Stromelysin-3 Is a Potent Negative Regulator of Adipogenesis Participating to Cancer Cell-Adipocyte Interaction/Crosstalk at the Tumor Invasive Front

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Abstract

The initial invasive processes during cancer development remain largely unknown. Stromelysin-3/matrix metalloproteinase 11 (ST3/MMP11) is associated with tumor invasion and poor prognosis. We present novel evidence that adipocytes present at human breast tumor invasive front are induced by cancer cells to express ST3. Using mouse syngeneic model, light and electron microscopy showed that in ST3-deficient mice but not in wild-type mice, forced cancer cell-adipocyte interaction/crosstalk results in adipocyte membrane alteration, allowing cancer cell fat infiltration and death. Thus, adipocytes are involved in initial cancer cell survival into connective tissue, and this effect is ST3 mediated. This suggested that ST3 might play a role in adipocyte metabolism. Accordingly, ST3-deficient mice exhibited fat excess and increased mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ) and adipocyte protein 2 (aP2) adipogenic markers, indicating that, in vivo, ST3 negatively regulates fat homeostasis. Moreover, ST3-deficient mouse embryonic fibroblasts exhibited a dramatic enhanced potential to differentiate into adipocytes associated with increased PPARγ and aP2 expression, and recombinant ST3 treatment reverted their differentiation. Thus, in vitro, ST3 reduces adipocyte differentiation in an autocrine manner. High fibroblasts/adipocytes ratio is a stroma feature, and peritumoral fibroblast origin remains debated. Our results support the concept that invading cancer cells aberrantly restore the negative ST3 function on adipogenesis into proximal adipocytes/preadipocytes, leading to the accumulation/maintenance of a particular peritumoral fibroblast population. Accordingly, in human breast tumors, we observed that ST3-expressing peritumoral fibroblasts are distinct from α-smooth muscle actin expressing myofibroblasts. This constitutes the first report of implication of a MMP in cancer cell-adipocyte interaction/crosstalk during early steps of connective tissue invasion. (Cancer Res 2005; 65(23): 10862-71)

Introduction

Carcinoma is deleterious due to the systemic dissemination of the disease and the constitution of metastases. The prerequisite to this process is the local colonization of adjacent connective tissues by epithelial cancer cells. The mesenchymal cells normally present in connective tissues include several cell types, most notably fibroblasts, endothelial cells, and adipocytes; it is becoming increasingly clear that these cells, although not transformed, actively participate in tumor development (1–4). At the initial invasive stage, pioneer invasive cancer cells remain as individual cells or groups of very few cells and establish epithelial/mesenchymal heterotypic cell interactions. This event is ephemeral because features of invaded connective tissue are rapidly and dramatically modified in response to the cancer cells. Later, cancer cells aggregate and invasive carcinomas most often appear as a “lump” due to the embedment of cancer cells into a dense collagen stroma that takes place via the desmoplastic response. This extracellular matrix (ECM) adaptation is associated with a modification of the nature and relative ratio of the stromal cell compartment, such as notable adipocyte disappearance and fibroblastic cell accumulation. The early biological events sustaining initial survival and implantation of cancer cells into the adjacent connective tissues remain unclear.

Previous in vivo data suggest that stromelysin-3/matrix metalloproteinase 11 (ST3/MMP11) might be a key factor in these initial processes. ST3 is a connective tissue–derived factor normally expressed in association with intense tissue remodeling during embryogenesis, tissue involution, wound healing, and metamorphosis (5). Whereas most MMPs require the presence of additive proteinases to be extracellularly activated, ST3 is activated before secretion by Golgi-associated furin-like proteinases (6, 7). Active ST3 is therefore secreted at the right time and placed in a rapid and focused fashion. Importantly, clinical studies have shown that, at the primary tumor level, high levels of ST3 expression by fibroblasts located in the vicinity of cancer cells (8) are associated with aggressiveness of numerous human carcinomas (breast, colon, prostate, etc.) and poor patient clinical outcome (9). Using several tumor models done in either nude, wild-type, or ST3-deficient mice, ST3 was shown to be not only a biomarker but a key factor for tumor development (10–12). ST3 does not seem to increase cancer cell proliferation or invasion (11), or tumor angiogenesis (13), but it has a paracrine antiapoptotic function, which allows for cancer cell survival (13, 14). Interestingly, using a syngeneic tumor model, this antiapoptotic effect was observed using light microscopy in small invasive tumors but was absent when tumors became clinically detectable, suggesting that ST3 acts early (13).
Thus, ST3 plays a deleterious function that is shared by human tumors of various origins and that presumably occurs at the initial steps of the invasive process (15). The present study was undertaken to test this hypothesis and identify the biological mechanism underlying this ST3 action. Collectively, our data show a new physiologic function for ST3 as a negative regulator of adipogenesis and indicate that this function is aberrantly restored subsequently to cancer cell- adipocyte interaction/crosstalk during the initial steps of cancer cell invasion.

