Loss of tumorigenicity of stably ERβ-transfected MCF-7 breast cancer cells

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Abstract

Proliferation of breast cancer cells is mediated by estrogen receptors (ER) - ERα and ERβ. At present, contradictory observations complicate the understanding of involvement of ERβ in breast cancer and functional definition of ERβ as a prognostic marker.

A stable expression of full length ERβ was established in the ERα-positive MCF-7 breast carcinoma cell line to evaluate the role for ERβ in maintenance of cell viability and estrogenic response, as well as proliferation, morphology and cell cycle progression. In order to verify *in vivo* tumourigenicity of ERβ transfectants were transplanted into nude mice.

Transfection of ERβ in MCF-7 resulted in a marginal increase of gelsolin protein expression. Constitutive expression of ERβ resulted in a significant 30% inhibition of cellular growth compared with transfection of the mock vector alone (p=0.043). This reduction in growth was associated a retardation of transition into S-phase of the cell cycle. The *in vitro* response to 17β-estradiol was reversed in cells over-expressing ERβ (p=0.016). However, no difference in response to the antiestrogens tamoxifen and ICI 182,780 were observed in the presence of ERβ. Importantly, over-expression of ERβ prevented establishment and growth of tumours as subcutaneous xenografts in immunodeficient mice *in vivo*.

These observations support the notion that ERβ is a tumour suppressor and be exploitable in terms of cancer prevention, improving therapeutic response or predicting disease progression.

Introduction

Estrogens are potent mitogens and are essential for normal development of the mammary gland. As a consequence of their mitogenic activity estrogens are also involved in tumourigenesis of the breast. Estrogenic response is mediated via binding and activation of the estrogen receptor, a ligand-inducible transcription factor belonging to the steroid hormone receptor superfamily (Diel et al., 2002). Two forms of estrogen receptor (ER) have been identified, ERα in 1986 and ERβ in 1996 (Green et al., 1986; Kuiper et al., 1996). These ERs are encoded on different chromosomes (Saunders et al., 2002) but share a high degree of primary structure similarity. The main structural differences between ERα and ERβ are found in the N-terminal domain and ligand-binding domain, suggesting binding to similar estrogen response elements but different ligand preferences (Kuiper et al., 1996). These differences suggest that the two receptors could serve distinct actions.

ERα and ERβ can form biologically functional heterodimers in tissues in which they are co-expressed (Jarvinen et al., 2000). However, both receptors are distinct in cellular distribution, regulate different sets of genes and can act in opposition to regulate activity of several target genes (Palmieri et al., 2002). In addition, ERβ lacks activation function-1 (AF-1) which in ERα is responsible for permitting ERα ligand-independent activity and eliciting the agonistic activity of tamoxifen (Watanabe et al., 1997). Unlike ERα expression, which is an efficient indicator of responsiveness to hormonal therapy in clinical breast cancer, the role or significance of ERβ in breast cancer still remains unclear (Gustafsson, 2000).

Since its identification, ERβ has been reported to co-express with ERα, to be both a positive and negative prognostic marker and both repress and induce malignant transformation in the breast (Speirs et al., 2004). The observed down-regulation of ERβ during carcinogenesis and progression (Roger et al., 2001) and response of ERβ-positive breast cancers to antihormonal therapy (Mann et al., 2001) would indicate that ERβ acts as a tumour suppressor and is a marker for a good prognosis (Jarvinen et al., 2000). In support of this hypothesis, ERβ expression in patient tumour samples is associated with negative axillary node status, low-grade tumours, reduced DNA synthesis and a greater disease-free survival (Jarvinen et al., 2000; Omoto et al., 2001). Similarly, immunohistochemical localisation of ERβ has been shown to correlate to both progesterone receptor levels and increased cellular differentiation in breast cancer, strongly supporting ERβ as a valuable independent indicator of favourable prognosis (Skliris et al., 2001; Nakopoulou et al., 2004).

Conversely, ERβ-positive tumours have been demonstrated to exhibit increased expression of cyclin A and the Ki-67 proliferation-related marker, be recurrently negative for progesterone receptor and associate with a poorer prognosis compared to tumours expressing ERα alone (Dotzlaw et al., 1999; Jensen et al., 2001; Speirs et al., 1999). In addition, the observation that ERβ mRNA levels were significantly elevated in tamoxifen-resistant tumour samples questioned the suggestion that ERβ could be used as a marker for endocrine therapy, although this was only based on a small study of 17 patients (Speirs et al., 1999). One explanation for the discrepancy in use of ERβ as a reliable prognostic marker was given by Gustafsson and Warner (2000) who suggested that prognosis may rely on expression and levels of ERβ variants. This is supported by the identification of several distinct ERβ variants and the observation that one such variant, the C-terminal truncated form ERβcx can act as a dominant negative regulator of ERα function (Ogawa et al., 1998).

In order to identify functional and mechanistic action of ERβ in proliferation and tumourigenesis we created ERβ-over-expressing MCF-7 breast carcinoma cells and assessed *in vitro* growth characteristics, response to (anti)estrogens and expression of cell cycle proteins. Further on, we investigated the effect of ERβ upon *in vivo* tumour establishment and growth. In contrast to other reports, we used a GFP-encoding expression vector, lipofection and antibiotic selection for establishment of stably transfected MCF-7 cells. Several studies used different cloning vectors and cells lines, including mammary, ovarian and prostate cell lines, as well as variant transfection methods to manipulate ERβ levels in cells *in vitro* (Bardin et al., 2004; Cheng et al., 2004; Lazennec et al., 2001; Paruthiyil et al., 2004; Ström et al., 2004; Tonetti et al., 2003). Only one study reported the engraftment of ERβ-transfected cells *in vivo* (Paruthiyil et al., 2004). As shown in the present report and in the majority of published studies, ERβ was associated with reduced cell growth, reduced invasiveness and motility or induction of apoptosis.

Material and methods

Cell culture and chemicals

The human breast carcinoma cell line MCF-7 was obtained from the tumour bank of the NCI (Bethesda, MD) and cultivated in RMPI 1640 media with 10 % foetal bovine serum (FBS). 7 days prior to experiments cells were maintained in phenol-red free RPMI containing 10 % dextran-coated charcoal (DCC)-stripped FBS and 2 mM glutamine (Life Technologies, Karlsruhe, Germany). 17β-estradiol (E2) and tamoxifen (tam) were purchased from Sigma (Deisenhofen, Germany) and the antiestrogen ICI 182,780 from Tocris (Bristol, UK).

Stable Transfection

For stable transfection of ERβ, full-length cDNA of the human ERβ gene (a gift of Organon, Oss, Netherlands) was cloned into pEGFP-N1 (Clontech, Heidelberg, Germany) to create pEGFP-ERβ. Lipid mediated transfection of pEGFP-ERβ into MCF-7 cells was performed using FuGene 6 (Roche, Mannheim, Germany) following the supplier's protocol. Cells were seeded into 6-well plates with 10^4 per well. A control cell line was established by transfecting an empty expression plasmid (MCF-7/GFP). The selection of positive clones was carried out with 800 µg/ml Geneticin (Life Technologies). After 3 weeks several clones of MCF-7 cells expressing stably ERβ were maintained. All these clones differed from wild-type cells regarding their growth rate and response to E2, but this study focussed on only one clone. Expression of both ERα and ERβ in transfected and control cell lines was monitored by RT-PCR and Western Blot. To monitor cell size and growth as well as the translocation of the ERβ-GFP-fusion protein an inverted contrasting microscope (DM IL, Leica, Wetzlar, Germany) equipped with a digital camera (DFC320, Leica) and fluorescence device was used.

MTT assay

Cell survival was determined by MTT assay. MCF-7, MCF-7/GFP and MCF-7/ERβ were treated with the indicated concentrations of either 17β-estradiol, tamoxifen or ICI-182,780 for 96 hours. Cells were seeded and assessed by standard MTT methods (Tantivejkul et al., 2003). Concentrations of drugs inhibiting cell growth by 50 % (IC50) were calculated.

