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ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · JANUARY 2006

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Int. J. Cancer: **118,** 290–301 (2006)

Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of β-catenin phosphorylation

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We have previously demonstrated that, following acquisition of endocrine resistance, breast cancer cells display an altered growth rate together with increased aggressive behaviour *in vitro*. Since dysfunctional cell–cell adhesive interactions can promote an aggressive phenotype, we investigated the integrity of this protein complex in our breast cancer model of tamoxifen resistance. In culture, tamoxifen-resistant MCF7 (TamR) cells grew as loosely packed colonies with loss of cell–cell junctions and demonstrated altered morphology characteristic of cells undergoing epithelial-to-mesenchymal transition (EMT). Neutralising E-cadherin function promoted the invasion and inhibited the aggregation of endocrine-sensitive MCF7 cells, whilst having little effect on the behaviour of TamR cells. Additionally, TamR cells had increased levels of tyrosine-phosphorylated β-catenin, whilst serine/threonine-phosphorylated β-catenin was decreased. These cells also displayed loss of association between β-catenin and E-cadherin, increased cytoplasmic and nuclear β-catenin and elevated transcription of β-catenin target genes known to be involved in tumour progression and EMT. Inhibition of EGFR kinase activity in TamR cells reduced β-catenin tyrosine phosphorylation, increased β-catenin–E-cadherin association and promoted cell–cell adhesion. In such treated cells, the association of β-catenin with Lef-1 and the transcription of *c-myc, cyclin-D1, CD44* and *COX-2* were also reduced. These results suggest that homotypic adhesion in tamoxifen-resistant breast cancer cells is dysfunctional due to EGFR-driven modulation of the phosphorylation status of β-catenin and may contribute to an enhanced aggressive phenotype and transition towards a mesenchymal phenotype *in vitro*.

Key words: β-catenin; epithelial-to-mesenchymal transition; epidermal growth factor receptor; invasion; endocrine resistance

Tamoxifen resistance presents a major challenge in the management of breast cancer patients. Despite an initial response to such therapies, the majority of patients will ultimately relapse and present with disease progression.[1,2] We have previously shown that upon acquisition of tamoxifen resistance, breast cancer cells display significant augmentation of the EGFR signalling pathway in an autocrine fashion,[3] which imparts greater *in vitro* metastatic ability to these cells as demonstrated by an increase in their motile and invasive behaviour.[4]

Loss of intercellular adhesion has been identified as a key process in the development of an aggressive tumour cell phenotype, with *in vitro* studies demonstrating a correlation between reduced cell–cell adhesion and increased invasive capacity.[5–7] Furthermore, deficient cell–cell adhesion is also a hallmark of EMT, a process consistently associated with tumour cell scattering and spread. Intercellular adhesion is primarily mediated through the AJ; is important for the maintenance of tissue architecture, cell structure and polarity; and can limit cell movement and proliferation. AJs assemble *via* the homophilic interactions between calcium-dependent transmembrane cadherin receptors, the cytoplasmic domains of which are linked to the actin cytoskeleton through interaction with α- and β-catenin proteins.[8] Significantly, it is emerging that β-catenin plays a dual function within the cell: in addition to its structural role within the AJ, β-catenin can act as a transcription cofactor along with the TCF/LEF family of DNA-binding proteins. Activation of β-catenin/TCF/LEF transcriptional activity drives the expression of a subset of genes which promote tumour establishment, growth and invasion;[9] and a direct relationship between β-catenin-mediated gene transcription and establishment of EMT is becoming clear.[10–12]

Increased levels of β-catenin within the cytoplasm and/or nucleus in tumour cells are suggestive of stabilization of the β-catenin protein and can result in enhanced β-catenin-mediated transcription.[13] Cytoplasmic β-catenin levels are regulated though its phosphorylation on serine and threonine residues through the action of GSK3β in a protein complex with axin and APC.[14,15] This facilitates the subsequent degradation of β-catenin through a ubiquitin-dependent pathway. Inhibition of GSK3β activity results in stabilization of the protein and its accumulation in the nucleus, where it associates with members of the TCF/Lef family of transcription factors to mediate gene transcription.

β-Catenin function may also be modulated though its phosphorylation on tyrosine, which results in the disruption of cell–cell junctions through perturbation of β-catenin–E-cadherin binding.[16] This has been demonstrated to occur following activation of growth factor receptors including c-erbB2 and the EGFR, which are known to associate with and phosphorylate β-catenin in a number of cell types.[17–19] In addition to this, several studies have reported elevated levels of tyrosine-phosphorylated β-catenin in invasive cancer types.[20,21]

Here, we report on the role of the AJ adhesion system in tamoxifen-resistant MCF7 breast cancer (TamR) cells, which display enhanced motile and invasive behaviour *in vitro* compared to their endocrine-sensitive counterparts. Our data demonstrate that development of endocrine insensitivity in MCF7 breast cancer cells is accompanied by a loss in cell–cell adhesion and a partial transition from an epithelial to a mesenchymal phenotype, events which are underpinned by the EGFR-induced modulation of the β-catenin phosphorylation state and an EGFR-dependent increase in nuclear β-catenin distribution and transcription of β-catenin target genes, which are implicated in both tumour establishment and EMT.

Material and methods

Cell lines and reagents

Tamoxifen-responsive, wtMCF7 breast cancer cells were a gift from AstraZeneca (Macclesfield, UK). These cells were routinely cultured in phenol red-free RPMI medium supplemented with 5% FCS, antibiotics (10 IU/ml penicillin, 10 μg/ml streptomycin) and 2.5 μg/ml fungizone. Cultures were maintained at 37°C in a

Abbreviations: AJ, adherens junction; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; FAM, 6-carboxyfluorescein; LEF, lymphoid enhancer factor; NF-κB, nuclear factor κB; 4-OH-TAM, 4-hydroxytamoxifen; PI3K, phosphatidylinositol-3 kinase; PKB, protein kinase B; PPARδ, peroxisome proliferator–activated receptor δ; TCF, T-cell factor; TGF, transforming growth factor; TTBS, TRIS-buffered saline + 0.05% Tween-20; wt, wild type.

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Received 24 February 2005; Accepted after revision 13 May 2005
DOI 10.1002/ijc.21355
Published online 3 August 2005 in Wiley InterScience (www.interscience.wiley.com).

humidified atmosphere with 5% CO_2. wtMCF7 cells were used to establish a tamoxifen-resistant variant cell line (TamR), as described previously,[3] which was subsequently maintained in phenol red-free RPMI medium supplemented with 5% charcoal-stripped and steroid-depleted FCS, antibiotics as above, glutamine (200 mM) and 4-OH-TAM (10^{-7} M in ethanol). wtMCF7 cells used for experimental analysis in the assays described below were maintained in the same cell culture medium used for the tamoxifen-resistant cell line (detailed below), with the omission of 4-OH-TAM.

All tissue culture media and constituents were obtained from Life Technologies (Paisley, UK), and tissue culture plastic ware was obtained from Nunc (Roskilde, Denmark). All reagents were from Sigma (Poole, UK) unless otherwise stated. The selective EGFR inhibitor gefitinib (Iressa, ZD1839)[22,23] was a gift from AstraZeneca Pharmaceuticals (Cheshire, UK), and PD098059 was from Calbiochem-Merck Biosciences (Beeston, UK).

Antibodies

The antibodies used in the study were anti-E-cadherin (HECD-1; R &D Systems, Oxford, UK), anti-β-catenin (pan-antibody) and anti-β-actin (Sigma), anti-α-catenin (Santa Cruz Biotechnology, Calne, UK) and antiphosphotyrosine (4G10) and anti-Lef-1 (Upstate Biotechnology, Milton Keynes, UK). Antibodies that recognised a phosphorylated form of β-catenin (ser33, 37 and thr41), activated EGFR (tyr1068) and activated PKB/AKT (ser473) were from Cell Signalling Technologies (Hitchin, UK). Anti-phospho-GSK3β (ser9) was from Upstate Biotechnology.

