[CANCER RESEARCH 64, 612–621, January 15, 2004]

Induction of Syndecan-1 Expression in Stromal Fibroblasts Promotes Proliferation of Human Breast Cancer Cells

Takashi Maeda,[2] Caroline M. Alexander,[3] and Andreas Friedl[1,2]

[1]Pathology and Laboratory Medicine Service, Department of Veterans Affairs Medical Center, Madison, Wisconsin; [2]Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison, Wisconsin; and [3]Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin

ABSTRACT

Infiltrating carcinomas characteristically elicit a reactive stromal response, and accumulating evidence indicates that tumor stroma fibroblasts reciprocally promote tumor development and growth. The cell surface heparan sulfate proteoglycan, syndecan-1 (Sdc1), is thought to function as a coreceptor for growth factor and extracellular matrix interactions, and Sdc1 expression is induced in reactive stromal cells in both mice and man. Mice with a targeted mutation in Sdc1 show reduced tumor development in response to oncogene expression and altered responses to other pathological stimuli that are associated with the induction of stromal Sdc1. Here, we test the hypothesis that Sdc1 is required for the growth-promoting activities of reactive stroma. We found that when highly invasive carcinoma cells (MDA-MB-231) were placed in contact with mouse embryonic fibroblasts (MEFs) in a coculture model, Sdc1 expression was induced. Sdc1 was not induced by less invasive or normal cell lines (T47D and NMuMG). Furthermore, the growth of MDA-MB-231 cells was enhanced by 42% when cocultured with Sdc1+/+ MEFs compared with Sdc1−/− MEFs. When T47D cells were cocultured with fibroblasts that expressed transfected Sdc1, these Sdc1-positive fibroblasts stimulated growth of the breast epithelial cells by 85% compared with untransfected controls. The growth-promoting effect was completely abolished when fibroblasts were transfected with mutant Sdc1 lacking heparan sulfate attachment sites. In conclusion, we have demonstrated that a growth-promoting loop exists between breast cancer cells and their stroma that depends on the activity of glycanated Sdc1.

INTRODUCTION

The importance of the microenvironment for tumor growth was recognized as early as 1889, when Paget formulated his "seed and soil" hypothesis on cancer growth and metastasis (reviewed in Ref. 1). Nevertheless, modern research efforts have focused largely on investigating genetic and functional abnormalities in the tumor cells. More recently, it has become evident that tumors have to be regarded as complex organ systems and that a better understanding of the relationship between tumor cells and their stromal environment is needed.

Reactive tumor stroma is formed at the site of primary invasion as a result of complex interactions between host stromal cells and invading tumor cells. The reactive stroma is an integral part of the tumor and contributes to the destructive effects of the disease. For example, tumor growth is not determined solely by the tumor cells but is rather governed by multifaceted communications between tumor cells and surrounding benign but altered host stromal tissue (2). However, the molecular mechanisms through which reactive stroma regulates tumor development and growth remain unknown.

Infiltrating ductal carcinoma, which represents the most common type of breast cancer, exhibits a particularly pronounced degree of stromal response. Compared with normal breast, the reactive stroma of infiltrating carcinoma shows remarkable alterations in the morphology and differentiation of fibroblasts and the composition of its extracellular matrix (ECM) (3–8). A recent report described elevated immunoreactivity for syndecan-1 (Sdc1) in the reactive stroma of infiltrating breast carcinoma tissues (9). The syndecans are a family of transmembrane cell surface heparan sulfate proteoglycans (HSPGs), which regulate cell-cell and cell-ECM adhesion, cell migration, and growth factor activity. The biological effects of syndecans are thought to be mediated through the binding of growth factors; *e.g.*, fibroblast growth factors (FGFs), hepatocyte growth factor, vascular endothelial growth factor, heparin-binding epidermal growth factor-like growth factor, and ECM molecules via the heparan sulfate (HS) chains (10, 11). Among syndecan family members, Sdc1 is expressed mainly in normal epithelial cells and tissues, but it is also transiently expressed in condensing mesenchyme during embryonal morphogenesis (12–14). The general pattern is transient loss of Sdc1 expression in the epithelium, with intense expression appearing in the condensed mesenchyme adjacent to the epithelium. Interestingly, the elevated levels of Sdc1 in mesenchyme-derived stromal cells and decreased levels in epithelial cells within infiltrating ductal carcinomas strikingly resemble the manner of Sdc1 expression observed at zones of active epithelial-mesenchymal interactions during fetal development (9).

In concert, the reported experimental evidence points toward Sdc1 as a key regulator of epithelial stromal interactions in development and cancer and prompted us to investigate the functional consequences of Sdc1 expression in tumor-associated fibroblasts. In the present study, we characterized stromal Sdc1 expression in human and murine tumor samples and developed an *in vitro* coculture model to simulate the Sdc1 induction observed *in vivo*. We find that tumors are heterogeneous in their ability to induce Sdc1. Furthermore, we observe that Sdc1 expression in stromal cells promotes carcinoma cell growth. These data suggest that Sdc1 induction in the reactive stroma of human breast carcinoma may play a critical role in tumor progression.

MATERIALS AND METHODS

Cells. The human breast carcinoma cell lines MDA-MB-231, MDA-MB-436, Hs578T, BT549, and MCF-7 were provided by Dr. A. C. Rapraeger (University of Wisconsin, Madison, WI). T47D breast carcinoma cells were obtained from Dr. M. Gould (University of Wisconsin). The normal mouse mammary gland epithelial cell line NMuMG (15) was provided by Dr. A. C. Rapraeger, and NIH-3T3 fibroblasts were obtained from Dr. J. S. Malter (University of Wisconsin). Primary mouse embryonic fibroblasts (MEFs) from wild-type animals (Sdc1+/+ MEFs) and from Sdc1-deficient mice (Sdc1−/− MEFs) were isolated according to standard methods from day 9.5 embryos and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (16, 17). All other cells were also cultured in DMEM plus 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Human and Mouse Tissue Samples and Immunohistochemistry. Twenty-nine randomly selected cases of human infiltrating breast carcinomas were used for immunohistochemical analysis. Institutional Human Subjects Committee approval was obtained for these studies (Protocol No. 1995-314). Paraffin-embedded tissue sections were subjected to immunostaining for Sdc1,

Received 8/6/03; revised 9/19/03; accepted 11/5/03.

Grant support: Supported by Cancer Center Core Grant CA14520.

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.

Requests for reprints: Andreas Friedl, University of Wisconsin-Madison, Department of Pathology and Laboratory Medicine, Clinical Sciences Center K4/850, 600 Highland Avenue, Madison, Wisconsin 53792-8550. E-mail: afriedl@wisc.edu.

syndecan-4, and glypican-1 as described by us previously (18). A monoclonal antibody (mAb) directed against human Sdc1 or Sdc3 cytoplasmic domain [clone 2E9; a gift from Dr. G. David (University of Leuven, Leuven, Belgium); Ref. 19] was applied at 5 μg/ml.

Normal adult and developing mouse mammary glands, Wnt-1-induced hyperplastic glands, and Wnt-1-induced mammary gland tumors (17) from Sdc1+/+ and Sdc1−/− animals were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin for sectioning. After epitope retrieval (pressure cooker heating for 2 min in 0.01 M citrate buffer) and blocking, the sections were incubated with rat mAb 281-2 against mouse Sdc1 ectodomain (4 μg/ml; a gift from Dr. A. C. Rapraeger; Ref. 20) at 4°C overnight. Total HSPGs were detected with anti-ΔHS antibody 3G10 as described previously (18, 19). Alexa 568-conjugated goat antirat IgG (1:400 dilution; Molecular Probes, Eugene, OR) served as secondary detection reagent. Tissue sections were then washed again and incubated with FITC-conjugated mouse mAb PCK-26 against pan-cytokeratin (1:25 dilution; Sigma Chemical Co., St. Louis, MO). Immunofluorescence was visualized using an epifluorescence microscope (Olympus BX51) equipped with a SPOT RT slider chilled charge-coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI). Sections for the negative control were prepared using normal rat IgG (Sigma Chemical Co.) instead of the primary antibody.

FGF Receptor (FGFR) Complex Reconstitution *in Situ*. The assays were performed with human infiltrating breast carcinoma sections as described by us previously (18, 21). Briefly, after blocking, sections were first incubated with FGF-2 (10 nM) and then incubated with FR1-AP (30 nM), a soluble fusion protein, containing the extracellular domain of FGFR-1 and alkaline phosphatase as enzyme tag (22). Signal was visualized with the peroxidase-based Envision Plus detection kit (DAKO, Carpinteria, CA). For negative controls, tissue sections were treated with heparitinase (Seikagaku, Falmouth, MA) before growth factor incubation, or the FGF-2 incubation step was omitted.

Coculture Experiments. Direct cocultures of epithelial cells and MEFs were performed using 8-well glass chamber slides coated with fibronectin (BD Biosciences, Bedford, MA). Sdc1+/+ MEF cells were seeded at a density of 4×10^4 cells/well. After 48-h incubation, breast cancer cell lines (MDA-MB-231, MDA-MB-436, Hs578T, BT549, MCF-7, and T47D) or normal mammary gland epithelial cell line (NMuMG) was seeded (1×10^4 cells) in each well and left to grow for 4 days in DMEM with 10% FBS. At the end of the experiments, cells were washed with cold PBS, and Sdc1 expression in the fibroblasts population was examined by immunocytochemistry.

For fluorescence-activated cell-sorting (FACS) analysis, Sdc1+/+ MEF cells were seeded at a density of 1×10^5 cells/well in 24-well plates. After a 48-h incubation, breast cancer cell lines (MDA-MB-231 and T47D) were seeded at a density of 2.5×10^4 cells/well and left to grow for 4 days. At the end of the experiments, cells were washed with cold PBS, and Sdc1 expression in fibroblasts was analyzed by flow cytometry.