RT-PCR

The total mRNA of 10^6 cells or 30 mg tumour-tissue was isolated using a RNA isolation Kit-System (Qiagen, Hilden, Germany). The RT-PCR was carried out using the one step EZrTth-system and related reagents from Applied Biosystems (Foster City, USA). Primers used to amplify ERα were 5'-TGGAGTCTGGTCCTGTGAGGG-CTG-3' (pos. 951-974) and 5'-GAAGAGGGTGCCAGGCTTTGTGGA-3' (pos. 1443-1496, EMBL Acc.-No. X03635), giving an amplified product of 545 bp. For ERβ primer sequences were 5'-AGTGCCGCTCTTGGAGAGCTG-3' (pos. 840-860) and 5'-CCTGGGTCGCTG-TGACCAGA-3' (pos. 1095-1114, EMBL Acc.-No. X99101), giving a PCR product of 274 bp. For detection of gelsolin RNA (NM_000177) the following primers were used: 5'-GTGAGACCCCACTGTTCAAGCA-3' (forward, pos. 1198 - 1219) and 5'-CTGTC GCCTCCATAGA ACTGTC-3' (reverse, pos. 1444 - 1465), giving a fragment size of 268 bp. Finally, the reference gene hydroxymethylbilane synthase (HMBS, NM_000190) was monitored using the following primer pairs: 5'-GGCTGCAACGGCGGAA-3' (forward, pos. 178 – 193) and 5'-CCTGTGGTGGACATAGCAATGATT-3' (reverse, pos. 313 – 336) giving a PCR product of 159 bp.

The final reaction volume of 50 µl contained 1x EZ-buffer, 300 µM dNTPs, 5 units rTth, 2.5 mM $MN(OAc)_2$, 250 ng RNA and 0.25 µM primer. The reverse transcription reaction was performed at 62°C for 30 min subsequently followed by PCR-amplification involving a denaturation step at 94°C for 2:00 min. The remaining PCR was carried out as following: 94°C for 1 min, 60°C for 0:30 min and annealing at 72°C for 0:30 min (10 cycles). Then 92°C for 1 min, 60°C for 0:30 min and 72°C for 0:30 min (25 cycles) and a final elongation at 72°C for 10 min. PCR products were analysed by electrophoresis through a 2 % agarose gel and visualised by ethidium bromide staining under UV illumination.

Quantitative real-time PCR (TaqMan)

Relative RNA quantification was performed by using standard protocols and pre-mixed PCR reagents (TaqMan; Applied Biosystems, Weiterstadt, Germany). Total RNA was extracted from approximately 10^6 cells following suppliers protocol (RNeasy; QIAGEN, Hilden, Germany) and transcribed into cDNA using TaqMan Reverse Transcription reagents. The cDNA was used for the fluorescent quantitative PCR assay and pre-developed probe/primer mixes were designed by the Assay-on-Demand service at Applied Biosystems (Table 1). Hydroxymethylbilane synthase (HMBS) was used as housekeeping gene. RT and PCR reactions were carried out as described before (Becker et al., 2005).

Gene expression was calculated using the comparative delta-delta-Ct method (Livka et al., 2001). Briefly, each sample was assayed for the number of PCR cycles required to cross the threshold of the linear range of the reaction (C_t). The amplification efficiency of target gene and reference gene were approximately equal. The three C_t values for each sample were averaged, whereas the difference between C_t values was below 1. Samples were normalized for the total template amount by subtracting the average *HMBS* C_t from the average *ERα/ERβ/Gelsolin* C_t (ΔC_t). The normalized gene expression (ΔC_t) of the control (MCF-7/wt) was then subtracted from the normalized expression of ERβ- and GFP-transfected cells ($\Delta\Delta C_t$). The relative gene expression of ERα/ERβ/gelsolin of transfectants in relation to control cells was calculated: $2^{-(\Delta\Delta Ct)}$.

Western Blot and Antibodies

Cells were harvested from sub-confluent flasks using RIPA buffer (containing 1 mM dithiothreitol, 1 mM sodium fluoride and a protease inhibitor cocktail (Sigma)) as previously described (Jensen et al., 2003). Snap frozen tissue pieces of approximately 30 mg were lysed in the same buffer using an Ultra-Turrax dispersing tool. In brief, after centrifugation at 13,000 x g for 10 min at 4°C and collection of the supernatant, aliquots of 25 µg of total protein were separated through 10 % SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were probed with following anti-human antibodies: ERα and cathepsin D (Santa Cruz Biotechnology, Santa Cruz, CA); Erb-B2 (DPC Biermann, Bad Nauheim, Germany); ERβ (gift from Akzo Nobel, Oss, The Netherlands); gelsolin (BD Biosciences, Heidelberg, Germany); $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cyclin D1, GFP (Cell Signaling, Beverly, MA); cyclin A, β-Actin (Sigma); Ki-67 (Dako, Glostrup, Denmark) and Cdk2 (Dianova, Hamburg, Germany).

FACS

For flow cytometric analysis, MCF-7 cells were harvested by trypsinisation from sub-confluent culture flasks into cold PBS, fixed in ice cold 70 % ethanol and stored at – 20°C. Cells were washed, treated with 100 µg/ml RNAse A and stained with 50 µg/ml Propidium iodide for 30 min at 37°C. Analysis of DNA content was performed using a Becton Dickinson FACScan (BD, Heidelberg, Germany) with a minimum of 10,000 events collected for analysis with BD Cellquest Software.

Immunofluorescence

Cells were grown for 48 hours in chambered coverslides (Nunc, Wiesbaden, Germany), fixed with 4 % paraformaldehyde in PBS and blocked with normal rabbit or goat serum. Incubation with the primary

antibodies G-/F-actin, α-tubulin (Acris, Hiddenhausen, Germany) and vimentin (Abcam, Cambridge, UK) was carried out for one hour at ambient temperature. Primary antibody binding was detected using an anti-rabbit-Cy3 antibody (Abcam) and anti-goat labelled Texas-Red antibody (Vector Laboratories, UK). Nucleus staining was performed using Hoechst-33258 (Sigma). Coverglasses were mounted in Vectorshield (Vector Laboratories, UK) and analysed by confocal laser scanning microscopy (Zeiss, Jena, Germany).

Animal experiments

Wild-type, ERβ- or GFP-transfected MCF-7 cells (10^7 cells/mouse) were transplanted subcutaneously into the flank of female NCR:*nu/nu* mice (Charles River Laboratories, Frederick, USA) without Matrigel. Mice were held in laminar flow shelves in germ-poor conditions at 22°C, 50 – 60 % relative humidity and a 12 hrs light-dark rhythm. They received autoclaved food and bedding (Sniff, Soest, Germany) and acidified drinking water *ad libitum.*

Animals were treated with 0.5 mg/kg estradiol (*i.m.*) immediately after transplantation once a week during the experiment to stimulate the growth of MCF-7 tumours and experiment was performed twice. Within a third animal experiment the 3 cell lines were transplanted as described, but E2 supplementation was omitted. Body weight and tumour diameters were measured twice weekly. Tumour volumes were calculated according to equation ($width^2$ length)/2. All animal experiments were performed with permission of the responsible local authorities (G 0221/03).

Densitometric and statistical analysis

Intensity of mRNA and protein bands of RT-PCR and Western blot assays was determined using the Histogram tool of Adobe Photoshop (Adobe Systems, San Jose, CA). The mRNA and protein expression of the molecule of interest was calculated in relation to β-Actin mRNA and protein levels.

Statistical differences ($p<0.05$) were calculated using the Mann-Whitney/U-Test within the software STATISTICA (Statsoft, Hamburg, Germany).

Results

Stable expression of ERβ in MCF-7 cells

Since the majority of clinical breast tumours are positive for both ERα and ERβ (Watanabe et al., 1997) we chose to use MCF-7 for our investigations, in order to mimic the clinical scenario. MCF-7 cells used in these experiments are ERα-positive and ERβ-negative. Cells were stably transfected with human ERβ which was N-terminally tagged with green fluorescent protein (GFP). Following antibiotic selection, positive cells could be identified by the presence of green fluorescence localised predominantly in the cytoplasm of these cells (Fig. 1a). In order to confirm the functionality of the ERβ-GFP protein 10^{-9} M E2 was added to MCF-7/ERβ cells maintained in a chamber slide. 3 hours after E2 treatment a translocation of the GFP signal into the nucleus was observed (Fig. 1c). In Fig. 1b and 1d the corresponding light microscopic photographs are shown. In mock-transfected MCF-7 cells GFP expression was spread throughout the whole cell and treatment with E2 did not induce a translocation of the GFP protein (data not shown). Several clones were identified and tested for ERβ expression and functionality to exclude effects caused by clonal selection or transfection procedure (data not shown). A clone expressing high levels of transfected ERβ was chosen for further studies, termed MCF-7/ERβ. As a control, MCF-7 was transfected with the "empty" expression vector and termed MCF-7/GFP.

