Shedding of Syndecan-1 by Stromal Fibroblasts Stimulates Human Breast Cancer Cell Proliferation via FGF2 Activation*§

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The cell surface heparan sulfate proteoglycan syndecan-1 is induced in stromal fibroblasts of breast carcinomas and participates in a reciprocal feedback loop, which stimulates carcinoma cell growth in vitro and in vivo. To define the molecular mechanism of carcinoma growth stimulation, a three-dimensional coculture model was developed that combines T47D breast carcinoma cells with immortalized human mammary fibroblasts in collagen gels. By silencing endogenous syndecan-1 induction with short interfering RNA and expressing mutant murine syndecan-1 constructs, it was determined that carcinoma cell mitogenesis required proteolytic shedding of syndecan-1 from the fibroblast surface. The paracrine growth signal was mediated by the syndecan-1 heparan sulfate chains rather than the ectodomain of the core protein and required fibroblast growth factor 2 and stroma-derived factor 1. This paracrine pathway may provide an opportunity for the therapeutic disruption of stroma-epithelial signaling.

We and others have recently observed a dramatic overexpression of the cell surface heparan sulfate proteoglycan (HSPG) syndecan-1 (Sdc1) in stromal fibroblasts of invasive breast carcinomas (9, 10). Sdc1 consists of an extracellular domain carrying heparan sulfate glycosaminoglycan (HSGAG) and chondroitin sulfate glycosaminoglycan (CSGAG), a transmembrane domain and a cytoplasmic domain. The biological functions of Sdc1 are not fully understood, but co-receptor roles in signaling of HSGAG-dependent growth factors and in cell adhesion events are well documented. Most of these activities are mediated through the HSGAG chains, but core protein-specific functions have also been described (11, 12). The Sdc1 ectodomain can be shed from the cell surface by proteolytic cleavage at a juxtamembrane site (13). The shed Sdc1 ectodomain retains its biologically active heparan sulfate chains and has been found to promote growth of myeloma tumors in vivo (14).

Syndecan-1 is normally expressed in many types of epithelia and plasma cells. Mesenchymal Sdc1 expression has been observed during the development of various organ systems and pathologically in stromal fibroblasts of several carcinoma types (9, 15–18). Sdc1 expression in carcinoma-associated fibroblasts is seen in the majority of infiltrating breast carcinomas and resembles Sdc1 induction in fibroblasts surrounding terminal end buds during mouse mammary gland development (9). This suggests that Sdc1 is involved in the reactivation of an oncofetal signaling pathway in breast carcinomas.

Our previous study has demonstrated that highly invasive MDA-MB-231 breast carcinoma cells induce Sdc1 expression in fibroblasts in a two-dimensional co-culture system and that Sdc1 overexpression in fibroblasts promotes cancer cell proliferation (10). The stimulating effect of stromal Sdc1 on mammary tumor cell growth has also been confirmed in vivo. Using mixed fibroblast-carcinoma cell xenografts, we have observed that forced expression of Sdc1 in fibroblasts promotes tumor growth and tumor angiogenesis (19).

Although these studies have advanced our understanding of the importance of HSPGs in epithelial-stromal interactions, they also pose a series of new questions regarding the require-

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The abbreviations used are: HSPG, heparan sulfate proteoglycan; HMF, human mammary fibroblasts; G6PDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; GFP, green fluorescent protein; siRNA, short interfering RNA; PBS, phosphate-buffered saline; m.o.i., multiplicity of infection; CSGAG, chondroitin sulfate glycosaminoglycan; FACs, fluorescence-activated cell sorter; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GAG, glycosaminoglycan; FGF, fibroblast growth factor; FGFR, FGFR receptor; RPE, (R)-phycoerythrin; MMP, matrix metalloprotease; m, murine; WT, wild type.
ment of specific Sdc1 molecular domains and the molecular mechanisms involved. To maintain a physiologically relevant milieu, we have developed a three-dimensional co-culture system that combines organ site-specific fibroblasts with breast carcinoma cells in a collagen matrix. We found that the growth-stimulating effect is mediated by the HSGAG chains on Sdc1, requires cleavage of the Sdc1 core protein at the cell surface, and also requires FGF2 activity. Because expression of the FGF2 receptor FGFR1c was up-regulated in T47D breast carcinoma cells, a model is proposed that involves the paracrine activity of fibroblast Sdc1-derived HSGAG forming a complex with FGF2 and FGFR1c on the carcinoma cell surface, resulting in accelerated growth.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse anti-delta heparan sulfate (3G10) antibody was purchased from Seikagaku America (Associates of Cape Cod, Falmouth, MA). Goat anti-human FGF2 neutralizing antibody was purchased from R & D Systems (Minneapolis, MN). Anti-human SDF1 polyclonal antibody and recombinant mouse SDF1 were bought from PeproTech Inc. (Rocky Hill, NJ). Rat monoclonal antibody directed against mouse syndecan-1 extracellular domain was kindly provided by Dr. A. C. Rapraeger (University of Wisconsin, Madison). Mouse anti-human Sdc1 (B-B4) conjugated to (R)-phycocyanine (RPE) was bought from Serotec Immunological Excellence (Oxford, UK). Heparitinase and chondroitinase ABC were purchased from IBEX Pharmaceuticals Inc. (Montreal, Quebec, Canada). Type I rat tail collagen was purchased from BD Biosciences.

