Published in final edited form as:

Biochem Biophys Res Commun. 2010 May 28; 396(2): 571–577. doi:10.1016/j.bbrc.2010.04.152.

Slit-Robo signaling mediates lymphangiogenesis and promotes tumor lymphatic metastasis

Xiao-Mei Yang^a, Hai-Xiong Han^a, Fei Sui^a, Yu-Min Dai^a, Ming Chen^a, and Jian-Guo Geng^{b,*}

^a Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, The Graduate School of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China

^b Vascular Biology Center, Divisions of Hematology, Oncology and Transplantation and Cardiology, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455, USA

Abstract

The Slit family of guidance cues binds to Roundabout (Robo) receptors to modulate neuronal, leukocytic and endothelial migration. Slit-Robo signaling had been reported to function as chemoattractive signal for vascular endothelial cells during angiogenesis. In this study, we found that Robo1 was expressed in lymphatic endothelial cells to mediate the migration and tube formation of these cells upon Slit2 stimulation, which were specifically inhibited by the function-blocking antibody R5 to Slit2/Robo1 interaction. To further explore the lymphangiogenic effect and significance mediated by Slit-Robo signaling, we intercrossed Slit2 transgenic mice with a non-metastatic RIP1-Tag2 mouse tumor model, and found that transgenic overexpression of Slit2 significantly enhanced tumor lymphangiogenesis and subsequently promoted mesenteric lymph node metastasis of pancreatic islet tumors. Taken together, our findings reveal that through interacting with Robo1, Slit2 is a novel and potent lymphangiogenic factor and contributes to tumor lymphatic metastasis.

Keywords

Slit; Robo; Lymphangiogenesis; Tumor metastasis; β -cell carcinoma

Introduction

Metastasis is the major cause of death associated with solid tumors [1], and lymphatic metastasis is the major route for cancer cell dissemination [2]. A common pattern for carcinomas is that regional lymph nodes are the first sites to develop metastases, either draining via pre-existing afferent lymphatic vessels and/or via newly formed lymphatic capillaries. The lymphatic vascular system has multiple functions in normal physiology including regulation of interstitial pressure, lipid absorption and inflammation [3-5]. Recent researches have indicated that excessive lymphangiogenesis correlates with lymphatic metastasis, and have

© 2010 Elsevier Inc. All rights reserved.

* Corresponding author. Address: MMC 480, 420 Delaware Street S.E. Minneapolis, MN 55455. USA. Tel: 86-21-5492-1352. Fax: 86-21-5492-1435. genglab@gmail.com (J.-G. Geng).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

identified growth factors including VEGF-C, VEGF-D and PDGF-BB as drivers of lymphangiogenesis [6-9].

The Slit family of guidance cues interacts with the Roundabout (Robo) family of transmembrane receptors in physiological and pathological processes requiring cell migration. Mammals have 3 Slit proteins (Slit1-3) and 4 Robos (Robo1-4) [10]. Slit2 interacts with Robo1 to mediate repulsive cues in axon guidance [11], neuronal migration [12] and leukocyte chemotaxis [13]. Also, Slit2 was widely expressed in various human cancers, and the interaction of Slit2 with Robo1 could induce tumor angiogenesis [14]. But whether Slit2 is involved in tumor metastasis remains to be answered. In this study, we found that Robo1 was expressed in human primary lymphatic endothelial cells (hLECs) and Slit2-Robo1 signaling promotes in vitro lymphangiogenic activity of cultured hLECs. Furthermore, we generated Slit2 transgenic mouse lines and intercrossed these mice with a well-characterized transgenic model of non-metastatic β -cell carcinogenesis (RIP1-Tag2), to examine the role of Slit2 in endogenous tumor metastasis. Our findings demonstrated that elevated expression of Slit2 in RIP1-Tag2 transgenic mice enhanced tumor lymphangiogenesis and increased regional lymph node metastasis. These data indicate that Slit2 is a regulator of adult lymphangiogenesis and reveal a causal role for Slit2-mediated lymphangiogenesis in the dissemination of tumor cells.

