Membrane Type 1 Matrix Metalloproteinase–Mediated Stromal Syndecan-1 Shedding Stimulates Breast Carcinoma Cell Proliferation

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Abstract

Mounting evidence implicates stromal fibroblasts in breast carcinoma progression. We have recently shown in three-dimensional coculture experiments that human mammary fibroblasts stimulate the proliferation of T47D breast carcinoma cells and that this activity requires the shedding of the heparan sulfate proteoglycan syndecan-1 (Sdc1) from the fibroblast surface. The goal of this project was to determine the mechanism of Sdc1 ectodomain shedding. The broad spectrum matrix metalloproteinase (MMP) inhibitor GM6001 specifically blocked Sdc1-mediated carcinoma cell growth stimulation, pointing toward MMPs as critical enzymes involved in Sdc1 shedding. MMP-2 and membrane type 1 MMP (MT1-MMP) were the predominant MMPs expressed by the mammary fibroblasts. Fibroblast-dependent carcinoma cell growth stimulation in three-dimensional coculture was abolished by MT1-MMP expression silencing with small interfering RNA and restored either by adding recombinant MT1-MMP catalytic domain or by expressing a secreted form of Sdc1 in the fibroblasts. These findings are consistent with a model where fibroblast-derived MT1-MMP cleaves Sdc1 at the fibroblast surface, leading to paracrine growth stimulation of carcinoma cells by Sdc1 ectodomain. The relevance of MT1-MMP in paracrine interactions was further supported by coculture experiments with T47D cells and primary fibroblasts isolated from human breast carcinomas or matched normal breast tissue. Carcinoma-associated fibroblasts stimulated T47D cell proliferation significantly more than normal fibroblasts in three-dimensional coculture. Function-blocking anti–MT1-MMP antibody significantly inhibited the T47D cell growth stimulation in coculture with primary fibroblasts. In summary, these results ascribe a novel role to fibroblast-derived MT1-MMP in stromal-epithelial signaling in breast carcinomas. [Cancer Res 2008;68(22):9558–65]

Introduction

The importance of the stroma in cancer development and progression is being increasingly recognized. Stromal fibroblasts participate in reciprocal interactions with carcinoma cells, which modulate carcinoma cell proliferation (1). The cell surface proteoglycan syndecan-1 (Sdc1), which in adult tissues is expressed mainly in epithelial and plasma cells, is induced in breast carcinoma stromal fibroblasts, recapitulating the stromal expression seen during mammary morphogenesis (2, 3). We have shown that stromal Sdc1 stimulates breast carcinoma cell proliferation *in vitro* and *in vivo* (3, 4). Recent work from our group has shed light on the mechanisms of stromal Sdc1-dependent growth stimulation (5). The induction of carcinoma cell proliferation in collagen gels requires the proteolytic release of the Sdc1 ectodomain, effectively converting the proteoglycan cell surface receptor into a diffusible, paracrine mediator. The growth-promoting activity requires the heparan sulfate (HS) chains and the presence of stromal cell–derived factor 1 (SDF1) and fibroblast growth factor 2 (FGF2). This finding suggests that HS from the Sdc1 ectodomain either stabilizes these growth factors in the three-dimensional coculture environment or more likely participates in a ternary complex with ligands and signaling receptors. Sdc1 shedding has been shown to regulate a multitude of biological functions in a cell autonomous and nonautonomous fashion (6–8). Apart from mediating the matrix and growth factor coreceptor functions of cell membrane–anchored Sdc1, the Sdc1 ectodomain seems to have some unique roles in biological events. Soluble Sdc1, which forms a complex with chemokines, is required to create chemotactic gradients in a model of lung inflammation (7). Shed Sdc1 modifies the tumor microenvironment and promotes tumor progression and metastasis in myelomas (9). Cleavage of Sdc1 by membrane type 1 matrix metalloproteinase (MT1-MMP) stimulates HT1080 fibrosarcoma cell migration (8). Elevated amounts of Sdc1 shedding in the blood stream indicate an ominous prognosis in several malignancies (10, 11).

Sdc1 is released constitutively from the cell surface, and shedding is enhanced in response to a number of different stimuli, such as treatment with SDF1 or HS degradation with heparanase (12, 13). The identity of the enzyme(s) responsible for Sdc1 cleavage and the release of the ectodomain have been the topic of some debate. There is a general consensus that members of the MMP family mediate Sdc1 cleavage. A tissue inhibitor of metalloproteinase-3 (TIMP-3)–sensitive metalloproteinase (14), a non-MMP (15), MMP-7 (7, 16), MMP-9 (12), and MT1-MMP (8, 17) have all been implicated in Sdc1 shedding. It is unclear whether these enzymes constitute alternative direct Sdc1 cleavage mechanisms or whether some of them promote shedding indirectly.

The goal of this study was to investigate the mechanism of Sdc1 shedding in our three-dimensional collagen gel coculture system. We found that MT1-MMP expressed by stromal fibroblasts directly cleaves Sdc1 at the surface of the same cell type and, thus, releases Sdc1 ectodomain as a paracrine mediator. Sdc1 ectodomain stimulates breast carcinoma cell proliferation, as we have previously shown. This finding shows a novel, protumorigenic role for MT1-MMP in breast cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Antibodies and reagents. Mouse anti-human MMP-2 monoclonal antibody was purchased from Chemicon International. Rabbit polyclonal antibody against MT1-MMP hinge region and rabbit anti–MT1-MMP catalytic domain polyclonal antibody were from Abcam. Mouse anti-human mucin-1 antibody was purchased from Santa Cruz Biotechnology. Anti-human pankeratin mouse monoclonal antibody was from Lab Vision. Rat monoclonal antibody (clone 281.2) directed against mouse Sdc1 extracellular domain and rabbit polyclonal antibody against syndecan cytoplasmic domain (S1CD) were kindly provided by Dr. A.C. Rapraeger (University of Wisconsin-Madison). Type I rat tail collagen was purchased from BD Biosciences. Human recombinant MT1-MMP catalytic domain, MMP-2 inhibitor III, and GM6001 MMP inhibitor were from Calbiochem. Recombinant human MMP-2 was from R&D Systems, Inc. Collagenase I and hyaluronidase were purchased from Sigma.

