Clinical Cancer Research

Tumor-associated Lymphatic Endothelial Cells Promote Lymphatic Metastasis By Highly Expressing and Secreting SEMA4C

Jun-Cheng Wei[1], Jie Yang[1], Dan Liu[1], Ming-Fu Wu[1], Long Qiao[1], Jun-Nai Wang[1], Quan-Fu Ma[1], Zhen Zeng[1], Shuang-Mei Ye[1], En-Song Guo[1], Xue-Feng Jiang[1], Lan-Ying You[1], Ying Chen[1], Li Zhou[1], Xiao-Yuan Huang[1], Tao Zhu[1], Li Meng[1], Jian-Feng Zhou[1], Zuo-Hua Feng[2], Ding Ma[1], and Qing-Lei Gao[1]

Abstract

Purpose: Lymphatic vessels are mainly regarded as passive conduits for the dissemination of cancer cells. In this study, we investigate whether and how the tumor-associated lymphatic vessels may play an active role in tumor metastasis.

Experimental Design: *In situ* laser capture microdissection of lymphatic vessels followed by cDNA microarray analysis was used to determine the expression profiling of lymphatic endothelial cells (LEC). Gene expression levels and activity of signaling pathways were measured by real-time RT-PCR, ELISA, or immunoblotting. Lymphangiogenesis was assessed by IHC. Lymph node metastasis was measured using fluorescence imaging. The effects of SEMA4C on lymphangiogenesis *in vitro* were evaluated using migration assay and tube-formation assay of LECs.

Results: Tumor-associated LECs are molecularly and functionally different from their normal counterparts. In addition to expressing high levels of membrane-bound SEMA4C, tumor-associated LECs also produced soluble SEMA4C (sSEMA4C). Increased serum sSEMA4C was detected in patients with breast cancer and cervical cancer. Patients with metastasis had much higher levels of serum sSEMA4C. sSEMA4C promoted lymphangiogenesis by activating PlexinB2-ERBB2 signaling in LECs, and promoted the proliferation and migration of tumor cells by activating PlexinB2-MET signaling, thus promoting lymphatic metastasis. Although the SEMA4C signaling pathways differ between LECs and tumor cells, RHOA activation was necessary for the effects of SEMA4C in both types of cells.

Conclusions: Tumor-associated LECs produce sSEMA4C to promote lymphatic metastasis of tumors. Our results suggest that SEMA4C and RHOA might be potential therapeutic targets, and that higher serum sSEMA4C could be a marker for breast cancer and cervical cancer. *Clin Cancer Res; 23(1); 214–24.* ©*2016 AACR.*

Introduction

Lymphatic vessels are one of the major routes by which cancer cells disseminate, especially during the initial stage of tumor growth (1, 2). The extent of lymph node metastasis is of major prognostic significance for many types of cancers, including breast cancer and cervical cancer. Lymphatic vessels are classically viewed as passive conduits for metastasis. A higher lymphatic vessel density (LVD) is thought to increase the chance that invasive tumor cells will enter the lymphatic vasculature (3, 4). However, recent studies on the immunoregulatory function of lymphatic endothelial cells (LEC) in inflammation (i.e., in wound healing and tumor metastasis) have included a new focus on LECs. In addition to providing a physical route for leukocyte transport, LECs have emerged as active players that control transport functions and directly communicate with immune cells (5–7). Nevertheless, whether and how LECs actively regulate lymphangiogenesis or communicate with tumor cells remains unclear.

Semaphorins are a large family of membrane-bound and secreted proteins originally identified as regulators of axon growth during the development of central nervous system (8). Classes 3–7 of the semaphorins appear in vertebrates, with SEMA3s being the only secreted type. Semaphorins signal through two major receptor families, Neuropilins and Plexins, and have either pro- or antitumor functions by which they directly target tumor cells and interact with endothelial cells in the vasculature (9, 10). Neuropilins are the receptors of SEMA3s, and mediate the antiangiogenic and antitumor effects of SEMA3s, including SEMA3A, SEMA3B, and SEMA3F (11). In the vasculature, Neuropilins also act as coreceptors of the VEGF family; SEMA3s are therefore likely to interfere with these key signals during the development of vascular and lymphatic systems (9, 12–14). Plexins also participate in regulating

[1]Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China. [2]Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

J.-C. Wei, J. Yang, and D. Liu contributed equally to this article.

Corresponding Authors: Ding Ma and Qing-Lei Gao, Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Anv, Wuhan, China. Phone: 8627-8366-3351; Fax: 8627-8366-2681; E-mail: dma@tjh.tjmu.edu.cn and Qing-Lei Gao, qingleigao@hotmail.com

doi: 10.1158/1078-0432.CCR-16-0741

Translational Relevance

A broad array of human malignancies disseminate through lymphatic vessels. Our clinical and mechanistic findings indicate that tumor-associated lymphatic endothelial cells (LEC) harbor molecular differences (compared with normal LECs), and actively regulate lymphangiogenesis and lymphatic metastasis. By releasing high levels of soluble SEMA4C (sSEMA4C), tumor-associated LECs not only promote lymphangiogenesis but also promote the proliferation and migration of tumor cells, thus facilitating lymphatic metastasis. Our results suggest that sSEMA4C could be a potential serum marker for the diagnosis as well as the risk of metastasis of breast cancer and cervical cancer. Moreover, high levels of SEMA4C expression were found in tumor-associated LECs. Therefore, antitumor therapies based on SEMA4C may preferentially target tumor sites while having a minimal influence on normal tissue. Taken together, our results highlight the potential diagnostic and therapeutic significance of SEMA4C.

angiogenesis. Plexins A1–A4 have been described as transducers of antiangiogenic signals conveyed by class-3 semaphorins such as sema3F, while the SEMA4D-PlexinB1 has been known as a potent inducer of angiogenesis and is associated with poor prognosis in various solid tumors (15, 16). Angiogenesis and lymphangiogenesis intersect in the regulation of tumor microenvironment, and several recent studies also suggested that semaphorins may affect lymphangiogenesis (17, 18). Nevertheless, the knowledge about the role of the semaphorin-mediated regulation of tumor lymphangiogenesis is still limited.

In this study, we investigated whether the molecular differences between tumor-associated LECs and normal LECs may include the differential expression of semaphorins that affect the lymphatic metastasis of tumors. The resulting data showed that SEMA4C, the role of which is currently unclear, was highly expressed by tumor-associated LECs and that SEMA4C played an important role in modulating both LECs and tumor cells. The membrane-bound SEMA4C (mSEMA4C) was cleaved by MMPs to release a soluble form. Soluble SEMA4C (sSEMA4C) functioned not only as an autocrine factor to promote lymphangiogenesis but also promoted the proliferation and migration of tumor cells, thereby promoting lymphatic metastasis. Therefore, tumor-associated lymphatic vessels can actively participate in lymphatic metastasis by highly expressing SEMA4C in LECs.

Materials and Methods

Cells

The human breast cancer cell line MDA-MB-231 and the human cervical cancer cell line HeLa were purchased from ATCC and cultured according to their guidelines. MDA-MB-231 and HeLa cells were authenticated at Shanghai Paternity Genetic Testing Center in November 2013 using short tandem repeat (STR) DNA profiling (ABI 3130xl Genetic Analyzer; Life Technologies). The cells were used for the experiments within 20 passages. Human normal lymphatic endothelial cells (LEC) were purchased from ScienCell and cultured in endothelial cell medium (ECM; ScienCell) with 5% FBS and endothelial growth medium supplements. The protocols for the primary culture and immunomagnetic isolation of normal and tumor LECs from patients are described in the Supplementary Methods. The primary culture was examined by inverted light microscopy (Olympus CK40) and showed homogeneous cobblestone-like morphology (typical LEC morphology; Supplementary Fig. S1A). Using flow cytometry, almost 100% of the primary culture expressed LYVE-1 and podoplanin (lymphatic markers), and less than 1% of the cells expressed CD34 (vascular endothelial cell marker), EPCAM (epithelial marker), or PDGFRα (fibroblasts marker; Supplementary Fig. S1B). LECs could be easily expanded from passage 2 to 8 without changes in their morphology or evidence of cellular senescence. In all related experiments, the fourth passage of LECs was used.

