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Growth of hormone-dependent MCF-7 breast cancer cells is promoted by constitutive caveolin-1 whose expression is lost in an EGF-R-mediated manner during development of tamoxifen resistance

Nicholas B. P. Thomas · Iain R. Hutcheson ·
Lee Campbell · Julia Gee · Kathryn M. Taylor ·
Robert I. Nicholson · Mark Gumbleton

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Abstract Caveolin-1 displays both tumour-suppressor and tumour-promoter properties in breast cancer. Using characterised preclinical cell models for the transition of oestrogen-sensitive (WT-MCF-7 cells) to a tamoxifen-resistant (TAM-R cells) phenotype we examined the role caveolin-1 in the development of hormone-resistant breast cancer. The WT-MCF-7 cells showed abundant expression of caveolin-1 which potentiated oestrogen-receptor (ER α) signalling and promoted cell growth despite caveolin-1 mediating inhibition of ERK signalling. In TAM-R cells caveolin-1 expression was negligible, repressed by EGF-R/ERK signalling. Pharmacological inhibition of EGF-R/ERK in TAM-R cells restored caveolin-1 and also resulted in the emergence of pools of phosphorylated caveolin-1. WT-MCF-7 cells exposed to tamoxifen for up to 12 weeks displayed increased caveolin-1 (peaking by week 2) followed (after week 8) by a marked decrease as the cells progress to develop a stable tamoxifen-resistant phenotype. The targeted down-regulation (siRNA) of caveolin-1 in WT-MCF-7 cells reduced growth but did not affect their sensitivity to tamoxifen, suggesting loss of caveolin-1 alone

is not sufficient to confer tamoxifen-resistance. Hyperactivation of EGFR/ERK is a feature of tamoxifen-resistant breast cancer cells, a principal driver of cell growth. Recombinant expression of caveolin-1 in TAM-R cells did not affect EGFR/ERK activity, potentially due to mislocalisation of caveolin-1 through hyperactivation of the mTOR pathway or altered caveolin-1 phosphorylation. This work defines a novel role for caveolin-1 with implications for the clinical course of breast cancer and identifies caveolin-1 as a potential drug target for the treatment of early oestrogen-dependent breast cancers. Further, the loss of caveolin-1 may have benefit as a molecular signature for tamoxifen resistance.

Keywords Caveolin · Tamoxifen · Breast cancer · Resistance · Hormone-dependent · EGFR · ERK1/2 · Phosphorylated-caveolin · mTOR · Oestrogen receptor ER α

Introduction

Tamoxifen is the most frequently prescribed anti-hormonal drug for the systemic treatment of oestrogen receptor (ER)-positive breast cancer. Its use has led to significant decreases in breast cancer mortality within the last decade [1, 2]. However, resistance to the drug occurs both in patients who initially benefited from the drug (acquired resistance), and in patients who at the onset of treatment were non-responsive despite an intact ER status (de novo resistance) [3]. Recent evidence has unequivocally shown that the development and progression of endocrine resistant breast cancer involves complex interactions between steroid and growth factor signalling pathways [4, 5]. In particular, increased EGF-R and hyper-activation of its

Nicholas B. P. Thomas, Iain R. Hutcheson and Lee Campbell have contributed equally to this work.

N. B. P. Thomas · L. Campbell · M. Gumbleton (✉)
Experimental Therapeutics, Welsh School of Pharmacy, Cardiff
University, Redwood Building, , King Edward VII Avenue,
Cardiff CF10 3XF, UK
e-mail: gumbleton@cf.ac.uk

N. B. P. Thomas · I. R. Hutcheson · J. Gee ·
K. M. Taylor · R. I. Nicholson
Tenovus Centre for Cancer Research, Cardiff University,
Redwood Building, , King Edward VII Avenue, Cardiff CF10
3XF, UK

down-stream signalling elements (Ras, MEK-1, ERK 1/2) invariably correlate with hormonal resistance, aggressive clinicopathology, metastatic disease and poor prognosis [6–9]. Indeed, in MCF-7 breast cancer cell lines displaying acquired resistance to the growth inhibitory effects of either tamoxifen, or the pure anti-oestrogen fulvestrant ('Faslodex'), increased EGFR/c-erbB2/ERK signalling activity is evident. As a corollary these tamoxifen-resistant cells display enhanced sensitivity to the growth inhibitory actions of the EGFR tyrosine kinase inhibitor gefitinib, as well as the anti-c-erbB2 monoclonal antibody trastuzumab, and the MEK inhibitor, PD098059 [10, 11].

The caveolins serve as membrane scaffolding proteins that compartmentalise various signalling molecules within discrete domains at the plasmalemma, termed caveolae [12]. Specifically, caveolin-1 is known to generally repress the inherent kinase activity of a number of proto-oncogenes through binding of the kinase catalytic regions to a contiguous twenty amino acid motif situated within the N-terminus of the caveolin-1 molecule, termed the caveolin scaffolding domain [13]. Examples of kinases shown to be inhibited by this association include amongst others the epidermal- and platelet-derived growth factor receptors (EGFR and PDGFR), several members of the ERK1/2 MAP kinase cascade, heterotrimeric G-proteins and src family tyrosine kinases [14–16]. Furthermore, several independent studies have demonstrated a reciprocal interaction between the expression of caveolin-1 and activated components of the ERK1/2 MAP kinase signalling pathway [17–20]. While, caveolin-1 mRNA and protein has been shown to be present in normal human mammary epithelial cells it has also been reported to be greatly reduced or absent in an extensive range of transformed cells derived from human breast cancers [21–24]. Caveolin-1 has been proposed as the elusive tumour suppressor protein residing at chromosomal loci 7q31.1, a fragile site frequently deleted in a wide spectrum of human cancers [25].

In contrast to its general inhibitory effects on growth factor mediated signal transduction, caveolin-1 has been shown to promote the activity of steroid receptor signalling pathways, in particular the ER pathway [26, 27]. Specifically, in both the presence and absence of oestrogen ligand caveolin-1 appears to promote the transactivation of ER α , but not ER β , via direct interaction with the AF-1 ligand-independent domain located in the N-terminal portion of the ER α molecule [27]. The potentiation of ER α signalling arising from caveolin-1-induced translocation of the ER α from the cytoplasm to the nucleus has been shown to result in increased transcription of ER-dependent genes [26]. This caveolin-ER α interaction has been demonstrated *in vitro* to confer a five-fold increase in resistance to tamoxifen in MCF-7 cells over-expressing recombinant caveolin-1 [26].

Similarly, the recombinant expression of caveolin-1 has been shown to induce androgen insensitivity in previously hormone sensitive prostatic cancer cell lines [28], an observation consistent with clinical samples showing caveolin-1 expression to positively correlate with androgen refractive prostate cancer [29].

Molecular adaptations occur during resistance to hormone ablation therapy that are common to cancers of the breast and prostate [30]. Therefore we may hypothesise that upregulation of caveolin-1 may facilitate hormone-independent growth of breast tumours that have become resistant to the growth inhibitory actions of tamoxifen. Alternatively, the loss of caveolin-1 in the tamoxifen-resistant subtype may afford increased EGFR/ERK signalling and breast tumour growth. To discriminate between these possibilities and to fully define a role for caveolin-1 in tamoxifen-resistant breast cancer we undertook a series of mechanistic studies using a well characterised preclinical human tamoxifen-resistant breast cancer cell model [10, 11, 31].

