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Estrogen receptor α regulates the expression of syndecan-1 in human breast carcinoma cells

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Abstract

Breast cancer (BC) is the primary cause of cancer-related mortality among women. Patients who express the estrogen receptor (ER), which mediates the tumorigenic effects of estrogens, respond to antihormonal therapy. Loss of ER expression or acquired resistance to E2 is associated with aggressive malignant phenotypes, which lead to relapse. These BC subtypes overexpress syndecan-1 (SDC1), a transmembrane heparan sulfate proteoglycan that mediates angiogenesis as well as the proliferation and invasiveness of cancer cells. We showed here that the activation of ER- α (ER α) by estrogens induces downregulation of SDC1 expression in ER(+) MCF7 cells but not in T47D cells. Loss of ER α expression, induced by RNA interference or a selective ER downregulator, led to subsequent SDC1 overexpression. E2-dependent downregulation of SDC1 expression required *de novo* protein synthesis and was antagonized by treatment with BAY 11-7085, an irreversible inhibitor of I κ B α phosphorylation, which inhibits the activation of NF κ B. Downregulation of SDC1 expression required ER α and activation of IKK, but was independent to downstream transcriptional regulators of NF κ B. BAY 11-7085 prevented E2-mediated phosphorylation of ER α on Ser118, increasing its proteasomal degradation, suggesting that IKK stabilized E2-activated ER α , leading to subsequent downregulation of SDC1 expression. Our results showed that sustained ER signaling inhibits SDC1 expression. Such antagonism elucidates the inverse correlation between SDC1 and ER expression in ER(+) BC as well as the overexpression of SDC1 in hormone receptor-negative BC subtypes with the most aggressive phenotypes. These results identify SDC1 as an attractive therapeutic target for BC as well as for other endocrine-associated cancers.

Key Words

- ▶ syndecan-1
- ▶ estrogen
- ▶ breast cancer
- ▶ MCF7

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Introduction

Breast cancer (BC) is a heterogeneous disease with distinct clinical outcomes which represents 25% of cancers of women worldwide (Torre *et al.* 2015). BC is the primary cause of cancer-related mortality among women (Torre *et al.* 2015) and are classified into subtypes according to the findings of histological, genetic and molecular analyses. Subtypes are defined according to the expression

of estrogen (ER) or progesterone receptors (PR), with or without the expression of human erb-b2 receptor tyrosine kinase 2 (HER2) (Malhotra *et al.* 2010). ER(+) tumors represent approximately 70% of BCs, indicating that tumor progression is mediated by estrogens (DeSantis *et al.* 2011).

The ligand-activated nuclear transcription factors ER α and ER β (encoded by ESR1 and ESR2, respectively) mediate

the biological functions of estrogens, directly through binding to estrogen response elements (ERE) of target genes or through binding to transcription factors such as AP-1, SP1 or NF κ B (Galien & Garcia 1997, Saville *et al.* 2000, O'Lone *et al.* 2004). ER activity is regulated as well by posttranslational modifications such as phosphorylation, acetylation, sumoylation or ubiquitination (Le Romancer *et al.* 2011). Estrogen markedly influences the patterns of gene expression of BC cells, and the expression of ERs, their functions or both contribute to tumorigenesis and tumor progression and therefore serve as targets for therapy and diagnostic markers (DeSantis *et al.* 2011). Although antiestrogens are used to treat BCs, they are not effective inhibitors of the proliferation of all ER(+) tumor cells. Thus, *de novo* or acquired resistance leads to the relapse of approximately 20–30% of patients. The triple-negative BC (TNBC) is the subtype that is the most resistant to therapies that target hormones (Castrellon 2017).

Complex interactions between the mitogenic signal transduction pathways and the components of the extracellular matrix govern the development and acquisition of resistance to cancer chemotherapy. Transmembrane heparan sulfate proteoglycan syndecan-1 (SDC1) is an essential component of the cellular microenvironment, and although it lacks catalytic activity, SDC1 mediates cytoskeletal organization as well as cellular adhesion, migration, proliferation and differentiation (Bernfield & Sanderson 1990). SDC1 may act as a coreceptor that regulates intracellular signal transduction pathways through binding the components of the extracellular matrix or growth factors (Multhaupt *et al.* 2009). Additionally, SDC1 translocates to the nucleus where it interacts with proteins such as transcription factors or histones to influence the expression of target genes (Brockstedt *et al.* 2002, Szatmári *et al.* 2012).

Evidence indicates that SDC1 expression is stringently regulated, and its deregulation contributes to numerous diseases, including cancer (Szatmári *et al.* 2015). SDC1 is overexpressed in the most aggressive subtypes of BC and is associated with tumor progression, angiogenesis, higher histological grade of tumors and shorter relapse-free survival (Maeda *et al.* 2006). Moreover, SDC1 is a marker of aggressiveness and chemoresistance of human BCs (Barbareschi *et al.* 2003, Götte *et al.* 2006). Interestingly, overexpression of SDC1 inversely correlates with ER expression (Barbareschi *et al.* 2003) and most ER(–) BCs, including the TNBC subtype, overexpress SDC1 (Baba *et al.* 2006).

SDC1 overexpression by the human granulosa tumor cell line KGN drastically affects estrogen synthesis and

signaling through transcriptional inhibition of the expression of CYP19A and ESR2 (Colombe *et al.* 2017). Considering these data, we hypothesized that antagonism between E2-mediated signaling and SDC1 metabolism may contribute to mammary tumorigenesis. To this end, we studied the regulation of SDC1 expression in response to estrogen stimulation in both ER(+) MCF-7 and T47D cell lines and in the ER(–) MDA-MB-231 mammary carcinoma cell line.

