Myoepithelial-specific expression of stefin A as a suppressor of early breast cancer invasion

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Abstract

Mammography screening has increased the detection of early pre-invasive breast cancers, termed ductal carcinoma *in situ* (DCIS), increasing the urgency of identifying molecular regulators of invasion as prognostic markers to predict local relapse. Using the MMTV-PyMT breast cancer model and pharmacological protease inhibitors, we reveal that cysteine cathepsins have important roles in early stage tumorigenesis. To characterize the cell-specific roles of cathepsins in early invasion, we developed a DCIS-like model, incorporating an immortalized myoepithelial cell line (N1ME) that restrained tumor cell invasion in 3D culture. Using this model, we identified an important myoepithelial-specific function of stefin A in suppressing invasion, whereby decreased stefin A promoted breast cancer cell invasion. Enhanced invasion was reliant on cathepsin B activation and interrogation of protein expression at the myoepithelial cell membrane revealed a major impact of stefin A loss on adhesion proteins including laminins, known substrates of cathepsin proteases. Use of a 138 patient cohort confirmed that myoepithelial stefin A is abundant in normal breast ducts and low-grade DCIS lesions, yet reduced in higher-grade DCIS lesions, supporting myoepithelial stefin A as a candidate marker of decreased risk of invasive relapse. We have therefore identified myoepithelial cell stefin A as a suppressor of early tumor invasion and a candidate marker to distinguish patients who are at a decreased risk of developing invasive breast cancer, and can therefore be spared further treatment.

Key words: myoepithelial cells, stefin A, breast cancer, cysteine cathepsins, 3D culture

Introduction

Ductal carcinoma *in situ* (DCIS) is a non-invasive breast cancer where malignant cells are confined to the ducts of the mammary gland (1). Before 1980, only 1% of all diagnosed breast cancer cases were pure DCIS, where it was usually identified as a mass-forming lesion (2). However due to the recent increased use of mammographic screening, between 15-25% of newly diagnosed breast cancers are pure DCIS (reviewed in (2)). Patients diagnosed with DCIS have a high survival rate of 98-99% and an overall risk of less than 1% for micro-invasion or metastasis to ipsilateral axillary lymph nodes (2). Breast-conserving surgery along with radiotherapy is a common option for DCIS-patients, and although five randomized trials found that radiotherapy reduced local recurrence rates by up to 50%, it did not appear to impact overall survival (reviewed in (2)). This highlights the need for markers that predict a good prognosis and those patients who can be spared adjuvant therapies.

Invasive breast cancer occurs when cancer cells break through the boundary of the duct (1). The 'boundary' of the ducts is composed of myoepithelial cells and the basement membrane, and the presence of these features is a distinguishing factor between DCIS and invasive breast cancer (3). Myoepithelial cells are spindle-shaped cells adjacent to the basement membrane that separate the inner layer of luminal epithelial cells from the interstitial stroma (4, 5). This single layer of cells is involved in the deposition of the basement membrane and contributes to the maintenance of polarity and mammary gland architecture in the normal breast (6). The loss of myoepithelial cells is sufficient to reduce epithelial polarity and promote cancer invasion (3, 6). The hypothesis that myoepithelial cells are natural tumor suppressors that resist malignant tumor transformation is supported by their ability to suppress tumor growth and invasion *in vitro* and *in vivo* (7, 8). Further, myoepithelial cells exhibit a proteinase inhibitor-dominated phenotype (7) that contributes to tumor suppression via the inhibition of proteases that have multiple pro-tumorigenic functions including invasion and angiogenesis (9). The interaction between tumor and myoepithelial proteases and inhibitors is not well understood, partly due to the lack of models that recapitulate this interaction.

A class of proteases prominently linked to tumorigenesis are the cathepsins, which are divided into serine, cysteine, and aspartyl types. The cysteine cathepsins are a group of lysosomal proteases that are involved in functions such as autophagy, apoptosis, antigen presentation and bone remodelling (reviewed in (10)). There are currently 11 identified human cysteine cathepsins: B, H, L, S, C, K, O, F, V, W and X/Z, most of which are optimally active in the acidic environment within lysosomes (10). The cysteine cathepsins (the focus of this article) are inhibited by their endogenous inhibitors, including the cystatin superfamily, comprising stefin A, stefin B and cystatin C (11). It is evident that the delicate balance between cathepsins and their inhibitors is important in tumorigenesis and metastasis (reviewed in (10)), where altered cathepsin localization to the plasma membrane or pericellular space (12) has been found to promote tumor cell invasion (via degradation of components of the extracellular matrix (ECM) including laminin, collagen IV and fibronectin (13) or cell-cell adhesion proteins such as E-cadherin (14)), angiogenesis (15) and epithelial-to-mesenchymal transition (16). The cancer-promoting roles of cathepsins are not restricted to tumor cell expression, with stromal cell-derived cathepsins also having important functions, such as macrophages (12, 14, 17). Of the cysteine cathepsins, cathepsin B has been widely implicated in tumor progression. In support of the pro-tumorigenic and metastatic functions of cathepsin B, cathepsin B deficient mice exhibit reduced metastatic burden in the MMTV-PyMT mouse model of mammary cancer (17). Furthermore, our group has demonstrated that tumor cell knockdown or selective pharmacological inhibition of cathepsin B resulted in a significant reduction of metastasis in transplantable mammary tumor models (18).

It is clear that cathepsin activation plays an important role in tumorigenesis, yet the cell-specific role of cysteine cathepsins and their inhibitors in early breast tumorigenesis is unclear. In this study, we utilize an *in vivo* model along with 3D models developed in the laboratory to investigate the cell specific contribution of protease inhibitors in the DCIS-to-invasive carcinoma transition. To date, incorporation of myoepithelial cells into 3D models has been rare, largely due to the limited number of stable normal breast myoepithelial cell lines available. We therefore developed a 3D model incorporating a hTERT immortalized myoepithelial cell line and demonstrated that these myoepithelial cells were sufficient to prevent invasive growth of MDA-MB-231 and CAL120 cell lines. Further analysis revealed that stefin A expression in the myoepithelial cells was critical for this function. For the first time, we confirm in patient-derived tissues that the expression of stefin A is highly abundant in myoepithelial cells surrounding normal ductal epithelium and low-grade DCIS lesions, yet is reduced in DCIS lesions with increasing grade and in micro-invasive lesions, supporting myoepithelial stefin A as a candidate myoepithelial-specific tumor suppressor.

