Slit2 Overexpression Results in Increased Microvessel Density and Lesion Size in Mice With Induced Endometriosis

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Abstract

We recently reported that Slit/Roundabout (ROBO) 1 pathway may be a constituent biomarker for recurrence of endometriosis, likely through promoting angiogenesis. In this study, we sought to determine as whether Slit2 overexpression can facilitate angiogenesis, increase lesion size, and induce hyperalgesia in mice with induced endometriosis. We used 30 Slit2 transgenic (S) and 29 wild-type (W) mice and cross-transplanted endometrial fragments from S to W (group SW) and vice versa (group WS), and also within the S and W (groups SS and WW, respectively), into the peritoneal cavity, inducing endometriosis. We also performed a sham surgery within both S and W mice (groups Sm and Wm, respectively). The size of the ectopic implants, microvessel density (MVD) and immunoreactivity to ROBO 1, and vascular endothelial cell growth factor (VEGF) in ectopic and eutopic endometrium, along with hotplate and tail-flick tests in all mice, were then evaluated. We found that the induction of endometriosis resulted in generalized hyperalgesia, which was unaffected by Slit2 overexpression. Slit2 overexpression did increase the lesion size significantly and correlated positively with the MVD in ectopic and eutopic endometrium. Slit2 expression levels appear to correlate with the MVD, but not with VEGF immunoreactivity, in ectopic endometrium. Consequently, we conclude that Slit2 may play an important role in angiogenesis in endometriosis. The increased angiogenesis, as measured by MVD, but not VEGF immunoreactivity, likely resulted in increased lesion size in induced endometriosis. Thus, SLIT2/ROBO 1 pathway may be a potential therapeutic target for treating endometriosis.

Keywords

angiogenesis, endometriosis, hyperalgesia, microvessel density, Slit2, transgenic mice

Endometriosis is a benign gynecological disorder reportedly affecting 1% to 22% of women of reproductive age.^{1,2} It is characterized by the ectopic presence of endometrial glands and stroma outside of the uterine cavity and is closely associated with dysmenorrhea, pelvic pain, and subfertility. While the pathogenesis of endometriosis is still poorly understood, angiogenesis is recognized as being of critical importance in the growth and survival of endometriotic lesions since it must be an absolute requirement for any lesion to become clinically relevant. As in tumor metastases, endometriotic lesions require nutritional and oxygen supply and thus neovascularization to invade into ectopic sites within the host and to maintain proliferation,³⁻⁵ hence angiogenesis has been recognized as an attractive target for novel medical therapeutics.⁶⁻⁹

In endometriosis, the involvement of vascular endothelial cell growth factor (VEGF) and other angiogenic mediators has long been recognized.^{4,5,10,11} Preclinical studies have demonstrated promising potentials of antiangiogenic therapies for endometriosis.¹²⁻¹⁴ Since endometriotic angiogenesis involves several pathways and the blockade of just one single pathway may not effectively suppress angiogenesis,¹⁵ the identification of all possible angiogenic molecules and pathways would

conceivably help devise more effective strategies to suppress angiogenesis in endometriosis. In addition, the reports that drugs with antiangiogenic potential can impact negatively on reproductive functions¹⁶⁻¹⁸ would mandate a thorough understanding of the angiogenic mechanisms involved in both normal and ectopic endometrium.⁷ This also requires, by necessity, the identification of all possible angiogenic molecules and pathways involved in endometriosis.

Supplementary material for this article is available on the *Reproductive Sciences* website at <http://rsx.sagepub.com/supplemental>.

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The Slit is a secretory glycoprotein consisting of 3 members, Slit1, Slit2, and Slit3, and was originally found to be secreted repellents in axon guidance and neuronal migration.¹⁹⁻²¹ It has been shown to be an endogenously available inhibitor of leukocyte chemotaxis.²² The receptor for Slit is the transmembrane protein Roundabout (ROBO), which currently consists of 4 members (ROBO1-4).²³ Wang et al demonstrate that the purified recombinant human Slit2 protein attracted the migration of human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner, indicating that Slit2 is a chemoattractant for HUVECs²⁴; disrupting the Slit2 signaling would significantly neutralize the migration of HUVECs. In addition, they convincingly show that Slit2 guided the direction of endothelial cell migration, rather than simply increasing the motility of endothelial cells. They also show that Slit is secreted by various cancer cells and ROBO1 is expressed in vascular endothelial cells, where Slit can attract vascular endothelial cells in vitro and promote tumor-induced angiogenesis in a xenograft model of human malignant melanoma cells.²⁴ Recently, Dunaway et al show that Slit2 stimulates endothelial cell assembly and migration in vitro and induces subcutaneous blood vessel remodeling in vivo.²⁵ Consistent with these findings, Slit-ROBO4 is reported to function as a chemoattractant to recruit vascular endothelial cells to sites for vasculogenesis.^{26,27} Increased Slit expression is associated with higher tumor angiogenesis as reflected by increased VEGF expression and microvessel density (MVD).²⁸

We have recently reported that increased immunoreactivity to SLIT in ectopic endometrium may be a constituent biomarker for the recurrence of ovarian endometriomas.²⁹ Thus, Slit2 may be involved in the development of endometriosis.

