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Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells

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ABSTRACT

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is an intercellular adhesion molecule that is overexpressed in a wide variety of human cancers, including colon, breast and lung and is associated with tumourigenesis, tumour cell adhesion, invasion and metastasis. In this study, we showed that CEACAM6 was overexpressed in a panel of oestrogen receptor (ERα)-positive human breast cancer cell lines (MCF-7:5C and MCF-7:2A) that have acquired resistance to oestrogen deprivation, and this overexpression was associated with a more aggressive invasive phenotype *in vitro*. Expression array analysis revealed that MCF-7:5C and MCF-7:2A cells overexpressed CEACAM6 mRNA by 27-fold and 12-fold, respectively, and were 6–15-times more invasive compared to non-invasive wild-type MCF-7 cells which expressed low levels of CEACAM6. Suppression of CEACAM6 expression using small interfering RNA (siRNA) completely reversed migration and invasion of MCF-7:5C and MCF-7:2A cells and it significantly reduced phosphorylated Akt and c-Src expression in these cells. In conclusion, our findings establish CEACAM6 as a unique mediator of migration and invasion of drug resistant oestrogen-deprived breast cancer cells and suggest that this protein could be an important biomarker of metastasis.

1. Introduction

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a glycosylphosphatidylinositol-anchored cell surface protein that functions as a homotypic intercellular adhesion molecule.[1] It is overexpressed in a number of human malignancies including pancreatic cancer, gastrointestinal cancer and breast cancers[2,3], and increased levels of CEACAM6 are inversely correlated to the differentiation state of cancer cells. Previous studies have shown that CEACAM6 is overexpressed in pancreatic adenocarcinoma cells, and its overexpression is associated with greater *in vivo* metastatic ability and increased invasiveness and migration.[4,5] More recently, Poola and co-workers[6] reported that the expression of CEACAM6 in atypical ductal hyperplasia was associated with the development of invasive breast cancer (IBC). Currently, however, the role of CEACAM6 overexpression in breast cancer migration and invasion is not known.

Invasion and metastasis are the hallmarks of cancer malignancy, and they are the primary cause of patient mortality during breast cancer progression.[7] Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels, and metastasis refers to the spreading of cancer cells to other parts of the body.[8] In order for a transformed cell to metastasize, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system and adhere to distant organs.[8] These processes require extensive alterations in gene expression profiles,

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including the down-regulation of genes involved in cell anchorage and the up-regulation of genes involved in cell motility and matrix degradation.[7,9,10]

Aromatase inhibitors (AIs) are anti-oestrogen agents that suppress oestrogen production in peripheral tissues and breast tumours by inhibiting or inactivating aromatase, the enzyme which catalyses the conversion of androgens to oestrogens in post-menopausal women.[11] Several randomized trials[12–15] have shown that third generation AIs are superior to adjuvant tamoxifen in terms of improved disease-free survival and less side-effects. Unfortunately, one of the consequences of prolonged oestrogen deprivation/suppression is the development of drug resistance.[16,17] Previous studies have shown that acquisition of tamoxifen resistance in breast cancer cells is associated with a significant increase in motility and invasion[18,19] along with increased CEACAM6 expression[20]; however, it is unknown whether acquired resistance to oestrogen deprivation affects tumour cell migration and invasion and whether CEACAM6 plays a role in this process.

In this study, we investigated the role of CEACAM6 in cellular migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation. We found that CEACAM6 was significantly overexpressed in oestrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells and that these cells were markedly more migratory and invasive than parental MCF-7 cells. Suppression of CEACAM6 expression by small interfering RNA (siRNA) completely reversed the invasive phenotype of MCF-7:5C and MCF-7:2A cells. E-cadherin and β-catenin were also significantly reduced in these cells. The mechanism of action of CEACAM6 appears to involve, in part, the c-Src and Akt signalling pathways.

2. Materials and methods

2.1. Reagents

17 Beta-oestradiol was purchased from Sigma Chemical Co. (St Louis, MO); PP2 was purchased from EMD Biosciences Inc. (La Jolla, CA); LY294002 was purchased from Promega (Madison, WI); fulvestrant was obtained as a generous gift from AstraZeneca (Macclesfield, United Kingdom); Affymetrix Human Genome U133 Plus 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA); foetal bovine serum (FBS), cell culture medium and other reagents were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell lines and culture conditions

Wild-type MCF-7 human breast cancer cells[21] were obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and were maintained in fully oestrogenized medium (RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1× non-essential amino acids and bovine insulin at 6 ng/mL (Sigma–Aldrich, St. Louis, MO). MCF-7:5C[21–23] and MCF-7:2A[24] cells were clonally selected from parental MCF-7 cells following long-term culture (>1 year) in phenol red-free RPMI 1640 media containing 10% dextran-coated charcoal stripped FBS (SFS).

2.3. RNA preparation and microarray hybridisation

Total RNA was prepared using the Qiagen RNeasy Mini kit. A DNase I digestion step was included to eliminate DNA contamination. cRNA was generated, labelled, and hybridised to the Affymetrix Human Genome U133 Plus 2.0 Arrays by the Northwestern University Genomics Core (Chicago, IL). Arrays were washed, stained and scanned according to the directions detailed in the Affymetrix GeneChip® Expression Analysis Technical Manual.

