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# Tumor-Secreted Vascular Endothelial Growth Factor-C Is Necessary for Prostate Cancer Lymphangiogenesis, but Lymphangiogenesis Is Unnecessary for Lymph Node Metastasis

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## Abstract

**Dissemination to draining lymph nodes is a frequent first step in prostate cancer metastasis. Although tumors metastasize to lymph nodes via the lymphatics, the importance of lymphangiogenesis in mediating the process remains controversial. Here, we inhibit intratumoral lymphangiogenesis in s.c. and surgical orthotopic implantation mouse models of human prostate cancer using several strategies. Stable expression of small interfering RNAs (siRNA) targeted against human vascular endothelial growth factor-C (VEGF-C) in PC-3 cells reduced intratumoral lymphatics by 99% in s.c. tumors, indicating that tumor-secreted VEGF-C is necessary for lymphangiogenesis. Expression of siRNAs against human VEGF-A somewhat reduced tumor lymphangiogenesis. Secretion of a soluble VEGF receptor-3/Flt4 fusion protein by PC-3 cells reduced intratumoral lymphatics by 100% in s.c. tumors. Combination of soluble Flt4 and VEGF-C siRNA yielded >92% reduction of intratumoral lymphatics in orthotopic prostate tumors. However, metastasis to lymph nodes was not significantly affected regardless of intratumoral lymphatic vessel density. The abundance of marginal lymphatics at the tumor-stromal interface was unchanged in orthotopic tumors whose intratumoral lymphatics were inhibited, suggesting that these marginal vessels could be sufficient for lymph node metastasis. Hematogenous metastasis (blood tumor burden, lung metastasis) correlated with degree of lymph node invasion. We also analyzed the lymphatics in spontaneous transgenic adenocarcinomas of the mouse prostate which metastasize to lymph nodes. Progression from well-differentiated prostate intraepithelial neoplasia to metastatic, undifferentiated adenocarcinoma was accompanied by loss of lymphatics. These results suggest that tumor-secreted VEGF-C and, to a lesser extent, VEGF-A, are important for inducing prostate cancer intratumoral lymphangiogenesis but are unnecessary for lymph node metastasis. (Cancer Res 2005; 65(21): 9789-98)**

## Introduction

In prostate cancer, metastasis to regional lymph nodes is a frequent early event that is correlated with poor clinical prognosis

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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(1, 2). Typically, pelvic lymphadenectomy is done prior to radical prostatectomy to assess lymph node status. In patients with lymph node-positive prostate cancer, 75% will possess bone metastases within 5 years regardless of treatment (2).

Analysis of metastasis patterns in human prostate cancer (1, 3) as well as lymphatic mapping studies using tracking dyes in breast and melanoma (4, 5) have shown that the pattern of tumor-to-lymph node dissemination is nonrandom. Tumors first invade draining (sentinel) lymph nodes before seeding more distant nodes (6). If the sentinel node is free of metastasis, other lymph nodes will also likely be uninvaded (7). Therefore, lymphatic vessels within or in proximity to tumors mediate dissemination to draining lymph nodes, which may then allow further seeding to more distant sites.

Although lymphatic vessels can be detected in prostate cancer (8–10), the role of intratumoral lymphatics in mediating lymph node metastasis has been controversial. While numerous clinical studies have correlated lymphatic vessel density (LVD) with lymph node metastasis in various cancers, nearly as many have failed to detect such associations (for a summary of clinical data, see ref. 11). In prostate cancer, increased LVD has been correlated both with lymph node metastasis (9, 10) and with higher Gleason score (8, 9), an indicator of more aggressive tumors. Consequently, it is unclear whether tumoral lymphatics actually facilitate lymph node metastasis, or are simply markers of tumors prone to disseminate regardless of LVD.

Tumor lymphangiogenesis is thought to rely on preexisting lymphatics (12). The major lymphangiogenic cytokines are vascular endothelial growth factors-C and -D (VEGF-C and VEGF-D), although platelet-derived growth factor-BB has also recently been implicated (13). VEGF-C and VEGF-D primarily bind VEGF receptor-3 (VEGFR-3, or Flt4) on the surface of lymphatic endothelial cells (14). Levels of VEGF-C/D have generally correlated with lymph node metastasis in human patients (13), and experimental overexpression of VEGF-C (15–17), VEGF-D (18), and platelet-derived growth factor-BB (19) in cell lines has resulted in increased tumor LVD and lymph node metastasis in tumor implantation models. Similar results were also obtained when VEGF-C was overexpressed in spontaneous Rip-Tag tumors (20). Whether the effects are due to increased lymphatic permeability or activation and/or increased abundance of intratumoral and/or peritumoral lymphatics remains unclear (13, 21).

Other studies have suggested that intratumoral lymphatics may be nonfunctional (17, 22, 23), or display abnormal function at the periphery (24), implying that lymphangiogenesis plays little role in facilitating primary tumor dissemination. In contrast, others have shown that interfering with ligand binding to VEGFR-3/Flt4 using a soluble receptor can inhibit tumor lymphangiogenesis and reduce lymph node metastasis (25–29). In most cases, both peritumoral

and intratumoral lymphatic vessels were affected, although some have speculated that the soluble receptor may have little or no effect on preexisting lymphatics (28, 30). Consequently, the requirement for tumor lymphangiogenesis, and the relative roles of intratumoral and peritumoral—preexisting or induced—lymphatics in mediating lymph node metastasis have remained controversial (13).

To address these questions, we investigated the contributions of intratumoral, tumor-induced lymphatics and peritumoral lymphatics in facilitating lymph node metastasis by inhibiting lymphangiogenesis in a surgical orthotopic implantation (SOI) model of human prostate cancer. Our results show that, although intratumoral lymphangiogenesis can be selectively ablated, this has no effect on lymph node metastasis. We also found that spontaneous transgenic adenocarcinoma of the mouse prostate (TRAMP) tumors do not induce lymphangiogenesis but nevertheless metastasize to lymph nodes. These results argue that peritumoral lymphatic vessels, perhaps preexisting at the tumor margins—and not intratumoral lymphatics induced by lymphangiogenesis—are critical for mediating lymph node dissemination.

## Materials and Methods

**Cell culture and mice.** A subline of the human prostate adenocarcinoma cell line PC-3 (American Type Culture Collection, Manassas, VA) was derived in our laboratory and used in these studies (designated PC3-#82). Cells were cultured in F-12K medium (Kaign's modification; Life Technologies-Invitrogen, Frederick, MD) containing 10% fetal bovine serum, glutamine, and antibiotics. Small interfering RNA (siRNA) experiments used PC3-#82 cells expressing ecotropic receptor (plasmid provided by H. Lodish, Biology, Massachusetts Institute of Technology, Cambridge, MA). Immunodeficient CD-1 nude mice, 30 to 35 days old (Charles River Laboratories, Wilmington, MA), were used for xenograft experiments. TRAMP mice (The Jackson Laboratory, Bar Harbor, ME; ref. 31) in a C57BL/6 background were obtained from A. Bai, Biology, Massachusetts Institute of Technology, Cambridge, MA.

**Plasmids.** PC3-#82 cells were transfected with Flt4-Ig expression plasmid (originally "VEGFR-3-Ig/pEBS7", K. Alitalo, Biomedicum, University of Helsinki, Helsinki, Finland; refs. 25, 32) using Effectene reagent (Qiagen, Valencia, CA) and selected with hygromycin (100-200 µg/mL) for stable expression. Ig-Neg control plasmid was made by removing the Flt4 coding sequence. All siRNAs were inserted into the retroviral vector pSIRIS (W.C. Hahn, Dana Farber Cancer Institute, Boston, MA; ref. 33). The siRNA plasmids were transfected with Effectene into Phoenix cells (American Type Culture Collection), and the secreted virus was subsequently used for stable infection of PC3-#82 cells expressing ecotropic receptor. After infection, cells were selected on puromycin (2.5 µg/mL) for stable siRNA expression. Please refer to Supplementary Materials for siRNA sequences.