Materials and methods

Materials

DMEM, Ham-F12 medium and antibiotics were purchased from Dominique DUTSCHER SAS (Brumath, France). Goat polyclonal (AF2780) anti-human Syndecan-1 antibody was obtained from R&D Systems. ESR1-antibody (F10) was from Santa Cruz Biotechnology and anti-Actin (Ab-1) from Millipore. Fetal bovine serum (FBS), Opti-MEM, Lipofectamine RNAiMax, ECL were purchased from Thermo-Fisher Scientific. M-MLV-RT, RNasin, GoTaq qPCR Master-Mix were from Promega. Positively charged nylon membrane (Amersham Hybon-N+, GE Healthcare, Life Sciences). E2, propyl-pyrazole-triol (PPT), diaryl-propionitrile (DPN), G1, G15 and SP600,125, fluoroshield were from Sigma. Ly290,004, UO126, BAY 11-7085 (Bay) and MG-132 were purchase from Selleckchem.

Cell line and culture conditions

The human mammary adenocarcinoma cells MCF7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were obtained from the European Collection of Authenticated Cell Cultures (ECACC). T47D cells are generous gift from Dr Le Romancer (INSERM-U1052, Centre de Recherche en Cancérologie de Lyon, France). Cells were cultured in Ham's/DMEM (HD) medium (1:1/v:v) supplemented with 10% (w/v) FBS, 2mmol/L glutamine, antibiotics (50IU/mL penicillin, 50 μ g/mL streptomycin, 100 μ g/mL kanamycin) and 0.25 μ g/mL amphotericin B and incubated at 37°C with 5% CO₂.

Hormonal and chemical treatments

Before treatments cells were cultured during 5 days in DMEM without phenol red, supplemented with 4.5g/L glucose and 10% (w/v) of charcoal-treated steroid-free FBS (MEM) for steroid deprivation. Concentration of 17 β -estradiol (E2) ranging from 10^{–12}mol/L to 10^{–8}mol/L

was used, and 10 ng/mL TNF α during a time of incubation ranging from 30 min to 96 h. Anti-estrogen ICI182,780 (ICI) and 4-OH-tamoxifen (Tam) were used at 10⁻⁸ mol/L as well as selective estrogen agonist and antagonist (PPT, DPN, G1 and G15) or for kinase pathway inhibitors (H89, Ly290,004, UO126, SP600,125 and BAY 11-7085) a concentration of 10⁻⁵ mol/L. For experiments with cycloheximide (CHX; 10 μ g/mL), actinomycin D (ACT; 2 μ mol/L) cells were pre-incubated for 30 min and then further incubated with E2 for indicated period.

Small-interfering RNA studies

MCF-7 cells were seeded in 24-well plates in MEM without antibiotics 24 h before transfection. RelA-siRNA (5'-CCA-UCA-ACU-AUG-AUG-AGU-UdTdT-3'; 5'-AAC-UCA-UCA-UAGUUG-AUG-GdTdT-3'), ESR1-siRNA (5'-UCA-UCG-CAU-UCC-UUG-CAA-AdTdT-3'; 5'-UUU-GCA-AGG-AAU-GCG-AUG-AdTdT-3') and siRNA-negative control (Medium-GC duplex#2) were diluted in Opti-MEM medium and transfected using Lipofectamine RNAiMax. At 6 h after transfection, medium was renewed with medium-containing antibiotics.

RNA extraction and real-time RT-PCR

Total RNA (250 ng) was isolated using TRI-Reagent Kit and reverse transcribed during 1 h 30 at 37°C with 200 U M-MLVRT, 20 U RNasin, 0.5 μ g oligo dT and 100 μ mol/L dNTP following by 5 min at 65°C. Semi-quantitative PCR was performed with Mx3005P real-time PCR system (Agilent Technologies) and the detection was done by the fluorescent dye SYBRGreen with GoTaq qPCR Master-Mix.

Primer sequences are listed in Table 1. GAPDH mRNA level was used as an internal control for normalizing different samples. Cycle threshold values were normalized by housekeeping gene GAPDH. The relative mRNA expression level was calculated as fold changes relative to untreated cells using the comparative threshold cycle value ($2^{-\Delta\Delta CT}$).

Western blotting

Cell lysates were obtained after incubation of cell layer with SDS-lysis buffer (62.5 mM Tris-HCl pH 6.8; 2% (w/v) SDS, 10% (v/v) glycerol; 50 mmol/L DTT; 0.01 % (w/v) bromophenol blue). Total proteins were sonicated and denatured 5 min at 95°C before loading on SDS-PAGE and electro-transferred to Hybond-ECL nitrocellulose membrane or cationic Nylon membrane (Hybond-N+, GE Healthcare Life Sciences). Membranes were blocked in 5% nonfat dry milk in TTBS at RT for 1 h before probing with primary antibody (1:1000 in TTBS-1% BSA) overnight at 4°C. Membranes were washed with TTBS and then horseradish peroxidase-conjugated secondary IgG was used for detection. The signal was visualized with an enhanced chemiluminescence reaction kit (ECL), revealed by autoradiography and quantified by densitometry using ImageJ software. For loading control, the membranes were stripped and re-probed with mouse anti-actin antibody (Ab-1).

