ADAM12 Produced by Tumor Cells Rather than Stromal Cells Accelerates Breast Tumor Progression

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Abstract
Expression of ADAM12 is low in most normal tissues but is markedly increased in numerous human cancers, including breast carcinomas. We have previously shown that overexpression of ADAM12 accelerates tumor progression in a mouse model of breast cancer (PyMT). In this study, we found that ADAM12 deficiency reduces breast tumor progression in the PyMT model. However, the catalytic activity of ADAM12 seems to be dispensable for its tumor-promoting effect. Interestingly, we show that ADAM12 endogenously expressed in tumor-associated stroma in the PyMT model does not influence tumor progression, but that ADAM12 expression by tumor cells is necessary for tumor progression in these mice. This finding is consistent with our observation that in human breast carcinoma, ADAM12 is almost exclusively located in tumor cells and, only rarely, seen in the tumor-associated stroma. We hypothesized, however, that the tumor-associated stroma may stimulate ADAM12 expression in tumor cells, on the basis of the fact that TGF-β1 stimulates ADAM12 expression and is a well-known growth factor released from tumor-associated stroma. TGF-β1 stimulation of ADAM12-negative Lewis lung tumor cells induced ADAM12 synthesis, and growth of these cells in vivo induced more than 200-fold increase in ADAM12 expression. Our observation that ADAM12 expression is significantly higher in the terminal duct lobular units (TDLU) adjacent to human breast carcinoma compared with TDLUs found in normal breast tissue supports our hypothesis that tumor-associated stroma triggers ADAM12 expression. Mol Cancer Res; 9(11); 1449–61. © 2011 AACR.

Introduction
Breast cancer continues to be one of the leading causes of death among women in Western countries. Each year, close to 200,000 women are diagnosed with breast cancer in the United States, and, despite improved screening for early detection as well as improved treatment modalities, approximately 40,000 U.S. women die of the disease (1).

Tumors are composed not only of neoplastic cells but also of stromal cells, including myofibroblasts, angiogenic, and inflammatory cells that reside in the extracellular matrix. Cell–cell and cell–matrix interactions between tumor cells, the surrounding stromal cells, and the extracellular matrix ignite cascades of molecular signals in and out of cells, modulating cell behavior and contributing to tumor progression (2–4). Metalloproteases are among the key proteins associated with malignancy (4, 5). One such metalloprotease, ADAM12 (Meltrin alpha), has recently been found to be a candidate breast cancer susceptibility gene (6). ADAM12 is a member of the large ADAM family of transmembrane proteins and has several potentially important biological functions in cancer (reviewed in refs. 7, 8). At the molecular level, ADAM12 can—as many other ADAMs (8, 9)—mediate proteolytic ectodomain shedding of growth factors and cell adhesion molecules (7, 8, 10). Importantly, ADAM12 is also involved in nonproteolytic protein–protein interactions—for example, it binds to integrins, syndecans, and TGF-β receptor II (TGF-βRII) at the cell surface, and its intracellular tail associates with adapters and signaling molecules (such as Grb2, Tks5, and Src kinases; refs. 7, 8).

In normal tissue, expression of ADAM12 is generally low; however, several studies have reported that the expression of ADAM12 is markedly increased in many human cancers, including breast (11–15), liver (16), bladder (17), and lung (18, 19) carcinomas as well as glioblastomas (20) and that the level of expression often relates to tumor stage. The potential use of ADAM12 as a biomarker for tumor progression was further evidenced by the finding that soluble ADAM12 in urine from breast and bladder cancer patients correlates with disease stage (17, 21, 22). We have
previously shown that overexpression of ADAM12 promotes tumor progression in the mouse mammary tumor virus (MMTV)-polyoma middle T antigen (PyMT) model (12). This discovery correlates with the finding that ADAM12 deficiency reduces tumor progression in the TRAMP mouse model of prostate cancer (23).

