Activation of Robo1 signaling of breast cancer cells by Slit2 from stromal fibroblast restrains tumorigenesis via blocking PI3K/Akt/β-catenin pathway

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Abstract

Tumor microenvironment plays a critical role in regulating tumor progression by secreting factors that mediate cancer cell growth. Stromal fibroblasts can promote tumor growth through paracrine factors; however, restraint of malignant carcinoma progression by the microenvironment also has been observed. The mechanisms that underlie this paradox remain unknown. Here, we report that the tumorigenic potential of breast cancer cells is determined by an interaction between the Robo1 receptor and its ligand Slit2, which is secreted by stromal fibroblasts. The presence of an active Slit2/Robo1 signal blocks the translocation of β-catenin into nucleus, leading to down-regulation of c-myc and cyclin D1 via the PI3K pathway. Clinically, high Robo1 expression in the breast cancer cells correlates with increased survival in breast cancer patients, and low Slit2 expression in the stromal fibroblasts is associated with lymph node metastasis. Together, our findings explain how a specific tumor microenvironment can restrain a given type of cancer cell from progression and demonstrate that both stromal fibroblasts and tumor cell heterogeneity affect breast cancer outcomes.
Introduction

The development of mammary gland requires interplay of multiple cell types including luminal, epithelial and myoepithelial cells, as well as stromal cells composed of fibroblasts, endothelial cells, adipocytes and immune cells. Previous studies have demonstrated that surrounding stroma is essential for normal mammary gland development such as proliferation, differentiation and branching (1, 2). For example, amphiregulin-producing epithelial cells elicit paracrine activation of EGFR in stromal cells to dictate mammary ductal morphogenesis (3), and TGF-β mediates inhibition of mammary ductal and alveolar growth via epithelial-stromal interaction (4).

Stromal cells also play a critical role in tumor initiation and progression (5). Especially, stromal fibroblasts have a predominant role in cancer progression (6, 7). It is well established that tumor associated stromal fibroblasts secrete high levels of HGF and SDF-1 to stimulate cancer cell growth through its receptors c-Met and CXCR4, respectively (8-10). TGFβ and metalloproteinase are also released from fibroblasts to promote tumorigenesis, regardless the normal function of TGFβ in inhibiting mammary gland growth (11, 12). Although most studies demonstrate that stromal cells positively regulate cancer growth, it has been observed that the microenvironment can restrain malignant progression of carcinoma (13, 14). For instance, small, early stage carcinomas have been found in random autopsy sections of prostate tissues from subjects who died of causes unrelated to cancer (15). Such observations suggest that restrained growth of cancer cells is clinically prevalent. However, the mechanisms underlying these observations remain to be elucidated. Here, we demonstrated that expression of Robo1 receptor in breast cancer cells coupling with Slit2 ligand secreted from stromal fibroblasts led to inhibition of tumor progression. These results explain, in part, how a specific microenvironment can restrain a given kind of cancer cell from further progression.
Materials and Methods

Ethics statement

Human breast cancer specimens and normal tissues were collected from National Taiwan University Hospital. All specimens were encoded to protect patients under protocols approved by the Institutional Review Board of Human Subjects Research Ethics Committee of Academia Sinica and National Taiwan University, Taipei, Taiwan. Written informed consent was obtained from each participant.

Cell lines and primary fibroblast isolation

Human breast cancer cell lines BT20, HCC1937, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-468, Hs578T, and SKBR3 were obtained from the American Type Culture Collection and maintained in DMEM or DMEM/F12 supplemented with 10% FBS and antibiotics. Tumor tissues from patients were cut and digested with trypsin (9). Tissues were cultured until fibroblast grew and attached in petri dish. Primary fibroblasts were maintained in DMEM/F12 supplemented with 10% FBS.

Gene expression using microarray analysis, quantitative real-time PCR (qRT-PCR) and immunoblotting

Total RNAs were extracted from breast cancer cell lines and fibroblasts with TRI reagent (Ambion). All cDNA were reversely transcribed with Superscript II transcriptase (Invitrogen). Affymetrix U133 Plus 2.0 human oligonucleotide microarrays were used to detect gene expression in MDA-MB-231, MDA-MB-361, Hs578T and SKBR3 cells. qRT-PCR was performed using ABI Step-One SYBR-Green system (Applied Biosystems) according to the manufacture’s instruction and primers sequences are listed in Supplementary Table 1. For immunoblotting, whole cell lysate was obtained using RIPA lysis buffer. Nuclear and
cytoplasmic proteins were extracted using ProteoJET cytoplasmic and nuclear protein extraction kit (Thermo Scientific, USA). The proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by immunoblotting with various antibodies. Antibodies against β-catenin, α-tubulin, β-actin, p85 and Slit2 were purchased from GeneTex (Irvine, CA), Robo1, Robo2, and Robo4 from Abcam (England), HDAC from Millpore (Billerica, MA) and phospho-Akt (Ser473) from Cell Signaling (Danvers, MA). Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used to detect signals. NIH Image J was used for quantification.

