Altered Microenvironment Promotes Progression of Pre-Invasive Breast Cancer: myoepithelial expression of αvβ6 integrin in DCIS identifies high-risk patients and predicts recurrence

Michael D Allen¹, Gareth J Thomas², Sarah Clark¹, Marwa M Dawoud¹, Sabarinath Vallath¹, Sarah J Payne¹, Jennifer J Gomm¹, Sally A Dreger¹, Sarah Dickinson¹, Dylan R Edwards³, Caroline J Pennington³, Ivana Sestak⁴, Jack Cuzick⁴, John F Marshall¹, Ian R Hart¹, J Louise Jones¹*

¹Barts Cancer Institute - a CR-UK Centre of Excellence, Centre for Tumour Biology, Queen Mary University of London, John Vane Science Centre, Charterhouse Square, London, EC1M 6BQ.
²Cancer Research UK Clinical Centre, Somers Cancer Research Building, Experimental Pathology Group, Southampton General Hospital, Southampton, SO16 6YD.
³Biomedical Research Centre, School of Biological Sciences, University of East Anglia, Norwich, Norfolk, NR4 7TJ
⁴Wolfson Institute of Preventive Medicine, Barts and The London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ

*To whom correspondence should be addressed: l.j.jones@qmul.ac.uk

Financial support:

MDA was funded by the Breast Cancer Campaign

Conflict of Interest: None

Running Title: Altered Myoepithelial cells in DCIS

Key words:

Breast Cancer, invasion, ITGβ6, TGFβ, DCIS, myoepithelial cells, microenvironment

Word Count: 5354
Translational Relevance:

Ductal carcinoma in-situ (DCIS) is the main precursor of invasive breast cancer and now accounts for 25-30% 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 up-regulation 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.
Abstract

**Purpose:** This study investigated the functional and clinical significance of integrin αvβ6 up-regulation in myoepithelial cells of ductal carcinoma in-situ (DCIS).

**Experimental Design:** Archival samples of DCIS and DCIS with associated invasion (n=532) were analysed for expression of αvβ6 by immunohistochemistry, and ability to predict recurrence and progression assessed in an independent, unique cohort of DCIS cases with long term follow up. Primary myoepithelial cells and myoepithelial cell lines, with and without αvβ6 expression, were used to measure the effect of αvβ6 on growth and invasion of tumor cell lines *in vitro*, and in a xenograft mouse model. Involvement of TGFβ signaling was established using MLEC assay and antibody inhibition, and expression and activation of matrix-metalloproteinase (MMP)-9 established by RT-PCR and zymography.

**Results:** Expression of αvβ6 is significantly associated with progression to invasive cancer (p<0.006) and with recurrence over a median follow-up of 114 months in a series of matched DCIS cases treated with local excision. We show that expression of αvβ6 drives myoepithelial cells to promote tumor cell invasion *in vitro* and enhances mammary tumor growth *in vivo*. The tumor promoting effect of αvβ6-positive myoepithelial cells is dependent on TGFβ-driven up-regulation of MMP9, and can be abrogated by inhibiting this pathway.

**Conclusion:** These findings indicate that altered myoepithelial cells in DCIS predict disease progression and recurrence, and demonstrate that up-regulation of αvβ6 on myoepithelial cells generates a tumor-promoter function through TGFβ up-regulation of MMP-9. These data suggest expression of αvβ6 may be used to stratify patients with DCIS.
Introduction

The majority of invasive breast cancers arise from the precursor lesion ductal carcinoma in-situ (DCIS) (1), whereby neoplastic cells proliferate and fill the breast ductal tree but are separated from the surrounding stroma by the intact myoepithelial-basement membrane (BM) wall of the ducts (Figure 1a). At some point during progression, the tumor cells penetrate the myoepithelial-BM interface and move into the interstitial matrix becoming an established invasive carcinoma with metastatic potential (Figure 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 one in four breast cancer diagnoses (3), contributing to recent concerns surrounding over-diagnosis and over-treatment within the breast screening setting (4, 5). Indeed, the need to understand more about the biological and molecular mechanisms underlying progression of DCIS to invasive disease and to generate robust risk stratification methods for 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), though one gene array-based study
identified a subset of DCIS cases with an expression profile more similar to invasive cancer, and found 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 demonstrate for the first time, up-regulation 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 dependent manner. Analysis of 532 DCIS cases demonstrates 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 up-regulation 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.
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 performed following patient consent and approval from the local research ethics committee (ref: 05/Q0403/199 and 09/H075/39). Further analysis was carried out 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 2x2 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). Clinopathological details are provided in supplementary methods.

