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17-β-Estradiol Inhibits Transforming Growth Factor-β Signaling and Function in Breast Cancer Cells via Activation of Extracellular Signal-Regulated Kinase through the G Protein-Coupled Receptor 30

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ABSTRACT

Breast cancer development and breast cancer progression involves the deregulation of growth factors leading to uncontrolled cellular proliferation, invasion and metastasis. Transforming growth factor (TGF)-β plays a crucial role in breast cancer because it has the potential to act as either a tumor suppressor or a pro-oncogenic chemokine. A cross-communication between the TGF-β signaling network and estrogens has been postulated, which is important for breast tumorigenesis. Here, we provide evidence that inhibition of TGF-β signaling is associated with a rapid estrogen-dependent nongenomic action. Moreover, we were able to demonstrate that estrogens disrupt the TGF-β signaling network as well as TGF-β functions in breast cancer cells via the G protein-coupled receptor 30 (GPR30). Silencing of GPR30 in MCF-7 cells completely reduced the ability of 17-β-estradiol (E2) to inhibit the TGF-β pathway. Likewise, in GPR30-deficient MDA-MB-231 breast cancer cells, E2 achieved the ability to suppress TGF-β signaling only after transfection with GPR30-encoding plasmids. It is most interesting that the antiestrogen fulvestrant (ICI 182,780), which possesses agonistic activity at the GPR30, also diminished TGF-β signaling. Further experiments attempted to characterize the molecular mechanism by which activated GPR30 inhibits the TGF-β pathway. Our results indicate that GPR30 induces the stimulation of the mitogen-activated protein kinases (MAPKs), which interferes with the activation of Smad proteins. Inhibition of MAPK activity prevented the ability of E2 from suppressing TGF-β signaling. These findings are of great clinical relevance, because down-regulation of TGF-β signaling is associated with the development of breast cancer resistance in response to antiestrogens.

Estrogens are pleiotropic hormones that influence a wide range of physiological processes such as the growth and differentiation of many tissues. Because of this crucial role, it is not astonishing that estrogens are also involved in the development and progression of many diseases. Estrogens enhance the proliferation of mammary cells, which is associated with an increase of mutations interfering with normal cellular processes, such as apoptosis and DNA repair (Russo and Russo, 2006). A variety of clinical studies implicate estrogens as risk factors for developing breast and endometrial tumors, which account for almost 40% of cancer incidence among women (Pike et al., 2004).

It was traditionally believed that 17-β-estradiol (E2) acts exclusively through nuclear estrogen receptors (ERs), such as ERα and ERβ. This pathway includes binding of E2 to the nuclear ER, resulting in the formation of a steroid-receptor complex, which interacts with DNA at estrogen-response elements (ERE) in the promotors of target genes (Nilsson et al., 2001). Besides this nuclear action, it is well accepted that E2 mediates cellular functions via nongenomic effects that do not involve the classic nuclear pathway. These nongenomic actions result in fast cellular responses such as second-messenger generation, Ca^{2+}-mobilization, or mitogen-

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ABBREVIATIONS: E2, 17-β-estradiol; BSA, bovine serum albumin; E2-BSA, 17-β-estradiol covalently linked to bovine serum albumin; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response elements; ERK, extracellular-regulated kinase; FCS, fetal calf serum; GPR30, G protein coupled receptor 30; HRP, horseradish peroxidase; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PTX, pertussis toxin; siRNA, small interfering RNA; TGF, transforming growth factor; PCR, polymerase chain reaction; TBS, Tris-buffered saline; ICI 182,780, fulvestrant; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene.

activated protein kinase (MAPK) activation (Filardo et al., 2002; Revankar et al., 2005). However, the identity of membrane-localized ERs, which mediate the nongenomic actions, remains controversial. On the one hand, there is evidence that the membrane-localized ERs are variants of nuclear ERs, such as ERα and ERβ, which may be transported to the plasma membrane by adaptor proteins (Evinger and Levin, 2005). On the other hand, it has been suggested that the orphan G protein-coupled receptor 30 (GPR30) is a putative membrane receptor mediating a wide range of nongenomic signals from E2. It is very interesting that ERα and GPR30 possess different pharmacological properties, because the pure antiestrogen ICI 182,780 has been described as an agonist at the orphan receptor GPR30 (Thomas et al., 2005).

There is increasing evidence that there exists also a cross-communication between ERs and growth factor receptor signaling, such as insulin-like growth factor-1, epidermal growth factor (EGF), TGF-α, and TGF-β (Ignar-Trowbridge et al., 1995; Thorne and Lee, 2003). Thus, it has been postulated that estrogens negatively modulate TGF-β signaling in breast cancer cells (Malek et al., 2006; Yoo et al., 2008). This is of importance because TGF-β acts as a potent inhibitor of tumor progression because of an induction of growth arrest and apoptosis of most normal and transformed epithelial cells, including those of the mammary gland (Li and Liu, 2007). Moreover, it has been suggested that antiestrogens mediate their growth inhibitory effects, at least in part, under the involvement of TGF-β (Buck et al., 2004). However, TGF-β concomitantly functions as a tumor promoter, because the chemokine is also a potent inductor of tumor cell migration contributing to an enhanced metastasis (Muraoka-Cook et al., 2005). In particular, in late-stage tumors, the balance between positive and negative effects of TGF-β on tumor progression may be shifted in favor of its promoting properties, because the sensibility of cells toward the antiproliferative and proapoptotic effects of the chemokine declines because of dysfunctional TGF-β receptors or TGF-β signaling (Leivo et al., 1998).

TGF-β mediates its biological effects mainly via two types of serine/threonine kinase receptors, TGF-β receptor I and TGF-β receptor II, followed by an activation of the Smad signaling pathway. After ligand binding and dimerization of TGF-β receptor I and TGF-β receptor II, receptor-regulated Smad2 and Smad3 proteins are phosphorylated and interact with the common mediator Smad4 (Massague, 1998). These activated Smad complexes translocate into the nucleus to regulate the transcriptional activity of Smad-responsive genes. It has been reported that there exists a cross-communication between estrogens and Smad activation resulting in a reduction of TGF-β functions; however, the underlying cellular processes have not been completely elucidated (Matsuda et al., 2001; Malek et al., 2006; Cherlet and Murphy, 2007; Yoo et al., 2008).

Therefore, we further examined the cross-communication between estrogens and TGF-β signaling in breast cancer cells. Here, we demonstrate that estrogens inhibit Smad signaling by activation of the extracellular-regulated kinase (ERK1/2). It is interesting that this estrogen-mediated action is transduced via the orphan receptor GPR30.

Materials and Methods

Chemicals. E2 and charcoal Norit A were purchased from Fluka (Buchs, Switzerland) and ICI 182,780 was from Tocris Cookson (Avonmouth, UK). For cell culture experiments, E2 and ICI 182,780 were dissolved in ethanol (stock solution, 5×10^{-3} M). E2 covalently linked to bovine serum albumin (E2-BSA), TGF-β, dextran, aprotinin, BSA, deoxycholic acid, Eagle's minimal essential medium, McCoy's 5A medium, Dulbecco's minimal essential medium, EDTA, EGTA, fetal calf serum (FCS), fibronectin, Giemsa dye, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, SDS, sodium fluoride, sodium orthovanadate, ethanol, Tris/HCl, Tris-base, sodium pyruvate, sodium chloride, magnesium chloride, L-glutamine, magnesium- and calcium-free phosphate-buffered saline (PBS), Tween 20, U0126, 4′,6-diamidino-2-phenylindole dihydrochloride, Fluoromount, Saponin, and Nonidet P-40 were purchased from Sigma-Aldrich (Taufkirchen, Germany). SDS-sample buffer, dithiothreitol, and 100-base pair DNA ladder were from New England Biolabs (Frankfurt/Main, Germany). GPR30-specific small interfering RNA (siRNA), control-siRNA, and Dharmafect1 transfection reagent were from Dharmacon RNA Technologies (Lafayette, CO), and rabbit-anti-GPR30 antibodies were from Biozol (Eching, Germany). Anti-phospho-Smad2 antibodies, anti-phospho-p44/42-MAPK antibodies, anti-rabbit and anti-mouse IgG-horseradish peroxidase-conjugated antibodies, and molecular weight markers were from Cell Signaling Technology (Danvers, MA). Anti-KDEL antibodies and anti-dopamine receptor D_2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 594 anti-rabbit antibodies, Alexa Fluor 488 anti-mouse antibodies, and Alexa Fluor 488 Concanavalin A were from Invitrogen GmbH (Karlsruhe, Germany). LumiGlo reagent and peroxide were from New England Biolabs. Fugene6 was from Roche Diagnostics (Mannheim, Germany). Polyvinylidene fluoride Immobilon transfer membrane was purchased from Millipore (Schwalbach, Germany). Sodium hydrogen carbonate and protein G plus agarose were purchased from Merck (Darmstadt, Germany). Gentamicin sulfate was from PAN Biotechnology (Aidenbach, Germany). *p*-Formaldehyde was from VWR (Vienna, Austria). Acrylamide and 18-mm coverslips were from Roth (Karlsruhe, Germany). Syringes were supplied by B/Braun Melsungen AG (Melsungen, Germany). Dual-Luciferase assay system was purchased from Promega (Mannheim, Germany).