Cell transfection

The tumor cells were transduced with CMV-luciferase-IRES-RFP lentiviral particles (GeneChem). RFP/luciferase–expressing cells were isolated by FACS and used for living imaging (IVIS SPECTRUM system, Caliper, Xenogen). Lentiviral particles containing shPlexinB2(1), which targeted 5′-CCAAGAGAGGCAGCGTGAAAGAGAA-3′, and shPlexinB2(2), which targeted 5′-GAGATCTCCACCTACAAGA-3′, were used to knockdown the expression of PlexinB2 in tumor cells and LECs. After selection with puromycin, the cells with stable transfection of shRNA were used for further experiments. To downregulate the expression of SEMA4C, PlexinB1, PlexinB2, or PlexinB3, the corresponding siRNA (RiboBio) was transfected into tumor cells using Lipofectamine 2000 according to standard protocols. For each target, two nonoverlapping siRNAs were used. Similar results were obtained using the paired shRNAs/siRNAs in all related experiments. Representative data or images are therefore shown for concision.

Animals

Female athymic nude (nu/nu) mice (4 weeks old) were purchased from SLAC Laboratory Animal Co. Ltd for studies approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College. The mice were maintained in the accredited animal facility of Tongji Medical College. Luciferase/RFP–expressing tumor cells (1×10^6) were washed, suspended in 50 μL of PBS, and injected into the mammary fat pads of 6- to 7-week-old mice. Three days after inoculation with tumor cells, the mice were treated with recombinant SEMA4C protein (20 μg/kg, subcutaneously around the tumor, three times per week), or anti-SEMA4C mAb (10 μg/kg, subcutaneously around the tumor, three times per week), or inhibitors lapatinib (100 mg/kg, orally twice daily), K252a (500 μg/kg, intraperitoneally three times per week), C3 toxin (50 μg/kg, subcutaneously around the tumor, three times per week), fasudil (2.5 mg/kg, intravenously once per day), and Y27632 (10 mg/kg, intraperitoneally three times per week). At the indicated time, the mice were euthanized, tumors and lymph nodes (axillary, submandibular, inguinal) were excised, and tumors were weighed. The metastases of tumor cells in the lymph nodes were confirmed by detecting tumor-expressed RFP under an Olympus SZX16 (Olympus Inc.) dissecting microscope. The average number of positive lymph nodes in each mice and the incidence of lymphatic metastasis (the ratio of the mice with lymph node metastasis to total mice) in each group were calculated.

RNA isolation and cDNA microarray analysis

Methods for rapid IHC, laser capture microdissection (LCM), RNA isolation, and cDNA microarray analysis of LECs are described in detail in our previously published article (19). Normal mammary gland tissues (besides benign–hyperplasic mammary gland) were obtained from breast hyperplasia patients undergoing surgery. Primary invasive breast carcinoma tissues were also obtained from patients undergoing surgery. Samples were approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained from all subjects. Patients were not pregnant or lactation. Intact RNA extracted from microdissected LECs was subjected to two rounds of linear amplification and then analyzed with an Affymetrix GeneChip array (HG U133 Plus 2.0). Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE73613.

Assay of gene expression by real-time RT-PCR

The relative quantity of mRNA was determined by real-time RT-PCR as described previously (20). The expression levels of genes were quantified using the comparative C_t method. The expression level of each mRNA was normalized to the level of *GAPDH* mRNA and expressed as an n-fold difference relative to the control. The primer sequences are shown in the Supplementary Methods.

IHC

Specimens from normal/benign-hyperplasic mammary glands (45 cases), primary invasive breast carcinoma (160 cases), normal cervix (40 cases), and cervical carcinoma (70 cases) were acquired by surgeries as approved by the Ethical Committee of the Medical Faculty of Tongji Medical College (Wuhan, P.R. China). The tumor specimens were acquired from patients with cancer who had not undergone preoperative radiotherapy or chemotherapy. Tissue sections were subjected to immunohistochemical analysis using the Avidin–Biotin Complex (ABC) Vectastain Kit (Zsgb-Bio) according to the manufacturer's protocol. Anti-human CD34 (Abcam, ab81289), anti-human SEMA4C (R&D Systems, AF6125), and anti-human/mouse LYVE-1 (Abcam, ab14917) antibodies were used as primary antibodies. The staining intensity of SEMA4C was graded as weak, moderate, or strong. Lymphatic vessel density (LVD) was determined by the hotspot method as described previously (21). Briefly, slides were scanned at low power and the areas with the highest density of LYVE-1–positive vessels were identified. LVD was determined by counting the number of LYVE-1–positive vessels in five high-power fields (200× magnification) in the selected areas by two independent pathologists and the mean values of vessel counts were obtained. The pathologic analyses were done double blinded.

Flow cytometric analysis

Cells were incubated with anti-human SEMA4C (R&D Systems, AF6125), EPCAM (Abcam, ab20160), PDGFRα (Abcam, ab65258), LYVE-1 (Abcam, ab14917), CD34 (Abcam, ab81289), and CD45 (Abcam, ab10558) antibodies or isotype controls at 4°C for 0.5 hour. The secondary antibody was a FITC-conjugated antibody. Parameters were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software. The percentage of positive cells was defined as the percentage of cells in the gate, which was set to exclude ∼99% of isotype control cells. The expression index of SEMA4C was calculated using the formula: mean fluorescence × percentage of positively stained cells (22, 23).

In-house Sema4C ELISA assay

Soluble SEMA4C levels in serum and culture supernatants were measured using a double antibody sandwich ELISA method using SEMA4C detection kits prepared by our own researchers. The concentrations of the capture antibody (R&D Systems, catalog # AF6125) and the detection antibody (R&D Systems, catalog # MAB6125) were 4 μg/mL and 0.4 μg/mL, respectively. The biotin-labeled detection antibody was prepared with a Biotin Labeling Kit-NH2, which was purchased from DoJinDo Molecular Technologies. SEMA4C recombinant protein was provided by Genescript company and was used as the standard. The standard curve covered a range of 200 to 1.56 ng/mL. The preparation of the ELISA Kit and the assays was performed according to the ELISA Development Guide of R&D Systems. All assays were performed in triplicate. Optical density (OD) is measured at a wavelength of 450 nm on a microtiter plate reader.

Western blot assay

Western blot assays were performed as described previously (24). The antibodies that were used are described in the Supplementary Methods.

Assay of RHOA activity

The level of activated RHOA in LECs and tumor cells was determined using a Rhotekin-binding assay (25). The isolation of activated RHOA was based on its high affinity to a recombinant fusion protein with the Rho-binding domain of Rhotekin (RBD) and glutathione-S-transferase protein (GST). In brief, cells were preincubated in medium 199 with 1% human serum albumin for 1 hour and then lysed. Lysates were cleared by centrifugation and then incubated at 4°C for 30 minutes with RBD-GST–immobilized glutathione-coupled Sepharose beads. Beads were washed and eluted with Laemmli sample buffer. The eluate was analyzed by Western blot analysis with a monoclonal anti-RhoA antibody.

Assay of tube formation of LECs

Matrigel (BD Biosciences) and serum-free ECM were mixed at a ratio of 3:2. A 50 μL volume of the mixture was polymerized in the wells of a 96-well plate. Then, 6×10^3 LECs in 100-μL ECM were placed onto the layer of Matrigel in each well. After 3 hours of incubation, tube morphogenesis was assessed by phase contrast microscopy. Tube formation was quantified by counting the number of cells in branch point capillaries (≥3 cells per branch) in 3 random fields per replicate (26). Tube formation was quantified by ImageJ software.

Other methods

Liquid chromatography - Electrospray ionization tandem mass spectrometry (LC-ESI/MS), migration assays, wound-healing assays, and cell proliferation assays were performed using standard protocols. For details, see Supplementary Methods.

