Identification of MAGEA antigens as causal players in the development of tamoxifen-resistant breast cancer

P-P Wong1,6, CC Yeoh1,6, AS Ahmad2, C Chelala3, C Gillett4, V Speirs5, JL Jones1 and HC Hurst1

The antiestrogen tamoxifen is a well-tolerated, effective treatment for estrogen receptor-α-positive (ER+) breast cancer, but development of resistance eventually limits its use. Here we show that expression of MAGEA2, and related members of this cancer-testis antigen family, is upregulated in tamoxifen-resistant tumor cells. Expression of MAGEA2 in tumors grown in vitro or as xenografts led to continued proliferation in the presence of tamoxifen. At the molecular level, we demonstrate that MAGEA2 protein localizes to the nucleus and forms complexes with p53 and ERα, resulting in repression of the p53 pathway but increased ER-dependent signaling. In a series of ER+, tamoxifen-treated breast cancer patients, we show a highly significant ($P = 0.006$) association between MAGEA (melanoma-associated antigen) expression and reduced overall survival, confirming the clinical significance of our observations.

INTRODUCTION

The use of the selective estrogen receptor modulator, tamoxifen, to treat hormone receptor-positive early breast cancer has had significant positive impact on the risk of recurrence and on long-term patient survival rates.1 Tumor expressions of estrogen receptor-α (ER) and progesterone receptor are currently the best predictors of response to antiestrogen therapy. However, all patients with metastatic disease and 30–40% of early disease patients will relapse while on tamoxifen treatment owing to de novo or acquired drug resistance.2

In an attempt to circumvent resistance, additional selective estrogen receptor modulators and the total estrogen antagonist, fulvestrant, plus a series of aromatase inhibitors (AIs), which act to block the synthesis of estrogens in postmenopausal women, have been developed. However, these newer compounds also eventually elicit tumor resistance.3,4 Several large clinical trials have compared tamoxifen treatment with newer endocrine therapies, and, in particular, the use of AIs as first-line treatment has been reported to show improvements in disease-free survival in postmenopausal women (reviewed in Hughes-Davies et al.5). However, meta-analyses have suggested that significant improvements in overall survival (OS) may only be obtained by using switching or sequencing protocols whereby patients receive tamoxifen for 2–3 years before moving to an AI.6 This suggests that, far from being surpassed by newer therapies, tamoxifen remains a vital drug in the treatment of breast cancer, and therefore studies to further biological understanding of tamoxifen resistance prolong its clinical effectiveness remain highly relevant.

In tumor cells resistant to tamoxifen, ER expression is usually retained and continues to regulate breast cancer cell proliferation and survival through cross-talk with other signaling pathways, leading to ligand-independent ER activation (reviewed in Ali and Coombes3 and Musgrove and Sutherland4). Here we have examined gene expression profiles in tamoxifen-resistant (TR) breast tumor-derived lines and report the identification and functional validation of MAGEA2 as a novel TR-associated gene. MAGEA2 is a member of the class I melanoma-associated antigen (MAGEA) family of cancer-testis antigens encoded by 12 highly homologous genes located on the X chromosome. They are defined by their lack of expression in somatic adult tissues, but frequent upregulation in a range of solid tumors.7 These proteins are also highly immunogenic, and their ability to induce spontaneous cytotoxic T-lymphocyte-dependent immune responses in cancer patients particularly marks them out as useful targets for immunotherapy.8 Cancer vaccine phase II trials based on recombinant MAGEA3 antigen have shown promise for the treatment of lung cancer and melanoma,9,10 and this has led to increased interest in the expression profile and activity of these proteins in other tumor types.

MAGE proteins are characterized by a ~170-amino-acid MAGE homology domain, which forms a tandem winged-helix structure that can act as a scaffold for protein–protein interactions.7,11 Their precise biological role is still emerging; however, they have been reported to interact with key cellular proteins, notably p53. In a study examining MAGEA2 expression in the acquisition of resistance to the chemotherapy drug, etoposide, MAGEA2 was shown to complex directly with p53 and recruit the histone deacetylase, HDAC3, to repress p53 transcriptional activity, thereby protecting cells from apoptosis.2 In a separate study, a number of class I MAGE antigens (from the A, B and C subfamilies) were found to interact indirectly with p53 via another scaffolding protein, the transcriptional corepressor, KAP1 (also termed TRIM28; TIF14), again resulting in apoptosis suppression.12 In multiple myeloma, the interaction of MAGEA proteins with p53 was shown to inhibit apoptosis through repression of Bax and...
stabilization of survivin. All of these studies have suggested that tumor expression of MAGE antigens is linked to treatment failure, prompting us to determine if overexpression of MAGEA2 can contribute to TR in breast tumors.

RESULTS

MAGEA2 expression in TR breast tumor lines

The ER+ human breast tumor lines T47D and ZR75-1 were maintained in media containing 10^{-7} M tamoxifen until they re-entered the cell cycle and could be expanded. At this point, they were considered as separate, TR lines. In addition, estrogen-deprived (mimicking resistance to an AI), TR (ODTR) lines were also established (see Materials and methods). Gene expression profiles for these derived lines were compared with wild-type (WT) cells using Affymetrix arrays, and the data were analyzed to identify up- or downregulated genes common to the derived lines.