Purification of E2-BSA with Dextran-Coated Charcoal. Charcoal Norit A (400 mg) was suspended in 50 mM Tris-buffer, pH 7.4, to a final concentration of [8% (w/v)] and stirred overnight at 4°C. Then, 40 mg of dextran [0.8% (w/v)] was added, stirred at room temperature for 20 min, and centrifuged at 3500*g* for 10 min. The charcoal pellet was resuspended in 5 ml of a 1.33×10^{-5} M E2-BSA/Tris (50 mM), pH 8.5, solution and stirred overnight at 4°C. Charcoal was then removed by centrifugation at 8000*g* for 20 min.

Preparation of Charcoal-Treated FCS. FCS was inactivated for 45 min at 56°C. Then, endogenous steroids were removed by treatment with dextran-coated charcoal. In brief, 10 g of charcoal Norit A was suspended in Tris-buffer, pH 7.4, and kept overnight at 4°C. Then, 100 mg of dextran was added, stirred for 20 min, and centrifuged for 10 min (3500*g*). The supernatant was decanted, and the charcoal pellet was loaded with FCS. The suspension was stirred for 3 h and centrifuged for 20 min (8000*g*). The procedure was repeated with the supernatant. For sterilization, the supernatant was filtrated through 0.2 μm membrane filters and kept at −20°C.

Cell Culture. MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Manassas, VA). MCF-7-2a cells were derived from MCF-7 cells (Hafner et al., 1996). MCF-7 and MCF-7-2a cells were maintained as a monolayer culture in Eagle's minimal essential medium with L-glutamine (2 mM) and 10% FCS. MDA-MB-231 cells were cultured in McCoy's 5A medium with L-glutamine (1.5 mM) and 5% FCS. Media were supplemented with sodium hydrogen carbonate (25 mM), sodium pyruvate (1 mM),

and gentamicin sulfate (50 mg/l), and all cell lines were cultured at 37°C in 5% CO_2.

Quantitative Real-Time PCR. RNA from MCF-7 and MDA-MB-231 cells were collected using an RNeasy kit (QIAGEN, Valencia, CA). cDNA was generated from total RNA using the FermentasAid First Strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a LightCycler480 and the SYBR Green PCR master mix (Roche Applied Science, Mannheim, Germany). Cyclophilin A was used as a normalization control for all experiments. For the measurement of ERα, ERβ, GPR30, and Cyclophilin A, the following primers from TIB Molbiol (Berlin, Germany) were used: ERα 5′-TCCAAAGAGAAGACCCTATCAATGTA-3′ (forward) and 5′-AGTAAGTCCCTTATTTGTTCAGC-3′ (reverse); ERβ 5′-TCGCTAGAACACACCTTACC-3′ (forward) and 5′-TTCACACGACCAGACTCCATA-3′ (reverse); GPR30 5′-CTCATGTGGACTGGGACC-3′ (forward) and 5′-TGAACGTCACCAGCCTC-3′ (reverse); Cyclophilin A 5′-TTTGCTTAATTCTACACAGTACTTAGAT-3′ (forward) and 5′-CTACCCTCAGGTGGTCTT-3′ (reverse). Total RNA of three different sets of cells were used to analyze gene expression. Data were obtained in triplicate, and the specific mRNA levels were expressed as the mean ± S.E.M. of relative mRNA expression relative to control cells.

Chemotaxis Assay. Cells (10^6/well) were preincubated with the indicated concentrations of E2, ICI 182,780, or E2-BSA for 30 min, trypsinized, and seeded in the upper well of a modified Boyden chamber with 8-μm pore width (TPP, Trasadingen, Switzerland). The lower chamber, separated by a fibronectin-coated (3 μg/ml) membrane, contained TGF-β or vehicle in the indicated concentrations. After 5 h of incubation at 37°C, media were aspirated, and the cells on the upper side of the filter were removed, whereas migrated cells were fixed with 96% ice-cold ethanol followed by staining with 500 μl of Giemsa [0.04% (w/v)] solution. Migrated cells were visualized by light microscopy. For each determination, 10 randomly chosen fields were counted and averaged. The specific migration index was calculated as ratio after setting the control vehicle random migration at 1.0.

Preparation of Membrane Proteins. MCF-7 and MDA-MB-231 cells, grown and transfected in 75-cm^2 cell culture flasks, were treated with prechilled membrane lysis buffer (20 mM Tris/HCl, 5 mM magnesium chloride, 1 mM EDTA, 0.6 mM EGTA freshly supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 50 mM sodium fluoride). Cells were lysed by passing through 20- and 30-gauge syringes, 10 times each. The suspension was centrifuged at 400*g* for 5 min at 4°C. The supernatant, containing the undissolved membrane proteins, was then centrifuged at 30,000*g* for 60 min at 4°C. The pellets were resuspended in ice-cold membrane lysis buffer, and aliquots (20 μg of protein) were separated by SDS-polyacrylamide gel electrophoresis and detected by Western blot analysis.

Immunoblotting and Immunoprecipitation. MCF-7 (3×10^5/well) or MDA-MB-231 (1.2×10^5/well) cells were seeded in six-well plates and cultivated for 24 h. For detection of ERK1/2 phosphorylation, cells were washed three times with PBS and then cultivated in medium without serum for 3 days before stimulation with E2, ICI 182,780, and/or TGF-β. For detection of Smad2 phosphorylation, cells were preincubated for 24 h with E2 in medium without FCS followed by stimulation with TGF-β for the indicated time periods. Then, cells were rinsed twice with ice-cold PBS and harvested in radioimmunoprecipitation assay buffer (50 mM Tris/HCl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 50 mM sodium fluoride). Lysated protein (15 μg) was eluted in SDS-sample buffer supplemented with dithiothreitol (200 mM), boiled at 95°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis. Gels were blotted overnight onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS)-Tween 20 (0.1%) for 1 h at 37°C, membranes were washed three times with TBS-Tween and incubated with anti-phospho-Smad2, anti-GPR30, or anti-dopamine D_2 receptor antibodies (1 μg/ml) overnight at 4°C or anti-phospho-p44/42-MAPK antibodies (0.5 μg/ml) for 2 h at room temperature. The membranes were washed three times with TBS-Tween followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h, washed, and developed with LumiGlo reagent and peroxide solution according to the manufacturer's protocol.

Abrogation of GPR30 by siRNA. Gene silencing was performed using sequence-specific GPR30-siRNA and Dharmafect1 as transfection reagent (Dharmacon). Cells were transfected with 100 nM GPR30 or control siRNA in medium without antibiotics according to the manufacturer's instructions. The silencing efficiency was verified by real-time PCR and Western blot analysis of membrane enriched protein fractions using anti-GPR30 antibodies. After 72 h, cells were sensitized with E2, and chemotaxis or Western blot experiments were performed in response to TGF-β.

