

# Effects of exon-deleted estrogen receptor $\beta$ transcript variants on growth, apoptosis and gene expression of human breast cancer cell lines

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**Abstract** Estrogen receptor  $\beta$  gene codes for a variety of transcript isoforms resulting from alternative splicing, which are expressed both in mammary gland and in breast cancer cells. We studied the function of two exon-deleted ER $\beta$  isoforms recently identified by our group in comparison to ER $\beta$ 1 in regulation of growth, apoptosis and gene expression of two breast cancer cell lines with different ER $\alpha$  status. Overexpression of ER $\beta$ 1, but not of the exon-deleted variants exerted strong antitumoral effects both on ER $\alpha$ -positive MCF-7 and ER $\alpha$ -negative SK-BR-3 cells. ER $\beta$ 1 overexpression slowed growth of MCF-7 and SK-BR-3 cells in the absence of E2 and also inhibited E2-triggered growth stimulation of MCF-7 cells, but overexpression of the exon-skipped variants did not affect cell growth. Whereas overexpression of ER $\beta$ 1 triggered an increased basal and tamoxifen-induced apoptosis of MCF-7 and SK-BR-3 cells, the isoforms ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 did not affect cellular tamoxifen response. The observed lack of function of the exon-deleted variants in terms of regulation of proliferation was accompanied both by their inability to affect expression of cyclins D1 and A2, p21 (WAF1) and PR and their disability to modulate estrogen response element (ERE) activation. In contrast, our results demonstrating antitumoral effects of ER $\beta$ 1 on breast cancer cells with different ER $\alpha$ -status support the hypothesis

that ER $\beta$  is able to exert antitumoral actions both on ER $\alpha$ -positive and -negative breast cancer cells.

**Keywords** Estrogen receptor beta · Splice variant · Breast cancer cell · Proliferation · Estradiol · Tamoxifen · Gene expression

## Introduction

Estrogens are pivotal in the growth and development of both normal and neoplastic mammary tissues, and mediate most of their action via ligand-dependent transcription factors called estrogen receptors (ER). Despite the fact that ER status is already an important biomarker in breast cancer [1] situation is now appreciated to be far more complex. Identification of a second ER, called ER $\beta$  [2] has led to a re-evaluation of estrogen action in target tissues such as breast tumours. ER $\beta$  is clearly expressed in both normal and neoplastic human breast tissue [3, 4] although its role in either is unknown. In animal studies, while ER $\alpha$  has been shown to be essential for normal mammary gland development, ER $\beta$  effects are more subtle, with roles in terminal differentiation [5] and modulation of ER $\alpha$  activity being described [6–8]. In contrast to ER $\alpha$ , published data suggest that ER $\beta$  expression declines during breast tumorigenesis [3, 9]. This downregulation of ER $\beta$  in breast tumors compared with normal breast tissue suggests a role for ER $\beta$  as a tumor suppressor [10]. Nevertheless, ER $\beta$  expression in breast tumors varies widely [4, 11] and attempts to correlate ER $\beta$  with various biomarkers have resulted in varied, often contradictory conclusions [12]. This might also be due to differential detection of variant non-ligand binding ER $\beta$  proteins which have been detected in breast tissues [13, 14] and which code for proteins

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exerting functions distinct from that of the full-length ER $\beta$ 1 protein [8, 15]. While the role of ER $\beta$  in breast cancer is unclear, one important currently emerging hypothesis is that increased expression of ER $\beta$  is associated with increased likelihood of response to endocrine therapy. Several studies report that increased levels of ER $\beta$  were associated with a better disease outcome and consistent with the breast tumour being tamoxifen sensitive [16–20].

However, when considering ER $\beta$  expression in breast cancers in vivo there are two groups, one where ER $\beta$  is coexpressed with ER $\alpha$  (ER $\beta$ +/ER $\alpha$ +) and the other where ER $\beta$  is expressed alone (ER $\beta$ +/ER $\alpha$ -). The first coexpressing group comprises ~59% of primary human breast cancers [17, 21], while the ER $\beta$  alone expressing group comprises ~17% of breast cancers [17, 21]. Emerging data support a differential function of ER $\beta$  when it is expressed alone compared with when it is co-expressed with ER $\alpha$  [22–25]. These observations might be explained by the fact that in coexpressing cells, the predominant mechanism of ER $\beta$  action might be negative modulation of ER $\alpha$ , whereas ER $\beta$  forms homodimers in cells not expressing ER $\alpha$ , thereby exerting alternative functions [6, 8, 26].

Another feature of both ERs is the variety of their mRNA isoforms resulting from differential splicing [27–31]. The so far identified ER $\beta$  splice variants are characterized by alternative 3'-exons (ER $\beta$ 2, ER $\beta$ 3, ER $\beta$ 4, ER $\beta$ 5) or by deletion of single or multiple exons (e.g. ER $\beta$  $\delta$ 2, ER $\beta$  $\delta$ 5/6). Some of these mRNA isoforms were demonstrated to code for ER $\beta$  proteins which are characterized by impaired estrogen or DNA binding or altered cofactor interaction [32, 33]. The emerging picture of multiple ER $\beta$  mRNA isoforms, and thus also the multitude of differentially built proteins, strongly suggests their synthesis to be considered as another level of complexity of estrogen signaling.

Previously we succeeded in molecular cloning of two novel ER $\beta$  splice variants from MDA-MB-231 breast cancer cells lacking exons 1, 2 and 5 or 1, 2, 5, and 6, respectively, which could be demonstrated to be expressed in a variety of human tissues [34]. In this study, we examined the function of these exon-deleted ER $\beta$  isoforms in comparison to ER $\beta$ 1 in two breast cancer cell lines with different ER $\alpha$  status.

## Materials and methods

### Materials

Phenol red-free DMEM culture medium was obtained from Invitrogen (Karlsruhe, Germany), FCS was purchased from PAA (Pasching, Austria). 17- $\beta$  estradiol, 4-OH tamoxifen, ICI 182,780, staurosporine and serum replacement 2 (SR2)

were obtained from Sigma (Deisenhofen, Germany), MCF-7 and SK-BR-3 breast cancer cells were obtained from American Type Culture Collection (Manassas, USA). M-MLV-P reverse transcriptase, Cell Titer Blue kit, Caspase-Glo 3/7 kit and ImProm-II<sup>TM</sup> Reverse Transcriptase were purchased from Promega (Mannheim, Germany). RNeasy Mini Kit, RNase Free DNase Set and Quantitect SYBR Green PCR Kit were obtained from Qiagen (Hilden, Germany). PCR primers were synthesized at Metabion (Planegg-Martinsried, Germany). Transfectin reagent was obtained from BioRad (Hercules, USA). Platinum Pfx Polymerase and OptiMEM medium were purchased at Invitrogen (Karlsruhe, Germany). Rapid-Scan gene expression panel was obtained from Origene (Rockville, USA).

### Plasmids

Vector pTARGET (Promega, Mannheim, Germany) allows cloning in *E.coli* and additionally carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. This vector also contains the neomycin phosphotransferase gene, a selectable marker for mammalian cells. pTARGET derivatives containing ORFs of ER $\beta$ 1, ER $\beta$  $\delta$ 125 or ER $\beta$  $\delta$ 1256 were used for overexpression in MCF-7 and SK-BR-3 cells. Vector pEGFP-N2 (Clontech) codes for the GFP protein for visualization of transfection efficacy using a fluorescence microscope. Vector pTAL-SEAP (Clontech) constitutively codes for the secreted alkaline phosphatase (SEAP) protein and served as positive control for the SEAP assay, and pTAL-ERE-SEAP is a reporter gene vector containing EREs in the promoter of the SEAP gene. Both vectors were used for the reporter gene assays performed in this study. Vector pSV- $\beta$ -GAL (Promega) constitutively codes for the  $\beta$ -galactosidase enzyme and was used as internal control for transfection efficacy in the reporter gene assays.

### Cell culture and transfections

MCF-7 and SK-BR-3 breast cancer cells were maintained in phenol red-free DMEM/F12 medium supplemented with 10% FCS or 1  $\times$  serum replacement (SR2). Cells were cultured with 5% CO<sub>2</sub> at 37°C in a humidified incubator. For transfection, 4  $\times$  10<sup>5</sup> cells per well of a 6-well dish were seeded in DMEM/F12 10% FCS. The next day, 2 ml fresh culture medium was added to the cells and transfection solution was prepared by mixing 5  $\mu$ l Transfectin reagent (BioRad) and 1  $\mu$ g plasmid DNA in OptiMEM reduced serum medium (Invitrogen) and added to the

cultured cells. For generation of stable clones, G418 selection (300 µg/ml) was started 48 h after transfection.