Several known ER target genes, including PGR, GREB1 and PDZK1, were among the downregulated genes (Figure 1a), but additional genes with no previous link to ER signaling were also significantly regulated. Of these, MAGEA2 showed a consistent fourfold upregulation on the arrays, which was validated by quantitative real-time polymerase chain reaction analysis. On immunoblotting a panel of TR lines and their WT, tamoxifen-sensitive counterparts, WT ER+ cells showed little or no MAGEA2 protein expression, but all had significant induction in their TR derivatives (Figure 1b, lanes 1–14). ER- lines are inherently TR, but most of these lines assayed were also negative for MAGEA2 expression, even when cultured in the presence of tamoxifen (exemplified by MDA-MB-453 cells; Figure 1b, lanes 17 and 18); however, the SKBR3 line did express elevated levels of MAGEA2, which were maintained when the cells were grown in tamoxifen media (Figure 1b, lanes 15 ad 16). As MAGEA genes are often coinduced in tumor cells, we also probed blots with a ‘pan-MAGE’ antibody able to recognize several members of the MAGE family. As illustrated for the T47DTR line (Figure 1c), upregulation of additional MAGEA-related proteins (including MAGEA10, A3 and A1) was observed in MCF-7 and ZR75-1TR lines (data not shown).

Figure 1. MAGEA2 is upregulated in TR breast cancer cell lines. (a) Heat map of gene expression analysis of TR derivatives of the human breast tumor lines ZR75-1 and T47D. The most significantly up- (red) and downregulated (green) genes compared with WT, sensitive (TS) lines are shown (false discovery rate-corrected P-value < 0.05) after unsupervised hierarchical clustering. Information includes Affymetrix probe ID, fold change, cytoband location, gene symbols and SWISSPROT keywords; data for MAGEA2 highlighted in blue color. (b) Whole-cell extracts (20 μg) from WT and TR breast cell lines were western blotted for MAGEA2 expression, as indicated. For T47D and ZR75-1 lines, estrogen-deprived (OD and ODTR) sublines were also available. Blots were reprobed for Hsc70 (loading control). (c) T47D (WT and TR) cell lysates (20 μg) were blotted using the pan-MAGE antibody 6C1. Identity of bands marked was inferred from size (A10 and A1) or comparison with lysates from cells expressing exogenous genes (A2 and A3).
As induction of MAGEA2 overexpression was a characteristic of all of our resistant cell lines, we tested if overexpression of this gene alone could have a functional role in resistance to tamoxifen. Stable MAGEA2-overexpressing lines were generated in WT, tamoxifen-sensitive MCF-7 and T47D breast tumor cells. Several individual colonies were expanded and checked for MAGEA2 expression (see Figure 2a and Supplementary Figure S1A); vector alone (VA)-transfected, non-expressing control lines were also established. Figure 2b illustrates changes in cell number for two individual MAGEA2-expressing MCF-7 derived lines when cultured with tamoxifen, compared with WT and VA control cells. There was a highly significant ($P < 0.01$) difference between growth of the MAGEA2-expressing lines, which continued to proliferate in tamoxifen-containing media, and the controls, which underwent growth arrest. Similar results were also obtained when T47D-derived lines were analyzed: the MAGEA2-expressing clones were again able to sustain their growth in tamoxifen-containing media ($P < 0.001$; Supplementary Figure S1B). In addition, we generated MAGEA3-expressing lines, as this family member was also found to be upregulated in several TR lines, and these also continued to proliferate in the presence of tamoxifen ($P < 0.001$; Supplementary Figure S2). Similar growth assays were used to compare the proliferation capacity of control and MAGEA2-expressing cells over a range of tamoxifen concentrations or in the presence of the total estrogen antagonist, fulvestrant. Expression of MAGEA2 allowed cells to continue to proliferate in $10^{-6}$ M tamoxifen ($P < 0.01$; Figure 2c), the highest dose tested; however, neither these cells nor the controls grew in media.

Figure 2. MAGEA2 overexpression is functionally linked to TR. (a) Western blot analysis of lysates (20 μg) from MCF-7-stable clones (C18 and C24) expressing exogenous MAGEA2, plus the VA control line, was probed for MAGEA2, p53, acetylated p53 and p21cip, as indicated. Blots were reprobed for Hsc70 (loading control). (b) MCF-7 (WT and VA) and clones C18 and C24 were split into six-well plates (5 × 10^5 cells per well) and treated with $10^{-7}$M tamoxifen (TAM) 24 h later for 8 days. Triplicate wells were harvested and counted daily. (c) MCF-7 VA and C24 lines were split into 12-well plates (5 × 10^4 cells per well) and treated with a range of tamoxifen concentrations, as indicated, over 8 days. For statistical analysis, data from all the C24 samples were averaged and compared with the averaged VA data. (d) MCF-7 control (WT and VA) and clones C18 and C24 were plated in triplicate on six-well plates (10^5 cells per well), grown with or without $10^{-7}$M tamoxifen for 144 h, and then assayed for Annexin V binding and PI uptake. The graph shows the percentage of early apoptotic (AnnV−PI−) cells for each line and the condition (see Supplementary Figure S4A for full analysis). (a–d) Error bars indicate the s.e. Student’s t-test was used to compare data from the indicated sample with VA control; *$P < 0.05$, **$P < 0.01$. (e) Western blot analysis of lysates (20 μg) from T47DTR cells transiently transfected with siRNA targeting MAGEA2 or non-silencing control (NSC) for 48 h probed for the proteins indicated. (f) T47DTR cells were transfected with NSC or siRNA targeting MAGEA2. After 48 h, transfected cells and WT T47D were split into 24-well plates (3 × 10^4 cells per well in triplicate) and treated with $10^{-6}$M tamoxifen 24 h later for 5 days. Error bars indicate the s.e. Student’s t-test was used to compare data from NSC- and siMAGEA2-transfected cells; *$P < 0.05$. 
containing fulvestrant, even at $10^{-8}$ M, the lowest dose tested (Supplementary Figure S3).