Since in the vertebrate the vascular and nervous systems overlap substantially and share several anatomical and functional parallels³⁰ and since both angiogenesis and innervation are well documented in endometriosis, it is plausible that Slit2 might also be involved in neurotrophic activities in and around ectopic implants, endometrium, and perhaps also in vagina, resulting in innervation and, consequently, endometriosis-associated pain.

Therefore, through cross-transplantation of endometrial tissues within and between Slit2 transgenic and wild-type (WT) mice, we sought in this study to investigate the role of Slit2 in the development of endometriosis and its impact on endometriosis-associated hyperalgesia. We evaluated the effect of Slit2 overexpression on the size of ectopic lesions and on endometriosis-induced pain by evaluating the thermal withdrawal latency of the hindpaws. To see any resultant changes in angiogenesis and the expression of the known angiogenic factor, VEGF, we also performed immunohistochemistry analysis of VEGF, and CD31 and CD34, the latter 2 being markers for MVD and thus an indirect measure of angiogenesis.³¹

Materials and Methods

Animals

Slit2 knockout mice were either embryonically lethal or die within a week or 2 weeks after birth,³² hence 1 way to

investigate the role of Slit2 in the development of endometriosis would be the use of Slit2 transgenic (Tg) mice. Slit2 Tg mice with C57BL/6 background were generated according to standard procedures and were characterized as reported previously.³²⁻³⁴ Briefly, the transgene was constructed by cloning the complementary DNA (cDNA) encoding the full-length of human Slit2 cDNA into the pCEP4F vector, which contains the CMV promoter and was injected into the pronuclei of fertilized C57 × CBA F1 oocytes. Genotypes were confirmed by Southern blot and polymerase chain reaction analysis, and additional validation came from genotyping the offspring of Slit2 Tg mice (which were homozygotes with a genotype, say, SS, as confirmed by genetic method) crossed with C57BL/6 mice (also homozygotes, but different from Tg, say, ss), which were Slit2 heterozygotes (Ss). The Tg mice showed no apparent phenotypic changes and were physiologically comparable to their WT counterpart.³³ The Slit2 expression in the uterus was detected by immunohistochemistry. Thirty female Slit2 Tg mice, 8 to 9 weeks old and about 18 to 20 g in body weight, were used in this study. Since the Tg mice were derived from C57BL/6 (ie, WT) mice and back-crossed for 10 generations, we thus used C57BL/6 mice as control in our experiments.

It should be noted that although this study did not examine human Slit2 binding to mouse Robo per se, their interaction has been extensively demonstrated by many previous studies, including our own ones.^{24,34} The Slit2 gene has over 90% homology among different species, reflecting the fact that it has an evolutionarily conserved role in repulsive axon guidance.¹⁹

Twenty-nine adult female C57BL/6 mice (SCXK-2008-0002), 8 to 9 weeks old and about 18 to 20 g in body weight, were purchased from the Laboratory Animal Science of Shanghai Medical College, Fudan University (Shanghai, China) and used in this study.

All mice were maintained under climate- and light-controlled conditions with a room temperature of 24°C and a light/dark cycle of 12/12 hours, with access to chows and water ad libitum. All experiments were performed under the guidelines of the National Research Council's *Guide for the Care and Use of Laboratory Animals*³⁵ and approved by the institutional experimental animals review board of Shanghai Obstetrics and Gynecology Hospital, Fudan University.

Experiment Protocol

After 3 days of acclimatization and before surgery, a baseline hotplate test and tail-flick test (described below) were performed in all the mice. Endometriosis was surgically induced (see surgical procedures below) when mice were in estrus as determined by microscopic examination of vaginal smear. In essence, endometriosis was induced through allotransplant of endometrial fragments into the peritoneal cavity. Depending on the donor and recipient status and the type of surgery (endometriosis-inducing or sham), there were 6 groups:

1. Group SS (n = 11) consisted of Slit2 Tg mice (recipients) transplanted with endometrial fragments from different

- Slit2 Tg mice (donors). Here, SS means that the endometrial tissues harvested from the S (the first S in SS, ie, Slit2 Tg) mice were cross-transplanted to the S (the second S in SS) mice. In other words, the donor of endometrial fragments was an S mouse and the recipient of the transplantation also was an S (but different) mouse (thus, the S→S or, for short, the SS notation);
2. Group WS (n = 10), consisted of Slit2 Tg mice (recipients) transplanted with endometrial fragments taken from WT mice (donors). In other words, the donor was a W mouse and the recipient was an S mouse (thus, the W→S or, for short, the WS notation).
 3. Group Sm (n = 9), consisted of Slit2 Tg mice that received a sham surgery.
 4. Group WW (n = 10), consisted of WT mice (recipients) transplanted with endometrial fragments from different WT mice (donors).
 5. Group SW (n = 9), consisted of WT mice (recipients) with endometrial fragments from Slit2 Tg mice (donors) transplanted into WT mice.
 6. Group Wm (n = 10), consisted of WT mice that received a sham surgery.

