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Estrogen receptor α attenuates transforming growth factor- β signaling in breast cancer cells independent from agonistic and antagonistic ligands

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Abstract To investigate a presumed crosstalk between estrogen receptor α (ER α) and the TGF- β signaling pathway in breast cancer, we analyzed the TGF- β -induced expression of the plasminogen activator inhibitor 1 (PAI-1) gene in ER-positive MCF-7 cells. After siRNA-mediated knock-down of endogenous ER α , the transcription level of PAI-1 was upregulated, pointing to an attenuation of TGF- β signaling by the presence of ER α . We verified these findings by a vice versa approach using a primary ER-negative cell model transiently overexpressing either ER α or ER β . We found that ER α , but not ER β , led to a strong inhibition of the TGF- β 1 signal, monitored by TGF- β reporter assays. This attenuation was completely independent of receptor stimulation by β -estradiol (E2) or inhibition by the pure antagonist ICI 182.780 (ICI). Our results indicate a permanent repression of PAI-1 by ER α and suggest a ligand-independent crosstalk between ER α and TGF- β signaling in breast cancer cells.

Keywords Transforming growth factor β ·
Estrogen receptor · Crosstalk

Introduction

Breast cancer is the most common cancer in women and is characterized by several, highly variable oncogenic stadia

relating to clinical, pathological, and molecular parameters. These different phases are defined by rearrangements of therapeutic marker patterns as well as alterations in response to chemo- and endocrine therapy [1, 2]. Reproductive hormones, particularly estrogens, are major key factors in breast cancer etiology and progression, consequently resulting in estrogen receptors (ER) being an important target for anti-cancer drug therapy. Moreover, the ER status is a basic prognostic marker for primary invasive breast cancer and an indicator for an individual hormonal therapy [1]. The ER isoforms ER α and ER β are members of the nuclear receptor superfamily and products of distinct genes [3]. In breast cancer, ER α plays an important role as a proliferative agent, thus determining tumor progression. The later identified ER β fulfills overlapping but also unique tasks and, in contrast to the ER α isoform, its function in breast cancer is not clear yet. The classical mechanism of ER action starts with ligand recognition and leads to DNA binding of the receptor to estrogen response elements (ERE) located in estrogen-responsive genes. This DNA-protein interaction also comprises the dimerization of ERs and an induction of conformational changes of the molecules which allows co-activator proteins to be recruited [4]. Besides, there exists an alternative mode of action of nuclear receptors, referred to as crosstalk. The underlying mechanism is predominantly based on protein–protein interactions, whereas DNA binding appears to be secondary, as only one recognition site in the target gene for one of the factors is sufficient. This ER containing protein complex may then act as a positive or negative regulator of transcription [5].

4-OH-tamoxifen and ICI 182.780 (ICI, Fulvestrant, Faslodex), the most commonly used antiestrogens, block estrogen-stimulated tumor growth and have demonstrated efficacy for treatment and prevention of ER-positive breast

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cancer [1]. In addition to the direct antagonistic effects of antiestrogen treatment, we have formerly shown that antiestrogens also induce an intensified secretion and activation of TGF- β [6]. This multifunctional cytokine plays a dual role in tumorigenesis, reflected by the two opposing properties of growth inhibition and tumor promotion [7]. The antiproliferative and thereby tumor suppressive character is based on mechanisms involved in cell cycle regulation, differentiation, cell proliferation, genome stability, suppression of telomerases, senescence, and apoptosis [8]. In the later stages of breast cancer, TGF- β may lose this potential and shift to a tumorigenic phenotype [9]. In this state, the activities of TGF- β are mainly characterized by growth stimulation, invasiveness, and metastasis. Even though the cytokine is in the focus of numerous studies, neither the initial trigger nor the underlying molecular mechanisms of this change are well understood.

Previous studies of our group emphasized the crucial role of TGF- β signaling in antiestrogen therapy and in the progression of breast cancer in general [6, 10–13]. By Kaplan–Meier analysis, we identified a correlation between the expression of TGF- β receptor II and a highly reduced overall survival in ER-negative breast cancer patients [14]. These findings indicate a coupling of both groups in a functional network and strongly presume a crosstalk between ER α and components of the TGF- β pathway in breast cancer cells. In this work, we established a cell model to investigate the putative crosstalk between ER and TGF- β signaling. We could show a ligand-independent influence of ER α on TGF- β 1 signaling mediated by Smad3 and c-fos.

Materials and methods

Cell culture

The ER-positive human breast cancer line MCF-7 and the well known ER α - and ER β -negative epithelial cancer line MDA-MB-435 [15] were propagated in DMEM containing 4.5 g of glucose/l (Invitrogen, Carlsbad, CA) supplemented with 1 mM sodium pyruvate (Invitrogen), 50 μ g/ml gentamicin (Invitrogen), and 10% fetal calf serum (FCS, Sigma–Aldrich, Deisenhofen, Germany). Cells were passaged twice per week. Before their use in experiments, cells were maintained for one passage in the same medium as described above but with 5% of steroid-depleted FCS (sulfatase and charcoal-treated FCS). All media contained phenol red, which is known to have a weak estrogenic effect [16].

Chemicals, plasmids, and siRNAs

β -estradiol (E2), ICI and purified human TGF- β 1 were obtained from Sigma–Aldrich, Tocris Bioscience (Bristol,

UK), and R&D Systems (Wiesbaden, Germany). ER α specific and c-jun specific antibodies were from Cell Signaling Technology (Danvers, MA), antibodies raised against Smad4 and c-fos were from Santa Cruz Biotechnology (Heidelberg, Germany), a Smad2 specific antibody from BD Biosciences (Erembodegem, Belgium), and an antibody against Smad3 from Invitrogen. The TGF- β -specific reporter plasmids p3TP [17] and p6SBE [18] were kindly provided by Jens Würthner (Macclesfield, UK) and Werner Hilgers (Paris, France). Expression constructs for the human ER isoforms (pER α , pER β) were generously provided by Francois Vignon (Montpellier, France) and Jan-Ake Gustafsson (Huddinge, Sweden). An expression vector for Smad4 (pSmad4) was a gift from Mark de Caestecker (Nashville, TN). The empty vectors pGL3-basic (Promega, Mannheim, Germany), phRL-TK (Promega), pcDNA3.1(+) (Invitrogen), pSG5 (Stratagene, La Jolla, CA), and pCMV5 [19] served as control plasmids.