Here, we explored how endogenous ADAM12 impacts breast tumor progression. In particular, we investigated the role of ADAM12 localization in the stromal compartment versus the tumor compartment in promoting tumor progression. To answer this question, we established an in vivo model system to investigate whether ADAM12 produced by the stromal cells in mouse mammary gland tumors influences tumor progression. The data show that ADAM12 produced by tumor cells promotes tumor progression, whereas ADAM12 expressed by the tumor-associated stroma did not. Interestingly, however, we observed that TGF-β1, known to be produced by the tumor-associated stroma, influences expression of ADAM12 in the tumor cell compartment. In addition, we observed that the proteolytic activity of ADAM12 seems to be dispensable for this tumor-promoting effect.

Thus, we hypothesize that the effect of ADAM12, that is, increased proliferation and dedifferentiation could be mediated through enhanced TGF-β1 signaling provided by the surrounding stroma.

Materials and Methods

Mice

Male FVB/N-TgN(MMTV-PyMT)634Mul (Jackson Laboratory) mice were mated with ADAM12 (Meltrin alpha)-deficient (ADAM12−/−) female mice (24) that had been previously backcrossed for 9 generations into the FVB/N background. Male PyMT-ADAM12+/− offspring were mated with female ADAM12−/− mice to obtain PyMT-ADAM12−/− and littermate PyMT-ADAM12+/− mice. All animals analyzed in this study were females and examined as previously described (12). Wild-type, ADAM12+/−, and ADAM12−/− mice on C57/Bl6 and FVB/N genetic backgrounds were used for injections of tumor cells. Cell lines (10⁶ cells per mouse) were injected subcutaneously into the right flank or into the mammary gland. Tumor size (length and width) was measured over time. Mice were sacrificed when the first tumor reached 1.2 cm². One hour before sacrifice, 5-bromo-2′-deoxyuridine (BrdU; 50 μg/g body weight) was injected intraperitoneally (i.p.); subsequently, mice were anesthetized by i.p. administration of a 1:1 mixture of Dormicum and Hypnotil. Mice were sacrificed, minced into small pieces, and tumor cells isolated as described (27). Freshly isolated cells were plated on collagen type I (PureCol from Advanced Biomatrix) and grown for 24 hours in DMEM, penicillin/streptomycin, and 10% FBS. Vital and adherent cells were harvested and immediately frozen or used for immortalization.

To generate immortalized PyMT cell lines, pBABE-PyMT (Addgene; plasmid 22305; ref. 27) was retrovirally transduced as described previously (28) into primary tumor cells isolated from PyMT-ADAM12+/− and PyMT-ADAM12−/− mice. Cells were kept in DMEM with 50 μg/mL hygromycin B. The established cell line deficient in ADAM12 expression was termed PyMT-A12null, whereas the control cell line expressing ADAM12 was termed PyMT-A12h (hz for heterozygous). PyMT-A12null cells stably expressing human ADAM12-L lacking the cytoplasmic tail (hADAM12Δcyt), hADAM12Δcyt with a catalytic site mutation (hADAM12Δcyt-E351Q), or vector control were generated by retroviral transduction as described (12). The ADAM12-E351Q mutant was previously shown to be proteolytically inactive (29–31). The cytoplasmic tail of ADAM12 retains ectopically expressed ADAM12 in the trans-Golgi network; hence, to ensure accurate ADAM12 expression at the plasma membrane, hADAM12Δcyt was used (32). The established cell lines were designated PyMT-A12, PyMT-A12EQ, and PyMT-VC, respectively, and kept in DMEM containing 4 μg/mL puromycin and 50 μg/mL hygromycin B.

Staining and analysis of mouse tissue and cells

Mammary gland tumors were obtained from PyMT-ADAM12−/− mice and PyMT-ADAM12+/− littermates and processed for histologic analysis as described (12, 33, 34). Briefly, the histologic evaluation was based on a 4-stage classification scheme that includes hyperplasia, adenoma, and early and late carcinoma (34). These stages are in agreement with the recommendations for classifications of mouse mammary tumor pathology (33) but improved for the PyMT model (34). Areas of early and late carcinoma, as well as hyperplasia and adenoma areas, were marked and the areas estimated by 2 independent observers as described (12, 34). To evaluate cell proliferation, DNA synthesis was determined using BrdU in vivo labeling and subsequent detection with an anti-BrdU antibody (Roche). Immunohistochemical staining of mouse tissue was done with the
rb109 antibody against ADAM12 or affinity purified ADAM12 polyclonal antibody from ProteinTech Group as described (28).