Statistical analysis

SPSS (version 13.0) software package was used for statistical analysis. The results are expressed as the mean value ± SD and were interpreted by one-way ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

Results

Tumor-associated lymphatic vessels highly express SEMA4C

To identify the molecular differences between tumor-associated LECs and their normal LEC counterparts, lymphatic vessels were isolated using *in situ* laser capture microdissection and verified by the detection of the mRNA of specific markers (Supplementary Fig. S2). Then, the gene expression profiles of tumor LECs and normal LECs were analyzed using a cDNA microarray (Fig. 1A). The differential expression of 10 representative genes (top 10) was verified using real-time RT-PCR in both breast cancer and cervical cancer (Fig. 1B). As we focused on semaphorins in this study, SEMA4C, among the top 10 genes, was chosen for further investigation.

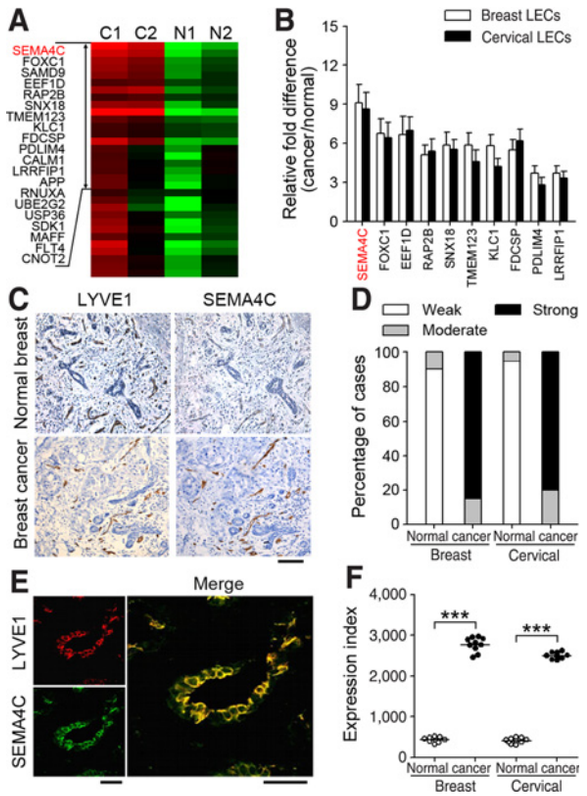

Figure 1.

Tumor-associated lymphatic vessels highly express SEMA4C. **A,** The transcriptional profiles of LECs isolated from breast cancer specimens (C1, C2) and normal mammary glands (N1, N2) were analyzed by cDNA microarray. A heatmap shows the representative genes that were significantly upregulated in tumor LECs. **B,** The expression levels of the indicated genes in LECs were detected by real-time RT-PCR. **C,** Immunohistochemical analysis of SEMA4C and the lymphatic marker LYVE-1 in serial sections of human breast and cervical specimens. Representative micrographs are shown at 200× magnification (left). Scale bar, 100 μm. **D,** The percentage of the cases with different intensity of SEMA4C staining is also shown (right). **E,** Immunofluorescence analysis of SEMA4C and LYVE-1 in tumor specimens. Representative images are shown at 400× magnification. Scale bar, 50 μm. **F,** The isolation and primary culture of LECs was performed as described in the Supplementary Methods. The protein levels of cell surface SEMA4C in the indicated LECs were detected by flow cytometric analysis. ***, $P < 0.001$.

Immunohistochemical analysis of serial sections showed that SEMA4C colocalized with the lymphatic marker LYVE1, but was not expressed in blood vessels (CD34-positive; Fig. 1C and Supplementary Fig. S3). Compared with lymphatic vessels in normal tissues, the expression of SEMA4C protein in tumor-associated lymphatic vessels was significantly increased (Fig. 1D). Using immunofluorescence, we showed that SEMA4C was localized on the membrane of tumor LECs and was expressed in 100% of the tumor lymphatic vessels (LYVE1-positive; Fig. 1E). Cell surface SEMA4C was increased in tumor LECs (Fig. 1F). These results suggest that tumor lymphatic vessels might functionally differ from normal lymphatic vessels due to high level of SEMA4C expression.

LECs release soluble SEMA4C

It has been shown that some types of semaphorins can be secreted from cells (SEMA3s) or released by proteolytic cleavage (SEMA4D; refs. 27, 28). We therefore analyzed whether SEMA4C can be released as a soluble form. Using LC-ESI/MS, we identified SEMA4C-specific peptide fragments in the culture supernatants of tumor LECs (Supplementary Fig. S4). The presence and the higher level of soluble SEMA4C (sSEMA4C) in the culture supernatants of tumor LECs were further confirmed by Western blot analysis (Fig. 2A) and ELISA (Fig. 2B). sSEMA4C was not observed in the cell lysates, and showed lower molecular weight than that of membrane SEMA4C (mSEMA4C; Fig. 2A), suggesting that sSEMA4C might be released from mSEMA4C. We therefore used different protease inhibitors to determine the protease responsible for sSEMA4C production. The results showed that the release of sSEMA4C was significantly decreased by treatment with GM6001, a general metalloproteinase inhibitor (Fig. 2C). Consistently, MMPs could induce sSEMA4C production in a dose-dependent manner (Fig. 2D). These results indicate that MMPs could cleave mSEMA4C to release sSEMA4C.

Because lymphatic fluid eventually converges into blood circulation, we then analyzed clinical samples to determine whether sSEMA4C could be detected in the serum of patients. Compared with healthy people, serum sSEMA4C levels were significantly increased in both breast cancer patients and cervical cancer patients (Fig. 2E; Supplementary Table S1) and further increased in patients with lymph node metastasis (Fig. 2F). The increase of serum sSEMA4C levels in breast cancer patients was related to lymph node metastasis, regardless of their ER, PR, or HER2 status (Fig. 2G).

SEMA4C modulates both tumor cells and tumor LECs

We next wondered whether sSEMA4C was released as a functional molecule or nonfunctional peptide. To clarify this, we first investigated whether sSEMA4C could bind to the cells in tumor microenvironment. Soluble SEMA4C was purified from the culture supernatants of Sf9 insect cells (Supplementary Fig. S5A). The cells from tumor tissues were incubated with biotin-labeled sSEMA4C, and then stained with anti-biotin antibody and the antibodies for different cell markers (Supplementary Fig. S5B–S5F). The results showed that the sSEMA4C-binding cells were mainly positive for lymphatic marker (Supplementary Fig. S5B) and epithelial marker (Supplementary Fig. S5C), but not the markers of fibroblasts, vascular endothelial cells, and leukocytes (Supplementary Fig. S5D–S5F). Therefore, sSEMA4C

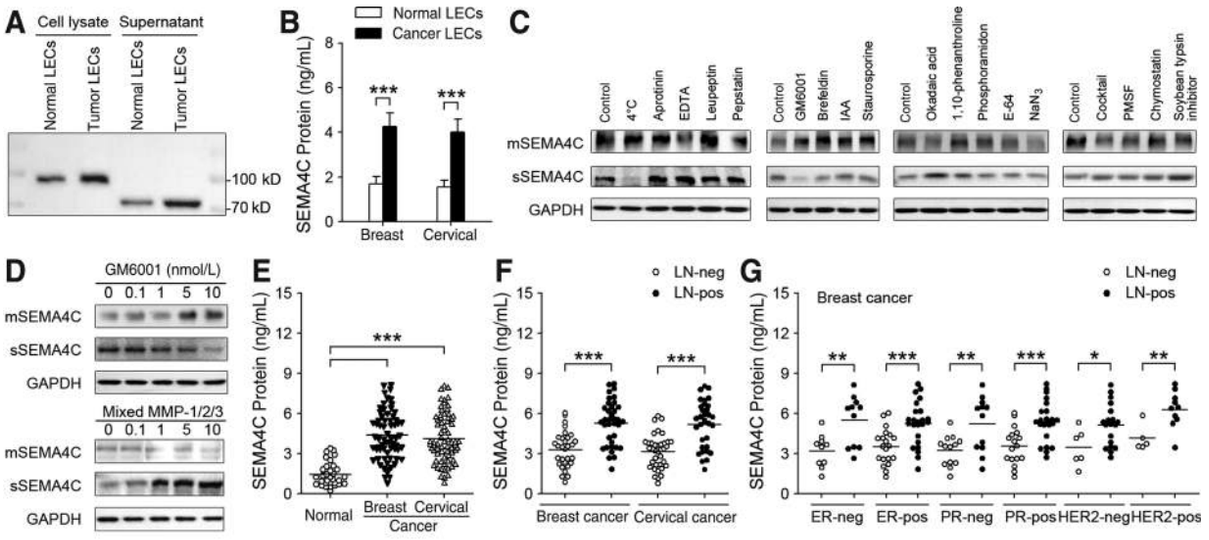

Figure 2.

