Multiple Functions of Sushi Domain Containing 2 (SUSD2) in Breast Tumorigenesis

Allison P. Watson, Rick L. Evans, and Kristi A. Egland

Abstract

Routinely used therapies are not adequate to treat the heterogeneity of breast cancer, and consequently, more therapeutic targets are desperately needed. To identify novel targets, we generated a breast cancer cDNA library enriched for genes that encode membrane and secreted proteins. From this library we identified SUSD2 (Sushi Domain Containing 2), which encodes an 822-amino acid protein containing a transmembrane domain and functional domains inherent to adhesion molecules. Previous studies describe the mouse homolog, Susd2, but there are no studies on the human gene associated with breast cancer. Immunohistochemical analysis of human breast tissues showed weak or no expression of SUSD2 in normal epithelial cells, with the endothelial lining of vessels staining positive for SUSD2. However, staining was observed in pathologic breast lesions and in lobular and ductal carcinomas. SUSD2 interacts with galectin-1 (Gal-1), a 14-kDa secreted protein that is synthesized by carcinoma cells and promotes tumor immune evasion, angiogenesis, and metastasis. Interestingly, we found that localization of Gal-1 on the surface of cells is dependent on the presence of SUSD2. Various phenotype assays indicate that SUSD2 increases the invasion of breast cancer cells and contributes to a potential immune evasion mechanism through induction of apoptosis of Jurkat T cells. Using a syngeneic mouse model, we observed accelerated tumor formation and decreased survival in mice with tumors expressing Susd2. We found significantly fewer CD4 tumor infiltrating lymphocytes in mice with tumors expressing Susd2. Together, our findings provide evidence that SUSD2 may represent a promising therapeutic target for breast cancer.

Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer-related death in women worldwide (1). Tumor-specific immunotherapies offer the potential for high specificity toward cancerous cells, while omitting normal tissues, thus minimizing the systemic side effects of conventional chemotherapy. Immunotherapies use the binding specificity of antibodies to target molecules that are upregulated or specific to tumor cells. One such therapy uses trastuzumab (Herceptin), a humanized monoclonal antibody that binds to HER-2/neu (ErbB2) and inhibits the proliferation and survival of HER2-dependent tumors. When administered concurrently with adjuvant chemotherapy, trastuzumab significantly increases disease-free survival (2). However, only 25% to 30% of human breast cancers overexpress HER-2 (3, 4); therefore, there is a great need for the identification of additional breast tumor-specific immunotherapy targets. One limitation is the availability of unique protein targets that are present on cancer cells but are not expressed in normal essential tissues.

To identify genes that encode membrane and secreted proteins useful for the diagnosis or treatment of breast cancer, a cDNA library was generated from membrane-associated polyribosomal RNA (5). To remove ubiquitously expressed genes, the library was subtracted with RNA from normal brain, liver, kidney, lung, and muscle (5). After sequencing 25,000 library clones and aligning the sequences associated polyribosomal RNA (5). To remove ubiquitously expressed genes, the library was subtracted with RNA from normal brain, liver, kidney, lung, and muscle (5). After sequencing 25,000 library clones and aligning the sequences to the human genome, the genes represented in the library were ordered by abundance. HER-2 was the eighth most abundant gene (5), and here we report the identification of a new human breast cancer gene, SUSD2 (Sushi Domain Containing 2), the 18th most abundant gene in the membrane-associated polyribosomal cDNA library (MAPcL). SUSD2 is located on chromosome 22 and encodes an 822-amino acid type I membrane protein containing somatomedin B, AMOP, von Willebrand factor type D, and Sushi domains, which are frequently found in molecules playing important roles in cell–cell and cell–matrix adhesion.

Two articles have been published describing the mouse homolog, Susd2 (also known as mSVS-1 or SYS-1), which was identified by cDNA microarray analysis comparing global gene expression levels of v-K-ras–transformed Ki3T3 cells with mouse NIH3T3 cells (6, 7). The Susd2 gene was found to be downregulated in Ki3T3 cells compared with...
Characterization of SUSD2 in Breast Tumorigenesis

SUSD2 in humans is limited. A recent publication detailing methods used to map human protein–protein interactions by mass spectrometry identified a high-confidence interaction between SUSD2 and galectin-1 (Gal-1; ref. 8). No further characterization of the interaction has been reported. Gal-1, encoded by the LGALS1 gene, is a 14-kDa protein that is part of a phylogenetically conserved family of proteins characterized by their ability to bind β-galactoside residues on many cellular glycoproteins. Gal-1 has been extensively studied and is implicated in tumor transformation, cell-cycle regulation, apoptosis, cell adhesion, migration, and inflammation (7).