Transfection Experiments. The GPR30 expression plasmid was kindly provided by R. Weigel (University of Iowa, Iowa City, Iowa). The dopamine receptor D_2 (long version) plasmids were purchased from Missouri S&T cDNA Resource Center (Columbia, MO). Plasmids were amplified using the Qiafilter maxi plasmid kit (QIAGEN) (Carmeci et al., 1997). Cells (3×10^4/well) were seeded in a six-well plate for the measurement of Smad2-phosphorylation or in 10-cm dishes for migration assays and were allowed to grow for 24 h. Cell transfection was performed over 48 h using Fugene6 according to the manufacturer's instructions.

Detection of GPR30 Expression by Immunofluorescence Microscopy. MDA-MB-231 cells (5×10^4) were seeded on coverslips for 24 h before transfection. After an additional 48 h, cells were fixed with 4% *p*-formaldehyde for 10 min, blocked with 1% BSA in PBS, and treated with primary antibodies for 60 min. As primary antibodies, 10 μg/ml rabbit-anti-GPR30 antibodies alone or in combination with 1 μg/ml mouse-anti-KDEL antibodies were used. Then, secondary antibodies conjugated with different fluorophores (2.5 μg/ml Alexa Fluor 594 anti-rabbit antibodies or 2.5 μg/ml Alexa Fluor 488 anti-mouse antibodies) or Concanavalin A Alexa Fluor 488 (100 μg/ml) were applied for 45 min. Primary and secondary antibodies were diluted in 1% BSA in PBS containing 0.1% saponin. After staining with 1 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride, coverslips were mounted on an object slide using Fluoromount, and images were taken on a Keyence BZ8000 (Keyence, Neu-Isenburg, Germany).

Smad Reporter Gene Assays. MCF-7 cells (9×10^4 cells/well) were seeded in 24-well plates 24 h before transfection. Cells were transfected with 0.5 μg/well Smad2-specific ARE-luc/FAST-1 plasmid (kindly provided by Dr. Anita Roberts, National Institutes of Health, Bethesda, MD) by the use of 1 μl/well Fugene6 reagent. *Renilla reniformis* luciferase plasmid (25 ng/well) was used to normalize transfection activity. After 24 h, media were replaced, and cells were incubated with U0126 and treated with TGF-β and/or E2 or E2-BSA in the indicated concentrations. Cells were harvested after 24 h and assayed for luciferase activity using the Dual-Luciferase assay system. Total light emission was measured during the initial 10 s of the reaction using a luminometer (PerkinElmer Life and Analytical Sciences, Rodgau, Germany). Firefly luciferase activities were corrected using *R. reniformis* luciferase activities.

Determination of ERE Activation. MCF-7-2a cells, stably transfected with the $ERE_{wt\ luc}$ plasmid, were used for the measurement of ERE activation. $ERE_{wt\ luc}$ contains the ERE as enhancer sequence and the reporter gene *luc* encoding the enzyme luciferase. Cells (1×10^4 cells/well) were disseminated in 96-well plates in Dulbecco's minimal essential medium with 5% charcoal-treated FCS. Cells were stimulated with E2 or BSA-E2 for 18 h. Luciferase activity was measured according to the manufacturer's protocol in a

luminometer (PerkinElmer). Cells stimulated with 10^{-8} M E2 were used as positive control and account for 100% ERE activation.

Statistics. Data are expressed as the mean ± S.E.M. of results from at least three experiments, each run in triplicate. Statistics were performed using Student's *t* test. *, $P < 0.05$ and **, $P < 0.01$ indicate a statistically significant difference versus control experiments.

Results

17-β-Estradiol Inhibits TGF-β Signaling via Membrane-Associated Receptors. Although it has been well established that E2 inhibits TGF-β signaling in MCF-7 cells, resulting in an interruption of TGF-β-mediated chemotaxis, the molecular mechanism of this cross-talk is less characterized (Malek et al., 2006). To assess whether E2 mediates its inhibitory action on TGF-β signaling via the classic nuclear estrogen receptors or through transmembrane localized estrogen receptors, E2 covalently linked to plasma membrane-impermeable BSA (E2-BSA) was used (Taguchi et al., 2004). As presented in Fig. 1, A and B, incubation of MCF-7 cells with E2-BSA resulted in a significant repression of TGF-β-mediated migration comparable with E2. Thus, E2-BSA at a hormone content of 10^{-7} M reduced the ability of TGF-β to induce MCF-7 migration by more than 70% (Fig. 1B). Higher concentrations did not further increase the inhibitory effect of E2-BSA. To verify that E2-BSA does not antagonize chemotaxis in general but is a specific inhibitor of TGF-β-mediated migration, chemotactic activity of MCF-7 cells in

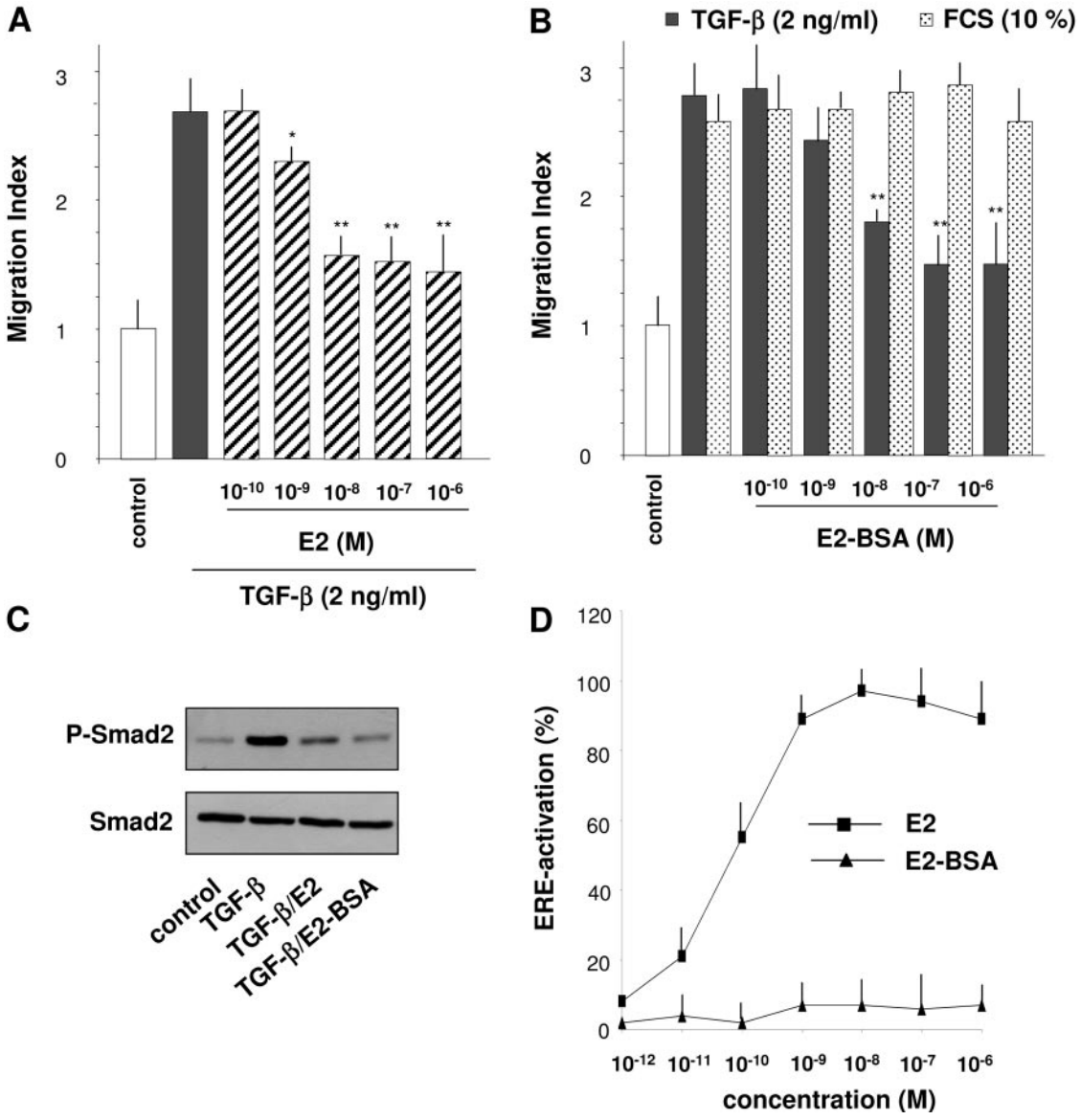

Fig. 1. E2 inhibits TGF-β signaling and function via membrane associated receptors. MCF-7 cells were treated with the indicated concentrations of E2 (A) or E2-BSA (B) for 30 min. Then, chemotaxis of cells in response to TGF-β or FCS was measured in a modified Boyden chamber. Data are expressed as arbitrary migration index after setting random migration of the vehicle controls to 1.0 ± S.E.M. from three experiments. *, $P < 0.05$, and **, $P < 0.001$ indicate a statistically significant difference versus control cells (A and B). To measure the influence of E2 and E2-BSA on Smad2 phosphorylation, MCF-7 cells were preincubated with E2 (10^{-7} M) or E2-BSA (10^{-7} M) for 30 min and then stimulated for 60 min with 2 ng/ml TGF-β. C, lysate protein (15 μg) was electrophoresed and immunoblotted with anti-phospho-Smad2- (top) or anti-Smad2-antibodies (bottom). D, to measure transcriptional activity of E2-BSA, luciferase activity under the control of ERE was measured after stimulation with the indicated concentrations of E2-BSA or E2 for 18 h in MCF-7-2a cells. Data are expressed as the percentage of luciferase activity ± S.E.M. from three experiments.