We report for the first time that caveolin-1 is constitutively expressed in oestrogen-dependent MCF-7 breast cancer cells (WT-MCF-7) where it promotes ER α signalling and cell proliferation despite caveolin-1 mediating inhibition of ERK signalling. The siRNA-mediated inhibition of caveolin-1 in WT-MCF-7 cells inhibited basal levels of phosphorylated ER α without any effect on total ER α levels. Further, proliferation of the WT-MCF-7 cells was significantly reduced by caveolin-1 knockdown to the same extent as tamoxifen inhibition of WT-MCF-7 cell growth. Conversely in cells that have specifically acquired a stable tamoxifen resistance phenotype (TAM-R cells), caveolin-1 expression was negligible. The siRNA-mediated knockdown of caveolin-1 in the WT-MCF-7 cells did not, however, affect the sensitivity of this cell to the growth-inhibitory actions of tamoxifen, suggesting loss of caveolin-1 alone is not sufficient to confer tamoxifen-resistance. Nevertheless, WT-MCF-7 cells exposed to tamoxifen for upto 12 weeks displayed initial increases in caveolin-1 followed by a marked decrease as the cells progress to develop a stable tamoxifen-resistance phenotype. The transient increase in caveolin-1 potentially imparting a survival advantage in the early/developmental stages of resistance. In TAM-R cells caveolin-1 expression is repressed by EGFR/ERK signalling. The pharmacological inhibition of EGFR/ERK in the TAM-R cells restored caveolin-1 and also led to the emergence of pools of phosphorylated caveolin-1. Recombinant expression of caveolin-1 in TAM-R cells did not affect EGFR/ERK activity, potentially due to mislocalisation of caveolin-1 through hyperactivation of the mTOR pathway and/or altered caveolin-1 phosphorylation.

Materials and methods

Cell culture

Tissue culture medium and constituents were purchased from Gibco Europe Ltd. (Paisley, Scotland) and tissue culture plastic ware was obtained from Nunc (Roskilde, Denmark). The wild type MCF-7 (WT-MCF-7) breast cancer cells (originally a gift from AstraZeneca Pharmaceuticals, Cheshire UK) were routinely cultured in phenol red-free RPMI medium supplemented with 5% foetal calf serum (FCS) plus penicillin-streptomycin (10iU/ml–10 µg/ml), fungizone (2.5 µg/ml) and glutamine (4 mM). The TAM-R cell line was derived from WT-MCF-7 as previously reported [11] and was grown in phenol-red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS, antibiotics, glutamine (4 mM) and 4-hydroxytamoxifen (4-OH-TAM, 100nM in ethanol). Their respective growth media were replaced every 4 days and cultures were maintained at 37°C in a humidified, 5% CO₂ atmosphere. The TAM-R cell line represents a stable tamoxifen-resistant phenotype acquired between 6 and 12 months of continual tamoxifen exposure [11].

Experimental cell culture for Western blotting, immunocytochemistry, PCR and cell growth studies

Following 4 days growth in their respective growth mediums (as described above) cells were transferred to phenol-red/steroid free, serum growth factor-free DCCM medium (Biosynergy Europe, Cambridge, UK) for 24 h. Cells were then treated for 7 days with either gefitinib (1 µM in ethanol, AstraZeneca, Macclesfield, UK), PD184352 (10 µM), oestradiol (1 nM), 4-OH-TAM (100 nM) or appropriate vehicle as control. The long-term tamoxifen studies involving WT-MCF-7 cells were performed with 4-OH-TAM (100 nM) present in the culture medium which was replenished with fresh media every 48 h for the duration of the 12 week study. The effect of increasing concentrations of EGF (0.01–10 ng/ml in PBS, Merck Biosciences, Nottingham, UK) for 10 min following a 7 day treatment with either tamoxifen (100 nM) or vehicle control (ethanol) was also assessed in WT-MCF-7 cells. All studies involving TAM-R cells were performed in the presence of 4-OH-TAM (100 nM). All cell culture studies were performed at least three times.

Transfection studies

The ecdysone-inducible mammalian expression system, with the pIND plasmid containing the full-length caveolin-1 cDNA (with all exons) (kind gift of Dr. D. Black, Beatson Institute, Glasgow, UK), was used according to the

manufacturers instructions. Briefly, TAM-R cells were grown until 70–80% confluent in phenol red/steroid free, serum free DCCM media containing 4-OH-TAM (100 nM) and the antibiotics penicillin G (100 units/ml) and streptomycin (100 µg/µl). Lipofectin reagent was complexed in antibiotic/serum free DCCM for 45 min at room temperature with the two plasmids comprising the caveolin-1 ecdysone inducible expression system, i.e. pIND-cav and pVgRXR. Cells were incubated for 6 h with transfection solution, then washed and incubated for 4 h in fresh media. Cells were again washed and incubated for a further 20 h with the ecdysone Ponasterone A (5–20 µM). All relevant controls were included: pIND-empty vector + pVgRXR + lipid; lipid only; Ponasterone A only; ethanol (vehicle for the Ponasterone A) only, and untransfected cells. Recombinant caveolin-1 protein expression was assessed by immunocytochemistry and Western blotting analysis.

Growth studies

Cell population growth was evaluated by means of trypsin dispersion of the cell monolayers (performed in triplicate) following a 7 day incubation with either EGF (10 ng/ml), gefitinib (1 µM), PD184352 (10 µM) or fulvestrant (100 nM). Controls were incubated for the same period of time with the appropriate vehicle. Cell counts were undertaken by use of a Coulter-counter (Luton, UK). All proliferation studies were performed at least 3 times.

Protein cell lysis

Cells were washed three times with PBS and lysed using ice-cold lysis buffer. The cellular contents were transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 15 min at 4°C and supernatant aliquots were stored at –20°C until required. Total protein concentrations were determined using the DC BioRad protein assay kit (BioRad, Hertfordshire, UK). Absorbances were analysed at 750 nm on a CECIL CE 2041 spectrophotometer.

Western blotting

Cell lysate samples (20–50 µg) were denatured, subjected to electrophoresis separation on 7.5% SDS polyacrylamide gel and trans-blotted onto a 0.2 µm nitro-cellulose membrane (Schleicher and Schuell, Dassel, Germany). Blots were blocked at room temperature overnight, in 10% BM Chemiluminescence Blotting Substrate (Roche diagnostics) made up in Tris buffered saline (TBS)-Tween 20 (0.05%). The blots were then incubated in one of the following primary antibodies for a minimum of 1 h; caveolin-1 (Transduction Labs, Lexington, KY), phospho-caveolin (Tyr14, #3251S; New England Biolabs, Hertfordshire, UK)