**Xenografts.** s.c. tumors were obtained by injecting  $2 \times 10^6$  cells into CD-1 nude mice anesthetized with avertin/tribromethanol. Tumors were removed for analysis and/or used as donor material for SOI ~3.5 weeks after injection, as described previously (34, 35). Briefly, a peripheral portion of the tumor was removed and sliced into ~1 mm<sup>3</sup> cubes under a dissecting microscope. CD-1 mice were anesthetized, and the abdominal regions exposed with an incision along the lower midline. A single tumor fragment was embedded into the right dorsolateral capsule and secured with 9-0 microsutures (Ethicon, Somerville, NJ). The peritoneum and overlying skin were each closed with one set of 5-0 sutures (United States Surgical, Norwalk, CT). The entire protocol was done in sterile conditions inside a fume hood, in accordance with animal care guidelines. Mice were analyzed when moribund, as judged by bladder/abdominal distension and/or severe weight loss (typically 2-3 months after implantation). Primary tumors were flash-frozen or fixed for immunohistochemistry. Lymph nodes were removed, fixed, weighed, and sectioned. A lymph node set was considered macroscopically invaded if its total mass exceeded 30 mg (by histology, typically >80% of the node is tumor material at this size; see Supplementary Table S1).

**Vascular endothelial growth factor-C and -A RNA quantitation.** Total RNA was extracted using RNeasy (Qiagen). RNA was digested with DNase (Ambion, Austin, TX), then recleaned with RNeasy. One microgram of total RNA was reverse-transcribed into cDNA using TaqMan reverse transcription reagent (Applied Biosystems, Branchburg, NJ). cDNAs were analyzed by quantitative PCR using SYBR Green PCR amplification kit (Applied Biosystems), measured in a Bio-Rad iCycler (Bio-Rad, Richmond, CA). Target gene message levels were normalized to glyceraldehyde-3-phosphate dehydrogenase levels, and then to the control sample. See Supplementary Materials for real-time PCR primer sequences.

**Vascular endothelial growth factor-C and -A protein quantitation.** PC3-#82 cells ( $5 \times 10^5$ ) were plated into 10 cm<sup>2</sup> plates and grown for 72 hours. Medium was replaced, conditioned for the times specified, collected, and spun to remove debris. Frozen s.c. and orthotopic tumors were thawed and homogenized in 1 mL cold Cellytic-MT mammalian cell lysis buffer (Sigma-Aldrich, St. Louis, MO) per gram of tumor material. Lysis buffer contained protease inhibitors (Roche, Mannheim, Germany). After homogenization, the lysate was chilled for >30 minutes, then spun to remove debris. Total soluble protein was quantitated by bicinchoninic acid protein assay (Pierce, Rockford, IL) to normalize ELISA results. The supernatant was diluted 1:4 or 1:10 in PC-3 medium for ELISA. Diluted tumor supernatant (200 µL) or undiluted conditioned medium was analyzed by human VEGF-A Quantikine ELISA (R&D Systems, Minneapolis, MN); 100 µL of the same were analyzed by human VEGF-C ELISA (IBL, Tokyo, Japan).

**Immunoblotting.** Soluble Flt4-Ig was detected by immunoprecipitating conditioned medium with Protein A beads (Invitrogen, Carlsbad, CA). The beads were spun, washed, and boiled in Laemmli SDS buffer containing 5% β-mercaptoethanol. The protein was run on 8% SDS gel and detected with goat anti-human VEGFR-3 antibody (clone AF349; 1:100 diluted; R&D Systems) or rabbit anti-human antibody conjugated to horseradish peroxidase (1:1,000 diluted; DAKO, Glostrup, Denmark). Tumor Flt4-Ig was detected in tumors by homogenizing in Cellytic-MT lysis buffer, as above, and running supernatant on SDS-PAGE.

**Histology and immunohistochemistry.** For immunohistochemistry, 2- to 3-mm-thick portions were removed near the periphery of the anterior-facing end of the tumor. For wild-type and TRAMP prostates, the dorsolateral lobes were dissected. In most cases, the tissue was fixed in zinc (Becton Dickinson, San Diego, CA) for 48 hours. For short-term orthotopic analysis, prostate tissue was fixed in 3.7% formaldehyde overnight. Primary antibodies for immunohistochemistry included rabbit anti-LYVE-1 (Ruoslahti lab; 1:450; ref. 36), goat anti-mouse VEGFR-3 (clone AF743, R&D Systems; 1:25) and rat anti-CD34 (clone RAM34, BD PharMingen, San Diego, CA; 1:25). Sections were dewaxed, microwaved in BD Retrieval buffer, and stained using standard protocols. Biotin-conjugated secondary antibodies included swine anti-rabbit immunoglobulin (DAKO) and rabbit anti-rat immunoglobulin (Vector Labs, Burlingame, CA), both diluted 1:250. Staining was amplified with Vectastain ABC kit (Vector Labs), developed with Vector VIP peroxidase substrate and counterstained with methyl green. Lymphatic and blood vessels were quantitated by counting the number of LYVE-1 or CD34-positive vessels, respectively, in two random, low-power fields (2.25 × 1.7 mm) per tumor. About 30% to 100% of the tumor area is covered with this approach, and the LVD from a minimum of seven independent tumors was typically quantitated for each cell line. In TRAMP and normal prostates, a single low-power field was used for lymphatic quantitation, typically covering 70% to 100% of the sample. TRAMP tumor grading was based on a system described by Hurwitz et al. (37). Two pathologists (M. Barry and R. Bronson) independently graded H&E TRAMP sections, and then together arrived at an agreed upon grade. For short-term SOI analysis, the length of the tumor periphery at ×105 final magnification was quantitated in pixel units by OpenLab software (Improvision Inc., Lexington, MA), and the number of lymphatics at the periphery was normalized to a 1,000-pixel perimeter. We defined "intratumoral" lymphatics as LYVE-1-positive vessels completely surrounded by tumor cells, and "marginal" or "peritumoral" lymphatics as vessels in contact with both tumor cells and stroma.

**Statistics.** All statistical comparisons were calculated with the unpaired Student's *t* test. All error bars show  $\pm$  SE.

