Organ-Specific Lymphangiectasia, Arrested Lymphatic Sprouting, and Maturation Defects Resulting From Gene-Targeting of the PI3K Regulatory Isoforms p85α, p55α, and p50α

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The phosphoinositide 3-kinase (PI3K) family has multiple vascular functions, but the specific regulatory isoform supporting lymphangiogenesis remains unidentified. Here, we report that deletion of the Pik3r1 gene, encoding the regulatory subunits p85α, p55α, and p50α, impairs lymphatic sprouting and maturation, and causes abnormal lymphatic morphology, without major impact on blood vessels. Pik3r1 deletion had the most severe consequences among gut and diaphragm lymphatics, which share the retroperitoneal anlage, initially suggesting that the Pik3r1 role in this vasculature is anlage-dependent. However, whereas lymphatic sprouting toward the diaphragm was arrested, lymphatics invaded the gut, where remodeling and valve formation were impaired. Thus, cell-origin fails to explain the phenotype. Only the gut showed lymphangiectasia, lymphatic up-regulation of the transforming growth factor-β co-receptor endoglin, and reduced levels of mature vascular endothelial growth factor-C protein. Our data suggest that Pik3r1 isoforms are required for distinct steps of embryonic lymphangiogenesis in different organ microenvironments, whereas they are largely dispensable for hemangiogenesis. Developmental Dynamics 238:2670–2679, 2009. © 2009 Wiley-Liss, Inc.

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INTRODUCTION

Phosphoinositide 3-kinases (PI3K) control cell size, metabolism, differentiation, survival, migration, and proliferation (Engelman et al., 2006; Fruman and Bismuth, 2009). PI3K-mediated pathways are being pursued as pharmacological targets for halting tumor growth, metastasis, and angiogenesis (Stephens et al., 2005; Garcia-Echeverria and Sellers, 2008), but drug-specificity remains challenging due to widespread expression of PI3K proteins. Identify-
ing and targeting specific subunits may help address those concerns.

There are four subgroups of PI3K enzyme (class Ia, Ib, II, and III) that differ in their regulation and substrate selectivity. Growth factor receptors activate primarily the class Ia subgroup, which are dimeric proteins containing a catalytic subunit (p110α, p110β, or p110δ) and a regulatory subunit (p85α, p55α, p50α, p85β, or p55γ). The dimers are targeted to membrane-associated signaling complexes through several protein interaction domains in the regulatory subunits, and through a Ras-binding domain in the catalytic subunit. Vascular cells express multiple class Ia catalytic and regulatory isoforms. A reasonable hypothesis is that different regulatory subunits afford specificity to the multiple (lymph)angiogenic signaling receptors linked to PI3Ks, including Tie1/Tie2 (Peters et al., 2004), and vascular endothelial growth factor receptor-1 (VEGFR-1),

Fig. 1. Intestinal lymphatic defects in Pik3r1 null newborns. A,B: Gross anatomy of a wild-type (+/+) and Pik3r1 (-/-) null newborn littermates. Chyloous ascites was evident in the intact peritoneum of Pik3r1 null mice (Supp. Data). Intestinal edema (white arrow), and liver necrosis (black arrows) are indicated in the Pik3r1 null organs. C–F: Intestinal lymphatics stained by whole-mount immunofluorescence for LYVE1 (C,D), vascular endothelial growth factor receptor-3 (VEGFR-1; green, E,F), and alpha smooth muscle actin (SMA; orange, that bleeds through the green channel [E,F]). D: The white arrow highlights the lack of intestinal lymphatics. E,F: The white arrows indicate segments of the mesenteric collectors highlighting the difference in lymph vessel diameter and morphology; the red arrow in E highlights a lymphatic valve of the sort lacking in the null mouse specimens. G,H: Intestinal lymphatics LYVE1-stained by whole-mount immunoperoxidase method. LYVE1+ presumptive macrophages in the mesentery (Kim et al., 2007; Kubota et al., 2009) are marked with asterisks. H: Brown-stained lymphatic vessels (lacteals) inside the villi are indicated with white arrows. I,J: Confocal immunofluorescence for VEGFR3 (green) and SMA (red). The white arrows highlight the increased diameter of the lymphatic collectors of Pik3r1 null (J) compared with wild-type mice (I). K,L: Whole-mount immunofluorescence of newborn small intestine stained for SMA. Blood vessels are highlighted by this method because they have higher numbers of smooth muscle cells (arteries>veins>>lymphatics). Increased blood vessel branching (arrows, K vs. L) at the level of the arcades is visible within the white boxes. Scale bars are as indicated in the panels.
-2, and -3 (Saharinen et al., 2004). However, no step in lymph vessel development has been ascribed to a specific PI3K regulatory subunit.

