Genetic Dissection of Tie Pathway in Mouse Lymphatic Maturation and Valve Development

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Objective—The genetic program underlying lymphatic development is still incompletely understood. This study aims to dissect the role of receptor tyrosine kinase with immunoglobulin-like and EGF (epidermal growth factor)-like domains 1 (Tie1) and Tie2 in lymphatic formation using genetically modified mouse models.

Approach and Results—We generated conditional knockout mouse models targeting Tie1, Tie2, and angiopoietin-2 in this study. Tie1ICD/ΔICD mice, with its intracellular domain targeted, appeared normal at E10.5 but displayed subcutaneous edema by E13.5. Lymph sac formation occurred in Tie2ICD/ΔICD mice, but they had defects with the remodeling of primary lymphatic network to form collecting vessels and valvulogenesis. Consistently, induced deletion of Tie1-ICD postnatally using a ubiquitous Cre deleter led to abnormal lymphangiogenesis and valve formation in Tie1ICD/ΔICD mice. In comparison with the lymphatic phenotype of Tie1 mutants, we found that the diameter of lymphatic capillaries was significantly less in mice deficient of angiopoietin-2, besides the disruption of collecting lymphatic vessel formation as previously reported. There was also no lymphedema observed in Ang2−/− mice during embryonic development, which differs from that of Tie1ICD/ΔICD mice. We further investigated whether Tie1 exerted its function via Tie2 during lymphatic development. To our surprise, genetic deletion of Tie2 (Tie2ICD/ΔICD) in neonate mice did not affect lymphatic vessel growth and maturation.

Conclusions—In contrast to the important role of Tie2 in the regulation of blood vascular development, Tie1 is crucial in the process of lymphatic remodeling and maturation, which is independent of Tie2. (Arterioscler Thromb Vasc Biol. 2014;34:1221-1230.)

Key Words: knockout mice ■ lymphatic abnormality ■ lymphatic vessel ■ Tie-1 receptor tyrosine kinase ■ Tie-2 receptor tyrosine kinase ■ valve

The formation of a functional lymphatic system, including initial lymphatics and collecting vessels with smooth muscle cell (SMC) coverage and intraluminal valves, is crucial for maintaining tissue fluid homeostasis.1–4 The key events of lymphatic development include lymphatic endothelial cell (LEC) differentiation,5 lymphoneovenous separation,6,7 the formation of primary lymphatic network,8,9 followed by the remodeling of the network to form a mature lymphatic system. The process of lymphatic remodeling and maturation involves the recruitment of peri-lymphatic SMCs, as well as valve development, in collecting vessels.10–14 The cellular and molecular mechanism underlying the events is still incompletely understood.

Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1) is a member of receptor tyrosine kinase family with a high degree of homology with Tie2. They are differentially required during vascular development. Tie2-mediated signals are crucial for the endothelial cell survival, maturation, and maintenance of blood vessel integrity.15–17 However, Tie2 expression is inhibited in LECs with high expression of prospero homeobox protein 1 (Prox1).18,19 In contrast, Tie1 is coexpressed with Prox1 by LECs.20 Mice null for Tie1 exhibit edema and hemorrhage with microvessels.16,20,21 Deletion of Tie1 was reported to result in abnormal lymphangiogenesis,20,22 including lymphatic overgrowth at initial stage but LEC apoptosis later with disrupted lymphatic network formation during embryonic development.20 Both angiopoietin-1 (Ang1) and Ang2 are ligands for Tie2 but do not bind to Tie1. It has been shown that Ang1 could induce Tie1 phosphorylation in cultured cells when Tie1 is coexpressed with Tie2.23 In vitro
biochemical analysis has shown that Tie1 could heterodimerize with Tie2.24 Therefore, Tie1 activation may be initiated indirectly by ligands binding to Tie2 in endothelial cells. However, there is also evidence suggesting that Tie1 may modulate blood vessel morphogenesis by downregulating Tie2-mediated signaling.25 Ang1-deficient mice die before the lymphatic development.26 However, Ang2 knockout mice show normal blood vascular development during embryogenesis27 but display abnormal recruitment of SMCs with lymphatic capillaries and defects with collecting lymphatic vessel formation and valve development.27,28 Interestingly, the lymphatic phenotype in Ang2 null mice could be rescued in the Ang1 knockin mice.27