Immunohistochemistry
Sections were dewaxed in xylene and antigen retrieved in pepsin solution; followed by incubation with 0.5mg/ml αvβ6 mouse monoclonal antibody (6.2G2 Stromedix, Cambridge, MA, USA) (24, 25), MMP9 (My Biosource, San Diego, USA, MBS 240189), p63 (Dako, Cambridge, M7247), SMA (Dako, M0851) or CK5/6 (Dako, M7237) overnight. Sections subsequently were incubated with Rabbit anti-Mouse biotinylated F(ab’)2 for 30 min, developed using ABC reagent and superDAB (Dako, Cambridge, UK) 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 HFFF2 cells, obtained from the American Type Culture Collection, were maintained in DMEM with 10% Fetal Calf Serum (FCS) and 2mM glutamine, AM12 cells transfected with pBABE puro β6 were maintained in DMEM with 10% Fetal Calf Serum (FCS) and 2mM glutamine with puromycin (1µg/ml, Sigma, Dorset, UK). Mink Lung Epithelial Cells (a kind gift from D. Rifkin, New York University) were maintained in DMEM 10% Fetal Calf Serum (FCS) and 2mM glutamine with puromycin (1µg/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, Paisley, UK), to purify the myoepithelial cell population (1089), and maintained in Hams-F12 media with 10% FCS, 2mM glutamine, Hydrocortisone (1µg/ml) EGF (10ng/ml) and Insulin (5µg/ml). All cell lines were verified by STR profiling (LGC Standards, Teddington, UK, tracking number 710081047). The 1089 cell line demonstrated 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 discarded, 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 pre-coated with collagen (10mg/ml) at a density of 2x10⁴ per well. Cells were transfected 72h later using the standard JetPrime protocol (PolyPlus, Illkirch, France) and 2µg of integrin β6 pcDNA1 neo (Addgene plasmid 13580) (28) or pcDNA1 empty vector. After 24h media was changed for serum free Hams-F12 and conditioned for 48h.

**Stable expression of β6 in 1089 myoepithelial cells**
Conditioned medium containing retrovirus from AM12/pBABE-β6 or AM12/pBABE-Puro cells, was incubated with 1089 cells (29). β6 positive 1089 and puromycin control cells were selected using puromycin (1μg/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**

2x10⁴ cells were plated onto glass coverslips and grown for 48h prior to 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, Billerica, MA, USA) which was diluted in 0.1% BSA in DMEM. Slides then were stained with a secondary Rabbit anti-Mouse 488 (Invitrogen, A11059) and counterstained with DAPI. Images were viewed on a Zeiss LSM 510 Meta microscope.

**Flow Cytometry**

3x10⁵ cells were incubated with primary antibody diluted in 0.1% BSA in PBS, except for the anti-αvβ6 antibody (10D5) which was diluted in 0.1% BSA in DMEM. Cells subsequently were stained with secondary Rabbit anti-Mouse FITC conjugated antibody (Dako, F0313) before reading on a Flow Cytometer (BD LSR II).

**Western Blotting**

Cells were trypsinized, centrifuged at 1200rpm, re-suspended in RIPA lysis buffer (Millipore) with ‘Complete’ protease inhibitors (Roche, Burgess Hill, UK). Protein concentrations were determined using the Dc Protein Assay (Bio-Rad, Hercules, CA, USA) and 50μg of protein was loaded on to an 8% Tris/Glycine gel, electrophoresed and transferred on to nitrocellulose.
membranes which were blotted with αvβ6 antibody (1:1000, SC-6632, Santa Cruz, Dallas, TX, USA), β-actin (1:10000, Santa Cruz, SC47778) or HSC70 (1:10000, SC-53935, Santa Cruz,) and Donkey anti-Goat HRP conjugated secondary (1:2000, Dako) or Goat anti-Mouse HRP conjugated secondary (1:2000, Dako) diluted in 5% Milk in TBS/T.

TGFβ Quantitation Assay

Mink Lung Epithelial Cells (MLEC) transfected with a PAI-1 promoter fused to a luciferase reporter gene were used to determine the amount of active TGFβ (30). Briefly, 4x10^4 MLEC’s were seeded in a 96 well plate and incubated overnight then serum starved for 4h before 4x10^4 test cells were plated on top of the MLEC’s and co-cultured for 16h. Medium was removed, lysis buffer (Promega, Madison, WI, USA) 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 LAP

Latency Associated Peptide (LAP, Sigma) was diluted to 0.5μg/ml, aliquots were added to the wells of a 96 well plate and incubated for 1h at room temperature. 2x10^4 cells were incubated with IgG isotype control antibody or αvβ6-blocking antibody (10D5) at 10μg/ml in Serum Free Media (SFM) for 20 mins at 4°C before plating onto LAP. Cells were incubated at 37°C for 45mins before fixing with 30% Methanol and staining with 0.1% Crystal Violet. Stained cells were dissolved with 30% Acetic Acid and absorbance read at 550nm.