LECs release soluble SEMA4C. **A,** The protein levels and molecular weights of SEMA4C in the cell lysates (mSEMA4C) and culture supernatants (sSEMA4C) of LECs were analyzed by Western blot analysis. LECs were isolated from breast cancer specimens and normal mammary glands. **B,** sSEMA4C in culture supernatants of the indicated LECs was detected by ELISA. **C** and **D,** After treatment with the indicated reagents for 1.5 hours, the protein levels of mSEMA4C and sSEMA4C in tumor LECs were detected by Western blot analysis. Applications of these reagents are described in Supplementary Methods. **E–G,** Serum sSEMA4C in the peripheral blood of healthy people or patients with breast cancer or cervical cancer was detected by ELISA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mainly binds to tumor cells and LECs in tumor microenvironment (Fig. 3A).

Treatment with recombinant sSEMA4C significantly enhanced the migration and proliferation of tumor cells (Fig. 3B and Supplementary Fig. S6). *In vivo* studies indicated that sSEMA4C treatment could promote tumor growth, while a function-blocking antibody against SEMA4C (anti-SEMA4C) resulted in a decrease in tumor size (Fig. 3C). As the migration and tube formation of LECs are crucial for tumor lymphangiogenesis (29), here, we found the migration (Supplementary Fig. S7A) and tube formation (Supplementary Fig. S7B) of LECs were also promoted by sSEMA4C and inhibited by the SEMA4C function-blocking antibody. These results suggest that sSEMA4C might promote tumor lymphangiogenesis.

Although GM6001 efficiently inhibited the production of sSEMA4C (Supplementary Fig. S8A), the promoting effect of SEMA4C on migration and tube formation of tumor LECs was not completely abrogated by GM6001. It was, however, abrogated by a function-blocking antibody that inhibits both mSEMA4C and sSEMA4C (Supplementary Fig. S8B), suggesting that the effect of mSEMA4C on lymphangiogenesis is also promotional. This was further confirmed by knocking down SEMA4C expression with siRNA (Supplementary Fig. S8C–S8E).

The above results showed that SEMA4C is a regulatory factor of both tumor cells and LECs. We therefore further investigated the effect of sSEMA4C on lymphangiogenesis and lymph node metastasis *in vivo*. The lymphatic vessel density (LVD) in sSEMA4C-treated tumors was significantly higher than that in control tumors (Fig. 3D). Because lymphatic metastasis positively correlates with tumor size (30), metastasis was analyzed when primary tumors reached comparable sizes, based on the dynamic monitoring of tumor growth in living mice. When primary tumors reached comparable sizes (Supplementary Fig. S9), the average number of positive lymph node in each mice and the ratio of lymph node metastasis in each group were all significantly increased by sSEMA4C (Fig. 3E). Corresponding with these results, blocking the function of SEMA4C resulted in a decrease in LVD in tumors and a decrease in lymph node metastasis (Fig. 3D and E). Taken together, these results suggest that increased expression of SEMA4C in tumor LECs could promote lymphangiogenesis and lymph node metastasis *in vivo*.

PlexinB2-ERBB2 signaling in LECs mediates SEMA4C-induced lymphangiogenesis

Plexin family receptors (type A–D) are responsible for semaphorin signal transduction, and B-family Plexins are thought to mediate a wide range of SEMA4 functions, including cell migration and angiogenesis (31). We therefore investigated whether PlexinB signaling mediated SEMA4C-induced lymphangiogenesis. PlexinB1, B2, or B3 was knocked down using corresponding siRNAs (Supplementary Fig. S10). Biotin-labeled sSEMA4C could not bind to LEC membranes only when PlexinB2 siRNA was used, indicating that PlexinB2 is the receptor of sSEMA4C on LECs (Fig. 4A). PlexinBs have been reported to stimulate the tyrosine kinase activity of MET and ERBB2, and regulate the activation of RHOA by interacting with Rho guanine nucleotide exchange factor (RhoGEF). Interestingly, sSEMA4C increased the phosphorylated level of ERBB2, but not MET, and activated RHOA in LECs (Fig. 4B). When PlexinB2 was knocked down by shRNA in LECs, the sSEMA4C-induced activation of ERBB2 and RHOA was completely abrogated (Fig. 4C), indicating that ERBB2 and RHOA were the subsequent targets following SEMA4C-

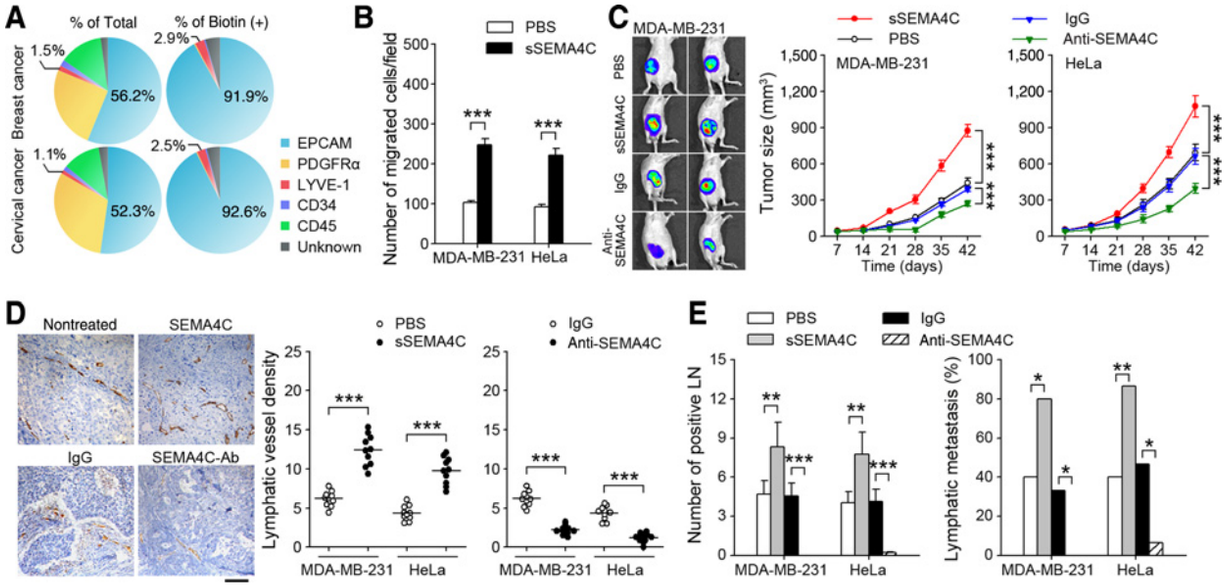

Figure 3.