Materials and Methods

Breast cancer histology and immunohistochemistry. Histologic examination of human normal breast tissue and breast invasive tumors were done using H&E staining. ST3 immunohistologic analysis was done using the 5ST-4A9 mouse monoclonal antibody (1:2,000) directed against the human ST3 as previously reported (9). For costaining, sections were pretreated with protease-3 (Ventana protocol, NexES; Tucson, AZ). They were first processed with the 5ST-4A9 antibody and stained using the kit LSAB-3.3-diaminobenzidine (Ventana, brown color). After washing, they were incubated (automat Benchmark, Ventana) with the 1A4 monoclonal antibody (Dako, Carpinteria, CA; 1:100) directed against the human α-smooth muscle actin (α-SMA) and revealed with phosphatase alcaline kit (Ventana, red color). Counter coloration was for 4 nm (blue color).

Breast cancer in situ hybridization. Paraffin-embedded, formalin-fixed sections (5 μm) were deparaffinized, rehydrated, and air-dried. After pretreatment with proteinase K, sections were hybridized with denatured digoxigenin-labeled ST3 sense or antisense probes (nucleotides 346-2105; ref. 8). The sections were incubated using goat anti-digoxigenin antibody coupled to alkaline phosphatase (Roche, Indianapolis, IN; 1:2,000) and revealed with alkaline phosphatase substrate solution containing levamisole (dark blue color). Sections were counterstained with nuclear fast red (Vector Labs, Burlingame, CA; nucleus in pink color).

Mouse fat tissue collection. Abdominal, inguinal, and thoracic mammary fat deposits of white adipose tissue were collected from twenty 8- to 9-week-old females and males.

Cancer cell/adipocyte interaction assay. These assays were conducted as previously described (13). Briefly, 5 × 10^5 C26 cells (in 50 μl of PBS 1×), derived from colon carcinoma induced in BALB/c mice by repeated intra rectal instillations of N-nitroso-N-methylurethane, were s.c. injected on both shaved flanks of 8- to 9-week-old females. Six wild-type (ST3+/+) and six ST3-deficient (ST3−/−) mice were used in each experiment. The experiments were done in triplicate. Mice were anesthetized in a CO2 chamber for 30 minutes followed by post-fixation for 2 hours at 4°C.

For histologic examination of human normal breast tissue and breast invasive tumors, specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer and embedded in Epon 812. For light microscopic analysis, semithin sections (2 μm) were deparaffinized, rehydrated, and air-dried. After pretreatment with proteinase K, sections were hybridized with denatured digoxigenin-labeled ST3 sense or antisense probes (nucleotides 346-2105; ref. 8). The sections were incubated using goat anti-digoxigenin antibody coupled to alkaline phosphatase (Roche, Indianapolis, IN; 1:2,000) and revealed with alkaline phosphatase substrate solution containing levamisole (dark blue color). Sections were counterstained with nuclear fast red (Vector Labs, Burlingame, CA; nucleus in pink color).

Toluidine blue staining and electron microscopy. For histologic evaluation, specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L, pH 7.2) overnight at 4°C and washed in same buffer for 30 minutes followed by post-fixation for 2 hours at 4°C in 1% osmium tetroxide (OsO₄) with cacodylate buffer. Then they were dehydrated through graded alcohol and embedded in Epon 812. For light microscopic analysis, semithin sections (2 μm) were stained with toluidine blue. Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate and examined with Philips 208 transmission electron microscope.

Primary culture and adipocyte differentiation of wild-type and stromelysin-3-deficient mouse embryonic fibroblasts. Pregnant females were sacrificed at 14.5 day after coitum. The embryos were removed and their heads were removed to serve for Southern blot genotyping. The rest of the embryos were then minced. The resulting slurry was plated in 10-cm dishes and mouse embryonic fibroblasts (MEF) allowed to grow to confluence in DMEM with 10% FCS. To initiate differentiation, at confluence, a dedifferentiation-inducing mix (10 μg/mL insulin, 0.5 μmol/L dexamethasone, and 0.5 mmol/L methylsobutyxanthine) was added. After 2 days, medium was replaced with fresh culture medium containing insulin (10 μg/mL).