Migration Assays

24 well Transwells (8μm pore, BD Bioscience, San Jose, CA, USA) were coated on the underside with 0.5μg/ml LAP, for 1h at room temperature then placed in a 24-well plate with
DMEM, lacking serum, in the lower chamber. 3x10⁴ cells were incubated with either an IgG isotype antibody or an αvβ6-blocking antibody (10D5) diluted in serum free media (20 mins at 4°C). Cells then were placed in the upper chamber and incubated for 24h. 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 β6-1089 by culturing in serum free Ham’s-F12 for 48h and was stored at -80°C. The upper-surface of 24-well Transwell was coated with 70μl growth factor-reduced Matrigel (diluted 1:3 in Hams-F12 without Serum) and incubated for 1 hour at room temperature. 500μl conditioned medium from either N-1089 or β6-1089 was then placed in the lower well. For inhibitor experiments either DMSO or MMP9 inhibitor (Millipore, 50nM, 444278) was added to conditioned media. 200μl of 3x10⁴ tumor cells was added to each well and incubated for 24-48h. 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 et al (31) was adapted by Nystrom et al (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 with 1 volume 10x DMEM, 1 volume FCS and 1 volume DMEM+10% FCS in which 5x10⁵ cells per ml HFFF2 fibroblasts had been suspended. Then 1 ml of this solution was placed into wells of a 24-well plate and allowed to polymerize for 30 min at 37 °C. 1ml of DMEM + 10% FCS and glutamine was added to each well and incubated overnight.
The next day $2.5 \times 10^4/500\mu l$ (DMEM+10% FCS) N-1089 or β6-1089 cells were plated on top of the gel and allowed to settle for 6hrs at 37°C, then $2.5 \times 10^4/500\mu l$ MDA-MB-231 cells were plated on each gel. Gels were fixed in formal saline and placed into paraffin blocks, 5μm 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 % area of the field the particles occupied and the depth the particles penetrated the gel (determined by three measurements per field of view) using ImageJ (NIH). The product of the three 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-h 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 volume 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 pre-incubated with IgG antibody, β6 blocking antibody (10μg/ml, 10D5) or TGFβRII blocking antibody (10μg/ml, AF-241-NA, R&D systems, Abingdon, UK) in SFM before they were plated on coverslips coated with fibronectin (1μg/ml) or LAP (0.5μg/ml). Media were collected after 24h 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 β6-1089 cell pellets using a kit (Qiagen, 74104). Analysis was performed on RNA from three separate experiments. TaqMan real-time PCR for MMP9 (Invitrogen, Hs00234579_m1) was carried out as previously described (34).

*Statistical Analysis*

We determined statistical significance of the differences between experimental groups by the two-tailed Student's T-test with PRISM software (Graphpad Software, San Diego, USA). P values less than 0.05 were considered significant. The relationship between staining patterns and DCIS grade, between MMP9 and αvβ6, and between αvβ6 and outcome was analyzed using Pearson Chi-square test using SPSS software (SPSS Inc, Chicago, USA) 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 un-adjusted for tumor grade and size. Animal studies were assessed for statistical significance between two medians by Mann-Whitney U test.
Results

Myoepithelial expression of αvβ6 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 αvβ6. This revealed no significant staining for αvβ6 integrin in normal or hyperplastic cases (Figure 1b), whereas 69% of high grade and 52% of non-high grade DCIS cases without invasion showed myoepithelial staining for αvβ6 (Table 1), with a significantly higher frequency of positivity in high grade cases (p<0.001). The frequency of myoepithelial cell αvβ6 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 αvβ6, we examined expression in an unique series of DCIS with long-term follow-up, derived from the UK/ANZ DCIS study, the end-point assessed was the appearance of any new breast cancer, either DCIS or invasive cancer, as the numbers are insufficient to asses 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 1), with cases scored using an H score to take account of the extent and intensity of myoepithelial-associated αvβ6 positivity, according to the criteria described by Allred et al (35). Analysis demonstrated a significant association between extent of αvβ6 expression (evaluated as an H score (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 αvβ6 recurred more quickly than those lacking the integrin, with the αvβ6 positive cases showing a median time to recurrence of 2.3 years and those negative for αvβ6 having a median of 11.4 years to recurrence.