Confocal immunocytochemistry

Cells were grown on glass coverslips in 24-well plates, fixed with 4% paraformaldehyde and permeabilized with 100% methanol. Non-specific binding sites were blocked

Table 1 Primers used for real-time PCR.

Genes	ID	Primer set	Length (bp)
GAPDH	NM_002046	GAAGGTGAAGTCTGGAGTC GAAGATGGTGATGGGATTC	225
ESR1	NM_000125	AGACATGAGAGCTGCCAACC GCCAGGCATTCTAGAAGG	299
ESR2	NM_001437.2	GAGTCTGGTCGTGTGAAGGA ACTTCTCTGTCTCCGCACAA	218
GPER1	NM_001505	GCAGGTCCAGCAGAGGTACA GAGCTGCTCACTCTCTGGGT	128
IL6	NM_000600.4	TGGGAATCCAGTGTGTGAA CACAGCATTCAAGTCGTAGT	183
PGR	NM_001202474	TCGAGCTCAGCGTTTCTA CACCATCCCTGCAATATCT	183
RELA (p65)	NM_021975.3	GCCATGGACGAAGTGTCCCC TGGTATCTGTGCTCCTCTCG	166
SDC1	NM_001006946	GAAACCTCGGGGAGAAATAC TACAGCATGAAACCCACCAG	188

with PBS-5% FBS before overnight incubation with primary antibody (diluted 1:50 in PBS containing 0.3% Triton X-100 and 1% BSA) in a humidified dark chamber. After washing, AlexaFluor-conjugated secondary antibody (1:100) was added at RT for 1 h. Coverslips were mounted with Fluoroshield medium containing 4',6-diamidino-2-phenylindole (DAPI) for DNA counterstaining and cells were observed with a confocal laser scanning unit (FluoView-FV1000, Olympus). Immunofluorescence intensities were measured using FV10-ASW-1.7 Olympus software. For each assay, three fields/coverslip were analyzed containing around 40 individualized cells/fields and repeated for three independent experiments.

Statistical analysis

Data were analyzed using the Student's *t*-test or the one-way ANOVA followed by Dunnett's (for dose or time response) or Tukey's test (GraphPad Prism5 software) when appropriate. Data were expressed as the mean $\pm$ S.E.M. Differences were considered statistically significant at *P < 0.05; **P < 0.01; ***P < 0.001.

Results

E2 mediates the downregulation of SDC1 expression in MCF7 cells

MCF7 cells were treated for 24 h with increasing concentrations of E2 (10^{-12} – 10^{-8} mol/L) in phenol red-free medium supplemented with 10% charcoal-stripped FBS (MEM). E2 caused dose-dependent inhibition of SDC1 mRNA levels, reaching 70% inhibition at 10^{-10} mol/L (Fig. 1A). Similarly, progesterone receptor (PGR) expression was upregulated in a dose-dependent manner (Fig. 1A). E2-mediated repression of *SDC1* mRNA expression occurred after 12 h, consistent with a decrease of the immunocytochemical detection of SDC1 protein after 24 h (Fig. 1B and C). Such long-term regulation was confirmed by Western blot analysis (Fig. 1D). Downregulation of *SDC1* mRNA and SDC1 levels was detected in cells cultured in medium containing phenol red and untreated FBS (HD) (Fig. 1E and F). Upregulation of *PGR* expression under the same conditions confirmed the steroid-dependent inhibition of SDC1 expression.

Figure 1

Estradiol induces a dose- and time-dependent inhibition of SDC1 expression in MCF7 cells. (A) Expression of *SDC1* (and *PGR* in the insert panel) mRNA in MCF-7 cells cultured in estrogen-deprived medium (MEM-10% FBSdest) and treated for 24 h with increasing concentration of estradiol (10^{-12} – 10^{-8} mol/L) (*n* = 4). (B) Expression of *SDC1* mRNA measured after addition of E2 (10^{-8} mol/L) for increasing period of time (1–24 h). (C) Immunocytochemical observation of SDC1 expression using goat polyclonal anti-SDC1 (AF2780 from R&D) in cells treated without or with E2 (10^{-8} mol/L) during 24 h. In same experimental conditions, the content of SDC1 protein was estimated by Western blot (D) (C and D are representative results from three independent experiments). (E) Expression of *SDC1* and *PGR* measured by QRT-PCR in estrogen-deprived medium MEM or in medium containing phenol red and non-charcoal-treated FBS (HD) (*n* = 3). (F) Immunocytochemical observation of SDC1 expression after 48 h of cell culture in MEM or HD medium (*n* = 3) (scale bar = 50 μ m).

SDC1 expression by Western blot can give rise to variable results sometimes not enough demonstrative according to the protocol used (membrane used for blotting, preparation of protein extracts, antibody) but also due to the existence of glycosylated or multimeric form of protein. Furthermore, considering that the extracellular domain harbors attachment sites for heparan sulfate and chondroitin sulfate chains, molecular weight of SDC1 can range from 33 kDa for SDC1 core-protein to 130–200 kDa with GAGs chains. Specificity of the goat polyclonal anti-human SDC1 antibody (AF2780) was confirmed using MCF7 cells overexpressing SDC1 or in MCF7 cells transfected with an *SDC1*-siRNA, by immunocytochemistry and Western blot (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article). The correlation between SDC1 levels and intensity of fluorescence was further confirmed by fluorescence-activated cell sorter (FACS). Additionally, the heparinase I and III treatment of whole MCF7 cells extract reduced the molecular weight of SDC1 in Western blotting experiments onto positively charged nylon membrane and revealed with the anti-SDC1 antibody (AF2780) confirming definitely its specificity. However, after heparinase treatment the SDC1 band still appear as a smear because SDC1 also contains heterogeneous chondroitin sulfate chains which are not removed by such treatment.

