**Abstract**

Recent studies have shown that lymphangiogenesis or the growth of lymphatic vessels at the periphery of tumors promotes tumor metastasis to lymph nodes. We show here that the fibronectin-binding integrin α4β1 and its ligand fibronectin are novel functional markers of proliferative lymphatic endothelium. Tumors and lymphangiogenic growth factors, such as vascular endothelial growth factor-C (VEGF-C) and VEGF-A, induce lymphatic vessel expression of integrin α4β1. Integrin α4β1 then promotes growth factor and tumor-induced lymphangiogenesis, as genetic loss of integrin α4β1 expression in Tie2Cre+ α4Y991A knock-in mice blocks growth factor and tumor-induced lymphangiogenesis, as well as tumor metastasis to lymph nodes. In addition, antagonists of integrin α4β1 suppress lymphangiogenesis and tumor metastasis. Our studies show that integrin α4β1 and the signals it transduces regulate the adhesion, migration, invasion, and survival of proliferating lymphatic endothelial cells. As suppression of α4β1 expression, signal transduction, or function in tumor lymphatic endothelium not only inhibits tumor lymphangiogenesis but also prevents metastatic disease, these results show that integrin α4β1-mediated tumor lymphangiogenesis promotes metastasis and is a useful target for the suppression of metastatic disease.

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**Introduction**

Tumor metastases are a leading cause of cancer-related mortality, and both tumor cell intrinsic and extrinsic factors can promote metastasis (1–3). Metastases can be detected in draining lymph nodes before they are detected in distant organs, and for most tumors, the clinical record suggests that lymph node metastases progress to distant metastases (4). Lymph nodes are thus the initial or frequent sites of metastasis for many tumors, including human pancreatic, gastric, breast, and prostate carcinomas, and melanomas. Tumor lymphangiogenesis, the growth of new lymphatic vessels, has been linked to the formation of lymph node metastases (1–7). Lymphatic capillaries, unlike typical blood capillaries, lack pericytes and a continuous basal lamina. Due to their greater permeability, lymphatic capillaries may be more effective than blood capillaries in allowing passage of tumor cells into and out of vessels. Peritumoral growth of lymphatic vessels has thus been associated with lymphatic metastasis (3, 5–7). Accordingly, increased expression of vascular endothelial growth factor-C (VEGF-C) or VEGF-A, which promotes lymphangiogenesis in primary tumors, correlates closely with increased incidence of regional lymph node and distant metastases in both humans and animals (3, 8–10). Systemic administration of antagonists of the VEGF-C receptor, VEGF-R3, blocks primary tumor lymphangiogenesis and metastasis (11–14).

The recent identification of selective markers of lymphatic endothelial cells (LEC) has allowed identification of mechanisms that regulate lymphangiogenesis. LECs selectively express Lyve-1, a member of the CD44 hyaluronic acid receptor family (15); Prox-1, a lymphatic vessel-specific homeobox transcription factor (16); and podoplanin (17). Whereas growth factors and their receptors play critical roles in angiogenesis and lymphangiogenesis, little is known about the roles of the integrin family of cell adhesion proteins in tumor lymphangiogenesis (18). The integrin family of membrane receptors for ECM proteins and immunoglobulin superfamily molecules includes Arg-Gly-Asp (RGD) binding integrins αvβ3, α5β1, αvβ3, and αvβ3, as well as the Glu-Leu-Asp-Val (EILDV) binding integrin αvβ1 (19, 20). A number of endothelial cell integrins, including α1β1, α2β1, α4β1, α5β1, α6β1, α2β1, αvβ3, and αvβ5, have been implicated in the regulation of cell growth, survival, and migration during vascular angiogenesis (21). However, little is known about the adhesion mechanisms that regulate...
pathologic lymphangiogenesis, although integrin α9β1 has been shown to promote embryonic development of the lymphatic system (22). In the studies presented here, we found that the fibronectin-binding integrin α4β1 promotes the adhesion and migration of LECs during growth factor and tumor-induced lymphangiogenesis, thereby facilitating tumor metastasis.