**Soft agar colony formation assay**

Fifty thousand primary fibroblasts were seeded in a 12-well plate and cultured for 2-3 days to reach 60-70% confluence. After washing with PBS, a layer of DMEM/F12/FBS containing 0.5% agar was laid on top of the fibroblasts. 2500 breast cancer cells were seeded in a layer of 0.35% agar in DMEM/FBS over the bottom layer. After 14 to 21 days, crystal violet-stained colonies were counted. For experiments with addition of recombinant protein, 50 ng/ml of rSlit2 was added to the top layer every 3 days.

**Lentivirus expression system**

shRNAs in pLKO-puro vectors were obtained from the National RNAi Core Facility, Taiwan. 293T cells were transfected with pMD.G, pCMVR8.91 and pLKO-puro-shRNA using lipofectamine 2000 (Invitrogen) for lentivirus packaging. For Robo1 overexpression, Lenti-X bicistronic lentiviral vector expression system (Clontech, Mountain view, CA) was used according to the manufacture’s instruction.

**Mouse Tumorigenicity assay**

Animal experimental protocols were approved by the Institutional Animal Care and Utilization
Committee, Academia Sinica. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were implanted with $1-2 \times 10^6$ 199Ct into fat pads a week before injection of $2 \times 10^5$ MDA-MB-231 or $5 \times 10^5$ HCC1937 cancer cells mixed with Matrigel (BD bioscience). Tumor volumes were measured every 4-5 days.

**Immunochemistry (IHC)**

Formalin-fixed paraffin embedded primary tumor tissue sections were used for IHC. Heat induced antigen retrieval was performed using EDTA buffer (Trilogy) at $100^\circ C$. Endogenous peroxidase was eliminated with 3% H$_2$O$_2$. Slides were blocked in PBS containing 10% FBS and then incubated with primary antibodies against Slit2 and $\beta$-catenin overnight at $4^\circ C$. After washing, slides were incubated with HRP Rabbit/mouse polymer before visualization with liquid diaminobenzidine tetrahydrochloride plus substrate DAB chromogen from Dako REAL EnVision (Carpinteria, CA). All slides were counterstained with hematoxylin. For Robo1 staining, rabbit anti-goat IgG Alkaline Phosphatase (Southern Biotech, Birmingham, AL) and NBT/BCIP were used for visualization. Robo1 expression was scored as ‘high’ when more than 50% tumor cells were positive for membrane staining and ‘low’ otherwise.

**Co-immunoprecipitation Assay**

Robo1 overexpressing BT20 cells were serum starved for 6 hr and treated with rSlit2 for 10, 20 and 30 min. Cells were lysed and incubated in lysis buffer (150 mM NaCl, 10 mM Tris pH 7.5, 2 mM Mg$_2$Cl$_2$ and 1% Triton X-100 ) for 30 min at $4^\circ C$. Cell lysates were pre-cleaned with normal mouse/rabbit IgG and A/G beads (Santa Crus) for 2 hr at $4^\circ C$. Immunoprecipitation was performed using antibodies against Robo1, p85 and mouse/rabbit IgG overnight at $4^\circ C$. The
antigen-antibody complex was immobilized with A/G beads for 2 hr at 4°C. After washed with wash buffer (150 mM NaCl, 10 mM Tris pH 7.5, 2 mM MgCl₂ and 0.1% Triton X-100), proteins were eluted by addition of loading dye and boiling at 95°C for 2 min. The proteins were subjected to SDS-PAGE analysis followed by immunoblotting with various antibodies.

