Semaphorin 4C Promotes Macrophage Recruitment and Angiogenesis in Breast Cancer

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Abstract

Semaphorins are a large family of evolutionarily conserved morphogenetic molecules that are associated with repelling axonal guidance. Intriguingly, recent researches indicate that semaphorins are involved in cancer progression. Semaphorin 4C (SEMA4C) has long been considered a neuronal migration gene, but we detected that it is also highly expressed in many malignant human cancers. During an investigation of subcancous tumor models, we found that SEMA4C expression promoted tumor growth and progression. We discovered that SEMA4C was involved in maintaining tumor cell self-renewal, likely by regulating the p53 pathway. Inhibiting the expression of endogenous SEMA4C in tumor cells impaired growth and induced senescence and cell-cycle arrest in the G2-phase. In addition, we found that SEMA4C induced the production of angiogenin and colony-stimulating factor-1 (CSF-1) in tumor cells by activating the NF-κB pathway in a plexinB2-dependent manner. In conclusion, SEMA4C expression in breast cancer cells promotes cancer cell proliferation, macrophage recruitment, and angiogenesis. Thus, inhibition of SEMA4C activity may be a novel therapeutic strategy for human breast cancer.

Implications: In breast cancer, therapeutic targeting of the SEMA4C pathway may prevent tumor growth, angiogenesis, metastasis, and progression.

Introduction

Breast cancer is currently the fastest growing cancer and the leading cause of cancer-related death among women worldwide, accounting for 25% of cancer diagnoses (1.7 million women) and 15% of cancer-related deaths (521,900 women) annually. Although the incidence of breast cancer is significantly higher in developed countries, half of new breast cancer diagnoses and 60% of breast cancer-related deaths occur in developing countries (1). Aside from being classified into different histologic subtypes, increasing evidence suggests that breast cancer can be broadly classified into five subtypes based on molecular features: basal-like (which generally corresponds to triple-negative disease), luminal-A [generally estrogen receptor (ER)-positive and low grade], luminal-B (also ER-positive but high grade), HER2-positive tumors, and normal breast-like. Triple-negative breast cancer is significantly correlated with high mitotic count, low tubule formation, strong nuclear pleomorphism, and high histologic grade, which have been similarly documented in previous studies of basal-like breast cancer. However, it is not a complete substitute of basal-like breast cancer because 6% of triple-negative tumors have been found to be sparsely intertwined within the luminal-rich cluster carrying low basal-like mRNA (2). Identifying and characterizing the driver genes inducing these alterations will provide insights into the development of novel therapeutic strategies for this aggressive disease.

Semaphorins are a highly conserved clan of molecular signals initially considered as guiding signal of axon navigation in neurodevelopment (3, 4). However, some semaphorins have been illustrated in angiogenesis, immunoregulation, and cancer (5). The functions of Semaphorin 4C (Sema4C) in biological progression are also diverse. For example, Sema4C has been shown to elicit functions in neurogenesis induced by cerebral ischemia (6), to modulate ureteric branching in the developing kidney (7), to promote terminal myogenic differentiation (8, 9), and to play an important role in TGF-β1–induced epithelial–mesenchymal transition (EMT) through the activation of p38 MAPK in proximal tubular epithelial cells (10). However, knowledge of the functions of Sema4C in tumor progression is still limited. Upregulation of Sema4C has been detected in esophageal cancer, gastric cancer, and rectal cancer and correlated with lymphatic metastasis (11). Several studies have demonstrated that Sema4C is crucial in regulating EMT in tumors. For example, Sema4C induced EMT through suppressing E-cadherin and induction of vimentin in hepatocellular carcinoma (12), breast cancer (13, 14), cisplatin-resistant cervical cancer (15), and pacitaxel-resistant lung cancer (16, 17). Moreover, in our previous study, we have found that tumor-associated lymphatic endothelial cells promote lymphatic metastasis by highly expressing and secreting SEMA4C, through activating the RhoA pathway (18). Tumors are composed of both cancer cells and stromal cells that are recruited into or activated in the local microenvironment. Current researches have shown that the growth and dissemination of tumors are the results of dynamic interaction between stromal
Yang et al.
cells and cancer cells in the tumor microenvironment (19). Macrophages are one of the main infiltrating cell groups in cancer stroma, and accumulating evidence has suggested that macrophages recruited in tumors are unable to present antigens and promote the progression of tumors by releasing the growth and angiogenic factors (20, 21). Tumor angiogenesis is also an important component of cancer development, involving the multistep process of endothelial cell migration and tubular formation (22). Angiogenesis and macrophage recruitment usually share common pathways, and these two biological processes are closely related to cancer progression (23).

In this study, we analyzed Sema4C activity in tumor cell proliferation, and its potential effects on tumor angiogenesis and metastatic dissemination were also addressed. We showed that the expression of Sema4C in tumor cells accelerates tumor growth and angiogenesis, and it also boosts metastatic dissemination. Our data reveals that this activity of Sema4C is mediated by plexinB2-dependent inactivation of the p53 pathway, leading to a reduction in p21 growth-inhibitory protein and its potential effects on tumor angiogenesis and metastasis through the bloodstream.

