Altered Microenvironment Promotes Progression of Preinvasive Breast Cancer: Myoepithelial Expression of αvβ6 Integrin in DCIS Identifies High-risk Patients and Predicts Recurrence

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Introduction

The majority of invasive breast cancers arise from the precursor lesion ductal carcinoma in situ (DCIS; ref. 1), whereby neoplastic cells proliferate and fill the breast ductal tree but are separated from the surrounding stroma by the intact myoepithelial–basement membrane wall of the ducts (Fig. 1A). At some point during progression, the tumor cells penetrate the myoepithelial–basement membrane interface and move into the interstitial matrix becoming an established invasive carcinoma with metastatic potential (Fig. 1A). However, DCIS does not invariably progress to invasive breast cancer: an early study of untreated low-grade DCIS indicated that 39% (11 of 28 patients) progressed to invasive breast cancer after a median follow-up of 31 years (2). High-grade DCIS is considered to progress more rapidly to invasive disease, but overall it is estimated that between one-third and half of DCIS diagnosed will progress to invasive breast cancer during a woman’s lifetime (3). The frequency of DCIS has increased dramatically with the widespread use of mammographic screening, accounting now for 1 in 4 breast cancer diagnoses (3), contributing to recent concerns surrounding overdiagnosis and overtreatment within the breast screening setting (4, 5). Indeed, the need to understand more about the biologic and molecular mechanisms underlying progression of DCIS to invasive disease and to generate robust risk stratification methods for...
Materials and Methods

Human tissue specimens

Clinical samples were obtained from surgical specimens from patients undergoing breast surgery between 2004 and 2009 at Barts Health NHS Trust London. Further details are provided in the Supplementary Methods.

The study was conducted following patient consent and approval from the local research ethics committee (ref: 05/Q0403/199 and 09/H075/39). Further analysis was conducted on a series of cases derived from the UK-DCIS study (22, 23) to determine relationship between αvβ6 and progression/recurrence. The UK/ANZ DCIS trial was a randomized 2 × 2 factorial trial recruiting women between May 1990 and August 1998. Inclusion criteria were diagnosis of DCIS, undergoing breast conserving surgery, and with confirmed clear margins. Patients were randomized to receive radiotherapy, tamoxifen, or both (22). Clinicopathologic details are provided in Supplementary Methods.

Immunohistochemistry

Sections were dewaxed in xylene and antigen retrieved in pepsin solution followed by incubation with 0.5 mg/mL αvβ6 mouse monoclonal antibody (6.2G Stromedix; refs. 24, 25), MMP9 (My Biosource, MBS 240189), p63 (Dako, M7247), SMA (Dako, M0851), or CK5/6 (Dako, M7237) overnight. Sections subsequently were incubated with rabbit anti-mouse biotinylated F(ab′)2 for 30 minutes, developed using ABC reagent and superDAB (Dako), and then counterstained with hematoxylin. Details of scoring method are given in Supplementary Methods.

Cell lines and tissue culture conditions

T47D, MCF-7, MDA-MB-231, and HFF2 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) with 10% fetal calf serum (FCS) and 2 mmol/L glutamine, AM12 cells transfected with pBABE puro β6 were maintained in DMEM with 10% FCS and 2 mmol/L glutamine with puromycin (1 μg/mL, Sigma). Mink lung epithelial cells (MLEC; a kind gift from D. Rifkin, New York University) were maintained in DMEM 10% FCS and 2 mmol/L glutamine with puromycin (1 μg/mL). Primary myoepithelial cells were isolated as described previously (26). An immortalized myoepithelial cell line (1089) (provided by M. O’Hare and P. Jat, Institute of Neurology, UCL) was generated from normal myoepithelial cells isolated from reduction mammoplasty material (27). Cells were sorted using integrin β4–labeled sheep anti-mouse magnetic beads (Invitrogen), to purify the myoepithelial cell population (1089) and maintained in Ham’s/F12 media with 10% FCS, 2 mmol/L glutamine, hydrocortisone (1 μg/mL), EGF (10 ng/mL), and insulin (5 μg/mL). All cell lines were verified by STR profiling (LGc Standards, tracking number 710081047). The 1089 cell line showed no similarity to any other cell line in the ATCC database (LGc Standards, tracking number U10010392). Frozen stocks were banked, and cell lines were grown for no longer than 6 months then

Translated Relevance

Ductal carcinoma in situ (DCIS) is the main precursor of invasive breast cancer and now accounts for 25% to 30% of all breast cancers detected; however, only half will progress to invasive disease during a woman’s lifetime. Currently, there are no robust markers to distinguish between those that will progress and those that will not. In this study, we show that the tumor-suppressive myoepithelial cells are altered in a subset of DCIS with upregulation of the integrin αvβ6, which is almost universal in DCIS that is associated with an invasive component, and the presence of αvβ6 is significantly associated with recurrence and progression. In vitro studies show that αvβ6–positive myoepithelial cells promote tumor invasion through a TGFβ– and MMP9–dependent mechanism. This suggests that detection of αvβ6 on myoepithelial cells may be of prognostic importance and provide new therapeutic avenues.

Patients with DCIS was highlighted in a recent NIH State-of-the-Science conference on DCIS (3).

A number of studies have aimed to identify markers or signatures that may predict progression of DCIS, most of which have focused on comparing DCIS tumor cells with its invasive counterpart. These studies generally show no specific changes associated with transition to invasion (6–8) and suggest that DCIS is genetically as advanced as invasive breast cancer (9). Other studies have shown that DCIS and invasive breast cancer largely cluster according to intrinsic molecular subtypes (10–12), although one gene array–based study identified a subset of DCIS cases with an expression profile more similar to invasive cancer and found that the genes characterizing this subset were related to reorganization of the microenvironment (13). The microenvironment of DCIS is complex, comprising the myoepithelial population as well as the stromal compartment. Myoepithelial cells have been shown to be potent tumor suppressors (14) exerting their effects through autocrine and paracrine pathways (15–18). However, several recent studies (19, 20) have suggested that DCIS-associated myoepithelial cells may lose their tumor suppressor ability and actually promote breast cancer progression (21).