All Western blot experiments have been done with nitrocellulose membrane and cationic nylon membrane (Supplementary Fig. 2). The upper band around 90 kDa was used for quantitative analysis.

Cell type-specific differential regulation of SDC1 expression by E2

The effects of estradiol were evaluated in the ER(+), luminal subtype-A T47D cell line and in the triple-negative cell line MDA-MB-231. Although MCF7 and T47D cells expressed similar levels of *SDC1* and *ESR1* mRNAs (Fig. 2A), estradiol induced a slight but dose-dependent increase in *SDC1* mRNA levels in T47D cells (Fig. 2B). In contrast, estradiol treatment did not affect *SDC1* mRNA levels in ER(–) MDA-MB-231 cells (Fig. 2B). Consistent with the findings that T47D cells express higher level of *PGR* than MCF7 cells (Fig. 2A), we found that progesterone (P4) increased *SDC1* mRNA expression in T47D cells but not in MCF7 cells (Fig. 2C). These data suggest that *SDC1* expression was not regulated by estradiol in ER(+)/PR(+) T47D cells. Therefore, we focused our attention on estrogen-sensitive MCF7 cells.

ER α is required for E2-mediated downregulation of SDC1 expression in MCF7 cells

MCF7 cells were treated with selective agonists of ER α , ER β and GPER (PPT, DPN and G1 at 10^{-8} mol/L, respectively). Only the ER α -agonist PPT mimicked the inhibitory effects of E2 on *SDC1* expression (Fig. 3A). Despite the absence of ER β (data not shown), inhibitory effects of DPN on *SDC1* expression were observed but judged as not significant, in agreement with the partial agonist effect of DPN on ER α (Harrington *et al.* 2003). We found that inhibition of *SDC1* expression did not require GPER signaling. In contrast, the ER-selective antagonist tamoxifen completely blocked the E2-dependent inhibition of *SDC1* mRNA and protein expression in MCF7 cells without affecting basal levels of expression (Fig. 3B and C), demonstrating that ER α

Figure 2

E2-dependent regulation of syndecan-1 expression in MCF7, T47D and MDA-MB-231 cells. (A) Relative expressions of *SDC1*, *ESR1*, *ESR2*, *GPER* and *PGR* in MCF7, T47D and MDA-MB-231 (MDA) cells cultured in MEM-10% FBSdest ($n = 3$). (B) Expression of *SDC1* in T47D and MDA-MB-231 cells cultured in MEM-10% FBSdest and treated for 24 h with increasing concentration of estradiol (10^{-12} – 10^{-8} mol/L). (C) MCF7 and T47D cells were incubated for 24 h with E2, P4 or both (10^{-8} mol/L) and *SDC1* mRNA level measured by QRT-PCR ($n = 3$).

was the main mediator of the downregulation of SDC1 expression by E2.

We next investigated the effects of ER deficiency on SDC1 expression. After a 24-h treatment with the anti-estrogen ICI182,780 (ICI), a significant increase in the basal level of SDC1 mRNA and a decrease in E2-induced inhibition of the levels of SDC1 mRNA were detected

(Fig. 3D). ICI treatment reversed the activation of PGR expression induced by E2, confirming its efficacy (data not shown). Time-dependent degradation of ER α was detected after ICI treatment, which inversely correlated with SDC1 levels ($r^2 = -0.926$, $P = 0.002$) (Fig. 3E). Moreover, immunocytochemical analysis detected a 30% decrease in ER α levels associated with a 45% increase in SDC1

Figure 3

ER α mediates ligand-dependent and -independent downregulation of SDC1 expression in MCF7 cells. (A) Expression of SDC1 estimated by QRT-PCR in MCF7 cells cultured in MEM before addition of E2 (10^{-8} mol/L) for 24 h or specific agonist of ER α (PPT), ER β (DPN) or GPER (G1) used at 10^{-8} mol/L. GPER antagonist G15 was added 30 min before E2 stimulation ($n = 3$). (B) MCF7 cells were incubated for 24 h with E2, tamoxifen (Tam) at 10^{-6} mol/L or both and SDC1 mRNA level measured by QRT-PCR ($n = 3$). (C) Immunocytochemical observation of SDC1 after 24 h of treatment with E2 (10^{-8} mol/L) and Tam (10^{-6} mol/L) (Representative experiments from $n = 3$). (D) SDC1 mRNA expression in MCF-7 cells cultured in MEM and treated with E2, ICI (10^{-8} mol/L) or both for 24 h. (E) Representative Western blot showing ER α and SDC1 protein expression after ICI treatment for increasing period of time. Expression levels were normalized with actin as loading control. (F) Immunocytochemical observation of SDC1 and ER α in MCF-7 treated for 24 h with or without ICI (10^{-8} mol/L). (G) Expression of SDC1 mRNA estimated by QRT-PCR 24 h after transfection of siRNA (10 nM) followed by an additional incubation 24 h without or with E2 ($n = 3-5$). (H) ER α and SDC1 expression were estimated by Western blot 72 h after transfection with increasing concentration of siRNA. (I) Immunocytochemical observation of SDC1 and ER α in MCF7 transfected with 10 nM of siNeg or siER α ($n = 3$) (scale bar = 50 μ m).

levels (Fig. 3F and Supplementary Fig. 3A). Similarly, silencing *ESR1* expression, which completely abrogated the E2-mediated overexpression of *PGR* mRNA (data not shown), was associated with a significant increase in the basal level of *SDC1* mRNA (63%) (Fig. 3G) and in the levels of SDC1 (Fig. 3H, I and Supplementary Fig. 3B). These results confirm the inverse correlation between ER α and SDC1 levels in MCF7 cells.