Cell culture. The human breast carcinoma cell line T47D was obtained from Dr. M. Gould (University of Wisconsin-Madison). Normal mammary fibroblasts immortalized with human telomerase and GFP-labeled were generously provided by Dr. C. Kuperwasser (18). These cells were originally named RMF/EG and are called human mammary fibroblasts (HMF) throughout this paper. T47D cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and penicillin/streptomycin (100 units/mL). HMF cells were cultured in DMEM supplemented with 10% calf serum, 2 mmol/L L-glutamine, and penicillin/streptomycin (100 units/mL). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Collagen gel coculture and cell growth assay. Three-dimensional collagen gel coculture was established, as previously described (5). Briefly, 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. T47D growth was quantified as pixel area of mucin-1 staining. Anti–mucin-1 antibody specifically stains T47D cells. The collagen gel was fixed and stained with mouse anti-human mucin-1. Images were acquired using a SPOT imaging system (Diagnostic Instruments, Inc.) on an Olympus inverted microscope. The mucin-1–positive area was measured using Image J software.[3]

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) was performed to measure MMP mRNA levels. Cells were dissociated from the collagen gel by collagenase and enzyme-free cell dissociation buffer. HMF and T47D cells were sorted with a triple-laser FACSVantage SE flow cytometer equipped with FACSDiva software. Then, total RNA was isolated from the two cell populations using the RNeasy Mini kit (Qiagen). Cell number equivalents of total RNA from T47D or HMF cells were reverse transcribed into cDNA using the ThermoScript RT-PCR System (Invitrogen). Primers were purchased from SuperArray Bioscience. Five percent of the reverse transcription product was used in the qRT-PCR reaction with SYBR Green PCR master mix on an iCycler instrument (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference.

Small interfering RNA transfection. MT1-MMP small interfering RNA (siRNA) oligonucleotides were purchased from Ambion. Five micrograms of siRNA oligonucleotides were delivered to 5×10^5 HMF using the Basic Nucleofector kit for primary mammalian fibroblasts and the Nucleofector device (Amaxa Biosystem) according to the manufacturer's protocol. After 48 to 72 h posttransfection, the HMF cells were lifted in trypsin (0.25% w/v) and cocultured with T47D cells in collagen gels. MT1-MMP siRNA oligonucleotides were validated by qRT-PCR and Western blot.

Gel electrophoresis and Western blotting. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproducts) containing protease inhibitor cocktail (Pierce) for 30 min on ice. The cell lysates were centrifuged at 10,000 rpm. The supernatants were collected, and protein concentration was measured. Samples and prestained molecular mass markers (Bio-Rad) were denatured in sample buffer (2% SDS, 10% glycerol, bromophenol blue, 2.5% β-mercaptoethanol) and heated to 100°C for 1 min before gel electrophoresis. Samples were then electrophoretically separated on Criterion XT precast gels (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were probed with anti MT1-MMP antibody (1 μg/mL). A horseradish peroxidase–conjugated secondary IgG (Sigma) was used for detection. The signal was visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Sdc1 shedding assay. HMFs overexpressing mouse Sdc1 (mSdc1; cells generated in our laboratory by Dr. Ning Yang) were cultured to 90% confluence. The culture medium was replaced with HBSS. Cells were treated with recombinant MT1-MMP catalytic domain at concentrations of 0 or 500 ng/mL at 37°C for 1 h in the presence or absence of 2.5 μmol/L GM6001. The supernatant was then collected and diluted with one volume of TUT buffer [10 mmol/L Tris, 8 mol/L urea, 0.1% Triton X-100, 1 mmol/L Na_2SO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L *N*-ethylmaleimide (pH 8.0)]. Then, 100 μL of DEAE beads preequilibrated with TUT were added. Tubes containing DEAE beads and the medium were rotated overnight at 4°C. Sdc1 was eluted with high salt HEPES buffer [30 mmol/L HEPES, 1 mol/L NaCl (pH 7.4)]. After the salt was removed with a desalting column (Pierce), the sample was digested with heparitinase and chondroitinase ABC (0.002 units/mL) twice for 2 h to remove all glycosaminoglycan chains and analyzed by Western blot.

Flow cytometry. HMFs overexpressing mSdc1 were cultured to 90% confluence. Cells were lifted with enzyme-free cell dissociation buffer (Life Technologies, Inc.-Invitrogen Corp.) and washed twice with HBSS. The cells (0.5×10^6) were subsequently resuspended in HBSS and treated with 1 μg rMT1-MMP at 37°C for 1 h. In the control samples, the enzyme was either omitted, or the MMP inhibitor GM6001 was added at 2.5 μmol/L. HMF cells were then collected and incubated with 5 μg rat monoclonal antibody (clone 281.2) directed against mSdc1 extracellular domain at 4°C for 1 h in FACS buffer (2% FBS in PBS). After three washes with FACS buffer, HMF cells were incubated with goat anti-rat IgG conjugated with R-phycoerythrin at 4°C for 1 h. After washing, the samples were analyzed on a FACS Caliber bench top cytometer (BD Biosciences). Cell scatter and propidium iodide (Sigma-Aldrich; 1 μg/sample) staining profiles were used to gate live, single-cell events for data analysis.

HA-tagged mouse Sdc1 *in vitro* digestion assay. An expression plasmid for amino-terminal HA-tagged mSdc1 (HA-mSdc1; generated in our laboratory by Dr. Dianhua Qiao) was transfected into 293A cells, and 48 h later, the cells were lysed in RIPA buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 7.4)]. The cell lysate (500 μg protein) was incubated with 50 μL anti-HA affinity beads (Roche Applied Science) at 4°C overnight. The beads were washed and treated with 0 or 1 μg rMT1-MMP with or without 2.5 μmol/L of GM6001 or with 1 μg rMMP-2 in hydrolysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 0.025% Brij 35 (pH 7.5)] at 37°C for 1 h. Then the beads were boiled in sample buffer for 5 min, and the supernatant was electrophoretically separated on 12% Bis-Tris Criterion XT precast gels (Bio-Rad). The gel was either silver-stained or transferred to a PVDF membrane for probing with rabbit polyclonal antibody against S1CD.

Primary breast fibroblast culture. The use of human tissue was approved by the institutional review board. Human breast tissue was minced into 1-mm to 2-mm pieces and subsequently digested with collagenase I (2 mg/mL) and hyaluronidase (2 mg/mL) in DMEM for 2 h at 37°C. Digested tissue was spun down at 3,000 rpm for 5 min and washed with HBSS. For normal tissue, the digested tissue was resuspended in HBSS and filtered through 100-μm and 40-μm cell strainers (BD Bioscience). The flow-through was collected and the cells were harvested by centrifugation (3,000 rpm, 5 min) and seeded in tissue culture plates in complete DMEM containing 10% FBS, 2 mmol/L L-glutamine, and penicillin/streptomycin (100 units/mL). Breast tumor tissue was further digested in 0.25% trypsin at 37°C for 10 min. Then, the digested tumor tissue was processed in the same manner as normal breast tissue. The cells were collected and seeded in tissue culture plates. On the next day, dead cells, unattached cells, and tissue debris were removed. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The epithelial cell contamination in primary fibroblast cultures was estimated as the percentage of cells displaying positive mucin-1 or cytokeratin staining. Because the culture

[3] http://rsb.info.nih.gov/ij/

medium favors fibroblast cell growth, confluent primary fibroblast cultures contain less then 1% of epithelial cell contamination.