### Reverse transcription and PCR

Total RNA was isolated by means of the RNeasy kit (Qiagen) according to the manufacturer's instructions. From 1 µg total RNA, cDNA was synthesized using 100 U M-MLV-P reverse transcriptase (Promega), 2.5 mM dNTP mixture and 50 pM random primers (Invitrogen). For detection of ERβ splice variants by standard RT-PCR, 2 µl of cDNA was amplified in a reaction mix of 1 U Platinum Polymerase (Invitrogen), 20 pmol of each primer, 1× PCR-buffer, 1.5 mM MgCl<sub>2</sub> and 2.5 mM of each dNTP. The cDNA was amplified in 35 cycles (1 cycle = 1 min at 94°C melting, 2 min at 56°C annealing, 3 min at 72°C extension). All PCR primers were designed intron-spanning, sequences are indicated in Table 1, position of ERβ primers is illustrated in Fig. 1a.

For *real time* PCR detection of ERβ isoforms or estrogen target genes (primer sequences in Table 1), 2 µl cDNA were amplified using the Quantitect SYBR Green PCR Kit (Qiagen) and the LightCycler PCR device (Roche Diagnostics, Mannheim, Germany). The PCR program was 95°C for 15 min, followed by 35 PCR cycles (95°C for 10 s, 56°C for 30 s, 72°C for 30 s) and a final extension for 5 min at 72°C, followed by a standard melting curve analysis. In all RT-PCR experiments, a 190 bp β-actin fragment was amplified as reference gene using intron-spanning primers

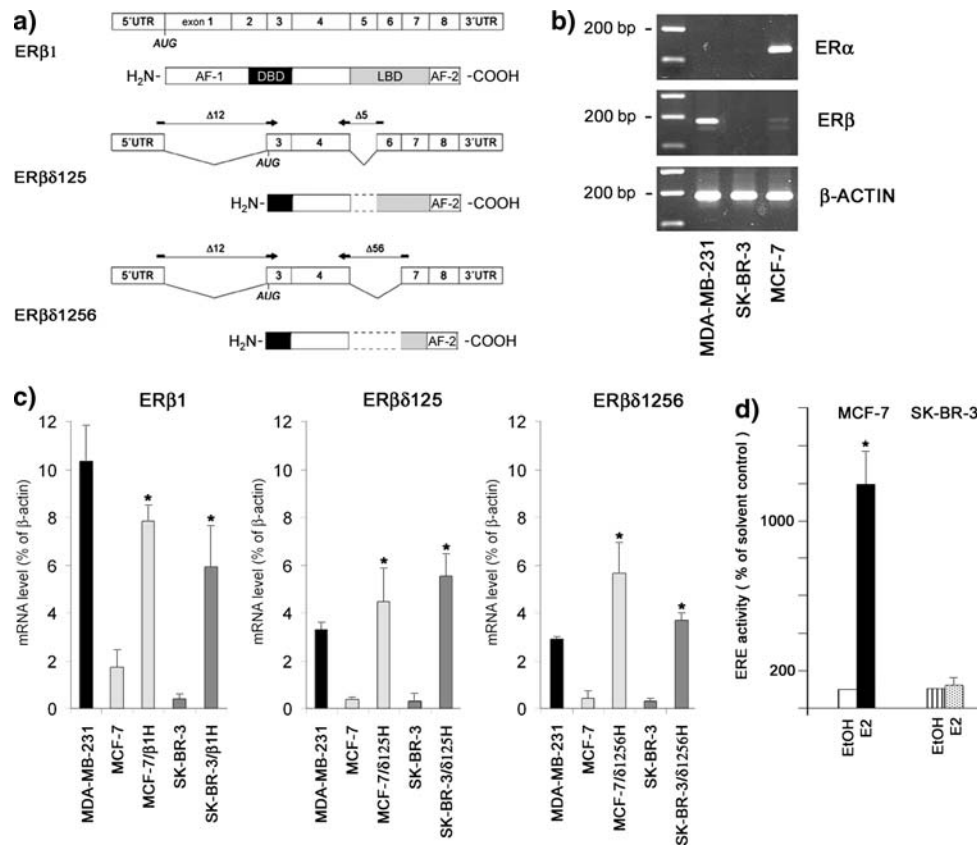
actin-2573 and actin-2876. After performing dilution experiments with sample cDNA over a 100-fold range confirming the PCR efficiencies of all primer pairs to be approximately equal [35], data were analyzed using the comparative ΔΔC<sub>T</sub> method [36] calculating the difference between the threshold cycle (C<sub>T</sub>) values of the target and reference gene of each sample and then comparing the resulting Δ C<sub>T</sub> values between different samples. In these experiments, mRNA not subjected to reverse transcription was used as a negative control to distinguish cDNA and vector or genomic DNA amplification.

### Cell viability assay

SK-BR-3 or MCF-7 wildtype (WT) cells or ERβ clones cultured in DMEM containing 1× serum replacement 2 (SR2) were seeded in 96-well plates in triplicates (1000 cells/well), and were treated with 100 nM DPN, 100 nM PPT or 1 nM 17-β estradiol alone or in combination with 4-OH tamoxifen (100 nM). After 0, 72, 96 and 120 h, relative numbers of viable cells were measured in comparison to the untreated control and the solvent control using the fluorimetric, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer's instructions at 560<sub>Ex</sub>/590<sub>Em</sub> nm in a Victor3 multilabel counter (PerkinElmer, Germany). Cell growth was expressed as percentage of day 0 or percentage of the untreated medium control. Statistical analysis of the data was performed by one-way ANOVA using Prism 2.0

**Table 1** Primer sequences used for RT-PCR amplification

Target	Oligonucleotides	Sequences 5'–3'
ERα	ERα1	TGATGAAAGGTGGGATACGA
	ERα2	AAGTTGGCAGCTCTCATGT
ERβ1	β1	CAAGGCCGGTGTGTTTATCT
	β2	GGCGTCACTGAGACTGTGG
ERβ-δ125	δ12	GGTGTGTTTATCTGCAAGGACA
	δ5	CTCATCCCGGAATCTTCTT
ERβ-δ1256	δ12	GGTGTGTTTATCTGCAAGGACA
	δ56	CCAGAGGGTACATACCGGGAA
β-Actin	actin-2573	CTGTGGCATCCACGAAACTA
	actin-2876	CGCTCAGGAGGAGCAATG
PR	PR-1	AACTTGCATGATCTTGTCAAACA
	PR-2	CACCATCCCTGCCAATATCT
Cyclin A2	CYCA-1	CTGCTGCTATGCTGTTAGCC
	CYCA-2	TGTTGGAGCAGCTAAGTCAAAA
Cyclin D1	CYCD-1	CTGGAGGTCTGCGAGGAA
	CYCD-2	GGGGATGGTCTCCTTCATCT
p21(WAF1)	WAF1	GCATGACAGATTTCTACCACTCC
	WAF2	AAGATGTAGAGCGGGCCTTT



**Fig. 1** Characterization of the splice variants and breast cancer models used in this study. **(a)** mRNA and expected protein structure of the exon-deleted isoforms in comparison to ERβ1. Arrows indicate the position of PCR primers used in this study. UTR = untranslated region; AUG = translation initiation codon; DBD = DNA binding domain; LBD = ligand binding domain; AF-1/2 = activation function 1/2. **(b)** Expression of estrogen receptors in MCF-7 and SK-BR-3 breast cancer cells was determined by RT-PCR. MDA-MB-231 cells were used as a positive control for ERβ expression, β-actin as a positive control for cDNA integrity. **(c)** Relative transcript levels of ERβ1 and the exon-deletion variants in MCF-7 and SK-BR-3 breast cancer cells. In comparison to wildtype cells, the relative mRNA levels detected after overexpression of the respective ERβ variant are shown. Specific ERβ mRNA levels of clones isolated after G418 selection were determined by means of *real time* RT-PCR using a Light Cycler device (Roche, Germany) in comparison to samples

which were not reversely transcribed as described in the materials and methods section and are expressed as % of the corresponding β-actin mRNA level ( $n = 3$ ). **(d)** Estrogen response of MCF-7 and SK-BR-3 cells: activation of estrogen response elements (ERE) after stimulation with 1 nM 17β-estradiol for 24 h. ERE activation was determined by luminometric quantification of secreted SEAP protein by means of the Phospha Light Assay (Applied Biosystem). Cells were lysed using the Beta-Glo Assay (Promega) and subjected to this assay for luminometric determination of transfected β-galactosidase enzyme as internal control for the transfection efficacy. Both luminometric SEAP and β-GAL quantification were carried out using a VICTOR3 multilabel plate reader (PerkinElmer). To normalize the data, SEAP values are expressed in relation to the measured β-GAL values ( $n = 3$ ). Data are expressed in % of the untreated (solvent EtOH) control. \* $P < 0.05$  vs. untreated control

Software (Graph pad, San Diego, USA), with statistical significance accepted at  $P < 0.05$ .