response to serum, a nonspecific migratory stimulus, was measured. Actually, pretreatment with E2-BSA did not reduce the capacity of serum to enhance migration, indicating a specific inhibitory role of E2-BSA on TGF-β-induced migration (Fig. 1B). Because Smad2/3 proteins have been identified as essential signaling molecules to mediate TGF-β-induced migration, it was also of interest to examine whether E2-BSA is able to modulate Smad phosphorylation (Malek et al., 2006). Indeed, when MCF-7 cells were treated with TGF-β in the presence of E2-BSA, phosphorylation of Smad2 was drastically reduced, which is in congruence with the inhibitory effect of E2-BSA on TGF-β-induced migration (Fig. 1C). To exclude potential artifacts due to contamination of E2-BSA preparations with unconjugated E2, a luciferase assay under the control of ERE served as means to exclude agonistic effects of E2-BSA on intracellular ERs. As shown in Fig. 1D, only E2 but not the conjugated E2-BSA induced luciferase activity in MCF-7-2a cells, which are stably transfected with the $ERE_{wtc\ luc}$ plasmid, confirming that the inhibitory effect of E2 on TGF-β function and signaling is mediated by membrane-associated receptors. Different types of receptors responsive to estrogens have been detected in plasma membranes of MCF-7 cells, which are either related to nuclear estrogen receptors or the orphan receptor GPR30 (Filardo et al., 2007). Indeed, quantitative real-time PCR analysis revealed that mRNA transcripts of ERα, ERβ, and GPR30 are present in MCF-7 cells. It is of interest that MDA-MB-231 cells, in which E2 possesses no inhibitory effect on TGF-β signaling, did not express mRNA of ERα and GPR30 (Fig. 2A). To further substantiate the role of trans-

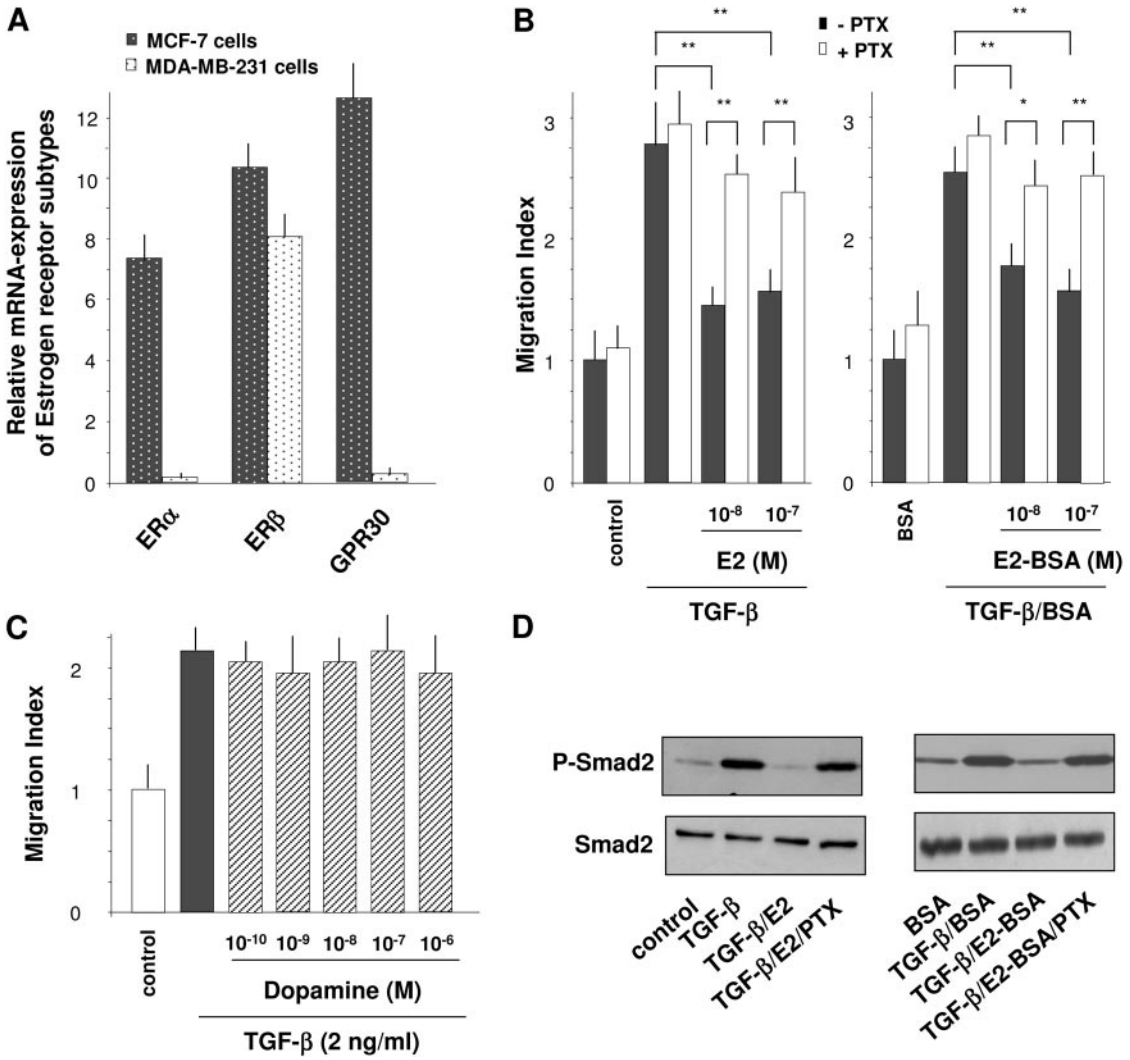

Fig. 2. PTX inhibits E2-mediated inhibition of TGF-β signaling and function. Quantitative real-time PCR analysis of ERα, ERβ, and GPR30 in MCF-7 and MDA-MB231 cells was examined. Real-time PCR starting with 10 ng of total RNA of three different sets of cells was performed with specific primers as indicated and described under *Materials and Methods* using Cyclophylin A as reference gene. A, relative mRNA expression was quantified using the comparative threshold cycle method. MCF-7 cells were pretreated with PTX (200 ng/ml) for 2 h followed by an incubation of E2 or E2-BSA in the indicated concentrations for 30 min. For control experiments BSA was used in a concentration of 0.3×10^{-8} M. Then, migration of cells in response to TGF-β (2 ng/ml) or TGF-β/BSA was measured in a modified Boyden chamber. Data are expressed as arbitrary migration index ± S.E.M. from three experiments. *, $P < 0.05$ and **, $P < 0.001$ indicate a statistically significant difference versus control cells. B, MCF-7-cells were transfected with dopamine receptor D_2 plasmids and sensitized with E2 after 48 h. Chemotaxis experiments were performed in response to TGF-β. Values are expressed as arbitrary migration index ± S.E.M. from three experiments. *, $P < 0.05$ and **, $P < 0.001$ indicate a statistically significant difference versus control cells. C, MCF-7 cells were pretreated with PTX (200 ng/ml) for 2 h after an incubation with E2 (10^{-7} M), BSA (0.3×10^{-8} M), or E2-BSA (10^{-7} M) for 30 min. D, cells were stimulated for 60 min with 2 ng/ml TGF-β or TGF-β/BSA, and 15 μg of lysate protein was electrophoresed and immunoblotted with anti-phospho-Smad2- or anti-Smad2-antibodies.