Because SUSD2 was highly abundant in the MAPcL, we investigated whether expression of the gene is associated with breast cancer. To study the phenotypic and biologic significance of SUSD2 in breast cancer, we conducted several in vitro and in vivo analyses using stable cell lines. We have identified several mechanisms by which SUSD2 may contribute to breast tumorigenesis.

Materials and Methods
Cell culture

MDA-MB-231, SK-BR-3, 293T, A2780, SKOV3, and 66CL4 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS (Atlanta Biologicals). Jurkat cells were maintained in RPMI with 10% FBS. All cell lines were grown at 37°C for 3 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute, and elongation at 72°C for 1 minute, with a final 5-minute extension at 72°C. The same PCR conditions were used with the Human Rapid-Scan gene expression panels (OriGene) for analysis of gene expression in normal tissue cDNA.

Western immunoblot analysis

Tissue culture cell lines were washed twice with PBS and lysed in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris–HCl pH 7.5, 1.0% sodium deoxycholate, 1.0% Igepal CA-630, 0.1% SDS, 5 mmol/L EDTA, and protease inhibitors. Protein extracts were separated by SDS-PAGE and transferred to polyvinylidine difluoride membranes (Millipore). Blots were incubated sequentially with Western Blocking Reagent (Roche), primary antibody, and then secondary antibody, all for 1 hour. Colorimetric detection was conducted using solutions of nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyl phosphate (NBT/BCIP 1-Step Solution; Pierce). Equal loading was verified by incubating the membranes with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. Primary antibodies used include polyclonal rabbit anti-SUSD2 (Pre捨て Antibodies), polyclonal goat anti-Gal-1 (R&D Systems), and monoclonal mouse anti-GAPDH (Sigma).

Immunofluorescence and confocal microscopy

Cells grown on glass coverslips were washed and fixed with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100, samples were blocked with 10% goat serum in PBS for 1 hour. Samples were exposed to primary antibodies for 1 hour followed by fluorescently conjugated secondary antibody staining for 1 hour. Coverslips were mounted on slides using Pro-Long Gold anti-fade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were obtained by the Olympus Fluoview FV1000 confocal laser microscope. Omission of the primary antibody was carried out to discriminate background staining. Primary antibodies used include polyclonal mouse anti-SUSD2 (Novus Biologicals) and polyclonal rabbit anti-Gal-1 (Cell Signaling).

Immunohistochemical staining

Blocks of formalin-fixed, paraffin-embedded human breast tissue and mouse tumors were prepared for immunohistochemical (IHC) analysis. Sections of 5-μm thickness were subjected to immunostaining by heat-induced epitope retrieval in citrate buffer followed by detection using a Vector ABC kit (Vector Laboratories), as described previously (12). Accumax breast tissue arrays (ISU Abxis) were also immunostained by the same protocol. Antibodies used include anti-SUSD2 (Pre捨て Antibodies), anti-mouse Ki67 (Biocare Medical), and anti-mouse CD31 (Abcam). The immunostained sections were reviewed by a trained pathologist. Staining for Ki67 and CD31 was quantitated using ImageJ software (http://imagej.nih.gov/ij/download.html; ref. 13).

Construction of stable cell lines

The open reading frame of SUSD2 was cloned into the pLXSN retroviral vector and transfected into Phoenix packaging cells using Effectene (Qiagen), as recommended by the manufacturer. After 48 hours, the supernatant containing nonreplicating forms of amphotropic virus was harvested. Target cell lines were infected at 70% confluence with virus supernatant in the presence of 10 μg/mL Polybrene (Sigma). Stable clones were generated with antibiotic selection in 600

www.aacrjournals.org Mol Cancer Res; 11(1) January 2013 75

Published OnlineFirst November 6, 2012; DOI: 10.1158/1541-7786.MCR-12-0501-T

Downloaded from mcr.aacrjournals.org on November 13, 2021. © 2013 American Association for Cancer Research.
μg/mL G418. Cells transduced with the empty pLXSN vector were used as controls.

SK-BR-3 cells were used to generate stable SUSD2 knockdown cell lines using SUSD2 short hairpin RNA (shRNA)-expressing lentiviral particles (pLKO.1 vector, MISSION shRNAs; Sigma). Cells were infected according to the manufacturer’s instructions and selected with 0.8 μg/mL puromycin. Stable clones were selected for further study based on the extent of SUSD2 knockdown determined by flow cytometry and Western immunoblot analysis (data not shown). A nontargeting shRNA sequence and the empty pLKO.1 vector were used as controls. (Sequences: SUSD2 #1, CCGGGAGCATTCATTCTGCAACTTTCTCAGGAAATGCAGAATGATCGTTTTTTG; SUSD2 #2, CCGGGCCATGGATGATCGTTTTTTG; SUSD2-19, CCGGGCGATGATCGTCTTTTTTG; SUSD2-14, CCGGGCGATGATCGTCTTTTTTG).