Materials and methods

Cell and recombinant proteins

The human lymphatic microvascular endothelial cells (Lonza, walkersville, MD) were cultured on gelatin-coated plastic in endothelial basal medium (EBM) supplemented with EGM-2 MV SingleQuots (Cambrex-Lonza). Growth factor VEGF-C was purchased from GenWay, San Diego, CA. The pVL1393-hSlit2.1-His plasmid was constructed by cloning the cDNA encoding the NH₂-terminal fragment of human Slit2 (1-267 amino acids) into a pVL1393 expression vector. The pVL1393-hRobo1-Fc plasmid was constructed by cloning the NH₂-terminal fragment of human Robo1 (1-820 amino acids) and human IgG Fc fragment into pVL1393. The constructs were verified by DNA sequencing. Recombinant Slit2 and Robo1-Fc were expressed in *Sf9* insect cells using the baculovirus expression system (BaculoGold®, Pharmingen) and purified by nickel affinity chromatography (GE Healthcare Biosciences) or Protein A affinity chromatography (Amersham).

RT-PCR analysis

Total RNA of cells was extracted with Absolutely RNA kit (Stratagene) and reverse transcribed by Reverse Transcription System (Invitrogen). Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed by the use of SYBR Green PCR master mix (Superarray). Primer pairs were purchased from Superarray. Cycle threshold values from triplicate assays were collected to calculate fold expression.

Immunohistochemistry and immunofluorescence

Tumor-bearing mouse was sacrificed and the pancreas was excised and fixed overnight in Bouin's solution or 4% paraformaldehyde. Samples were then processed, embedded in paraffin or OCT compound and sectioned at 6 μ m. Antibodies against Robo1 (10 μ g/ml), LYVE-1 (Angiobio; 1:100 dilution), and T-antigen (Research diagnostics, 1:50 dilution) were used for immunohistochemical or immunofluorescent staining. For immunohistochemical staining, primary antibody reaction products were visualized with the respective peroxidase-conjugated secondary antibody (Jackson Immunoresearch; 1:100 dilution) and DAB substrate (Pierce). For immunofluorescent staining, primary antibody reaction products were visualized with the appropriate FITC- or TRITC-conjugated secondary antibody (Jackson Immunoresearch; 1:500 dilution) and images were captured on Leica TCS SP2 confocal laser scanning microscope.

Tube formation assay

Tube formation assay was conducted as described in [14]. 96-well cell culture plates were coated with 50 μ l growth factor reduced Matrigel (BD Biosciences) for each well and incubated at 37°C for 30 min to promote gelling. Cells were suspended at a concentration of 1.3×10^5 cell/ml in DMEM medium supplemented with 2% fetal calf serum (FCS). Aliquots of cells (0.1 ml per aliquot) were added to each Matrigel-containing well and incubated at 37°C for 4-6 hr. Then the tubular structures were identified and photographed. The inhibition experiments of R5, a function-blocking mouse IgG_{2b} to Slit2/Robo1 interaction [14], were carried out as above.

Boyden chamber assay

Cells were starved in EBM-2 medium containing 2% heat inactivated FCS for 3 hr. The cell migration assay was conducted in a 48-well micro-chemotaxis chamber (Neuro Probe, Inc.). PVP-free polycarbonate membranes (8 μ m pore) were coated with 1% gelatin. The bottom chambers were loaded with or without testing factors, while the upper chambers were seeded with hLECs (5×10^5 cells/ml), suspended in EBM-2 supplemented with 2% FCS. For antibody inhibition experiments, hLECs were pretreated with the indicated amounts of mouse IgG_{2b} control or R5 at 37°C for 30 min, prior to adding to the upper chambers. They were incubated at 37°C for 4 hrs. The filters were then fixed, stained with 0.5% crystal violet, and the cells that had migrated through the filters were counted.