Caveolin-2 (Transduction Labs), phospho-ER α (ser118) (#sc-12915-R, Santa Cruz), total ER α (#sc-543, Santa Cruz), phospho-ERK1/2 (#9101; New England Biolabs), total ERK1/2 (#9102 New England Biolabs) and phospho-EGFR (Y1068, #2234; New England Biolabs), total EGFR (Upstate Biotechnology), phospho-AKT (#4058P; New England Biolabs) total AKT (#9272; New England Biolabs), phospho-tuberin/TSC-2 (#3617S; New England Biolabs) total tuberin/TSC-2 (#3612; New England Biolabs), phospho-mTOR (#2971S; New England Biolabs), total mTOR (#2983; New England Biolabs) and TGN-46 (used at 1:500 was a kind gift from Dr Arwyn Jones, Cardiff University) all at a 1/1,000 dilution (unless where stated) and made up in 1% milk. Membranes were washed three times over 15 min in TBS-Tween and then incubated for 1 h with the required secondary IgG horseradish peroxidase labelled antibody (donkey anti-rabbit or sheep anti-mouse; Amersham Biosciences UK Ltd., Buckinghamshire, UK) diluted 1/10,000 in 10% BM Chemiluminescence Blotting Substrate and made up in (TBS)-Tween. Membranes were again washed three times in TBS-Tween for 30 min. Detection was performed by applying a thin film of SuperSignal[®] WEST DURA/PICO chemiluminescent substrate [Pierce, and Warriner Ltd, Chester, UK] to the membrane for 5 min. Hyperfilm ECL film was exposed to the membrane for between 2 and 30 min as appropriate. The film was then removed and placed in developer and fixer. Results were scanned using a BioRad model GS-700 densitometer (Bio-rad Laboratories, Hertfordshire, UK).

Immunocytochemistry

WT-MCF7 and TAM-R cells were seeded onto 3-aminopropyltriethoxysilane (APES)-coated glass coverslips, contained in 35 mm culture dishes at a density of 1×10^5 cells/dish. Phosphorylated EGFR, phosphorylated ERK1/2, caveolin-1 and caveolin-2 immunostaining was carried out as described below. Cells were washed with PBS and fixed in paraformaldehyde (2% v/v in PBS) overnight at room temperature (r/t), then twice PBS washed and stored at -20°C in a sucrose medium (0.25 M sucrose, 0.005 M magnesium chloride prepared in 1:1 PBS:glycerol). After washing in PBS, they were incubated in a humidity chamber with a 10% normal human serum block to prevent non-specific antibody binding. The cells were then exposed overnight to one of the following: rabbit phospho-EGFR (Y1068, #2234; New England Biolabs, 1/1,000 dilution in PBS), rabbit phospho-ERK1/2 (#9101; New England Biolabs, 1/1,000 dilution in PBS), mouse caveolin-1 monoclonal antibody (#2511, New England Biolabs, Hertfordshire, UK, 1/500 dilution in PBS) or the mouse caveolin-2 monoclonal antibody (Transduction Labs, Lexington, KY, 1/500 dilution in PBS). Using PBS washes between steps,

either 1/20 of the biotinylated anti-rabbit or anti-mouse secondary antibodies in 0.1% BSA/PBS (Rabbit Unitect[™] and Mouse Unitect[™] ABC kit; Oncogene Research Products, Cambridge, MA, USA) and freshly formed avidin-biotinylated horseradish peroxidase complex (Mouse Unitect[™] ABC kit; 1/20 in 0.1% BSA/PBS) were then applied sequentially to all coverslips followed by liquid diaminobenzidine tetrahydrochloride (DAB) + substrate-chromogen (K3468; Dako A/S Ltd.). All slides were either methyl green or 10% haematoxylin counterstained, air-dried and coverslipped using non-aqueous mountant. Immunostaining for each assay was assessed by two personnel using a dual-viewing attachment to an Olympus BH-2 light microscope. Cytoplasmic and membrane caveolin-1 and caveolin-2 staining was determined.

Fluorescent microscopy

About 2×10^5 cells were grown on 0.17 mm thick coverslips for 24 h prior to transfection, as above. At pre-determined times cells were fixed with 4% formaldehyde for 15mins, permeabilised with 0.4% saponin in PBS with 1% BSA, blocked with 10% normal goat serum and then incubated with both anti-phospho-ERK1/2 rabbit polyclonal antibody (1/1,000, New England Biolabs, Hertfordshire, UK) and anti-caveolin-1 mouse monoclonal antibody (1/500, Transduction Labs, Lexington, KY) for 1 h at room temp. The cells were then incubated with Alexa Flour 488-conjugated anti-rabbit secondary antibody and Alexa Flour 594-conjugated anti-mouse antibody (both 1/1,000, Molecular Probes) for 1 h, and assembled onto slides using Vectorshield (Vector Laboratories) with DAPI (4',6-diamidino-2-phenylindole). All coverslips were viewed on a Leica RPE automatic microscope using a 100 $\times$ oil immersion lens. The fluorescent superimposed images were acquired using a multiple bandpass filter set as well as bright field for Differential Interference Contrast.

RT-PCR

Cell monolayers were lysed and the total RNA was isolated using an RNA isolator kit (Genosys Biotech Inc., Cambridge, UK). Total RNA (1 μg) was reverse transcribed and the resulting cDNA amplified using specific caveolin-1 and caveolin-2 primers. RT-PCR conditions were optimized for the primer set using standard conditions as described previously [11]. Co-amplification PCR reactions were performed using both primers for the gene of interest and β -actin (internal control). For detection of caveolin-1, cDNA samples were co-amplified for 26 cycles, whereas, for caveolin-2 cDNA samples were co-amplified for 28 cycles. All amplicons were then separated by gel electrophoresis, visualized under UV illumination, photographed, and scanned where

necessary using a Bio-Rad Laboratories Ltd. model GS-690 Imaging Densitometer.

Primers used were designed manually using OLIGO (MedProbe AS, Oslo, Norway) primer pair design software. Primers were designed where possible to span intron/exon borders and specificity was checked using the European Molecular Biology Laboratory (EMBL)-GenBank database software using the BLAST program. The respective Forward and Reverse primers were:

Caveolin-1: 5' TCA ACC GCG ACC CTA AAC ACC 3' and 5' TGA AAT AGC TCA GAA GAG ACA T 3' (561 bp)

Caveolin-2: 5' GGC TCA ACT CGC ATC TCA A 3' and 5' AAA ACA GAC AAA CAA ATA AGA A 3' (753 bp)

β -Actin: 5'-GGA GCA ATG ATC TTG ATC TT and 3'-CCT TCC TGG GCA TGG AGT CCT (204 bp).

Targeted (siRNA) down-regulation of caveolin-1 and tamoxifen sensitivity assays

As previously described [32, 33] a siRNA duplex (21 nucleotides) targeting the caveolin-1 mRNA sequence 5'-AACCAGAAGGGACACACAGUU-3' was used to down-regulate caveolin-1 protein expression and a siRNA duplex targeting the firefly luciferase (GL2) mRNA sequence 5'-AACGUACGCGGAUACUUCGA-3' was used as a negative control. The duplexes were purchased from MWG (Ebersberg, Germany) as unprotected, desalted, and purified siRNA.

WT-MCF-7 and TAM-R cells were seeded at a density of $3 \times 10^3 \text{ cm}^{-2}$ in a 24-well format. After 24 h, growth media containing 4-OH-TAM was supplemented to some of the WT-MCF-7 cells. At day 2 both WT-MCF-7 and TAM-R cells were transfected with 250 nmol (2.5 μg) of siRNA annealed with Lipofectamine 2000 (Invitrogen) for 4 h as recommended by the manufacturer. Following transfection the cells were supplemented with growth containing 5% FCS with or without tamoxifen as appropriate. The siRNA transfection procedure was repeated at day 5 and the cells harvested at 72 h later (day 8 post-seeding) for cell counts and Western blot analysis.