An estrogen-specific reporter plasmid (pERE) was cloned by insertion of the estrogen response element (ERE) sequence into the luciferase vector pGL3-basic (Promega) by standard techniques. A PCR product was generated using self-hybridizing primers (restriction sites in lowercase, ERE-specific sequences in uppercase, hybridization sequences are underlined: ERE forward 5'-ctcagAGGTCACAGTGACCTAGGTCACAGTGACCT-3', ERE reverse 5'-aagcttATATACCCAGATCTAGGTCAGTGTGACC-3'). Expression plasmids for Smad3, c-jun, and c-fos were constructed by reverse transcription using an oligo-dT primer followed by a specific PCR (Smad3 forward 5'-CCATGTCGTCCATCCTGCCTTT-3', Smad3 reverse 5'-CTCGAGTTAAGACACACTGGAACAGCG-3', c-jun forward 5'-CACGTGAAGTGACGGACTGT-3', c-jun reverse 5'-TTTTTCTCTCCGTCGCAACT-3', c-fos forward 5'-CCTACCCAGCTCTGCTTCAC-3', c-fos reverse 5'-CACAGCCTGGTGTGTTTCAC-3'). The resulting PCR products were cloned into the Invitrogen plasmids pcDNA3-flag (pSmad3), pcDNA3.1(+) (pc-jun) and pcDNA3.1(-) (pc-fos), respectively. For expression of Smad2, we subcloned the open reading frame from the expression vector pCMV6-Smad2 (clone IMAGp998C076351, Imagenes, Berlin, Germany) into the vector pcDNA3 (Invitrogen). For ER α knock-down experiments, we used the ER α ShortCut siRNA Mix (New England Biolabs, Mannheim, Germany) and a Control siRNA (Qiagen).

Transfection experiments

One day before transfection, MCF-7 and MDA-MB-435 cells were seeded into 24-well culture plates at a density of 5×10^4 per well. Cells were transfected using Effectene Transfection Reagent (Qiagen) with a total amount of 0.2 or 1 μ g DNA. The 1 μ g approach contained 200 ng pERE

[pcDNA3.1(-)] or p3TP (pGL3-basic) or p6SBE (pGL3-basic) + 1 ng phRL-TK + 500 ng pER α (pCMV5) or pER β (pSG5). The 0.2 μ g approach consisted of 40 ng p3TP (pGL3-basic) + 5 ng phRL-TK + 100 ng pER α (pCMV5) + 60 ng pSmad2 (pcDNA3) or pSmad3 (pcDNA3) or pSmad4 (pcDNA3) or pc-jun [pcDNA3.1(+)] or pc-fos [pcDNA3.1(-)]. The total amount of DNA was kept constant by addition of the corresponding control vector DNA (control vectors are given in brackets). For real-time RT-PCR analysis, cells were seeded as described above. After 24 h, transfection was performed with 500 ng pER α DNA using Effectene Transfection Reagent (Qiagen). Knock-down experiments were carried out in MCF-7 cells that were plated with 5×10^4 cells per well in 24-well plates and incubated for 24 h at 37°C. Afterwards, cells were transfected using the RNAiFect Transfection Reagent (Qiagen) and ER α -specific siRNA (ER α siRNA) or control siRNA (Con siRNA) at a final concentration of 50 nM. At the time of transfection, cells were also treated with various substances as indicated in the results part.

Luciferase assay

After 48 h of transfection, cells were harvested. Luciferase activity was measured on an AutoLumat Plus (Berthold Technologies, Bad Wildbad, Germany) using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturers instructions. Co-transfection of the *Renilla* luciferase vector phRL-TK was used as an internal control. Transfections were done in triplicates and repeated at least thrice in independent experiments.

Western blot analysis

For verification of the protein composition, cells were lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 10 μ l/ml Protease Inhibitor Cocktail Set III (Merck, Darmstadt, Germany). Protein concentration was determined using Bradford Reagent (Bio-Rad, Munich, Germany). Subsequently, equal amounts of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl, 5% nonfat dry milk, and 0.05% Tween 20, the transferred proteins were incubated with a primary antibody over night, followed by incubation with a peroxidase-conjugated secondary antibody for 1 h. Visualization of the proteins was carried out by chemoluminescence using the Phototope-HRP Western Blot Detection System (Cell Signaling).

Quantitative RT-PCR analysis

Quantification of target mRNAs from transfected cells was performed by RT-PCR, monitoring the increase in fluorescence of the SYBR Green dye (Roche Applied Science, Mannheim, Germany) on a Light Cycler (Roche Applied Science) in real time. 24 h after transfection, cells were harvested and total RNA was isolated with the RNeasy Mini Kit (Qiagen) as described by the supplier. Reverse transcription was performed with 500 ng of total RNA and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using an oligo-dT primer. Sequences of primers were as follows: PAI-1 forward 5'-TGCTGGTGAATGCCCTCTACT-3', PAI-1 reverse 5'-CGGTCATTCCCAGGTTCTCTA-3', ITGB5 forward 5'-CTGGAACAACGGTGAGATT-3', ITGB5 reverse 5'-CCATCTTGGCAGGTAGCAGT-3', TIMP-1 forward 5'-CTGTTGTTGCTGTGGCTGATA-3', TIMP-1 reverse 5'-CCGTCCACAAGCAATGAGT-3', GAPDH forward 5'-CGGAGTCAACGGATTTGGTCGTAT-3', GAPDH reverse 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Statistical analysis

Data are given as mean \pm SEM. Statistical comparisons were performed by unpaired Student's *t* test. A value of $P < 0.05$ was considered significant.

Results

Knock-down of ER α in ER-positive MCF-7 cells by siRNA enhanced PAI-1 gene expression

According to our preclinical observations we postulated an interference of ER α with components of the TGF- β signaling pathway [14]. To verify this hypothesis, we carried out knock down experiments using ER-positive breast cancer cells MCF-7. Endogenously expressed ER α was downregulated by ER α specific siRNA molecules and the efficiency of the ER α knock-down was controlled by western blotting. Transfection of MCF-7 cells with ER α siRNA led to a clear reduction of the ER α protein level in contrast to the cells transfected with a non-silencing control siRNA (Fig. 1a).