The effect of SEMA4C on tumor cells and LECs. **A,** sSEMA4C binds to tumor cells and LECs. Single-cell suspensions were prepared from tumor tissues, and incubated with biotin-labeled sSEMA4C or biotin. The cells were then stained with anti-biotin antibody and the antibodies for LYVE-1, EPCAM, PDGFRα, CD34, CD45, or isotype control. After flow cytometric analysis, the percentages of positive cells in total cells and in biotin-positive cells were calculated. The mean value of three independent experiments is shown. EPCAM indicates epithelial cells (tumor cells); PDGFRα, fibroblasts; LYVE-1, lymphatic endothelial cells; CD34, vascular endothelial cells; and CD45, leukocytes. See also Supplementary Fig. S5. **B,** Migration assays were performed as described in the Supplementary Methods. The bottom chambers were filled with 0.5 μg/mL recombinant sSEMA4C protein or PBS. **C–E,** Three days after inoculation with tumor cells, sSEMA4C (20 μg/kg), anti-SEMA4C (10 μg/kg), or control solution (PBS or IgG) was injected subcutaneously around the tumor, three times per week. The size of the tumors was dynamically monitored using *in vivo* bioluminescence images (**C,** left). Tumor volume was measured (**C,** right; $N = 10$ per group). Lymphatic vessels were visualized using staining for LYVE-1 (**D,** left). Scale bar, 100 μm. LVD was quantified (**D,** right). Metastases were determined when primary tumors reached comparable sizes. The average number of positive lymph node in each mice and the incidence of lymphatic metastasis (the ratio of the mice with lymph node metastasis to total mice, interpreted by χ^2 test) in each group were calculated (**E**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

PlexinB2 binding. To further examine the roles of ERBB2 and MET in SEMA4C-mediated signaling, the ERBB2 inhibitor lapatinib and the MET inhibitor K252a were used. The results showed that lapatinib, but not K252a, inhibited sSEMA4C-induced activation of RHOA (Fig. 4C). Consistent with these findings, lapatinib suppressed the promotional effect of sSEMA4C on migration (Supplementary Fig. S11) and tube formation (Fig. 4D) of LECs, same as the effect of PlexinB2 shRNA. Moreover, lapatinib also suppressed sSEMA4C-induced lymphangiogenesis *in vivo* (Fig. 4E), while K252a did not have any effect on LECs *in vitro* or *in vivo*. In addition, because VEGF-C is considered the key drivers of lymphangiogenesis, we investigated the relationship between SEMA4C and VEGFC-related signaling. The result showed that VEGF-C stimulation did not affect the protein levels of mSEMA4C and sSEMA4C in LECs, and that sSEMA4C stimulation also did not the protein levels of VEGF-C, VEGFR-3, and neuropilin-2 in LECs (Supplementary Fig. S12). These results indicate that PlexinB2-ERBB2 signaling in LECs is necessary for SEMA4C-induced lymphangiogenesis.

Soluble SEMA4C promotes the migration and proliferation of tumor cells through PlexinB2-MET signaling

PlexinB2 was also the receptor of sSEMA4C in both a breast cancer cell line and a cervical cancer cell line (Fig. 5A). Distinct from its function in LECs, sSEMA4C stimulation resulted in higher phosphorylated levels of MET in the ERBB2-negative MDA-MB-231 cells (Supplementary Fig. S13A). In HeLa cells that express ERBB2, sSEMA4C also induced the phosphorylation of MET instead of ERBB2 (Supplementary Fig. S13B). Consistent with these results, the MET inhibitor K252a abrogated the sSEMA4C-induced activation of MET and RHOA in tumor cells, same as the effect of PlexinB2 shRNA (Fig. 5B). The treatment with K252a also abolished the promoting effects of sSEMA4C on tumor cell migration (Fig. 5C), tumor cell proliferation (Fig. 5D), and tumor growth (Fig. 5E). These effects of sSEMA4C were not influenced by the ERBB2 inhibitor lapatinib. Similar results were obtained when MET expression was knocked down using shRNA (Supplementary Fig. S14). These results suggest that PlexinB2-MET signaling is required for the effects of sSEMA4C on tumor cells.

RHOA signaling is crucial for SEMA4C-induced lymph node metastasis

The above data indicate that although SEMA4C signaling pathways are different between LECs and tumor cells, they both result in the activation of RHOA. Moreover, both the ROCK I and II, downstream factors of RHOA, were activated by sSEMA4C stimulation (Supplementary Fig. S15). Therefore, we further investigated whether RHOA might play an important role in SEMA4C functions. A RHOA inhibitor (C3 toxin) and the inhibitors of the downstream factors of RHOA (Y-27632

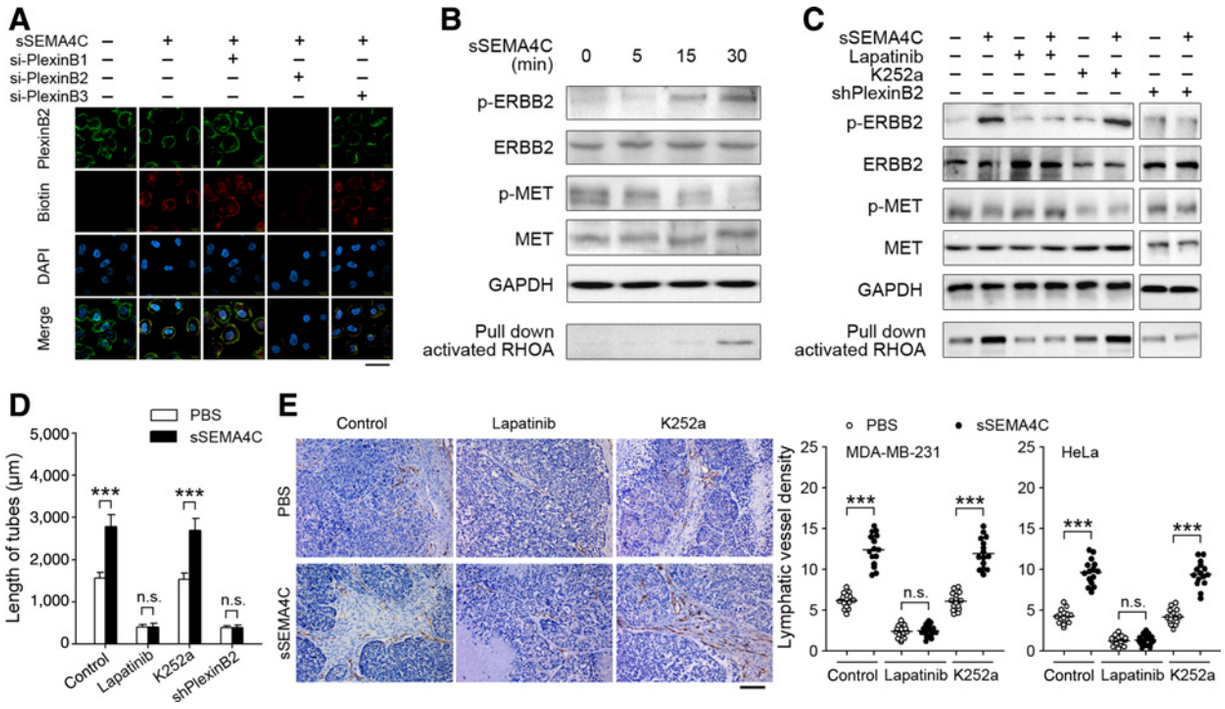

Figure 4.