Generation of Slit2 transgenic and Slit2;RIP1-Tag2 double transgenic mice

Slit2 transgenic mice were generated according to standard procedures. The transgene was constructed by cloning the full-length human Slit2 cDNA into the pCEP4F vector, which contains the CMV promoter, and was injected into the pronuclei of fertilized C57 $\times$ CBA F1 oocytes. Genotypes were confirmed by dot blotting and Southern blotting. PCR screening of Slit2 heterozygotes was performed using the following primers: Slit2 FW, 5'-CCCTCCGGATCCTTTACCTGTCAAGGTCCT-3'; Slit2 RV, 5'-TGGAGAGAGCTCACAGAACAAGCCACTGTA-3'. Transgenic founders were maintained by breeding with C57 mice. RIP1-Tag2 mice were purchased from NCI (National Cancer Institute, USA), and phenotypic characterization of RIP1-Tag2 mice was as described previously [15]. The Institutional Animal Care and Use Committee of the Chinese Academy of Sciences approved all experiments. All mice were euthanized after their experimental periods.

Immunoblotting

Each pancreas was excised and lysed in radioimmunoprecipitation buffer containing protease inhibitors, followed by homogenization and clearing by centrifugation. S1 (an IgG_{2a} mAb to the NH₂-terminal portion of human Slit2) was used as the primary antibody for detection of Slit2 expression. Horseradish peroxidase-conjugated anti-mouse IgG was used as the secondary antibody and the protein bands were detected using the electrochemiluminescence system.

Determination of lymphangiogenesis

The total pancreatic or tumoral lymphatic length of each pancreas was photographed and measured from 200 $\times$ magnification fields of LYVE-1 staining sections, and the statistic data were from at least five fields per pancreas and four animals per group.

Statistical analyses

Statistical analyses for most of the experiments were performed using a standard two-tailed Student's *t*-test. *P*-values below 0.05 (*) or 0.01 (**) were considered as statistically significant or highly significant, respectively. Survival curves were analyzed using the Kaplan-Meier method, with groups compared by respective median survival. Two-tailed *P* values were calculated using log-rank test. Incidences of mesenteric lymph node metastasis and intestinal metastasis between two groups were analyzed by Chi-square test.

Results

Robo1 is expressed in primary lymphatic endothelial cells

Robo1 was observed to express in vascular endothelial cells; but its expression profiles in lymphatic endothelial cells are unknown. We examined this by semiquantitative real-time RT-PCR analysis at first. As shown in Fig. 1A and 1B, Robo1 was expressed in primary hLECs at a comparable level with that in human umbilical vein endothelial cells (HUVECs). Using immunofluorescence method, we found Robo1 was predominantly expressed in the cytoplasm and membrane of hLECs, partially co-localized with the lymphatic marker LYVE-1 but in contrary to the nucleus localization of homeobox gene Prox-1 (Fig. 1C).

Slit2-Robo1 signaling induces lymphangiogenic activity of hLECs in vitro

Slit2 is known to modulate cell motility through binding to Robo1, which is conserved among various cell types, including neuronal cells, leukocyte and vascular endothelial cells. So it is interesting to explore whether Slit2 regulates the motility of lymphatic endothelial cells, regarding the expression of Robo1 on the surface of hLECs. Using the baculovirus expression system, we expressed recombinant human Slit2 and human soluble Robo1-Fc (hRobo1-Fc) as described in Methods. The proteins were verified by SDS-PAGE staining and immunoblotting analysis with respective antibodies (Fig. 2A). To determine whether recombinant Slit2 can recognize Robo1, an enzyme-linked immunosorbent assay (ELISA) was developed in which recombinant Slit2 protein was coated onto plates and bound hRobo1-Fc was detected with HRP-conjugated anti-human IgG antibody. Our results confirmed the successful interaction of recombinant Slit2 with Robo1 (Fig. 2B).