Relative expression of ERα and ERβ protein and mRNA

The expression of both ERα and ERβ mRNA was monitored by conventional endpoint RT-PCR (35 cycles) and real-time-PCR. Visualisation of RT-PCR products by ethidium bromide staining was performed to demonstrate the presence and absence of ERα and ERβ mRNA: While ERα mRNA was found in wild type and transfected cells, ERβ mRNA was only detected in ERβ-transfected MCF-7 cells (Fig. 2). Using real-time PCR and $\Delta\Delta C_T$ calculation the change in copy number of both ERs normalised to an internal reference (HMBS) and related to non-transfected MCF-7 cells was determined. Table 2 summarises the results of real-time-PCR. Three independent PCR runs showed that the relative gene expression of ERβ is about 282- and 442-fold higher in transfected cells compared to non-transfected MCF-7/wt cells. The amount of ERα mRNA marginally varies between transfected and wild type cells (Tab. 2).

ERα and ERβ proteins were detected by Western Blot and semi-quantified in relation to β-Actin levels (Fig. 2). Wild-type (non-transfected) and MCF-7/GFP were solely positive for ERα, the relative protein expression (RPE) was 0.43 and 0.32, respectively. Transfection of ERβ cDNA did not affect protein level of ERα in these cells (MCF-7/ERβ: $RPE_{ER\alpha}$ = 0.37). MCF-7/ERβ expressed both receptor proteins (Fig. 2c and 2d, Lane 4), while control cells were ERβ-negative. Due to distinct properties of the antibodies used for ERα and ERβ detection a direct comparison of RPE is not appropriate. The expression of GFP protein was found to be equal in ERβ- and empty-vector-transfected cells (Fig. 2e). Continued expression of ERα and ERβ was routinely verified throughout the complete study.

ERβ is transcriptionally active in MCF-7/ERβ cells

In order to confirm the functionality of ERβ expressed in MCF-7/ERβ cells we assessed their ability to modulate protein expression of the well known estrogen-related gene cathepsin D. MCF-7/wt and MCF-7/GFP cells showed an obvious cathepsin D level increased by treatment with 10^{-9} M 17β-estradiol for 6 hours (Fig. 3a). Cathepsin D protein was significantly induced in response to ERβ transfection (Fig. 3a), in a similar manner to the ERα dependency previously reported (Xing et al., 1998). The relative cathepsin D protein expression normalised to β-Actin was calculated out of 3 repeat experiments and a RPE value of 0.97 was detected in MCF-7/ERβ, while wild-type cells and GFP transfectants showed a RPE of 0.45 and 0.39, respectively (Fig. 3b). However, cathepsin D level in ERβ-transfectants was not additionally elevated after treatment with E2 (Fig. 3a and Fig. 3b).

Alteration in the gelsolin protein level of MCF-7 by ERβ expression

During daily maintenance of the cells a marginal change of cellular appearance of ERβ-transfected MCF-7 cells was observed. But neither FACS-based analysis of cell size nor immunohistological investigation of the cytoskeleton (G/F-actin, α-tubulin and vimentin) evidenced significant differences between transfectants and control cells (data not shown).

Gelsolin is known to be an actin-binding protein with dysregulated expression in several human tumour types (Kothakota et al., 1997). Analysis of protein (Fig. 4a) and mRNA (Table 3) expression of gelsolin in the cells demonstrated an ERβ-associated increase in protein but not mRNA levels. Calculation of relative protein (Fig. 4b) imply normalisation of sample band intensity to β-Actin. We found an observable increase of gelsolin protein in MCF-7/ERβ cells, that was not significant (p =

0.078). The relative gelsolin gene expression of ERβ- and mock-transfected cells was not modified in relation to wild-type MCF-7 (Table 3).

Inhibition of MCF-7 growth and modulation of cell cycle progression by ERβ expression

To investigate whether expression of ERβ influenced the growth rate and survival of MCF-7 cells, we assessed the growth rate of MCF-7, MCF-7/GFP and MCF-7/ERβ by MTT assay. As shown in Fig. 5, ERβ-expressing cells demonstrated a reduced growth rate compared to ERβ-negative cells. After 96 hrs, MCF-7 and MCF-7/GFP showed a 2.7- and 2.6-fold increase in cell number post seeding, respectively. In contrast, MCF-7/ERβ demonstrated only a 1.9-fold increase in cell number, representing a growth retardation of 30 % compared to MCF-7 and MCF-7/GFP (p=0.043).

By flow cytometric analyses, ERβ-expressing MCF-7 demonstrated no cell cycle phase arrest or increased level of cell death to account for the retardation of cellular growth compared to MCF-7/GFP. But entry of MCF-7/ERβ cells into S-phase of the cell cycle following serum-withdrawal mediated cell synchronisation was retarded compared to MCF-7 and MCF-7/GFP (Table 4). Both wild-type and MCF-7/GFP demonstrated maximum numbers of cells in S-phase at 18 hrs, compared to 24 hrs in MCF-7/ERβ (Table 4).

In order to explain the retarded growth and retarded S-phase entry of MCF-7/ERβ cells we analysed cell cycle proteins associated with proliferative status and S-phase progression by Western blot analyses, specifically cyclins A and D1, Cdk2, $p21^{Waf1}$, $p27^{Kip1}$ and Ki-67. Expression of ERβ resulted in an apparent but not significant decrease in expression of both cyclin A and Cdk2, both factors associated with S-phase entry and progression. This observation is consistent with the flow cytometric analyses and probably accounts for the observed retardation of S-phase transition (Fig. 6a). No change of either cyclin D1, $p21^{Waf1}$, $p27^{Kip1}$ or Ki-67 were observed in consequence to ERβ expression (Fig. 6a). We semi-quantified the intensity of Western Blot band by using the histogram tool of Adobe Photoshop and calculated a relative protein expression in relation to β-Actin expression (Fig. 6b). These calculations approved findings concluded from Western blot bands (Fig. 6a): cyclin A and Cdk-2 protein expression was slightly decreased in ERβ-transfected MCF-7 cells (not significant, p = 0.094), while levels of the other assayed cell cycle modulators were nearly unmodified.

Response to 17β-estradiol and the antiestrogens tamoxifen and ICI 182,780

Sensitivity of wild-type, GFP- and ERβ-transfected MCF-7 cells *in vitro* to 17β-estradiol, tamoxifen and ICI 182,780 was measured by MTT assay. For these experiments cells were maintained in steroid-depleted medium containing charcoal-stripped FBS. Antiestrogens tamoxifen and ICI 182,780 (Fulvestrant) were chosen because of their different mechanism of action, tamoxifen being an ER-modulator and ICI 182,780 an inhibitor of ER-protein (Piccart et al., 2003). Initially, experiments started with a multi-dose-response analysis (range of concentration: 10^{-4} to 10^{-11} M) (data not shown), than analysis focused on one concentration per compound. As expected, treatment of MCF-7 and MCF-7/GFP with 17β-estradiol for 96 hrs stimulated cellular proliferation resulting in a 1.53-fold and 1.42-fold increase in cell number compared to untreated cells, respectively (Table 5). In MCF-7/ERβ, treatment with 10^{-9} M 17β-estradiol did not raise proliferation rate, but cells were slightly growth inhibited in relation to untreated control. Moreover, comparison of increase in cell number of E2-treated ERβ-transfectants and E2-treated mock-transfectants giving a significant difference of cell survival of 35 % (p=0.016). However, no differential response to the antiestrogens tamoxifen or ICI 182,780 was observed between MCF-7/wt, MCF-7/GFP and MCF-7/ERβ (Table 5).

Expression of ERβ prevents establishment and growth of MCF-7 as xenografts in immunocompromised mice

In response to the obvious differences in growth of wild-type and ERβ-expressing MCF-7 *in vitro,* it was essential to examine the tumourigenicity and engraftment of these tumour cells *in vivo* in order to reflect *in vitro* findings. MCF-7, MCF-7/GFP and MCF-7/ERβ cells were transplanted into the flank of immunodeficient NCR:nu/nu mice. In three independent experiments, MCF-7 and MCF-7/GFP inoculation resulted in 29/30 and 30/30 tumours developing, respectively (Fig. 7). In contrast, only 1/36 tumours arose from inoculation with MCF-7/ERβ cells (Fig. 7). According to the E2-mediated growth inhibiting effect on MCF-7/ERβ observed in *in vitro* experiments, one of three transplantation experiments was performed without E2-supplementation (data not shown). However, deficiency of E2 had no influence on engraftment and tumour formation of MCF-7/ERβ cells. Wild-type and GFP transfectants engrafted without E2 stimulation, but tumour growth was delayed (data not shown). As a consequence of the loss of tumourigenicity resulting from ERβ expression, no studies could be undertaken to assess the response of these cells to estrogens or antiestrogens *in vivo*.