Cell proliferation in cultured cells was determined using the Clik-it EdU Imaging Kit (Invitrogen) that allows incorporation of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) into DNA during active DNA synthesis. Briefly, PyMT-A12hz, PyMT-A12null, PyMT-A12, PyMT-A12EQ, and PyMT-VC cells were seeded on collagen-coated dishes and allowed to recover for 16 hours. The cells were treated with 10 μmol/L EdU and incubated for 30 minutes at 37°C to enable EdU incorporation. Subsequently, the cells were fixed, permeabilized, and stained with Alexa Flour azide and cell nuclei were detected with 4',6-diamidino-2-phenylindole (DAPI). The fraction of cells with incorporated EdU was detected by fluorescence microscopy and analysed by MetaMorph software.

Cells plated on collagen-coated 35-mm dishes were fixed (4% paraformaldehyde), permeabilized (0.1% Triton X-100), incubated with anti-mouse pan-cytokeratin antibody (Dako; 1:200), or normal mouse IgG (100), incubated with anti-mouse pan-cytokeratin antibody (Dako) and fluorescence imaging was done using an inverted Zeiss Axiovert 220 Apotome system equipped with a 63x/1.4 Plan-Apochromat water immersion objective. The images were processed using the Axiovision program (Carl Zeiss).

Reverse transcription PCR and quantitative PCR
Total RNA was extracted and isolated from mouse tissue and mouse cell lines using Trizol (Invitrogen). RNA (1 μg) was treated with DNase I and reverse transcribed using the High-Capacity cDNA RT-kit from Applied Biosystems. The Reverse transcription PCR (RT-PCR) was conducted using the Axiovision program (Carl Zeiss).

Table 1. ADAM12 immunostaining of 6 different breast cancer tissue arrays

<table>
<thead>
<tr>
<th>Arrays</th>
<th>Cases</th>
<th>Positive (%)</th>
<th>Diagnosis</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>YTMA 12</td>
<td>338</td>
<td>227 (67.2)</td>
<td>322 IDC, 12 ILC, 4 others</td>
<td>Yale University, CT</td>
</tr>
<tr>
<td>YTMA 10A</td>
<td>325</td>
<td>167 (51.4)</td>
<td>271 IDC, 45 ILC, 9 others</td>
<td>Yale University, CT</td>
</tr>
<tr>
<td>Zymed 57-7043</td>
<td>51</td>
<td>42 (82.4)</td>
<td>51 IDC</td>
<td>Zymed, CA</td>
</tr>
<tr>
<td>Zymed ID10011</td>
<td>47</td>
<td>34 (72.3)</td>
<td>44 IDC, 3 ILC</td>
<td>Zymed, CA</td>
</tr>
<tr>
<td>PAK1</td>
<td>22</td>
<td>17 (77.3)</td>
<td>18 IDC, 4 ILC</td>
<td>Turku University, Finland</td>
</tr>
<tr>
<td>PAK2</td>
<td>22</td>
<td>18 (81.8)</td>
<td>20 IDC, 2 ILC</td>
<td>Turku University, Finland</td>
</tr>
<tr>
<td>Total</td>
<td>805</td>
<td>505 (62.7)</td>
<td>726 IDC, 66 ILC, 13 others</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: IDC, infiltrating ductal carcinomas; ILC, infiltrating lobular carcinomas.
resections of 61 patients who underwent conservation surgery for a breast lump at Turku University Hospital (Turku, Finland) were analyzed. To prepare macrosections, fresh surgical breast tissue was sliced at 5-mm intervals through the specimen. Each tissue slice was visualized with mammography to ensure excision of the whole tumor area. All tissue slices were then separately and in sequence fixed in formalin, embedded in paraffin in 5 × 5-cm macroblocks, cut into 5-μm sections, and hematoxylin and eosin (H&E) stained. From these macroblocks, 1 to 3 representative areas were excised, reembedded in paraffin, and 5-μm sections cut for staining. From the same hospital, normal breast tissue was obtained from 10 patients who underwent aesthetic breast-reduction surgery. The experimental use of the surgical specimens was in accordance with the hospital ethical guidelines of Turku University Hospital.