Proliferation assays in normal media suggested that MAGEA2 expression did not confer an inherent proliferation advantage (data not shown). We therefore examined if MAGEA2 expression can protect cells from apoptosis, particularly in the presence of tamoxifen, which has been found to induce both cytostasis and programmed cell death in ER+ cells. MCF-7-derived control (WT) and two independent MAGEA2-expressing lines were grown in the presence and in the absence of tamoxifen-containing media for 6 days and then assayed for surface expression of Annexin V (AnnV), a recognized hallmark of early apoptotic cells. The samples were stained additionally with propidium iodide (PI) to differentiate between intact cells (AnnV−PI−), early apoptotic (AnnV+PI−), late apoptotic/necrotic cells (AnnV+PI+, using fluorescence-activated cell sorting analysis (full cell cycle profile; Supplementary Figure S4a).

When the percentage of apoptosing cells in each population was examined (Figure 2d), both MAGEA2-expressing clones showed a significantly ($P < 0.01$) lower proportion of dead and dying cells in the presence of tamoxifen compared with the controls. Again, similar results were also found for the T47D-derived MAGEA2-expressing clones (Supplementary Figure S4B).

As a further functional test, we transiently transfected one of the derived TR cell lines with control or MAGEA2-targeted small interfering RNA (siRNA), and then monitored growth in tamoxifen-containing media over 5 days. Silencing MAGEA2 in T47DTR cells occurred with reasonable efficiency (Figure 2e) and these showed a significantly ($P < 0.05$) reduced ability to proliferate in $10^{-6}$ M tamoxifen, compared with cells transfected with control siRNA (Figure 2f). In our gene expression profiling of the T47DTR line, nearly 2000 genes were identified as differentially regulated compared with WT cells. Therefore, our finding that growth of these cells in tamoxifen is compromised by silencing MAGEA2 alone strongly implicates it as a causal gene in the TR phenotype.

MAGEA2 regulates the transcriptional activity of p53 and ER

Published data have suggested that, in chemoresistant cells, MAGEA proteins can interact with, and thereby inhibit the activity of, p53. We therefore used co-immunoprecipitation (CoIP) to examine if MAGEA2 can complex with p53 in breast tumor cells, and whether this impacts on p53 signaling, initially analyzing a T47DTR-derived line (C30 cells) expressing exogenous MAGEA2. MAGEA2 was detected in p53 complexes in C30 cells but not in control cells (Supplementary Figure S5A, upper left panel). Similarly, p53 was found in MAGEA immunoprecipitates (Supplementary Figure S5A, middle lower panel), thus confirming formation of a complex between p53 and exogenous MAGEA2. Additional western blots demonstrated equal expression of total and VA-α expressing cells after tamoxifen treatment (Figure 4a, lanes 5–8), whereas silencing MAGEA2 in T47DTR cells had the reverse effect, reducing total ERα load (Figure 2e).

As acetylation of p53 is a hallmark of transcriptional activity, to complex with p53 and repress its activity, MAGEA2 must either directly interact with p53 or cause the relocalization of p53. Control and MAGEA2-expressing MCF-7 cells were grown with and without tamoxifen and subfractionated to isolate cytoplasmic and nuclear extracts for analysis by western blotting. As illustrated in Figure 4a, MAGEA2 protein was detected chiefly in the cytoplasm of overexpressing cells grown in normal media, but was increased in the nucleus in cells exposed to tamoxifen. This correlated with loss of acetylated p53, whereas the levels of total p53 remained constant. Similar observations concerning MAGEA2 subcellular localization were also made in fractionated T47DTR cells (Supplementary Figure S5B) and on MCF-7 C24 cells using confocal microscopy (Supplementary Figure S5C).

Taken together, these data suggest that in tamoxifen-treated cells MAGEA2 is increased and localizes to the nucleus, which in turn prevents p53-dependent cell cycle arrest. Another protein known to be increased by tamoxifen treatment is ERα itself (Laios et al., and this effect was also observed in our VA control cells; Figure 4a, first 4 lanes). Total ERα and its active form, phospho-ERα (serine 118) levels were even further enhanced, however, in the MAGEA2-overexpressing lines, particularly upon tamoxifen treatment (Figure 4a, lanes 5–8), whereas silencing MAGEA2 in T47DTR cells had the reverse effect, reducing total ERα load (Figure 2e). Furthermore, our data also indicate that phospho-p42/44 mitogen-activated protein kinase (MAPK) activity is enhanced in MAGEA2-overexpressing cells after tamoxifen treatment (Figure 4a, lanes 5–8). As MAPK also regulates ERα transcriptional activity, these observations together suggest strongly that MAGEA2 can also impact on ER-mediated pathways. To determine whether MAGEA2 affects ERα transcriptional activity, we silenced MAGEA2 by transfecting cells with its expression vector in the presence of tamoxifen; the silenced MAGEA2-overexpressing clones were pre-treated with estrogen-free media for 48 h, and then transiently transfected with anERE-dependent luciferase reporter construct in the presence or in the absence of β-estradiol (E2) or tamoxifen. Compared with observations in VA cells, overexpression of MAGEA2 significantly enhanced the transcriptional activity of ERα in the presence of tamoxifen alone and reversed the suppression of E2-mediated activation in cells treated with both reagents (Figure 4b). Further CoIP assays were then performed to investigate if MAGEA2 and ERα are able to form a complex in these cells. As illustrated in Figure 4c, ERα was found in MAGEA2 immunoprecipitates and MAGEA2 was found in ERα precipitates, confirming that these two proteins can associate, in particular, with tamoxifen-treated cells. Again, this
interaction was verified using CoIP with endogenous proteins expressed in the T47DTR line (see Supplementary Figure S6).