E2-mediated downregulation of *SDC1* expression requires *de novo* protein synthesis

Treatment with the protein synthesis inhibitor cycloheximide (CHX) significantly reduced the E2-dependent inhibition of *SDC1* expression (83.4% untreated cells vs 44.8% CHX-treated cells) (Fig. 4A) without detectable effects on growth and cell viability

(Supplementary Fig. 4). Long-term treatment (24 h) with the RNA polymerase inhibitor actinomycin D (ACT) or ACT+CHX completely abrogated the inhibition of *SDC1* expression induced by estradiol (Fig. 4A) but significantly increased cell death (Supplementary Fig. 4). During a shorter treatment with ACT, which did not detectably affect the viability of MCF7 cells, the stability of *SDC1* mRNA was not significantly affected by E2 (Fig. 4B).

The potential involvement of the MAPK/ERK, PI3K/AKT or JNK signal transduction pathways on the regulation of *SDC1* expression was investigated. Although EGF induced rapid phosphorylation and activation of ERK1/2 and AKT (Fig. 4C), it had no effect on *SDC1* expression (Fig. 4D). Further, in the presence of inhibitors of MEK, AKT and JNK (UO126, Ly294,002 or Sp600,125, respectively), inhibition of *SDC1* expression after estradiol treatment was not reversed.

Figure 4

E2-dependent transcriptional regulation of *SDC1* expression requires a *de novo* protein synthesis and is independent to ERK/AKT/JNK pathways. (A) Cycloheximide (CHX at 10 μ M) and actinomycin D (ACT at 2×10^{-6} mol/L) or both were first added for 30 min before subsequent E2 treatment (10^{-8} mol/L). Expression of *SDC1* was evaluated by QRT-PCR after 24 h of incubation ($n = 3$). (B) Cells were treated with ACT for 30 min and stimulated for additional 1, 3 or 6 h with E2. (C) Activation/phosphorylation of ERK, AKT and JNK pathways evaluated by Western blot 20 min after E2 (10^{-10} mol/L) or EGF (10 ng/mL) treatment (representative experiments). (D) MCF-7 cells were first treated with MEK, AKT and JNK inhibitors (UO126, Ly294,002 and Sp600,125, respectively) for 30 min before treatment for 24 h with E2 (10^{-10} mol/L) or EGF (10 ng/mL). *SDC1* mRNA expression was quantified by QRT-PCR. Data were means $\pm$ s.e.m. from four independent experiments ($n = 4$). $^{*}P < 0.05$ compared to untreated control cells.

Inhibition of the NF κ B signal transduction pathway by Bay 11-7085 (Bay) reverses ER α -mediated downregulation of SDC1 expression

TNF α downregulates the expression of SDC1 (Day *et al.* 2003). TNF α triggers the canonical NF κ B signal transduction pathway, and the NF κ B and ER-signal transduction pathways interact in BC cells (Frasor *et al.* 2009). Therefore, we further investigated the involvement of the NF κ B signal transduction pathway in the E2-dependent downregulation of the expression of SDC1. We detected a 20% reduction (judged not statistically significant) in SDC1 mRNA expression after cells were treated with TNF α (Fig. 5A). This effect was reversed by addition of Bay, an irreversible inhibitor of I κ B α phosphorylation, which prevents the activation of the NF κ B signal transduction pathway. However, Bay treatment significantly inhibited ($\geq$ twofold) estradiol-mediated downregulation of SDC1 expression without altering the basal level of SDC1 mRNA.

Immunocytochemistry detected a significant reduction (-42% , $p=0.035$) of SDC1 in MCF7 cells after TNF α treatment but not with Bay (Fig. 5B). Thus, Bay partially reversed the effect of TNF α and completely abrogated the downregulation of SDC1 expression induced by E2. RelA is a terminal effector of the NF κ B signal transduction pathway. In MCF7 cells transfected with siRelA (Fig. 5C), the basal level of SDC1 expression significantly increased, although E2-mediated inhibition was detected (Fig. 5D).

There was no significant difference in the level of SDC1 mRNA after cells were treated only with TNF α or in combination with E2 in siNeg- or siRelA-transfected cells (Fig. 5F). In MCF7 ER α knockouts, E2-dependent inhibition of SDC1 mRNA was not detected, and Bay treatment had no effect on SDC1 mRNA expression even in the presence of E2, suggesting that Bay-dependent effects required ER α .

We found that TNF α treatment led to a 100-fold increase in the level of IL6 mRNA, and this effect was inhibited by Bay or E2 (Supplementary Fig. 5). Further, silencing of ESR1 or RelA expression significantly reduced the E2- and TNF α -dependent overexpression of IL6 mRNA. These data suggest that E2-dependent downregulation of SDC1 expression involves a mechanism requiring the activity of ER α and that activation of the IKK complex, but is independent of the downstream regulatory components of the NF κ B signal transduction pathway.