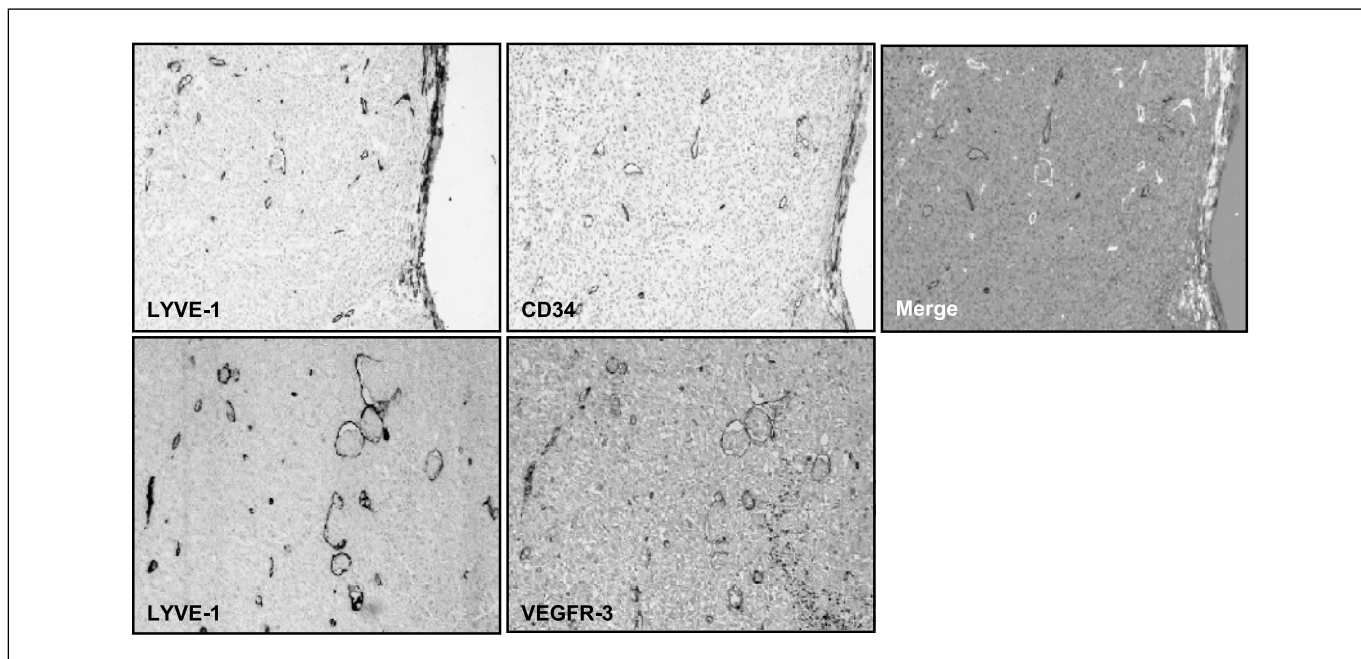
## Results

**Stable small interfering RNAs specifically reduce vascular endothelial growth factor-C or -A expression and protein secretion.** s.c. tumors formed by a subline of PC-3 prostate cancer cells (designated PC3-#82) possessed abundant intratumoral lymphatic vessels, as confirmed by immunohistochemical staining for the lymphatic markers LYVE-1 and VEGFR-3/Flt4, and absence of staining for the blood vessel marker CD34 (Fig. 1). Because blood vessels in some tumors have been reported to express VEGFR-3/Flt4 (38), we used LYVE-1 and CD34 for the rest of these studies.

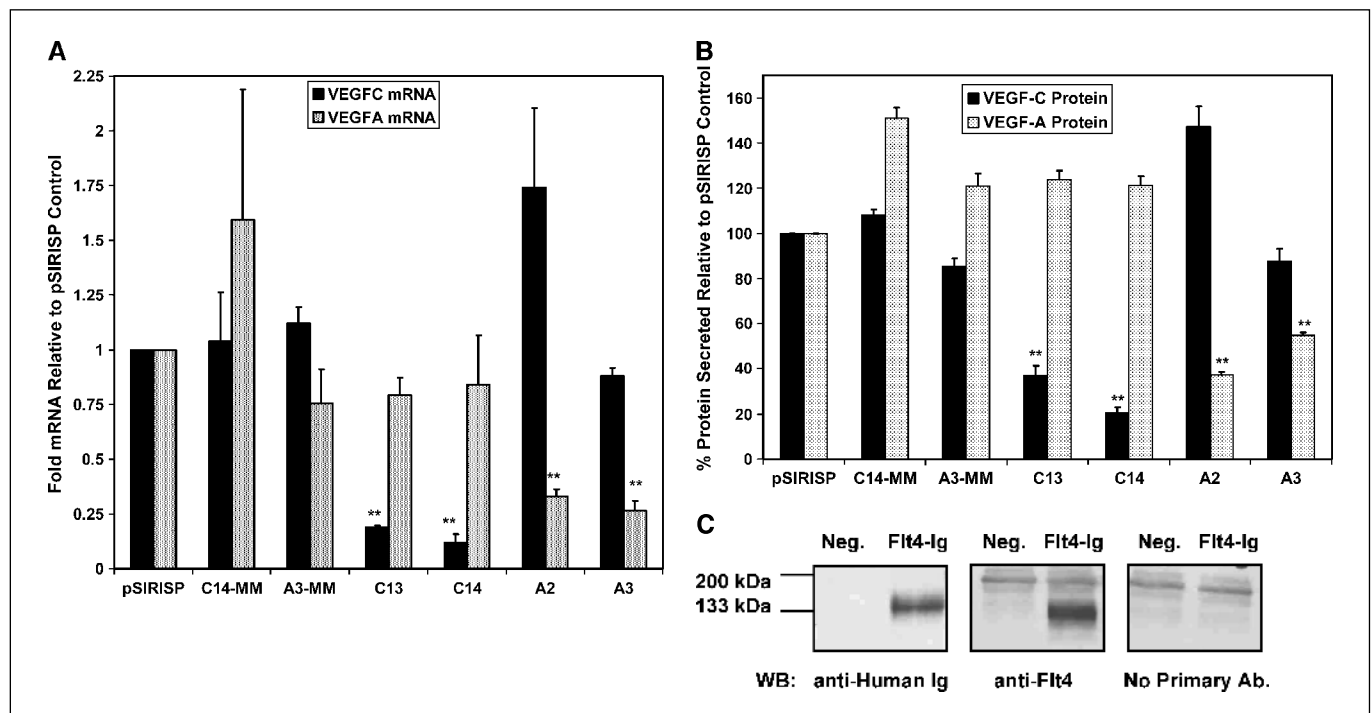
PC3-#82 cells expressed and secreted VEGF-C (Fig. 2A and B), but not VEGF-D (data not shown). To examine the importance of tumor-secreted VEGF-C in promoting lymphangiogenesis, we stably expressed siRNAs against VEGF-C in PC3-#82 cells. We also generated siRNAs against VEGF-A, a potent inducer of angiogenesis previously reported to stimulate lymphatic growth in the mouse ear (39). C13 and C14 siRNAs knocked down VEGF-C mRNA by 81% and 88%, respectively, relative to vector control (Fig. 2A). A2 and A3 siRNAs reduced VEGF-A mRNA by 67% and 74%, respectively (Fig. 2A). Quantitative PCR showed that VEGF-C siRNAs had little effect on VEGF-A expression, and vice versa. C14-MM and A3-MM mismatch (MM) controls showed little siRNA efficacy. Relative RNA message levels for VEGF-C/A were reflected in their relative protein abundance in conditioned medium, as assayed by ELISA (Fig. 2B). Cells expressing C14 siRNA accumulated virtually no VEGF-C in conditioned medium after 72 hours, whereas cells expressing C13 showed modest accumulation (Supplementary Fig. S1). VEGF-A secretion was not significantly reduced by either C13 or C14 siRNAs, but was slightly increased in C14-MM control.

As an additional approach for ablating lymphatics, we expressed the soluble VEGFR-3/Flt4-human Fc-Ig fusion protein (Flt4-Ig) in PC3-#82 cells, as described previously (25, 32). Protein secretion was confirmed by immunoprecipitation from conditioned medium and Western blot against VEGFR-3 (Fig. 2C).