RNA extraction and RT-PCR

Total RNA was extracted from wtMCF7 or tamoxifen-resistant cells using Trizol reagent (Life Technologies) and converted to cDNA using random hexamers and Super-Script II reverse transcriptase (Invitrogen, Paisley, UK). cDNA was amplified using PCR primer pairs (MWG Biotech, Milton Keynes, UK) specific for human *E-cadherin* (forward, AAC ATG GTT CAG ATC AAA TC; reverse, AAG CTT GAA GAT CGG AGG ATT ATC G), *α-catenin* (forward, CTG ATA TAC AAG CAG CTG; reverse, GCC AAG AAG TCA TCA ATG GA), *β-catenin* (forward, GCT GAT TTG ATG GAG TTG GA; reverse, TCA GCT ACT TGT TCT TGA GTG AA), *c-myc* (forward, TTG CAG CTG CTT AGA CGC TG; reverse, CCA CAT ACA GTC CTG GAT GA), *cyclin D1* (forward, GGA TGC TGG AGG TCT GCG AG; reverse, GAG AGG AAG CGT GTG AGG CG), *CD44* (forward, GTG ATC AAC AGT GGC AAT GG; reverse, GGG CCC TAA TTT CAG AAA GC), *COX-2* (forward, GCA GTT GTT CCA GAC AAG CA; reverse, CAG GAT ACA GCT CCA CAG CA) and *uPAR* (forward, GCT GGT GGA GAA AAG CTG TA; reverse, CCT TCT TCA CCT TCC TGG AT). All PCRs were performed as multiplex reactions with PCR primers specific for β-*actin* (forward, 5′-CCT TCC TGG GCA TGG AGT CCT-3′; reverse, 5′-GGA GCA ATG ATC TTG ATC TT-3′) as an internal control. PCR was performed in a semiquantitative manner using 25 cycles so that products were in the linear range of amplification. PCR products were separated on a 1.0% agarose gel, stained with ethidium bromide and photographed under UV light.

Real-time PCR

The transcript level of the respective molecules was determined using real-time quantitative PCR based on Amplifluor technology and modified from a method previously reported.[24] Briefly, pairs of PCR primers were similarly designed using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA). One of these primers contained an additional sequence, known as the Z sequence (5′-ACT GAA CCT GAC CGT ACA-3′), which is complementary to the universal Z probe (Intergen, Livingston, UK). A Taqman detection kit for β-actin was purchased from Perkin-Elmer (Beaconsfield, UK). The reaction was carried out using the hot-start Q-master mix (Abgene, Epsom, UK) under the following conditions: 10 pmol specific forward primer, 1 pmol reverse primer containing the Z sequence, 10 pmol FAM-tagged probe (Intergen) and cDNA from approximately 50 ng RNA. The reaction was carried out using an IcyclerIQ (Bio-Rad, Hemel Hempstead, UK), equipped with an optic unit that allows real-time detection of 96 reactions and the following cycling parameters: 94°C for 12 min and 50 cycles at 94°C for 15 sec, 55°C for 40 sec and 72°C for 20 sec. Levels of mRNA in the sample were quantified with reference to a standard that was simultaneously amplified with the samples.

Fluorescent microscopy

wtMCF7 or TamR cells were cultured on glass coverslips and fixed with 3.7% formaldehyde, permeabilised with 0.1% Triton X-100 and blocked with 10% normal goat serum for 30 min. Staining was performed using the antibodies described above together with fluorescently labelled secondary IgG conjugates (AlexaFluor 488 and 594; Molecular Probes, Eugene, OR), both in PBS containing 1% BSA. Cells were then washed, mounted onto microscope slides with Vectashield (Molecular Probes) and viewed at ×63 magnification with an oil-immersion objective.

Western blotting and immunoprecipitation

Cells were cultured in 10 cm dishes until they reached log-phase growth. They were subsequently serum-starved for 24 hr prior to addition of growth factor or inhibitor treatments as indicated. After washing twice in 5 ml PBS on ice, lysis was performed by scraping in 150 μl ice-cold lysis buffer [50 mM TRIS (pH 7.5), 5 mM EGTA, 150 mM NaCl and 1% Triton X-100] containing protease inhibitors (2 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM PMSF, 20 μM phenylarsine, 10 mM sodium molybdate, 10 μg/ml leupeptin and 8 μg/ml aprotinin). Lysates were clarified by centrifugation for 15 min at 15,000 g 4°C; and the protein concentration of the resultant supernatants was quantified using the DC protein assay kit (Bio-Rad). For immunoprecipitation experiments, 500 μg of total cell protein were incubated with 1 μg of primary antibody overnight at 4°C, then incubated for 2 hr at 4°C with protein A/G:agarose conjugate (Upstate Biotechnology). Following extensive washing in fresh lysis buffer, the resultant pellet was briefly boiled in SDS-PAGE sample buffer and subjected to electrophoresis as follows. Equal amounts of cell protein were separated on 8% SDS-PAGE gels and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking the membrane in 5% skimmed milk powder in TTBS, blots were incubated with primary antibody (diluted in 5% milk/TTBS solution) prior to detection using horseradish peroxidase–tagged secondary antibodies and chemiluminescence (Pierce and Warriner, Chester, UK). After exposing the blots to X-ray film (Amersham Biosciences, Chalfont St. Giles, UK), quantitation was performed by scanning the films obtained from a minimum of 3 independent experiments with an imaging densitometer (Bio-Rad).

Cell fractionation

Cytoplasmic and nuclear extracts from cells were prepared by first washing cell monolayers with PBS (4°C) prior to scraping into PBS and pelleting at 100 g for 3 min. Approximately 50 μl of packed cells were lysed by gentle resuspension in hypotonic lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and protease inhibitors as detailed above] on ice for 15 min prior to addition of detergent (Igepal, at a final concentration of 0.4%). After centrifugation, for 30 sec at 11,000 g, the supernatant was removed and stored as the cytoplasmic fraction. To obtain a nuclear preparation for protein analysis, the remaining pellet was resuspended in nuclear extraction buffer [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT and protease inhibitors as previously described] and centrifuged for 5 min at 20,000 rpm. The resultant supernatant contained the nuclear proteins. The concen-

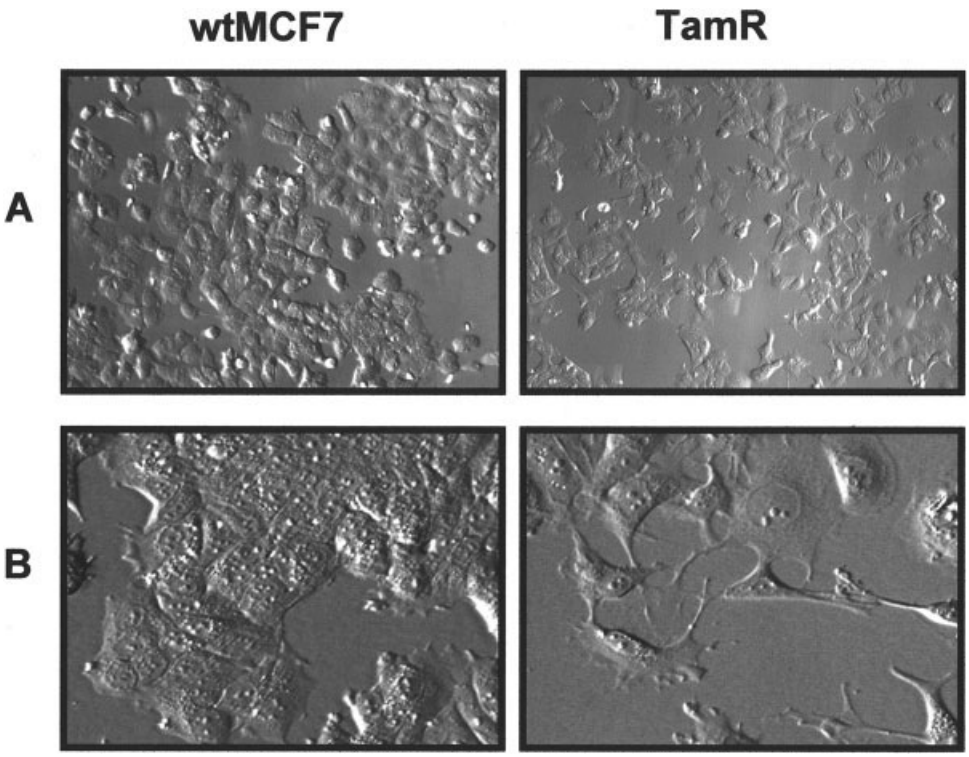

FIGURE 1 – Tamoxifen-resistant breast cancer cells display altered morphology. wtMCF7 cells and their tamoxifen-resistant counterparts (TamR cells) were cultured for 7 days under routine conditions. Resultant cell colonies were subsequently viewed and photographed under bright field illumination at low (×10) magnification (*a*). To better observe individual cell morphology, further images were taken at high (×20) magnification using a Hoffman condenser (*b*). Whilst wtMCF7 cells grew as discrete cell colonies with tight cell–cell junctions, TamR cells grew as loosely packed colonies in which there was significant loss of cell–cell adhesion and increased membrane activity (ruffling and lamellipodia formation), giving an altered morphology characteristic of actively spreading cells.

tration of proteins in both the cytoplasmic and nuclear fractions was determined, and equal amounts of protein separated by SDS-PAGE were transferred to nitrocellulose and immunoprobed as outlined above. Blots were subsequently exposed to X-ray film and quantitated using a scanning densitometer.