The interference of ER α with the TGF- β signal was measured by RT-PCR quantification of the plasminogen activator inhibitor 1 (PAI-1) mRNA level, a TGF- β -dependent protein. Analysis of mock transfected cells treated with 10^{-10} M TGF- β 1 revealed a four-fold increase of the PAI-1 mRNA level when compared with vehicle treated cells (Fig. 1b) reflecting a predicted response of this

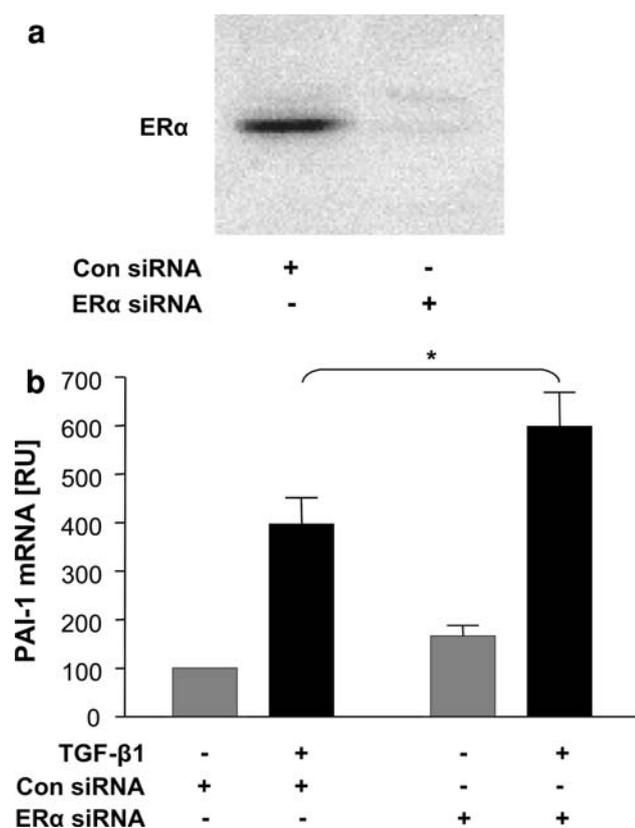


Fig. 1 Effect of ER α knock-down on TGF- β 1 signal transduction. For knock-down experiments MCF-7 cells were transiently transfected with Con siRNA or ER α siRNA and treated with 10^{-10} M TGF- β 1 or vehicle. **a** 24 h after transfection, cells were lysed and obtained lysates were analyzed by Western Blotting to monitor the silencing efficiency. **b** 24 h after transfection, cells were harvested for RNA preparation followed by cDNA synthesis. The amount of PAI-1 mRNA was measured by real-time RT-PCR and normalized to PCR standards with known DNA concentrations. The value of vehicle treated cells was set to 100. All values represent means \pm SEM of at least three independent experiments. * $P < 0.05$

TGF- β target gene in ER α positive cells. After siRNA knock-down of ER α , the stimulation of PAI-1 scaled up six-fold when compared with BSA treated cells representing a significant difference between ER α positive MCF-7 cells and siRNA-mediated ER α knock-down cells ($P = 0.0396$). These results strongly verified our hypothesis of an attenuating effect of ER α on the TGF- β 1 response. The house-keeping gene GAPDH mRNA level was not influenced by TGF- β 1 stimulation and furthermore was unaffected by transfection with siRNA (data not shown).

Generation and characterization of a model system appropriate to study the ER/TGF- β crosstalk

To avoid well known difficulties of simultaneously transfected siRNA and DNA molecules, we designed a model cell system of primary ER-negative cells transiently

overexpressing both ER isoforms. Cells were transfected with either pER α or pER β for overexpression of the receptors and transiently expressed ER α was detected by Western Blotting (Fig. 5) and reporter assays (Fig. 2a, c). The detection of ER β was restricted to the specific response to the pERE reporter plasmid (Fig. 2b). The biological activity of the ectopic expressed proteins was measured by a co-transfected, ERE containing reporter plasmid pERE (Fig. 2a), reflecting the pharmacological response to E2. Both proteins exhibited full transcriptional functionality and a weak basal activity which could be stimulated in a dose dependent manner by E2. ER α activity reached a plateau at 10^{-11} M, ER β activity at 10^{-9} M E2 (Fig. 2a, b). The respective E2 concentrations were used in the following experiments to ensure full transcriptional activity of each receptor. The basal activity of overexpressed ER α could be completely blocked by addition of the antiestrogen ICI at a concentration of 10^{-9} M (Fig. 2c). This concentration was also used in the following experiments to study effects of ER protein, independent of ER-driven transcriptional activity. In all cases, mock transfected cells showed no significant pERE activity.

Expression of ER α but not ER β diminished the TGF- β 1 signal of a reporter plasmid

We investigated the effect of ER α and ER β expression on TGF- β signaling by analyzing the activation of the two established TGF- β dependent reporter vectors p3TP and p6SBE. The plasmid p3TP was constructed using a part of the promoter region of the TGF- β target gene PAI-1 [17]. Experiments were conducted in the presence of the above mentioned E2 concentrations.

In MDA-MB-435 cells, the intrinsic p3TP-driven luciferase activity was increased five-fold by treatment with 10^{-10} M TGF- β 1. Transient overexpression of ER α significantly reduced this activation to only two-fold ($P = 0.0328$, Fig. 3a). Remarkably, the TGF- β 1-mediated activity in the presence of ER α was suppressed to a level similar to the basal TGF- β 1 response in the absence of the receptor. The TGF- β 1 response of unstimulated but ER α transfected MDA-MB-435 cells, however, was slightly lower than in control vector transfected cells.

In MCF-7 cells with endogenous ER α expression, basal activity of p3TP was three-fold lower than in ER α negative MDA-MB-435 cells (Fig. 3b). p3TP was induced two-fold by addition of TGF- β 1. Transient overexpression of ER α significantly reduced the basal activity ($P = 0.0489$) and completely abrogated the TGF- β 1 dependent induction of p3TP ($P = 0.0378$). The reporter plasmid activities of both unstimulated and TGF- β 1 induced MCF-7 cells transiently overexpressing ER α were clearly reduced when compared with mock transfected cells.