2.4. Microarray data analysis

Assessment of data quality was conducted following default guidelines in the Affymetrixs GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Data were extracted and normalised using *Affymetrix Microarray Suite* (MAS5.0) following recommended protocols for background and chip-correction. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the three cell lines were arrayed to determine consistent and reproducible patterns of gene expression. All but one array showed a high degree of reproducibility within a set of replicate hybridisations, leaving at least three array replicates per cell line for further analysis. Genes across all arrays with an expression intensity <70 were removed. To eliminate genes with variable expression within a group of replicates, normalised gene intensity ratios (signal intensities divided by the median gene intensity all hybridisations) were derived, then the standard deviation of the log-transformed normalised intensity ratios were calculated for each group of replicates. Genes with a standard deviation >0.15 were excluded. Lastly, to filter for genes with variable expression between cell lines, genes were retained that showed a standard deviation of >0.3. A total of 904 genes met the filtering criteria described and were examined by hierarchical clustering using resources available at TGen.[c] Uncentred Pearson's correlation with average linkage was used on $\log_2$-transformed data, with induced genes indicated in red and repressed genes in green. Random permutation analysis was performed as previously described [25] using 10,000 permutations. Genes with a p-value <0.01 and an alpha value <0.01 were used for gene ontology analysis.

2.5. Cell proliferation assay

Cell proliferation assay was performed as previously described.[22] The DNA content of the cells was determined using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). For each analysis, three replicate wells were used, and at least three independent experiments were performed.

2.6. Western blot analysis

Western blot analyses were performed as previously described.[22] Separated proteins were transferred onto nitrocel-

[c] Internet address: http://biodiscovery.tgen.org/microarray/.

lulose membranes (Milllipore) and incubated overnight at 4 °C with the respective primary antibodies; CEACAM6 and CEACAM5 (Signet Laboratories, Dedham, MA); ERα, N-cadherin, β-catenin, CXCR4, MMP9, E-cadherin and CD44 (Santa Cruz Biotechnology, Santa Cruz, CA); fibronectin (Chemicon International, Temecula, CA); c-Src and p-Src^{Tyr529} (Biosource International, Carmarillo, CA); AKT and p-AKT^{Ser473} (Cell Signaling Technology, Beverly, MA); and β-actin (Sigma Chemical Co., St Louis, MO). Secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) to visualise the resolved proteins.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR) for ERα and CEACAM6

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Ten micrograms of total RNA for each sample were converted to first-strand cDNA using SuperScript III with a combination of random hexamers and oligo(dT) as primers (Invitrogen). Quantitative real-time PCR assays were done as previously described[22] with the Taqman Universal or SYBR Green PCR Master Mixes and an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The ERα forward and reverse primers were 5′-AAGAGGGTGCCAGGCTTTGT-3′ and 5′-CAGGATCTCTAGCCAGGCAC AT-3′, respectively. The *ERα* probe was 5′-[FAM]-ATTTGACCCTCCATGATCAGGTCC ACC-[TAMRA]-3′. The forward and reverse primers for *CEACAM6* were synthesised by Sigma Genosys (Sigma–Aldrich). The sequences for *CEACAM6* forward and reverse primers were 5′-GACGTTTGTGTGGATTGCTGGAACGC-3′ and 5′-TGCCACGCAGCCTCTAACC-3′, respectively. The reporter dye at the 5′-end of each probe was FAM and the quencher dye at the 3′-end was TAMRA. The 18S ribosomal RNA (*18S rRNA*) gene was used as an endogenous control to normalise for differences in the amount of total RNA in each sample, *18S rRNA* primers and probes were purchased from Applied Biosystems. Relative expression of the target gene was calculated using the 2 delta CT method described previously[26] (Relative expression = $2^{-\Delta CT}$; where $\Delta CT = C_{T\ (\text{Target gene})} - C_{T\ (\text{endogenous control gene})}$), where *18S rRNA* is the endogenous control gene. To determine relative RNA levels within the samples, standard curves for the PCR were prepared by using cDNA from one sample and making twofold serial dilutions covering the range equivalent to 20–0.625 ng RNA (for 18S rRNA analyses, the range was 4–0.125 ng).

2.8. Cell migration and invasion assays

Cell migration was measured in a Boyden chamber using Transwell filters obtained from Corning (Cambridge, MA). Cells (1×10^5) in 0.5 mL serum-free medium were placed in the upper chamber, and the lower chamber was loaded with 0.8 mL medium containing 10% SFS. Cells that migrated to the lower surface of filters were stained with Wright Giemsa solution, and five fields of each well were counted after 24 or 48 h of incubation at 37 °C with 5% CO_2. Three wells were examined for each condition and cell type, and the experiments were repeated in triplicate. Cell invasion assay was performed using the Chemicon cell invasion kit (Chemicon International, Temecula, CA) in accordance with the manufacturer's protocol. Cells (1×10^5/ml) were seeded onto 12-well cell culture chamber using inserts with 8 μM pore size polycarbonate membrane over a thin layer of extracellular matrix. Following incubation of the plates for 48 h at 37 °C, cells that had invaded through the ECM layer and migrated to the lower surface of the membrane were stained and counted under the microscope in at least 10 different fields and photographed.