Aggregation assay

wtMCF7 and TamR cells were harvested, washed and resuspended at a concentration of 5×10^4/ml in tissue culture medium containing 1 mM EGTA, neutralising E-cadherin antibody (HECD-1, 10 μg/ml), 2 mM $CaCl_2$, 1 mM EGTA plus 2 mM $CaCl_2$ or 10 μl PBS vehicle. Cells were then divided into microcentrifuge tubes and rotated to allow constant agitation of the samples. Portions of each cell suspension were removed after 40 min and fixed by addition of formaldehyde (4% final concentration). Cells were viewed using a hemocytometer, and the total number of particles (cell aggregates consisting of 4 or more cells) was counted per random field of view.

Cell invasion assay

Cell invasion was quantified using a modification of a previously described method.[25] Briefly, ThinCert tissue cell culture inserts (31.2 mm^2 culture surface, 8.0 μm pore size; Greiner Bio-One, Gloucester, UK) were coated with Matrigel (0.4 μg/ml) overnight at room temperature in a sterile tissue culture hood. After rehydrating the matrix with serum-free RPMI for 1 hr at 37°C, cells were seeded into the chambers at 5×10^4/well with or without treatment as indicated, whilst 500 μl of culture medium were added to the outside of the well. Inserts were cultured at 37°C for 72 hr, after which the noninvasive cells and Matrigel were removed from the inside of the wells with a cotton swab. After fixation of the invaded cells with 4% formaldehyde, porous membranes were removed from the invasion chamber using a scalpel blade and mounted onto glass microscope slides using Vectashield (Molecular Probes) containing the nuclear stain DAPI. Cell invasion was quantified with a fluorescent microscope by viewing 5 separate fields per membrane at ×20 magnification and counting the number of cells (identified by stained cell nuclei) in each field. Data were then plotted as mean cells per field ± SD.

Cell motility assay

ThinCert multiwell plate inserts (as described above) were coated on their underside with 10 μg/ml fibronectin (Sigma) for 2 hr at 37°C. After coating, membranes were rinsed in 1 × PBS before immersion into the lower chamber containing 500 μl of tissue culture medium. Serum-starved cells were then harvested and resuspended in culture medium at 5×10^5/ml, and 100 μl of cell suspension was added to the top of each migration chamber in the presence or absence of treatment as indicated. Cells were then allowed to migrate to the underside of the membrane for 20 hr. Nonmigratory cells on the upper surface of the membrane were then removed with a cotton swab, and the cells that had migrated to the underside of the membrane were fixed in 3.7% formaldehyde in PBS and stained with 0.5% crystal violet. The number of migratory cells per membrane was subsequently counted using an inverted microscope and a ×20 objective. Each determination represents the average of 3 individual experiments, each performed in duplicate, with error bars showing the SD.

Results

TamR cells display altered morphology and increased β-catenin expression

We have previously demonstrated that upon acquisition of resistance to tamoxifen, MCF7 cells display enhanced invasive capacity *in vitro*.[4] When the morphology of endocrine-sensitive (wtMCF7) and endocrine-resistant (TamR) cells was investigated by microscopy of cells routinely growing in culture, we observed that whilst wtMCF7 cells grew as tightly packed colonies characteristic of epithelial cells and demonstrated limited cell spreading (Fig. 1*a,* compare images taken at ×10 magnification), TamR cells appeared flattened and actively spreading and had lost the majority of their cell–cell contacts (Fig. 1*b,* compare images at ×20 magnification). Since loss of components of the AJ can compromise cell–cell adhesive interactions and contribute to the

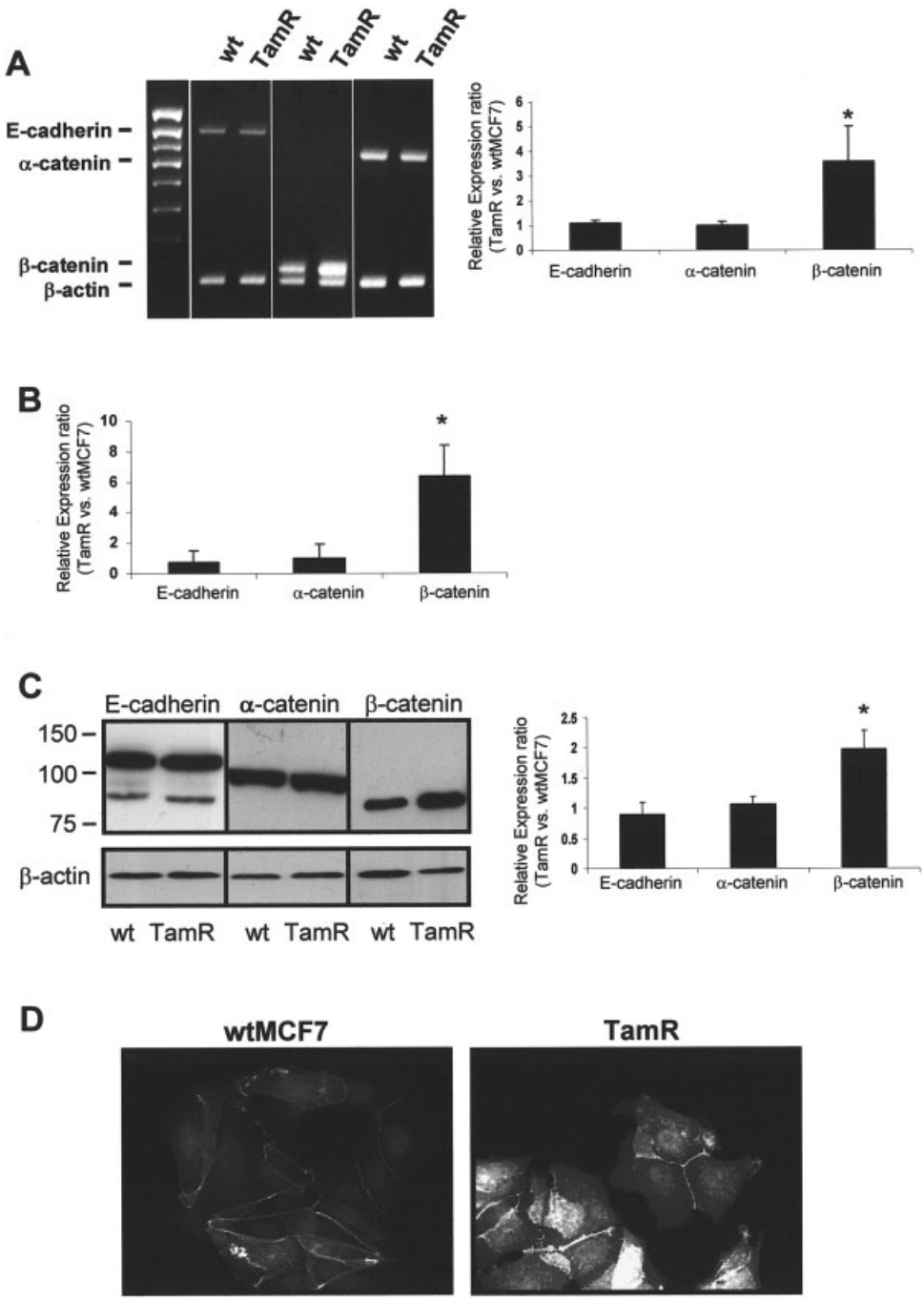

FIGURE 2 – Expression of E-cadherin and catenin in wtMCF7 and TamR cells. (*a*) RT-PCR analysis of E-cadherin, α-catenin and β-catenin mRNA. Reactions were performed as coamplifications along with β-actin as a loading control. PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized with UV illumination. A 1 kb DNA marker was included to determine PCR product size (E-cadherin, 751 bp; α-catenin, 600 bp; β-catenin, 230 bp; β-actin 204 bp). Densitometric analysis revealed that levels of β-catenin mRNA were increased in TamR cells compared to wtMCF7 cells. $*p < 0.05$. (*b*) Elevated β-catenin mRNA was confirmed following real-time PCR analysis of mRNA levels. $*p < 0.05$ (TamR cells *vs.* wtMCF7 cells). (*c*) Equal amounts of cell protein were separated by SDS-PAGE and subsequently Western-blotted and immunoprobed with anti-E-cadherin, α- and β-catenin antibodies. Scanning densitometry of the resultant blots demonstrated that β-catenin protein levels were elevated in TamR cells. Data presented as mean expression ratio between TamR and wtMCF7 cells: $*p < 0.02$. (*d*) Immunofluorescent staining was performed on formaldehyde-fixed, permeabilised cells to determine the location of β-catenin. Observation at ×63 magnification revealed that cytoplasmic and nuclear levels of β-catenin were significantly elevated in TamR cells compared to their wtMCF7 counterparts.

aggressiveness of tumour cells *in vitro,* we further investigated expression of the AJ components E-cadherin and α- and β-catenin in these cells.

