



Vascular Endothelial Growth Factor C Disrupts the Endothelial Lymphatic Barrier to Promote Colorectal Cancer Invasion

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BACKGROUND & AIMS: Colorectal cancer (CRC) is highly metastatic. Metastases spread directly into local tissue or invade distant organs via blood and lymphatic vessels, but the role of lymphangiogenesis in CRC progression has not been determined. Lymphangiogenesis is induced via vascular endothelial growth factor C (VEGFC) activation of its receptor, VEGFR3; high levels of VEGFC have been measured in colorectal tumors undergoing lymphangiogenesis and correlated with metastasis. We investigated VEGFC signaling and lymphatic barriers in human tumor tissues and mice with orthotopic colorectal tumors. **METHODS:** We performed immunohistochemical, immunoblot, and real-time polymerase chain reaction analyses of colorectal tumor specimens collected from patients; healthy intestinal tissues collected during operations of patients without CRC were used as controls. CT26 CRC cells were injected into the distal posterior rectum of BALB/c-nude mice. Mice were given injections of an antibody against VEGFR3 or an adenovirus encoding human VEGFC before orthotopic tumors and metastases formed. Lymph node, lung, and liver tissues were collected and evaluated by flow cytometry. We measured expression of vascular endothelial cadherin (CDH5) on lymphatic vessels in mice and in human intestinal lymphatic endothelial cells. **RESULTS:** Levels of podoplanin (a marker of lymphatic vessels), VEGFC, and VEGFR3 were increased in colorectal tumor tissues, compared with controls. Mice that expressed VEGFC from the adenoviral vector had increased lymphatic vessel density and more metastases in lymph nodes, lungs, and livers, compared with control mice. Anti-VEGFR3 antibody reduced numbers of lymphatic vessels in colons and prevented metastasis. Expression of VEGFC compromised the lymphatic endothelial barrier in mice and endothelial cells, reducing expression of CDH5, increasing permeability, and increasing trans-endothelial migration by CRC cells. Opposite effects were observed in mice and cells when VEGFR3 was blocked. **CONCLUSIONS:** VEGFC signaling via VEGFR3 promotes lymphangiogenesis and metastasis by orthotopic colorectal tumors in mice and reduces lymphatic endothelial barrier integrity. Levels of VEGFC and markers of lymphatic vessels are increased in CRC tissues from patients, compared with healthy intestine. Strategies to block VEGFR3 might be developed to prevent CRC metastasis in patients.

Keywords: Colon Cancer; Mouse Model; Tumor Progression; VEGFC.

lymphatic vessel (LV) invasion and metastasis are common in CRC's early stages.² Previous studies have shown that vascular endothelial growth factor (VEGF) family members and their receptors (VEGFR) contribute to lymphangiogenesis and the metastatic process in CRC.² In fact, high levels of VEGFC and its receptor have been described to promote tumor-associated LV expansion, thus becoming one of the most efficient pathways in regulating lymphangiogenesis, during CRC dissemination.^{3–5} However, functional studies regarding lymphangiogenesis and lymphatic metastasis are still lacking in CRC. The lymphatic system plays critical roles in the maintenance of fluid homeostasis, immune response, and cancer progression,⁶ and to exert its biological functions it undergoes lymphangiogenesis, a process of growth and expansion of the lymphatic vessels. Lymphangiogenesis is activated by the binding of VEGFC and VEGFD to their receptor VEGFR3,⁷ even if no association of VEGFD expression has been found with CRC lymphatic spread.⁸ Even though VEGFC interacts with VEGFR2, which is primarily involved in the induction of angiogenesis,⁹ one of the major physiological functions of this growth factor is generally accepted to be the inducer of LV growth.¹⁰

Tumor metastasis is responsible for most cancer-related deaths. The metastatic process can be described in several steps, which involve the transmigration of cancer cells from the primary tumor into the microvascular or the lymphatic endothelium (intravasation and/or extravasation), and subsequently, the formation of a secondary tumor at a distant targeted organ.¹¹ The experimental manipulation of VEGFC, together with the blocking of VEGFR3 activation, has been studied in several cancer animal models, providing functional evidences that VEGFC is able to induce tumor lymphangiogenesis, and concomitantly promote

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Abbreviations used in this paper: CRC, colorectal cancer; HIBEC, human intestinal blood endothelial cell; HILEC, human intestinal lymphatic endothelial cell; LN, lymph node; LV, lymphatic vessel; MFI, mean fluorescence intensity; SAR, SAR131675; VE-Cad, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Colorectal cancer (CRC) is the second most common cause of cancer-related mortality in the Western world.¹ Although it can spread in a variety of ways,

metastasis formation in various distant organs.^{12–14} One of the main restrictions of tumor cell intravasation through the lymphatic system is LV integrity,¹⁵ which is strictly related to the organization of interendothelial adherens junction proteins. These junctions are formed by the homotypic interaction of vascular endothelial cadherin (VE-Cad).¹⁶ Upon cell-to-cell adhesion, cadherin units form a core adhesion complex on adjacent cells via their extracellular domains.¹⁶ In greater detail, the VE-Cad intracellular region forms a complex with an intracellular protein network, including α -, β -, and γ -catenin and p120,¹⁷ thus contributing to the strength of intracellular adhesion.¹⁸ Phosphorylation of VE-Cad on Tyr residues disrupts endothelial cell adherens junctions by causing the dissociation of catenins from VE-Cad, facilitating the diapedesis of leukocytes and metastatic cancer cells.¹⁹ It has been reported that an aberrant localization of VE-Cad on blood vessels is responsible for cancer cell intravasation and extravasation, thus increasing the metastatic spread of several tumors, including CRC.¹⁹ VE-Cad expression on the microvascular endothelial cell membrane depends on its internalization and degradation, and one of the mechanisms mediating these 2 events is the activation of VEGFR2 by its ligand VEGFA.¹² No mechanistic data are available however, regarding the role of VEGFR3 signaling on lymphatic endothelial cell adherens junctions during CRC spreading. In the present study, with the use of an orthotopic xenotransplantation mouse model of CRC, we demonstrate that VEGFC binding to VEGFR3 controls the metastatic spread through the maintenance of lymphatic endothelial barrier integrity.

Material and Methods

For additional information on Materials and Methods, please see the [Supplementary Material](#).

Patients

Tumoral colon tissues were obtained from surgical specimens of CRC patients at different TNM stages, ranging from pT1N0 to pT4N2bM1b. Healthy tissues from the intestine of patients admitted for bowel resection due to diverticulosis or polyps were used as controls. Specimens were formalin-fixed and paraffin-embedded or frozen in Cryoblock Compound (DiaPath, Bergamo, Italy) or frozen on dry ice and stored at -80°C . Human studies were approved by the ethics committee of the Istituto Clinico Humanitas.

Animals and Animal Handling

Mice used in this study were male BALB/c-nude (CAnN.Cg-Foxn1nu/Crl) (Charles River Laboratories Italia, Lecco, Italy) 7–11 weeks old. All animals were maintained under pathogen-free conditions. Housing was temperature controlled, with a 12-hour light/12-hour dark cycle. Procedures involving mice conformed to institutional guidelines in agreement with national and international law and were approved by the ethics committee of the Humanitas Research Hospital.

Preparation of Cell Suspensions and Transanal Mucosal Injection

CT26 mCherry-positive cells were harvested from near-confluent cultures by a brief exposure to 0.5% trypsin and 0.02% EDTA (Lonza; Verviers, Belgium). Cells were washed with phosphate-buffered saline $1 \times^{-/-}$ (Lonza) and analyzed by fluorescence-activated cell sorting for mCherry positivity before injection. Mice were anesthetized with 100 mg/kg ketamine and 50 mg/kg xylazine. With the use of a 29-gauge syringe, 15000 CT26 mCherry-positive cells were injected submucosally into the distal posterior rectum in a final volume of 50 μL , as described previously.²⁰ Tumor growth was visually monitored daily. Mice were sacrificed on post-injection day 19.

Statistical Analyses

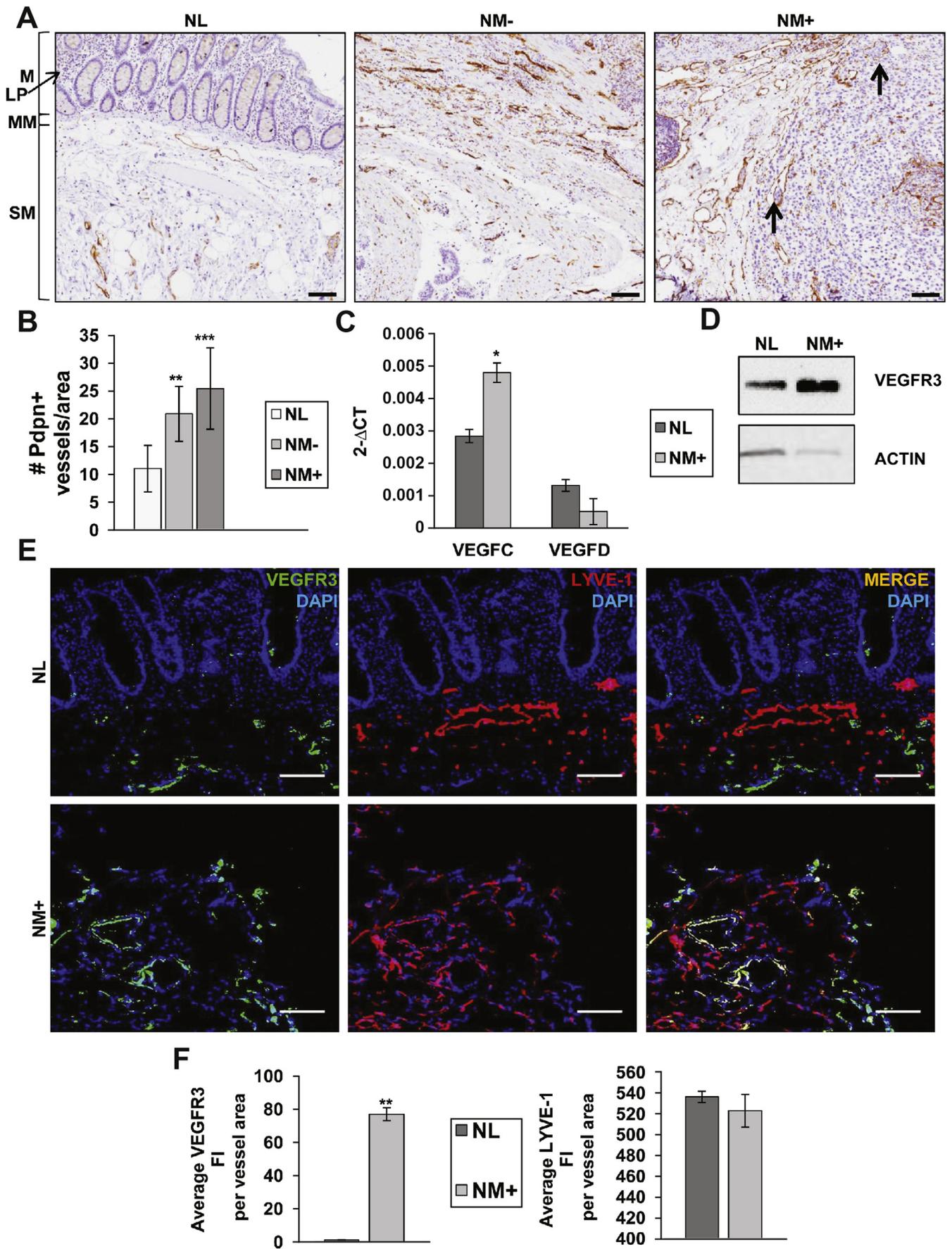
Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA), with a 2-tailed, unpaired Student *t* test or with the analysis of variance test with Bonferroni's or Dunnett's Multiple Comparison Test, as appropriate. Data were presented as mean \pm SD and differences were considered statistically significant when $P \leq .05$.

Results

Lymphatic Vessel Density and the Vascular Endothelial Growth Factor C/Vascular Endothelial Growth Factor Receptor 3 Pathway Are Altered in Human Metastatic Colorectal Cancer

Increased lymphatic vessel density in CRC is a key step for metastatic progression.^{5,21} For this reason, we first evaluated lymphangiogenesis in colon tissues derived from CRC patients with negative ($n = 13$) or positive ($n = 13$) NM stage, and compared them with healthy controls ($n = 15$). We performed an immunohistochemical staining for Podoplanin, a widely accepted marker of LVs.² Normal colonic biopsies showed sporadic thin LVs in the lamina propria and the submucosa, and CRC tissues from both nonmetastatic (NM $-$) and metastatic (NM $+$) patients contained numerous LVs ([Figure 1A](#)). Importantly, LVs within NM $+$ tumoral sections were loaded with metastatic cells ([Figure 1A, black arrows](#)). Quantitative analysis revealed that LV density was significantly higher both in NM $-$ and NM $+$ CRC patients, when compared with healthy tissue ([Figure 1B](#)), with no differences between NM $-$ and NM $+$ subjects, revealing that lymphangiogenesis occurs in human CRC independently from the metastatic process.