Our previous studies prompted us to investigate the hypothesis that regulatory isoforms encoded by the Pik3r1 gene (p85α/p55α/p50α) have more critical functions in lymphangiogenesis than in hemangiogenesis. Specifically, we previously showed that Pik3r1 null mice survive to birth but develop chylos ascites, a hallmark of lymphatic insufficiency (Fruman et al., 2000). Mice lacking only p85α (p55α/p50α isoforms intact; Fruman et al., 1999), p55α/p50α (p85α isoform intact; Chen et al., 2004), or p85β encoded by Pik3r2 (Ueki et al., 2002) showed no lymphatic defects. However, chylos ascites was also reported in newborn mice with a point mutation abrogating the interaction between p110α and Ras (Gupta et al., 2007), implicating p110α as an important Ras effector in perinatal lymphatic development. Here, we report that the class Ia regulatory isoforms encoded by Pik3r1 are required for organ-specific steps of lymphangiogenesis.

RESULTS AND DISCUSSION

We sought to determine if abnormal lymphatic development underlies chylos ascites (Fruman et al., 2000) and intestinal edema (Fig. 1A,B) in Pik3r1 null mice as follows: Using LYVE1 (Schledzewska et al., 2006), VEGFR-3 (Lymboussaki et al., 1998), and Prox1 (Rodriguez-Niedenfuhr et al., 2001) as lymphatic endothelial cell markers (Kim et al., 2007), we analyzed lymphatic density and morphology in Pik3r1-targeted newborns, all of which presented with chylos ascites (Supp. Fig. S1, which is available online). To visualize the complete vascular tree, we used the pan-endothelial markers VEGFR-2 and PECAM1 (platelet endothelial cell adhesion molecule-1), whose expression did not change upon Pik3r1 deletion (data not shown). As shown by LYVE1 whole-mount immunohistochemistry (IHC), there was a paucity of serosal lymphatics at birth in Pik3r1 null mice (Fig. 1C,D), as determined by the mean number of vessel branch points/area (n = 5/group; wild-type 11.6 vs. null 3.8; P < 1 × 10⁻⁵). In contrast, the lymphatic capillaries (lacteals; Papp et al., 1962) were normal in number and size (Fig. 1H; Kim et al., 2007). The morphology of the remaining Pik3r1 null mesenteric and serosal lymphatics was abnormal (Fig. 1E-G). Confocal microscopy indicated that segments of the mesenteric collectors of Pik3r1 null mice were more variable than wild-type; that is, some were twice as wide as those of wild-type littermates (Fig. 1I,J) and others were less than half the normal size (Fig. 1E,F). Wider distribution of diameter classes in null vs. wild-type lymphatics resulted in a failure to achieve statistical significance for mean diameter (data not shown). Hemangiogenesis appeared normal except that the duodenal arcades exhibited additional branching as visualized by alpha smooth muscle actin (SMA) staining (Fig. 1K,L).

Overall, the phenotype of the Pik3r1-targeted newborns was reminiscent of intestinal lymphangiectasia, a clinical condition in which inflammation or obstructing tumors (Kolbjornsen et al., 1994) results in poor lymphatic drainage. Our data suggest that, in the absence of Pik3r1, lymphangiectasia resulted from abnormal lymphatic development. Liver and heart necrosis (Fruman et al., 2000) may also contribute to impaired lymphatic function in Pik3r1 null mice by impairing venous function, although widespread venous insufficiency is unlikely because (i) the newborns lacked pleural effusions (Fig. 2A–F) and (ii) despite significantly decreased dermal lymphatic branching and increased diameter (Fig. 2G), subcutaneous edema was barely detectable. Of interest, Angiopoietin2 null newborn mice have chylos ascites, but also show pleural effusions, subcutaneous edema, with fewer and smaller lacteals than control littermates, thus their lymphatic defects differ significantly from Pik3r1 null mice (Gale et al., 2002). Because angiopoietins signal by means of PI3K (Peters et al., 2004), it is still possible that aspects of the Pik3r1 null phenotype reflect a deficit in this pathway. Postnatal blood vessel remodeling is also defective in Angiopoietin2 null mice (Gale et al., 2002), but this could not be studied in Pik3r1 null mice, because they died by postnatal day 1. However, Pik3r1 null newborn mice had normal blood vessel density and morphology, exemplified by whole-mount IHC for SMA in visceral (Fig. 2H,I), with the exception of the additional branching of the arcades noted already (Fig. 1K,L).