RT-PCR of mRNA extracted from wtMCF7 and TamR cells did not reveal any significant changes in either E-cadherin or α-catenin mRNA expression between the 2 cell types. In contrast to this, a small but significant elevation in the level of β-catenin mRNA was observed in the endocrine-resistant cells (Fig. 2*a*). These observations were confirmed using quantitative PCR (Fig. 2*b*). Analysis of cellular proteins in lysates from wtMCF7 and TamR cells by Western blotting demonstrated that whilst levels of E-cadherin and α-catenin protein were similar, the amount of β-catenin protein detectable in TamR cells was on average 2-fold higher than that in wtMCF7 cells (Fig. 2*c*). Immunofluorescent staining of β-catenin demonstrated plasma membrane staining in both cell types (Fig. 2*d*). However, TamR cells showed a significant increase in cytoplasmic β-catenin as well as more intense nuclear staining.

β-catenin phosphorylation is altered in TamR cells and accompanies a reduction in its association with E-cadherin and increased association with LEF-1 and EGFR

The observed elevation in the levels of cytoplasmic and nuclear β-catenin in TamR cells may be suggestive of altered protein turnover.[26] Ubiquitin-mediated degradation of β-catenin occurs following the phosphorylation of β-catenin on serine/threonine residues through the action of GSK3β in concert with APC and

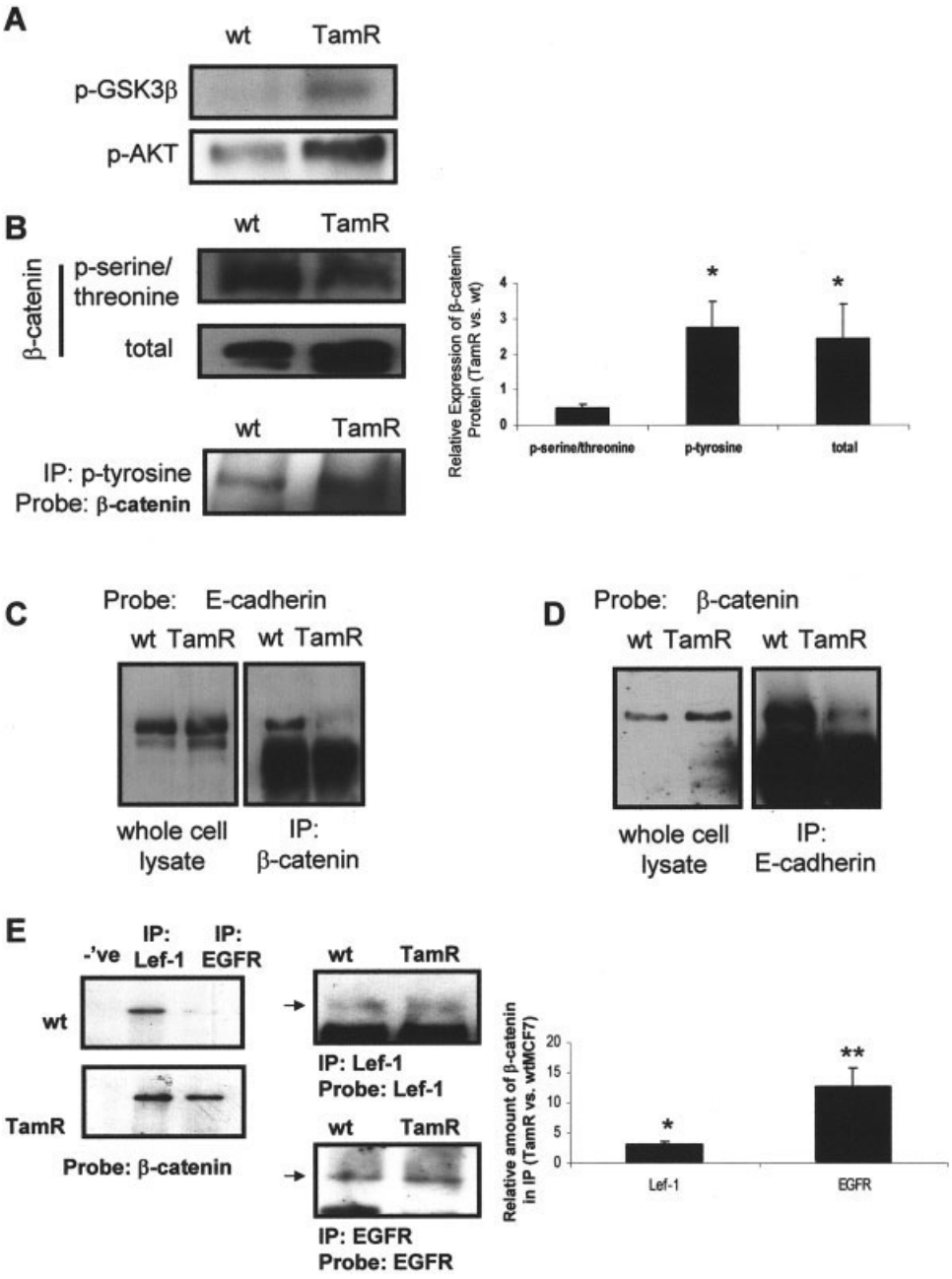

FIGURE 3 – TamR cells reveal differences in β-catenin phosphorylation and association with E-cadherin, Lef-1 and EGFR. (*a,b*) Immunoprobing of cell lysates with an antibody specific for β-catenin phosphorylated on serine 33 and 37 and threonine 41 revealed lower levels of serine/threonine-phosphorylated β-catenin in TamR cells than wtMCF7 cells. Immunoprecipitation with antiphosphotyrosine antibodies (4G10) and immunoprobing with β-catenin (pan) antibody demonstrated an increased level of β-catenin phosphorylated on tyrosine in TamR cells. Scanning densitometry was used to calculate the mean fold increase/reduction in phosphorylated β-catenin ± SD in TamR cells *vs.* wtMCF7 cells. $*p < 0.05$. (*c,d*) Immunoprobing of both whole-cell lysates and β-catenin immunoprecipitates from wtMCF7 and TamR cells with E-cadherin antibodies revealed a reduction in the amount of β-catenin associating with E-cadherin in TamR cells (*b*). Confirmatory results were obtained following immunoprobing of E-cadherin immunoprecipitates with β-catenin (*c*). (*e*) Lysates from wtMCF7 or TamR cells were immunoprecipitated with antibodies against Lef-1 or EGFR. Subsequent probing with β-catenin antibodies revealed that a greater amount of β-catenin was associated with Lef-1 and EGFR in TamR cells compared to their wtMCF7 counterparts. Densitometry was performed on blots ($n = 3$), and the mean expression ratio in TamR cells (with respect to wtMCF7 cells) ± SD is shown in the accompanying graph. $*p < 0.05$ TamR cells *vs.* wtMCF7; $**p < 0.02$ TamR cells *vs.* wtMCF7 cells.

axin.[27,28] Using an antibody that specifically detects β-catenin phosphorylated on serine 33 and 37 and threonine 41, we revealed that basal levels of the serine/threonine-phosphorylated form of β-catenin were reduced in TamR cells compared to their nonresistant parental cells (Fig. 3*a*). Lysates from wtMCF7 and TamR cells were subsequently probed using antibodies against APC, activated AKT, a major regulator of GSK3β activity and the inactive form of GSK3β (GSK3β phosphorylated on serine-9). Whilst no difference in the level of APC protein was observed in either cell line (data not shown), TamR cells had consistently greater amounts of both activated AKT and, as an expected consequence of this, inactive GSK3β (Fig. 3*a*).

Given that we have previously reported significant levels of endogenous EGFR activity in TamR cells[3] and that tyrosine phosphorylation of β-catenin can occur following activation of various growth factor receptors, including the EGFR,[29,30] we hypothesised that the tyrosine phosphorylation status of β-catenin would be altered in TamR cells. In the absence of specific antibodies that recognise this form of β-catenin, we immunoprecipitated phosphotyrosine-containing proteins from wtMCF7 and TamR cell lysates with antiphosphotyrosine antibodies and subsequently probed these samples for β-catenin. Immunoprecipitates from TamR cells contained significantly more β-catenin than their wtMCF7 counterpart, suggesting an increased amount of the tyrosine-phosphorylated form of β-catenin in these cells (Fig. 3*b*).