### Materials and Methods

#### Cell lines

Cell lines (MDA-MB-231, MCF-7, MDA-MB-435s, T47D, MCF-10A, PC-3, A375, SKMEL-2, A431, A549, BEAS-2B, SKOV3, SW626, HeLa, Siha, PANCl, SW1990, HT29, and SW480) were purchased from ATCC in 2012 and were cultured for <1 month in our laboratory before being frozen in stock vials in liquid nitrogen. In 2013, we resuscitated the abovementioned 19 cancer cell lines, cultured them for <1 month, and obtained the results shown in Fig. 1A; then, we froze these cells in liquid nitrogen again except for MDA-MB-231. HUVEC (primary umbilical vein endothelial cells) and LEC (human lymphatic endothelial cells) were purchased from ATCC in 2013 and immediately cultured for the experiments. Before we started the in vivo experiments, the MDA-MB-231 cell line was tested and authenticated by detecting the short tandem repeat locus and sex gene amelogenin on November 1, 2013. The test report has been uploaded.

#### Patients

A total of 83 samples of breast cancer and 20 samples of adenofibroma were obtained from patients who were admitted to Tongji Hospital, Tongji Medical College, the Huazhong University of Science and Technology (Hubei, P. R. China). Average age of patients with breast cancer was 52.7 years. All patients signed written informed consent according to the Declaration of Helsinki.

#### Antibodies and reagents

Anti-VWF, anti-PCNA, anti-p100/52, anti-p105/50, anti-IKBα, anti-p-IKBα-s36, anti-p-IKBα-s32, anti-p65, anti-p53, anti-Rb, anti-pRb, anti-p16, anti-p-p53, anti-Cyclin D1, anti-Cyclin E, anti-Cyclin A2, anti-Cyclin B1, and anti-Cdc2 were purchased from Proteintech Group Inc. Anti-Cyclin B1, anti-Cdc2 were purchased from RayBiotech Inc. Anti-VWF antibodies were purchased from Abcam.

#### qRT-PCR

Total RNA was extracted from cells with TRizol (Invitrogen) according to the instructions of the manufacturer and 2 μg RNA was reverse transcribed with random primers. Power SYBR Green PCR reactions were performed three times for each sample and the ABI prism 7900HT sequence detection system was used for analysis.

### Xenograft tumor model

Four-week-old BALB/c nu/nu mice were purchased from the SLAC Laboratory Animal Co. Ltd. A total of 3.6 × 10^6 MDA-MB-231 tumor cells expressing shSEMA4C or EV were implanted into the mammary fat pad of BALB/c nu/nu mice. Tumor size was measured every third day with calipers. The volume (v) of the tumor was calculated by the formula v = (l × w^2)/2, where l is the length and w is the width of the tumors (24). Statistical methods of tumor weight and superficial lung metastasis were performed as described previously (24). Histologic analysis was performed on paraffin-embedded sections stained with hematoxylin and eosin. All animal procedures were approved by the Ethical Commission of the Huazhong University of Science and Technology (Hubei, P. R. China).

#### Tissue analysis

Four-µm-thick sections of primary human breast tumors and MDA-MB-231 xenograft tumors were incubated with the proper antibodies. Streptavidin and all second-fluorescence antibodies used were bought from The Jackson Laboratory. Cell nuclei were marked with DAPI (Invitrogen) and apoptotic cells were detected by TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) Assay Kit (Roche). Independent fields were quantified for every section, and different sections of every tumor were analyzed by a microscope (Leica) and quantified by ImageJ software.

### Monocyte migration assays

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycodenz) from heparinized blood of healthy volunteers. Monocytes were purchased from Epitomx. The NF-κB inhibitors, MG132 and parthenolide, were obtained from Sigma-Aldrich. A Dual Luciferase Reporter Gene Assay Kit was purchased from Promega. ELISA kits for testing ANG and CSF-1 were purchased from RayBiotech Inc. Anti-NG2 antibody was purchased from Abcam.
positively purified by a magnetically labeled anti-CD68 mAb (Miltenyi Biotec). Purity of the CD68⁺ cells was >94% and a Transwell System (8-μm pores; Costar) was used to test migration. In short, 2 × 10⁵ monocytes were placed on the top layer of a cell culture insert with permeable membrane and conditioned medium was placed below the cell permeable membrane. Following an incubation period (8 hours), the cells that have migrated through the membrane were stained with Giemsa and counted by ImageJ software.

In vitro permeability assay

In vitro permeability assay was performed as described previously (25). In our article, HUVECs and human pericytes were bought from Procell. In the product instructions, HUVECs were imported from ATCC and sold to us, and the pericytes were produced from human microvessel using the collagenase-neutral protease digestion, stainless steel mesh filtration, and density gradient centrifugation method. The total number of pericytes was about 5 × 10⁵ cells/bottle. The purity of cells was over 90%, and identified by alpha-SMA, NG2, and GFAP immunofluorescence, and it does not contain human immunodeficiency virus-1, HBV, HCV, Mycoplasma, bacteria, yeast, and fungi. The In vitro Vascular Permeability Assay Kit (catalog no. ECM644) was purchased from Millipore, and used it in accordance with instructions.