Discussion

Clinically, the presence of ERα is used as a diagnostic and prognostic marker in breast cancer (Speirs, 2002). In contrast, no such association has yet been proved for ERβ in breast cancer, mainly due to the large variation in reported levels of ERβ-positive tumours, ranging from 26 % to 92 % (Speirs et al., 2004). This broad range in values has been at least partially attributed to the different determination methods, expression threshold value and relatively small sample sizes. In clinical specimens of breast cancer ERβ has been shown to mostly co-express with ERα. It showed an inverse correlation to progesterone receptor expression, was associated with increased disease-free survival and low-grade well-differentiated tumours (Cullen et al., 2001; Dotzlaw et al., 1999; Knowlden et al., 2000; Omoto et al., 2001; Shaw et al., 2002; Skliris et al., 2001). Furthermore, malignant breast tumours have been shown to either lose ERβ expression or exhibit significantly lower protein expression levels compared to ERα (Roger et al., 2001; Saunders et al., 2002), implying ERβ as a tumour suppressor (Saji et al., 2005). Data of retrospective studies of ERβ protein expression in clinical samples, summarised by Murphy and Watson (2006), demonstrating its potential role as predictor of treatment response when co-expressed with ERα.

The breast carcinoma cell line MCF-7 used in this study expresses ERα but is devoid of ERβ protein expression. We have stably expressed ERβ in this cell line to further characterise the functional significance and role of ERβ in human breast cancer. The functionality of transfected ERβ was demonstrated by ERβ protein translocation from cytoplasm into nucleus and cathepsin D gene regulation. Over-expression of ERβ in MCF-7 cells was shown to alter cellular morphology, estrogenic response and cell cycle progression, specifically S-phase entry and transit *in vitro*. Importantly in terms of the clinical situation, we have shown that expression of ERβ prevented tumour establishment *in vivo*.

Expression of the actin-binding protein and cell motility factor gelsolin is decreased in several human neoplastic lesions, including breast, and associates with increased cellular motility and invasion (Afify et al., 1998; Asch et al., 1999; De Corte et al., 2002; Lee et al., 1999; Shieh et al., 1999). SiRNA mediated knockdown of gelsolin expression in the MCF-10A breast tumour cell lines was shown to induce an epithelial-mesenchymal transition (EMT), a process associated with

tumourigenesis (Tanaka et al., 2006). EMT is characterised by change of epithelial cells to a fibroblastic cell morphology, loss of contact inhibition, increase in actin filaments and enhanced motility and invasion *in vitro* (Tanaka et al., 2006). A slight morphological modification was also observed in our ERβ-transfectants. Because of its relevance for cellular architecture and tumor biology, we determined gelsolin expression in control and ERβ-transfected cells. Although gelsolin mRNA levels did not appear to differ, higher protein levels of gelsolin were observed in our ERβ-transfected MCF-7 cells compared to control. The discrepancy between mRNA and protein levels of gelsolin although unexpected had previously been reported in several other studies (Audic et al., 2004). The higher gelsolin protein levels in ERβ-transfected MCF-7 suggest an ERβ-associated induction of gelsolin. In a study utilising the MDA-MB-231 breast cell line, ERβ over-expression also was associated with a change in cellular morphology (Lazennec et al., 2001). The authors concluded that ERβ was inducing a differentiation-like process in MDA-MB-231, but did not looked at gelsolin expression in their cells. Possibly, our observations and the published hypotheses (Lazennec et al., 2001; Tanaka et al., 2006) support a link between gelsolin and ERβ expression.

In our study, ERβ was able to induce protein expression of the known estrogen-regulated gene cathepsin D in a ligand-independent manner indicating functional transcription machinery. Association of cathepsin D protein levels with hormone-dependent breast cancer and estrogen receptor expression has been described previously (Rochefort et al., 2000. The promoter of cathepsin D contains an estrogen response element (ERE) for transcriptional activation through an ERα-transcription-complex (Xing et al., 1998). Our results support the idea that cathepsin D is a shared genetic target between ERα and ERβ (Hyder et al., 1999).

The ERβ-mediated inhibition of *in vitro* and *in vivo* cell growth supports both the tumour suppressor function of ERβ and the use of ERβ expression as a good prognostic marker in human breast cancer (Balfe et al., 2004). Analyses of the cell cycle in MCF-7 ERβ-transfected cells identified that ERβ expression resulted in an increase in the time required for cells (synchronised at G_0/G_1 of the cell cycle) to transit into and enter S-phase. This delayed transit into S-phase was not due to either arrest at the G_1/S checkpoint or significant increase in cell death. A similar study by Paruthiyil et al. (2004) suggested that ERβ inhibited growth of estradiol treated MCF-7 via induction of a G_2 cell cycle arrest, an observation not noticed in our study. Although explanations for this discrepancy maybe the use of estradiol induced growth stimulation or the different ERβ expression levels, another feasible possibility is the presence or absence of cell cycle synchronisation prior to analysis. Our study utilised

cells synchronised at the same stage of the cell cycle prior to determination of cell cycle profiles. In contrast, it is not unreasonable to suggest that a G_2 arrest could be connected with an accumulation of cells in S-phase. Further studies are required to address the question of an ERβ induced G_2/M arrest rather than just a slow S-phase transit. In relation to cell cycle proteins, Paruthiyil et al. (2004) demonstrated repression of cyclin A and cyclin D1 and increase in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ expression in ERβ-positive MCF-7 cells. In our study we observed decrease in expression of cyclin A and Cdk2 protein but no significant change in expression of $p27^{Kip1}$, $p21^{Waf1/Cip1}$ or cyclin D1 protein in ERβ-expressing MCF-7. The discrepancies in observations in MCF-7 maybe due to the difference in transfection methods (viral versus non-viral). Furthermore, variances of MCF-7 populations and experimental conditions between our and published studies account probably for the effect, that in our MCF-7/wt cells cyclin D1 level is not increased within 6 hours after E2 treatment. Expression of ERβ in the ERα-positive T47D breast tumour cell line resulted in up-regulation of $p27^{Kip1}$ and down-regulation of cyclin E (Ström et al., 2004). In the ER-negative MDA-MB-231 breast cell line, ERα and ERβ expression induced TGFα and $p21^{Waf1}$ expression (Lazennec et al., 22001). ERβ expression in MDA-MB-231 decreased proliferation, cell invasion and motility in a ligand dependent manner. Unlike ERα, expression of ERβ did not regulate *c-myc* proto-oncogene expression in MDA-MB-231 cells (Lazennec et al., 2001). Taken together the observations in breast cancer cell lines of repressed expression of protein complexes involved in cell cycle (Yam et al., 2002) further support the tumour suppressor nature of ERβ and suggest functionality of ERβ involved with DNA replication rather than mitosis or cell division.

Exposure of MCF-7 cells to the estrogen 17β-estradiol has been shown to induce proliferation (Simard et al., 1997). Expression of ERβ in MCF-7 reversed in our studies this proliferative response resulting in a retardation of cell growth of about 40 % compared to wild-type and MCF-7/GFP cells. Although ERα and ERβ have been shown to form heterodimers when co-expressed in cells *in vitro* differences in their transcriptional functionality have been reported (Cowley et al., 1997): 17β-estradiol activated ERα binding to activation site-1 (AP-1) operates as a transcriptional activator whereas 17β-estradiol activated ERβ binding to AP-1 suppresses transactivation of target genes (Webb et al., 1995). An ERβ-triggered inhibition of ERα in the presence of 17β-estradiol has also been previously reported (Paech et al., 1997). Therefore, it is apparently the balance in levels between ERα and ERβ which dictates the overall outcome of 17β-estradiol treatment. As a consequence when ERβ levels exceed ERα levels the resulting outcome could be suppression of ER-mediated gene transcription and

subsequently growth inhibition. In contrast to E2, expression of ERβ did not influence response of MCF-7 to the antiestrogens tamoxifen or ICI 182,780. Unlike ERα, ERβ lacks a functional AF-1 domain which is required for estrogen-ligand independent activity and the agonistic effect of tamoxifen (Delaunay et al., 2000; Watanabe et al., 1997). Consequently, ERβ expression did not induce loss of sensitivity to tamoxifen. It remains to be seen whether expression of ERβ at higher levels may alter response to ERα-antiestrogen complexes via competition at the AP-1 binding site or sequestration of ERα.