Cell Culture—The human breast carcinoma cell line T47D was obtained from Dr. M. Gould (University of Wisconsin, Madison). The normal mammary fibroblasts immortalized with human telomerase and labeled with GFP (RMF/EG) were generously provided by Dr. A. C. Kuperwasser (20). Throughout this study, RMF/EG cells will be referred to as human mammary fibroblasts (HMF). T47D cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin/streptomycin. HMF cells were cultured in DMEM supplemented with 10% calf serum, 2 mM L-glutamine, and penicillin/streptomycin. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

Collagen Gel Co-culture and Cell Growth Assay—Three-dimensional collagen gel co-culture was established based on a method described previously by Wozniak and Keely (21). T47D cells and HMF cells were mixed at a ratio of 2:1 in collagen type I gel at a final collagen concentration of 1.3 mg/ml. Collagen gel co-culture was maintained in DMEM containing 10% calf serum at 37 °C in a humidified atmosphere containing 5% CO2. Twenty four hours after transduction, HMF-siRNA treated with 0.15M glycine in PBS at 4 °C overnight to reduce autofluorescence. After washing with PBS for 30 min, cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with 3% FBS in PBS for 2 h at room temperature. Collagen gels were then incubated with anti-human Sdc1 antibody (10 μg/ml, B-B4-RPE) at 4 °C overnight. After washing, the samples were analyzed on a FACSCalibur benchtop cytometer (BD Biosciences). Cell scatter and propidium iodide (1 μg/sample; Sigma) staining profiles were used to gate live, single-cell events for data analysis. Cells were sorted under sterile conditions on a triple-laser FACSVantage SE equipped with the FACSDiva digital electronics analyzer.

Quantitative RT-PCR—Quantitative real time PCR was performed to measure FGF receptor, syndecan-1, syndecan-2, and syndecan-4 mRNA levels. Cells were dissociated from collagen gels by collagenase treatment (2 mg/ml; Worthington) and enzyme-free cell dissociation buffer (Invitrogen) treatment (37 °C, 1 h) and washed with cold PBS containing 2% FBS. 106 cells were subsequently incubated for 30 min on ice with 1 μg of mouse anti-human syndecan-1 IgG conjugated to RPE (B-B4-RPE). After washing, the samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cell scatter and propidium iodide (1 μg/sample; Sigma) staining profiles were used to gate live, single-cell events for data analysis. Cells were sorted under sterile conditions on a three-laser FACSVantage SE equipped with the FACSDiva digital electronics analyzer.

Immunofluorescent Staining—Collagen gel cultures were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and washed for 20 min in PBS. Collagen gels were then treated with 0.15 M glycine in PBS at 4 °C overnight to reduce autofluorescence. After washing with PBS for 20 min, cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with 3% FBS in PBS for 2 h at room temperature. Collagen gels were then incubated with anti-human Sdc1 antibody (10 μg/ml, B-B4-RPE) at 4 °C overnight. After extensive washing with PBS, the secondary antibody, Alexa 594-conjugated anti-mouse (1:100; Invitrogen), was added for 1 h at room temperature. The gels were then washed four times with PBS. In the negative control, collagen gels were only stained with the secondary antibody. Immunostaining was then analyzed with a laser-scanning confocal microscope (MRC 1024, Bio-Rad).

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siRNA Transfection—siRNA transfection was performed as described (12). Three siRNAs designed to specifically silence human syndecan-1 were purchased from Ambion (Austin, TX). 400 nM siRNA was added to 1 × 10⁶ HMF cells using Lipofectamine 2000 and Opti-MEM 1 transfection medium (Invitrogen). Control cells were transfected with lipid-based vehicle alone. At 24–48 h after transfection, the HMF cells were lifted in trypsin (0.25% w/v) and co-cultured with T47D cells in collagen gel.