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 (36)). This “pure” α6β4 expressing cell population, (N-1089), was characterized by immunofluorescent staining for key myoepithelial cell markers (Figure 1c, upper panel) following introduction of the puromycin resistance gene. Integrin β4 (36), P-Cadherin (P-Cad) (37), cytokeratin 17 (CK17) (38) and Desmoglein 3 (Dsg3) (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 (Figure 1c, middle panel). 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 (Figure 1d and data not shown). Expression of αvβ6 was also confirmed by Western blotting (Figure 1e). Primary myoepithelial cells were isolated from normal reduction mammoplasty 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 (Figure 1c, lower panel). αvβ6 over-expressing 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 Figure 1 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 Latency Associated Peptide (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-D-Lysine (PDL) or Bovine Serum Albumin (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) prior to 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, demonstrating 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 co-cultured with Mink Lung Epithelial Cells (MLEC) stably transfected with a TGF-β responsive Plasminogen Activator Inhibitor 1 (PAI-1) promoter fused to a luciferase gene. Co-culture 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 (Figure 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 RNAi to αvβ6 (Supplementary Figure 2 a-d).
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 to normal (αvβ6 negative) myoepithelial cells. Breast cancer cell lines were placed in Transwells coated with Matrigel®, and conditioned medium, (CM) from primary myoepithelial cells 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 αvβ6 (Figure 3a). The same assays were performed 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 (Figure 3b) compared with CM from N-1089 cells.

We have modified the organotypic skin model, developed by Fusenig et al (31), to establish a more physiologically relevant model of breast cancer invasion. Fibroblasts (HFFF2) 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 H+E (Figure 3c), p63 (to label myoepithelial cells), pan-Cytokeratin (to show myoepithelial cells and MDA-MB-231) and αvβ6 (Supplementary Figure 3 and data not shown). The “invasion index” was determined as described in Materials and Methods. Gel analysis demonstrated a significant increase in the invasion index of MDA-MB-231 cells when co-cultured with β6-1089 compared with N-1089 cells (Figure 3d, p<0.0001).
Myoepithelial cells expressing αvβ6 promote breast tumor growth in vivo

The mammary fat pads of female 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 three 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; 174mm³) than those produced when mixed with N-1089 (N-1089 median; 75mm³, p=0.018) (Figure 3e & f). These data indicate 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.

αvβ6 expressing myoepithelial cells promote invasion in a TGFβ and MMP9 dependent manner

Previously we demonstrated that αvβ6 expression by keratinocytes results in up-regulation of MMP9 and a concomitant increase in invasive capacity (29). Normal myoepithelial cells have been shown to down regulate MMP9 in breast cancer cell lines (17). Therefore we investigated whether αvβ6 expression by myoepithelial cells modulates MMP9 levels. Conditioned media from N-1089, β6-1089, MDA-MB-231 and MCF-7 cells was generated over 24h, after which medium was collected and run on a gelatin zymogram. A clear band at 92KDa, corresponding to MMP9, was identified in the CM from β6-1089 cells but not in CM from breast cancer cells (Figure 4a). This was confirmed by qPCR (Figure 4b), which showed significant up-regulation of MMP9 RNA in β6-1089 cells, versus their normal counterparts (p=0.01).
The β6-1089 cells were pre-incubated with a β6-blocking antibody (10D5) or IgG isotype control before plating onto LAP 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 if αvβ6 up-regulated MMP9 through TGFβ activation, the β6-1089 cells were pre-incubated with a TGFβRII blocking antibody before plating onto LAP. 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 (Figure 4c).

Transwell invasion assays were performed using N-1089 or β6-1089 CM in the presence of an MMP9 inhibitor (50nM, Millipore, 444278) or DMSO control (Figure. 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 up-regulation of myoepithelial cell αvβ6 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 αvβ6. Staining for MMP9 was heterogeneous, with some tumor cell cytoplasmic staining as well as stromal and inflammatory cell positivity, while there frequently was enhanced staining at the myoepithelial-basement membrane interface (Figure 4e). Strong staining for αvβ6 was restricted to myoepithelial cells (Figure 4e). Overall, 64% (n=101) of DCIS ducts exhibited myoepithelial positivity for MMP9 whilst 59% (n=93) showed αvβ6 staining, with a highly
significant association between expression of both molecules (p<0.0001) (Figure 4f and Table 3).

Discussion

Breast cancer develops through defined clinical and pathological 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 seven-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 breast ducts but are separated from the surrounding stroma by an intact myoepithelial-BM interface. Myoepithelial cells have been shown to express several tumor suppressor proteins (e.g. p63, p73, 14-3-3-σ, Maspin) (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) (46) and neuropillin 1(47).

Allinen et al 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), though 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 up-regulation of integrin αvβ6 by myoepithelial cells
in 52-69% of non-high grade and high grade DCIS, respectively, but 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 demonstrated 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 avb6, with median time to recurrence of 2.3 years 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 Latency Associated Peptide 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 matrix metalloproteinases (e.g. MMP3 and MMP9 (50-52)). Activation of MMPs plays an important role in the remodeling of the ECM and de-regulation 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 (53-55).
However, this is the first study to describe up-regulation of $\alpha\nu\beta6$ in the non-tumorigenic microenvironment of a tumor.