**Tumor-secreted vascular endothelial growth factor-C is necessary for lymphangiogenesis.** PC3-#82 cells expressing siRNAs against VEGF-C or VEGF-A, or control siRNAs were injected s.c. into CD-1 immunodeficient mice. We did not see consistent tumor growth effects correlated with VEGF-C or VEGF-A inhibition. Tumors were removed  $\sim$ 3.5 weeks postinjection, sectioned and stained for LYVE-1 and CD34. PC3-#82 cells expressing C14 siRNA showed a  $>$ 99% reduction in LVD (Fig. 3A, c) relative to controls (Fig. 3A, a and b). Tumors expressing C13, a less effective siRNA against VEGF-C, yielded an 83% reduction in LVD relative to controls (Fig. 3B). Interestingly, both siRNAs against VEGF-A (A2, A3) yielded a nearly 50% reduction in LVD versus controls (Fig. 3A, d for A3 and data not shown for A2; combined  $P = 0.052$ ). As both VEGF-A siRNAs were only partially effective in reducing VEGF-A gene expression, it is possible that more potent VEGF-A siRNAs would have yielded greater reduction in LVD. LVD quantitation of siRNA- or siRNA control-expressing tumors is shown in Fig. 3B. In agreement with results by others (25, 26), expression of soluble Flt4-Ig (Fig. 3A, f) yielded complete inhibition of lymphangiogenesis versus Ig-Neg control (Fig. 3A, e and C). In all cases, blood vessel density was not consistently affected (Fig. 3B and C; images not shown), although C14-MM control tumors had somewhat increased angiogenesis. Staining also appeared slightly lighter in some tumors expressing siRNA. Taken together, these results indicate that tumor-secreted VEGF-C is necessary for intratumoral lymphangiogenesis. To a lesser extent, tumor-secreted VEGF-A may also be important. The lack of reduction in blood vessels, especially by A2 and A3, might



**Figure 1.** PC3-#82 cells form s.c. tumors with intratumoral lymphatic and blood vessels. Serial tumor sections probed with antibodies against LYVE-1 or CD34 show specific, nonoverlapping staining for lymphatics or blood vessels, respectively (top). In the merged image, lymphatics are colored white, and blood vessels black. Serial sections probed with LYVE-1 and anti-mouse VEGFR-3/Flt4 show coincident staining (bottom).



**Figure 2.** PC3-#82 cell expression and secretion of VEGF-C or VEGF-A can be reduced by siRNA. *A*, relative mRNA levels for VEGF-C (black) or VEGF-A (gray) were measured by quantitative PCR, and normalized to pSIRISP vector control. C13 and C14 cells express siRNAs against VEGF-C. A2 and A3 cells express siRNAs against VEGF-A. C14-MM and A3-MM (MM, mismatch) are siRNA specificity controls. siRNAs specifically down-regulated expression of the target gene and reduced accumulation of either VEGF-C or VEGF-A protein in conditioned medium, as assessed by ELISA (*B*). Control samples typically secreted ~2,000 pg VEGF-C/mL conditioned medium/24 hours, and ~200 pg VEGF-A/mL conditioned medium/24 hours. *C*, secretion of soluble VEGFR-3/Flt4-Ig fusion protein in PC3-#82 cells was confirmed by immunoprecipitating fusion protein from conditioned medium and Western blot (\*\*,  $P < 0.001$ ).

reflect the limited mRNA knock-down, and/or suggest that other angiogenic factors are sufficient for inducing tumor angiogenesis.

**Ablation of prostate intratumoral lymphatics does not inhibit lymph node metastasis.** PC-3 cells have been reported to metastasize infrequently from s.c. tumors (40). In contrast, prostate tumor cells introduced orthotopically metastasize in patterns similar to human prostate cancer, including invasion of lymph nodes, lung, and bone (34, 35). To model the early stages of metastasis, where cells must detach from the primary tumor before intravasating into vasculature, we used SOI to graft a single piece of solid tumor into the dorsolateral prostate lobes of CD-1 mice (Fig. 4A). Graft material was derived from s.c. tumors. As an advantage over other xenograft techniques, SOI minimizes the artificial dispersal of cells away from the site of implantation, as is often seen when tumor cells are injected as a suspension (41). As expected, the tumors introduced by SOI develop initially from a single focus in the interluminal spaces of the prostate (Fig. 6A, a). Between 2 and 3 months after implantation, ~50% of mice develop lymph node macrometastases. We find that hematogenous spread (circulating tumor cells in blood and lung metastasis) is strongly associated with lymphatic invasion (Supplementary Fig. S2), and primary tumors possess abundant blood and lymphatic vessels (data not shown; similar to Fig. 1).

To determine the metastatic effects of ablating lymphatic vessels in orthotopic prostate tumors, we used SOI to transplant PC3-#82 tumors expressing either VEGF-C siRNA (C14), Flt4-Ig, or controls. LVD was reduced by ~50% in both C14- and Flt4-Ig-expressing tumors (Fig. 4B). Although statistically significant ( $P = 0.012$  for C14 versus control;  $P = 0.0046$  for Flt4-Ig versus control), this reduction

was far less severe than was seen in s.c. tumors. Expression of either C14 siRNA or Flt4-Ig in orthotopic tumors did not affect the incidence of macroscopic lymph node metastasis (Fig. 4C), the average mass of macroscopically invaded lymph nodes (Fig. 4C;  $P = 0.92$ ), or the average size of the primary tumors (data not shown). For lymph nodes without obvious macrometastases, histologic analysis identified micrometastases in virtually all samples analyzed, regardless of cell line (Supplementary Table S1).

To understand why inhibition of lymphangiogenesis was less effective in orthotopic versus s.c. tumors, we used ELISA to measure the human VEGF-C protein levels in s.c. and orthotopic tumors expressing C14 or siRNA control. The concentration of human VEGF-C protein in C14 orthotopic tumors was increased relative to C14 s.c. tumors and was correlated with LVD in both C14 and control tumors (Supplementary Fig. S3). Reduced siRNA-mediated inhibition of VEGF-C secretion over the duration of the experiment possibly accounted for less severe inhibition of tumor lymphangiogenesis. In the case of Flt4-Ig-expressing tumors, Western blot for VEGFR3/Flt4 indicated that, in some orthotopic tumors, expression of the fusion protein was also severely reduced (Supplementary Fig. S4).

To further ablate orthotopic intratumoral lymphatics, we generated a derivative of PC3-#82 that combined expression of VEGF-C siRNA and Flt4-Ig (Flt-C14), in addition to a cell line expressing both empty-vector controls (Ig-pSIRISP). Specific knock-down of VEGF-C mRNA and secretion of Flt4-Ig were again confirmed (Fig. 5A). As expected, Flt-C14 s.c. tumors possessed no lymphatic vessels, whereas Ig-pSIRISP control tumors had abundant LYVE-1 staining (Fig. 5B). When implanted orthotopically, Flt-C14 tumors ( $n = 11$ ) exhibited a 92% reduction in LVD

versus controls ( $n = 8$ ;  $P < 0.001$ ). A single Flt4-C14 tumor possessed moderate LVD, and without this outlier, inhibition of LVD increases to 98% versus control. Interestingly, blood vessel density was also reduced  $\sim 35\%$  in Flt-C14 tumors versus Ig-pSIRISP control.