Immunofluorescence staining. Cells growing on chamber slides were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed for 20 min in PBS. Cells were then treated with 0.15 mol/L glycine in PBS for 30 min 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 10% goat serum in PBS for 1 h at room temperature. Cells were then incubated with primary antibody at room temperature for 1.5 h. After extensive washing with PBS, the secondary antibody was added for 1 h at room temperature. Immunostaining was then analyzed with a fluorescence microscope.

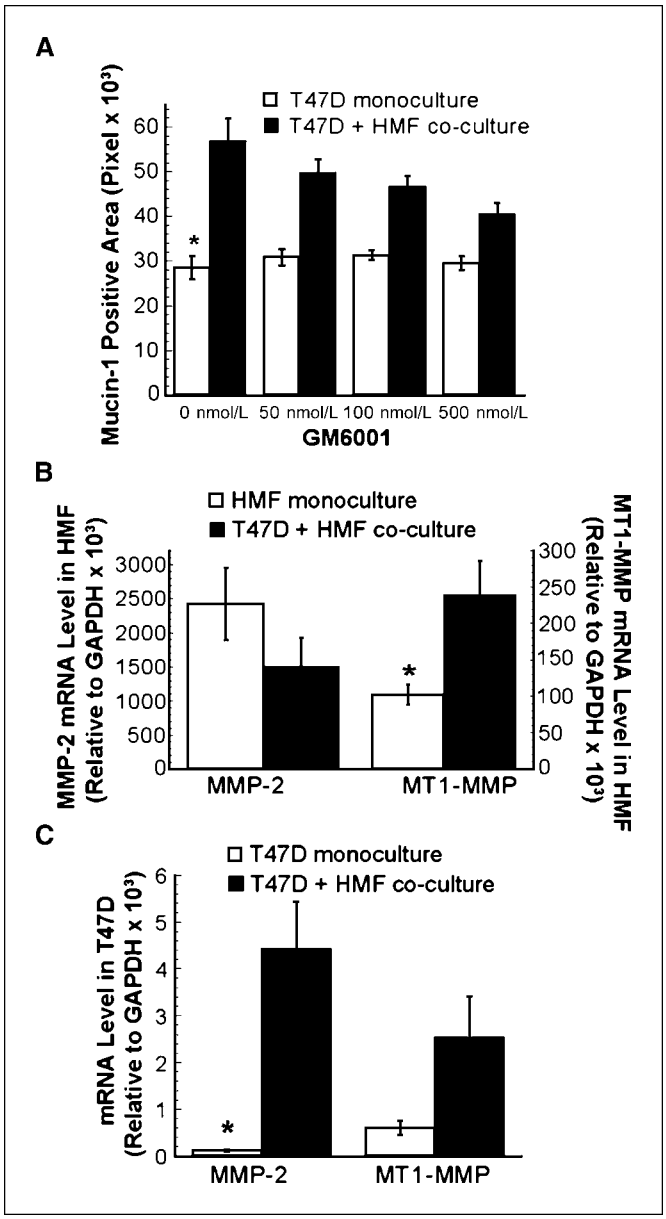

Figure 1. GM6001 inhibits T47D cell growth stimulation in coculture. MMP-2 and MT1-MMP mRNA levels are altered by coculture. *A,* 0, 50, 100, or 500 nmol/L of GM6001 were added in both collagen gel and the medium at the day of seeding. Collagen gel coculture was fixed and stained with anti-human mucin-1 antibody on the third day of culture. T47D cell growth was analyzed as the pixel area of mucin-1–positive staining on the collagen gel. *, $P < 0.05$ versus T47D + HMF coculture without GM6001. *B,* MMP-2 and MT1-MMP mRNA levels of HMF. *C,* MMP-2 and MT1-MMP mRNA levels of T47D cells. Total RNA was isolated from HMF or T47D cells harvested from collagen gel culture. qRT-PCR was performed, as described in Materials and Methods. mRNA levels relative to a housekeeping gene (GAPDH) were calculated as $2^{(\text{cycle threshold of GAPDH} - \text{cycle threshold of MMP})} \times 1{,}000$. *, $P < 0.05$ versus T47D + HMF coculture.

Results

T47D mammary carcinoma cell growth stimulation by fibroblasts requires MMP activity. HMFs stimulate growth of T47D mammary carcinoma cells in three-dimensional coculture, an activity that requires the induction of Sdc1 expression in the fibroblasts and the proteolytic release of this proteoglycan from the fibroblast surface (5). A number of different enzymes have been implicated in Sdc1 shedding, all of which belong to the MMP family (7, 8, 12, 16, 17). Therefore, one would predict that the inhibition of Sdc1 cleavage with an MMP inhibitor would abolish fibroblast-mediated carcinoma growth stimulation. Indeed, when GM6001, a broad spectrum MMP inhibitor, is added to the cocultures, T47D cell growth is inhibited in a dose-dependent manner, whereas T47D monoculture growth is unaffected (Fig. 1*A*). This result indicates that MMP activity is required for HMF-mediated T47D cell growth stimulation.

HMF-derived MMP-2 and MT1-MMP are the most abundant MMPs in T47D/HMF coculture. MMP-2, MMP-7, MMP-9, MMP-11, MMP-13, and MT1-MMP are the MMP family members that have been implicated in breast cancer progression (19, 20). Therefore, we analyzed mRNA levels of these MMPs in HMF and T47D cells. T47D cells produce extremely low mRNA levels for each of these MMPs (Table 1). This finding is consistent with reports by other investigators that poorly invasive breast cancer cells, such as T47D or MCF7, do not express MMPs at significant levels (21). In HMF cells, MMP-2 and MT1-MMP were the predominant MMPs (Table 1). Because MMP expression in stromal fibroblast can be modulated by carcinoma cells (22–24), we compared expression levels in monoculture and coculture. We discovered that the MMP-2 mRNA level in HMF is decreased under coculture conditions. In contrast, the fibroblast MT1-MMP mRNA level is significantly increased during coculture (Fig. 1*B*). In T47D cells, both MMP-2 and MT1-MMP mRNA levels remain low during coculture, although the MMP-2 level is significantly increased in coculture compared with monoculture (Fig. 1*C*).