We used both primary human myoepithelial cells and a myoepithelial cell line (N-1089) engineered to over-express $\alpha\nu\beta6$ as a model of DCIS associated myoepithelial cells. The functionality of $\alpha\nu\beta6$ was confirmed by enhanced attachment and migration to the $\alpha\nu\beta6$ ligand LAP which is blocked by an $\alpha\nu\beta6$ blocking antibody (10D5) and siRNA to $\alpha\nu\beta6$. Furthermore, we show $\alpha\nu\beta6$ activates a PAI-1 TGF$\beta$ 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 demonstrated that conditioned media from the $\beta6$-1089 cell line promotes invasion of MDA-MB-231 (basal-like), T47D (ER-) and MCF-7 (ER+) when compared to the model of normal myoepithelial cells (N-1089), with a similar activity demonstrated 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 $\alpha\nu\beta6$ positive myoepithelial cells in breast cancer. We further demonstrate enhanced invasion in the presence of $\alpha\nu\beta6$-positive myoepithelial cells in a modified organotypic assay used previously in our laboratory (32). In this assay MDA-MB 231 cells exhibited significantly increased invasion into a matrigel:collagen:fibroblast mixture when co-cultured with $\beta6$-1089 compared to N-1089 cells. In order 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 $\beta6$-1089 or N-0189. The tumors that formed in the $\beta6$-1089 cohort grew with faster kinetics and the median tumor size was significantly increased compared with the N-1089 group after 13 weeks (Figure 3f and e respectively).
Since \( \alpha \nu \beta 6 \) activates TGF-\( \beta \) 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 \( \alpha \nu \beta 6 \) is able to activate MMP9 in Oral Squamous Cell Carcinoma to drive increased invasion. In keeping with this, we show that \( \beta 6-1089 \) cells produce more MMP9 than the N-1089 (Figure 4a) and that this could be blocked both by ITGB6 blocking antibody 10D5 and by inhibiting TGFBRII with a blocking antibody. We further demonstrate that an MMP9 inhibitor can significantly reduce the invasion of MDA-MB-231 cells in presence of \( \beta 6-1089 \) conditioned media. These data show for the first time a paracrine pro-tumor effect from \( \alpha \nu \beta 6 \) expressing myoepithelial cells on breast cancer cells and that this effect is dependent on \( \alpha \nu \beta 6 \)-TGF-\( \beta \) mediated activation of MMP9.

TGF\( \beta \) is the archetypal pleiotropic cytokine, which has been shown to be both a tumor suppressor and promoter (56). The role of TGF\( \beta \) 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 Loss of Heterozygosity or allelic loss (57). However, high expression of TGF\( \beta \) also correlates with 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 TGF\( \beta \) pathway mutations and may use TGF\( \beta \) for a growth advantage. Indeed low level expression of TGFBRII in ER- breast cancer is associated with better outcome (59) whereas over-expression of TGF\( \beta \) is associated with increased incidence of metastasis (60). A bioinformatic approach by Padua et al to examine breast cancer for a TGF\( \beta \) Responsiveness Signature (TBRS) indicated that as many as 40\% of breast cancers are capable of responding to TGF\( \beta \) signals (61). Here we demonstrate that TGF\( \beta \) signaling can also influence earlier stage disease.
We have demonstrated that a subset of pure DCIS and almost all DCIS with invasive disease exhibit myoepithelial cells that express $\alpha\nu\beta_6$ integrin, and show that this change results in a switch in the myoepithelial cells from tumor suppressive to tumor promoting activity via TGF$\beta$ and MMP9 signaling. Our data indicate a significant association between $\alpha\nu\beta_6$ expression and disease recurrence, and shows that $\alpha\nu\beta_6$-positive DCIS recurs more rapidly than $\alpha\nu\beta_6$-negative disease. Further studies are needed to establish whether $\alpha\nu\beta_6$ could be used in the clinical setting to stratify patient care, and if TGF$\beta$ 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.
Acknowledgements

We thank; Dr Paul Weinreb (Biogen IDEC) and Dr Shelia Violette (Stromedix) for supplying the 6.2G2 antibody; Professor Dean Sheppard, who provided the integrin pcDNA1 neo via Addgene and Professor D. Rifkin, who provided the MLEC TGF-β reporter cell line.

