

Molecular and Cellular Characterization of Two Patient-Derived Ductal Carcinoma in Situ (DCIS) Cell Lines, ETCC-006 and ETCC-010

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Abstract

Background: Currently it is unclear how *in situ* breast cancer progresses to invasive disease; therefore, a better understanding of the events that occur during the transition to invasive carcinoma is warranted. Here we have conducted a detailed molecular and cellular characterization of two, patient-derived, ductal carcinoma *in situ* (DCIS) cell lines, ETCC-006 and ETCC-010.

Methods: Human DCIS cell lines, ETCC-006 and ETCC-010, were compared against a panel of cell lines including the immortalized, breast epithelial cell line, MCF10A, breast cancer cell lines, MCF7 and MDA-MB-231, and another DCIS line, MCF10DCIS.com. Cell morphology, hormone and HER2/ERBB2 receptor status, cell proliferation, survival, migration, anchorage-independent growth, indicators of EMT, cell signalling pathways and cell cycle proteins were examined using immunostaining, immunoblots, and quantitative, reverse transcriptase PCR (qRT-PCR), along with clonogenic, wound-closure and soft agar assays. RNA sequencing (RNAseq) was used to provide a transcriptomic profile.

Results: ETCC-006 and ETCC-010 cells displayed notable differences to another DCIS cell line, MCF10DCIS.com, in terms of morphology, steroid-receptor/HER status and markers of EMT. The ETCC cell lines lack ER/PR and HER, form colonies in clonogenic assays, have migratory capacity and are capable of anchorage-independent growth. Despite being isogenic, less than 30% of differentially expressed transcripts overlapped between the two lines, with enrichment in receptor tyrosine kinase pathways and DNA replication/cell cycle programs.

Conclusions: For the first time, we provide a molecular and cellular characterization of two, patient-derived DCIS cell lines, ETCC-006 and ETCC-010, facilitating future investigations into the molecular basis of DCIS to invasive ductal carcinoma transition.

Background

Biomedical research relies heavily on the use of cellular *in vitro* models. In cancer research, a wide variety of cell lines are available to study the molecular details of cancer initiation and progression and are enormously beneficial to develop and test therapeutics drugs. For breast cancer research, MCF-7, T47D and MDA-MB-231 are utilized in more than two thirds of the studies using cell lines [1], with MCF-7 and T47D cell lines representing luminal type A breast cancer [2] and MDA-MB-231 representing basal-like type B [3]. MDA-MB-231 cells are used extensively as a model for triple-negative breast cancer, based on the lack of expression of cell surface receptors, estrogen receptor (ER), progesterone receptor (PR) and the growth factor receptor, human epidermal growth factor receptor-2 (HER2/ERBB2) [4]. While numerous cell lines have been developed to represent invasive ductal carcinoma (IDC), fewer lines are available for ductal carcinoma *in situ* (DCIS), a non-obligate precursor to invasive breast cancer [5]. DCIS results from an abnormal proliferation of epithelial cells in the duct of the breast and is associated with a lack of invasive growth into the local stroma [6, 7].

Some cellular models of early stage breast cancer have been developed. MCF10DCIS.com is the DCIS cell line most used by researchers according to the literature. MCF10DCIS.com was derived from a xenograft lesion following two trocar passages of the premalignant cell line MCF-10AT [8, 9]. In xenografts, MCF10DCIS.com forms lesions resembling comedo DCIS with central necrotic areas [9]. SUM225 is another available model for DCIS; it is derived from a chest wall recurrence in a patient diagnosed and treated for DCIS six years prior [10]. In 3D cultures, SUM225 forms atypical clusters [11] and using a mouse mammary intraductal-transplantation method, SUM225 formed lesions similar to human DCIS [12]. While those DCIS cell lines and a few others (reviewed by Brock et al. [13]) exist, none are directly derived from a primary DCIS tumor.

In 2014, Yong et al. [14] developed five DCIS sub-cell lines derived from a primary tumor by overexpressing the human telomerase reverse transcriptase (hTERT). For this study, we acquired two of those five cell lines, ETCC-006 and ETCC-010. We selected these two lines based on the original publication where the authors showed that each cell line had different abilities in proliferation and tumor growth when injected into mice, with ETCC-010 showing the most aggressive phenotype *in vivo* and ETCC-006 *in vitro*. Those cell lines have not been used in the literature other than in the original publication [13].

Yong et al. stated that the ETCC lines that they derived were ER positive, based on their observations using immunoblotting and immunofluorescence [14]. Leading on from their observations, we first aimed to stimulate those cells with estradiol and to analyze the changes in gene expression of estrogen responsive genes. However, in preliminary experiments we observed no difference in expression of estrogen responsive genes after estradiol stimulation; therefore, we considered whether our results might be explained by a lack of expression of ER in those cells. In consequence, we decided to conduct a molecular and cellular characterization of ETCC-006 and ETCC-010 DCIS cell lines to clarify and extend results published by Yong et al. [14] and to provide comprehensive information for our subsequent studies and to the research community. This work was conducted by comparing ETCC-006 and ETCC-010 to MCF10DCIS.com [9], the conventional cell line model of DCIS, and to the widely characterized breast cancer cell lines, MCF-7, a hormone-receptor positive breast cancer model [15, 16], MDA-MB-231, a triple-negative breast cancer model [4], and the immortalized, breast epithelial cell line MCF-10A [17].

In detail, we determined the cellular phenotypes of the two ETCC DCIS cell lines by examining general cell morphology, along with proliferation, survival, migration and anchorage-independent growth. Next we examined molecular features including, hormone-receptor status, markers for epithelial-mesenchymal transition (EMT) and protein expression levels of several signalling proteins in the AKT and ERK pathways and in the cell cycle. In parallel, RNA expression of several DCIS markers was measured by qRT-PCR and the transcriptomic profile of ETCC-006 and ETCC-010 was determined by RNA sequencing (RNAseq) and compared to an existing RNAseq data set from MCF-10A cells [17]. Overall, our results suggest that the ETCC-006 and ETCC-010 DCIS cell lines may represent a more aggressive form of DCIS and provide a valuable resource to examine the transition between non-invasive and invasive ductal carcinoma of the breast.

Methods

Cell culture

ETCC-006 and ETCC-010 were purchased from Leibniz-Institute DSMZ (Braunschweig, Germany). MCF10DCIS.com cells were obtained from Wayne State University (Michigan, USA). MCF-10A, MCF-7, MDA-MB-231, originally from ATCC, were a kind gift from Prof Rosemary O'Connor (UCC, Cork, Ireland). Cell lines were authenticated using short, tandem repeat (STR) profiling (Eurofins Genomics). Cells were cultured according to the following media requirements. MCF-10A cells were maintained in DMEM/F12 supplemented with 5% horse serum, 10 ng/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin and 0.5 ng/ml hydrocortisone. MCF-7 and MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. MCF10DCIS.com were cultured in DMEM/F12 supplemented with 5% horse serum, 1.05 mM calcium chloride and 10 mM HEPES. ETCC-006 and ETCC-010 were cultured in RPMI supplemented with 10% FBS and 1% P/S. Cells were maintained at 37°C with 5% CO₂ and were mycoplasma-free.

Antibodies

Western blotting and indirect immunofluorescence were performed using the following primary antibodies: β actin (A5441, Sigma-Aldrich, Ireland); α 1 integrin (#1952, Merck Millipore, Ireland); cyclin B1 (#4135), estrogen receptor α (#8644), HER2 (#4290), EGFR (#4267), IGF-1R (#3027), pan AKT (#2920), pan ERK (#4696), pan p-AKT (#4060), pan p-ERK (#4377), p-EGFR (#3777), progesterone receptor A/B (#8757) [all from Cell Signaling Technology Europe, Leiden, Netherlands]; cyclin D1 (ab16663) and N-cadherin (ab19348) [both from Abcam, Cambridge, UK]; E-cadherin (#610181, BD Biosciences, San Jose, CA, USA); GAPDH (sc47724) and vimentin (sc32322) [both from Santa Cruz Biotechnology, Dallas, TX, USA]. Secondary antibodies for western blots were IRDye 800CW or 680RD (LI-COR Biosciences UK Ltd, Cambridge, UK); and the secondary antibody used for β actin indirect immunofluorescence was DyLight 488 donkey anti-mouse (Jackson ImmunoResearch Europe Ltd, Ely, UK).

Immunofluorescence

Cells were seeded in a 6-well plate onto sterile coverslips and allowed to grow for 48 hours. Cells were then fixed with 4% paraformaldehyde (PFA) in PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgCl₂, pH 6.9), quenched with 50 mM ammonium chloride and permeabilized with 0.1% Triton X in PHEM buffer for 5 min. Non-specific antibody binding was blocked by incubating the coverslips with 5% goat serum/PHEM for 30 min. Cells were stained with anti- β actin (1:400) overnight at 4°C in an humidified chamber and washed with PHEM. The coverslips were then incubated with donkey, anti-mouse, 488-fluorophore (1:1000) conjugated secondary antibody and DAPI (4',6-diamidino-2-phenylindole) at room temperature for one hour then washed with PHEM. The coverslips were mounted onto Fluoroshield histology mounting medium (Sigma-Aldrich, Ireland). Images were acquired using a Leica DM6000 upright fluorescence microscope.