Of interest, lymphatics from the abdominal surface were significantly reduced (Fig. 3A–D,G), but were present in the thoracic surface (Fig. 3E,F,G), appearing capable of extending from the body wall but not from the retroperitoneal sac (Oliver, 2004). These results are consistent with Pik3r1 having different roles on lymph and blood vessel development, suggesting that Pik3r1 isoforms are required for proper lymphatic patterning but are dispensable for, or to some extent inhibitory of, blood vessel branching.

To gain insight into the cellular basis for the organ-specific dependency of lymphangiogenesis, we first questioned whether Pik3r1 loss causes vessel regression, or arrests vessel growth during development. Seminal work by Sabin (Sabin, 1913; for a re-
Fig. 2.

PIK3R1 REQUIRED FOR EMBRYONIC LYMPHANGIOGENESIS

**Fig. 2.**

**Fig. 3.**

**Fig. 3.**
Pharmacological inhibitors of PI3K activity and PI3K mutants decrease endothelial cell proliferation and survival in vitro (Makinen et al., 2004). However, loss of \( \text{Pik3r1} \) alone is not always sufficient to impair proliferation; for example, in T cells, \( \text{Pik3r2} \) (p85\( ^\alpha \)) compensates (Deane et al., 2007). Indeed, mesenteric lymphatics of \( \text{Pik3r1} \) null mice and wild-type littermates did not differ in proliferation or cell death, as assessed by bromodeoxyuridine (BrdU) incorporation and phosphohistone-H3 analyses, or by TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling) staining, respectively (data not shown). Instead, our results are consistent with mouse lymphatic development studies (Kim et al., 2007) that support a vessel-branchoing mechanism driving mesenteric plexi remodeling without changes in cell number (Patan, 2004). Alternatively, \( \text{Pik3r1} \) null mice may lack a subpopulation of lymphatic progenitors, like those detected in quail–mouse chimera (Pudliszewski and Pardanaud, 2005). Although descriptions of (lymphangiogenic mechanisms in the diaphragm are lacking, decreased invasion was the most visible defect in \( \text{Pik3r1} \) null diaphragms. Collectively, these results suggest that \( \text{Pik3r1} \) is required for distinct steps of lymphangiogenesis in distinct organ microenvironments, despite common vessel origins.

VEGF-C is needed for embryonic lymphangiogenesis (Karkkainen et al., 2004), and its tumor expression is PI3K-dependent (Tang et al., 2003). Of interest, we found only decreased levels of mature \( \Delta \text{NzVEGF-C} \) in the \( \text{Pik3r1} \) null gut, without obvious differences in the levels of precursor pro-VEGF-C, which is the substrate for N-terminal and C-terminal cleavages by plasmin, furin, and proconvertases (Fig. 6A; Jeltsch et al., 2003). Interpreting these results will require a better understanding of posttranslational processing of VEGF-C in vivo, and of the role of the different products, which signal through different VEGFRs in lymph vessel growth, remodeling, and maturation (Jeltsch et al., 2003).

To better understand the impact of \( \text{Pik3r1} \) deletion on maturation, we assessed smooth muscle/pericyte investment and valve formation. Mesenteric lymphatics of \( \text{Pik3r1} \) null mice lacked valves (Fig. 6B–E); instead of endothelial luminal flaps, we found abnormal vessel-wall constrictions (Figs. 1–5, 6B,D). Only two other mutants show valve-less phenotypes: the knockout of \( \text{Foxc2} \) (a member of the forkhead family of transcription factors; Petrova et al., 2004), and the knock-in of a PDZ-less \( \text{ephrinB2} \) mutant (Makinen et al., 2005). Although Eph-receptors may signal by means of PI3K (Makinen et al., 2005), it is not understood how \( \text{ephrinB2} \) affects valve formation.