PlexinB2-ERBB2 signaling mediates SEMA4C-induced lymphangiogenesis. **A,** Identification of the SEMA4C receptor in LECs. The expression of PlexinBs was knocked down using corresponding siRNA, and LECs were then incubated with biotin-labeled sSEMA4C and anti-biotin fluorescent antibody. The binding of sSEMA4C to the membrane of LECs was analyzed using immunofluorescence assays. Representative images are shown at 400× magnification. Scale bar, 50 μm. **B,** LECs were stimulated with sSEMA4C (0.5 μg/mL) for the indicated times. ERBB2, p-ERBB2, MET, p-MET, and activated RHOA were detected by Western blot analysis. **C** and **D,** LECs were pretreated with lapatinib (10 nmol/L) or K252a (50 nmol/L) or transfected with PlexinB shRNA and then stimulated with sSEMA4C (0.5 μg/mL). ERBB2, p-ERBB2, MET, p-MET, and activated RHOA were detected by Western blot analysis (**C**). LECs were used for the tube-formation assays as described in the Materials and Methods (**D**). **E,** Three days after inoculation with tumor cells, the mice were treated with lapatinib (100 mg/kg, orally twice daily) or K252a (500 μg/kg, intraperitoneally three times per week). Meanwhile, sSEMA4C (20 μg/kg) or PBS control was injected subcutaneously around the tumor three times per week. Lymphatic vessels were visualized by staining for LYVE-1 (left). Scale bar, 100 μm. LVD was quantified (right). ***, $P < 0.001$; n.s., not significant.

and fasudil) suppressed SEMA4C-induced lymphangiogenesis, which was similar to the effect of lapatinib (Fig. 6A and Supplementary Fig. S16). Blocking RHOA signaling also suppressed tumor cell migration (Supplementary Fig. S17) and tumor growth (Fig. 6B; Supplementary Fig. S18), which was the same as the effect of K252a. In the experiments *in vivo*, the number of positive lymph nodes (Fig. 6C) and the incidence of lymphatic metastasis (Fig. 6D) were increased by the treatment with sSEMA4C. Intriguingly, the effects of sSEMA4C were only partially attenuated by either lapatinib or K252a, but almost completely abolished by double treatment of lapatinib and K525a (Supplementary Fig. S19), suggesting that sSEMA4C promoted lymphatic metastasis through its effects on both LECs and tumor cells. Importantly, the promoting effect of sSEMA4C on lymph node metastasis could be fully suppressed by blocking RHOA signaling. Taken together, these results demonstrate that RHOA signaling is critical for SEMA4C-induced lymph node metastasis.

Discussion

For decades, lymphatics have been portrayed as passive participants in metastasis. Higher lymphangiogenic activity is mainly regarded as an increased opportunity for tumor cells to access the lymphatic system (4). The data in this study show that tumor-associated lymphatic vessels could play an active role in tumor metastasis, which was, at least in part, due to the higher expression of SEMA4C by tumor-associated LECs. Previous studies described the gene expression profile of tumor LECs in mouse models, and suggested that the differentially expressed genes might associate with tumor growth and nodal metastasis (32, 33). In this study, our data show that the gene expression profile of human tumor LECs differs from that of normal LECs, and that tumor-associated LECs acquired an activated phenotype, which was associated with the increased sprouting and tube formation activity. Soluble SEMA4C was released from LECs due to proteolytic cleavage of membrane-bound SEMA4C. The increased production of soluble SEMA4C not only promoted lymphangiogenesis but also promoted the proliferation and migration of tumor cells. Therefore, the upregulation and release of sSEMA4C might be an important mechanism by which tumor LECs can actively modulate the metastatic capacity of tumor cells. These results also reveal that SEMA4C might be a valuable marker for the identification and characterization of tumor LECs that are different from normal LECs.

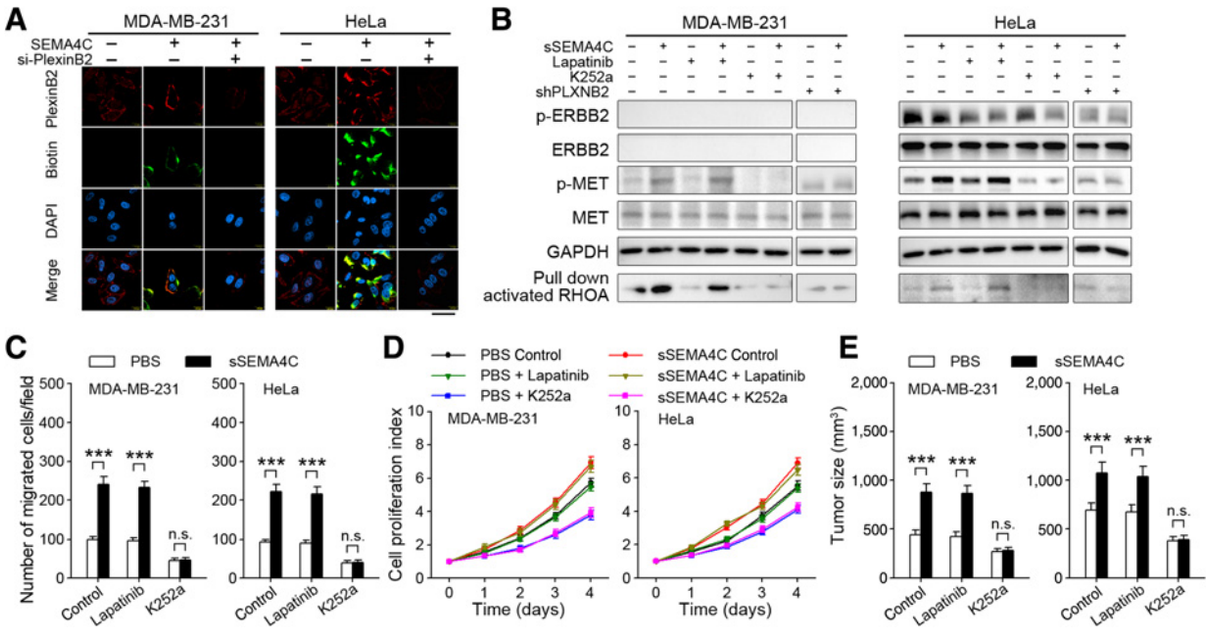

Figure 5.

sSEMA4C promotes the migration and proliferation of tumor cells through PlexinB2-MET signaling. **A,** The expression of PlexinB2 in MDA-MB-231 and HeLa cells was knocked down using siRNA, and then the tumor cells were incubated with biotin-labeled sSEMA4C and anti-biotin fluorescent antibodies. The binding of sSEMA4C to the membranes of tumor cells was analyzed using immunofluorescence assays. Representative images are shown at 400× magnification. Scale bar, 50 μm. **B,** Tumor cells were pretreated with lapatinib or K252a or transfected with PlexinB shRNA and then stimulated with sSEMA4C. ERBB2, p-ERBB2, MET, p-MET, and activated RHOA were detected by Western blot analysis. **C** and **D,** Tumor cells were pretreated with lapatinib (10 nmol/L) or K252a (50 nmol/L) or transfected with PlexinB shRNA and then used for the migration assays (**C**) and cell proliferation assays (**D**) as described in the Supplementary Methods. **E,** Three days after inoculation with tumor cells, the mice were treated with lapatinib (100 mg/kg, orally twice daily) or K252a (500 μg/kg, intraperitoneally three times per week), respectively. Meanwhile, sSEMA4C (20 μg/kg) or PBS control was injected subcutaneously around the tumor three times per week. The size of tumor was measured at 6 weeks after tumor inoculation ($N = 10$ per group). ***, $P < 0.001$; n.s., not significant.

Lymphangiogenesis has been shown to be influenced by cytokines and growth factors in the tumor microenvironment (34). However, the producers of lymphangiogenic factors are primarily reported to be tumor cells, mesenchymal cells, and immunocytes (3, 29, 35, 36). For instance, oncogenes such as *RAS*, *MYC*, and *SIX1* lead to the increased expression of lymphangiogenic factors, including members of the VEGF, FGF, and PDGF families (37–39). So far, it has not been demonstrated whether tumor LECs secret the factor(s) that can regulate tumor lymphangiogenesis. Data in this study show that tumor-associated LECs express high levels of SEMA4C and that they release soluble SEMA4C, which in turn promotes lymphangiogenesis in an autocrine manner. The release of soluble SEMA4C is due to the cleavage of membrane-bound SEMA4C by MMPs, which are widely expressed and are activated at higher levels in the tumor milieu than in normal tissues. Thus, the increase of MMPs in the tumor microenvironment might increase sSEMA4C production to a higher level *in vivo*. Consistent with these results, a function-blocking antibody against SEMA4C and the inhibitors of SEMA4C signaling dramatically reduced lymphangiogenesis both *in vitro* and *in vivo*. Taken together, our results demonstrate that the expression of SEMA4C in tumor LECs is critical to tumor lymphangiogenesis.