Adipocyte dedifferentiation of stromelysin-3-deficient mouse embryonic fibroblasts. At day 8 of differentiation, buffer containing or not mouse recombinant ST3 (3.5 μg/mL) was added daily to the culture medium. Dedifferentiation experiments were conducted for 60 hours. Preparation of recombinant active and inactive ST3 (Glu²⁵⁴-Ala substitution; ref. 11), as well as control of their enzymatic activity were done as previously described (16). Briefly, the recombinant proteins corresponding to amino acids 102 to 492 and containing the catalytic and hemopexin domains of mouse ST3 were produced in Escherichia coli using the prokaryote expression vector pET3b. Protein was extracted from inclusion bodies. After protein purification and renaturation, the ST3 enzymatic activity was tested in vitro.

Oil Red O staining. To estimate the adipocyte phenotype, cultures were fixed in 10% formalin in PBS 1× and for 30 minutes, rinsed thrice with distilled water, and then air-dried. The fixed cells were stained with 0.5% Oil Red O for 20 minutes, washed with distilled water, and were counterstained with hematoxylin (17).

RNA isolation and Northern blot analysis. Specimens of thoracic and inguinal mammary fat pads and from abdominal fat from wild-type and ST3-deficient mice, or cultured cells were homogenized (using an Omni 2000 Polytron). Total RNA was isolated using acid guanidinium thiocyanate lysis buffer (18). Ten micrograms of RNA were fractionated by electrophoresis through 1% agarose gel in the presence of formaldehyde and transferred to nylon membranes (Hybond N, Amersham, Arlington Heights, IL). Filters were acidified for 10 minutes in 5% CH₃COOH and stained for 10 minutes [0.004% maleic acid, 0.5 mol/L CH₃COONa (pH 5.0)] before hybridization. Northern blots were hybridized 8 hours at 42°C under stringent conditions (50% formamide, 5 × SSC, 0.1% SDS, 0.1% polyvinyl pyrrolidone, 0.1% Ficoll, 20 mmol/L sodium pyrophosphate, 10% dextran sulfate, and 100 μg/mL single-stranded DNA) and either with ST3, peroxisome proliferator-activated receptor γ (PPARγ), adipocyte protein 2 (aP2), MMP2, or 36B4 cDNA probes randomly primed. Washings were done in 2 × SSC, 0.1% SDS twice at room temperature for 15 minutes followed by twice in 0.1× SSC, 0.1% SDS at 55°C. Blots were stripped in boiled 0.1% SDS between different probes. The PPARγ and aP2 probes were kindly provided by J. Auwerx (Institut de Génétique et de Biologie Moléculaire et Cellulaire; ref. 19). The ST3, MMP2 and 36B4 probes were described previously (9). 36B4 was used as positive control of loading.

Results

Stromelysin-3 expression by adipocytes located at the invasive front of human tumors. Study of ST3 expression at the initial steps of cancer cell invasion is difficult in human because localization of such events is quite impossible. To circumvent this problem, we presumed that similar dynamic events should occur later, in larger tumors, at the tumor invasive front (Fig. 1B). In this area (Fig. 1B, area F), cancer cells may also be sparsely distributed, and the connective tissues at the edge may still contain all the mesenchymal cell types present in normal tissues (Fig. 1A) that are most often missing in constituted invasive tumors (Fig. 1C). We thus focused on the invasive front (Fig. 1D) of human breast carcinomas. Using the 5ST-4A9 ST3-specific monoclonal antibody, we observed that ST3 was expressed by adipocytes proximally located to the invasive cancer cells (Fig. 1F and G) but not by distally located adipocytes (Fig. 1E). This was observed in all adipocyte cancer front samples examined (>20), indicating that this is a common event during local invasion. As expected from previous data (9), the few fibroblasts located in the same areas were also ST3 positive. ST3 expression in adipocytes was further confirmed by in situ hybridization; ST3 mRNA was observed in the adipocyte cytoplasm (Fig. 1H). Interestingly, we observed major morphologic changes in adipocytes located at the cancer cell contact [compare in Fig. 1B the adipocytes present in tumor invasive front (area F) and in adipose tissue (area A) and Fig. 1E and F; same magnification]. They exhibited a drastic