Recombinant Adenoviruses Transduction—cDNAs for mSdc1 (from A. C. Rapraeger, University of Wisconsin), mGpc1 (from G. David, University of Leuven, Belgium), mSdc1-TDM, sol-mSdc1, and uc-mSdc1 (all from R. Sander-son, University of Alabama at Birmingham) were cloned into the Adeno-X™ Expression Systems 2 according to the manufacturer’s protocol (Clontech). Different doses (5, 10, and 50 multiplicities of infection (m.o.i.)) of recombinant adenoviruses were applied to HMF cells prior to co-culture. Protein expression was confirmed by Western blotting.

HSPG Extraction—Total HMF cell HSPGs were purified as described previously (23). In brief, cultured cells were placed on ice, washed three times with cold HEPES-buffered saline, and extracted with 1 ml of TUT buffer per 10-cm dish (10 mM Tris, 8 mM urea, 0.1% Triton X-100, 1 mM Na₂SO₄, 1 mM phenylmeth-ylsulfonfluoride, 1 mM N-ethylmaleimide, pH 8.0) for 5 min and scraped into a conical tube. After sonication, 80–100 μl of DEAE beads pre-equilibrated with TUT were added to the tube. Tubes containing DEAE beads and extraction solution were rotated overnight at 4 °C. HSPGs were eluted with high salt buffer with a ratio of 1:1. Then 150 μl of DEAE beads pre-equilibrated with TUT were added. The rest of the procedure was the same as total HSPG extraction.

Isolation of GAGs—Glycosaminoglycans (GAGs) were isolated as reported by Lee et al. (24), with some minor modifications. Briefly, 80–100% confluent HMF cells were extracted with extraction buffer. The proteins and GAGs were precipitated by addition of 3 volumes of 95% ethanol containing 1.3% potassium acetate. The precipitate was digested exhaustively with proteinase K (Invitrogen) at 65 °C for 24 h. Proteins remaining in the digest were precipitated by 5% trichloroacetic acid, and the supernatant was once again precipitated by 3 volumes of 95% ethanol containing 1.3% potassium acetate. The precipitate was dried and desalted on a PD-10 column using pyridine acetate buffer, pH 5.0, as an eluent. The eluted fractions were dried by speedvac and pooled. Quantitation of sulfated GAGs was carried out by the dye-binding assay of Chandrasekhar et al. (25) using dimethyl-methylene blue (Aldrich), except that the absorbance was read at 525 nm.

Gel Electrophoresis and Western Blotting—Extracted HSPGs were digested with heparitinase and chondroitinase ABC (0.002 units/ml) twice for 2 h to remove all glycosaminoglycan chains. Samples and pre-stained molecular mass markers (Bio-Rad) were denatured in sample buffer (SDS 2%, glycerol 10%, bromphenol blue, β-mercaptoethanol 2.5%) and heated to 100 °C for 1 min before gel electrophoresis. Samples were then electrophoretically separated on a Criterion™ XT precast gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane. The blots were probed with anti-delta HS (3G10) antibody (0.03 μg/ml), which reacts with HS “stubs” generated by heparitinase treatment or rat anti-mouse Sdc1 (m281.2). A horseradish peroxidase-conjugated secondary IgG (Sigma) was used for detection. The signal was visualized with SuperSignal West Femto maximum sensitivity substrate (Pierce).

RESULTS

Mammary Stromal Fibroblasts Stimulate T47D Breast Carcinoma Cell Growth—In an organotypic co-culture model, T47D human breast carcinoma cells were combined with HMF and embedded in three-dimensional collagen gels. HMF cells had previously been immortalized by transduction with human telomerase and labeled by expression of GFP (20). Collagen was chosen because it resembles the extracellular matrix milieu of invasive breast carcinoma better than Matrigel, which models the laminin-rich basement membrane-type extracellular matrix surrounding normal breast epithelium (21, 26). T47D carcinoma cells in monoculture arranged themselves into small spheroids or irregular clusters, which did not significantly increase in size throughout a 7-day culture period (Fig. 1A). In contrast, the presence of fibroblasts induced progressive carcinoma cell growth, resulting in large, irregular, and partially branched epithelial cell clusters. A quantitative assessment of epithelial cell numbers revealed that the presence of fibroblasts caused a highly significant growth stimulation of the T47D cells from day 3 onward (Fig. 1B). The overall growth rate of the fibroblasts was low, and their growth rate was slightly suppressed by the presence of T47D cells.
T47D Breast Carcinoma Cells Induce Sdc1 Expression in Mammary Fibroblasts in Three-dimensional Co-culture—In a prior study, we had observed that the poorly differentiated MDA-MB-231 breast carcinoma cells induced expression of Sdc1 in mouse fibroblasts, whereas better differentiated carcinoma cells, including T47D, failed to do so (10). Using the three-dimensional co-culture system described here, T47D cells induced the fibroblast expression of Sdc1 mRNA ~5-fold (Fig. 2A). In contrast, expression of the syndecan family members Sdc2 and Sdc4 remained relatively unchanged. The induction of Sdc1 was confirmed at the protein level by immunofluorescence staining (Fig. 2B) and by flow cytometric analysis (Fig. 2C and supplemental Table 2) and closely resembled stromal Sdc1 induction in human and mouse mammary tumors in vivo.