To construct coculture in vitro vascular permeability models, pericytes (1.5 × 10⁴ cells/cm²) were seeded on the bottom side of the collagen-coated polyester membrane of the Transwell inserts. The cells were let to adhere firmly overnight, then endothelial cells (7.5 × 10⁴ cells/cm²) were seeded on the top side of the inserts placed in the well of the 24-well culture plates containing no cells, pericytes, or astrocytes. The models were maintained in HUVECs culture medium and then treated with EV-conditioned media or shSEMA4C-conditioned media. After treatment, a high molecular weight FITC-Dextran is added on top of the cells, allowing the fluorescent molecules to pass through the endothelial cell monolayer/cocultures. The extent of permeability can be determined by measuring the fluorescence of the receiver plate well solution. The concentration of FITC-Dextran was analyzed by an EnVision fluorescence multiwell plate reader [Ex (l) 480 nm; Em (l) 535 nm, BD Biosciences]. The percentages of control were quantified.

In vitro angiogenesis assay

A total of 5 × 10⁴ cells of a given genotype were plated in 1 mL of RPMI1640 with 2% FCS for 2 days and conditioned media was...
collected. A total of $5 \times 10^4$ HUVECs were seeded in 100 µL of conditioned media on Matrigel per well (96-well dishes, Costar). Cells were incubated at 37°C in a tissue culture incubator for 4–6 hours and were inspected by an inverted light microscope at 4–10 × magnification. Angiogenesis was quantified with ImageJ by counting the number of junctions in capillaries in five random fields per replicate.

Growth curve assay
A total of $1 \times 10^4$ tumor cells were seeded per well in multiple 96-well dishes (Costar), resuspended cells, and then counted by Automated Cell Counter at 24, 48, 72, and 96 hours.

Proliferation index assay
Another way to detect cell proliferation is flow cytometry. CFSE (eBioscience No.65-0850) is widely used for cell tracking and proliferation studies, and the proliferation index was detected by flow cytometry, which was following the manual and experimental protocol.

Reporter assay
Luciferase activity was determined by dual-Glo Luciferase Assay Regent (Promega) and measured in a moonlight 2010 luminometer. Firefly Luciferase was normalized to constitutive Renilla luciferase.

Western blotting
Western blot assays were performed as described previously [18]. Finally, enhanced chemiluminescence (GE Healthcare) was used for final detection. Quantity One (Bio-Rad Laboratory) was used to measure the detected signals digitally.

Analysis of Oncomine data
To determine the coexpression pattern of SEMA4C and ANC/CSF-1 in breast cancer, we used the datasets in the Oncomine database (https://www.oncomine.org). Oncomine is a cancer microarray database and web-based data-mining platform aimed at facilitating discovery from genome-wide expression analyses. The analysis enables multiple comparisons of gene expression (DNA or RNA) between different studies and the significance of the gene expression across the available studies was also taken into account.

Statistical analysis
Statistical significance for parametric data was assessed by the Student t test. Fisher exact test was used to analyze clinicopathologic factors. $P < 0.05$ was considered to be statistically significant.

Results
SEMA4C is commonly expressed in human cancer cells and its elevated expression is associated with poor prognosis of patients
Previous studies have identified Sema4C as a presumed tumor promoter whose expression may be upregulated in the invasion and lymphatic metastasis of esophageal cancer, rectal cancer, and gastric cancer [11]. Interestingly, in screening of 17 human tumor-derived cell lines, evidently high levels of SEMA4C protein were found in cells originated from different types of tumors, including mammary carcinoma, prostate carcinoma, melanomas, lung cancer, cervical cancer, and gastrointestinal cancer (Fig. 1A). To further investigate the correlation between SEMA4C expression and tumor progression, gene expression in 1,402 clinically annotated breast cancer samples, 1,435 ovarian cancer samples, 1,926 lung cancer samples, and 876 gastric cancer samples were analyzed and we discovered a significant correlation between high levels of SEMA4C and poor prognosis (Fig. 1B, by http://kmplot.com). Furthermore, we analyzed the SEMA4C protein expression profile in breast cancers and fibroadenoma (Fig. 1C). Moreover, higher levels of SEMA4C seemed to be associated with tumor stage and metastatic progression in a subset of breast cancers (Table 1; $P = 0.004$; $P = 0.006$). These findings seemed to suggest that SEMA4C acts as a tumor promoter and prompted us to detect the functional role of SEMA4C in the progression and metastasis of tumors in vivo. Thus, we selected the MDA-MB-231 cell line, derived from human breast cancer, for further experiments, characterized by high SEMA4C expression, ER/PR/Erbb2 negativity, a fast growth rate, and high metastatic potential.