2.9. CEACAM6 siRNA-mediated gene knockdown

CEACAM6-specific siRNA (Silencer™ Predesigned siRNA; sense: GCCCUGGUGUAUUU UCAUtt, antisense: AUCGAAAAUACAC CAGGGCtg) (AM16704) and scramble sequence control siRNA (Silencer™ Negative Control siRNA) were purchased from Ambion (Austin, TX). Transfection complexes were prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 0.3 μL of siPORT NeoFX transfection reagent (Ambion) and 10 nM CEACAM6 siRNA or negative control siRNA (Ambion). Cells (9×10^4 cells per well) were reverse-transfected in 12-well plates simultaneously with addition of transfection complexes. The medium was replaced with phenol red-free RPMI supplemented with 10% SFS 24 h after transfection and cultures were harvested for CEACAM6 protein and mRNA analyses.

2.10. Statistical analyses

Statistical analyses were performed using Microsoft Excel (Seattle, WA). Differences between groups were evaluated using Student's *t*-test. Data were considered significant if $p < 0.05$.

3. Results

3.1. Characterisation of long-term oestrogen-deprived breast cancer cells

The growth of oestrogen-deprived MCF-7:5C and MCF-7:2A cells is compared to parental MCF-7 cells in Fig. 1A. Both MCF-7:5C and MCF-7:2A cells grew robustly in the absence of oestrogen whereas MCF-7 cells grew minimally without oestrogen. The doubling times were 2.7, 3.4, and 6 d for MCF-7:5C, MCF-7:2A and MCF-7 cells, respectively. We also examined cell morphology changes associated with resistance to long term oestrogen deprivation using phase-contrast microscopy. Fig. 1B shows that MCF-7 cells grew as a uniform monolayer of tightly associated cells with limited cell spreading but distinct cellular boundaries, whereas oestrogen-deprived MCF-7:5C and MCF-7:2A cells grew in a less uniform monolayer with cellular boundaries that were obscured. ERα mRNA and protein expression were also significantly increased in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells and treatment with oestradiol or the pure anti-oestrogen fulvestrant significantly down-regulated its expression (Fig. 1C and D) in all three cell lines. Overall, these results show that oestrogen deprivation increases ERα expression and alters the morphology of MCF-7:5C and MCF-7:2A cells.

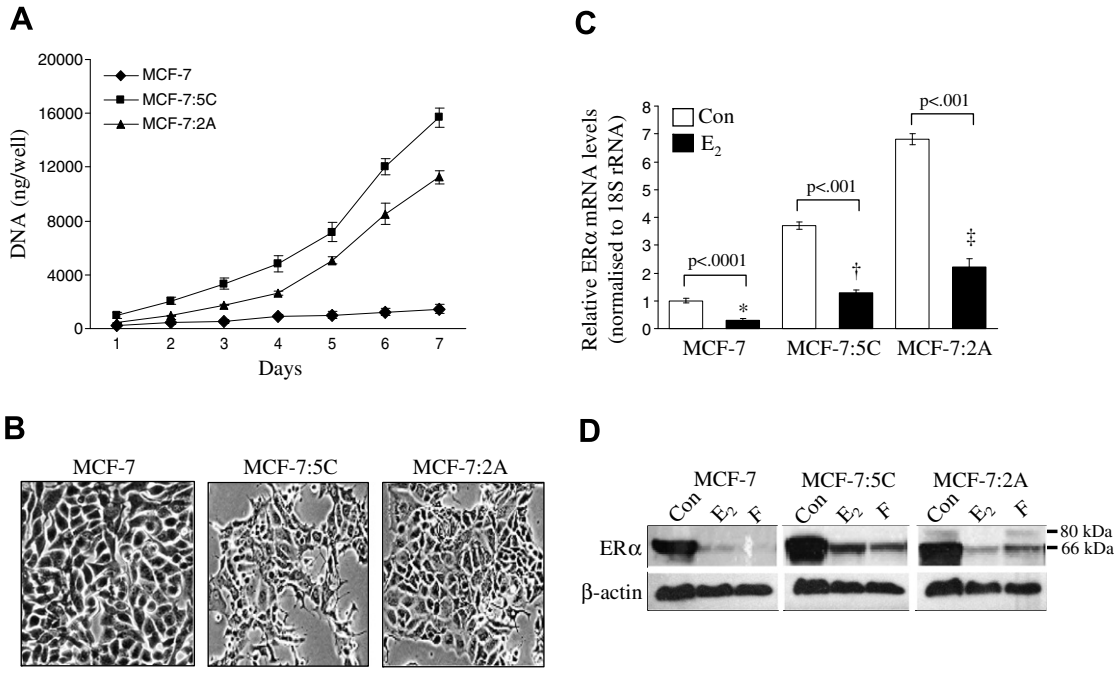

Fig. 1 – Characterisation of long-term oestrogen-deprived breast cancer cells. (A) For proliferation assays, cells were seeded in 24-well dishes (30,000 per well) in oestrogen-free RPMI media and total DNA was quantitated at the indicated time points. (B) Phase-contrast microscopy pictures of MCF-7, MCF-7:5C and MCF-7:2A cells. Images were produced by the Olympus DP-3030 camera and Olympus IX-70 software. Magnification, ×10. (C) ERα mRNA level was determined by quantitative RT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (D) ERα protein levels were determined by immunoblotting with a specific ERα antibody. Cells were treated with 1 nM oestradiol or 1 μM fulvestrant for 48 h and 50 μg of protein lysates was analysed. β-Actin was used as a loading control.