MT1-MMP expression in HMF cells is required for T47D carcinoma cell growth stimulation. Next, we tested whether MMP-2, MT1-MMP, or both are required for T47D growth stimulation in collagen gel coculture. Neither the small molecule MMP-2 inhibitor III [2-((isopropoxy)-(1,1′-biphenyl-4-ylsulfonyl)-amino)-*N*-hydroxyacetamide] nor neutralizing anti–MMP-2 antibody suppresses T47D cell growth in monoculture or coculture (Supplementary Fig. S1). Conversely, when MT1-MMP is inhibited by adding a neutralizing antibody directed against the MT1-MMP catalytic domain, T47D cell growth in coculture is reduced to the level of monoculture (Fig. 2*A*). The neutralizing activity of the anti–MMP-2 and anti–MT1-MMP antibodies was validated with an *in vitro* fluorogenic substrate digestion assay (Supplementary Fig. S2).

To further ascertain the importance of fibroblast MT1-MMP for carcinoma cell growth stimulation, we knocked down expression of this enzyme in fibroblasts with siRNA. Before mixing the two cell types for the coculture experiments, HMF cells were transiently transfected with MT1-MMP siRNA or control siRNA oligonucleotides. The efficacy of the MT1-MMP siRNA treatment was validated by both qRT-PCR and immunoblot analysis. MT1-MMP siRNA

Table 1. MMP mRNA levels in HMF or T47D cells relative to GAPDH

	MMP-2	MMP-7	MMP-9	MMP-11	MMP-13	MT1-MMP
HMF	485 ± 44	0.01 ± 0.001	0.00061 ± 0.0002	ND	0.0055 ± 0.0011	94 ± 7
T47D	0.0018 ± 0.0005	0.006 ± 0.0011	0.0008 ± 0.00044	ND	0.029 ± 0.0026	0.045 ± 0.0022

NOTE: Total RNA was isolated from HMF or T47D cells. Quantitative RT-PCR was performed as described in Materials and Methods. MMP mRNA levels, relative to the house keeping gene GAPDH, were calculated as $2^{(CT_{GAPDH} - CT_{MMP})} * 1000$. ND, not detected.

reduces the mRNA level by almost 80% (Fig. 2*B*) and decreases the protein level considerably (Fig. 2*C*). The siRNA treatment of HMF cells reduces the growth stimulation of T47D cells to the level of T47D cell growth in monoculture, whereas control siRNA has no effect (Fig. 2*D*). Importantly, the addition of recombinant MT1-MMP catalytic domain (rMT1-MMP) to the coculture completely restores T47D growth stimulation to the control level (Fig. 2*D*). These results indicate that fibroblast-derived MT1-MMP is required for T47D cell growth stimulation in coculture and that soluble enzyme can substitute for endogenous, membrane-bound MT1-MMP.

Secreted Sdc1 ectodomain bypasses the requirement for MT1-MMP activity in coculture. We hypothesize that fibroblast MT1-MMP stimulates T47D carcinoma cell growth indirectly by cleaving and releasing Sdc1. If this model is correct, one would anticipate that adding Sdc1 ectodomain to the coculture should reverse the T47D cell growth inhibition caused by blocking fibroblast MT1-MMP activity. To test this hypothesis, we forcibly expressed a soluble, secreted form of mouse Sdc1 in HMF (25) in coculture with T47D cells. Soluble Sdc1 expressed in HMF completely restores the T47D cell growth stimulation in the presence of the MMP inhibitor GM6001 (Fig. 3*A*). Similarly, T47D cell growth inhibition in response to MT1-MMP neutralizing antibody treatment or as a result of MT1-MMP siRNA knockdown in fibroblasts is entirely reversed by the expression of soluble Sdc1 in HMF (Fig. 3*B* and *C*). These data suggest that, in this coculture model, MT1-MMP stimulates carcinoma cell growth primarily by releasing Sdc1 ectodomain as a paracrine mediator from the fibroblast surface.

Sdc1 is an MT1-MMP substrate. To verify that MT1-MMP participates in Sdc1 shedding, we applied rMT1-MMP to monolayers of mSdc1-overexpressing HMF. The activity of rMT1-MMP was confirmed with fluorogenic substrate (data not shown). mSdc1 was detected in the medium after the cells were exposed to rMT1-MMP, whereas mSdc1 was absent from the medium in the presence of the MMP inhibitor GM6001 (Fig. 4*A*). Most of the shed mSdc1 is detected as an apparent SDS-resistant dimer migrating at ~150 kDa, which is consistent with reports by other investigators (26). To further show that MT1-MMP induces Sdc1 shedding, we performed flow cytometric analysis of cell surface mSdc1 with and without rMT1-MMP exposure. rMT1-MMP treatment decreases the level of cell surface mSdc1 by approximately two-thirds, although a significant amount of mSdc1 remains on the cell surface (Fig. 4*B*). The MMP inhibitor GM6001 reverses this decrease. This result indicates that MT1-MMP promotes Sdc1 shedding from the surface of stromal fibroblasts but is not definitive evidence for direct proteolytic cleavage of Sdc1 by MT1-MMP. One possibility is that MMP-2 or some other enzyme, which is also present in the cocultures, is activated by MT1-MMP and that this activated enzyme then cleaves Sdc1. However, such an indirect mechanism of Sdc1 release by MMP-2 is unlikely given that MMP-2 inhibition had no effect on fibroblast-mediated growth stimulation of T47D carcinoma cells (Supplementary Fig. S1).

To show directly that mSdc1 is a substrate of MT1-MMP, we performed a digestion assay with isolated HA-mSdc1 *in vitro*. HA-mSdc1 was produced in 293A cells and enriched with anti-HA affinity beads. After exposure to rMT1-MMP or active rMMP-2, digested HA-mSdc1 was analyzed by silver staining and Western blot. Intact HA-mSdc1 migrates by SDS-PAGE consistent with its molecular weight of ~40 kDa (Fig. 4*C* and *D*), contrasting with the anomalous slow migration typically observed with wild-type Sdc1 (5, 27). In our experiments, the HA sequence may interfere with the "extended" conformations thought to be responsible for the anomalous migration of Sdc1 (27). A fragment of ~25 kDa is also seen in all samples containing HA-mSdc1. This fragment is not modified by the addition of rMT1-MMP or rMMP-2 (Fig. 4*C*), does not react with an antibody directed against the cytoplasmic domain of mSdc1 (Fig. 4*D*), and, therefore, is likely nonspecific.

