−/− intestinal lymphatic vessels expressed a mosaic of blood and lymphatic markers (Figure 7D and data not shown).
shown). Inducible T-syn+/− lymphatic vessels had no expression of podoplanin, and the loss of lymphatic endothelial podoplanin occurred before abnormal lymphatic development (Figure 7D and data not shown), suggesting a causal relationship during postnatal tissue remodeling. These data demonstrate that O-glycosylated podoplanin is important for maintaining the separation of these systems in postnatal physiology. Therefore, abnormal O-glycosylation of podoplanin may contribute to lymphatic abnormalities in pathological conditions.

Abnormal connections of portal venous and intestinal lymphatic systems cause aberrant transport of lipids with consequent fatty liver disease. Separation of blood and lymphatic vascular systems is essential for proper transport of lipids. At P7, T-syn+/− mesenteric collecting lymphatics were filled with lymph; in contrast, EHC T-syn+/− mesenteric collecting lymphatics were filled with blood (Figure 8A). We thus examined whether these abnormalities caused abnormal lipid transport in EHC T-syn+/− mice. Similar to T-syn+/− livers, E18.5 and P0 EHC T-syn+/− livers had normal morphology (Figure 8, B and C). However, immediately after pups began nursing on milk, the P1 EHC T-syn+/− livers displayed abnormal accumulation of vacuoles of triglycerides (Figure 8C), which resembles microvesicular steatosis in human steatosis hepatitis (34). The phenotype worsened progressively. At P7, EHC T-syn+/− livers had extensive steatosis, inflammatory infiltrates, and hepatocyte ballooning (Figure 8, B and D, and Supplemental Figure 10A). EHC T-syn+/− mice that survived beyond neonatal development developed liver cirrhosis and splenomegaly (Supplemental Figure 10, B and C, and data not shown). EHC T-syn−/− adult mice were not obese (Supplemental Figure 10, D and E), differing from mice with Prox1 haploinsufficiency (35). The plasma lipid levels of T-syn+/− and EHC T-syn−/− mice were comparable (Figure 8E), suggesting no major differences in lipid metabolism. But triglyceride levels were significantly elevated in EHC T-syn+/− compared with T-syn+/− liver (Figure 8E). Given that triglycerides are the major components of
chylomicrons, these data suggested that misconnections between intestinal blood and lymphatic vessels in EHC T-syn−/− mice allowed a portion of chylomicrons to be directly transported from small intestine into the liver through the portal vein system. To test this possibility, both T-syn+/+ and EHC T-syn−/− mice were gavaged with the fluorescent lipid BODIPY FL C16. One hour later they were examined for the presence of fluorescent lipid in the portal vein, which normally should not be observed. As expected, the fluorescent lipids were only visualized in EHC T-syn−/− portal vein (Figure 8F), providing direct evidence that some chylomicrons are directly deposited into liver because of misconnections between intestinal blood and lymphatic vessels (Figure 8G). Inducible T-syn−/− livers also had an increase in lipid deposition (data not shown), which is consistent with the fatty liver phenotype in EHC T-syn−/− mice.

**Discussion**

Our results demonstrate that endothelial O-glycans control the separation of blood and lymphatic vessels during embryonic and postnatal development, at least in part by regulating podoplanin expression. Moreover, we show that intestinal blood and lymphatic
mice have defects in lymphatic development. Podoplanin is likely a cause rather than consequence of lymphatic variations in genetic backgrounds and the temporal difference remains to be investigated.

Independent of identity loss and that the confused endothelial identity is the primary cause of the formation of the hybrid vessels in EHC T-syn/–/– mice. The observed marker profile further suggests that endothelial O-glycans are dispensable for the early commitment phase of endothelial cells to the lymphatic lineage but are indispensable for the subsequent endothelial differentiation and maturation that are required for development and maintenance of segmented blood and lymphatic vessels. Although unlikely, a potential alternative explanation could be that misconnections initially arise independent of identity loss and that the confused endothelial marker profile is a consequence of altered flow dynamics in EHC T-syn/–/– lymphatic vessels resulting from the misconnections. Whether misconnections arise at specific sites or sporadically remains to be investigated.