MAGEA2-expressing xenografts are resistant to tamoxifen treatment
We next tested the ability of MAGEA2 to confer resistance to tamoxifen in an in vivo model of breast cancer. MAGEA2-expressing MCF-7 C24 and VA control cells were injected subcutaneously into opposite flanks of ovariectomized nude mice in the presence or in the absence of E2 pellets. In the presence of E2, both control and MAGEA2-overexpressing cells established tumors that grew at a similar rate (Figure 5a). In contrast, in the absence of E2 supplementation, VA control cells were unable to form tumors, whereas MAGEA2-expressing cells formed small tumors (Figure 5b).

To evaluate the effect of antiestrogens on xenograft growth, we implanted the mice with tamoxifen pellets after establishing
E2-induced tumors. In agreement with the cell culture assays, the growth of MAGEA2-overexpressing tumors was not inhibited by tamoxifen, whereas the control tumors regressed upon tamoxifen treatment ($P < 0.05$; Figure 5a). One cohort of mice was implanted with tamoxifen pellets alone. Surprisingly, tamoxifen supplementation accelerated growth of MAGEA2-overexpressing xenografts threefold relative to the same cells grown without tamoxifen pellets ($P < 0.01$; Figure 5b). This response may reflect the increased MAGEA2 nuclear expression observed in tamoxifen-treated cells noted above (Figure 4a) coupled with activation of ER-dependent proliferation and survival pathways.

**Figure 4.** MAGEA2 interacts with the ER. (a) MCF-7-derived MAGEA2-expressing (C18 and C24) and VA lines were incubated with and without $10^{-7}$ M tamoxifen (TAM) for 8 days. Nuclear (N) and cytoplasmic (C) extracts (20 μg) were analyzed by western blotting for the indicated antigens. Blotting for lamin A/C and actin confirmed extract integrity. (b) MCF-7-VA and C18/C24 cells were transiently transfected with an ERE-luciferase reporter construct (100 ng) and left untreated or given $10^{-8}$ M E2 and/or $10^{-6}$ M TAM, as indicated, 24 h later. Luciferase values were corrected for transfection efficiency and expressed as fold activity compared with untreated cells (set at 1) using data from three independent experiments, each performed in triplicate; error bars indicate the s.e. Student’s t-test was used to compare data from the indicated samples, *$P < 0.05$; **$P < 0.01$. (c) WCE lysates from MCF-7VA and C18/C24 cells treated as in (a) were immunoprecipitated (IP) for ER (middle panels) or MAGEA2 (right panels), and then blots were probed for ER (top row) or MAGEA2 (middle row), as indicated. Control blots (left panels) carrying 5% of the protein used for the IP were additionally probed for Hsc70 as a loading control.

Immunohistochemical analysis confirmed the persistence of MAGEA2 overexpression in the C24 xenografts (Figure 5c). Overall, our data demonstrate that overexpression of MAGEA2 lowers the E2 requirement for MCF-7 cells to grow as xenografts and renders the tumors insensitive to antiestrogen therapy.

MAGEA expression is associated with reduced OS in patients with ER+ primary breast cancer treated with tamoxifen.

To determine if these observations have clinical significance, pan-MAGEA immunostaining was assessed on sections from...
formalin-fixed, paraffin-embedded material from 144 cases of ER+ primary breast carcinoma collected from three separate centers (see Materials and methods). All the patients had been treated with adjuvant tamoxifen alone until relapse. Tumors positive for MAGEA (35%) all showed moderate or strong intensity staining with both nuclear and cytoplasmic distribution (Figure 6a). As noted previously,23 there was significant intertumor variation as to which compartment predominated. Positive MAGEA staining was associated with a highly significant reduction in OS (P = 0.006) as shown in Figure 6b, where a marked separation in survival curves is evident after 5 years of follow-up. This therefore suggests a link between MAGEA expression in the primary tumor and clinical resistance to tamoxifen therapy, consistent with our in vitro and xenograft data.

**DISCUSSION**

Although initially identified over 20 years ago, the functional role and mode of action of MAGE antigens is still highly controversial. Here we demonstrate, for the first time, the pleiotropic effects of MAGEA2 antigen on ER+ breast tumor cells, leading to their continued proliferation and survival in the presence of physiological levels of the therapeutic antihormone, tamoxifen.