Western blotting

Cells were plated onto 10cm tissue culture dishes and were harvested when reaching about 70% confluence. In one experiment, cells were either grown in complete media or serum starved for 24 hours before harvesting. Cells were scraped from the plates and lysed using NP-40 lysis buffer (150 mM NaCl, 50 mM Tris HCl pH=8.0, 1% NP-40 and 1X cOmplete EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, Ireland) for 30 minutes on ice. Cleared lysates were prepared by centrifugation for 30 minutes at 12,000g at 4°C. Protein levels were quantified using Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Biosciences Ltd, Ireland) and equal amounts of protein samples (40-50 µg) were resuspended in SDS loading buffer and run on 10% SDS-PAGE at 100V for 2 hours. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Ireland) for 1 hour at 100V then blocked with 5% milk or bovine serum albumin (BSA) for 1 hour at room temperature and incubated with the primary antibody overnight at 4°C. The next day, the membrane was washed with Tris-buffered saline + 0.1% Tween-20 (TBST) for 5 minutes, 3 times, incubated with secondary antibody for 1 hour at room temperature, washed with TBST for 5 min 3 times and visualized using an Odyssey Imaging System (LI-COR Biosciences).

RNA analysis by quantitative, reverse transcriptase, polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol (Thermo Fisher Scientific). Briefly, 0.2 mL of chloroform was added per 1 ml of TRIzol reagent, samples were homogenized and then left at room temperature for 3 minutes. The aqueous phase was separated by centrifugation, and RNA was precipitated using isopropanol. After two washes using 75% ethanol, the RNA pellet was air dried, resuspended in water and incubated at 58°C for 10 min.

1 µg of RNA was treated with DNase to eliminate contaminating DNA using TURBO DNase (Invitrogen, Biosciences Ltd, Ireland) then used in a cDNA synthesis reaction using Superscript II (Thermo Fisher Scientific, Biosciences Ltd, Ireland) as per manufacturer instructions. Reactions lacking reverse transcriptase enzyme were also run in the same condition as controls. The cDNA synthesized was diluted 1:5 and used for qRT-PCR. The diluted cDNA was used in qRT-PCR reactions. Briefly, 25 ng cDNA was combined with SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, Ireland) in 20 µl reactions and run using the following conditions:

- 95°C 10 min
- 94°C 30 sec, 57°C 45 sec, 72°C 1 min repeated 39 times
- 94°C 30 sec, 57°C 45 sec, 72°C 15 min
- Melting curve stage

qRT-PCR was performed using the StepOnePlus™ Real-Time PCR System and StepOnePlus software (Applied Biosystems, Foster City, CA, USA). After analysis of the melting curve, results were normalized to

the expression of ubiquitin C, *UBC* [18], using the $\Delta\Delta C_T$ method. Technical duplicates were done for each reaction, and three biological replicates were processed. qRT-PCR primers used for each RNA target are listed in Table 1.

Table 1: DNA primers for qRT-PCR analyses of gene expression

Target Gene	Forward primer	Reverse primer
<i>BIRC5</i>	ACCGCATCTCTACATTCAAG	CAAGTCTGGCTCGTTCTC
<i>CCNB1</i>	CCAAATCAGACAGATGGAAAT	GCCAAAGTATGTTGCTCGA
<i>ESR1</i>	CCACCAACCAGTGCACCATT	GTCTTCCGTATCCCACCTTC
<i>GSTM1</i>	CTATGATGTCCTTGACCTCCACCGTA	ATGTTCACGAAGGATAGGGTAGCT
<i>HER2</i>	TCCTGTGTGGACCTGGATGAC	CCAAAGACCACCCCCAAGA
<i>MK167</i>	CCACACTGTGTCGTCGTTG	CCGTGCGCTTATCCATTCA
<i>MYBL2</i>	AAAACAGTGAGGAGGAAC	CAGGGAGGTCAAATTAC
<i>STK15</i>	GGAGAGCTTAAATTGCAGATT	GCTCCAGAGATCCTCTCAT
<i>PR</i>	TACCCGCCCTATCTCAACTACCTG	GGACACCATAATGACAGCCTGATG
<i>UBC</i>	TCGAGAATGTCAAGGCAAAGATC	GAGTGGACTCTTCTGGATGTTGTA

RNA sequencing of ETCC-006 and ETCC-010 transcriptomes

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The RNA integrity was measured using the RNA 6000 Nano Kit (Agilent). Libraries were prepared using the New England Biolabs (NEB) Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760) and NEBNNext Poly (A) mRNA Magnetic Isolation Module (NEB #E7490). Libraries were sequenced using the Illumina NextSeq 500 sequencer and read 85 bases from library inserts (Genomics Core Facility, EMBL). The bioinformatic analysis was performed using the Galaxy platform [19]. FASTQ reads were trimmed and filtered for quality and adapter using Trimmomatic [20] and aligned to the human reference genome (hg19) using RNA Star [21]. FeatureCounts was used to quantify gene expression [22] and differential expression analysis was performed with DESeq2 [23] using RNAseq datasets generated for ETCC-006 and ETCC-010 against an MCF-10A RNAseq dataset (Project: PRJEB2623; Sample Accession: SAMEA1484687; Experiment Accession: ERX016686; European Nucleotide Archive (ENA), EMBL-EBI). Overexpressed genes with p-value < 0.05 were selected for pathway-based analysis using ReactomePA [24].

Cell proliferation monitoring

MCF-10A, MCF-7, MDA-MB-231, MCF10DCIS.com, ETCC-006 and ETCC-010 cells were seeded at a density of 50,000 cells per 12-well plate in triplicate. Media was replaced every third day. Cells were trypsinized, resuspended into medium and counted every 24 hours over a period of 6 days. Trypan blue was used to monitor the viability of the cells. Population doubling time was calculated (16) using values at day 2 and day 5 with the following formula:

$$\text{Population Doubling Time} = \frac{\text{duration} \times \log(2)}{\log(\text{final cell number}) - \log(\text{initial cell number})}.$$

Clonogenic assay

MCF-10A, MCF-7, MDA-MB-231, MCF10DCIS.com, ETCC-006 and ETCC-010 cells were seeded at a density of 2,000 cells per six-well plate in triplicate. Media was replaced every third day. Cells were washed with PBS (phosphate buffered saline) and fixed with 96% ethanol for 10 minutes, then stained with 0.05% crystal violet in 20% ethanol for 30 minutes. Excess crystal violet was removed by washing with sterile, distilled water and plates were dried. Staining was assessed using an Odyssey Imaging System and quantified using ImageJ software.

Wound healing assay

MCF-10A, MCF-7, MDA-MB-231, MCF10DCIS.com, ETCC-006 and ETCC-010 cells were seeded on plates with two-well, culture inserts (#80209, Ibidi GmbH, Gräfelfing, Germany) the day before performing the assay to form a monolayer. Removal of the insert created a wound of 500 µm width. Wound closure was monitored 5 hours, 9 hours and 12 hours after removal of the insert. Images were acquired using the EVOS FL Auto Imaging System (Invitrogen, Biosciences Ltd, Ireland). Wound width was quantified using the ImageJ software.

Anchorage-independent growth

MCF-10A, MCF-7, MDA-MB-231, MCF10DCIS.com, ETCC-006 and ETCC-010 cells were seeded at a density of 300,000 cells in an agarose suspension (0.4% agarose) over an agarose underlay (0.8% agarose) for assessment of anchorage-independent growth. Media was changed every 5 days. After 3 weeks of growth, the agarose layers were stained with 1% crystal violet at 4°C overnight and destained through sterile, distilled, water washes every 24 hours until single colonies appeared. Staining was assessed using an Odyssey Imaging System and counted by hand.

Results

Morphologic characterization of the DCIS cell lines ETCC-006 and ETCC-010, compared to MCF10A, MCF7, MDA-MB-231 and MCF10DCIS.com

The primary goal of our work was to further characterize the recently developed ETCC-006 and ETCC-010 DCIS cell lines [14]. Before comparing the molecular details of those cell lines, we first examined overall

cell morphology by comparing ETCC-006 and ETCC-010 to another DCIS cell line, MCF10DCIS.com, a normal-like breast epithelial cell line, MCF10A, and two breast cancer cell lines, MCF7 and MDA-MB-231.

Interestingly the two ETCC DCIS cell lines showed evident morphologic differences with each other, despite being isogenic and derived from the same tumor; yet, they displayed some striking similarities with the other cell lines examined. We studied their morphologic differences while growing onto a culture plate (Figure 1A) or onto coverslips after b-actin immunofluorescence and DAPI staining of cell nuclei (Figure 1B). b-actin is a highly conserved and expressed protein, implicated in the control of cell growth and migration [25]. It is important for the maintenance of the cellular architecture; therefore, we chose it as a marker to better visualize differences in cell morphology. In Figure 1, differences in size, shape and organization can be noticed in the panel of cell examined.

Similar to MCF-7, MCF10DCIS.com cells displayed high cellular contact among cells, with cells forming clusters with each other. This suggests that those cells are highly dependent on cellular contacts for growth to be possible. Although cell size was not quantified, observation under the microscope suggests that MCF10DCIS.com cells are of similar size as MCF-7 cells.

Considering the ETCC lines, ETCC-006 cells seem to show the largest cell size of all cells analyzed in this study, with many cytoplasmic projections observed. Moreover, ETCC-006 cells were phenotypically striking because of their apparent flatness when visualized under the microscope; thus, observation under bright field microscopy proved to be more difficult than for other cell lines (Figure 1A).