Phosphorylation of β-catenin on tyrosine can promote its dissociation from E-cadherin, with a subsequent reduction in cell–cell adhesion.[31,32] To determine whether the association of β-catenin with E-cadherin differed between endocrine-sensitive and endocrine-insensitive cells, β-catenin was immunoprecipitated from lysates of wtMCF7 and TamR cells and subsequently probed for E-cadherin (Fig. 3*c*). Less E-cadherin was detectable in β-catenin immunoprecipitates from TamR cells compared to wtMCF7 cells. These results were confirmed by reverse immunoprecipitations

with E-cadherin and subsequent probing with β-catenin antibodies (Fig. 3*d*). In each case, these observations were not due to differences in protein loading since probing of the immunoprecipitates with β-catenin demonstrated similar amounts of the protein in each sample (data not shown). These immunoprecipitation experiments were extended to determine whether a direct association of β-catenin with the EGFR existed in TamR cells. Upon probing of EGFR immunoprecipitates with β-catenin antibody, a signal was detectable in TamR cells but not in wtMCF7 cells (Fig. 3*e*). In addition to this, we assessed whether β-catenin associated with members of the TCF/Lef transcription family as might occur as a consequence of increased nuclear accumulation of β-catenin in TamR cells. Figure 3*d* demonstrates that the amount of β-catenin associating with Lef-1 is greater in TamR cells compared to wtMCF7 cells (up to 3-fold increase by densitometric analysis of 3 separate experiments). In both cases, these changes were not due to differences in protein levels between the groups as immunoblotting Lef-1 and EGFR immunoprecipitates with Lef-1 and EGFR antibodies, respectively (Fig. 3*e,* arrow), did not reveal any significant differences.

In vitro *invasion and aggregation is modulated by inhibition of E-cadherin function in wtMCF7, but not TamR, cells*

To address the question of the functionality of the AJ system in these cells, we performed invasion assays following experimental inhibition of E-cadherin-mediated adhesion using either Ca^{2+} chelation by addition of 1 mM EGTA or antibody-mediated neutralisation with HECD-1 (10 μg/ml). Inhibition of E-cadherin function by both antibody and chemical treatment of the cells significantly increased the number of wtMCF7 cells invading through an artificial basement membrane, whilst having only a small effect on TamR cell invasion (Fig. 4*a,b*). In the case of EGTA (Fig. 4*b*), these effects were abolished by inclusion of excess Ca^{2+}. To further investigate E-cadherin function in endocrine-sensitive and endocrine-resistant breast cancer cells, we investigated the ability of both cell types to spontaneously form aggregates *in vitro*. In the absence of any treatment, more cell aggregates were observed in wtMCF7 cell samples than TamR cells (Fig. 4*c*), whereas experimental neutralisation of E-cadherin function using the HECD-1 antibody significantly reduced the number of aggregates formed by wtMCF7 cells but had little effect on TamR cell aggregation. Similar inhibitory effects on aggregation were observed only in wtMCF7 cells using EGTA (data not shown).

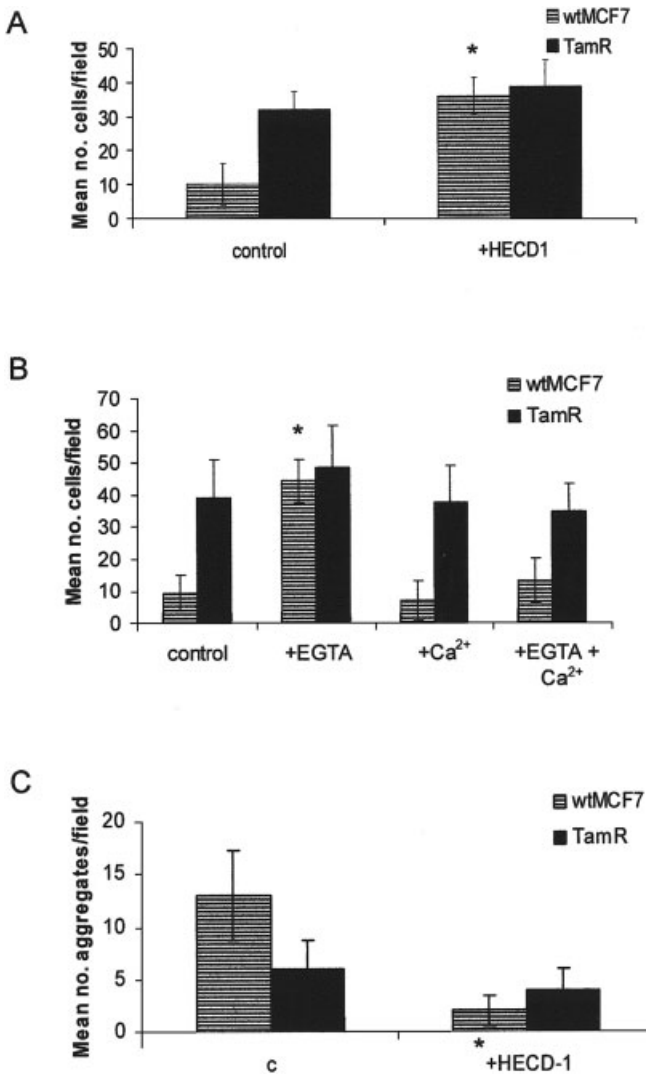

FIGURE 4 – Analysis of E-cadherin function in wtMCF7 and TamR cells. wtMCF7 and TamR cell invasion through Matrigel (*a,b*) or cell–cell aggregation (*c*) was quantified with and without disruption of cadherin-mediated cell–cell adhesion by neutralising antibody to E-cadherin (HECD-1, 10 μg/ml) or Ca^{2+} chelation (EGTA, 1 mM). Both antibody-mediated (*a*) and EGTA-mediated (*b*) neutralisation of E-cadherin promoted wtMCF7 cell invasion, whereas no significant increase in cell invasion was seen with TamR cells. Neutralisation of E-cadherin with HECD-1 reduced the ability of wtMCF7 cells to spontaneously form aggregates (*c*). TamR cells, which formed fewer aggregates under basal conditions, were not affected by E-cadherin neutralisation. $*p < 0.05$ *vs*. control.

Signalling through the EGFR modulates β-catenin phosphorylation, association with E-cadherin and Lef-1 and nuclear distribution in tamoxifen-resistant breast cancer cells

Probing of wtMCF7 and TamR cell lysates confirmed that TamR cells display elevated levels of activated EGFR (Fig. 5*a*). Given that β-catenin associates with the EGFR in TamR cells, we investigated whether β-catenin function could be modulated through this receptor. Western blot analysis was performed on either whole-cell lysates or immunoprecipitates from TamR cells using a range of antibodies as indicated (Fig. 5). In some cases, the endogenous activity of the EGFR was inhibited using the selective EGFR inhibitor gefitinib (1 μM, 24 hr). Further samples were obtained from cells stimulated with TGF-α (10 ng/ml, 60 min) in the presence or absence of gefitinib.

Activation of the EGFR, as observed by an increase in phosphorylation at Tyr1068, was greatly enhanced after stimulation of TamR cells with TGF-α, an effect that was inhibited by the inclusion of gefitinib (Fig. 5*b*). TGF-α also caused an increase in the amounts of activated AKT (Ser473) and inactive GSK3β (Ser9 phosphorylated) and a reduction in the amount of serine/threonine-phosphorylated β-catenin. No changes in total β-catenin levels were observed following TGF-α stimulation. Immunoprecipitation experiments (Fig. 5*c*) revealed that TGF-α stimulation led to an increase in the amount of tyrosine-phosphorylated β-catenin within the cells together with a reduction in the amount of E-cadherin detectable in β-catenin immunoprecipitates. Furthermore, activation of the EGFR increased the amount of β-catenin associating with Lef-1. These effects appeared to be EGFR-specific since they were abrogated by inclusion of gefitinib.

The observed increased association of β-catenin with the Lef-1 transcription factor following EGFR activation may implicate this receptor in promoting nuclear redistribution of β-catenin in TamR cells. To determine whether activation of the EGFR signalling pathway altered β-catenin distribution, cell fractionation was performed on cells in which EGFR activity was inhibited (1 μM gefitinib, 24 hr) or stimulated by TGF-α (10 ng/ml, 60 min). Following Western blotting of cytoplasmic and nuclear fractions from these cells and subsequent densitometry, our data revealed that inhibition of basal EGFR activity in TamR cells significantly