In TR ER+ breast cancer cells, and lines engineered to overexpress MAGEA2, we were able to detect a complex between MAGEA2 protein and p53, which was consistent with the observed inhibition of the p53 pathway in these cells. Previous studies have also noted a functional interplay between p53 and several MAGEA antigens including MAGEA2; however, the nature of this interaction at the molecular level is disputed. Two studies suggested that MAGEA and p53 proteins do not interact directly but via binding to different regions of RING (Really Interesting New Gene) domain-containing proteins, particularly in the transcriptional coressor, KAP1 (also termed TRIM28, TIF-1β). The association with KAP1 has...
been suggested variously to recruit histone deacetylases to p53, to lead to p53 ubiquitination and degradation. In contrast, others have used p53-derived peptides or bacterially made proteins to map a direct interaction between MAGEA antigens and the DNA-binding surface of p53. Using published reagents, we have not been able to detect an interaction between MAGEA2 and KAP1 or histone deacetylases in the breast tumor lines studied here (unpublished data), nor did we see any reduction in either WT or mutant p53 levels in a range of MAGEA2-expressing breast lines; however, we did observe a clear diminution in the expression of p53 acetylation both in MAGEA2-overexpressing cells and in lines selected for TR (Figures 2a, e and 4a and Supplementary Figures S1A and 5A), which was completely consistent with the reduced expression of the cell cycle inhibitor, p21cip/CDKN1A, and the continued growth in tamoxifen exhibited by these lines. Our demonstration that MAGEA2 represses the transcriptional activity of p53 (Figures 3b and d) agrees with work by Meek and co-workers who linked MAGEA binding to the p53 DNA-binding domain to a lack of p53 interaction at its endogenous target genes, including p21cip/CDKN1A, as monitored using chromatin immunoprecipitation. As found here, p53 stability was not altered by coexpression with MAGEA antigens. This suggests therefore that MAGEA2 expression in resistant cells reduces the ability of tamoxifen-induced p53 to bind to its target genes, resulting in less efficient G1/S arrest and apoptosis and continued proliferation in the presence of the antihormone, as observed using cell culture (Figures 2b and d) and xenografts (Figure 5b). Reduced p53 association with chromatin will also limit its access to histone acetyl transferases, thus accounting for the lower levels of acetylated p53 in our TR- and MAGEA2-expressing cells. Consistent with this proposed molecular interplay between MAGEA2 and the p53 pathway, we have also confirmed that MAGEA2 protein is increased in the nucleus, and, in particular, in tamoxifen-treated cells (Figure 4a).

Although MAGEA2-expressing cells were found to be resistant to a range of tamoxifen concentrations (Figure 2c), they were not cross-resistant to the total estrogen antagonist, fulvestrant. As fulvestrant also acts as a selective estrogen receptor degrader, stimulating cellular turnover of ERα, this demonstrates that intact ER signaling is also essential for MAGEA2 activity in TR breast cancer cells. Using reporter assays, we show that ERα transcriptional activity is no longer inhibited in tamoxifen in MAGEA2-expressing lines (Figure 4b); instead, tamoxifen acts as an ER agonist in these cells. This change in cellular response to tamoxifen is a hallmark of resistant breast cancer and results from alterations in the activity, abundance or stoichiometric balance of ER coexpressors and coactivators in TR cells (reviewed in McDonnell and Wardell and Green and Carroll). Significantly, we were able to show that MAGEA2 and ERα can form complexes in tamoxifen-treated MAGEA2-expressing lines and TR cells (Figure 4c and Supplementary Figure S6). Given the fact that the tamoxifen treatment induces nuclear expression of MAGEA2 (Figure 4a), it is tempting to speculate that the interaction of ERα with MAGEA2 is predominantly in the nucleus, as it is not observed in non-tamoxifen-treated cells (Figure 4c). At the same time, the interaction of ERα with MAGEA2 could be either direct or could involve ER cofactors. Given that our data show an increase in MAPK activity, which correlates with increased ER phosphorylation (Figure 4a), this pathway may act to aid downstream ERE promoter activity via MAGEA2 (Figure 4b).

We examined MAGEA expression in a series of ER+ breast cancers with an antibody validated for use on formalin-fixed, paraffin-embedded material, which detects several members of the MAGE family. In our panel of derived resistant lines, although MAGEA2 was the most consistently overexpressed, we also readily detected the expression of MAGEA3 and A10 and to a lesser extent MAGEA1 (Figure 1c). Furthermore, silencing MAGEA2 alone in these cells led to a significant but incomplete reversal of the TR phenotype (Figure 2f). We have also shown that MAGEA3 can cooperate with MAGEA2 in the transcriptional repression of p53 (Figures 3b and c), and lines selected to overexpress MAGEA3 were also resistant to tamoxifen (Supplementary Figure S2). Taken together, these observations suggest that several members of this highly homologous family can functionally contribute to TR, in agreement with the overlap in MAGEA activity reported in other studies.

Analysis of our tumor study revealed a highly significant association (P = 0.006) between positive MAGEA staining and reduced OS (Figure 6b). Previous studies have shown that a low percentage of ER-positive breast cancers are MAGEA positive. However, these studies were restricted to cancers that were not TR. In contrast, although all the patients in our study were treated with tamoxifen only, 66% of them were TR substantiating our results. This strongly implies a link between expression of members of the MAGEA family and failure of tamoxifen therapy. When logistic regression was used to impute the missing values (see Supplementary Methods) as an exploratory analysis, both grade (P = 0.001) and MAGEA expression (P = 0.006) could be shown to be significant covariates with an increased hazard ratio of 2.027 (95% confidence interval: 1.228–3.345) for MAGEA expression, implying that patients with MAGEA-positive primary tumors were two times as likely to progress. Relating these findings to the clinic suggests that MAGEA-positive cases are unlikely to respond to tamoxifen; indeed, from our observations on MAGEA2-expressing xenografts (Figure 5b), treatment may even accelerate tumor growth. However, patients with MAGEA-negative tumors could be treated and then monitored for MAGEA upregulation as a marker of TR onset. In this regard, it is significant that the reliable detection of MAGEA3 expression in circulating breast tumor cells has recently been described. Patients with de novo, or acquired MAGEA expression could be switched to treatment with an AI; however, we show here that a selective estrogen receptor degrader such as fulvestrant may be more effective (Supplementary Figure S3). Unfortunately, the poor bioavailability of fulvestrant has meant that its effectiveness in vitro has, to date, not been replicated in the clinic (reviewed McDonnell and Wardell). As an alternative, current MAGEA immunotherapy regimens, given either as cotherapy with tamoxifen or at the onset of MAGEA expression, may be a viable approach to treating hormone-resistant breast cancer.