Methods

Mouse models

Mouse investigations were performed after approval by the La Trobe University Animal Ethics Committee. Bl/6 MMTV-PyMT positive female mice were injected (intraperitoneal, 200 µl/20g mouse) daily with 50mg/kg CA-074 (synthesized and purified in the Bogyo laboratory, CA) or vehicle (5% DMSO/saline) from day 30 - 49. On day 50, mice were culled and second, third and fourth mammary glands were resected and sent for histology. Mouse tissues were fixed in 10% buffered formalin for 6 hours and paraffin-embedded. Sections at three different depths throughout the mammary gland were taken and were stained with hematoxylin and eosin according to standard protocols. Alternatively, sections were stained with 1 µg/ml anti-α-smooth muscle actin (Abcam, ab66133) or with isotype control antibodies, as below (IHC). Sections were scored by a pathologist blinded to treatment groups (Sandra O'Toole) for the presence of invasive regions of cancer growth within the mammary gland. Experiment included 8 mice per group.

Derivation of the N1ME myoepithelial cell line

Fresh human breast reduction mammoplasty tissue was obtained and digested to single cell suspension. Myoepithelial cells were immunopurified using anti-CD10 magnetic beads (CD10 antibody, Dako cat#M0727, Beads, Dynal Pan mouse IgG cat# 110.23) as previously described (19). The retroviral expression vector pMSCV-CMV-puro-hTERT was transfected into Phoenix packaging cells using Fugene6 (Promega, WI, USA). Conditioned media was filtered and incubated with the myoepithelial primary cells along with polybrene. Myoepithelial cells were then selected with 0.4µg/ml puromycin and named N1ME. Initially the cells were grown in Medium 171 (Cascade Biologics, M-171-500) supplemented with mammary epithelial growth supplement (MEGS, Cascade Biologics, S-015-5), penicillin/streptomycin and puromycin. Recently, the N1ME cell line has been maintained in Mammary Epithelial Cell Growth Medium (MEGM) (Lonza, Switzerland, CC3151) with Single Quot supplements (Lonza, CC-4136). After some passaging, the N1ME cell line was retrovirally infected with pMSCV-mCherry vector, as described above but with the PT67 packaging cell line transfected using Lipofectamine (Invitrogen, Carlsbad, CA, USA), and sorted by flow cytometry performed using standard techniques.

Cell culture

The MCF10.DCIS.com (DCIS) cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM): Nutrient Mix F-12/5% Fetal Bovine Serum (FBS)/1% penicillin/streptomycin. The MDA-MB-231, MDA-MB-231-GFP and CAL-120 cell lines were maintained in DMEM/10% FBS/1% penicillin/streptomycin. All cell lines were maintained at 37 °C, 5% CO_2. In 2013, MCF10.DCIS.com (DCIS) (20) and MDA-MB-231 human breast carcinoma cells were provided by B. Sloane. In 2014, MDA-MB-231 cells were obtained from ATCC and labelled with GFP by A. Möller. The CAL-120 human breast carcinoma cells were obtained from DSMZ by Dr. Elgene Lim in 2014. Cell lines were tested and authenticated by short tandem repeat (STR) profiling and mycoplasma tested in 2015 and 2016. TALEN and siRNA constructs used to disrupt expression or knockdown proteins of interest are described in supplementary material.

FACS analysis

N1ME, DCIS and MDA-MB-231 cells were assessed for basal, luminal and myoepithelial markers as previously described (21). In brief, cells were stained with a cocktail of lineage markers (PE conjugated CD45, CD235a, CD31) and then with epithelial subpopulation specific markers (EpCAM-PB and CD49f-PE-Cy7). All cells were resuspended in propidium iodide to allow gating on viable cells only. The BD LSR Fortessa X20 was used to analyze all samples. Compensation was completed manually at the time of sample acquisition, using single color controls in each experiment. All data files were analyzed using the free software program FlowLogic.

3D Cell culture

All 3D cultures were performed using a reconstituted basement membrane, Cultrex® (Trevigen, Gaithersburg, MD, 3433-005-01). Glass bottom 8 well chambers (ThermoFisher, NUN155409) were coated with 100% Cultrex and allowed to solidify at 37 °C for 20 minutes. Cells (pre-mixed at a predetermined ratio) were seeded on top of the solidified Cultrex and allowed to adhere for 60-90 minutes before 2% Cultrex in MEGM media (Lonza) was overlaid. Media was changed every 4 days unless otherwise stated. Inhibitor 3D studies were performed by the addition to the media of 50 µM of the highly selective cathepsin B inhibitor CA-074 or the pan-cysteine cathepsin inhibitor JPM-OEt (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, MD) reconstituted in dimethyl sulfoxide (DMSO), or DMSO as control, this was refreshed every 48 hours. Microscopy techniques are described in supplementary material.

Quantification of circularity of 3D cultures

Bright field images of 3D cultures were processed and analyzed using the Fiji distribution of ImageJ (22) as follows. Extraction and mask generation of the individual cultures was carried out by applying an edge filter followed by an unsharp mask (radius=6, mask=0.8). The resulting image was blurred using a Gaussian filter (sigma=4) to make subsequent thresholding more accurate. A threshold was applied, manually adjusted if required and used to create a binary mask. The resulting mask was filtered by size to remove small, erroneous, debris. Each individual mask was then measured for its perimeter and convex hull lengths. The result for the convex hull length was divided by the perimeter length to generate the convex hull to perimeter ratio used for the subsequent data analysis.