Bay regulates ER α expression and its activity through the E2-dependent phosphorylation of ER α -Ser118

The results of the preceding experiments indicate that the IKK inhibitor Bay might alter SDC1 mRNA expression by regulating ER α activity through an NF κ B-independent mechanism. Here, we detected similar and significant repression of ESR1 mRNA levels after treatment with Bay, E2 or Bay+E2 (Fig. 6A). However, Bay significantly reversed the E2-mediated upregulation of PGR,

Figure 5

The inhibitor of IKK, Bay-11-7085 reduces the ER-dependent inhibition of SDC1 expression. (A) Cells were first treated for 30 min with the NF κ B inhibitor, Bay 11-7085 (Bay) (10^{-5} mol/L) and E2 or TNF α (10 ng/mL) was added for additional 24 h before SDC1 mRNA quantification by qRT-PCR. (B) Representative immunocytochemical observation of SDC1 in MCF7 after Bay pre-treatment and E2 or TNF stimulation. (Scale bar = 50 μ m). (C) Expression of RELA mRNA 48 h after RelA-silencing ($n=3$). (D) MCF7 was transfected with siRelA for 48 h and stimulated with E2, TNF α or both for additional 24 h. SDC1 mRNA expression was measured by QRT-PCR ($n=3$). (E) Expression of ESR1 mRNA 48 h after transfection with siESR1 ($n=3$). (F) SDC1 mRNA expression was measured in MCF7 transfected 48 h with siESR1 and stimulated with E2, Bay or both for an additional period of 24 h ($n=3$). For D and F $^{*}P<0.01$; $^{***}P<0.001$ compared to respective transfected control cells by one-way ANOVA with Tukey multiple comparison tests. $^{*}P<0.05$ using Student's t -test.

although it had no detectable effect on basal levels (Fig. 6B). The expression of *ESR1* and *PGR* mRNAs after estrogen treatment differed in their kinetics (Fig. 6C). Thus, the E2-induced downregulation of *ESR1* mRNA levels was observed after 12 h of treatment, consistent with the levels of *SDC1* mRNA. In contrast, the upregulation of *PGR* mRNA was detected from 3 h after E2 treatment. However, the expression of these genes was affected by Bay treatment, suggesting that an IKK inhibitor affected early and late responses to E2 treatment. Thus, 6 h of Bay treatment were sufficient to induce a significant reduction of ER α levels in MCF7 cells, although E2 had no detectable effect (Fig. 6D). Moreover, treatment with MG-132, an inhibitor of the proteolytic activity of the 26S proteasome complex, abrogated the effects of Bay treatment (Fig. 6E).

We next studied the phosphorylation of ER α on Ser118, Ser167 and Ser104/106, which affect the stability, DNA-binding activity or transcriptional activity of ER α . Phosphorylation of ER α on Ser118 was very low in unstimulated MCF7 cells but was induced 15-fold by treatment with E2 for 30 min (ER α -Ser118/ER α 66 ratio compared with the control). However, treating cells first with Bay abrogated E2-induced phosphorylation at Ser118

without affecting the basal level of Ser118 phosphorylation (Fig. 6F). In contrast, phosphorylated ER α -Ser167 was detectable in untreated MCF7 cells, but treatment with Bay or E2 did not induced a significant change in the ratio of phosphoER:ER. Thus, whether phosphorylation of Ser167 contributes to ER α activity, it was not induced by E2 treatment (Le Goff *et al.* 1994, present data) and may therefore not be involved in mediating the E2-dependent inhibition of *SDC1* expression.

Discussion

Overexpression of SDC1 and ER(–) status serve as markers of an aggressive malignant phenotype and poor prognosis of patients with BC. However, we are unaware of convincing evidence that identifies the underlying mechanism. Therefore, we aimed here to investigate the effects of estrogens and consequences of the estrogen signaling deregulation on the expression of *SDC1* in cell lines derived from human BCs.

The present study demonstrates that treatment of ER(+) MCF7 cells with E2 led to time- and dose-dependent

Figure 6

Bay regulates *ESR1* expression and E2-dependent phosphorylation of ER α on Ser118. Expression of *ESR1* (A), *PGR* (B) mRNA in MCF7 pre-treated or not with Bay (30 min) before 24 h of E2 treatment ($n = 3$). (C) Expression of *ESR1* and *PGR* mRNA in MCF-7 cells treated with E2 for 1–24 h of incubation. (D) ER α content in MCF-7 cells was determined by Western blot 6 h after incubation with Bay or E2 or after pre-treatment with Bay (30 min) before the E2 treatment. Upper panel is a representative experiment and lower panel is a densitometric quantification of ER α /Actin ratio ($n = 3$). (E) Same experiments than in D were performed but with a first treatment of 30 min with 10 mM of the proteasome inhibitor, MG-132. (F) Phosphorylation of ER α on Ser118, Ser167 and Ser104/106 observed by Western blot in MCF7 cells treated for 30 min with Bay or E2 or first treated with Bay for 30 min prior to E2 stimulation (representative experiment). Quantitative analysis of Ser118/ER α and Ser167/ER α ratio ($n = 3$).

downregulation of the expression of *SDC1* mRNA and protein. We present convincing evidence that identifies ER α as the mediator of the effects of E2. Thus, in MCF7 cells, depletion of ER α was associated with the upregulation of SDC1 expression. However, we detected an opposing effect of E2 on SDC1 expression in the T47D ER(+) luminal A subtype BC cell line. A difference in response to E2 treatment between these two cell lines have been reported, expression of 50% of E2-target genes are downregulated in MCF7 cells, while most are upregulated in T47D cells (Rangel *et al.* 2017). Additionally, PR modulates ER α action directly through a physical interaction that regulates the binding of ER α to chromatin and the transcriptional activity of ER α (Mohammed *et al.* 2015), which may explain the differential expression of *SDC1* in response to E2 treatment in PR(+) or PR(−) BC cell lines. Moreover, T47D cells are more sensitive to progesterone (Yu *et al.* 2017), leading to the downregulation of *ESR1* expression in T47D cells but not in MCF7 cells (Savoldi *et al.* 1995).