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Figure 1. Histology and ST3 expression of normal human breast tissue and invasive front of breast carcinomas. Histologic examination of human normal breast tissue (A) and invasive breast tumor (B). C and D, higher magnification of tumor center and invasive front, respectively. E-G, ST3 immunohistochemistry of normal (E) and peritumoral (F and G) adipocytes. H and I, ST3 in situ hybridization of tumor invasive front using antisense (H) and sense probe (I). Notice that compared with normal adipose tissue, the number and the size of adipocytes are greatly reduced at the invasive front of the tumor (B, compare areas A and F; compare E and F) and that adipocytes have totally disappeared in the tumor center, whereas numerous peritumoral fibroblasts are seen (B, area C). ST3 protein (brown) was visualized in adipocytes (lipids in white, nucleus in blue) located adjacent to invading cancer cells (F and G) but not in normal tissue (E). G, note that in adipocytes ST3 protein is located within the rim of cytoplasm surrounding the flat nucleus, at one side of the cell. H, ST3 mRNA (dark blue) show similar localization (nucleus in pink). I, sense control probe gave a negative result. Original magnification: ×200 (A and B), ×400 (C-F), ×1,000 (oil immersion, G-I). Abbreviations: A, adipose tissue; C, tumor center; F, tumor invasive front; G, normal mammary gland.
reduction in size and those located more proximal to the tumor center exhibited the larger reduction (Fig. 1D). There were also modifications in shape from polyhedral cells (with supple membranes) to more rounded cells (with stiff membranes).

Thus, invading cancer cells altered adjacent differentiated adipocytes and are able to induce them to express ST3. This suggests that ST3 might play a role related to cancer cell-adipocyte interaction.

**Dramatic alteration of adipocytes and cancer cells resulting from their forced interaction in stromelysin-3-deficient host mice.** To test this hypothesis, we took advantage of a mouse tumor model in which a few C26 cancer cells are s.c. injected in syngeneic mice, thereby mimicking the human situation, because tumors devoid of adipocytes are rapidly constituted. Moreover, using this model in ST3-deficient mice, we previously observed a smaller tumor size due to increased cell apoptosis at 6 days after injection (13). To study the effect of ST3 on cancer cell/adipocyte interactions, we did light microscopic morphologic analyses of the injection sites during the initial steps of cancer cell implantation, at day 4 after injection in wild-type or ST3-deficient mice (Fig. 2). Regardless of the ST3 status of the host mice (A-C), we observed at this stage that the cells did not form compact tumors but remained more or less sparsely distributed in the connective tissue. However, in ST3-deficient connective tissues, the cellularity was lower with fewer cancer cells. There were striking histologic differences between wild-type and ST3-deficient recipients. In the ST3-deficient context (B and C), cancer cells seemed attracted towards the host adipocytes, establishing intimate contact with them. Numerous adipocytes were surrounded by a row of cancer cells giving rise to rosette-like (B) and granuloma-like (C) arrangements. Adipocyte decay and infiltration of lipid droplets of variable numbers and sizes into cancer cells were seen. Lipid droplets were also present in the ECM (B). Such cellular arrangements and fat infiltration were never observed in the wild-type context (A).

These results prompted us to further study the cancer cell/adipocyte interface using electron microscopy (D-I). In wild-type mice (D, F, and H), the unilocular adipocytes were well defined with intact plasma and basement membranes. Fibroblasts were seen in close proximity to the adipocytes (H), and the ECM contained many typical parallel collagen fibrils and fibers (D, F, and H). In ST3-deficient mice (E, G, and I), the adipocyte membranes were greatly altered and/or disrupted. The interface lining between the cancer cells and adipocytes were sometimes enlarged and presented numerous electron dense granular deposits in the periphery of decaying adipocytes whose nature remains unknown. Lipid droplets were also trapped into this abnormal layer (E). Electron microscopy examination further confirmed the uptake of lipid droplets by the cancer cells and by the ECM. In some cancer cells, the endoplasmic reticulum was dilated (I). Furthermore, there were dead or dying cancer cells in the vicinity of the depleting adipocytes, and some of these cells seemed vacuolated with loss of cell morphology. Finally, the ECM was greatly affected, notably at the collagen level, which seemed less abundant and disorganized.

Thus, ST3 deficiency alters the reaction of host adipocytes to cancer cells. These data indicate that ST3 is required for cancer cell survival via adipocyte function, suggesting that ST3 might normally play a role in adipocyte metabolism.