In some experiments, designated noncontact coculture, Sdc1+/+ MEF cells were seeded in 24-well plates and grown for 48 h as described above. Then, cell culture inserts (0.4-μm pore size; BD Biosciences) were placed in each well, and 5.0×10^4 MDA-MB-231 cells in 300 μl of medium were added to each insert and incubated for 4 days. At the end of the experiments, the inserts were removed, Sdc1+/+ MEF cells were washed with cold PBS, and Sdc1 expression was analyzed by flow cytometry.

To evaluate the effect of stromal Sdc1 expression on carcinoma cell growth, Sdc1+/+ MEF cells and Sdc1−/− MEF cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After a 48-h incubation, breast cancer cell lines were seeded (5×10^2 cells) to each well and left to grow for 5 days in DMEM with 10% FBS. Similarly, cocultures were performed with T47D and NIH-3T3 cells. NIH-3T3 cells transfected with wild-type Sdc1 (NIH-3T3/Sdc1), HS-deficient $Sdc1^{TDM}$ (NIH-3T3/$Sdc1^{TDM}$), or empty vector (NIH-3T3/vector; described below) were seeded in 96-well plate at a density of 2×10^4 cells/well and grown to confluence. T47D cells were seeded (1×10^3 cells/well) and left to grow for 6 days in DMEM with 5% FBS before the growth assays.

Immunocytochemistry. Cells on glass chamber slides coated with fibronectin (BD Biosciences) were fixed in 3.7% formaldehyde at room temperature for 15 min. After washing and blocking, the slides were incubated with rat mAb 281-2 (1 μg/ml) followed by Alexa 568-conjugated goat antirat IgG as a secondary antibody (1:400 dilution).

Cytokeratin ELISA. Epithelial cell number was evaluated in the cocultures by cytokeratin ELISA. The 96-well plates were washed, and cells were fixed in methanol-acetone (1:1) at −20°C for 10 min. After washing, the cells were blocked for 15 min and then incubated with rabbit polyclonal antibody NCL-CKp against human multicytokeratin (1:200 dilution; Novocastra, Newcastle, United Kingdom) at room temperature for 1 h. After washing, peroxidase-conjugated donkey antirabbit IgG (1:200 dilution; Amersham Pharmacia Biotech Inc., Piscataway, NJ) was added to each well and incubated for 30 min. The plates were washed and incubated with 100 μl of 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped by the addition of 25 μl of 1 M H_2SO_4, and plates were read at 450 nm. Results were blanked against fibroblast cultures alone.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay. DNA fragmentation of individual cells was detected *in situ* by TUNEL assay with the *In Situ* Cell Death Detection kit, Fluorescein (Roche Molecular Biochemicals, Mannheim, Germany). Cytokeratin/TUNEL dual staining was performed to allow identification of MDA-MB-231 cells. MDA-MB-231 cell cocultures with Sdc1+/+ MEF or Sdc1−/− MEF cells on 8-well chamber slides were prepared as described above. Cells grown for 2 or 5 days were washed with PBS and fixed in methanol-acetone at −20°C for 10 min. After blocking, the slides were incubated with rabbit polyclonal antibody NCL-CKp against human multicytokeratin (1:200 dilution; Novocastra), followed by Alexa 568-conjugated goat antirabbit IgG as a secondary antibody (1:400 dilution; Molecular Probes). After washing, incubation in freshly prepared TUNEL reaction mixture was performed for 60 min at 37°C in the dark, and nuclei were counterstained with Hoechst 33258 (Sigma). The slides were examined by fluorescence microscopy using appropriate filter sets. For the TUNEL-positive control, cells were treated with DNase I (Promega, Madison, WI) at a concentration of 10 units/μl for 10 min at room temperature after fixation. An apoptotic index was calculated as the ratio of the number of red cells with green nuclei (cytokeratin and TUNEL positive) and the number of total red cells (cytokeratin positive). This experiment was performed in quadruplicate, and at least 2×10^2 MDA-MB-231 cells were counted per well.

Proliferation Marker Analysis. Proliferation of MDA-MB-231 cells in coculture was measured by determining Ki-67 labeling. Cytokeratin/Ki-67 dual staining was carried out to allow MDA-MB-231 cells to be distinguished from fibroblasts. Cocultures grown on 8-well chamber slides for 2 or 5 days were washed with PBS and fixed in methanol-acetone at −20°C for 10 min. After blocking, the cells were incubated with rabbit polyclonal antibody NCL-CKp against human multicytokeratin and mouse mAb 7B11 against human Ki-67 (1:80 dilution; Zymed Laboratories Inc., South San Francisco, CA). Incubation with Alexa 488-conjugated goat antirabbit IgG (1:400 dilution; Molecular Probes) and Alexa 546-conjugated goat antimouse IgG (1:400 dilution; Molecular Probes) supplemented with Hoechst 33258 completed the procedure. The proliferation index was calculated as the percentage of the number of green cells with orange nuclei (cytokeratin and Ki-67 positive) divided by the number of total green cells (cytokeratin positive). This experiment was performed in quadruplicate. At least 3×10^2 MDA-MB-231 cells were counted per well.

Stable Transfection of Sdc1 cDNA. Mouse Sdc1 cDNA (22) containing the coding region (gift from Dr. A. C. Rapraeger) was inserted into the *Eco*RV site of pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Mouse Sdc1 with a mutation of all three HS attachment sites ($Sdc1^{TDM}$) was kindly provided by Dr. R. Sanderson (23). Proper orientation of the insert was confirmed by DNA sequencing. The transfection reagent TransIT-LT1 reagent (Mirus Co., Madison, WI) was used according to the manufacturer's instructions. After selection in G418 (500 μg/ml; Mediatech Inc., Herndon, VA), cells expressing the transfected proteoglycan were enriched by two consecutive rounds of FACS (see below). The enriched populations were routinely cultured with 250 μg/ml G418.

FACS. FACS was performed to examine Sdc1 expression on the cell surface and select for cells expressing high levels of core protein. Cells were dissociated from plates using enzyme-free cell dissociation buffer (Life Technologies, Inc.-Invitrogen Corp., Grand Island, NY), washed with culture medium, and counted. After resuspension in FACS buffer (PBS with 2% FBS), cells were incubated with rat mAb 281-2 (0.5 μg/10^6 cells) on ice for 30 min. Then, the cells were washed two times and incubated with phycoerythrin-conjugated antirat IgG (BD Biosciences) for 30 min on ice. After washing, samples were analyzed using a Becton Dickinson FACS instrument. Samples