membrane localized estrogen receptors, the inhibitory effect of E2 and E2-BSA on TGF-β-evoked migration and Smad phosphorylation was measured in the presence of pertussis toxin (PTX) to eliminate functional $G\alpha_i$ signaling of membrane-associated ERα as well as GPR30. Actually, preincubation of MCF-7 cells with PTX almost completely reversed the inhibitory effect of E2 and E2-BSA on TGF-β-induced migration and Smad2 phosphorylation, confirming the participation of $G\alpha_i$ protein-coupled receptors in E2-mediated repression of TGF-β signaling (Fig. 2, B and D). To examine whether the inhibitory action of E2 on TGF-β signaling is a receptor-specific effect, MCF-7 cells were transfected with cDNA plasmids encoding the long version of the dopamine D2 receptor subtype, which also couples to $G\alpha_i$. Although Western blot analysis confirmed the expression of the dopamine D_2 receptor (data not shown), stimulation of MCF-7 cells with dopamine did not inhibit TGF-β-induced migration (Fig. 2C).

Expression of GPR30 Is Critical for Repression of TGF-β Signaling by 17-β-Estradiol. With respect to the contribution of membrane-associated receptors to E2- and E2-BSA-caused reduction of TGF-β-provoked effects, it was of interest to further discriminate between a membrane-localized ERα and GPR30. In contrast to an antagonistic effect of ICI 182,780 on ERα, it has been clearly indicated that this antiestrogen has an opposing agonistic activity on GPR30 (Thomas et al., 2005). Therefore, we measured whether ICI 182,780 exhibits similar actions as E2. In analogy to the natural ligand, ICI 182,780 also inhibited chemotaxis of MCF-7 cells as well as Smad2 activation in response to TGF-β, implicating that GPR30 seems to be an appropriate candidate to mediate the cross-communication between E2 and the TGF-β cascade (Fig. 3, A and B). To further corroborate the crucial role of GPR30, we used siRNA interfering with GPR30 expression in MCF-7 cells. Abrogation of

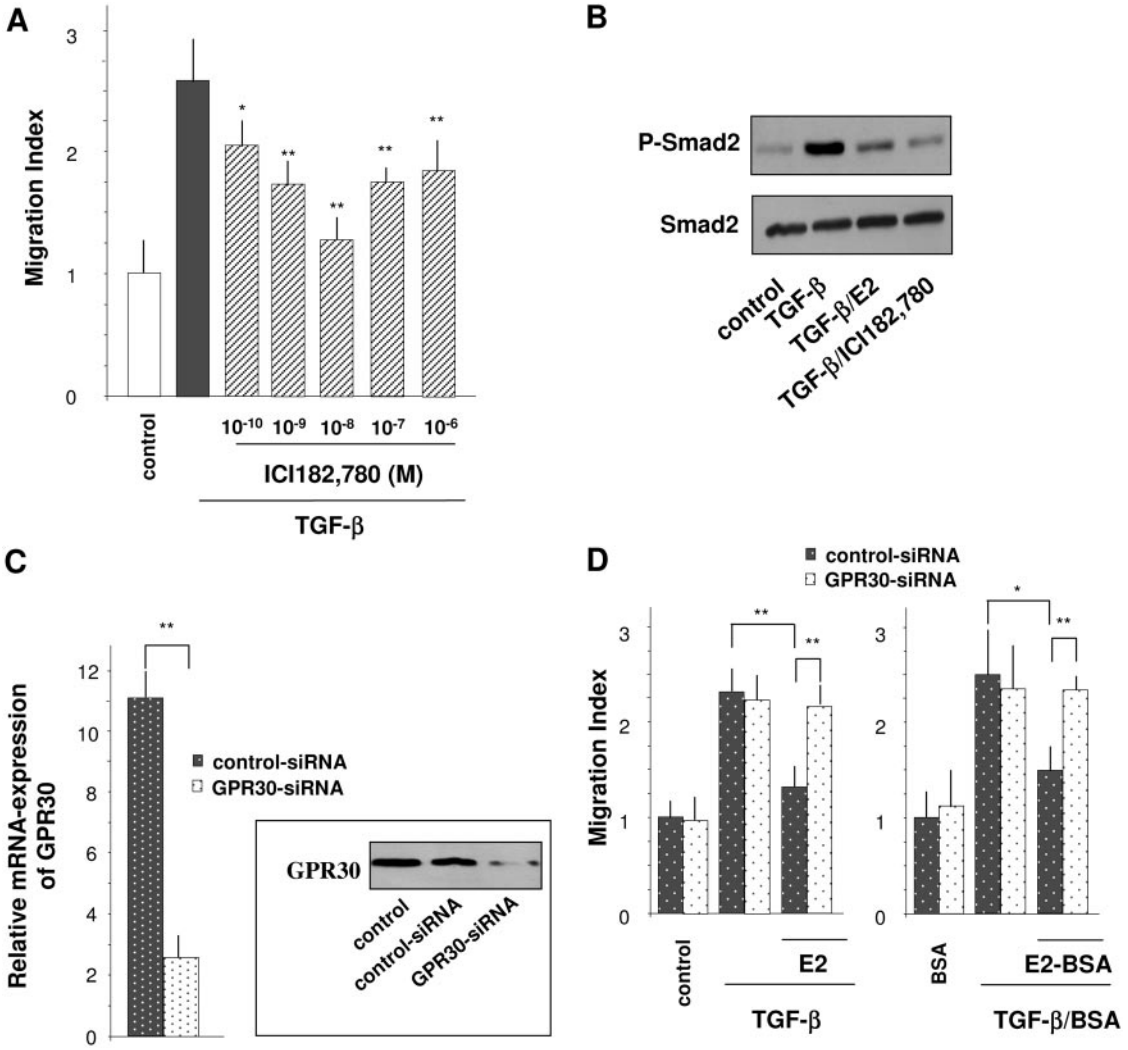

Fig. 3. GPR30 is critical for repression of TGF-β signaling and function by E2 in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of ICI 182,780 for 30 min. A, chemotaxis of cells in response to TGF-β (2 ng/ml) was measured. Values are expressed as arbitrary migration index ± S.E.M. from three experiments. *, $P < 0.05$ and **, $P < 0.001$ indicate a statistically significant difference versus control cells. B, MCF-7 cells were treated with ICI 182,780 (10^{-8} M) for 30 min. Then, cells were stimulated for 60 min with 2 ng/ml TGF-β and 15 μg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2- or anti-Smad2-antibodies. To evaluate the role of GPR30 in E2-mediated inhibition of TGF-β signaling, MCF-7 cells were transfected either with GPR30 siRNA or control siRNA (100 nM). C, the silencing efficiency was verified by real-time PCR and Western blot analysis of membrane enriched protein fractions using anti-GPR30-antibodies. D, after 72 h, cells were sensitized with E2 (10^{-7} M), BSA (0.3×10^{-8} M), or E2-BSA (10^{-7} M) for 30 min, and chemotaxis experiments were performed in response to TGF-β (2 ng/ml). Values are expressed as arbitrary migration index ± S.E.M. from three experiments. *, $P < 0.05$ and **, $P < 0.001$ indicate a statistically significant difference versus control cells.

GPR30 was verified by means of Western blot analysis of membrane-enriched fractions as well as real-time PCR analysis. Indeed, both methods confirmed that treatment with siRNA resulted in a serious reduction of protein and mRNA levels (Fig. 3C). It is very interesting that in GPR30-deficient MCF-7 cells, E2 and E2-BSA almost completely lost their ability to suppress migration after treatment with TGF-β, whereas in MCF-7 cells incubated with noncoding siRNA, the behavior of E2 and E2-BSA on TGF-β mediated actions was not affected (Fig. 3D). Because GPR30-silencing in MCF-7 cells abolished E2 and E2-BSA effects, we next examined whether an inhibitory effect of the steroid hormone on TGF-β signaling could be established in nonresponsive MDA-MB-231 cells upon transfection with a cDNA encoding GPR30. Measurement of GPR30 by Western blot analysis of membrane-enriched fractions indicated a prominent expression of the GPR30 in transfected cells (Fig. 4E). GPR30 immunoreactivity was mainly located at the endoplasmic reticulum because the GPR30 immunosignal colocalized with KDEL staining, a marker for the endoplasmic reticulum (Fig. 4, A–D). No significant overlap of immunosignals of GPR30 and Concanavalin A, a marker for the plasma membrane immunosignals, could be observed (data not shown).