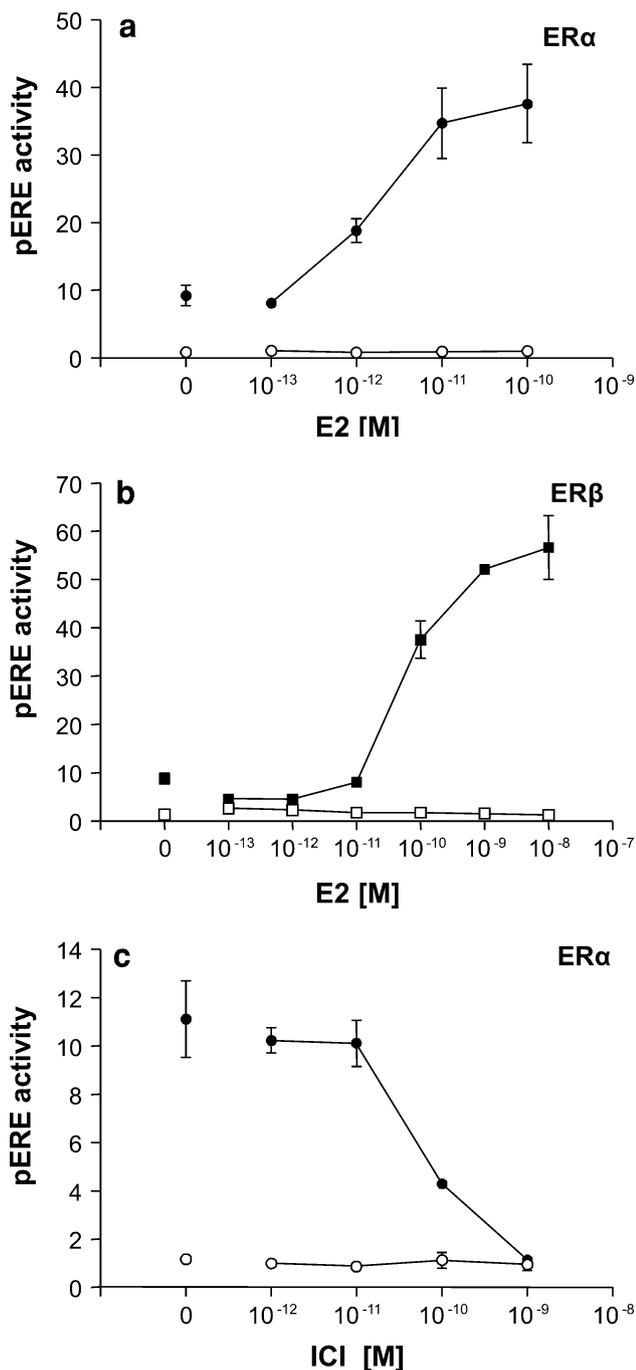


Fig. 2 Transcriptional activity of ER α and ER β in MDA-MB-435 cancer cells. ER α and ER β negative MDA-MB-435 cells were transiently transfected with expression vectors for ER α (●) or ER β (■) or empty control vectors (○ and □, respectively). Cells were co-transfected with a reporter vector containing an ERE (pERE) and, incubated with varying concentrations of E2 or ICI. 24 h after transfection, cells were harvested and assayed for luciferase activity. pERE activity is given in arbitrary units (firefly luciferase activity normalized to *Renilla* luciferase activity). All values represent means \pm SEM of at least three independent experiments. **a** Transiently ER α expressing MDA-435 cells treated with E2. **b** Transiently ER β expressing MDA-MB-435 cells treated with E2. **c** Transiently ER α expressing MDA-MB-435 cells treated with ICI

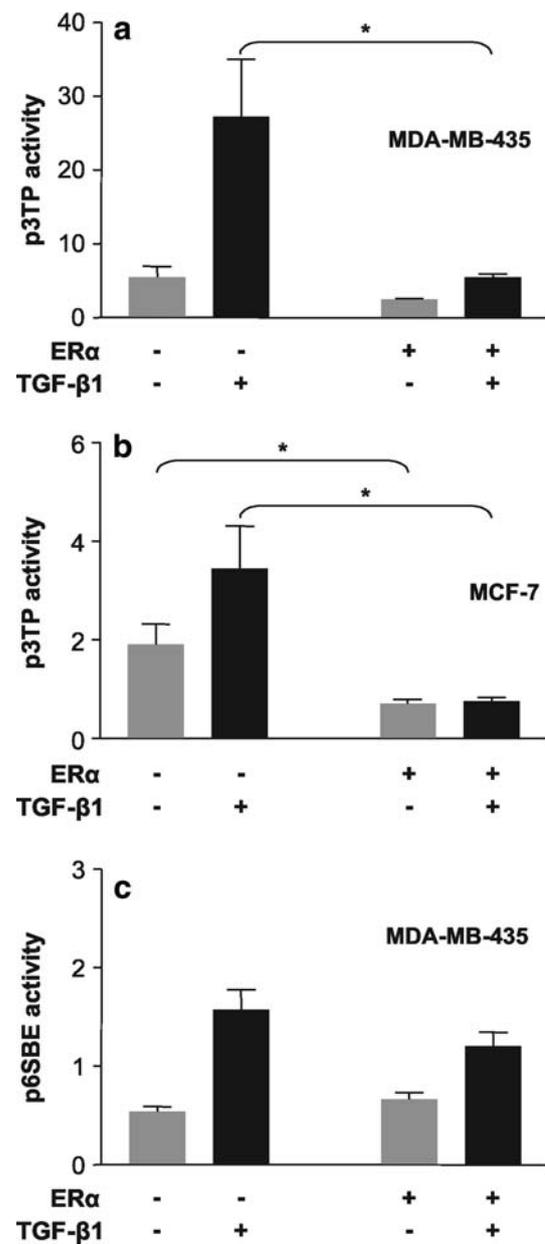


Fig. 3 Effect of ER α expression on TGF- β 1 signal transduction. MDA-MB-435 or MCF-7 cells were transiently transfected with p3TP or p6SBE and treated with 10⁻¹⁰ M TGF- β 1 or vehicle for 24 h. For analysis of ER α effects on TGF- β 1 signal transduction, an expression vector for ER α or control vector was co-transfected. For full ER α transcriptional activity all experiments were carried out in the presence of 10⁻¹¹ M E2. 24 h after transfection, cells were harvested and assayed for luciferase activity. p3TP and p6SBE activity is given in arbitrary units (firefly luciferase activity normalized to *Renilla* luciferase activity). All values represent means \pm SEM of at least three independent experiments. * $P < 0.05$. **a** p3TP activity in MDA-MB-435 cells. **b** p3TP activity in MCF-7 cells. **c** p6SBE activity in MDA-MB-435 cells