In agreement with its proposed role as a tumour suppressor, ERβ expression inhibited establishment and growth of MCF-7 cells as tumour xenografts in immunodeficient mice in three independent experiments. A previous study which involved viral infection of ERβ into MCF-7 supported this finding, with a positive relationship between the virus-mediated multiplicity of infection of ERβ and loss of tumour formation capacity being noted (Paruthiyil et al., 2004). Although an explanation for this significant finding can only be hypothesised, reasons may include the effect of ERβ on cell cycle associated genes like $p21^{Waf1}$, $p27^{Kip1}$ and cyclin D1. In vitro, we could show an estradiol-mediated decrease in proliferation of ERβ-over-expressing MCF-7 cells, so that is supposable, that MCF-7/ERβ cells transplanted into nude mice, are growth inhibited due to the estrogenic microenvironment of the mouse. A recent study of Hartmann et al. (2006) showed anti-angiogenetic effects of ERβ during tumor engraftment of breast cancer cells. T47D cells transfected with ERβ and transplanted into mice exhibited a lowered intratumoral blood vessel number as wells as reduced VEGF expression (Hartmann et al., 2006). These results can also serve as reason for the ERβ-associated loss of tumorigenicity found in our study. But due to unavailable tumor material it is presently unfeasible to reproduce this finding.

In summary, our data provide strong evidence to support ERβ as a tumour suppressor in breast cancer via its growth inhibitory functions, induction of morphological change and ability to prevent *in vivo* tumour growth. Taken together in combination with the clinical situation demonstrating lower ERβ expression in advanced and aggressive breast tumours our results strongly suggest re-expression of ERβ as a potential therapeutic direction.

Expression of ERβ in breast tumours is known to be epigenetically silenced by both deacetylation of histone tails and gene methylation (Duong et al., 2005; Sasaki et al., 2002; Zhao et al., 2003). Recently, the use of histone deacetylase (HDAC) inhibitors has been suggested as one such approach to re-induce ERβ expression (Duong et al., 2005). Treatment of breast tumour cells *in*

vitro with HDAC inhibitors has been shown to both decrease expression of ERα and induce expression of ERβ in breast tumour cell lines 15 and sensitised them to the antiestrogen tamoxifen (Jang et al., 2004). Moreover, the ERβ gene promoter is methylated in approximately 80% of cancers but not normal breast tissue (Sasaki et al., 2002; Zhao et al., 2003) confirming ERβ gene silencing and reduced transcriptional control of ERβ. Similarly to the HDACs, hypermethylation of ERβ and its associated lack of transcriptional activity could be revoked by use of DNA methyltransferase inhibitors such as 5-aza-2-deoxycytidine (Yang et al., 2001).

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Abbreviations

EMT, epithelial-mesenchymal transition; E2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; GFP, green fluorescence protein; ICI, ICI 182,780; tam, tamoxifen; wt, wild-type

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Legends to figures

Figure 1 Expression of ERβ-GFP fusion protein in MCF-7 cells *in vitro* shown by fluorescence microscopy. Stable ERβ-transfected MCF-7 cells express an ERβ protein that is N-terminally flagged with GFP and localised in the cytoplasm of the cells when cultivated in steroid-depleted media (a). 6 hours after addition of 17β-estradiol GFP signal translocates into the nucleus (c).
Pictures were taken at day 33 after transfection using an inverted microscope from Leica (DM IL). (b) and (d) corresponding light micrographs. Magnification 200 x.

Figure 2 Expression of ERα and ERβ mRNA and protein. Both receptors were analysed in MCF-7/wt, MCF-7/GFP and MCF-7/ERβ cells by RT-PCR (a and b) and Western Blot (c and d), respectively. Detection of β-Actin was used as loading control in both assays. RT-PCR for detection of ERα (a) and ERβ (b) mRNA: Lane **1**, Molecular weight marker, Lane **2**, Negative control (H_2O); Lane **3**, Positive control (ERα/ERβ-cDNA); Lane **4**, MCF-7 wild-type; Lane **5**, MCF-7/ERβ; Lane **6**, MCF-7/GFP. Expression of ERα (c) ERβ (d) and GFP (e) determined by Western blotting. Lane **1**, recombinant ERα or ERβ protein (Panvera, MD); Lane **2**, negative control (RIPA buffer); Lane **3**, MCF-7 wild-type; Lane **4**, MCF-7/ERβ; Lane **5**, MCF-7/GFP.

Figure 3 Expression of cathepsin D protein in wild-type (wt), control (GFP) and ERβ-transfected MCF-7 cells. (a) Illustration of Western blot bands of mature cathepsin D and β-Actin. Total protein lysates were separated by SDS/Page and transferred onto nitrocellulose membranes. Incubation with specific antibodies against human cathepsin D was performed over night at 4°C. (b) Semi-quantitative calculation of relative protein expression (RPE) of cathepsin D normalised to β-Actin levels using the histogram tool of Adobe Photoshop. Cells were treated for 6 hours. Each experiment was performed 3-times independently.
** significant to corresponding MCF-7/wt*

Figure 4 Expression of gelsolin protein in wild-type, ERβ- and GFP-transfected MCF-7 cells. (a) Protein expression was monitored by Western blotting using an anti-human gelsolin antibody. (b) Band intensity was measured using histogram tool of Adobe Photoshop. Each signal was standardised to β-

Actin. Mean values of relative protein or gene expression (RPE) was calculated out of 3 experiments. Cells were treated with 17β-estradiol for 6 hours. ERb – MCF-7ERβ-transfectants, GFP – MCF-7/GFP-transfectants.

Figure 5 Effect of ERβ-transfection on *in vitro*-proliferation of MCF-7 cells. Increase of cell numbers was determined by MTT assay 1, 2, 3 and 4 days after seeding into a 96-well plate (5 x 10^3 cells/ well). ERβ-transfected cells showed a significantly slower rise in cell viability at day 4 than wild-type or GFP-transfected MCF-7 cells. Experiments were performed triply.

Figure 5 Western Blot analysis of cell cycle associated proteins. (a) Cyclins A and D1, Cdk2, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ as well as Ki-67 and β-Actin were detected in wild-type, GFP- and ERβ-transfected MCF-7 cells. 25 µg total protein of untreated or 10^{-9} M 17β-estradiol treated (6 hours) cells were used for these investigations. (b) Quantification of band intensity was carried out using Adobe Photoshop (Histogram tool). Each signal was normalised to β-Actin expression and calculated out of 2 independent experiments.

Figure 7 Effect of ERβ-transfection on tumourigenicity of wild-type, ERβ- and GFP-transfected MCF-7 cells. 10^7 cells of each MCF-7 line were transplanted *s.c.* into the flank of immunodeficient nude mice and engraftment of cells was either supplemented twice a week with 0.5 mg/kg/d 17β-estradiol (*i.m.*) beginning one day after inoculation or not stimulated with E2. The tumour volume was measured with a calliper-like instrument and calculated according to equation ($width^2$ length)/2. 8 animals were transplanted per cell line; experiments were repeated three times.