**Immunofluorescence**

Control and shRobo1 of MDA-MB-231 cells were seeded on coverslips and treated with rSlit2. The cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, washed with PBS and permeabilized in 0.2% Triton X-100 for 15 min. After washed with PBS, cells were blocked with PBS containing 10% FBS for 2 hr before incubation with the primary antibody as indicated anti-β-catenin overnight at 4°C. The cells were incubated for 1 hr with a fluorochrome-conjugated secondary antibody (Alexa Fluor 488 anti-mouse). Coverslips with stained cells were then washed with PBS, stained with 4’,6-Diamidino-2-phenylindole (DAPI), and mounted onto glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For the primary tumor tissue sections, 100°C EDTA buffer was used for heat induced antigen retrieval. Slides were blocked in PBS containing 10% FBS and then incubated with primary antibodies against Slit2 (Sigma) and α-smooth muscle actin (α-SMA, Dako) overnight at 4°C. The slides were incubated for 1 hr with fluorochrome-conjugated secondary antibody (Alexa Fluor 488 anti-mouse or Alexa Fluor 594 anti-rabbit). Slides were then stained with DAPI, and mounted onto glass slides with mounting medium.

**Statistical methods**

For soft agar colony formation and tumorigenicity assay, all data were presented as means ± SD,
and Student’s t-test was used to compare control and treatment groups. Asterisk (*) indicated statistical significance with \( p \)-value < 0.05. Asterisk (**) indicated statistical significance with \( p \)-value < 0.01. The association between Robo1 gene expression and survival of breast cancer patients was estimated using univariate Cox proportional-hazards regression analysis, while the association among survival, Robo1 gene expression and other clinical predictors including age, tumor size, distant metastasis, lymph-node status, tumor grade, estrogen receptor expression, and Her2/neu expression was analyzed by multivariate Cox regression analysis. Hazard ratio was evaluated using the method of Grambsch and Therneau. No violation of the proportional assumption was detected. The optimal cutoff point of Robo1 gene expression for 5-year survival was determined by ROC analysis. The survival curve and the statistics were generated by Kaplan-Meier method and log-rank test, respectively (16). The correlation between Slit2 gene expression and lymph node metastasis after adjustment for other clinical predictors was estimated using partial Pearson's correlation.

**Results**

Stromal fibroblasts can either suppress or promote tumorigenicity of breast cancer cells

Initially, we determined how stromal fibroblasts affect breast cancer cell tumorigenic activity by performing a series of soft agar colony formation assays using a bottom agar layer containing fibroblasts and an upper agar layer containing breast cancer cells (hereafter referred to as coculture). MDA-MB-231 and SKBR3 were co-cultured with primary fibroblasts (carcinoma-associated fibroblasts, CAF; normal breast-associated fibroblasts, NAF) isolated from breast cancer patients. Surprisingly, two opposite phenotypes were observed that stromal fibroblasts suppressed colony formation in MDA-MB-231 cells but promoted it in SKBR3 (Fig.1A and 1B).
Suppression was also observed in additional breast cancer cell lines including MDA-MB-361, Hs578T and MDA-MB-157 (Fig. 1C), while promotion was observed in BT20, HCC1937 and MDA-MB-468 cell lines (Fig. 1D). Furthermore, these opposite phenotypes were also observed when cancer cells were co-cultured with immortalized human CAFs (199Ct) and human fibroblast cell lines (WI38 and Hs68). The colony formation of MDA-MB-231 was reduced (Fig. 1E), but increased in SKBR3 (Fig. 1F). These results suggested that fibroblasts could either promote or suppress tumorigenic activity of different breast cancer cells.

**Identification of Robo1 as a candidate receptor for fibroblast-associated tumor suppression**

The design of the co-culture system strongly suggested that the suppression mechanism is most likely to be mediated by signaling through soluble factors secreted by fibroblasts to the receptors on breast cancer cells. To identify potential receptor, we used human cDNA microarrays to compare the mRNA expression profiles among MDA-MB-231, MDA-MB-361, Hs578T and SKBR3 cells (Fig. 2A). Of the 610 genes that are up-regulated in MDA-MB-231, MDA-MB-361 and Hs578T cells compared to SKBR3 cells, eight encoded receptors (Fig. 2B). qRT-PCR analysis confirmed the expression profiles of these eight receptors to be similar to that of the cDNA microarrays (Fig. 2C). Within these eight candidate receptors, Robo1 had an expression profile consistent with the contrasting phenotypes of the fibroblast-suppressed cell lines MDA-MB-231, MDA-MB-361 and MDA-MB-157 (high Robo1 expression) versus the fibroblast-promoted cancer cell lines BT20, HCC1937 and SKBR3 (low Robo1 expression). Since there are multiple Robo receptor members been reported to be involved in tumorigenesis (17, 18), we then examined the expressions of Robo1, Robo2 and Robo4 at both mRNA and protein levels among these breast cancer cell lines. It appeared that only Robo1 had high expression in MDA-MB-231, MDA-MB-361 and MDA-MB-157 but low in BT20, HCC1937 and SKBR3 cell lines, while
Robo2 and Robo4 were ubiquitously expressed in all breast cancer cell lines tested (Fig. 2D and E), suggesting that Robo1 may be the differentially expressed receptor mediating fibroblast suppression in certain breast cancer cell lines.