Cell proliferation and colony-forming assays
To observe cellular proliferation rates, 2 × 10^4 cells were plated in 100-mm tissue culture plates. Cells were collected and counted every 24 hours using an automated cell counter (Coulter Particle Counter, Beckman Coulter). Colony formation was analyzed by plating 500 cells in 100-mm tissue culture plates (in triplicate). After 8 days, colonies were fixed in 2% paraformaldehyde and stained with 0.5% crystal violet. Colonies were defined as a minimum of 50 cells in a group and were counted using image analysis software (AlphaInnotech).

Migration and invasion assays
Cellular invasion and migration were analyzed using Boyden chamber-style cell culture inserts with or without Matrigel, respectively (BD Falcon). Cell culture inserts containing a polyester membrane with 8.0-μm pores were placed in 12-well cell culture dishes and seeded with 2 × 10^4 cells per well in the top chamber, in serum-free media. Culture medium with 10% FBS was used as a chemoattractant in the lower chamber. After 22 hours, migrated cells were fixed with 2% paraformaldehyde and stained with 0.05% crystal violet. Inserts were removed and mounted on glass microscope slides. Cells in 10 random fields were counted.

In vitro apoptosis assay
We adapted a method for detection of Jurkat T cell apoptosis during coculture with breast cancer cells, originally described by Kovacs-Solyom and colleagues (14). Cancer cell lines were plated on glass coverslips and allowed to adhere overnight. Jurkat cells were prewashed with 200 ng/mL Hoechst 33342 (Sigma) and added to the coverslips at a 1:1 Jurkat-to-breast cancer cell ratio. After coinubcation for 16 hours, coverslips were fixed and stained with Annexin V-FITC for analysis by confocal microscopy. The percentage of apoptotic Jurkat cells was calculated by counting the number of Hoechst-stained cells (blue) that also stained with Annexin V-FITC (green). The following formula was used for calculations: (number of green and blue cells)/(number of blue cells) × 100 = percentage apoptosis.

Coimmunoprecipitation reactions
Cell extracts were immunoprecipitated using polyclonal rabbit anti-SUSD2 (Prestige Antibodies) or monoclonal mouse anti-Gal-1 (Santa Cruz Biotechnology) antibodies. Briefly, 1 mg of cellular lysate was incubated with Protein A agarose beads (GE Healthcare Life Sciences) to remove nonspecific binding proteins. The supernatant was then subjected to immunoprecipitation by adding 1.5 μg of anti-SUSD2 antibody (Prestige Antibodies) or 3 μg of anti-Gal-1 antibody (Santa Cruz Biotechnology). The mixtures were gently agitated overnight at 4°C followed by the addition of Protein A agarose beads and agitation for 1 hour at 4°C. Immunocomplexes associated with the beads were recovered by centrifugation and washed by 3 cycles of resuspension in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris–HCl pH 7.5, 1.0% sodium deoxycholate, 1.0% Igepal CA-630, 0.1% SDS, and 5 mmol/L EDTA) followed by centrifugation. The immunocomplexes retained on the beads were resuspended in buffer containing 20% glycerol, 2% SDS, 250 mmol/L Tris pH 6.8, β-mercaptoethanol, and 0.1% bromophenol blue, then boiled and subjected to SDS-PAGE followed by Western immunoblot analysis for anti-SUSD2 and anti-Gal-1, as described earlier.

Flow cytometry
Cells were harvested using nonenzymatic cell-dissociation buffer to avoid enzymatic digestion of cell surface proteins. To show the presence of SUSD2 and Gal-1 on the cell surface, nonpermeabilized cells were resuspended in PBS supplemented with 1% heat-inactivated FBS and 0.1% NaN_3 and stained with anti-SUSD2 (Novus Biologicals) and anti-Gal-1 (R&D Systems) antibodies, followed by fluorescently conjugated secondary antibodies. Samples were analyzed using an Accuri Flow Cytometer and gated on the basis of forward- and side-scatter profile to exclude debris and cellular aggregates from the analysis.

In situ proximity ligation assay
Cells cultured in 16-well chamber slides were used with the Duolink In Situ Proximity Ligation Assay (Duolink-PLA) kit according to the manufacturer’s instructions. Cells were stained with primary antibodies and incubated with the appropriate pair of Plus and Minus oligonucleotide-conjugated secondary antibodies from Olink Bioscience. After hybridization, ligation, and amplification, a detection solution containing Texas Red-labeled oligo-linkers (Duolink Detection Kit; Olink Bioscience) was added to allow visualization of protein–protein interactions as red dots. Coverslips were mounted using mounting media with DAPI and sealed with nail polish. Control experiments were carried out with primary antibodies to proteins that do not interact with SUSD2 or Gal-1. Images were obtained using a FV1000 confocal microscope.