Here, we show for the first time upregulation of integrin αvβ6 in DCIS-associated myoepithelial cells. This change results in a switch in the myoepithelial cells from tumor suppressive to tumor promoting in a TGFβ– and MMP9–dependant manner. Analysis of 532 DCIS cases shows that αvβ6 is more frequently expressed in DCIS associated with invasion and in a separate cohort of DCIS with long-term follow-up αvβ6 expression significantly predicts both progression and recurrence. We suggest that upregulation of αvβ6 may be a key marker of transition of DCIS to invasive disease, is predictive of prognosis, and that TGFβ and MMP9 could be targeted in early breast cancer for therapeutic benefit.
discarded and fresh cells were then recovered from liquid nitrogen.

**Transient transfection of primary myoepithelial cells**

Primary myoepithelial cells were recovered from liquid nitrogen and plated onto a 6-well plate precoated with collagen (10 mg/mL) at a density of 2 x 10^4 per well. Cells were transfected 72 hours later using the standard JetPrime protocol (PolyPlus) and 2 μg of integrin β6 pcDNA1 neo (Addgene plasmid 13580; ref. 28) or pcDNA1 empty vector. After 24 hours, media were changed for serum-free Ham’s/F12 and conditioned for 48 hours.

**Stable expression of β6 in 1089 myoepithelial cells**

Conditioned medium containing retrovirus from AM12/pBABE-β6 or AM12/pBABE-Puro cells, was incubated with 10^8 1089 cells (29). β6-positive 1089 and puromycin control cells were selected using puromycin (1 μg/mL, Sigma). Control, puromycin-resistant 1089 cells were designated normal (N-) 1089; β6-transduced cells were further enriched using integrin αvβ6-labeled anti-mouse IgG magnetic beads (Invitrogen) and designated β6-1089.

**Immunofluorescence**

A total of 2 x 10^5 cells were plated onto glass coverslips and grown for 48 hours before fixation with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS before staining. All antibodies were diluted in 20% normal rabbit serum in PBS, except for the αvβ6 antibody (MAB2077Z, Clone 10D5; Millipore), which was diluted in 0.1% bovine serum albumin (BSA) in DMEM. Slides then were stained with a secondary rabbit anti-mouse.
488 (Invitrogen, A11059) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were viewed on a Zeiss LSM 510 Meta microscope.

**Flow cytometry**

A total of 3 \times 10^5 cells were incubated with primary antibody diluted in 0.1% BSA in PBS, except for the anti-\(\beta6\) antibody (10D5), which was diluted in 0.1% BSA in DMEM. Cells subsequently were stained with secondary rabbit anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Dako, F0313) before reading on a Flow Cytometer (BD LSR II).

**Western blotting**

Cells were trypsinized, centrifuged at 1,200 rpm, re-suspended in radioimmunoprecipitation (RIPA) lysis buffer (Millipore) with "Complete" protease inhibitors (Roche). Protein concentrations were determined using the DC Protein Assay (Bio-Rad), and 50 \mu g of protein was loaded on to an 8% Tris/Glycine gel, electrophoresed and transferred on to nitrocellulose membranes that were blotted with \(\beta6\) antibody (1:1,000, SC-6632, Santa Cruz), \(\beta\)-actin (1:10,000, Santa Cruz, SC47775), or HSC70 (1:10,000, SC-53935, Santa Cruz,) and donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary (1:2,000, Dako) or goat anti-mouse HRP-conjugated secondary (1:2,000, Dako) diluted in 5% milk in TBS/T.

**TGF\(\beta\) quantitation assay**

MLECs transfected with a PAI-1 promoter fused to a luciferase reporter gene were used to determine the amount of active TGF\(\beta\) (30). Briefly, 4 \times 10^6 MLEC's were seeded in a 96-well plate and incubated overnight then serum starved for 4 hours before 4 \times 10^4 test cells were plated on top of the MLEC's and cocultured for 16 hours. Medium was removed, lysis buffer (Promega) added, and the plate incubated at –80°C for 1 hour before thawing at room temperature. Cells were scraped to ensure lysis and the luciferase substrate added according to manufacturer’s instructions (Promega). Luminescence was recorded on a Victor microplate reader.

**Adhesion to latency-associated peptide**

Latency-associated peptide (LAP, Sigma) was diluted to 0.5 \mu g/mL, aliquots were added to the wells of a 96-well plate and incubated for 1 hour at room temperature. A total of 2 \times 10^4 cells were incubated with IgG isotype control antibody or \(\beta6\)-blocking antibody (10D5) at 10 \mu g/mL in serum-free media (SFM) for 20 minutes at 4°C before plating onto LAP. Cells were incubated at 37°C for 45 minutes before fixing with 30% methanol and staining with 0.1% crystal violet. Stained cells were dissolved with 30% acetic acid and absorbance read at 550 nm.

**Migration assays**

Twenty-four-well Transwells (8-\mu m pore, BD Bioscience) were coated on the underside with 0.5 \mu g/mL LAP, for 1 hour at room temperature, and then placed in a 24-well plate with DMEM, lacking serum, in the lower chamber. A total of 3 \times 10^5 cells were incubated with either an IgG isotype antibody or an \(\beta6\)-blocking antibody (10D5) diluted in SFM (20 minutes at 4°C). Cells then were placed in the upper chamber and incubated for 24 hours. Migrating cells were quantified by applying trypsin to the underside of the Transwell and counting the detached cells on a CASY counter.