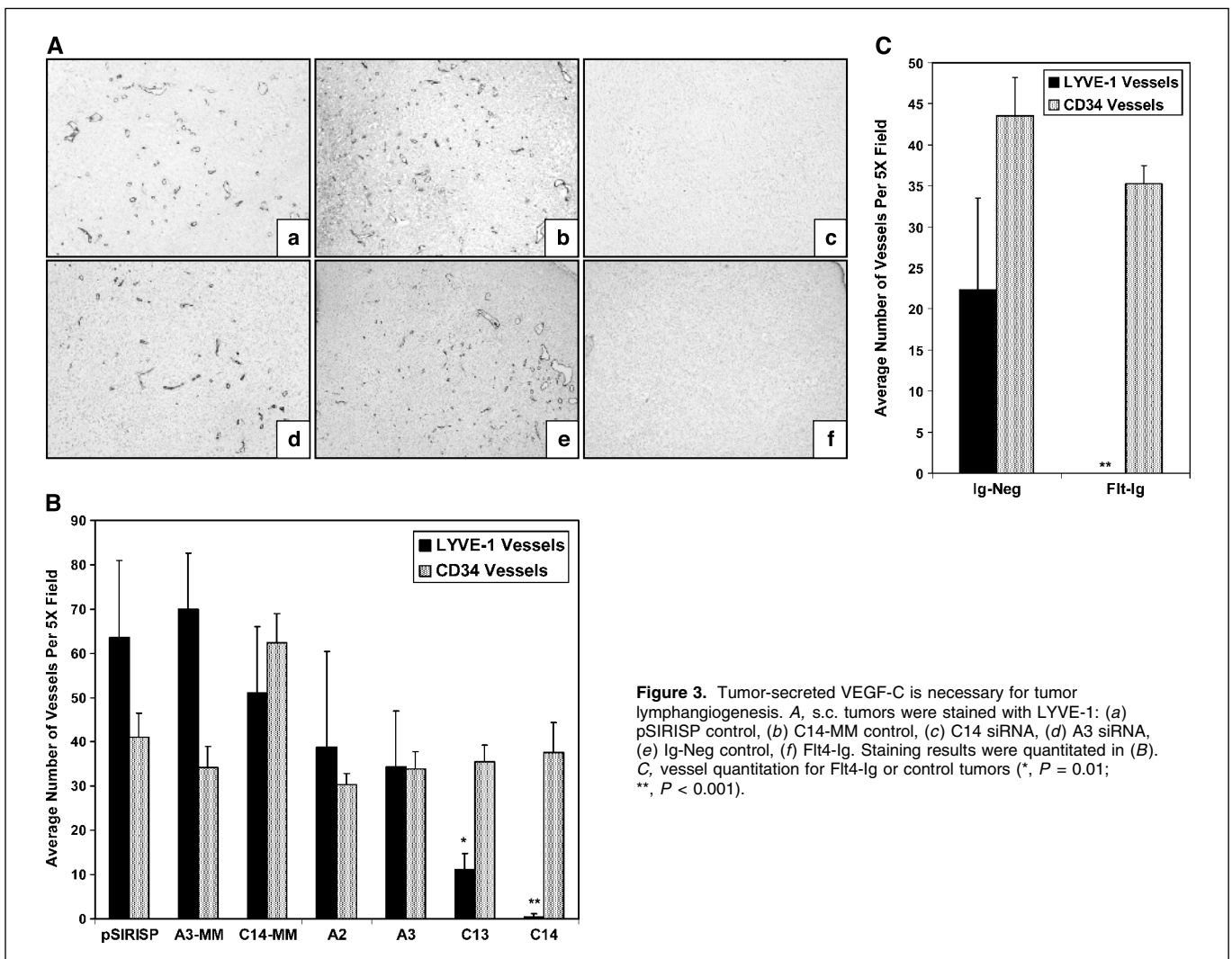
Despite a  $>92\%$  reduction in intratumoral lymphatics, the incidence of microscopic and macroscopic lymph node invasion, and the mass of macroscopically invaded lymph nodes were again largely unaffected in Flt-C14 tumors versus Ig-pSIRISP control (Fig. 5C;  $P = 0.15$ ), or other controls used in this study (Figs. 4C and Fig. 5C). As before, nearly all local lymph nodes evaluated harbored micro- or macrometastatic tumor invasion (Supplementary Table S1). Also, we found no significant correlation between LVD and lymph node metastasis in individual orthotopic tumors whose lymphatics were ablated (Fig. 5D). Our data argue that intratumoral lymphangiogenesis is unnecessary for prostate cancer metastasis to lymph nodes.

**Abundance of preexisting marginal lymphatics is unaffected in Flt-C14 orthotopic tumors.** At least two possible explanations could account for how orthotopic prostate tumors metastasized efficiently to lymph nodes despite a  $>98\%$  inhibition of intratumoral lymphangiogenesis in 10 of 11 Flt-C14 tumors. Formally, it is possible that a minority of lymphatic vessels ( $<2\%$  of total) is

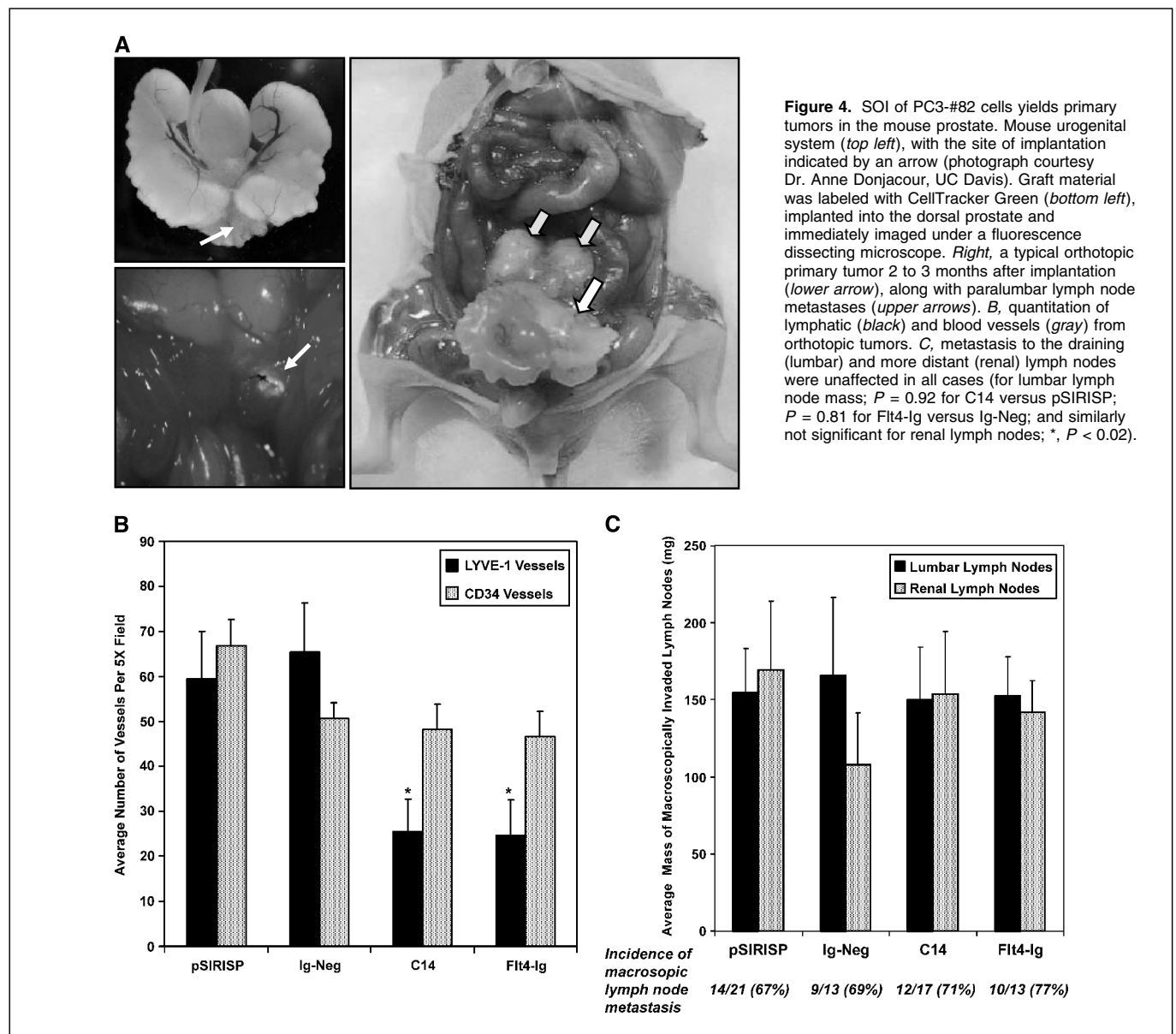
sufficient for metastasis. A more likely explanation is that marginal lymphatic vessels at the tumor-stromal margin—and not intratumoral lymphatics—are responsible for mediating lymph node metastasis.