Because MT1-MMP has been reported to cleave mSdc1 at a juxtamembrane site (Ala^{243}-Ser^{244}; ref. 28), one predicts that after rMT1-MMP treatment, a small fragment containing transmembrane and cytoplasmic domains of mSdc1, with a size of ~10 kDa, would appear. Indeed, after incubation with rMT1-MMP, bands corresponding to fragments of ~30 and 10 kDa replace full-length HA-mSdc1 in the silver-stained gel (Fig. 4*C, lane 3*). Conversely, when incubated with activated rMMP-2, full-length HA-mSdc1 remains at a high level, although bands of ~30 kDa appear (Fig. 4*C, lane 5*). No short peptide corresponding to a fragment containing the transmembrane and cytoplasmic domains of mSdc1 is detected. This suggests that rMMP-2 does not cleave HA-mSdc1 at the juxtamembrane site but instead might cleave HA-mSdc1 at a different site located closer to the amino-terminus.

To verify that the small fragment generated by rMT1-MMP digestion contains the cytoplasmic domain of mSdc1, we probed the membrane with a rabbit polyclonal antibody (S1CD) directed against this molecular region (Fig. 4*D*). Indeed, the small fragment with a size of ~10 kDa reacts with the anticytoplasmic domain antibody. Interestingly, a larger ~30 kDa fragment is also recognized by this antibody, suggesting that mSdc1 contains another MT1-MMP–sensitive cleavage site closer to the amino-terminus. As a matter of fact, Endo and coworkers localized an MT1-MMP–sensitive site at Gly^{82}-Leu^{83} in human Sdc1 (8). The S1CD antibody also reacts with a ~30-kDa sized fragment generated by rMMP-2; however, consistent with the silver staining result, a smaller ~10-kDa fragment is not detected. This further supports our conclusion that rMMP-2 does not cleave mSdc1 at the juxtamembrane site.

Primary cancer-associated fibroblasts stimulate T47D cell growth significantly more than normal fibroblasts in an MT1-MMP activity–dependent manner. To evaluate the relevance of stroma cell–derived MT1-MMP in human breast cancer, we isolated stromal fibroblasts from both human breast carcinoma tissue (CAF) and matched adjacent normal breast tissue (NF) and compared their interaction with T47D cells in collagen gel matrix coculture. Twelve pairs of CAF and NF, each derived from a different patient, were tested in coculture. The samples included 10 infiltrating ductal carcinomas, one mucinous carcinoma, and one infiltrating lobular carcinoma. To test the purity of the isolated cells, we stained with antibodies to the fibroblast marker S100A4/

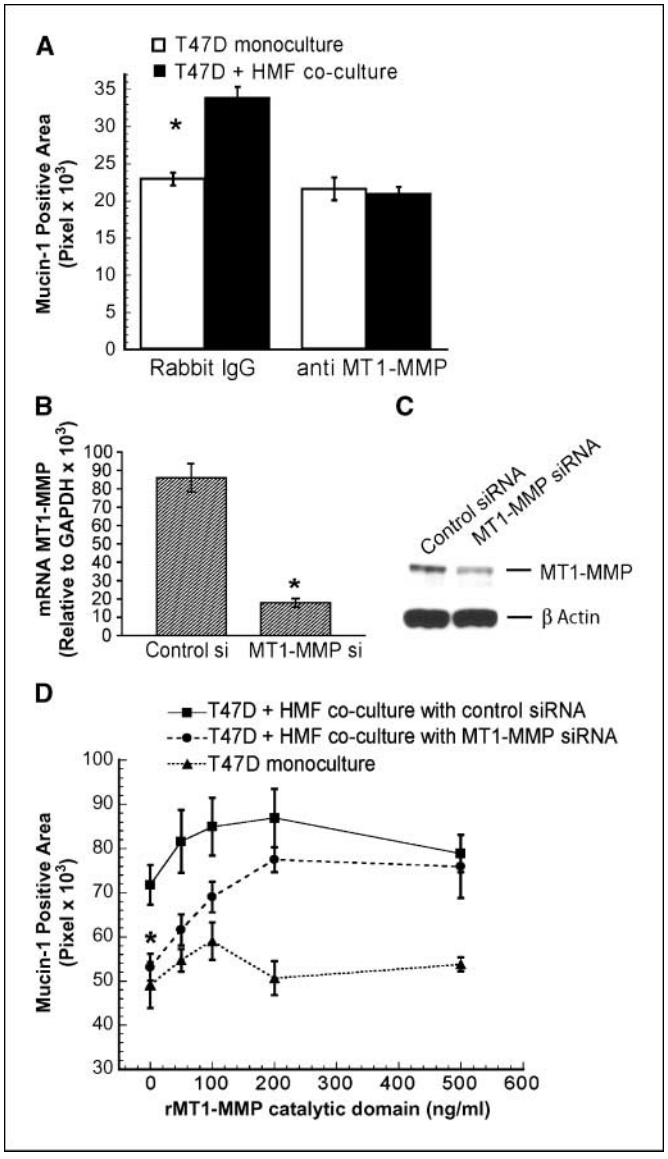

Figure 2. Inhibition of MT1-MMP blocks T47D cell growth stimulation in coculture. *A,* MT1-MMP neutralizing antibody abolishes T47D cell growth in coculture. Collagen gel culture was treated with 10 μg/mL of anti–MT1-MMP catalytic domain antibody or rabbit IgG for 3 d. *, $P < 0.05$ versus T47D + HMF coculture. *B,* MT1-MMP siRNA oligonucleotides reduce the MT1-MMP mRNA level. HMFs were transiently transfected with MT1-MMP siRNA oligonucleotides or control oligonucleotides. Four days after transfection, total RNA was isolated. The relative MT1-MMP mRNA level was quantified, as described in Materials and Methods. *, $P < 0.05$ versus control siRNA. *C,* MT1-MMP protein expression is reduced by siRNA oligonucleotide treatment. Whole cell lysate (40 μg) was loaded on gel and analyzed by immunoblot. The blot was probed with an antibody against the MT1-MMP hinge region. The data are representative of several independent experiments. *D,* silencing of fibroblast MT1-MMP expression by siRNA oligonucleotides significantly decreases T47D cell growth in coculture and rMT1-MMP restored the stimulatory effect. Collagen gel culture was treated with 0, 50, 100, 200, or 500 ng/mL of rMT1-MMP catalytic domain for 3 d. T47D cell growth was analyzed, as described in Materials and Methods. *, $P < 0.05$ versus T47D + HMF coculture with control siRNA and without rMT1-MMP.