Author Contributions

MDA performed all of the experiments. JLJ with MDA designed the study, scored IHC, analyzed data and prepared the manuscript. GJT assisted with gelatin zymography. SC and MMD collected the cases and SAD and SV carried out immunohistochemistry. SD assisted with PAI-1 luciferase assays. SJP and IH carried out animal experiments. JC and IS provided access to the UKDCIS tissue collection and patient data and performed statistical analysis. DRE and CJP carried out MMP QPCR analysis. JIG isolated and provided all the primary myoepithelial cell lines. JFM and IH critically evaluated the study and, with JLJ and MDA wrote the final version of the manuscript. All authors participated in discussion of the work and gave final approval.
References

33. Jensen MM, Jorgensen JT, Binderup T, Kjaer A. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. BMC medical imaging. 2008;8:16.
Figure Legends

Figure 1. Characterization of normal and αvβ6 overexpressing myoepithelial cell line and primary cells.

a) The normal breast duct comprises an inner luminal epithelial cell layer surrounded by a myoepithelial cell layer (stained for CK5/6), separating the epithelial compartment from the surrounding duct. In DCIS, the lumen of the duct becomes filled with neoplastic cells, but still surrounded by an intact myoepithelial cell layer. With progression to invasive cancer, the tumor cells breach the myoepithelial cell layer (arrow) and invade the surrounding stromal compartment.

b) Normal breast ducts, ducts showing epithelial hyperplasia and examples of low grade and high grade DCIS demonstrate an intact myoepithelial cell layer, as shown by SMA immunoreactivity. No staining for αvβ6 is seen in the normal or hyperplastic ducts, or in this example of low grade DCIS, but myoepithelial expression of αvβ6 is seen in the duct showing high grade DCIS. Magnification x100 and x400, Scale bar, 100μm and 20μm respectively.

c) Immunofluorescent staining montage; N-1089, β6-1089 and primary myoepithelial cells stained for a panel of markers identifying myoepithelial cells: Integrin β4 (β4), P-Cadherin (P-Cad), Cytokeratin 17 (CK17) and Desmoglein 3 (Dsg3). All cell types exhibited positive staining for these markers; however, staining for αvβ6 was only detectable in β6-1089. These images are representative of at least 3 different experiments. Magnification x630, scale bar, 20μm.
d) Flow cytometry profile of N-1089 and β6-1089 stained for the αvβ6 integrin (Millipore, clone 10D5), the dotted line indicates N-1089 (Mean Fluorescence Intensity [MFI] = 3.23), and the dashed line indicates β6-1089 (MFI = 40). The black line indicates the IgG Isotype control staining (MFI = 3.48). These data are representative of at least 3 independent experiments.

e) Western blot; the upper band shows expression of integrin αvβ6 (Santa Cruz, Clone I098, 106KDa) in β6-1089 and N-1089 cell lines, markedly higher levels of αvβ6 were detected in the β6-1089 lane. The lower band shows HSC70 (Santa Cruz, 70KDa) to indicate equal loading. Representative of 3 independent Western blots.

**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 Latency Associated Peptide (LAP, 0.5μg/ml), Poly-D-Lysine (PDL, 1μg/ml) or Bovine Serum Albumin (BSA, 0.1%) for 45 min at 37°C. The cells were fixed and stained with crystal violet, which was solubilized and the absorbance read at 550nm. The background binding to BSA was subtracted from the PDL and LAP values and Adhesion was normalized to IgG controls. β6-1089 cells adhesion to LAP was blocked in the presence of the αvβ6-blocking antibody. Error bars show standard deviation of triplicate data, the graph is representative of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, ***<0.001.

b) Migration assay; N-1089 and β6-1089 cells were incubated with either an IgG antibody (IgG) or an anti-αvβ6 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 24h. 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 αvβ6-blocking antibody. Error bars show standard deviation of triplicate data, the graph is representative of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, * <0.05.

c) TGFβ luciferase assay; N-1089 and β6-1089 cells were incubated with no antibody, an IgG antibody (IgG) or an anti-β6 antibody (10D5) then plated on top of MLEC cells containing a TGFβ responsive PAI-1 luciferase reporter construct for 16h. 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 αvβ6-blocking antibody, MLEC cells alone indicate background luminescence. Error bars show standard deviation of triplicate data, the graph is representative of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, * <0.05.

**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 serum free media). Conditioned media (CM) from primary myoepithelial cells transfected with pcDNA1 or pcDNA1-β6 grown in serum free media 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 standard deviation, the graph is a pool of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, * <0.05, ** <0.01, ***<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 serum free media). Conditioned media (CM) from N-1089 and β6-1089 cells grown in serum free media 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 standard deviation, the graph is a pool of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, * <0.05, ** <0.01, ***<0.001.

c) Organotypic assay for invasion; fibroblasts were mixed into collagen:Matrigel (50:50), (i) N-1089 or (ii) β6-1089 cells were plated on top of the gel and allowed to attach before MDA-MB-231 cells were overlaid. Organotypics were then raised onto a metal grid and fed from
below with media. These were cultured for 10 days, changing the media every two days; gels were then fixed in formal saline and embedded in paraffin. 5\(\mu\)m serial sections were stained with H + E, p63, pan-cytokeratin and \(\alpha\)v\(\beta\)6 (not shown). The p63 and \(\alpha\)v\(\beta\)6 positive cells remained on the gel surface. Magnification x100, scale bar 100\(\mu\)m.