**Increased adipose tissues in stromelysin-3-deficient mice.** Consistent with the above hypothesis, we observed that the mean body weight of ST3-deficient mice maintained on a normal diet was significantly higher than that of control littersmates, independent of gender and genetic background differences (i.e., 10 females, 27.5 g versus 23.8 g; P < 0.0003). Moreover, the weight of surgically isolated abdominal fat was also higher in the ST3-deficient mice (i.e., 10 females, 440 mg versus 220 mg; P < 0.0003). Histologic analysis of abdominal fat deposits and of inguinal and thoracic mammary fat pads revealed that the diameter of the adipocytes in ST3-deficient fat (Fig. 3A, b) was larger than in the wild-type fat (Fig. 3A, a), indicating adipocyte hypertrophy. However, because it was impossible to count the adipocytes present in the fat samples, we cannot exclude that the adipocyte cellularity might also be increased. Using Northern blot analysis (Fig. 3B), expression of two adipogenic markers, the PPARγ (19) and the aP2 (20), was observed in all fat samples studied, but their levels were higher in ST3-deficient mice (lanes 4-6), compared with wild-type mice (lanes 1-3). Low ST3 expression was detected in all wild-type fat samples studied (lanes 1-3). Finally, expression of MMP2 (gelatinase A), another member of the MMP family known to be expressed during adipogenesis (21), was also tested. MMP2 was expressed in all fat tissues studied and was slightly increased in ST3-deficient mammary fat samples. These results indicate that, in vivo, ST3 is involved in a negative regulatory process that controls fat mass homeostasis.

**Increased adipocyte differentiation potential of stromelysin-3-deficient mouse embryonic fibroblasts.** To test if ST3 is a negative regulator of normal adipogenesis, we studied MEFs, which have been reported to have the capacity to differentiate into adipocytes (22). We did primary cultures and compared the efficacy of wild-type and ST3-deficient MEFs to differentiate into adipocytes in response to a differentiation-inducing mix. This treatment is known to induce the expression of proteins associated with adipocyte differentiation and the accumulation of lipids (23). Cell differentiation was phenotypically evaluated by Oil Red O staining of lipids (ref. 17; Fig. 4A), and at molecular level by Northern blot analysis of the expression of PPARγ, aP2, ST3, and MMP2 (Fig. 4B). At every time point tested, ST3-deficient MEFs (f) showed higher frequency and size of intracellular red-stained lipid droplets compared with wild-type MEFs (a-e). At 6 days after differentiation-inducing mix treatment, >50% of ST3-deficient MEFs had differentiated into adipocytes (i), whereas <25% of ST3 wild-type MEFs had differentiated (d). Consistently, PPARγ and aP2 mRNA levels were higher in ST3-deficient MEFs (Fig. 4B, lanes 7-12) compared with wild-type MEFs (lanes 1-6). Interestingly, ST3 deficiency led to constant high expression of PPARγ even in confluent ST3-deficient MEFs, which had not started to differentiate, suggesting that it could be constitutive. By contrast, aP2 expression remained differentiation-inducing mix dependent. ST3 was expressed at cell confluence (lane 1) in wild-type MEFs, and addition of differentiation-inducing mix strongly repressed this expression for 2 days (lanes 2 and 3). MMP2 was expressed in both ST3-positive and ST3-negative MEFs. In ST3-negative MEFs, its expression was first lower than in wild-type MEFs (lanes 7-9) but higher after day 6 (lanes 10-12).

Taken together, these data show that the potential of ST3-deficient MEFs to differentiate into adipocytes is markedly increased compared with that of wild-type MEFs, confirming that ST3 is a physiologic negative regulator of adipogenesis.

**Reversion of differentiation-inducing mix–induced adipocyte phenotype of stromelysin-3-deficient mouse embryonic fibroblasts by recombinant stromelysin-3 treatment.** Finally, because dedifferentiation of preexisting adipocytes has been reported (24), we investigated if ST3 can revert adipocyte
Figure 2. Optical and electron microscopy analysis of wild-type and ST3-deficient adipocyte-cancer cell interfaces. Examination were done at 4 days after s.c. injection of C26 cells in either wild-type or ST3-deficient mice. Optical analyses of wild-type (A) or ST3-deficient (B and C) mice using toluidine blue coloration of semithin sections. Rosette-like (B) and granuloma-like (C) organization of cancer cells around adipocytes were observed in ST3-deficient mice. Note the adipocyte decay and the fat infiltration in ECM and cancer cells located at the periphery of adipocytes. Lipids (gray). D-I, electron photomicrographs. In wild-type injection sites (D, F, and H), there are well-delineated adipocytes and abundant production of parallel collagen fibers. Active fibroblasts were seen in close proximity of adipocytes. In ST3-deficient injection sites (E, G, and I), the membranes of adipocytes were altered allowing lipid droplet infiltration. Numerous fat droplets (*) were seen in cells and ECM. Adipocyte membranes sometimes exhibited dense granular deposition with lipid inclusions. Collagen fibers were reduced in quantity and sometimes grossly altered. Endoplasmic reticulum was enlarged. Original magnification: ×100 (A-C), ×4,000 (D and E), ×5,000 (F and G), ×6,000 (H and I). Abbreviations: A, adipocytes; C, collagen fibers; F, fibroblasts; D, deposition; E, endoplasmic reticulum.
fibroblast-like spindle-cell shape (b and c). This was accompanied by decreased numbers and size of lipid droplets, as shown by Oil Red O staining. Northern blot analysis (Fig. 5b) showed that ST3 treatment led to a dramatic reduction of PPARγ mRNA level but not aP2 (lane 3). MMP2 expression was also slowly decreased. To test if this ST3 function was dependent on its enzymatic activity, similar experiments were done using an inactive recombinant ST3 in which the glutamic residue of the catalytic site was changed to an alanine (Glu220-Ala; ref. 11). This inactive ST3 was unable to revert MEF adipocyte differentiation (data not shown), indicating that ST3 enzymatic activity is required in this process.