The molecular mechanism of the lymphatic defects in EHC T-syn/–/– mice appears to be related to the impaired expression of podoplanin. The observed lymphatic abnormalities in EHC T-syn/–/– mice strongly resemble the phenotypes of Pdpn–/– mice. Postnatal deletion of O-glycans in inducible T-syn/–/– mice also resulted in impaired expression of endothelial podoplanin that occurs prior to the onset of lymphatic defects. Thus, the loss of podoplanin is likely a cause rather than consequence of lymphatic defects. It is worth noting that the lymphatic phenotype in inducible T-syn/–/– mice is primarily observed in small intestines, where tissue remodeling and angiogenesis are active postnatally. These observations suggest that the maintenance of segregated blood and lymphatic vessels requires persistent expression of O-glycoproteins, such as podoplanin, in tissues of continuous turnover of lymphatic endothelial cells in adult mice. In addition, these findings underscore the potential pathophysiological relevance of this unique phenotype. Taken together, these results indicate that O-glycans are specifically required in lymphatic endothelial cells, primarily to ensure proper function of podoplanin for the establishment and maintenance of distinct blood and lymphatic systems during embryonic development and postnatal tissue remodeling. Although lack of O-glycosylation might affect the functions of other glycoproteins that regulate lymphangiogenesis and thus contribute to the vascular defects of EHC T-syn/–/– mice, our results indicate that abnormal O-glycosylation of endothelial podoplanin is sufficient to result in the formation of hybrid vessels and blood/lymphatic vessel misconnections.

Prox1 is expressed in both transitional and mature lymphatic endothelial cells and controls the establishment of lymphatic systems during embryonic development (11, 13, 38). Yet it remains unclear how Prox1 controls the switch from venous endothelial cells to lymphatic endothelial cells and how it maintains lymphatic endothelial cell identity. Intriguingly, lymphatic endothelial cells that lack O-glycans have impaired expression of podoplanin and express a mosaic of blood endothelial and lymphatic endothelial markers. This result suggests that O-glycosylation of podoplanin contributes to lymphatic endothelial cell identity. The Pdpn gene is one of the target genes whose expression is upregulated by Prox1 (39). Therefore, it is plausible that Prox1 controls the segregation of blood and lymphatic systems, at least in part by upregulating podoplanin expression. Podoplanin induces aggregation of platelets that express Syk and SL-76 by interacting with a lectin-like receptor named CLEC-2 on platelets (40–42). However, podoplanin likely controls additional processes that are independent of platelets, as mice having either no or less than 15% circulating platelets do not display lymphatic defects (43–45). Therefore, precisely how podoplanin regulates lymphatic development remains to be defined.

Lipid transport is one of the primary functions of lymphatic vessels (10, 11). Dietary lipids are transported in the form of chylomicrons from small intestine to systemic circulation via the intestinal lymphatic vessels and thoracic duct. In circulation, triglyceride components of chylomicrons are unloaded to adipose and muscle tissues by the activity of lipoprotein lipase (46). Consequently, only chylomicron remnants are transported to liver through the hepatic artery. In EHC T-syn/–/– mice, because of aberrant intestinal vein and lymphatic connections, chylomicrons are directly transported to liver via the portal vein system, which causes fatty liver disease (steatohepatitis) (Figure 8G). Nonalcoholic fatty liver disease is prevalent (34, 47). However, the pathogenesis of this disease remains poorly understood (34, 48). Our findings provide what we believe to be a novel potential mechanism for the pathogenesis of this common disease.

In conclusion, endothelial O-glycans control the separation of blood and lymphatic vessels during embryonic and postnatal development, at least in part by regulating podoplanin expression. Aberrant connections between intestinal blood and lymphatic vessels contribute to the pathogenesis of fatty liver disease in mice.
Methods

Mice, microscopy, semiquantitative RT-PCR, and morpholino knockdown in Xenopus laevis and zebrafish. See Supplemental Methods.