ADAM12 immunohistochemistry of human breast tissue

ADAM12 immunostaining was done as described earlier (11, 12), using either polyclonal antibodies to human ADAM12 (rb109, rb116, or rb122; 1:250 dilution) or normal rabbit IgG fraction (10 μg/mL) prepared in PBS. Detection was done using the DakoChemMate Detection Kit (Dako). Examination of the 805 cases was done as an overall evaluation of positive or negative ADAM12 immunostaining. Examination of the 61 macroblocks was done in more detail. The intensity and distribution of ADAM12 staining in benign and malignant breast epithelial cells were evaluated independently by 2 pathologists. Staining of the surrounding stroma for ADAM12 was evaluated in all cases, and evaluation of breast terminal duct lobular units (TDLU) further than 5 mm away from the carcinoma cells was done.

Statistical analysis

Statistical analysis was done using Kaplan–Meier to determine tumor-free periods, and the log-rank test, the Wilcoxon signed rank test, and the Student’s t test for comparing 2 independent groups. P < 0.05 was considered statistically significant.

Results and Discussion

ADAM12 deficiency delays PyMT-induced mammary tumorigenesis

To elucidate the role of ADAM12 in breast cancer, we analyzed the effect of ADAM12 deficiency on mammary tumor growth in the PyMT mouse model of breast cancer. ADAM12-deficient mice were cross-bred with MMTV-PyMT mice and analyzed for tumor onset and progression. PyMT-ADAM12+/− mice developed palpable breast tumors at a significantly slower rate than did PyMT-ADAM12+/+ mice (Fig. 1A). In the PyMT-ADAM12−/− cohort, tumors in female offspring appeared at day 73 ± 12 (mean ± SD), whereas PyMT-ADAM12+/+ mice developed tumors by day 61 ± 8. The tumor burden was significantly decreased in PyMT-ADAM12−/− mice at 12 weeks of age, as compared with age-matched PyMT-ADAM12+/+ mice (Fig. 1B). Histopathologic examination of H&E-stained sections of breast tissue was done to analyze tumor grade (degree of differentiation), as described (12, 34). We evaluated the percentage of early and late carcinoma, as well as hyperplasia and adenoma, areas within the mammary gland tumors (34) and found a significant reduction of areas of early and late carcinoma in the tumors from PyMT-ADAM12−/− mice as compared with PyMT-ADAM12+/+ mice (Fig. 1C and D). In addition to developing spontaneous breast tumors, PyMT mice have a high incidence of lung metastasis. Thus, the percentage of mice bearing at least one tumor nodule was determined by histologic examinations as exemplified in Figure 1E. The incidence of lung metastasis was 55% in PyMT-ADAM12hz mice (6/11) and 9% in PyMT-ADAM12null mice (1/11; Fig. 1F). Taken together, these data show that ADAM12 significantly influences tumor initiation and progression, confirming and extending previous reports (12, 15, 23).