T47D Breast Carcinoma Cell Growth Stimulation by Mammary Fibroblasts Requires Sdc1 Induction in Fibroblasts—We have recently described that Sdc1 induction in mouse fibroblasts stimulates breast carcinoma cell growth in vitro and in vivo, which prompted us to examine whether stromal Sdc1 induction in human fibroblasts was also responsible for breast carcinoma cell growth stimulation in three-dimensional culture (10, 19). Transfecting fibroblasts with Sdc1 siRNA constructs prior to co-culture significantly diminished Sdc1 induction compared with the vehicle-only controls. Western blots probed with an anti-HS stub antibody, which recognizes all HSPGs after heparitinase treatment, showed a selective reduction of Sdc1 without any effect on the levels of other HSPGs (Fig. 3B). Mammary fibroblasts pretreated with siRNA oligonucleotide 3 will be referred to as HMF-siRNA cells throughout the rest of the study. The suppression of Sdc1 induction in HMF-siRNA cells was confirmed by immunocytochemistry, which also demonstrated that Sdc1 expression in the carcinoma cells was unaffected (Fig. 3A).

Silencing of Sdc1 induction in fibroblasts abolished the T47D carcinoma cell growth advantage provided by the presence of fibroblasts but had no effect on fibroblast growth (Fig. 3C). Importantly, forced expression of murine Sdc1 (mSdc1, which is not targeted by the siRNA oligonucleotides) in the HMF-siRNA cells by adenoviral transduc-

![FIGURE 2. T47D breast carcinoma cells induce Sdc1 expression in mammary fibroblasts. A. Sdc1 mRNA level in HMF is increased in co-culture compared with monoculture. HMF cell total RNA was isolated and analyzed by quantitative RT-PCR as described under “Experimental Procedures.” Relative cycle threshold (RCT) was calculated by subtracting G6PDH cycle threshold from the raw cycle threshold. Fold mRNA level change in co-cultured HMF cells relative to monocultured cells was calculated as (2^((-ΔΔCT_{cocculture}) − ΔΔCT_{monoculture})). Sdc2 and Sdc4 mRNA level changes were measured as controls. *, p < 0.05 versus Sdc1. B. Immunofluorescent staining of Sdc1 in T47D and HMF cells. Collagen gel cultures were fixed and stained with mouse anti-human Sdc1 antibody (B-B4, 10 μg/ml) on the 5th day of culture (red channel). HMF cells were identified by GFP expression (green channel). Images were captured with a laser-scanning confocal microscope (MRC 1024, Bio-Rad). Scale bar, 25 μm. C, flow cytometry analysis of Sdc1 expression in T47D and HMF cells. Cells grown in collagen gels were released by collagenase and incubated with B-B4-RPE antibody for 30 min on ice. Data were collected with a FACSCalibur benchtop cytometer and analyzed using FlowJo software (Ashland, OR). Results are representative of several independent experiments.](image-url)
The Sdc1 Core Protein Is Dispensable for the Mitogenic Effect on T47D Breast Carcinoma Cells—The Sdc1 molecule is composed of a core protein and covalently linked HSGAG and CSGAG chains. Recent reports, which attributed important Sdc1 activities to its core protein domains (12, 27, 28), prompted us to investigate whether the core protein was required for carcinoma cell growth promotion by fibroblasts. HMF-siRNA cells were transduced to overexpress either murine glypican-1 (mGpc1, Fig. 4B) or mSdc1 as a control. Gpc1, which is expressed by mammary fibroblasts at baseline levels but is not induced in breast carcinoma stroma, was chosen because, like Sdc1, the molecule localizes to the cell surface but possesses no homology to Sdc1 apart from the GAG attachment consensus sites (Fig. 4A). Surprisingly, mGpc1 overexpression in the HMF-siRNA fibroblasts restored carcinoma cell growth stimulation similar to mSdc1 expression, indicating that the Sdc1 core protein is not specifically required (Fig. 4C). The base-line Gpc1 levels present in mammary fibroblasts in mono- and co-culture are insufficient to maintain carcinoma cell mitogenesis, but forced mGpc1 overexpression evidently compensates for suppressed Sdc1 induction.