Mice. To generate EHC T-syn+/− mice, Cigaltf/f mice were crossed with Tie2Cre Tg mice [Tg(Tek-cre)1Ywa] (provided by Masashi Yanagisawa, Boston University School of Medicine, Boston, Massachusetts, USA) (50). To induce the deletion of Cigalt1 in vivo, mice were injected intraperitoneally with 250 μg of poly-I:C (Sigma-Aldrich) once every other day over 6 days (3 injections total) starting at age 2 weeks. Pdpw+/− mice (129/SvEv background) were generated as described previously (33) and provided by M.C. Williams (Boston University School of Medicine, Boston, Massachusetts, USA).

Endothelial cell–specific Cigalt1 Tg mice (Tie2–T-syn Tg) on an FVB/N genetic background were generated with standard procedures. In brief, full-length murine Cigalt1 was inserted into the vector that contained the 2.1-kb promoter and 10-kb enhancer of the gene C1galt1 (provided by M.C. Williams (Boston University School of Medicine, Boston, Massachusetts, USA) (30). The purified DNA fragment was microinjected into a fertilized FVB/N mouse oocytes, and offspring were produced. Mice were genotyped as described previously (19, 22).

Endothelial cell–specific Cigalt1 Tg mice were cultured in complete endothelial cell medium (Invitrogen) that were coated with rat anti-mouse CD102 (clone 3C4; BD) according to the manufacturer’s instructions. Subconfluent primary endothelial cells were purified from culture with Dynabeads M-450 (Dynal, USA) (30). The purified DNA fragment was microinjected into a fertilized FVB/N mouse oocytes, and offspring were produced. Mice were genotyped as described previously (19, 22).

O-glycan structures from cultured endothelial cells were analyzed as described previously (52). Briefly, T-syn+/− and T-syn−/− endothelial cell lines were collected and vacuum dried, and O-glycans were released by amnios-based β-elimination. The glycans were labeled with 2-amino benzamide (2-AB) and collected from phase columns (Zorbax NH2; Agilent Technologies) using HPLC equipped with a fluorescence detector (excitation, 330 nm; emission, 420 nm). Glycans were analyzed for composition/structure by MALDI-TOF-MS (Applied Biosystems) in a linear positive mode.

Microscopy. Embryos and organs were photographed at autopsy. For routine histology, tissues were fixed with 10% formalin and embedded in paraffin. Sections (5 μm) were stained with H&E.

For immunohistochemistry, deparaffinized sections were blocked with a serum-free protein block (Dako) and streptavidin/biotin blocking kit (Vector Laboratories). Sections were then incubated overnight with biotinylated goat anti-mouse Lyve-1 (5 μg/ml; R&D Systems) or biotinylated anti-Tn antigen mAb (2 μg/ml; clone Ca3638, IgM) as the primary antibody overnight at 4°C. The staining was visualized using HRP-conjugated streptavidin (Dako) and diaminobenzidine substrate (Vector Laboratories).

For confocal microscopy, mouse tissues were fixed in 4% PFA at room temperature for 1–2 hours, washed, cryoprotected with 15% sucrose at 4°C overnight, and mounted in OCT compound (Sakura Finetek). Cryosections (approximately 20–50 μm) were incubated with biotin-conjugated goat anti-mouse Lyve-1 (5 μg/ml), rat anti-mouse CD31 mAb (clone MEC13.3, 1:50; BD), and Cy3-conjugated anti–α-SMA mAb (clone 1A4; 1:50; Sigma-Aldrich) overnight at 4°C; developed with streptavidin Cy5 (1:50; Invitrogen) and Alexa Fluor 488–conjugated donkey anti-rat IgG (1:100; Invitrogen); and mounted with ProLong Gold mounting medium supplemented with ToPro3 (Invitrogen). Sections were also stained with biotin-conjugated goat anti-mouse Lyve-1, rat anti-mouse CD31 mAb, hamster anti-podoplanin mAb (clone 8.1.1; 4 μg/ml; Santa Cruz Biotechnology Inc.), rat anti-mouse CD105 mAb (clone M7/18; 5 μg/ml; ebioscience), rat anti-mouse endomucin mAb (clone V7C7; 2 μg/ml; Santa Cruz Biotechnology Inc.), and rabbit anti-Prox1 (1:100; Abcam) in various combinations. For whole-mount staining, small intestines were fixed overnight in 2% PFA, and the tissues were treated with 3% BSA in serum-free protein block with 0.3% (v/v) Triton X-100 and subsequently incubated with rat anti-mouse CD31 mAb, hamster anti-mouse podoplanin mAb, biotinylated goat anti-mouse Lyve-1, or rabbit anti-Prox1. The samples were analyzed by confocal laser scanning microscopy using a Nikon C1 scanning head mounted on a Nikon ECLIPSE 2000U inverted microscope (Plan Apochromats dry objective lens, ×20, NA 0.75; Nikon Instruments Inc.). Z-stack images were collected at 1-μm steps with sequential laser excitation to eliminate bleed-through and with confocal parameters selected to minimize the thickness of the calculated optical section. Volume images from the confocal data sets were processed with IMARIS software (Bitplane) for 3-dimensional views of detailed vascular morphology. Images are presented as maximum-intensity projections of the Z-stacks. Hybrid vessels in EHC T-syn−/− mice were identified based on their mixed expression of blood and lymphatic markers. Confocal images at ×20 were used for quantification.