The use of Sm and Wm groups was intended to control possible effect of surgery and also provide a reference to other groups.

Four weeks after implantation, the second round of hotplate and tail-flick tests was performed. Thereafter, all mice were sacrificed through cervical dislocation. The abdominal cavity was immediately reopened through the original incision, and the lesions were measured by 2 perpendicular diameters (D_1 and D_2) with a caliper, and the cross-sectional lesion area was calculated using the formula ($D_1 \times D_2 \times \pi/4$) as reported previously.³⁶ The total size (in mm²) of ectopic lesions in each group was evaluated without any knowledge of the group identity (blinded evaluation). Ectopic and eutopic endometrial tissue samples in all mice were harvested and fixed immediately after collection in 10% formalin-acetic acid and embedded in paraffin for histopathologic examination and immunohistochemical (IHC) analysis. These measurements and the IHC analysis were performed in a blind fashion, without knowledge of the group identity of the mouse in question.

Surgical Procedures

Surgery was performed under aseptic precautions to transplant small pieces of uterine fragments to peritoneum of lower parts of the abdomen and pelvic cavity, identical to published studies.³⁷⁻³⁹ Prior to any invasive procedure, the mice were anesthetized with 100 mg/kg ketamine hydrochloride. For each group, laparotomy was performed and the left uterine horns were removed. The excised horns, with connecting fat tissues removed as much as possible, were immersed in a sterile lactate solution⁴⁰ and opened longitudinally. Each uterine segment was cut into 4 smaller fragments of roughly equal size ($\sim 2 \times 2$ mm). For mice in groups SS and WW, the uterine fragments were allografted into the peritoneum of lower parts

of the abdomen and pelvic cavity. For mice in group SW, we sutured the uterine fragments of the Slit2 Tg mice into the peritoneum of WT mice, while mice in group WS, the uterine fragments of the WT mice were sutured into the peritoneum of Slit2 Tg mice. A total of 4 uterine fragments were sutured to the peritoneal wall of the lower part of the lateral abdominal and pelvic cavity with a 6/0 braided silk suture. For mice in Sm and Wm groups, laparotomy was performed and the left uterine horn was removed and 4 pieces of fat tissues, roughly equal in size (also $\sim 2 \times 2$ mm) to the endometrial fragments used in the endometriosis-inducing groups, were separated and removed from the left uterine horn and sutured to the peritoneal wall of the lower part of the lateral abdominal and pelvic cavity with a 6/0 braided silk suture. Then the midline incision was closed with a 3/0 braided silk suture. After surgery, all mice, including those received sham surgery, were fed with 2 mg/L 17 β -estradiol (Sigma, St Louis, Missouri) solution daily for 2 weeks, as reported previously.⁴¹ Penicillin of 40 000 U/d was administered intramuscularly to all mice for 5 days to prevent infection after surgery.

Hotplate and Tail-Flick Test Procedures

The hotplate test,⁴² or the test of sensitivity to the thermal nociception, was evaluated with a commercially available Hot Plate Analgesia Meter (Model BME-480; Institute of Biomedical Engineering, Chinese Academy of Medical Sciences, Tianjin, China) consisting of a metal plate of 25 $\times$ 25 cm in size, which can be heated to a constant temperature of $54.0 \pm 0.1^\circ\text{C}$, on which a plastic cylinder (20 cm in diameter, 18 cm in height) was placed. Mice were brought to the testing room and allowed to acclimatize for 10 minutes before the test begins.

The latency to respond to thermal stimulus defined to be the time (in second) elapsed from the moment when the mouse was inserted inside the cylinder to the time when it licked or flicked its hindpaws or jerked off or jumped off the hot plate. Each animal was tested only once in 1 session. The latency was calculated as mean of 2 readings recorded at intervals of 24 hours.

The tail-flick test was evaluated with a commercially available tail-flick Analgesia Meter (Model ZH-LUO/B; Zhenghua Co, Ltd, Huaibei, Anhui, China). Thermal stimulation was given by a beam of high-intensity light focused on the tail 2 to 3 cm distal to the end. The time between the start of the stimulation and tail withdrawal was measured as the tail-flick latency. The intensity of the beam was adjusted to produce mean control reaction time within 60 seconds before the test begins, after which the heat source was held constant for all mice and not adjusted for individual animals. All mice to be tested were placed in a metallic mesh cage with their tails extended through a slot located at the rear of the cage and allowed to acclimatize for 10 minutes before the test begins. Withdrawal of the tail exposed to the light automatically turned off the thermal stimulus, automatically stopped the time-counting, and the latency time was automatically displayed. Each animal was tested only once in 1 session. The latency was calculated as mean of 2 readings recorded at intervals of 12 hours.