Mouse model
All animal experiments were approved by the Institutional Animal Care and Use Committee at Sanford Research (Sioux Falls, SD). Seven-week-old female Balb/c mice (Charles...
The 293T cells do not express SUSD2, and pcDNA3.1-SUSD2-Myc-His transiently transfected did not affect cellular proliferation or anchorage-dependent growth. Overexpression of SUSD2 was observed in the cytoplasm and along the cytoplasmic membrane of various pathologic lesions of the breast, including papillary metaplasia (Fig. 1A, 4–5) and sclerosing adenosis (Fig. 1A, 6). Positive staining for SUSD2 was observed in lobular and ductal breast carcinoma, as well as in both in situ and invasive breast carcinomas (Fig. 1A, 7–9). On higher magnification, cancerous cells stain distinctly positive for SUSD2 around the cell membrane (Fig. 1A, 9). To increase the sample size, a breast cancer tissue array was analyzed for SUSD2 staining by IHC for a total of 75 patient tumor samples studied. Overall, 82% of the samples stained positively for SUSD2, including 41 of 47 estrogen receptor–positive (ER+), 30 of 36 HER-2 amplified, and 4 of 7 triple-negative subtypes.

To investigate the expression of SUSD2 in normal tissues, we used reverse-transcriptase PCR (RT-PCR) analysis on a cDNA panel derived from 28 different normal tissues. Many normal tissues did not express SUSD2; however, expression was detected in several normal tissues, such as adipose, adrenal gland, kidney, lung, mammary gland, placenta, thyroid, trachea, and uterus (Fig. 1B). These data indicate that SUSD2 has restricted expression in normal tissues.

Because the MAPcL was generated from pooled membrane-associated polyribosomal RNA derived from 6 cell lines, we determined which of the cell lines contain SUSD2. Whole-cell lysates harvested from the MAPcL cell lines and 293T cells transiently transfected with pcDNA3.1-SUSD2-Myc-His were separated by SDS-PAGE. After immunoblotting using an anti-SUSD2 antibody, 2 strong bands at 60 and 110 kDa were present in SK-BR-3 cells and the positive control (Fig. 1C, top). Comprising 822 amino acids, the predicted size of SUSD2 is 90.4 kDa. The 110-kDa band is most likely a glycosylated form of SUSD2, which is consistent with previous studies showing Susd2 has at least 4 glycosylation sites (6). The 60-kDa band suggests SUSD2 is posttranslationally cleaved. Lower levels were detected in MCF7 and hTERT-HME1 cells, whereas there was almost no detectable SUSD2 protein in ZR-75-1, MDA-MB-231, or LNCaP cells (Fig. 1C, top). Similar expression patterns were observed with RT-PCR analysis using total RNA from the cell lines as a template to generate cDNA (Fig. 1C, bottom). Finally, an anti-SUSD2 antibody was used for IHC analysis to stain for SUSD2 in the 4 breast cancer cell lines used to generate the MAPcL. Three of the 4 breast cancer cell lines had very weak to no detectable SUSD2, MCF7 (luminal A subtype), ZR-75-1 (luminal A subtype), and MDA-MB-231 (basal subtype; ref. 15; Supplementary Fig. S1C, S1E, and S1F). However, SK-BR-3, a HER-2 subtype (15), showed distinct SUSD2 cell membrane staining by IHC (Supplementary Fig. S1D). The significance of SUSD2 being expressed in SK-BR-3 cells that have amplification of HER-2 will be further investigated in future studies.

**Overexpression of SUSD2 increases invasion, but does not affect cellular proliferation or anchorage-dependent growth**

To determine the function of SUSD2 in breast tumorigenesis, stable SUSD2-expressing cell lines were generated.
Figure 1. Analysis of SUSD2 in human tissues. A, paraffin-embedded blocks of tissue were collected from women undergoing a bilateral mastectomy for breast cancer. Tissue sections were immunostained with anti-SUSD2 antibody, counterstained with hematoxylin, and analyzed by a trained pathologist. Images 1 through 8 were taken at an original magnification of ×200, and image 9 was taken at ×400. Scale bars indicate 100 μm. B, expression of SUSD2 in normal tissues was analyzed by RT-PCR using a panel of cDNAs derived from 28 different normal tissues. C, expression of SUSD2 in MAPcL cell lines was determined by Western immunoblot analysis (top) using whole-cell lysates harvested from MAPcL cell lines and pcDNA3.1-SUSD2-myc-His-transfected 293T cells as a positive control. RT-PCR analysis (bottom) was conducted using primers to SUSD2 that amplify a 489-bp fragment.
MDA-MB-231 cells, which do not endogenously produce SUSD2 (Fig. 1C), were transfected with either pLXSN-SUSD2 or the empty vector, and antibiotic selection was used to generate stable cell lines. To examine whether SUSD2 affects the growth of breast cancer cells, we conducted cell proliferation and colony-forming assays. Cellular proliferation was assayed by cell counting using a Coulter Counter. There was no significant difference in growth rates with SUSD2 overexpression compared with the empty vector controls (data not shown). The colony-forming ability was assessed by seeding 500 cells in a 100-mm cell culture dish and counting the number of colonies formed in 8 days. Morphologic analysis of the colonies showed looser, less circular colonies in the SUSD2 overexpressing cell lines compared with the empty vector control, but the total number of colonies did not significantly differ (Fig. 2A). In general, the plating efficiency of all 4 cell lines tested was approximately 80%. Similar results for proliferation and colony-forming ability were observed using additional clones (data not shown). These results together indicate that SUSD2 does not play a role in cellular proliferation or anchorage-dependent growth of breast cancer cells.