In spite of these findings, it is still poorly understood about how Tie1 and Tie2 are involved in the process of lymphatic formation and remodeling. To investigate further the role of Tie1- and Tie2-mediated signals in lymphatic development, we generated conditional knockout mouse models targeting Tie1, Tie2, and Ang2. Findings from this study indicate that Tie1 is important in the process of remodeling primary lymphatic network to form collecting vessels and valvulogenesis, which is independent of Tie2.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Generation of Conditional Tie1-ICD\textsuperscript{Floxed} Mice
To investigate the function of Tie1 intracellular kinase domain in lymphatic development, we generated a conditional knockout model with its exon 15 and exon 16 flanked by loxP sites (Tie1\textsuperscript{Floxed}, Figure 1A). The 2 exons encode part of Tie1 intracellular domain (ICD) immediately after the transmembrane domain. To examine the floxed allele, we crossed Tie1-ICD\textsuperscript{Floxed} with Ella\textsuperscript{-}Cre mice to excise exon 15 and 16. Transcripts encoding the truncated Tie1 lacking the ICD (Tie1\textsuperscript{ΔICD}) were detected by reverse transcription polymerase chain reaction (Figure 1B), and its protein was confirmed by Western blot analysis (Figure 1C). The truncated Tie1 consists of the extracellular and transmembrane region plus a small intracellular fragment (ATCILLSNTPMETYWTS). As shown in Figure 1C, the size of the truncated Tie1 (Tie1\textsuperscript{ΔICD}) is \(~90\text{ kDa (817 amino acids). We noticed that the expression level of Tie1\textsuperscript{ΔICD} decreased dramatically in Tie1\textsuperscript{ΔICD/ΔICD} mice compared with that of wild-type mice. This may be because of the nonsense-mediated mRNA decay, which is triggered when ribosomes encounter the premature translation-termination codon.29,30 By immunostaining, we could detect weak staining of the truncated Tie1 (Tie1\textsuperscript{ΔICD}) in blood vessels, which is triggered when ribosomes encounter the premature translation-termination codon.29,30 By immunostaining, we could detect weak staining of the truncated Tie1 (Tie1\textsuperscript{ΔICD}) in blood vessels, which is triggered when ribosomes encounter the premature translation-termination codon.

[Figure 1. Generation of tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1) conditional knockout mice (Tie1-ICD\textsuperscript{Floxed}) and analysis of the vascular development in Tie1\textsuperscript{ΔICD/ΔICD} mice. A, Tie1-ICD\textsuperscript{Floxed}-targeting construct. Two loci of crossover in P1 bacteriophage (loxp) sites (red triangles) together with a neomycin resistance gene (neo) flanked by 2 flippase recognition target (FRT) sites (blue triangles) were introduced into introns 14 and 16, respectively, to flank exon 15 to 16 of Tie1. Founders were mated with Cre- or FLP (flippase)-expressing mice to generate models with the modified allele. DNA between the 2 loxp sites is removed by Cre recombinase-mediated excision and FRT-flanked neo cassette is excised by flippase. B, Analysis of the wild-type and mutant Tie1 (Tie1\textsuperscript{ΔICD}) transcripts by reverse transcription polymerase chain reaction. C, Immunoprecipitation and Western blot analysis of wild-type and mutant Tie1 protein in lung tissue lysate (E18.5, embryonic day 18.5). Arrow points to the truncated Tie1 protein band (Tie1\textsuperscript{ΔICD}). D, Immunostaining analysis of blood vascular development and Tie1 expression in Tie1\textsuperscript{ΔICD/ΔICD} mice in comparison with the heterozygous and wild-type mice (E10.5). As shown by whole-mount immunostaining for platelet endothelial cell adhesion molecule 1 (PECAM1), blood vessels formed normally in Tie1\textsuperscript{ΔICD/ΔICD} mice. E, Edema developed in Tie1\textsuperscript{ΔICD/ΔICD} mice compared with that of control mice. White arrow points to the hemorrhage in the tail tip of Tie1\textsuperscript{ΔICD/ΔICD} mice. Scale bar, 200 \(\mu\text{m}\) in D and 100 \(\mu\text{m}\) in E.]
vascular endothelial cells sustained for platelet endothelial cell adhesion molecule 1 (Figure 1D; E10.5, embryonic day 10.5) and also in LECs shown in the following figures. Interestingly, Tie1ΔICD/ΔICD mice displayed less severe vascular phenotypes in comparison with that of Tie1 complete knockout mice16,20,21 and survived to the later stage during embryonic development. Approximately two thirds of Tie1ΔICD/ΔICD mice of 10 litters were alive at E18.5 (total 69 embryos; Tie1ΔICD/ΔICD: 55.07%; Tie1ΔICD/ΔICD: 27.54%). Tie1ΔICD/ΔICD mice could be born alive, but none of them survived.

Abnormal Formation of Primary Lymphatic Network in Tie1ΔICD/ΔICD Mice

We then analyzed the vascular formation of Tie1ΔICD/ΔICD mice at different stages of embryogenesis. Mice homozygous for Tie1-ICD deletion appeared normal at E10.5, with the proper formation of blood vessels (Figure 1D) but developed back edema by E13.5 (Figure 1E). However, lymph sac formation occurred without obvious abnormality in Tie1ΔICD/ΔICD mice. Further analysis of LEC proliferation by BrdU (5-bromo-2'-deoxyuridine) labeling showed that there was no significant difference in LEC proliferation between Tie1ΔICD/ΔICD and control mice at E12.5 (Figure 2A and 2B). The LEC proliferating index was 21.03±3.03% (Tie1ΔICD/ΔICD, n=5), 23.91±4.66% (Tie1ΔICD/ΔICD, n=5), and 24.07±3.85% (Tie1+/−, n=5). Note that total Prox1 positive LEC number in embryo tissue sections decreased in Tie1ΔICD/ΔICD mice (47.88±5.29 cells/grid, n=5) compared with the heterozygous (56.30±5.68 cells/grid, n=5; P=0.0415) and wild-type control mice (53.10±5.03 cells/grid, n=5; P=0.1485; Figure 2C). Furthermore, edema in Tie1ΔICD/ΔICD mice became more severe at E15.5. By whole-mount immunostaining, we showed that the lymphatic network became disorganized in Tie1ΔICD/ΔICD mice as shown in Figure 2D. There was a significant increase in the number of abnormal lymphatic connections in Tie1ΔICD/ΔICD mice (per grid, white arrow in Figure 2D; 2.08±0.93, n=6) compared with Tie1ΔICD/ΔICD (0.27±0.14, n=6; P=0.0008) and wild-type control (0.19±0.14, n=4; P=0.0042). Also we observed isolated lymphatic fragments in the skin of Tie1ΔICD/ΔICD mice (per grid, arrowhead in Figure 2D; Tie1ΔICD/ΔICD, 0.44±0.29, n=6), which were rarely observed in the heterozygous (0.07±0.08, n=6; P=0.0131) and wild-type control (0.05±0.07, n=4; P=0.0321). Consistently, we observed a decrease of Prox1 positive LEC number in Tie1ΔICD/ΔICD mutants (305.72±64.31 cells/grid, n=6) in comparison with that of heterozygous (440.91±65.33 cells/grid, n=6; P=0.0074) and wild-type control at E15.5 (438.80±55.73