Utilizing the recombinant Slit2 protein, we then examined whether Slit2 could induce the differentiation, specifically tube formation of primary lymphatic endothelial cells. VEGF-C, a growth factor known to promote lymphangiogenesis, was used as a positive control. Recombinant Slit2 protein increased the generation of tubular networks in a dose-dependent manner (Fig. 2D). And pre-incubation of hLECs with R5 (a monoclonal antibody specifically binds to the first immunoglobulin domain of Robo1 and blocks Slit2-Robo1 interaction, ref. [14]) but not control IgG_{2b} neutralized the effect of Slit2, but not of VEGF-C, resulting in fewer and shorter tube structures (Fig. 2C and 2D). The Boyden chamber assay was performed to test whether Slit2 had chemotactic effect on the migration of hLECs. We found that recombinant Slit2 protein attracted the migration of hLECs dose-dependently (Fig. 2E). Pre-incubation of hLECs with R5, but not with an IgG_{2b} control, significantly neutralized Slit2-induced migration but not VEGF-C-induced migration (Fig. 2F). We also examined whether Slit2 is able to induce proliferation of hLECs. However, no detectable activity was found (data not shown). These results demonstrate that Slit2 is a potent lymphangiogenic factor, promoting lymphangiogenic activity through interaction with Robo1.

Transgenic overexpression of Slit2 enhances tumor lymphangiogenesis of β -cell carcinomas

Transgenic mouse lines overexpressing human full-length Slit2 under the control of the CMV promoter were generated (Fig. 3A), which efficiently expressed the FLAG-tagged hSlit2 transgene. Three transgenic mouse lines with C57BL/6 background were established that exhibited stable transmission of the transgene to their progeny. One of the three lines that exhibited higher levels of Slit2 in the pancreas was extensively analyzed and referred as Slit2 transgenic mice (Slit2 Tg, Fig. 3B and Supplementary Fig. 1). Notably, Slit2 transgenic mice were viable, fertile and did not exhibit detectable changes in islet development. Analysis of pancreatic lymphatic vessel profiles by LYVE-1 immunoreactivity showed no obvious aberrance in the disposition of lymphatics in the pancreas which co-existed with the bile ducts and blood vessels mostly, but revealed significantly increased total length of LYVE-1-positive lymphatics in pancreas of Slit2 transgenic mice compared with wild-type littermates (Fig. 3C), supporting a pro-lymphangiogenic activity of Slit2 in vivo.

To investigate whether Slit2 promotes lymphangiogenesis or metastasis of endogenous tumors, Slit2 transgenic mice were crossed with RIP1-Tag2 transgenic mice, a well-characterized transgenic model of non-metastatic β -cell carcinogenesis that displays morphological features typical of human pancreatic β -cell tumors. Of note, β -cell tumors that develop in RIP1-Tag2 mice are capable of local invasion, but do not induce extensive lymphangiogenesis [16]. The resultant Slit2;RIP1-Tag2 double transgenic mice displayed increased levels of Slit2 expression in the pancreas as determined by immunoblotting (Fig. 3B and Supplementary Fig. 1). Examination of lymphatics by LYVE-1 immunohistochemical staining revealed aggregates of tumor cells within the lumen of lymphatic vessels in Slit2;RIP1-Tag2 mice at 14 weeks of age, which is the late stage of this islet carcinoma (Fig. 3D). These aggregates of tumor cells were detected in lymphatic vessels both inside (Fig. 3D, left) and around (Fig. 3D, right) the primary tumor in Slit2;RIP1-Tag2 mice, which were not observed in RIP1-Tag2 mice. Using a mouse anti-Robo1 antibody and a rabbit anti-LYVE-1 antibody in the immunofluorescent detection, we observed that Robo1 was expressed in LYVE-1-positive tumor lymphatics (Fig. 3E). Robo1-positive intra-tumoral blood vessels were concurrently detected (Fig. 3E). Statistical analysis of LYVE-1 staining revealed increased tumor lymphangiogenesis in Slit2;RIP1-Tag2 mice compared with RIP1-Tag2 mice at 14 weeks of age (Fig. 3F). And while lymphatics in RIP1-Tag2 mice hardly penetrate into the tumor, in Slit2;RIP1-Tag2 mice lymphatics were found to extend into the tumor body significantly (Fig. 3D and 3F). These results demonstrate that overexpression of Slit2 enhances tumor lymphangiogenesis of islet β -cell carcinomas in vivo.