Sucrose density membrane fractionation under detergent-free conditions

A detergent-free method was used to isolate caveolae-enriched domains and prepare subcellular fractions as previously described [34] but with slight modifications. Treated and control cells from T-75 cm^2 flasks were washed in icecold PBS (pH 7.3) and the cells scraped into 1 ml of 150 mM Na_2CO_3 with 1 mM EDTA (pH 11.0).

The cell solution was then passed through a 25-g syringe needle and homogenised with six strokes of a Dounce pestle, followed by sonication in a water bath with $3 \times 20 \text{ s}$ bursts interspersed with 30 s incubations on ice. Approximately 1 ml of 80% sucrose in MES-buffered saline containing protease inhibitors (MBS: 25 mM MES, 150 mM NaCl, 2 mM EDTA-MBS, pH 6.5) was added to the homogenate to form a 40% sucrose lysate and placed at the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was then generated by the sequential layering of 1.4 and 0.8 ml of 35 and 5% sucrose (prepared in MBS), respectively, onto the top of the lysate. The gradient was then centrifuged at 180,000g using a SW41Ti rotor (Beckman Instruments-Fullerton, CA) for 18 h at 4°C. Twelve 300 μl fractions were collected (1–12; beginning from the top of the tube) and 20 μl aliquots from each fraction then mixed with an equal volume of 2 $\times$ loading buffer and subjected to Western blot analysis.

Statistics

For immunocytochemical analysis, comparisons of H-scores were determined using the Mann–Whitney 'U' test for non-parametric data. Overall differences between control and treatment groups were determined by two-way ANOVA. Direct comparisons between control and treatment effects were assessed using a Student's *t*-test (2-groups) or ANOVA (for comparisons of greater than 2 groups) with a post-hoc Bonferroni test. Differences were considered significant at the $P < 0.05$ level.

Results

Inverse relationship between EGFR/ERK signalling activity and caveolin-1 expression in WT-MCF-7 cells and TAM-R cells

Western blotting and immunocytochemical analysis confirmed previous findings [10, 11, 31] that under basal growth conditions, i.e., in the absence of exogenous growth factors, total and phosphorylated EGFR protein expression is higher in TAM-R compared to the parental WT-MCF-7 cells (Fig. 1a); indeed WT-MCF-7 cells displayed negligible levels of total- and activated-EGFR. The phosphorylated EGFR in TAM-R cells was observed at both plasma membrane and perinuclear regions of the cell (Fig. 1b). Basal phosphorylated ERK1/2 protein levels were also significantly increased in TAM-R cells compared to WT-MCF-7 cells, but total ERK1/2 protein levels were equivalent in the two cell lines (Fig. 1a). Phosphorylated ERK1/2 in TAM-R cells was most apparent in the nucleus, however, increased levels of cytoplasmic phosphorylated

Fig. 1 Basal levels of activated (phosphorylated) EGFR and ERK1/2 in the parental hormone-sensitive WT-MCF-7 cells and in the tamoxifen resistant TAM-R cells. **a** Western blotting and **b** immunocytochemistry. Western blot analysis of total EGFR and ERK1/2 expression was also performed alongside β -actin protein expression to demonstrate equivalent sample loading. Results are representative of at least three separate experiments

ERK1/2 were also observed in TAM-R cells compared to WT-MCF-7 cells (Fig. 1b).

Under the same basal growth conditions caveolin-1 mRNA (Fig. 2a) and caveolin-1 protein (Fig. 2b) levels were significantly reduced in the TAM-R compared to WT-MCF-7 cells. Immunocytochemical analysis also revealed TAM-R cells to be generally devoid of caveolin-1 expression, while WT-MCF-7 cells displayed caveolin-1 in both cytoplasmic and plasma membrane compartments (Fig. 2c); in the occasional TAM-R cells where low levels

Fig. 2 Caveolin-1 in the WT-MCF-7 and TAM-R cells. **a** mRNA by RT-PCR, and **b** Western blotting, **c** Immunocytochemistry. In WT-MCF-7 cells caveolin-1 appeared as patches along peripheral cell membrane (arrows) and within the cytoplasm (arrowheads). β -actin was used as an internal control for RT-PCR and in Western blotting. Results are representative of at least three separate experiments

of caveolin-1 were observed it was clearly localised to perinuclear regions (Fig. 4a ‘control’). The expression of caveolin-2 was found not to be different between the WT-MCF-7 and TAM-R cell lines (Fig 2b, c) with caveolin-2 present at both plasmalemmal and cytoplasmic locations.

Differential effect of altering EGFR/ERK1/2 signalling in WT-MCF-7 and TAM-R cells upon caveolin-1 expression and cell proliferation

Figure 3a shows the effect of pre-treatment with 1 μ M gefitinib (a selective inhibitor of the EGFR tyrosine kinase). In

Fig. 3 Inhibition of activated EGFR and activated ERK1/2 in the WT-MCF-7 and TAM-R cells. **a** Western blot for caveolin-1, total and phosphorylated EGFR, and total and phosphorylated ERK1/2 prior to and following incubation with gefitinib (1 μ M) for 1 h. **b** Western blot for caveolin-1, total and phosphorylated EGFR, and total and phosphorylated ERK1/2 prior to and following incubation with PD184352 (10 μ M) for 1 h. Data are representative of at least three separate experiments

WT-MCF-7 cells gefitinib decreased the levels of p-EGFR and of downstream p-ERK1/2, with a concomitant slight increase in caveolin-1 protein and mRNA (data not shown). As expected the elevated basal levels of p-EGFR and p-ERK1/2 in the TAM-R cells were substantially reduced by gefitinib without effects upon the expression levels of total EGFR or total ERK1/2. Gefitinib treatment in the TAM-R cells led to a substantial up-regulation in caveolin-1 protein expression (Fig. 3a) which appeared localised to perinuclear regions (Fig. 4a ‘gefitinib’, panel ii), a similar subcellular restriction to that seen in the minimal caveolin-1 expressing untreated TAM-R cells (Fig. 4a ‘control’, panel i). The loss of pEGFR and pERK1/2 and induction of caveolin-1 by gefitinib was associated with a significant inhibition (85%) of TAM-R cell growth ($P < 0.05$, $n = 4$, Fig. 4c), while the growth of the parental WT-MCF-7 cells, expressing negligible pEGFR, was unaffected by gefitinib (Fig. 4b).

Figure 3b shows the effect of pre-treatment with 10 μ M PD184352 (a selective inhibitor of MEK1/2). In WT-MCF-7 cells PD184352 led to a loss of basal (i.e. unstimulated) p-ERK1/2 with a concomitant slight increase in caveolin-1. Treatment with PD184352 in TAM-R cells led to a complete loss of basal p-ERK1/2 but also to a substantial increase in caveolin-1 (Fig. 3b) which appeared localised to perinuclear regions (Fig. 4a ‘PD184’, panel iii) and notably absent from the plasma membrane. The growth of WT-MCF-7 cells (Fig. 4b) remained essentially unchanged with PD184352 treatment, while that of TAM-R cells (Fig. 4c) was reduced by 90% ($P < 0.05$, $n = 4$).