**Invasion assays**

Conditioned medium was generated from N-1089 or \(\beta6\)-1089 by culturing in serum-free Ham’s/F12 for 48 hours and was stored at –80°C. The surface of 24-well Transwell was coated with 70 \mu l growth factor–reduced Matrigel (diluted 1:3 in Ham’s/F12 without serum) and incubated for 1 hour at room temperature. Five hundred microliters conditioned medium from either N-1089 or \(\beta6\)-1089 was then placed in the lower well. For inhibitor experiments, either dimethyl sulfoxide (DMSO) or MMP9 inhibitor (Millipore, 50 nmol/L, 444278) was added to conditioned media. Two hundred microliters of 3 \times 10^4 tumor cells was added to each well and incubated for 24 to 48 hours. Invading cells were quantified by applying trypsin to the bottom of the Transwell and counting the detached cells on a CASY counter.

**Organotypic invasion assays**

The skin organotypic method developed by Fusenig and colleagues (31), was adapted by Nystrom and colleagues (32), and further modified. Matrigel/collagen gels were prepared as follows: 3.5 volumes of collagen type 1 (BD Bioscience) and 3.5 volumes of Matrigel were mixed on ice and was stored at -20°C. Cells then were placed into wells of a 24-well plate and allowed to polymerize for 30 minutes at 37°C. One milliliter of DMEM + 10% FCS and glutamine was added to each well and incubated overnight.

The next day 2.5 \times 10^7/500 \mu l (DMEM + 10% FCS) N-1089 or \(\beta6\)-1089 cells were plated on top of the gel and allowed to settle for 6 hours at 37°C; then 2.5 \times 10^7/500 \mu l MDA-MB-231 cells were plated on each gel. Gels were fixed in formal saline and placed into paraffin blocks, 5-\mu m sections were cut and analyzed by immunohistochemical staining. Invasion was determined by taking images of 3 random fields of view from triplicate gels and analyzing the number of invasive particles, the percentage area of the field the particles occupied, and the depth the particles penetrated the gel (determined by 3 measurements per field of view) using ImageJ (NIH). The product of the 3 values gave an overall "invasive index" (32).

**Mice**

Female 6-week-old CB17 SCID mice were purchased from Harlan and were maintained in IVC cages in a pathogen-free, temperature-controlled, 12-hour light and dark cycle environment. All animals were used in accordance
with United Kingdom Home Office regulations and ethical approval by the local ethics committee.

Tumor volumes based on caliper measurements were calculated by the modified ellipsoidal formula: tumor volume = \(1/2(length \times width^2)\) (33). Further information on the mouse experiments are in the Supplementary Methods.

Zymography

Cells (\(1 \times 10^5\)) were preincubated with IgG antibody, \(\beta_6\)-blocking antibody (10 \(\mu\)g/ml, 10D5), or TGF\(\beta\)-RII-blocking antibody (10 \(\mu\)g/ml, AF-241-NA, R&D Systems) in SFM before they were plated on coverslips coated with fibronectin (1 \(\mu\)g/ml) or LAP (0.5 \(\mu\)g/ml). Media were collected after 24 hours, and MMP9 activity was analyzed using SDS-PAGE substrate gels. Gelatin (Bloom 300, Sigma) was added to a 10% acrylamide separating gel at a final concentration of 1 mg/ml. The intensity of the bands was measured by densitometric analysis. Direct comparisons between separate gels were not made, as the intensity of background staining was variable. Experiments were repeated a minimum of 3 times in triplicate. Further information on the zymogram protocol is in the Supplementary Methods.

Real-time PCR

RNA was isolated from N-1089 and \(\beta_6\)-1089 cell pellets using a kit (Qiagen, 74104). Analysis was conducted on RNA from 3 separate experiments. TaqMan real-time PCR using a kit (Qiagen, 74104). Analysis was conducted on blocking antibody (10 \(b\) \(b\)g/ml) or LAP (0.5 \(b\) \(b\)g/ml). Media were collected after 24 hours, and MMP9 activity was analyzed using SDS-PAGE substrate gels. Gelatin (Bloom 300, Sigma) was added to a 10% acrylamide separating gel at a final concentration of 1 mg/ml. The intensity of the bands was measured by densitometric analysis. Direct comparisons between separate gels were not made, as the intensity of background staining was variable. Experiments were repeated a minimum of 3 times in triplicate. Further information on the zymogram protocol is in the Supplementary Methods.

Statistical analysis

We determined statistical significance of the differences between experimental groups by the 2-tailed Student \(t\) test with PRISM software (GraphPad Software). \(P < 0.05\) was considered significant. The relationship between staining patterns and DCIS grade, between MMP9 and \(\alpha\)\(\beta_6\), and between \(\alpha\)\(\beta_6\) and outcome was analyzed using Pearson \(\chi^2\) test using SPSS software (SPSS Inc.) with \(P < 0.05\) considered as significant. Cases from UK-DCIS were matched on treatment received, age and time of follow-up and analyzed by logistical regression, adjusted and unadjusted for tumor grade and size. Animal studies were assessed for statistical significance between 2 medians by Mann–Whitney \(U\) test.

Results

**Myoepithelial expression of \(\alpha\)\(\beta_6\) in DCIS is associated with progression to invasive cancer and predicts disease recurrence**

A total of 583 clinical breast samples comprising normal breast tissue, epithelial hyperplasia of usual type, “pure” DCIS and DCIS with invasion were analyzed for myoepithelial-associated expression of \(\alpha\)\(\beta_6\). This revealed no significant staining for \(\alpha\)\(\beta_6\) integrin in normal or hyperplastic cases (Fig. 1B), whereas 69% of high-grade and 52% of non–high-grade DCIS cases without invasion showed myoepithelial staining for \(\alpha\)\(\beta_6\) (Table 1), with a significantly higher frequency of positivity in high-grade cases (\(P < 0.001\)). The frequency of myoepithelial cell \(\alpha\)\(\beta_6\) positivity in DCIS with associated invasion is significantly higher than in pure DCIS (\(P < 0.001\)) with 96% of high-grade and 87% of non–high-grade DCIS exhibiting staining.