Overexpression of Slit2 facilitates lymphatic metastasis of β -cell carcinomas

Compared with RIP1-Tag2 mice, Slit2;RIP1-Tag2 mice displayed almost the same tumor burden and tumor number at 14 weeks of age (Fig. 4A). Interestingly, obvious regional mesenteric lymph node metastases and intestinal metastases were detected in 14-week-old Slit2;RIP1-Tag2 mice (Fig. 4B-a). Analysis of the metastasis by hematoxylin and eosin (H&E) staining (Fig. 4B-b and 4B-c) or SV40 T-antigen immunohistochemical staining (Supplementary Fig. 2) revealed that these islet β -cells form metastases under the epithelium of the intestine mucous membrane as well as in the mesenteric lymph nodes. Metastatic rates in RIP1-Tag2 and Slit2;RIP1-Tag2 mice were analyzed with dozens of 14-week-old mice (Table 1). Although RIP1-Tag2 mice had been reported to be a non-metastatic model of pancreas insulinoma [17], we found a few exceptions of lymph node metastasis similar to those in Slit2;RIP1-Tag2 mice. Nevertheless, the rate of mesenteric lymph node metastasis was significantly higher and the rate of intestine metastasis was also much higher in Slit2;RIP1-Tag2 mice than in RIP1-Tag2 mice (Table 1). No overt metastases were observed in distant lymph nodes or other organs in either group of mice. Consequent analysis of the overall survival

revealed a significantly lower survival rate in Slit2;RIP1-Tag2 mice (Fig. 4C). As RIP1-Tag2 mice typically succumb to a combination of tumor burden and hyperinsulinemia, blood insulin levels in both mice were detected by ELISA analysis and demonstrated no obvious difference (Fig. 4D). These results indicate that transgenic overexpression of Slit2 promotes lymphatic metastasis of islet tumors in RIP1-Tag2 mice, which leads to their accelerated mortality.

Discussion

Growth factors like VEGF-A, VEGF-C and PDGF-BB were documented to be angiogenic or lymphangiogenic under specific circumstances [9,16,18-20]. Axon guidance molecules have also been implicated in vascular system development [21] and even lymphatic vessel development, such as the neuropilin-2 [22]. Here we provide evidences that axon guidance molecule Slit2 is a lymphangiogenic factor and reveal a causal role for Slit2-mediated lymphangiogenesis in the dissemination of tumor cells.

Tumor cell metastasis to regional lymph nodes is an early event in metastatic tumor spread, and is frequently used as a prognostic factor to predict disease outcome. The existence and functionality of intratumoral lymphatics in human and experimental rodents remain controversial [9,23]. In our model of pancreatic islet tumorigenesis, tumor lymphangiogenesis in RIP1-Tag2 mice hardly penetrate into the tumor, in contrary to the observed intra-tumoral lymphatics in Slit2;RIP1-Tag2 mice. This is different from the peripheral location of lymphangiogenesis induced by VEGF-C overexpression in RIP1-Tag2 mice [16], suggesting a guidance role of Slit2 in tumor lymphangiogenesis. Furthermore, aggregates of tumor cells within the lumen of lymphatic vessels were occasionally seen in Slit2;RIP1-Tag2 mice. These findings indicate that Slit2-promoted lymphangiogenesis provides portals for tumor metastasis, and that invasion of tumor cells into the lymphatic vessels is a critical step in metastasis. Given that Slit2 was expressed in various human malignant cancers, it would be meaningful to explore the potential correlation between Slit2 expression profile in human primary cancer cells and occurrences of regional lymph node metastasis.

