Slit-2 Induces a Tumor-suppressive Effect by Regulating β-Catenin in Breast Cancer Cells*

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SLIT-2 is considered as a candidate tumor suppressor gene, because it is frequently inactivated in various cancers due to hypermethylation of its promoter region and allelic loss. However, the exact mechanism of its tumor-suppressive effect has not been elucidated. Here, we observed that Slit-2-overexpressing breast cancer cells exhibited decreased proliferation and migration capabilities compared with control cells under in vitro conditions. These results were confirmed in vivo in mouse model systems. Mice injected with MCF-7/Slit-2 cells showed a 60–70% reduction in tumor size compared with mice injected with MCF-7/VC cells both in the absence and presence of estrogen. Upon further elucidation, we observed that Slit-2 mediates the tumor-suppressive effect via a coordinated regulation of the β-catenin and PI3K signaling pathways and by enhancing β-catenin/E-cadherin-mediated cell-cell adhesion. Our study for the first time reveals that Slit-2-overexpressing breast cancer cells exhibit tumor suppressor capabilities through the novel mechanism of β-catenin modulation.

Slit-2, a protein that belongs to the Slit family of large extracellular matrix-secreted glycoproteins, has been shown to exhibit tumor-suppressive effects in various human cancers (1–7). However, the exact mechanism of its tumor suppressor ability is not well characterized. SLIT-2 is located in chromosome 4p15.2 and encodes the human orthologue of the Drosophila Slit-2 protein (8). Slit consists of a family of 3 genes (SLIT-1, SLIT-2, and SLIT-3), and these genes are candidate ligands for the repulsive guidance receptors, members of the ROBO gene family (1, 9–12). Slit-2 has four leucine-rich repeats at its N-terminal end, followed by nine epidermal growth factor (EGF) repeats, a laminin G domain, and a cysteine-rich C-terminal region (13, 14). Slit-2 interacts with its receptor, Roundabout-1 (Robo-1), through its leucine-rich repeat domain (13). Initially, the role of the Slit/Robo complex was described in the nervous system, where it regulated axon guidance, branching, and neural migration (9, 15–18). Recently, the importance of the Slit/Robo pathway was reported during the embryonic development of various organs such as the lung, kidney, and heart and systems such as the CNS (1, 19–23). Slit-2 expression has also been detected in normal skin, as well as in epithelial and endothelial cells (12, 20, 24–27). These results suggest that Slit/Robo may be involved in the regulation of organogenesis.

SLIT-2 promoter region hypermethylation has been detected in various cancers such as breast cancer, non-small cell lung cancer, small cell lung cancer, colorectal carcinoma, and gliomas (2–7, 28). It has been also shown that, in various cancer types, Robo expression is also altered (19, 29). This indicates that Slit/Robo signaling may play important roles in cancer development. Mutation studies and expression analysis of the SLIT-2 gene have revealed loss of heterozygosity, a couple of missense substitutions in one of the EGF-like domains, and rearrangement at its genomic locus (4p15.2) in a significant proportion of human cancers (3–6). Moreover, primary breast tumors and a majority of breast tumor cell lines have been reported to exhibit reduced or absent SLIT-2 expression (3). Furthermore, analysis of the Slit-2 promoter region in these tumor cells showed the presence of extensive hypermethylation of the SLIT-2 5′-cytosine-guanine (CpG) island (3). Lack or reduced expression of SLIT-2 was correlated with CpG hypermethylation, and further treatment with the demethylating agent 5′-azacytidine restored SLIT-2 expression (3). In addition, Slit-2-overexpressing breast cancer cells or Slit-2-treated breast cancer cells showed decreased colony formation (3).