Previous data indicate that \( \text{Foxc2} \) ablation causes lymph valve agenesis and abnormal pericyte/SMC investment, due to up-regulation of \( \text{Pdgfb} \) and endoglin transcription in lymphatics (Petrova et al., 2004). Therefore, to test the hypothesis that PI3K regulates endoglin transcription, luciferase reporter assays were conducted on cultured human primary lymphatic endothelial cells transfected using human-derived endoglin promoter-luciferase constructs (Rius et al., 1998; Botella et al., 2002). First, a series of endoglin promoter deletion mutations comprising sequential human 5′ noncoding promoter regions extending from the minimal promoter (Fig. 7A, base pairs –148–281, relative to the endoglin transcription start site) to a position 782 base pairs upstream of the transcription start site (Fig. 7A, 782–281) were cotransfected with either the wild-type or dominant-negative p85\( ^\alpha \) expression construct. These constructs were characterized elsewhere (Rius et al., 1998; Botella et al., 2002). Analysis of the endoglin promoter deletion mutations indicated that PI3K-mediated repression of endoglin expression is governed at least in part by elements contained in the proximal promoter DNA segment 5′ to position –281, relative to the transcription initiation site in the minimal endoglin promoter (Fig. 7A). Moreover, cotransfection of the wild-type p85\( ^\alpha \) subunit expression inhibited the shortest maximally responsive endoglin luciferase reporter construct (–397/+281; Fig. 7B) in a dose-dependent manner. In contrast, the domi...
nant-negative construct, p85αdn, was significantly less effective in terms of inhibition of endoglin promoter reporter expression (Fig. 7B).

Fig. 6. Decreased vascular endothelial growth factor-C (VEGF-C) levels are associated with altered lymphatic wall and valve structure. A: The p85α and VEGF-C protein levels in Western blots of homogenates prepared from newborn mesenteries and diaphragm crura (three mice were pooled per lane). β-actin was used as a total protein loading control. B–E: Whole-mount staining for lymphatic endothelial cell vascular endothelial growth factor receptor-3 (VEGFR-3; green) and mural cell alpha smooth muscle actin (SMA; red). Red arrowheads point to lymph valves in mesenteric collectors of a wild-type newborn and the corresponding region in a Pik3r1-null, which display an abnormal wall structure instead of a valve. The white arrowhead indicates the normal direction of lymph flow.

Fig. 7. Increased lymphatic endothelial endoglin expression in the mesentery of Pik3r1 null mice. A,B: Endoglin promoter luciferase constructs were tested in primary human lymphatic endothelial cell culture for regulation by p85α. A: Successful endoglin promoter 5’ deletion constructs were transfected along with p85α protein expression construct corresponding to maximal endoglin promoter inhibition (300 ng/ml). The endoglin promoter segment numbers represent base pairs numbered from the 5’-terminus to the transcriptional start site. B: Lymphatic endothelial cell cultures were transfected with the 397/281 endoglin promoter luciferase construct and either wild-type or dominant-negative p85αdn protein expression constructs. Histogram black bars, wild-type p85α; gray bars, dominant-negative p85α, p85αdn. For expression plasmid dosage studies, empty pcDNA was used to maintain constant total DNA levels. C–F: Anti-endoglin whole-mount staining of embryonic day (E) 19 mesenteries from a Pik3r1 null mouse and a wild-type littermate. Black arrow highlights the increased endoglin protein expression within the abnormal lymphatic collectors of Pik3r1 null mice.
A limitation of this approach is that the efficiency of transfection of the primary human lymphatic endothelial cells with p85 constructs, while sufficient for the reporter assays, was not sufficient to produce a statistically significant change in endogenous endoglin protein levels overall, as determined by Western blotting. However, consistent with the reporter data shown, luciferase reporter experiments using constitutively active forms of the PI3K p110 catalytic subunit and constitutively active Akt, but not dominant-negative forms of these proteins, showed increasing inhibition of the endoglin promoter with plasmid dosage (data not shown).

Overexpression of wild-type p85α may exhibit a dominant-negative effect due to overproduction of p85α monomers, which could compete with endogenous p85p110 dimers. Thus, in vitro systems, p85α overexpression could produce a similar effect as the p85α dominant-negative mutant that lacks any ability to bind p110. Our results suggest that this does not occur in this system, but are consistent with loss of repression of endoglin expression in Pik3r1 null lymphatics, providing support for the view that endoglin expression is inhibited by PI3K at the transcriptional level, and suggesting that the regulation of endoglin by PI3K in mice is recapitulated in human lymphatic endothelial cells. To further test the hypothesis that PI3K regulates endoglin expression in developing lymphatics, we prepared whole-mount sections, which were immunostained with anti-endoglin antibody. Consistent with the preceding results, we found increased endoglin expression in gut lymphatics of Pik3r1 null mice (Fig. 7C–F).