All statistics were calculated and graphed using Prism version 7. Frequency distributions for each sample group (n=3) were generated to show the distribution of convex hull to perimeter ratios for each sample group. Each distribution was then fitted with a Log Gaussian fit and the centers of each fit were compared for statistical variation. Variation in mean convex hull to perimeter ratio was determined using a two-tailed unpaired t-test (for 2 groups) or a one-way ANOVA followed by Tukey's post hoc analysis (for >2 groups).

Protease labelling and Western blotting

Cells were lysed by freezing in citrate buffer (50 mM citrate, pH 5.5, 0.5% CHAPS, 0.1% Triton X-100, 4 mM DTT). For cathepsin B activity gels, activity-based probes [GB123 (1 μM) (23) or BMV109 (0.1 μM) (24)] were added to lysates from a 100x stock, and proteins were incubated for 30 minutes at 37 °C. The addition of 4x sample buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 0.04% bromophenol blue, 5% beta-mercaptoethanol) stopped the reaction with boiling for 10 minutes. Proteins (30-50 μg) were separated by SDS-PAGE (on either 15% acrylamide or gradient 4-20% gels, precast from Bio-Rad, CA, USA) and visualized using a Typhoon flatbed laser scanner (GE Healthcare, UK) for Cy5 fluorescence. For immunoblotting, proteins were transferred to nitrocellulose membranes and subjected to standard Western blotting protocols. Membranes were incubated with 1 μg/ml primary antibodies against stefin A (Abcam, Cambridge, UK, ab61223), stefin B (Abcam, ab53725), cystatin C (R&D Systems, MN, USA, AF1196) and cathepsin B (R&D Systems, AF965), or 1:10,000 dilution of β-actin (Sigma-Aldrich, MO, USA, A22280) or GAPDH (D16H11, Cell Signalling, MA, USA, 8884). Signal was detected using ECL (GE Healthcare), G:Box GelDoc (Syngene, CBG, UK) and GeneSys (Syngene) software with automatic exposure. Method for densitometry analysis is described in supplementary material.

Mass spectrometry

Isolation and enrichment of cell lysate, plasma membrane and soluble fractions

Cell lysates were isolated from human breast N1ME, DCIS.com and MDA-MB-231 cells ($1x10^6$ cells approx.) using detergent cell lysis and centrifugation, as detailed in supplementary material. Plasma membrane and soluble fractions were isolated using a combination of carbonate extraction, cell lysis, ultracentrifugation, and acetone precipitation, as detailed in supplementary material.

Proteomic analysis of cellular, membrane and soluble profiles of human breast N1ME, DCIS and MDA-MB-231 cells.

Cellular lysates, and enriched fractions for cellular membrane and soluble samples from human breast N1ME, DCIS and MDA-MB-231 cells were analyzed by mass spectrometry-based proteomics using an in-gel digestion approach followed by nanoliquid chromatography (Ultimate 3000 RSLCnano) coupled directly to a Q-Exactive HF Orbitrap (ThermoFisher Scientific) mass spectrometer operated in data-

dependent acquisition mode, as described in supplementary material. The mass spectrometry proteomics data have been deposited to the PeptideAtlas repository with the data set identifier PASS01048.

Relative quantification of cellular, membrane and soluble profiles by mass spectrometry

The relative abundance of cellular, plasma membrane and soluble profiles by mass spectrometry were determined by label-free quantitation using either precursor intensity (MaxQuant, Andromeda, LFQ) or peptide spectral matches/spectral counting (Proteome Discoverer, Scaffold Q+S, spectral counts), as described in the Extended Experimental Procedures.

Immunohistochemistry (IHC)

For human tissues, normal breast sections and primary breast carcinoma samples were obtained from Sandra O'Toole at the Royal Prince Alfred Hospital (RPAH) either as full-faced slides (for the micro-invasive carcinoma) or in a tissue microarray (TMA) (25). The use of archived human tissues was approved by the HREC of RPAH (approval number X15-0388 (SSA/16/RPAH/397)). Sections (formalin-fixed, paraffin embedded) were stained with 1 µg/ml anti-human stefin A (Abcam, Cambridge, UK, ab61223), p63 (Dako, Denmark, DAK-p63, following antigen retrieval), anti-human α-smooth muscle actin (Abcam, ab66133) or with isotype control antibodies, overnight at 4 °C and detected with a biotin-conjugated secondary antibody (Vector Laboratories, CA, USA) for 1 hour at room temperature. Peroxidases were blocked and ABC reagent (Vector) and DAB peroxidase substrate kit (Vector) were used to visualize specific staining. Human stefin A staining was scored by an independent pathologist, Elizabeth Robbins (see supplementary material). Data input was performed by Cristina Selinger, and statistics were performed by Dr. Timothy Molloy.

Statistical Analysis

Statistics were conducted using the data analysis software package within GraphPad Prism v7 for Windows (GraphPad Software) and PASW Statistics 18 (SPSS). Error bars indicate SEM unless otherwise stated.

Results

Treatment with cathepsin B inhibitors decreases invasive growth in vivo

It has been well documented that cysteine cathepsins and their inhibitors have important roles in breast cancer; however, their role in early breast cancer is not well studied. To test the therapeutic efficacy of cathepsin inhibitors in the DCIS-to-invasive transition in an *in vivo* model of early tumorigenesis, we treated MMTV-PyMT mice (which spontaneously develop mammary gland tumors (26)) with the cathepsin B-selective inhibitor CA-074 for the time period between DCIS and invasive carcinoma development in this model (30-50 days). At the time of treatment cessation, mammary glands were histologically evaluated, including assessment of smooth muscle actin expression as a myoepithelial marker (Fig. 1A). Comparison of the treatment (CA-074) versus control (DMSO) groups revealed that cathepsin B inhibition decreased the number of invasive regions detected in the mammary glands (Fig. 1B). CA-074-treated mice developed DCIS yet rarely progressed to invasive disease at experimental endpoint (2/8), in contrast to the development of invasive disease in most of the control group (6/8) (Fig. 1C). This was independent of tumor cell proliferation, as confirmed by equivalent Ki67 staining in control and treatment groups (Supplementary Fig. S1). Together, these data supported cathepsins in early tumorigenesis yet the cell specific functions could not be tested in this model.