It has been reported that the biological properties and molecular mechanisms of some molecules differ between their membrane and soluble forms. For instance, soluble TNFα (sTNFα) induces necrocytosis, whereas membrane TNFα induces apoptosis and shows cytotoxicity, even in sTNFα-resistant tumor cells (40, 41). It was recently reported that cleaved SEMA3C (p65-SEMA3C) is a protumorigenic factor but that full-length SEMA3C functions as an inhibitor of tumor angiogenesis, lymphangiogenesis, and lymphatic metastasis (17). Nevertheless, sSEMA4C promotes lymphangiogenesis, and our results also show that the inhibitory effect of blocking SEMA4C cleavage (by GM6001) on LECs was weaker than the effect of using SEMA4C siRNAs or a function-blocking antibody, suggesting that the effect of mSEMA4C on lymphangiogenesis is also promotional. Thus, the effects of mSEMA4C and sSEMA4C may be consistent, conforming to the general pattern that the membrane form and soluble form of cytokines mediate similar functions in different ways. However, mSEMA4C is highly expressed on parts of tumor LECs (Supplementary Fig. S3), and direct cell–cell contact is needed for mSEMA4C to function. Therefore, the soluble form of SEMA4C expands its sphere of influence and plays an important role in regulating tumor progression.

In this study, we demonstrate that the effects of SEMA4C on LECs and tumor cells are mediated by the same receptor, PlexinB2, and that RHOA is critical to SEMA4C-mediated

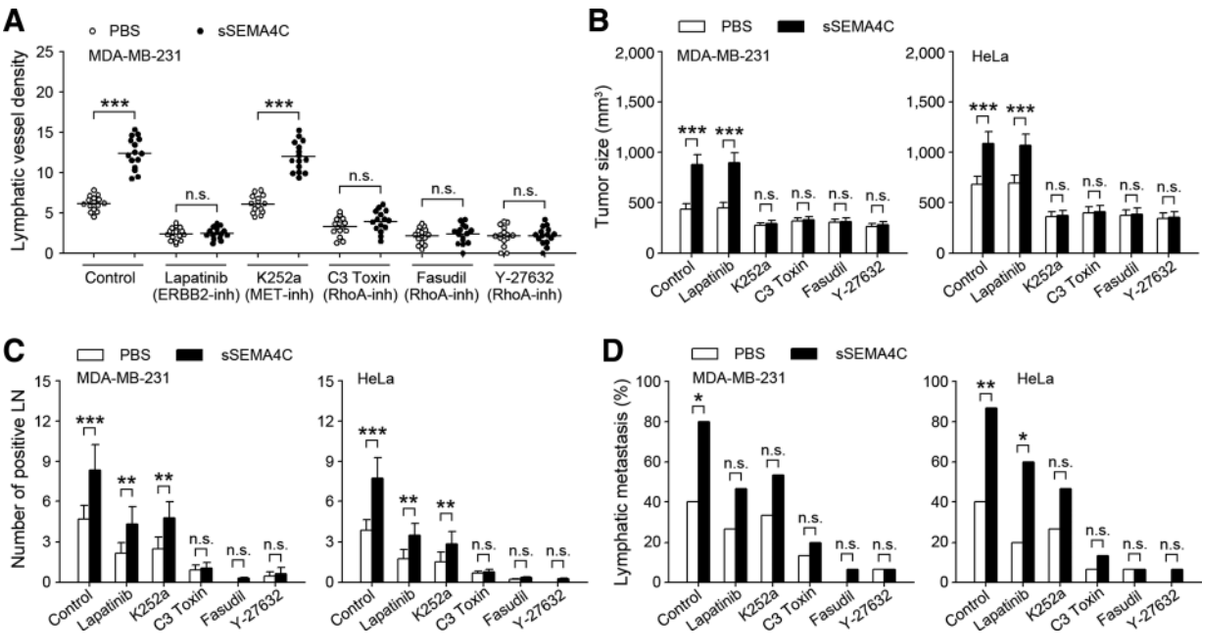

Figure 6.

RHOA signaling is required for SEMA4C-induced lymph node metastasis. Three days after inoculation with tumor cells, the mice were treated with lapatinib (100 mg/kg, orally twice daily), K252a (500 μg/kg, intraperitoneally three times per week), C3 toxin (50 μg/kg, subcutaneously around the tumor three times per week), fasudil (2.5 mg/kg, intravenously once per day), or Y27632 (10 mg/kg, intraperitoneally three times per week). Meanwhile, sSEMA4C (20 μg/kg) or PBS control solution was injected subcutaneously around the tumor three times per week. The mice were euthanized at 6 weeks after tumor inoculation ($N = 10$ per group). **A,** LVD was quantified. **B**, The size of tumor was measured. **C** and **D,** The average number of positive lymph node in each mice (**C**) and the incidence of lymphatic metastasis (the ratio of the mice with lymph node metastasis to total mice) in each group were calculated (**D**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

signaling in both cell types. However, the pathways that activate RHOA are different. In LECs, SEMA4C-PlexinB2 activates RHOA through ERBB2 signaling. Blocking ERBB2 can abolish SEMA4C-induced lymphangiogenesis, but it does not block the effect of SEMA4C on tumor cells. In tumor cells, SEMA4C-PlexinB2 activates RHOA through MET signaling. Blocking MET abrogates the promotional effects of SEMA4C on the proliferation and migration of tumor cells, but does not block the effect of SEMA4C on LECs. The activation of RHOA acts as a critical junction in SEMA4C signal transduction. While blocking ERBB2 or MET alone only partially reduced the promoting effect of SEMA4C on lymph node metastasis, RHOA inhibitors completely abolished the effects of SEMA4C *in vivo*. Therefore, targeting RHOA signaling might play an important role in antitumor therapies.

The detection of tumor-associated biomarker in the peripheral blood of cancer patients provides an opportunity to analyze the changes in tumor burden and monitor the responses to treatment. The results in this study suggest that sSEMA4C could potentially be a serum marker for the diagnosis and the risk of metastasis of breast cancer and cervical cancer. Moreover, high levels of SEMA4C expression were found in tumor-associated LECs. Therefore, antitumor therapies based on SEMA4C may preferentially target tumor sites while having a minimal influence on normal tissue. Taken together, our results highlight the potential clinical significance of SEMA4C, suggesting that further studies are needed to investigate the diagnostic and therapeutic applications of SEMA4C.

In summary, this study demonstrates that tumor-associated lymphatic vessels actively regulate lymphatic metastasis by expressing high levels of SEMA4C in LECs. SEMA4C not only promoted lymphangiogenesis but also enhanced the metastatic capacity of tumor cells, thus coordinating these processes to facilitate lymphatic metastasis. On the other hand, higher serum levels of soluble SEMA4C were detected in breast cancer patients and cervical cancer patients. These results suggest that targeting SEMA4C/RHOA might have significant therapeutic value in cancer treatment and also imply that sSEMA4C might be a potential marker for the noninvasive diagnosis and assessment of cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Q. Gao, J.-C. Wei, J. Yang, D. Liu, D. Ma

Development of methodology: J.-C. Wei, J. Yang, D. Liu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-C. Wei, J. Yang, D. Liu, M.-F. Wu, J.-N. Wang, Z. Zeng, L.-Y. You, L. Zhou, X.-Y. Huang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Gao, J.-C. Wei, J. Yang, D. Liu, L. Qiao, S.-M. Ye, E.-S. Guo, Y. Chen, T. Zhu, L. Meng, J.-F. Zhou

Writing, review, and/or revision of the manuscript: Q. Gao, J.-C. Wei, J. Yang, D. Liu, Z.-H. Feng

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Gao, J. Yang, Q.-F. Ma

Study supervision: Q. Gao, D. Ma

Acknowledgments

We thank Dr. Qi-Lin Ao and Dr. Shuang Guo (Department of Pathology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology) for reviewing histology data, and thank Dr. Jun-Xiang Wan of the Department of Pediatrics (University of California, Los Angeles) for her kind help in setting up the In-house Sema4C ELISA assay.