Robo1 mediates fibroblast-associated tumor suppression

To directly test whether Robo1 plays such a role in fibroblast-mediated suppression of breast tumorigenicity, we established two independent clones of MDA-MB-231 cells with Robo1 depleted using a lentivirus shRNA system (Fig 3A). The colony formation of these two clones was greatly enhanced in fibroblast co-culture assay compared to the parental control (Fig. 3B). Furthermore, co-injection of Robo1-depleted MDA-MB-231 cells with 199Ct into fat pads of NOD/SCID mice generated significantly larger tumors compared to the parental MDA-MB-231 control (Fig. 3C and Supplementary Fig. S1A). Identical results were also observed when MDA-MB-157 cells were used (Fig. 3D and E). Moreover, depletion of Robo2 had no effect on colony forming activity of MDA-MB-231 cells (Supplementary Fig. S2), confirming the differentially expressed Robo1 is the key player. Conversely, ectopic expression of Robo1 in BT20 and HCC1937 cells (Fig. 3F), which had low endogenous Robo1 expressions and failed to suppress colony forming abilities, resulted in a significant decrease in colony formation (Fig. 3G). Consistently, co-injection of HCC1937 cells expressing Robo1 with fibroblasts into NOD/SCID mice fat pads generated much smaller tumors compared to the parental HCC1937 cells (Fig. 3H and Supplementary Fig. S1B). Taken together, these results indicated that the presence of Robo1 in cancer cells plays a critical role in fibroblast-mediated suppression of tumorigenesis.

Slit2 secreted from stromal fibroblasts inhibits tumorigenesis via Robo1 receptor

It has been reported that Slit2 serves as a Robo1 ligand in central nervous system to exhibit midline repellent (19, 20). Expression of Slit2 from fibroblasts may be responsible for the Robo1
suppression of tumorigenesis. We first examined Slit2 expression in breast cancer cell lines (MDA-MB-231, MDA-MB-361, SKBR3 and BT20), fibroblast cell lines (199Ct, WI38 and HS68) and primary fibroblasts (221C, 222N, 288N and 428N). We found that Slit2 level was 4-15 fold higher in fibroblasts than in MDA-MB-231 cells, which expressed the highest level of Slit2 among the breast cancer cell lines (Fig. 4A). Consistently, immunostaining analysis of primary cancer specimens showed that Slit2 expression was higher in stromal fibroblasts than in tumor cells (Fig. 4B and Supplementary Fig. S3). To test the potential role of secreted Slit2 in tumorigenesis, we added conditioned medium harvested from Slit2-expressing 199Ct to MDA-MB-231 cells cultured in soft agar. Addition of conditioned medium can reduce colony formation in a dose dependent manner (Fig. 4C). In contrast, co-culture with Slit2-depleted 199Ct cells did not inhibit colony formation of MDA-MB-231 (Fig. 4D and E). Furthermore, when purified recombinant Slit2 protein (rSlit2) was added to MDA-MB-231 cells, the colony numbers of MDA-MB-231 cells decreased about 60%, while the Robo1-depleted cells showed no response to rSlit2 (Fig. 4F). Together, these results demonstrated that Slit2 secreted from fibroblasts suppresses tumor growth via Robo1 receptor.