To assess the predictive value of \(\alpha\)\(\beta_6\), we examined expression in a unique series of DCIS with long-term follow-up, derived from the UK/ANZ DCIS study, the endpoint assessed was the appearance of any new breast cancer, either DCIS or invasive cancer, as the numbers are insufficient to assess progression to invasion only. Cases and controls were matched for treatment protocol. The median follow-up was 114 months (22, 23). Staining was evaluated in 52 case–control pairs (details of cases shown in Supplementary Table S1), with cases scored using an \(H\) score to take account of the extent and intensity of myoepithelial cell staining. Analysis showed a significant association between extent of \(\alpha\)\(\beta_6\) expression (evaluated as an \(H\) score; ref. 35) and disease recurrence or progression (\(P = 0.006\)). This association was maintained following adjustment for DCIS grade and size (\(P = 0.02\), \(H\) score; Table 2). All cases were confirmed as having clear margins as part of the inclusion criteria to the trial. We found that those cases positive for \(\alpha\)\(\beta_6\) recurred more quickly than those lacking the integrin, with the \(\alpha\)\(\beta_6\)-positive cases showing a median time to recurrence of 2.3 years and those negative for \(\alpha\)\(\beta_6\) having a median of 11.4 years to recurrence.

**Table 1. Expression of \(\alpha\)\(\beta_6\) in pure DCIS and DCIS associated with invasive disease**

<table>
<thead>
<tr>
<th>Myoepithelial (\alpha)(\beta_6) expression</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>0</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Pure DCIS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>104 (69%)</td>
<td>47 (31%)</td>
<td>151</td>
</tr>
<tr>
<td>Non–high grade</td>
<td>44 (52%)</td>
<td>40 (48%)</td>
<td>87</td>
</tr>
<tr>
<td>DCIS from invasive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>189 (96%)</td>
<td>8 (4%)</td>
<td>197</td>
</tr>
<tr>
<td>Non–high grade</td>
<td>84 (87%)</td>
<td>13 (13%)</td>
<td>97</td>
</tr>
</tbody>
</table>

NOTE: Formalin-fixed, paraffin-embedded sections of normal/benign tissue, pure DCIS, and DCIS with associated invasion were stained for \(\alpha\)\(\beta_6\) integrin, and the absence or presence of myoepithelial cell staining was recorded, the number and percentage of cases in each category is indicated. In 189 of 197 (96%) of high-grade DCIS with invasive cancer cases, the myoepithelial cell compartment stained positively for \(\alpha\)\(\beta_6\), whereas 104 of 157 (62%) of high-grade pure DCIS cases exhibit \(\alpha\)\(\beta_6\) expression. There is no detectable staining of \(\alpha\)\(\beta_6\) in normal or hyperplastic breast tissue.
LAP, which is part of the trimeric complex that forms shown by several myoepithelial cells were generated (see Materials and Methodial markers (Supplementary Fig. S1 and data not shown).

normal reduction mammaplasty material (Fig. 1E). Primary myoepithelial cells were isolated from Expression of markers were detected (Fig. 1D and data not shown).

1089 with regard to other integrin levels or myoepithelial markers (Fig. 1C, bottom). A considerable increase in the amount of β6 integrin by flow cytometry, consistent with successful transduction, was observed but no differences between N-1089 and β6-1089 with regard to other integrin levels or myoepithelial markers were detected (Fig. 1D and data not shown). Expression of αvβ6 was also confirmed by Western blotting (Fig. 1E). Primary myoepithelial cells were isolated from normal reduction mammaplasty material (n = 2) and purified as previously described (26). Cells were plated onto collagen-coated glass coverslips and shown by immunofluorescence to maintain expression of myoepithelial markers (Fig. 1C, bottom). αvβ6-overexpressing primary myoepithelial cells were generated (see Materials and Methods), and further characterization indicated significant increase in β6 integrin with no change in other myoepithelial markers (Supplementary Fig. S1 and data not shown).

Expressed αvβ6 in the β6-1089 cell line was functional as shown by several in vitro assays. Integrin αvβ6 binds to the LAP, which is part of the trimeric complex that forms secreted TGF-β (40). Therefore, β6-1089 and N-1089 cells were plated on to LAP, poly-ν-l-lysine (PDL), or BSA, to determine background adhesion. Figure 2A shows β6-1089 cells adhered preferentially to LAP compared with N-1089 cells (P = 0.003); this adhesion was reduced significantly after incubation of cells with an αvβ6-blocking antibody (10D5) before plating onto matrix (P = 0.008). Figure 2B indicates β6-1089 cells migrate significantly more to LAP than do N-1089 cells (P = 0.02) in modified Boyden chamber (Transwell) assays (41). Migration of β6-1089 cells to LAP was ablated significantly (P = 0.04) in the presence of blocking antibody whereas N-1089 cells were unaffected, showing that migration to LAP was mediated exclusively by αvβ6.

Binding of αvβ6 to LAP causes TGF-β activation (40); therefore, N-1089 or β6-1089 cells were cocultured with MLECs stably transfected with a TGF-β responsive plasminogen activator inhibitor 1 (PAI-1) promoter fused to a luciferase gene. Coculture with the β6-1089 cells caused a significant increase in luciferase activity (P = 0.02), compared with N-1089 cells, which was abolished with a monoclonal antibody to αvβ6 (Fig. 2C). No TGF-β activation was detected when MLEC cells were cultured with media conditioned on β6-1089 or N-1089 cells (data not shown), indicating that direct cell–cell contact is required. Adhesion, migration, and reporter assay results were replicated using RNA interference to αvβ6 (Supplementary Fig. S2a–S2d).