To verify the involvement of VEGFC/VEGFR3 pathway in metastatic CRC-associated lymphangiogenesis, human healthy and malignant CRC biopsies were analyzed for the expression of these 2 molecules, by real-time polymerase chain reaction, Western blot, and immunofluorescence. [Figure 1C](#) showed that VEGFC-expression levels were up-regulated in mucosal extracts of CRC patients, when compared with control tissues, while the other VEGFR3 ligand, VEGFD did not change. This is in accordance with other studies, showing reduced levels of VEGFD in metastatic CRC⁸; for this reason, the role of this specific growth factor was not taken into consideration for this study.



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Notably, VEGFR3 expression levels were up-regulated in mucosal extracts from CRC patients, when compared with healthy counterparts (Figure 1D). In addition, frozen sections were double-stained for LYVE-1 and VEGFR3 (Figure 1E) and their mean of fluorescence intensity (MFI) quantified, as described recently.²² A lymphatic-specific increase of VEGFR3 (Figure 1F, left panel), but not LYVE-1 (Figure 1F, right panel) in CRC sections was observed compared with control tissues, indicating that the tumor microenvironment increases VEGFR3 expression on individual LVs. Collectively, these data demonstrate that lymphatic vascularization is increased in human metastatic CRC, and suggest that the tumor microenvironment stimulates the VEGFC/VEGFR3 pathway on LVs, rather than VEGFD/VEGFR3 pathway, a finding compatible with tumor-induced lymphangiogenesis.

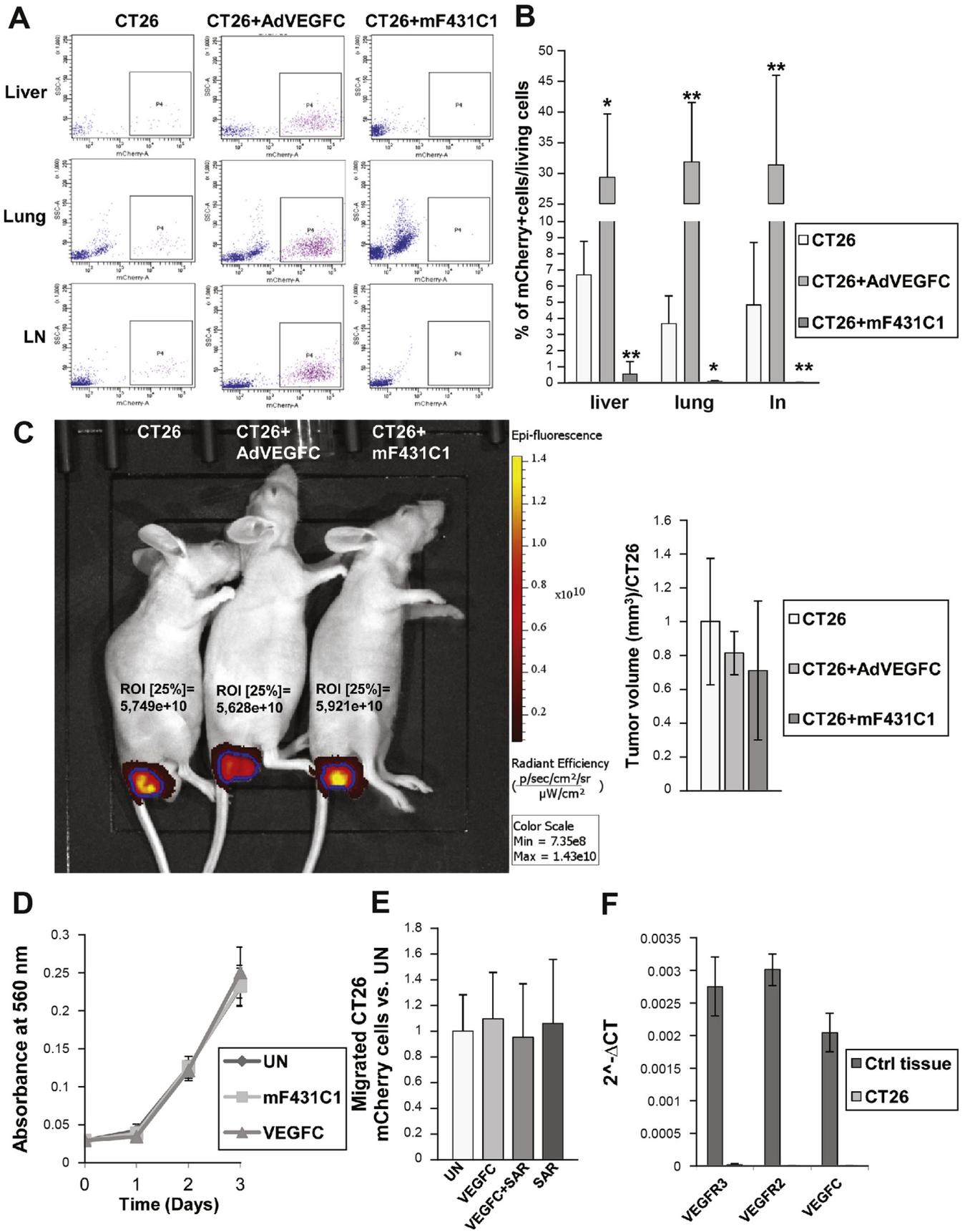
The Vascular Endothelial Growth Factor C/Vascular Endothelial Growth Factor Receptor 3 Pathway Is Actively Involved in the Metastatic Process of a Colorectal Cancer Orthotopic Model

In several cancer animal models, VEGFC has been found to enhance the passage of metastasis through LVs.^{13,23} To evaluate the functional role of the VEGFC/VEGFR3 pathway in metastatic CRC, we modulated these 2 proteins in a well-known CRC mouse model.²⁰ Mice that underwent mCherry-positive CT26 cell orthotopic injection in the rectal mucosa were injected with an adenovirus encoding hVEGFC or with an anti-VEGFR3 antibody (mF431C1). Western blot and immunoprecipitation analyses of tissue lysates obtained from the colons of each experimental group clearly showed the efficient inhibition of VEGFR3 phosphorylation (Supplementary Figure 1A, left panel) and up-regulation of hVEGFC (Supplementary Figure 1A, right panel) at the indicated time points. At 19 days of treatment lymph nodes (LNs), lungs, and livers were processed for metastasis evaluation by fluorescence-activated cell sorting analysis (Figure 2A and B). Quantification of mCherry-positive cells showed that systemic delivery of VEGFC significantly increased metastasis dissemination to draining LNs, lungs, and livers (Figure 2B). On the contrary, mF431C1 completely ablated CT26 migration to LNs, with only few cells detected in lungs and liver (Figure 2B). Notably, the effect observed on metastasis was independent from

primary tumor growth (Figure 2C) and was unaltered by the effects of the VEGFC/VEGFR3 signaling pathway on CT26 biological functions (Figure 2D and E). In fact, tumor size was unaltered upon VEGFC or mF431C1 treatment (Figure 2C), and in vitro CT26 cells stimulated with VEGFC and/or VEGFR3 inhibitor, did not show any differences in both cell growth (Figure 2D) and invasion (Figure 2E). In addition, CT26 cells did not express VEGFR2, VEGFR3, or VEGFC, as revealed by real-time polymerase chain reaction analysis (Figure 2F), which confirms that the CT26-related metastatic process was not due to a direct effect of the VEGFC/VEGFR3 pathway on cancer cell proliferation and invasion.

We then evaluated whether the hastened VEGFC/VEGFR3-dependent metastatic dissemination correlated with altered lymphangiogenesis. For this purpose, colons from CRC-bearing mice treated with VEGFC or mF431C1 were evaluated for LV expansion both in terms of LV density and size. Untreated tumor bearing mice (CT26) displayed increased LVs density (Figure 3A and B) and size (Figure 3D and E), when compared with control tumor-free animals. Systemic delivery of the pro-lymphangiogenic factor VEGFC resulted in increased LV density (Figure 3A and B) and dimension (Figure 3D and E), compared with untreated tumor-bearing mice. On the contrary, mF431C1-administered animals, displayed impaired lymphangiogenesis, in fact, only few and small LVs were visible compared to untreated tumor-bearing mice (Figure 3). Notably, the systemic delivery of VEGFC or VEGFR3 blocking antibody affected only LYVE-1-positive vessels, while these treatments did not alter the number (Figure 3A and C) and dimensions (Figure 3D and E) of CD31-positive LYVE-1-negative blood vessels, indicating that the VEGFC/VEGFR3 pathway has no impact on angiogenesis in our CRC model. In addition, to exclude a possible influence of VEGFC and the virus itself on blood endothelial cells, human intestinal blood endothelial cells (HIBECs) were efficiently infected with the same adenoviruses used in vivo (Supplementary Figure 1B). Results showed that HIBECs proliferation (Supplementary Figure 1C), migration (Supplementary Figure 1D), and tubulogenesis (Supplementary Figure 1E) were not affected by the virus itself or by VEGFC released in the culture medium upon infection. Overall, these data demonstrate that VEGFR3 signaling is crucial for CRC progression through the lymphatic system, which

Figure 1. Lymphatic vessel density and VEGFC and VEGFR3 signaling are increased in the colon of CRC patients. (A, B) Immunohistochemical staining with an antibody recognizing Podoplanin was performed on healthy (NL, n = 15), nonmetastatic (NM-, n = 13), or metastatic (NM+, n = 13) CRC colon tissues (A), and quantitative analysis of LV density is shown in (B). Data are expressed as mean percentage of LV per area \pm SD. Bar = 100 μ m. ***P* < .01; ****P* < .001 by analysis of variance test with Bonferroni's correction for multiple comparisons. (C, D) Healthy (NL, n = 13) and metastatic CRC (NM+, n = 13) patients were measured for VEGFC and VEGFD, and VEGFR3 content in mucosal extracts, by real-time polymerase chain reaction (C) and Western blot analysis (D), respectively. (E) Representative images of frozen sections from the colon of control (NL, n = 8) or metastatic CRC (NM+, n = 8) patients stained for LYVE-1 (red), VEGFR3 (green), and 4',6-diamidino-2-phenylindole (blue). Bar = 100 μ m. (F) Mean fluorescence intensity (FI) per vessel was analyzed on VEGFR3+ (left panel) and LYVE-1+ (right panel) LVs (10–15 vessels/patient). FI are relative units normalized per vascular area expressed in square micrometers. Values are mean \pm SD. **P* < .05; ***P* < .01 vs NL by Student *t* test. LP, lamina propria; M, mucosa; MM, mucularis mucosae; SM, submucosa.



correlates with the modulation of lymphangiogenesis at the tumor site.

The Adherens Junction Protein Vascular Endothelial Cadherin Is Modulated During Colorectal Cancer Metastasis on Lymphatic Vessels in a Vascular Endothelial Growth Factor C/Vascular Endothelial Growth Factor Receptor 3–Dependent Manner