We next examined whether SUSD2 expression influences the ability of breast cancer cells to migrate and/or invade using Boyden chamber-style cell culture inserts without or with Matrigel, respectively. MDA-MB-231-SUSD2 cell lines and vector-only controls were plated on the membranes, and allowed to migrate toward a chemoattractant in the lower chamber. As shown in Fig. 2B, SUSD2 overexpression did not significantly alter cellular migration. We carried out a similar experiment to compare the ability of MDA-MB-231 cells to invade through Matrigel-coated cell culture inserts. As shown in Fig. 2C, SUSD2 overexpression significantly increased invasion by almost 7-fold.

**Interaction of SUSD2 with galectin-1**

A previous study using mass spectrometry to screen for human interacting proteins identified an interaction between SUSD2 and Gal-1 (16). To verify that SUSD2 and Gal-1 interact, we carried out coimmunoprecipitation using anti-SUSD2 antibodies followed by Western immunoblot analysis with anti-Gal-1 antibodies. The reciprocal order of the antibodies was also conducted. Gal-1 was detected by Western immunoblot analysis when anti-SUSD2 antibodies were used for immunoprecipitation, and SUSD2 was detected by Western immunoblot analysis of proteins pulled down by immunoprecipitation with anti-Gal-1 antibodies (Fig. 3A). No bands were detected using

![Figure 2](image-url)
Subcellular localization of SUSD2 and galectin-1

To determine whether SUSD2 and Gal-1 colocalize in breast cancer cells, immunofluorescence confocal microscopy was conducted. Cells were fixed, permeabilized, incubated with anti-SUSD2 and anti-Gal-1 antibodies followed by fluorescently labeled secondary antibodies, and visualized for SUSD2 and Gal-1 staining. Gal-1 had a similar staining pattern in all of the studied cell lines and was predominately located in the cell cytoplasm (Fig. 4A), consistent with the findings by PLA (Fig. 3B). We further verified this interaction by flow cytometry to analyze cell surface presentation of SUSD2 and Gal-1. Because the cells were not permeabilized, only surface proteins were detected. A large shift in fluorescence of cell surface SUSD2 above the secondary antibody-only control indicates that SUSD2 is localized on the cell surface (Fig. 4C, top). When cells are labeled for Gal-1, an increase in the amount of Gal-1 on the surface of MDA-MB-231-SUSD2 cells is detected compared with the vector-only control (Fig. 4C, top). Similar results were observed in stable SK-BR-3 SUSD2 knockdown cell lines generated using lentiviral transduction particles containing SUSD2-specific shRNA constructs. Cells were screened for cell surface SUSD2 and Gal-1 by flow cytometry and compared with the empty vector control cell line (Fig. 4C, middle). Cell surface Gal-1 was completely abrogated in the cell line displaying the greatest SUSD2 knockdown.

Figure 3. Interaction of SUSD2 and galectin-1. A, co-immunoprecipitation was conducted using anti-SUSD2 antibodies followed by Western immunoblot analysis with anti-Gal-1 antibodies (top). The reciprocal order of antibodies was also carried out (bottom). As a control for the Western blot analysis, 50 µg of total protein from MDA-MB-231-SUSD2 cells was loaded onto the gel but not subjected to immunoprecipitation. B, in situ PLA was conducted using anti-SUSD2 and anti-Gal-1 antibodies. Negative controls were carried out with antibodies to GAPDH and RAP80. Red dots indicate protein–protein interactions. Images shown are 20 stacked optical sections taken at ×300 magnification using the same laser intensity, gain, and offset settings. C, SUSD2 and Gal-1 expression in MDA-MB-231 stable cell lines was determined by Western immunoblot analysis.
knockdown (SK-BR-3kd #2; Fig. 4C, middle). Consistently, the cell line that exhibited a partial knockdown of SUSD2 displayed a decreased amount of Gal-1 on the cell surface compared with SK-BR-3-vector cells (SK-BR-3kd #1; Fig. 4C, middle). These results show that the amount of Gal-1 on the cell surface correlates with expression of SUSD2 and confirm that SUSD2 is essential for cell surface presentation of Gal-1.