Figure 2. Defects with the formation of primary lymphatic network in Tie1ΔICD/ΔICD mice. A. Immunostaining for BrdU (green) and prospero homeobox protein 1 (Prox1; red) to examine lymphatic endothelial cell (LEC) proliferation in jugular lymph sac of E12.5 embryos. White arrows point to the double positive LECs (yellow). Quantification of proliferating LECs (BrdU/Prox1−) and total LECs (C) in the tissue sections across the lymph sac region of homozygous Tie1ΔICD/ΔICD, heterozygous Tie1ΔICD/ΔICD, and wild-type mice at E12.5. There is no significant difference in LEC proliferating index between Tie1ΔICD/ΔICD and control mice in spite of a trend toward less LECs in Tie1 mutant embryos. B. Whole-mount immunostaining for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1; green) and Prox1 (red) with the back skin of E16.5 embryos. Note that deletion of Tie1-ICD disrupts proper formation of lymphatic network compared with that of control mice (white arrowhead points to an isolated lymphatic fragment and arrows point to abnormal lymphatic connection). E and F. Quantification of LECs (Prox1+) and lymphatic branch points in the skin of homozygous Tie1ΔICD/ΔICD, heterozygous Tie1ΔICD/ΔICD, and wild-type mice. Scale bar, 25 μm in A and 200 μm in D.
cells/grid, n=4; P=0.0138; Figure 2E). There was no significant difference in lymphatic branch points in skin tissue at this stage (Tie1ΔICDΔCD: 16.96±5.27 points/grid, n=6; Tie1ΔICDΔCD, 18.27±3.27 points/grid, n=6; wild-type Tie1+/+: 17.26±2.61 points/grid, n=4; Figure 2F). Interestingly, at a later stage of embryonic development (E17.5), Tie1ΔICDΔCD mutants appeared swollen with increased lymphatic vessel branching points and diameter (Figure I in the online-only Data Supplement) compared with wild-type and heterozygous mice. This may be secondary to the subcutaneous edema. A similar observation was also made with lymphatic capillaries in the intestine (Shang Z, He Y, unpublished data, 2014).

Failure of Collecting Lymphatic Vessel Formation and Valvulogenesis in Tie1ΔICDΔCD Mice
We found that Tie1 was highly expressed in lymphatic valves in embryos (Figure 3A) and also in postnatal mice (Figure 3B). As shown in Figure 3C, white arrow points to one collecting lymphatic vessel with strong staining for Prox1 but weak for lymphatic vessel endothelial hyaluronan receptor 1 in the heterozygous Tie1ΔICDΔCD mice (E15.75). However, the remodeling of lymphatic capillaries to form collecting vessels did not occur in Tie1ΔICDΔCD mice (Figure 3C). Lack of collecting lymphatic formation in Tie1 mutant mice was further confirmed by Prox1 staining of back skin at E18.5 (Figure 3D). There were also no lymphatic valves detected in Tie1ΔICDΔCD mice as visualized by integrin-α9 staining (Figure 3D). Consistent with the observation with mesentery lymphatic development,10,12,31 the process of collecting vessel formation and valvulogenesis starts at the same time in skin. As shown in Figure II in the online-only Data Supplement, the valve morphogenesis started with the formation of LEC clusters, which was first detected at E15.5 (arrow). Valve forming region with condensed LECs became more obvious by E16.5 (arrow). LEC condensation was not observed in Tie1ΔICDΔCD mice. Similar defects with collecting lymphatic vessel formation and valve development were also observed in the mesentery of intestine of Tie1ΔICDΔCD mice (Figure 4A–4C).