Characterization of a myoepithelial cell line for modelling DCIS

The presence of an intact myoepithelial layer is the key distinguishing factor between DCIS and invasive pathologies, hence to investigate this interaction *in vitro* a myoepithelial cell line is necessary. The N1ME myoepithelial cell line was derived via immunopurification of CD10 positive cells from normal breast tissue and hTERT immortalization. To ensure the N1ME cell line had standard myoepithelial cell characteristics, extensive characterization and analysis was completed. N1ME cells have smooth muscle cell-like morphology when grown in 2D and grow in spheroids in 3D (Supplementary Fig. S2A and B), as expected. To confirm that N1ME cells expressed basal cell markers we used flow cytometry to measure the cell surface expression of EpCAM and CD49f, previously accepted markers to distinguish luminal, basal and stromal populations (21). The N1ME cells had high CD49f and low EpCAM, characteristic of basal cells (Fig. 2A). This also confirms lack of contaminating myofibroblasts, that could have potentially been purified from the normal breast; myofibroblasts have previously been identified in the EpCAM low/CD49f low stromal compartment (21). As controls, we used DCIS.com and MDA-MB-231 cells. As expected, the DCIS.com cells expressed markers (EpCAM high/CD49f^{+}) consistent with luminal breast progenitors, whilst the MDA-MB-231 cells were also basal-like (Fig. 2A), as expected given that this tumor line is phenotypically basal. To further characterize the identity of the N1ME line and distinguish it from the basal MDA-MB-231 line, we compared the proteome of these cell lines using mass spectrometry. This revealed 388 proteins uniquely identified in the N1ME cells in comparison to the DCIS and MDA-MB-231 cells (Supplementary Fig. S2C, Supplementary Table S1). Interrogation of unique proteins and comparison with protein signatures previously identified for purified normal breast myoepithelial and luminal cells (27) revealed that the N1ME cells indeed expressed myoepithelial markers and lacked the epithelial and tumor cell markers expressed in the DCIS.com and MDA-MB-231 cell lines (Fig. 2B, Supplementary Table S2). Together, these data supported the myoepithelial identity of the N1ME cell line.

Recapitulating DCIS in vitro *using a 3D co-culture model*

We next incorporated the N1ME cell line into 3D tumor cell cultures. When grown in 3D overlay culture on a reconstituted basement membrane (Cultrex®), the DCIS.com cell line grows in a non-invasive

spheroid-like structure (Supplementary Fig. S2D). Co-culture of these non-invasive cells with N1ME cells results in no apparent alterations in invasive phenotype or overall cell density (Supplementary Fig. S2D). Confocal microscopy of these cultures revealed that the N1ME cells surround the DCIS cells (Supplementary Fig. S2E).

To analyze the impact of myoepithelial cells on more invasive cells, the triple negative MDA-MB-231 breast cancer cell line was used. In 3D culture, the MDA-MB-231 cells were distinctly more invasive than the DCIS.com line with distinctive invasive protrusions spreading through the cultrex (Fig. 2C, blue Hoechst stained). Co-culture of these cells with N1ME revealed a clear reversion of this invasive phenotype, whereby the addition of N1ME (red) cells reverted growth of this cell line to a DCIS-like phenotype (Fig. 2C). This phenotype was maintained for over 14 days (Supplementary Fig. S2F). This restriction of invasion by N1ME cells was also observed using the CAL120 triple negative invasive breast cancer cell line (Fig. 2F). This is the first time reversion of invasive breast cancer cells by myoepithelial cells has been visualized in 3D culture. This phenotype was specific to myoepithelial cells in 3D culture and could not be recapitulated in 2D culture (Supplementary Fig. S2G) or using non-myoepithelial cell lines (Supplementary Fig. S2H).

To statistically compare the difference in tumor cell invasion when cultured in 3D alone or in combination with N1ME cells, we used a measure of circularity (the perimeter to convex hull ratio) in which a reduction of circularity provides a measure of invasive phenotype (Supplementary Fig. S2I-K). These calculations revealed that the addition of N1ME myoepithelial cells to invasive cancer cells (MDA-MB-231 or CAL120) resulted in more circular colonies, and hence less invasive structures in these co-cultures (Fig. 2D, E, G, H). This method therefore provides a quantitative measurement of the circularity/invasiveness of 3D co-cultures.

Reduction of stefin A promotes breast cancer cell invasion

Given our *in vivo* results implicating cathepsins in early invasion, we investigated the expression of cathepsin B and the cystatin family of cathepsin inhibitors in the tumour and myoepithelial cell lines incorporated in the 3D model. Interestingly, N1ME cells have a cathepsin inhibitor-dominant phenotype, with high levels of stefin A and stefin B detected and to a lesser extent cystatin C (Fig. 3A). In tumor cell lines, levels of stefin A were inversely correlated with invasive phenotype, with MDA-MB-231 cells having the lowest expression (Fig. 3A). Stefin B was expressed at similar levels in all cell lines, while cystatin C expression was elevated in the more invasive cell lines (MDA-MB-231 and CAL120) (Fig. 3A). Although the levels of mature (25/30 kDa) and pro (50 kDa) cathepsin B was similar in all cells lines (Fig. 3Bi), use of the activity-based probe GB123 (23) confirmed that cathepsin B activity was increased in tumor lines with highest metastatic potential (MDA-MB-231, Fig. 3Biii), as expected in view of its pro-tumorigenic roles. In contrast, cathepsin L activity did not correlate with metastatic potential or cystatin expression. Importantly, the N1ME cells had very low cathepsin B activity (Fig. 3Biii), most likely due to inhibition by the cystatins which are abundantly expressed in these cells.