Myoepithelial cells expressing αvβ6 promote breast tumor cell invasion

Normal myoepithelial cells exert a tumor suppressor function (14, 15, 17, 19, 20, 42) but cancer-derived myoepithelial cells may lose this capacity (21). We examined whether myoepithelial cells expressing αvβ6 exhibited compromised tumor suppressor activity compared with normal (αvβ6-negative) myoepithelial cells. Breast cancer cell lines were placed in Transwells coated with Matrigel, and conditioned medium (CM) from primary myoepithelial cells

| Table 2. Relationship between staining for αvβ6 and disease recurrence and/or progression |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Mean (SD)       | Controls (n = 52) | Recurrence (n = 52) |
| H score                        | 128.8 (103.7)   | 77.4 (81.8)      | 0.006            |
| Matched logistic regression   |                 |                 |                 |
| OR (95%CI) unadjusted         | 1.01 (1.00–1.01)| 0.01            | 1.01 (1.00–1.01) | 0.02            |
| P                              |                 |                 |                 |

NOTE: DCIS samples from the UKCCCR DCIS trial were stained for αvβ6 and scored according to the extent and intensity of staining using the H score method. In total, 104 case–control pairs were available for study, of which 40 cases showed recurrent DCIS, 11 cases progressed to invasive disease, and 1 case developed further malignant breast disease of unknown status. Median follow-up was 114 months. Those cases that recurred showed a significantly higher αvβ6 H score than the control cases (P = 0.006).

Abbreviation: CI, confidence interval.

aAdjusted for grade and tumor size.

Myoepithelial cells expressing αvβ6 as a model of DCIS-associated cells

We obtained an immortalized cell line, derived from normal breast tissue (27), which was positively selected for expression of integrin α6β4 (a marker of myoepithelial cells; ref. 36). This “pure” α6β4-expressing cell population, (N-1089), was characterized by immunofluorescent staining for key myoepithelial cell markers (Fig. 1C, top) following introduction of the puromycin resistance gene. Integrin β4 (36), P-cadherin (P-Cad; ref. 37), cytokeratin 17 (CK17; ref. 38), and desmoglein 3 (Dsg3; ref. 39) expression is detected exclusively in myoepithelial cells in the normal breast and maintained in the N-1089 cells. Low levels of αvβ6 expression by N-1089 cells likely were induced because of culture of the cells on plastic (29). We then retrovirally transduced the N-1089 cell line with β6 integrin subunit cDNA, and the cell line was re-examined to confirm maintenance of myoepithelial-restricted markers in the correct subcellular localizations (Fig. 1C, middle). A considerable increase in the amount of β6 integrin by flow cytometry, consistent with successful transduction, was observed but no differences between N-1089 and β6-1089 with regard to other integrin levels or myoepithelial markers were detected (Fig. 1D and data not shown). Expression of αvβ6 was also confirmed by Western blotting (Fig. 1E). Primary myoepithelial cells were isolated from normal reduction mammaplasty material (n = 2) and purified as previously described (26). Cells were plated onto collagen-coated glass coverslips and shown by immunofluorescence to maintain expression of myoepithelial markers (Fig. 1C, bottom). αvβ6-overexpressing primary myoepithelial cells were generated (see Materials and Methods), and further characterization indicated significant increase in β6 integrin with no change in other myoepithelial markers (Supplementary Fig. S1 and data not shown).

Expressed αvβ6 in the β6-1089 cell line was functional as shown by several in vitro assays. Integrin αvβ6 binds to the LAP, which is part of the trimeric complex that forms
Figure 2. αvβ6 functional assays in the β6-1089 cell line using blocking antibodies. A, adhesion assay; N-1089 and β6-1089 cells were incubated with either an IgG isotype antibody (IgG) or an anti-αvβ6 antibody (10D5) then plated onto TGF-β LAP (0.5 μg/mL), PDL (1 μg/mL), or BSA (0.1%) for 45 minutes at 37°C. The cells were fixed and stained with crystal violet, which was solubilized and the absorbance read at 550 nm. The background binding to BSA was subtracted from the PDL and LAP values, and adhesion was normalized to IgG controls. (Continued on the following page.)
transfected with pcDNA1 or pcDNA1-β6 was placed in the lower well. After varying incubation times, invading cells were quantified (see Materials and Methods). MDA-MB-231 and MCF7 tumor cells showed a significant increase in invasion (P < 0.0001) in the presence of CM from primary myoepithelial cells expressing ovβ6 (Fig. 3A). The same assays were conducted with MDA-MB-231, MCF7, and T47D cell lines, using N-1089 or β6-1089 CM in the lower well. Invasion of MCF-7, T47D, and MDA MB 231 cells was increased significantly (P = 0.0007, P = 0.02, and P = 0.006, respectively) when exposed to CM from β6-1089 cells (Fig. 3B) compared with CM from N-1089 cells.

We have modified the organotypic skin model, developed by Fusenig and colleagues (31), to establish a more physiologically relevant model of breast cancer invasion. Fibroblasts (HFF2) were embedded in a 1:1 collagen:Matrigel mixture onto which the N-1089 or β6-1089 cells were plated with MDA-MB-231 cells layered on top. After 10 days, culture gels were processed and embedded in paraffin and sections stained with hematoxylin and eosin (H&E; Fig. 3C), p63 (to label myoepithelial cells), pan-Cytokeratin (to show myoepithelial cells and MDA-MB-231), and ovβ6 (Supplementary Fig. S3 and data not shown). The "invasion index" was determined as described in Materials and Methods. Gel analysis showed a significant increase in the invasion index of MDA-MB-231 cells when cocultured with β6-1089 compared with N-1089 cells (Fig. 3D, P < 0.0001).