Figure 1

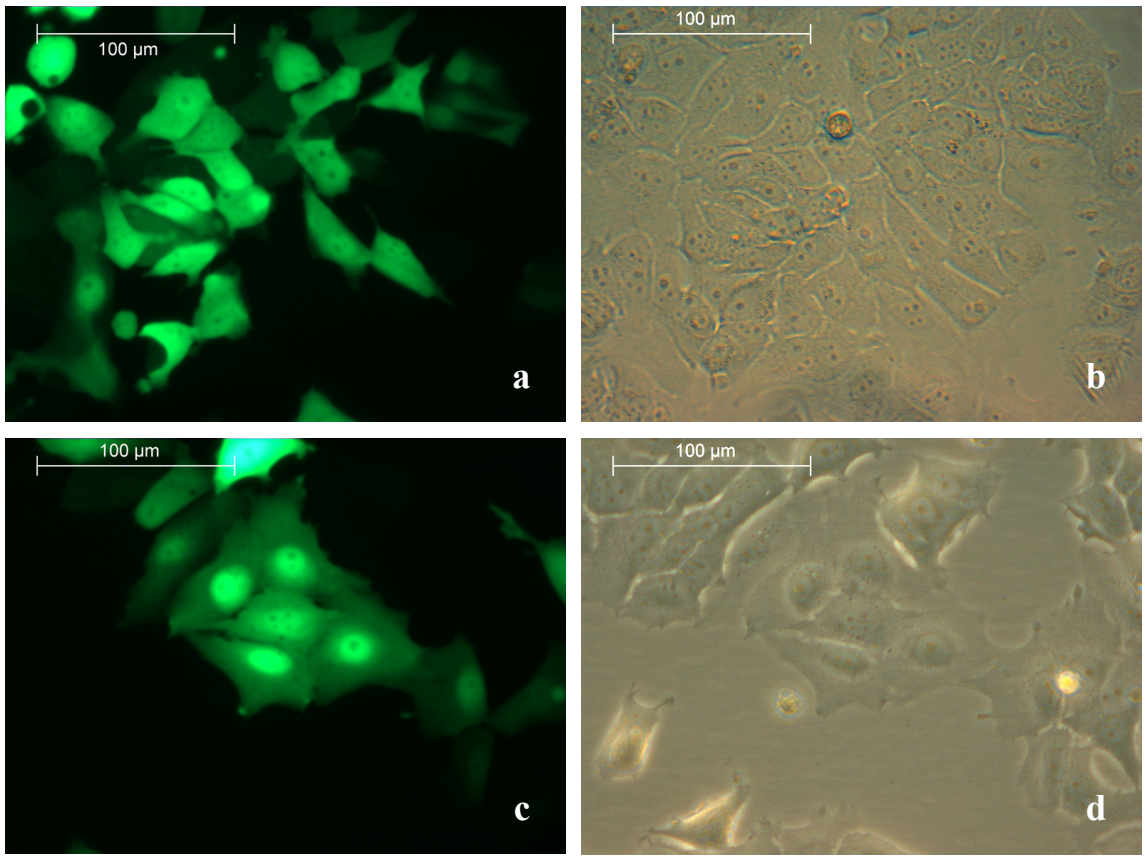

Figure 2

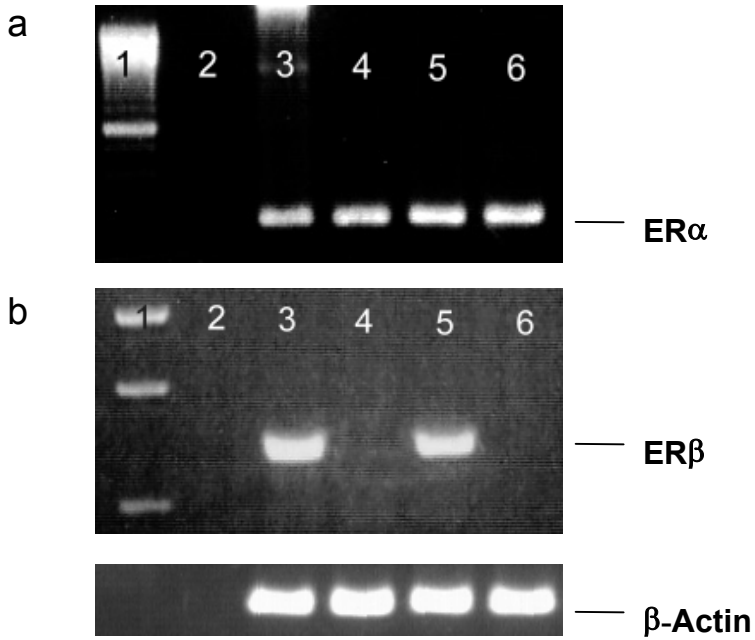
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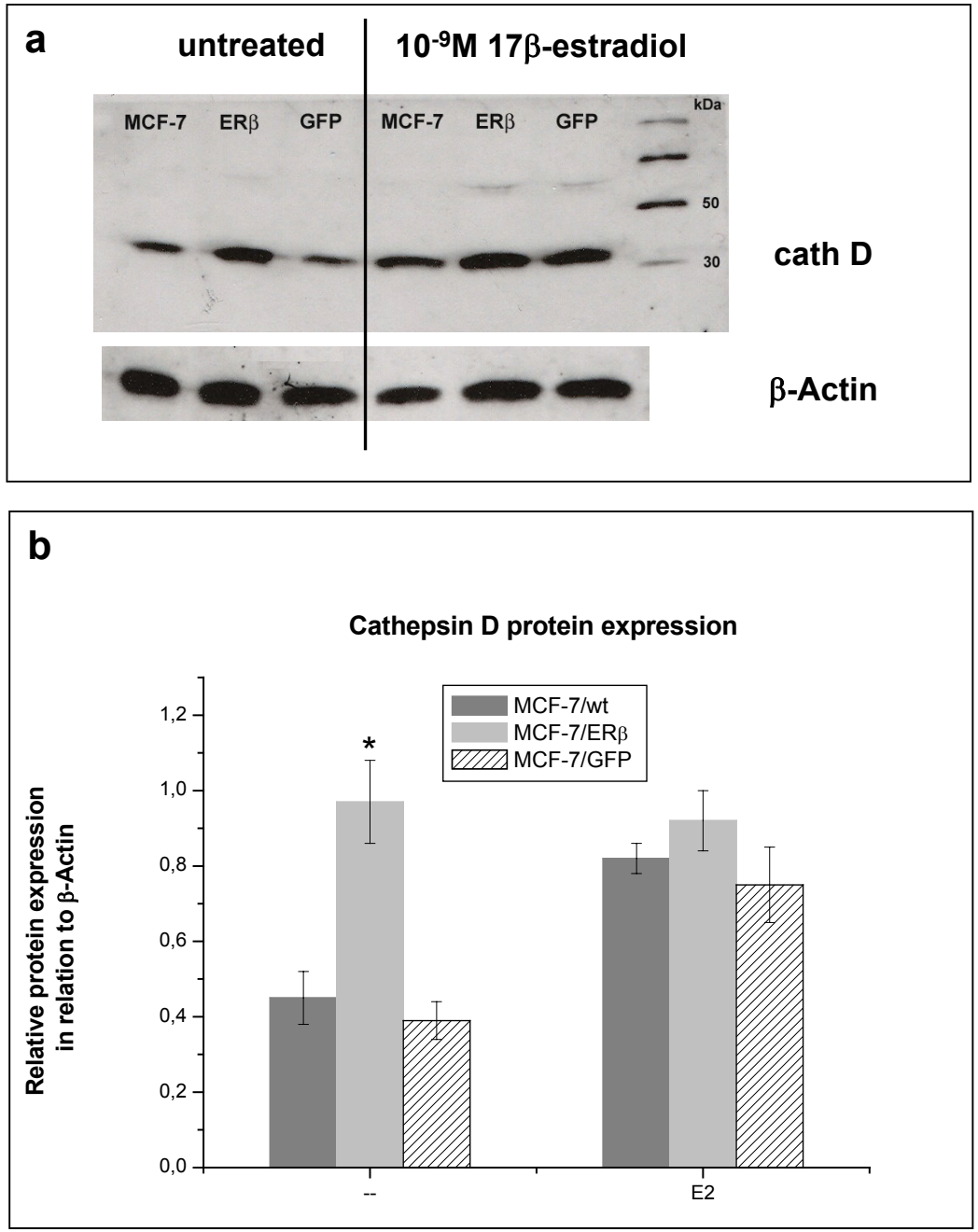
Figure 3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b
Cathepsin D protein expression
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MCF-7/ERβ
MCF-7/GFP
Relative protein expression in relation to β-Actin
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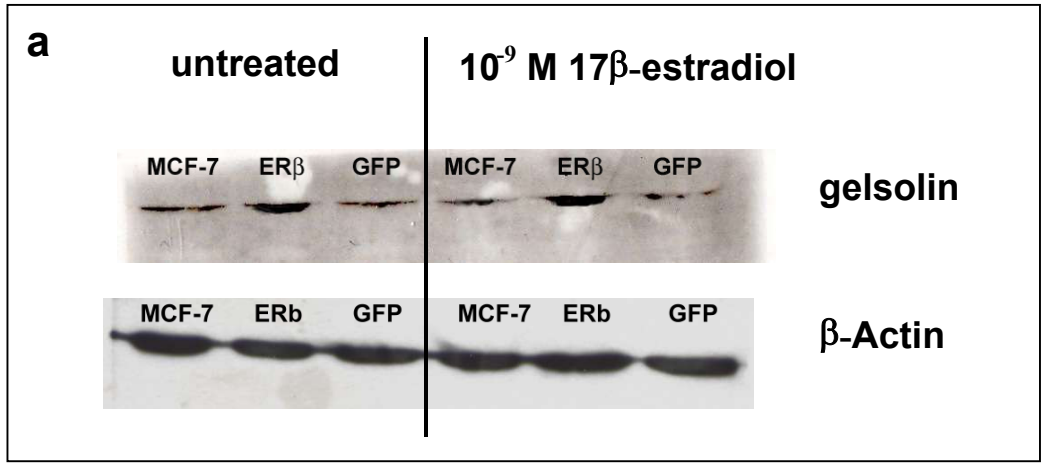
E2

Figure 4


a
untreated
10-9 M 17β-estradiol
MCF-7
ERβ
GFP
MCF-7
ERβ
GFP
gelsolin
MCF-7
ERb
GFP
MCF-7
ERb
GFP
β-Actin