ETCC-010 cells were derived from the same DCIS tumor as ETCC-006, yet they show little morphologic similarities to each other. Instead, ETCC-010 cells displayed a high degree of resemblance to MDA-MB-231 cells (Figure 1B). ETCC-010 cells were elongated, with the nucleus occupying most of the available space within the cell. The difference in morphology between ETCC-006 and ETCC-010 is interesting since they both have the same genetic background, highlighting tumor heterogeneity within DCIS lesions [26].

Molecular subtype of DCIS cell lines

In breast cancer care, testing of the ER, PR and HER2 receptor status is routinely done to assess the prognosis and the treatment that will be given to the patient [1]. In breast cancer research, the ER, PR and HER2 receptor status of cell lines is verified to determine which breast cancer subtype those cell lines represent. Before analyzing the ER, PR and HER2 receptor status of the DCIS cell lines, we confirmed the published molecular subtype of the control cell lines by immunoblot analysis. MCF-10A and MDA-MB-231 are known to have no expression of ER, PR and HER2. MCF-7 is a hormone receptor-positive breast cancer cell line, so it expresses ER and PR and modest levels HER2 [4] Those results were confirmed at both protein and RNA levels (Figure 2).

MCF10DCIS.com has the same genetic background as MCF-10AT, which was derived from MCF-10A cells through oncogenic transformation with an activated *HRAS* gene [8, 27]; therefore, it is not surprising that similar to MCF-10A, this cell line shows little expression of ER at the protein and RNA level. PR was

detected at the protein level (Figures 2A and B) but not at the RNA level (Figure 2C). Since levels of ER and PR in MCF10DCIS.com are low, those receptors are likely not functionally relevant. High levels of HER2 were detected in MCF10DCIS.com, explained by the presence of two gene copies of *ERBB2* [28, 29].

While ETCC-006 showed no expression of ER, PR and HER2, we detected very low levels of ER and no expression of PR and HER2 at protein and RNA level in ETCC-010 cells. These results contradict the previous work by Yong et al. (2014) [14]; however, our work shows directly that HER2 is not expressed in these two DCIS cell lines and agrees with previous work showing that treatment with Herceptin (monoclonal antibody directed against HER2) was not cytotoxic to ETCC cell lines [14]. Taken together, our work indicates that ETCC-006 and ETCC-010 are functionally similar, based on receptor status, to triple-negative breast cancer cell lines.

Analysis of the gene expression of Oncotype DX DCIS markers in DCIS cell lines

Since we found that ETCC-006 and ETCC-010 display different characteristics to what was published by Yong et al. (2014) [14] we decided to characterize the cell lines using previously suggested DCIS markers. However, defining DCIS using a small set of known markers has been difficult [30], mainly because medical research on DCIS is more limited compared to that of IDC.

The Oncotype DX DCIS score is a 12-gene panel that generates predicted 10-year risk of local DCIS and invasive recurrence following treatment by breast conserving surgery [31]. It includes five gene markers of proliferation – *MKI67* (Ki67), *STK15* (AURKA), *BIRC5* (survivin), *CCNB1* (cyclin B1), *MYBL2* (MYB proto-oncogene like 2); *PGR* (progesterone receptor), *GSTM1* (glutathione S-transferase M1) and five reference genes. While it is not relevant to calculate a DCIS score for cell lines, we decided to assess the expression levels of those markers in the DCIS cell lines, ETCC-006 and ETCC-010, compared to the other cell lines in our panel.

Figure 3 shows the results of qRT-PCR to analyse the expression of *MKI67*, *STK15*, *BIRC5*, *CCNB1*, *MYBL2*, *GSTM1* and *PGR*. MCF-7 cells show high expression of the proliferation markers (Figure 3A) along with high expression of *GSTM1* and *PGR* (Figure 3B). MDA-MB-231 cells show high expression of four proliferation markers, very low expression of *PGR* and reduced *GSTM1* expression compared to MCF-7 cells. Regarding MCF10DCIS.com, we observed lower expression of the proliferation markers, when compared to the breast cancer cell lines, and low *GSTM1* and *PGR* expression. It is important to note that while *PGR* RNA levels appeared low as determined by qRT-PCR; PR protein was detectable by immunoblotting (Figure 2). Meanwhile, ETCC-006 showed high expression of four proliferative markers and low expression of *PGR* and *GSTM1*. ETCC-010 displayed a similar trend to MCF10DCIS.com cells, with comparable levels of *MKI67*, *STK15* and *BIRC5* expression. However, ETCC-010 showed slightly higher expression of *GSTM1* and lower expression of *PGR*, compared to ETCC-006. Analysis of this small panel of genes suggests that differences in gene expression observed between the ETCC DCIS lines likely reflects the cellular heterogeneity of the original tumor.

Genome-wide comparison of the ETCC-006 and ETCC-010 DCIS model to MCF-10A

In parallel to analyzing the molecular subtype of our cells, we performed RNA sequencing (RNAseq) of ETCC-006 and ETCC-010 cell lines. RNAseq allows the identification of transcripts present in the ETCC-006 and ETCC-010 cells at a genome-wide level. By comparing the level of expression of transcripts in ETCC-006 and ETCC-010 to those in MCF-10A cells, we aimed to identify if genes in some cancer-related pathways showed altered expression. Before performing RNAseq, we analyzed the quality of the total RNA extracted from our cell lines using bioanalyzer traces (Agilent) to calculate an RNA integrity (RIN) value that is indicative of the level of degradation of the RNA. RIN values range from 0 (degraded) to 10 (intact). We obtained RIN values of 10 for RNA extracted from ETCC-006 and ETCC-010, each with two replicates. This indicated that the RNA was of good quality and could be used for library preparation.

After RNA sequencing, reads were trimmed and filtered for quality with Trimmomatic [20] and aligned to the human reference genome (hg19) with RNA STAR [21]. Gene expression was quantified with FeatureCounts [22] and differential expression was performed using DESeq2 [23]. We compared the transcriptome of the ETCC lines to that of MCF-10A, using an available dataset, as detailed in the Methods. After the differential expression, we excluded genes with a p-value over 0.05. Figure 4A is a Venn diagram of the number of genes with higher expression in ETCC-006 and ETCC-010 in orange and lower expression in ETCC-006 and ETCC-010 in blue. In total, 235 and 246 genes were over-expressed in ETCC-006 and ETCC-010, with 99 genes in common; while 194 and 240 genes were under-expressed in ETCC-006 and ETCC-010, with 91 genes in common (Figure 4A). All genes identified from our RNAseq analysis are listed in Additional File 1.

Next, reactome pathway-based analyses were performed using an R/Bioconductor package, ReactomePA [24], using the lists of over-expressed genes in the ETCC DCIS cell lines to identify enriched pathways (Figure 4B and 4D). The most enriched pathways common to ETCC-006 and ETCC-010 related to cell proliferation: mitotic G1-G1/S phases, G1/S transition, unwinding of DNA (S phase), etc. Interestingly, integrin-cell surface interaction gene products were enriched in both cell lines compared to MCF-10A. Since those regulate cell motility, it possibly indicates that ETCC-006 and ETCC-010 have migratory abilities. ReactomePA also allowed us to generate String analyses of the genes implicated in the pathways mentioned (Figure 4C and 4E). Proliferation in both cell lines seems to rely mostly on commonly upregulated genes (*CCND1*, *MCM* family, *MYBL2*, etc.). *CCND1* is an important regulator of the G1/S transition [32], the MCM proteins constitute a family of protein involved in the regulation of DNA replication [33], and *MYBL2* is a transcription factor involved in the regulation of the G1/S transition [34]. Interestingly, an enrichment of “signalling by VEGF” was observed in ETCC-006 (Figure 4B), and VEGF (vascular endothelial growth factor) signalling has been shown to be upregulated in DCIS [35]. Overall, most enriched pathways in ETCC-006 and ETCC-010 are related to DNA replication and proliferation.

Monitoring of cell proliferation and survival in DCIS cell lines, ETCC-006 and ETCC-010

To confirm the observation from the transcriptomic study that suggest that proliferation is promoted in the ETCC lines, cell growth was monitored to determine population doubling time (PDT) using trypan blue staining. Figure 5A shows proliferation curves for each of the cell lines examined. MCF-10A, MCF-7 and

MDA-MB-231 showed different rates of proliferation with PDT of 20 hours, 24.4 hours and 36.2 hours respectively. The rate of proliferation of MCF10DCIS.com was comparable to that of MCF-10A, with 20.5 hours PDT. ETCC-006 and ETCC-010 showed intermediate proliferative rates to that of MCF-7 and MDA-MB-231, with 30.7 hours and 29.7 hours respectively. Interestingly, while most cell lines seemed to undergo a lag phase after plating, the ETCC-010 growth profile displayed high proliferative abilities even at low cell number (Figure 5A).

To extend our characterization of the ETCC cell lines, we used the clonogenic assay to monitor cell survival by testing the cells' ability to go through cell division with no limit in time [36, 37]. The clonogenic assay reflects cell survival *in vitro* by measuring the staining intensity of colonies formed with crystal violet. Following growth and replenishing cultures with fresh media, cells were fixed after ten days and colonies were stained (Figure 5B). Compared to MCF7 cells, MDA-MB-231 and ETCC-006 cells showed reduced colony formation, which was quantitated by crystal violet staining intensity (Figure 5C). For ETCC-006, the reduction in signal intensity might be explained by the characteristic flatness of the cells that makes them unsuitable for crystal violet staining. Interestingly, while MCF-7 and MDA-MB-231 showed distinct colonies, all DCIS cell lines appeared more like a monolayer, with an observable reduction in colony formation particularly for the ETCC DCIS lines (Figure 5C).