**Slit2/Robo1 signaling blocks nuclear translocation of β-catenin via PI3K/Akt pathway**

Slit2/Robo1 signaling has been associated with decreasing Akt phosphorylation and down-regulation of β-catenin activity (21, 22). Moreover, PI3K/Akt/β-catenin signaling is highly activated in breast cancers (23, 24). Slit2 secreted from the surrounding fibroblasts may suppress tumorigenic ability of cancer cells by inhibiting the PI3K/Akt/β-catenin pathway through Robo1 receptor. To examine this possibility, we performed co-immunoprecipitation (Co-IP) assay with anti-Robo1 antibody and found that Robo1 was immunoprecipitated with p85, a subunit of PI3K, within 10-30 min upon rSlit2 treatment (Fig. 5A). Reciprocal Co-IP with anti-p85 antibody
further confirmed the Robo1-p85 interaction (Supplementary Fig. S4). The phospho-Akt activity was also decreased in rSlit2 treated control cells, but not the Robo1-depleted cells (Fig. 5B, lanes 1 vs. 2 and lanes 3 vs. 4, respectively). Consistently, using cellular fractionation and immunoblotting, we found that the amount of nuclear β-catenin was reduced in the control cells but not the Robo1-depleted cells when co-cultured with 199Ct (Fig. 5C, lanes 7 & 8) although the β-catenin expression levels were comparable between the control and Robo1-depleted cells (Supplementary Fig S5). Supportively, upon rSlit2 treatment, an enhanced nuclear localization of β-catenin in the Robo1-depleted cells was detected by immunofluorescence assay (Fig. 5D), whereas the expression of cyclin D1 or c-myc in cells expressing Robo1 was reduced about 2 folds (Fig. 5E). Conforming to these findings, reduction of nuclear β-catenin was observed in tumor specimens with either high Robo1 expression in cancer cells or high Slit2 expression in stromal fibroblast (Supplementary Fig. S6). Together, these results suggested that activation of Slit2/Robo1 signaling inhibits the PI3K/Akt pathway to block β-catenin nuclear translocation and down-regulate cyclin D1 and c-myc expression (Fig. 5F).

**High expression of either Robo1 in breast cancer cells or Slit2 in stromal fibroblasts is associated with better breast cancer prognosis**

The above results imply that the Slit2/Robo1 pathway plays an essential role in fibroblast-mediated tumor suppression. We then analyzed the expression levels of Robo1/Slit2 in three cohorts of breast cancer patient specimens (Supplementary Table 2-5). The expression of Robo1 mRNA measured by qRT-PCR in breast cancer tissues was positively correlated with survival of breast cancer patients ($P=0.006$, Table 1) in the first cohort (characteristics of patients were provided in Supplementary Table 2). The correlation remained significant after adjustment for age, tumor size, distant metastasis, lymph node involvement, estrogen-receptor expression, and
Her2/neu gene expression ($P=0.02$, Table 1). Furthermore, patients with high Robo1 mRNA expression showed better prognosis than those with low Robo1 expression (log-rank $P=0.002$, Fig. 6A) when those patients were grouped based on a cutoff value determined by receiver-operating characteristic curve (ROC) analysis (25). Consistently, patients with high Robo1 protein expression determined by IHC (Fig. 6B) had positively correlation with survival of breast cancer patients ($P=0.019$, Supplementary Table 3) in the second cohort (characteristics of patients were provided in Supplementary Table 4). The correlation remained significant after adjustment for age, tumor size, distant metastasis, lymph node involvement, estrogen-receptor expression, and Her2/neu gene expression ($P=0.02$, Supplementary Table 3). Patients with high Robo1 protein expression showed better prognosis than those with low Robo1 protein expression (log-rank $P=0.0156$, Fig. 6C). Supportively, a cDNA microarray analysis revealed that low Slit2 expression in stromal cells correlated with poor outcome in breast cancer (26). Consistent with this observation, high Slit2 mRNA expression in stromal fibroblasts was correlated with low frequency of lymph node metastasis in the third cohort (characteristics of patients were provided in Supplementary Table 5) of freshly obtained samples ($P=0.03$, Fig 6D). The correlation remained significant after adjustment for age, tumor size and estrogen-receptor expression ($P=0.04$). Taken together, these results further suggested that high expression of either Robo1 in breast cancer cells or Slit2 in stromal fibroblasts is associated with better prognosis in breast cancer.

**Discussion**

Cancer originates from genetic alterations in progenitor cells and progresses with constant modulation from its microenvironment composed of fibroblasts, endothelial cells and immune
cells (27). In this communication, we found that stromal fibroblasts enhanced tumorigenic activity of some breast cancer cells including BT20, HCC1937 and SKBR3, while suppressed others including MDA-MB231, MDA-MB361 and Hs578T (Fig. 1). The latter cancer cell group expressed Robo1 receptor, which recognizes ligand, Slit2, predominantly secreted from stromal fibroblasts (Fig 4). Presence of an active Slit2/Robo1 signaling inhibited the PI3K pathway by interacting with its subunit, p85, which led to inhibition of Akt phosphorylation, β-catenin translocation and c-myc and cyclin D1 expression (Fig. 5). These results explain, in part, how specific stromal fibroblasts expressing Slit2 can restrain a given kind of cancer cell expressing Robo1 from progression.