Although these studies indicate that SLIT-2 can act as a tumor suppressor gene, its tumor-suppressive effect under in vivo conditions and the exact mechanisms of its anti-tumor property are not yet known. One of the pathways through which Slit-2 mediates its function may be by modulating β-catenin/wnt signaling, because the Slit family of proteins were identified as conserved targets of the β-catenin/wnt signaling pathway and interaction between Slit/Robo signaling and wnt signaling was observed during ureteric bud development (30, 31). β-Catenin is a critical regulatory molecule of the wnt signaling pathway and also plays an important role in cad-

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§ The abbreviations used are: EGF, epidermal growth factor; Robo-1, Roundabout-1; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’ medium; siRNA, small interference RNA; PBS, phosphate-buffered saline; TCF, T-cell factor; LEF, lymphoid enhancer factor; VC, vector control; GSK-3β, glycogen synthase kinase-3β; SCID, severe combined immunodeficiency disease; DAPI, 4′,6-diamidino-2-phenylindole.
herin-based cell-cell adhesion by indirectly linking cadherins to the actin cytoskeleton (32, 33). In the cytoplasm, serine and threonine phosphorylation regulated the stability of β-catenin by targeting it to GSK-3β/Axin/adenomatous polyposis coli complex-mediated proteasomal degradation or its translocation to the nucleus. In the nucleus, β-catenin interacts with members of the LEF/TCF family of transcriptional activators (34, 35). Several β-catenin/TCF target genes (such as matrix metalloproteases, cytoskeletal proteins, and angiogenic cytokines) have been implicated in cancer progression (36–41). Thus, β-catenin is considered to be an oncogene, and its deregulation or mutational activation can lead to cancer (42–44). Furthermore, up-regulation of β-catenin has been shown in various cancer tissues, including breast cancer (42).

Here, we report for the first time that the overexpression of Slit-2 in breast cancer cells inhibits breast cancer growth in two in vivo mouse model systems, both in the absence and presence of estrogen. We have also shown that Slit-2 induces its tumor suppressor activity by a coordinated regulation of the β-catenin/TCF and PI3K/Akt signaling pathways as well as by enhancing β-catenin/E-cadherin-mediated cell-cell adhesion.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The Slit-2-overexpressing MCF-7 (MCF-7/Slit-2) stable cell line and vector control cell line (MCF-7/VC) were provided by Dr. Farida Latif (University of Birmingham, Birmingham, UK). These cells were maintained in DMEM containing 10% fetal bovine serum, 50 µg/ml Zeocin, and 1% penicillin-streptomycin at 37°C in 5% CO₂ (3). MDA-MB-231 cells were generously provided by Hava Avraham (Beth Israel Deaconess Medical Center, Boston) and maintained in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO₂.

**Microarray Analysis**—Total RNA was collected from both MCF-7/Slit-2 and MCF-7/VC cell lines by using TRIZol reagent (Invitrogen), per the manufacturer’s protocol. Microarray analysis was performed at the Beth Israel Deaconess Medical Center genomics core facility by using an Affymetrix Microarray gene chip containing 40,000 human genes.

**Proliferation Assay**—Cells (5 × 10⁴), seeded on 96-well plates, were cultured in DMEM supplemented with 0.2% fetal bovine serum and 50 ng/ml EGF. The number of viable cells was quantified at various time points by using the CellTiter 96® Aqueous kit (Promega, Madison, WI), as per the manufacturer’s instructions.

**Chemotaxis Assay**—A chemotaxis assay was performed in 24-well cell culture chambers with 8-µm pore inserts, as described previously (45). Membranes were pre-coated with fibronectin (2.5 µg/ml). Breast cancer cells were suspended in chemotaxis buffer (DMEM/0.1% bovine serum albumin/12 mM HEPES) at 2.5 × 10⁶ cells/ml. 150 µl of cell suspension was loaded onto the upper chamber. 0.6 ml of medium containing CXCL12 (5 nM) was then loaded onto the lower chamber. The plates were incubated for 6 h at 37°C in 5% CO₂. After incubation, the inserts were removed carefully and cells were fixed, stained, and counted using standard procedures.

**Soft-agar Colony Formation Assay**—Cells (2 × 10⁴) suspended in medium containing 0.4% Ultra pure agarose (Invitrogen) were layered on top of 1 ml of medium containing 0.8% agarose in 6-well culture plates. Cells were incubated with 1 ml of DMEM supplemented with 10% fetal bovine serum. After 2 weeks of incubation, the colonies were stained with 0.005% Crystal Violet for 1 h, and colonies were counted by using a Nikon Diaphot 300 inverted microscope.

**Immunoprecipitation and Western Blot Analysis**—Immunoprecipitation and Western blot analysis were done as described elsewhere (45). According to the particular experiment, equal amounts of protein (1 mg) were bound with specific antibodies. Then, the immune complexes were precipitated with Protein A-Sepharose beads (Amersham Biosciences) and were Western blotted with the relevant antibodies.