We show here significant downregulation of *SDC1* mRNA in MCF7 cells only after a 12-h treatment with E2 and that *de novo* protein synthesis was required for maximal E2-dependent transcriptional repression of *SDC1* expression. Further, inhibition of the activity of RNA polymerase II had no significant effect on the half-life of *SDC1* mRNA, suggesting that E2-activated ER α signaling downregulated the expression of *SDC1* mRNA through transcriptional repression vs decreased mRNA stability. Thus E2-dependent post-transcriptional regulation of *SDC1* by miRNA is unlikely, although estrogens regulate miR expression in BC cells (Klinge 2012) and the fact that *SDC1* mRNA might be a potential target for some of them such as miR-10b which promotes the motility and invasiveness of BC cells (Ibrahim *et al.* 2012).

Numerous ER α -binding sites are present in BC cells, including MCF7 cells (Welboren *et al.* 2009, Ikeda *et al.* 2015), and some reside on the short arm of chromosome 2, which may be too far upstream of *SDC1* gene (Lin *et al.* 2007) and are therefore unlikely to regulate *SDC1* transcription. Consistently, *SDC1* has never been identified as ER-binding gene in microarray experiments or chromatin immunoprecipitation (ChIP) assays (Lin *et al.* 2007, Hah & Kraus 2014, Carroll 2016). Although *in silico* analysis does not detect putative ERE sequences in the proximal and distal promoters of *SDC1*, transcription factors binding sites such as AP-1, SP1 and NF κ B contribute to the transcriptional regulation of mouse *sdcl* (Hinkes *et al.* 1993, Vihinen *et al.* 1996, Iguchi-Ishiguro *et al.* 2012).

Estrogen signaling is induced through the interaction of ER α with such transcription factors (Hah & Kraus 2014),

although it is unlikely that a specific interaction between E2-activated ER α and a cis-acting DNA sequence located upstream of the *SDC1* promoter represses transcription, because such canonical genomic signaling rapidly regulates target genes and does not require *de novo* synthesis. Alternatively, nongenomic actions of estrogens mediated by kinase-dependent posttranslational modification of ER may occur. However, we show here that EGF-dependent activation of ERK and AKT, or the downregulation of their expression by selective inhibitors, failed to reverse E2-mediated repression of *SDC1* transcription in MCF7 cells.

In contrast, we found that BAY 11-7085, an irreversible inhibitor of NF κ B activation (Pierce *et al.* 1997), significantly decreased E2-dependent inhibition of *SDC1* expression without modifying the basal level. The NF κ B and ER signaling pathways interact (Van Laere *et al.* 2007, Frasor *et al.* 2009) and ER α can interact with NF κ B by various mechanisms depending on the stimulus or cell type (Kalaitzidis & Gilmore 2005). IKK is a complex of cytosolic factors composed of two canonical IKK factors, that is IKK α and IKK β , the regulatory subunit IKK γ and a non-canonical IKK ϵ (Verhelst *et al.* 2013). BAY 11-7085 prevents the activation and the phosphorylation of I κ B α by the IKK complex, leading to cytosolic sequestration of the NF κ B transcription factors RelA and p50.

Activation of NF κ B pathway by TNF α slightly influenced the expression of *SDC1* in MCF7 cells, but significantly reduced the levels of SDC1 protein. Interestingly, the same differential effect between the expression of *SDC1* mRNA and protein occurs in a human colorectal carcinoma epithelial cell line and human endothelial cells treated with TNF α (Kainulainen *et al.* 1996, Day *et al.* 2003). Here we show that E2-dependent downregulation of *SDC1* expression can be reversed by Bay and that TNF α treatment and silencing RelA expression had no detectable effect. In contrast, TNF α significantly increased the level of IL6 expression, and treatment with Bay or E2, as well as silencing the expression of ER α or RelA, attenuated or completely abrogated this activation. Thus, the mechanism of E2-mediated inhibition of IL6 expression involves tethering ER α to NF κ B to prevent their binding to the IL6 promoter (Galien & Garcia 1997), which may differ from the mechanism of E2-induced repression of SDC1 expression. Our present results show that in accordance with the presence of an NF κ B-responsive elements in the proximal promoter of *SDC1* (Iguchi-Ishiguro *et al.* 2012), NF κ B regulated the expression *SDC1*. However, E2-dependent repression of SDC1 expression requires an activated IKK complex and therefore is likely

independent of upstream or downstream components of the NF κ B signal transduction pathway.

Interestingly, a shorter treatment with the IKK inhibitor led to a decrease in ER α levels, which was reversed by first treating cells with an inhibitor of the 26S proteasome complex. Further, Bay pre-treatment prevented E2-induced phosphorylation of ER α on Ser118 without changing the level of ER α 66. The phosphorylation of ER α -Ser118 facilitates the direct binding of ER α to target genes by promoting the interactions of ER α with its coregulators and the nuclear localization of ER α (Marsaud *et al.* 2003, Le Romancer *et al.* 2011, Tian *et al.* 2015).