MATERIALS AND METHODS

Cell lines

HepG2, H1299 and all breast tumor-derived lines (apart from H3396) were obtained from the ATCC (Manassas, VA, USA) and grown according to their recommendations. H3396 cells were cultured in RPMI plus 10% fetal calf serum. TR sublines were derived by continuous culture in media supplemented with 10−7M 4-hydroxy tamoxifen (Sigma, Dorset, UK) for 3–6 months until the cells could be expanded. Estrogen-deprived (OD) lines were maintained in low estrogen conditions for 6 months as described previously. Using phenol red-free media supplemented with charcoal-stripped serum (Sigma), and ODTR versions were derived by subsequent culture in tamoxifen (10−9M). The following lines have been authenticated (May 2011) by STR profiling (LGIC Standards): MCF-7 (WT, TR, C18, C24, VA), T47D (WT, TR) and H3396 (WT, TR). All proliferation assays were repeated at least two times with the repeats showing similar results; representative experiments are presented. Media were supplemented with vehicle, tamoxifen or fulvestrant (Sigma) where indicated. Proliferation and apoptosis assays were carried out as detailed previously.

Plasmids, siRNA and transfection conditions

The insert from a MAGEA2 cDNA clone (IMAGE Consortium, clone ID: 8327628) was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Plasmid DNA (with and without MAGEA2 insert) was introduced into T47D and MCF-7 cells using nucleofection (Amaxa, Basel, Switzerland). Stable clones were selected in media containing 500 μg/ml G418, expanded and
characterized for MAGEA2 expression by western blot and quantitative polymerase chain reaction analyses. The p21cip/p21DNP4 promoter luciferase reporter vector and pCMV p53 expression plasmid were generous gifts from Prof Dennis McCance (Queen’s University, Belfast, UK). The p53 reporter PGL3PV Lucifer construct was purchased from Addgene (Cambridge, MA, USA) and the pGL3-ERE-luciferase vector was kindly provided by Professors Simak Ali (Imperial College London, UK). For reporter assays, cells were transfected using GeneJuice (Merck Bioscience, Darmstadt, Germany) and efficiency was monitored using the pRL-TK Renilla control vector (Promega, Madison, WI, USA). All samples in a series were transfected with an equal amount of DNA with an appropriate amount of pCDNA3.1 vector added as a mock control. Each sample was harvested at 48 h and assayed by Dual Luciferase assays following the manufacturer’s protocol (Promega). For siRNA transfection, subconfluent T47DTR cells were transiently transfected using INTERFERIN (Polyplus, Illkirch-Graffenstaden, France) with 40 ng of non-silencing control siRNA (Qiagen, Valencia, CA, USA; no.1022076) or On Target Plus Smartpool MAGEA2 targeting siRNA (Dharmacon, Lafayette, CO, USA; no. 00635001).

Gene expression profiling and data analysis
RNA was extracted from log-phase T47DTR, ZR75-1, OHT47DTR and ODRZ75-1TR cells using TRIZOL reagent (Invitrogen) and purified over an RNAeasy mini columns (Qiagen), including the DNAse I digestion step. Total RNA (5 μg) was prepared for hybridization to Affymetrix GeneChip Human Genome U133 Plus 2.0 oligonucleotide arrays following the manufacturer’s recommendations. The data can be viewed at http://www.ncbi.nlm.nih.gov/geo using accession number GSE22664. Details regarding data handling and analysis of differential gene expression compared with WT lines are given in the Supplementary information.

Western blotting
Cells were washed with phosphate-buffered saline and scraped into chilled lysis buffer (8% urea, 1% thioeur, 0.5% CHAPS (3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), 50 μM dithiothreitol and 24 μM spermine). Lysates were assayed for protein using Bradford reagent (Pierce, Rockford, IL, USA), resolved by denaturing sodium dodecyl sulfate—polyacrylamide gel electrophoresis, and then electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Proteins were detected with antibodies to: pan-MAGEA (clone 6C1; Santa Cruz, Dallas, TX, USA; sc-20034), MAGEA2 (sc-130164), MAGEA3 (sc-130809), p53 (DO1, sc-126), acetylated(Ac)-p53 (DO1), 4 g anti-p53 (DO1), 4 g anti-pan-MAGEA, 4 g anti-MAGEA2, 4 g anti-MAGEA3, 4 g anti-p53 (DO1), 4 g anti-p53 (DO1), 4 μg anti-MAGEA4, 4 μg anti-MAGEA5, 4 μg anti-MAGEA6, 4 μg anti-ERα or control immunoglobulin G (Santa Cruz; sc-2027) overnight at 4 °C. The next day, immunoprecipitates were washed three times with the wash buffer (Invitrogen) for 5 min at 4 °C, and resuspended in 45 μl of 2 × sodium dodecyl sulfate—polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride membranes and protein detection was achieved by western blotting.