d) Invasion of MDA-MB-231 cells in the presence of either N-1089 or \(\beta\)6-1089 cells was quantitated by taking images of 3 random fields of view from triplicate gels and analyzing the number of invasive particles, the % area of the field the particles occupied and the depth the particles penetrated the gel (determined by three measurements per field of view) using ImageJ (NIH). The product of the three values gave an overall “invasive index” and demonstrates significantly more invasion by MDA MD 231’s in the presence of \(\beta\)6-1089 compared to N-1089. Error bars show standard deviation, the graph is a pool of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, **<0.001.

e) MDA-MB-231 cells mixed with N-1089 (total cell number 3x10\(^6\)/mouse) were injected into the mammary fat pad of 10 SCID mice. A further 10 SCID mice were injected with MDA-MB-231 cells mixed with \(\beta\)6-1089 cells (total cell number 3x10\(^6\)/mouse). After 13 weeks tumor measurements were made and the longest (L) and the shortest (W) were used to calculate the tumor volume using the standard formula: \(1/2(L \times W^2)\). MDA-MB-231 cells injected with \(\beta\)6-1089 cells formed significantly larger tumors then those injected with N-1089 (p=0.0185). Statistical analysis by Mann-Whitney U test, p<0.05 considered significant, *<0.05.

f) MDA-MB-231 cells mixed with N-1089 (total cell number 3x10\(^6\)/mouse) were injected into the mammary fat pad of 10 SCID mice. A further 10 SCID mice were injected with MDA-
MB-231 cells mixed with β6-1089 cells (total cell number 3x10^6/mouse). After 13 weeks tumor measurements were made and the longest (L) and the shortest (W) were used to calculate the tumor volume using the standard formula: \( \frac{1}{2}(L \times W^2) \). MDA-MB-231 cells injected with β6-1089 cells (dashed and grey line) developed larger tumors more quickly than those injected with N-1089 (black lines). Thin lines show individual tumor sizes and thick lines show average tumor sizes.

**Figure 4. αβ6 induces MMP9 production through TGFβ activation in β6-1089 cells**

a) Gelatin zymogram on conditioned media from MDA-MB-231, MCF-7, β6-1089, N-1089 and T47D cells cultured for 24h. Medium was run on a 10% gelatin zymogram and incubated for 24h at 37ºC. The gel was stained with Coomassie blue and washed; a band detected at 92Kda indicates the source of the MMP9 is the β6-1089 cell line. rMMP9 = recombinant MMP9 (1μg/ml).

b) Real time PCR on RNA isolated from N-1089 or β6-1089 cells shows increased MMP9 RNA in β6-1089 samples. Data, from a pool of three separate experiments, show a significant increase in expression of MMP9 in β6-1089 cells relative to N-1089, which was set as 100%. Statistical analysis by Student’s t-test, p<0.05 considered significant, * <0.05.

c) Gelatin zymogram on conditioned media from β6-1089 pre-incubated with either IgG isotype antibody, anti-αvβ6 antibody (10D5) or TGFβRII blocking antibody, plated on TGF-β Latency Associated Peptide (LAP, 0.5μg/ml) and cultured for 24h. Medium was run on a 10% gelatin zymogram and incubated for 24h at 37ºC. The gel was stained with Coomassie blue and washed; a band was detected at 92Kda with control antibody but not in the presence of blocking antibodies indicating the secretion of MMP9 from β6-1089 cells is dependent on
activation of TGFβRII as well as binding of αvβ6; rMMP9 = recombinant MMP9 (100μg/ml).

d) Transwell invasion assay; MDA-MB-231 cells were plated in Transwells coated with Matrigel (diluted 1:3 in serum free media). Conditioned media from N-1089 and β6-1089 cells, grown in serum-free media for 48 hours, was placed in the bottom wells with either DMSO or MMP9 inhibitor (50nM). Plates were incubated for 24 hours and the number of invading cells quantitated by counting the cells on 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 tumor cells with β6-1089 CM is determined against this. Significantly higher invasion is observed where MDA-MB-231s were exposed to CM from β6-1089 cells with DMSO, which was ablated when the MMP9 inhibitor was added to the β6-1089 CM. Error bars show standard deviation, the graph is a pool of at least 3 independent experiments. Statistical analysis by Student’s t-test, p<0.05 considered significant, * <0.05, ** <0.01, ***<0.001.

e) Myoepithelial cells exhibit co-incident expression of MMP9 and αvβ6. Serial sections from 104 DCIS cases were stained and representative images are shown for (i) MMP9 and (ii) αvβ6. Staining for αvβ6 is limited to the myoepithelial cell layer in the DCIS duct and there is co-localized strong staining for MMP9. The upper panels were taken at x200 and the lower panels are the same section at x400.

f) The 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 Chi-Squared test, p<0.05 considered significant, * <0.05, ** <0.01, ***<0.001.
Table 1. Expression of αvβ6 in pure DCIS and DCIS associated with invasive disease.