Sema4C/PlexinB2 signaling blockade in breast cancer cells leads to growth arrest and cell senescence through the p53 signaling pathway
To evaluate the function of Sema4C, we knocked down the gene of breast cancer cells. We transduced MDA-MB-231 cells with lentiviral vectors encoding either short hairpin RNA (shRNA) sequences targeting SEMA4C or a noncoding empty vector. Real-time PCR and Western blotting confirmed that shSEMA4C had efficiently suppressed the protein expression of endogenous SEMA4C in MDA-MB-231 cells compared with the empty vector (Fig. 2A and B). Cell counting assays demonstrated that knockdown of SEMA4C significantly inhibited MDA-MB-231 cellular growth ability (Fig. 2C). Cell IHC also indicated that...
MDA-MB-231 cells expressing shSEMA4C showed a >50% reduction in proliferation rate as assessed by anti-human PCNA staining (Fig. 2D), whereas apoptosis was not affected as tested by TUNEL assay (Supplementary Fig. S1). Further investigation about the reason for the decrease in proliferation of SEMA4C knockdown tumor cells is necessary. Flow cytometry affirmed that there was no change in the apoptosis rate in MDA-MB-231 cells transfected with siRNA sequences targeting SEMA4C or PLEXINB2 with respect to cells transfected with negative control miRNA (siNC), SEMA4C miRNA (siSEMA4C), or PLEXINB2 miRNA (siPLEXINB2) were cultured in 10% FBS for 96 hours, and apoptosis was determined after labeling cells with recombinant Annexin V-PE and propidium iodide (PI). Cells were transfected with siNC, siSEMA4C, or siPLEXINB2 for 96 hours, and the cell-cycle profile was detected using FACS analyses following PI staining. Cellular senescence was detected using a SA-β-gal staining assay, the SA-β-gal activity (arrowhead) was increased in cells transfected with siSEMA4C or siPLEXINB2. Notably, shSEMA4C-transfected cells did not go through any significant morphologic change compared with controls in the first 5 days of culture. However, in the following days, some gradually acquired a flattened and enlarged cell shape, thus implying a prosenescent nonapoptotic state. As shown in Fig. 2G, the senescent marker of SA-β-gal increased significantly in MDA-MB-231 cells transfected with either siSEMA4C or siPLEXINB2 compared with control cells. Cellular senescence is a stress response that leads to permanent withdrawal from the cell cycle and p53-p21Cip1 and phospho-Rb-p16INK4a are two major pathways that indicated in the induction of senescence. The altered expression of cell-cycle regulators, such as phospho-p53, observed in MDA-MB-231 cells transfected with either siSEMA4C or siPLEXINB2 suggests growth arrest as a result of cellular senescence. In contrast, the level of phospho-RbSer798 remained unchanged (Supplementary Fig. S3A). Therefore, the protein level of the cyclin-dependent kinase inhibitor p21Cip1, often expressed by senescent cells, was determined. The ratio of phospho-p21Cip1-Thr145 to total p21Cip1 increased in the MDA-MB-231 cells transfected with either siSEMA4C or siPLEXINB2, suggesting that knockdown of SEMA4C or PLEXINB2 induced activation of p53, followed by activation of p21Cip1 (Supplementary Fig. S3A). In addition, the level of Cdc2

Figure 2.
SEMA4C regulates tumor cell proliferation through the p53 signaling pathway. A and B, Real-time PCR and Western blot analysis of Sema4C and β-actin expression. The mRNA and protein levels of SEMA4C were downregulated by SEMAC shRNA (shSEMA4C) in MDA-MB-231 cells compared with a nondcoding empty vector (EV). C, MDA-MB-231 cells transduced to express shSEMA4C or a nondcoding EV were grown in a 10% FBS culture for 4 days. Cell growth was evaluated daily in separate dishes by a cell counting assay. Data are presented as the mean ± SD from three separate experiments; **P < 0.01. D, Cell IHC was used to analyze the proliferation rate as assessed by anti-human PCNA staining in MDA-MB-231 cells expressing shSEMA4C or EV; P < 0.05. E, Cells with no transduction (CON) and Cells transduced with siNC, SEMA4C miRNA (siSEMA4C), or PLEXINB2 miRNA (siPLEXINB2) were cultured in 10% FBS for 96 hours, and apoptosis was determined after labeling cells with recombinant Annexin V-PE and propidium iodide (PI). F, Cells were transfected with siNC, siSEMA4C, or siPLEXINB2 for 96 hours, and the cell-cycle profile was detected using FACS analyses following PI staining. G, Cellular senescence was detected using a SA-β-gal staining assay, the SA-β-gal activity (arrowhead) was increased in cells transfected with siSEMA4C or siPLEXINB2.
was significantly decreased in the MDA-MB-231 cells transfected with either siSEMA4C or siPLEXINB2, whereas the other cell-cycle-related proteins, including cyclin A, B, D, and E and histone H3, were unchanged. Cdc2, the cyclin-dependent kinase required to enter mitosis, can be inhibited simultaneously by three transcriptional targets of p53: Gadd45, p21Cip1, and 14-3-3s. As shown in Supplementary Fig. S3A, the expression of Gadd45 and 14-3-3s remained unchanged. And in the MDA-MB-231 cells overexpressing SEMA4C, the ratio of phospho-p53 to total p53 and phospho-p21Cip1–Thr145 to total p21Cip1 decreased, while cdc2 expression increased, but the overall effects were reversed when PLEXINB2 was knocked down (Supplementary Fig. S3B). Collectively, the presence of a high ratio of phospho-p21Cip1–Thr145 to total p21Cip1 and a significantly higher quality of phosphor-p53 together with the low level of cdc2 indicate that silenced SEMA4C or silenced PLEXINB2 induces irreversible growth arrest and cellular senescence in MDA-MB-231 cells by activation of the p53–p21Cip1 pathway.