Assessment of migration and anchorage-independent growth potential of the DCIS cell lines

Since the integrin cell surface interactions pathway was enriched in ETCC-006 and ETCC-010 (Figure 4B and 4D), we monitored the migratory abilities of the DCIS cell lines using a wound healing assay. Wound width and wound area was examined after removal of the culture insert at time zero hours (T0), five hours after insert removal (T5), and then at nine and 12 hours post-insert removal (T9 and T12). Using bright-field microscopy, we observed wound closure within 12 hours in MCF-10A, MCF-7 and ETCC-006 cells (Figure 6A - C). In ETCC-010, after 12 hours the wound was close to closure. MCF10DCIS.com and MDA-MB-231 showed slower wound closure, a wound measurement over still over 100 µm after 12 hours.

Anchorage-independent growth, a property of most malignant cells to grow independently of a solid surface, was evaluated by a soft agar colony formation assay [38]. As expected, breast cancer cell lines, MCF-7 and MDA-MB-231, showed the highest ability to form colonies in soft agar. The three DCIS cell lines MCF10DCIS.com, ETCC-006 and ETCC-010 showed similar abilities to form colonies in agar, but with numbers intermediate to MCF-10A (lowest) and MCF-7 or MDA-MB231 (highest) (Figure 6D and 6E).

ETCC-006 and ETCC-010 DCIS cell lines express markers of epithelial-mesenchymal transition

Taken together, our data has shown that the DCIS cell lines ETCC-006 and ETCC-010 express migration markers (Figure 4) and have migration abilities (Figure 6). We next sought to consider whether those cell lines have undergone epithelial to mesenchymal transition (EMT). EMT is a common event in cancer progression where epithelial cells lose their epithelial phenotype, characterized by tight and adherent cells, to gain a mesenchymal phenotype (Figure 7A). Moreover, during EMT, cells lose expression of epithelial markers, such as E-cadherin, to gain expression of mesenchymal markers (vimentin, N-cadherin, etc.).

Along with cell motility [39], activation of EMT programs contribute to the events leading to metastasis [39–41].

To examine the level of expression of key EMT markers, whole cell lysates from each cell line were prepared. Vimentin, E-cadherin, N-cadherin, β -catenin and $\beta 1$ -integrin expression was analyzed by immunoblotting. High expression of vimentin was observed in ETCC-006 and ETCC-010 at similar levels to those observed in MDA-MB-231; while low expression of vimentin was detected in MCF10DCIS.com at level comparable to MCF-10A (Figure 7B and 7C).

Most strikingly, while MCF10DCIS.com cells expressed E-cadherin, we could not detect expression of E-cadherin in ETCC-006 and ETCC-010. Instead, expression of N-cadherin was observed in ETCC-006 and ETCC-010 lysates. β -catenin is a transcription factor induced by the WNT pathway that stimulates transcription of genes involved in the EMT [42], and its expression is associated with poor prognosis in breast cancer [43]. In our analysis, β -catenin protein levels were elevated in MCF-10A, MCF10DCIS.com and ETCC-006, while levels were more balanced in MCF-7, MDA-MB-231 and ETCC-010 cells. $\beta 1$ -integrin is a transmembrane protein, whose function is to bind extracellular matrix (ECM) [44] and regulate a cell's ability to invade [45]. $\beta 1$ -integrin was detected at high levels in MCF-10A, MDA-MB-231 and ETCC-006, with ETCC-010, MCF10DCIS.com and MCF-7 showing lower protein levels.

Overall, the co-expression of β -catenin, vimentin, $\beta 1$ integrin and N-cadherin suggests that ETCC-006 and ETCC-010 have undergone an EMT transition. Although MCF10DCIS.com cells express some markers of EMT, overall it is more representative of cells with an epithelial phenotype.

Signalling profile and cell cycle proteins in DCIS cell lines

Since signalling by receptor tyrosine kinases was most enhanced in ETCC-006 cells according to our RNAseq data and ReactomePA analysis (Figure 4), and ETCC-006 and ETCC-010 have undergone EMT (Figure 7), we next decided to investigate levels of some of proteins in major cell signalling pathways. We measured protein levels of two receptor tyrosine kinases, Epidermal Growth Factor Receptor (EGFR) and Insulin-like Growth Factor 1 Receptor (IGF-1R), both critical in the processes leading to metastasis in breast cancer [46, 47]. To do this, cell lysates were prepared using cells grown in normal conditions. Total levels of IGF-1R, EGFR and the active phosphorylated-EGFR were examined by immunoblotting (Figure 8A and 8B). Low protein levels of EGFR were detected in MCF-7, with the other cell lines showing similar levels of EGFR. In MCF-10A cells, phosphorylation of EGFR was detected; whereas, p-EGFR was not detected in other cell lines under normal conditions. IGF-1R showed high levels in MCF-7 and low levels in MDA-MB-231 cells. The DCIS cell lines showed similar levels of IGF-1R, with intermediate levels to those observed in MDA-MB-231 and MCF-7 cells.

Common downstream targets of EGFR and IGF-1R include the Ras/Raf/MEK/ERK and PI3K/ AKT pathways, known to stimulate proliferation, migration and EMT when activated after being phosphorylated [42]. While total levels of ERK were similar across DCIS cell lines (Figure 9 C and 9D, normalized to GAPDH protein), phosphorylated ERK (p-ERK) levels seem slightly higher in MDA-MB-231,

MCF10DCIS.com and ETCC-006. Similarly, total AKT levels were similar across cell lines; however, phosphorylated AKT (p-AKT) showed high disparity (normalized to β-actin). Low p-AKT was observed in MCF-7 and MDA-MB-231 cells. Higher levels of p-AKT were observed in MCF-10A, ETCC-006 and ETCC-010, while MCF10DCIS.com showed consistently high levels of p-AKT.

Next, we aimed to investigate whether AKT is constitutively activated in MCF10DCIS.com cells. To do this, we compared the phosphorylation levels of AKT under normal and serum starvation conditions (Figure 8E, 8F and 8G). In MCF7, MDA-MB-231, ETCC-006 and ETCC-010 cells, we found that p-AKT is lost after serum starvation conditions. We confirmed the constitutive activation of AKT in MCF10DCIS.com cells, as p-AKT levels remain unaffected under serum starvation conditions. This was also observed in MCF10A cells (Figure 8E) and could reflect variability in PI3K levels [48].

Since high levels of cyclin D1 are associated with DCIS [49], we examined the levels of cyclin B1 and cyclin D1, two regulatory proteins in the cell cycle (Figure 8H and 8I). While the cyclin B1 level seemed slightly lower in the DCIS cell lines compared to the control cell lines, cyclin D1 showed high expression in MCF-7 and ETCC-006, in agreement with our RNAseq results (Figure 5).

Discussion

Choosing the correct model, animal or cellular, is critical in research. To study DCIS in an *in vitro* cell model, we choose to use three DCIS cell lines: MCF10DCIS.com, a well characterized DCIS cell line model, and ETCC-006 and ETCC-010, two patient-derived cell lines. To date, other research groups have not used these recently established patient-derived lines; therefore, we conducted a baseline phenotypic and molecular characterization of DCIS cell lines, ETCC-006 and ETCC-010.

Despite some differences in *in vitro* and *in vivo* assays as highlighted in the original publication, ETCC-006 and ETCC-010 share the same genetic background. For many of the properties examined in this work, we mostly found that ETCC lines behave quite similar to each other *in vitro*, with similar rates of proliferation, colony formation and anchorage-independent growth. Still the two lines have very different transcriptional outputs, with less than 30% of differentially expressed transcripts being common between the two cell lines. Similarly, ETCC-006 shows more enrichment of proliferative markers than ETCC-010, with cyclin D1 more highly expressed in ETCC-006 compared to ETCC-010 in our RNAseq data (Additional File 1) and at protein levels (Figure 9H and I). A limitation of our RNAseq analysis is that both ETCC lines were compared to an existing dataset from MCF-10A cells that do not share the same genetic background. However, we believe that MCF-10A RNAseq data is the best choice for a comparison, and this is the first transcriptomic study of the ETCC DCIS cell lines.

In one key finding, we showed that ETCC-006 and ETCC-010 are more similar to triple-negative breast cancer cell lines, with no expression or limited expression of ER, PR and HER2 (Figure 2). This is contrary to what was described in the original publication [14]. Importantly, we have concerns regarding the original classification of ETCC cells as ER+, as the immunoblot using MCF7 cells, widely considered as an ERα-positive “prototype”, did not show ERα protein expression [14]. We did consider that expression of

ER and PR could be lost over time; however, at the time of our study, we started with cells on passage 15 for ETCC-006 and 19 for ETCC-010 and purchased directly from the cell line repository DMSZ at the Leibniz Institute. Additionally, it has been argued that loss of steroid receptor expression by cells in culture is unlikely [50].

One of our most interesting observations was that ETCC-006 and ETCC-010 cells express markers of EMT (vimentin, N-cadherin and β 1 integrin), suggesting that they have undergone a transition to a more mesenchymal-like phenotype. While they showed comparable proliferation abilities to other cell lines examined, both ETCC lines displayed greater migratory capacity compared to MCF10DCIS.com in a wound closure assay. Taken together, this suggests that ETCC-006 and ETCC-010 may represent models of high-grade DCIS.