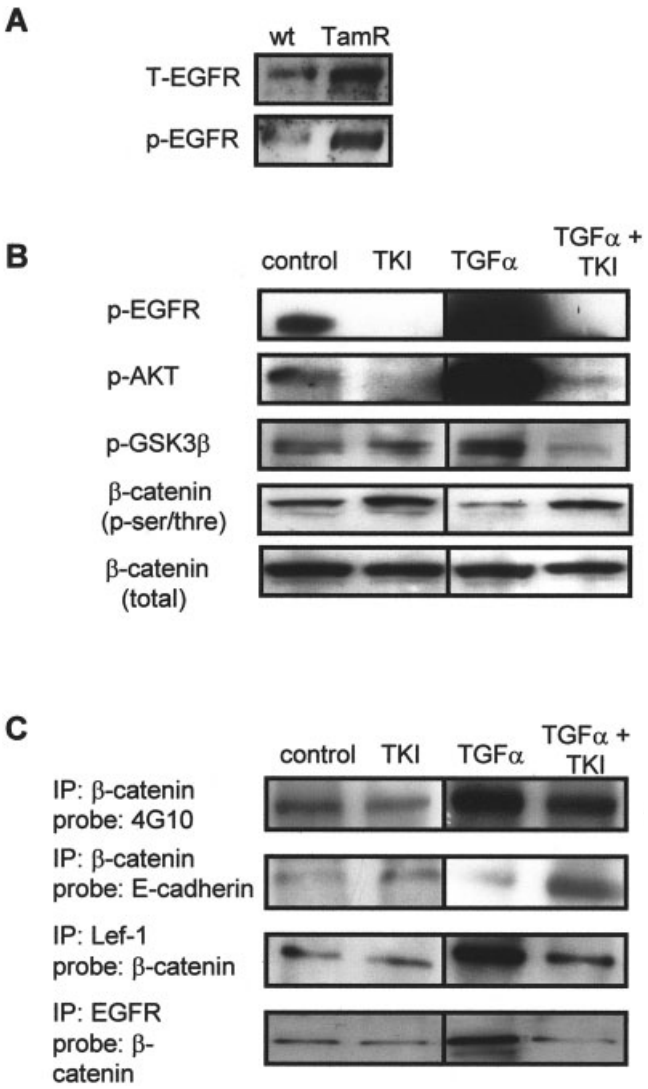

FIGURE 5 – EGFR activation modulates β-catenin phosphorylation status and association with Lef-1 in TamR cells. (*a*) wtMCF7 and TamR total cell lysates were probed for the presence of total and activated (phosphorylated at Y0168) EGFR. (*b*) TamR cells were left untreated or stimulated with TGF-α (10 ng/ml, 60 min) prior to Western blotting and immunoprobing with the antibodies shown. In some samples, basal or TGF-α-induced EGFR activity was inhibited by preincubation with 1 μM gefitinib (TKI). TGF-α-induced EGFR activation resulted in increased AKT activity and GSK3β phosphorylation and reduced the amount of serine/threonine-phosphorylated β-catenin detectable. No changes were observed in total β-catenin levels. (*c*) Immunoprecipitation was performed on TamR cells following treatments as above. Subsequent immunoprobing revealed that activation of the EGFR induced β-catenin phosphorylation on tyrosine and promoted loss of association with E-cadherin and gain in association with the EGFR and Lef-1.

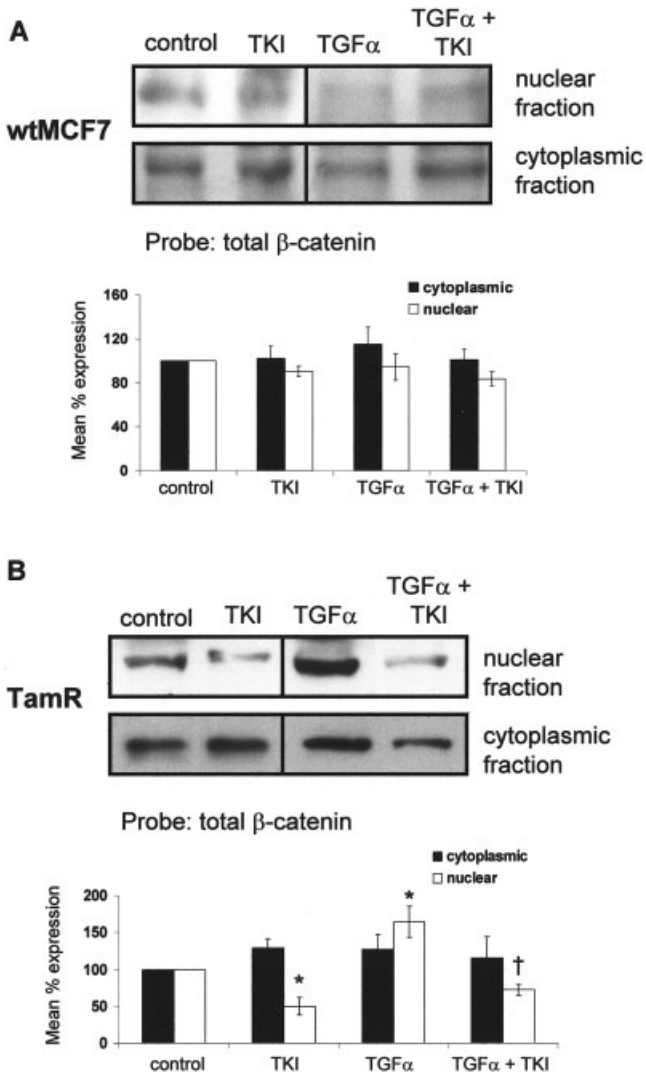

FIGURE 6 – EGFR promotes nuclear distribution of β-catenin in TamR cells. TamR (*a*) or wtMCF7 (*b*) cells were treated with gefitinib (TKI), TGF-α or a combination prior to cell fractionation; and the amount of β-catenin in the cytoplasmic and nuclear fractions was determined by Western blotting. Inhibition of EGFR activity reduced the amount of β-catenin observed in the nucleus of unstimulated TamR cells. TGF-α treatment promoted an elevation in nuclear β-catenin levels, an effect which was blocked by pretreatment with gefitinib. Data from scanning densitometry of blots ($n = 3$) are shown in the accompanying graphs. $*p < 0.05$ *vs.* control, $\dagger p < 0.05$ *vs.* TGF-α treatment.

reduced the amount of β-catenin detectable within the nucleus (Fig. 6), whilst TGF-α stimulation promoted an increase in nuclear β-catenin levels, an effect which was blocked by preincubation with gefitinib. No significant change was observed in the level of β-catenin detected in the cytoplasmic fraction following these treatments.

In contrast to this, fractionation of MCF7 cells revealed overall lower amounts of β-catenin in both cytoplasmic and nuclear fractions and, more significantly, that treatment with TGF-α did not alter β-catenin distribution in this cell type.

Modulation of EGFR-induced β-catenin function alters EMT-like phenotype of TamR cells

Our data show that inhibition of EGFR activity in TamR cells reduces β-catenin tyrosine phosphorylation and increases the association of β-catenin with E-cadherin. We thus determined whether modulation of β-catenin function would impinge on the EMT-like phenotype (loss of cell–cell adhesion, altered morphology and increased *in vitro* migration and invasion). Observation of TamR cells following treatment with gefitinib (1 μM, 24 hr) revealed a reduction in cell spreading and the reestablishment of tight cell–cell junctions in a large proportion of the cells, whereas TGF-α treatment (10 ng/ml, 24 hr) induced significant cell spreading, an effect which was inhibited by gefitinib (Fig. 7*a*). Little effect on wtMCF7 cell morphology was observed in any treatment. Further *in vitro* assays demonstrated that inhibition of EGFR function, and