Formalin fixed paraffin-embedded sections of normal/benign tissue, pure DCIS and DCIS with associated invasion were stained for αvβ6 integrin and the absence or presence of myoepithelial cell staining was recorded, the number and percentage of cases in each category is indicated. In 189/197 (96%) of high grade DCIS with invasive cancer cases the myoepithelial cell compartment stained positively for αvβ6. Whereas 104/157 (82%) of high grade pure DCIS cases exhibit αvβ6 expression. There is no detectable staining of αvβ6 in normal or hyperplastic breast tissue.

Table 2. Relationship between staining for αvβ6 and disease recurrence and/or progression

DCIS samples from the UKCCCR DCIS trial were stained for αvβ6 and scored as 1) positive or negative; or 2) 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 which recurred demonstrated a significantly higher αvβ6 H-Score than the control cases (p=0.006).

Table 3. Co-incident staining of MMP9 and αvβ6 in DCIS

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 90/156 (57%) DCIS cases the myoepithelial cell compartment stained positively for αvβ6 and MMP9. Whereas only 3/156 (2%) of DCIS cases positive for αvβ6 were MMP9 negative and 11/156 (7%) of αvβ6
negative cases were positive for MMP9. Pearson Chi-squared analysis demonstrated significant (p<0.0001) correlation between αvβ6 and MMP9 expression.
Table 1. Expression of αvβ6 in pure DCIS and DCIS associated with invasive disease.

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal</strong></td>
<td>0</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td><strong>Hyperplasia</strong></td>
<td>0</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td><strong>Pure DCIS</strong></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><strong>DCIS from Invasive</strong></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>
**Table 2. Relationship between staining for αvβ6 and disease recurrence and/or progression**

<table>
<thead>
<tr>
<th></th>
<th>Recurrence (n=52)</th>
<th>Controls (n=52)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-score</td>
<td>128.8 (103.7)</td>
<td>77.4 (81.8)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

**Matched logistic regression**

<table>
<thead>
<tr>
<th></th>
<th>P-value</th>
<th>OR* (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-score</td>
<td>0.01</td>
<td>1.01 (1.00-1.01)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*adjusted for grade and tumour size
Table 3. Co-incident staining of MMP9 and αvβ6 in DCIS

<table>
<thead>
<tr>
<th>Myoepithelial αvβ6 expression</th>
<th>MMP9 Positive</th>
<th>MMP9 Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoepithelial 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>
Figure 1

(a) Normal Duct → DCIS → DCIS/Invasion

(b) Normal
SMA  $\alpha v \beta 6$

Hyperplasia
SMA  $\alpha v \beta 6$

Low Grade DCIS
SMA  $\alpha v \beta 6$

High Grade DCIS
SMA  $\alpha v \beta 6$

(c) N-1089
β6-1089

(d) Graph showing fluorescence intensity vs. % of MAX.

(e) Western blot analysis:
- β6: 105 KDa
- HSC70: 70 KDa
Figure 2

(a) Relative Adhesion (vs IgG)

(b) No. of Migrating Cells

(c) Ave RLU

Downloaded from clincancerres.aacrjournals.org on April 20, 2017. © 2013 American Association for Cancer Research.
Figure 4.

(a) Shows a comparison of protein expression in MDA-MB-231, MCF-7, β6-1089, and N-1089 cell lines. T47D cells are also included for comparison.

(b) Graph illustrating the percentage of MMP9 expression in N-1089 and β6-1089 cells. Significant differences are indicated by an asterisk.

(c) Western blot analysis showing the expression of IgG, 10D5, TGF-βRII, and rMMP9.

(d) Bar graph showing the percentage of invasion in N-1089 + DMSO, β6-1089 + DMSO, N-1089 + MMP9 In, and β6-1089 + MMP9 In. Significant differences are indicated by asterisks.

(e) Images of tissue sections stained with MMP9 and αvβ6 antibodies, showing positive expression in both.

(f) Bar graph depicting the percentage of cases with avβ6+/MMP9+, avβ6+/MMP9-, avβ6-/MMP9+, and avβ6-/MMP9-. The graph indicates a significant difference with an asterisk.

Downloaded from clincancerres.aacrjournals.org on April 20, 2017. © 2013 American Association for Cancer Research.
Clinical Cancer Research

Altered Microenvironment Promotes Progression of Pre-Invasive Breast Cancer: myoepithelial expression of αvβ6 integrin in DCIS identifies high-risk patients and predicts recurrence

Michael D Allen, Gareth J Thomas, Sarah E Clark, et al.

Clin Cancer Res  Published OnlineFirst October 22, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-1504

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/10/22/1078-0432.CCR-13-1504.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.