Slit2-Robo1 signaling had been demonstrated to be angiogenic under certain circumstances [14]. As a matter of fact, overexpressed Slit2 indeed moderately increased tumor angiogenesis at an earlier stage of 10 weeks in our model, immunohistochemically determined by vWF, a marker for vascular endothelial cells (data not shown). But no increased angiogenesis was found in 14-week-old Slit2;RIP1-Tag2 double transgenic mice. And we didn't find any invasion of tumor cells into blood vessels. This selectivity towards lymphangiogenesis at late stage of islet carcinomas resembles what had been reported in VEGF-C-overexpressed RIP1-Tag2 mice [16]. Although currently there is no explanation for this selectivity, the fact that β -cell tumors that develop in RIP1-Tag2 mice already induced extensive angiogenesis but not lymphangiogenesis may partially account for this.

In conclusion, our studies reveal that the repulsive axon guidance molecule Slit2 is a novel regulator of adult lymphangiogenesis and functions to promote lymphatic metastasis of β -cell carcinomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Biao Wang for construction of the pCMV-hSlit2 expression plasmid. This work was supported by grants from National Science Foundation of China (30811120438, 30721065, 30700409 and 30630036), Ministry of Science and Technology of China (2007CB914501, 2007CB947102, 2009ZX09103-685 and 2010CB529700), Shanghai

Municipal Commission for Science and Technology (08JC1421400) and the National Institutes of Health (RO1AI064743, RO1CA126897).

Abbreviations

hLECs	human lymphatic endothelial cells
HUVECs	human umbilical vein endothelial cells
RT-PCR	reverse-transcription polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
H&E	hematoxylin and eosin
SV40 T	simian virus 40 Tag oncoproteins

References

- Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006;127:679–695. [PubMed: 17110329]
- Nathanson SD. Insights into the mechanisms of lymph node metastasis. *Cancer* 2003;98:413–423. [PubMed: 12872364]
- Swartz MA, Hubbell JA, Reddy ST. Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design. *Semin. Immunol* 2008;20:147–156. [PubMed: 18201895]
- Shin WS, Rockson SG. Animal models for the molecular and mechanistic study of lymphatic biology and disease. *Ann. NY. Acad. Sci* 2008;1131:50–74. [PubMed: 18519959]
- Jamieson T, Cook DN, Nibbs RJ, Rot A, Nixon C, Mclean P, Alcamí A, Lira SA, Wiekowski M, Graham GJ. The chemokine receptor D6 limits the inflammatory response in vivo. *Nat. Immunol* 2005;6:403–411. [PubMed: 15750596]
- Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Ylä-Herttuala S, Jäättelä M, Alitalo K. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001;61:1786–1790. [PubMed: 11280723]
- Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat. Med* 2001;7:186–191. [PubMed: 11175849]
- Chang LK, Garcia-Cardena G, Farnebo F, Fannon M, Chen EJ, Butterfield C, Moses MA, Mulligan RC, Folkman J, Kaipainen A. Dose-dependent response of FGF-2 for lymphangiogenesis. *Proc. Natl. Acad. Sci. USA* 2004;101:11658–11663. [PubMed: 15289610]
- Cao R, Björndahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsarlis K, Dissing S, Ohhashi T, Jackson DG, Cao Y. PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell* 2004;6:333–345. [PubMed: 15488757]
- Wong K, Park HT, Wu J, Rao Y. Slit proteins: guidance cues for cells ranging from neurons to leukocytes. *Curr. Opin. Genet. Dev* 2002;12:583–591. [PubMed: 12200164]
- Dickson BJ, Gilestro GF. Regulation of commissural axon pathfinding by slit and its Robo receptors. *Annu. Rev. Cell Dev. Biol* 2006;22:651–675. [PubMed: 17029581]
- Wu W, Wong K, Chen J, Jiang Z, Dupuis S, Wu JY, Rao Y. Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* 1999;400:331–336. [PubMed: 10432110]
- Prasad, Qamri Z, Wu J, Ganju RK. Slit2/Robo1 modulates the CXCL12/CXCR4-induced chemotaxis of T cells. *J. Leukoc. Biol* 2007;82:465–476. [PubMed: 17565045]
- Wang B, Xiao Y, Ding B, Zhang N, Yuan X, Gui L, Qian K, Duan S, Chen Z, Rao Y, Geng J. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell* 2003;4:19–29. [PubMed: 12892710]
- Hanahan D. Heritable formation of pancreatic β -cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 1985;315:115–122. [PubMed: 2986015]

16. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO. J* 2001;20:672–682. [PubMed: 11179212]
17. Hanahan D, Christofori G, Naik P, Arbeit J. Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. *Eur. J. Cancer* 1996;32:2386–2393. [PubMed: 9059326]
18. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J. Exp. Med* 2002;196:1497–1506. [PubMed: 12461084]
19. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J. Exp. Med* 2005;201:1089–1099. [PubMed: 15809353]
20. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, Qi JH, Claesson-Welsh L, Alitalo K. Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc. Natl. Acad. Sci. USA* 1998;95:14389–14394. [PubMed: 9826710]
21. Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. *Nature* 2005;436:193–200. [PubMed: 16015319]
22. Caunt M, Mak J, Liang WC, Stawicki S, Pan Q, Tong RK, et al. Blocking Neuropilin-2 Function Inhibits Tumor Cell Metastasis. *Cancer Cell* 2008;13:331–342. [PubMed: 18394556]
23. He YL, Rajantie L, Ilmonen M, Makinen T, Karkainen MJ, Alitalo KY. Preexisting Lymphatic Endothelium but not Endothelial Progenitor Cells Are Essential for Tumor Lymphangiogenesis and Lymphatic Metastasis. *Cancer Res* 2004;64:3737–3740. [PubMed: 15172976]

Fig. 1.

Robo1 is expressed in primary lymphatic endothelial cells. (A-B) Semiquantitative real-time RT-PCR analysis of the expression of Robo1 in hLECs relative to HUVECs. PCR products were run in agarose gel to confirm the proper size of the amplified fragments, with β -actin as internal control (A). NTC is no template negative control. (C) Robo1 (green) was detected on the membrane and cytoplasm of hLECs, with LYVE-1 (red, upper) or Prox-1 (red, lower) as markers of hLECs. Nuclei were identified by DAPI. Scale bar: 50 μ m. Results are representatives of three independent experiments.

Fig. 2.

Slit2 induces tube formation and migration of lymphatic endothelial cells, which are inhibited by function-blocking antibody R5 to Slit2/Robo1 interaction. (A) Affinity purified recombinant Slit2 was silver stained and immunoblotted with S1 (an IgG_{2a} mAb to the NH₂-terminal portion of human Slit2), and recombinant hRobo1-Fc was analyzed by Coomassie Brilliant Blue (CBB) staining and immunoblotted with HRP-conjugated secondary antibody. (B) Recombinant Slit2 interacts with hRobo1-Fc in an ELISA assay. Plate wells were coated with recombinant Slit2 in different concentrations or buffer control (-). The experiment was carried out in triplicate and was repeated three times. (C) Tube formation of hLECs on Matrigel visualized by phase-contrast microscopy. Cells were respectively treated with VEGF-C, Slit2 or Slit2 plus function-blocking Ab R5. Scale bar, 50 μm. (D) Normalization analysis of the effects of recombinant Slit2 and VEGF-C on the tube formation of hLECs, with R5 for inhibition and mouse IgG for control. Results were calculated as mean ± SD from measurements of photographed tubular structures. (E-F) The effects of recombinant Slit2 and VEGF-C on hLECs migration were measured using the Boyden chamber assay. Recombinant Slit2 induced migration in a dose-dependent manner (E). Comparing to control IgG, R5 effectively inhibited Slit2-induced but not VEGF-C-induced migration of hLECs (F). Results

were calculated as mean $\pm$ SD from measurements of three separate experiments. * $P < 0.05$; ** $P < 0.01$.