#### Apoptosis assays

Wildtype cells or ERβ-clones cultured in DMEM supplemented with 1 × serum replacement 2 (SR2, Sigma) were seeded in 96-well plates (5000 cells/well) and treated with 1 nM 17-β estradiol in combination with different concentrations of 4-OH tamoxifen. After 6 h of treatment, cellular apoptosis was determined by measurement of caspase 3 and 7 activity by means of the luminometric

Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol using a Victor3 multilabel counter (PerkinElmer, Germany). Additionally, apoptosis was measured by means of the Annexin V-FLUOS Staining Kit (Roche, Germany). Cells were treated with Annexin V and propidium iodide (PI) according to the manufacturer's protocol, and apoptotic cells exhibiting positive green Annexin V fluorescence but no red PI staining were counted. Cellular apoptosis was expressed as percentage of the untreated control cells. Statistical analysis of the data was performed by one-way ANOVA using Prism 2.0 Software (Graph pad, San Diego, USA), with statistical significance accepted at  $P < 0.05$ .

## Reporter gene assays

MCF-7 wildtype cells, ER $\beta$ -modulated MCF-7 clones and vector transfected MCF-7 control cells were seeded in 6-well plates in DMEM/F12 supplemented with 5% FCS ( $4 \times 10^5$  cell per well), five hours later serum concentration was reduced to 1% and  $0.5 \times$  serumfree SR2 medium was added. The next day, prior to transfection medium was changed to  $1 \times$  SR2. Transfections were carried out mixing 10  $\mu$ l Transfectin reagent (BioRad, Hercules, USA) in a total volume of 250  $\mu$ l OptiMEM medium with 5  $\mu$ g pEGFP-N2 vector (Clontech) for easy visualization of transfection efficacy using a fluorescence microscope, 5  $\mu$ g pTAL-SEAP vector (Clontech) as positive control for the SEAP assay, or 10  $\mu$ g of reporter gene vector pTAL-ERE-SEAP (Clontech). Generally, 5  $\mu$ g of the pSV- $\beta$ -GAL vector (Promega) was added to the transfection solution serving as internal control for transfection efficacy. 24 h after adding the 250  $\mu$ l transfection solution to the medium, cells were stimulated with 100 nM DPN, 100 nM PPT or 1 nM 17- $\beta$  estradiol alone or in combination with 100 nM 4-OH tamoxifen or 100 nM ICI 182,780 in fresh DMEM/F12 containing  $1 \times$  SR2. The next day, medium was removed and 20  $\mu$ l of it were subjected to the Phospha Light Assay (Applied Biosystem) for luminometric quantification of secreted SEAP protein in the culture supernatant according to the instructions of the manufacturer. Cells were lysed using the Beta-Glo Assay (Promega) and subjected to this assay for luminometric determination of transfected  $\beta$ -galactosidase enzyme as internal control for the transfection efficacy. Both luminometric SEAP and  $\beta$ -GAL quantification were carried out using a VICTOR3 multilabel plate reader (PerkinElmer). To normalize the data, SEAP values are expressed in relation to the measured  $\beta$ -GAL values.

## Results

### Overexpression of ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 in breast cancer cell lines

Recently we identified two new ER $\beta$  splice isoforms lacking ER $\beta$ 1 exons 1, 2 and 5 (termed ER $\beta\delta$ 125) or exons 1, 2, 5 and 6 (termed ER $\beta\delta$ 1256) from human MDA-MB-231 breast cancer cells, which are expressed in a variety of human tissues [34]. The deduced variant ER $\beta$  proteins are predicted to use an alternative translation start codon in exon 3 in the same reading frame as ER $\beta$ 1, do not contain the AF-1 domain and have deletions both in ligand binding domain (LBD) and DNA binding domain (DBD) (Fig. 1a). Given that MCF-7 breast cancer cells express ER $\alpha$  but only small amounts of ER $\beta$ 1 (Fig. 1b, c) and SK-BR-3 cells do

not express notable amounts of both receptors (Fig. 1b, c), we first examined expression of ER $\beta\delta$ 125 and ER $\beta\delta$ 1256 in both cell lines by real time PCR. To confirm specificity of amplification of the exon-deleted variants, a set of isoform-specific PCR primers was used annealing at the junction of exons 0 and 3 (primer  $\delta$ 12) and the junction of exon 4 and 6 (primer  $\delta$ 5) or exon 4 and 7 (primer  $\delta$ 56), respectively, and identity of the resulting amplicons was confirmed by sequencing. Both cell lines exhibited a very weak expression of ER $\beta$ 1, ER $\beta\delta$ 125 and ER $\beta\delta$ 1256 when compared to MDA-MB-231 breast cancer cells (Fig. 1c). Thus, we used these cell lines to elucidate the function of the exon-deleted ER $\beta$  splice variants in comparison to ER $\beta$ 1 by means of overexpression. MCF-7 and SK-BR-3 cells were transfected with pTARGET mammalian expression vectors (Promega) containing the coding region of ER $\beta$ 1, ER $\beta$ - $\delta$ 125 or ER $\beta$ - $\delta$ 1256 [34] or the original pTARGET vector as negative control. After verification of their expression in transient transfection assays on mRNA level by means of RT-PCR (data not shown), MCF-7 and SK-BR-3 clones stably expressing the transfected pTARGET derivatives were generated by G418 selection (300  $\mu$ g/ml). About 6 weeks after transfection, 3 to 6 MCF-7 or SK-BR-3 clones per derivative were isolated using cloning disks and propagated. In these clones, mRNA levels of ER $\beta$ 1, ER $\beta\delta$ 125 or ER $\beta\delta$ 1256, respectively, was relatively quantified in relation to  $\beta$ -actin expression by means of *real time* RT-PCR, avoiding false positive signals from vector DNA by comparison to a sample which was not reversely transcribed. The success of overexpression of these ER $\beta$  isoforms was additionally verified by sequencing of the amplified cDNA. MCF-7 and SK-BR-3 clones mock transfected with the original pTARGET vector as negative control were identified by detection of mRNA transcribed from the neomycin resistance gene of this vector by means of RT-PCR (primers pTAR1 and pTAR2). Additionally, overexpression of ER $\beta$ 1, ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 in the transfected clones was verified by means of immunoblot-analysis using ER $\beta$  antibody 5197P (Acris Antibodies, Hiddenhausen, Germany) (data not shown). Whereas we did not detect notable expression of all three protein isoforms in MCF-7 wildtype cells, MCF-7/ER $\beta$ 1 cells exhibited a strong band of about 55 kDa, whereas the expected bands of 28 and 35 kDa size were detected in MCF-7 cells overexpressing ER $\beta\delta$ 1256 or ER $\beta\delta$ 125.

Three clones from each cell line stably exhibiting higher mRNA levels of the transfected ER $\beta$  subtypes similar the respective levels in MDA-MB-231 breast cancer cells (termed MCF-7 or SK-BR-3/ $\beta$ 1H, MCF-7 or SK-BR-3/ $\delta$ 125H, MCF-7 or SK-BR-3/ $\delta$ 1256H) were chosen for further characterization. Since these three clones turned out to exhibit nearly identical properties in terms of proliferation, apoptosis, ERE activity and gene regulation, in the



following data from the analysis of one clone is shown representatively.