Because orthotopic tumors were analyzed 2 to 3 months after implantation, the tumors tended to be large ( $\sim 1$  g; see Fig. 4A) and almost completely devoid of stromal tissue. To examine tumor interaction with preexisting marginal lymphatics, we transplanted Flt-C14 or control tumors using SOI and analyzed them 2 to 3 weeks after implantation. In most cases, tumors were not palpable and were found by sectioning through the dorsolateral prostate (Fig. 6A, a). As expected, primary tumors consistently arose from a single focus.

We stained microscopic Flt-C14 or control orthotopic tumors with LYVE-1 and found that both were in contact with lymphatics located at the tumor-stromal margin (Fig. 6A, b-d; data not shown for control tumors). In Flt-C14 tumors, all stages of lymphatic invasion were observed, including tumor growth up against individual lymphatic vessels without compression (Fig. 6A, b), intravasation of tumor cells into lymphatics (Fig. 6A, c), and crushing of vessels (Fig. 6A, d). Typically, these marginal lymphatics delineated the exact region of contact between the expanding tumor periphery and the surrounding prostatic stroma.



**Figure 3.** Tumor-secreted VEGF-C is necessary for tumor lymphangiogenesis. A, s.c. tumors were stained with LYVE-1: (a) pSIRISP control, (b) C14-MM control, (c) C14 siRNA, (d) A3 siRNA, (e) Ig-Neg control, (f) Flt4-Ig. Staining results were quantitated in (B). C, vessel quantitation for Flt4-Ig or control tumors (\*,  $P = 0.01$ ; \*\*,  $P < 0.001$ ).



Quantitation of marginal lymphatics revealed little difference between Flt-C14 and control orthotopic tumors (Fig. 6B;  $P = 0.55$ ). However, intratumoral lymphatics were present in control tumors but completely absent in Flt-C14 tumors (Fig. 6B). These results indicate that a combination of VEGF-C siRNA and Flt4-Ig fusion protein selectively inhibited intratumoral lymphangiogenesis without affecting marginal, possibly preexisting, lymphatics, and suggest that these vessels at the periphery are sufficient for mediating lymph node metastasis.

**Spontaneous TRAMP tumors do not induce lymphangiogenesis.** To extend our observations, we examined the lymphatics in spontaneous TRAMP tumors. TRAMP transgenic mice express the SV40 large T antigen driven by the prostate-specific rat probasin promoter (31). Prostatic intraepithelial neoplasia (PIN), a precursor of prostate cancer, appears as early as 10 weeks of age, and progresses to undifferentiated adenocarcinoma (42). TRAMP prostates are graded 1 to 6 (1 being normal prostate, and 6 being undifferentiated adenocarcinoma), based on

variables including cell differentiation and invasion through the basement membrane (37, 43). By 28 weeks, 100% of TRAMP mice were reported to harbor lymph node and/or lung metastases (44). Importantly, local and distant dissemination is predominantly seen only in mice with primary tumors of grade 4 or higher (37).

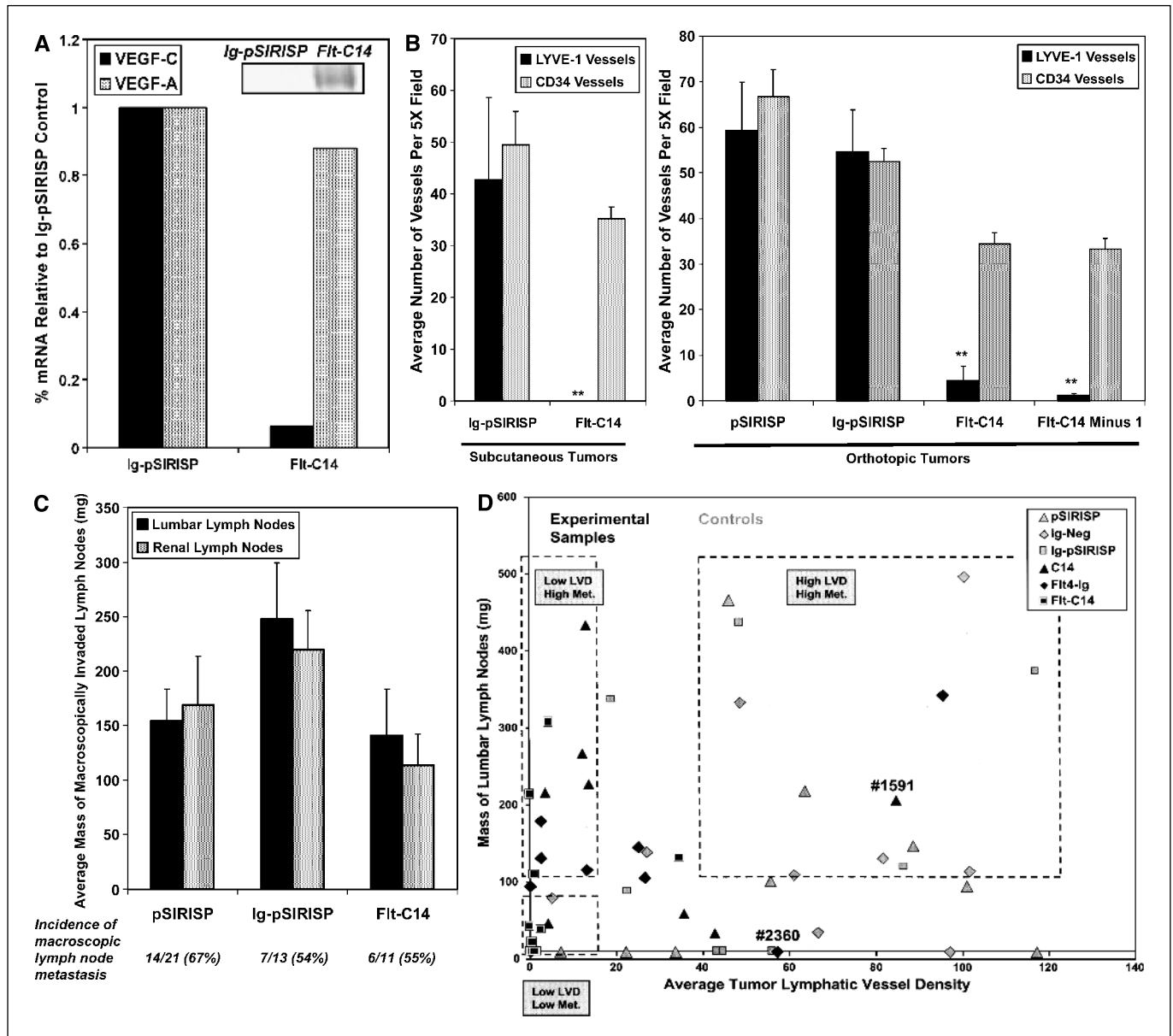
We examined the prostatic lymphatics in 8 normal C57BL/6 mice and 14 TRAMP mice at different ages and/or tumor stages. The lymphatics in normal prostates were located in the interluminal spaces outside individual ductal structures (Supplementary Fig. S5), and their abundance and location did not differ in mice between 15 and 35 weeks of age (data not shown). In TRAMP prostates, PIN develops from the initial expansion of luminal cells within ductal structures. As with wild-type prostates, lymphatics in TRAMP prostates were consistently located outside of ductal structures and did not infiltrate into tumorigenic areas (Fig. 6C). Tumorigenic prostates graded from 1 to 4 did not exhibit significant differences in LVD versus normal

prostates (Fig. 6D). In the most severe cases of prostate cancer (grades 5 and 6), the tumorigenic regions had overtaken the surrounding stroma, and lymphatic density in the prostate was reduced 7-fold versus either normal prostates or low-grade tumorigenic prostates (Fig. 6C and D;  $P < 0.001$ ). Because metastasis to lymph nodes is predominantly seen only in high-grade TRAMP tumors (37), it is likely that these spontaneous tumors also use preexisting lymphatics located at the tumor-

stromal border prior to vessel compression and destruction. These results support our findings in the xenograft SOI model that intratumoral lymphangiogenesis is not required for lymph node metastasis.