Myoepithelial cells expressing ovβ6 promote breast tumor growth in vivo

The mammary fat pads of female severe combined immunodeficient mice (SCID) mice were injected with 3 million cells comprising a 50% mixture of the breast cancer cell line MDA-MB-231 and either N-1089 or β6-1089. Tumor measurements were taken 3 times a week and the subcutaneous volume determined. It was observed that the MDA-MB-231 cells mixed with β6-1089 cells formed established tumors more rapidly than did those tumor cells injected with N-1089 cells (data not shown). Those tumors produced in the presence of β6-1089 were significantly larger in volume (β6-1089 median; 174 mm3) than those produced when mixed with N-1089 (N-1089 median; 75 mm3), P = 0.018; Fig. 3E and F). These data indicate that a distinct growth advantage was conferred upon the MDA-MB-231 mixed with β6-1089; possibly due to increased MMP secretion allowing more unrestricted growth and invasion.

(Continued) β6-1089 cells adhesion to LAP was blocked in the presence of the ovβ6-blocking antibody. Error bars show SD of triplicate data, the graph is representative of at least 3 independent experiments. Statistical analysis by the Student t test, P < 0.05 considered significant; **, <0.01. B, migration assay; N-1089 and β6-1089 cells were incubated with either an IgG antibody (IgG) or an anti-ovβ6 antibody (10D5). The cells were then plated in Transwell chambers, the undersides of which were coated with either BSA (0.1%) or LAP (0.5 μg/mL), and incubated in serum-free conditions for 24 hours. The number of migrating cells was quantified by counting the cells on the underside of the Transwell. Significantly more β6-1089 cells migrated to LAP than the N-1089 control cell line and this was ablated in the presence of the ovβ6-blocking antibody. Error bars show SD of triplicate data, the graph is representative of at least 3 independent experiments. Statistical analysis by the Student t test, P < 0.05 considered significant; **, <0.01. C, TGFβ-luciferase assay; N-1089 and β6-1089 cells were incubated with no antibody, an IgG antibody (IgG) or an anti-ovβ6 antibody (10D5) and then plated on top of MLEC cells containing a TGFβ-responsive PAI-1 luciferase reporter construct for 16 hours. Cells were lysed and the luminescence read after addition of the substrate. Significantly more luminescence was detected when β6-1089 cells were plated with the MLEC cells compared with the N-1089 cells; this was suppressed significantly in the presence of the ovβ6-blocking antibody. MLEC cells alone indicate background luminescence. Error bars show SD of triplicate data, the graph is representative of at least 3 independent experiments. Statistical analysis by the Student t test, P < 0.05 considered significant; **, <0.01.

Altered Myoepithelial Cells in DCIS

Previously, we showed that ovβ6 expression by keratinocytes results in upregulation of MMP9 and a concomitant increase in invasive capacity (29). Normal myoepithelial cells have been shown to downregulate MMP9 in breast cancer cell lines (17). Therefore, we investigated whether ovβ6 expression by myoepithelial cells modulates MMP9 levels. Conditioned media from N-1089, β6-1089, MDA-MB-231, and MCF-7 cells were generated over 24 hours, after which medium was collected and run on a gelatin zymogram. A clear band at 92 kDa, corresponding to MMP9, was identified in the CM from β6-1089 cells but not in CM from breast cancer cells (Fig. 4A). This was confirmed by quantitative PCR (Fig. 4B), which showed significant upregulation of MMP9 RNA in β6-1089 cells, versus their normal counterparts (P = 0.01).

The β6-1089 cells were preincubated with a β6-blocking antibody (10D5) or IgG isotype control before plating and incubating for 24 hours. The resultant CM was collected and run on a gelatin zymogram. A clear band, corresponding with the recombinant MMP9, was detected from β6-1089 treated with IgG antibody, but not with β6-blocking antibody. To determine whether ovβ6 upregulated MMP9 through TGFβ activation, the β6-1089 cells were preincubated with a TGFβRII-blocking antibody before plating. The CM was run on a gelatin zymogram and a clear band corresponding with the recombinant MMP9 was detected from the β6-1089 cells treated with IgG antibody but not TGFβRII antibody-treated cells (Fig. 4C).

Transwell invasion assays were conducted using N-1089 or β6-1089 CM in the presence of an MMP9 inhibitor (50 nmol/L, Millipore, 444278) or DMSO control (Fig. 4D). MDA-MB-231 cell invasion was decreased significantly, to control levels, in the presence of β6-1089 CM plus MMP9 inhibitor (P = 0.005), whereas the presence of the MMP9 inhibitor had no significant effect on invasion mediated by N-1089 CM. Thus, upregulation of myoepithelial cell ovβ6 expression caused a TGFβ-dependent increase in MMP9 secretion and a concomitant increase in tumor cell invasion.