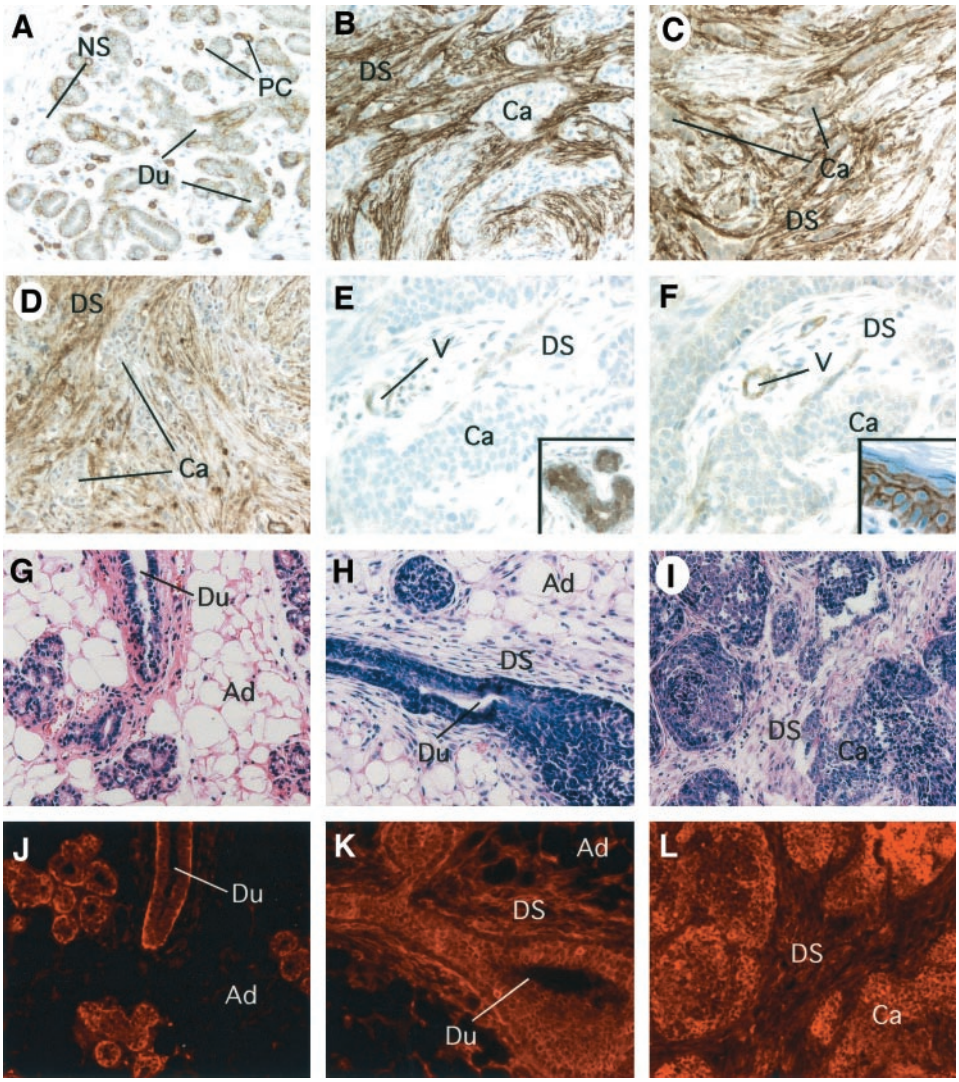

Fig. 1. Syndecan-1 (Sdc1) is induced in stromal fibroblasts of human and murine breast carcinoma samples. *A–F*, human mammary gland tissue samples. *A*, immunoperoxidase staining of normal terminal duct lobular unit for Sdc1 using antibody against the extracellular domain. No immunoreactivity is present in normal stromal fibroblasts, whereas epithelial cells of ductules (*Du*) and plasma cells (*PC*) serve as internal positive control. *B*, immunoperoxidase staining of infiltrating ductal carcinoma for Sdc1 using antibody against the extracellular domain. Strong staining is seen in the desmoplastic stroma (*DS*). In this case, the carcinoma cells (*Ca*) are Sdc1 negative. *C*, immunoperoxidase staining of infiltrating ductal carcinoma for Sdc1 using antibody against the cytoplasmic domain. Strong staining is seen in the desmoplastic stroma. *D*, reconstitution of the fibroblast growth factor (FGF)-2/heparan sulfate proteoglycan/FGFR1c complex *in situ*. Section was incubated with FGF-2 and subsequently incubated with FR1c-AP, a fusion protein containing the extracellular domain of FGF receptor 1 and alkaline phosphatase. Bound FR1c-AP was detected with anti-alkaline phosphatase antibody and visualized with horseradish peroxidase. Heparan sulfate proteoglycans in the desmoplastic stroma bind FGF-2 and promote binding of FGF-2 to FGF receptor 1c. *E*, immunoperoxidase staining of infiltrating ductal carcinoma for syndecan-4. Note weak staining of blood vessel (*V*), but negative stroma. *Inset* shows strong syndecan-4 positivity in normal breast acini as positive control. *F*, staining of infiltrating ductal carcinoma for glypican-1. Note moderate staining of blood vessels and mild staining of carcinoma cells, but negative stroma. *Inset* shows strong glypican-1 positivity in epidermal keratinocytes as positive control. *G–L*, tissue samples from mice overexpressing Wnt-1 in their mammary glands. *G* and *J*, serial sections of normal mammary glands were stained with H&E (*G*) or with antimouse Sdc1 monoclonal antibody 281-2, detected with Alexa 568-conjugated antirat IgG (*red channel*; *J*). No Sdc1 immunoreactivity was found in stroma surrounding normal mammary glands, whereas epithelial cells are positive. *H* and *K*, serial sections of Wnt-1-induced hyperplastic/preinvasive glands were stained with H&E (*H*) or with antimouse Sdc1 monoclonal antibody (*K*). A restricted pattern of Sdc1 expression was observed in the reactive stroma adjacent to the abnormal duct. *I* and *L*, serial sections of Wnt-1-induced mammary gland tumors were stained with H&E (*I*) or with antimouse Sdc1 monoclonal antibody (*L*). Immunostaining for Sdc1 was observed in stroma associated with invasive carcinomas. The carcinoma cells were also Sdc1 positive. Original magnification of all panels, ×200. *Du*, normal duct/ductule; *NS*, normal stroma; *PC*, plasma cell; *Ca*, carcinoma cells; *DS*, desmoplastic stroma; *Ad*, adipose tissue; *V*, blood vessel.

for the negative control were prepared using isotype rat IgG2aκ (BD Biosciences) at the same concentration instead of the primary antibody.

Bromodeoxyuridine Assay. To determine the growth-stimulatory effect of NIH-3T3/Sdc1-conditioned medium on T47D cells, confluent NIH-3T3/Sdc1 cells or control transfectants were cultured for 2 days in the presence of 5% FBS, and conditioned media were harvested. Carcinoma cells were seeded at a density of 1×10^4 cells/well in 96-well plates and grown for 24 h in DMEM supplemented with 10% FBS. The cells were then washed with PBS, and medium was replaced with serum-free DMEM for 24 h. Subsequently, the medium was replaced with conditioned media described above, incubated for 18 h, and labeled with bromodeoxyuridine during the last 3 h of incubation. Incorporated bromodeoxyuridine was detected according to the manufacturer's instructions. Results were blanked against cell cultures without bromodeoxyuridine labeling.

Statistical Analysis. Student's *t* test was used for statistical analysis of the experiments. $P < 0.05$ was taken as the level of significance.

RESULTS

Sdc1 Is Induced in Stromal Fibroblasts of Mammary Carcinomas. Sdc1 expression in stromal cells was measured by immunohistochemistry in 29 randomly selected infiltrating ductal carcinoma samples using an antibody directed against the extracellular domain of

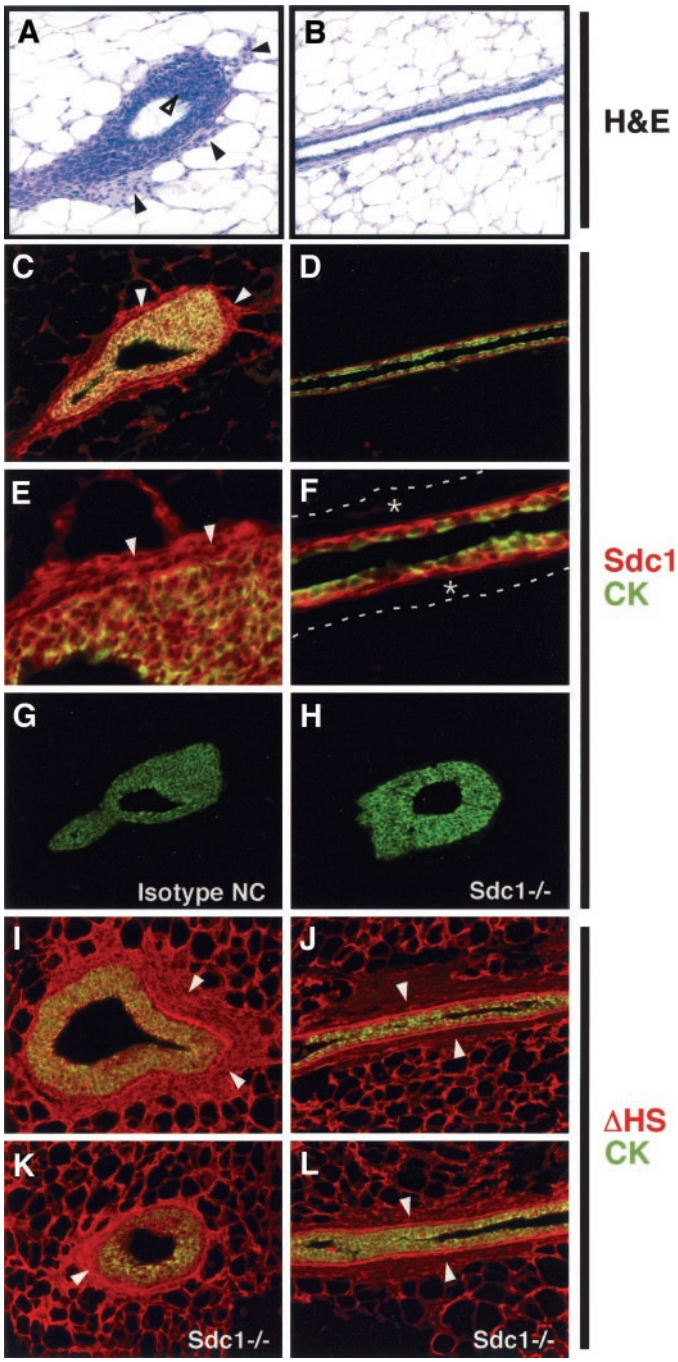

Fig. 2. Syndecan-1 (Sdc1) is induced in stromal cells of developing mouse mammary gland. Paraffin sections of mammary gland tissue from pubescent 4-week-old mice were stained with H&E (*A* and *B*), with antimouse Sdc1 antibody 281 (*red signal*; *C*−*F* and *H*), and with anti-ΔHS antibody 3G10 (*red signal*; *I*−*L*). Antibody 3G10 reacts with HS stubs generated by heparitinase digestion and allows the detection of all heparan sulfate proteoglycans regardless of the core protein. All slides were double labeled with FITC-conjugated anti-pan-cytokeratin antibody (*green signal*). *A*, terminal end bud (TEB). *B*, mature duct in same section. *C*, TEB of Sdc1+/+ mouse stained for Sdc1 (*red*) and cytokeratin (*green*). Note Sdc1 expression not only by the epithelial cells but also by stromal fibroblasts (*white arrowheads*). *D*, duct of Sdc1+/+ mouse stained for Sdc1 (*red*) and cytokeratin (*green*). Note Sdc1 expression by luminal epithelial cells and myoepithelial cells, but not by stromal fibroblasts. *E*, same TEB as shown in *C*, but at ×600 magnification. Note strong Sdc1 expression by stromal fibroblasts surrounding the TEB (*white arrowheads*). *F*, same duct as shown in *D*, but at ×600 magnification. Fibroblastic and collagenous stroma is marked by *asterisk* and outlined by *dashed line*. Note only minimal Sdc1 expression in stromal tissue compartment. *G*, negative control, where anti-Sdc1 antibody has been replaced with isotype-matched rat IgG at same concentration. *H*, TEB of Sdc1−/− mouse stained with anti-Sdc1 antibody (*red*) and cytokeratin (*green*). As expected, Sdc1 is not detected in the Sdc1-deficient mice. *I*, TEB of Sdc1+/+ mouse stained with anti-ΔHS antibody (*red*) and anti-cytokeratin antibody (*green*). Note strong signal in stromal compartment indicated by *white arrowheads*. *J*, duct of Sdc1+/+ mouse stained with anti-ΔHS antibody (*red*) and anti-cytokeratin antibody (*green*). *White arrowheads* indicate stromal compartment. *K*, TEB of Sdc1−/− mouse stained with anti-ΔHS antibody (*red*) and anti-cytokeratin antibody (*green*). Note strong signal in stromal compartment (*white arrowheads*). *L*, duct of Sdc1−/− mouse stained with anti-ΔHS antibody (*red*) and anti-cytokeratin antibody (*green*). *White arrowheads* indicate stromal compartment. All images ×200 magnification unless otherwise indicated.

this molecule. In 22 cases (76%), moderate to strong immunoreactivity was observed at least focally in stromal fibroblasts. Applying more stringent evaluation criteria, 10 cases (34%) showed diffuse and strong stromal staining (Fig. 1*B*). Sdc1 expression by carcinoma cells was variable, ranging from strongly positive to completely negative (Fig. 1*B*; Ref. 18). In contrast to the carcinoma tissues, no stromal Sdc1 staining was detected in normal breast tissues, whereas normal epithelium and plasma cells stained strongly for Sdc1 in this location (Fig. 1*A*). The presence of Sdc1 in the stromal compartment could be the result of synthesis by stromal cells or shedding from carcinoma cells. Proteolytic release of Sdc1 from the cell surface is well documented, and other investigators hypothesized that this mechanism may also be responsible for Sdc1 accumulation in breast carcinoma stroma (9, 24). To distinguish between these possibilities, we repeated immunohistochemical staining on a limited number of carcinoma samples with strong stromal Sdc1 positivity using an antibody directed against the cytoplasmic domain of Sdc1 (19). A signal with this antibody would only be expected in case of Sdc1 production by stromal cells. Indeed, the staining pattern observed with the anti-cytoplasmic domain antibody is identical to that generated by the anti-extracellular domain antibody (Fig. 1*C*). This result shows that Sdc1 is expressed by stromal cells; however, the possibility that shed Sdc1 ectodomain is also present in the stroma cannot be excluded. Interestingly, no stromal Sdc1 induction was observed in any of 20 prostate carcinoma cases present on a tissue microarray (data not shown).