Requirement of Tie1 in Postnatal Lymphatic Growth and Maturation
To examine the role of Tie1 in postnatal lymphangiogenesis, we generated the doubly transgenic mice (Tie1-ICDαα; UBC-CreERT2) by mating Tie1-ICDαα mice with the transgenic mouse line ubiquitously expressing CreERT2 (a Cre recombinase fused to a mutant estrogen ligand-binding domain, UBC-CreERT2).32 Tie1 deletion was induced by the intragastric injection of tamoxifen into new-born pups from postnatal day 1 to 3, and Tie1-ICDαα; UBC-CreERT2 mice were used as control. Mice were analyzed at P7 or at a later stage. As shown in Figure 5A, Tie1-ICDαα; UBC-CreERT2 mice displayed defective formation of lymphatic network in tail skin. Tie1-ICD deletion efficiency is shown in Figure 5B. By quantification analysis, we showed that there was a significant decrease in lymphatic ring structure in tail skin of Tie1 mutant mice compared with that of control mice (Tie1-ICDαα; 7.75±3.04 lymphatic ring/grid, n=3; Tie1-ICDαα; 31.38±2.49 lymphatic ring/grid, n=4; P<0.0001; Figure 5F). The abnormal lymphatic network in Tie1-ICDαα; mice was validated by fluorescence microlymphography using fluorescein isothiocyanate-dextran (Figure 5C), and the development of feet edema was also observed in Tie1 mutant mice (Figure 5D). We further demonstrated that lymphatic vessels became sparse and disorganized in the ear skin of Tie1-ICDαα; mice (Figure 5E). There was a significant decrease of lymphatic density in Tie1-ICD mutants.
compared with control mice (Tie1-ICDΔICD−/−: 31.4±6.62×10^4 μm^2/grid, n=3; Tie1-ICDΔICD+/+: 42.83±3.09×10^4 μm^2/grid, n=4; P=0.0268; Figure 5G). To examine collecting lymphatics and valves in Tie1-ICDΔICD−/− mice, we performed immunostaining for platelet endothelial cell adhesion molecule 1, lymphatic vessel endothelial hyaluronan receptor 1, and integrin-α9 with ear skin from the mutant and control mice. We found that lymphatic vessels became sparse and valves in the precollecting lymphatic vessels were not properly formed in the ventral side of ear skin (Figure 6A). There was a significant decrease of lymphatic valves in Tie1-ICDΔICD−/− mutants (2.13±0.74 valves/grid, n=4) in comparison with that of control mice (6.14±0.68 valves/grid, n=5, P<0.0001). In the dorsal side of ear skin, valves in the large collecting vessels were formed in Tie1-ICDΔICD−/− mice (Figure 6B). However, the collecting lymphatics in Tie1-ICDΔICD−/− mice showed strong staining with lymphatic vessel endothelial hyaluronan receptor 1, which did not occur in those of control mice (Figure 6B). This suggests that the deletion of Tie1 postnatally may impair the maturation of collecting lymphatic vessels.

**Distinct Lymphatic Abnormality in Ang2 Null Mice**

To explore the connection between Tie1 and Ang2 in lymphatic development, we generated a conditional knockout mouse model targeting Ang2, with its exon 3 flanked by loxP sites (Ang2Flox, Figure 7A). To examine the floxed allele, we crossed Ang2Flox/Flox with EIIa-Cre mice to excise exon 3, and there was no Ang2 expression detected in Ang2 null mice by Western blot analysis (Figure 7B). In addition to the defective formation of collecting lymphatic vessels as previously reported, we found that the diameter of lymphatic vessels was altered in Ang2−/− mice compared with that of control mice (Figure 7C, 7D, and 7G). By quantification, there was a significant decrease in lymphatic vessel diameter (Ang2−/−: 12.97±0.93 μm, n=4; Ang2+/−: 18.51±0.83 μm, n=4; P=0.0001) and LEC number in vessel wall (Ang2−/−: 6.90±0.84 LECs/100 μm lymphatic vessel, n=4; Ang2+/−: 9.56±0.76 LECs/100 μm lymphatic vessel; P=0.0033) of Ang2−/− mice compared with control mice at E18.5 (Figure 7D–7F). Consistent with previous findings, there was abnormal SMC recruitment with lymphatic capillaries (Figure III in the online-only Data Supplement) and no formation of lymphatic valves in Ang2 null mice as visualized by integrin-α9 staining (Figure 7G). Similar observations with defects in collecting vessel formation were also made in the mesentery of intestine (Figure III in the online-only Data Supplement). However, there was no lymphedema observed in Ang2 null mice during embryogenesis in spite of the lymphatic abnormalities, which differed from those of Tie1ΔICD/ΔICD mice.
Tie2Flox/− line to generate the doubly transgenic mice (UBC-CreERT2 in lymphangiogenesis, we used the mouse died by E10.5 as previously reported. To study the role of Tie2 EIIa-Cre mice to excise exon 1 and found that Tie2 null mice development in there were no obvious defects observed with lymphatic development in mice homozygous for Tie1 ΔICD, which is similar to the reported hypomorphic Tie1 neo/neo mice.20 In spite of the low level of Tie1 ΔICD expression, we noticed that Tie1 ΔICD mice displayed less severe vascular phenotype in comparison with that of Tie1 null mice.16,20,21 It is, therefore, possible that Tie1 ΔICD retains the function of Tie1 and that the lethality of Tie1 AICD/ΔICD mice may result from the low expression level of Tie1 ΔICD. It remains to be validated whether the biological function of Tie1 relies on its intracellular kinase domain. Furthermore, we showed that the formation of lymph sac occurred in Tie1 AICD/ΔICD mice, butLEC number decreased in embryo sections E12.5, as well as whole-mount skin tissues (E15.5) in Tie1 mutants, compared with that of control mice.