3.2. *Global gene expression profiles of oestrogen-deprived breast cancer cells*

Transcriptional profiling of parental MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A cells was performed using Affymetrix Human Genome U133 Plus 2.0 Array. Two-dimensional hierarchical clustering was performed to analyse differences in gene expression patterns between MCF-7 cells and MCF-7:5C and MCF-7:2A cells. Data filtering identified 904 genes that were significantly altered between MCF-7:5C and MCF-7:2A cells and parental MCF-7 cells (Fig. 2A and Supplementary Fig. S1). The sample dendogram showed that MCF-7:2A cells and MCF-7 cells clustered more closely, whereas MCF-7:5C cells clustered on a more distant branch, suggesting that MCF-7:2A cells are more similar to parental MCF-7 cells than MCF-7:5C cells (Fig. 2A). In order to define cell signalling mechanisms that differed significantly between parental MCF-7 and MCF-7:5C and MCF-7:2A cells, random permutation weighted gene analysis was performed as described in Section 2. A comparison of MCF-7 expression data with that of MCF-7:5C and MCF-7:2A revealed that 4068 genes were highly differentially expressed (Supplementary Table 1). Gene Ontology analysis showed a significant number of genes associated with cell cycle control, proliferation, growth factor signalling, cell adhesion and motility and invasion. In particular, we found that CEACAM6 was overexpressed by 27-fold in MCF-7:5C cells and 12-fold in MCF-7:2A cells (Fig. 2B), and it was highly weighted in our random permutation analysis (p-value < .0001) (Supplementary Table 1).

3.3. *Oestrogen deprivation increases CEACAM6 expression and enhances migration and invasion of oestrogen-deprived breast cancer cells*

To confirm our microarray data, CEACAM6 mRNA expression was determined by quantitative RT-PCR. Fig. 3A shows that CEACAM6 mRNA was significantly upregulated in oestrogen-deprived MCF-7:5C and MCF-7:2A cells compared with parental MCF-7 cells. Similarly, by Western blotting, CEACAM6 protein was undetectable in MCF-7 cells but was strongly expressed in MCF-7:5C and MCF-7:2A cells (Fig. 3B). Other invasion proteins such as CEACAM5, MMP9, CXCR4 and CD44 were also markedly elevated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells (Fig. 3B). This finding is consistent with a recent study by Mackay and coworkers[27] which revealed that many genes associated with extracellular matrix remodelling were significantly upregulated following aromatase inhibitor treatment of primary breast tumours.

3.4. *Oestrogen deprivation increases migration and invasion of breast cancer cells*

Since MCF-7:5C and MCF-7:2A cells overexpressed several invasion genes, we next assessed the migratory and invasive

potential of these cells *in vitro*. Cell migration was measured using a modified Boyden chamber assay with 10% SFS as a chemoattractant. As shown in Fig. 3C, MCF-7:5C and MCF-7:2A cells had the highest numbers of migrating cells compared to MCF-7 cells; a phenotype that correlated with CEACAM6 expression. Similar results were obtained when the different cell lines were tested for their ability to invade through membranes coated with Matrigel. Fig. 3D shows that MCF-7:5C and MCF-7:2A cells had the highest number of invading cells, while MCF-7 cells were non-invasive. The invasive ability of the cell lines was as follows: MCF-7:5C > MCF-7:2A > MCF-7.

3.5. *CEACAM6 suppression inhibits invasion and migration of MCF-7:5C cells*

To test the hypothesis that CEACAM6 is required for cell migration and invasion, we used siRNA to suppress CEACAM6 expression. MCF-7:5C cells were transfected with CEACAM6-specific or control (scrambled sequence) siRNA, and Western blot analysis was performed 72 h post-transfection. Fig. 4A (top) shows that CEACAM6 protein was significantly suppressed (75–85%) in MCF-7:5C cells transfected with the CEACAM6-specific siRNA but not the control siRNA. siRNA suppression of CEACAM6 expression was also confirmed at the transcript level using qRT-PCR at 48 h following transfection (Fig. 4A, bottom). To clarify the role of CEACAM6 in cell invasion, MCF-7:5C cells were pretreated with CEACAM6 siRNA or control siRNA for 48 h and invasion was measured over the subsequent 48 h. Fig. 4B shows that CEACAM6 siRNA almost completely reversed the invasiveness of MCF-7:5C cells, whereas control siRNA did not affect cell invasion. The invasiveness of MCF-7:5C cells was inhibited by nearly 80% when CEACAM6 expression was suppressed. A similar trend was observed for cell migration (data not shown). Suppression of CEACAM6 also significantly reduced phosphorylated Akt and phosphorylated c-Src in MCF-7:5C cells (Fig. 4C). E-cadherin and β-catenin were also significantly reduced in MCF-7:5C and MCF-7:2A cells, whereas pAkt and N-cadherin were significantly upregulated in these cells compared to parental MCF-7 cells (Fig. 4D). Similar experiments performed in MCF-7:2A cells also showed a dramatic reduction (60%) in invasion following CEACAM6 suppression (data not shown).