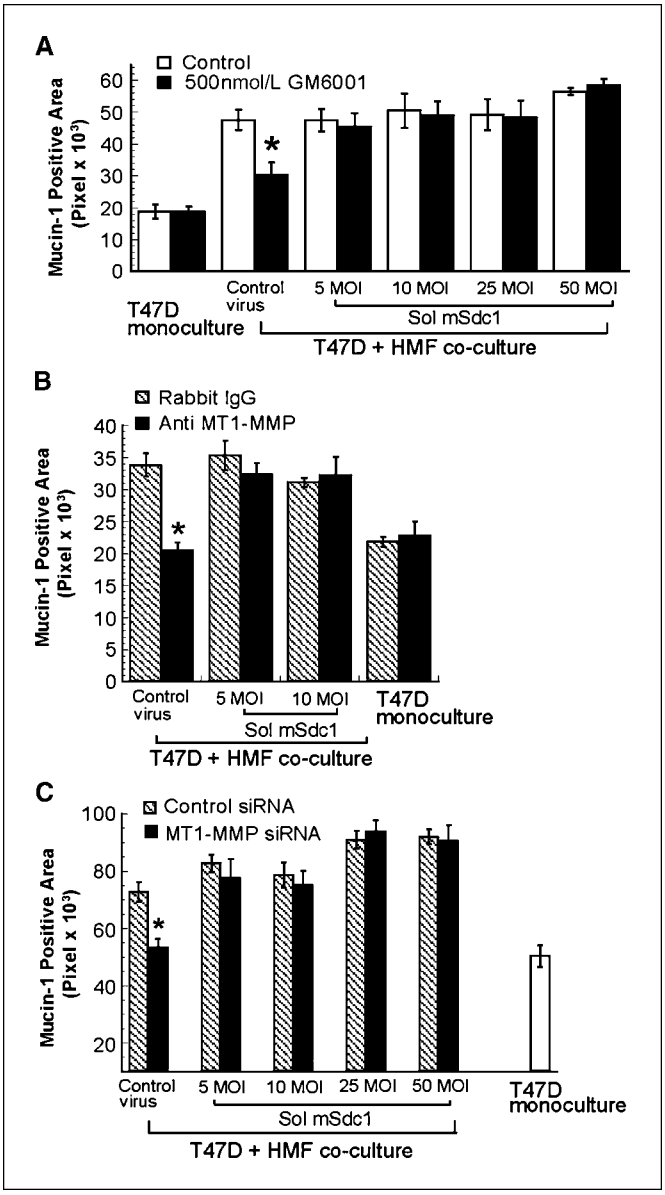

Figure 3. Soluble mSdc1 restores T47D cell growth stimulation in coculture when MT1-MMP activity is inhibited. *A,* soluble mSdc1 rescues the T47D cell growth inhibition caused by GM6001 in coculture. HMFs were transduced with adenovirus at multiplicity of infection (*MOI*) of 5, 10, 25, or 50 to force expression of a soluble mSdc1 mutant (*Sol Sdc1*) before cell mixing for the coculture. Collagen gel culture was treated with 500 nmol/L of GM6001 for 3 d. *, $P = 0.008$. *B,* soluble mSdc1 reverses the T47D cell growth inhibited by MT1-MMP neutralizing antibody. HMFs expressing soluble mSdc1 were cocultured with T47D in the presence of 10 μg/mL of anti–MT1-MMP or rabbit IgG for 3 d. T47D cell growth was then analyzed as the anti-human mucin-1 staining area. *, $P < 0.05$ versus rabbit IgG control. *C,* soluble mSdc1 rescues T47D cell growth in coculture with siRNA-treated HMF. Soluble mSdc1 was expressed in MT1-MMP siRNA-treated HMF in the same manner, as shown in *A*. Collagen gel culture was analyzed after a 3-d growth period. *, $P < 0.05$ versus control siRNA.

FSP1 and the myofibroblast marker smooth muscle actin (SMA). Both CAF and NF were uniformly positive for S100A4/FSP1, and a subset of cells from either source also expressed SMA (Fig. 5*A*). When CAF or NF were cocultured with T47D cells in collagen gel matrix, CAF stimulated T47D proliferation to a significantly greater extent than NF (Fig. 5*B*). Neutralizing anti–MT1-MMP antibody abolishes the growth advantage induced by CAF over NF (Fig. 5*B*), indicating that in primary human breast carcinomas, fibroblasts produce MT1-MMP with a carcinoma growth–promoting activity.

Discussion

MT1-MMP is expressed in both breast carcinoma cells and peritumor stromal fibroblasts (29–32) and has been implicated in breast carcinoma progression. Most mechanistic work has focused on MT1-MMP expressed by carcinoma cells and has established roles in invasion, growth, and metastasis. The role of stroma cell–derived MT1-MMP in cancer progression is less well known. In the present study, we found that MT1-MMP from stromal fibroblast is responsible for the shedding of stromal Sdc1, which subsequently stimulates breast carcinoma cell proliferation in three-dimensional collagen gel coculture.

MT1-MMP expression is regulated by cell autonomous and microenvironmental mechanisms. In mammary carcinoma cells, MT1-MMP levels are under the control of the transcriptional modulator Id-1 and zonula occludens protein 1. Contact with collagen 1 and hypoxic conditions stimulate MT1-MMP expression (33, 34). Interestingly, myoepithelial cells, which are physiologically located between mammary epithelial cells and the surrounding stroma, reduce MT1-MMP expression (35). Conditioned medium from the highly aggressive MDA-MB-231, but not the less aggressive MCF-7 breast carcinoma cells, stimulates MT1-MMP expression in human fibroblasts, suggesting an induction by secreted factors (22). Conversely, Selvey and coworkers were unable to detect MT1-MMP induction in fibroblasts during noncontact coculture with breast carcinoma cells (23). We observed a significant induction of fibroblast MT1-MMP mRNA levels in direct coculture with well-differentiated T47D breast carcinoma cells (Fig. 1*B*). *In vivo*, high-level stromal MT1-MMP expression is limited to fibroblasts in the immediate vicinity of tumor islands, suggesting that either direct tumor cell contact or a short range or labile paracrine factor mediates the induction (32).