ADAM12 affects the proliferative capacity of PyMT tumor cells

On the basis of previous studies proposing that ADAM12 may influence tumor cell proliferation (13, 20, 38, 39), we hypothesized that endogenous ADAM12 in the PyMT mouse model regulates cancer cell proliferation. Therefore, BrdU incorporation was assessed in PyMT-ADAM12−/− and PyMT-ADAM12+/+ tumors. A significant decrease in tumor cell proliferation was observed in the PyMT-ADAM12−/− tumors compared with PyMT-ADAM12+/+ tumors (Fig. 2A). To study the observed difference in tumor cell proliferation in more detail, tumor cells from both PyMT-ADAM12−/− and PyMT-ADAM12+/− mice were isolated, immortalized, and grown in vitro. Immunofluorescence staining using an antibody against cytokeratins confirmed that the immortalized PyMT-ADAM12null and PyMT-A12hz cell lines were of epithelial origin (Supplementary Fig. S1A). RT-PCR and Western blotting confirmed the endogenous expression of ADAM12 in PyMT-A12hz tumor cells and a lack of ADAM12 expression in the PyMT-A12null tumor cells (Fig. 2B and C). Next, the proliferative capacity of the two tumor cell lines was compared by in vitro and in vivo assays. The PyMT-A12null tumor cells showed a significantly reduced proliferative capacity in vitro compared with PyMT-A12hz tumor cells (Fig. 2D). Accordingly, when the PyMT-A12null and PyMT-A12hz cells were injected into the mammary gland of wild-type or ADAM12+/− mice, the PyMT-A12null cells grew significantly slower than the PyMT-A12hz tumor cells (Fig. 2E and F). Although there is a significant difference between proliferation of PyMT-A12hz and PyMT-A12null tumor cells in vitro, we reason that the more pronounced difference in in vivo tumor cell growth relates to the tumor–stroma interactions. Similarly, PyMT-A12hz cells injected subcutaneously gave rise to significantly larger tumors compared with PyMT-A12null cells (Supplementary Fig. S1B). Intriguing, we
observed that orthotopic injection of the tumor cells gave rise to larger tumors when compared with subcutaneously injected tumor cell (Fig. 2E and F and Supplementary Fig. S1B). A potential explanation could be that the microenvironment and, thereby, growth conditions in the mammary fat pad of mice are different and more advantageous for breast tumor cells than when these cells are injected subcu-
taneously in the dorsal flank as previously suggested (40).

To begin to unravel the molecular mechanism behind the decreased tumor cell proliferation in the absence of ADAM12, we tested the hypothesis that ADAM12 stimulates Akt signaling, a known inducer of cell proliferation. Interestingly, levels of phosphorylated Akt were decreased by approximately 50% in PyMT-A12null compared with PyMT-A12hz tumor cells (Fig. 2G), suggesting that ADAM12 expression in PyMT tumor cells stimulates growth via the Akt signaling pathway.

The proteolytic activity of ADAM12 is dispensable for ADAM12-accelerated PyMT tumor progression

The proteolytic activity of ADAM12 has been shown to involve ectodomain shedding and the consequent release of...
EGFR ligands (20, 24, 29, 31, 39, 41, 42). Ligand binding of EGFR contributes to the activation of numerous intracellular signaling pathways, such as proliferation and differentiation, which are important for tumor progression (43). Instantly, the key question remaining to be answered is whether the proteolytic activity of ADAM12 is required for its tumor-promoting effect in the PyMT model. Hence, we expressed human ADAM12, a proteolytically inactive mutant form of human ADAM12 (ADAM12EQ; refs. 29–31) or the empty vector (as a control) into PyMT-A12null tumor cells. The resulting 3 new cell lines—PyMT-A12, PyMT-A12EQ, and PyMT-VC—were studied. Western blotting showed that ADAM12 was expressed in both the PyMT-A12 and PyMT-A12EQ cells but not in the PyMT-VC cells (Fig. 3A). The proliferative capacity of the 3 tumor cell lines was compared by in vitro and in vivo assays. Interestingly, both PyMT-A12 and PyMT-A12EQ tumor cells showed a significantly increased proliferative capacity in vitro compared with PyMT-VC tumor cells (Fig. 3B). However, the proliferative index between PyMT-A12 and PyMT-A12EQ tumor cells was not significantly different. The 3 cell lines were subsequently injected into wild-type mice.
Figure 3. The proteolytic capacity of ADAM12 is dispensable for tumor growth. PyMT-A12null cells stably expressing vector control (PyMT-VC), human ADAM12 lacking the cytoplasmic tail (PyMT-A12), or human ADAM12 lacking the cytoplasmic tail and with a catalytic site mutation (PyMT-A12EQ) were examined. A, Western blot analysis of ADAM12 expression in the 3 cell lines. B, mean (±SD) cell proliferation assessed by EdU incorporation in immortalized PyMT-VC (n = 2,936), PyMT-A12 (n = 948) and PyMT-A12EQ (n = 1,811) tumor cells. C, mean (±SD) tumor volumes over time in ADAM12+/− mice on FVB/N genetic background injected with PyMT-A12 (n = 8), PyMT-A12EQ (n = 5), or PyMT-VC (n = 7) cells into the mammary-gland (m.gland). D, mean (±SD) tumor volumes over time in ADAM12+/− mice on FVB/N genetic background injected subcutaneously (s.c) with PyMT-A12 (n = 5), PyMT-A12EQ (n = 4), or PyM-VC (n = 5) cells. E, left, EGFR was immunoprecipitated (IP) from PyMT-VC, PyMT-A12, PyMT-A12EQ total tumor cell lysate and were blotted with phosphotyrosine (pTyr). Right, Western blot analysis of pTyr, total EGFR, and actin from input of PyMT-VC, PyMT-A12, and PyMT-A12EQ tumor cell lysate used for IP (representative from 3 experiment). F, Western blot analysis of pAkt and total Akt in PyMT-VC, PyMT-A12, and PyMT-A12EQ tumor cells. The data are representative of 3 independent experiments. n.s. = nonsignificant; WB, Western blot. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C and D; based on final volumes).