Immunohistochemistry

Serial 4- μ m sections were obtained from each paraffin-embedded tissue block, with the first resultant slide being stained for hematoxylin and eosin to confirm pathologic diagnosis, and the subsequent slides stained for Slit2, ROBO1, VEGF, CD31, and CD34. Routine deparaffinization and rehydration procedures were performed following published protocols.⁴³

The rabbit polyclonal antibodies against Slit2 (LS-C19086; LifeSpan, Seattle), ROBO1, VEGF (ab7279 and ab44724; Abcam, Cambridge, UK), and the rat monoclonal antibodies against CD34 and CD31 (ab8158 and ab56299; Abcam), diluted to 1:200, 1:100, 1:200, 1:100, and 1:400, respectively, were used as primary antibodies. For antigen retrieval, the slides were heated at 98°C in an EDTA buffer (pH 9.0) for a total of 30 minutes and cooled naturally at room temperature. The endogenous peroxidases were blocked by immersing slides in 3% hydrogen peroxide-methanol solution (v/v) for 15 minutes at room temperature. Sections were then incubated overnight with the primary antibody at 4°C. After slides were rinsed, biotinylated anti-rat secondary antibody was applied and detected using the DAB kit (85-9643; Invitrogen, California) and biotinylated anti-rabbit secondary antibody was applied and detected using the DAB kit (MR-SPR120; MingRui Biotech, Shanghai). The bound antibody complexes were stained for 3 to 5 minutes or until appropriate for microscopic examination with diaminobenzidine and then counterstained with hematoxylin and mounted. Myometrial tissues were specifically excluded. Each staining run incorporated a positive control slide. Slit2 and ROBO1 staining in retina tissue, VEGF staining in eutopic endometrium, CD31 staining in kidney tissue, and CD34 staining in lung tissue were used as positive control. A negative control was also incorporated by replacing the primary antibody with immunoglobulins of the same isotype.

Immunoreactivity staining was characterized quantitatively by digital image analysis using the Image Pro-Plus 6.0 (Media Cybernetics, Inc, Bethesda, Maryland) as reported by Wang-Tilz et al⁴⁴ without prior knowledge of any information on group assignment. Briefly, images were obtained with the microscope (Olympus BX51; Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70, Olympus). A series of 10 random images on several sections were taken for each immunostained parameter to obtain a mean value. Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images, and measurement readings were obtained. Immunohistochemical parameters assessed in the area detected included (a) integrated optical density (IOD); (b) total stained area (S); and (c) mean optical density (MOD), which is defined as $MOD = IOD/S$, equivalent to the intensity of stain in all positive cells. All sections were inspected independently by 2 persons (Y.Z. and Y.L.). Discrepancies, if occurred, were resolved by consensus.

Quantification of Angiogenesis

The MVD was assessed following Weidner et al⁴⁵ and was assessed on CD34 (denoted as CD34-MVD) and, as a confirmation, also on CD31 (CD31-MVD)-stained slides by light microscopy in areas containing the highest numbers of capillaries and small venules (neovascular hot spots), as described previously.^{29,46} Each count was expressed as the highest number of microvessels identified in ~ 0.25 mm² fields of the “hot spots” at a magnification of $\times 400$. At least 5 fields were examined for each slide. All counts were evaluated independently by 2 investigators (Y.L. and Y.Z.) without any knowledge of the group identity of the mouse at hand using a double-headed light microscope; both having a consensus on what constituted a single microvessel before a vessel was included in the count. Endothelial cells or cell cluster clearly separated from adjacent microvessels, ectopic endometrial cells, and other connective tissue elements were included for microvessel counting. Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. The MVD was defined to be the mean of the vessel counts obtained in these fields, as reported previously.⁴³

Statistical Analysis

For descriptive statistics, we used boxplots⁴⁷ to graphically depict groups of data, in which the bottom and top of the box represent the lower and upper quartiles, respectively, the band near the middle of the box represents the median, the ends of the whiskers represent the smallest and the largest nonoutlier observations (no more than 1.5 times the interquartile range from the box), and the dots outside the whiskers are considered outliers by convention. In all data analyses, outliers were not excluded from analysis unless otherwise explicitly stated in the results. The comparison of distributions of continuous variables between or among 2 or more groups was made using the Wilcoxon and Kruskal test, respectively, and the paired Wilcoxon test was used when the before-after comparison was made for the same group of animals. Pearson correlation coefficient was used when evaluating correlations between 2 variables. To see whether induction of endometriosis and/or SLIT2/ROBO1/VEGF immunoreactivity, MVD and other possible factors were responsible for the change in hotplate and tail-flick latency before and after the surgery, a multiple linear regression analysis was performed. The presence of endometriosis or not was depicted by a dummy variable with 1 if endometriosis is induced or 0 if not. To see whether the donor and recipient status (S vs W) has any effect on the size of ectopic lesions, a multiple linear regression model was used with a dummy variable coding for donor status (1 if S or 0 if W) and the other coding for recipient status (1 if S or 0 if W). To ensure the normality of the linear regression analysis, a Q-Q plot was examined using the residuals of the regression.

P values of less than .05 were considered statistically significant. All computations were made with R 2.14.1⁴⁸ (www.r-project.org).