Treatment of GPR30-transfected cells with TGF-β resulted in an enhanced migration and a Smad2 activation comparable with vehicle-transfected MDA-MB-231 cells (Fig. 5, A and B). Actually, when MDA-MB-231 cells were incubated with E2, an inhibitory effect on migration and Smad activation induced by TGF-β was only visible in GPR30-transfected cells (Fig. 5, A and B). In agreement with silencing experiments in MCF-7 cells, the results in MDA-MB-231 cells confirm the crucial role of GPR30 for estrogen-mediated suppression of the TGF-β cascade.

GPR30 Induces Inhibition of TGF-β Signaling via ERK1/2 Activation. Next, it was of interest to further examine intracellular signaling pathways between GPR30 and TGF-β signaling. In epithelial cells, it has been proposed that activation of ERK1/2 (p44/42 MAPK) has a negative regulatory effect on Smad signaling because of an inhibition of the nuclear translocation of Smad proteins (Kretzschmar et al., 1999). Moreover, E2 is known to activate ERK1/2 in several cell types (Filardo et al., 2002). Therefore, we examined whether E2 activates ERK1/2 via GPR30, resulting in an inhibition of TGF-β function and signaling. In analogy to further studies, the addition of E2 to MCF-7 cells resulted in a concentration-dependent phosphorylation of ERK1/2 reaching a maximum at 1 to 10 nM E2 (Fig. 6A). Activation of ERK1/2 by E2 was transient with a maximum after 10 to 15 min, which then returned to basal levels after approximately 60 min (data not shown). In agreement, E2-BSA also induced ERK1/2 activation (Fig. 7A). To further examine the role of GPR30 on ERK1/2 activation, MCF-7 cells were stimulated

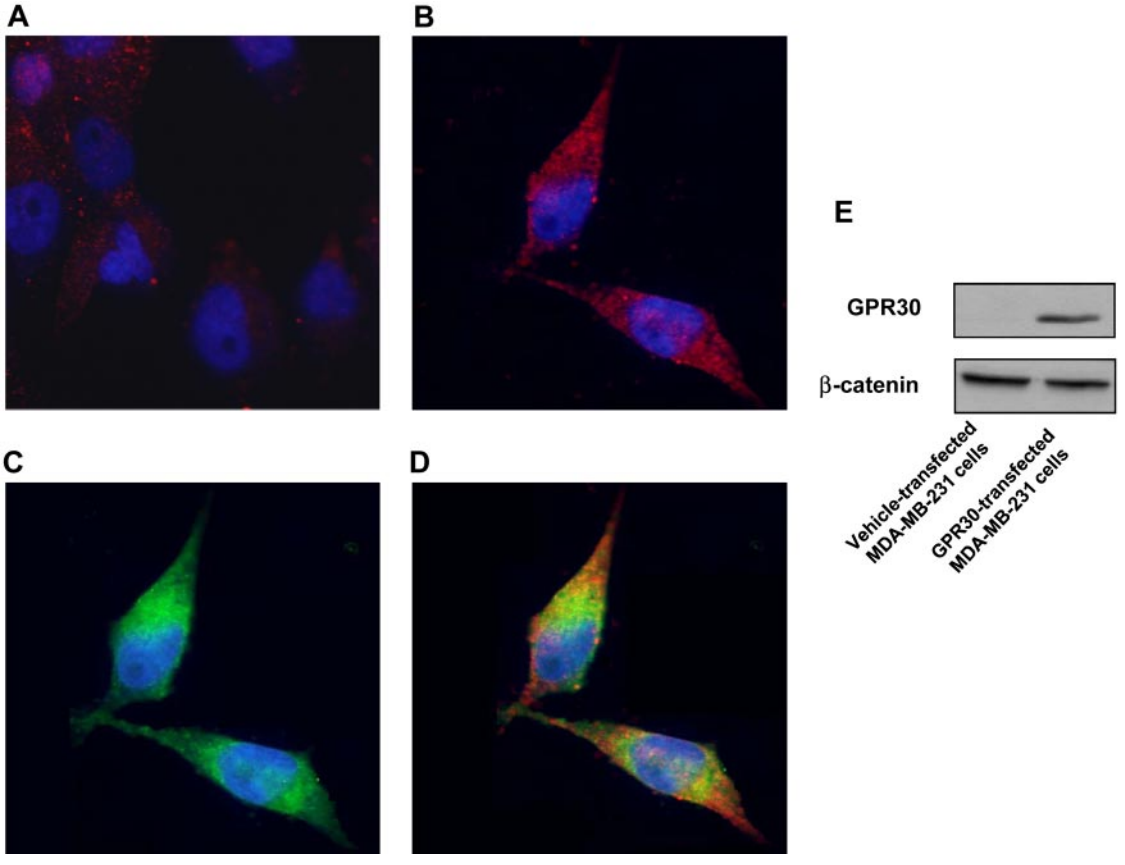

Fig. 4. Expression and localization of GPR30 in transfected MDA-MB-231 cells. MDA-MB-231 cells were transfected with GPR30 plasmids as described under *Materials and Methods*. E, Western blot analysis of membrane-enriched protein fractions confirmed an expression of GPR30 in transfected MDA-MB-231 cells. Immunofluorescence microscopy indicated a localization mainly at the endoplasmic reticulum, Western blot analysis of membrane-enriched protein fractions, and fluorescence-activated cell sorting analysis using anti-GPR30 antibodies. Vehicle-transfected MDA-MB-231 cells (A) or GPR30-transfected cells were stained with 4′,6-diamidino-2-phenylindole dihydrochloride and anti-GPR30 (B) or/and anti-KDEL (C and D) antibodies.

with ICI 182,780. Indeed, a pronounced activation of ERK1/2 was also detected in response to ICI 182,780 comparable with E2, suggesting that ERK1/2 activation is mediated via GPR30 (Fig. 6B). To more rigorously prove the involvement of the orphan receptor in E2-induced activation of ERK1/2, we used MDA-MB-231 cells that lack both ERα and GPR30. As expected, E2 failed to induce ERK activation in vehicle-transfected MDA-MB-231 cells (Fig. 6C). But when MDA-MB-231 cells were transfected with plasmids encoding GPR30, E2 achieved the ability to activate ERK1/2, which clearly indicates that GPR30 contributes to the E2-induced activation of ERK1/2 (Fig. 6D). Next, the capacity of activated ERK1/2 on E2- and E2-BSA-induced inhibition of TGF-β function and signaling was determined using the specific MAPK inhibitor U0126. As expected, pretreatment of MCF-7 cells with U0126 in a concentration of 3 μM before the addition of E2-BSA resulted in a complete inhibition of ERK1/2 phosphorylation (Fig. 7A). Thus, the inhibitory effect of E2 and E2-BSA on TGF-β-mediated Smad2 phosphorylation was examined in the presence of U0126. As presented in Fig. 7B, E2 and E2-BSA lost their ability to inhibit TGF-β-induced Smad2 phosphorylation in MCF-7 cells when U0126 was present. Because Smad proteins mediate the chemotactic behavior of MCF-7 in response to TGF-β, we also tested whether ERK1/2 phosphorylation is essential for the repression of TGF-β-induced migration by E2 and E2-BSA. In fact, pretreatment of MCF-7 cells with U0126 almost completely prevented the inhibitory effect of E2 and E2-BSA on TGF-β-induced motility (Fig. 7C). To further substantiate the inhibitory action of ERK1/2 on Smad activation, transient transfection experiments