Growth factor coreceptor activity is a well-characterized function of cell surface HSPGs, a role best documented for FGF-2. HS chains are thought to promote signaling by participating in a ternary complex with FGF ligand and FGFR. As an example for HS-regulated growth factor signaling, we examined the ability of stromal HSPGs to promote the FGF-2-FGFR1c interaction by reconstituting the ternary complex *in situ*. Indeed, in the presence of FGF-2, soluble FGFR1c binds to HSPGs in the stromal compartment, colocalizing with Sdc1 expression (Fig. 1*D*). Complex formation *in situ* is dependent on HS chains and FGF-2 because treatment with heparitinase greatly reduces the binding signal, and omission of the FGF-2 incubation step abolishes the binding signal (data not shown; Ref. 18). These data suggest that Sdc1 promotes FGF-2 binding activity in stromal fibroblasts, although we cannot exclude the possibility that other HSPGs also contribute.

To test the possibility that stromal fibroblasts of breast carcinomas generally up-regulate HSPG expression, we examined expression of other cell surface HSPGs. Syndecan-4 is found in tumor microvessels, but not in stromal fibroblasts (Fig. 1*E*). Glypican-1 is also detected in some breast carcinoma microvessels and focally immediately surrounding carcinoma cell islands, but it does not colocalize with stromal fibroblasts (Fig. 1*F*). These observations suggest that Sdc1 overexpression is specific to this HSPG.

We further examined peritumoral stromal Sdc1 expression in Wnt-1-induced mouse mammary tumor tissues (17). Strong immunostaining for Sdc1 was observed in stroma associated with invasive carcinomas (Fig. 1, *I* and *L*). The carcinoma cells also expressed Sdc1 in this model. In contrast, no Sdc1 immunoreactivity was found in stroma surrounding normal mammary glands (Fig. 1, *G* and *J*). In hyperplastic/preinvasive glands, a more regionally restricted but pro-

nounced pattern of stromal Sdc1 overexpression was observed (Fig. 1, *H* and *K*).

Sdc1 Expression in Mesenchyme Occurs Normally during Mammary Gland Development. In several organ systems, Sdc1 is expressed at sites of intense epithelial-mesenchymal interactions during development, similar to the pattern of Sdc1 expression in mammary carcinomas. This led us to speculate that Sdc1 induction in breast tumor stroma may represent an oncofetal reactivation of an epithelial-mesenchymal signaling pathway. In mice, at the beginning of puberty at about 3 weeks of age, cells at the ductal tips start proliferating and form bulbous structures known as terminal end buds (TEBs). The TEBs advance through the mammary fat pad, generating the arborizing ductal/alveolar system via growth and branching morphogenesis. The advancing growth of the TEB is not dissimilar to the infiltrative growth occurring at the leading edge of carcinomas. The TEB is surrounded by a small amount of fibroblast-rich stroma (Fig. 2*A*), which strongly expresses Sdc1 (Fig. 2, *C* and *E*). In contrast, stroma associated with mature ducts found at a small distance from the end bud on the same tissue section (Fig. 2*B*) contains essentially no Sdc1 (Fig. 2, *D* and *F*). As expected, Sdc1 is absent from either mammary stroma or epithelium of Sdc1−/− mice (Fig. 2*H*). Next, we evaluated total HSPG content in TEB stroma, using an antibody that reacts with HS stubs (ΔHS) generated by heparitinase treatment, regardless of the core protein. Total HSPG content does not differ significantly between Sdc1+/+ and Sdc1−/− mice, suggesting that either Sdc1 represents a HSPG of minor abundance or that a compensatory increase of other HSPGs occurs in Sdc1−/− mice at this tissue location (Fig. 2, *I* and *K*). The total HSPG content is higher in TEB stroma than in periductal stroma in either animal type (Fig. 2, *I*−*L*).

In a Coculture Model, Sdc1 Is Induced in Response to Contact with MDA-MB-231 Breast Carcinoma Cells. In direct coculture, we used a number of human breast carcinoma cell lines (MDA-MB-231, MDA-MB-436, Hs578T, BT549, MCF-7, and T47D) and normal mouse NMuMG cells. These epithelial cells were cocultured with embryonic fibroblasts from wild-type mice (Sdc1+/+ MEF cells) to examine whether Sdc1 induction in fibroblasts occurs *in vitro*. MDA-MB-231 and T47D cells formed tumor island surrounded by fibroblasts highly reminiscent of infiltrating ductal carcinoma *in vivo* (Fig. 3). There was a well-defined epithelial-stromal interface, which was more evident in coculture with T47D cells. MCF-7 cells formed small tumor islands, but their growth on the fibroblast layer was slow (data not shown). NMuMG cells also formed clear epithelial islands surrounded by fibroblasts (Fig. 3). However, Hs578T, BT549, and MDA-MB-436 cells lacked the ability to form clear tumor islands on the surface of fibroblasts (data not shown).

Immunocytochemical staining revealed that among the carcinoma cell types capable of forming epithelial islands, only highly invasive MDA-MB-231 cells induced Sdc1 expression in fibroblasts growing in contact with tumor cells in direct coculture (Fig. 3). Because the antimouse Sdc1 mAb 281-2 does not react with human Sdc1, the increase in immunoreactivity for Sdc1 in tumor-associated fibroblasts is the result of induction rather than shedding from MDA-MB-231 cells, which is in keeping with our observations in human carcinoma tissues. T47D carcinoma cells, which are better differentiated than MDA-MB-231 cells (25, 26), and benign NMuMG cells failed to induce Sdc1 in adjacent fibroblasts (Fig. 3). All breast carcinoma cell types lacking the ability to form epithelial islands in our coculture system similarly failed to induce Sdc1 expression in surrounding fibroblasts (data not shown). Induction of cell surface Sdc1 expression in fibroblasts was also confirmed by FACS analysis. Coculture of Sdc1+/+ MEF cells with MDA-MB-231 cells resulted in a subpopulation with significantly elevated Sdc1 levels (Fig. 4*A*), whereas T47D cells lacked the ability to produce this change in the fibroblasts (Fig. 4*B*). To examine the possibility that soluble factors derived from tumor cells are involved in Sdc1 induction, fibroblasts were indirectly cocultured with MDA-MB-231 cells using cell culture membrane inserts to prevent direct cell-cell contacts. FACS analysis revealed that Sdc1 was not induced in Sdc1+/+ MEF cells in this culture setting (Fig. 4*C*). These results suggest that direct cell-cell contact is necessary for Sdc1 induction in this coculture system, but induction by a short-range or labile diffusible factor cannot entirely be excluded.

Stromal Sdc1 Induction Promotes MDA-MB-231 Breast Carcinoma Cell Growth. Next we sought to test the hypothesis that a tumor stroma rich in Sdc1-expressing fibroblasts acts as a permissive microenvironment for tumor growth. To this end, carcinoma cell growth in the presence of wild-type (Sdc1+/+ MEFs) and genetically Sdc1-deficient (Sdc1−/− MEFs) fibroblasts was compared. Carcinoma cell growth was specifically measured using a cytokeratin ELISA. Cytokeratin is a marker of epithelial cells, and therefore, the signal generated in this assay is a measure of the number of carcinoma cells only. Earlier pilot experiments had confirmed a linear relation-