Given the high levels of cystatins in the myoepithelial cell line, we next wanted to test their function in our 3D model. To do this, a small siRNA screen was conducted to selectively knockdown these cystatin cathepsin inhibitors in N1ME cells (Supplementary Fig. S2L-N). Although the siRNA control lines blocked MDA-MB-231 cell invasion, knockdown of stefin A could not restrain tumor invasion (Fig. 3C and D). The impact of stefin A was greater than that observed with knockdown of stefin B and cystatin C, where only very minor tumor outgrowths, or no invasion was observed, respectively (Fig. 3C and D). Given that stefin A expression was high in the N1ME cells, correlated with cathepsin B activity (Fig. 3A and B), and that

knockdown had the greatest impact on tumor cell invasion, we wanted to further confirm its invasion suppressive function by using stable gene editing of the N1ME cell lines.

Stefin A low (heterozygote null) N1ME cell lines were created using transcription activator-like effector nucleases (TALENs), resulting in a 60-80% decrease in stefin A expression (Supplementary Fig. S3). As expected, reduction in stefin A expression resulted in an increase in cathepsin B activity (Supplementary Fig. S3Aiii). Such a correlation was not observed with cathepsin L activity. Although a reduction in stefin A expression did not impact myoepithelial cell proliferation or morphology (Supplementary Fig. S3D and E), it had a dramatic effect in 3D co-culture. The stefin A low N1ME cells failed to inhibit MDA-MB-231 cell invasion to the extent observed with wild type (WT) N1ME cells (Fig. 4), confirming the results achieved with the siRNA experiments. This result suggests that a reduction in stefin A within the myoepithelial cells allows tumor cell invasion even though the myoepithelial cells remain physically present. This was observed using both unlabeled and GFP-labeled MDA-MB-231 cells and the CAL120 cell line (Fig. 4, Supplementary Fig. S3G-I). Together, these findings demonstrate the importance of stefin A in the myoepithelial-driven suppression of tumor cell invasion.

To confirm that the alteration in phenotype was due to the role of stefin A as a cathepsin inhibitor, we treated MDA-MB-231 cells alone, or co-cultured with the stefin A low N1ME line with cathepsin B specific (CA-074) and pan-cysteine cathepsin (JPM-OEt) inhibitors. We reasoned that, given stefin A is secreted from N1ME cells (Supplementary Fig. S4A), addition of inhibitors to the media was feasible. Indeed, we observed that CA-074 treatment rescued the phenotype caused by stefin A loss, reverting the invasive protrusions of the co-cultures back to the DCIS like state observed using WT N1ME cells (Fig. 5 top panels). JPM-OEt also reverted the invasive protrusions in the co-cultures, however this was not significant compared to vehicle control treatment (Fig. 5). Importantly, this phenotype was not observed in the absence of myoepithelial cells. Use of the inhibitor did not inhibit invasion of the MDA-MB-231 cells cultured in the absence of N1ME cells; in fact, it made the breast cancer cells more invasive (Fig. 5 bottom panels). This was also observed using N1ME conditioned media or recombinant stefin A (Supplementary Fig. S4B, C), where tumor cell invasion was not suppressed. These results indicate that both the physical presence of myoepithelial cells and intact stefin A expression is required to block invasion, suggesting that stefin A loss alters the tumor suppression function of the myoepithelial cells by increasing the proteolysis of a myoepithelial cell substrate.

Altered adhesion protein profile in stefin A low myoepithelial cells

A number of cathepsin B substrates have been implicated in tumor invasion, including membrane associated and extracellular matrix proteins. Membrane preparations of N1ME WT cells and N1ME stefin A low cells were compared by label-free quantitative mass spectrometry. For proteins differentially expressed (ratio of spectral count [Rsc] ±2), gene ontology analysis (biological process) revealed that N1ME stefin A low cells displayed a loss in expression of cell adhesion-associated proteins (Fig. 6A and B, Supplementary Table S3). Of note, three laminin subunits were amongst those most reduced in the N1ME stefin A low cells compared to WT cells (Fig. 6B). Laminin has previously been identified as a substrate of cathepsin B at both neutral and slightly acidic pH (28), suggesting that enhanced cathepsin B activity in the stefin A low cells could be responsible for decreased cell surface expression of these adhesion proteins. Together, these results suggest that the function of stefin A in N1ME cells is to conserve cell-cell and cell-matrix adhesion, reducing tumor cell invasion. Such a phenotype is observed in the 3D cultures where tumor-myoepithelial cell adhesion is clearly lost upon stefin A reduction. Together, this data suggests that the physical constraints of myoepithelial cells on inhibiting cancer cell invasion via cell-cell and cell-matrix adhesion are reliant on stefin A expression. This also supports our findings that cathepsin

inhibitors alone cannot inhibit tumor cell invasion in the absence of myoepithelial cells, as the likely cathepsin target that promotes invasion is of myoepithelial origin.

Stefin A expression is abundant in myoepithelial cells surrounding normal ductal epithelium and low-grade DCIS lesions, yet is lost in high-grade DCIS lesions

Our studies utilising the N1ME cell line suggest that stefin A is highly abundant in myoepithelial cells. To confirm our findings clinically, stefin A expression was assessed in breast tissue derived from cancer-free women. Indeed, we detected abundant stefin A expressed in the myoepithelial cells surrounding normal ducts (Fig. 7A and B). Expression patterns were confirmed by the use of two independent stefin A antibodies (Supplementary Fig. S5A). We then interrogated cell-specific stefin A expression in early stage tumorigenesis using a tissue microarray (TMA) comprising sections of >800 lesions encompassing benign ducts, usual ductal hyperplasia, low, intermediate or high nuclear grade DCIS (derived from 138 patients diagnosed with DCIS only). The myoepithelial expression of stefin A was retained in hyperplastic and low grade DCIS lesions (Fig. 7C and D), yet was reduced or absent in many intermediate and high-grade DCIS lesions (Fig. 7E, G). The distinction between DCIS and invasion is the presence of the myoepithelial cell layer (3) and myoepithelial marker immunohistochemistry (IHC) is used widely in diagnostic clinical practice to aid in this distinction. To rule out loss or attenuation of the myoepithelial layer in stefin A negative lesions, serial sections were stained with p63 (Fig. 7F), a nuclear myoepithelial marker. Only p63 positive samples were included in the analysis. Importantly, stefin A expression inversely correlated with DCIS grade (Fig. 7G), yet did not correlate with ER, PR, histological grade or tumor size (Supplementary Table S4A). A fraction (35%) of normal ducts lacked myoepithelial stefin A, and currently the implications of this loss on future breast cancer risk is unknown.