Normal Lymphatic Development in Tie2-Deficient Mice

We found that Tie2 was weakly expressed in lymphatic vessels (Figure 8A). To investigate the biological role of Tie2 in lymphatic vessel formation, we generated a conditional knockout mouse model with exon 1 of Tie2 gene flanked by loxP sites (Tie2Flox, Figure 8B). We crossed Tie2FloxFlox with Elha-Cre mice to excise exon 1 and found that Tie2 null mice died by E10.5 as previously reported. To study the role of Tie2 in lymphangiogenesis, we used the UBC-CreERT2 mouse line to generate the doubly transgenic mice (Tie2FloxFlox; UBC-CreERT2), and Tie2FloxFlox;UBC-CreERT2 mice were used as control. Tie2 deletion was induced by intragastric injection of tamoxifen into new-born pups as described above, and the deletion efficiency was shown in Figure 8C. Surprisingly, there were no obvious defects observed with lymphatic development in Tie2FloxFlox mice (Figure 8D and 8E). Furthermore, lymphatic valves (white arrow) in precollectors and large collecting vessels also formed normally in Tie2 mutants compared with those of control mice (Figure 8G).

Discussion

We have shown in this study that the remodeling of primary lymphatic network to form collecting vessels including valve development was disrupted in mice homozygous for Tie1 mutation (Tie1 ΔICD/ΔICD), in addition to the abnormal lymphangiogenic growth as previously reported.20,22 Although mice null for Ang2 also displayed defective formation of lymphatic vessels, the phenotypes were different from that of Tie1 ΔICD/ΔICD mice, including thinner lymphatic capillaries with abnormal SMC coverage and lack of lymphedema. We have further demonstrated that postnatal deletion of Tie2 (Tie2 ΔICD/ΔICD) did not affect lymphatic growth. Therefore, independent of Tie2, Tie1 is important in the process of lymphatic remodeling and maturation, which differs from that of Ang2 deficiency.

It is worth pointing out that Tie1 allele (Tie1 ΔICD/ΔICD) of the mutant mice developed in this study appeared to be hypomorphic with reduced expression of Tie1 ΔICD, which is similar to the reported hypomorphic Tie1 neo/neo mice.20 In spite of the low level of Tie1 ΔICD expression, we noticed that Tie1 ΔICD/ΔICD mice displayed less severe vascular phenotype in comparison with that of Tie1 null mice.16,20,21 It is, therefore, possible that Tie1 ΔICD retains the function of Tie1 and that the lethality of Tie1 ΔICD/ΔICD mice may result from the low expression level of Tie1 ΔICD. It remains to be validated whether the biological function of Tie1 relies on its intracellular kinase domain. Furthermore, we showed that the formation of lymph sac occurred in Tie1 ΔICD/ΔICD mice, but LEc number decreased in embryo sections E12.5, as well as whole-mount skin tissues (E15.5) in Tie1 mutants, compared with that of control mice.

Figure 5. Requirement of tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1) in postnatal lymphangiogenesis. **A** and **B**, Analysis of lymphatic vessels by immunostaining for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1; green), platelet endothelial cell adhesion molecule 1 (PECAM1; red), and Tie1 (white) in the tail skin (P7, postnatal day 7). Note that Tie1 deletion is efficient as shown in **B**, **C**, Fluorescein isothiocyanate (FITC)-dextran microlymphangiography to examine the lymphatic patterning and functionality in the ear skin of Tie1 ΔICD ΔICD/ΔICD and control mice (10-week-old). Dotted lines indicate the injection site of FITC-dextran. Note that the draining lymphatic vessels of Tie1 ΔICD ΔICD/ΔICD mice are disorganized compared with those of Tie1 ΔICD ΔICD/ΔICD mice. **D**, Lymphedema in Tie1 ΔICD ΔICD/ΔICD mice was observed as the swelling of their feet. Asterisk points to the swollen toe. **E**, Analysis of lymphatic vessels by immunostaining for LYVE1 showed that lymphatic network became much sparse in the ear skin of Tie1 ΔICD ΔICD/ΔICD mutants compared with that of control mice. **F**, Quantification of lymphatic ring structures in the tail skin of Tie1 ΔICD ΔICD/ΔICD and control mice. **G**, Quantification of lymphatic vessel density in the ear skin of Tie1 ΔICD ΔICD/ΔICD- and control mice. Scale bar, 100 μm in **A** and **B** and 500 μm in **E**.

Normal Lymphatic Development in Tie2-Deficient Mice

We found that Tie2 was weakly expressed in lymphatic vessels (Figure 8A). To investigate the biological role of Tie2 in lymphatic vessel formation, we generated a conditional knockout mouse model with exon 1 of Tie2 gene flanked by loxP sites (Tie2Flox, Figure 8B). We crossed Tie2FloxFlox with Elha-Cre mice to excise exon 1 and found that Tie2 null mice died by E10.5 as previously reported. To study the role of Tie2 in lymphangiogenesis, we used the UBC-CreERT2 mouse line to generate the doubly transgenic mice (Tie2FloxFlox; UBC-CreERT2), and Tie2FloxFlox;UBC-CreERT2 mice were used as control. Tie2 deletion was induced by intragastric injection of tamoxifen into new-born pups as described above, and the deletion efficiency was shown in Figure 8C. Surprisingly, there were no obvious defects observed with lymphatic development in Tie2FloxFlox mice (Figure 8D and 8E). Furthermore, lymphatic valves (white arrow) in precollectors and large collecting vessels also formed normally in Tie2 mutants compared with those of control mice (Figure 8G).
This is also consistent with results from our postnatal study where induced deletion of Tie1-ICD resulted in decreased lymphatic density as shown in Figure 5. Furthermore, lymphatic network was disorganized in Tie1 mutants during embryonic and postnatal development. The findings imply an important role of Tie1 in the formation and maintenance of lymphatic integrity. In contrast to the previous reports, we did not detect a significant difference in LEC proliferating index between Tie1ΔICD/ΔICD and control mice at E12.5. However, we did observe that there was an increase in the diameter and density of lymphatic vessels in Tie1ΔICD/ΔICD mice at later stage of embryonic development (E17.5). It is likely that this may be secondary to tissue edema resulting from increased vascular leakage and abnormal lymphatic draining in Tie1 mutants.