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FIGURE 3. T47D cell growth stimulation requires stromal Sdc1 expression in fibroblasts. A, immunofluorescent staining for Sdc1. siRNA or mock-transfected HMF cells were co-cultured with T47D cells in collagen gels for 3 days and then fixed and stained with Alexa Fluor antibody (red channel). HMF cells were identified by GFP expression (green channel). Images were collected at high magnification (×60). B, Sdc1 expression in HMF cells is significantly decreased by siRNA transfection. Four days after transfection with siRNA, total HSPGs were extracted, treated with heparitinase and chondroitinase, and analyzed by Western blot using anti-delta HS (3G10) antibody. C, silencing Sdc1 expression in HMF cells with different siRNAs decreases T47D cell growth stimulation in co-culture. HMF-siRNA cells were co-cultured with T47D cells for 3 days. Cell growth was analyzed as indicated under “Experimental Procedures.” *, p < 0.05 versus control. D, Western blot analysis of mouse Sdc1 expression in HMF cells. HMF cells were transduced with adenovirus encoding mouse Sdc1. The next day, the cells were transfected with lipid alone or siRNA targeting human Sdc1. Twenty four hours after siRNA transfection, the HMF cells were cultured on Petri dishes or co-cultured with T47D cells in collagen gel for an additional 3 days. Total HSPGs were extracted from HMF cells grown on Petri dishes and analyzed by Western blot using rat anti-mouse Sdc1 antibody (m281.2; 2.5 μg/ml). E, HSPG sample from D was analyzed by Western blot and probed with antibody 3G10 (0.03 μg/ml). F, expression of mouse Sdc1 in HMF-siRNA cells rescues the T47D cell growth. HMF cells were transfected with adenovirus encoding mouse Sdc1 and transfected with siRNA targeting endogenous human Sdc1. 24 h after siRNA transfection, the HMF cells were co-cultured with T47D cells in collagen gel for 3 days. Collagen gels were then fixed and stained with Hoescht dye. The T47D cell number was determined as described above. Data represent mean ± S.E.* p < 0.05 versus control and mSdc1 5 or 10 m.o.i.

FIGURE 4. Glypican-1 overexpression in stromal fibroblasts restores T47D cell growth in co-culture. A, schematic representation of wild-type mSdc1 and mGpc1 constructs. Gpc1 is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. B, Western blot analysis of HSPG expression in HMF cells. HMF cells were transduced with adenovirus encoding mGpc1 and transfected with siRNA for human Sdc1. Total HSPGs were analyzed by Western blot using 3G10 antibody (0.03 μg/ml). C, mGpc1 overexpressed in HMF cells restores T47D cell growth in co-culture with HMF-siRNA cells. Collagen gel co-cultures were fixed and stained with Hoescht dye on the 3rd day of culture. T47D cell number was determined as described above. Data represent mean ± S.E. * p < 0.05 versus groups with recombinant virus treatment.
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**FIGURE 6.** Heparan sulfate chains are required for T47D cell growth stimulation by mammary fibroblasts. A, schematic representation of the TDM mSdc1 mutant (29). In this mutant, heparan sulfate attachment sites at 37, 45, and 47 have been eliminated. B, Western blot analysis of TDM mSdc1 expression in HMF cells. HMF cells were transduced with adenovirus encoding wild-type or TDM mSdc1 and transfected with siRNA targeting human Sdc1. Total HSGAGs were extracted and analyzed by Western blot using rat anti-mSdc1 antibody (m281.2; 2.5 μg/ml). C, TDM mSdc1 expressed in HMF-siRNA cells only partially rescues T47D cell growth in co-culture. Collagen gel co-cultures were grown for 3 days in 0.1 μg/ml intact or heparitinase-digested HSGAG. D, HSGAGs extracted from HMF cells rescue T47D cell growth in co-culture with HMF-siRNA cells. HSGAGs were extracted from HMF cells and quantified. Co-cultured cells were grown for 3 days in 0.1 μg/ml intact or heparitinase-digested HSGAG. T47D cell number was then quantified. Data represent mean ± S.E.

**HSGAG Chains on Sdc1 Are Necessary but Not Sufficient for T47D Carcinoma Cell Growth Stimulation**—This result suggests that carcinoma cell growth stimulation may be related to HSGAG chains. To test this hypothesis more directly, we transduced HMF-siRNA cells with the mSdc1 mutant, TDM mSdc1. In this mutant, three serine residues, which constitute the three N-terminal HSGAG attachment sites, have been changed to alanine, rendering the molecule HS-deficient (29) (Fig. 5A). Western blots with and without prior heparitinase digestion verified that TDM mSdc1 was expressed and that its glycanation was significantly reduced compared with WT mSdc1 (Fig. 5B). Forced TDM mSdc1 expression in HMF-siRNA cells incompletely restored growth stimulation of T47D carcinoma cells, indicating that full glycanation of this HSPG is required for its activity (Fig. 5C). The partial activity seen with this mutant may be due to the remaining two GAG attachment sites near the transmembrane domain (serines 210 and 220), which are preferentially decorated with CSGAG chains but can also carry HSGAGs (29). The dependence of the growth-promoting effect on Sdc1 HSGAGs was further tested by adding isolated HSGAGs to co-cultures of HMF-siRNA and T47D cells. Purified HSGAGs were able to restore the growth-promoting effects at concentrations similar to those expected during Sdc1 induction in the fibroblasts, but this activity was sensitive to heparitinase treatment (Fig. 5D). Interestingly, HSGAG chains did not promote T47D cell growth in monoculture, suggesting that other fibroblast-derived factors are required.