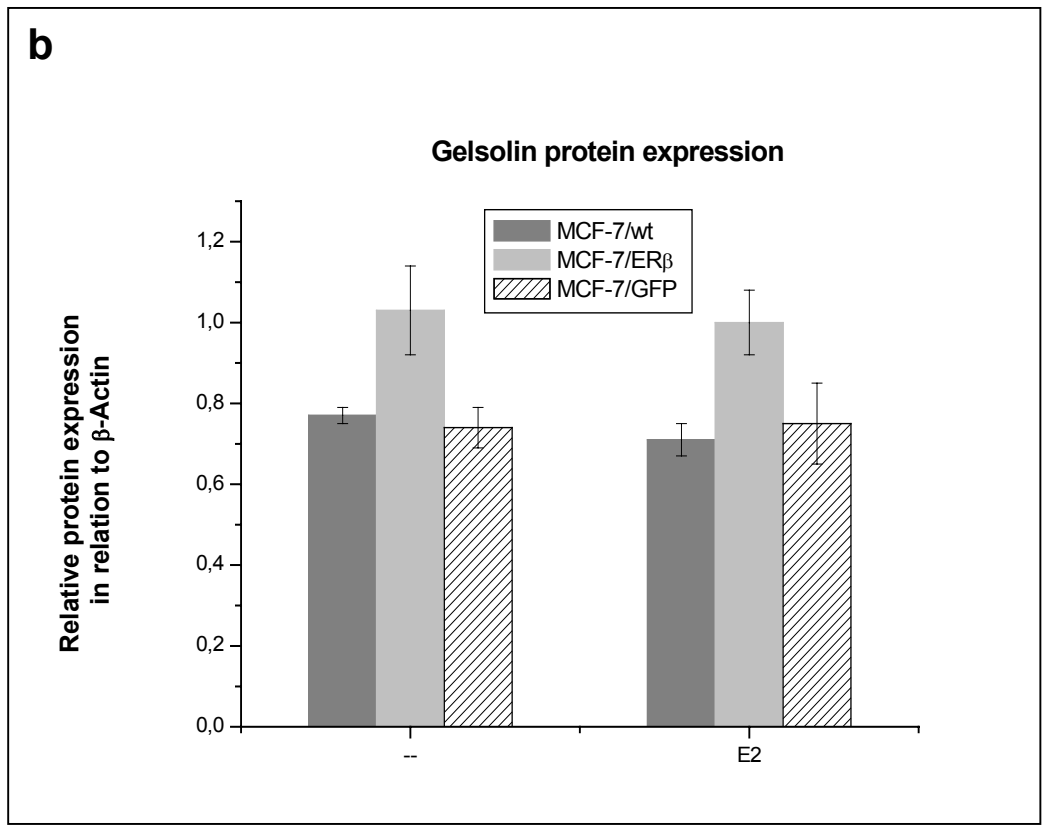

b
Gelsolin protein expression
MCF-7/wt
MCF-7/ERβ
MCF-7/GFP
Relative protein expression
in relation to β-Actin
1,2
1,0
0,8
0,6
0,4
0,2
0,0
--
E2

Figure 5

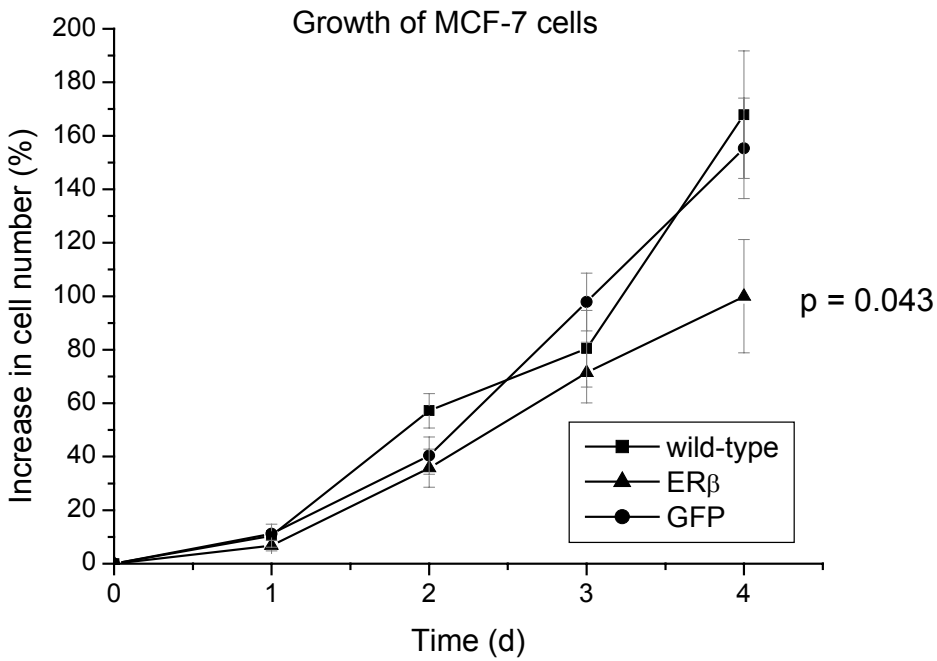
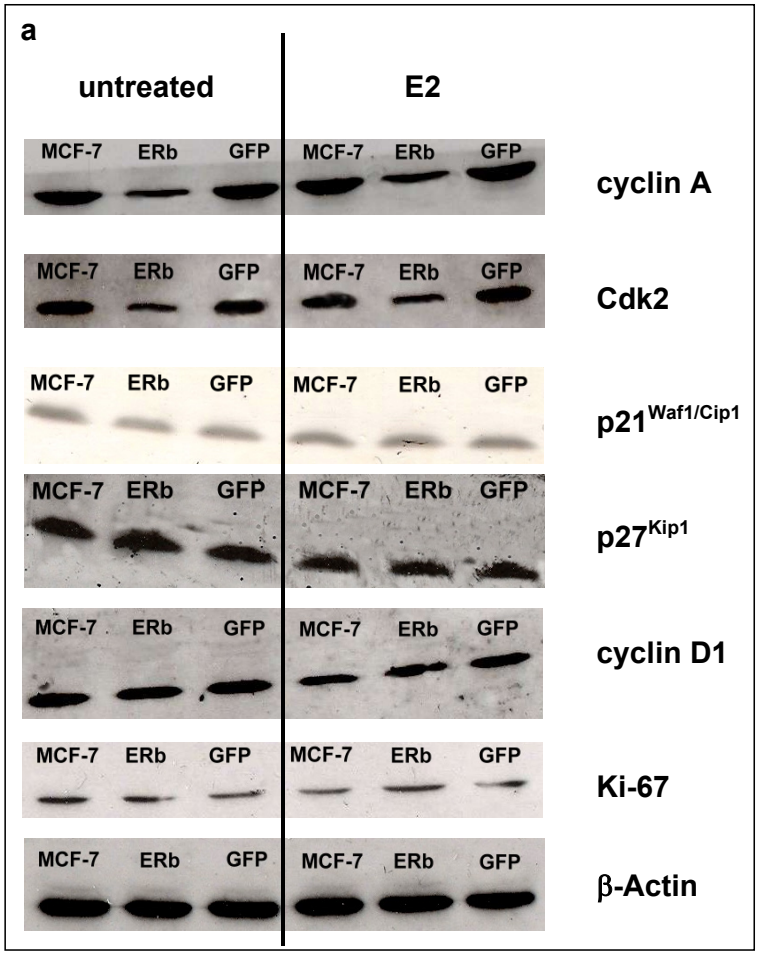

Growth of MCF-7 cells
Increase in cell number (%)
Time (d)
0
20
40
60
80
100
120
140
160
180
200
0
1
2
3
4
wild-type
ERβ
GFP
p = 0.043

Figure 6


a
untreated
E2
MCF-7 ERb GFP
cyclin A
Cdk2
p21Waf1/Cip1
p27Kip1
cyclin D1
Ki-67
β-Actin