the mammary gland (Fig. 3C) or subcutaneously in the right flank (Fig. 3D) of ADAM12+/− mice. Similarly to the in vitro proliferation data, both PyMT-A12 and PyMT-A12EQ cells produced tumors of significantly increased final tumor volume as compared with the PyMT-VC cells. The growth pattern of tumors produced by PyMT-A12EQ and PyMT-A12 cells were not significantly different. In support of these data, overexpression of
ADAM12 in PyMT cells did not induce phosphorylation of EGFR (Fig. 3E), suggesting that ADAM12 is not involved in ectodomain shedding and the consequent release of EGFR ligands, such as EGF and HB-EGF, in these cell lines. However, we found that both PyMT-A12 and PyMT-A12EQ tumor cells exhibited higher levels of phosphorylated Akt compared with PyMT-VC tumor cells (Fig. 3F), indicating that ADAM12, independent of its proteolytic activity, may stimulate Akt signaling to support tumor cell proliferation. Taken together, these results suggest that the effect of ADAM12 on tumor progression in the PyMT model system is not dependent on its proteolytic activity. Although these data contrast with the finding that ADAM12 positively correlates with the proliferative index in human glioblastomas and the ability to shed proheparin binding EGF (20), they substantiate the reports that ADAM12 mRNA is endogenously expressed in carcinoma cells but that it is not detected in normal mouse mammary gland expressing atypical ductal hyperplasia (ADH) and/or ductal carcinoma in situ (DCIS) (11, 12, 14, 17, 18). To verify this conclusion, we analyzed the ADAM12 distribution pattern by immunohistochemistry (data not shown). To further support these observations, we analyzed ADAM12 expression in breast carcinomas from 805 cases, arranged in 6 separate tissue arrays. A total of almost 63% of the cases exhibited positive ADAM12 immunoreactivity (Table 2), whereas stromal cells were generally not positive for ADAM12 immunostaining (data not shown). To further test the putative role of ADAM12 produced by murine stromal cells, B16F10 melanoma cells were injected subcutaneously into wild-type, ADAM12+/−, and ADAM12−/− mice. No difference was observed in the growth of tumors injected into the 3 different types of recipient mice (Supplementary Fig. S2), indicating that ADAM12 expressed by the stromal compartment has no impact on the growth of the B16F10 melanoma cells. Collectively, these data reveal that stromal expression of ADAM12, as shown by 3 different cell lines (PyMT-A12hz, PyMT-A12null, and B16F10) in two different stromal compartments (subcutaneous space and the mammary gland), does not influence tumor growth. Although the stromal compartment is the major source of several matrix metalloproteinases with important roles in tumor progression and invasion (4, 58), our data strongly indicate that only tumor cell expression of ADAM12 contributes to tumor progression.