The TGF- β reporter plasmid p6SBE was activated three-fold in MDA-MB-435 cells by stimulation with TGF- β 1 (Fig. 3c). A slight attenuation of this induction by

overexpressing ER α was statistically not significant. Expressed in MCF-7 cells, p6SBE showed no induction by TGF- β 1, and thus, no effect of overexpressed ER α was detectable (data not shown).

Overexpression of ER β had no effect on the TGF- β 1 dependent induction of p3TP, neither in MDA-MB-435 cells, nor in MCF-7 cells (data not shown).

ER α -mediated down-regulation of the TGF- β 1 signal affects TGF- β target genes regulated by members of the Smad and AP-1 transcription factor families

For a more detailed characterization of TGF- β 1 signaling, we studied the influence of transiently overexpressed ER α on TGF- β target genes which are known to be co-activated by different sets of transcription factors. PAI-1 [9], integrin β 5 (ITGB5, [20]), and the tissue inhibitor of metalloproteinase 1 (TIMP-1, [21]) were shown to be regulated by TGF- β 1, whereas GAPDH was used as a TGF- β independent gene.

Real-time RT-PCR examinations of TGF- β 1 stimulated MDA-MB-435 cells overexpressing the receptor exhibited an upregulation of all three TGF- β target genes, expressed as relative activations (TGF- β 1 activated mRNA level per basal mRNA level). ITGB5 and TIMP-1 showed a 1.5- to 2-fold activation (Fig. 4b, c), whereas PAI-1 was induced by a factor of 30 (Fig. 4a). As expected, the regulation of GAPDH was independent from TGF- β 1 and revealed no induction (Fig. 4d).

Overexpression of ER α caused a significant reduction of the PAI-1 mRNA level when compared with TGF- β 1 stimulated but non-transfected MDA-MB-435 cells ($P = 0.0489$, Fig. 4a). These findings are in accordance with the previously shown experiments (Figs. 1b, 3a, b). Regulation of PAI-1 gene expression after TGF- β 1 stimulation is mediated by Smad proteins and members of the AP-1 family (reviewed in [22]). In contrast, TGF- β -dependent transcription of the ITGB5 gene needs a collaboration of the transcription factors Smad and Sp1 [20]. This transcriptional system, however, was not influenced by ER α overexpression after TGF- β stimulation (Fig. 4b). TIMP-1 is transcribed by an interaction of AP-1 proteins and additional but non-Smad proteins [23], and was also not affected by the overexpression of ER α (Fig. 4c). The house-keeping gene GAPDH is known to be poorly regulated and is not regulated by TGF- β [24]. Consequently, the GAPDH mRNA level was not influenced by overexpression of ER α (Fig. 4d).

ER α -mediated down-regulation of the TGF- β 1 signal depended on regulatory sequences specific for Smad3 and c-fos

The observations above indicated that the inhibitory effect of ER α might be dependent on members of the Smad and

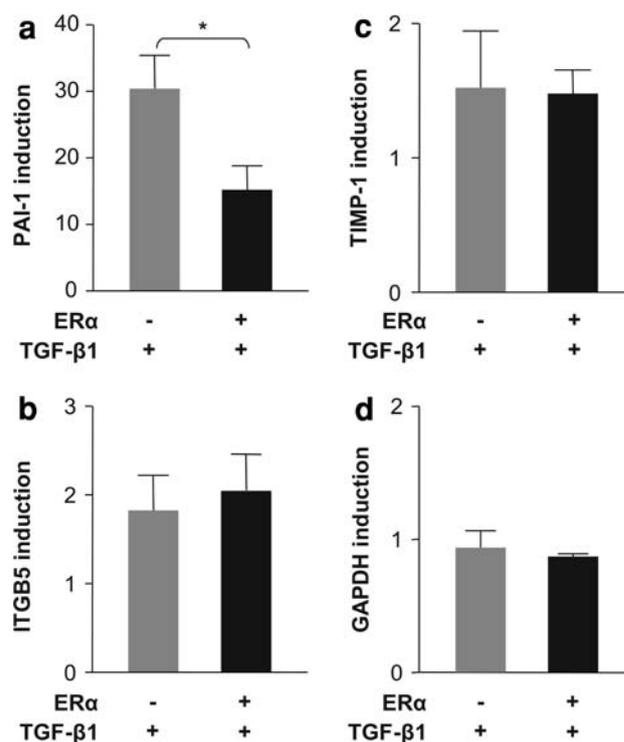


Fig. 4 Effect of ER α expression on TGF- β target gene expression. MDA-MB-435 cells were transiently transfected with an expression vector for ER α or control vector and treated with 10^{-10} M TGF- β 1 or vehicle. 24 h after transfection, cells were harvested for RNA preparation and cDNA synthesis. mRNA levels of the genes were detected by real-time RT-PCR and related to DNA standards of known concentrations. All values are expressed as relative units (RU: mRNA after TGF- β 1 stimulation/basal mRNA) and represent means \pm SEM of at least three independent experiments. * $P < 0.05$. **a** mRNA induction of PAI-1. **b** mRNA induction of ITGB5. **c** mRNA induction of TIMP-1. **d** mRNA induction of GAPDH

AP-1 transcription factor families. Until now, there was no evidence for an upregulation of TGF- β signaling proteins by ER α except for c-fos [25]. Thus, we examined the influence of overexpressed ER α on endogenously synthesized Smad and AP-1 proteins in the absence and presence of TGF- β 1 in MDA-MB-435 cells. Western Blot analysis showed no significant impact on Smad2, Smad3, Smad4 and c-jun expression, but an E2-driven upregulation of the c-fos protein level due to the transient expression of ER α (Fig. 5).