MT1-MMP stimulates cancer growth and invasion by a variety of direct and indirect mechanisms. One of the recognized cardinal

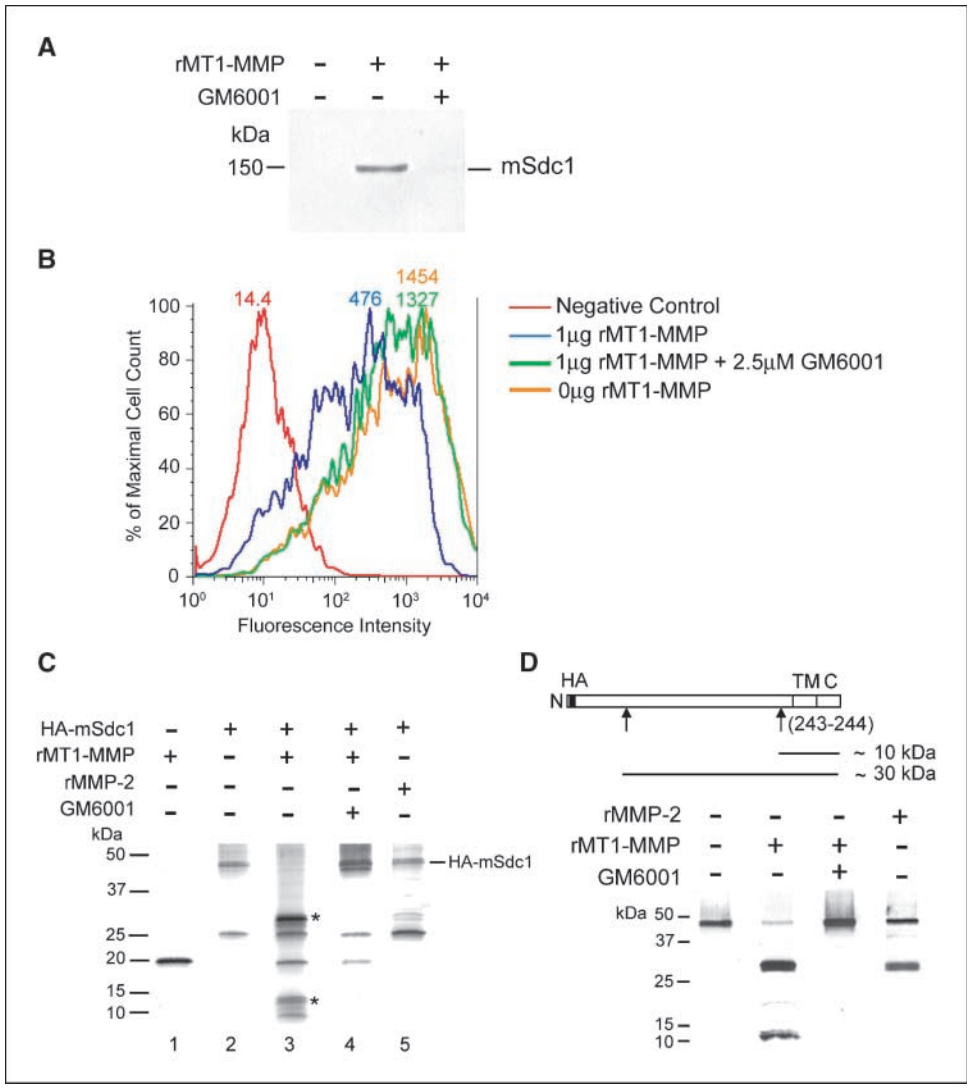

Figure 4. Recombinant MT1-MMP induces Sdc1 shedding and cleaves Sdc1 *in vitro*. *A,* rMT1-MMP induces Sdc1 shedding from HMF. HMF overexpressing mSdc1 were treated with 0 or 500 ng/mL of rMT1-MMP with or without 2.5 μmol/L GM6001 at 37°C for 1 h. The medium was collected after the treatment and analyzed for the presence of shed mSdc1, as described in Materials and Methods. *B,* rMT1-MMP decreases cell surface mSdc1 of HMF overexpressing mSdc1. Cells were lifted and exposed to 0 or 1 μg of rMT1-MMP with or without 2.5 μmol/L GM6001 at 37°C for 1 h. After washing, cells were labeled with rat anti-mSdc1 ectodomain antibody (clone 281.2) and analyzed by flow cytometry. The numbers represent the mean fluorescence intensity of the samples. *C,* rMT1-MMP cleaves HA-mSdc1. HA-mSdc1 was exposed to 0 or 1 μg of rMT1-MMP with or without 2.5 μmol/L GM6001 or 1 μg of activated rMMP-2 at 37°C for 1 h. Then samples were separated on a 12% Bis-Tris Criterion XT precast gel (Bio-Rad) and silver-stained. The asterisks indicate the major digested fragments. *D,* two fragments resulting from rMT1-MMP digestion contain the cytoplasmic domain of mSdc1. HA-mSdc1 was digested, as described in *C*. The samples were separated on 12% Bis-Tris Criterion XT precast gel (Bio-Rad) and transferred to a PVDF membrane. The blot was probed with rabbit anti-Sdc1 cytoplasmic domain antibody (S1CD). The schematic drawing depicts the predicted pattern of rMT1-MMP–mediated HA-mSdc1 cleavage. Arrows indicate the potential cleavage sites. The lines represent the possible fragments containing the cytoplasmic domain generated by digestion, which can be recognized by antibody S1CD. The data are representative of several experiments. *N,* amino-terminus; *TM,* transmembrane domain; *C,* cytoplasmic domain.

functions of MT1-MMP is the activation of other proteases, particularly MMP-2. In an elegant xenograft study, Taniwaki and colleagues showed that carcinoma cell growth stimulation by MT1-MMP depended on the stromal supply of MMP-2 (36). Conversely, Hotary showed that MT1-MMP–stimulated carcinoma cell growth in three-dimensional collagen matrices did not require MMP-2 or other proteases of the MMP family (37). Similarly, in our study, MMP-2 inhibition with a neutralizing antibody or a small molecule inhibitor had no effect on carcinoma cell growth stimulation by fibroblasts, arguing against a role of this enzyme in our model.

MT1-MMP can stimulate carcinoma cell invasion and proliferation directly by collagen degradation (37–39). In Hotary's work, MT1-MMP–mediated carcinoma cell growth was abolished when the cells were embedded in mutant, protease-resistant collagen (37). Despite the fact that T47D cells were suspended in collagen in our study, it is unlikely that collagen degradation played a role in fibroblast-induced carcinoma cell growth stimulation. The effect of MT1-MMP inhibition on carcinoma cell growth was reversed by the addition of Sdc1 ectodomain, indicating that Sdc1 cleavage was the only required MT1-MMP activity (Fig. 3). Also, in contrast to the work by Hotary, which showed that accelerated growth required anchorage of the enzyme at the cell surface (37), soluble MT1-MMP catalytic domain was sufficient to restore growth in our study (Fig. 2*D*). Thus, it seems that the prevailing MT1-MMP mechanism of action depends on cell source and target cell type. For instance, MT1-MMP on tumor cells cleaves cell surface molecules, such as integrins, to facilitate cell adhesion and migration (40, 41). Stromal cell–derived MT1-MMP may contribute to tumor growth and invasion indirectly by releasing paracrine mediators from the pericellular environment. These diverse functions of MT1-MMP are facilitated by a surprisingly wide spectrum of substrates (42). A recent proteomics study identified a number of cell surface extracellular matrix (ECM) constituents, receptors, and cytokines as MT1-MMP substrates (43).