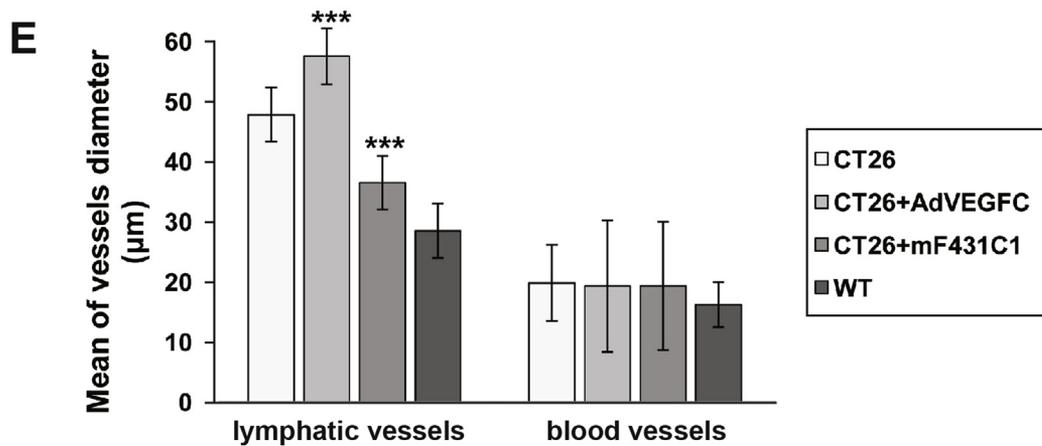
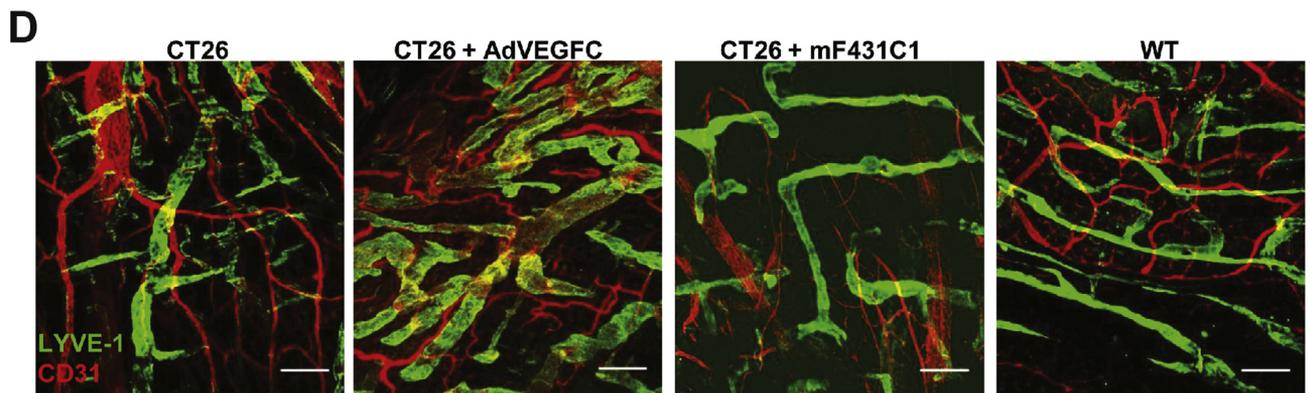
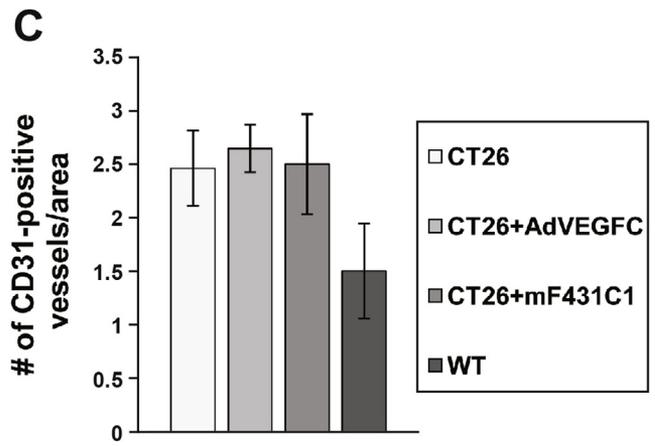
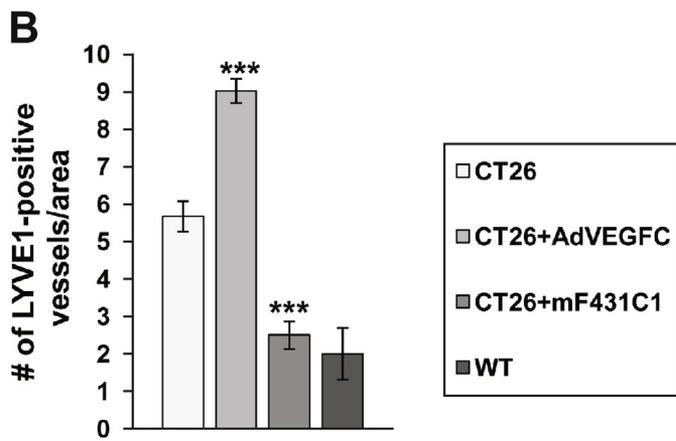
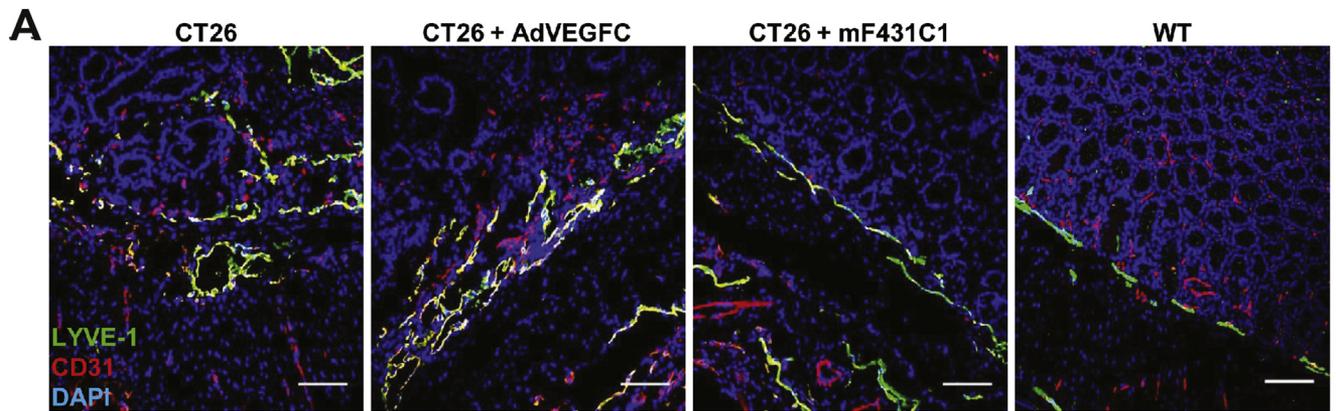
Although many efforts have been made to elucidate the role of VE-Cad expressed on blood vessels during cancer progression, little is known about its expression on LVs during tumor invasion, and no published data exist regarding its potential link to CRC. We hypothesized that VEGFR3 activation by VEGFC could lead to VE-Cad internalization on the lymphatic vasculature. To verify our hypothesis, tumor-bearing mice receiving AdVEGFC or mF431C1 treatment were evaluated for VE-Cad expression and distribution on colonic LVs. Results revealed that untreated tumor-bearing mice (CT26 group) had a discontinuous VE-Cad distribution on LVs, compared with control animals (without tumor group), which displayed the canonical button-like and zipper-like junctions²⁴ (Figure 4A). Interestingly, alteration of VE-Cad expression on LVs was even more evident in AdVEGFC-treated mice, while upon VEGFR3 blocking, was observed a partial restoration of VE-Cad distribution on LVs, compared with healthy animals (Figure 4A). Frozen sections of colons derived from each experimental group, were stained for LYVE-1 and VE-Cad (Figure 4B) and MFI was calculated. Quantification analysis in Figure 4C demonstrated that while untreated tumor-bearing mice displayed a significant reduction in VE-Cad MFI compared with healthy controls, systemic delivery of VEGFC significantly decreased VE-Cad expression, when compared with the CT26-only group. On the contrary, mice treated with mF314C1 had an increased expression of VE-Cad on LYVE-1–positive vessels, comparable with healthy controls (Figure 4C). Importantly, similar results were obtained in VEGFC- or mF431C1-treated healthy mice (Supplementary Figure 1F), indicating that the VEGFC/VEGFR3 pathway is actively involved in regulating VE-Cad expression and distribution on intestinal LVs both in physiological conditions and during metastatic CRC.

Modulation of Vascular Endothelial Growth Factor Receptor 3 Signaling Leads to Vascular Endothelial Cadherin Internalization and β -Catenin Cytoplasmic Release in Human Intestinal Lymphatic Endothelial Cell

To directly study the link between VE-Cad and VEGFR3 signaling in intestinal LVs, we isolated and functionally characterized human intestinal lymphatic endothelial cells (HILEC).²² For this purpose, HILECs were stimulated with VEGFC and/or with SAR131675 (SAR), a tyrosine kinase inhibitor that specifically targets VEGFR3.²⁵ Cells were stained for the lymphatic marker Prox-1 and VE-Cad, and cell membrane VE-Cad MFI was quantified. Stimulation with VEGFC increased cytoplasmic VE-Cad localization (Figure 5A, white arrows), when compared with unstimulated cells and altered VE-Cad distribution on HILEC's cell membrane (Figure 5A, red arrows), resulting in a discontinuous expression pattern of this adherens junction molecule, and a reduced membrane MFI (Figure 5B). The VEGFC-dependent effects were abolished by inhibition of VEGFR3 (Figure 5A and B). HILECs treated only with SAR displayed similar cell membrane VE-Cad localization and MFI, in comparison with unstimulated cells, with no internalization of the protein (Figure 5A and B). These findings show that VE-Cad distribution and internalization directly depends on VEGFR3 activation by VEGFC, as observed in vivo (Figure 4).

Because in quiescent cells, β -catenin interacts with VE-Cad in complexes mainly at the plasma membrane, maintaining a low level of β -catenin at the cytoplasm,¹⁶ we stained HILECs also for this protein (Figure 5A). VEGFC-treated cells displayed a reduction in β -catenin at the plasma-membrane compared with unstimulated HILECs. In the presence of SAR, the effects of VEGFC on β -catenin membrane localization were lost (Figure 5A). We therefore measured β -catenin cell membrane MFI. Notably, HILECs treated with SAR alone did not display a significant increase in β -catenin, when compared with untreated cells. VEGFC-stimulated cells had a lower plasma-membrane β -catenin MFI compared with untreated controls, and SAR abolished these effects (Figure 5C). MFI of β -catenin in cells treated with the VEGFR3 inhibitor alone was comparable with that of untreated cells. Importantly, HIBECs infected with AdVEGFC displayed comparable distribution and internalization of both VE-Cad and β -catenin

Figure 2. VEGFC/VEGFR3 pathway activation increases metastatic dissemination of CRC without targeting CT26 cells. Mice receiving mCherry CT26 orthotopic injection were administered with an adenovirus encoding hVEGFC, or anti-VEGFR3 antibody (mF431C1). (A, B) Fluorescence-activated cell sorting analysis shows mCherry-positive cells in the liver, lung, and LNs. (C) Tumor growth and dimension were measured by IVIS in vivo imaging system (left panel) and caliper (right panel). Results are expressed as mean tumor volume (mm³) vs untreated (CT26) from 3 independent experiments (n = 6 mice/group). (D) CT26 cell proliferation was quantified by crystal violet and data are expressed as 560 nm absorbance. Values are mean \pm SD of 3 independent experiments with 6 replicates per group. (E) CT26 cells were subjected to migration through Matrigel-coated filters for 16 hours and the number of migrated cells vs unstimulated per filter was reported. (F) Confluent CT26 cells were examined for the expression of VEGFR3, VEGFR2, and VEGFC by real-time polymerase chain reaction and were compared with healthy mouse colons (Ctrl tissue). Values are means \pm SD of 3 independent experiments with 3 replicates/group. **P* < .05; ***P* < .01 vs control (CT26) by analysis of variance test with Dunnett's correction for multiple comparisons.



(Supplementary Figure 2A), indicating that VEGFC has a direct effect only on LECs.

Taken together, these results suggest that VEGFC-dependent VEGFR3 activation impairs cell-cell adhesion by inducing the disassembly of the VE-Cad- β -catenin complex at intercellular junctions.

Vascular Endothelial Growth Factor Receptor 3 Activation by Vascular Endothelial Growth Factor C Alters Human Intestinal Lymphatic Endothelial Cell Permeability and Affects CT26 Cell Transendothelial Migration

Cell-to-cell adherens junctions are important for endothelial barrier function and integrity.¹⁶ For this reason, we assessed the capability of the VEGFC/VEGFR3 signaling pathway to modulate HILECs permeability through induction of intercellular junction opening. HILECs were stimulated with VEGFC and/or SAR and permeability was assessed by measuring the transendothelial electrical resistance. VEGFC-treated HILECs showed a significant decrease in transendothelial electrical resistance across the cell monolayer, when compared with unstimulated cells (Figure 6A). This effect was abolished by SAR, resembling untreated control cells. Notably, SAR-treated HILECs displayed the same transendothelial electrical resistance as untreated cells, demonstrating the specific dependence of intestinal lymphatic endothelial cell permeability on VEGFC/VEGFR3 activation.

To mimic the cancer cell intravasation/extravasation process in vitro, we performed a transendothelial migration assay using a co-culture of HILECs and mCherry CT26 cells (Figure 6B). HILECs were treated with VEGFC and/or SAR, and mCherry CT26 transendothelial migration was quantified after 16 hours of co-culture. Results showed that VEGFC induced an increase in CT26 migration, when compared with untreated HILEC. Interestingly, treatment with both VEGFC and the VEGFR3 inhibitor showed similar CT26 migration, in comparison with untreated controls (Figure 6C and D). In addition, HILECs treated with SAR alone displayed a slight reduction in CT26 transmigration (Figure 6C and D), revealing a direct effect of VEGFC-dependent-VEGFR3 activation on CRC cell transendothelial migration. Notably, AdVEGFC has no effect on CT26 transendothelial migration through HIBECs (Supplementary Figure 2B), indicating that VEGFC does not play a role in hematogenous metastatic spread.