Subsequently, we transfected MDA-MB-435 cells to transiently express one of the Smad or AP-1 proteins beside the luciferase assay plasmids and the ER α expression vector. These co-transfection experiments were carried out using cells pre-incubated with E2 or ICI to determine a conceivable influence of the pharmacological activity of ER α . The overexpression of the transcription factors was confirmed by Western Blotting (data not shown).

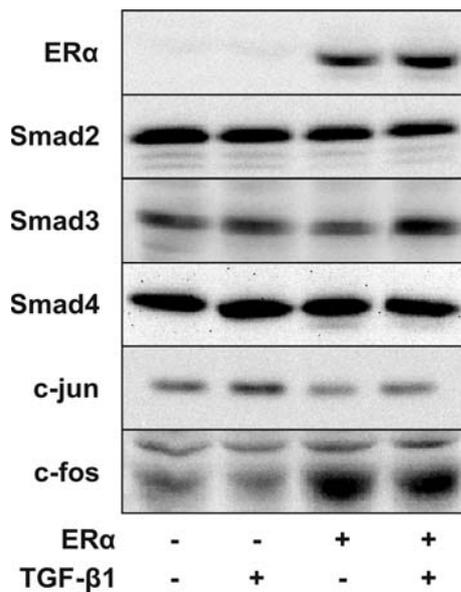


Fig. 5 Effect of ER α expression on Smad and AP-1 protein levels. MDA-MB-435 cells were mock transfected or transiently transfected with ER α and stimulated with 10^{-10} M TGF- β 1 or vehicle in the presence of 10^{-9} M E2 for 24 h. For protein analysis, cells were lysed and equal amounts of total protein were subjected to Western Blot analysis

In cells treated with 10^{-11} M E2, overexpression of Smad2 (Fig. 6a, column 5–8) showed no significant influence on TGF- β 1 response compared to control samples without overexpressed transcription factor (Fig. 6a, column 1–4). Neither the level of TGF- β 1 induction nor the effect of overexpressed ER α was significantly affected by transiently expressed Smad2. In contrast, after co-transfection of a Smad3 expression vector, the basal p3TP activity was significantly increased (three-fold, $P = 0.0016$, Fig. 6a, columns 1 and 9) and so was the activity after TGF- β 1 stimulation (four-fold, $P = 0.0010$, Fig. 6a, columns 2 and 10). By accessory overexpression of ER α , the TGF- β response was strongly reduced compared with the p3TP activity of ER α free cells ($P = 0.0060$, Fig. 6a, column 11 and 9). This activity was equal to the level of ER α , TGF- β 1 and Smad3 negative control cells (Fig. 6a, column 11 and 1). The Smad3 overexpressing TGF- β induced sample also showed an activity reduction comparable to the activity of untreated cells (Fig. 6a, columns 12 and 9). Briefly depicted, after co-transfection of a Smad3 expression vector, the TGF- β response showed almost the same distribution of p3TP activity as the mock transfected cells but on an approximately three-fold higher level (Fig. 6a, columns 9–12 compared to 1–4). As already shown for Smad2, co-transfection of a Smad4 expression vector revealed no statistically significant change of p3TP activity (Fig. 6a, columns 13–16 compared to 1–4).

Similar results were obtained after E2 and TGF- β 1 co-stimulation of AP-1 transfected cells. Overexpression of c-jun did not result in any significant activity shift in comparison with the control cells (Fig. 6c, columns 1–8). After co-transfection of a c-fos expression plasmid, the basal activity of the p3TP reporter was enhanced (three-fold, $P = 0.0133$, Fig. 6c, columns 1 and 9) and so was the TGF- β 1 induced activity (3.5-fold, $P = 0.0128$, Fig. 6c, columns 2 and 10). This increase was completely abolished after co-expression of ER α . The value without TGF- β 1 stimulation decreased to approximately one-third of the corresponding sample without ER α expression ($P = 0.0106$, Fig. 6c, columns 11 and 9) and the TGF- β induced activity of cells with overexpressed ER α was also reduced to the level of the unstimulated, ER α -free, c-fos expressing cells (Fig. 6c, columns 12 and 9).

Subsequently, we carried out the same set of experiments, this time inhibiting overexpressed ER α transcriptional activity by 10^{-9} M ICI. In Smad3 overexpressing cells, a significant increase of p3TP activity was detected compared with control cells (2.5-fold, Fig. 6b, basal activity $P = 0.0165$, columns 1 and 9, TGF- β 1 activation $P = 0.0337$, columns 2 and 10) as well as a reduction by ER α (Fig. 6b, untreated reduction $P = 0.0107$, columns 9 and 11, TGF- β 1 reduction to the range of ER α free activity, columns 10 and 12). Also in c-fos overexpressing cells, the increase of the TGF- β 1 response and the ER α -mediated switch of this TGF- β 1 signal could be observed (Fig. 6d, basal activity $P = 0.0027$, three-fold, columns 1 and 9, TGF- β 1 activation $P = 0.0038$, 3.5-fold, columns 2 and 10, untreated reduction $P = 0.0010$, columns 9 and 11, TGF- β 1 reduction to the range of ER α free activity, columns 10 and 12). Smad2 (Fig. 6b, columns 5–8), Smad4 (Fig. 6b, columns 13–16), and c-jun (Fig. 6d, columns 5–8) overexpression did not lead to significant alterations of the TGF- β response in cells transfected or not transfected with the ER α expression vector. Strikingly, the effect of pharmacologically inactive ER α was nearly the same as shown for E2-activated ER α , pointing to a mechanism independent of the formation of a ligand–receptor complex.

Discussion

To analyze a potential correlation between ER α and the TGF- β pathway, we transfected ER α -specific siRNA into ER α -positive MCF-7 cells and monitored the effect on the regulation of the TGF- β responsive gene PAI-1 [17]. Due to the decreased expression of ER α the PAI-1 gene was significantly upregulated. These data provide strong evidence that constitutively expressed ER α leads to a continuous repression of PAI-1.