The negative correlation between stefin A expression and DCIS grade was restricted to myoepithelial cells. Evaluation of stefin A expression in the tumor epithelium (Supplementary Fig. S5B) revealed an increase in DCIS lesions in general, and an increase with grade (Supplementary Fig. S5C, Supplementary Table S4B). This suggests that the role of stefin A in early tumorigenesis is likely cell dependent and therefore it is the loss of myoepithelial cell stefin A surrounding DCIS lesions that is most implicated in the DCIS-invasive transition. In support of this, knockout of stefin A in the DCIS.com cell line did not affect cell growth or invasion in 3D culture (Supplementary Fig. S5D-F).

Patients diagnosed with high-grade DCIS have an increased risk of local invasion compared to low grade lesions (29). However, as clinical follow-up on the subsequent development of invasive carcinoma (fortunately a rare event as patients received modern treatment) was not available, we investigated stefin A expression in high-grade DCIS lesions with associated micro-invasive regions, the earliest phase of invasion. Micro-invasion is defined as an invasive focus measuring no more than 1mm. In this study alpha smooth muscle actin (SMA), a cytoplasmic/cytoskeletal myoepithelial marker, was used to highlight the presence of the myoepithelial cells, including identification of any small focal breaks in the myoepithelial cell boundary (Fig. 7H, white arrows). In line with an association between stefin A loss and tumor invasion, it was observed that DCIS lesions with micro-invasion did not express myoepithelial stefin A (Fig. 7H, Supplementary Fig. S6). Suggesting that the decrease in myoepithelial stefin A expression predicts invasion and that loss of stefin A may precede myoepithelial cell loss in invasive lesions. This supports our findings with the 3D co-culture models in that intact stefin A expression is important in myoepithelial-specific suppression of tumor invasion.

Discussion

This study implicates myoepithelial stefin A in preventing the progression of DCIS-to-invasion. We reveal that targeted loss of stefin A in myoepithelial cells is sufficient to promote or restore tumor cell invasion

in a 3D DCIS-like model developed in the laboratory. This function relies on the cathepsin inhibitory role of stefin A as cathepsin inhibitors could rescue this phenotype. Critically, here we report for the first time that stefin A is highly expressed in myoepithelial cells of low-grade DCIS lesions, those that have the lowest risk of local recurrence within 10 years (29). These data suggest that myoepithelial stefin A has an important suppressive function in the DCIS-to-invasive transition and that it is worthy of further investigation as a prognostic marker, to distinguish patients who are at a decreased risk of developing invasive breast cancer, and could therefore be spared from adjuvant therapies.

In our MMTV-PyMT study, we confirmed that cathepsin inhibition was sufficient to reduce the development of invasive lesions *in vivo*. Previously, increased expression of cathepsin B in the MMTV-PyMT model did not affect palpable tumor onset, but accelerated tumor progression (30). In the same model, genetic ablation of cathepsin B resulted in decreased primary tumor growth and increased tumor latency (17). These findings correlate with the results of our *in vivo* experiment and further validate the role of cathepsin B in tumor onset and progression. However, these previous studies did not investigate cathepsin B during the early progression of DCIS-to-invasion. As up to 25% of new breast cancer patients are diagnosed at the DCIS stage (2), we believe identifying the role for cathepsins and their inhibitors i.e. stefin A at this early time point is critical.

Previous studies on the involvement of stefin A in tumorigenesis are contradictory with reports that it is a tumor-suppressor in some cancers (31-33), yet a malignant marker in others (34-36). However, investigations into the cell-specific expression and function of stefin A in early tumorigenesis are limited. Here we report that a critical source of stefin A at the DCIS stage is the myoepithelial cells. A study by Lee et al. reported that stefin A expression decreases in tumor cells of invasive lesions compared to DCIS and that stefin A reduction promotes tumor invasion (37). The comparison of DCIS samples to invasive lesions did not allow an assessment of whether stefin A loss can occur in DCIS lesions before invasion, nor did it assess changes to the myoepithelial compartment as we have investigated in the current study.

Our work supports others that suggest myoepithelial cells have very important roles in dictating invasion. Certainly, changes to these cells that may promote invasion have been described. Myoepithelial cells associated with DCIS lesions have been documented to have a different gene expression profile than normal myoepithelial cells (19, 38). Specifically, these data demonstrated that DCIS-associated myoepithelial cells had upregulated angiogenesis and invasion genes and a decrease in the expression of a number of differentiation markers (19, 38). Therefore, the need for myoepithelial cells derived from normal tissue is critical to allow for functional studies that interrogate the role of such genes/proteins in cancer cell invasion. Our study has used myoepithelial cells derived from normal tissue and by developing 3D *in vitro* models allowed investigations into the structural and functional interactions between cell types, a phenomenon that is often lost in 2D monolayers. We have revealed that myoepithelial cells can in fact restrict the outgrowth of highly invasive breast cancer cells in a 3D co-culture model, recapitulating early breast cancer lesions, and that stefin A is an integral factor in this reversion. Helping to define the mechanism by which myoepithelial cells inhibit tumour development. This speaks to the critical role of the tumor microenvironment in impacting tumor behavior. We were also able to develop methods to quantify this reversion of invasion, providing a template for future studies investigating molecular drivers of the myoepithelial-derived suppressive function. It has previously been revealed that conditioned media from myoepithelial cells can suppress the invasion of tumorigenic cell lines (7, 39, 40), however in our experiments the presence of myoepithelial cells along with stefin A expression was required to suppress invasion. Suggesting that the expression of stefin A in myoepithelial cells is required for its function as a physical barrier. This was further reinforced when the addition of cathepsin inhibitors to cultures of breast cancer cells alone had no effect, yet was able to reverse the effects of stefin A reduction in N1ME cells.