Throughout this work, we have compared ETCC-006 and ETCC-010 to MCF10DCIS.com cells, a well-characterized DCIS cell line that produces comedo DCIS lesions in xenografts [9, 12].

Compared to the ETCC lines, MCF10DCIS.com appears to have conserved an epithelial phenotype with cells showing tight contact, no expression of EMT markers and limited migratory abilities. We did show constitutive activation of AKT, likely explained by a mutation in the PI3K gene [51, 52]. Indeed, MCF10DCIS.com has been shown to have a gain of function mutation, H1047R, in the catalytic domain of PI3K [51]. This mutation is one of the most oncogenic mutations and occurs frequently in other types of cancer [53]. Despite this activating mutation of the PI3K/AKT pathway, taken together our data suggests that the MCF10DCIS.com cell line may be less aggressive than the ETCC lines.

Where do DCIS cell lines fit in terms of the clinical representation of DCIS? As previously described, DCIS is an early stage of breast cancer, with a large spectrum of cases classified from low-grade disease to high-grade DCIS. High-grade DCIS is associated with a higher risk of developing IDC [54]; therefore if the ETCC lines truly represent high-grade DCIS, they could be a valuable tool to study the mechanisms leading to the transition between DCIS and IDC. In addition to ETCC-006 and ETCC-010, four more cell lines were derived from the same DCIS lesion, ETCC-001, ETCC-007, ETCC-008 and ETCC-011 [14]. It would be interesting to include these lines for future studies and further characterization. However, the overall number of DCIS cell lines is still limited, highlighting an ongoing need for more DCIS cell lines to be established and characterized, as pointed out recently by Brock et al. [13].

In the clinical DCIS spectrum, MCF10DCIS.com cells have been characterized as a model of human comedo DCIS in xenografts [9, 12]. In terms of histopathology, comedo DCIS is considered high-grade DCIS [55]. While we were unable to compare what type of tumor is induced by MCF10DCIS.com, ETCC-006 and ETCC-010 when injected in mice, our study does suggest that MCF10DCIS.com is an *in vitro* model of DCIS that is less aggressive than the ETCC lines. Together with established DCIS lines, we propose that continued use of the ETCC lines in xenografts, 2D co-culture and 3D culture systems (reviewed by Brock et al. [13]) may facilitate the search for the specific molecular requirements that permit breast cancer cells to transition from DCIS to IDC.

Conclusions

Patient-derived DCIS cell lines, ETCC-006 and ETCC-010, exhibit molecular markers and cellular behaviour consistent with a more aggressive form of DCIS. Although both lines were derived from the same DCIS lesion, they display markedly different transcriptomic profiles, supporting intra-tumoral heterogeneity within DCIS affected areas of the breast. Our results provide a baseline phenotypic and molecular characterization of these newly established DCIS cell lines for further exploration by the research community.

List Of Abbreviations

AKT/PKB Protein kinase B

DCIS Ductal carcinoma *in situ*

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

EMT Epithelial to mesenchymal transition

ER Estrogen receptor

ERK Extracellular signal-regulated kinase

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

HER2 Human epidermal growth factor receptor 2

IDC Invasive ductal carcinoma

IGF-1R Insulin-like growth factor 1 receptor

MEK Mitogen-activated protein kinase kinase

PDT Population doubling time

PI3K Phosphoinositide 3-kinase

PR Progesterone receptor

PTEN Phosphatase and tensin homolog

qRT-PCR Quantitative, reverse transcriptase, polymerase chain reaction

RIN RNA integrity

RNAseq RNA sequencing

TNBC Triple negative breast cancer

UBC Polyubiquitin-C

VEGF Vascular endothelial growth factor

Declarations

Ethics approval and consent to participate

Specific ethical approval and consent to participate was not required for this work.

Consent for publication

All authors read and approved the manuscript.

Availability of data and materials

The datasets supporting the conclusions of this article are available in the European Genome-phenome Archive (EGA) repository, under study accession number EGAS00001004553, and included within the article as Additional File 1.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

J.S.: project design, data acquisition, data analysis, interpretation of data and manuscript writing; K.D.: project conception, project design, interpretation of data and manuscript writing. All authors read and approved the manuscript.

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Figures

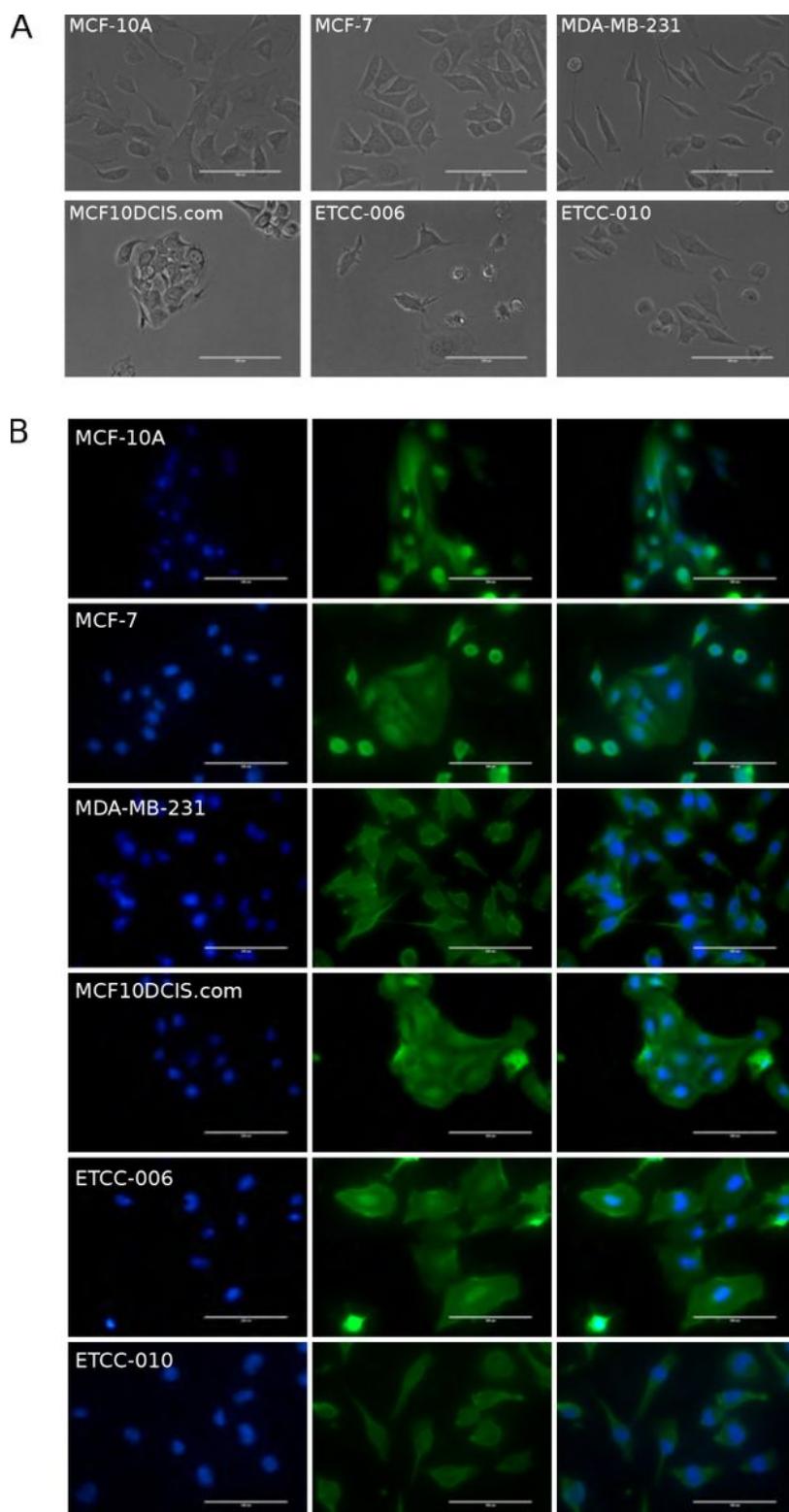


Figure 1

DCIS cell lines, MCF10DCIS.com, ETCC-006 and ETCC-010, show distinct cellular morphologies compared to other breast cancer cell lines and a normal-like breast epithelial cell line. Phenotypic and morphological analysis of MCF10DCIS.com, ETCC-006 and ETCC-010 in comparison with MCF-10A, MCF-7 and MDA-MB-231 cell lines by (A) bright field microscopy and (B) immunofluorescence using a primary antibody against β -actin, with an anti-mouse Alexa Fluor 488 secondary antibody. DAPI was used to stain cell

nuclei in the immunofluorescence analysis. Images were captured using the EVOS FL Auto Imaging System at 40X magnification. Scale bar represents 100 μ m.