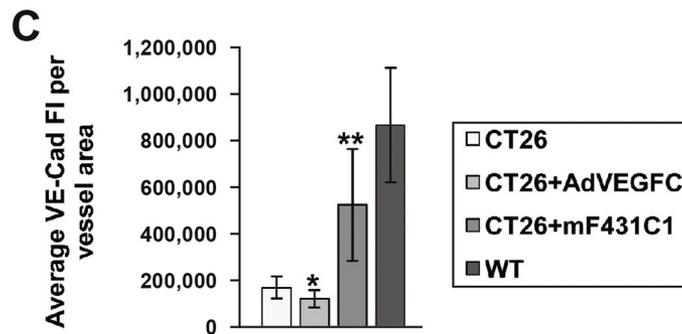
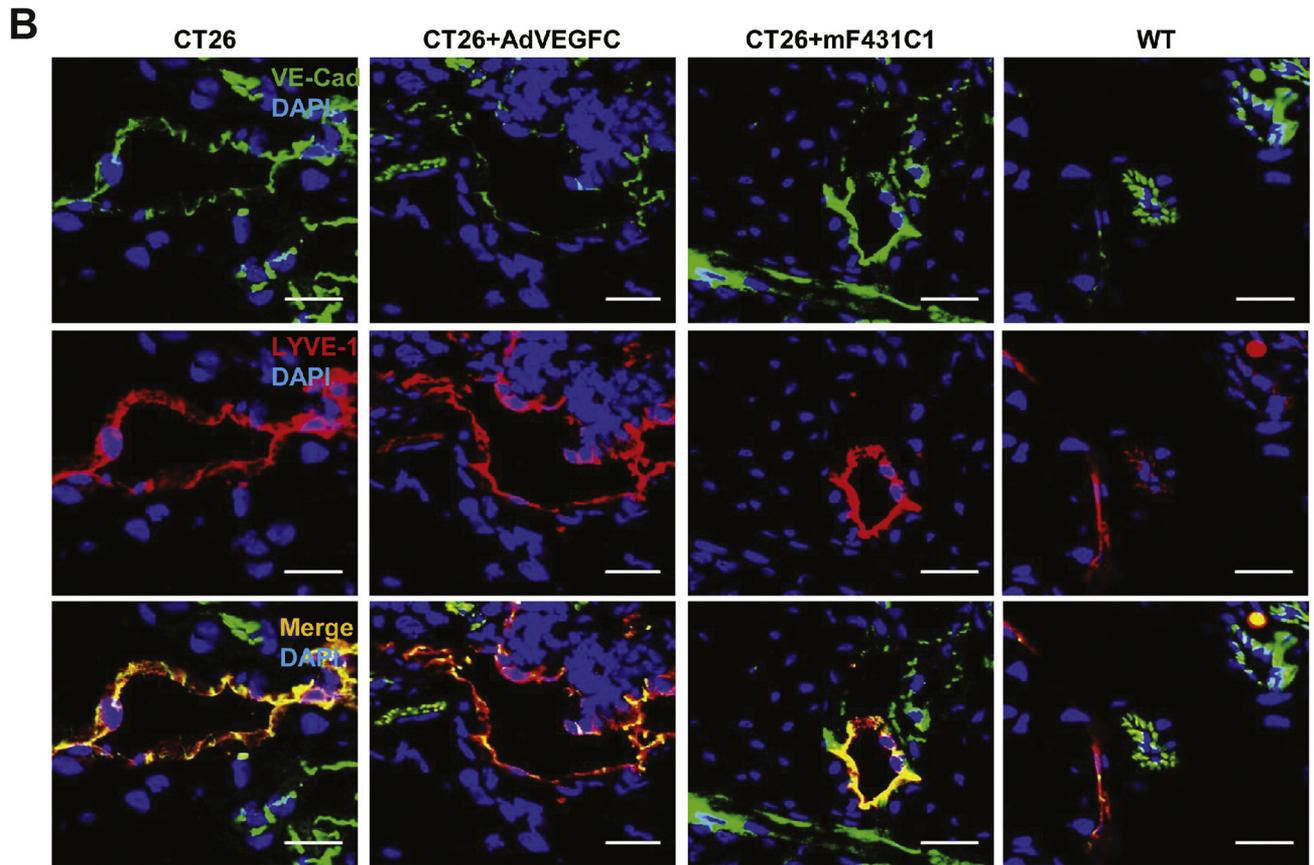
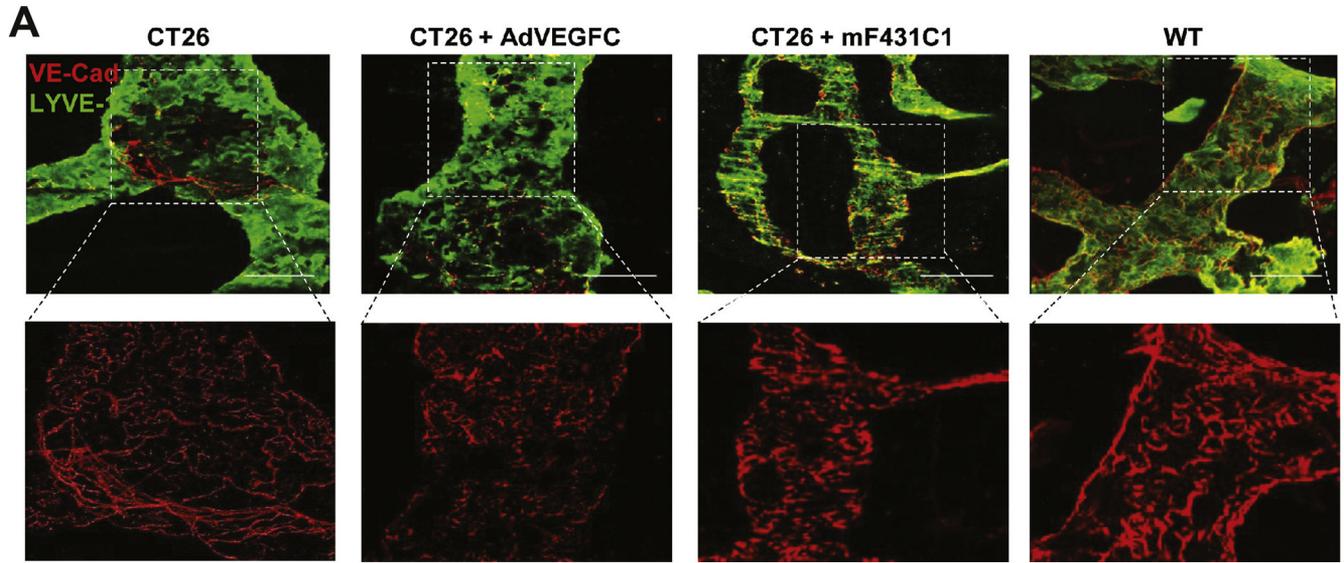
Lymphatic Vessels in Tumor Tissue of Metastatic Colorectal Cancer Patients Displayed Decreased Vascular Endothelial Cadherin Levels

To confirm what was observed in vivo and in vitro, we further evaluated VE-Cad expression levels on LYVE-1-positive vessels in metastatic CRC patients, and compared them with controls. Normal colon tissues showed a strong expression of VE-Cad on LVs, and CRC sections had a significant cadherin decrease (Figure 7A). Co-expression of VE-Cad and LYVE-1 was quantified revealing a lymphatic specific decrease of this cadherin in CRC sections compared with controls (Figure 7B). This result correlated with high levels of both VEGFC and VEGFR3 in the CRC microenvironment. Overall, these data demonstrate that lymphatic vascularization is increased in CRC tissues, and that the tumor microenvironment stimulates the VEGFC/VEGFR3 pathway, in association with decreased VE-Cad on LVs. These elements contribute to an impaired lymphatic endothelial barrier function and facilitate lymphatic metastatic spread, as described in Figure 7C. Stimulation of VEGFC leads to the induction of VEGFR3 signaling causing VE-Cad- β -catenin dissociation, VE-Cad internalization and β -catenin cytoplasmic release resulting in cell-junction opening and increased metastatic spread (Figure 7C).

Discussion

Tumor-induced angiogenesis and lymphangiogenesis are thought to expedite cancer cell entry into the blood and lymphatic vasculature and foster metastatic dissemination. However, although the role of the vascular endothelium in cancer progression is well described,²⁶ the contribution of tumoral LVs is still unclear. In many types of human cancers, including CRC,³⁻⁵ the expression of VEGFC² and its receptor, VEGFR3,^{25,27} correlates with lymphangiogenic sprouting, metastases, and poor prognosis. In accordance with these studies, we confirmed that active lymphangiogenesis takes place in CRC patients displaying a high expression of VEGFC and increased VEGFR3 levels on LVs. VEGFD is also known to be a VEGFR3 ligand and is considered an important pro-lymphangiogenic factor²⁸; however, unlike VEGFC, it was not altered in CRC patients, and for this reason our study only focused on VEGFC. Several cancer animal models have highlighted the importance of the lymphatic vasculature in metastatic dissemination thanks to the modulation of the VEGFC/VEGFR3 pathway. In fact, He et al¹⁴ demonstrated in a mouse model of lung cancer, that VEGFC-overexpressing LNM35

Figure 3. The activation of VEGFR3 signaling fosters lymphangiogenesis in CRC. (A) Representative images of frozen sections from colons of animals without tumor (WT) and CT26-bearing mice, with or without adenoviruses encoding hVEGFC, or anti-VEGFR3 antibody (mF431C1). Tissues were stained for LYVE-1 (green), CD31 (red), and 4',6-diamidino-2-phenylindole (blue), and LYVE-1+ (B) and CD31+ LYVE-1- (C) vessels were counted in 10 comparable regions per section. Results are expressed as mean values \pm SD, and are representative of 3 independent experiments (n = 3 mice/group). Scale bar = 100 μ m. (D, E) Whole mounts of distal colonic segments from the indicated experimental groups were stained for LYVE-1 (green) and CD31 (red). (D) Representative images of whole mount staining of distal colons from the indicated groups. (E) LV and blood vessels diameters were quantified and reported as mean values \pm SD of 3 independent experiments (n = 3 mice/group with 5 fields/colonic segment for each experiment). Scale bar = 200 μ m. ***P < .001; vs CT26 by analysis of variance test with Dunnett's correction for multiple comparisons.



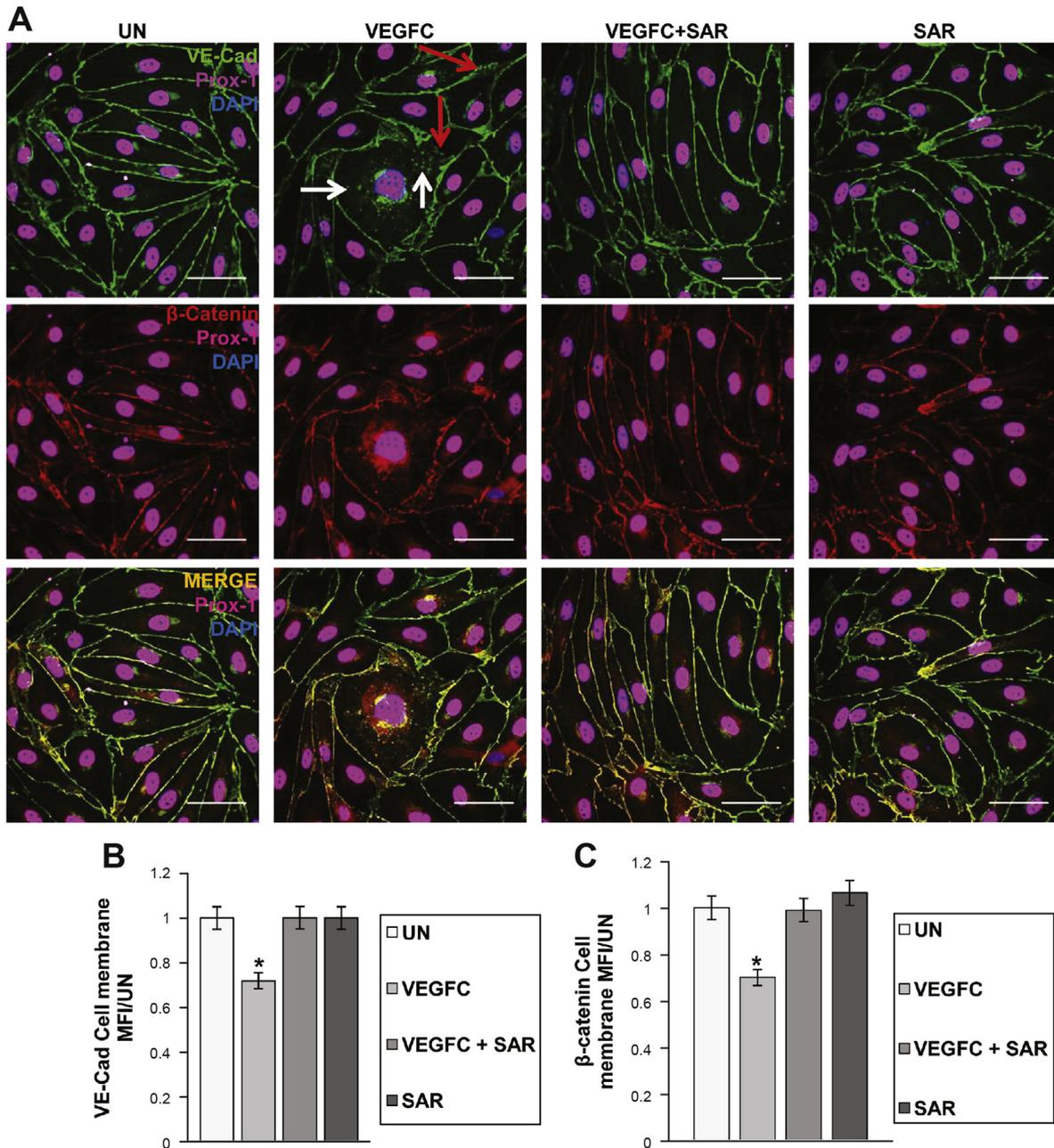


Figure 5. Modulation of the VEGFR3 signaling pathway mediates VE-Cad and β -catenin internalization on HILEC. (A) Representative merges of HILECs stained for the lymphatic marker Prox-1 (magenta), 4',6-diamidino-2-phenylindole (DAPI; blue), β -catenin (red), and VE-Cad (green). Cells were left untreated (UN), stimulated for 30 minutes with VEGFC, in the presence or absence of the VEGFR3 inhibitor SAR. Representative merges of HILECs stained for Prox-1 (magenta), DAPI (blue), β -catenin (red), and VE-Cad (green). VEGFC stimulation induced VE-Cad internalization (white arrows) along with a discontinuous VE-Cad membrane distribution (red arrows). (B, C) Cell surface mean FI was analyzed for VE-Cad (B) and β -catenin (C). Results are representative of 3 independent experiments each in triplicate/group. Scale bar = 50 μ m. Values are expressed as mean \pm SD. * $P < .05$; vs UN by ANOVA test with Dunnett's correction for multiple comparisons.