Given these data, we hypothesised that stefin A inhibits cathepsin B degradation of a myoepithelial target, preserving adhesion or polarization of epithelial and myoepithelial cells resulting in a DCIS-like state. Alterations in cell-cell and cell-matrix adherence are considered some of the first processes that cells undergo to initiate the process of invasion (41) and cell adhesion proteins such as E-cadherin and laminin have been identified as substrates of cathepsins (14, 28). It is plausible that the increase in cathepsin B activity caused by the reduction of myoepithelial stefin A could enhance cleavage of cell adhesion proteins, promoting tumor cell invasion and outgrowth. This is consistent with our observation of a loss of cell adhesion proteins in stefin A low myoepithelial cells, including laminin. Strongly correlating with this data is a study revealing tumour-associated myoepithelial cells had reduced laminin-1, and these cells were unable to instill polarity in acini cultures in comparison to myoepithelial cells from normal glands (6). Therefore, we hypothesize that the increased activity of cathepsin B in the stefin A low N1ME cell line allows for cleavage of adhesion proteins, resulting in decreased cell adhesion of myoepithelial cells and permitting cancer cell invasion, again highlighting the need for both stefin A expression and myoepithelial cell physical presence. Similar to our results with stefin A, matrix metalloproteinase-8 (MMP-8) is expressed by normal myoepithelial cells, but is lost in DCIS, and overexpression of MMP-8 in myoepithelial cells resulted in an increase in adhesion (42).

If diagnosed and treated early, patients with DCIS have a very high chance of survival (Burstein et al., 2004), in fact many may not need treatment at all. Identifying prognostic markers in DCIS lesions is necessary for individualizing treatment options for the increasing number of patients diagnosed with early breast cancer. To date, there are limited biomarkers that allow prediction of risk of relapse in patients diagnosed with DCIS. Despite considerable effort to identify tumor cell markers that predict DCIS progression, there are limited markers to date that warrant further evaluation. This is in part due to studies that reveal limited genetic and transcriptional differences between tumor cells in DCIS and invasive lesions (43, 44). A commercial test currently available for predicting disease recurrence in women with early-stage breast cancer is the Onco*type* DX ® Recurrence Score (RS), based on the expression of 21 genes (45). This has now been adapted for DCIS, whereby an Onco*type* DX DCIS® score has been developed (46). While this test can aid in patient treatment decisions for those with low or high scores, 16-25% of patients will fall into the 'intermediate' score range, indicating it's 'unclear' whether they will receive benefits from adjuvant therapy (45, 46). Ongoing trials are therefore required to determine the utility of these scores in discriminating indolent and high-risk DCIS lesions. Given the similarities between tumor cells of DCIS and invasive lesions, prognostic markers in the surrounding microenvironment may hold great promise. To date, although protease inhibitors in myoepithelial cells have been implicated in tumor progression, none have shown promise as prognostic markers. Our finding that stefin A is decreased in high-grade and micro-invasive lesions, yet abundant in low-grade DCIS lesions, suggests its potential as a prognostic marker for discriminating DCIS lesions with a decreased risk of local recurrence. Culmination of stefin A into an affordable next generation assay or even an IHC based assay may be beneficial, and will need to be tested in larger follow-up cohorts.

By combining a DCIS patient cohort and 3D co-cultures, this study demonstrates that myoepithelial stefin A loss promotes invasion even in the presence of an intact myoepithelial layer. We therefore suggest that stefin A expression may serve as a prognostic marker to predict DCIS patients with minimal risk of invasive relapse, and those that could be spared adjuvant therapies such as radiotherapy. Prognostic studies in DCIS cohorts with follow-up data will be important to confirm stefin A as a biomarker to aid in individualized treatment decisions.

Author Contributions

Conceptualization, H.M.D., L.E.E.M. and B.S.P.; Methodology, H.M.D., J.R., L.E.E.M., D.S. and B.S.P.; Software, C.J.N.; Formal analysis, H.M.D., T.J.M., D.W.G. and C.J.N.; Investigation, H.M.D., J.R., A.S., E.R., N.K.B., P.H., P.F., K.L.B., M.C. and D.W.G.; Resources, K.P., M.B., A.M., B.S., C.S.L., A.H., C.I.S., S.A.O.T., B.F.S. and B.S.P.; Writing – original draft, H.M.D. and B.S.P.; Writing – Review and Editing, H.M.D., B.S.P., S.A.O.T., L.E.E.M., J.R., D.A.S., D.W.G., K.L.B., T.J.M. and C.S.L.; Funding Acquisition, B.S.P.; Supervision, B.S.P. and L.E.E.M.

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Figure 1. Cysteine cathepsin inhibition *in vivo* decreases development of invasive lesions in mouse mammary glands. (A) Representative images of second, third or fourth mammary glands with DCIS/invasive regions from mice treated with 50mg/kg CA-074 or DMSO (control) in saline for 20 days. At day 50, mice were culled and mammary glands harvested, sectioned, and stained by H&E. Serial sections were stained with anti-smooth muscle actin (myoepithelial marker) and visualized with DAB. These sections were counterstained with hematoxylin. Representative images from eight mice per group. Scale bars represent 25 µm. Mammary glands of all mice were blindly scored by a pathologist and were

determined to be invasive or non-invasive (normal, hyperplasia, DCIS). (B) Percentage of mammary gland with each diagnosis per group were graphed. (C) The final diagnosis for each mouse was determined and compared between groups. *p<0.05 by Chi-square test.