Figure 1. The Slit2 staining in endometrium in Slit2 Tg and wild-type mice. Immunohistochemical staining of Slit2 in endometrium of Slit2 transgenic (A) and C57BL/6 (B) mice. C, Positive control (POS CTL). Slit2 staining in a retina tissue sample; D, negative control (NEG CTL). The primary antibody was replaced with immunoglobulins of the same isotype.

Results

Surgical Induction of Endometriosis

In our experiment, the Slit2 Tg mice had higher Slit2 expression in the endometrium when compared with the WT mice (Figure 1), just as expected.

The endometriosis-inducing surgery successfully induced endometriosis in SS, SW, WS, and WW mice but not in Sm and Wm mice. There was no death resulting from either the endometriosis-inducing or sham surgery.

Lesion Size in Different Groups

We first evaluated the total lesion surface areas in all groups of mice and found that the lesion size differed significantly among the 4 groups of mice with induced endometriosis (Figure 2; $P = .005$). It can be seen from Figure 2 that, on average, the SS group had the largest lesion sizes while the WW group had the smallest among the 4 groups. Of note, the average lesion size in SS mice was 3 times of that in the WW mice. The multiple linear regression analysis confirmed this impression: after deleting an apparent outlier in the WS group (Figure 2), we found that both the donor and recipient statuses were significantly associated with the lesion size ($P = .026$ and $.004$, respectively). That is, Slit2 overexpression either in the donor or in the recipient tissues was associated with increased lesion size.

Effect on the Response to Noxious Stimuli

To see whether Slit2 overexpression affects mice's generalized hyperalgesia as result of induced endometriosis or surgery, we tested the response to noxious thermal stimuli in all groups of mice before and after the induction of endometriosis or the sham surgery using hotplate and tail-flick tests. Before the

Figure 2. Boxplot of total lesion areas among different groups of mice. S denotes Slit2 transgenic mouse and W, wild-type mouse. SS: the endometrial fragments from S were cross-transplanted into the peritoneal cavity of another S mouse; WW: wild-type mouse to another wild-type mouse; SW: S mouse to W mouse; WS: W mouse to S mouse.

surgery that transplanted either endometrial tissues or fat tissues to the pelvic cavity, there was no difference in response latency in hotplate test among all groups ($P = .67$). There was no difference either between Slit2 transgenic and WT mice ($P = .49$).

Yet after surgery, a significant difference among the 6 groups was found ($P = .029$). When the before–after change in latency was evaluated, the difference among them did not

Figure 3. A, Boxplot of the change in hotplate response latency before and after the surgery. B, Boxplot of the change in tail-flick response latency before and after the surgery. The dashed line designates no change. The group designations are the same as in Figure 2, but Sm denotes Slit2 transgenic mice that received a sham endometriosis-inducing surgery. Similarly, Wm denotes wild-type mice that received a sham endometriosis-inducing surgery (see text for more details).

reach statistical significance (Figure 3A; $P = .076$). All mice, as a whole, had significantly reduced response latency ($P = .0005$, paired test), which was most visible in mice with induced endometriosis but not in mice received sham surgery (Figure 3A). However, neither hotplate nor tail-flick latency correlated with the size of lesions (all P values $> .68$).

With the change of latency as a dependent variable, the multiple linear regression using donor and recipient status, presurgery latency, and the indicator indicating as whether the mouse had induced endometriosis or sham surgery as covariates revealed that induced endometriosis and presurgery (baseline) latency were significantly and positively associated with the change ($P = 9.0 \times 10^{-14}$ and $P = .0024$, respectively, $R^2 = .67$). This suggests that neither donor nor recipient status (ie, Slit2 expression level) was associated with the change, but the induction of endometriosis and the baseline latency did.

For tail-flick test, no difference in baseline level was found ($P = .90$). In contrast to the hotplate test, there was no difference in response latency after surgery ($P = .36$). In fact, there

was no significant change in response latency before and after surgery (Figure 3B; $P = .55$, paired test). With change of latency as the dependent variable, the multiple linear regression using donor and recipient status, presurgery latency, and the indicator indicating as whether the mouse had induced endometriosis or sham surgery as covariates indicated that presurgery (baseline) latency was the only covariate that was significantly associated with the change ($P = 1.1 \times 10^{-11}$, respectively, $R^2 = .56$). Indeed, the baseline latency correlated closely with the after-surgery level ($r = .57$, $P = .2.2 \times 10^{-6}$).

Immunoreactivity to Slit2, ROBO1, VEGF, CD31-MVD, and CD34-MVD in Ectopic Endometrium

We next examined immunoreactivity to Slit2, ROBO1, VEGF, CD34-MVD, and also CD31-MVD in ectopic endometrium. As shown in Figure 4, Slit2 immunoreactivity was seen mostly in glandular epithelial cells and was localized in the cytoplasm. ROBO1 and VEGF immunoreactivity was seen mostly in glandular epithelial cells as well as in vascular endothelial cells and was localized in the cytoplasm. As expected, both CD34 and CD31 immunostainings were seen mostly in vascular endothelial cells (figures for positive and negative controls are provided in Supplemental Materials).