**Extractions of Nuclear and Cytoplasmic Fractions**—Nuclear and cytoplasmic fractions were collected by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce), as per the manufacturer’s protocol. The purity of fractionation was determined with Oct-1 antibody.

**Small Interfering RNA (siRNA)-mediated Knockdown**—siRNA-mediated knockdown of β-catenin and Robo-1 was performed using β-catenin SMART pool siRNA reagents and ON-TARGETplus SMARTpool Robo-1 siRNA reagents (Dharmacon, Inc., Boulder, CO) respectively, according to the manufacturer’s protocol. Briefly, MCF-7 cells were electroporated with 100 nM siRNA using the Amaxa Nucleofector™ device (Amaxa Biosystems, Cologne, Germany), as per the manufacturer’s protocol. The respective non-targeted siRNA SMARTpool was used as a control. β-Catenin siRNA-mediated knockdown was estimated by detecting β-catenin expression 48 h after the initial transfection by using Western blot analysis. Robo-1 siRNA-mediated knockdown was estimated by detecting Robo-1 receptor expression 48 h after the initial transfection by using flow cytometry.

** Constructs and Transfections**—ORF Clone of Homo sapiens slit homolog 2 (Drosophila) (SLIT2) was purchased from OriGene Technologies Inc. (Rockville, MD) and subcloned into pcDNA 3.1/V5-His Vector (Invitrogen). 2 µg of pcDNA 3.1/V5-His-SLIT-2 plasmid was mixed with the cellular suspensions, transferred to a 2.0-mm electroporation cuvette, and nucleofected using the Amaxa Nucleofector™ device (Amaxa Biosystems) as per manufacturer’s protocol. Transfection efficiency was monitored by Western blotting procedures by using anti-V5 antibody (Invitrogen).

**Confocal Microscopy**—MCF-7/VC and MCF-7/Slit-2 cells were cultured in chamber slides. Then slides were fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were washed thrice with PBS, blocked with 5% normal goat serum in PBS/Triton for 60 min, and treated with rabbit anti-β-catenin antibody (Cell Signaling Technology, Beverly, MA), and monoclonal anti-E-cadherin antibody (Chemicon, Temecula, CA) overnight at 4°C. The slides were washed thrice with PBS and stained with Alexa Fluor 568-labeled anti-rabbit IgG antibodies, Alexa Fluor 568-labeled anti-mouse IgG antibody (Molecular Probes, Eugene, OR), or fluorescein isothiocyanate-labeled anti-rabbit IgG antibody. The cells were washed thrice in PBS, and slides were mounted by using Pro-long Gold Antifade with DAPI (Invitrogen). The cells were then examined under a Zeiss confocal microscope, and the images were acquired by using LSM510 software. Nuclear staining was
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FIGURE 1. Slit-2-overexpressing MCF-7 cells show decreased proliferation and chemotactic capability. Slit-2 expression (A, upper panel) and Robo-1 expression (A, second panel) in MCF-7/VC and MCF-7/Slit-2 clones were analyzed by Western blotting using anti-c-myc and anti-Robo-1 antibodies. Equal protein was confirmed in each sample by stripping and re-probing the blot with anti-β-Actin antibody (A, third panel). Slit-2-myc expression also analyzed in cell culture supernatant concentrates. Cell culture supernatants were concentrated by using Amicon Ultra-100K filters (A, lower panel). B, cells were subjected to a proliferation assay after seeding in 96-well plates in DMEM supplemented with 0.2% fetal bovine serum and 50 ng/ml EGF. Then the number of viable cells was quantified at various time points by using the CellTiter 96®Aqueous kit (Promega), as per the manufacturer’s instructions. C, cells were subjected to chemotaxis assay toward CXCL12 (50 ng/ml) by using chemotaxis chambers as described under "Experimental Procedures." D and E, for the soft agar colony formation assay, cells were mixed in medium containing 0.4% Ultra pure agarose (Invitrogen) and layered on top of 1 ml of medium containing 0.8% agarose in 6-well culture plates. Then cells were incubated with 1 ml of DMEM supplemented with 10% fetal bovine serum. After 2 weeks of incubation, the colonies were stained with 0.005% Crystal Violet for 1 h and counted by using a Nikon Diaphot 300 inverted microscope. All of the above experiments were repeated three times, and a representative one is shown. LPF = low power field. *p < 0.05 for all experiments. VC, Vector Control-MCF-7; Slit-2 (c1), Slit-2-overexpressing MCF-7 clone 1; Slit-2 (c2), Slit-2-overexpressing MCF-7 clone 2; SN, cell supernatant concentrate.