Thus, ST3 can revert mature adipocyte phenotype. This also clearly indicates that ST3 acts intrinsically on adipocytes. **Stromelysin-3 defines a subpopulation of peritumoral fibroblasts.** Until now, whatever the carcinomas studied, ST3 expression was reported to be restricted to a subset of fibroblasts located at proximity of cancer cells in the stroma of invasive tumors (8, 9). Among the peritumoral fibroblasts, the myofibroblasts that expressed α-SMA constitute the only well-characterized subset (25). Interestingly, it has been shown that myofibroblasts express MMPs, most notably MT1-MMP and MMP2 (26). To date, the nature of ST3-expressing fibroblasts remains unclear, because only one study reported that a subset of cells producing ST3 seems to be myofibroblasts (27). Taken together, our results suggested that they might derive from adipocytes. It was therefore of interest to further investigate the nature of ST3-expressing peritumoral fibroblasts. Immunostaining of adjacent breast cancer sections with antibody directed against α-SMA or ST3 showed that, although often located in the same tumor areas, ST3-expressing and α-SMA-expressing cells were not the same (data not shown). To definitely conclude, we therefore did costaining of several breast cancer sections. No costained fibroblast was observed. Thus, the ST3-expressing fibroblasts were distinct from the α-SMA-positive fibroblasts (Fig. 6C-F). Moreover, we noticed that the ST3-expressing adipocytes present at the tumor invasive front never express α-SMA (Fig. 6A and F).

These data clearly indicated that in the tumors presenting constituted stroma, ST3 is specific of a discrete subset of peritumoral fibroblasts.

**Discussion**

Numerous clinical and experimental observations have argued for a deleterious ST3 function during the initial steps of cancer cell invasion (reviewed in ref. 15). The present results provide evidence that this activity is mediated via a new ST3 physiologic function that is aberrantly expressed in the malignant context.

**Stromelysin-3 participates to cancer cell-adipocyte interaction/crosstalk.** In human carcinomas, we observed an unexpected ST3 expression in adipocytes in addition to the classic peritumoral fibroblastic expression (8). ST3 expression was not up-regulated in all adipocytes but was restricted to adipocytes proximal to the cancer cells, as those located more distally were ST3 negative, indicating that the expression efficiency is proportional to the distance between the two cell types. This suggests that, as previously established for ST3-expressing fibroblasts (28, 29), cancer cell-secreted soluble factors or cancer cell-adipocyte contacts are required for ST3 expression in adipocytes. This is the first report of ST3 induction by cancer cells in adipocytes. This observation is important because adipocytes are the most numerous mesenchymal cells in numerous organs, filling a large part of connective tissues.
Normal ST3-deficient mice show a higher body weight and fat deposits compared with control littermate mice due to increased size of adipocytes (32). The higher body weight of mice fed a high fat diet is consistent with previous studies showing that ST3-deficient mice exhibit higher body weight and fat deposits (30, 33). Consistently, ST3-deficient mice have a higher capacity to differentiate in adipocytes than wild-type MEFs, indicating that ST3 limits adipogenesis. We noticed that PPARγ expression was more regulated in both ST3-deficient MEFs and tissues deficient for ST3 than the expression in wild-type MEFs, suggesting that this ST3 function occurs very early in the tumor development process. Accordingly, in the present work, we observed that ST3 is required for adipocyte and cancer cell survival subsequent to their interaction as early as 4 days after cancer cell injection. In ST3-deficient animals, both the basement and plasma membranes surrounding the adipocytes (30) were altered allowing the passage of lipids from the adipocytes to the cancer cells and ECM and ultimately leading to the progressive decay of adipocytes. This is accompanied by lower numbers of cancer cells. Interestingly, lipid-induced cancer cell apoptosis and necrosis have previously been reported (31).