We found that among the 4 groups of mice with induced endometriosis, a statistically significant difference in immunoreactivity to SLIT2, VEGF, CD34-MVD, and CD31-MVD in ectopic endometrium was found ($P = .038$, $P = .020$, $P = .002$, and $P = .0001$, respectively; Figure 5). The difference in ROBO1 immunostaining did not reach statistical significance ($P = .093$). Multiple linear regression, however, indicated that, for both Slit2 and ROBO1, only the donor status as being the Slit2 Tg mouse (ie, when the implants originated from SLIT2 Tg mouse) was significantly and positively associated with the immunoreactivity level in ectopic endometrium ($P = .003$ and $P = .048$, respectively). This is consistent from what the boxplots revealed in Figure 5: when the recipient statuses are the same, the donor status makes a difference. When the endometrial tissues came from a Slit2 Tg (S) mouse, then the SLIT2 or ROBO1 staining level in ectopic implants was higher than from a WT (W) mouse.

For VEGF immunoreactivity, however, the multiple linear regression indicated that donor and recipient status and their interaction were all significantly associated with the VEGF staining level ($P = .045$, $P = .037$, and $P = .003$, respectively). It is interesting to note that the VEGF immunoreactivity was lower in mice with transplanted endometrial tissues originating from a mouse with a different Slit2 gene expression status than those when donor and recipient mice were the same strain (Figure 5C).

For CD31-MVD, the multiple regression indicated that both donor and recipient statuses were significantly associated with the CD31-MVD ($P = .00016$, and $P = 2.2 \times 10^{-6}$, respectively). The CD31-MVD was the highest in the SS mice and lowest in the WW mice (Figure 5D), suggesting that CD31-MVD was dependent on both donor and recipient statuses.

Figure 4. Immunohistochemical staining of SLIT2, ROBO1, VEGF, CD34, and CD31 in ectopic (EC) endometrium. The rows are for SS, WS, WW, and SW groups, respectively while the columns are for SLIT2, ROBO1, VEGF, CD34, and CD31, respectively. All magnifications were $\times 400$. The group designations are the same as in Figures 2 and 3. The scale bar is 100 μm . ROBO indicates Roundabout; VEGF, vascular endothelial cell growth factor.

Among all 5 IHC measures, only CD31-MVD and CD34-MVD values were found to be significantly correlated with the lesion size ($r = .35$, $P = .037$, and $r = .37$, $P = .027$, respectively). Regressing CD34-MVD on SLIT2, VEGF, CD31-MVD, and ROBO1 immunoreactivity levels indicated that only CD31-MVD was associated with CD34-MVD ($P = 5.5 \times 10^{-7}$, $R^2 = .52$).

For CD34-MVD, its values correlated positively with that of CD31-MVD ($r = .72$, $P = 1.1 \times 10^{-6}$). The highest and lowest CD34-MVD was found in the SS and WW groups, respectively, similar to CD31-MVD (Figure 5E). The multiple linear regression indicated that both donor and recipient statuses were significantly associated with the CD34-MVD level ($P = .006$, and $P = .002$, respectively), hence providing the explanation as

Figure 5. Boxplot of immunoreactivity to SLIT2 (A), ROBO1 (B), VEGF (C), CD31-MVD (D), and CD34-MVD (E) in ectopic endometrium in mice with induced endometriosis. The group designations are the same as in Figure 2. ROBO indicates Roundabout; VEGF, vascular endothelial cell growth factor; MVD, microvessel density.

why the highest and the lowest MVD level in the SS and WW groups, respectively. Regressing of CD34-MVD values on SLIT2, ROBO1, and VEGF, but not CD31, immunostaining levels indicated that both SLIT2 and ROBO1 immunoreactivities, but not VEGF, were associated with CD34-MVD ($P = .0003$, $P = .001$, and $P = .002$ [for interaction term], respectively). Similarly, regressing CD31-MVD on the same set of covariates found that both SLIT2 and ROBO1 staining levels are positively associated with the MVD count ($P = .004$, $P = .004$, and $P = .015$ [for interaction term], respectively). In fact, after removing an apparent outlier, the correlation coefficient

between CD31-MVD and SLIT2 and between CD34-MVD and SLIT2 was 0.30 ($P = .08$) and 0.37 ($P = .029$), respectively, when compared with $r = .05$ ($P = .77$) and $r = .18$ ($P = .29$), respectively, for VEGF.

Immunoreactivity to SLIT2, ROBO1, VEGF, CD31-MVD, and CD34-MVD in Eutopic Endometrium

We next examined immunoreactivity to SLIT2, ROBO1, and VEGF and counted MVD by CD31 and CD34 staining in eutopic endometrium (Figure 6; positive and negative controls are

Figure 6. Immunohistochemical staining of Slit2, ROBO1, VEGF, CD34, and CD31 in eutopic (EU) endometrium. The rows are for SS, WS, Sm, WW, SW, and Wm groups while the columns for Slit2, ROBO1, VEGF, CD34, and CD31. All magnifications were $\times 400$. The group designations are the same as in Figures 2 and 3. The scale bar is 100 μ m. ROBO indicates Roundabout; VEGF, vascular endothelial cell growth factor.