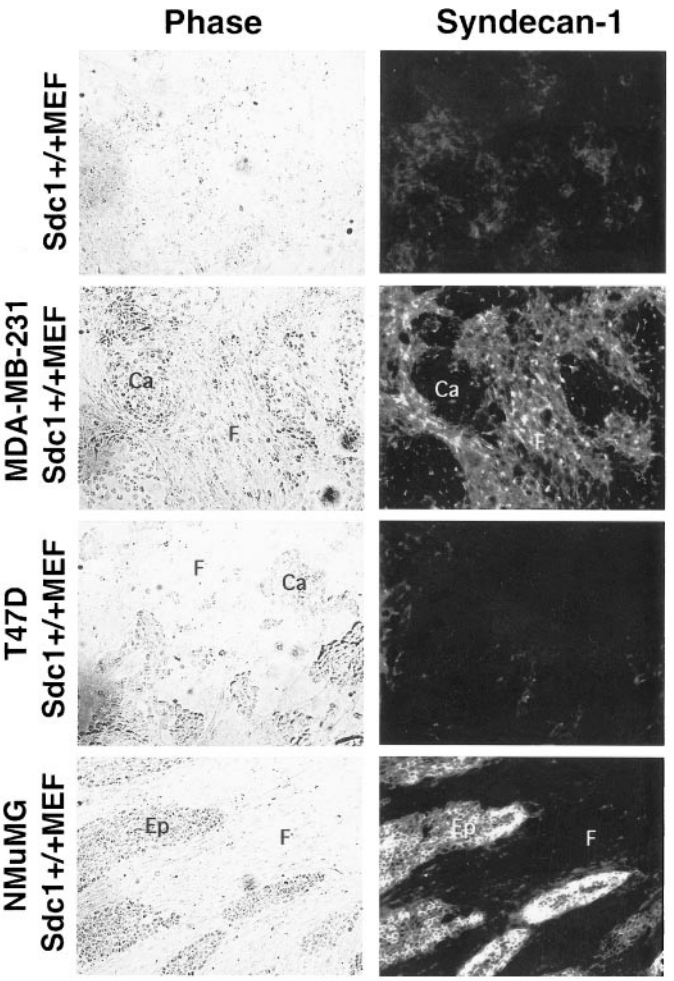

Fig. 3. Syndecan-1 (Sdc1) is induced in mouse embryonic fibroblasts (MEFs) by direct coculture with highly invasive MDA-MB-231 breast carcinoma cells. Phase-contrast and fluorescence photomicrographs of Sdc1+/+ MEF cells cocultured with human MDA-MBA-231 (Sdc1+/+ MEFs/MDA231), T47D breast carcinoma cells (Sdc1+/+ MEFs/T47D), or NMuMG normal mouse mammary epithelial cells (Sdc1+/+ MEFs/NMuMG) are shown. Cocultures were performed on glass chamber slides for 4 days. Antimouse Sdc1 monoclonal antibody 281-2 was visualized with Alexa 568-conjugated antirat IgG. Induction of Sdc1 expression was seen in Sdc1+/+ MEF cells surrounding MDA-MB-231 cells. NMuMG cells showed strong Sdc1 expression. Note that monoclonal antibody 281-2 does not react with human Sdc1. Original magnification, ×100. *Ca*, carcinoma cells; *F*, fibroblasts; *Ep*, epithelial cells.

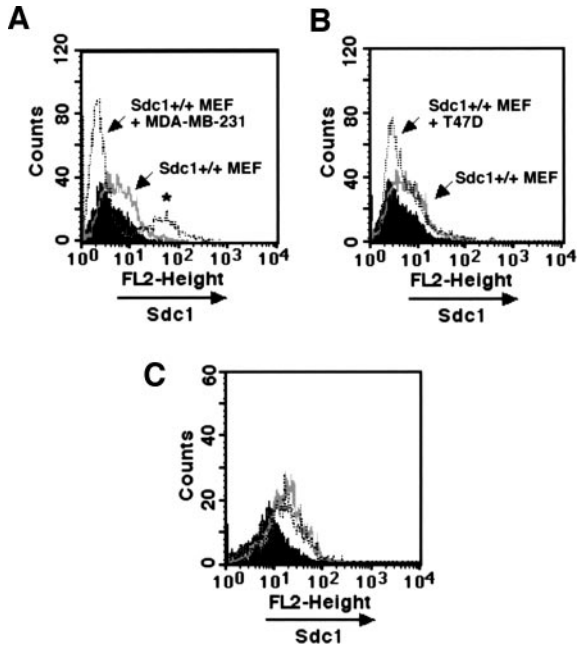

Fig. 4. Flow cytometric analysis of syndecan-1 (Sdc1) in mouse embryonic fibroblasts (MEFs). Sdc1 cell surface expression was examined in Sdc1+/+ MEF cells directly or indirectly cocultured with breast carcinoma cells. *A* and *B*, Sdc1+/+ MEF cells were directly cocultured with MDA-MB-231 cells (*A*) or T47D cells (*B*) in 24-well plates for 4 days. Cells were dissociated and stained with rat anti-Sdc1 monoclonal antibody 281-2. After incubation with goat antirat IgG-R-PE, Sdc1 cell surface expression was examined by flow cytometry. Data are presented as the overlays of isotype control (*filled*), control Sdc1+/+ MEF cells (*gray-shaded*), and Sdc1+/+ MEF cells cocultured with cancer cells (*dotted*) histograms. The *asterisk* in *A* indicates a population of Sdc1-expressing fibroblasts. *C*, Sdc1+/+ MEF cells were cocultured with MDA-MB-231 cells separated by cell culture inserts for 4 days. Sdc1 expression was examined by flow cytometry in a similar fashion. Data are presented as the overlays of isotype control (*filled*), control Sdc1+/+ MEF cells (*gray-shaded*), and Sdc1+/+ MEF cells indirectly cocultured with MDA-MB-231 cells (*dotted*) histograms. Results are representative of two different experiments.

ship between cell number and absorbance signal (data not shown). Coculture of MDA-MB-231 cells with Sdc1+/+ MEF cells yielded significantly increased carcinoma cell numbers, compared with parallel cocultures with Sdc1−/− MEF cells (Fig. 5). In contrast, other breast cancer cell lines, which were not able to induce Sdc1 in surrounding Sdc1+/+ MEF cells, grew at a similar rate in coculture with either Sdc1+/+ MEF or Sdc1−/− MEF cells. These results indicate that Sdc1 expression in surrounding fibroblasts promotes growth of MDA-MB-231 cells in direct coculture.

Sdc1 Expression by Fibroblasts Modulates Cell Proliferation but Not Apoptosis of MDA-MB-231 Breast Carcinoma in Direct Coculture. Tumor growth is the result of a relative balance between cell proliferation and cell death. Therefore, it is possible that Sdc1-expressing stromal fibroblasts exert their growth-promoting effect by inducing proliferation or by reducing apoptosis. To distinguish between these possibilities, we measured proliferation index and apoptotic index of MDA-MB-231 cells in coculture with Sdc1+/+ MEF cells or Sdc1−/− MEF cells.

Ki-67, a nuclear proliferation-associated antigen, was measured to examine cell proliferation. After direct coculture with Sdc1+/+ MEF or Sdc1−/− MEF cells for 2 or 5 days, MDA-MB-231 cells were immunostained for Ki-67. Epithelial MDA-MB-231 cells were distinguished from fibroblasts using cytokeratin double staining (Fig. 6). At day 2, a significantly increased MDA-MB-231 proliferation index was observed in the presence of Sdc1+/+ MEF compared with Sdc1−/− MEF cells (Table 1). At day 5, when the cells had achieved a higher degree of confluence, this difference had largely disappeared. We further examined cell death of MDA-MB-231 cells in coculture with the two types of fibroblasts using the TUNEL assay. The type of surrounding fibroblasts (Sdc1+/+ MEFs or Sdc1−/− MEFs) did not affect cell death of MDA-MB-231 cells at either day 2 or 5 (Table 1). These results suggest that Sdc1-expressing fibroblasts stimulate carcinoma cell proliferation rather than reduce carcinoma cell death.

Stable Expression of Sdc1 in NIH-3T3 Cells Promotes Proliferation of T47D Breast Carcinoma Cells in Direct Coculture, and HS Is Required for Carcinoma Cell Growth Stimulation by Stromal Sdc1. T47D cells have a lower invasive capacity and failed to induce Sdc1 expression within surrounding Sdc1+/+ MEF cells as described above. Consequently, T47D cells gained no growth advantage from coculture with Sdc1+/+ MEF *versus* Sdc1−/− MEF cells. To provide further evidence for the growth-promoting effect of Sdc1 in tumor-associated fibroblasts on breast cancer cells and to determine whether HS required for this effect, we cocultured T47D cells with fibroblasts in which Sdc1 had been forcibly expressed. We chose NIH-3T3 cells because this immortal cell line can be stably transfected. As in Sdc1+/+ MEF cells, T47D cells failed to induce Sdc1 in wild-type NIH-3T3 cells (data not shown).

NIH-3T3 cells were stably transfected with mouse Sdc1 cDNA or with a cDNA coding for a mutant form of the Sdc1 core protein, which lacks all three HS attachment sites (23). To avoid potential clonal variation, pooled populations of sorted cells rather than single clones of transfected cells were used in all experiments. Expression of transfected protein (NIH-3T3/Sdc1 and NIH-3T3/Sdc1^{TDM}) in the sorted cells was verified by immunocytochemistry and FACS analysis. In contrast, vector-transfected control cells (NIH-3T3/vector) produced Sdc1 at a low baseline level (Fig. 7*A*). The growth rates of the three fibroblastic cell types did not differ significantly (Fig. 7*B*).

T47D cells were cocultured with NIH-3T3/Sdc1, NIH-3T3/Sdc1^{TDM}, and NIH-3T3/vector cells, and epithelial cell number was evaluated by cytokeratin-ELISA after 6 days in culture. The number of T47D cells in the presence of NIH-3T3/Sdc1 fibroblasts was significantly higher than that in the presence of control NIH-3T3/

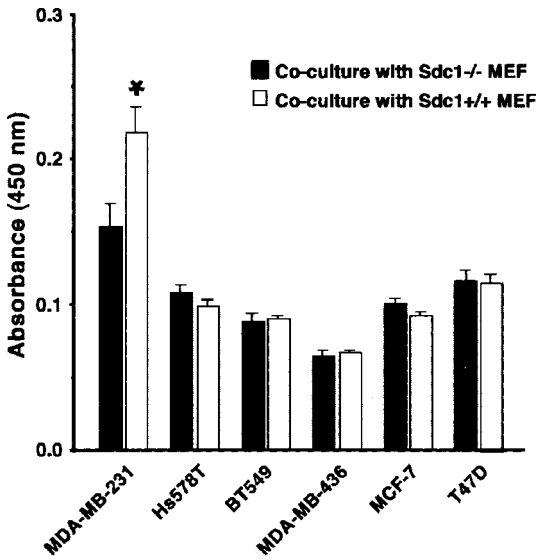

Fig. 5. Growth of MDA-MB-231 breast carcinoma cells is enhanced by coculture with wild-type mouse embryonic fibroblasts (MEFs) compared with coculture with syndecan-1 (Sdc1)-deficient fibroblasts. Breast cancer cell lines (MDA-MB-231, Hs578T, BT549, MDA-MB-436, MCF-7, and T47D) were directly cocultured with Sdc1+/+ MEF or Sdc1−/− MEF cells in 96-well plates for 5 days. Cells were fixed, and the number of cancer cells was evaluated by cytokeratin ELISA using antihuman multicytokeratin antibody. The number of MDA-MB-231 cells cocultured with Sdc1+/+ MEF cells was significantly increased compared with that cocultured with Sdc1−/− MEF cells. Results are representative of three independent experiments. Each *bar* represents the mean ± SD ($n = 5$). *, $P < 0.01$.