Modulation of ER α signalling alters caveolin-1 expression in WT-MCF-7 cells without effect on EGF-ligand induced ERK1/2 activity

Figure 5a shows the effect of oestradiol or tamoxifen treatments in WT-MCF-7 cells. Oestradiol (1 nM) for 7 days decreased slightly caveolin-1, and to a greater extent total ER α , protein levels compared to no treatment control, while having no effect upon phosphorylated or total ERK1/2 expression. Tamoxifen for 7 days led to a clear increase in caveolin-1 expression and a marginal increase in ER α , while having no significant effect on the basal protein levels of either phosphorylated or total ERK1/2. Oestradiol significantly increased the cell growth rate over the 7 day treatment period ($P < 0.01$, $n = 3$) while tamoxifen led to a significant decrease in growth ($P < 0.01$, $n = 3$) (Fig. 4b).

Figure 5b shows the effect of EGF stimulation in WT-MCF-7 cells in the presence or absence of tamoxifen. In the absence of tamoxifen (control; lanes 1–5) a 10 min incubation with EGF led to activation of ERK1/2 in a concentration-dependent manner with maximal p-ERK1/2 levels observed at an EGF concentration of between 1 and 10 ng/ml. There was no effect of EGF upon caveolin-1 levels, at least upto 1 ng/ml EGF concentration, although a small increase in caveolin-1 was evident at the highest EGF concentration (10 ng/ml; lane 5). In contrast with tamoxifen treatment (7 days) (‘Tamoxifen’ lanes 6–10, Fig. 5b) we noted substantial increases in caveolin-1 expression in the WT-MCF-7 cells compared to the respective non-tamoxifen treated control cells (lanes 1–6). The tamoxifen-induced increase in caveolin-1 appeared to occur independent of EGF ligand. As expected EGF stimulation increased p-ERK1/2 activity in a concentration-dependent manner in both control and tamoxifen-treated cells, with tamoxifen treatment per se not effecting the pattern or magnitude of EGF stimulated p-Erk1/2 expression.

Fig. 4 Caveolin-1 immunohistochemistry in TAM-R cells and the effect of various treatments upon WT-MCF-7 and TAM-R cell growth.

a Immunocytochemistry for endogenous caveolin-1 in TAM-R cells (panel i), in TAM-R cells treated with 1 μ M gefitinib (panel ii), in TAM-R cells treated with 10 μ M PD184352 (panel iii). Re-expressed caveolin-1 (panels ii and iii) appeared predominantly within the cytoplasm (arrowheads); **b** Histogram of WT-MCF-7 cell growth at 7 day post-treatment with oestradiol (E_2 , 1 nM), tamoxifen (100 nM), EGF (10 ng/ml), gefitinib (1 μ M) or PD184352 (10 μ M); **c** Histogram of TAM-R cell growth at 7 day post-treatment with EGF (10 ng/ml), gefitinib (1 μ M) or PD184352 (10 μ M). Results are expressed as mean $\pm$ SEM of triplicate wells and are representative of at least three separate experiments. * denotes significance $P < 0.01$ versus control

Caveolin-1 shows a temporal pattern of expression during the development of endocrine resistance in WT-MCF-7 cells treated longterm with tamoxifen

Figure 5c shows caveolin-1 expression in WT-MCF-7 cells treated with tamoxifen over a period of 12 weeks, with cells harvested at various pre-determined time points. Compared to the untreated control (–) cells the tamoxifen treated (+) cells displayed an increased (3–4 fold) expression of caveolin-1 by 2 weeks with levels remaining relatively constant thereafter upto 8 weeks. By week 10 a marked decrease in caveolin-1 expression was observed

which diminished further by week 12 as the WT-MCF-7 cells, initially sensitive to the effects of tamoxifen, progressed to acquire an endocrine-resistant phenotype.

Targeted inhibition of caveolin-1 protein in WT-MCF-7 cells leads to reduced cell proliferation, reduced ER α signalling and increased ERK signalling without altering sensitivity to tamoxifen

The targeted down-regulation of constitutively expressed caveolin-1 by siRNA (Cav siRNA) resulted in the profound loss of caveolin-1 protein compared to control siRNA

Fig. 5 Protein expression in WT-MCF-7 cells. **a** Western blot of caveolin-1, total oestrogen receptor α (ER α) and ERK1/2 (total and phosphorylated) in untreated WT-MCF-7 cells and following incubation with either oestradiol (1 nM) or tamoxifen (100 nM) for 7 days; **b** Western blot of caveolin-1 and phosphorylated ERK1/2 in WT-MCF-7 cells prior to and following incubation with tamoxifen (100 nM) for 7 days, and subsequently treated with either increasing concentrations of EGF (0.01–10 ng/ml) or vehicle control (0 ng/ml) for 10 min; **c** Western blot of caveolin-1 during endocrine resistance development in WT-MCF-7 cells treated longterm (upto 12 weeks) with tamoxifen [(100 nM) (+)] compared to vehicle control untreated (–) cells. Data are representative of at least three separate experiments

(Fig. 6a). We also noted caveolin-1 siRNA treatment led to reductions in phosphorylated ER α ^{Ser118} (Fig. 6a lanes 3 vs. 5) comparable to that induced by tamoxifen (lanes 1 vs. 2), but with levels of total ER α remaining unchanged. Further, targeted down-regulation of caveolin-1 led to significant increases in basal pERK both in the presence (lanes 4 vs. 6) or absence (lanes 3 vs. 5) of tamoxifen but with total ERK remaining unchanged.

siRNA-mediated loss of caveolin-1 was accompanied by a significant 31% reduction in WT-MCF-7 cell

Fig. 6 Effect of siRNA-mediated down-regulation of caveolin-1 on p-ERK activity, cell growth and tamoxifen sensitivity in WT-MCF-7 cells. Treatments are caveolin-1 siRNA or control siRNA (see methods) and incubation in the presence or absence of tamoxifen (100 nM) for 7 days. NT = no treatment. **a** Representative Western blot of caveolin-1, ER α (total and phosphorylated) and ERK1/2 (total and phosphorylated) in WT-MCF-7 cells, **b** Histogram of WT-MCF-7 cell growth. Results are expressed as mean $\pm$ SEM of four separate experiments. * denotes significance $P < 0.05$ versus respective control

proliferation (vs. Control siRNA; Fig. 6b). Of note, the caveolin-1 siRNA mediated inhibition of WT-MCF-7 cell growth was similar to that achieved by treatment with tamoxifen (29% reduction in growth at 100 nM over 7 days; Figure 6b). Greater reductions were evident in WT-MCF-7 growth rate when cells were exposed to simultaneous tamoxifen and siRNA (either Cav or Control), this possibly due to the lipid-siRNA delivery systems modulating to some extent the activity of tamoxifen. Importantly cells co-treated with caveolin-1 siRNA and tamoxifen did not show any significant changes in proliferation rate compared to co-treatment with control siRNA and tamoxifen, indicating downregulation of caveolin-1 alone is not sufficient to alter the sensitivity of cells to the growth inhibitory effects of tamoxifen.