Luciferase Reporter Assay—To determine luciferase reporter activity, TCF luciferase constructs (0.5 μg), containing the wild-type (pTOPFLASH) or mutant (pFOPFLASH; Upstate, Charlottesville, VA) TCF binding sites, were transfected into MCF-7/VC and MCF-7/Slit-2 cells (5 × 10^5 per well). Transfection experiments were carried out in triplicate using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. In addition, the cells were cotransfected with an internal control (0.1 μg of pRL-TK Renilla luciferase vector, Promega, Madison, WI). The cells were incubated for 48 h after the transfection and then treated with EGF (100 ng/ml) for 24 h. The cells were washed with PBS and lysed in 1× passive lysis buffer (Dual Luciferase kit, Promega). To measure the activities of firefly and Renilla luciferase, 20-μl aliquots of the supernatant were transferred into a 96-well plate and assayed in a Wallac Victor® counter luminometer (PerkinElmer Life Sciences) using reagents from the Dual Luciferase kit (Promega). The firefly (TOPFLASH or FOPFLASH) luciferase activity was corrected for Renilla luciferase activity (pRL-TK) to control for transfection efficiency. TOPFLASH activity was also normalized to the FOPFLASH activity. Data are expressed as the mean of triplicate values of the normalized TOPFLASH activity.

In Vivo Tumor Models—Female CB17/SCID and nude (nu/nu) mice, obtained from Charles River Laboratories (Wilmington, MA), were housed under specific pathogen-free conditions. The in vivo experiments were performed in accordance with the guidelines of our Institutional Animal Care and Use Committee. Cells from each stable cell line were concentrated to 3 × 10^6 per 200 μl and injected subcutaneously into the flank of each mouse. One group of mice was injected subcutaneously with 2.5 μg of β-estradiol 17-valerate in 50 μl of Sesame oil twice a week. Tumor size was assessed once a week, and tumor volume was calculated. At the end of the 4th week for the SCID mice and 6th week for nude mice, the animals were scanned under a Micro CT scanner (GE eXplore Locus Micro CT), and the final tumor volume was assessed. Tumors were excised, minced homogenized in modified RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100, and a mixture protease inhibitors, Roche Applied Science), and clarified by centrifugation at 13,000 × g for 15 min at 4 °C. Supernatants were subjected to Western blot analysis.

RESULTS

In this study, to analyze the tumor suppressor property of Slit-2 gene, we have used myc-tagged human SLIT-2 plasmid stably expressing MCF-7 breast cancer cells. Initially, we confirmed Slit-2 overexpression in two clones of Slit-2-overexpressing MCF-7 cells. Both cell lysates (Fig. 1A, upper panel) showed the overexpression of Slit-2-myc. Clone 2 exhibited more Slit-2-myc expression than clone 1. Because Slit-2 mediates its function by binding to specific receptor roundabout-1 (Robo-1), we also analyzed the expression of this receptor in control and Slit-2-overexpressing MCF-7 cells and observed that MCF-7 cells and Slit-2-overexpressing MCF-7 cells express moderate level of Robo-1 receptor (Fig. 1A, middle panel). Because Slit-2 is a secretory protein we also confirmed its expression in cell supernatant concentrates of both clones (Fig. 1A, lower panel).