Together, these results show that adipocytes permit initial cancer cell survival into connective tissues and indicate that this effect is, at least partially, mediated via ST3.

**Stromelysin-3 negatively regulates fat homeostasis.** The first evidence of a role of ST3 in adipocyte metabolism came from a previous study on obesity showing that ST3-deficient mice fed with high fat diet exhibited higher body weight and fat deposits due to increased size of adipocytes (32). The higher body weight and excess of adipose tissues observed in the present study in normally fed ST3-deficient mice compared with control littermate are in agreement with this result. Formation of adipose tissue requires the conversion of preadipocytes into mature adipocytes (30, 33). This differentiation switch activates a specific program of gene expression that is followed by the accumulation of lipids. Accordingly, the increased adipogenesis observed in ST3-deficient fat tissues was accompanied by increased expression of the two adipogenic markers, PPARγ (19) and aP2 (20). Altogether, these data indicate that, in vivo, ST3 constitutes a negative regulatory mechanism to assure fat homeostasis.

The most evident way to limit adipose tissue mass is to limit preadipocyte differentiation. Consistently, we show that ST3-deficient MEFs have a higher capacity to differentiate in adipocytes than wild-type MEFs, indicating that ST3 limits adipogenesis. We noticed that PPARγ expression was no more regulated in both MEFs and tissues deficient for ST3. Because similar data were not observed for the second adipogenic gene aP2, this suggests that ST3-induced inhibition of adipogenesis might be mediated through the down-regulation of PPARγ expression. In fact, PPARγ activity has been reported to be required not only for adipocyte differentiation but also for maintaining the metabolic function of fully differentiated adipocytes (22, 34). Strongly supporting this hypothesis, PPARγ expression was dramatically reduced by ST3 treatment of adipocyte-differentiated ST3-deficient MEFs. As ST3 is not a transcription factor, this effect is probably indirect. The nature of the intermediate molecules remain to be established.

The expression of several MMPs has already been examined in the 3T3L1 model (35, 36). Because some of them have been shown to be associated with cancer invasion, we examined the expression of MMPs and found that their expression was dramatically reduced by ST3 treatment (19) and aP2 (20). Altogether, these data suggest that ST3 function occurs very early in the tumor development process. Accordingly, in the present work, we observed that ST3 is required for adipocyte and cancer cell survival subsequent to their interaction as early as 4 days after cancer cell injection. In ST3-deficient animals, both the basement and plasma membranes surrounding the adipocytes (30) were altered allowing the passage of lipids from the adipocytes to the cancer cells and ECM and ultimately leading to the progressive decay of adipocytes. This is accompanied by lower numbers of cancer cells. Interestingly, lipid-induced cancer cell apoptosis and necrosis have previously been reported (31).

**Figure 4.** Oil Red O staining and Northern blot analysis of MEF adipocyte differentiation. A, Oil Red O staining of confluent wild-type and ST3-deficient MEFs (a and f, respectively), d2, d4, d6, and d8 differentiation-inducing mix–treated wild-type MEFs (b-e) showing increasing number and size of lipid droplets. d2, d4, d6, and d8 differentiation-inducing mix–treated wild-type MEFs (g-j) showing a dramatically higher number and size of lipid droplets than that of wild-type MEFs. B, Northern blot analysis of differentiation-inducing mix–induced differentiation of wild-type (ST3+/+), lanes 1-6) and ST3-deficient (ST3−/−) MEFs (lanes 7-12). C, confluent cells. Differentiation-inducing mix treatment led to a decrease of ST3 expression for 2 days and to an increase of PPARγ and aP2. In ST3-deficient MEFs, the absence of ST3 expression was accompanied by a constantly high expression of PPARγ and an increase of aP2 expression, consistent with the higher propensity of these cells to differentiate into adipocytes. Compared with the wild-type MEFs, MMP2 expression was first decreased (lanes 7-9) and then increased (lanes 10-12) in ST3-deficient MEFs.
to be absent or decreased during adipogenesis, whereas numerous others remain highly expressed during adipocyte differentiation (i.e., MMP2), it was suggested that MMPs may exert opposing functions in adipocyte metabolism. In this context, ST3 should be considered as a potent inhibitor of adipocyte differentiation.