3.6. *Oestradiol down-regulates CEACAM6 expression and blocks migration and invasion of MCF-7:5C cells*

We also examined whether CEACAM6 expression is hormonally regulated in MCF-7:5C and MCF-7:2A cells. As shown in Fig. 5A and B, oestradiol completely down-regulated CEACAM6 mRNA and protein expression in MCF-7:5C and MCF-7:2A cells. This down-regulation was an ERα-mediated event since pretreatment with the anti-oestrogen fulvestrant, which is known to degrade ERα[28,29], was able to reverse the inhibitory effect of oestradiol on CEACAM6 protein in both cell lines (Fig. 5B). Fulvestrant also completely counteracted the anti-invasive effects of oestradiol in MCF-7:5C cells (Fig. 5C). Interestingly, oestradiol enhanced the invasiveness of parental MCF-7 cells (Fig. 5D) without significantly changing CEACAM6 protein level in these cells (Fig. 5B).

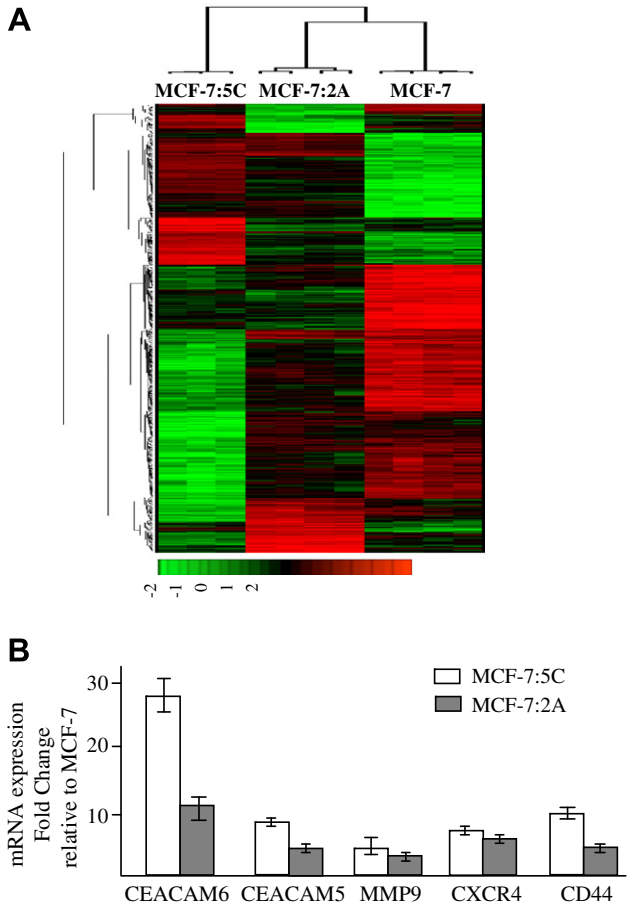

Fig. 2 – Overview of global gene expression patterns in wild-type MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A variant clones. (A) Unsupervised hierarchical clustering dendogram of 904 genes most differentially expressed across the three cell lines. Each row represents a single gene. Red, genes with high expression levels and green, genes with low expression levels. The similarities in the expression pattern amongst the three cell lines are presented as a "condition tree" on the top of the matrix. (B) Expression levels of invasion genes in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells, as identified by microarray analysis.

3.7. *Inhibition of c-Src reduces the invasiveness of MCF-7:5C and MCF-7:2A cells*

Previous studies have reported that CEACAM6 cross-linking initiates c-Src-dependent cross-talk between CEACAM6 and αvβ3 integrin, leading to increased ECM-adhesion and invasion.[30] We therefore determined c-Src kinase activity in oestrogen-deprived MCF-7:2A and MCF-7:5C cells by measuring phosphorylation of c-Src at Tyr^{529}. Both MCF-7:5C and MCF-7:2A cells showed significantly elevated levels of phosphorylated c-Src^{Y529} compared to parental MCF-7 cells, and treatment with the c-Src kinase inhibitor PP2 significantly reduced the invasiveness of MCF-7:5C and MCF-7:2A cells (Supplementary Fig. S2). Inhibition of Akt phosphorylation using the PI3K inhibitor LY294002 also significantly reduced cell growth and invasion of these cells (Supplementary