#### Proliferation of breast cancer cells overexpressing ER $\beta\delta$ 125, ER $\beta\delta$ 1256 or ER $\beta$ 1

Given that estrogen receptors are known to regulate cellular proliferation by different molecular mechanisms, we examined proliferation of our breast cancer models transfected with ER $\beta$ 1 or the splice isoforms. For this purpose, both vector-transfected and ER $\beta$ -transfected clones from ER $\alpha$ -positive MCF-7 and estrogen-unresponsive SK-BR-3 cells were cultured in serumfree SR2 medium without E2 or in serumfree medium supplemented with 1 nM of this steroid hormone for up to five days. In the absence of E2, MCF-7 and SK-BR-3 cells overexpressing ER $\beta$ 1 exhibited a significantly reduced proliferation when compared to growth of vector-transfected control cells, an effect which was more pronounced in the estrogen-unresponsive SK-BR-3 cell line (Fig. 2a, b). In contrast, overexpression of ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 did not affect cell growth of the breast cancer cell lines tested. Addition of 17 $\beta$ -estradiol as expected only increased growth of ER $\alpha$ -positive MCF-7, but not of SK-BR-3 control cells, and dose response analysis revealed that growth stimulation of MCF-7 cells was largest at a E2 concentration of 1 nM (Fig. 2c). This E2-triggered proliferation was observed in MCF-7 control cells and also in MCF-7 cells overexpressing the ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 isoforms, but not in MCF-7 cells overexpressing ER $\beta$ 1 (Fig. 2a). A similar growth stimulatory effect as triggered by E2 was observed after treatment of MCF-7 cells with selective ER $\alpha$  agonist PPT (1,3,5-tris (4-Hydroxyphenyl)-4-propyl-1H-pyrazole). Like E2, this substance was not able to stimulate proliferation of MCF-7 cells overexpressing ER $\beta$ 1. In contrast, treatment with selective ER $\beta$  agonist DPN (diarylpropionitrile) clearly reduced cell growth of all MCF-7 clones tested. Whereas DPN slightly weakened proliferation of MCF-7 control cells and MCF-7 cells overexpressing the ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 isoforms, cell numbers of ER $\beta$ 1-overexpressing MCF-7 cells after 5 days of DPN treatment were even decreased down to 79% of the seeded cell number (Fig. 2a).

To examine, whether overexpression of the exon-deleted ER $\beta$  isoforms would be able to affect the response of breast cancer cell lines to selective estrogen receptor modulator tamoxifen, cells were treated with this substance (0.5–25  $\mu$ M) in combination with 1 nM E2 for five days. Treatment with 4-OH tamoxifen clearly reduced cell numbers of all MCF-7 and SK-BR-3 clones tested in a dose-dependent manner, and response to this drug was much stronger in MCF-7 cells. However, overexpression of

ER $\beta$ 1 or the exon-deleted variants did not affect tamoxifen response of this cell line. In contrast, overexpression of ER $\beta$ 1 in SK-BR-3 cells resulted in a slightly increased growth inhibitory effect of 4-OH tamoxifen (5 and 10  $\mu$ M) (Fig. 3a).

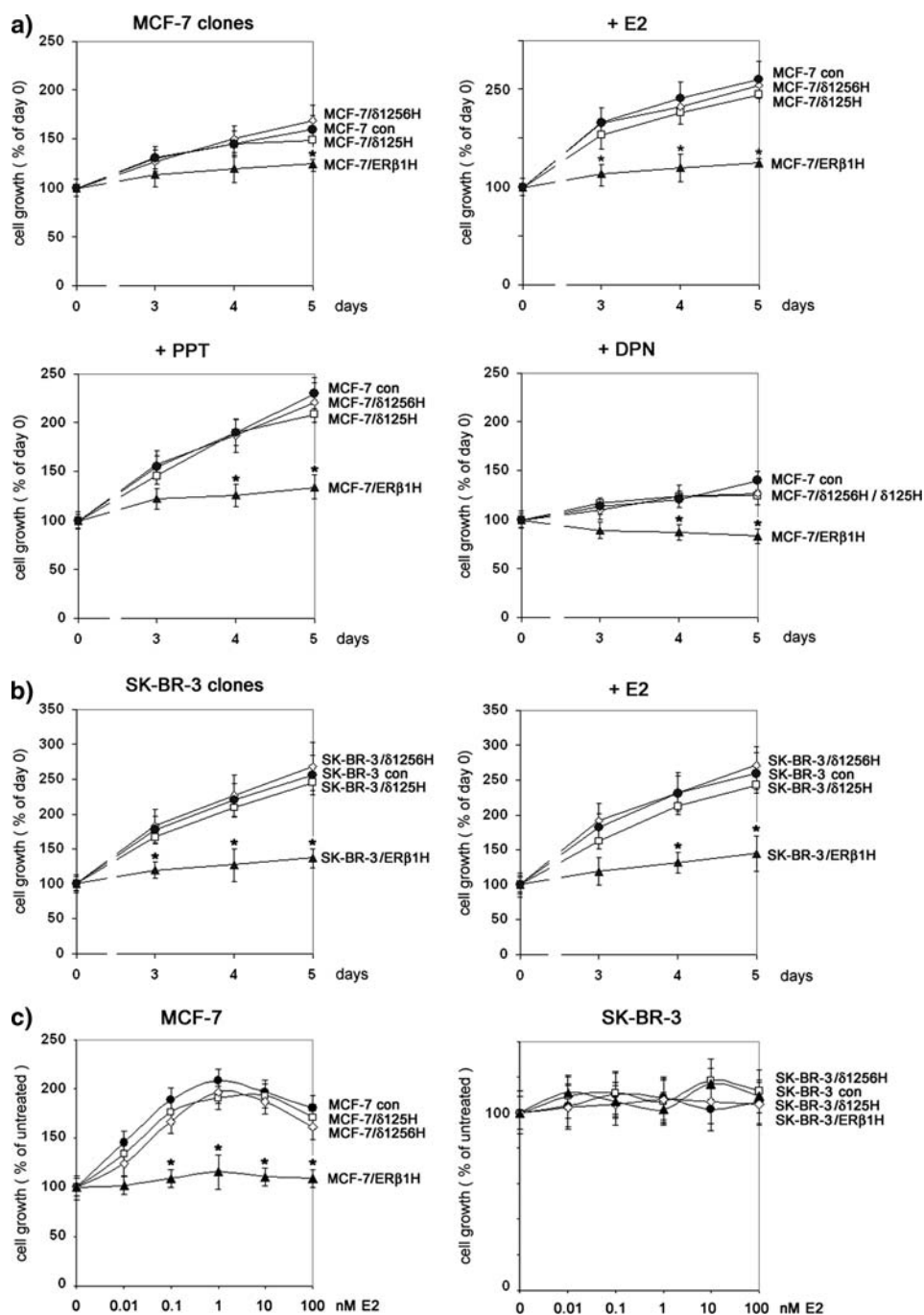
To examine, whether ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 would be able to modulate function of ER $\beta$ 1, we overexpressed these isoforms in a breast cancer cell line strongly expressing ER $\beta$ 1. For this purpose, the transcript level of the exon-deleted variants in MDA-MB-231 cells was increased 20.9-fold (ER $\beta\delta$ 125 or 34.7-fold (ER $\beta\delta$ 1256) by means of transfection and G418 selection as described before. Proliferation assays with these MDA-MB-231 clones analyzing their growth in comparison to mock-transfected cells in the presence or absence of E2, DPN or 4-OH tamoxifen did not reveal any effect of overexpression of the exon-skipped isoforms (data not shown).

#### Apoptosis of breast cancer cells overexpressing ER $\beta\delta$ 125, ER $\beta\delta$ 1256 or ER $\beta$ 1

Given that tamoxifen is known to trigger apoptosis in breast cancer cells and ER $\beta$  recently was reported to affect apoptosis in different cellular systems [37, 38], we also tested whether ER $\beta$  isoforms could modulate apoptotic response to tamoxifen. Cultured in serumfree medium supplemented with 1 nM E2, MCF-7 and SK-BR-3 cells overexpressing ER $\beta$ 1, but not the exon-deleted isoforms exhibited an increased apoptosis rate when compared to control cells even in absence of tamoxifen. After treatment with different concentrations of this drug, a similarly elevated caspase activity of ER $\beta$ 1-overexpressing cells was observed. In contrast, 4-OH tamoxifen concentrations from 0.5 to 10  $\mu$ M did not trigger apoptosis in MCF-7 control cells or cells overexpressing the ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 isoform, a significant apoptotic response of these MCF-7 and SK-BR-3 clones was only observed after treatment with 25  $\mu$ M 4-OH tamoxifen (Fig. 3b).