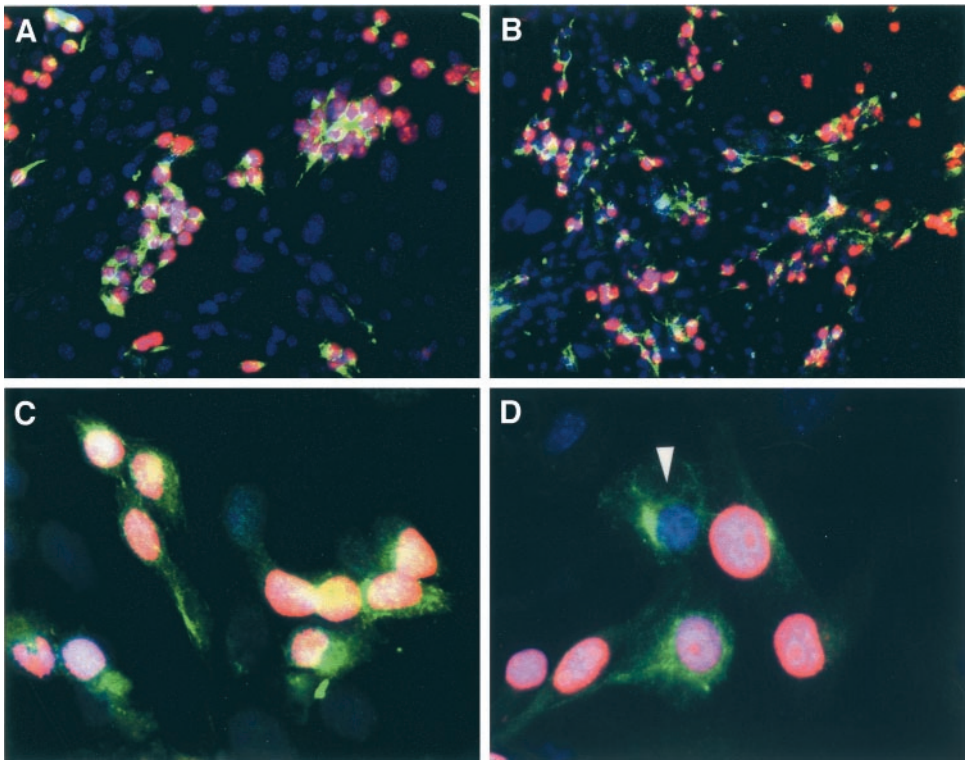

Fig. 6. Syndecan-1 (Sdc1)-expressing fibroblasts stimulate proliferation of MDA-MB-231 breast carcinoma cells. MDA-MB-231 cells were directly cocultured with Sdc1+/+ MEFs or Sdc1−/− MEFs on glass chamber slides for 2 days. Cells were fixed with methanol-acetone and immunostained for Ki-67 (*orange*). Epithelial MDA-MB-231 cells were distinguished using cytokeratin double staining (*green*). The nuclei were counterstained with Hoechst 33258 (*blue*). Three-color merged images are shown. *A* and *C*, MDA-MB-231 and Sdc1+/+ MEF cells in coculture. The carcinoma cell islands are recognizable as cells with *green cytoplasm* and *pink nuclei*, the fibroblasts are seen as *blue nuclei* only. *B* and *D*, MDA-MB-231 and Sdc1−/− MEF cells in coculture. The *arrowhead* indicates a cytokeratin-positive but Ki-67-negative cell. Original magnification: *A*, ×200; *B*, ×100; *C* and *D*, ×600.

Table 1 *Apoptosis and proliferation of MDA-MB-231 breast carcinoma cells in coculture with MEFs*[a]

The apoptotic index represents the fraction of TUNEL-positive carcinoma cells, and the proliferative index represents the fraction of Ki67-positive carcinoma cells.

	Apoptotic index (%)		Proliferative index (%)	
Coculture of MDA-MB-231	Day 2	Day 5	Day 2	Day 5
with Sdc1+/+ MEF	1.3 ± 0.7[b]	1.6 ± 0.5	93.2 ± 1.5	97.8 ± 0.2
with Sdc1−/− MEF	1.7 ± 0.6	1.4 ± 0.2	81.2 ± 1.7[c]	94.4 ± 1.5

[a] MEF, mouse-derived embryonic fibroblast; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; Sdc, syndecan.
[b] Each value represents the mean ± SD.
[c] Significant difference between Sdc1+/+ and Sdc1−/− MEF ($P < 0.01$).

vector cells (Fig. 8*A*). In concert with evidence provided above, this result indicates that Sdc1 expression in fibroblasts promotes growth of neighboring breast carcinoma cells in direct coculture. Importantly, the growth-promoting effect was abolished when fibroblasts expressed HS-deficient Sdc1 (Fig. 8*A*), demonstrating that HS chains are required. Interestingly, conditioned media of wild-type Sdc1-expressing fibroblasts did not affect cell proliferation of T47D cells (Fig. 8*B*), suggesting that direct cell-cell contact is required for the growth-promoting effect. However, a short-range or labile diffusible mediator cannot be ruled out.

DISCUSSION

Malignant tumors characteristically acquire the ability to invade adjacent normal host tissues. The process of invasion places tumor cells and normal host stromal cells in direct contact and leads to dramatic morphological and functional alterations in stromal cells (3, 27). Pathologists refer to the formation of a reactive tumor stroma as desmoplastic response, which is one of the hallmarks of infiltrating carcinomas.

However, signals clearly do not flow unidirectionally from tumor cells to stromal cells because the stromal environment also reciprocally influences tumor cell behavior. Olumi *et al.* (2) have demonstrated a striking tumor-promoting ability of carcinoma-associated stromal cells. Other investigators showed that, conversely, normal mammary stromal cells inhibited the growth of MCF7 breast carci-

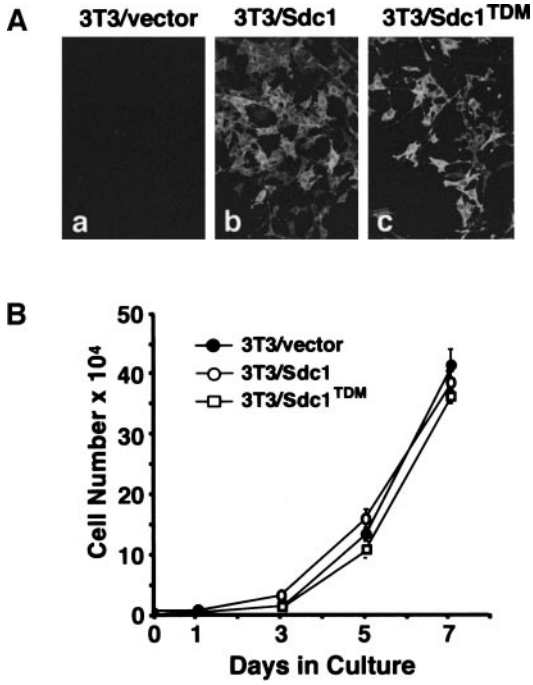

Fig. 7. Characterization of syndecan-1 (Sdc1)-transfected NIH-3T3 fibroblasts. *A*, immunofluorescence analysis of cell surface Sdc1 in NIH-3T3 fibroblasts. NIH-3T3 fibroblasts were transfected either with control plasmid (3T3/vector; *a*), wild-type Sdc1 (3T3/Sdc1; *b*), or mutant Sdc1 lacking heparan sulfate (3T3/Sdc1^{TDM}; *c*) and cultured on glass camber slides. Cells were fixed and stained with anti-Sdc1 monoclonal antibody, followed by Alexa 568-conjugated goat antirat IgG. Original magnification: ×200. *B*, growth of Sdc1 transfectants *in vitro*. 3T3/vector, 3T3/Sdc1, or 3T3/Sdc1^{TDM} cells were seeded into 24-well plates. Cells were counted in triplicate at the indicated days. Results are shown as mean ± SD.

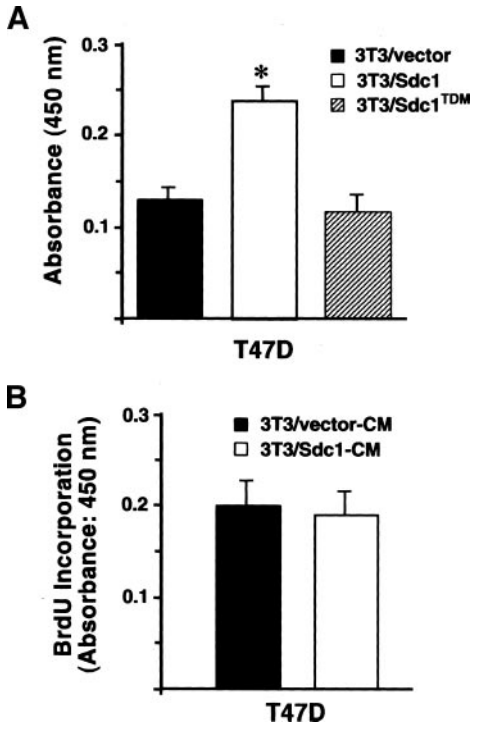

Fig. 8. *A*, Sdc1-transfected fibroblasts promote growth of T47D cells in direct coculture in a heparan sulfate-dependent fashion. T47D cells were directly cocultured with 3T3/vector, 3T3/Sdc1, or 3T3/Sdc1^{TDM} cells in 96-well plates for 6 days. Cells were fixed, and the number of T47D cells was evaluated by cytokeratin ELISA. Results are representative of three independent experiments. Each *bar* represents the mean ± SD ($n = 5$). *, $P < 0.01$. *B*, conditioned media of Sdc1-transfected cells does not promote proliferation of T47D cells. T47D cells were incubated with conditioned media from control transfectants (*3T3/vector-CM*) or wild-type Sdc1 transfectants (*3T3/Sdc1-CM*) for 18 h as indicated. Cell growth was determined by the bromodeoxyuridine incorporation assay. Each *bar* represents the mean ± SD ($n = 5$).

noma cells (28). These findings suggest that stromal cells can generate positive as well as negative growth signals.