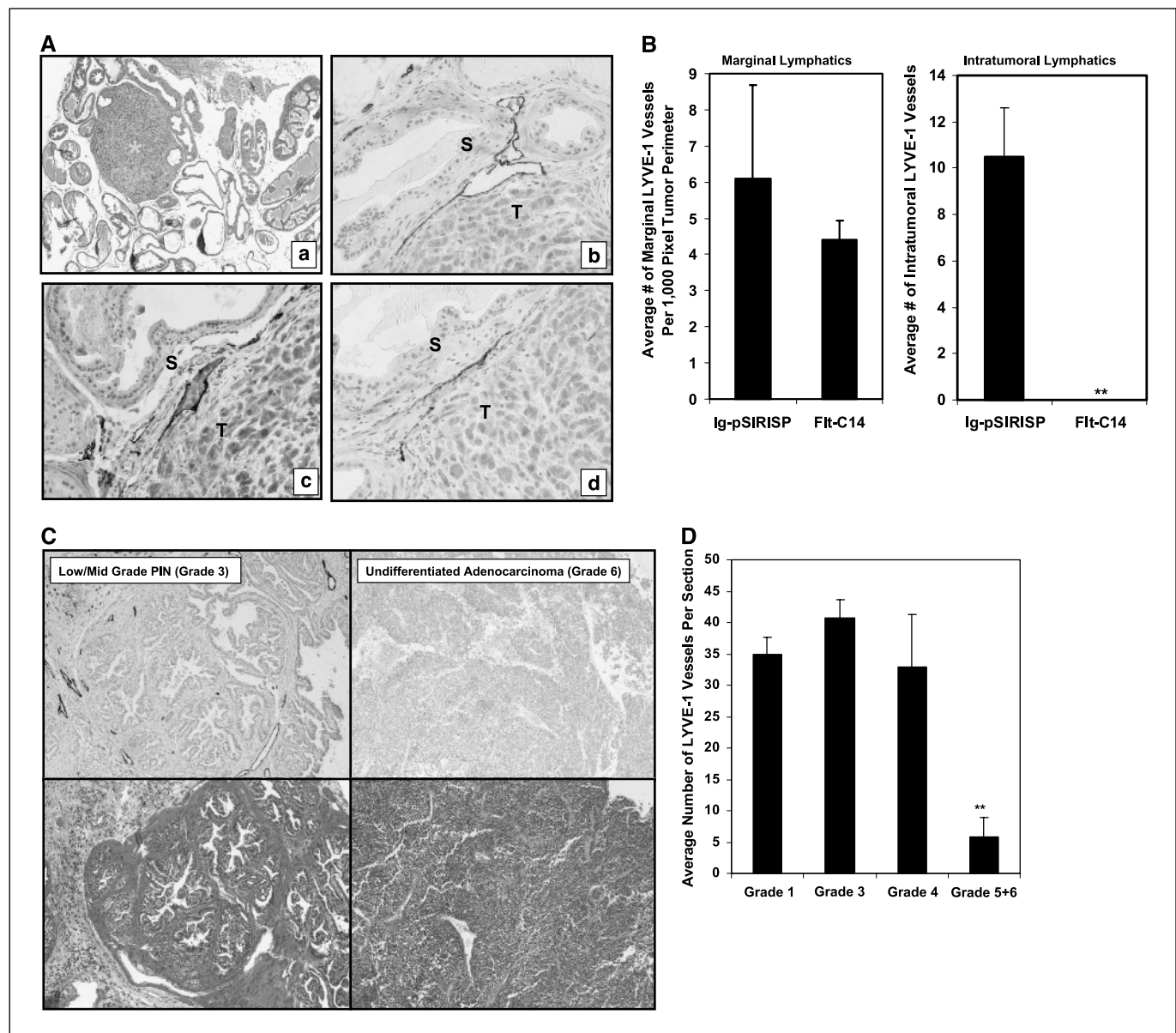
## Discussion

Lymph node status has traditionally been used as a prognostic indicator of prostate cancer aggressiveness, dissemination to



**Figure 5.** Combined expression of VEGF-C siRNA and Fit4-Ig in PC3-#82 cells effectively reduces orthotopic tumor LVD without markedly affecting lymph node metastasis. *A*, combined Fit-C14-expressing cells were validated for VEGF-C mRNA knock-down and secretion of fusion protein (*inset*). *B*, lymphatic and blood vessel densities were quantitated from s.c. (*left*) and orthotopic (*right*) primary tumors. Combination of Fit4-Ig with C14 siRNA inhibited average LVD 100% in s.c. tumors and >92% in orthotopic tumors ( $n = 11$ ). In 10 of 11 Fit-C14 orthotopic tumors, average LVD was reduced >98% ("minus 1"), compared with Ig-pSIRISP control ( $n = 8$ ). For reference, vessel density for pSIRISP vector control from Fig. 4B is also shown. *C*, metastasis to draining and more distant lymph nodes was not significantly affected in Fit-C14 tumors compared with Ig-pSIRISP control ( $P = 0.15$  for lumbar lymph nodes) or pSIRISP control ( $P = 0.80$ ; data reproduced from Fig. 4C). *D*, metastasis is not correlated with LVD in individual orthotopic tumors. Points, data from a single tumor/mouse. Control tumors (*gray*) and experimental tumors (*black*; refer to labels in *D*) for cell line identification. *Top right box*, tumors with heavy lymph node metastatic burdens (>100 mg mass, or  $\sim 10\times$  the typical mass of uninvaded lymph nodes) and high LVD. Note that tumors in this zone consist predominantly of control samples (*gray*). *Bottom left box*, tumors with low lymph node metastatic burdens and low LVD. Note that tumors in this zone predominantly consist of samples where LVD was experimentally reduced (*black*). *Top left box*, tumors with heavy lymph node metastatic burdens despite low LVD. Note that all samples in this zone are experimentally manipulated (*black*). Tumor #1591 (C14) was subsequently found to have lost siRNA inhibition of VEGF-C (Supplementary Fig. S3), whereas tumor #2360 showed reduced Fit4-Ig protein levels (Supplementary Fig. S4; \*\*,  $P < 0.001$ ).





**Figure 6.** Marginal lymphatics are found at the tumor-stromal interface of Flt-C14 orthotopic tumors, and in the interluminal spaces outside regions of PIN in TRAMP prostates. **A**, H&E staining of the prostate 2 to 3 weeks after SOI reveals a single focal microscopic tumor (*star*) surrounded by prostatic ductal acinar structures (**a**). All stages of lymphatic invasion were seen in Flt-C14 orthotopic tumors stained with LYVE-1, including tumor growth against lymphatic vessels (**b**), intravasated vessels containing tumor cells (**c**), and compression of vessels (**d**). Note that, in all cases, marginal lymphatics delineated the tumor-stromal junction ("T," tumor region; "S," stromal region). **B**, quantitation of marginal lymphatics in Flt-C14 and control orthotopic tumors (*left*), and quantitation of intratumoral lymphatics (*right*). Flt-C14 tumors possessed marginal, but not intratumoral lymphatics. **C**, regions of grade 3 PIN (*left*), or grade 6 undifferentiated adenocarcinoma (*right*) from TRAMP prostates were stained with LYVE-1 (*upper*) or H&E (*bottom*). In samples with PIN, lymphatics were located in the stroma and excluded from tumorigenic regions. Lymphatics were mostly absent in regions of undifferentiated adenocarcinoma. **D**, quantitation of total lymphatics in normal and TRAMP prostate sections. Grades 5 to 6 TRAMP prostates possess significantly fewer lymphatic vessels than lower grade or normal (grade 1) prostates ( $P < 0.001$ ).

distant sites and likelihood of recurrence after therapy (1–3, 45). Although recent clinical studies have examined the abundance of lymphatics and/or lymphatic growth factors in prostate cancer, the results have been difficult to interpret. In most cases, VEGFR-3/Flt4 was up-regulated in advanced or node-positive prostate cancer (9, 10, 46, 47). One study found augmented tyrosine phosphorylation of VEGFR-3/Flt4 in advanced versus early stage (node-negative) prostate cancer (46), whereas another found up-regulation of a truncated form of VEGFR3/Flt-4, but not full-length receptor (48). Furthermore, VEGF-C was up-regulated in some cases of metastatic prostate cancer (9, 10) but not in others

(46, 48). VEGF-D was also increased in node-positive versus node-negative prostate cancer in some studies (46, 48) but was unchanged in another (9).