Loss of SEMA4C decreases tumor growth and metastasis in vivo

In vivo, the tumorigenic potential of shSEMA4C-transduced cells was studied by subcutaneous transplantation in nude mice. Tumor volume was measured regularly, and tumor weight was obtained at the given time in the experiment. The in vivo stability of SEMA4C knockdown in MDA-MB-231 cells was determined by IHC assay of the SEMA4C protein. SEMA4C expression was consistently strong in EV control tumors and low or absent in microscopic, dormant shSEMA4C tumors (Fig. 3A and B). ShSEMA4C tumor xenografts displayed a significant reduction in tumor volume and weight (Fig. 3C and D), and also with a significant decrease of spontaneous metastatic dissemination in lung and liver (Fig. 3E and F). Their metastasis potential was quantified by scoring the micrometastasis and macrometastasis in the lung and liver. Collectively, these results indicate that the presence of Sema4C in the tumor contributes to tumor growth and metastasis.

SEMA4C regulates angiogenesis and macrophage recruitment

Because proliferation is important for the rapid growth of solid tumors, we analyzed tumors 15 days after transplant; as expected, shSEMA4C tumor cells showed a >60% reduction in proliferation rate (Fig. 4A), whereas apoptosis was only moderately increased (Fig. 4B).

As we all know, tumor growth is highly dependent on its vascular supply. The likelihood of reduced angiogenesis being another primary cause for delayed tumor growth was investigated. Slices of tumors grown either in EV or in shSEMA4C xenografts were stained with anti-vWF antibody, and it was found that the total vessel number was obviously lesser in shSEMA4C tumors (Fig. 4C). To further elucidate the effect of SEMA4C loss on tumor vascular system, a short-term in vitro experiment was used to measure angiogenesis. This assay assesses the ability of primary endothelial cells to form three-dimensional capillary-like structures, in response to appropriate clues. Conditioned media from MDA-MB-231 with shSEMA4C significantly decreased tubular formation compared with that from cells with empty vector and control cells (Fig. 5A). Similarly, conditioned medium from MDA-MB-435 expressing shSEMA4C also showed decreased...
tubular formation, compared with that from cells with empty vector. However, conditioned medium from MCF-7 overexpressing SEMA4C increased the tubular formation compared with that from cells with empty vector (Supplementary Fig. S4A–S4D). These results revealed that SEMA4C has an impact on angiogenesis in multiple cancer cell types.

Invading cancer cells could migrate through blood vessel walls directly and enter into the circulation (intravasation), which requires destroying endothelial connections. Therefore, we detected the vessel integrity. As shown in Supplementary Fig. S5A, the shSEMA4C tumor vasculature was completely encapsulated by parietal cells, which were identified by NG2 immunostaining. In comparison, a local smooth muscle cell envelope defect was observed around the vessels of EV tumors. To explore EC-pericyte interaction in vitro, a blood–brain barrier model was used to coculture HUVECs and the primary pericytes (25). In the tracer permeability assays, when monolayer HUVECs were plated on the Transwell membrane, there was no significant difference between shSEMA4C and EV-conditioned media, but when the HUVECs cocultured with pericytes the permeability reduced significantly in the EV-conditioned media compared to shSEMA4C-conditioned media (Supplementary Fig. S5B). Therefore,

Figure 4.
Tumor cell proliferation, angiogenesis, and macrophage recruitment in SEMA4C knockdown tumors. A, Control or shSEMA4C-expressing tumors (15 days after transplant) were immunostained to reveal PCNA proliferation markers (red), whereas nuclei were visualized by DAPI (blue). Representative fields are shown. The bar graph shows the percentage of PCNA-positive cells (mean ± SD) in five tumors per experimental group. For all of the quantifications shown in this figure, at least three sections and 10 fields were counted for each tumor. *, *P < 0.05. B, Tumor sections were analyzed to reveal apoptotic cells using a TUNEL green kit. Micrographs showing representative fields. The bar graph indicates the percentage of TUNEL-positive cells (mean ± SD) in five tumors per experimental group. C, Representative pictures of the peritumoral vasculature. As shown, the degree of vascularization was significantly lower in mice carrying shSEMA4C tumors. Micrographs showing sections from tumors stained with vwf (red) to reveal endothelial cells. The graphs indicate the percent area occupied by vessels or the vessel number calculated in five fields per section. D, Sections from tumors excised at day 15 after transplant were immunostained with an anti-F4/80 antibody to selectively detect macrophages (green), whereas nuclei were revealed with DAPI (blue). Macrophages were quantified in the periphery and center of the tumors. Values represent the mean ± SD from five tumors per experimental group (*, *P < 0.05; **, *P < 0.01).
Figure 5. Effect of SEMA4C on the tubular formation of endothelial cells and migration of macrophages. A, Representative photographs (left) and mean numbers of tube-forming structures (right) at 10× magnification. HUVECs (1 x 10⁴) were incubated in conditioned medium for 4–6 hours. Tubular formation of HUVECs was significantly reduced by conditioned medium from SEMA4C-knockdown MD-MB-231 cells compared with EV transfection. B, Representative photographs (left) and mean numbers of migrating monocytes (right) purified from human peripheral blood analyzed via a Transwell migration assay. Conditioned medium derived from tumor cells transduced with shSEMA4C or EV was included in the bottom chamber. Migrated monocytes were stained with Giemsa and counted in low magnification micrographs of the filters. C, Angiogenesis cytokine antibody arrays were carried out with conditioned medium from either MDA-MB-231 cells expressing EV or cells stably expressing SEMA4C shRNA. Real-time PCR (D) and ELISA (E) were performed to validate the mRNA and protein expression levels of ANG and CSF-1. Values in the graph represent the mean ± SD of three independent experiments (*, P < 0.05; **, P < 0.01).
these results suggested that the microenvironment of SEMA4C-expressing tumors result in poor contact of EC-mural cells, which, in turn, might lead to a decline in mechanical stability, and make it easier for tumors to cross the blood vessel barrier, thus leading to tumor metastasis.