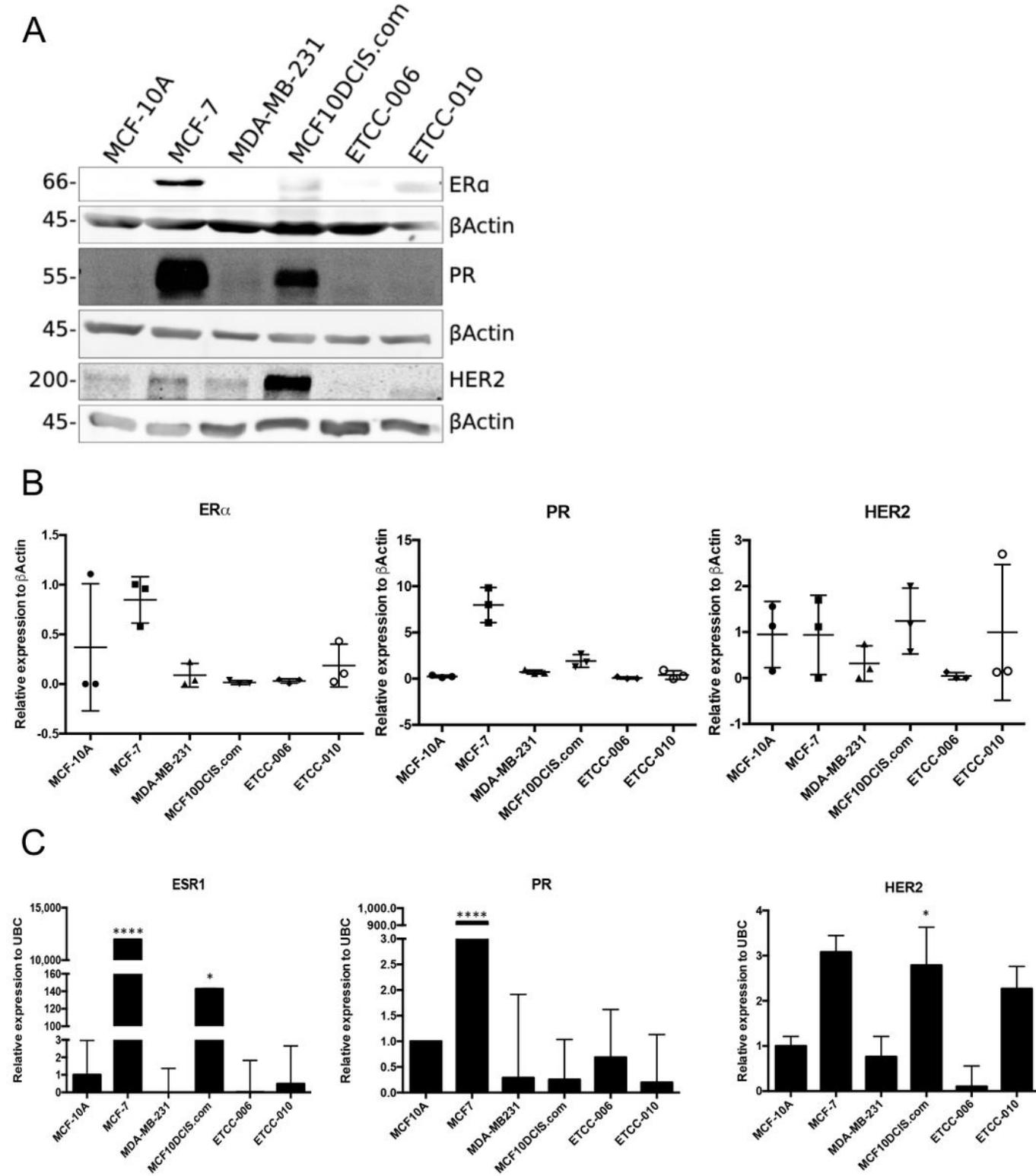
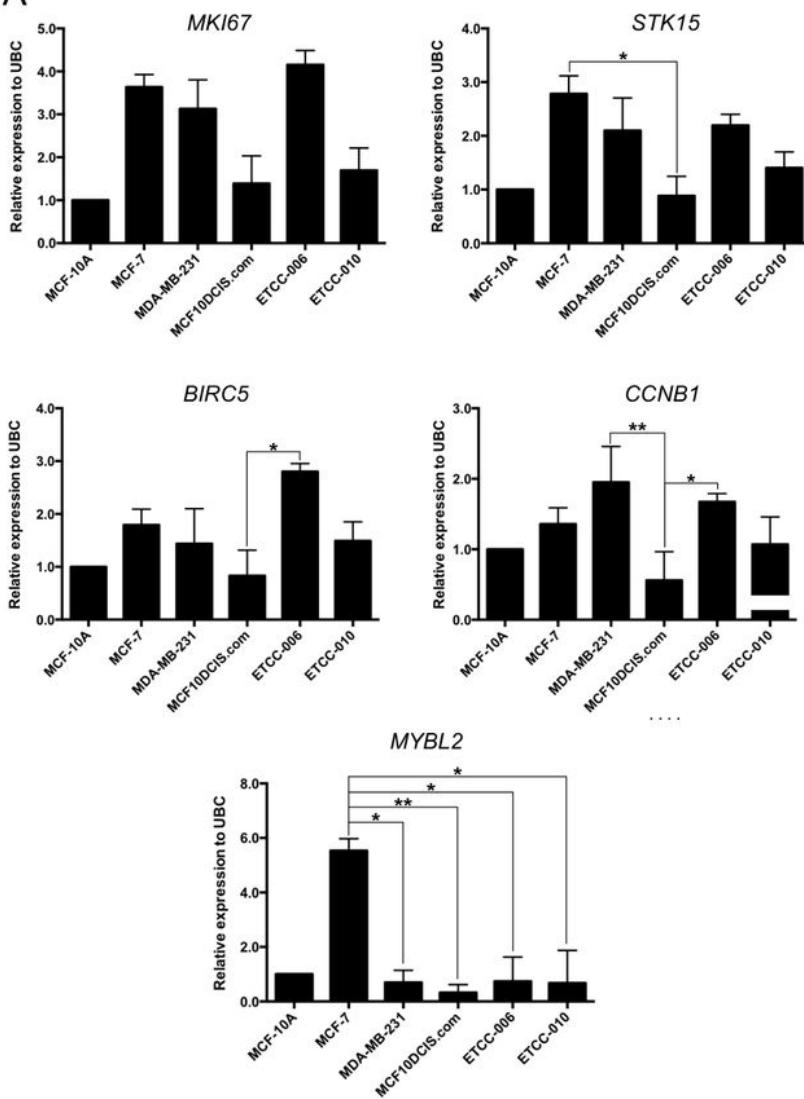


Figure 2

Molecular subtype based on hormone-receptor status of DCIS cell lines, ETCC-006 and ETCC-010. Estrogen receptor alpha (ER α), progesterone receptor (PR) and receptor tyrosine kinase ErbB-2 (HER2) levels were measured at the protein level (A) and (B) and RNA level (C) by immunoblotting and qRT-PCR.

For the immunoblot, 42 µg of total protein was loaded per lane onto the gel. Protein level was quantified and normalized against βactin using ImageJ. This experiment was repeated in triplicate, this is a representative immunoblot. Full-length blots are presented in Supplementary Figure 1.

A



B

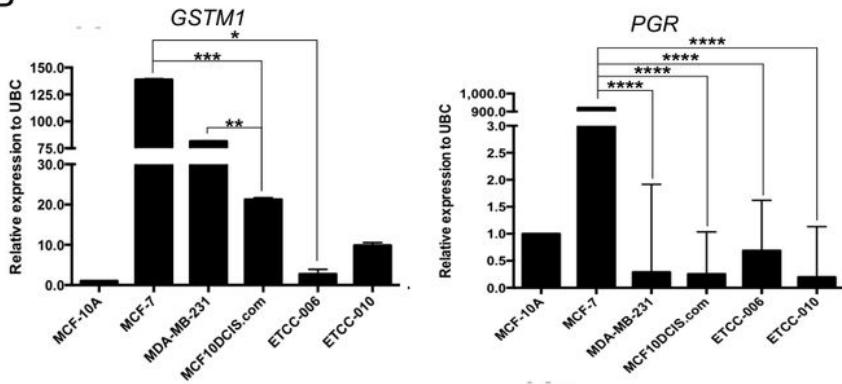
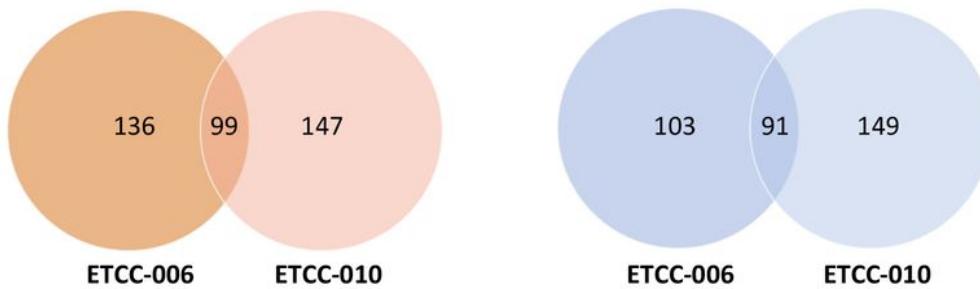


Figure 3

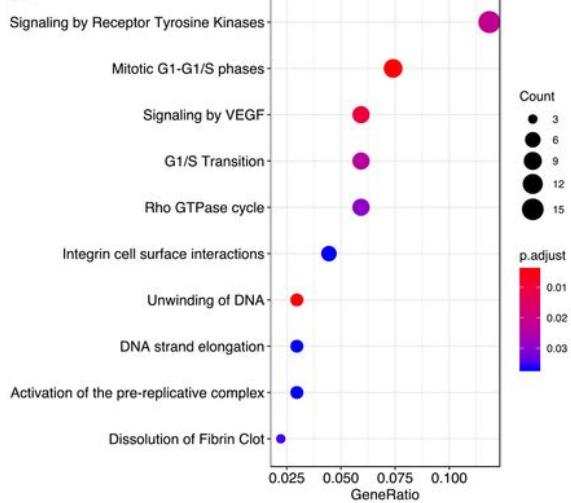
Expression level of Oncotype DX DCIS score markers in the DCIS cell lines analyzed by qRT-PCR. (A) Relative expression of the proliferation markers is associated with an increased risk of DCIS recurrence

whereas (B) GSTM1 and PGR have a protective role against the DCIS risk of recurrence. qRT-PCR was performed using cDNA synthesized from total RNA isolated from normal-like and breast cancer cell lines, MCF-10A, MCF-7, MDA-MB-231, and DCIS cell lines, MCF10DCIS.com, ETCC-006 and ETCC-010. All data was normalized to UBC (polyubiquitin-C) and statistical analysis was done using one-way ANOVA (GraphPad Prism v.8.3.0). **** p-value < 0.0001; *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05.