The clinical relevance of these findings was verified by staining serial sections of pure DCIS and DCIS with invasion (n = 108) for MMP9 and ovβ6. Staining for MMP9 was heterogeneous, with some tumor cell
Figure 3. Effect of myoepithelial cell expression of αvβ6 on in vitro and in vivo tumor cell behavior. A, Transwell invasion assay with primary myoepithelial cells: MCF-7 and MDA-MB-231 cells were plated in Transwells coated with Matrigel (diluted 1:3 in SFM). CM from primary myoepithelial cells transfected with pcDNA1 or pcDNA1-β6 grown in SFM for 24 hours was placed in the lower wells. The plates were incubated for 48 hours (or 24 hours for MDA-MB-231), and the number of invading cells was quantified by counting the cells on the underside of the Transwell. Results are plotted as a ratio where the invasion of the tumor cells in the presence of CM from primary myoepithelial cells transfected with pcDNA1 is set as 100% and the invasion of tumors cells with CM from primary myoepithelial cells transfected with pcDNA1-β6 CM is determined against this. Significantly higher invasion is seen in MCF-7 and MDA-MB-231 cells exposed to CM from primary myoepithelial cells transfected with pcDNA1-β6 cells. Error bars show SD, the graph is a pool of at least 3 independent experiments. Statistical analysis by the Student t test, *P < 0.05 considered significant; **P < 0.01; ***P < 0.001. B, Transwell invasion assay with 1089 myoepithelial cell line: T47D, MCF-7 and MDA-MB-231 cells were plated in Transwells coated with Matrigel (diluted 1:3 in SFM). CM from N-1089 and β6-1089 cells grown in SFM for 48 hours was placed in the lower wells. The plates were incubated for 48 hours (or 24 hours for MDA-MB-231) and the number of invading cells was quantified by counting the cells on the underside of the Transwell. Results are plotted as a ratio where the invasion of the tumor cells in the presence of N-1089 CM is set as 100%, and the invasion of tumors cells with β6-1089 CM is determined against this. Significantly higher invasion is seen in MCF-7, T47D, and MDA-MB-231 cells exposed to CM from β6-1089 cells. Error bars show SD, the graph is a pool of at least 3 independent experiments. (Continued on the following page.)
cytoplasmic staining as well as stromal and inflammatory cell positivity, whereas those frequently was enhanced staining at the myoepithelial–basement membrane interface (Fig. 4E). Strong staining for αvβ6 was restricted to myoepithelial cells (Fig. 4E). Overall, 64% (n = 101) of DCIS ducts exhibited myoepithelial positivity for MMP9 whereas 59% (n = 93) showed αvβ6 staining, with a highly significant association expression of both molecules (P < 0.0001; Fig. 4F and Table 3).

Discussion
Breast cancer develops through defined clinical and pathologic stages, from hyperplasia to DCIS and finally invasive disease and metastasis. Since the introduction of mammographic screening programs, the incidence of detection of DCIS has increased 7-fold (3), and recent reviews suggest that only one-third to half of these cases will progress to invasive breast cancer during a woman’s lifetime (3, 4). Current management strategies treat all DCIS lesions as potentially invasive as there are no robust markers to distinguish between those cases with the potential to progress and those unlikely to. In DCIS, neoplastic cells fill and distend ducts and are separated from the surrounding stroma by an intact myoepithelial–basement membrane interface. Myoepithelial cells have been shown to express several tumor suppressor proteins (e.g., p63, p73, 14-3-3, Maspin; refs. 43–45), consistent with their established tumor suppressor function (14). However, it has been shown that tumor-derived myoepithelial cells differ from normal and lose the ability to polarize luminal epithelial cells (20). Further studies have shown differences in gene expression between normal and DCIS-associated myoepithelial cells such as increased lysyl oxidase (LOX; ref. 46) and neuropilin 1 (47). Allinen and colleagues examined the microenvironment of normal and tumor breast tissue and found that myoepithelial cells exhibited the most numerous changes in gene expression of all the microenvironmental cell types in DCIS (19), although the predictive and functional relevance of such changes has not been established.

In this study, we show that myoepithelial cells exhibit functionally relevant changes in their phenotype and behavior in a subset of DCIS cases, leading to acquisition of invasion-promoting properties. We have shown upregulation of integrin αvβ6 by myoepithelial cells in 52% to 69% of non–high-grade and high-grade DCIS, respectively, but it is present in almost 100% of DCIS associated with invasive disease. These data suggest that αvβ6 might be a marker of DCIS that is more likely to progress to invasive disease. It already has been established that high-grade DCIS progresses to invasion and local recurrence more quickly than low-grade (48, 49). To more directly address whether expression of αvβ6 can predict recurrence and progression, we assessed expression in a cohort of DCIS cases treated with local excision, confirmed margins free of disease and with long-term follow-up as part of the UK/ANZ DCIS trial (22), and showed a significant correlation between αvβ6 expression in myoepithelial cells and recurrence of breast cancer either as in situ or invasive disease, independent of patient age, disease grade, or extent. Furthermore, those with αvβ6 positivity developed recurrence more quickly than those lacking αvβ6, with median time to recurrence of 2.3 versus 11.4 years, respectively. This work supports the proposal that expression of αvβ6 in the DCIS-associated myoepithelial cells is a marker of DCIS cases more likely to progress to invasion and recurrence. The integrin αvβ6 is restricted to epithelial cells and is usually expressed only in embryogenesis and wound healing in adult humans. αvβ6 primarily binds to the RGD motif of LAP of pro-TGFβ leading to activation of the protein. The downstream consequence of αvβ6 activation has so far been shown to lead to activation of MMPs (e.g., MMP3 and MMP9; refs. 50–52). Activation of MMPs plays an important role in the remodeling of the ECM and deregulation of these proteins in cancer leads to destruction of basement membrane that would otherwise constrain tumor cells from invading.

Previous studies have identified αvβ6 expression in invasive carcinomas, and these indicate that expression is associated with aggressive tumor behavior and reduced survival.
TGF-β siRNA. to a blocked by an and migration to the a determined by Pearson a function of a tumor. a of a tumor. a determined against this. Significance determined by Student t test, P < 0.05 considered significant; **, <0.01; ***, <0.001. E, myoepithelial cells exhibit coincident expression of MMP9 and αvβ6. Serial sections from 102 DCIS cases were stained and representative images are shown for (a) MMP9 and (b) αvβ6. Staining for αvβ6 is limited to the myoepithelial cell layer in the DCIS duct and there is colocalized strong staining for MMP9. Top, taken at ×200 and bottom are the same section at ×400. F, graph shows the percentage of cases stained positive or negative for αvβ6 and MMP9 on serial sections, this was scored and the correlation was determined by Pearson χ² test, P < 0.05 considered significant; **, <0.01; ***, <0.001.

(53–55). However, this is the first study to describe upregulation of αvβ6 in the nonneoplastic microenvironment of a tumor.