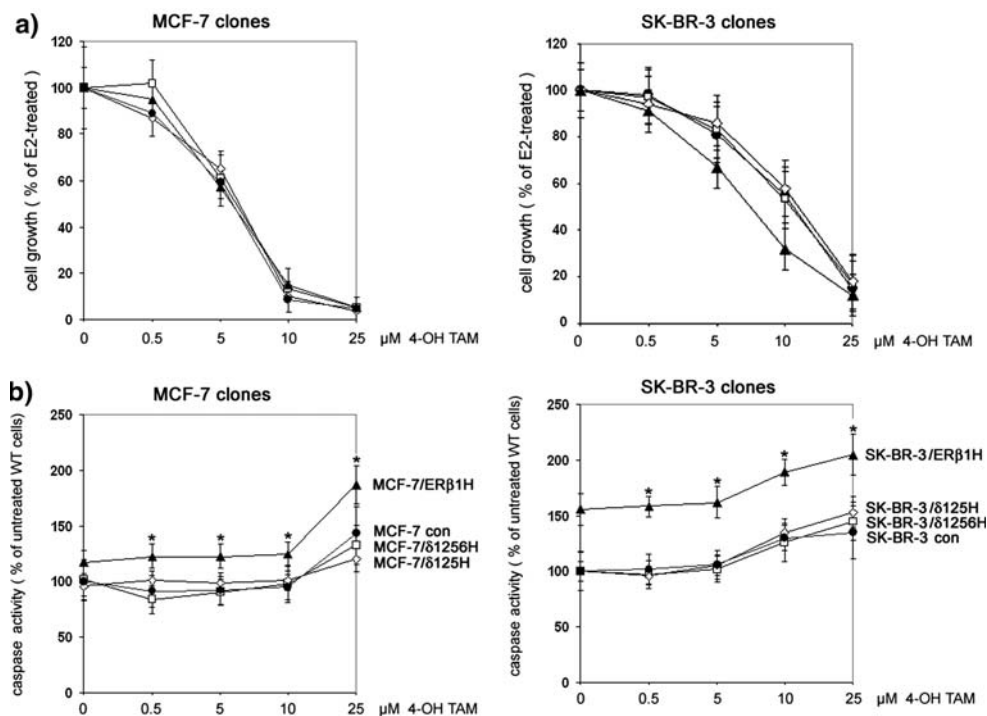
#### Expression of estrogen-responsive genes in breast cancer cells overexpressing ER $\beta\delta$ 125, ER $\beta\delta$ 1256 or ER $\beta$ 1

Given that estrogen receptors are ligand inducible transcription factors directly regulating gene transcription, we studied the effect of the exon-deleted ER $\beta$  splice variants in comparison to ER $\beta$ 1 on expression of 14 estrogen responsive genes in MCF-7 and SK-BR-3 cells (progesterone receptor (PR), cyclin D1, cyclin A2, cyclin-dependent kinase 2 (CDK2), autotaxin, PS2, ER $\alpha$ , FAS ligand, HER2, cathepsin D, EGFR, IGFBP-4, WISP-2, p21



**Fig. 2** Growth of breast cancer cells overexpressing ER $\beta\delta$ 125, ER $\beta\delta$ 1256 or ER $\beta$ 1. (a) Transfected MCF-7 cells overexpressing ER $\beta$ 1, ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 were cultured up to 5 days in serumfree (SR2) medium in the absence of estradiol or supplemented with 1 nM 17- $\beta$  estradiol (E2), 100 nM of ER $\beta$ -agonist DPN or 100 nM of ER $\alpha$ -agonist PPT. Cell growth was compared to mock-transfected cells (MCF-7 con). Black circle = MCF-7-vector control; black triangle = MCF-7/ER $\beta$ 1H; open rhombus = MCF-7/ER $\beta\delta$ 1256H, open square = MCF-7/ER $\beta\delta$ 125H. \* $P < 0.01$  vs. vector transfected control cells. (b) Transfected SK-BR-3 cells overexpressing ER $\beta$ 1, ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 were cultured up to 5 days in serumfree medium in the absence of estradiol or supplemented with 1 nM 17- $\beta$  estradiol (E2). Cell growth was compared to mock-transfected cells (SK-BR-3 con).

Black circle = SK-BR-3-vector control; black triangle = SK-BR-3/ER $\beta$ 1H; open rhombus = SK-BR-3/ER $\beta\delta$ 1256H, open square = SK-BR-3/ER $\beta\delta$ 125H. \* $P < 0.01$  vs. vector transfected control cells. (c) Dose-response analysis of the generated MCF-7 and SK-BR-3 clones. Cells grown in serumfree (SR2) medium were treated with the indicated concentrations of 17- $\beta$ -estradiol and cell growth was determined on day 5. Generally, relative viable cell numbers were measured using the resazurin-based Cell Titer Blue fluorescence assay as described in the materials and methods section on day 0, 3, 4, and 5. Viable cell numbers are expressed as indicated in percentage of day 0 (a, b) or in percentage of the untreated control (c). Results were obtained from four separate experiments and are expressed as means  $\pm$  SD



**Fig. 3** Tamoxifen effects on breast cancer cells overexpressing ER $\beta$ 1 or the exon-deleted splice variants. **(a)** For determination of antiproliferative action of tamoxifen, cells were incubated in serum-free medium supplemented with 1 nM E2 in combination with the indicated concentrations 4-OH tamoxifen for 5 days and relative cell growth was assessed by means of the Cell Titer Blue assay as described in the material and methods section. Growth is expressed in percentage of the E2-treated control ( $n = 3$ ). **(b)** For examination of apoptotic effects of tamoxifen, cells cultured in serumfree medium

supplemented with 1 nM E2 were treated with the indicated concentrations of 4-OH tamoxifen for 6 h and apoptosis was determined by measurement of caspase 3 or 7 activation by means of the luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol using a Victor3 multilabel counter (PerkinElmer, Germany). Apoptosis is expressed as means  $\pm$  SD in percent of the untreated control cells ( $n = 3$ ) \* $P < 0.05$  vs. control cells