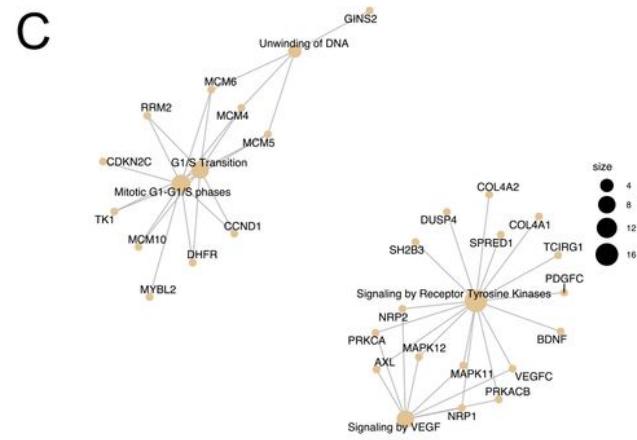
A



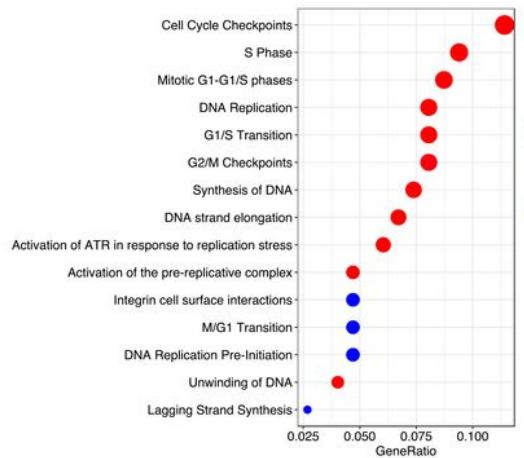
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C



D



E

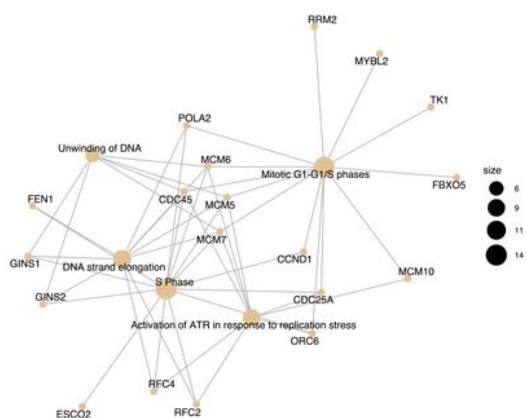


Figure 4

Transcriptomic comparison of the DCIS cell lines ETCC-006 and ETCC-010 to MCF-10A. (A) Venn diagram of the number of genes with higher expression in orange and lower expression in blue in ETCC-006 and ETCC-010 compared to MCF-10A (p -value < 0.05). (B) ETCC-006 reactome-pathway analysis and (D) ETCC-010 reactome-pathway analysis; (C) ETCC-006 and (E) ETCC-010 associated String pathway analyses of the differentially over-expressed genes in ETCC-006 and ETCC-0010 (p -value < 0.05) to identify enriched pathways in those cells and the genes associated with those pathways.

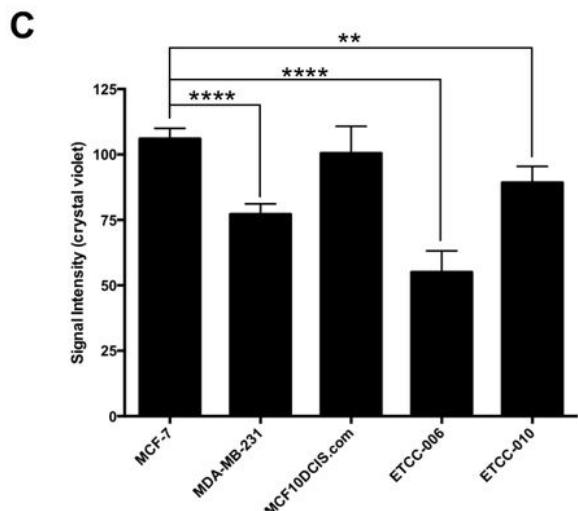
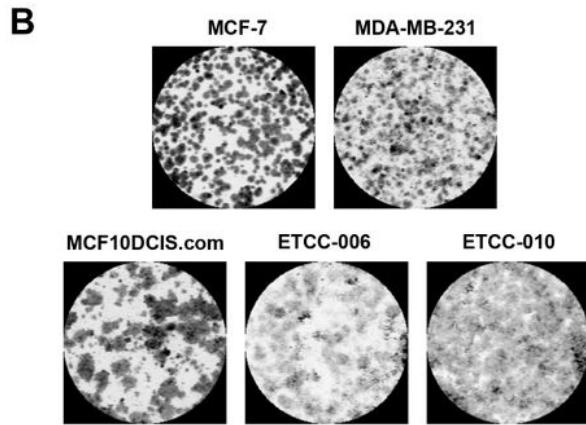
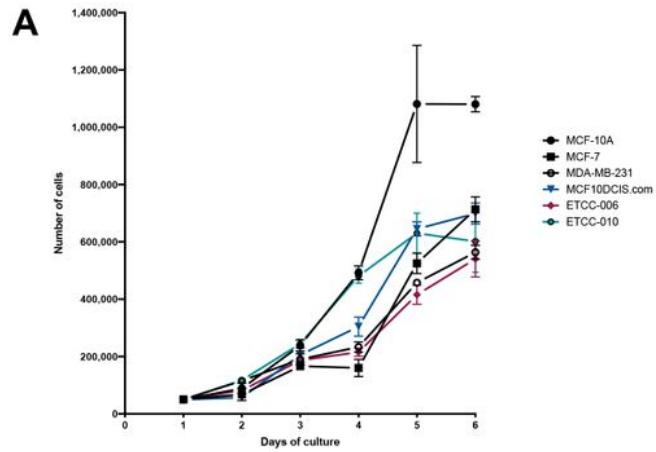
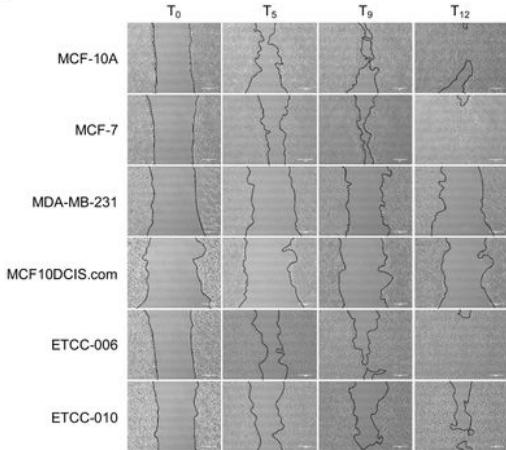


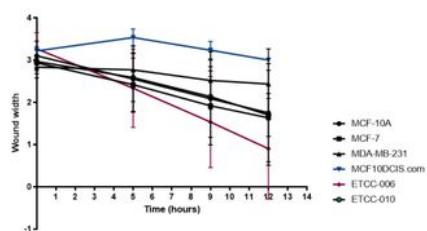
Figure 5

Proliferation and survival assessment of the DCIS cell lines. Cell proliferation was monitored by trypan blue staining over six days (A); while cell survival was assessed by a clonogenic assay, followed by crystal violet staining (B) and quantification using ImageJ (C). Statistical analysis of the clonogenic assay was done using one-way ANOVA (GraphPad Prism v.8.3.0). **** p-value < 0.0001; *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05.