**ADAM12 is produced by tumor cells in human breast cancer**

The relevance of our findings to human breast carcinoma is significant. Previous studies of ADAM12 expression in breast carcinomas have reported that it is highly expressed in carcinoma cells but that it is not detected in the stroma (11, 12, 14, 17, 18). To verify this conclusion, we analyzed the ADAM12 distribution pattern by immunohistochemistry in human breast carcinoma specimens from 805 cases, arranged in 6 separate tissue arrays. A total of almost 63% of the cases exhibited positive ADAM12 immunoreactivity of the tumor cells (Table 1), whereas stromal cells were generally not positive for ADAM12 immunostaining (data not shown). To further support these observations, we analyzed ADAM12 immunoreactivity in malignant breast lesions in sections obtained from 61 cases in which macroblocks had been prepared (Supplementary Fig. S3). Detailed semiquantitative immunohistochemical analyses (Table 2) showed that ADAM12 immunoreactivity was found in 47% of infiltrating ductal carcinomas (IDC) and 97% of infiltrating lobular carcinomas (ILC) samples studied. Furthermore, areas containing atypical ductal hyperplasia (ADH) and/or ductal carcinoma in situ (DCIS) in addition to tumor cells were selected (see insert in Supplementary Fig. S3A). We observed ADAM12 immunoreactivity in 77% of the epithelial cells in ADH and 84% of epithelial cells in DCIS. Of all the tissue specimens that exhibited...
positive ADAM12 immunoreactivity, only the breast epithelial cells were found to be positive for ADAM12 (Fig. 5A and B). In few cases, a few stromal cells exhibited positive staining for ADAM12 (data not shown). These data confirm previous observations that, in human breast cancer, ADAM12 expression is almost exclusively located in tumor cells, and only rarely seen in the tumor-associated stroma (11–13).

### Table 2. ADAM12 immunohistochemical evaluation on whole-mount breast material from 61 cases

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Positivity, %</th>
<th>Intensity (0-3; ± SE)</th>
<th>Membr, %</th>
<th>Cytopl (%)</th>
<th>Membr + Cytopl, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>9</td>
<td>77</td>
<td>2.6 (±0.2)</td>
<td>0</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>DCIS</td>
<td>7</td>
<td>84</td>
<td>2.5 (±0.2)</td>
<td>0</td>
<td>57</td>
<td>43</td>
</tr>
<tr>
<td>IDC</td>
<td>33</td>
<td>47</td>
<td>1.8 (±0.1)</td>
<td>42</td>
<td>19</td>
<td>39</td>
</tr>
<tr>
<td>ILC</td>
<td>9</td>
<td>97</td>
<td>3.0 (±0.0)</td>
<td>0</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>96</td>
<td>2.3 (±0.3)</td>
<td>33</td>
<td>33</td>
<td>34</td>
</tr>
</tbody>
</table>

**NOTE:** Evaluation consisted of distribution, staining intensity (0, no staining; 1, weakly; 2, moderate; 3, strongly stained) membrane, and cytoplasmic localization.

**Abbreviations:** ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ.
Tumor-associated stroma induces ADAM12 expression in the epithelium