The molecular mechanisms underlying these stromal-epithelial communications are currently unclear. A comparison of gene expression profiles between preinvasive and invasive breast carcinomas using serial analysis of gene expression recently revealed a cluster of "invasion-specific" genes (29). Expression of some of these genes or expressed sequence tags localized to the juxtatumoral stroma. The functional role of most of these molecules in tumor growth and progression remains unresolved.

With our present work, we establish the molecular basis for a feedback loop between breast carcinoma cells and stromal fibroblasts. Using fibroblasts isolated from Sdc1 knockout mice, we show that stromal cell expression of the cell surface HSPG Sdc1 is required for the growth-promoting effect on carcinoma cells. Of course, it is likely that Sdc1 is not sufficient but that additional molecules participate in this signal exchange. Our observations raise several questions: is stromal Sdc1 induction a unique feature of breast carcinomas, and what is the prognostic impact of stromal Sdc1 expression? What carcinoma cell-derived factor(s) is (are) responsible for stromal Sdc1 induction? What molecular mechanism mediates the tumor growth-promoting effect of Sdc1-expressing stromal fibroblasts?

In our experiments, stromal Sdc1 expression occurs both in human carcinomas and in murine Wnt-1-induced tumors, indicating its relevance across species. Whether or not lack of stromal Sdc1 contributes to the reduced tumor incidence observed in Sdc1 knockout mice (17) is currently unknown. An immunohistochemical survey of a small number of prostate carcinomas failed to reveal stromal Sdc1 induction, although this tumor type shares some morphological and many functional characteristics with breast carcinomas. However, stromal Sdc1 induction is not unique to breast carcinomas. Stromal Sdc1 expression has been reported in basal cell carcinomas of skin and in gastric carcinomas, where its presence is associated with a poor clinical outcome (30, 31). The impact of stromal Sdc1 on breast carcinoma prognosis is currently under investigation in our laboratory.

Another essential open question is the mechanism of stromal syndecan induction. A line of study by other investigators has examined the regulation of Sdc1 expression in 3T3 fibroblasts. They found that members of the FGF family (in particular, FGF-2), but not other growth factors such as epidermal growth factor, platelet-derived growth factor, or insulin-like growth factor, induced Sdc1 in this cell type (32). Sdc1 induction occurred via a FGF-inducible response element, located far upstream of the proximal promoter (33). A paracrine induction mechanism is also an attractive hypothesis for the breast carcinoma model, considering the location of Sdc1-expressing fibroblasts in the juxtatumoral stroma. Therefore, we tested the hypothesis that soluble carcinoma cell-derived factors are responsible for Sdc1 induction by using an indirect noncontact coculture system (transwell system). In contrast to direct coculture, breast carcinoma cells failed to induce Sdc1 in fibroblasts when separated by a membrane, suggesting that direct cell-cell contact is required. In support of this notion, it has been shown that during experimental organ culture recombination of early dental epithelium and mesenchyme, induction and spread of the Sdc1-positive zone in the dental mesenchyme required close and continuous contact with the epithelium (34). Also, MDA-MB-231 cells lack FGF-2 expression, ruling out this growth factor as paracrine mediator (35). Although we cannot exclude the possibility of diffusible factors participating in Sdc1 induction, they do not appear to be sufficient in our assay system.

In the simple coculture system used for our experiments, stromal Sdc1 induction was restricted to breast carcinoma cells capable of forming epithelial islands on the fibroblast layer. Among a series of breast carcinoma cells tested, only MDA-MB-231 cells both formed epithelial islands and induced Sdc1. The low rate of Sdc1 induction in coculture likely reflects the limitations of the *in vitro* system rather than the true biological occurrence of this event, considering that 76% of human breast carcinoma samples showed at least moderate stromal Sdc1 induction.

Equally important as the induction mechanism is the nature of the growth signal generated by Sdc1-expressing stromal fibroblasts. Interestingly, growth stimulation was not limited to carcinoma cells capable of inducing stromal Sdc1. Instead, increased growth was also observed when T47D cells, which themselves lack the ability to induce stromal Sdc1, were cocultured with fibroblasts in which Sdc1 had been forcibly expressed. This result suggests that the growth signal generated by Sdc1-expressing fibroblasts is more general in nature. Our coculture experiments also provide evidence that the increase in carcinoma cell number is caused by elevated mitogenesis rather than reduced apoptosis. This finding contrasts with observations by Olumi *et al.* (2), who identified both increased mitogenesis and reduced apoptosis in their prostate epithelium-cancer stroma recombination experiments. Considering our Sdc1 staining data in prostate cancer and the fact that their stromal cells were not defined at the molecular level, it is likely that factors other than Sdc1 were at play.

The growth-promoting effect of stromal Sdc1 on carcinoma cells in our coculture experiments was HS dependent. Via its HS component, Sdc1 interacts with a number of ECM constituents and growth factors (11). It is tempting to speculate that Sdc1 expression in reactive stromal fibroblasts creates a favorable microenvironment for accelerated tumor cell growth by storing and presenting these factors to the carcinoma cells. A multitude of epithelial mitogens, including FGFs,

hepatocyte growth factor, and heparin-binding epidermal growth factor-like growth factor, bind to Sdc1 HS chains (11, 36). For FGF family members, it has been shown that the active signaling complex is a ternary assembly of the growth factor ligand, FGFR, and HS (37). Therefore, stromal cell-derived Sdc1 may present an active HS/growth factor complex to the FGFR on the carcinoma cells. FGFR activation by heterotypic cell-cell contacts indeed occurs *in vitro* (38, 39), and our *in situ* binding experiments demonstrate that breast carcinoma stroma HSPGs can promote binding of FGF-2 to FGFR1. Delehedde *et al.* (40) demonstrated that the mitogenic response of MDA-MB-231 cells is regulated by HSPGs. The cells do not respond to FGF-2, unless HS sulfation has been reduced by chlorate treatment, suggesting that inhibitory HS species on the carcinoma cell surface suppress a mitogenic response. A role for stromal Sdc1 as growth factor reservoir and autocrine activator has also been proposed for multiple myeloma. Here, Sdc1 is shed abundantly from myeloma cells and accumulates in the fibrotic bone marrow stroma (41, 42). Proteolytic cleavage of Sdc1 from stromal fibroblasts and paracrine stimulation of carcinoma cells as growth factor chaperone would also be an appealing explanation for the Sdc1 effect in our model. However, noncontact coculture with Sdc1-overexpressing fibroblasts fails to demonstrate a mitogenic response, suggesting that direct cell-cell contact is required for this part of the feedback loop as well.

Although it is likely that growth factors are the major determinators of carcinoma cell growth, ECM molecules can also be of crucial importance. Sdc1 has been shown to bind a host of ECM molecules, including thrombospondins, tenascin, fibronectin, and collagens type I, II, and V (43–46). Enhanced presentation of ECM components to carcinoma cells by Sdc1-expressing fibroblasts is an attractive hypothesis, especially because many of these ECM molecules are aberrantly expressed in breast carcinomas. Fibronectin in particular has been reported to positively modulate the response of mammary epithelial cells to growth factors (47), and binding of fibronectin to fibroblasts is enhanced by Sdc1 overexpression (48).

The pattern of Sdc1 expression in breast carcinoma stroma is highly reminiscent of mesenchymal Sdc1 expression seen during development in several organs. With stroma representing the adult mesenchyme counterpart, Sdc1 expression in tumor stroma could represent an example for the oncofetal reactivation of an important regulatory pathway. This hypothesis is supported by our finding of Sdc1 overexpression in stromal cells surrounding the advancing TEB of developing mouse mammary gland.

In summary, our observations establish stromal Sdc1 as a crucial factor regulating epithelial-stromal interactions in breast carcinoma and place this HSPG at the center of a reciprocal feedback loop. Further analysis of these communication pathways may lead to the development of prognostic markers and novel therapeutic targets.

ACKNOWLEDGMENTS

We thank Kathy Schell for help with the flow cytometry analyses and Sally Drew for assistance with immunohistochemistry of human tissue samples.