Figure 4. Systemic delivery of human VEGFC alters VE-Cad pattern distribution and expression on LVs. (A) Representative images of whole mount tissues from colons of animals without tumor (WT) and CT26-bearing mice, with or without AdVEGFC, or mF431C1 and stained for LYVE-1 (green) and VE-Cad (red). Lower panels are higher magnifications of the white dashed squares in the upper panel. Scale bar = 50 μ m. (B, C) Frozen-sections from the colon of all experimental groups were stained with both anti-LYVE-1 and anti-VE-Cad antibodies, with representative images expressed in (B). VE-Cad mean fluorescence intensity (FI) per vessel was analyzed on LYVE-1+ vessels, and FI is expressed in (C) as relative units per vascular area. Scale bar = 20 μ m. Results are representative of 3 independent experiments with 3 mice per group. Values are expressed as mean \pm SD. * $P < .05$; ** $P < .01$; vs CT26 by analysis of variance test with Dunnett's correction for multiple comparisons.

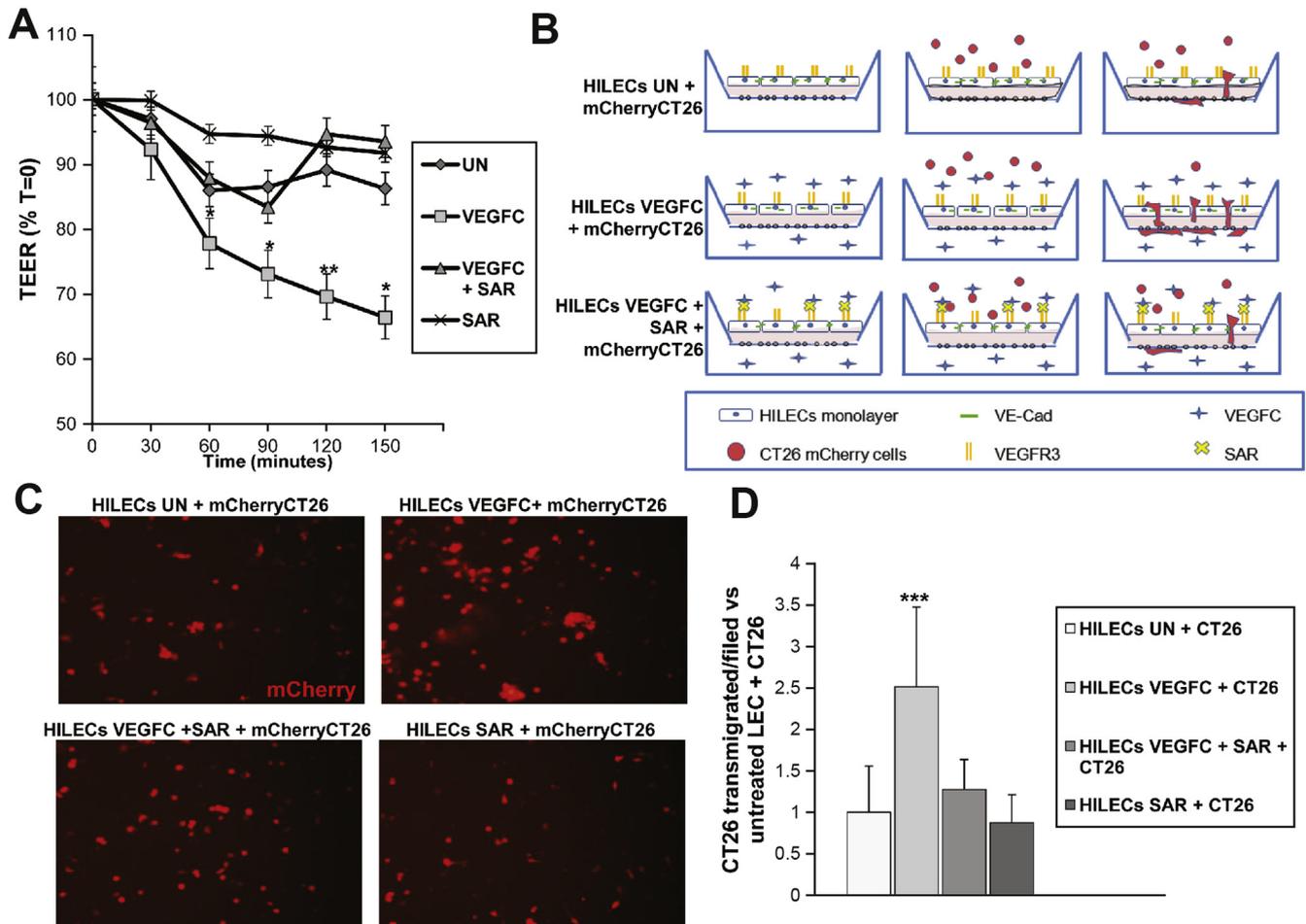


Figure 6. VEGFR3 activation by VEGFC alters HILEC permeability and modulates CT26 cell transendothelial migration. (A) HILECs permeability was quantified through transendothelial electrical resistance. Inhibition of VEGFR3 was achieved by adding SAR (23 μ M) 30 minutes before VEGFC (100 ng/mL). Bars represent mean values per group \pm SD. Bar = 50 μ m. * P < .05; ** P < .01 vs untreated (UN) by analysis of variance test with Dunnett's correction for multiple comparisons. (B) Cartoon of the transendothelial migration assay: mCherry-CT26 cells were seeded on HILEC confluent monolayers left untreated, treated with VEGFC, and/or with SAR131675, VEGFC+SAR, and SAR alone, respectively. The mCherry-positive transmigrated cells were counted (D) and representative images are shown in (C). Data are expressed as mean \pm SD of 3 experiments performed in triplicate. Magnification: 10 \times . *** P < .001; vs UN by analysis of variance test with Dunnett's correction for multiple comparisons.

cells induce massive lymphangiogenesis together with lymphatic activation facilitated tumor cell entry in LVs and promoted metastatic dissemination to LNs.¹⁴ Notably, in this study, LV destabilization and tumor metastases were blocked using an adenovirus producing a soluble VEGFR3-Ig fusion protein.¹⁴ In addition, Matsumoto et al²⁷ showed that in a mouse model of breast cancer, VEGFR3 signaling was required for the early events of the metastatic process and that mF431C1 was able to block both tumor lymphangiogenesis and metastases. In a similar way, our study shows that LVs undergo dramatic lymphangiogenic changes in response to nearby CT26 mCherry cells, including sprouting and dilation of the larger collecting vessels draining the tumor area. We demonstrate for the first time that, upon VEGFR3 inhibition, tumor-lymphangiogenesis represents a rate-limiting step for CRC lymphogenous metastases, which are significantly suppressed at distant organs. In contrast, upon systemic VEGFC delivery, metastasis is fostered in

association with active lymphangiogenesis. Consistent with other findings, in the tumoral context we do not observe any effects on blood vessel density and enlargement upon VEGFC treatment^{29,30} or VEGFR3 inhibition.⁷ It is worthy of note that these experiments do not exclude the possibility that VEGFC could act on other cells other than lymphatic endothelial cells, such as VEGFR3-expressing macrophages.²⁵

It is still unclear whether tumor cell intravasation/extravasation requires the transmigration of cancer cells through the cytoplasm of endothelial cells, as occurs for leukocytes, or whether they transmigrate between 2 adjacent endothelial cells after the disruption of cell-to-cell connections.¹⁹ The endothelial barrier integrity is maintained by a complex balance between cell-to-cell adherens junctions. In some pathologic conditions, such as inflammation and cancer, endothelial junctions can be disrupted leading to the opening of intercellular gaps and inducing

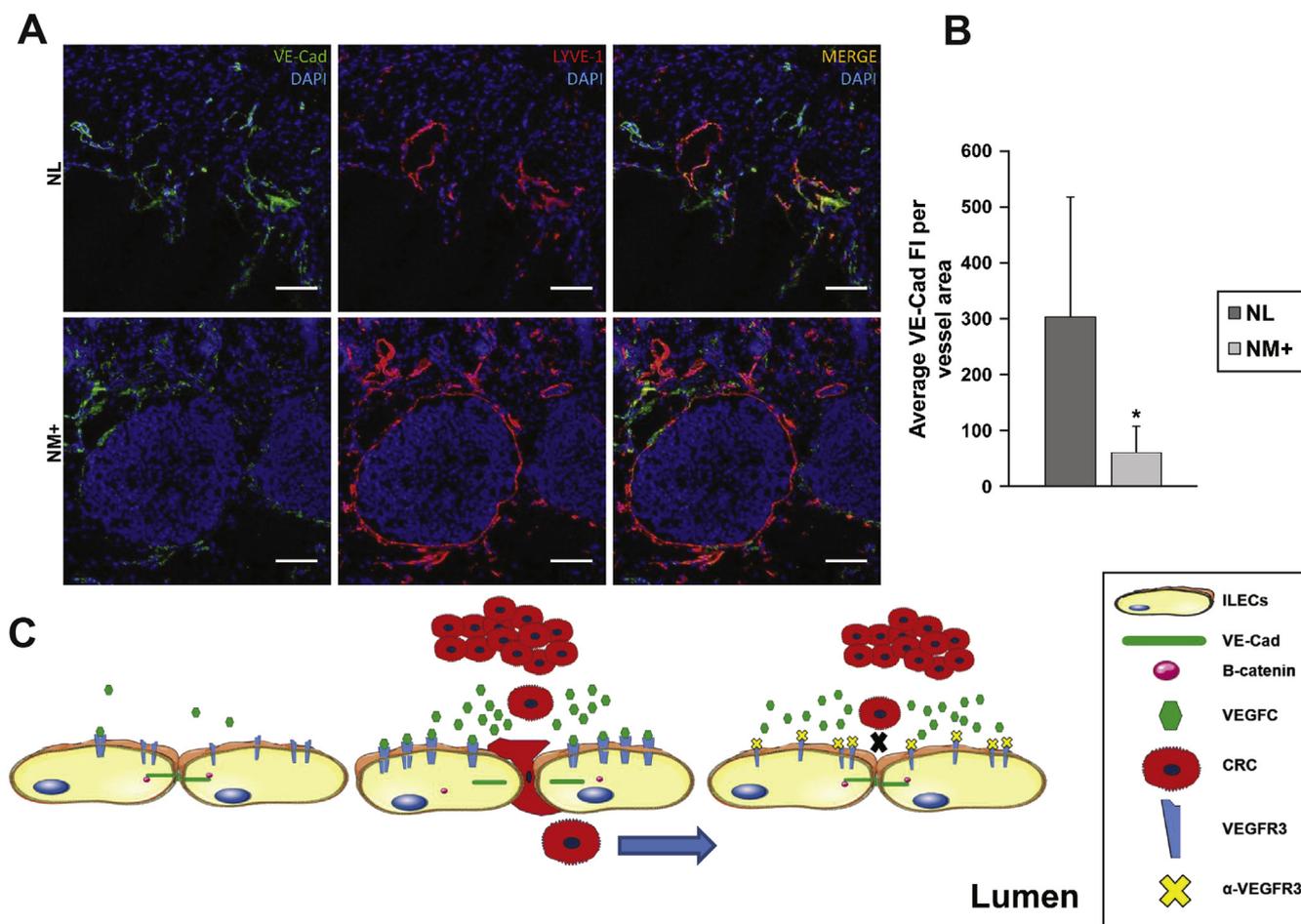


Figure 7. VE-Cad expression is decreased on LVs of metastatic CRC patients. (A, B) Representative frozen sections from the colon of control (NL) (n = 8) or metastatic CRC (NM+) (n = 8) patients were stained for both LYVE-1 and VE-Cad (A). Bar = 100 μm. (B) VE-Cad mean fluorescence intensity (FI) per vessel was analyzed on LYVE-1+ LVs. FI is expressed as relative units per vascular area (10–15 vessels/patient). Values are expressed as mean ± SD. *P < .05; vs NL by Student t test. (C) The left panel shows that under physiological conditions, intestinal lymphatic endothelial cells (ILECs) are quiescent and the barrier integrity is maintained. In the central panel, VEGFC secreted in the tumor microenvironment stimulates VEGFR3 overexpressed on ILECs and impairs endothelial barrier integrity, which promotes lymphogenous metastasis formation. In the right panel, inhibition of VEGFR3 signaling maintains lymphatic barrier functionality and abates metastasis dissemination.