The expression of ADAM12 is low in most normal tissues but relatively high in many human cancers. Given our observation that the tumor cells in breast cancers are responsible for the increased ADAM12 expression, we investigated potential factors that could trigger induction of ADAM12 in these tumor cells. Specifically, we hypothesized that tumor-associated stroma influences ADAM12 expression in tumor cells because it is known that interactions between cancer cells and stromal components influence tumor growth and progression (4). The tumor-associated stromal cells encompass a number of cells with a broadly similar phenotype, known as reactive fibroblasts, myofibroblasts, or carcinoma-associated fibroblasts (2, 3). These cells have received increased attention because of their contribution to tumor development and progression through the release of a number of cytokines. Among these cytokines, TGF-β1 (59) is frequently referred to as having strong effects on both tumor growth and invasion by increased proliferation, dedifferentiation, and enhanced EMT (4, 59, 60). Interestingly, TGF-β1 is a known transcriptional regulator of ADAM12 (16) and has been shown to upregulate ADAM12 transcription in several cancer cell lines, including the breast cancer cell line MDA-MB-231 (61). Hence, we analyzed whether tumor–stromal interactions or the tumor-associated stroma could regulate the expression of ADAM12 in cells normally not expressing the protein, potentially through TGF-β1 action. For this purpose, we took advantage of Lewis lung cells, which express no or low levels of ADAM12 in vitro (Fig. 6A), and which are syngeneic with the ADAM12-deficient mouse strain. Initially, we showed that stimulation of Lewis lung cells with TGF-β1 in vitro increased the expression of ADAM12, as assessed by RT-PCR, qPCR, and Western blot (Fig. 6B–D). Subsequently, Lewis lung cells were injected subcutaneously into wild-type, ADAM12+/−/C0, or ADAM12−/−/C0 mice and, as expected, no difference in tumor growth was seen (Fig. 6E). However, the ADAM12 transcript was more than 200-fold upregulated in Lewis lung tumors formed in ADAM12−/−/C0 mice compared with Lewis lung cells grown in vitro (Fig. 6F), indicating that the stroma compartment or tumor–stroma interaction initiate tumor cell expression of ADAM12. In comparison, the level of ADAM12 mRNA in the Lewis lung tumor tissue in wild-type and ADAM12+/−/C0 mice was much higher than in tumors from ADAM12−/−/C0 mice (Fig. 6G), suggesting that ADAM12 is expressed in the stroma compartment as also shown in Figure 4A. Immunohistochemistry staining of ADAM12 in Lewis lung cells grown as subcutaneous tumors confirm the presence of ADAM12 protein in the tumor cells (Supplementary Fig. S4). These data support the hypothesis that tumor-associated stroma induces ADAM12 expression in tumor cells, and they suggest the possibility that TGF-β1 may be one stromal factor that contributes to ADAM12 expression (46).

To analyze whether changes in tumor-associated stroma have the ability to influence expression of ADAM12 in...
human breast tissue, we analyzed the macroblocks from human breast cancer patients described above. These macroblocks enabled us to carry out immunohistochemical evaluation of ADAM12 expression in extended areas of the surrounding tissue from 45 cases of invasive carcinomas. In particular, we were able to examine the TDLUs adjacent to the carcinomas in greater detail (Supplementary Fig. S3). ADAM12 immunostaining was clearly increased both in distribution and intensity in the TDLUs adjacent to the invasive tumor, compared with the normal TDLUs from

Table 3. ADAM12 immunohistochemical evaluation of terminal ductal lobular units in whole-mount material in 10 nontumorous cases and in 45 cases of invasive carcinomas

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Positivity (cells; %)</th>
<th>Intensity (0-3; ± SE)</th>
<th>Membr, %</th>
<th>Cytopl, %</th>
<th>Membr + Cytopl, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontumorous TDLU</td>
<td>28</td>
<td>1.6 (±0.22)</td>
<td>60</td>
<td>0</td>
<td>40</td>
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<tr>
<td>Invasive carcinomas TDLU</td>
<td>89</td>
<td>2.9 (±0.06)</td>
<td>0</td>
<td>81</td>
<td>19</td>
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</tbody>
</table>

NOTE: Evaluation consisted of distribution, staining intensity (0, no staining; 1, weakly; 2, moderately; 3, strongly stained) membrane, and cytoplasmic localization. Abbreviation: TDLU, terminal ductal lobular units.
breast reductions (Fig. 5C and D). Whereas 89% of epithelial cells in the TDLUs adjacent to invasive carcinomas were positive for ADAM12, only 28% in the normal TDLUs in breast-reduction tissue showed positive ADAM12 immunostaining (Table 3). This result could imply that humoral factors (i.e., TGF-β1) from the malignant tumor or the tumor-associated stroma might influence expression of ADAM12 in the breast epithelium in the TDLUs adjacent to the malignant tumor. Indeed, a recent study showed that TGF-β1 is upregulated at the interface between the periphery of breast tumor tissue and normal adjacent tissue compared with normal tissue (62).

Together, the data presented here strongly emphasize the importance of endogenous ADAM12 for efficient tumor development. Intriguingly, the protumorigenic effect of ADAM12 seems to involve nonproteolytic mechanisms. Moreover, our data imply that the stroma may trigger ADAM12 expression in tumor cells and TDLUs adjacent to tumor cells, even though ADAM12 expression in the stroma is not important for tumor growth in the mouse models studied. On the basis of these findings, we propose that ADAM12 expression in tumor cells contributes to their proliferation and dedifferentiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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