Slit-2-overexpressing Cells Exhibit Inhibited Proliferation and Migration—Incorporated proliferation, migration, and adhesion are important properties of tumorigenesis and the metastasis of cancer cells (46). Dallol et al. (3) have shown that Slit-2-overexpressing breast cancer cells and breast cancer cells
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MCF-7/VC cells, which had a tumor volume of ~650–800 mm³. Because MCF-7 cells are estrogen receptor-positive, we further examined whether Slit-2 can overcome the tumor-sustaining effects of estrogen. For this study, we injected mice with 2.5 μg of β-estradiol 17-valerate twice weekly starting from 1 week prior to cell injection. Although tumor volume in mice injected with the MCF-7/VC cells was high (~1050–1200 mm³), the tumor volume in mice injected with the MCF-7/Slit-2 cells was much lower (250–320 mm³). We confirmed these results by repeating the experiments in nude mice (nu/nu) (Fig. 3B). SCID and nude mice injected with the MCF-7/Slit-2 cells also showed a drastic decrease in tumor size even in the presence of estrogen as compared with mice injected with the control cells (MCF-7/VC) (Fig. 3, C–G).

Microarray Analysis—To investigate the mechanisms involved in the Slit-2-mediated tumor-suppressive effect, we performed microarray analysis by using a gene chip containing 40,000 human genes. Among the various genes that are downregulated in MCF-7/Slit-2 cells compared with control cells (MCF-7/VC), we selected the β-catenin/TCF family of transcription factors for our further investigation and then confirmed these results by using Western blot analysis.

Slit-2 overexpression in MCF-7 cells shows increased degradation of β-catenin—β-catenin/LEF/TCF transcriptional activity has been shown to regulate the expression of a subset of genes that promote tumor establishment, growth, and invasion (34–38, 47). Increased levels of β-catenin within the cytoplasm and/or in the nucleus were observed in breast cancer cells (48, 49). Further, mammalian Slit family proteins have also been identified as evolutionarily conserved targets of the wnt/β-catenin signaling pathway (30). When we analyzed the expression of β-catenin in the total cell lysates of both vector control and Slit-2 overexpressing MCF-7 cells, we observed a decrease in β-catenin expression in the Slit-2 overexpressing MCF-7 clones (Fig. 4A). Because we found drastic down-regulation of β-catenin in clone 2, we used clone 2 for our further experiments. Upon further analysis of the mechanisms involved in the decreased expression of β-catenin, we observed an increase in β-catenin phosphorylation at the serine 45 site (Fig. 4B). It has been shown that β-catenin phosphorylation at serine 45 initiates the phosphorylation of other serine and threonine residues, which are necessary for the ubiquitination and proteasomal-mediated degradation of β-catenin (50). Our coimmunoprecipitation study revealed an increase in both the association of GSK-3β with β-catenin and in the ubiquitination of β-catenin in MCF-7/Slit-2 cells compared with MCF-7/VC cells (Fig. 4, C and D). These results indicate that there is increased proteasomal degradation of β-catenin in the Slit-2 overexpressing cells compared with vector control cells.

To correlate that β-catenin down-regulation is responsible for inhibited soft agar colony formation, we knocked down β-catenin from MCF-7 cells by using an siRNA technique (Fig. 4E) and performed a soft agar colony formation assay. As shown in Fig. 4F, β-catenin knock-down MCF-7 cells exhibited decreased colony-forming ability compared with non-targeted siRNA-transfected cells. Further, we also analyzed β-catenin expression in lysates of tumor derived from MCF-7/VC- and MCF-7/Slit-2 (c2)-injected mice by Western blot analysis and
Slit-2 overexpression suppresses the tumorigenic capability of MCF-7 breast cancer cells in vivo models. MCF-7/VC and MCF-7/Slit-2 (c2) cells were injected (3 × 10^6 cells) into the flanks of female SCID mice (n = 5). One group of mice was injected subcutaneously with 2.5 μg of β-estradiol 17-valerate in 50 μl of Sesame oil twice a week. Tumor size was measured by using digital calipers once a week, and volume was determined according to the formula (W^2 × L)/2. A, the graph represents the tumor volume in mice injected with MCF-7/VC or MCF-7/Slit-2 (c2) cells with or without estrogen treatment. Tumor volume was assessed up until 5 weeks after injection. B, the experiments in panel A were repeated in nude mice with or without estrogen treatment. Tumor size was measured by using digital calipers once a week for 6 weeks, and volume was determined as indicated above. At the end of the fifth week in the case of SCID mice (G) and at the end of the sixth week in the case of nude mice (D), the tumor volume was analyzed and calculated by using the Micro CT scan imaging system. *p < 0.05 for all experiments. E, photographs of the tumors derived from SCID mice 5 weeks after the injection of MCF-7/VC or MCF-7/Slit-2 (c2) cells in the presence or absence of estrogen. F, photographs of representative nude mice 6 weeks after the injection of MCF-7/VC or MCF-7/Slit-2 (c2) cells in the presence or absence of estrogen. G, Micro CT-scanned photographs of SCID mice 5 weeks after the injection of MCF-7/VC or MCF-7/Slit-2 (c2) cells in the presence or absence of estrogen. UN = untreated.
It has been shown that EGF stimulation reduced TOPFLASH activity by 47% compared with vector control-transfected cells (Fig. 7). The Slit-2-overexpressing MCF-7 cells also showed decreased expression of β-catenin and cyclin D1 (Fig. 7C) and increased β-catenin/E-cadherin association (Fig. 7D) compared with vector control-transfected cells.