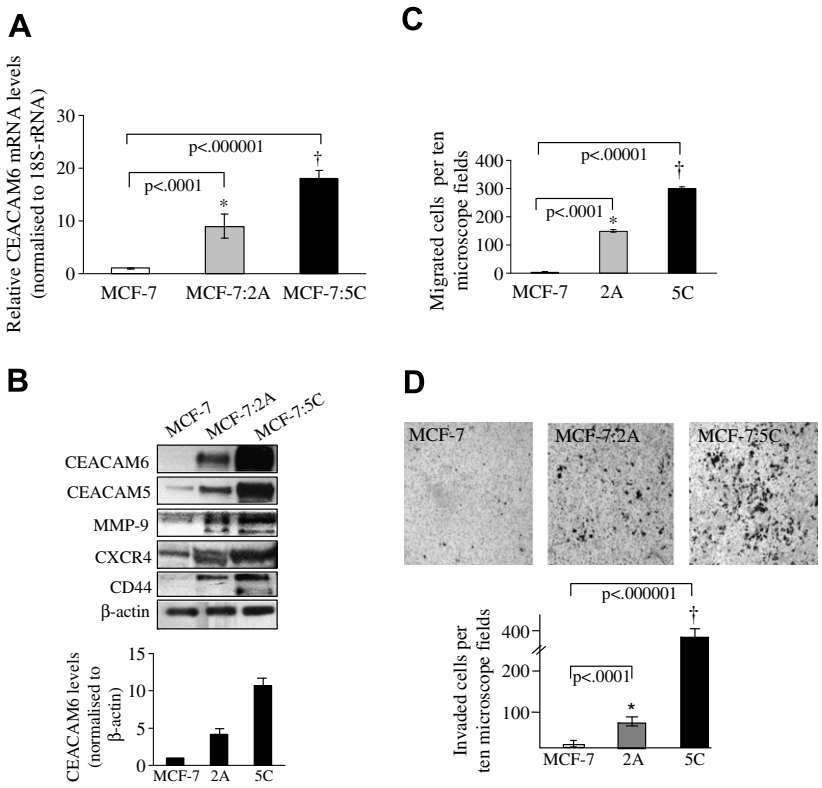

Fig. 3 – CEACAM6 promotes cell migration and invasion of oestrogen-deprived breast cancer cells. (A) CEACAM6 mRNA levels in parental MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A cells were measured by qRT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates, and the error bar represents the standard deviation. (B) Western blot analysis of CEACAM6 and other invasion proteins in MCF-7, MCF-7:5C and MCF-7:2A cells. The relative ratio of CEACAM6 was calculated by densitometry (bottom). The *bar graph* (bottom) depicts the averages of the data obtained from three individual experiments, and data are expressed as means ± SE. (C) Quantification of cells migrating across Transwell filters. (D) Cells that invaded through the Matrigel-coated transwells were fixed, stained, visualised at 20× magnification by light microscopy and photographed. Each panel represents an example of three replicates. Ten random fields were counted per insert at 20×.

Fig. S2), thus suggesting an important role for the c-Src and Akt signalling pathways in invasion.

4. Discussion

Despite advances in detection and treatment of metastatic breast cancer, mortality from this disease remains high because current therapies are limited by the emergence of therapy-resistant cancer cells. In this study, we showed that oestrogen deprivation significantly increased the motility and invasiveness of two ERα-positive human breast cancer cell lines that have acquired resistance to oestrogen deprivation, and that these cells overexpressed the invasive gene CEACAM6. Furthermore, knockdown of CEACAM6 expression completely inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells and caused a reduction in phosphorylated c-Src and pAkt expression. A significant reduction in E-cadherin and β-catenin was also observed in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells. To our knowledge, this study is the first to demonstrate a critical role for CEACAM6 in migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation.

Previous studies have reported that overexpression of CEACAM6 in pancreatic adenocarcinoma cells is associated with enhanced cellular invasiveness and increased metastatic potential *in vivo*, and that this effect is completely attenuated by suppression of CEACAM6 expression.[4] Recently, Scott and coworkers[20] reported that CEACAM6 was upregulated by 20-fold in tamoxifen-resistant MCF-7 cells compared to tamoxifen-sensitive cells, and that hormone sensitivity could be partially restored in the tamoxifen-resistant cells by siRNA silencing of CEACAM6. This *in vitro* data were substantiated in clinical breast cancer where it was demonstrated that CEACAM6 was overexpressed in primary breast tumours that subsequently relapsed following adjuvant tamoxifen and in a multivariate analysis, only CEACAM6 remained a significant predictor of recurrence.[31] These findings are consistent with our present study which shows that CEACAM6 is significantly upregulated in oestrogen-deprived breast cancer cells that have acquired resistance to oestrogen suppression, and knockdown of CEACAM6 expression reverses the invasive phenotype of these cells. The fact that CEACAM6 is identified independently in two model systems using endocrine agents with distinct modes of action suggests that it may play an important role in endocrine