Slit2 and Robo1 were first identified in the development of central nervous system (28). Interestingly, Slit2/Robo1 signaling plays a role in regulating outgrowth of mammary branches by inhibiting nuclear translocation of β-catenin in the basal myoepithelial cells (21), suggesting the regulatory role of Slit2/Robo1 in mammary gland development. Loss of either Slit2 from fibroblasts or Robo1 from epithelial progenitor cells disrupts normal development of mammary gland. Homozygous deletion and promoter methylation of Robo1 have been observed in breast and lung cancer implicating a potential tumor suppressor role of Robo1 (29, 30). A recent study reported that endothelial cell-secreted Slit2 suppresses tumor growth and motility in mouse mammary adenocarcinoma (31). Moreover, our clinical data demonstrated that breast cancer patients with low Robo1 expression in the cancer cells have worse prognosis (Fig. 6). Consistent with these findings, we suggested that regulation of Slit2 in surrounding cells and Robo1 in cancer cells are important for cancer progression. Interestingly, up-regulation of Robo1 has been reported to be correlated with poor prognosis in nasopharyngeal cancer (NPC) (32). Although the
precise mechanism to explain this apparent discrepancy remains unclear, it is likely that Robo1 may have other distinctive function in NPC or the microenvironment of NPC is substantially different from that of breast cancer.

Activation of the PI3K/Akt signaling pathway results in aberrant cell proliferation, tumorigenesis and metastatic competence (33). In many types of human cancer, Akt-mediated β-catenin nuclear accumulation and increased transcriptional activity that promotes tumor development (34). Our observation showed that activation of Slit2/Robo1 suppresses Akt phosphorylation and indirectly blocks β-catenin translocation from cytoplasm to nucleus. Furthermore, we also demonstrated that β-catenin accumulates in membrane instead of nucleus in breast cancer patients with high Robo1 and Slit2 expression, supporting the importance of the Slit2/Robo1-mediated suppression of Akt/β-catenin activity in breast tumorigenesis. It has been shown that the Slit2/Robo1 signal serves as an inhibitory cue to block β-catenin activity (22, 35). Consistent with these reports, fibroblast-secreted Slit2 blocked β-catenin activity in tumorigenesis of Robo1 expressing cancer cells. It was noted that the Src homology (SH) domain of PI3K-associated p85 is critical for binding to tyrosine kinase receptors to activate downstream Akt signaling (36). Our observation that Robo1 bound to p85 and suppressed phosphorylation of Akt upon addition of Slit2 suggests a potential inhibitory effect for tyrosine kinase receptors. Whether Robo1/Slit2 signaling suppressing tumorigenesis acts on additional pathways remains to be further explored.

The restraint of cancer cells by fibroblasts has to be disrupted as cancer progresses. This disruption can occur in different steps of the signaling pathway mediated by Slit2/Robo1. In addition to down-regulate Slit2 and Robo1 expression, amplification of c-myc and cyclin D1 has been seen in many breast cancers (37, 38). Also, constitutively activation of Akt and
augmentation of β-catenin-TCF/LEF transcriptional activity by mutation have been frequently seen in cancer cells (39, 40). These oncogenic alterations along with other oncogenic signal transduction pathways could help cancer cells to overcome the Slit2/Robo1 mediated restraint.

Our data that high Robo1 expression in breast cancer cells correlated with better patient survival and low Slit2 expression in stromal fibroblasts associated with lymph node metastasis provide useful and novel prognosis markers for breast cancer. Besides the genetic alteration in cancer cells, epigenetic or genetic alterations in cancer-associated stromal cells appear to have significant roles in regulating cancer progression. Therefore, these findings offer potential therapeutic targets for inducing tumor dormancy based on the observation that stromal fibroblasts can suppress tumorigenicity of cancer cells via Slit2/Robo1 pathway.

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induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast

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the vascular network by inhibiting pathologic angiogenesis and endothelial


Figure Legends

Fig. 1. Stromal fibroblasts can either promote or suppress soft agar colony formation of breast cancer cells.

A, B. Soft agar colony formation assays using MDA-MB-231 (A) and SKBR3 cells (B) co-cultured with primary carcinoma-associated fibroblasts (CAF) or normal breast-associated fibroblasts (NAF). C, D. Soft agar colony formation assays using breast cancer cell lines other than MDA-MB-231 and SKBR3 co-cultured with immortalized human CAF (199Ct). Colony formation in MDA-MB-361, Hs578T and MDA-MB-157 cells was inhibited (C), but increased in HCC1937, BT20 and MDA-MB-468 cells (D) when co-cultured with 199Ct. E, F. Soft agar colony formation assays using MDA-MB231 (E) and SKBR3 cells (F) co-cultured with two other human fibroblast cell lines, WI38 and Hs68. All data points were performed in triplicates and all experiments were performed at least three times with similar results. Data show means ± standard deviation. **, p < 0.01

Fig. 2. Robo1 is identified as a candidate receptor involved in fibroblast-mediated tumor suppression.