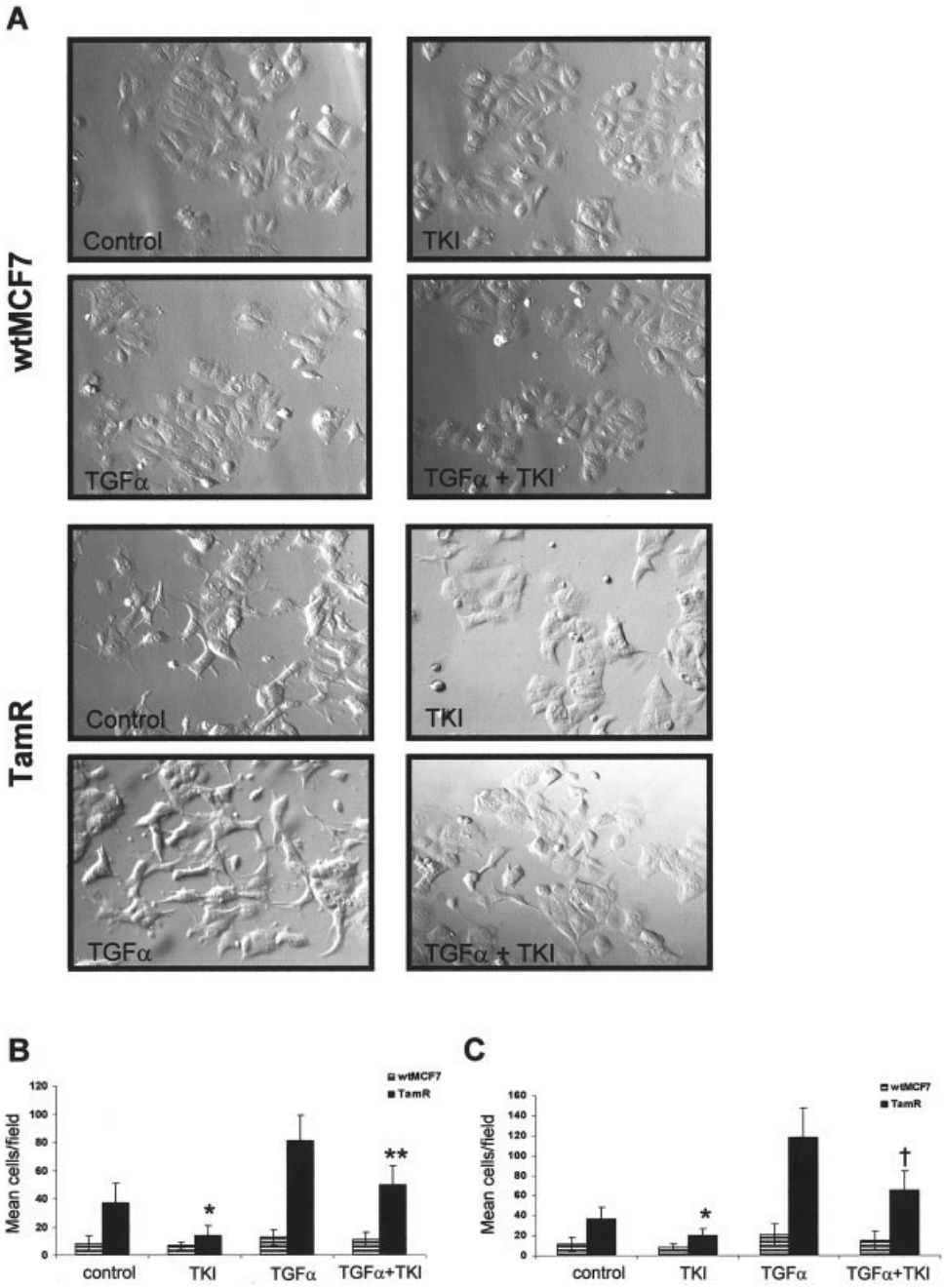

FIGURE 7 – EGFR blockade inhibits EMT-like behaviour in TamR cells. The role of the EGFR in mediating TamR cell morphology was determined by treating cell colonies with gefitinib (TKI, 1 μM, 24 hr), TGF-α (10 ng/ml, 24 hr) or a combination of both agents and imaging the resultant colonies at ×20 magnification. Images are representative of each sample. (*b*) Cell invasion in response to EGFR modulation was assessed by seeding 5×10^5 cells onto Matrigel-coated porous membranes and incubating for 72 hr at 37°C. Invasive cells were stained with DAPI and counted. (*c*) The migratory ability of the cells in the presence of TKI, TGF-α or both agents was determined by seeding 5×10^5 cells onto fibronectin-coated membranes. Following 24 hr incubation, migrating cells were fixed, stained with crystal violet and counted. For both invasion (*b*) and motility (*c*) experiments, the mean number of cells per field of view (at ×5 magnification) was determined ± SD for a minimum of 3 separate experiments. $*p < 0.05$ *vs.* control, † < 0.05 *vs.* TGF-α alone.

thus modulation of β-catenin activity, reduced basal and TGF-α-stimulated TamR invasion (Fig. 7*b*) and motility (Fig. 7*c*).

EGFR regulates expression of β-catenin/TCF/LEF genetic targets in TamR cells

To further investigate the importance of β-catenin in promoting the altered phenotype of tamoxifen-resistant breast cancer cells and the involvement of EGFR, we used RT-PCR to analyse the expression of a panel of genes reported to be transcribed through a β-catenin/TCF/LEF-dependent mechanism.[9] Expression of *c-myc, cyclin-D1, CD44* and *COX-2* was elevated in TamR cells compared to wtMCF7 cells under basal growth conditions (Table I). A small but nonsignificant increase in *uPAR* was also observed in this cell type. Further analysis was performed on cells that had been treated with gefitinib (1 μM), TGF-α (10 ng/ml) or a combination of both agents for 24 hr. These experiments revealed that

TABLE I – RT-PCR ANALYSIS OF β-CATENIN REGULATED GENE EXPRESSION

Gene investigated	Mean fold increase in expression ± SD (TamR *vs.* wt)
c-myc	$2.02 \pm 0.46^{**}$
cyclin D1	$1.77 \pm 0.40^{*}$
CD44	$2.77 \pm 0.38^{*}$
COX-2	$2.62 \pm 0.34^{**}$
uPAR	1.47 ± 0.54

mRNA was extracted from replicate samples ($n = 4$ for each treatment) of wtMCF7 and TamR cells and RT-PCR performed to determine the expression of genes known to be regulated through a β-catenin-mediated mechanism. PCR products were separated on 1% agarose gels containing ethidium bromide, and densitometric analysis was performed on the resultant images. Levels of *c-myc, cyclinD1, CD44* and *COX-2* were all increased in TamR cells compared to wtMCF7 cells. $*p < 0.05$ and $**p < 0.02$ *vs.* wtMCF7 cells.

TABLE II – RT-PCR ANALYSIS OF EGFR MODULATION OF β-CATENIN TARGET GENES IN TamR CELLS

Gene investigated	Mean fold increase in expression ± SD in TamR cells				
	Control	TKI	TGF-α	TGF-α + TKI	TGF-α + PD
c-myc	1	0.46 ± 0.17**	2.44 ± 0.69**	0.85 ± 0.22†	0.76 ± 0.13
cyclin D1	1	0.65 ± 0.22*	1.87 ± 0.31*	0.73 ± 0.04†	1.17 ± 0.22
CD44	1	0.64 ± 0.04*	3.47 ± 1.06**	1.55 ± 0.53†	2.31 ± 0.41††
COX-2	1	0.70 ± 0.39*	1.78 ± 0.41*	0.92 ± 0.06†	1.37 ± 0.34††

RT-PCR was performed on reverse-transcribed mRNA from TamR cells following treatment with gefitinib (TKI), TGF-α, PD098059 (PD) or TGF-α in combination with TKI or PD; data were obtained as for Table I. Inhibition of EGFR signalling in TamR cells reduced basal and TGF-α-stimulated levels of *c-myc, cyclinD1, CD44* and *COX-2* genes. PD-mediated ERK1/2 suppression had a reduced effect on suppression of TGF-α-stimulated *CD44* expression. $^{*}p < 0.05$ and $^{**}p < 0.02$ *vs.* control; $^{\dagger}p < 0.05$ *vs.* TGF-α alone; $^{\dagger\dagger}p < 0.05$ *vs.* TGF-α + TKI treatment.

expression of these genes could be modulated through stimulation or inhibition of the EGFR in TamR cells (Table II). No significant change was observed in wtMCF7 cells (not shown). Since the EGFR can activate gene transcription through ERK1/2 stimulation[33–35] thus masking potential β-catenin involvement, RT-PCR was performed on TGF-α-stimulated cells pretreated with the ERK1/2 inhibitor PD098059. Whereas expression of *c-myc* and *cyclin D1* remained suppressed, *CD44* and *COX-2* expression showed only partial inhibition.

Discussion

We have previously identified that upon development of resistance to tamoxifen, MCF7 cells acquire an increased proliferation rate together with an increased capacity for *in vitro* invasion and migration.[3,4] Here, we demonstrate that endocrine-resistant MCF7 cells lose their epithelial features and acquire mesenchymal characteristics, including a spreading/migrating phenotype and loss of cell–cell adhesive interactions. These are events characteristic of cells undergoing EMT.[36,37] To elucidate mechanisms that may underlie the EMT-like transition that accompanies endocrine resistance, we provide evidence of a role for β-catenin as both a mediator of E-cadherin dysfunction and a regulator of gene expression using an *in vitro* MCF7 model of tamoxifen resistance. Additionally, we demonstrate the importance of the EGFR as a modulator of β-catenin function in these cells.

Loss or dysfunction of E-cadherin-mediated cell–cell adhesion is a hallmark of EMT in tumour cells, with numerous studies highlighting the inverse relationship between strong intercellular adhesion and tumour invasion and spread.[38,39] Indeed, based on evidence such as this, E-cadherin itself has been proposed as a prognostic indicator.[40,41] Given the role of cadherin-based cell–cell adhesion in suppressing tumour spread, it is possible that the aggressive phenotype of tamoxifen-resistant breast cancer cells *in vitro* may occur as a result of modulation of cadherin/catenin expression. To this end, we investigated the expression of major AJ components (E-cadherin, α- and β-catenin) in both wt and TamR MCF7 cells. Whereas our data did not reveal any significant difference in either E-cadherin or α-catenin expression between the 2 cell types, levels of β-catenin mRNA were increased in TamR cells. Although it is currently unclear how regulation of β-catenin occurs at the transcriptional level, recent evidence has demonstrated the presence of NF-κB and E2F1 binding sites within the β-catenin gene (*CTNNB1*) promoter.[42] E2F1 expression is enhanced by cyclin D1, itself shown to be a target of β-catenin/TCF/LEF-mediated transcription; thus, our observation that *cyclin D1* is increased in TamR cells (Table I) might be evidence of a self-propagating mechanism for β-catenin gene expression in these cells.