Figure 7. Boxplot of immunoreactivity to Slit2 (A), ROBO1 (B), VEGF (C), CD31-MVD (D), and CD34-MVD (E) in eutopic endometrium in all groups of mice. The group designations are the same as in Figures 2 and 3. ROBO indicates Roundabout; VEGF, vascular endothelial cell growth factor; MVD, microvessel density.

provided as Supplemental Materials). We found a statistically significant difference in immunoreactivity to SLIT2, ROBO1, VEGF, CD31-MVD as well as in CD34-MVD in eutopic endometrium (Figure 7; $P = .002$, $P = .008$, $P = .002$, $P = .009$ and $P = .002$, respectively). Using donor and recipient statuses and the indicator for endometriosis as covariates, the multiple linear regression analysis found that, for both SLIT2 and ROBO1, only the Slit2 recipient status was significantly associated with the immunoreactivity level ($P = 3.5 \times 10^{-5}$, and $P = .001$,

respectively). In contrast, the presence of endometriosis (as opposed to sham surgery) as well as Slit2 recipient status were significantly and positively associated with the VEGF ($P = .015$ and $P = .0007$, respectively) and with the CD31-MVD immunoreactivity levels ($P = .032$ and $P = .0001$, respectively). For all mice, the CD31-MVD correlated significantly with the Slit2 immunoreactivity ($r = .32$, $P = .02$). However, CD34-MVD did not seem to correlate with the Slit2 staining levels ($r = .20$, $P = .17$).

Using log-transformed CD34-MVD value (to improve normality) as the dependent variable, the multiple linear regression analysis found that both Slit2 donor and recipient statuses and the presence of endometriosis were all significantly and positively associated with CD34-MVD values ($P = .029$, $P = .003$, and $P = .012$, respectively).

We also found that the CD34-MVD value in WW mice was significantly higher than that of Wm mice ($P = .03$), so did CD31-MVD ($P = .03$). However, neither CD34-MVD nor CD31-MVD values in SS mice were statistically different from that of Sm mice ($P = .23$ and $P = .21$, respectively).

Determinants of Lesion Size

From previous analysis, we found that Slit2 overexpression either in the donor or the recipient tissues was associated with increased lesion size. With the IHC data, we carried out a multiple regression analysis attempting to identify which protein is associated with the lesion size (square root transformed in order to improve normality, as judged by the Q-Q plot). Among the 5 immunostaining measurements, we found that CD34-MVD was the only covariate that was significantly associated with the lesion size ($P = .02$).

Discussion

Angiogenesis is the physiological process involving the growth of new blood vessels from preexisting vessels.⁴⁹ In the female reproductive tract, physiological angiogenesis occurs in wound healing and endometrial regeneration. Extensive work has shown that endometrial implants require neovascularization to proliferate and invade into ectopic sites.⁵ Following the first report on the relationship between peritoneal angiogenic activity and endometriosis⁵⁰ and also on the dense vascularization surrounding endometriotic implants,⁵¹ VEGF overexpression in endometriotic implants has been demonstrated⁵² and higher concentrations were found in endometriosis.^{4,53} Other angiogenic factors, such as fibroblast growth factor (FGF), are also reported to be involved in endometriosis.⁵⁴ Thus, several animal studies have shown that antiangiogenic agents are effective in suppression of endometrial implants.^{12,36,55–57}

This study demonstrates that, in endometriosis, Slit2 may also be involved in angiogenesis. Indeed, we and others have previously shown that Slit2 promotes angiogenesis.^{24,25} In this study, we found a close correlation between SLIT2 immunoreactivity and MVD in ectopic endometrium and a close correlation between MVD and lesion size (Figures 2 and 5). The increased angiogenesis in ectopic endometrium, as measured by elevated MVD levels, was associated with increased lesion size in induced endometriosis. However, Slit2 overexpression does not seem to be associated with any increase in generalized hyperalgesia resulting from endometriosis.

The finding that neither CD31-MVD nor CD34-MVD in ectopic endometrium is associated with the VEGF expression is somewhat surprising, but not entirely unexpected, for several reasons. First, the use of the Slit2 transgenic mice might

somehow eclipse the role of VEGF in angiogenesis in ectopic endometrium. Second, this study only examined VEGF or VEGF-A, as did in most human studies of endometriosis. It is plausible that other VEGF family members, such as VEGF-C, may also be responsible for MVD in ectopic endometrium. Finally, Laschke et al reported that selective blockade of VEGF only results in a slight reduction in MVD in a dorsal skinfold chamber model in hamsters.¹⁵ They concluded that vascularization of endometriotic lesions is not solely driven by VEGF but depends on the cross talk between VEGF, FGF, and PDGF.¹⁵ In addition, as WW mice had induced endometriosis but Wm mice did not, the result that MVD—likely to be independent from Slit2 expression in this case—in eutopic endometrium was higher in WW mice than that in Wm mice appeared to be consistent with reportedly higher MVD in eutopic endometrium in women with endometriosis⁵⁸ and with reported increased proliferation of endothelial cells.⁵⁹ Indeed, the presence of endometriosis (in the WW group) may have disrupted various vascular events in the endometrium, resulting in an overall increase in angiogenesis (ie, higher MVD levels in WW mice than that in Wm mice),⁶⁰ even though neither WW nor Wm group expresses Slit2. These data, taken together and along with data published by our groups that Slit2 is involved in angiogenesis in tumors,^{28,61} appear to suggest that Slit2 may be involved in the pathogenesis of endometriosis, possibly independent of VEGF expression in ectopic endometrium as seen by the lack of correlation between SLIT2 and VEGF immunostaining and between VEGF and MVD.