The activity of the 26S proteasome controls the level of ER α , and the reduction of phosphorylation of ER α -Ser118 phosphorylation correlates with faster elimination of E2-induced ER and inhibition of ER α -mediated gene regulation (La Rosa *et al.* 2012). Estrogen-induced phosphorylation of ER α and its transcriptional activation through phosphorylation of ER α on Ser118 preferentially involves I κ B kinase- α (IKK- α) (Park *et al.* 2005, Weitsman *et al.* 2006). However, IKK β and IKK ϵ regulate as well, ER α activity and the expression of its target genes (Guo *et al.* 2016, El-Shennawy *et al.* 2018). Interestingly, decreased phosphorylation of I κ B α occurs after estrogen treatment of HeLa cells stably transfected with a cDNA encoding the human ER α (Sun *et al.* 1998) or in rat brain (Wen *et al.* 2004), suggesting that E2 directly regulates IKK activity. Moreover, Bay had no effect on the basal levels of ER α phosphorylation and the expression of *SDC1*. Therefore, we believe it is reasonable to conclude that in MCF7 cells, the regulation of IKK activity and subsequent phosphorylation of ER α -Ser118, confers upon E2 the ability to regulate the stability of ER α as well as its transcriptional activity, and therefore, the expression of transcriptional co-regulator(s) that subsequently downregulate *SDC1* expression.

Estrogen can signal through IKK-dependent phosphorylation of histones bound in the vicinities of the promoters of target genes in MCF7 cells (Perillo *et al.* 2014). Thus, delayed downregulation mediated by ER α may occur through the upregulation or *de novo* synthesis of nuclear corepressors or associated HDACs that subsequently deacetylate chromatin to prevent recruitment of RNAPII and the transcriptional machinery to secondary ER α target genes (Carroll 2016, Stossi *et al.* 2009). Interestingly, a specific HDAC inhibitor, trichostatin A (TSA), strongly decreases *ESR1* and *SDC1* expression in MCF7 cells (unpublished data). Although ER α expression is downregulated in a TSA-dependent manner in MCF7 cells (Margueron *et al.* 2004),

HDAC-mediated downregulation of *SDC1* expression has not been shown, to our knowledge. However, HDACs may contribute to the activity of transcriptional corepressors that regulate the expression of ER-driven genes in MCF7 (Vanneste *et al.* 2017). This hypothesis is consistent with the paradigm that upregulation of gene expression occurs during acute exposure to estrogens, while most genes transcriptionally downregulated by E2 require sustained estrogenic treatment and their expression may be regulated through indirect mechanisms (Frasor *et al.* 2003).

Consistent with its structure and function as a coreceptor for growth factors and cytokines, SDC1 modulates numerous biological processes in BC cells that influence tumor progression, such as cell proliferation, adhesion, migration and angiogenesis (Nikolova *et al.* 2009). Accordingly, high levels of SDC1 are associated with negative prognostic parameters, shorter overall survival and resistance to chemotherapy (Baba *et al.* 2006, Götte *et al.* 2006) as well as with the loss of ER expression that occurs in most aggressive, hormone receptor-negative subtypes of BC (Barbareschi *et al.* 2003). Interestingly, inflammatory BC (IBC) is associated with the loss of ER expression and activation of NF κ B pathway (Van Laere *et al.* 2007). SDC1 can act as a coreceptor for IL6 via its glycosaminoglycan chains and promotes the activation of the inflammatory NF κ B pathway (Ibrahim *et al.* 2013, 2017) as well as the generation of more aggressive and inflammatory BC (IBC) (Hassan *et al.* 2013).

The malignant progression of BC coincides with a shift from estrogen dependence to independence, which is associated with increases in SDC1 expression (Baba *et al.* 2006). Interestingly, the SDC1-binding protein (SDCBP) expression is inversely correlated with ER α expression, and through the activation of focal adhesion kinase, SDCBP enhances the ability of SDC1 to induce the migration and invasiveness of BC cells (Qian *et al.* 2013). The resistance of SDC1-deficient mice to Wnt-induced breast tumorigenesis (Alexander *et al.* 2000) confirms that SDC1 metabolism may be involved in the progression of BC and that its overexpression is a hallmark of an aggressive malignant phenotype.

In summary, we demonstrate here that ER α downregulates SDC1 expression in MCF7 cells. Estradiol, through a mechanism requiring an activated IKK complex, although independent to NF κ B, increased the downregulation of SDC1 by ER α . Inhibition of IKK by BAY-11-7085 prevented the E2-induced phosphorylation of ER α -Ser118, leading to its subsequent proteasomal degradation. These findings suggest that IKK increased

the stability and activity of ER α , thereby contributing to the *de novo* synthesis of repressor(s) involved in the transcriptional regulation of *SDC1*. Although additional experiments are required to fully identify the mechanism of ER α -dependent inhibition of *SDC1* expression, our results indicate the existence of an antagonism between ER α signaling and *SDC1* expression, thereby contributing to new insights into the mechanism responsible for the inverse correlation between *SDC1* expression and ER signaling in ER(+) BC as well as the overexpression *SDC1* in the triple-negative subtype. Novel therapeutic strategies are required to treat drug-resistant ER (+) and ER (–) BCs. Our present findings strongly suggest that *SDC1* may serve as a potential novel target for designing effective therapeutic strategies.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0285>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

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