REFERENCES

1. Fidler, I. J. Seed and soil revisited: contribution of the organ microenvironment to cancer metastasis. Surg. Oncol. Clin. N. Am., *10:* 257–269, vii–viiii, 2001.
2. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res., *59:* 5002–5011, 1999.
3. Ronnov-Jessen, L., Petersen, O. W., and Bissell, M. J. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol. Rev., *76:* 69–125, 1996.
4. Barsky, S. H., Rao, C. N., Grotendorst, G. R., and Liotta, L. A. Increased content of type V collagen in desmoplasia of human breast carcinoma. Am. J. Pathol., *108:* 276–283, 1982.
5. Clezardin, P., Frappart, L., Clerget, M., Pechoux, C., and Delmas, P. D. Expression of thrombospondin (TSP1) and its receptors (CD36 and CD51) in normal, hyperplastic, and neoplastic human breast. Cancer Res., *53:* 1421–1430, 1993.
6. Mackie, E. J., Chiquet-Ehrismann, R., Pearson, C. A., Inaguma, Y., Taya, K., Kawarada, Y., and Sakakura, T. Tenascin is a stromal marker for epithelial malignancy in the mammary gland. Proc. Natl. Acad. Sci. USA, *84:* 4621–4625, 1987.
7. Takeuchi, J., Sobue, M., Sato, E., Shamoto, M., and Miura, K. Variation in glycosaminoglycan components of breast tumors. Cancer Res., *36:* 2133–2139, 1976.
8. Ricciardelli, C., Brooks, J. H., Suwiwat, S., Sakko, A. J., Mayne, K., Raymond, W. A., Seshadri, R., LeBaron, R. G., and Horsfall, D. J. Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. Clin. Cancer Res., *8:* 1054–1060, 2002.
9. Stanley, M. J., Stanley, M. W., Sanderson, R. D., and Zera, R. Syndecan-1 expression is induced in the stroma of infiltrating breast carcinoma. Am. J. Clin. Pathol., *112:* 377–383, 1999.
10. Steinfeld, R., Van Den Berghe, H., and David, G. Stimulation of fibroblast growth factor receptor-1 occupancy and signaling by cell surface-associated syndecans and glypican. J. Cell Biol., *133:* 405–416, 1996.
11. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem., *68:* 729–777, 1999.
12. Vainio, S., Lehtonen, E., Jalkanen, M., Bernfield, M., and Saxen, L. Epithelial-mesenchymal interactions regulate the stage-specific expression of a cell surface proteoglycan, syndecan, in the developing kidney. Dev. Biol., *134:* 382–391, 1989.
13. Vainio, S., Jalkanen, M., and Thesleff, I. Syndecan and tenascin expression is induced by epithelial-mesenchymal interactions in embryonic tooth mesenchyme. J. Cell Biol., *108:* 1945–1953, 1989.
14. Solursh, M., Reiter, R. S., Jensen, K. L., Kato, M., and Bernfield, M. Transient expression of a cell surface heparan sulfate proteoglycan (syndecan) during limb development. Dev. Biol., *140:* 83–92, 1990.
15. David, G., Van der Schueren, B., and Bernfield, M. Basal lamina formation by normal and transformed mouse mammary epithelial cells duplicated *in vitro*. J. Natl. Cancer Inst. (Bethesda), *67:* 719–728, 1981.
16. Todaro, G. J., and Green, H. G. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol., *17:* 299–313, 1963.
17. Alexander, C. M., Reichsman, F., Hinkes, M. T., Lincecum, J., Becker, K. A., Cumberledge, S., and Bernfield, M. Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice. Nat. Genet., *25:* 329–332, 2000.
18. Mundhenke, C., Meyer, K., Drew, S., and Friedl, A. Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 receptor binding in breast carcinomas. Am. J. Pathol., *160:* 185–194, 2002.
19. Lories, V., Cassiman, J. J., Van den Berghe, H., and David, G. Differential expression of cell surface heparan sulfate proteoglycans in human mammary epithelial cells and lung fibroblasts. J. Biol. Chem., *267:* 1116–1122, 1992.
20. Jalkanen, M., Nguyen, H., Rapraeger, A., Kurn, N., and Bernfield, M. Heparan sulfate proteoglycans from mouse mammary epithelial cells: localization on the cell surface with a monoclonal antibody. J. Cell Biol., *101:* 976–984, 1985.
21. Friedl, A., Filla, M., and Rapraeger, A. C. Tissue-specific binding by FGF and FGF receptors to endogenous heparan sulfates. Methods Mol. Biol., *171:* 535–546, 2001.
22. Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., and Leder, P. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol. Cell. Biol., *12:* 240–247, 1992.
23. Langford, J. K., Stanley, M. J., Cao, D., and Sanderson, R. D. Multiple heparan sulfate chains are required for optimal syndecan-1 function. J. Biol. Chem., *273:* 29965–29971, 1998.
24. Subramanian, S. V., Fitzgerald, M. L., and Bernfield, M. Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. J. Biol. Chem., *272:* 14713–14720, 1997.
25. Westley, B., and Rochefort, H. A secreted glycoprotein induced by estrogen in human breast cancer cell lines. Cell, *20:* 353–362, 1980.
26. Keely, P. J., Fong, A. M., Zutter, M. M., and Santoro, S. A. Alteration of collagen-dependent adhesion, motility, and morphogenesis by the expression of antisense α_2 integrin mRNA in mammary cells. J. Cell Sci., *108:* 595–607, 1995.
27. Ronnov-Jessen, L., Petersen, O. W., Koteliansky, V. E., and Bissell, M. J. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J. Clin. Investig., *95:* 859–873, 1995.
28. Dong-Le Bourhis, X., Berthois, Y., Millot, G., Degeorges, A., Sylvi, M., Martin, P. M., and Calvo, F. Effect of stromal and epithelial cells derived from normal and tumorous breast tissue on the proliferation of human breast cancer cell lines in co-culture. Int. J. Cancer, *71:* 42–48, 1997.
29. Iacobuzio-Donahue, C. A., Argani, P., Hempen, P. M., Jones, J., and Kern, S. E. The desmoplastic response to infiltrating breast carcinoma: gene expression at the site of primary invasion and implications for comparisons between tumor types. Cancer Res., *62:* 5351–5357, 2002.
30. Bayer-Garner, I. B., Dilday, B., Sanderson, R. D., and Smoller, B. R. Syndecan-1 expression is decreased with increasing aggressiveness of basal cell carcinoma. Am. J. Dermatopathol., *22:* 119–122, 2000.
31. Wiksten, J. P., Lundin, J., Nordling, S., Lundin, M., Kokkola, A., von Boguslawski, K., and Haglund, C. Epithelial and stromal syndecan-1 expression as predictor of outcome in patients with gastric cancer. Int. J. Cancer, *95:* 1–6, 2001.
32. Jaakkola, P., Vihinen, T., Maatta, A., and Jalkanen, M. Activation of an enhancer on the syndecan-1 gene is restricted to fibroblast growth factor family members in mesenchymal cells. Mol. Cell. Biol., *17:* 3210–3219, 1997.

33. Jaakkola, P., Maatta, A., and Jalkanen, M. The activation and composition of FiRE (an FGF-inducible response element) differ in a cell type- and growth factor-specific manner. Oncogene, *17:* 1279–1286, 1998.
34. Vainio, S., and Thesleff, I. Coordinated induction of cell proliferation and syndecan expression in dental mesenchyme by epithelium: evidence for diffusible signals. Dev. Dyn., *194:* 105–117, 1992.
35. Luqmani, Y. A., Graham, M., and Coombes, R. C. Expression of basic fibroblast growth factor, FGFR1 and FGFR2 in normal and malignant human breast, and comparison with other normal tissues. Br. J. Cancer, *66:* 273–280, 1992.
36. Derksen, P. W., Keehnen, R. M., Evers, L. M., van Oers, M. H., Spaargaren, M., and Pals, S. T. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood, *99:* 1405–1410, 2002.
37. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J., and Mohammadi, M. Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. Mol. Cell, *6:* 743–750, 2000.
38. Richard, C., Liuzzo, J. P., and Moscatelli, D. Fibroblast growth factor-2 can mediate cell attachment by linking receptors and heparan sulfate proteoglycans on neighboring cells. J. Biol. Chem., *270:* 24188–24196, 1995.
39. Filla, M. S., Dam, P., and Rapraeger, A. C. The cell surface proteoglycan syndecan-1 mediates fibroblast growth factor-2 binding and activity. J. Cell. Physiol., *174:* 310–321, 1998.
40. Delehedde, M., Deudon, E., Boilly, B., and Hondermarck, H. Heparan sulfate proteoglycans play a dual role in regulating fibroblast growth factor-2 mitogenic activity in human breast cancer cells. Exp. Cell Res., *229:* 398–406, 1996.
41. Bayer-Garner, I. B., Sanderson, R. D., Dhodapkar, M. V., Owens, R. B., and Wilson, C. S. Syndecan-1 (CD138) immunoreactivity in bone marrow biopsies of multiple myeloma: shed syndecan-1 accumulates in fibrotic regions. Mod. Pathol., *14:* 1052–1058, 2001.
42. Yang, Y., Yaccoby, S., Liu, W., Langford, J. K., Pumphrey, C. Y., Theus, A., Epstein, J., and Sanderson, R. D. Soluble syndecan-1 promotes growth of myeloma tumors *in vivo*. Blood, *100:* 610–617, 2002.
43. Saunders, S., and Bernfield, M. Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. J. Cell Biol., *106:* 423–430, 1988.
44. Koda, J. E., Rapraeger, A., and Bernfield, M. Heparan sulfate proteoglycans from mouse mammary epithelial cells. Cell surface proteoglycan as a receptor for interstitial collagens. J. Biol. Chem., *260:* 8157–8162, 1985.
45. Salmivirta, M., Elenius, K., Vainio, S., Hofer, U., Chiquet-Ehrismann, R., Thesleff, I., and Jalkanen, M. Syndecan from embryonic tooth mesenchyme binds tenascin. J. Biol. Chem., *266:* 7733–7739, 1991.
46. Sun, X., Mosher, D. F., and Rapraeger, A. Heparan sulfate-mediated binding of epithelial cell surface proteoglycan to thrombospondin. J. Biol. Chem., *264:* 2885–2889, 1989.
47. Woodward, T. L., Xie, J., Fendrick, J. L., and Haslam, S. Z. Proliferation of mouse mammary epithelial cells *in vitro*: interactions among epidermal growth factor, insulin-like growth factor I, ovarian hormones, and extracellular matrix proteins. Endocrinology, *141:* 3578–3586, 2000.
48. Mali, M., Elenius, K., Miettinen, H. M., and Jalkanen, M. Inhibition of basic fibroblast growth factor-induced growth promotion by overexpression of syndecan-1. J. Biol. Chem., *268:* 24215–24222, 1993.

Induction of Syndecan-1 Expression in Stromal Fibroblasts Promotes Proliferation of Human Breast Cancer Cells

Takashi Maeda, Caroline M. Alexander and Andreas Friedl

Cancer Res 2004;64:612-621.