Slit-2 Overexpression Also Inhibits Akt and GSK-3β Phosphorylation in Breast Cancer Cells—The coordination of β-catenin pathways and PI3K pathways has been reported in breast and lung cancer cells. Furthermore, the PI3K/Akt pathway also plays an important role in the stabilization of β-catenin by regulating GSK-3β activity (52, 53). In our previous study, we observed that exogenous Slit-2 stimulation inhibits PI3K activity (45). In the present study, we analyzed EGF-induced Akt phosphorylation and observed that the phosphorylation of Akt was significantly inhibited in the Slit-2-overexpressing cells compared with vector control cells (Fig. 8A). We also analyzed the phosphorylation of GSK-3β, which is a substrate of Akt, and found that its phosphorylation was decreased in the MCF-7/Slit-2 cells (Fig. 8B). Hence, it appears that, in Slit-2-overexpressing cells, the decreased activity of Akt results in the decreased phosphorylation of GSK-3β. Interestingly, the dephosphorylated form of GSK-3β has been shown to possess enhanced β-catenin phosphorlyating activity (54).

DISCUSSION

The Slit family of large extracellular matrix-secreted and membrane-associated glycoproteins are known to be crucial regulators of the repulsive cues on axons and growth cones during neural development (1, 3–7, 9, 14). Recently, Slit-2 has been proposed as a "tumor suppressor gene" because of its decreased expression and loss of heterozygosity in most of the human cancers, including breast cancer, due to hypermethylation at the CpG islands of its promoter region (Sundareshan et al. 55). In addition, the SLIT-2 gene is mapped to chromosome 4p15.2 and ~63% of breast tumors also show loss of heterozygosity at the 4p15.1–15.3 region (3–5). Recent studies have confirmed that epigenetic events such as hypermethylation of CpG sites in regulatory regions may be critical alternative mechanisms of tumor suppressor gene inactivation (56). These studies indicate that Slit-2 appears to function as a novel tumor suppressor gene. However, the exact mechanisms of its tumor suppressive function are not well defined.

In the present study, we characterized the tumor-suppressive effect of the SLIT-2 gene in Slit-2-overexpressing MCF-7 breast cell lines. We further confirmed some of signaling results in Slit-2 transiently overexpressing MDA-MB-231 cells. Slit-2-transfected MDA-MB-231 cells showed decreased expression of β-catenin and cyclin D1 (Fig. 7C) and increased β-catenin/E-cadherin association (Fig. 7D) compared with vector control-transfected cells.

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For the experiments, we used MCF-7 breast cancer cells and Slit-2 transiently expressing MDA-MB-231 cells. We observed a decreased rate of proliferation in Slit-2-overexpressing cells compared with control cells by using an in vitro proliferation assay. We also confirmed this phenomenon in a soft agar colony forming assay. This assay revealed that Slit-2-overexpressing cells almost lost their colony-forming ability. Dallol et al. (3) have demonstrated nearly similar results by showing that Slit-2-conditioned medium suppresses the growth of several breast cancer cell lines. Furthermore, our Robo-1 knockdown experiment suggests that Slit-2 may exert its function in an autocrine manner. We have also analyzed the tumorigenic effect of Slit-2-overexpressing cells in mouse model systems. Slit-2-overexpressing cells showed a remarkable decrease in their tumorigenic capability compared with control cells when xenografted into SCID mice. Several previous studies have demonstrated that continuous estrogen supplementation sustains and enhances the tumorigenic effect of MCF-7 cells in nude mice (57). Our study in both SCID and nude mice indicates that Slit-2 overexpression significantly overcomes the tumor-enhancing and -sustaining effects of estrogen by exhibiting a marked inhibition of tumor size even in the presence of estrogen. These results provide further evidence for the antitumor activities of the SLIT-2 gene.