increased vascular permeability.³¹ We focused our attention on the most important player in maintaining barrier integrity: VE-Cad. In fact, blocking VE-Cad is sufficient to destabilize endothelial cell contacts in vivo and to increase vascular permeability.³² In contrast, stabilizing endothelial cell junctions by enhancing the VE-Cad adhesive function in knock-in mice interferes with the induction of vascular permeability and leukocyte extravasation.^{3,33} One of the mechanisms involved in adherens junction destabilization is the activation of VEGFR2 by VEGFA, which induces VE-Cad tyrosine phosphorylation and its internalization.¹⁶ In this scenario, VEGFA facilitates cancer cell intravasation by disrupting the VE-Cad–β-catenin complex and inducing adherens junction weakness and formation of transcellular holes.³⁴ Weis et al¹⁹ provided evidence that VEGFA-mediated breakdown in blood endothelial integrity through VE-Cad disruption hastens metastases.¹⁹ In addition, they demonstrated that VE-Cad inhibition compromises endothelial barrier function and promotes tumor cell

extravasation.¹⁹ We hypothesized that, similar to blood vessels, the lymphatic vasculature could respond to the modulation of the VEGFC/VEGFR3 pathway with VE-Cad redistribution. In vitro results on HILEC confirmed our hypothesis, in fact, VEGFC-induced VEGFR3 signaling led to VE-Cad–β-catenin complex dissociation, VE-Cad internalization, and β-catenin cytoplasmic release. Breslin et al³⁵ demonstrated that VEGFC-dependent VEGFR3 activation significantly increased lymphatic endothelial cell permeability. In accordance with this study, we provide in vivo evidence that VEGFR3 signaling induces major alterations in the intestinal lymphatic barrier and strongly impairs its functionality. Importantly, we propose a mechanism of CRC cell intravasation/extravasation that is dependent on VEGFR3 activation on lymphatic endothelial cells that facilitates tumor cell entry/exit in a VEGFC-dependent manner. The most intriguing and novel finding of our study is that the increased metastasis formation in VEGFC-treated mice during CRC progression is not only a

consequence of enhanced lymphangiogenesis, but could also be a result of VE-Cad distribution and localization on LVs. In fact, VEGFC stimulates LVs sprouting and dilation, but also modulates VE-Cad expression on intestinal lymphatics. These results are in accordance with Tammela et al,³⁶ who observed that VEGFC delivery induces VE-Cad down-regulation on lymphatic capillaries. Interestingly, upon VEGFR3 inhibition VE-Cad localization in tumor-bearing mice was comparable with healthy animals and directly linked VE-Cad distribution with the VEGFC/VEGFR3 pathway and lymphogenous metastases. These findings confirm our in vitro data and are consistent with what we observed in human metastatic CRC specimens. As in the mouse, human CRC samples have consistent overexpression of VEGFC and VEGFR3 in tumor tissue, which overlaps with increased lymphangiogenesis and diminished lymphatic VE-Cad expression. At present, the lymphatic route of tumor metastases is less understood than the vascular route.³⁷ Our results help to better understand metastatic dissemination through lymphatics; however, they do not exclude that other molecules released in the tumor microenvironment could be involved in lymphatic barrier alteration, such as some inflammatory mediators.³⁸

LVs are largely quiescent in adults,³⁹ and lymphangiogenesis may provide a safe target for cancer metastases. The use of neutralizing antibodies targeting VEGFC and/or VEGFR3 in clinical studies that have progressed to phase 1 clinical trials in patients with advanced solid tumors¹⁵ have provided encouraging results. In addition, several small-molecule protein kinase inhibitors targeting various kinases, including VEGFR3, are now available, and some of them have been approved for the treatment of various cancers,¹⁵ showing an overall survival benefit in patients with refractory metastatic CRC.⁴⁰

In summary, our findings strongly support and encourage a VEGFC/VEGFR3-targeted therapy for the prevention of CRC metastatic dissemination. The inhibition of this signaling pathway may reduce tumor lymphangiogenesis and restore lymphatic barrier integrity through the maintenance of VE-Cad distribution on LVs. The lymphatic vasculature may thus represent a potent target to abate and prevent CRC lymphogenous spread.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2015.03.005>.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Immunohistochemical Staining of Human Colonic Tissues

To quantify LV density, the number of Podoplanin-positive LVs was assessed in tumor areas of surgical specimens from patients with CRC. Normal areas of the intestine from patients admitted for bowel resection due to diverticulosis or polyps were used as controls (NL, n = 15). Tissues were fixed in 4% buffered formalin, processed, and stained with H&E or immunostained with specific antibodies. Two-micrometer-thick sections of formalin-fixed paraffin-embedded tissues were deparaffinized in Bioclear (Bioptica; Milan, Italy) and rehydrated in a descending ethanol series. After antigen retrieval by heating 10 minutes in a microwave with EDTA buffer (pH 8.0; 0.25 mM), sections were incubated for 20 minutes with Peroxidase-1 solution (Biocare Medical; Pike Lane Concord, CA) to quench endogenous tissue peroxidase. Tissue slides were then incubated for 60 minutes at room temperature in a humid chamber with mouse antihuman Podoplanin monoclonal antibody (clone D2-40; 1:100; ABD Serotec; Puchheim, Germany) and staining was completed using the Biocare Medical HRP-Conjugated Mouse Detection Kit and diaminobenzidine chromogen as substrate, according to the manufacturer's instructions. Lastly, sections were counterstained with CAT hematoxylin (Biocare Medical) for 5 minutes. After staining, the slides were dehydrated through graded alcohols and mounted with a cover slip using a Eukitt Quick Hardening Mounting Media (Fluka; Milan, Italy). Negative controls with an omission of the primary incubation were also included. The slides were analyzed randomly by two of the authors, blinded to clinical data.

LV density was quantified in the lamina propria and submucosal layers, both areas richly vascularized by lymphatic capillaries. Only vessels stained with Podoplanin and visible lumina were considered countable because small nerve fibers and fibroblasts also expressed Podoplanin,¹ and could potentially be confused with collapsed lymphatic capillaries. The mean vessel density of 10 areas with an elevated number of LVs in the 20× magnification objective field, were assessed separately using the VS-ASW software (Olympus; Milan, Italy).

Real-Time Polymerase Chain Reaction

RNA was extracted from human tumor and healthy specimen or from healthy mouse colon and CT26 cell line using the PureZOL RNA isolation reagent (Bio-Rad, Hercules, CA) according to manufacturer's instructions. RNA retrotranscription was performed with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA). Real-time polymerase chain reaction analyzes were performed starting with complementary DNA obtained from human NM+ tumor (n = 13) or healthy (n = 13) tissues or from CT26 cell line and mouse colon used as control. The primer pairs used are summarized in the [Supplementary Table 1](#).

The reactions were performed on ViiA7 Real-Time PCR System (Applied Biosystems). glyceraldehyde-3-phosphate

dehydrogenase expression was used as housekeeping gene. Data were calculated using the $2^{-\Delta Ct}$ method.

Cell Line Culture Condition and Infection

The murine colon cancer CT26 cells were kindly provided by Dr Giuseppe Celesti (Humanitas Clinical and Research Center, Milan, Italy). As demonstrated, the murine colon cancer CT26 cells are highly metastatic to liver and lungs.² Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, glutamine, sodium pyruvate, nonessential amino acids, and a penicillin-streptomycin amphotericin mixture (Lonza). Adherent monolayer cultures were maintained on plastic at 37°C in 5% CO₂ and 95% air. All cultures were *Mycoplasma* free.

The lentiviral vector pRRLsin.PPT.hPGK.mcherry_pre (kindly provided by Dr Marco Erreni, Humanitas Clinical and Research Center, Milan, Italy) was produced by transient transfection of 293T cells according to standard protocols.³ Briefly, subconfluent 293T cells were co-transfected with 11.7 μg of the transfer plasmid, 3.5 μg of the packaging plasmid, 3.5 μg of envelope plasmid, and 5 μg of re-expressing plasmid (ViraPower lentiviral packaging mix; Invitrogen, Carlsbad, CA) by lipofectamine (Invitrogen). After 16 hours, medium was changed, and 24 hours later recombinant lentiviral vectors were collected, filtered through 0.22-μm pore size cellulose acetate filters and used immediately. After having determined the maximum viral efficiency 10⁵ CT26 cells in 6-well plates were used. After 24 hours, CT26 cells were incubated with complete medium and 48 hours later analyzed for infection efficiency, by LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). CT26 mCherry cells maintained mCherry production for several passages.

Vascular Endothelial Growth Factor Receptor 3 Antibody Blockade

Mice were given intraperitoneal injections of an anti-mVEGFR3 antibody, mF431C1 (clone 31C1; Eli Lilly and Company, 800 μg/100 μL phosphate-buffered saline [PBS] per mouse).^{4,5} Mice were administered intraperitoneal injections 3 times per week for the entire experiment, starting from the first day after CT26 mCherry cell injections. A group of mice were sacrificed at 0, 5, 12, and 19 days after CT26 injection and mucosal extracts were analyzed by Western blot for assessment of VEGFR3 inhibition. All experiments were performed in triplicate, using 4–8 mice per experimental group.

Adenoviral Human Vascular Endothelial Growth Factor C Administration

Mice were given intravenous injections of an engineered adenovirus encoding human VEGFC (AdVEGFC), or control virus encoding green fluorescent protein (AdGFP) (not shown) (5×10^8 PFU/mouse; ViraQuest; North Liberty, IA)⁵ 7 days before CT26 mCherry cell injections. A group of mice were sacrificed at –7, 5, 12, and 19 days from CT26 injection, and mucosal extracts were analyzed by Western blot for assessment of human VEGFC production. Adenoviral preparation

was free from contamination with helper viruses, bacteria, or lipopolysaccharide. All experiments were performed in triplicate, using 4–8 mice per experimental group.

Tumor Dimension

Before being sacrificed, mice were anesthetized with 100 mg/kg ketamine and 50 mg/kg xylazine and fluorescent imaging were performed using a Caliper Xenogen IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA). A light image was obtained for anatomical orientation and fluorescent imaging was performed to observe mCherry using standard parameters, including 12.9-cm field of view to observe 3 mice simultaneously, excitation filter = 570 nm, emission filter = 620 nm, f-stop 1, pixel binning 8, and 0.5 seconds exposure time. Images were analyzed using Living Imaging software and the region of interest function. At the indicated time point mice were sacrificed and tumor volume was measured by caliper and calculated with the formula $V = 0.5 \times \text{length} \times \text{width}^2$.

CT26 Proliferation and Invasion Assay

A crystal violet assay was used to assess CT26 proliferation, as described previously.⁶ Briefly, CT26 cells were seeded in 96-well cell culture plates (1×10^3 cells/well) with complete growth medium containing 1% fetal calf serum, and either left untreated or treated with human recombinant VEGFC (100 ng/mL; R&D Systems, Minneapolis, MN) or mF431C1 (300 μ g/mL; Eli Lilly and Company). Medium was changed every other day and after 3 days in culture, cells were stained with 0.2% crystal violet (Sigma, St Louis, MO) dissolved in ethanol. Uptake of dye by cells on plates was eluted with 33% acetic acid in water. Plates were gently shaken for 20 minutes and the absorbance at 560 nm was measured by a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). The optical density of each sample was then compared with a standard curve, in which the optical density was directly proportional to known cell numbers. Invasion assay was assessed by using a BD Bio-Coat Matrigel invasion chamber with polycarbonate filters (8- μ m pore size; BD Biosciences), 24 hours serum starved CT26 80×10^3 /filter were plated in the upper chamber in Dulbecco's modified Eagle medium, both the upper and the lower chamber were supplemented with human VEGFC (100 ng/mL; R&D Systems) and/or SAR131675 (23 nM dissolved in dimethyl sulfoxide; Selleckchem, Houston, TX). After 16 hours, medium was removed from both chambers, and cells that had migrated onto the lower surface of the porous membrane were washed twice in PBS and stained with Diff Quick (Medion Diagnostics, Miami, FL), according to manufacturer's instruction. Triplicates of migrated cells were counted in 5 consecutive fields using a $10\times$ objective. All experiments were performed in triplicate.