### Materials and Methods

**Reagents.** Recombinant human basic fibroblast growth factor (bFGF), VEGF-A, and VEGF-C were from R&D Systems. Rabbit anti-human, rabbit anti-mouse Ly-1 antibodies (RDI-102PA50 and RDI-103PA50), and hamster anti-murine podoplanin (103-M40) were from Research Diagnostics, Inc. Rat anti-mouse CD31 (MEC 13.3) was from BD Bioscience. Rabbit anti-human/mouse podoplanin (D240) was from Biocare Medical LLC. Murine anti-pan-species Prox-1 (MAB5652, clone 5G10) and anti-human fibronectin (TEV-1) were from Millipore. Goat anti-human αvβ1 (HP1/2), rat anti-murine αvβ3 (PS2), and rat isotype-matched control antibody (IgG2b) were gifts from Biogen-Idec. Anti-murine VEGF-R3 (AFL4) was from eBioscience. Alexa 488–conjugated murine anti-pan-cytokeratin (Clone C11) was from Cell Signaling Technology. Donkey anti-rabbit, rabbit, and mouse IgGs conjugated with Alexa Fluors 488, 568, or 647 were from Jackson ImmunoResearch. Growth factor–depleted Matrigel was from Becton Dickinson. Anti-murine VEGF-R3 (AFL4) was from Biogen-Idec. Anti-murine VEGF-R3 (AFL4) was from EMD Millipore. The Alexa Fluor 488 goat anti-murine IgG antibody was from Jackson ImmunoResearch. Alexa Fluor 488 anti-mouse CD31 (MEC 13.3) was from BD Bioscience. Rabbit anti-mouse CD31 (MEC 13.3) was from BD Bioscience. Rabbit anti-mouse podoplanin (D240) was from Biocare Medical LLC. Murine anti-pan-species Prox-1 (MAB5652, clone 5G10) and anti-human fibronectin (TEV-1) were from Millipore. Goat anti-human αvβ1 (HP1/2), rat anti-murine αvβ3 (PS2), and rat isotype-matched control antibody (IgG2b) were gifts from Biogen-Idec. Anti-murine VEGF-R3 (AFL4) was from eBioscience. Alexa 488–conjugated murine anti-pan-cytokeratin (Clone C11) was from Cell Signaling Technology. Donkey anti-rabbit, rabbit, and mouse IgGs conjugated with Alexa Fluors 488, 568, or 647 were from Jackson ImmunoResearch. Growth factor–depleted Matrigel was from Becton Dickinson.

**Cell culture.** LECs (HMVEC-dyNeo, Cambrex) were cultured in endothelial growth medium-2 containing 10% fetal bovine serum (FBS; Cambrex/Lonza). Lewis lung carcinoma (LLC) and B16 melanoma cells were obtained from American Type Culture Collection, Panc02 pancreatic ductal carcinoma cells were obtained from the National Cancer Institute DCTD Tumor Repository, and each was cultured in DMEM containing 10% FBS and antibiotics.

**Cell adhesion assays.** Adhesion assays were performed by coating non-tissue culture–treated plates with 10 μg/mL CS-1 fibronectin or rsVCAM overnight at 4°C. Plates were blocked for 2 h with 3% heat denatured bovine serum albumin (BSA). LECs were incubated in plates for 15 min at 37°C in the presence of 25 μg/mL of isotype-matched anti-α5β1 (JBS5), anti-αvβ3 (LM609), anti-αvβ5 (P1F6), or anti-αvβ1 (HP1/2) antibodies. Plates were washed thrice, stained with crystal violet, and extracted, and absorbance at 560 nm was measured. Assays were performed thrice with triplicate samples per group.

**Migration assays.** LEC monolayers were scratched using a 20-μL pipette tip. Plates were washed, and media containing 100 ng/mL VEGF-C and function blocking anti-integrin α4β1, anti-α5β1, anti-αvβ3, or anti-αvβ5 antibodies (25 μg/mL) were added for 8 to 24 h. "Wound" closure was quantified from digital images using Metamorph imaging software (Version 6.3r5, Molecular Devices). Experiments were performed thrice.

**LEC tube formation.** LECs (5 × 104) were added to chamber slides containing Matrigel in the presence of 50 ng/mL VEGF-C and medium or 25 μg/mL of anti-α4β1 (HP1/2), anti-αvβ5 (P1F6), anti-αvβ3 (LM609), and anti-α5β1 (JBS5), and plates were incubated at 37°C for 24 h. The mean number of vessel branchpoints ± SEM was determined for triplicate samples. Experiments were performed thrice.

**Thoracic duct sprouting assay.** Thoracic ducts were carefully dissected from mice and cultured as described (23). Ducts were cut into 1-mm-long rings, embedded in type I collagen gels for 4 d in DMEM containing 10% FBS, and then fixed in 4% paraformaldehyde. Sprouting area was measured using Metamorph imaging software.