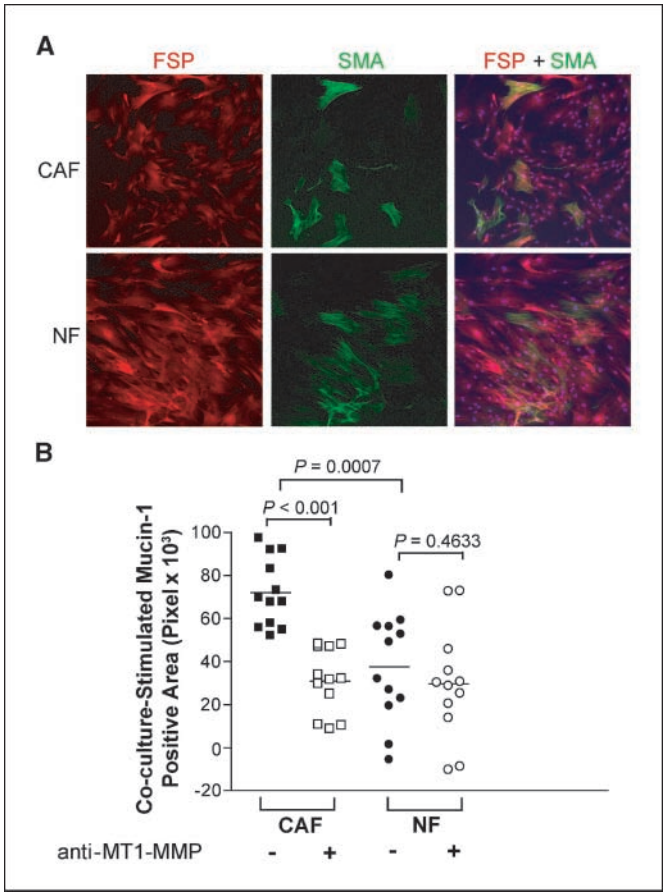

Figure 5. CAF stimulate T47D carcinoma cell proliferation significantly more than NF, and inhibition of MT1-MMP reverses this growth advantage. *A,* immunofluorescence staining of CAF and NF. CAF or NF were grown on chamber slides until they reached ~80% confluency, fixed, and stained with anti-S100A4/FSP1 and anti–α-SMA antibodies. Images are representative of 12 pairs of CAF and NF. *B,* CAF induce T47D proliferation significantly more than NF in an MT1-MMP activity–sensitive manner. CAF and NF pairs were cocultured with T47D cells. The coculture was treated with 10 μg/mL of anti–MT1-MMP or rabbit IgG for 3 d. Coculture-stimulated T47D cell growth was calculated as mucin-1–positive area of T47D + CAF or NF coculture minus mucin-1–positive area of T47D monoculture.

A variety of enzymes have been implicated in Sdc1 shedding. These include a TIMP-3–sensitive unidentified MMP (14), MMP-7 (7, 16), and MMP-9 (12). The most conclusive evidence for a direct enzymatic degradation of Sdc1 has been presented for MT1-MMP (8). The MT1-MMP consensus cleavage site contains the amino acid sequence PXX↓L (ideally PXP↓L or PXG↓L; ref. 44). $P^{80}TG\downarrow L^{83}$ represents the only such consensus site in human Sdc1 (hSdc1). However, MT1-MMP has also been found to cleave hSdc1 at $S^{243}QG\downarrow L^{246}$, an alternative site not conforming with the consensus sequence (44). MT1-MMP cleaves mSdc1 at $A^{243}\downarrow SQSL^{247}$, another site sharing no similarity with the consensus sequence described above (44). According to its amino acid sequence, mSdc1 contains an MT1-MMP consensus cleavage site at $P^{53}DT\downarrow L^{56}$ and our results suggest that MT1-MMP indeed cleaves mSdc1 at this location. Our *in vitro* digestion results indicate that MMP-2 is not involved in Sdc1 shedding, although the enzyme displays some digestive activity toward the amino-terminal portion of HA-mSdc1.

The evidence presented here and in recent reports by our group suggests a complex, reciprocal interplay between breast carcinoma cells and stromal fibroblasts, involving the induction of Sdc1 and MT1-MMP expression in fibroblasts by carcinoma cells, followed by MT1-MMP–mediated shedding of Sdc1 ectodomain from the fibroblast surface (3–5). The HS chains attached to the released Sdc1 ectodomain fragments stimulate breast carcinoma cell proliferation by augmenting SDF1 and FGF2 activity (5).

Because this model was devised using an assay system with an immortalized mammary fibroblast cell line, the question of the relevance of the described pathways in human breast cancer can be raised. Our observations on cocultures of primary fibroblasts with T47D breast carcinoma cells suggest that stromal MT1-MMP plays a role in the majority of breast carcinomas (Fig. 5*B*). Consistent with reports by other investigators (45), CAFs stimulate carcinoma cells to a greater degree than NFs. Apparently, the differences between CAFs and NFs were maintained during the brief culture period, despite the fact that some NFs in culture displayed an activated, myofibroblast-like, SMA-positive phenotype (Fig. 5*A*). Surprisingly, NFs show a more pronounced heterogeneity than CAFs as far as their carcinoma cell growth–promoting capability is concerned (Fig. 5*B*). The inactivation of MT1-MMP activity with a neutralizing antibody significantly reduced carcinoma cell growth stimulation by CAFs.

Broad spectrum MMP inhibitors have been a disappointment in clinical trials (46). One reason for this failure could be the fact that MMPs may have dual roles in tumor promotion and suppression (20, 46). MMPs release antiangiogenic peptides, such as endostatin, angiostatin, or endorepellin, from their ECM precursor molecules.

Therefore, the unselective inhibition of all MMPs may have either a neutral or even an adverse effect on disease outcome. A specific blockage of MT1-MMP seems a promising therapeutic strategy to inhibit both breast carcinoma cell invasion and disrupt the novel paracrine, growth-promoting signaling pathway described here.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interests.

Acknowledgments

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