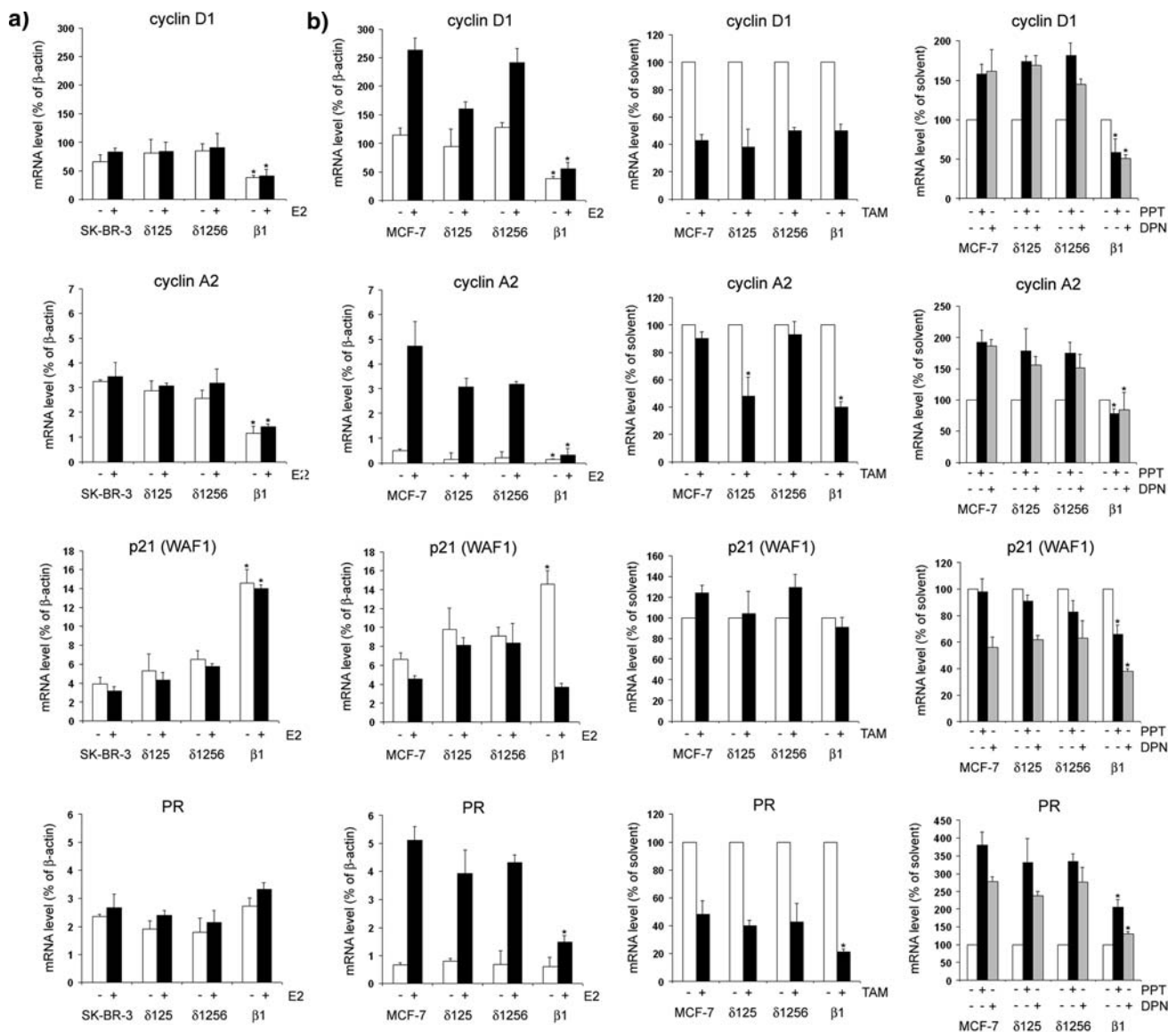
(WAF1). For this purpose, we analyzed expression of these genes in ER $\beta$ - or mock-transfected MCF-7 and SK-BR-3 cells cultured in serumfree medium or treated with 1 nM E2, 10 nM PPT or 10 nM DPN for 24 h on mRNA level by means of real time RT-PCR.

Four of the analyzed genes, p21(WAF1), cyclin A2, cyclin D1 and PR exhibited altered mRNA levels in ER $\beta$ 1-transfected MCF-7 cells (Fig. 4). In ER $\alpha$ -positive MCF-7 cells, but not in SK-BR-3 cells, transcript levels of cell cycle regulator cyclin D1 were 2.4-fold induced after E2 treatment. Both in MCF-7 and SK-BR-3 cells, overexpression of ER $\beta$ 1, but not of the exon-deleted isoforms resulted in a significantly reduced basal cyclin D1 mRNA level, which was not significantly increased after E2 treatment. Treatment with 5  $\mu$ M 4-OH tamoxifen resulted in a decrease of cyclin D1 mRNA levels down to 40–50% in MCF-7 cells irrespective their ER $\beta$  status. Treatment with ER $\alpha$ -agonist PPT and ER $\beta$ -agonist DPN slightly increased cyclin D1 transcript levels in MCF-7 control cells and in cells overexpressing the exon-deleted variants, but decreased mRNA levels of this gene in MCF-7 cells overexpressing ER $\beta$ 1.

Cyclin A2 mRNA levels were 8.3-fold elevated after E2 treatment in MCF-7 cells, but not in SK-BR-3 cells. A similar E2-triggered increase of cyclin A2 transcript levels was observed in MCF-7 cells overexpressing the ER $\beta$  $\delta$ 125 or ER $\beta$  $\delta$ 1256 isoform, but overexpression of ER $\beta$ 1 nearly abolished estrogenic cyclin A2 induction. In SK-BR-3 cells, overexpression of ER $\beta$ 1, but not of the exon-deleted isoforms resulted in a significantly reduced basal cyclin A2 mRNA level, which was not significantly increased after E2 treatment. Treatment of MCF-7 clones with 4-OH tamoxifen did not affect cyclin A2 transcript levels in MCF-7 control and ER $\beta$  $\delta$ 1256-overexpressing cells, but reduced mRNA levels of this gene by about 50% in MCF-7 cells overexpressing ER $\beta$ 1 or ER $\beta$  $\delta$ 125. Whereas treatment with DPN or PPT elevated cyclin A2 mRNA levels in vector-transfected MCF-7 cells and in MCF-7 cells overexpressing the exon-deleted isoforms, no such effect was observed in MCF-7 cells overexpressing ER $\beta$ 1.

Transcript levels of antiproliferative cell cycle regulator and tumor suppressor p21(WAF1) were significantly increased in MCF-7 and SK-BR-3 cells overexpressing ER $\beta$ 1, but E2 stimulation led to strong decrease





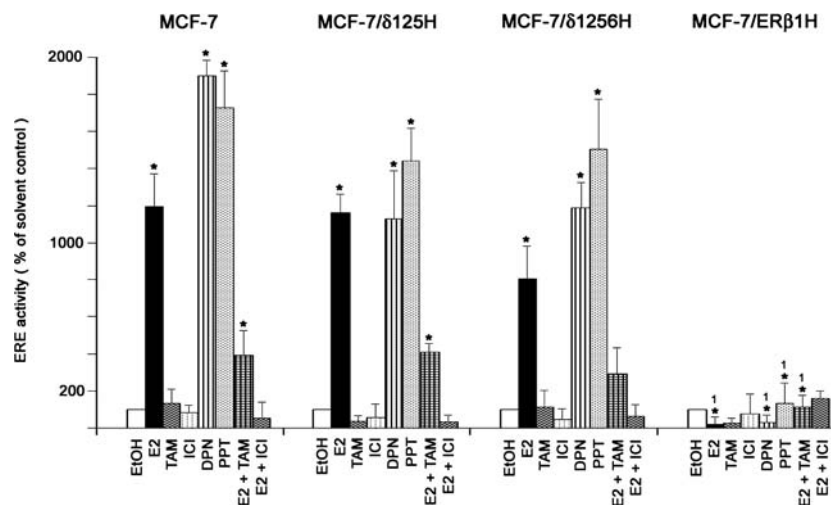
**Fig. 4** Transcript levels of four genes in breast cancer cells overexpressing ER $\beta\delta125$ , ER $\beta\delta1256$  or ER $\beta1$ . **(a)** SK-BR-3 clones cultured in serumfree medium were treated with 1 nM E2 24 h prior to total RNA isolation. **(b)** MCF-7 cells cultured in serumfree medium were treated with 100 nM DPN, 100 nM PPT or 1 nM E2 alone or in combination with 100 nM 4-OH tamoxifen for 24 h prior to total

RNA isolation. Shown are the relative expression levels as determined by real time RT-PCR expressed in percentage of the corresponding  $\beta$ -actin transcript level (left panel) or as percentage of the solvent control (right panel). Results were obtained from five separate experiments and are expressed as means  $\pm$  SD. \* $P < 0.05$  vs. mock-transfected control cells

of p21(WAF1) expression in MCF-7 cells. In contrast, overexpression of the ER $\beta\delta125$  or ER $\beta\delta1256$  isoform did not significantly affect p21 (WAF1) transcript levels which were not regulated by E2 in these clones and in MCF-7 control cells. Whereas tamoxifen treatment did not affect p21(WAF1) expression in all MCF-7 clones tested, treatment with ER $\beta$  agonist DPN resulted in a significant decrease of transcript levels, an effect which was even more pronounced in MCF-7/ER $\beta1$  cells.

Progesterone receptor (PR) expression is known to be regulated by estrogens in an ER $\alpha$ -dependent manner. As

expected E2 treatment resulted in a strong increase of PR mRNA levels in MCF-7 control cells. This effect was not significantly different in MCF-7 cells overexpressing the exon-deleted isoforms, but it was clearly diminished in MCF-7 cells overexpressing ER $\beta1$ . Tamoxifen treatment led to downregulation of PR mRNA levels by about 50% in MCF-7 control cells or cells expressing the ER $\beta$  splice variants, an effect which was even more pronounced in MCF-7 cells expressing ER $\beta1$ . Overexpression of ER $\beta1$ , but not of the exon-deleted isoforms diminished the PPT- or DPN-triggered upregulation of PR transcript levels.