**Shedding of Sdc1 from the Fibroblast Surface Is Required for T47D Cell Growth Stimulation**—We noticed that mammary fibroblasts and T47D cells had only limited contact in the three-dimensional collagen gels (Fig. 3A), raising the possibility that Sdc1 may act at a distance from its cell surface attachment site. Proteolytic cleavage of Sdc1 with release of the ectodomain from the cell surface has been described *in vitro* and *in vivo*. In a myeloma model, shed Sdc1 ectodomain accelerates tumor growth *in vivo* in an autocrine manner (14). Interestingly, Gpc1, which can substitute for Sdc1 in our assay, is also readily released from the cell surface by cleavage of its glycosylphosphatidylinositol anchor. This led us to investigate the possibility that Sdc1 shedding may also play a role in paracrine breast carcinoma growth stimulation. When an Sdc1 mutant, which lacks a transmembrane and cytoplasmic domain (Fig. 6A) and is efficiently secreted (sol mSdc1, Fig. 6B), was forcibly expressed in HMF-siRNA cells, the mitogenic effect on T47D cells was restored, indicating that the soluble Sdc1 ectodomain is fully active in this assay (Fig. 6C). Western blots with and without prior heparitinase digestion verified that soluble mSdc1 is expressed at levels similar to WT mSdc1 and is glycanated with HSGAG chains (Fig. 6B). To address the question whether shedding is required for Sdc1 activity, we expressed an Sdc1 mutant in which the putative juxtamembrane proteolytic cleavage site from Gln-238 to Gln-252 had been replaced with the corresponding domain of human CD4 (13) (Fig. 6A). This uncleavable Sdc1 mutant was expressed at levels similar to wild-type mSdc1 (Fig. 6B) and was fully glycanated but did not restore T47D cell growth stimulation (Fig. 6C). In concert, these data indicate that Sdc1 is shed under co-culture conditions, that Sdc1 ectodomain is active, and that shedding is required for T47D cell growth stimulation.
forms are expressed in epithelial cells and are activated by stromally derived FGF3, FGF7, and FGF10. Conversely, the c isoforms are generally expressed in mesenchymal type cells and are activated by FGF1, FGF2, and FGF4. Surprisingly, in the presence of fibroblasts, the expression of the “mesenchymal” FGFR1c and FGFR2c in T47D cells was significantly up-regulated in addition to FGFR1b (Fig. 7A). This finding is reminiscent of the aberrant overexpression of FGFR1c during malignant progression of prostate epithelial cells (30). Because FGF2 is the principal FGFR1c ligand present in breast carcinomas, we investigated the role of this growth factor in stromal to epithelial signaling in breast cancer. A neutralizing anti-FGF2 anti-body abolished the growth stimulatory activity of mammary fibroblasts, whereas control serum had no effect, indicating that FGF2 is participating in an autocrine or paracrine signaling loop (Fig. 7B). To determine whether exogenous FGF2 can substitute for fibroblasts, fibroblast-derived HSGAGs were added to T47D cells in the presence or absence of FGF2. The results showed that FGF2 stimulated T47D cell growth in a dose-dependent manner (Fig. 7C). At the concentration of 100 pM FGF2 and 0.1 μg/ml fibroblast-derived HSGAGs, T47D cell growth in monoculture was stimulated to the same extent as in co-culture with fibroblasts (Fig. 7D).