In addition to elevated mRNA levels, our data demonstrate a significant increase in β-catenin protein in both the cytoplasm and nucleus of TamR cells, where β-catenin may interact with members of the TCF/Lef family of transcription factors to promote gene expression. The ability of β-catenin to act as part of the adhesive and transcriptional machinery within the cell involves modulation of the protein through phosphorylation on serine/threonine and tyrosine residues: reduced serine/threonine phosphorylation of β-catenin deregulates turnover, destabilises the cadherin adhesion complex and facilitates the nuclear translocation of β-catenin and subsequent gene transcription,[7,43–45] whereas tyrosine phosphorylation of β-catenin can result in its dissociation from E-cadherin and loss of association with the cytoskeleton, promoting reduced cell–cell adhesion and cell scattering.[16,46] Accompanying the redistribution of β-catenin was a reduction in the phosphorylation of β-catenin on serine/threonine and an increase in its phosphorylation on tyrosine. This may thus suggest that the degradation of β-catenin in TamR cells is impaired or occurs at a reduced rate.

Activation of the PI3K/AKT signalling pathway results in inactivation of GSK3β through its AKT-mediated phosphorylation.[47] Further analysis of TamR cell lysates revealed high levels of both activated AKT and inactive GSK3β, which would implicate this pathway as an underlying mechanism at least partially responsible for the stabilization of, and increase in, β-catenin. Tumour cell lines producing truncated APC proteins are unable to effectively phosphorylate β-catenin and have high levels of cytosolic β-catenin;[48] however, upon examining our cells for the APC protein, neither its size nor level of expression appeared altered between wtMCF7 cells and their endocrine-resistant counterparts (S. Hiscox, unpublished observations). Although we have suggested a role for AKT in reducing the serine/threonine phosphorylation of β-catenin in TamR cells, a role for Wnt signalling cannot be ruled out since activation of this pathway results in inhibition of GSK3β activity, leading to catenin stabilization and nuclear translocation.[49,50] The role of Wnt signalling in TamR cells is thus remains unclear, and studies are currently under way in our laboratory to address this.

The observations that (*i*) significantly more β-catenin is phosphorylated on tyrosine in TamR cells compared to wtMCF7 cells, (*ii*) these cells overexpress members of the erbB2 family of growth factor receptors[3] and (*iii*) the EGFR can promote β-catenin phosphorylation[17,51] suggest an interplay between these molecules in TamR cells. Additionally, since levels of β-catenin–erbB complexes are significantly elevated in more aggressive tumour types and metastases in humans,[29] a relationship between growth factor–induced phosphorylation of β-catenin and a change towards an EMT phenotype *in vitro* would be plausible. Indeed, we show evidence here that β-catenin associates with the EGFR in TamR cells but not wtMCF7 cells, and we observed a loss of association between β-catenin and E-cadherin, as has been previously demonstrated upon tyrosine phosphorylation of β-catenin.[16] Together, these observations may go some way to explain why TamR cells display reduced cell–cell contacts and the apparent lack of effect on *in vitro* invasion following experimental neutralisation of E-cadherin function, contrary to our observations for wtMCF7 cells.

Our data here provide evidence for a role of the EGFR as a mediator of β-catenin function in TamR cells. Following inhibition of basal EGFR activity using gefitinib,[22,23] we observed a reduction in tyrosine-phosphorylated β-catenin and its association

with the EGFR together with a rise in serine/threonine-phosphorylated β-catenin, an effect that can be attributed to the inhibition of AKT and thus elevation of GSK3β activity. Moreover, the amount of β-catenin associating with E-cadherin was seen to increase, whilst β-catenin association with Lef-1 was reduced. Although stimulation of these cells with TGF-α (10 ng/ml, 1 hr) inhibited serine/threonine phosphorylation of β-catenin, an effect that might be predicted to result in elevated β-catenin levels, we did not observe any significant change in total β-catenin in cell lysates. Elevated β-catenin levels have been reported after 60 min stimulation of the EGFR in dermal fibroblasts[52] and are coupled with an increase in TCF activity. Our data suggest that in TamR cells levels of cytoplasmic β-catenin may increase over a greater time period. Cell fractionation experiments, however, did demonstrate a small rise in cytoplasmic levels of β-catenin following 1 hr TGF-α treatment of TamR cells. Using cell fractionation techniques, we demonstrated that β-catenin was redistributed within the cell, particularly into the nuclear fraction, following EGFR stimulation in TamR cells only. Correspondingly, inhibition of EGFR activity reduced the amount of nuclear β-catenin detectable in these cells. These observations may thus help to explain the observed EGFR-mediated increase in association of β-catenin with the Lef-1 transcription factor, presumably brought about by the increased amount of β-catenin within the nucleus available for interaction with such proteins.

To elucidate potential consequences of the altered β-catenin phosphorylation state and its resultant redistribution in TamR cells, we used RT-PCR to analyse the expression of several genes known to be driven through a β-catenin/TCF/Lef-dependent mechanism. This is important in light of recent evidence which has implicated β-catenin-mediated transcription events in the induction of EMT.[53–55] Target genes of the β-catenin signalling pathway include *c-myc,*[56] *cyclin D1,*[57,58] *COX-2,*[59,60] *uPAR*[61] and *CD44*.[62] Evidence for TCF/Lef binding sites have also been demonstrated within the *MMP7* promoter.[63] Expression of these genes is pro-oncogenic, contributing to the proliferation, progression and spread of tumours, and thus may play significant roles in the induction and progression of EMT.[64–66] Our data revealed that *c-myc, cyclin D1, CD44* and *COX-2* transcripts were all increased in TamR cells compared to wtMCF7 cells under basal conditions. A small but nonsignificant increase was also observed for *uPAR*. Although TamR cells are significantly more invasive than wtMCF7 cells,[4] no specific product was detectable for *MMP7* in either cell type. Since MMP7 is well characterised as having an invasion-promoting role, these data suggest that its expression in these cells may occur *via* other pathways, which may involve, *e.g.,* integrin expression, as has been suggested by Pozzi *et al*.[67] Interestingly, data obtained from microarray analysis of TamR cell mRNA have demonstrated a significant elevation in *PPARδ* gene expression in TamR cells (R. Burmi, personal communication). He *et al*.[68] established the presence of a TCF response element within the *PPARδ* gene and demonstrated that β-*catenin/TCF upregulates expression of PPARδ* in colon carcinoma cells. However, although there are suggestions that elevated *PPARδ* expression is required to induce target genes involved in the regulation of tumour proliferation,[69,70] other reports have suggested that *PPARδ* may reduce carcinogenesis.[71] Thus, the contribution of *PPARδ* to the TamR cell phenotype is unclear, and further investigations are under way to address this. Expression of *c-myc, cyclin D1, COX-2* and *CD44* was EGFR-dependent in TamR cells but not wtMCF7 cells, which may be indicative of the effects of the EGFR as a regulator of β-catenin transcription function in these cells. The mechanisms controlling expression of the genes investigated here is complex and does not depend on β-catenin/TCF/Lef activity alone. Thus, our current observations present circumstantial evidence for a role of β-catenin in EGFR-mediated gene expression. Increased gene transcription can occur through other pathways, including EGFR-mediated ERK1/2 activation.[34] Interestingly, TGF-α stimulation of TamR cells pretreated with the ERK1/2 inhibitor PD098059 was still able to influence β-catenin phosphorylation (S. Hiscox, unpublished observations) and expression of CD44 and COX-2 (Table II). However, to fully define β-catenin as playing a critical role in gene expression in our model of endocrine resistance, manipulation of β-catenin expression at the genetic level will be necessary.

Since we suggest here a role for β-catenin and the EGFR in the development of an aggressive phenotype in endocrine-resistant wtMCF7 cells, we determined whether inhibition of β-catenin activity brought about through modulation of EGFR function would affect their EMT-like characteristics. Treatment of TamR cell colonies with gefitinib reduced TamR cell spreading, giving rise to colonies in which tight cell–cell junctions appeared to be restored. TamR colony scattering induced by TGF-α was also inhibited by gefitinib. In MCF7 cells, which possess very low levels of endogenous EGFR protein, no significant changes in cell behaviour were observed following gefitinib and/or TGF-α challenge. Further experiments demonstrated that inhibition of EGFR activity, and thus reduced β-catenin signalling, led to a reduction in both basal and TGF-α-induced TamR cell invasion and migration.

In conclusion, we have shown here that the development of endocrine resistance in MCF7 breast cancer cells is accompanied by a change in β-catenin phosphorylation, loss of AJ function and increased transcription of β-catenin target genes. Furthermore, we provide evidence that suggests that EGFR-induced modulation of β-catenin activity and function in these cells may promote the development and progression of EMT in endocrine-resistant cancer and thus contribute to an advanced disease state.

Acknowledgements

We thank Ms. K. Ingram, Ms. E. Mayfield and Ms. C. Dutkowski for expert technical assistance. Ms. K. Ingram and Ms. E. Mayfield are supported by a Wellcome Trust scholarship. We are grateful to Dr. J. Gee for permission to cite unpublished results.

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