**Stromelysin-3 reverts adipocyte differentiation.** Until recently, current views consider adult adipocytes to be terminally differentiated cells. However, several studies have shown that adipocytes can become fibroblasts after acute injury or in malignant situations (24, 37). Such transition can be induced in vitro by tumor necrosis factor-α (TNF-α) treatment of differentiated 3T3-L1 cells (38). ST3 treatment of differentiation-inducing mix–differentiated ST3-deficient MEFs reduces lipid droplets in number and size, and the cells lose their round adipocyte phenotype to adopt a spindle cell morphology, reminiscent of preadipocyte fibroblast-like phenotype. This finding suggests that ST3 can be involved in adipocyte dedifferentiation. Another possibility could be that ST3 induces lipolysis of existing mature adipocytes as reported for TNF-α (39), or that ST3 reduces preadipocyte differentiation. In any case, this indicates that, in adipocytes, ST3 exerts an autocrine function. Moreover, the enzymatic activity of ST3 is required in this process, because inactive mutated recombinant ST3 has no effect. This opens a new opportunity of finding ST3 target proteins. In fact, although ST3 function in tumor development is dependent on its catalytic domain (11), its substrate(s) remain(s) unknown. Adipocytes represent therefore a new target for such investigations.

**Stromelysin-3 participates to desmoplasia.** One of the main feature of tumor stroma edified through the desmoplastic response is an extremely high fibroblasts/adipocytes ratio (Fig. 1A). Peritumoral fibroblasts are composed of several subpopulations that are morphologically undistinguishable. Their origins remain debated (24, 40, 41). It has been proposed that besides the fact that tumorderived factors may induce an increased proliferation of resident fibroblasts, peritumoral fibroblasts might derived from preexisting mesenchymal cells. The most documented subpopulation is the αSMA-positive myofibroblasts that can derive from either fibroblasts, vascular smooth muscle cells, or pericytes (25).

Our data show that invasive cancer cells induce ST3 expression in adjacent adipocytes, and that ST3 is a potent negative regulator of adipogenesis, able to reduce and even to revert adipocyte differentiation. It is therefore tempting to speculate that ST3-expressing peritumoral fibroblasts observed once tumor stroma is constituted might derive, at least partially, from adipocytes and/or preadipocytes through a trans-differentiation process. In support of such a concept, we observed drastic morphologic changes of ST3-positive adipocytes at the breast tumor invasive front, and we showed that ST3-expressing peritumoral fibroblasts are not myofibroblasts but define a particular subset of fibroblasts. Interestingly, it has been recently proposed that peritumoral fibroblasts can result from adipocyte dedifferentiation and/or prevention of differentiation of preexisting preadipocytes to mature adipocytes (24, 41). However, the molecules involved in these processes have not been identified. Collectively, our data indicate that ST3 may be one such molecule and might, in response to cancer cell invasion, participate in the accumulation and/or maintenance of peritumoral fibroblasts. Because it is well established that peritumoral fibroblasts provide structural and biochemical support for invading cancer cells (2, 42), such a function is consistent with previous results indicating that ST3 favors cancer cell survival in connective tissues (13).

In conclusion, our results point out the essential role of adipocytes in tumor progression and the implication of ST3 in this process. To date, most of the in vivo studies on epithelial-mesenchymal interactions during cancer cell invasion have focused on fibroblasts, endothelial, and inflammatory cells in the context of a constituted stroma that contains very few, if any, adipocytes (43). Consequently, very little attention has been given to adipocytes. However, adipocytes are active endocrine cells producing various adipokines (44). The adipocyte is therefore an excellent candidate to influence tumor behavior through heterotypic signaling processes and might prove to be critical for tumor survival, growth, and metastasis (45, 46). In fact, epidemiologic studies have identified obesity as one of the major risk factors for cancer (47), and a large amount of adipose tissues has been associated with poor prognosis for breast cancer in obese women (46) and more recently with breast cancer risk (48). This constitutes the first in vivo evidence implicating an
MMP in cancer cell-adipocyte interaction. Indeed, to date, the relationship between adipocyte metabolism and the MMP system has been investigated during post-weaning mammary gland involution (49, 50) and obesity-mediated fat mass development (36, 51) but never in a malignant context.

Acknowledgments

Figure 6. ST3 and α-SMA expression in breast cancer peritumoral adipocytes and fibroblasts. ST3 (5ST-4A9 monoclonal antibody) and α-SMA (1A4 monoclonal antibody) coimmunostaining of sections of tumor invasive front (A and B) and tumor center (C-F) from several breast carcinomas. ST3 positivity (brown) was visualized in adipocytes (lipids in white; A and B) and fibroblasts (C-F) located adjacent to invading cancer cells. α-SMA positivity (red) was not observed in adipocytes but in fibroblasts (C-F) located adjacent to invading cancer cells. Notice that the ST3-expressing and α-SMA-expressing peritumoral fibroblasts belong to two discrete fibroblast subsets. As expected, strong α-SMA staining was observed in normal vascular structures (B). Original magnification, ×400.


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References


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