A. Summary of cDNA microarray analyses. Total six hundred and ten genes expressed at least 2-fold higher in MDA-MB-231 (I), MDA-MD-361 (II) and Hs578T (III) than in SKBR3 were found by Affymetrix microarray analysis. B. Eight transmembrane receptors were identified
among the 610 genes with their expression pattern matched with the profile that MDA-MB-231, MDA-MB-361 and Hs578T had a higher level than that of SKBR3. C. Reconfirming the expression profile of the eight transmembrane receptors in six different breast cancer cells. Their receptor mRNA expressions were measured directly by qRT-PCR with Gapdh as an internal control. D, E. mRNA and protein expressions of Robo1, 2 and 4 were measured by RT-PCR (D) and immunoblotting (E), respectively. Gapdh and α-tubulin served as loading controls for RNA and protein expression, respectively.

**Fig. 3. Robo1 plays an essential role in fibroblast-mediated suppression of breast tumorigenesis.**

A. Immunoblotting analysis of Robo1 expression in MDA-MB-231 cells infected by two independent lentiviruses, #180 and #248, carrying shRobo1. Cells infected with lentiviral sh-luciferease (shCtrl) or uninfected (Mock) served as controls. α-tubulin was used as a loading control. B. Soft-agar colony formation assays using Mock, shCtrl and shRobo1 MDA-MB-231 cells co-cultured with 199Ct. C. Tumor growth assay in NOD/SCID mice. shCtrl or shRobo1 MDA-MB-231 cells were injected into mammary fat-pad with or without 199Ct and the tumor volumes were measured every 4-5 days. D. Immunoblotting analysis of Robo1 expression in MDA-MB-157 cells infected by shRobo1 #180. Cells infected with shCtrl served as the control. α-tubulin was used as a loading control. E. Soft-agar colony formation assays using shCtrl and shRobo1 MDA-MB-157 cells co-cultured with 199Ct. F. Immunoblotting analysis of ectopic expression of Robo1 in BT20 and HCC1937 cells. Cells were infected either with lentiviruses carrying Robo1 cDNA (Robo1) or empty vector (LentiV). β-actin was used as a loading control. G. Soft agar colony formation assay for LentiV and Robo1 overexpressing BT20 and HCC1937
cells co-cultured with or without 199Ct. H. Tumor growth assay in NOD/SCID mice. HCC1937 cells expressing Robo1 or control (LentiV) were injected into mammary fat-pad with or without 199Ct and the tumor volumes were measured every 4-5 days. All data points were performed in triplicates and all in vitro experiments were performed at least three times. Data show means ± standard deviation. For tumor growth assay in NOD/SCID mice, six mice per group were used. *, $p < 0.05$; **, $p < 0.01$.

**Fig. 4. Slit2 secreted from stromal fibroblasts inhibits cancer cell tumorigenesis.**

A. *Slit2* mRNA expression in breast cancer cell lines (MDA-MB-231, MDA-MB-361, SKBR3 and BT20), fibroblast cell lines (199Ct, WI38 and HS68) and primary fibroblasts (221C, 222N, 288N and 428N) were examined by qRT-PCR. B. IHC staining with antibody against Slit2 in breast cancer specimen. Low magnification (left panel) scale bar: 25 μm. High magnification (right panel) scale bar: 50 μm. Arrows indicate stromal fibroblasts. Arrow heads indicate cancer cells. C. Soft-agar colony formation assay for MDA-MB-231 cells treated with conditioned medium (199Ct cm) collected from 199Ct culture. D. Immunoblotting analysis of Slit2 proteins in harvested conditioned medium (cm-Slit2) from shCtrl or shSlit2 199Ct. β-actin was used as a loading control. E. Soft agar colony formation assay for MDA-MB-231 cells co-cultured with shCtrl or shSlit2 199Ct. F. Soft agar colony formation of shCtrl and shRobo1 MDA-MB-231 cells treated with PBS or 50 ng/ml of recombinant Slit2 (rSlit2) protein. All data points were performed in triplicates and all experiments were performed at least three times. Data show means ± standard deviation. *, $p < 0.05$; **, $p < 0.01$.