Figure 2. Characterization and 3D modelling of breast myoepithelial and cancer cell lines. (A) Cell surface EpCAM and CD49f expression determined on cell lines using flow cytometry analysis. Plots show EpCam and CD49f expression on cells after gating on viable cells that are Lin negative (CD45- CD235a- CD31-); N1ME: CD49f^{Hi}/EpCAM^{low}; DCIS: CD49f^{Hi}/EpCAM^{Hi}; MDA-MB-231: CD49f^{Hi}/EpCAM^{low}. (B) A heat map depicting the correlation expression profile of select differentially expressed proteins in myoepithelial cellular proteomics (N1ME) in comparison to luminal (DCIS) and basal (MDA-MB-231) cellular models. Data represents differential abundance based on normalised LFQ intensity values (*n* = 3). Bright field and confocal images, rendered in Imaris of (C) MDA-MB-231 and (F) CAL120 invasive breast cancer cells (Hoechst stained - blue) grown on reconstituted basement membrane with overlay alone, and co-cultured with N1ME cherry-labelled myoepithelial cells (red) for 7 days. Representative images of *n* = 3. Scale bars represent 200 µm. (D, G) Differences in invasive growth of 3D cultures was determined by calculating the ratio between the perimeter and convex hull of each colony (circularity). A value of 1 indicates a smooth object, as the value moves away from 1 towards zero the number and/or size of protrusions from the colony is increased. (E, H) Frequency distribution of population data under log Gaussian fit. A bin center closer to 1 indicated a smooth colony surface. Comparison of center of each curve was statistically analyzed, ****p<0.0001. *n* = 3.

Figure 3. siRNA knockdown of cathepsin inhibitors affects myoepithelial cells' ability to control invasive breast cancer cells. (A) Expression of stefin A, stefin B and cystatin C detected by Western blotting in whole cell lysates of human breast myoepithelial and epithelial cell lines. β-actin was used as a loading control. (Bi) Expression of cathepsin B detected by Western blotting, the 28 and 30kDa bands reflect the heavy chain of double chain and single chain forms of mature cathepsin B. (Bii) β-actin was used as a loading control. (Biii) Cathepsin B and L activity were determined by the use of an activity based probe (GB123). Blots representative of 3 independent experiments. (C) 3D co-culture of MDA-MB-231 cells with N1ME cherry-labelled myoepithelial cells, with siRNA knockdown of cathepsin inhibitors stefin A, stefin B and cystatin C or siRNA control. Scale bars represent 200 µm. (D) Quantification of invasive outgrowths as described in Figure 2, **p<0.01, ****p<0.0001. *n* = 2.

Figure 4. Decreased myoepithelial stefin A expression promotes MDA-MB-231 invasion in 3D co-culture. Breast cancer cells cultured alone, co-cultured with N1ME stefin A wild type, or N1ME stefin A low cells. Top panel: Bright field images of MDA-MB-231 (not labelled) and co-cultured with myoepithelial cells. Bottom panels: Confocal images rendered in Imaris, of MDA-MB-231 (Hoechst stained - blue), MDA-MB-231-GFP (green) or CAL120 (Hoechst stained - blue) alone or co-cultured with myoepithelial cells (red). Scale bars represent 200 µm. (v) Quantification of invasive outgrowths as described in Figure 2. **p<0.01, ***p<0.001, ****p<0.0001. *n* = 3. Bright field images of other cultures and further quantification provided in Supplementary material.

Figure 5. Cysteine cathepsin inhibitors revert the invasive state of MDA-MB-231 cells in 3D co-culture with stefin A low myoepithelial cells. MDA-MB-231 cells alone or in 3D co-culture with N1ME stefin A low cells were treated with cysteine cathepsin inhibitors CA-074, JPM-OEt, or DMSO control. Inhibitors were replenished every 48 hours. Bright field images and confocal images, rendered in Imaris, of MDA-MB-231 cells (blue, Hoechst stain) alone or co-cultured with stefin A low myoepithelial cells (red). Scale bars represent 200 µm. Right panels: Quantification of invasive outgrowths as described in Figure 2. NS = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. *n* = 3.

Figure 6. Membrane proteome reflects alteration in adhesion and cell attachment in myoepithelial cells. (A) Gene Ontology (biological process) analysis of membrane proteins significantly differentially expressed in N1ME WT compared to stefin A low cell (normalized ratio spectral counts; Rsc ±2, $p<0.05$) reflect enrichment of cell adhesion/attachment protein networks (cell-matrix adhesion, substrate adhesion, cell adhesion, and regulation of cell shape, $p<0.05$). The number of differentially expressed proteins associated with each biological process is shown. Data representative of three independent biological replicates, performed in technical duplicates. (B) Volcano plot illustrates differentially expressed proteins associated with cell adhesion and attachment. The −log10 (Benjamini–Hochberg corrected P value) is plotted against the log2 (fold change/Rsc: N1ME_low/N1ME). Proteins identified as more abundant (red) or less abundant (green) in the N1ME stefin A low cell membrane compared to N1ME WT membrane preparation. Laminin proteins (LAMA3/C1/C3) are highlighted in yellow.

Figure 7. Stefin A expression in human normal and carcinoma tissue. Sections of formalin-fixed, paraffin-embedded tissue were stained with rabbit anti-human stefin A and visualized with DAB (brown). All sections were counterstained with hematoxylin (blue nuclei). Expression of stefin A in myoepithelial cells surrounding (A, B) normal breast ducts and (C) DCIS lesions. (D) Aberrant or (E) no myoepithelial stefin A expression in DCIS lesions. (F) Mouse anti-human p63 was used as a positive control for the presence of myoepithelial cells in all tissues. (G) Myoepithelial stefin A expression was pathologist scored and compared between groups: normal, usual ductal hyperplasia (UDH), DCIS grades low, intermediate (inter) and high. The percentage of patients with the scoring intensity is shown. Comparison is by Chi-square test on patient numbers in each group ****$p<0.0001$, **$p<0.01$. *n* = 138 patients. (H) DCIS tissue with identified micro-invasive regions were stained with rabbit anti-human stefin A and smooth muscle actin (SMA) and visualized with DAB (brown staining). The presence of myoepithelial cells was confirmed by SMA positivity on serial sections. White arrows indicate the focal break in the myoepithelial boundary. Black arrows indicate invasive cells. Scale bars represent 50µm.