**Fig. 5** Estrogen response element (ERE) activity in MCF-7 cells overexpressing ER $\beta$ 1 or the exon-deleted variants. MCF-7 clones cultured in 6-well plates in serumfree (SR2) medium were transfected with 10  $\mu$ g of reporter gene vector pTAL-ERE-SEAP (Clontech) and 5  $\mu$ g of the pSV- $\beta$ -GAL vector (Promega) as internal control for transfection efficacy. 24 h after transfection, cells were stimulated with the indicated substances. The next day, medium was removed and 20  $\mu$ l of it were subjected to the Phospha Light Assay (Applied Biosystem) for luminometric quantification of secreted SEAP protein

Irrespective of treatment, we did not observe any significant differences between the different ER $\beta$  clones and MCF-7 or SK-BR-3 control cells regarding expression of CDK2, autotaxin, PS2, ER $\alpha$ , FAS ligand, HER2, cathepsin D, EGFR, IGFBP-4 or WISP-2.

#### Estrogen response element (ERE) activity in MCF-7 cells overexpressing ER $\beta\delta$ 125, ER $\beta\delta$ 1256 or ER $\beta$ 1

To further confirm the observed lack of function of both exon-deleted isoforms, we analyzed their ability to modulate ERE activation by means of reporter gene assays. Like MCF-7 control cells, MCF-7 cells overexpressing ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 exhibited a strong ERE activation as response to treatment with 1 nM E2 (Fig. 5). These cells also showed strong ERE activation after treatment with ER $\beta$  agonist DPN or ER $\alpha$  agonist PPT. In these cell lines, E2-triggered ERE activation was significantly inhibited by co-treatment with 0.5  $\mu$ M 4-OH tamoxifen and to an even larger extent by co-treatment with pure antiestrogen ICI 182,780 (0.5  $\mu$ M). In contrast, overexpression of ER $\beta$ 1 totally inhibited ERE activation in MCF-7 cells.

#### Discussion

Recently we have identified the two novel human exon-deleted ER $\beta$  transcript variants ER $\beta\delta$ 125 and ER $\beta\delta$ 1256 in

in the culture supernatant according to the instructions of the manufacturer. Cells were lysed using the Beta-Glo Assay (Promega) and subjected to this assay for luminometric determination of transfected  $\beta$ -galactosidase enzyme as internal control. Both luminometric SEAP and  $\beta$ -GAL quantification were carried out using a VICTOR3 multilabel plate reader (PerkinElmer). To normalize the data, SEAP values are expressed in relation to the measured  $\beta$ -GAL values ( $n = 5$ ) \* $P < 0.01$  vs. untreated control. \*<sup>1</sup> $P < 0.01$  vs. vector-transfected MCF-7 cells

MDA-MB-231 breast cancer cells. Both transcripts previously have been demonstrated to be translated, they code for alternative ER $\beta$  proteins of about 35 kDa (ER $\beta\delta$ 125) and 28 kDa (ER $\beta\delta$ 1256) [39]. The aim of this study was to elucidate the function of both new ER $\beta$  isoforms in breast cancer cells with different ER $\alpha$  status in regulation of cell growth, apoptosis, gene expression and estrogen response element (ERE) activation. In contrast to MDA-MB-231 cells, both MCF-7 and SK-BR-3 breast cancer cells do not express notable amounts of ER $\beta\delta$ 125 and ER $\beta\delta$ 1256 transcripts. Whereas estrogen-responsive MCF-7 cells are a widely used model for hormone-dependent breast cancer expressing high levels of ER $\alpha$  and relatively low levels of ER $\beta$ , estrogen-unresponsive and HER-2 overexpressing SK-BR-3 cells do not express functional ER $\alpha$  and exhibit only marginal ER $\beta$  levels [40]. Our data demonstrating E2-triggered ERE activation in MCF-7, but not in SK-BR-3 cells confirm these differences on molecular level. However, the fact that treatment with ER $\beta$  agonist DPN was able to slightly reduce MCF-7 cell proliferation suggests that ER $\beta$ 1 levels in these cell line are low but sufficient to mediate some weak effects of this ligand.

In this study, we stably introduced cDNA coding for ER $\beta$ 1, ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 into MCF-7 and SK-BR-3 cells to compare the effect of overexpression of these receptors in human breast cancer cells with different ER $\alpha$  status. For further characterization, we have chosen not the stably transfected clones exhibiting the highest expression levels, but the ones with lower overexpression levels

comparable to the respective expression we measured in MDA-MB-231 cells and in single human breast cancer samples.

Several studies show evidence that ER $\beta$  negatively regulates cellular proliferation, promotes apoptosis and thus may have a protective role in normal breast and prostate [37, 41, 42]. Though also many ER $\beta$  splice variants are expressed both in mammary gland and in breast cancer [27], the specific role of ER $\beta$  splice isoforms in this tissue remains unclear. Three different ER $\beta$  variant mRNAs that have deletions in exon 5 or 6 or exons 5/6 have been identified in human breast, uterus and ovarian tissues [31, 43, 44]. A recent study examined the function of one of these exon-deleted variants, ER $\beta$ - $\delta$ 5, suggesting that this isoform might act as a dominant negative receptor on ER $\alpha$  and ER $\beta$  pathways [38]. In another study, an ER $\beta$  isoform lacking the exons 2, 5 and 6 was identified and it was stated that deletion of these exons would cause a frame shift mutation resulting in premature termination of translation [45]. The exon-deleted variants ER $\beta$  $\delta$ 125 and ER $\beta$  $\delta$ 1256 we examined here use a different translation initiation codon in the beginning of exon 3 allowing translation in the same reading frame as ER $\beta$ 1. The proteins coded by these variants are predicted not to contain the activation function 1 (AF-1) domain mediating the ligand-independent transcriptional activity of ER $\beta$  and are predicted to have deletions both in the DNA-binding domain (DBD) and in the ligand-binding domain (LBD). Thus, it is expected that both the ligand-dependent and ligand-independent activity of the deduced proteins are significantly diminished.

Our findings that ER $\beta$ 1 promotes antitumoral effects on breast cancer cells *in vitro* are in line with previous studies reporting similar observations. Omoto et al. [46] stably expressed ER $\beta$ 1 in MCF-7 cells under the control of a cytomegalovirus promoter and found that the receptor had a negative effect on proliferation of these cells and also reduced the number of colonies in an anchorage-independence assay. A similar study overexpressing ER $\beta$ 1 in MCF-7 cells also reported growth inhibition by ER $\beta$ 1 and reduced tumor formation in a mouse xenograft model [47]. ER $\beta$ 1 overexpression was also shown to inhibit E2-triggered stimulation of estrogen-responsive T47D cells, an effect accompanied by decrease of Cyclin A2 and E mRNA levels, and also reduced angiogenesis and growth of T47D breast cancer xenografts [48, 49]. Other studies also suggested that antitumoral effects of ER $\beta$  are not necessarily dependent on the presence of ER $\alpha$  [37, 41]. Supporting these reports, in this study we demonstrate that ER $\beta$ 1 exerts antitumoral effects not only on hormone-dependent MCF-7 breast cancer cells, but also on ER $\alpha$ -negative SK-BR-3 cells.

Recent studies suggest that many genes which are regulated by ER $\alpha$  are also regulated by ER $\beta$  in various tissues, but it is becoming increasingly clear that ER $\beta$  is also able

to counteract ER $\alpha$ -triggered gene activation in some settings and also exerts specific gene regulation [50, 51]. A study examining the impact of ER $\beta$  on gene networks regulated by estrogen receptor alpha in breast cancer cells revealed that ER $\beta$  had diverse effects on gene expression, enhancing or counteracting ER $\alpha$  regulation for distinct subsets of estrogen target genes. Whereas ER $\beta$  in the absence of E2 elicited the stimulation or suppression of many genes that were normally only regulated by E2-triggered ER $\alpha$  activation, in the presence of this steroid ER $\beta$  elicited the expression of a unique group of genes that were not regulated by E2-triggered ER $\alpha$  activation [52]. Another recent study examining the effect of ER $\beta$  overexpression on transcriptome of ER $\alpha$ -positive T-47D breast cancer cells identified a subset of 14 DNA replication and cell-cycle related genes to be down-regulated by ER $\beta$  [53]. In breast cancer cells, E2 is known to regulate expression of key cell cycle genes such as c-Myc, cyclin D1, cyclin E, cyclin A, cdc 25A, p45(Skip12) and p27(Kip1) [54–56]. The cyclin D promoter is one site where ER $\beta$  opposes ER $\alpha$ -mediated activations and the ER $\beta$ -antiestrogen complex can stimulate transcription [57]. Cyclin D transcription is known to be reduced by antiestrogens and its overexpression leads to resistance to antiestrogens [58, 59]. Cyclins E and A are important later in the G1 phase of the cell cycle when they participate in activation of CDK2, a crucial step in moving the cell into the S phase of cell cycle [60]. Cell cycle inhibitor and tumor suppressor gene p21 (WAF1) previously also has been demonstrated to be an important mediator of cellular estrogen response [61].