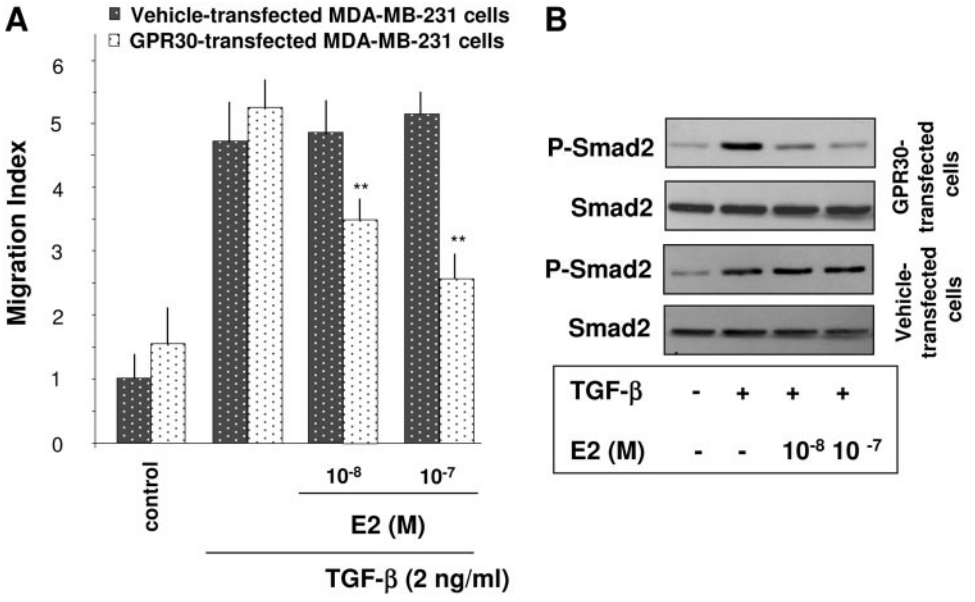

Fig. 5. Expression of GPR30 in MDA-MB-231 cells induces E2-mediated inhibition of TGF-β signaling and function. MDA-MB-231 cells were transfected with GPR30 plasmids and sensitized with E2 after 48 h. Chemotaxis experiments were performed in response to TGF-β. Values are expressed as arbitrary migration index ± S.E.M. from three experiments. *, $P < 0.05$ and **, $P < 0.001$ indicate a statistically significant difference versus control cells (B). To measure the influence of E2 on Smad2 phosphorylation in GPR30-transfected MDA-MB-231, cells were incubated with E2 for 30 min and then stimulated for 60 min with 2 ng/ml TGF-β. C, 15 μg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 or anti-Smad2 antibodies.

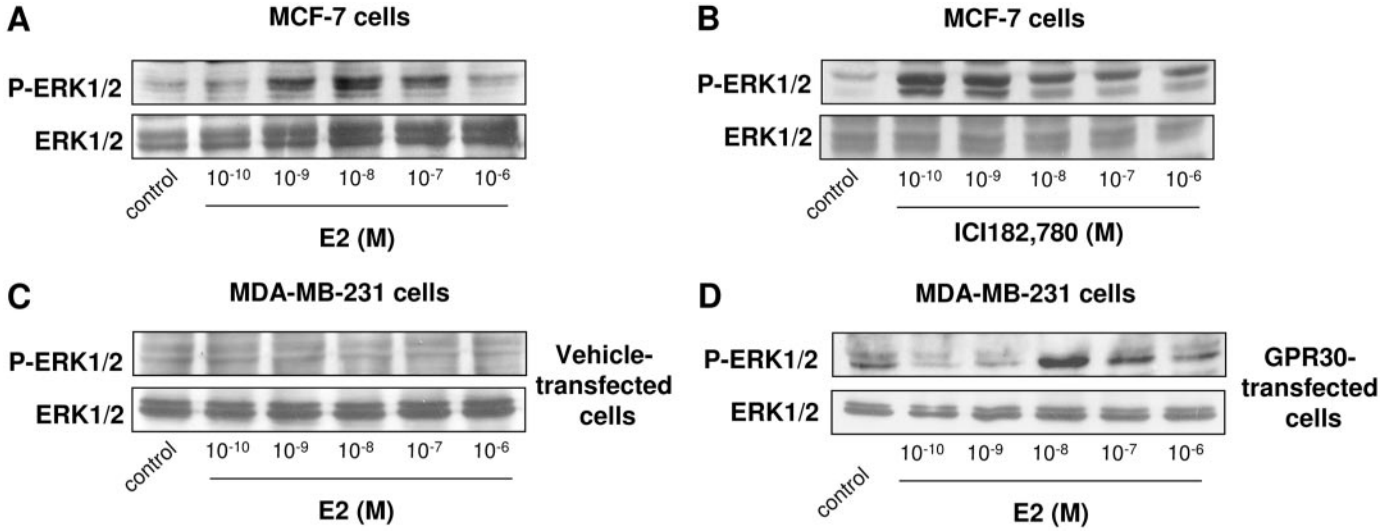

Fig. 6. ERK1/2 phosphorylation by E2 and ICI 182,780 in MCF-7 and MDA-MB-231-cells. MCF-7 cells were stimulated with E2 (A) or ICI 182,780 (B) in the indicated concentrations for 10 min. A and B, 15 μg of lysate protein was electrophoresed and immunoblotted with anti-phospho-p44/42-MAPK (top) or anti-p44/42-MAPK-antibodies (bottom). MDA-MB-231 cells were transfected with GPR30 plasmids as described under *Materials and Methods*. After 48 h GPR30- (C) or vehicle-transfected cells (D) were stimulated with E2 in the indicated concentrations for 10 min. C and D, 15 μg of lysate protein were electrophoresed and immunoblotted with anti-phospho-p44/42-MAPK (top) or anti-p44/42-MAPK-antibodies (bottom).

on the basis of a Smad2-responsive promoter with luciferase activity were performed. Indeed, a drastic increase of luciferase activity could be detected for the Smad2 promoter in response to TGF-β, which was drastically reduced when cells were sensitized with E2 or E2-BSA. But when MCF-7 cells were pretreated with U0126, E2 and E2-BSA lost their aptitude to inhibit TGF-β-induced Smad2 promoter activity (Fig. 7D).

In conclusion, these data lead to the assumption that E2 stimulates ERK1/2 phosphorylation via GPR30, which results in an inhibitory effect on TGF-β-induced Smad signaling and function.

Discussion

A pivotal aspect of tumor development promoted by estrogens is their ability to negatively modulate the expression or functional activity of tumor suppressors (Deroo and Korach, 2006). Indeed, TGF-β has been identified as a prominent tumor suppressor, although the cytokine functions paradoxically in several human cancers (Muraoka-Cook et al., 2005). Because of the cell growth inhibitory effect of TGF-β in many cell types, disruption of TGF-β signaling is a means by which cancer cells avoid cell growth arrest. Thus, it has been suggested that the initial loss of TGF-β-mediated growth inhibition by estrogens contributes to the development of human cancer (Parekh et al., 2002). Indeed, antiestrogens have been associated with an increased TGF-β secretion, which correlates with the inhibition of growth of some human breast cancer cells (Chen et al., 1996; Benson, 2004). On the contrary, activation of TGF-β signaling has been shown to mediate metastasis later in malignant progression because of