To determine if the influence of SUSD2 on the subcellular localization of Gal-1 is specific to breast cancer cells, similar techniques were used to analyze two different ovarian cancer cell lines generated to overexpress SUSD2. As expected, overexpression of SUSD2 in SKOV3 (data not shown) and A2780 ovarian cancer cells leads to an increased cell surface presentation of Gal-1, indicating that this interaction is not limited to breast cancer cells (Fig. 4C, bottom).

Mouse breast cancer model

The 4T1 mammary carcinoma cell line was originally derived from a spontaneously arising mammary tumor in a Balb/c mouse. This model is often used for its high propensity to metastasize to lungs, liver, bone, and brain, similar to human breast cancers (18). Syngeneic mouse models allow analysis of the immune system’s response to the tumor. We used a 4T1 sibling cell line (66CL4) to examine the effect of Susd2 overexpression on breast tumorigenesis (18). Both 4T1 and 66CL4 cell lines have been classified as triple negative (estrogen receptor negative, progesterone receptor negative, and HER-2 amplification negative; ref. 19) and represent very aggressive models for breast tumorigenesis. Because 66CL4 wild-type cells do not endogenously express Susd2 (data not shown), we generated stable cell lines overexpressing either Susd2 or the empty pLXSN vector. Wild-type 66CL4 cells endogenously express Gal-1, and expression levels of Gal-1 are similar in cell lines with or without expression of Susd2 (data not shown), consistent with the results from human breast cancer cell lines (Fig. 3C). Similarly, we observed no significant difference in growth rates or colony-forming ability of 66CL4 cells with overexpression of Susd2, and similar results were observed in multiple clones (data not shown). These results are consistent with the findings from SUSD2 overexpression in human breast cancer cell lines and confirm that Susd2 does not play a role in cellular proliferation or anchorage-dependent growth of mouse mammary carcinoma cells.

Seven-week-old female Balb/c mice were randomly divided into two groups of 12 mice and each group was injected with either 66CL4-pLXSN or 66CL4-Susd2 cells. After a palpable tumor was formed, measurements were taken every 2 to 3 days. Mice in the 66CL4-Susd2 group displayed accelerated tumor formation and decreased survival compared with the 66CL4-pLXSN controls (Fig. 5A). Median survival for 66CL4-Susd2 tumor-bearing mice was 36 days, compared with 41 days for the 66CL4-pLXSN tumor-bearing mice. In addition, more mice in the 66CL4-Susd2 group were sacrificed because of the development of moribund
titate aberrant blood vessel growth, we calculated the average appearance of the microvasculature, we did not observe density in the tumors. Despite the obvious differences in the extent of angiogenesis by calculating the microvessel density in 66CL4-Susd2 tumors (Fig. 6B). We assessed CD31, an endothelial cell marker, was markedly more higher proliferative rate of the tumor cells themselves.

The presence of metastases in lungs was analyzed by hematoxylin and eosin (H&E) staining. D, lymphocytes were prepared from spleen, tumors, and blood of tumor-burdened mice and stained with CD4-PE and CD8-FITC for analysis by flow cytometry. The lymphocytes were gated on the basis of FSC- and SSC-plots and the ratios of numbers of cells in the CD4+ (x-axis) and CD8+ (y-axis) cell populations were determined. Data are representative of independently carried out experiments (n = 6-9 for each group; P < 0.05).

We sought to determine if the accelerated tumor formation in the 66CL4-Susd2 mice was due to an increased proliferative rate of the tumor cells. We used IHC staining of paraffin-embedded tumors with Ki67 antibodies. The Ki67 nuclear antigen is associated with cellular proliferation and is expressed in glandular cells of breast carcinomas. The Ki67 index as a measure of the tumor growth rates. We observed similar Ki67 indexes for both 66CL4-Susd2 and 66CL4-pLXSN tumors (Fig. 6A), indicating that the difference in tumor growth rates in the mice was not due to a higher proliferative rate of the tumor cells themselves.

IHC staining of the tumors showed that the expression of CD31, an endothelial cell marker, was markedly more aberrant in the 66CL4-Susd2 tumors (Fig. 6B). We assessed the extent of angiogenesis by calculating the microvessel density in the tumors. Despite the obvious differences in the appearance of the microvasculature, we did not observe significant differences in the microvessel density. To quantitate aberrant blood vessel growth, we calculated the average length of vessel segments in between branch points. As shown in Fig. 6B, 66CL4-Susd2 tumors exhibited much shorter vessel segments between branch points than did the 66CL4-pLXSN tumors (63.2 vs. 91.3 pixels, respectively).