**Clinical specimen collection.** Patients at the Moores University of California-San Diego (UCSD) Cancer Center underwent breast or gastric surgical treatment using standard techniques. Normal tissue was obtained from patients undergoing breast reduction or prophylactic mastectomy. Specimens were reviewed by a pathologist to assess the surgical margin tissue. Tissues not needed for diagnosis were embedded in OCT for cryosectioning. 35 invasive tumors [24 ductal carcinomas (8 stage I, 9 stage II, 5 stage III, 2 stage IV) and 11 lobular carcinomas (6 stage I, 1 stage II, 4 stage III)], 5 non-invasive cancer, and 15 normal mammary glands.

**Immunohistochemistry.** Lymphatic vessels were detected by immunostaining of cryosections with 2 μg/mL anti-human Ly-1 (RDI-102PA50), anti-murine Ly-1 (RDI-103PA50), anti-Prox-1 (MAB5652, clone 5G10), anti-murine podoplanin (103M40), or anti-human podoplanin (D240). Integrin α4β1 was detected with 2 μg/mL anti-α4β1 (6590). Lymphatic vessels were quantified in 5 to 10 microscopic fields per cryosection by automated pixel density determination as the mean number of pixels ± SEM for each treatment group.

The mean number of mice with metastases in inguinal (LLC), brachial/axillary (PyMT), or hilar (Panc02) lymph nodes was determined by immunostaining cryosections of lymph nodes with 5 μg/mL Alexa 488 conjugated anti-murine cytokeratin (C11) in three replicate experiments. B16 melanoma metastases were detected by H&E staining of lymph node sections.

Thick cryosections (20 μm for Matrigel plugs and 50 μm for tumors) were fixed in 1% paraformaldehyde for 1 h at 4°C; washed in PBS for 5 min; blocked in 0.3% Triton X-100, 0.2% BSA, 5% normal goat serum, and 0.1% NaN3 in PBS for 2 h at room temperature; and incubated in 5 μg/mL primary antibody overnight at 4°C. Sections were washed four times for 1 h each at 4°C and then incubated in Alexa 568– or Alexa 488–conjugated donkey anti-rat IgG secondary antibody overnight at 4°C. Sections were washed four times for 1 h at room temperature, postfixed in 1% paraformaldehyde and rinsed in PBS, and coverslips were mounted.

**Transgenic animals.** PyMT+ transgenic female mice were derived as previously described (24). FVB and C57Bl6 mice were from Charles River, and C57Bl6 integrin αY991A mice were derived as previously described (25). Male Tie2Cre+ mice (26) from The Jackson Laboratory were crossed with female integrin α4κoop/κoop mice (27), and Tie2Cre+ α4κoop/κoop progeny were then crossed with α4κoop/κoop mice to obtain sibling Tie2Cre–α4κoop/κoop, Tie2Cre+ α4κoop/κoop, and Tie2Cre+ α4κoop/κoop mice.

**Tumor studies.** LLC or B16 cells (5 × 103) were injected s.c. into wild-type (WT) or integrin αY991A mice in a C57Bl6 background (n = 10–12). Animals were sacrificed 3 wk later.

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Alternatively, WT mice with 7-day-old palpable (30 mm³) tumors were treated by i.p. injections of 200 μg/25 g body weight of function-blocking anti-integrin α4 (n = 10), isotype-matched control rat IgG1 (n = 10), or saline (n = 10) every third day for 2 wk. Lymphatic vessels and metastases were quantified in tumors and lymph nodes, respectively, in three replicate experiments.

To study orthotopic pancreatic carcinomas, the abdominal cavities of mice (n = 10) were opened and the tails of the pancreas were exteriorized. One million syngeneic Panc02 cells were injected into the pancreatic tail, the pancreas was placed back into the abdominal cavity, and the incision was closed. Tumors and hilar lymph nodes were excised after 30 d. Experiments were performed thrice.

**Lymphangiogenesis assays.** Ice-cold Matrigel containing saline (400 μL) or VEGF-C, VEGF-A, or bFGF (400 ng) was injected into WT (n = 10), α4Y991A (n = 10), Tie2Cre+ α4loxp/loxp (n = 4), Tie2Cre+ α4loxp/+ (n = 2), or Tie2Cre− α4loxp/loxp (n = 4) mice for 10 d. In other studies, WT mice were treated by i.p. injection with 200 μg/mouse of function-blocking anti-α4β1 (PS2), anti-VEGF-R3 (AFL4), PS2 plus AFL4, rsVCAM, or isotype control anti-α5β1 antibodies on days 1, 3, and 6 (n = 10). After 10 d, Matrigel plugs were removed, and 5-μm sections were immunostained with anti-Lyve-1 antibodies. At least five fields per section were analyzed. Experiments were performed at least thrice.