Upon exploring the mechanisms of the tumor-suppressive function of the SLIT-2 gene, we observed the decreased expression of β-catenin in Slit-2-overexpressing MCF-7 cells. Elevated levels of β-catenin have been observed in various human cancers, including breast cancer. Moreover, β-catenin has been linked with a poor prognosis in human adenocarcinomas (58). Mammalian Slit proteins have been suggested as evolutionarily conserved targets of the wnt/β-catenin signaling pathway (30). Recently, it has become more evident that β-catenin plays a dual function.

FIGURE 5. Slit-2-overexpressing cells show decreased nuclear translocation of β-catenin as well as increased expression of E-cadherin, enhanced intercellular adhesions, and association between β-catenin and E-cadherin. Nuclear extracts (NE) and cytoplasmic extracts (CE) were collected from both MCF-7/VC and MCF-7/Slit-2 cells by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) as per the manufacturer’s protocol. The extracts were subjected to Western blotting using anti-β-catenin antibody (A, upper panel). The purity of fractionation and equal loading of protein in each lane were determined with Oct-1 antibody (A, lower panel). β-catenin is a pivotal component of adherens junctions and is regulated by the Wnt signaling pathway. A decrease in nuclear β-catenin has become more evident when Slit-2 is overexpressed. We confirmed that this decrease in nuclear β-catenin was not due to a decrease in total β-catenin levels by Western blotting (A). B, MCF-7/VC and MCF-7/Slit-2 cells were cultured in chamber slides. Cells were fixed and treated with rabbit anti-β-catenin antibody. After washing, cells were probed with Alexa 568-tagged anti-rabbit IgG antibody, and the slides were mounted by using Prolong Gold Antifade with DAPI (Invitrogen), and then examined under a Zeiss confocal microscope. The percentage of nuclear fluorescent staining was calculated by considering the fluorescence of the MCF-7/VC cells as 100%. Both MCF-7/Slit-2 and MCF-7/VC cells were lysed, and the cell lysates were immunoprecipitated with anti-β-catenin antibody and Western blotted with anti-E-cadherin antibody (D, upper panel). Equal protein was confirmed in each sample by stripping and re-probing the blot with anti-β-catenin antibody (D, lower panel). All of the above experiments were repeated three times, and a representative one is shown.

We also confirmed this phenomenon in a soft agar colony forming assay. This assay revealed that Slit-2-overexpressing cells almost lost their colony-forming ability. Dallol et al. (3) have demonstrated nearly similar results by showing that Slit-2-conditioned medium suppresses the growth of several breast cancer cell lines. Furthermore, our Robo-1 knockdown experiment suggests that Slit-2 may exert its function in an autocrine manner. We have also analyzed the tumorigenic effect of Slit-2-overexpressing cells in mouse model systems. Slit-2-overexpressing cells showed a remarkable decrease in their tumorigenic capability compared with control cells when xenografted into SCID mice. Several previous studies have demonstrated that continuous estrogen supplementation sustains and enhances the tumorigenic effect of MCF-7 cells in nude mice (57). Our study in both SCID and nude mice indicates that Slit-2 overexpression significantly overcomes the tumor-enhancing and -sustaining effects of estrogen by exhibiting a marked inhibition of tumor size even in the presence of estrogen. These results provide further evidence for the antitumor activities of the SLIT-2 gene.