Recombinant expression of caveolin-1 in TAM-R cells has no effect on basal and EGF-induced ERK1/2 activity

Using an ecdysone-inducible mammalian caveolin-1 expression system regulated by the exogenous inducer Ponasterone A (10–20 μ M), we observed an abundant and dose-dependent expression of recombinant caveolin-1 in TAM-R cells (Fig. 7a, b). As expected little to no caveolin-1 expression was seen in TAM-R cells transfected with caveolin-1 vector but not exposed to inducer (Fig. 7a, 0 μ M) or where treatment comprised lipid or empty vector only (Fig. 7b). Immunocytochemical analysis revealed recombinantly expressed caveolin-1 to be predominantly perinuclear/cytoplasmic with little peripheral plasma membrane localisation (Fig. 7b).

We observed that increasing the expression levels of recombinant caveolin-1 (Fig. 7a, inducer concentrations 2–20 μ M) did not effect the levels of activated ERK1/2.

Fig. 7 Ponasterone-regulated recombinant caveolin-1 expression in TAM-R cells. **a** Western blot of caveolin-1 and phosphorylated ERK1/2 in TAM-R cells transfected with pIND-caveolin-1 and subsequently treated with increasing concentrations of Ponasterone A (2–20 μ M); **b** Immunocytochemistry of caveolin-1 protein expression in TAM-R cells incubated with Ponasterone A (20 μ M) following transfection with either pIND-empty control vector (panel i) or pIND-caveolin-1 (panel ii). Results are representative of at least three separate experiments

Further, recombinant caveolin-1 did not alter the concentration-dependent profile for EGF-ligand activation of ERK1/2 (Fig. 8a). To rule out population averaging effects inherent in Western blot and RT-PCR, the recombinant caveolin-1 expression and activation status of EGFR and ERK1/2 within individual cells was examined by dual-label immunofluorescence microscopy (Fig. 8b). Immunocytochemistry revealed the localisation of recombinant caveolin-1 in TAM-R cells to be predominately perinuclear/cytoplasmic in nature with little co-localisation to plasma membrane p-EGFR and p-ERK1/2. Cells within the same field of view that either showed recombinant caveolin-1 expression or a lack of it did not differ in the level of basal p-ERK1/2 (Fig. 8b-i) or EGF stimulated p-ERK1/2 (Fig. 8b-ii), or in the level of EGF stimulated p-EGFR (Fig. 8b-iii).

Increased AKT/mTOR activity correlates with the presence of phosphorylated TSC-2 but phosphorylated TSC-2 appears not responsible for the mislocalisation of re-expressed caveolin-1 in TAM-R cells

Preceding experiments in TAM-R cells showed that recombinant caveolin-1, and both residual low levels as well as the TK inhibitor (gefitinib or PD184325)-mediated re-expression, of endogenous caveolin-1 is mislocalised away from the plasma membrane. Here we investigated the role of AKT/mTOR signalling and altered TSC-2 dynamics in this mislocalisation. Figure 9 shows the phosphorylated status of AKT/mTOR and TSC-2. Compared to WT-MCF-7 cells we noted increased p-AKT, p-mTOR and p-TSC-2 in the TAM-R cells. While WT-MCF-7 cells were devoid of p-TSC-2, the levels of total AKT, of total TSC-2 and of total mTOR did not differ between the two cell types. Using sucrose density-gradient centrifugation of membrane fractions we explored the co-localisation, or otherwise, of caveolin-1 and TSC-2 in the TAM-R and WT-MCF-7 cells grown in the presence (+) or absence (–) of gefitinib.

Figure 10a, b show respectively protein localisation in various membrane fractions from WT-MCF-7 and TAM-R cells treated with gefitinib (+gefitinib). In the WT-MCF-7 cells (+gefitinib) caveolin-1 was enriched in fractions 2–6 but showed a more restricted distribution in the TAM-R (+gefitinib) cells with enrichment in fractions 2–4; a distinction which may have functional implications. The distribution of Total TSC-2 showed an equivalent pattern between the WT-MCF-7 and TAM-R cells with enrichment in fractions 9–12. Although phosphorylated TSC-2 was absent in WT-MCF-7 cells (Fig. 10a) it was present at significant levels in the TAM-R cells (Fig. 10b) with enrichment in fractions 11–12. No co-localisation of

Fig. 8 Recombinant expression of caveolin-1 in TAM-R cells, and levels of basal and ligand-stimulated phosphorylated ERK1/2. **a** Western blot of recombinant caveolin-1 and phosphorylated ERK1/2 in TAM-R cells transfected with either pIND-caveolin-1 or pIND-empty control vector and treated with Ponasterone A (20 μ M). The cells were subsequently challenged with vehicle control (0 ng/ml) or increasing concentrations of EGF (0.01–10 ng/ml); **b** Dual immunofluorescence microscopy in TAM-R cells. Expression of recombinant caveolin-1 (*in red*) with: in panel i (vehicle control); panel ii phosphorylated ERK1/2 (*in green*) following EGF (10 ng/ml); panel iii phosphorylated EGFR (*in green*) following EGF (10 ng/ml). Note as in Fig. 4a recombinant caveolin-1 in TAM-R cells appeared predominantly within the cytoplasm. Results are representative of at least three separate experiments

caveolin-1 and TSC-2 was observed under any of the tested conditions in either the WT-MCF-7 or TAM-R cells.

Analysis of subcellular fractions taken from untreated WT-MCF-7 cells (–gefitinib; data not shown) revealed similar distributions in the panel of molecules investigated compared to the gefitinib treated WT-MCF-7 cells. However, a notable difference was seen for the TAM-R cells grown in the presence (+, Fig. 10b) or absence (–, Fig. 10c) of gefitinib. Specifically, beyond the predictable loss of pERK with gefitinib treatment, a clear distinction was detection of phosphorylated caveolin-1 (p-caveolin-1) in the TAM-R (+gefitinib) cells which was localised to fractions 5–9 (Fig. 10b), whereas p-caveolin-1 was completely absent in any of the fractions recovered from untreated (–gefitinib) TAM-R cells (Fig. 10c) or indeed from any of the WT-MCF-7 cells.

The protein content profile of the various fractions harvested in our work is consistent with profiles described by others, i.e., the more buoyant lipid raft membrane fractions (fractions 1–6) possessing a reduced protein content compared to that of the less buoyant non-raft membrane fractions (fractions 8–12) (Fig. 10d).

Discussion

In cancer caveolin-1 appears to be able to serve as both an oncogene and a tumour suppressor protein. This dual activity is typified in breast cancer, where the influence of caveolin-1 expression on disease progression appears both type and stage specific [35–37]. The use of cell lines derived from breast carcinomas [18, 22, 38] together with the development and study of caveolin-1 knockout mice [39–41] have concluded that the loss of caveolin-1 in mammary epithelial cells is sufficient to drive several pathological features of breast cancer. These include amongst others the activation of the EGFR/RAS/ERK signalling pathway, increased cell proliferation and cell migration, initiation of mammary gland dysplasia, enhanced anchorage independent growth and metastatic potential. Conversely, several clinico-pathological studies correlate caveolin-1 over-expression with aggressive features of certain breast cancers, most notably the basal-like and inflammatory invasive subtypes [42–44]. However, none of these studies have explored the functional role of caveolin-1 in the development of hormone resistant breast cancer. The

Fig. 9 Western blot showing direct comparison of phosphorylated and total levels AKT, TSC-2 and mTOR in WT-MCF-7 and TAM-R breast cancer cells. Data are representative of at least three separate experiments

development of hormone-resistance following initial response to tamoxifen therapy represents a step wise advancement in disease progression and is a leading cause of relapse and mortality in breast cancer patients.