**Statistical analysis.** All statistical analyses were performed with a two-tailed Student’s t test or ANOVA.

**Results**

**Integrin α4β1 and fibronectin are markers of tumor lymphatic endothelium.** To identify cell adhesion proteins that regulate tumor lymphangiogenesis, we evaluated the expression of several integrins on lymphatic vessels in normal and tumor tissues, including integrins α4β1, α5β1, αvβ3, and αvβ5. These integrins were selected for their well-defined roles in angiogenesis and wound healing (18). We found that only integrin α4β1, a cell surface receptor for fibronectin and VCAM-1 (vascular cell adhesion molecule;
refs. 28, 29), was highly upregulated on tumor lymphatic vessels (Fig. 1; Supplementary Figs. S1 and S2). Cryosections of breast tissue from patients with or without invasive carcinomas and from patients with gastric tumors were immuno-stained with antibodies to integrin \( \alpha_4\beta_1 \) and Lyve-1, podoplanin, or Prox-1, well-established markers of lymphatic endothelium (15, 17). We found that integrin \( \alpha_4\beta_1 \) (red) was strongly expressed on Lyve-1, Prox-1, and podoplanin+ lymphatic vessels (green) in mammary tumors but not in normal mammary glands (Fig. 1A and B; Supplementary Figs. S1–S3). Integrin \( \alpha_4\beta_1 \) was also expressed on lymphatic endothelium in human gastric tumors (Fig. 1A). Importantly, expression of integrin \( \alpha_4\beta_1 \) on lymphatic endothelium indicated the presence of invasive ductal or lobular tumors, as >60% of tumor lymphatic vessels were integrin positive whereas <20% of normal lymphatic vessels were integrin positive (Fig. 1A).

Integrin \( \alpha_4\beta_1 \) was also strongly expressed on lymphatic endothelium in tumors from mice with PyMT+ spontaneous breast tumors and was not expressed in normal breast tissue (Fig. 1C; Supplementary Fig. S2). This integrin was also expressed on lymphatic vessels in LLC tumors (Fig. 1C). Expression of integrin \( \alpha_4\beta_1 \) on lymphatic endothelium in murine mammary glands correlated with the presence of invasive ductal tumors, as 100% of lymphatic vessels in murine breast tumors were integrin \( \alpha_4\beta_1 \) positive whereas only 5% of lymphatic vessels in normal mouse breast tissue expressed this integrin (Fig. 1C).

To determine whether integrin \( \alpha_4\beta_1 \) expression is induced by purified lymphangiogenic factors, we stimulated lymphangiogenesis in mice by implanting VEGF-C saturated Matrigel plugs and immunostained cryosections of these tissues to detect integrin \( \alpha_4\beta_1 \) and three independent markers of lymphatic vessels: podoplanin, Lyve-1, and Prox-1 (15–17). Integrin \( \alpha_4\beta_1 \) expression colocalized extensively with each of these three distinct markers of lymphatic vessels (Fig. 1C, left). These results confirm that integrin \( \alpha_4\beta_1 \) is a marker of proliferative lymphatic vessels. We next investigated which lymphangiogenic factors can induce integrin \( \alpha_4\beta_1 \) expression in lymphatic vessels. VEGF-C, VEGF-A, and bFGF strongly induced \( \alpha_4\beta_1 \) expression on lymphatic vessels during lymphangiogenesis.

Figure 2. Inhibition of integrin \( \alpha_4\beta_1 \) function blocks LEC migration and lymphangiogenesis. A, images and quantification of Lyve-1+ lymphatic vessels per field ± SEM in VEGF-C saturated Matrigel from saline, anti-\( \alpha_4\beta_1 \), anti-\( \alpha_5\beta_1 \), or recombinant soluble VCAM-treated mice from these mice (\( n = 10; *, P < 0.002 \)). B, mean percentage of TUNEL+ Lyve-1+ vessels per field ± SEM (\( *, P < 0.0002 \)) in Matrigel from A. C, VEGF-C stimulated LEC migration in the presence of medium, anti-\( \alpha_4\beta_1 \), or anti-\( \alpha_5\beta_1 \) antibodies (clG; \( *, P < 0.01 \)). D, LEC in vitro “vessel” formation in the presence of anti-\( \alpha_4\beta_1 \) or isotype matched control (anti-\( \alpha_5\beta_1 \)) antibodies; mean vessel branch points per 100× field ± SEM (\( *, P < 0.0001 \)).
In addition, we found that integrin α4β1 is strongly expressed on the surface of 90% of cultured LECs (Supplementary Fig. S3). Thus, integrin α4β1 is a marker of proliferating lymphatic vessels.