Upon exploring the mechanisms of the tumor-suppressive function of the SLIT-2 gene, we observed the decreased expression of β-catenin in Slit-2-overexpressing MCF-7 cells. Elevated levels of β-catenin have been observed in various human cancers, including breast cancer. Moreover, β-catenin has been linked with a poor prognosis in human adenocarcinomas (58). Mammalian Slit proteins have been suggested as evolutionarily conserved targets of the wnt/β-catenin signaling pathway (30). Recently, it has become more evident that β-catenin plays a dual function.
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within the cells. In addition to its structural role of associating with the E-cadherin/actin cytoskeletal system during the regulation of cell-cell adhesion, β-catenin can act as a transcription factor along with the TCF/LEF family of DNA-binding proteins (34, 35). Increased levels of β-catenin within the cytoplasm and/or nucleus in tumor cells are suggestive of stabilization of the β-catenin protein and can result in enhanced β-catenin-mediated transcription (36–38, 47).

In our study, we observed the increased phosphorylation of β-catenin at its Ser-45 phosphorylation site. It has been established that Ser-45 phosphorylation by casein kinase I initiates phosphorylation at Thr-41, Ser-37, and Ser-33 by GSK-3β, and these sites are recognized by the ubiquitin ligase complex that mediates β-catenin degradation (50). Furthermore, we observed an increased association of β-catenin with GSK-3β and enhanced ubiquitination in Slit-2-overexpressing MCF-7 cells. These results confirmed that there is an increased degradation of β-catenin in the Slit-2-overexpressing cells, resulting in the reduced cytosolic concentration and decreased nuclear translocation of β-catenin in these cells. Additionally, our luciferase gene reporter assay revealed inhibition of β-catenin/TCF transcriptional activity in the Slit-2-overexpressing cells. Further, upon analysis of the expression of various β-catenin/TCF genes, we found decreased expression of cyclin D1, MMP-2, and MMP-9 in the Slit-2-overexpressing cells. These genes have been identified as critical mediators of proliferation, invasiveness, and tumor progression (36–38, 47). Slit-2 also blocked the expression of the TCF-4 transcription factor, which is responsible for activation of vimentin, a crucial mediator of epithelial-mesenchymal transition (59).

In many types of human cancer, it has been reported that the transcriptional activity of β-catenin is regulated either through
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the wnt/wingless-dependent pathway or via independent signaling pathways (35, 60). In our previous studies, we showed that Slit-2 inhibits PI3K and Akt phosphorylation (45). Akt has been demonstrated to phosphorylate GSK-3β. Our present study indicates that Slit-2-mediated inhibition of Akt activation might result in the decreased phosphorylation of GSK-3β, a substrate of Akt. Dephosphorylated GSK-3β is an active form that phosphorylates β-catenin, leading to its degradation in the ubiquitin-dependent proteasome pathway. Our study suggests that Slit-2 may regulate β-catenin function through a coordinated regulation of the β-catenin and PI3K pathways.

Recently, the β-catenin and PI3K signaling pathways have also been implicated in the regulation of cell-cell adhesion (58). Cell-cell adhesion, which is mediated by cadherins and catenins, is fundamentally involved in the organization of epithelial tissues (61). Loss of intercellular adhesion has been identified as a key process in the development of an aggressive tumor cell phenotype (61). This alteration of tumor cell phenotype is mediated through the expression and regulation of E-cadherin. Decreased expression of E-cadherin signifies the transformation of epithelial cells to a more invasive mesenchymal phenotype (58). Moreover, the Slit/Robo complex is known to regulate β-catenin/N-cadherin-mediated cell-cell adhesion in neuronal cells (62, 63). In our study, we observed increased localization of E-cadherin in cell borders at sites of cell-cell adhesion and its enhanced association with β-catenin in Slit-2-overexpressing cells. Moreover, we also observed increased translocation of β-catenin to the membrane in Slit-2-overexpressing cells. Slit-2-mediated up-regulation of E-cadherin might also play an important role in the mechanism to sequester β-catenin at the plasma membrane and may halt β-catenin transport to the nucleus. Further confirmation of some of the functional and signaling data in Slit-2 transiently expressing MDA-MB-231 cells indicates that the tumor-suppressive effect of Slit-2 is due to Slit-2 overexpression rather than clonal variation or heterogeneity of breast cancer cell lines.

We demonstrate, in two mouse model systems, that Slit-2 overexpression induces tumor suppressor activity in breast cancer cells. Furthermore, we show that Slit-2 mediates its tumor-suppressive effect via a novel mechanism, through the coordinated regulation of the β-catenin/TCF and PI3K/Akt signaling pathways and by enhancing cell-cell adhesion.

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