The profile of lymphocytes in the tumors, spleen, and blood was identified by immunostaining of cell surface markers and analysis by flow cytometry. Lymphocytes were gated on the basis of forward- and side-scatter profiles, and the proportion of CD4 to CD8 lymphocytes was calculated (Fig. 5D). These data together suggest that overexpression of Susd2 by mammary carcinoma tumors in mice alters the population of tumor-infiltrating CD4 lymphocytes.

Overexpression of SUSD2 increases apoptosis of Jurkat cells in coculture

To examine the possibility that a similar reaction occurs when human cell lines expressing SUSD2 are exposed to T cells, we carried out an in vitro coculture experiment using

Figure 5. Analysis of Susd2 in breast tumorigenesis. A, tumor volume was monitored and mice were sacrificed when tumor volumes reached 1,500 mm3 or mice exhibited moribund conditions. Analysis of survival indicates expression of Susd2 correlates with decreased survival (P = 0.0199). B, cause of death was recorded at the time of sacrifice. Numbers indicated in the table refer to the actual number of mice sacrificed because of each specific cause of death. Percentages indicate the percentage of each mouse model group that was sacrificed because of each specific cause of death. Significance was calculated by χ2 test (P = 0.0247). C, presence of metastases in lungs was analyzed by hematoxylin and eosin (H&E) staining. D, lymphocytes were prepared from spleen, tumors, and blood of tumor-burdened mice and stained with CD4-PE and CD8-FITC for analysis by flow cytometry. The lymphocytes were gated on the basis of FSC- and SSC-plots and the ratios of numbers of cells in the CD4+ (x-axis) and CD8+ (y-axis) cell populations were determined. Data are representative of independently carried out experiments (n = 6-9 for each group; P < 0.05).
Jurkat cells, an immortalized line of T cells. Adaptations of this model have been widely used to show that tumor cell lines can induce T cell apoptosis in coculture experiments (14, 20–23). In our model, Jurkat cells were cocultured with stable MDA-MB-231 cells overexpressing either SUSD2 or the empty vector control for 16 hours before staining of apoptotic cells with Annexin V. Coculture with SUSD2-expressing cells caused a significant increase of apoptosis of the Jurkat cells (Fig. 7). We observed an average of 82% apoptosis of Jurkat cells cocultured with MDA-MB-231-SUSD2, compared with 63% with MDA-MB-231-Vector cells (Fig. 7). This method detects one of the earliest events in apoptosis by using Annexin V, which has a strong and specific affinity for phosphatidyl serine. Phosphatidyl serine is translocated from the inner leaflet of the plasma membrane to the outer leaflet soon after apoptosis is induced (24). As such, this method does not allow evaluation of the various pathways that culminate in the induction of apoptosis. This preliminary study suggests that breast cancer cell lines expressing SUSD2 may stimulate a more robust apoptotic response in T cells, but no definitive conclusions can be made about which apoptotic pathways are affected.

Discussion

In the present study, we characterize a novel breast cancer gene, SUSD2, and investigate its function in tumorigenesis using in vitro functional assays and an in vivo syngeneic mouse model. SUSD2 is a type I transmembrane protein that localizes to the plasma membrane of breast cancer cells. We identified SUSD2 through generation of a breast cancer cDNA library enriched with genes encoding membrane and secreted proteins (5), and found that it is highly expressed in breast cancer, but shows a restricted expression pattern in normal tissues (Fig. 1). IHC analysis showed strong staining in all stages of breast cancer, as well as in nonmalignant pathologic breast lesions (Fig. 1A). Although 82% of breast tumors stained positive for SUSD2, limited staining was observed in benign breast tissue, with just the endothelial lining of blood vessels staining positive (Fig. 1A).

Characterization of SUSD2 in Breast Tumorigenesis
Because little is known about the function of SUSD2, we characterized the role of SUSD2 in breast cancer through the generation of stable cell lines. While overexpression of SUSD2 did not alter the ability of breast cancer cells to migrate in a Boyden chamber-style assay, it did significantly increase the ability of these cells to invade through Matrigel, a basement membrane-like substance used to simulate the metastatic potential of cancer cells (Fig. 2). Successful metastasis requires migration of the tumor cells through the basement membrane and invasion of the surrounding tissues. Our results indicate that breast cancer cells expressing SUSD2 may have enhanced metastatic potential, which was consistent with the increased incidence of lung metastases in the mouse model (Fig. 5C). Because metastatic disease is the main cause of death in patients with breast cancer, the presence of SUSD2 in a breast tumor may represent a poor prognosis for the patient.