Metastasis Dissemination Assessment

At the indicated time point, mice were sacrificed and liver, lungs, and draining LNs were taken for metastasis evaluation by fluorescence-activated cell sorting analysis, as described

previously,⁷ and a piece of mCherry tumor was used as a positive control. Briefly organs were disrupted through a 70- μ m filter (BD Falcon) with a 1-mL syringe plunger and washed with 10 mL RPMI medium supplemented with 10% fetal calf serum and 20 mM HEPES. Liver and lungs were then centrifuged at 800 rpm for 10 minutes to discard debris. ACK lysis buffer was used directly on pellets to remove red blood cells. All samples were washed with RPMI medium and centrifuged. Samples were then suspended in PBS and counted. Then 5×10^6 cells were stained with LIVE/DEAD Dead Cell Stain Kit (Life Technologies, Carlsbad, CA) and washed in phosphate-buffered saline. Stained cells were analyzed using an LSRFortessa cell analyzer (BD Biosciences), and data were analyzed using the FACSDiva software (BD Biosciences) considering mCherry-positive cells on total living cells. All experiments were performed in triplicate, using 4 mice per experimental group.

Immunofluorescence of Human and Murine Colon Tissues

Frozen sections (6 μ m) of human (tumor tissue = 8; healthy control = 8) and murine colon tissues (n = 8 per experimental group) were fixed in 4% paraformaldehyde solution for 10 minutes and then permeabilized in 0.1% Triton X-100 solution for 20 minutes at room temperature. Tissue sections were then blocked with PBS containing 2% bovine serum albumin, 0.05% Tween, and 2% goat (for human colon tissues) or donkey serum (for murine colon tissue) for 30 minutes at room temperature. After blocking, human colon sections were incubated with mouse anti-VEGFR3 (1:100; Millipore, Billerica, MA) or mouse anti-VE-Cad (1:50; Abcam, Cambridge, MA), and rabbit anti-LYVE-1 (1:200; Abcam) antibodies for 2 hours. Murine colon sections were incubated overnight at 4°C with rabbit anti-CD31 (1:100; Life Science Biotechnology) and/or anti-VE-Cad (1:100; eBioscience) and goat anti-LYVE-1 (1:50; R&D Systems) primary antibodies. All sections were subsequently incubated for 30 minutes with anti-mouse Alexa Fluor 647–conjugated, anti-rabbit Alexa Fluor 488–conjugated, anti-goat Alexa Fluor 488–conjugated, anti-rat Alexa Fluor 488–conjugated, and anti-rabbit Alexa Fluor 594–conjugated antibodies (1:1000; Molecular Probes), followed by incubation with 4',6-diamidino-2-phenylindole for nuclear staining (1:25000; Invitrogen). Sections were mounted with ProLong Gold mounting medium (Invitrogen) and analyzed with a laser scanning confocal microscope (Fluo-View FV1000; Olympus).

Whole Mount Staining

Mice were anesthetized with 100 mg/kg ketamine and 50 mg/kg xylazine, and 10 mL of 1% paraformaldehyde fixative solution was perfused into the heart. Colons were removed from untreated (UN) and treated mice subjected to orthotopic CRC protocol with the indicated treatments (n = 5–8 mice each experimental group), and immersed in 1% paraformaldehyde fixative solution, overnight at 4°C. Tissues were then washed and incubated with hamster anti-CD31 (1:500; Millipore), rabbit anti-LYVE-1 (1:500; Abcam),

and rat anti-VE-Cad (1:200; eBioscience) antibodies diluted in PBS containing 0.3% Triton X-100, 2% bovine serum albumin, 5% goat serum, 0.01% glycine, and 0.1% sodium azide, overnight at 4°C. Anti-hamster Alexa Fluor 647, anti-rabbit Alexa Fluor 488 (1:500; Molecular Probes) and anti-rat Alexa Fluor 647 (1:500; Molecular Probes) were used as secondary antibodies, and incubated overnight at 4°C. Samples were then mounted with Vectashield (Vector Laboratories, Burlingame, CA) and imaged with a laser scanning confocal microscope (Fluoview FV1000; Olympus).

Morphometric and Immunofluorescence Intensity Measurements

Morphometric analysis of colon whole mounts was performed by 3-dimensional visualization of LVs. Images were taken as a z series stack using a 10× objective, and analyzed by Imaris Bitplane software, which allowed visualization of the LVs in the submucosal and serosal-muscular layers. For each colon analyzed (n = 5–8 mice each experimental group), LV density and size were measured in 10 randomly chosen regions covered by vessels (each 1.0 mm² in area) and were represented as average number of vessels per area (mm²) and mean value of diameters (μm), respectively.

The number of LYVE-1-, VEGFR3-, or VE-Cad-positive vessels in single and double-stained murine colon tissues was established by stereological point counting of 10 regions per section (mucosal, submucosal and serosal layers), each 1.5 mm² in area, using the FluoView software (Olympus). We considered as LVs only LYVE-1-positive vessel with a visible lumen. Values are expressed as mean number of positive vessels per square millimeter. Analysis of human and mouse VEGFR3 and/or VE-Cad and LYVE-1 double-staining, was performed as described by Tammela et al,⁸ with slight modifications. In brief, murine colon sections (n = 8 mice each experimental group) were double-stained with rabbit anti-LYVE-1 (1:400; Abcam) and rat anti-VE-Cad (1:100; eBioscience) primary antibodies, while staining of human colon sections are described here. Fluorescent images were acquired at a constant exposure time at 20× or 60× magnification on a laser scanning confocal microscope (FluoView FV1000; Olympus). Colons stained with secondary antibodies alone were used to set the exposure time. Vascular structures with a lumen were analyzed with FluoView software (Olympus) and all images were within a linear intensity range between 0 and 6231. To exclude nonspecific staining, structures <8 μm (1 μm = 6.8 pixels) in diameter were excluded. To calculate mean vessel intensity, the sums of pixel intensities per vessel were divided by total vessel area (μm²). Mean vessel fluorescence intensity (FI) from 5 to 10 images per specimen were averaged and compared between treated and control groups (mouse) and healthy and tumor (human).

Isolation and Culture of Human Intestinal Lymphatic Endothelial Cells and Human Intestinal Blood Endothelial Cells

HILEC were obtained from HIMEC, which were isolated as described previously.^{5,9} Briefly, HIMEC were obtained

from normal areas of the intestine of patients admitted for bowel resection of colon cancer, polyps, or diverticulosis (n = 10). HIMEC were isolated by enzymatic digestion of intestinal mucosal strips, followed by gentle compression to extrude endothelial cell clumps, which adhere to fibronectin-coated plates and were subsequently cultured in MCDB131 medium (Sigma) supplemented with 20% fetal calf serum, antibiotics, heparin, and endothelial cell growth factor. After 9 days in culture, as reported previously,⁵ cells were trypsinized and incubated with the following antibodies: anti-human Podoplanin (1 mg per 10⁶ cells; Santa Cruz, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-human VEGFR3 (625 ng per 10⁵ cells; R&D Systems), and phycoerythrin-conjugated mouse anti-human CD31 antibody (10 μL per 10⁶ cells; BD Biosciences), followed by incubation with Alexa Fluor 647- and Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes). HILEC were then sorted on a FACSAria IIU (BD Biosciences), using a FACSDiva software (version 6.1.3; BD Biosciences), by positive selection for Podoplanin and VEGFR3 gated on a CD31-positive cell population cells (BD Pharmingen) and HIBEC by gating on CD31-positive and Podoplanin and VEGFR3-negative cell population. Cultures of HILEC and HIBEC were maintained at 37°C in 5% CO₂, fed twice a week, and split at confluence. HILEC and HIBEC were used between passages 2 and 6.

Human Intestinal Blood Endothelial Cell Adenoviral Infection

HIBEC were seeded on 6-well plates and infected with AdVEGFC or AdGFP (multiplicity of infection = 500), used as a control, or left untreated in MCDB131 complete medium supplemented with 2% fetal calf serum. After 6 hours medium wash replaced with fresh MCDB131 complete medium supplemented with 20% fetal calf serum and used after 2 days.

Immunoprecipitation and Western Blotting

Human colonic samples from CRC-involved area (n = 13) and healthy controls (n = 13) or mouse colon tissues from animals with the indicated treatments and at the indicated time points (n = 3/group/time) were mechanically homogenized in lysis buffer for protein extraction. Infected HIBECs and controls were scraped in lysis buffer for protein extraction, while medium was collected for VEGFC evaluation. Insoluble material was removed by centrifugation for 30 minutes at 14,000 rpm at 4°C and the concentration of proteins in each lysate was measured using the Bio-Rad protein assay (Bio-Rad Laboratories). For immunoprecipitation, mouse extracts were pre-cleared with lysis buffer-containing protein-G agarose beads for 1 hour at 4°C. Pre-cleared lysates (1 mg) were incubated with an antibody against mouse VEGFR3 (1 μg/mL in lysis buffer; R&D Systems) overnight at 4°C. After washing with lysis buffer, the immunoprecipitates were resuspended in 50 μL sodium dodecyl sulfate sample buffer, incubated at 95°C for 5 minutes and immunoblotted, as described here.

Proteins (80 μg for tissue homogenate and 50 μg for cell lysates), cell culture medium (50 μL) and immunoprecipitates were separated on a 10% Tris-glycine gel and electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories). Nonspecific binding was blocked with Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20, followed by overnight incubation at 4°C with the rabbit anti-human VEGFR3 antibody (1:500; Life Technologies), the rabbit anti-human VEGFC antibody (1:1000; Abcam), and an anti-actin antibody (1:1000; Santa Cruz Biotechnology) as loading control. Mouse anti-phosphotyrosine (1:1000; Millipore) and anti-mouse VEGFR3 (1:500; Eli Lilly and Company) antibodies were used to visualize immunoprecipitated samples. Membranes were washed for 1 hour with Tris-buffered saline containing 0.1% Tween 20 and then incubated for 1 hour with the appropriate horseradish peroxidase-conjugated secondary antibody (1:3000; GE Healthcare; Milan, Italy). The membranes were then incubated with Immobilon Western Chemilum (Millipore) for 1 minute, after which bands were detected by Chemidoc (Bio-Rad Laboratories), using Quantity One software.

Tubule Formation Assay, Migration Assay, and Proliferation Assay

HIBEC tube formation was assessed using Matrigel (BD Biosciences) as described previously.⁵ Briefly, multi-well dishes were coated with 250 μL of complete medium containing 5 mg/mL Matrigel for 30 minutes at 37°C. HIBEC were seeded in triplicate at a density of 5×10^4 in complete MCDB131 medium infected with AdVEGFC, AdGFP, or untreated. Cells were cultured on Matrigel for 4 hours, and inverted phase-contrast microscopy was used to assess and count endothelial tube-like structures. Five high-power fields per condition were examined. All experiments were repeated 3 times.

Migration assay was assessed as reported previously,⁹ with some modifications. By using a BD BioCoat Matrigel invasion chamber with polycarbonate filters (8- μm pore size; BD Biosciences), starved (MCDB131 for 18 hours) HIBEC (50×10^3 /filter) were plated in the upper chamber in MCDB131 medium. After 18 hours, medium was removed from both chambers, and cells that had migrated onto the lower surface of the porous membrane were washed twice in PBS and stained with Diff Quick (Medion Diagnostics), according to the manufacturer's instruction. Triplicates of migrated cells were counted in the entire membrane using a 20 \times objective. Proliferation for HIBEC was evaluated as described here for CT26 cells with brief modifications. Briefly, HIBEC were seeded in 96-well cell culture plates (1×10^3 cells/well) with MCDB131 medium. HIBEC were previously infected with AdVEGFC, AdGFP, or not infected. Medium was changed every other day and after 4 days in culture, cells were stained with 0.2% crystal violet (Sigma) dissolved in ethanol. Uptake of dye by cells on plates was eluted with 33% acetic acid in water. Plates were gently shaken for 20 minutes and the absorbance at 560 nm was measured by a Versamax microplate reader (Molecular

Devices). The optical density of each sample was then compared with a standard curve, in which the optical density was directly proportional to known cell numbers.