To determine whether LECs also express integrin α4β1 ligands, we immunostained cultured LECs with antibodies directed against the α4β1 ligands fibronectin or VCAM-1. We observed fibronectin expression in cultured LECs by Western blotting and in lymphatic vessels in vivo by immunostaining of PyMT+ spontaneous breast tumors (Supplementary Fig. S3). In contrast, we did not observe VCAM-1 expression in LECs by fluorescence-activated cell sorting analysis (Supplementary Fig. S3) or Western blotting (not shown). These results show that integrin α4β1 and its ligand fibronectin are novel markers of proliferative lymphatic endothelium in vitro and in invasive tumors in vivo.

Lymphangiogenesis depends on integrin α4β1 signal transduction. To determine whether integrin α4β1 regulates lymphangiogenesis, we s.c. implanted mice with Matrigel saturated with saline, VEGF-C, or VEGF-A and treated animals with antagonists of murine integrin α4β1, including function-blocking anti-α4β1 antibodies and recombinant soluble VCAM, and with isotype-matched control antibodies (Fig. 2A). Whereas VEGF-C and VEGF-A stimulated the growth of new lymphatic vessels in Matrigel, both integrin α4β1 antagonists, but not control antibodies, strongly suppressed lymphangiogenesis in VEGF-C (Fig. 2A) and VEGF-A (Supplementary Fig. S4) stimulated animals. Notably, antibody antagonists of α4β1 induced apoptosis of LECs in vivo, as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining of Lyve-1+ vessels in Matrigel plugs (Fig. 2B; Supplementary Fig. S5).

We also found that integrin α4β1 promotes adhesion and migration of LECs on cellular (CS-1) fibronectin. LECs adhered to CS-1 fibronectin and antibody antagonists of integrin α4β1, but not antibodies to other integrins, including

![Figure 3. Inhibition of VEGF-C lymphangiogenesis in integrin α4 mutant animals. A, top, genomic PCR analysis of Tie2Cre(+)/α4lox/lox, Tie2Cre(+)/α4lox/+, and Tie2Cre(-)/α4lox/lox mice for Cre recombinase (100 bp), intact integrin α4 (180 bp), floxed α4 (280 bp), and excised α4 (600 bp); bottom, Western blotting of Cre recombinase (38 kDa) and β-actin (42 kDa) in lung lysates from Tie2Cre(+)/α4lox/lox and Tie2Cre(-)/α4lox/lox mice. B, top, cryosections of VEGF-C saturated Matrigel plugs in Tie2Cre(+) and Tie2Cre(-) mice immunostained to detect Cre (red) and Lyve-1+ expression (green) and counterstained with DAPI (blue). Cre+ vessels are indicated by arrows. Bottom, Cryosections of VEGF-C saturated Matrigel plugs in Tie2Cre(+) and Tie2Cre(-) mice immunostained to detect integrin α4 (red) and Lyve-1 (green) positive vessels (arrows). C and D, mean Lyve-1+ lymphatic vessels per field ± SEM in Matrigel plugs from VEGF-C (C) or VEGF-A (D) stimulated Tie2Cre(+)/α4lox/lox, Tie2Cre(+)/α4lox/+, and Tie2Cre(-)/α4lox/lox mice (*, P < 0.007).](image-url)
anti-αvβ3 (Supplementary Fig. S5), anti-αvβ5, and anti-α5β1 (not shown), blocked LEC adhesion on CS-1 fibronectin-coated plates. We also observed that VEGF-C stimulated LEC migration, which was blocked by antibody antagonists of integrin α4β1, but not by antibody antagonists of other integrins, including anti-α5β1 (Fig. 2C; Supplementary Fig. S5). VEGF-C also stimulated LEC invasion and vessel ("tube") formation in three-dimensional matrices in vitro, which was blocked by antibody antagonists of integrin α4β1 but not by isotype-matched antagonists of α5β1 (Fig. 2D; Supplementary Fig. S5), αvβ3, or αvβ5. Together, these results suggest that integrin α4β1 promotes LEC invasion during lymphangiogenesis in vivo.