These observations indicate that FGF2 is required for T47D cell growth stimulation in co-culture with fibroblasts and plausibly support, but do not prove, the notion that FGF2 activity is sufficient for mitogenesis. Stromal cell-derived growth factor-1 (SDF1), a chemokine also known as CXCL12, signals via CXCR4 and plays a key role in the tumor microenvironment. Recently, SDF1 has been shown to be responsible for the growth-stimulating effect of tumor fibroblasts on breast carcinoma (56). SDF1 is an attractive candidate molecule for Sdc1-dependent paracrine growth stimulation because HSGAGs bind the chemokine and modulate its activity (31, 32). The small molecule CXCR4 inhibitor AMD3100 (5 ng/ml) or an SDF1-neutralizing antibody significantly reduces the growth-promoting effect of HMF cells on T47D cells (data not shown and see Fig. 7E), indicating that the SDF1/CXCR4 axis is a critical component of our model. One way to explain the dependence on both FGF2 and SDF1 is a sequential involvement of the signaling molecules. To determine whether SDF1 acts downstream of FGF2 or vice versa, rescue experiments with recombinant cytokines were performed. FGF2 rescues T47D carcinoma cell growth in co-cultures treated with anti-SDF1 antibody (Fig. 7E). In contrast, SDF1 fails to rescue T47D growth in co-cultures treated with anti-FGF2 antibody (Fig. 7F).

This result suggests that FGF2 signaling lies downstream of the SDF1 pathway.

DISCUSSION

The data presented here support a model in which T47D breast carcinoma cells induce expression of Sdc1 in mammary fibroblasts and that the fibroblasts reciprocally stimulate T47D cell growth by a mechanism that involves Sdc1 shedding from the fibroblast surface. The mitogenic stimulus depends on both SDF1 and FGF2, where FGF2 shedding is situated downstream of SDF1 (Fig. 8).

Shedding of the Sdc1 ectodomain by proteolytic cleavage, which is obligatory for this model, has been well documented both in vitro and in vivo (33–35). Through its release from the cell surface, Sdc1 is converted from a cell surface receptor to a diffusible mediator. However, comparing direct contact co-culture and noncontact co-culture with tissue culture inserts, we previously had concluded that diffusible factors were insufficient for T47D carcinoma cell stimulation and that direct carcinoma cell-fibroblast contact was required (10). This apparent discrepancy suggests that the diffusion range of released Sdc1 ectodomain may be limited in these experiments by binding to pericellular matrix components. Diffusion and convection under “bulk” media culture (e.g. culture dishes with tissue cul-
ture inserts) have been shown to have profound effects on growth kinetics, presumably because of differences in the availability of paracrine factors in the microenvironments (36). Sdc1 ectodomain diffusion in collagen gels would be predicted to be particularly restricted, given its binding interaction with collagens I and III (37).

Sdc1 shedding occurs at basal levels but can be significantly increased by a variety of stimuli, which include the protein kinase C activator phorbol 12-myristate 13-acetate, epidermal growth factor family members, stromal cell-derived factor-1 (SDF1, CXCL12), and thrombin (38, 39). Interestingly, it has been reported recently that FGF2 can induce Sdc1 shedding, suggesting a positive feedback loop in our model (40). A distinct proteolytic cleavage site is present between amino acids Ala-243 and Ser-244 in the juxtamembrane region of the Sdc1 ectodomain (33). Proteolytic Sdc1 cleavage has been attributed to a variety of enzymes, which include matrix metalloprotease (MMP) 7, MMP9, and the membrane type MMPs MT1-MMP (MMP14) and MT3-MMP (13, 39–41). The identity of the protease(s) responsible for Sdc1 shedding in our model is subject to ongoing research in our laboratory.

The effect of shed Sdc1 ectodomain on cell proliferation is controversial. Sdc1 ectodomain has been reported to inhibit the proliferation of myeloma and different breast carcinoma cells (S115 and MCF-7) in an HSGAG-dependent manner (42, 43). Conversely, expression of a soluble form of Sdc1 in myeloma cells causes accelerated tumor growth in vivo, consistent with our observations (14). Further support for a detrimental role of shed Sdc1 is provided by the clinical observations that the serum Sdc1 ectodomain level is an independent prognostic factor in myeloma and correlates with poor prognosis in lung cancer (44, 45). The effects of Sdc1 ectodomain on tumor cells are likely not limited to enhanced mitogenesis. Yang and co-workers (14) observed increased invasion of myeloma cells in response to secreted Sdc1 ectodomain. In our study, Sdc1-positive fibroblasts induced carcinoma cells to form branch-