Although Ang2-deficient mice had abnormal lymphatic development, as also shown by other researchers, we found that Ang2 null mice and Tie1 mutants displayed distinct lymphatic defects. Consistent with previous reports, we observed that there was abnormal coverage of SMC with lymphatic capillaries in Ang2-deficient mice (Figure III in the online-only Data Supplement) but not in Tie1 mutants. Interestingly we also showed that the diameter of lymphatic capillaries and the number of LECs in the vessel wall were significantly less in Ang2−/− mice than those of littermate controls. However, in spite of the lymphatic defects, there were...
no lymphedema observed in Ang2 null mice during embryonic development. Furthermore, although it has been shown that genetic insertion of Ang1 cDNA into Ang2 locus in the knockin model can rescue the lymphatic defects of Ang2 null mice, it is unclear how Ang1 exerts such an effect on LECs, where Tie2 is lowly expressed. Findings from this study have further demonstrated that induced deletion of Tie2 in neonatal mice did not affect lymphatic development. Because there is no direct interaction of Ang1 or Ang2 with Tie1, it remains to be explored how Tie1 and angiopoietins exert their function, independent of Tie2, during the lymphatic remodeling and valvulogenesis.

The formation of collecting lymphatics involves the fusion of lymphatic capillaries into larger ones containing intraluminal valves. In our previous study, we showed that integrin-α9–positive valve structures were observed mainly at sites where 2 lymphatic vessels converge. It is likely that valve morphogenesis may occur simultaneously during the lymphatic remodeling to form the collecting lymphatics and that the pre-existing endothelium of fused lymphatic...
capillaries may provide an important base during valvulogen-
esis. Although the mechanism underlying the formation of
collecting lymphatics and valves awaits further investigation,
one interesting observation is that primary lymphatic network
exerts fluid draining function even before the establishment of
a mature lymphatic network containing collecting lymphatics
with intraluminal valves. This has been shown in genetically
modified mouse models where lymphedema occurs because of
the defective formation of primary lymphatic network, as shown
in Vegfr3<sup>ΔLIVβ/ΔLIVβ</sup> or Vegfr3<sup>ΔKonut/ΔKonut</sup> mice,<sup>6</sup> at a stage
(eg, E13.5) when collecting lymphatics has not been formed.
The formation of valved collecting lymphatic vessels initi-
ates at approximately E15.5 during murine embryonic develop-
ment as shown in this study (Figure 3C; Figure II in the
online-only Data Supplement) and also by other research-
ers.<sup>10–12</sup> It is, therefore, reasonable to speculate that the initial
flow inducible Tie1 in lymphatic remodeling and maturation
by LECs, particularly in lymphatic valves. The mechanism of
Tie1.36,37 Tie1 is widely expressed by endothelial cells of
both blood vascular and lymphatic vessels during embry-
onic development. However, it is downregulated postnatally
in blood vasculature, and constitutive expression of Tie1 is
only maintained in selected areas of the adult vasculature<sup>18</sup>
or induced in areas constantly exposed to disturbed flow, such as
at vascular branching points.<sup>18</sup> Interestingly, consistent with
previous findings,<sup>10</sup> we found that Tie1 was highly expressed by
LECs, particularly in lymphatic valves. The mechanism of
flow inducible Tie1 in lymphatic remodeling and maturation is
still to be investigated. Furthermore, although several other
factors have been reported to participate in the process of lymph-
atic valve development,<sup>14</sup> it is still to be examined whether
Tie1 and the previously reported genes are directly required for
valve formation or the observed disruption of valvulogen-
esis may be secondary to the defective formation of collecting
lymphatic vessels. It is also interesting to find out how these
factors coordinate to control collecting vessel formation and
maturation and whether they work in the same pathway or in
different pathways controlling specific cellular programs.

In summary, we show in this study that Tie1, independent of Tie2, plays important roles in lymphatic formation, remod-
eling, and valvulogenesis. Although the detailed molecular mechanism underlying the process awaits further investiga-
tion, it is interesting to speculate that shear stress inducible
Tie1 may participate in sensing dynamic changes of fluid flow
when the lymph circulation is established during embryogen-
esis. Studies along the line will provide further insights into
the cellular and molecular events responsible for lymphatic
development.

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**Disclosures**

None.