Part of the complexity in analyzing these data arises from the difficulty of distinguishing whether VEGFR-3/Flt4 was up-regulated in tumor-associated lymphatics or in the tumor cells themselves (46–48). Indeed, *in vitro* studies have shown that prostate cancer cell lines can express the related receptors VEGFR-1/Flt1 (49, 50) and VEGFR-2/Flk1 (49–51). Furthermore, staining for VEGFR-3/Flt4 in one study of prostate cancer exclusively highlighted tumor and epithelial cells, but not endothelial vessels (46).

In studies where VEGFR-3/Flt4 staining identified lymphatics associated with prostate cancer, the localization of these vessels was either reported to be peritumoral (10), or both peritumoral and intratumoral (9). However, VEGFR-3/Flt4 has also been found to be expressed in some tumor blood vessels (38). One clinical study of prostate cancer reported lymphatics primarily in the tumor periphery and nontumorigenic stromal regions (8). Because lymphatics were significantly reduced in tumors, the authors speculated that prostate cancer progression causes lymphatic destruction. LYVE-1-positive vessels were also correlated with increased Gleason score (8), but a detailed study comparing node-positive- with node-negative prostate cancer using LYVE-1 has not been done.

Up-regulated VEGFR-3/Flt4, VEGF-C, and VEGF-D have also been correlated with other variables of prostate cancer progression, including Gleason score (9, 10, 47) and PSA level (10, 47). Consequently, it is difficult to distinguish whether increased lymphatics actually facilitate nodal metastasis or are simply markers of more aggressive primary tumors. Similarly, in experimental mouse models, whether lymphangiogenesis is required for lymph node metastasis may depend on the innate aggressiveness of the tumor in question. It is possible that for tumors already adept at colonizing distant sites, preexisting lymphatics may be sufficient for lymph node metastasis, whereas less aggressive cancers may require additional vessels to disperse more cells and increase the probability of metastasis. This may explain why overexpression of insulin-like growth factor receptor I in pancreatic islet tumors by Hanahan's group yielded aggressive tumors that metastasized to lymph nodes without significant lymphangiogenesis [as reported by Alitalo et al. (ref. 52)].

Functional studies using assays for microlymphangiography and interstitial fluid pressure have suggested that intratumoral lymphatics may be nonfunctional (17, 22). Tumor compression of intratumoral lymphatic vessels may be responsible for the absence of function, although tumor-induced lymphatics may inherently be physiologically abnormal (23, 24). Although this apparent absence of function has been interpreted to suggest that intratumoral lymphatics are unimportant for metastasis, that hypothesis needs further testing.

In contrast with our results, work by others has shown that inhibiting tumor lymphatics with soluble VEGFR-3/Flt4-Ig fusion protein can reduce metastasis to lymph nodes both in xenograft models (25, 26, 28, 53, 54) and in Rip-Tag spontaneous tumors (27). In most studies, both peritumoral and intratumoral lymphatics were inhibited, although some have suggested that Flt4-Ig may have no effect on preexisting lymphatics (28, 30, 53, 54), or may inhibit peripheral, but not intratumoral, lymphatics (27). The varying effectiveness of Flt4-Ig may reflect how and when the inhibitor was administered, its concentration, diffusion to surrounding tissues, abundance of preexisting lymphatics, and local concentration of VEGF-C/D ligands. Several articles report that high-level, systemic expression of Flt4-Ig fusion protein can suppress metastasis (27, 53, 54). Recent work by Pytowski et al. has suggested that VEGF-C-mediated VEGFR-3 signaling might be unnecessary for the maintenance of preexisting lymphatics in the mouse tail (55). In any case, the relative importance of peritumoral versus intratumoral lymphatics in mediating lymph node spread has remained unclear. In addition, inhibiting lymphangiogenesis through the use of soluble receptor, VEGFR-3/Flt4 antibody (29) or VEGF-D antibody (18), has not distinguished between the ligands required for the process and/or the source of the ligands. VEGF-C/D may be secreted by tumors or from stromal sources including tumor-associated macrophages (56).

In this study, we have used the SOI model of human prostate cancer to show that intratumoral lymphangiogenesis can be

inhibited in tumors (Flt-C14) without significantly affecting lymph node metastasis. In early stage Flt-C14 tumors, we found that despite the absence of intratumoral lymphangiogenesis, the abundance of peritumoral lymphatics was not statistically different from controls, and in all cases, tumor-intravasated lymphatic vessels were observed. These data suggest that intratumoral lymphangiogenesis is unnecessary for lymph node metastasis in prostate cancer, and that marginal, possibly preexisting, lymphatics are sufficient. He et al. (53) reported that VEGF-C can promote dilation and sprouting in preexisting lymphatics and that this could be inhibited by high levels of systemic Flt4-Ig, although the tumor cells still coopted the preexisting lymphatics and lymph node metastases still occurred, albeit at reduced levels. Those results could be reconciled with ours if the high levels of Flt4-Ig partially inhibited the intravasation of tumor cells into preexisting lymphatics.

We have also obtained corroborative results using the TRAMP spontaneous model of prostate cancer. In TRAMP, metastasis to lymph nodes is primarily observed only in tumors of grade 4 or higher (37). In TRAMP prostates, we found that lymphatics were typically located outside the luminal acinar regions where PIN and adenocarcinoma develop. Peritumoral, but not intratumoral, lymphatics were seen and, as the tumors invaded through the basement membrane into surrounding stromal regions (grades 5 and 6), significantly fewer lymphatics were observed, suggesting the destruction of preexisting lymphatics and the absence of lymphangiogenesis. This is similar to human clinical prostate cancer (8), and also indicates that preexisting peritumoral lymphatics are sufficient for lymph node metastasis.

It remains to be determined whether lymph node metastasis is important for hematogenous dissemination. In our SOI model, we observed that hematogenous metastasis was strongly associated with lymph node invasion (Supplementary Fig. S2). These data may indicate that tumors enter the blood circulation indirectly via lymphatics, or that blood and lymphatic vessel intravasation occur simultaneously. Others have proposed that lymph nodes may act as bridgeheads where tumor cells with limited metastatic capability can proliferate and acquire additional mutations that allow further dissemination (57). Whether this hypothesis is accurate remains to be seen.

In summary, we have shown that, in prostate cancer, lymph node metastasis relies on peritumoral, and not intratumoral, lymphatics, suggesting that the peritumoral lymphatics that preexist before tumor development may be sufficient for disseminating tumor cells to local and more distal lymph nodes. Our results also suggest that inhibiting lymphangiogenesis may be easier than ablating preexisting lymphatics. As targeting lymphatic vasculature has recently been proposed as an antimetastatic approach for limiting the spread of primary tumors (13), this study shows that the need to target the surrounding marginal lymphatics is especially imperative.

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