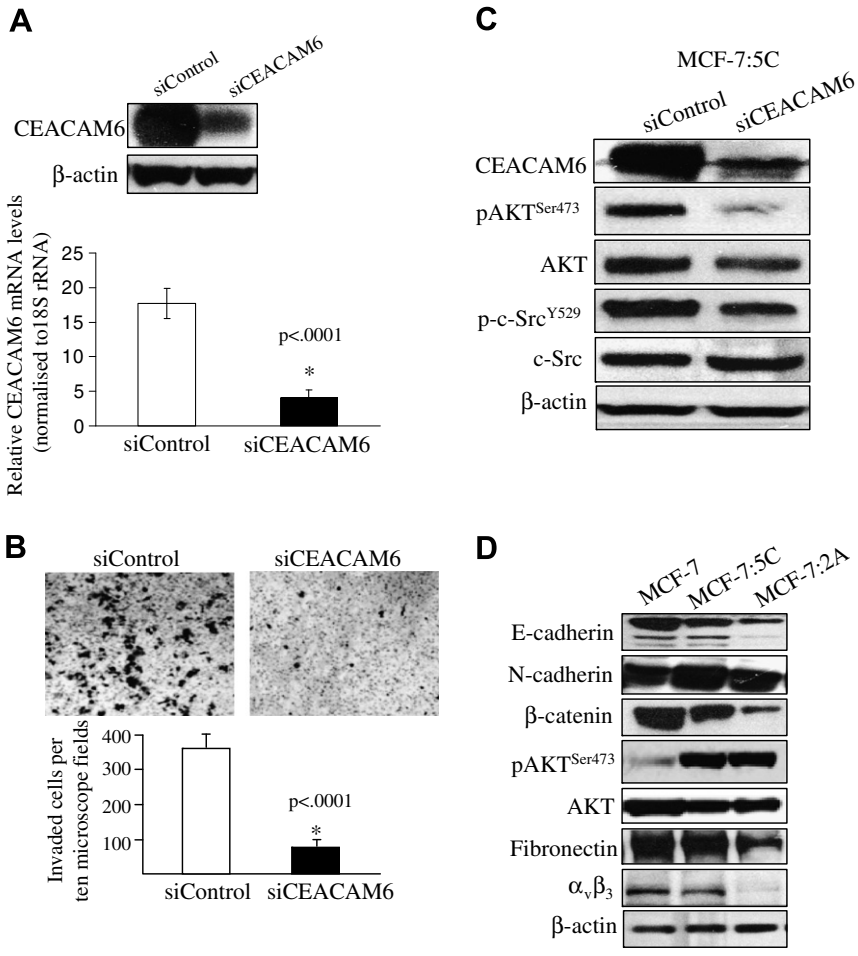

Fig. 4 – CEACAM6 suppression completely blocks invasion of MCF-7:5C breast cancer cells. (A) siRNA-mediated gene knockdown of CEACAM6 was verified by Western blot (top panel) and qRT-PCR (bottom panel). For qRT-PCR experiments, relative expression of CEACAM6 gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (B) Matrigel invasion assay of siControl and siCEACAM6-transfected MCF-7:5C cells. (C) Immunoblot analysis of MCF-7:5C cells transfected with CEACAM6 siRNA or control siRNA for 72 h. β-Actin was used as a loading control. (D) Western blot analyses of E-cadherin, β-catenin, N-cadherin, Akt and pAKT protein expression in MCF-7, MCF-7:5C and MCF-7:2A cells.

resistance. Currently, the mechanism by which CEACAM6 facilitates invasion is not fully understood. However, there is evidence that CEACAM6, along with other GPI-anchored proteins, is capable of modulating the activity of intracellular tyrosine kinases such as c-Src.[32,33] In particular, studies by Duxbury and coworkers[30,34] showed that c-Src activity was increased in CEACAM6-overexpressing BxPC3 human pancreatic cancer cells and decreased following suppression of CEACAM6 expression, and that inhibition of c-Src activity significantly suppressed CEACAM6-mediated cellular invasiveness. We found that phosphorylated c-Src was significantly elevated in MCF-7:5C and MCF-7:2A cells, and that suppression of CEACAM6 expression reduced its level in these cells. Pharmacological blockade of c-Src using the Src tyrosine kinase inhibitor pyrazolopyrimidine (PP2) also inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells. In addition, we found markedly elevated levels of phosphorylated Akt^{ser473} in MCF-7:5C and MCF-7:2A cells, which were dramatically reduced following CEACAM6 suppression. Akt is a serine/threonine protein kinase that mediates cell survival, proliferation[35,36], tumour cell migration and invasion and metastasis,[37] and previous studies have shown that c-Src activates the PI3K/Akt signalling pathway.[38] Thus, it is possible that activation of both c-Src and Akt might play a role in mediating CEACAM6-induced migration and invasion.

The epithelial-to-mesenchymal transition (EMT) plays a key role in metastasis and is characterised by the conversion of epithelial cancer cells to a more motile phenotype that facilitates invasion. A critical molecular feature of EMT is the down-regulation of E-cadherin,[39] a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. E-cadherin acts *de facto* as a tumour suppressor inhibiting invasion and metastasis and is frequently repressed or degraded during transformation. In our study, E-cadherin and β-catenin were significantly decreased, whereas N-cadherin was markedly increased in invasive MCF-7:5C and MCF-7:2A cells compared to non-invasive MCF-7 cells. In addition, our cell morphology studies showed EMT-like changes in