CoIP assays
Immunoprecipitation was carried out using the Immunoprecipitation Kit (Invitrogen) according to the manufacturer’s protocol. Briefly, cells were lysed in buffer (20 μg Tris-Cl (pH 7.6), 170 μM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP40, 1 mM dithiothreitol) supplemented with 5 μM trichostatin A and protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Cells lysate (1 mg) was incubated with 50 μl of Dynabeads (Invitrogen) and either 2 μg anti-p53 (DO1), 4 μg anti-pan-MAGEA, 4 μg anti-ERα or control immunoglobulin G (Santa Cruz; sc-2027) overnight at 4 °C. The next day, immunoprecipitates were washed three times with the wash buffer (Invitrogen) for 5 min at 4 °C, and resuspended in 45 μl of 2 × sodium dodecyl sulfate—polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes and protein detection was achieved by western blotting.

Xenograft studies
Control (VA) or MAGEA2-expressing (C24) MCF-7 cells were suspended in matrigel and injected subcutaneously (5 × 106 cells per mouse) in opposite flanks of 6-8-week-old ovariectomized athymic mice (Harlan, Oxfordshire, UK). Subcutaneous implantation with E2 pellets (17E, 0.25 mg, 60-day release; Innovative Research, Novi, MI, USA; 20 mice) or tamoxifen pellets (25 mg, 21-day release pellet, 10 mice) occurred a day before cell inoculation. A further 10 mice were not implanted with any pellets. After 3 weeks, 10 of the estrogen-treated mice were additionally implanted with tamoxifen pellets. Tamoxifen pellets were replaced by reinseion on day 21. Tumor size was measured in three dimensions in millimeters weekly and tumor volumes were calculated according to the formula: volume = π/6 × (length × width × height) and expressed in mm3. For histological analysis, dissected tumors were fixed overnight in formaldehyde, sectioned and stained with the pan-MAGE antibody C61.

Clinical material
Paraffin sections from formalin-fixed primary tumors from 144 patients with a confirmed diagnosis of ER+ breast cancer were analyzed from three separate cohorts as follows: 74 cases from the EDRTC 10850/1 trials,13,14 at Guys and St Thomas’ Hospital (London, UK); 32 cases identified from clinical records (by CCY and JLJ) of patients from Bart’s and the London Hospital (BLT); 38 cases representing a subset of ER+ cases from a series collected at St James’ University Hospital (Leeds, UK).15 All patients were operated on for their primary tumor and received adjuvant tamoxifen treatment for an average of 62 months (BLT and Leeds cases; Guys and St Thomas’ Hospital cases received tamoxifen for life) or until relapse when patients were switched to a range of chemotherapeutic regimens. Clinicopathological details are given in Supplementary Table S1.

Immunohistochemistry
For the BLT cases, a tissue microarray (TMA) was constructed from routinely fixed, paraffin-embedded tumor blocks taking 3 × 0.5 mm2 cores selected from representative tumor areas. A TMA for the Leeds cases was already available,15 the Guys and St Thomas’ Hospital cases were examined on whole sections. Sections from MCF-7 WT and C24 cell pellets embedded in paraffin were used as negative and positive controls, respectively. Sections were dewaxed and rehydrated using standard methods and antigen retrieval was performed by pressure cooking with antigen unmasking solution (Vector Labs, Peterborough, UK; H-3300). The pan-MAGEA mouse monoclonal antibody 6C1 was applied at 1:100 dilution for 40 min at room temperature. Slides were then processed using a DAKO autostainer and Visualis (Vector Labs) and DAB (3,3′-diaminobenzidine tetrahydrochloride) as chromagen and then counterstained. MAGEA staining was scored by two pathologists. Staining was either homogeneously positive throughout the tumor, both cyttoplasmic and nuclear, or absent (see Figure 6a).

Statistical analysis
Survival curves were calculated by Kaplan–Meier method for the clinical study. Student’s t-test was used for growth curves. All P-values were two-sided; P < 0.05 was considered significant.

Research ethics
All studies in mice were performed under license and followed UK Home Office regulations. Access to patient samples for immunohistochemistry was via ethical approval granted by the East London and City Research Ethics Committee (LREC Ref: 06/Q0603/25; Guys and St Thomas’ Hospital samples) or (LREC Ref: 05/Q043/199; BLT samples) or from the Leeds (East) Local Research Ethics Committee (LREC Ref: 06/Q1206/180; Leeds samples).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We are grateful to our colleagues Andrew Clear, Keyur Trivedi and Abeer Shaaban for technical assistance with the TMA for the BLT and Leeds cases, respectively, Dr Yaohe Wang for help with scoring the Immunohistochemistry study, Ian Hart and Steven Robinson for invaluable advice and help with the xenograft study and to Richard Grose for useful comments on the manuscript. We thank Tracey Chapman for expert handling of the Affymetrix arrays and Simak Ali, Dennis McCance and Hinrich Gronemer for the generous provision of reagents. This work was funded by Cancer Research UK and Barts and the London Charity.

AUTHOR CONTRIBUTIONS
P-PW and CCY designed and performed the experiments. ASA and CC performed statistical analysis of survival data. CG, VS and JLJ provided patient...
tissue material for survival studies. JLI and CCY performed immunohistochemistry and tumor scoring of the patient samples. P-PW and HCH wrote the paper with substantial input from CCY.