To explore the role of α4β1 in lymphangiogenesis further, we examined lymphangiogenesis in mice deficient for expression of integrin α4β1 in LECs using an in vivo Matrigel plug lymphangiogenesis assay. As integrin α4−/− mice die at E11.5 due to cardiac malformations (30), we examined lymphangiogenesis in a tissue-specific integrin deletion mutant, the Tie2Cre α4lox/lox mouse. We crossed Tie2Cre mice, which express Cre under the influence of the Tie2 promoter in endothelial cells, with integrin α4lox/lox mice (27). Cre+ α4lox/lox mice were identified by genomic PCR from tail DNA for Cre and integrin α4 alleles (Fig. 3A, top). Cre expression in Tie2Cre+ α4lox/lox mice in vivo was detected by Western blotting of lung tissue lysates from Tie2Cre+ α4lox/lox and Tie2Cre- α4lox/lox mice (Fig. 3A, bottom). Cre expression was also shown in LECs in vivo by immunostaining of VEGF-C-saturated Matrigel plugs to detect Cre and Lyve-1 (Fig. 3B, top). Integrin α4 expression on Lyve-1+ lymphatic vessels was reduced in Tie2Cre+ α4lox/lox animals compared with that of Tie2Cre− animals (Fig. 3B, bottom). To explore the role of integrin α4β1 in lymphangiogenesis, we stimulated Tie2Cre+ α4lox/lox, Tie2Cre- α4lox/lox, and Tie2Cre+ α4lox/lox littermates by implanting mice with Matrigel containing saline, VEGF-C (Fig. 3C), or VEGF-A (Fig. 3D). We found that lymphangiogenesis was induced in Tie2Cre+ α4lox/lox and Tie2Cre- α4lox/lox mice, but not in Tie2Cre+ α4lox/lox mice. Together, these results indicate that integrin α4β1 expression is required for lymphangiogenesis.

To determine how integrin α4β1 regulates lymphangiogenesis, we evaluated the interactions of VEGF-C and α4β1 in vitro. VEGF-C strongly stimulated integrin α4β1-mediated LEC adhesion and promoted integrin α4β1 association with the adaptor protein paxillin within focal adhesions at the leading edges of cells, as determined by immunocytochemistry and coimmunoprecipitation studies (Fig. 4A; Supplementary Fig. S6). As VEGF-C promotes integrin α4β1 expression in vivo (Fig. 1) and activity in vitro (Fig. 4A), it is likely that VEGF-C and integrin α4β1 function in the same molecular pathway. Importantly, whereas antagonists of integrin α4β1 and VEGF-R3 both substantially inhibit lymphangiogenesis, their effects are neither additive nor synergistic, suggesting that these two molecules function in the same molecular pathway (Supplementary Fig. S7).

To explore the importance of integrin α4β1 signaling in LECs, we isolated LECs from mice with an integrin α4Y991A knock-in mutation (25). This mutation in the cytoplasmic tail of integrin α4β1 disrupts integrin α4β1-mediated
association with paxillin and talin (31–33) and blocks α4β1-mediated leukocyte adhesion (33). Although LECs isolated from WT and integrin αY991A mice expressed similar levels of integrin α4β1 (Supplementary Fig. S6), LECs from αY991A mice did not polarize or develop mature paxillin-containing focal adhesions when adhering to CS-1 fibronectin (Fig. 4B, left). Importantly, αY991A LECs failed to migrate in response to VEGF-C (Fig. 4B, right). Additionally, VEGF-C stimulated ex vivo lymphatic vessel sprouting from isolated thoracic ducts (large lymphatic vessels) when isolated from WT but not αY991A animals (Fig. 4C; Supplementary Fig. S6). Finally, VEGF-C stimulated lymphangiogenesis in Matrigel plugs in vivo was completely inhibited in integrin αY991A mice (Fig. 4D). In fact, integrin α4β1 association with paxillin was suppressed in VEGF-C containing Matrigel plugs from αY991A mice, and few α+ paxillin+ vessels with well-formed lumen were observed in mutant mice (Supplementary Fig. S6). These results indicate that integrin α4β1 expression and signal transduction are required for LEC migration and invasive responses to lymphangiogenesis factors in vitro and during in vivo lymphangiogenesis.