Grant Support

This work was supported by the "973" Program of China (No. 2009CB521808) and National Natural Science Foundation of China (81572570, 81502250, 81372801, 81072135, 81172467, 81201639, 81072132, 81071663).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 22, 2016; revised June 25, 2016; accepted June 29, 2016; published OnlineFirst July 8, 2016.

References

1. Sundar SS, Ganesan TS. Role of lymphangiogenesis in cancer. J Clin Oncol 2007;25:4298–307.
2. Thiele W, Sleeman JP. Tumor-induced lymphangiogenesis: a target for cancer therapy? J Biotechnol 2006;124:224–41.
3. Sleeman JP, Thiele W. Tumor metastasis and the lymphatic vasculature. Int J Cancer 2009;125:2747–56.
4. Podgrabinska S, Skobe M. Role of lymphatic vasculature in regional and distant metastases. Microvasc Res 2014;95:46–52.
5. Swartz MA.Immunomodulatory roles of lymphatic vessels in cancer progression. Cancer Immunol Res 2014;2:701–7.
6. Card CM, Yu SS, Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. J Clin Invest 2014;124:943–52.
7. Shin K, Lee SH. Interplay between inflammatory responses and lymphatic vessels. Immune Netw 2014;14:182–6.
8. Kolodkin AL, Matthes DJ, Goodman CS. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 1993;75:1389–99.
9. Nasarre P, Gemmill RM, Drabkin HA. The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. Onco Targets Ther 2014;7:1663–87.
10. Worzfeld T, Offermanns S. Semaphorins and plexins as therapeutic targets. Nat Rev Drug Discov 2014;13:603–21.
11. Sakurai A, Doci CL, Gutkind JS. Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. Cell Res 2012;22:23–32.
12. Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M. VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. J Cell Biochem 2002;85:357–68.
13. Whitaker GB, Limberg BJ, Rosenbaum JS. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). J Biol Chem 2001;276:25520–31.
14. Xu Y, Yuan L, Mak J, Pardanaud L, Caunt M, Kasman I, et al. Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. J Cell Biol 2010;188:115–30.
15. Liu H, Yang Y, Xiao J, Yang S, Liu Y, Kang W, et al. Semaphorin 4D expression is associated with a poor clinical outcome in cervical cancer patients. Microvasc Res 2014;93:1–8.
16. Kato S, Kubota K, Shimamura T, Shinohara Y, Kobayashi N, Watanabe S, et al. Semaphorin 4D, a lymphocyte semaphorin, enhances tumor cell motility through binding its receptor, plexinB1, in pancreatic cancer. Cancer Sci 2011;102:2029–37.
17. Mumblat Y, Kessler O, Ilan N, Neufeld G. Full-length semaphorin-3C is an inhibitor of tumor lymphangiogenesis and metastasis. Cancer Res 2015;75:2177–86.
18. Doci CL, Mikelis CM, Lionakis MS, Molinolo AA, Gutkind JS. Genetic identification of SEMA3F as an antilymphangiogenic metastasis suppressor gene in head and neck squamous carcinoma. Cancer Res 2015;75:2937–48
19. Wu M, Han L, Shi Y, Xu G, Wei J, You L, et al. Development and characterization of a novel method for the analysis of gene expression patterns in lymphatic endothelial cells derived from primary breast tissues. J Cancer Res Clin Oncol 2010;136:863–72.
20. Liu D, Zhang XX, Xi BX, Wan DY, Li L, Zhou J, et al. Sine oculis homeobox homolog 1 promotes DNA replication and cell proliferation in cervical cancer. Int J Oncol 2014;45:1232–40.
21. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis–correlation in invasive breast carcinoma. N Engl J Med 1991;324:1–8.
22. Feng XX, Liu M, Yan W, Zhou ZZ, Xia YJ, Tu W, et al. beta3 integrin promotes TGF-beta1/H2O2/HOCl-mediated induction of metastatic phenotype of hepatocellular carcinoma cells by enhancing TGF-beta1 signaling. PLoS ONE 2013;8:e79857.
23. Liu D, Zhang XX, Wan DY, Xi BX, Ma D, Wang H, et al. Sine oculis homeobox homolog 1 promotes alpha5beta1-mediated invasive migration and metastasis of cervical cancer cells. Biochem Biophys Res Commun 2014;446:549–54.
24. Feng XX, Luo J, Liu M, Yan W, Zhou ZZ, Xia YJ, et al. Sirtuin 6 promotes transforming growth factor-beta1/H2O2/HOCl-mediated enhancement of hepatocellular carcinoma cell tumorigenicity by suppressing cellular senescence. Cancer Sci 2015;106:559–66.
25. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW. Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ Res 2000;87:335–40.
26. Chan DA, Kawahara TL, Sutphin PD, Chang HY, Chi JT, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. Cancer Cell 2009;15:527–38.
27. Basile JR, Castilho RM, Williams VP, Gutkind JS. Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. Proc Natl Acad Sci USA 2006;103:9017–22.
28. Basile JR, Holmbeck K, Bugge TH, Gutkind JS. MT1-MMP controls tumor-induced angiogenesis through the release of semaphorin 4D. J Biol Chem 2007;282:6899–905.
29. Alitalo K. The lymphatic vasculature in disease. Nat Med 2011;17:1371–80.
30. Cairns RA, Hill RP. Acute hypoxia enhances spontaneous lymph node metastasis in an orthotopic murine model of human cervical carcinoma. Cancer Res 2004;64:2054–61.
31. Zhou Y, Gunput RA, Pasterkamp RJ. Semaphorin signaling: progress made and promises ahead. Trends Biochem Sci 2008;33:161–70.
32. Albrecht I, Bieri R, Leu A, Granacher P, Hagmann J, Kilimann MW, et al. Paralemmin-1 is expressed in lymphatic endothelial cells and modulates cell migration, cell maturation and tumor lymphangiogenesis. Angiogenesis 2013;16:795–807.
33. Clasper S, Royston D, Baban D, Cao Y, Ewers S, Butz S, et al. A novel gene expression profile in lymphatics associated with tumor growth and nodal metastasis. Cancer Res 2008;68:7293–303.
34. Cao Y.Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. Nat Rev Cancer 2005;5:735–43.
35. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, et al. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest 2005;115:247–57.
36. Oka M, Iwata C, Suzuki HI, Kiyono K, Morishita Y, Watabe T, et al. Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. Blood 2008;111:4571–9.

37. Su WC, Shiesh SC, Liu HS, Chen CY, Chow NH, Lin XZ. Expression of oncogene products HER2/Neu and Ras and fibrosis-related growth factors bFGF, TGF-beta, and PDGF in bile from biliary malignancies and inflammatory disorders. Dig Dis Sci 2001;46:1387–92.
38. Ruddell A, Mezquita P, Brandvold KA, Farr A, Iritani BM. B lymphocyte-specific c-Myc expression stimulates early and functional expansion of the vasculature and lymphatics during lymphomagenesis. Am J Pathol 2003;163:2233–45.
39. Liu D, Li L, Zhang XX, Wan DY, Xi BX, Hu Z, et al. SIX1 promotes tumor lymphangiogenesis by coordinating TGFbeta signals that increase expression of VEGF-C. Cancer Res 2014;74: 5597–607.
40. Zhang H, Yan D, Shi X, Liang H, Pang Y, Qin N, et al. Transmembrane TNF-alpha mediates "forward" and "reverse" signaling, inducing cell death or survival via the NF-kappaB pathway in Raji Burkitt lymphoma cells. J Leukoc Biol 2008;84:789–97.
41. Vudattu NK, Holler E, Ewing P, Schulz U, Haffner S, Burger V, et al. Reverse signalling of membrane-integrated tumour necrosis factor differentially regulates alloresponses of CD4+ and CD8+ T cells against human microvascular endothelial cells. Immunology 2005; 115:536–43.