Human Intestinal Lymphatic Endothelial Cell Immunofluorescence and Evaluation of Vascular Endothelial Cadherin Internalization

Serum starved (MCDB131 for 24 hours) confluent HILEC were left unstimulated or stimulated with VEGFC (100 ng/mL; R&D Systems) and/or SAR131675 (23 nM dissolved in dimethyl sulfoxide; Selleckchem) for 30 minutes in starving medium. HIBEC infected for AdVEGFC, AdGFP or not infected were serum starved for 1 hour. Cells were fixed with 4% paraformaldehyde fixative solution for 10 minutes at room temperature, washed with PBS and blocked and permeabilized with 5% fetal calf serum, 5% human serum, 2% bovine serum albumin, 0.1% Triton X-100 solution for 30 minutes at room temperature. After blocking, HIBEC were incubated with mouse anti-VE-Cad (1:100; BD Bioscience), rabbit anti- β -catenin (1:600; Abcam), and HILECs also with goat anti-Prox1 (1:80; R&D Systems) antibodies overnight at 4°C. After washing, HILECs were subsequently incubated for 1 hour with anti-goat Alexa Fluor 647-conjugated, anti-rabbit Alexa Fluor 594-conjugated and anti-mouse Alexa Fluor 488-conjugated antibodies (1:1000; Molecular Probes), and HIBEC with anti-rabbit Alexa Fluor 594-conjugated and anti-mouse Alexa Fluor 647-conjugated antibodies followed by incubation with 4',6-diamidino-2-phenylindole for nuclear staining (1:25000; Invitrogen) for 10 minutes at room temperature. Cover glasses were mounted with ProLong Gold mounting medium (Invitrogen). Fluorescent images were acquired at a constant exposure time at 60 \times magnification on a laser scanning confocal microscope (FluoView FV1000; Olympus). To calculate mean membrane intensity, the sums of pixel intensities per cell membrane were subtracted from inner membrane pixel intensities. Mean membrane fluorescence intensity from 10 images per treatment were averaged and compared between treated and control groups. VE-Cad internalization experiments were performed in triplicates and are the result of 3 independent experiments.

Human Intestinal Lymphatic Endothelial Cell Permeability Assay

HILECs were seeded on fibronectin-coated 0.4- μm pore-size Transwell Permeable Supports (Corning Costar, Cambridge, MA), cultured in complete culture medium and let to form a monolayer, as described previously.¹⁰ After the establishment of a stable monolayer HILECs were serum starved for 24 hours and then were stimulated with VEGFC (100 ng/mL; R&D Systems) and/or with SAR (23 nM dissolved in dimethyl sulfoxide; Selleckchem) and assayed for permeability using a Millicell-ERS volt ohm meter (World Precision Instruments, New Haven, CT) to measure the transendothelial electrical resistance as described previously. The percentage of change in the transendothelial

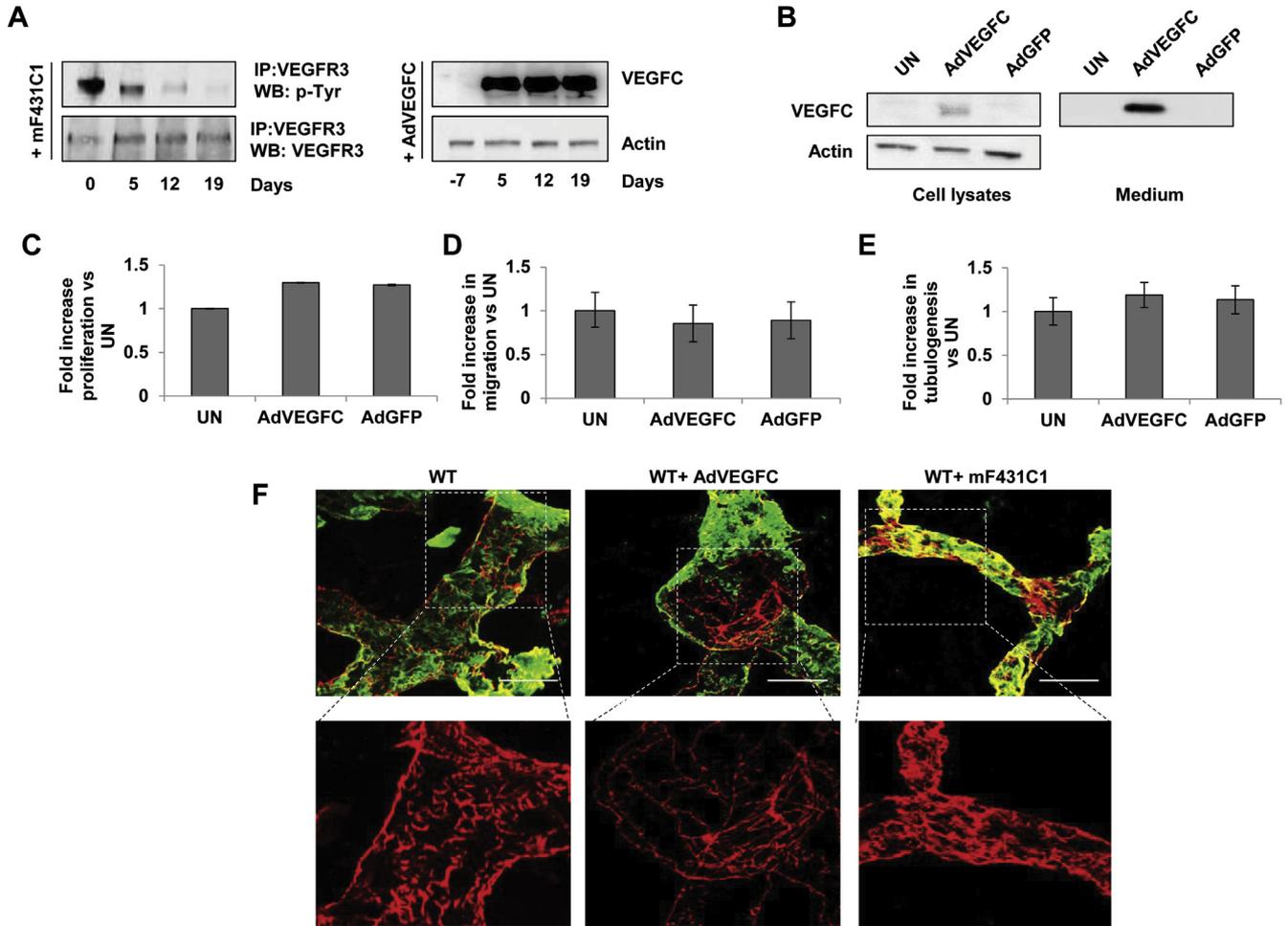
electrical resistance was calculated with respect to time 0 for each indicated time point. Experiments were performed in triplicates and are the result of 3 independent experiments.

Transendothelial Migration Assay

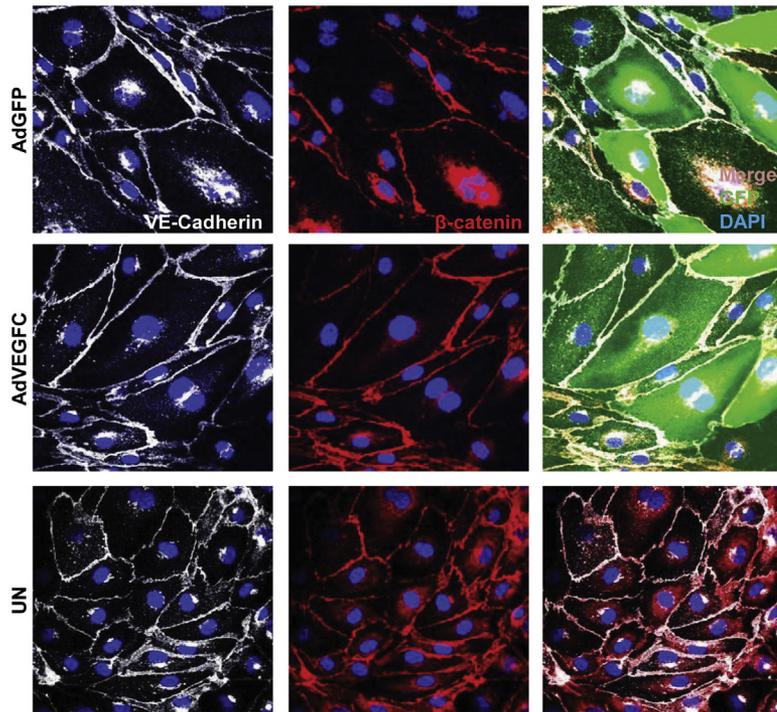
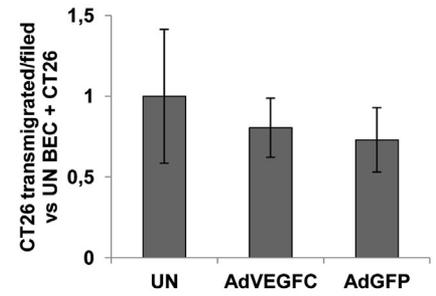
BioCoat Matrigel invasion chamber with polycarbonate filters (8- μ m pore size; BD Biosciences) were seeded with HILECs or HIBECs in complete medium. After the establishment of a stable monolayer HILECs were stimulated with VEGFC (100 ng/mL; R&D system) and/or with SAR131675 (23 nM dissolved in dimethyl sulfoxide; Selleckchem) for 24 hours. HIBECs infected with AdVEGFC or GFP or untreated were cultured in complete medium to establish a stable monolayer. The mCherry CT26 cells were trypsinized, counted and plated on top of HILEC monolayer in the presence of VEGFC (100 ng/mL; R&D Systems) and/or SAR (23 nM dissolved in dimethyl sulfoxide; Selleckchem) or on HIBECs in complete medium. After 16 hours, mCherry CT26 transmigrated cells were counted in 5 consecutive fields with a 10 \times zoom fluorescent microscope. Experiments were performed in triplicate and are the result of 3 independent experiments.

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Supplementary Figure 1. Mice receiving mCherry CT26 orthotopic injection were administered with an adenovirus encoding hVEGFC ($n = 3/\text{time point}$), or anti-VEGFR3 antibody (mF431C1, $n = 3/\text{time point}$). (A) Colonic samples from healthy and tumor-bearing mice with the indicated treatments and at the indicated time points were mechanically homogenized in lysis buffer for protein extraction. Representative blots for immunoprecipitation and immunoblotting are shown. Days are relative to CT26 injections. (B–E) HIBEC were infected with AdVEGFC, AdGFP, or not infected. (B) Western blot performed on cell lysates or medium demonstrated that VEGFC was produced at low levels by HIBEC and it was efficiently secreted when cells were infected with AdVEGFC. HIBEC infected with AdVEGFC, AdGFP, or left untreated (UN) displayed comparable proliferation (C), migration (D), and capillary formation (E). Results are representative of 3 independent experiments each in triplicate/group. Values are expressed as mean \pm SD. (F) Representative images of colonic whole mount staining of mice without tumor (WT) and groups administered with adenoviruses encoding hVEGFC, or anti-VEGFR3 antibody (mF431C1) stained for LYVE-1 (green) and VE-Cad (red). WT group (left upper panel), is visible the canonical “button and zippers” VE-Cad distribution; AdVEGFC-injected group, revealed a discontinuous VE-Cad LVs’ distribution; VEGFR3 inhibition with mF431C1 increased VE-Cad on LVs. Lower panels are higher magnifications of the white dashed squares in the upper panel. Scale bar = 50 μm .

A**B**

Supplementary Figure 2. (A) Representative merges of HIBECs stained for DAPI (*blue*), β -catenin (*red*), and VE-Cad (*white*) or expressing GFP (*green*). Cells were not infected (UN) or infected with AdVEGFC or AdGFP displayed similar VE-Cad and β -catenin distribution. Scale bar = 50 μ m. Transendothelial migration assay: mCherry-CT26 cells were seeded on HIBEC confluent monolayers not infected, infected with AdVEGFC or with AdGFP. The mCherry-positive transmigrated cells were counted (B). Results are representative of 3 independent experiments each in triplicate/group and values are expressed as mean \pm SD.

Supplementary Table 1.Real-time PCR primers

Gene product	Forward primer	Reverse primer
hVEGFC	5'-CTGCCGATGCATGTCTAAAC-3'	5'-CTTGTTGCTGCCTGACAC-3'
hVEGFD	5'-CAGTACCTGAATTAGTGCCTG-3'	5'-ATAGCATGTCAATAGGACAGAG-3'
hGAPDH	5'-CATGAGAAAGTATGACAACAGC -3'	5'-AGTCCTTCCACGATACCAAAG-3'
mVEGFR3	5'-TATGTCCGAAAGGGCAGTG-3'	5'-ACACCTTATCAAAGATGCTCTCG-3'
mVEGFR2	5'-GACCAAGAGTGACCAAGGGG-3'	5'-GATTGGACTTGACTGCCCA-3'
mVEGFC	5'-GCGCTGATCCCCAGTCCG-3'	5'- AGGACAGACATCAGCTCATC-3'
mGAPDH	5'-CGTGTTCTACCCCCAATGT-3'	5'-TGTCATCATACTTGGCAGGTTTCT-3'