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PRX1, and FOXC2 cooperate to control connexin37 and calcineurin
B is required for lymphatic network formation, remodeling, and valve develop-
GATA2 mutations in patients with MDS/AML or MonoMAC syndrome
and primary lymphedema reveal a key role for GATA2 in the lymphatic
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**Significance**

Tyrosine kinase with immunoglobulin-like and EGF-like domain (Tie) receptor tyrosine kinases play crucial roles in blood vascular development. However, their function in lymphatic development is poorly understood. We have shown in this study that the formation of collecting lymphatic vessels including valvulogenesis was disrupted in mice homozygous for Tie1 mutation during embryonic and postnatal development. Although mice null for angiopoietin-2 also displayed defective formation of lymphatic vessels, the phenotypes were different from that of Tie1−/− mice, including thinner lymphatic capillaries with abnormal smooth muscle cell coverage and lack of lymphedema. Surprisingly, we have found that postnatal deletion of Tie2 did not affect lymphatic network formation. These findings demonstrate the essential requirement of Tie1 but not Tie2 in the regulation of lymphatic remodeling and maturation and advance our current understanding of Tie pathway in vascular development.
Genetic Dissection of Tie Pathway in Mouse Lymphatic Maturation and Valve Development

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Supplemental Figure I. Abnormal lymphangiogenesis in \( \text{Tie}^\Delta \text{ICD}/\Delta \text{ICD} \) skin (E17.5). A-C.

Analysis of lymphatic vessels by immunostaining for LYVE-1 (red) in the back skin of embryos (E17.5). Note that there was increased lymphatic vessel branching points (\( \text{Tie}^{\Delta \text{ICD}/\Delta \text{ICD}} \): 70.85 ± 7.42, n=4; \( \text{Tie}^{+/\Delta \text{ICD}} \): 54.47 ± 3.53, n=3; \( \text{Tie}^{+/+} \): 57 ± 5.96, n=3) and diameter (\( \text{Tie}^{\Delta \text{ICD}/\Delta \text{ICD}} \): 29.41 ± 4.92 μm, n=4; \( \text{Tie}^{+/\Delta \text{ICD}} \): 20.40 ± 3.16 μm, n=3; \( \text{Tie}^{+/+} \): 19.05 ± 2.70 μm, n=3) in \( \text{Tie}^{\Delta \text{ICD}/\Delta \text{ICD}} \) mutants compared with those of control mice. Scale bar: 300 μm.
Supplemental Figure II. Analysis of the lymphatic valve formation in skin. Analysis of lymphatic vessels by immunostaining for PROX1 (green) in the back skin of embryos (E14.5-16.5). The process of valve morphogenesis initiated with the formation of LEC clusters (strong PROX1 expression, arrow), which was detected at E15.5 (not at E14.5). Valve forming region with condensed LECs became more obvious by E16.5 (arrow). LEC condensation was not observed in Tie1^{ ΔICD/ΔICD} mice. Scale bar: 50 μm.
Supplemental Figure III. Abnormal formation of collecting lymphatic vessels and valves.
in the mesentery of Ang2<sup>−/−</sup> mice. **A.** Analysis of collecting lymphatic vessel formation by immunostaining for PROX1 (green), αSMA (red) with mesentery (E18.5). Note that the remodeling of primary lymphatic network to form collecting vessels does not occur in the Ang2 null mice and that there is abnormal recruitment of smooth muscle cells with capillary lymphatic vessels (white arrow). **B.** Analysis of lymphatic vessels and valves (white arrows) by immunostaining for Prox1 (green), Integrin-α9 (red) in the mesentery (E18.5). There was also no formation of lymphatic valves in the mesentery of Ang2 null mice. Scale bar: 100 μm.
Material and Method

Generation of *Tie1-ICD*\(^{\text{Flox}}\), *Tie2*\(^{\text{Flox}}\), *Ang2*\(^{\text{Flox}}\) conditional knockout mice

All animal experiments were performed in accordance with the institutional guidelines of Soochow University Animal Center. For construction of targeting vectors, a 10 kb genomic fragment was retrieved from BAC clones bMQ-304L4, bMQ-383G19, or bMQ-440K9 for *Tie1*, *Tie2* and *Ang2* respectively. For *Tie1-ICD*\(^{\text{Flox}}\) targeting construct, the first loxP was inserted into intron 14, and the second loxP together with neomycin-resistant gene flanked by FRT sites was inserted into intron 16. For *Tie2*\(^{\text{Flox}}\) targeting construct, the first loxP was inserted into the 5' region upstream of transcription initiation site, and the second loxP together with neomycin cassette was inserted into intron 1. For *Ang2*\(^{\text{Flox}}\) targeting construct, the first loxP was inserted into intron 2, and the second loxP together with neomycin cassette was inserted into intron 3. The constructs were linearized by NotI and electroporated into R1 embryonic stem cells. ES cell screening and chimeric mice generation were performed as previously described. Genetic transmission was confirmed by backcrossing the chimera with C57BL/6J mice and the Neo-cassette was removed using FLPeR mice as previously described. The floxed mice used in this study were maintained in C57BL/6J with at least four backcrosses. In all the phenotype analysis, littermates were used as control.

To examine the floxed alleles, *Tie1-ICD*\(^{\text{Flox}}\), *Tie2*\(^{\text{Flox}}\) and *Ang2*\(^{\text{Flox}}\) mice were crossed with EIIA-Cre mice [23] respectively to remove the genomic DNA between the two loxP sites. Mice were genotyped by PCR using primers as follows. For the genotyping of *Tie1-ICD* knockout allele, the primers used were forward primer (5'-TTTGGCAACTTACCATGAACCTG-3'), reverse primer 1
(5’-TTAGACAGATAAGTCAGAGCCAGAC-3’), and reverse primer 2
(5’-GCATAGCTGGATCAGTCTCCAGGACC-3’), to amplify a 192 bp fragment for the wildtype allele and a 520 bp for the knockout allele. For the genotyping of Tie2 knockout allele, the primers used were forward primer (5’-GACATAATCAGTCTGTTGGGTC-3’), reverse primer 1 (5’-AATACTGAATCCGGTGAGCTTG-3’), and reverse primer 2 (5’-AGCTCCGACCAGATTCCACAGCCATTAGC-3’), to amplify a 574 bp fragment for the wildtype allele and a 341 bp for the knockout allele. For the genotyping of Ang2 knockout allele, the primers used were forward primer (5’-AAGCTTGCCATGTCCAAGCTC-3’), reverse primer 1 (5’-TGATCTTACAGTGCCCACGCAG-3’), and reverse primer 2 (5’-GAAGCCGCGGTGCATGCAAGTGAGTGAATGTG-3’), to amplify a 122 bp fragment for the wildtype allele and a 626 bp for the knockout allele.