**Integrin α4β1 promotes tumor lymphatic metastasis.** As integrin α4β1 promotes growth factor–induced lymphangiogenesis in vivo, we asked whether integrin α4β1 could promote tumor lymphangiogenesis and subsequent metastasis to lymph nodes. To test this possibility, we implanted integrin α4−/− negative LLC or B16 melanoma cells s.c. into syngeneic mice and treated them with intravascular injections of saline, function-blocking anti-α4β1, or isotype-matched control antibodies. Tumors and draining inguinal lymph nodes were removed after 21 days and analyzed for tumor lymphangiogenesis and metastasis to lymph nodes. We found that antagonists of integrin α4β1 significantly suppressed lymphangiogenesis (Fig. 5A) and metastasis (Fig. 5B) in LLC and B16 melanoma tumors, as analyzed by cytokeratin immunostaining (LLC), H&E staining, or macroscopic analysis (B16; Supplementary Fig. S8). These studies indicate that integrin α4β1 may play a key role in promoting lymphangiogenesis and thereby tumor metastasis to lymph nodes. These studies also suggest that integrin α4β1 antagonists may be useful in suppressing lymphatic metastasis by inhibiting tumor lymphangiogenesis.

To determine whether integrin signaling plays a role in tumor lymphangiogenesis and metastasis in vivo, LLCs were s.c. implanted into WT and integrin αY991A mice. Three weeks later, tumors and draining inguinal lymph nodes were removed and analyzed in thick (50 μm) and thin (5 μm) sections for tumor lymphangiogenesis and metastasis to lymph nodes. We found that tumor-induced lymphangiogenesis was substantially suppressed in αY991A mice (Fig. 5C; Supplementary Fig. S8). Similar reductions in lymphangiogenesis were observed when Panc02 pancreatic carcinoma tumor cells were implanted orthotopically in the pancreas of αY991A mutant mice (Fig. 5C; Supplementary Fig. S8). Importantly, cytokeratin-positive tumor metastasis to draining lymph nodes was also suppressed in integrin αY991A mutant mice (Fig. 5D; Supplementary Fig. S8). Additionally, spontaneous metastases of Panc02 cells to other organs were also reduced in αY991A mice (Supplementary Fig. S9). These studies indicate that integrin α4β1 signaling plays an important role in promoting lymphangiogenesis and thereby tumor metastasis to lymph nodes and other tissues. Taken together, these results indicate that integrin α4β1−/− mediated tumor lymphangiogenesis is associated with tumor metastasis.

Our studies show that integrin α4β1 promotes LLC migration, sprouting and vessel formation in vitro, as well as growth factor–induced lymphangiogenesis and survival in vivo. These results strongly suggest that integrin α4β1...
promotes tumor-induced lymphangiogenesis. However, as integrin \(\alpha_4\beta_1\) is also expressed on vascular endothelial cells (34) and immune cells (35), which have been shown to promote angiogenesis, lymphangiogenesis, tumor growth, and metastasis by expressing proangiogenic factors (36), it is not absolutely clear whether integrin \(\alpha_4\beta_1\) promotes tumor lymphangiogenesis and lymphatic metastasis. To decipher the relative roles of vascular, LEC, and hematopoietic cell integrin \(\alpha_4\beta_1\) in lymphangiogenesis, we examined tumor growth, angiogenesis, lymphangiogenesis, inflammation, and metastasis in animals transplanted with bone marrow transplanted from WT and \(\alpha_4\beta_1\) mice (Fig. 6; Supplementary Fig. S10). Integrin \(\alpha_4\beta_1\) mice were transplanted with WT or \(\alpha_4\beta_1\) bone marrow, whereas WT mice were transplanted with \(\alpha_4\beta_1\) or WT bone marrow, and LLC cells were subsequently implanted. Tumor lymphangiogenesis and metastasis were suppressed by 50% and 80%, respectively, in \(\alpha_4\beta_1\) mice with WT bone marrow, indicating that lymphangiogenesis depends significantly on host integrin \(\alpha_4\beta_1\) (Fig. 6A–C). However, angiogenesis and tumor growth were not affected in \(\alpha_4\beta_1\) mice with WT bone marrow, indicating that host endothelial cells do not require integrin \(\alpha_4\beta_1\) function (Supplementary Fig. S10). We did find that lymphangiogenesis and metastasis (as well as angiogenesis and tumor growth) were suppressed by 50% in WT mice with \(\alpha_4\beta_1\) bone marrow (Fig. 6; Supplementary Fig. S10), indicating that lymphangiogenesis results from the combined effects of bone marrow and host endothelial cell contributions. Importantly, our studies indicate that LEC rather than vascular cell integrin \(\alpha_4\) promotes tumor lymphangiogenesis, as tumor angiogenesis and growth are not reduced in \(\alpha_4\beta_1\) hosts yet are reduced in animals with \(\alpha_4\beta_1\) bone marrow. Therefore, our combined results indicate that LEC integrin \(\alpha_4\beta_1\) promotes tumor lymphangiogenesis and metastasis, whereas hematopoietic cell integrin \(\alpha_4\beta_1\) may contribute to lymphangiogenesis and metastasis by promoting tumor angiogenesis and growth. Taken together, our in vitro and in vivo studies indicate that LEC integrin \(\alpha_4\beta_1\) plays an essential role in promoting tumor lymphangiogenesis and metastasis.