Indeed, the Slit/ROBO pathway has been shown to be involved in normal development and pregnancy in endometrium.⁶² In particular, Slit2 expression is found to peak in the mid-secretory phase of the menstrual cycle.⁶² In view of overproduction of estrogen and proinflammatory cytokines in endometriosis² and our recent finding that higher SLIT/ROBO1 immunoreactivity is a likely constituent biomarker for recurrence of ovarian endometriomas,²⁹ these data suggest that Slit2 might be important in the development of endometriosis, likely involved in the angiogenesis of ectopic implants. While Slit2 may likely be involved in neoangiogenesis in endometriosis, the apparent differential expression patterns between normal and ectopic endometrium may raise the hope that Slit2 may be a tissue or disease-specific endothelial cell marker that could be targeted by vascular-disrupting agents.⁹

The dependence of donor status in SLIT2 and ROBO1 immunoreactivity in ectopic endometrium as we observed is sensible, since the ectopic endometrium came originally from the donor mice and thus the expression of Slit2 and its receptor ROBO1 should resemble that of the original tissue. This also seems to be consistent with the finding from a human–mouse xenograft model of endometriosis, in which the VEGF that stimulates angiogenesis is found to be derived from the human endometrial explants, whereas the vasculature supplying the growing human lesions was found to be of murine origin.¹² In contrast, the dependence on recipient status in SLIT2 immunoreactivity in eutopic endometrium is to be expected since the Slit2 expression in these 2 organs/tissues should not be affected

very much by the origin of the transplanted endometrial implants. The increased VEGF expression in eutopic endometrium in mice with induced endometriosis also is consistent with what is seen in humans.⁶³ What is interesting, but also somewhat puzzling, is that VEGF expression levels in eutopic endometrium also depend on the recipient status in addition to the presence of endometriosis. In fact, the dependence on the recipient status is seen also in ectopic endometrium. Whether this signals some interaction between SLIT2 and VEGF signaling is unclear and warrants future investigation.

The dependence of hotplate latency after induction of endometriosis on the baseline level and the presence of endometriosis are consistent with our previous report in a similar mouse model of endometriosis.⁴¹ The reduced hotplate latency as a result of endometriosis is consistent with endometriosis-induced vaginal hyperalgesia in rats as manifested by heightened sensitivity to vaginal distention⁶⁴ and also is consistent with our recent finding that women with endometriosis have generalized hyperalgesia when compared with women without.⁶⁵ We note that the endometriosis-induced hyperalgesia was independent of Slit2 expression.

In contrast, the tail-flick response latency does not seem to deteriorate as a result of endometriosis. This discrepancy may stem from the difference in nociceptive reaction between the 2 stimuli: it is well known that the hotplate test elicits reactions involves spinal–brain stem–spinal reflexes while the tail-flick test elicits the spinal reflexes.⁶⁶ That is, the hotplate stimulation elicits supraspinal responses, whereas tail-flick thermal stimulation elicits subspinal response.

While Slit2 interacts with Robo1 to mediate repulsive cues in axon guidance and neuronal migration,^{21,67} in our study, we found that Slit2 overexpression did not impact on generalized hyperalgesia resulting from induced endometriosis. In fact, the extent of hyperalgesia did not correlate with the lesion size, which seems to be consistent with the consensus that there is a poor correlation between pain severity and endometriosis stage or lesion type.⁶⁸ The homogeneity in the way in inducing endometriosis in these mice aside, our data appear to suggest that Slit2 overexpression in induced endometriosis does not affect the level of generalized hyperalgesia.

In summary, we found that Slit2 overexpression results in increased size of the endometrial implants in induced endometriosis, with concomitant increased angiogenesis as measured by MVD. These findings, in conjunction with the role of SLIT/ROBO1 in recurrence of endometriosis,²⁹ the correlation of SLIT immunoreactivity and the severity of dysmenorrhea in adenomyosis⁶⁹ strongly suggest that Slit2 may play an important role in angiogenesis in endometriosis and may be a therapeutic target in treating endometriosis.

Authors' Note

SWG conceived and designed the entire study, carried out data analysis and interpretation, drafted the manuscript, and revised it following reviewers' comments; YZ performed the IHC and pain testing and participated in writing; YL carried out the

entire experiment and participated in writing; XSL participated in study design and writing; JGG provided the transgenic mice and participated in writing; all authors approved the final version of this manuscript.

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