We have previously shown that the constitutive expression of an activated EGFR/RAS/ERK signalling pathway is the main mechanism by breast cancer cells circumvent the growth inhibitory effects of tamoxifen [10, 11, 31]. In this present study we have utilised the same pre-clinical model to examine a possible regulatory role for caveolin-1 in the development and maintenance of the acquired tamoxifen-resistant phenotype. We show that the increased levels of constitutively phosphorylated EGFR and ERK1/2 observed in the tamoxifen-resistant (TAM-R) breast cancer cells were paralleled by decreased levels of caveolin-1. The ability of gefitinib and PD184352, (specific inhibitors of EGFR and ERK, respectively) to significantly up-regulate caveolin-1 protein expression in TAM-R cells substantiates that the loss caveolin-1 in this cell type occurs as a consequence of increased EGFR/ERK signalling. While this represents the first report of such a relationship in tamoxifen-resistant breast cancer cells such a reciprocal link is recognised in several types of transformed cells, including those derived from breast tumours [18, 19, 41]. The ERK-mediated negative regulation of caveolin-1 in various transformed cells has been reported to occur at the transcriptional level with

direct inhibition of caveolin-1 promoter activity [19]. The loss of caveolin-1 mRNA we observed here in the TAM-R cells is consistent with such a mechanism although alterations in mRNA stability cannot be ruled out.

Earlier studies [26, 27] have shown that the recombinant expression of caveolin-1 is sufficient to mediate ligand-independent transactivation of ER α in MCF-7 cells. Our finding that TAM-R cells display a profound loss of caveolin-1 suggests a positive modulation of ER α signalling by caveolin-1 is unlikely to represent a clinically relevant mechanism of tamoxifen resistance. However, given that caveolin-1 is present in tamoxifen-sensitive WT-MCF-7 cells then a role for caveolin-1 in the early stages of the development of resistance, through sustaining ER α signalling following ER α blockade with ant-oestrogens, is clearly a possibility. In support of this conjecture, we observed increased expression of caveolin-1 in WT-MCF-7 cells following short term (7 days) exposure to tamoxifen. Longer term exposure (upto 12 weeks) of WT-MCF-7 cells to tamoxifen revealed progressively greater increases in caveolin-1 which remained elevated through to week 8, and thereafter displayed a marked continual decrease through to week 12 as the WT-MCF-7 cells gradually advanced toward the TAM-R phenotype, becoming more dependent upon EGFR/ERK mediated cell-proliferation; the stable form of the TAM-R phenotype is realised between 6 and 12 months of continuous tamoxifen exposure and within which a constitutively activated EGFR/RAS/ERK signalling pathway is a salient feature [10, 11, 31].

While the inverse relationship between caveolin-1 and EGF-R/ERK activity is reasonably well established the relationship between caveolin-1 expression and cellular ER α levels is more complicated. For example, in neuronal cells [45] and MCF-7 breast cancer cells [46], ER α signalling can induce the down-regulation of recombinant caveolin-1. Likewise mammary cells derived from caveolin-1 null mice exhibit higher levels of total ER α compared to mammary cells derived from their wild-type counterparts [47]. Alternatively, caveolin-1 expression in vascular smooth muscle cells is actually enhanced by increased ER α signalling [46]. In this current work we have confirmed TAM-R cells generally express lower levels of total ER α than WT-MCF-7 cells. We further show that in WT-MCF-7 cells the levels of total ER α remained unchanged (although activated ER α levels decreased) following siRNA-mediated down-regulation of constitutively expressed caveolin-1. Therefore in the context of hormone-related breast cancer the relationship between caveolin-1 and total ER α expression may be indirect.

Parallels can be made between the above and existing clinical data derived from breast cancer cohorts [47–49]. For example, inverse patterns of expression between caveolin-1 and EGF-R are recognised in clinical breast tumours [48, 49].

Indeed our own immunohistochemical analysis (data not shown) of primary breast tumours from 93 patients presenting for surgery also indicates a similar reciprocal relationship ($P < 0.027$) between the loss of caveolin-1 and gain of EGF-R. In addition positive clinical correlations

Fig. 10 Western blotting and co-fractionation of membrane fractions taken from WT-MCF-7 (**a**) and TAM-R cells (**b** and **c**). WT-MCF-7 (**a**) and TAM-R cells (**b**) treated with gefitinib (1 μ M) for 7 days. No differences were seen in WT-MCF-7 between gefitinib treated (+) and non-treated (–) cells. **c** Highlights those differences observed in TAM-R cells not treated with gefitinib compared to gefitinib treated TAM-R cells (**b**). Twelve fractions were collected (fraction 1, top of sucrose gradient; fraction 12, bottom of sucrose gradient) and an aliquot of each fraction (20 μ l) was resolved by SDS/PAGE. **d** Shows the % protein load of each fraction and in agreement with other co-fractionation studies shows protein to increase from fraction 1 to fraction 12. Representative data from three separate experiments are shown

between caveolin-1 and hormone receptor status [47, 49] exist. Indeed up to 35% of all ER α positive tumours also co-express caveolin-1 [47, 49]. Further, the clinical study of Gee et al. [9] has shown the presence of phosphorylated ERK-1/2 in ER α positive breast cancers can predict shorter duration of response and poor survival in patients who had received anti-hormonal agents. We therefore propose that the loss of caveolin-1 in ER α positive breast cancer may ultimately transpire to be a useful biomarker to predict the likely response of patients to initial tamoxifen therapy and/or the development of tamoxifen resistance in patients already receiving hormonal therapy.

To further delineate the roles of endogenous caveolin-1 in hormone-related breast cancer and examine if the loss of caveolin-1 can be the causal factor of tamoxifen resistance we undertook a series of caveolin-1 siRNA transfections in the hormone-dependent WT-MCF-7 cells. We observed the targeted down-regulation of endogenous caveolin-1 to result in significant inhibition in WT-MCF-7 cell growth, comparable to that achieved when the cells were treated with the anti-oestrogen tamoxifen. This result clearly defines a novel role for caveolin-1 in promoting the growth of oestrogen-dependent breast cancer cells. The siRNA-mediated reductions in caveolin-1 in the WT-MCF-7 cells also resulted in decreased ER α activity, a finding consistent with caveolin-1 potentiating ER α signalling [27]. The siRNA studies also revealed endogenous caveolin-1 to serve as a potent inhibitor of ERK signalling in WT-MCF-7 cells, consistent with caveolin-1's proposed role as a tumour suppressor protein. Collectively, these results suggest that in WT-MCF-7 cells caveolin-1 assumes a more prominent role in facilitating cellular proliferative pathways than it does in growth inhibition.