For transmission electron microscopy, specimens were fixed with 2% glutaraldehyde and 3% PFA solution, postfixed for 90 minutes with 1% osmium tetroxide, and then treated with 1% tannic acid (all in 0.1 M sodium cacodylate–HCl buffer, pH 7.2). After dehydration in graded ethanol and critical point drying, specimens were coated with Pt/C. For transmission electron microscopy, specimens were embedded in Epon 812 (TAAB), sectioned with an ultramicrotome, and examined with a J-7600 transmission electron microscope (Hitachi).

For intravital microscopy, mice were anesthetized with an intraperitoneal injection of tribromomethanol and amylene hydrate (Sigma-Aldrich) and placed on a heating pad (37°C) to maintain body temperature. A segment of small intestine was exteriorized, and 200 μl of lysine fixable dextran–FITC (150 kDa at 1 mg/ml; Invitrogen), India ink, or red fluorescent microbeads (diameter, 1 μm; Invitrogen) were injected via the left common carotid artery catheter. Intravital microscopy was conducted using an Eclipse E600FN microscope (Nikon) with a saline immersion ×4 objective. The microscope was equipped with a DC-330 CCD color camera (Dage-MTI) connected to a video recorder (Panasonic). Video was
recorded for subsequent frame-by-frame analysis to capture the arterial and venous phases of the flow.

**Immunoblotting.** Primary endothelial cells from lungs were lysed with RIPA buffer (Pierce) supplemented with complete protease inhibitor cocktail (Roche Applied Science). Protein concentration was quantified using the micro BCA protein assay kit (Pierce), and cell lysates containing 25 µg total protein were resolved by 5%–20% gradient SDS-PAGE. After blotting, Immobilon-P transfer membranes (Millipore) were blocked and probed with the following antibodies: anti-podoplanin, anti-Tie1, anti-angiopoietin-2, anti-Ang2, anti-VEGF-C (Santa Cruz Biotechnology Inc.), anti-lyve-1 (Novus Biologicals), anti-VEGFR3 (Abcam), anti-fibronectin (BD transduction laboratories), anti-integrin αv (R&D Systems), anti-FLA (Zymed, Invitrogen), and anti-β-actin (US Biologicals). For lectin blotting, cell lysates were immunoprecipitated with anti-podoplanin. The immunoprecipitated podoplanin was subjected to SDS-PAGE and transferred to a membrane and then probed with biotinylated goat anti-mouse IgG (Pierce). After washing, the membrane was reacted with HRP-conjugated streptavidin (Pierce) and developed using the ECL Western Blotting Detection System (Amersham).

**Lipid analysis.** For lipid staining, cryosections (10 µm thick) of lung were stained with Bodipy FL C_12_5 (40 µg in 200 µl solution; Invitrogen) (35). After 1 h, mice were anesthetized and the transport of fluorescent lipid was visualized under intravital microscopy.

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