**Induction of gene deletion**

Induction of gene deletion was performed as previously described by tamoxifen treatment (Sigma-Aldrich) ³. Briefly, new-born pups were treated by intragastric injection from postnatal day 1 for 3 days, and mice (1-week-old) were treated by intraperitoneal injection for 5 sequential days. Mice were analyzed at P7 or at a later stage up to 10-week-old.

**Analysis of Tie1ΔICD expression by RT-PCR**

Embryos (E10.5) were collected and homogenized in Trizol (Invitrogen). RNA extraction and reverse transcription was performed following standard procedures. For PCR amplification of the cDNA fragment coding for the intracellular region of Tie1, the following primers were
used: forward primer: 5′-GAAGAAGCTGCCTACATCGGAG-3′; reverse primer: 5′-TGTGAATGAACCTCTCACTAAG-3′. The PCR products were a 573 bp fragment for wildtype Tie1 and a 251 bp for Tie1^{ICD}.

**Immunoprecipitation and western blot analysis**

For the analysis of in vivo expression of wildtype Tie1 and Tie1^{ICD}, lung tissues from E18.5 embryos were collected and homogenized as previously described. Equal amount of protein was used for analysis and Tie1 was immunoprecipitated using goat anti-human Tie1 antibody (R&D Systems, AF619). The immunocomplexes were pulled down using Protein G-Sepharose 4B (Zymed, 101242), and analyzed by western blot using the same antibody or rabbit anti-human Tie1 (Santa Cruz, SC-9025). Antibodies used for Tie2 and Ang2 expression analysis were anti-Tie2 (Santa Cruz, SC-324), anti-Ang2 (a kind gift from Dr. Gou-Young Koh, KAIST, Korea) respectively. Beta-actin (Santa Cruz, SC-47778) was used as the loading control.

**Immunostaining**

For whole-mount immunostaining with the tail skin (P7), embryonic back skin, ear skin and mesentery, tissues were harvested and processed as previously described. The antibodies used were rat anti-mouse PECAM (BD Pharmigen, 553370), rabbit anti-mouse LYVE1 (Abcam, ab14917), rabbit anti-human Prox1 (Abcam, ab11941), goat anti-human Tie1 (R&D, AF619), goat anti-mouse Tie2 (R&D, AF762), goat anti-mouse Integrin a9 (R&D, AF3827), Cy3-conjugated mouse anti-mouse αSMA (Sigma, C6198). Appropriate Alexa488, Alexa594, Alexa633 (Invitrogen), or Cy5 (Jackson) conjugated secondary antibodies were used for
staining. Fluorescently labeled samples were mounted with Vectashield (VectorLabs), and analyzed with a confocal microscope (Olympus FluoView 1000), Olympus BX51 or Leica MZ16F fluorescent dissection microscope.

**Cell proliferation analysis**
Mice were injected intraperitoneally with 5-Bromo-2’-deoxyuridine (BrdU) (0.1 mg/gram body weight, Sigma-Aldrich) two hours before tissue collection. Mouse embryos (E12.5) were fixed in 4% PFA overnight at 4°C, then dehydrated and embedded in paraffin. BrdU incorporation was detected using rat anti-BrdU (ABD serotec, MCA2060B), and followed by staining with Alexa488-Donkey-anti-Rat (Invitrogen). LECs were identified by immunostaining for Prox1 as described above. BrdU⁺ / Prox1⁺ doubly positive cells were examined under Olympus BX51 fluorescence microscope and quantified.

**Quantification of lymphatic vessel parameter and valves**
For the quantification of lymphatic parameters in ear and embryonic back skin, eight or more images (with x 200 magnification) were taken in similar regions with a confocal microscope (Olympus FluoView 1000) and kept constant for all the samples. Lymphatic vessel density and capillary diameter were measured and analyzed using Image Pro Plus (MediaCybernetics, Inc., Bethesda, MD) as previously described ⁵. Lymphatic valves, branch points and lymphatic endothelial cell number were counted manually. For the quantification of tail lymphatic capillary rings, images were taken under a fluorescence microscope and lymphatic ring structures were quantified.
Fluorescence microlymphography

The functionality of the lymphatic network in Tie1-ICD<sup>UCK0</sup> (Tie1-ICD<sup>Flox</sup>; UBC-CreERT2) and control mice was assayed by fluorescence microlymphangiography using fluorescein isothiocyanate (FITC)-conjugated dextran (FITC-dextran 2000, Sigma-Aldrich), as previously described<sup>5</sup>. The lymphatic vessels were examined using a fluorescence dissection microscope.

Statistical analysis

Statistical analysis was performed with the unpaired $t$ test. All statistical tests were two-sided.

Reference