We used both primary human myoepithelial cells and a myoepithelial cell line (N-1089) engineered to overexpress αvβ6 as a model of DCIS-associated myoepithelial cells. The functionality of αvβ6 was confirmed by enhanced attachment and migration to the αvβ6 ligand LAP, which is blocked by an αvβ6-blocking antibody (10D5) and siRNA to αvβ6. Furthermore, we show that αvβ6 activates a PAI-1 TGFβ luciferase reporter, which is also blocked by 10D5 and siRNA.

Using our model system in Transwell invasion assays of several breast cancer cell lines, we have shown that CM from the β6-1089 cell line promotes invasion of MDA-MB-231 (basal-like), T47D (estrogen receptor [ER+]) and MCF-7 (ER−) when compared with the model of normal myoepithelial cells (N-1089), with a similar activity shown by primary myoepithelial cells. The breast cancer cell lines studied represent a cross-section of different breast cancer cell types indicating the broad relevance of the effect of αvβ6-positive myoepithelial cells in breast cancer. We further show enhanced invasion in the presence of αvβ6-positive myoepithelial cells in a modified organotypic assay...
the effect. It has already been shown that not release it from the matrix, and therefore we postulated that this could be blocked both by increased invasion. In keeping with this, we show that activate MMP9 in oral squamous cell carcinoma to drive tumor progression on non–small cell lung carcinoma (NSCLC), colorectal, prostate, and gastric cancer. Intense staining has been seen and positively correlated with metastasis in breast, prostate, and colorectal cancer (58). Breast cancer along with skin cancer and gliomas exhibit fewer TGFβ pathway mutations and may use TGFβ for a growth advantage. Indeed low-level expression of TGFβRII in ER− breast cancer is associated with better outcome (59), whereas overexpression of TGFβ is associated with increased incidence of metastasis (60). A bioinformatic approach by Padua and colleagues to examine breast cancer for a TGFβ-responsiveness signature (TRBS) indicated that as many as 40% of breast cancers are capable of responding to TGFβ signals (61). Here, we show that TGFβ signaling can also influence earlier stage disease.

We have shown that a subset of pure DCIS and almost all DCIS with invasive disease exhibit myoepithelial cells that express αvβ6 integrin and show that this change results in a switch in the myoepithelial cells from tumor-suppressive to tumor-promoting activity via TGFβ and MMP9 signaling. Our data indicate a significant association between αvβ6 expression and disease recurrence and shows that αvβ6-positive DCIS recurs more rapidly than αvβ6-negative disease. Further studies are needed to establish whether αvβ6 could be used in the clinical setting to stratify patient care and whether TGFβ and MMP9 could be targeted in early breast cancer for therapeutic benefit. This may represent a key stage in the evolution of breast cancer that could be used in a predictive and prognostic setting, allowing more tailored management of women with DCIS and may offer opportunities for therapeutic intervention.

Table 3. Coincident staining of MMP9 and αvβ6 in DCIS

<table>
<thead>
<tr>
<th>Myoepithelial αvβ6 expression</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoepithelial MMP9 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>90 (57%)</td>
<td>11 (7%)</td>
<td>101</td>
</tr>
<tr>
<td>Negative</td>
<td>3 (2%)</td>
<td>52 (34%)</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>93</td>
<td>63</td>
<td>156</td>
</tr>
</tbody>
</table>

NOTE: Formalin-fixed, paraffin-embedded serial sections of DCIS were stained for αvβ6 integrin or MMP9 and the absence or presence of myoepithelial cell staining was recorded, the number and percentage of cases in each category is indicated. In 9 of 156 (57%) DCIS cases, the myoepithelial cell compartment stained positively for αvβ6 and MMP9, whereas only 3 of 156 (2%) DCIS cases positive for αvβ6 were MMP9 negative and 11 of 156 (7%) of αvβ6 negative cases were positive for MMP9. Pearson χ² analysis showed significant (P < 0.0001) correlation between αvβ6 and MMP9 expression.

used previously in our laboratory (32). In this assay, MDA-MB-231 cells exhibited significantly increased invasion into a Matrigel:collagen:fibroblast mixture when cocultured with β6-1089 compared with N-1089 cells. To show that this effect was not simply an in vitro phenomenon, we injected the mammary fat pad of 20 SCID mice with a 50:50 mixture of MDA-MB-231 and either β6-1089 or N-1089. The tumors that formed in the β6-1089 cohort grew with faster kinetics and the median tumor size was significantly increased compared with the N-1089 group after 13 weeks (Fig. 3F and E, respectively).

As αvβ6 activates TGF-β in a localized manner and does not release it from the matrix, and therefore we postulated that there must be a secondary active agent(s) mediating the effect. It has already been shown that αvβ6 is able to activate MMP9 in oral squamous cell carcinoma to drive increased invasion. In keeping with this, we show that β6-1089 cells produce more MMP9 than the N-1089 (Fig. 4A) and that this could be blocked both by αvβ6-blocking antibody 10D5 and by inhibiting TGFBRII with a blocking antibody. We further show that an MMP9 inhibitor can significantly reduce the invasion of MDA-MB-231 cells in presence of β6-1089 CM. These data show for the first time a paracrine protumor effect from αvβ6-expressing myoepithelial cells on breast cancer cells and that this effect is dependent on αvβ6-TGF-β-mediated activation of MMP9.

TGFβ is the archetypal pleiotropic cytokine, which has been shown to be both a tumor suppressor and a tumor promoter (56). The role of TGFβ as a tumor suppressor is clearly evidenced by the frequency with which various components of the pathway are lost, TGFBR2, TGFBR1 and SMAD4 are commonly mutated by LOH or allelic loss (57). However, high expression of TGFβ also correlates with increased invasion and shows that this is dependent on αvβ6-mediated activation of MMP9.

Disclose of Potential Conflicts of Interest

J.F. Marshall is a Consultant/Advisory Board member of GSK. No potential conflicts of interest were disclosed by the other authors.
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References

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