Fig. 3.

Overexpression of Slit2 promotes lymphangiogenesis and lymphatic invasion of pancreatic islet tumors in Slit2;RIP1-Tag2 mice. (A) Schematic display of FLAG-tagged human Slit2 (hSlit2) transgene construct. The FLAG sequence was inserted in front of the Slit2 cDNA and the transgene was under the control of CMV promoter. The lengths of the DNA fragment are given in base pairs (bp). (B) Slit2 expression. Western blot analysis of pancreas extracts from age-matched wide-type (WT) versus Slit2 transgenic mice and RIP1-Tag2 versus Slit2;RIP1-Tag2 mice. Tubulin was used as loading control. (C) Representative immunohistochemical staining of LYVE-1 in pancreas of 14-week-old wild-type (WT) or Slit2 transgenic (Slit2 Tg) mice and the corresponding normalization analysis of pancreas lymphatics (right). Results are mean $\pm$ SD values of the total lymphatic lengths from a minimum of four mice per group. *

$P < 0.05$. Scale bar: 100 μm . (D) Representative images of tumor cells invading the intra-tumoral (left) or peri-tumoral lymphatic vessels (right) of 14-week-old Slit2;RIP1-Tag2 mice. Arrow heads indicate LYVE-1-labeled lymphatic vessels with invading tumor cells. Scale bar: 100 μm . (E) Robo1 (green) was expressed in LYVE-1-positive (red) tumor lymphatics. Arrows indicate co-localization of Robo1 with LYVE-1. T: tumor; LV: lymphatic vessel. Scale bar: 40 μm . (F) Representative immunohistochemical staining of LYVE-1 in pancreas of 14-week-old RIP1-Tag2 (left) or Slit2;RIP1-Tag2 (middle) mice and the corresponding normalization analysis of tumor lymphatics (right). Results are mean $\pm$ SD values of the total tumoral lymphatic lengths from a minimum of four mice per group. * $P < 0.05$. Scale bar: 100 μm .

Fig. 4.

Slit2;RIP1-Tag2 mice have increased regional lymph node tumor metastasis and decreased survival rate compared with RIP1-Tag2 mice. (A) Tumor burdens and tumor numbers (mean $\pm$ SD) of Rip1-Tag2 ($n = 8$) and Slit2;Rip1-Tag2 ($n = 9$) mice at 14 weeks showed no significant differences. (B) Identification of islet β -cell metastases. Macroscopic image of primary islet tumor and metastases was shown (a). Arrow head points to a primary tumor. Arrows point to a mesenteric lymph node metastasis and an intestinal metastasis. Microscopic images indicate tumor metastasis (arrows) colonized into the submucosal layers (arrow head) of the small intestine (b) and the mesenteric lymph nodes (c). (C) Kaplan-Meier survival curves of RIP1-Tag2 ($n = 35$) and Slit2;RIP1-Tag2 ($n = 13$) mice. $P = 0.0117$ analyzed by log-rank test. (D) Blood insulin levels in 14-week-old RIP1-Tag2 ($n = 5$) and Slit2;RIP1-Tag2 ($n = 5$) mice showed no significant difference, determined by ELISA.

Table 1

Incidences of mesenteric lymph node metastasis and intestinal metastasis in 14-week-old RIP1-Tag2 and Slit2;RIP1-Tag2 mice.

Mice	Lymph Node Metastasis ^c	Intestinal Metastasis
RIP1-Tag2 ^a	6.25% (2 in 32)	3.125% (1 in 32)
Slit2;RIP1-Tag2 ^b	34.0% (17 in 50)	16.0% (8 in 50)

^a
n = 32

^b
n = 50

^c $P < 0.05$ as analyzed by Chi-square test.