Additionally we determined the targeted siRNA-mediated down-regulation of caveolin-1 in WT-MCF-7 cells failed to alter their sensitivity to the growth inhibitory actions of tamoxifen. This implies the loss of caveolin-1 per se may not be the causal factor in tamoxifen resistance in breast cancer. Other molecular changes undoubtedly are required for the manifestation of a full tamoxifen resistant phenotype and a number of ER α co-activators are identified

as up-regulated in tamoxifen resistant breast cancers compared to tamoxifen sensitive tumours [30]. However, perhaps most significant with respect to our studies is the recognised upregulation of EGF-R levels evident in TAM-R cells [50], consolidating a model of coordinated gain of EGF-R coupled with the loss of caveolin-1 expression as a mechanism central to conferring hormone-resistance.

Previous studies [35–37] have used recombinant expression of caveolin-1 to restore caveolin-1 levels in various experimental cancer models in order to inhibit oncogenic signalling pathways, including the EGF-R/ERK cascade. Here we examined if the restoration of caveolin-1 in TAM-R cells through recombinant means was sufficient to inhibit EGF-R/ERK signalling, a key driver of the tamoxifen-resistant phenotype in this cell model. Using an ecdysone-inducible mammalian expression system that allowed varying levels of caveolin-1 to be expressed in a tightly regulated manner, we showed that the activation status of EGF-R and ERK (basal and EGF stimulated) remained unaltered in the TAM-R cells, even when the recombinant caveolin-1 was highly over-expressed. The lack of effect upon EGF-R/ERK signalling by caveolin-1 re-expression suggests in some way an altered caveolin-1 functioning in the TAM-R cells. We showed in various elements of our work here the mislocalisation of caveolin-1 in the TAM-R cells contrasting to the spatial pattern of caveolin-1 in WT-MCF-7 cells which was localised to both the plasma membrane and cytoplasmic compartments. Specifically in those TAM-R cells retaining very low levels of caveolin-1 or in TAM-R cells exposed to EGFR/ERK inhibition (respectively, gefitinib and PD184352) the endogenous caveolin-1 protein localised to perinuclear regions rather than the plasma membrane. Similarly, in the TAM-R cells expressing recombinant caveolin-1 the pattern was predominantly cytoplasmic. Such altered subcellular distribution a possible basis for caveolin-1 dysfunction.

Recent work has shown that caveolin-1 is correctly trafficked to the peripheral cell membrane through co-localisation with tuberlin (TSC-2) [51, 52]. As such caveolin-1 is retained within a post-Golgi cytoplasmic compartment when TSC-2 is rendered dysfunctional [51, 52]. The AKT/mTOR signalling pathway is the main upstream effector that mediates the inhibitory phosphorylation of TSC-2 and has previously been determined to cause caveolin-1 mislocalisation [52]. Consequently we examined the linkage between AKT/mTOR signalling and caveolin-1 mislocalisation in the TAM-R cells and show for the first time increased AKT/mTOR activity with attendant phosphorylated TSC-2 in the TAM-R cells compared to the WT-MCF-7 cells where in the latter AKT/mTOR activity was lower and where phosphorylated TSC-2 was absent. However, we were unable within any of our experimental treatments to show co-localisation of caveolin-1 with TSC-2 (either phosphorylated or non-phosphorylated TSC-2 forms)

indicating that phosphorylated TSC-2 is unlikely to be responsible for the apparent altered subcellular distribution of re-expressed caveolin-1 in TAM-R cells. However, AKT/mTOR mediated effects on the subcellular distribution of caveolin-1, independent of TSC-2 remain possible given the fundamental actions of increased AKT/mTOR signalling (specifically RICTOR-TORC2) on cytoskeletal dynamics [52].

We determined that re-expressed caveolin-1 in the TAM-R (gefitinib-treated) cells to exist in both non-phosphorylated and phosphorylated forms, contrasting to WT-MCF-7 cells (untreated or gefitinib treated) or indeed untreated TAM-R cells where phosphorylated caveolin-1 could not be detected. Phosphorylation of caveolin-1 at tyrosine 14 has been reported to result in movement of this protein from cell edges and the formation of aggregated caveolin-1 positive vesicles deep within the cytoplasm of cells [53, 54]. Indeed cellular distribution of non-phosphorylated and phosphorylated pools of caveolin-1 in the TAM-R cells (gefitinib treated) appeared spatially distinct as revealed by sucrose density gradient subcellular fractionation. Therefore caveolin-1 phosphorylation in the gefitinib-treated TAM-R cells may offer a possible explanation for the apparent mislocalisation of at least a pool of re-expressed caveolin-1. Further, the phosphorylation of caveolin-1 per se may explain the inability of this protein, or a pool thereof, to interact and inhibit EGF-R/ERK signalling in the TAM-R cells.

The factor(s) responsible for the phosphorylation of caveolin-1 in TAM-R cells are currently unknown, although the most probable candidate is c-Src, for which caveolin-1 is an established substrate [55]. Previous studies using this same pre-clinical model have indeed shown that TAM-R cells abundantly express c-Src and that this molecule facilitates the increased invasive behaviour of these cells [56]. Further, c-Src-mediated caveolin-1 phosphorylation is reported to stimulate the subcellular trafficking of activated EGF-R augmenting cell migration and anchorage-independent growth [57–59]. To this end, Joshi et al. [60] have recently shown phosphorylated caveolin-1 to co-operate with Rho/ROCK and FAK-dependent signalling pathways promoting tumour cell invasion and metastasis in breast cancer. Such studies are consistent with clinical data showing a positive correlation between caveolin-1 expression (although distinction between phosphorylated and non-phosphorylated forms not addressed) and poor patient survival in highly aggressive invasive breast cancers [42–44, 61]. The induction of caveolin-1 and the presence of a pool of phosphorylated caveolin-1 in gefitinib treated TAM-R cells may have profound implications for the overall function of caveolin-1 in these cells and for the clinical management of tamoxifen-resistant breast cancers involving the use of EGF-R inhibitors [62].

In summary, we present a model in which caveolin-1 contributes to the aetiology of breast cancer and endocrine resistance. In this, caveolin-1 facilitates the growth of early stage, hormone-dependent breast cancers via potentiation of ER α signalling. The transient increase in caveolin-1 during initial exposure to tamoxifen, during the early/developmental stages of resistance, may represent a compensatory mechanism to maintain ER α -mediated cell growth following acute withdrawal of oestrogen. However, upon long term oestrogen deprivation the EGF-R levels accrued during the acquisition of tamoxifen resistance ultimately raises the basal activity of ERK-1/2 to a threshold sufficient to repress caveolin-1. The resulting ERK-mediated inhibition of caveolin-1 allowing multiple components of the EGF-R/RAS/ERK signalling cascade to function unconstrained, and as such the tumour promoting roles of caveolin-1 are superseded by the EGF-R/ERK signalling cascade. By implication the co-ordinated gain in EGF-R/ERK signalling and down-regulation of caveolin-1 may be a necessary requirement for the maintenance of the full tamoxifen resistant phenotype. Finally, in a setting of tamoxifen-resistance the re-expression of caveolin-1 and its subsequent phosphorylation, through for example tyrosine kinase inhibition, may co-operate with the EGF-R/ERK pathway to further promote late-stage tumour progression and metastasis.

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