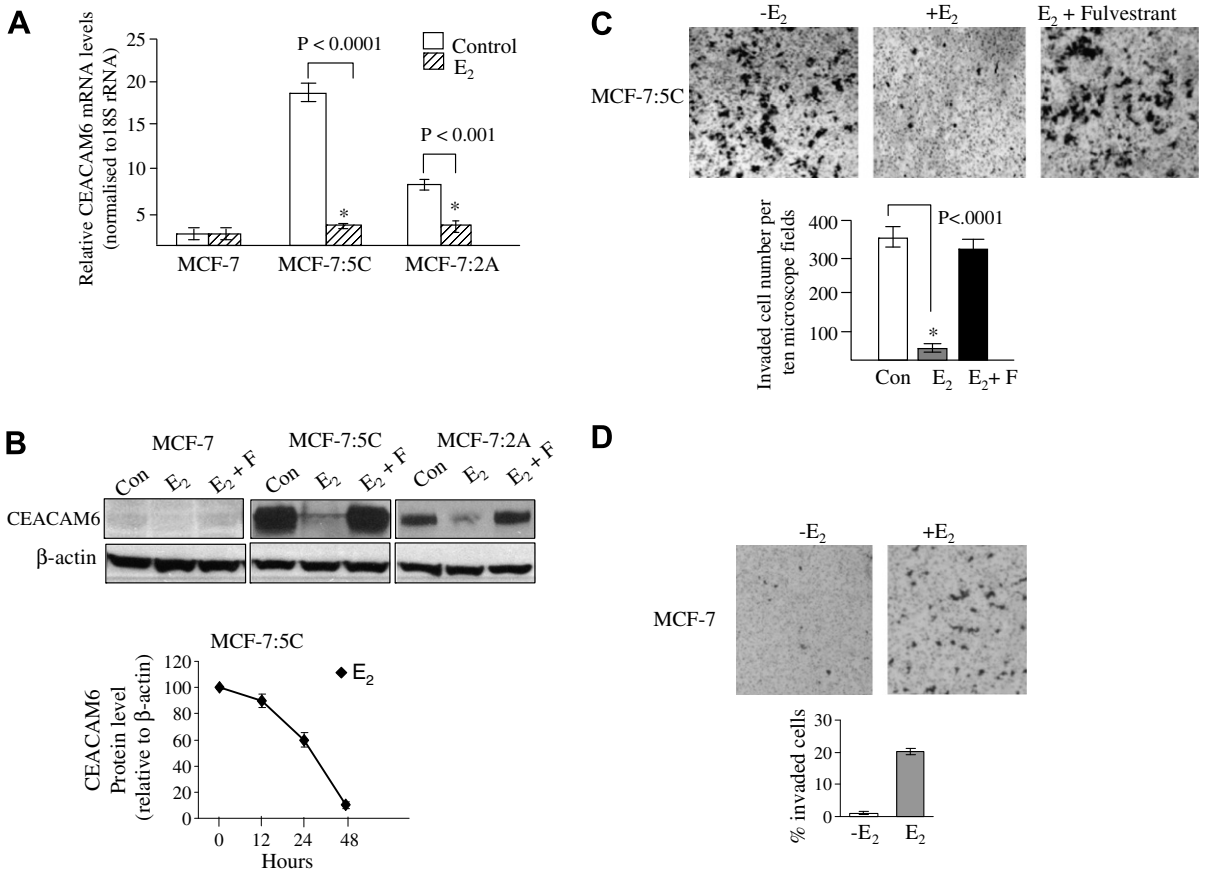

Fig. 5 – 17β-Oestradiol suppresses CEACAM6 expression and blocks invasion of oestrogen-deprived breast cancer cells. (A) Quantitative RT-PCR analyses of CEACAM6 mRNA expression in MCF-7:5C and MCF-7:2A cells following treatment with 1 nM oestradiol (E_2) for 48 h. Expression levels were internally normalised to the housekeeping gene 18S rRNA (error bars, SE) (B) Western blot analysis of CEACAM6 protein expression in MCF-7, MCF-7:2A and MCF-7:5C cells. *Line graph* shows the time-dependent effect of E_2 on CEACAM6 protein level in MCF-7:5C cells. (C) Invasion of MCF-7:5C cells is blocked by E_2 but not the pure anti-oestrogen fulvestrant. Invasion assay was performed as previously described in Fig. 3. (D) Effect of oestradiol on the invasiveness of wild-type MCF-7 cells. Each panel represents an example of three replicates.

MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells. A variety of signal transduction pathways impinge on the regulation of E-cadherin levels or subcellular distribution. In particular, Akt/PKB has been shown to repress transcription of the E-cadherin gene, which leads to conversion of epithelial cells into invasive mesenchymal cells.[40] We have found that MCF-7:5C and MCF-7:2A both cells overexpress phosphorylated Akt, and gene ontology analysis of expression data obtained for MCF-7:5C and MCF-7:2A cells reveals that the P13K/Akt signalling pathway is significantly ($p = 0.002$) altered compared to parental MCF-7 cells.

In conclusion, we have identified CEACAM6 as a critical gene in the regulation of migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation. Since aromatase inhibitors are now considered the standard of care for the hormonal treatment of early breast cancer in postmenopausal women, this finding has important clinical implications for these patients because it suggests that extended use of aromatase inhibitors may potentially lead to the development of metastatic disease. CEACAM6 can thus serve as a powerful predictor of future recurrence and may also represent a promising new therapeutic target for breast cancer.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.05.016.

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