To analyse the molecular mechanisms underlying the observed alterations in proliferation and apoptosis of MCF-7 and SK-BR-3 cancer cells overexpressing ER $\beta$ 1, we examined expression of a set of 14 estrogen-responsive genes on mRNA level. Overexpression of ER $\beta$ 1 in MCF-7 cells previously was reported to inhibit proliferation by repressing c-myc, cyclin D1 and cyclin A expression and by induction of antiproliferative p21(WAF1) and p27(Kip1) leading to a G<sub>2</sub> cell cycle arrest (Paruthiyil et al. 2004). Corresponding to these data, we observed both inhibition of E2-triggered upregulation of cyclin D1, cyclin A2 and PR in ER $\beta$ -overexpressing MCF-7 cells and ligand-independent decrease of cyclin D1 and increase of p21(WAF1) transcript levels. Our data also demonstrate ligand-independent inhibition of cyclin D1 and A2 expression and upregulation of p21(WAF1) in ER $\beta$ 1-overexpressing SK-BR-3 breast cancer cells suggesting that ER $\beta$ 1-triggered cell cycle arrest can also occur in an estrogen- and ER $\alpha$ -independent manner. Thus, gene regulation of cyclin D1, cyclin A2 and p21(WAF1) can be suggested to be important molecular mechanisms underlying the observed growth inhibitory effect of ER $\beta$ 1 both in ER $\alpha$ -positive MCF-7 and in ER $\alpha$ -negative SK-BR-3 breast cancer cells. In contrast,

overexpression of the exon-deleted ER $\beta$  variants did not significantly change mRNA levels of these cell cycle regulators, an observation explaining the lack of antiproliferative effects of these variants when overexpressed in breast cancer cells. PR gene transcript levels were not affected by ER $\beta$ 1 overexpression in a ligand-independent manner, but ER $\beta$ 1 blocked E2-triggered upregulation of this steroid hormone receptor, supporting the hypothesis that ER $\beta$ 1 can modulate gene regulation both by ligand-dependent and ligand-independent mechanisms. In line with previous reports [58, 59], treatment of MCF-7, but not SK-BR-3 cells with tamoxifen resulted in inhibition of E2-triggered increase of cyclin D1 and PR mRNA levels. Tamoxifen effect on expression of these genes was not affected by overexpression of ER $\beta$ 1 or the splice variants. In contrast, this drug did not affect p21(WAF1) mRNA levels and decreased cyclin A2 transcript levels only in MCF-7 cells overexpressing ER $\beta$ 1 or the  $\delta$ 125 variant, an effect obviously too small to bear on growth of these cells. Whereas DPN or PPT did not affect gene expression in SK-BR-3 cells, both substances triggered increased transcript levels of cyclins A2 and D1 and PR and decreased p21 (WAF1) mRNA levels both in MCF-7 control cells and cells overexpressing one of the exon-deleted ER $\beta$  variants in a manner very similar to the observed E2 effect suggesting that the levels of ER $\alpha$  and ER $\beta$ 1 present in these cells are sufficient to mediate agonistic effects on expression of these genes. The fact that DPN does not decrease cyclin D1 and A2 transcript levels in wildtype MCF-7 cells suggests that the inhibitory effect of ER $\beta$ 1 on cyclin expression is dependent on higher ER $\beta$ 1 expression levels. In contrast, treatment with these drugs did not increase mRNA levels of cyclins or PR in MCF-7 cells overexpressing ER $\beta$ 1, also resembling the observed E2 effects. However, why overexpression of ER $\beta$ 1 in breast cancer cells did not lead to stronger specific effects of DPN on gene expression in MCF-7 or SK-BR-3 cells remains unclear.

Further examining the molecular mechanisms underlying the observed differences between ER $\beta$ 1 and the exon-deleted isoforms in regulation of gene expression, we analysed ERE activation in MCF-7 cells. Our data demonstrating a drastical inhibition of E2-, DPN- and PPT-triggered ERE activation by ER $\beta$ 1 overexpression, but not by overexpression of ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 confirms the inability of the exon-deleted isoforms to affect ERE activity. Our observation that DPN is able to trigger notable ERE activation in MCF-7 wildtype cells expressing low levels of ER $\beta$ 1, whereas higher levels of ER $\beta$ 1 do not mediate this DPN effect suggests that DPN effects on ERE activity in this cell line are strongly dependent on ER $\beta$ 1 expression level.

Selective estrogen receptor modulator (SERM) tamoxifen is known to exert antitumoral effects on breast cancer

cells both by inhibition of proliferation and by induction of cellular apoptosis. Whereas ER $\alpha$  is an established molecular marker for success of tamoxifen therapy, it remains controversial whether ER $\beta$  also is a predictor of endocrine therapy responsiveness in human breast cancer. Whereas tamoxifen binding to ER $\alpha$  is known to act antagonistically both at estrogen response elements (EREs) and AP-1 sites, binding of this drug to ER $\beta$  has agonistic effects on AP-1 sites. Our data showing no effect of ER $\beta$ 1 or the novel isoforms on antiproliferative tamoxifen action in MCF-7 cells are in line with single clinical studies reporting no relevance of this receptor for endocrine responsiveness [25]. The observed slightly enhanced antiproliferative effect of tamoxifen on ER $\beta$ 1-overexpressing SK-BR-3 cells and the elevated apoptotic effect of this drug both on MCF-7 and SK-BR-3 cells overexpressing ER $\beta$ 1, however, would support the majority of clinical studies reporting that patients with positive ER $\beta$ -status show better response to tamoxifen therapy [16, 20, 62]. Our results suggesting that ER $\beta$ 1 has a stronger effect in potentiating tamoxifen-induced growth inhibition and apoptosis in ER $\alpha$ -negative SKBR-3 than in ER $\alpha$ -positive breast cancer cells also support a recent study showing a better response after adjuvant tamoxifen for ER $\beta$ -positive patients within the ER $\alpha$ -negative group than among ER $\alpha$ -positive patients [63]. If this holds true it would support the hypothesis that ER $\alpha$ -negative but ER $\beta$ -positive breast cancer patients could benefit from adjuvant tamoxifen.

In this study, we analyzed the function of two exon-deleted ER $\beta$  splice isoforms recently identified by our group in comparison to ER $\beta$ 1 in breast cancer cell lines with different ER $\alpha$  status. Overexpression of ER $\beta$ 1, but not of the exon-deleted variants exerted strong antitumoral effects both on ER $\alpha$ -positive MCF-7 and ER $\alpha$ -negative SK-BR-3 cells. Whereas ER $\beta$ 1 overexpression slowed growth of MCF-7 and SK-BR-3 cells in the absence of E2 and also inhibited E2-triggered growth stimulation of MCF-7 cells, overexpression of the exon-skipped variants did not affect cell growth. Whereas overexpression of ER $\beta$ 1 triggered a slightly increased antiproliferative effect of tamoxifen on SK-BR-3 cells and an increased basal apoptosis of MCF-7 and SK-BR-3 cells, an effect also present under tamoxifen treatment, the isoforms ER $\beta\delta$ 125 or ER $\beta\delta$ 1256 did not affect cellular tamoxifen response. The observed lack of function of the exon-deleted variants in terms of regulation of proliferation was accompanied both by their inability to affect expression of cyclins D1 and A2, p21 (WAF1) and PR and their disability to modulate ERE activation. In contrast, our results demonstrating antitumoral effects of ER $\beta$ 1 on breast cancer cells with different ER $\alpha$ -status support the hypothesis that this receptor also is able to act as a tumor suppressor in an ER $\alpha$ -independent manner.



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