Valve formation is either independently controlled by Foxc2 and Pik3r1, or Pik3r1 acts downstream of Foxc2. However, in contrast to Foxc2 null mice, Pik3r1 ablation was not accompanied by increased mural cell recruitment, as assessed with IHC for SMA (Figs. 1–5) and NG2 (data not shown). Foxc2 expression in adipocytes is p85/p110-dependent (Gronning et al., 2002), and so is the subcellular localization and phosphorylation status of other forkhead superfamily members (Abid et al., 2004). However, we found no difference in Foxc2 between Pik3r1 null mice and wild-type littermates by immunohistochemistry and Western blot analyses (data not shown). Our data suggest that Pik3r1 isoforms are dispensable for proper mural cell investment, but are necessary for lymph valve formation.

Endoglin has been implicated in blood vessel maturation and stability. Mutations in endoglin are responsible for hereditary hemorrhagic telangiectasia (McAllister et al., 1994), which is caused by reduced levels of endoglin protein expression (Pece-Barbara et al., 1999; reviewed in Bernabeu et al., 2007). Recent studies using endoglin transgenic mice indicate that endoglin overexpression in vascular precursor cells promotes vascular smooth muscle cell investment of major vessels (Mancini et al., 2007). The finding that endoglin, a prohemangiogenic endothelial cell marker, is up-regulated in Pik3r1 null mouse lymphatic tissues and human lymphatic endothelial cells suggests that PI3K-dependent endoglin repression opposes angiogenesis and plays a role in lymphangiogenic vessel identity.

These data not only suggest a mechanism whereby abnormal endoglin expression in Pik3r1 null lymphatic vessels contributes to the pathology observed in Pik3r1 null mice, but also provide the novel insight that PI3K-dependent regulation of endoglin may play a role in lymph vessel homeostasis. Endoglin expression is thought to repress transforming growth factor-β (TGFβ) signaling by inhibiting the TGFβ receptor ALK5 (Lastres et al., 1996; Bianco et al., 2005). Pharmacologic inhibition of ALK5-dependent TGFβ signaling in lymphatic cells accelerates lymphangiogenesis in a mouse model of chronic peritonitis (Oka et al., 2008). Thus, our study suggests that endoglin may play a role in regulating lymphangiogenic homeostasis. This view is supported by the observation that endoglin is absent in normal blood vascular smooth muscle, but is increased after vessel injury (Ma et al., 2000) and in atherosclerotic vascular smooth muscle (Conley et al., 2000).

The present study, combined with the absence of lymphatic defects in p85α null (p55α/p60α isoforms intact) and p55α/p50α null (p85α isoform intact) embryos, indicates that p85α/p50α isoforms have redundant but required functions in lymphatic development. These functions are most likely to involve phosphotyrosine-based associations with Tie1/Tie2, PDGFβRs, VEGFβRs, or Eph receptors, because what is preserved among the Pik3r1 isoforms are the SH2 domains. Based on the lymphatic defects of p110α-mutant mice (Gupta et al., 2007), an attractive model is that effective activation of class Ia PI3K requires at least two interactions downstream of receptor tyrosine kinases: SH2 domains of a regulatory isoform binding to phosphotyrosine, and binding of p110α to activated Ras. Further in vitro and in vivo studies to determine the lymphangiogenic signaling pathway(s) dependent on each isoform, and to elucidate the extent to which their function in vascular development is endothelial cell-autonomous will provide new insight into the mechanism of lymphangiogenesis.

**EXPERIMENTAL PROCEDURES**

Protocols were preapproved by our Institutional Animal Care and Use Committee. We followed published methods for generation of the Pik3r1 targeted mice (Fruman et al., 2000), Western blot analysis (Fruman et al., 2000), whole-mount IHC (Karkkainen et al., 2004; Petrova et al., 2004), lymphatic endothelial cell culture (Petrova et al., 2004), and luciferase reporter analyses (Rius et al., 1998; Botella et al., 2002). At least eight surviving mice of each genotype were analyzed per data point.

Image analysis of lymphatic vessel branching and diameter was estimated using a morphometric approach (Kumar et al., 1997). For vessel branching, the number of branch points was determined for a minimum of five independent samples each from wild-type and null mouse specimens. For vessel diameters, the width of the vessel lumen was obtained by delineating the luminal diameter, in triplicate, using Photoshop CS2 (Adobe Systems). Statistical significance was assessed using a two-tailed Student’s t-test and assuming unequal variances.
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