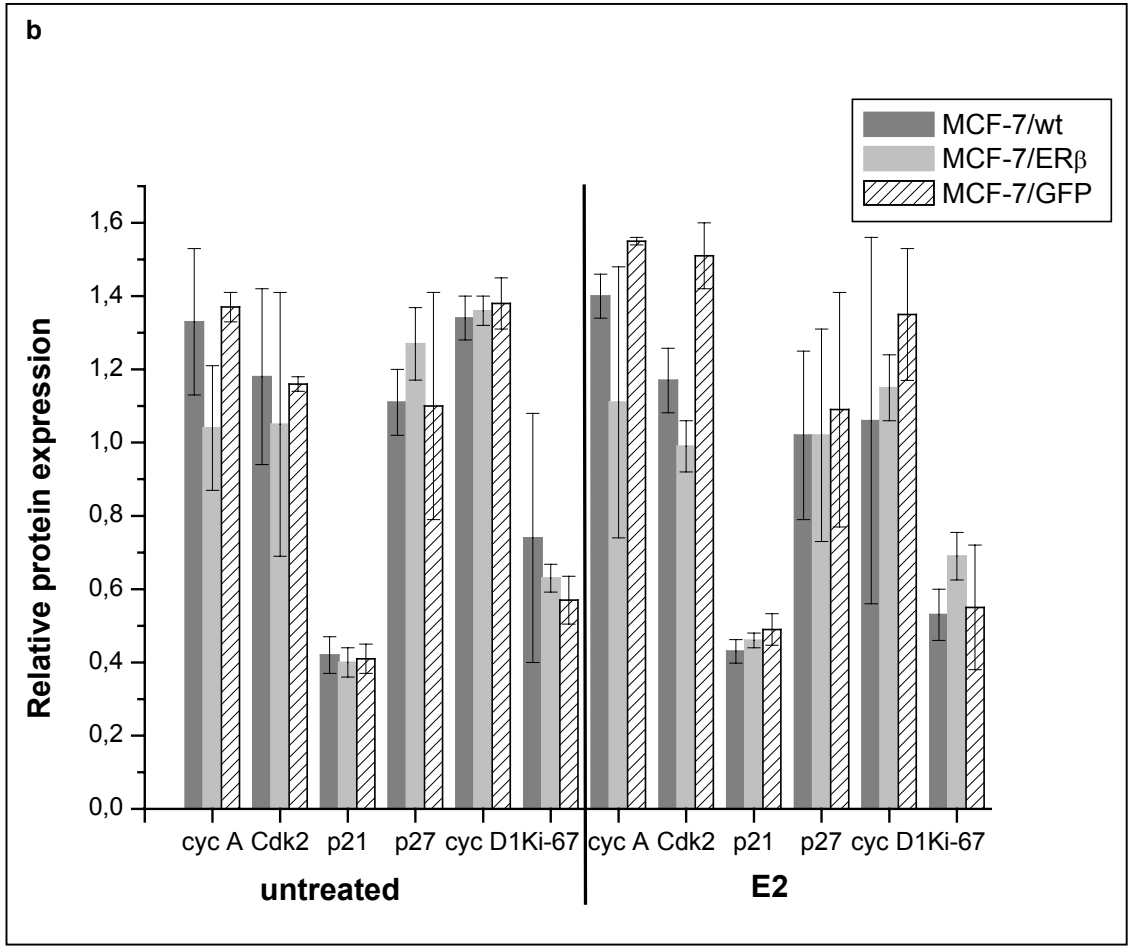

b
MCF-7/wt
MCF-7/ERβ
MCF-7/GFP
Relative protein expression
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
cyc A Cdk2 p21 p27 cyc D1 Ki-67
cyc A Cdk2 p21 p27 cyc D1 Ki-67
untreated
E2

Figure 7

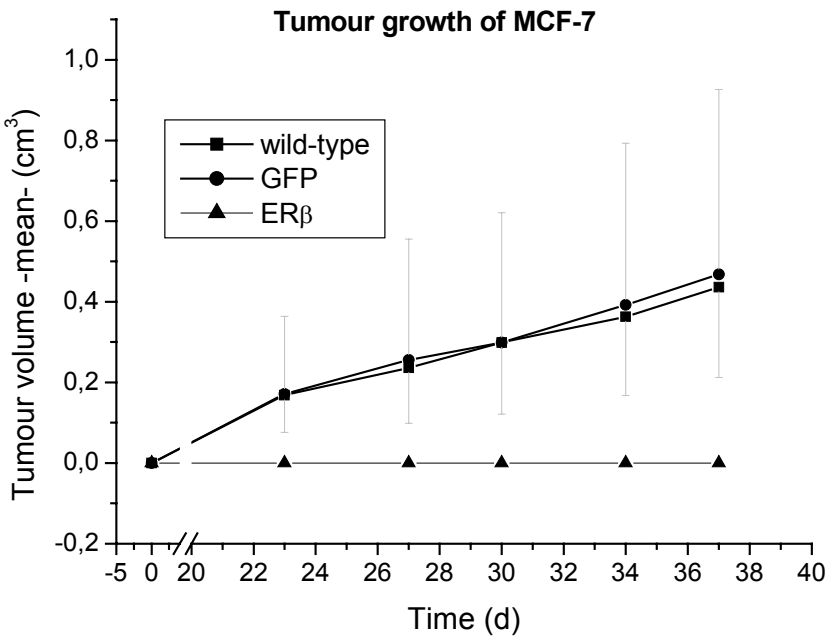

Tumour growth of MCF-7
Tumour volume -mean- (cm3)
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
wild-type
GFP
ERβ
-5
0
20
22
24
26
28
30
32
34
36
38
40
Time (d)

Table 1 TaqMan assays

Gene name	Gene symbol	Accession ID	Assay-on-Demand ID
Estrogen receptor α	ESR1	NM_000125	Hs00174860_m1
Estrogen receptor β	ESR2	NM_001437	Hs00230957_m1
Gelsolin	GSN	NM_198252	Hs00609276_m1
Hydroxymethylbilane synthase	HMBS	NM_000190	Hs00609297_m1

Table 2 Relative gene expression of ERα and ERβ (Quantitative real time PCR)

	ERα	ERβ
MCF-7/ERβ		
PCR 1	1.58 ± 0.92	303.37* ± 1.00
PCR 2	1.59 ± 0.57	442.63* ± 1.07
PCR 3	0.80 ± 2.12	282.01* ± 1.00
MCF-7/GFP		
PCR 1	0.82 ± 1.10	1.28 ± 0.78
PCR 2	1.09 ± 1.05	1.56 ± 0.94
PCR 3	0.70 ± 0.97	1.40 ± 0.92

The numbers represent the x-fold change in relative gene expression of ERβ- or GFP-transfected cells in relation to gene expression of MCF-7/wt. For the calculation the ΔΔCt method was used; each PCR was carried out in triplicate to receive 3 Ct values per sample.
* $p < 0,05$

Table 3 Relative gene expression of gelsolin (Quantitative real time PCR)

Treatment	Cell line	Fold change
-	MCF-7/ERβ	0.95 ± 1.22
-	MCF-7/GFP	0.80 ± 1.07
E2	MCF-7/ERβ	1.00 ± 1.73
E2	MCF-7/GFP	0.48 ± 1.18
	MCF-7(-) vs MCF-7(E2)	0.36 ± 0.56
	ERβ(-) vs ERβ (E2)	0.36 ± 0,77
	GFP(-) vs GFP (E2)	0.12 ± 0.6

The fold change of relative gene expression is related to MCF-7/wt without or with E2 treatment (6 hours), respectively. In the lower part of the table results of comparison of E2-treated versus non-treated cells are shown. The ΔΔCt method was used to quantify the difference in gene copy number.
- untreated, E2 17β-estradiol

Table 4 Ratio of wild-type, ERβ- and GFP-transfected MCF-7 cells (%) in S-phase at different time points.

	MCF-7/wt	MCF-7/GFP	MCF-7/ERβ
0h	4.25 ± 0.70	6.19 ± 1.30	8.20 ± 2.96
6 h	5.08 ± 0.96	6.35 ± 2.76	5.84 ± 1.47
18 h	**20.54 ± 1.09**	**21.04 ± 2.93**	8.32 ± 1.20
24 h	12.45 ± 3.87	15.48 ± 3.38	**22.88 ± 3.37**
30 h	11.90 ± 0.79	10.09 ± 0.85	10.22 ± 0.54
48 h	7.68 ± 0.63	10.07 ± 1.98	8.18 ± 1.16

Values were calculated from three independent FACS experiments. Cells were grown in T25 flasks maintained in serum-depleted and phenolred-free RPMI media for 24 hours, followed by subsequent addition of 10% dextran-charcoal-treated FBS. Cells were harvested at assigned time and prepared for FACS analysis. Figures in bold denote maximum cell number in S-phase.

Table 5 Change of cell number (x-fold) of wild-type, GFP- and ERβ-transfected MCF-7 cells after treatment with (anti)estrogens for 4 days.

	MCF-7/wt	**MCF-7/GFP**	**MCF-7/ERβ**	***P* value**
10^{-9} M 17β-estradiol	1.53 ± 0.57	1.42 ± 0.34	0.92 ± 0.26	0.016
10^{-7} M tamoxifen	0.99 ± 0.17	1.0 ± 0.14	0.85 ± 0.14	0.1 (ns)
10^{-7} M ICI 182,780	0.6 ± 0.22	0.55 ± 0.16	0.67 ± 0.22	0.3 (ns)

Increase is related to the cell number of untreated cells (set to 1) at day 4 of the treatment. Mean values from 6 experiments ± standard deviations are shown. P-value denotes the significance of the difference between wt/GFP transfectants and ERβ-transfected MCF-7. ns= not significant.