**Fig. 5. Slit2/Robo1 signaling blocks β-catenin translocation through PI3K/Akt pathway.**
A. Robo1 overexpressing BT20 cells were treated with rSlit2 (200 ng/ml) for 10, 20 and 30 min. Co-immunoprecipitation (Co-IP) of Robo1 and p85 was detected using immunoblotting analysis in response to the rSlit2 treatment. Normal IgG (IgG) was used as a negative Co-IP control. β-actin served as a loading control. B. Immunoblotting analysis of phospho-Akt in shCtrl and shRobo1 MDA-MB-231 cells treated with rSlit2 (200 ng/ml). Total Akt was used as a quantification control. β-actin served as a loading control. Relative expression (RE) in phospho-Akt to total Akt protein levels is indicated. C. Immunoblotting assay of β-catenin in cytosolic and nuclear fractions of the shCtrl and shRobo1 MDA-MB-231 cells co-cultured with or without 199Ct. α-tubulin and histone deacetylase (HDAC) were used as cytosolic and nuclear markers, respectively. D. Immunofluorescence staining with antibody against β-catenin for shCtrl and shRobo1 MDA-MB-231 cells after treated with rSlit2 (200 ng/ml). Scale bar: 20μm. Arrows indicate nuclei. E. Immunoblotting analysis of shCtrl and shRobo1 MDA-MB-231 cells treated with rSlit2 using antibodies against cyclin D1 and c-myc. α-tubulin as a loading control. RE in cyclin D1 and c-myc to α-tubulin protein levels is indicated. F. Diagram summarized the pathway of how Slit2/Robo1 signal is transmitted from stromal fibroblasts to breast cancer cells.

**Fig. 6.** Clinical relevance of Robo1/Slit2 expression in breast cancer specimens.

A. Kaplan-Meier survival analysis for 122 breast cancer patients divided into two groups based on their Robo1 mRNA expression levels. Blue line (n=93) represents Robo1 high and red line (n=29) represents Robo1 low patients. B. IHC staining of Robo1 in breast tumor specimens. Low magnification (left panel) scale bar: 100 μm. High magnification (right panel) scale bar: 50 μm. C. Kaplan-Meier survival analysis for 162 breast cancer specimens using IHC staining to detect Robo1 expression in cancer cell membrane. Blue line (n=63) represents Robo1 high and red line
(n=99) represents Robo1 low patients. D. Fifty-one cancer-associated fibroblasts isolated from freshly obtained specimens were used to determine the correlation between Slit2 mRNA expression and metastasis status of the patients. Slit2 mRNA levels were measured by qRT-PCR and compared with the internal control gene, Gapdh. Patients were divided into two groups according to the metastasis status to correlate with the relative Slit2 mRNA expression. N-, no lymph node metastasis (n=26) and N+, with lymph node metastasis (n=25).
Table 1: Univariate and multivariate proportional hazards analysis of mortality of breast cancer patients

<table>
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<th>Variables</th>
<th>Univariate</th>
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<th>P</th>
<th>Multivariate</th>
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<tr>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P</td>
<td>Hazard ratio (95% CI)</td>
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<td>Robo1 gene expression (per two-fold increase)</td>
<td>0.81 (0.71-0.94)</td>
<td>0.006</td>
<td>0.84 (0.74-0.98)</td>
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<td>Age (per decade)</td>
<td>0.99 (0.97-1.01)</td>
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<td>1.00 (0.97-1.03)</td>
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<td>Tumor size (per grade)</td>
<td>1.63 (1.24-2.26)</td>
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<td>1.33 (0.90-1.95)</td>
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<td>Distant metastasis</td>
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<td>5.25 (1.01-27.13)</td>
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<td>Lymph node positivity</td>
<td>3.56 (2.21-5.72)</td>
<td>&lt;0.0001</td>
<td>3.40 (1.84-6.26)</td>
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<td>Estrogen-receptor expression (per point)</td>
<td>0.71 (0.54-0.96)</td>
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<td>0.48 (0.32-0.72)</td>
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<td>Her2/neu gene expression (per point)</td>
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CI: confidence interval
Fig. 1 Chang et al. 2012
A

B

<table>
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+++ : > 10 fold, ++ : 5-10 fold, + : < 2-5 fold

C

D

E

Fig. 2 Chang et al. 2012
Fig. 3 Chang et al. 2012
Fig. 4 Chang et al. 2012
Fig. 5 Chang et al. 2012
Fig. 6 Chang et al. 2012
Activation of Robo1 signaling of breast cancer cells by Slit2 from stromal fibroblast restrains tumorigenesis via blocking PI3K/Akt/β-catenin pathway


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