**FIGURE 7.** T47D cell growth in co-culture is FGF2- and SDF1-dependent. A, FGF receptor (FR) mRNA levels in T47D cells change under co-culture conditions. T47D cell total RNA isolation and quantitative RT-PCR were performed as described under “Experimental Procedures.” Data were calculated as mRNA level relative to the reference gene, G6PDH, using the formula \( \frac{2^{(-\Delta\Delta CT)}}{100} \), where CT is cycle threshold. B, FGF2 neutralizing antibody blocks T47D cell growth in co-culture. One or 10 ng/ml of the FGF2 neutralizing antibody was added in both collagen gel and culture media. Goat serum and omission of the antibody were used as controls. Data represent mean ± S.E. *, p < 0.05 versus controls. C, FGF2 stimulates T47D cell growth in collagen gel monoculture. FGF2 (0, 100, 500, 1000, and 5000 pM) was added into collagen gel and media in the presence or absence of 0.1 µg/ml fibroblast HSGAGs. T47D cell growth was analyzed after 3 days of incubation. Heparitinase I- and II-digested HSGAGs were used as a control. D, comparison of T47D cell monoculture treated with FGF2 and HSGAGs with co-culture. The combination of FGF2 and HSGAGs restores T47D cell growth in monoculture. E, partial inhibition of T47D cell growth in co-culture by anti-SDF1 antibody is rescued by FGF2. Mouse recombinant SDF1 (0, 50, and 100 ng/ml) and FGF2-neutralizing antibody (1 µg/ml) were added in both collagen gel and media. Cell growth was analyzed after 3 days of incubation. *p < 0.05 versus control.
ing, irregular clusters, which resemble infiltrating ductal carcinoma (Fig. 1A).

Our data support a model in which FGF2 stimulates breast carcinoma growth and requires fibroblast-derived HSGAG as a cofactor. This conclusion is unexpected because FGF2 is generally not considered a growth stimulator of breast carcinoma cells. Furthermore, T47D cells are not HSPG-deficient and indeed express abundant Sdc1 (46). Mammary epithelial cells generally respond poorly to FGF2 because they express FGFR2b, an FGFR isoform that binds stroma-derived FGF3 and FGF7 instead of FGF2 (47). In co-cultures of T47D breast carcinoma cells and mammary fibroblasts, we observed a remarkable up-regulation of FGFR1c and FGFR1b, which (in contrast to FGFR2b) bind and are activated by FGF2. The aberrant expression of mesenchymal type FGFRs has been described in prostate carcinomas, where it accompanies malignant progression and stromal independence (48). In prostate cancer, the appearance of FGFR1c was accompanied by loss of FGFR2b. This receptor loss was not observed in our model. The up-regulation of FGFR1c and FGFR1b expression during co-culture may contribute to T47D growth stimulation but does not appear to be required because T47D cells respond to FGF2 in the absence of fibroblasts (Fig. 7C).

In order for an FGF ligand to generate a sustained signal, both an appropriate FGFR and a suitable HSGAG are required (49, 50). Specific, highly sulfated domains within HSGAG chains are thought to interact with domains within the FGF and FGFR, thus stabilizing a ligated FGFR dimer capable of signaling (51). Recent work suggests a link between HSPG expression and the proliferative potential in breast carcinoma cells. Our laboratory and others have shown that in large groups of human patients with breast cancer, high Sdc1 levels correlate with an aggressive, highly proliferative cancer phenotype and poor survival (52, 53). Compared with normal breast epithelium, breast carcinoma cell HSGAGs have an elevated ability to form a ternary complex with FGF2 and FGFR1c in vivo, an activity that correlates closely with Sdc1 expression levels (23). However, HSGAGs from almost half of the carcinomas tested had very limited or no activity as FGF2 co-factors, suggesting that in a subgroup of carcinomas HSPGs do not support FGF2 signaling.

Because T47D cells respond to FGF2 only weakly unless fibroblast-derived HSGAGs are present (Fig. 7C), it can be concluded that T47D HSGAGs are poorly suited as FGF2 co-factors. The concept of HSGAGs being the limiting factor in FGF signaling in breast carcinoma is supported by work from other groups. Delehede et al. (54) showed that MDA-MB-231 breast carcinoma cells produce HSGAGs that inhibit FGF2 signaling. When the degree of sulfation was reduced artificially by chlorate treatment, the cells became FGF2-responsive. Fibroblasts may supply HSGAGs, which compensate for carcinoma cell HSPGs with poor activity or compete with carcinoma cell HSPGs with inhibitory activity in FGF2 signaling. Interestingly, Sdc1 ectodomain HSGAGs can be modified in the tissue environment. Sdc1 ectodomain isolated from NMuMG mouse mammary epithelial cells or isolated from wound fluid inhibits FGF2 signaling via FGFR1c but is converted to a stimulator after degradation of poorly sulfated domains by bacterial heparitinase or mammalian heparinase (55). Whether similar post-synthetic modifications are required for fibroblast-derived Sdc1 ectodomain in our model is unknown.

In summary, we define a reciprocal growth-promoting signaling pathway between mammary fibroblasts and breast carcinoma cells that requires the proteolytic cleavage of Sdc1 from the fibroblast surface, the activity of Sdc1 HSGAG chains, SDF1, and FGF2. This model provides several opportunities for therapeutic intervention. The specific disruption of proteolytic Sdc1 shedding appears as a particularly attractive strategy.

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