Several important factors that regulate angiogenesis and metastasis of tumors were released by tumor-associated macrophages. (26–28). Interestingly, we detected a 2-fold reduction in macrophage infiltration into the periphery and the center of the shSEMA4C xenografts by staining the frozen sections with the selective antibody F4/80 for macrophages (Fig. 4D). In contrast, tumor-infiltrating granulocytes did not decrease when lacking SEMA4C in vivo based on cd45 immunostaining (Supplementary Fig. S6). These observations revealed that the expression of SEMA4C in tumor cells enhanced their ability to recruit monocytes/macrophages. To further study this effect, we performed in vitro migration assays with purified human peripheral blood mononuclear cells. As shown in Fig. 5B, the conditioned medium of MDA-MB-231 with shSEMA4C attracted fewer macrophages compared with the medium collected from empty vector–transduced control cells. Similar results were observed in conditioned medium from MDA-MB-435–expressing shSEMA4C, while conditioned medium from MCF-7–overexpressing SEMA4C increased macrophage migration compared with the medium collected from empty vector–transduced control cells (Supplementary Fig. S7A and S7B).

Of note, the analyzed sections derived from tumors of the same age, which means that masses of extremely different size could have been compared, and tumors of small size do not contact as many vessels as the exponentially growing tumors. To eliminate any potential confusion, we detected the tumors at the same size and ultimately found that the total vessel number and macrophage counts were obviously less in shSEMA4C tumors (Supplementary Fig. S8).

Intriguingly, although Sema4C is a transmembrane protein, its extracellular domain could be shed in the extracellular space (18). Thus, it is essential to test whether the purified Sema4C ectodomain can elicit the same functional effects on tumor cell proliferation, endothelial cells, and monocytes as seen with conditioned medium. As shown in Supplementary Fig. S9, recombinant Sema4C protein did not affect MDA-MB-231 proliferation and endothelial cells tubular formation despite the decrease in parthenolide-treated cells at 10 minutes (Fig. 6B). These results indicated that both pathways are involved in regulating the expression of ANG and CSF-1. We subsequently examined whether SEMA4C mediated the angiogenic phenotype through both pathways or not. Knockdown of SEMA4C or PLEXINB2, which is the high-affinity receptor of SEMA4C, compared with control cells.

NF-KB signaling can be divided into a “classical” pathway and an “alternative” NF-KB pathway, with IkB kinase b phosphorylates IkB-a and IKBa phosphorylates the p100 precursor of the NF-KB p52 subunit, respectively. Both of the two signaling activities lead to the accumulation of heterodimeric NF-KB transcription factors in the nucleus. The classical pathway mainly regulates p50/p65 and p50/p52 Rel dimers, while the alternative pathway mainly regulates p52/relB heterodimer. The proteasome inhibitor MG132 could reduce the processing of p100 to p52 (32) and parthenolide, a sesquiterpene lactone that can inhibit RelA (p65) activation by preventing the degradation of IkB-a and nuclear translocation of RelA, but does not prevent p100 processing (33). To map out which pathway plays an important role in regulating the secretion of ANG and CSF-1, we treated MDA-MB-231 cells with the two inhibitors. Compared with nontreated cells, the mRNA levels of ANG and CSF-1 were reduced to approximately 50% in the cells treated with MG132 for either 30 minutes or 3 hours, while they were reduced close to 100% in parthenolide-treated cells at 10 minutes (Fig. 6B). These results indicated that both pathways are involved in regulating the expression of ANG and CSF-1. We subsequently examined whether SEMA4C mediated the angiogenic phenotype through both pathways or not. Knockdown of either SEMA4C or PLEXINB2 decreased the nuclear translocation of p65 (RELA) and p50, as assessed by confocal microscopy (Fig. 6C), while not modulating the nuclear translocation of p52. The Western blot results identified that nuclear P65 and p50 and cytoplasmic p105 and IkB-a (p32 and p36) were decreased in MDA-MB-231 cells with siSEMA4C or siPLEXINB2, while nuclear p52 and cytoplasmic p100 showed no change (Fig. 6D). Nuclear P65 and p50 and cytoplasmic p105 and IkB-a (p32 and p36) increased in MDA-MB-231 cells overexpressing SEMA4C, but the effect diminished when PLEXINB2 was knocked down (Fig. 6E). These results indicated that SEMA4C induced the production of ANG and CSF-1 in MDA-MB-231 cells by activating the canonical NF-KB pathway in a plexinB2-dependent manner.