REFERENCES

1. Loi S, Haibe-Kains B, Desmedt C, Wirapati P, Lallemand F, Tutt AM et al. Predicting prognosis using molecular profiling in estrogen receptor-positive breast cancer treated with tamoxifen. BMC Genom 2008; 9: 239.
2. Butt AJ, Sutherland RL, Musgrove EA. Live or let die: oestrogen regulation of breast cancer. Nat Rev Cancer 2002; 2: 101–115.
3. Hughes-Davies L, Caldas C, Wishart GC. Tamoxifen: the drug that came in from the cold. Br J Cancer 2009; 101: 875–878.
4. McDonnell DP, Wardell SE. The molecular mechanisms underlying the pharmacological actions of ER modulators: implications for new drug discovery in breast cancer. Curr Opin Pharmacol 2010; 10: 620–628.
5. Green KA, Carroll JS. Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. Nat Rev Cancer 2007; 7: 713–722.
6. Simson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 2003; 3: 615–626.
7. Loi S, Haibe-Kains B, Desmedt C, Wirapati P, Lallemand F, Tutt AM et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010; 28: 509–518.
8. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combination therapy. Eur J Cancer 2008; 44: 1997–2015.
9. Atanackovic D, Altorki NK, Cao Y, Ritter E, Ferrara CA, Ritter G et al. A new family of tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. Proc Natl Acad Sci USA 2005; 102: 11616–11621.
10. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T. A new cancer vaccine of T lymphocytes on a human melanoma. J Exp Med 1995; 182: 689–698.
11. Nardiello T, Jungbluth AA, Mei A, Diliberto M, Huang X, Dabrowski A et al. MAGE-A3 protein by high-resolution missense mutation analysis. Proc Natl Acad Sci USA 2010; 107: 10362–10370.
12. McDonnell DP, Wardell SE. The molecular mechanisms underlying the pharmacological actions of ER modulators: implications for new drug discovery in breast cancer. Curr Opin Pharmacol 2010; 10: 620–628.
13. Green KA, Carroll JS. Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. Nat Rev Cancer 2007; 7: 713–722.
14. Grigoriadis A, Caballero OL, Hoek KS, da Silva L, Chen YT, Shin SJ et al. CT-X antigen expression in human breast cancer. Proc Natl Acad Sci USA 2009; 106: 13493–13498.
15. Markou A, Strati A, Malamos N, Georgoulis V, Lianidou ES. Molecular characterization of circulating tumor cells in breast cancer by a liquid bead array hybridization assay, Clin Chem 2011; 57: 421–430.
16. Clarke N, Jimenez-Lara AM, Voltz E, Gronenbeyer H. Tumor suppressor IRF-1 mediates retinoid and interferon anticancer signaling to death ligand TRAIL. EMBO J 2004; 23: 3051–3060.
17. Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME et al. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 2003; 144: 1032–1044.
18. Williams CM, Scibetta AG, Friedrich JK, Canosa M, Berlato C, Moss CH et al. AP-2gamma promotes proliferation in breast tumour cells by direct repression of the CDKN1A gene. EMBO J 2009; 28: 3591–3601.
19. Maas RA, Bruning PF, top B, Breedijk AJ, Peterse HL. Growth arrest associated changes of mRNA levels in breast cancer cells measured by semi-quantitative RT-PCR: potential early indicators of treatment response. Cancer Lett 1995; 97: 107–116.
20. Brooks CL, Gu W. Ubiquitination phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003; 15: 164–171.
21. Lain S, Hollick JJ, Campbell J, Staples OD, Higgins M, Aoubala M et al. Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. Cancer Cell 2008; 13: 454–463.
22. Liaois I, Joume F, Laurent G, Nonclercq D, Toillon RA, Seo HS et al. Mechanisms governing the accumulation of estrogen receptor alpha in MCF-7 breast cancer cells treated with hydroxymacrofen and related antiestrogens. J Steroid Biochem Mol Biol 2003; 87: 207–222.
23. Chen YT, Ross D, Chiu R, Zhou XK, Chen YY, Lee P et al. Multiple cancer/testis antigens are preferentially expressed in hormone-receptor negative and high-grade breast cancers. PLoS One 2011; 6: e17876.
24. Marcar L, Maclaine NJ, Hupp TR, Meek DW. Mage-A/cancer/testis antigens inhibit p53 function by blocking its interaction with chromatin. Cancer Res 2010; 70: 10362–10370.
25. Daouvis S, White R, Parker MG. The antiestrogen ICI 182780 disrupts estrogen receptor nucleo-cytoplasmic shuttling. J Cell Sci 1993; 106: 1377–1388.
26. Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME et al. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 2003; 144: 1032–1044.
27. Williams CM, Scibetta AG, Friedrich JK, Canosa M, Berlato C, Moss CH et al. AP-2gamma promotes proliferation in breast tumour cells by direct repression of the CDKN1A gene. EMBO J 2009; 28: 3591–3601.
28. Fentiman IS, Christiaens MR, Paridaens R, Van Geel A, Rutgers E, Berner J et al. Treatment of operable breast cancer in the elderly: a randomised clinical trial EORTC 10851 comparing tamoxifen alone with modified radical mastectomy. Eur J Cancer 2003; 39: 309–316.
29. Fentiman IS, van Zijl J, Karydas I, Chaudary MA, Margreeter R, Legrand C et al. Treatment of operable breast cancer in the elderly: a randomised clinical trial EORTC 10850 comparing modified radical mastectomy with tumorectomy plus tamoxifen. Eur J Cancer 2003; 39: 300–308.
30. Maraga L, Cummings M, Peter MB, Shaaban AM, Horgan K, Hanby AM et al. Carcinoembryonic antigen cell adhesion molecule 6 predicts breast cancer recurrence following adjuvant tamoxifen. Clin Cancer Res 2008; 14: 405–411.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)