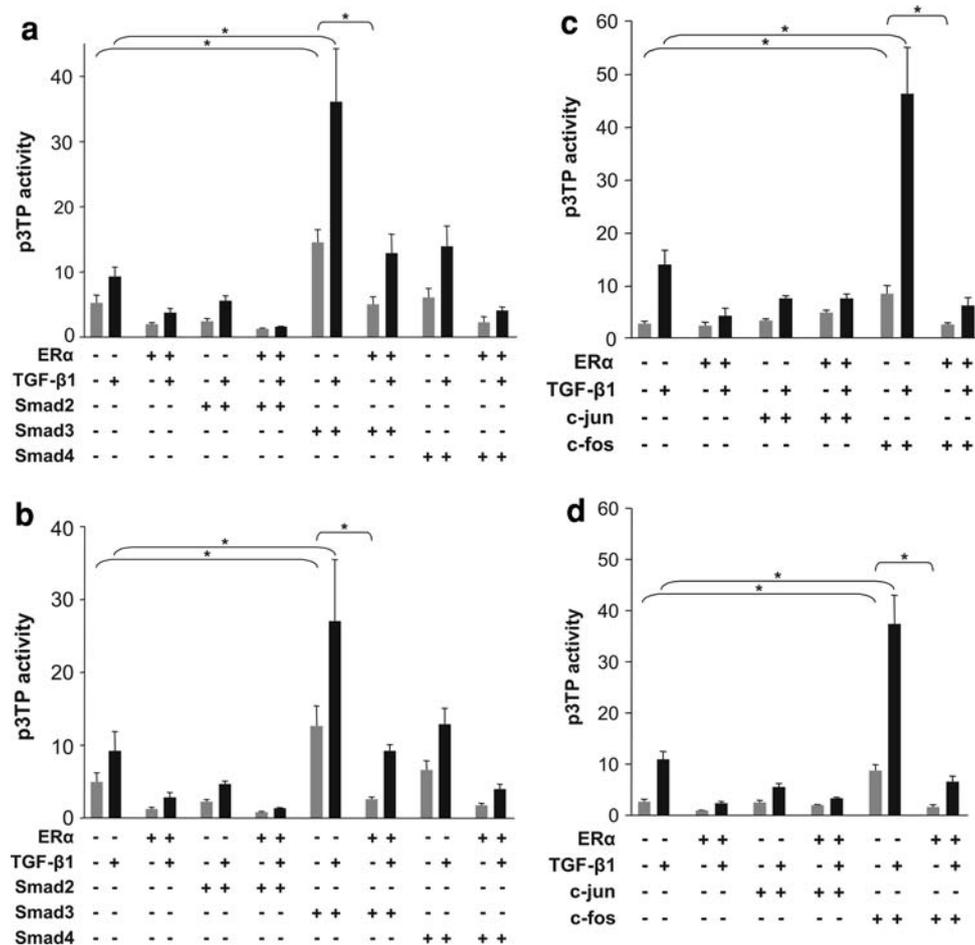


Fig. 6 Effect of ER α and transcription factor co-expression on TGF- β 1 signal transduction. MDA-MB-435 cells were transiently co-transfected with expression vectors for ER α or control vector and the transcription factors Smad2, Smad3, Smad4, c-jun, c-fos, or the corresponding control vectors. Transfected cells were treated with 10^{-11} M E2 or 10^{-9} M ICI. TGF- β 1 treatment and the luciferase assay were performed as specified in Fig. 2. All values represent means \pm SEM of at least three independent experiments. * $P < 0.05$. **a** Transient co-expression of ER α and the transcription factor Smad2,

Smad3, or Smad4 and treatment with 10^{-11} M E2 and 10^{-10} M TGF- β 1. **b** Transient co-expression of ER α and the transcription factor Smad2, Smad3, or Smad4 and treatment with 10^{-9} M ICI and 10^{-10} M TGF- β 1. **c** Transient co-expression of ER α and the transcription factor c-jun or c-fos and treatment with 10^{-11} M E2 and 10^{-10} M TGF- β 1. **d** Transient co-expression of ER α and the transcription factor c-jun or c-fos and treatment with 10^{-9} M ICI and 10^{-10} M TGF- β 1

We additionally confirmed this putative crosstalk with a vice versa approach using a model system of ER-negative cancer cells, transfected to transiently overexpress the ER isoform ER α or ER β . These transfected MDA-MB-435 cells were completely sensitive to agonist and antagonist treatment and maximal effects on ligand-dependent receptor activation or inactivation were achieved in concentration ranges similar to those determined in prior stimulation experiments using ER-positive MCF-7 breast cancer cells [13, 26]. Interestingly, these concentrations (10^{-11} M E2, 10^{-9} M ICI) also maximized the effects on growth induction or growth inhibition of untransfected MCF-7 cells [27, 28]. Our data showed an ER response of the cellular model system quite similar to established breast cancer cell lines naturally expressing ERs.

To study our previous observations in more detail, we used the p3TP reporter plasmid, which was derived from a PAI-1 TGF- β responsive sequence, and the reporter p6SBE. We have shown an interference of ER α with signaling components of the TGF- β system. While over-expressed ER α strongly reduced the TGF- β 1 signal in ER-negative MDA-MB-435 cells, ER β had no effect. Interestingly, Burdette and Woodruff [29] described a very similar effect of ER α protein to the activin signaling pathway by using the same TGF- β -sensitive reporter p3TP.

Still, the question remains why our reporter assays displayed ER α -sensitive signals using the p3TP construct while the p6SBE plasmid showed only a weak or actually no signal in MDA-MB-435 cells and MCF-7 cells, respectively. Both reporter plasmids contain different TGF- β -sensitive binding

sites. p3TP contains three 12-O-tetradecanoylphorbol 13-acetate (TPA) responsive elements (TRE) and a TGF β -specific promoter region of the PAI-1 gene [17], whereas the p6SBE reporter includes six sequential Smad binding elements (SBE) controlling a SV40 promoter [18]. We presume relevant differences in the transcription factor binding site setting of both constructs, leading to a more AP-1 controlled activation in case of the p3TP plasmid and a more Smad induced activation for the p6SBE construct. Depending on the cellular context these differences may draw a distinction in the cellular response after stimulation.