Finally, we wanted to know the impact of ANG or CSF-1 removal in SEMA4C-driven functional effects. As shown in Fig. 7A–D, using ANG or CSF-1 siRNA in the overexpressing Sema4C regulates ANG and CSF-1 through the NF-KB signaling pathway

Because ANG and CSF-1 are already known to be NF-KB targets (30, 31), we performed a dual-huciferase reporter assay using the NF-KB response element to confirm regulation of NF-KB signaling by SEMA4C. As shown in Fig. 6A, the NF-KB activity was diminished in the cells with silenced SEMA4C, as well as silenced PLEXINB2, which is the high-affinity receptor of SEMA4C, compared with control cells.

SEMA4C regulates secretion of proangiogenic factors

We attempted to make sure whether certain secretory factors regulated by SEMA4C have effects on tumor vasculature. Hence, we probed human angiogenesis cytokine antibody arrays with conditioned medium from control MDA-MB-231 cells and cells expressing SEMA4C shRNA (Fig. 5C). In consistent with the experimental phenomena, we discovered that SEMA4C does indeed regulate two angiogenic factors, namely, ANG and macrophage CSF-1. It is known that ANG is a potent angiogenic factor that binds to endothelial cells, inducing the formation of blood vessels (29). CSF-1, originally identified as a hematopoietic growth factor, stimulates the proliferation, differentiation, and survival of monocytes and macrophages (27). To validate the arrays results, real-time PCR and ELISA were performed. As shown in Fig. 5D and E, the mRNA and secreted protein levels of ANG and CSF-1 were significantly decreased in the MD-MB-231 cells expressing SEMA4C knockdown compared with the control cells with an empty vector. In addition, the secretion of ANG and CSF-1 recovered after the Sema4C cDNA was reexpressed in these siSEMA4C cells (Supplementary Fig. S10). Moreover, the mRNA and secreted protein levels of ANG and CSF-1 were significantly increased in the MCF-7 cells overexpressing SEMA4C compared with the control cells with empty vector (Supplementary Fig. S11). These results suggested that secretion of ANG and CSF-1 was regulated by the level of SEMA4C expression.
SEMA4C regulates ANG and CSF-1 through the NF-κB signaling pathway. A, Reporter assays with NF-κB sites. One day after transfection with miRNA, cells were transduced with reporter plasmids, and luciferase activity was determined after 48 hours. B, The mRNA levels of ANG and CSF-1 were detected in MDA-MB-231 cells that were treated with MG132 for 30 minutes and 3 hours or parthenolide for 10 minutes. C, Confocal microscopy. SEMA4C or PLEXINB2 knockdown reduced IkB-α degradation and nuclear translocation of RELA (P65) and p50 (both in green), but not p52 (red). Nuclei were revealed with DAPI (blue). D, SEMA4C or PLEXINB2 knockdown reduced the degradation of the inhibitory subunit IkB-α over a time course consistent with canonical NF-κB pathway inactivation. Representative Western blot analysis of three independent experiments. E, Canonical NF-κB pathway activation was increased in MDA-MB-231 cells overexpressing SEMA4C, but the effect was diminished when PLEXINB2 was knocked down. Representative pictures of three independent experiments (*, P < 0.05; **, P < 0.01).
Some extracted text is as follows:

**SEMA4C MCF-7 cells, conditioned medium from MCF-7 overexpressing SEMA4C increased tubular formation and macrophage migration compared with the medium collected from empty vector–transduced control cells, and knockdown of ANG or CSF-1 eliminated the effect in SEMA4C-driven cells. Moreover, we found a positive correlation between Sema4C and ANG or CSF-1 levels in large datasets of breast cancer cell lines (34), such as Oncomine (Fig. 7E).**

**Discussion**

Semaphorins, in addition to the function of signal elements in nerve guidance, are also related to tumorigenesis. Sema4A is a membrane-anchored semaphorin that uses the plexinD1 receptor for signaling and functions as an inhibitor in angiogenesis development. Sema4F was found to be downregulated in a panel of human neurofibromas, implying that it functions as an inhibitor of tumor progression. The study of the SEMA4C
Yang et al.

In this study, we confirmed that SEMA4C was ubiquitously expressed in many kinds of human cancer cell lines and found out that the expression of SEMA4C was significantly correlated with TN stages and lymphatic invasion in breast cancer cells. By analyzing public datasets, we observed a significant correlation between Sema4C elevated levels and poor breast cancer outcomes, which deserves further study. Next, we demonstrate that Sema4C/PlexinB2 signal activity is a special requirement for breast cancer cell proliferation by inhibiting p53 signaling. Blocking Sema4C/PlexinB2 signaling resulted in G2–M-phase arrest, tumor-suppressor genes upregulation, and cell senescence. Notably, the overexpression of SEMA4C could double suppress the p-p53 activation, and the knock-down of PLEXINB2 could reverse these effects. Thus, our data indicated that decreased p-p53 activity is crucial for cancer cell survival and proliferation, and Sema4C/PlexinB2 signal pathway activity is an essential mechanism for maintaining this function in breast cancer cells. The same phenomenon was also detected recently by Gurrapu and colleagues (13), which supports our findings.