Of great interest is the finding that SUSD2 interacts with Gal-1 (Fig. 3). Galectins are a family of carbohydrate-binding proteins that share an affinity for β-galactoside residues of various proteins and components of the extracellular matrix (25). Gal-1 has been extensively studied for its role in tumor immune-escape mechanisms (7) and angiogenesis (26–28). Our studies indicate that expression of SUSD2 leads to an increased amount of Gal-1 on the cell surface (Fig. 4). Previous studies have found that cellular localization of Gal-1 is essential for interaction with the immune system, and specifically that cell surface Gal-1 is necessary for Gal-1–mediated apoptosis of T cells (14). Our studies show that more Jurkat cells undergo apoptosis when cocultured with MDA-MB-231-SUSD2 cells compared with the vector-only control (Fig. 7). SUSD2-dependent cell surface localization of Gal-1 has not previously been reported and may play a significant role in modulating the body’s immune response to breast cancer. Two possible models for this interaction are that SUSD2 acts as a chaperone for Gal-1 to get to the surface of the cell or SUSD2 sequesters secreted Gal-1 on the cell surface. Future studies will define the exact interaction between the two proteins.

We generated stable 66CL4 cell lines to inject into the mammary fat pad region of mice. In general, mice in the 66CL4-Susd2 group displayed decreased survival and accelerated tumor growth compared with the 66CL4-vector control group, despite similar Ki67 indexes (Figs. 5 and 6). Although statistically significant (P = 0.0199), the difference in median survival rate between the 2 groups of mice was only 12%. This result may be explained by the fact that the 4T1 and 66CL4 mouse models are virulent models, and a larger difference in survival between the 2 groups may be observed using a less aggressive model. The difference in blood vessel growth between 66CL4-Susd2 and 66CL4-pLXSN tumors may partially account for the difference in tumor growth rates. The formation of tortuous and saccular vessels with haphazard patterns of excessive branching and interconnections is often correlated with accelerated tumor growth (29, 30).

Interestingly, significantly fewer CD4 lymphocytes were observed in the 66CL4-Susd2 tumors (Fig. 5D). The significance of CD4 lymphocytes within tumors is a controversial topic. CD4 cells are classified as “helper T cells,” and are generally thought to recruit and activate CD8 cytotoxic T cells and other lymphocytes to the site of immune activation. Evidence suggests that CD4 cells play a role in activating the host’s immune response to cancer by controlling the activation and persistence of CD8 T cell responses, and might also enhance the ability of the antigen-presenting cells to initiate an endogenous CD8 response (31). Recently, a subset of CD4+CD25+ T cells, classified as regulatory T cells, was identified and are now thought to be essential in controlling immune responses by suppression of effector T cell proliferation and cytokine production (32). Future studies are needed to categorize the mouse CD4 tumor-infiltrating lymphocyte populations into CD4+CD25− and CD4+CD25+; therefore, conclusions related to the effect of Susd2 on this particular subset of T cells are not in the scope of this study.

The major finding from this study indicates that expression of Susd2 by mammary tumors promotes many aspects of breast tumorigenesis, including tumor immune evasion, angiogenesis, and metastasis. We have shown that SUSD2 is a protein on the surface of breast cancer cells and may be a promising therapeutic target. By targeting SUSD2, multiple processes of breast tumorigenesis may be inhibited.

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

Authors’ Contributions
Conception and design: A.P. Watson, R.L. Evans, K.A. Egland
Development of methodology: A.P. Watson, R.L. Evans, K.A. Egland
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.P. Watson, R.L. Evans
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.P. Watson, R.L. Evans, K.A. Egland
Writing, review, and/or revision of the manuscript: A.P. Watson, R.L. Evans, K.A. Egland
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.P. Watson, R.L. Evans, K.A. Egland
Study supervision: K.A. Egland

Acknowledgments
The authors thank Drs. Keith Miskimins for helpful suggestions and critical reading of the article, and Sue Kane for excellent scientific support and outstanding ideas. The authors also acknowledge LCM Pathology (Sioux Falls, SD) and the Sanford Research Molecular Pathology Core run by Claire Evans for assistance with sample collection and immunohistochemical staining, and Jeffrey Sachs for his stellar laboratory skills and assistance with in vitro analyses of the mouse cell lines.

Grant Support
This research study was supported by a grant from Susan G. Komen for the Cure awarded to K.A. Egland. In addition, A.P. Watson was awarded a 2011 American Medical Association (AMA) Foundation Seed Grant Award. The Molecular Pathology, Flow Cytometry and Imaging Cores were supported by a grant from the NIH Center of Biomedical Research Excellence (COBRE; grant no. 1P20RR024219-01A2).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 24, 2012; accepted October 8, 2012; published OnlineFirst November 6, 2012.
References

Multiple Functions of Sushi Domain Containing 2 (SUSD2) in Breast Tumorigenesis

Allison P. Watson, Rick L. Evans and Kristi A. Egland


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-12-0501-T

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2012/11/06/1541-7786.MCR-12-0501-T.DC1

Cited articles
This article cites 32 articles, 12 of which you can access for free at:
http://mcr.aacrjournals.org/content/11/1/74.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/11/1/74.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mcr.aacrjournals.org/content/11/1/74.full#related-urls.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.