**Discussion**

Recent studies have shown that lymphangiogenesis develops in primary tumors or in the peritumoral space and promotes lymphatic metastasis, as expression of VEGF-A or VEGF-C stimulates tumor lymphangiogenesis and metastasis (3, 5–10) whereas antagonists of VEGF-C or VEGF-R3 suppress these events (11–14). The studies presented here indicate that the LEC integrin \(\alpha_4\beta_1\) plays a critical role in tumor lymphangiogenesis and metastasis by promoting LEC migration and survival in vivo.

Our studies show that integrin \(\alpha_4\beta_1\), rather than integrins \(\alpha_5\beta_1\), \(\alpha_\text{v}\beta_5\), and \(\alpha_\text{v}\beta_3\), is expressed on lymphatic endothelium in spontaneous and experimental tumors and in response to purified lymphangiogenic growth factors. Whereas other integrins may also participate in the regulation of tumor lymphangiogenesis, limited information is available...
about which integrins can regulate this process. Integrin α9β1 promotes developmental lymphangiogenesis as integrin α9 null mice exhibit chylothorax, an accumulation of milk in the abdomen of newborn mice, which results from improperly functioning lymphatic vessels (22, 37), whereas antagonists of integrin α5β1 blocked inflammatory lymphangiogenesis in the eye and trachea (38, 39). However, little is known about roles that these integrins may play in tumor lymphangiogenesis.

Four lines of evidence indicate that LEC integrin α4β1 plays a direct role in regulating lymphangiogenesis. First, integrin α4β1 is poorly expressed in normal lymphatic vessels but is upregulated during lymphangiogenesis in vivo. Second, antagonists of integrin α4β1 suppress VEGF-C and tumor-induced lymphangiogenesis, as well as tumor metastasis to lymph nodes. Third, lymphangiogenesis is suppressed in Tie2Creα4lox/lox mice, which are defective in endothelial cell expression of integrin α4, and in α4V991A animals, which exhibit defective LEC integrin α4 migration and invasion. Additionally, bone marrow transplant studies confirm that host integrin α4 is required for tumor lymphangiogenesis and metastasis but not as important for tumor angiogenesis and growth.

Numerous studies have indicated that tumor lymphangiogenesis promotes lymphatic metastases by providing a direct conduit for tumor cell escape to nearby draining lymph nodes (3, 5–10). These studies also indicate that breast, prostate, pancreatic, and melanoma metastases to distant organs generally arise indirectly from lymphatic metastasis, as prophylactic removal of lymph nodes can prevent widespread disease (4). Although Wong and colleagues found that knockdown of VEGF-C expression in tumor cells suppresses tumor lymphangiogenesis without affecting metastasis to lymph nodes (40), other studies indicate that VEGF-C increases delivery of tumor cells to lymph nodes via the lymphatics (41). Whereas it is possible that, in some tumor systems, de novo lymphangiogenesis is not required for tumor metastasis, most studies clearly show that tumor lymphangiogenesis does help promote tumor metastasis.

It is yet not clear whether integrin α4β1 also plays a role in the development of the lymphatic system. Integrin α4 null mice die before lymphatic vessels are established (30). Tie2Cre+ α4lox/lox mutant animals exhibit no defects in development, but these mice also exhibited mosaic Cre expression in endothelial cells. Integrin α4V991A mice also exhibit no developmental defects. As integrins α9β1 and α4β1 both bind to CS-1 fibronectin and VCAM-1, it is possible that integrin α9β1 can compensate for the loss of α4 during lymphatic development and may play a role in tumor lymphangiogenesis. Future studies will clarify the relative roles of these two integrins during developmental and tumor lymphangiogenesis. In conclusion, our studies show the important role of the integrin α4β1 in lymphangiogenesis and suggest that antagonists of this integrin may be useful in the clinical setting to suppress the spread of tumors through the lymphatic system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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