Subsequently, we could identify some players contributing to the ER α -TGF- β 1 interference. mRNA analysis of different TGF- β responsive genes showed a combination of Smad proteins, the primary signal transducers of TGF- β signaling [7], and AP-1 transcription factors being involved. More precisely, we identified the transcription factors Smad3 and c-fos as switch points of the TGF- β pathway. Both proteins led to an enhanced TGF- β 1 activity after overexpression, whereas Smad2, Smad4, and c-jun had no effect. Nevertheless, the stimulatory effect of Smad3 and c-fos diminished by ER α co-expression, demonstrating an interaction between the receptor and TGF- β 1 signal processing components.

Two types of interactions are conceivable. A molecular interaction between ER α and proteins involved in TGF- β signaling or regulation of gene expression by ER α . In the latter case one has to distinguish between a direct effect by downregulation of TGF- β signaling proteins or an indirect effect by upregulation of TGF- β inhibitory factors. Thus, a set of experiments was designed to differentiate between these two possible modes of action. Even though the recombinant ER α protein was transcriptionally competent, a significant role of the transcriptional activity of the receptor could be excluded. This was shown by the fact that the suppressor function of ER α overexpressed in our model MDA-MB-435 cells was insensitive to agonist and antagonist stimulation.

Several lines of evidence from other groups also suggest that a molecular interaction is responsible for this ER α function in MCF-7 cells. Qi and co-workers [30] found some hints on binding of ER α and c-jun as a suppressor of stress-induced cell death. Another group detected an *in vitro* binding of immobilized c-jun to recombinant ER α , simultaneously negating an interaction of ER α and c-fos [31]. Moreover, Matsuda et al. [32] demonstrated that ER α suppresses TGF- β signaling in the presence of estrogen by complex formation with Smad3, and Wu et al. [33] showed an ER α -Smad4 interaction. Nevertheless, there are some reports dealing with a repressor or co-repressor function of ER α on specific DNA sequences. Green and co-workers [34] postulate a mechanism of this ER α activity depending on antiestrogen binding and mediated by HDAC. This

explicit model is not consistent with our data, because the effect described in our study is independent of an antiestrogen impact. Several additional data on an ER α -mediated repressor function exist, but all of them are based on a ligand-bound state of the receptor (reviewed in [4]). Further experiments designed to investigate potential binding partners of ER α are necessary, taking into account both types of models, protein–protein as well as protein–DNA binding.

What is the potential role of both, ER α and TGF- β , embedded into the complex processes of breast cancer incidence and progression? With regard to breast cancer progression, PAI-1 as well as the transcription factors Smad3 and c-fos are known as pro-oncogenic regulators of invasive cell behavior and tumor metastasis [35, 36]. Our data provide strong evidence that constitutively expressed ER α leads to a continuous repression of PAI-1. This assumption is supported by retrospective studies showing a definite correlation between high amounts of ER α and low PAI-1 expression [35, 37]. The pro-invasive capacity of Smad3 is extensively reviewed by Roberts [38], and there is also evidence for a pro-metastatic potential of c-fos, demonstrated in hormone receptor negative breast cancer cells [39]. Both oncogenic proteins were strongly diminished in the presence of ER α . Thus, the anti-metastatic effect of ligand-free ER α might be mediated by knock-down of components of the TGF- β signaling pathway, namely by inhibition of the pro-invasive proteins PAI-1, Smad3, and c-fos. This mechanism also explains the role of TGF- β in ER α -positive and ER α -negative patients according to the overall survival, which we studied in 2004. The expression of TGF- β receptor type II in ER α -negative patients is correlated with highly reduced overall survival, whereas simultaneous loss of both ER α and TGF- β receptor type II is comparable with down-regulation of TGF- β signaling and was associated with longer overall survival [14]. Moreover, the inhibitory function of ER α is independent from the ligand-dependent receptor function and a loss of ER α would result in a poor prognosis, which actually was shown in several studies [1, 2, 40].

Our findings point to a possible mechanism which connects the level of ER α as a positive prognostic factor in breast cancer [2] with the protein level of PAI-1 defined as a marker for poor prognosis [35]. Both factors are negatively correlated. We suggest that, among other factors, a high level of ER α might implicate a good prognosis for a patient by a so far unknown mechanism of PAI-1 suppression. Foekens and co-workers [41] also discuss a relation between PAI-1 and resistance to tamoxifen therapy. The authors suppose a fine-tuned mechanism balancing the expression of ER α and PAI-1 [42]. PAI-1 is part of the urokinase-type plasminogen activator system consisting of urokinase type plasminogen activator (uPA), uPA receptor (uPAR), PAI-1, and PAI-2.

Activation of this system results in the activation of plasmin and subsequently in the activation of additional matrix metalloproteinases (MMP), finally leading to the degradation of the extracellular matrix. Thus, the uPA system is suspected to play a central role in metastasis [42]. It is known that TGF- β induces the expression of PAI-1 [43], which in turn is involved in activation and liberation of TGF- β [44]. In addition, TGF- β directly induces the expression of a MMP in fibroblasts [45]. In combination with antiestrogen induced TGF- β secretion [6], this regulatory network could precede tumor progression and a change to the invasive state.

One important finding of our study was that the TGF- β 1 signal strongly differs in the presence or absence of ER α , but not ER β . This conclusion, initially confirmed in breast cancer cells, was reproduced and explicitly studied in our epithelial cancer cell model and is most likely to execute a general mechanism in breast cancer progression. This influence on the TGF- β 1 activity, however, was neither regulated by E2 nor by receptor inactivation with the pure antagonist ICI. These interesting results clearly show that ER α exhibits an elementary regulatory impact on TGF- β signaling in breast cancer cells independent of pharmacological ligand-binding. Thus, a role of this additional ER α feature in the TGF- β switch-over from a tumor suppressor to a pro-oncogenic factor is conceivable. These findings might be a perspective for the design and development of peptide-based anti-tumor drugs, mimicking ER α protein binding capacities without ER α receptor functions. Pharmaceutical peptides could then be applied for prevention of breast cancer metastasis. Certainly, additional investigations to further depict the molecular mechanisms of this phenomenon and to explore the possible role of ligand-independent ER α action in antiestrogen resistance are necessary and still in progress.

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