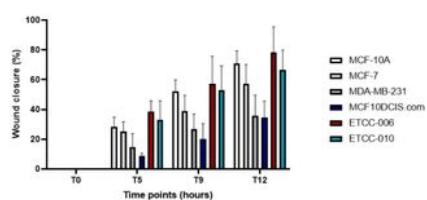
A



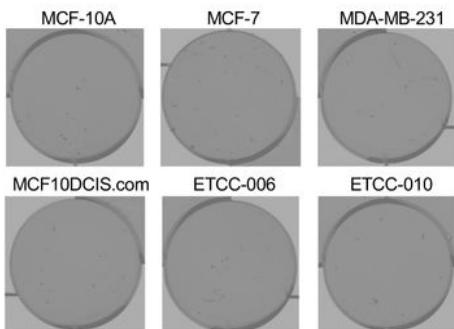
B



C



D



E

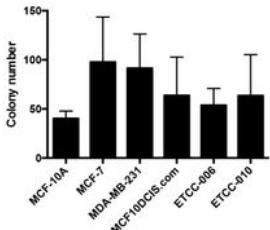
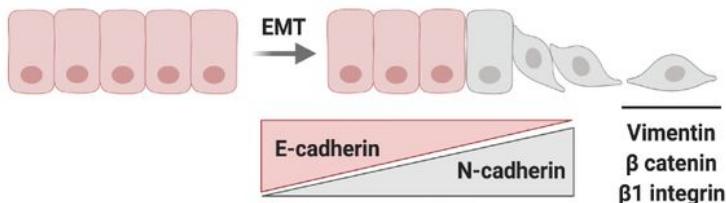


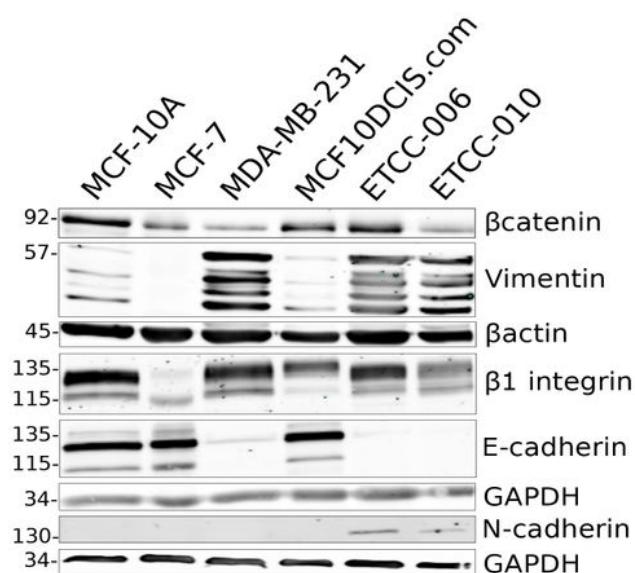
Figure 6

Migration and anchorage-independent growth assessment of the DCIS cell lines. Migratory capacity of the DCIS cell lines, in comparison to MCF10, MCF7 and MDA-MB-231 cells, was examined by a wound healing assay. The wound was imaged using bright field microscopy at 20X (A) and pseudo-marked in black. (B) wound width and (C) wound area was quantified in ImageJ in arbitrary units. Anchorage-independent growth was measured by soft agar assays; wells were imaged using the Odyssey Li-Cor system (D) and scored manually (E). The experiments were repeated in triplicate.

A



B



C

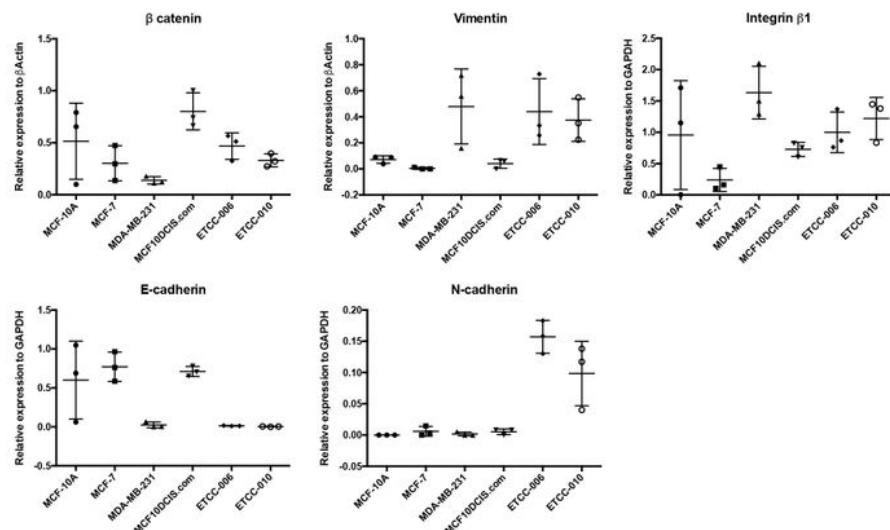
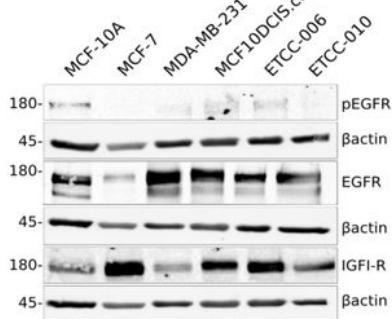


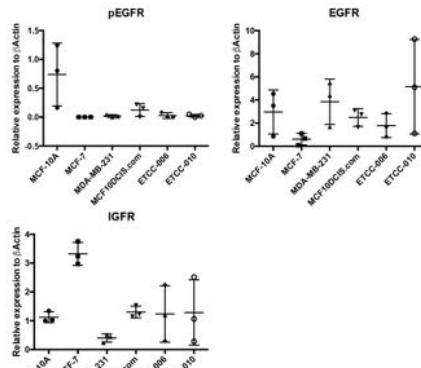
Figure 7

ETCC-006 and ETCC-010 display markers of epithelial to mesenchymal (EMT) transition. (A) Schematic illustrating the markers associated with EMT (B) Western blot analysis of EMT markers in the DCIS cell lines compared to breast cancer cell lines, MCF7 and MDA-MB-231, and normal-like breast epithelial cells, MCF10A. 42 µg of total protein was loaded per lane onto the gel. (C) Protein level was quantified and normalized against β actin or GAPDH using ImageJ. This experiment was repeated in triplicate, panel B is a representative immunoblot. Panel A was created with BioRender.com. Full-length blots are presented in Supplementary Figure 2.

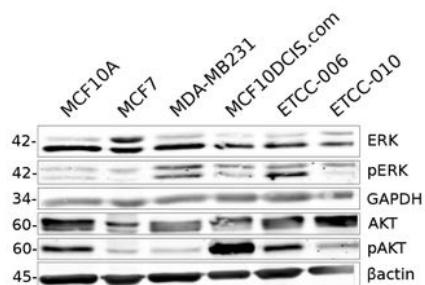
A



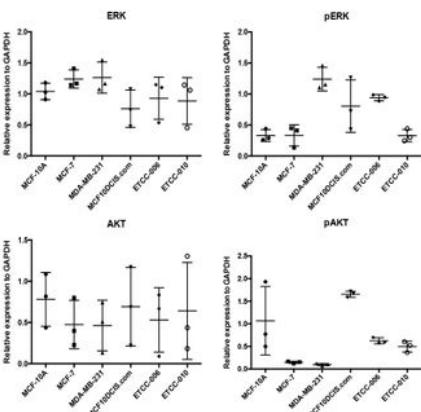
B



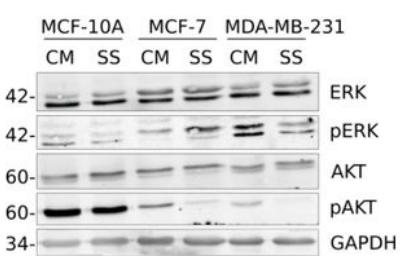
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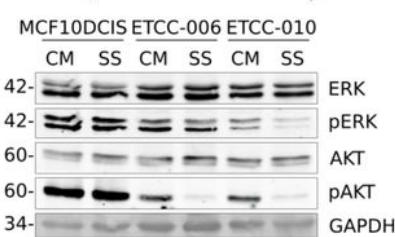
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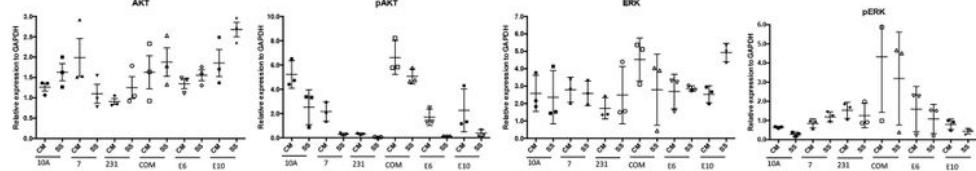
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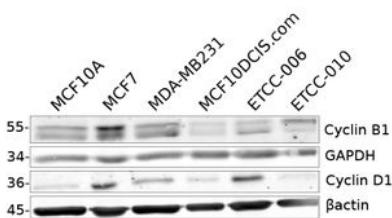
F



G



H



I

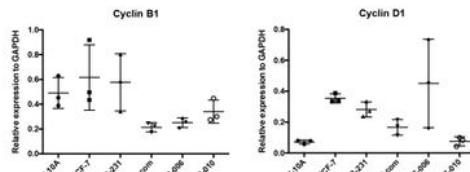


Figure 8

Profile of cell signalling and cell cycle proteins in the DCIS cell lines compared to breast cancer cell lines. (A) and (B) IGF-1R, EGFR and p-EGFR protein level. (C) ERK, p-ERK, AKT and p-AKT protein levels under normal conditions. (D), (E) and (F) Comparison of ERK, p-ERK, AKT and p-AKT protein levels under normal conditions and serum-starvation conditions. (H) and (I) Cyclin B1 and D1 protein levels. Protein level was quantified and normalized against β actin or GAPDH using ImageJ. This experiment was repeated in triplicate; panels A, C, E, F and H are representative immunoblots. Full-length blots are presented in Supplementary Figures 3 - 7.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalmaterial.docx](#)
- [ETCC6ETCC10DifferentialGenespval0.05.xlsx](#)
- [7SuppMatFig8HBMCCancerSamson.pdf](#)
- [6SuppMatFig8FBMCCancerSamson.pdf](#)
- [5SuppMatFig8EBMCCancerSamson.pdf](#)
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