These consistent findings inspired us to discover the functional role of SEMA4C in the progression of cancer in vivo, particularly as prior studies had not explored its undiscovered mechanism in tumor invasion and metastasis (11). Interestingly, it was observed that knockdown of SEMA4C in MDA-MB-231 cells evidently weakened the xenograft tumor growth and reduced tumor cell proliferation of macrophages and angiogenesis. Consistent with the data of clinical samples and in vitro experiments, knockdown of SEMA4C in MDA-MB-231 cells effectively reduced the proliferation of cancer cells, migration of macrophages, and tubular formation of HUVECs. Both the in vivo and in vitro studies powerfully supported our hypothesis that SEMA4C accelerate tumor growth not only by strengthening the proliferative capacity of cancer cells but also by macrophage recruitment and angiogenesis.

What are the macrophage recruitment and angiogenesis factors induced by Sema4C in cancer cells? Our current experimental data provided evidence that the release of ANG and CSF-1 declined in tumors with decreased SEMA4C and that these factors, other than the soluble purified Sema4C ectodomain, were important for both macrophage chemotaxis and local angiogenesis, despite the observation that soluble purified Sema4C could attract macrophage, which, in part, promote tumor progression. ANG, a 14 kDa basic heparin-binding protein, is one of the most effective angiogenic factors in various experimental models of angiogenesis (29).

It is clear that malignant tumors require it during development, as it has been shown that the initiation of revascularization in dormant lesions allows them to progress. In breast cancer, the increase of ANG expression has been related to the transition from normal to invasive breast carcinoma (35). Independent of hypoxia-inducible factor 1 and VEGF, the importance of ANG as an effective regulator of tumor angiogenesis has been emphasized in recent years (30). In the current literature, Yu and colleagues (36) showed that ANG can act as a ligand for PlexinB2 and also regulate cancer cell viability and tumor progression. However, in our study, knocking down ANG did decrease cell growth but did not affect cell cycle (Supplementary Fig. S12). CSF-1, which induces macrophage infiltration and activation in the tumor, is highly expressed and positively correlated with grade and progression in breast tumors (27, 37). Moreover, recruited macrophages can promote angiogenesis by producing angiogenic growth factors and proteinases, such as VEGF, MNP-9, and uPA (38), and can recruit and make cross-talk with other cells in the tumor microenvironment (39). Alternatively, they also have other functions, such as stimulation of tumor cell migration, invasion, and intravasation into the circulation, and ultimately leading to tumor progression (26, 40). Together, these findings indicated that ANG and CSF-1 not only have the ability to construct a vasculature with an increase in vascular density but also form escape pathways for tumor cells to enter circulation. And this is why the depletion of SEMA4C in the mouse model can significantly inhibit metastasis.

Semaphorins elicit intracellular responses through their receptors, which include neuropilins and plexins. Class 4 semaphorins are thought to be ligands for plexin-B receptors, and recent studies have demonstrated that PlexinB2 is the high-affinity receptor for Sema4C (41). The mechanisms by which SEMA4C restricts the release of ANG and CSF-1 remain to be determined. It has been reported that ANG can regulate angiogenesis and CSF-1 can regulate macrophage recruitment, and they are both regulated by NF-κB. The NF-κB family consists of five members, including NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel. The activation of NF-κB signaling induces the accumulation of the heterodimeric NF-κB transcription factors in the nucleus, with the classical pathway regulating mainly p50/p65 and p50/c-Rel dimers and the alternative pathway regulating p52/relB dimers. Moreover, we demonstrated that the classical NF-κB pathway was required for enhanced angiogenesis induced by Sema4C expression, which, in turn, regulates ANG and CSF-1. Thus, inhibiting ANG and CSF-1 may be a potential way to treat tumors that have developed resistance to anti-VEGF therapies. Our findings demonstrated that SEMA4C-PLEXINB2 signaling blockade led to impaired tumor growth by disrupting the blood supply and macrophage recruitment through NF-κB signal down-regulation, suggesting that this pathway may play a pivotal role in human breast cancer.

In summary, cancer cells acquire a number of characteristic alterations during the course of transformation, including the capacity to proliferate autonomously, to elicit angiogenesis, to invade surrounding tissues, and to metastasize to distant sites. Here, we confirmed that SEMA4C plays an important role in cancer cell proliferation and induces ANG and CSF-1 expression in tumor cells, thus supporting a new way of this semaphorin in promoting tumor progression, thereby suggesting that targeting SEMA4C could be of value in the treatment of human breast cancer. Further studies of the mechanism of SEMA4C will be crucial to gain new insights into which tumors can benefit clinically from antiproliferation and angiogenic therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References cited in this manuscript are available in the online version of this article.
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Acknowledgments
We thank Rajat Upadhyay for editing the English version of this article. The authors also thank the support of the Technical Innovation Special Project of Hubei Province (2018ACA138). This work was financially supported by a grant from the National Science Foundation of China (81802896 and 81771544). and National Science and Technology Major Sub-Project (2018ZX10301402-002).

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Received September 1, 2018; revised January 4, 2019; accepted July 10, 2019; published first July 15, 2019.

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www.aacrjournals.org Mol Cancer Res; 17(10) October 2019

Semaphorin 4C Promotes Breast Cancer Progression

Published OnlineFirst July 15, 2019; DOI: 10.1158/1541-7786.MCR-18-0933

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Semaphorin 4C Promotes Macrophage Recruitment and Angiogenesis in Breast Cancer

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doi:10.1158/1541-7786.MCR-18-0933

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