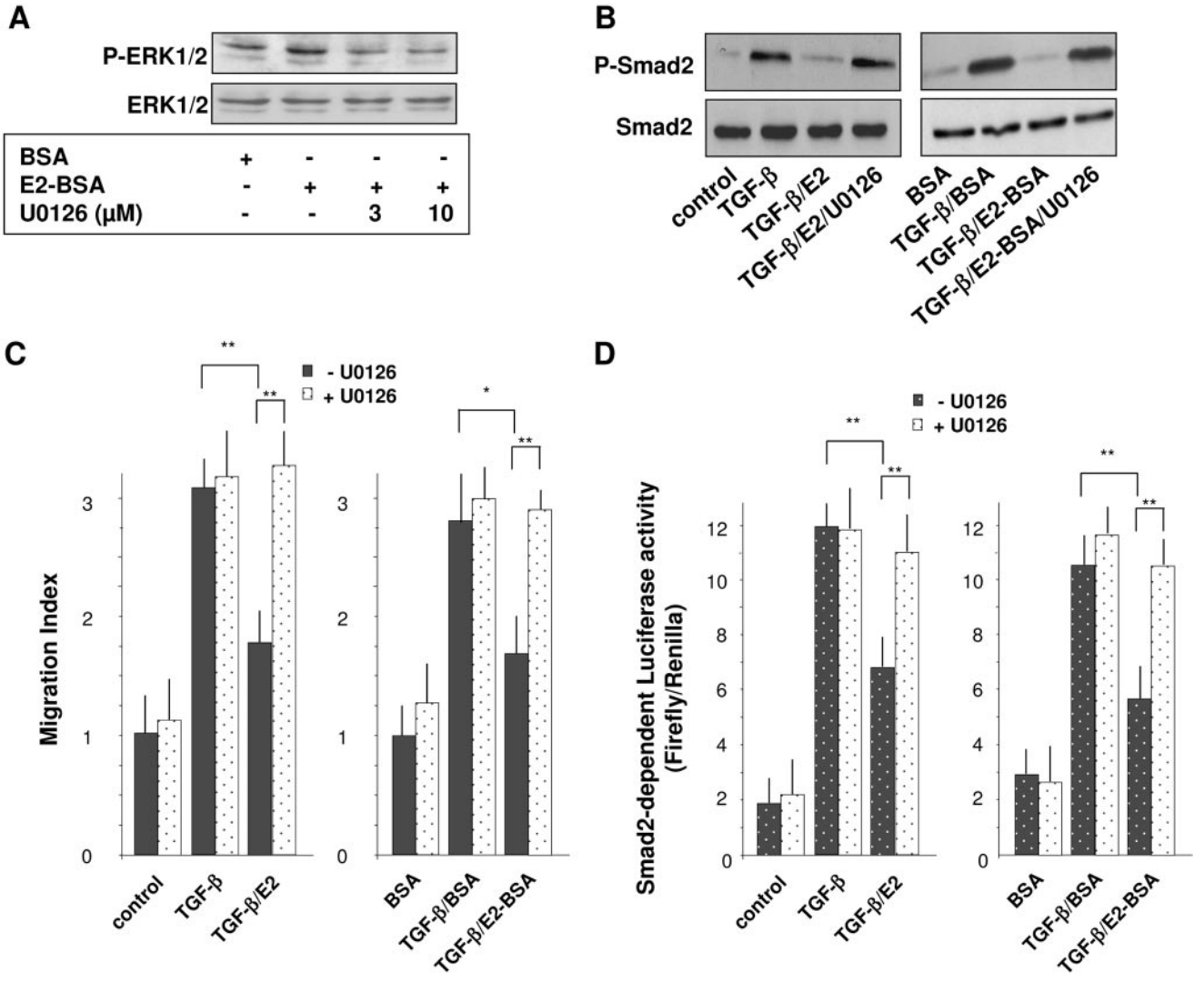

Fig. 7. Inhibition of ERK1/2 phosphorylation prevents the inhibitory effect of E2 on TGF-β signaling and function in MCF-7 cells. MCF-7 cells were pretreated with the indicated concentrations of U0126 for 90 min. A, cells were stimulated with BSA (0.3×10^{-8} M) or E2-BSA (10^{-7} M) for 10 min and 15 μg of lysate protein were electrophoresed and immunoblotted with anti-phospho-p44/42-MAPK (top) or anti-p44/42-MAPK-antibodies (bottom). MCF-7 cells were preincubated with U0126 (3 μM) followed by a treatment with E2 (10^{-7} M), BSA (0.3×10^{-8} M), or E2-BSA (10^{-7} M) for 30 min. B, cells were stimulated for 60 min with 2 ng/ml TGF-β and 15 μg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 or anti-Smad2 antibodies. Chemotaxis of U0126-pretreated (3 μM) and E2- (10^{-7} M), BSA- (0.3×10^{-8} M) or E2-BSA- (10^{-7} M)-sensitized MCF-7 cells in response to TGF-β (2 ng/ml) was measured in a modified Boyden chamber. Data are expressed as arbitrary migration index ± S.E.M. from three experiments. *, $P < 0.05$ and **, $P < 0.001$ indicate a statistically significant difference versus control cells (C). MCF-7 cells were transfected with a luciferase-coupled promoter construct responsive to Smad2. After 24 h, cells were pretreated with U0126 (3 μM) and sensitized with E2 (10^{-7} M), BSA (0.3×10^{-8} M), or E2-BSA (10^{-7} M). D, cells were stimulated with TGF-β (2 ng/ml) for 24 h, and luciferase activity was determined. Values present the average number of counts corrected by *R. reniformis* luciferase activity ± S.E.M. from three experiments (**, $P < 0.001$).

an enhancement of cell migration and invasion, angiogenesis, and the suppression of the immune system (Wakefield and Roberts, 2002; Miyazono et al., 2003). In fact, estrogens have been identified to inhibit TGF-β signaling in breast cancer cells, which is in agreement with studies indicating that estrogens decrease the in vitro invasiveness and migration of tumor cells (Platet et al., 2004). Despite the function of estrogens to modulate TGF-β secretion, a direct cross-talk between the steroid hormones and TGF-β signaling has been described previously (Malek et al., 2006; Cherlet and Murphy, 2007). However, the molecular mechanisms of this cross-communication are not well elucidated. It has been suggested that there exists a cross-communication of intracellular ER with *R*-Smad proteins via a direct physical binding resulting in an impaired TGF-β signaling (Matsuda et al., 2001). However, other laboratories could not observe such a direct physical interaction, when cells were transiently transfected with Smad3 and ERα expression vectors (Cherlet and Murphy, 2007).

Our data clearly indicate a nongenomic estrogen-dependent action via membrane-localized receptors as the inhibition of TGF-β signaling induced by E2 occurs also in response to membrane-impermeable BSA conjugates, and moreover, the effect is reversed in the presence of PTX. Indeed, the existence of functional ERs associated with the plasma membrane is widely appreciated, although there exist several concepts about the nature of membrane-localized ERs. Most studies refer to receptors that are related to either the classic nuclear types, ERα and ERβ, or to an orphan estrogen responsive receptor, GPR30 (Marino and Ascenzi, 2008; Prossnitz et al., 2008). In the present study, we provide fundamental evidence that the inhibition of TGF-β signaling triggered by E2 relies on the expression and activity of GPR30. Thus, E2 lost its ability to suppress TGF-β signaling in MCF-7 when GPR30 was down-regulated. Moreover, in GPR30-deficient MDA-MB-231 cells, E2 achieved the aptitude to inhibit TGF-β signaling only after transfection with GPR30-encoding plasmids. It should be mentioned that there exists some controversy regarding the localization of the GPR30. Thus, several studies indicate an expression of the GPR30 only in the endoplasmic reticulum and other tubuloreticular compartments (Revankar et al., 2005; Otto et al., 2008). Another group has demonstrated that GPR30 is also detectable in the plasma membrane, becomes sequestered from the cell surface, and codistributes into clathrin-coated vesicles (Filardo et al., 2007). This discrepancy may be explained by the observation that G protein-coupled receptors traffic between the endoplasmic reticulum, submembranous vesicles, and the plasma membrane during receptor biogenesis and their internalization in response to agonist stimulation. Moreover, it has also been shown that despite the impermeable nature of E2-BSA, the ligand gains access to the endoplasmic reticulum (Wang et al., 2008). Thus, it seems also possible that GPR30 signals from the endoplasmic reticulum, or other intracellular locations, by a currently undefined molecular mechanism.

Although GPR30 and the classic nuclear ERα have evolved ligand-binding pockets that display considerable overlap in their ligand-binding profiles, there exist fundamental differences concerning receptor activation. Thus, it has been demonstrated that the antiestrogens tamoxifen and ICI 182,780 not only have high binding affinities to the GPR30 but also mimic the actions of E2 (Thomas et al., 2005). This is in agreement with our results indicating that TGF-β signaling is inhibited by both, the estrogen E2 and the antiestrogen ICI 182,780.

Furthermore, it was of interest to identify the mechanisms, by which GPR30 interferes with the TGF-β cascade. It is well established that TGF-β mediates a variety of its action by the formation of activated Smad complexes, which translocate into the nucleus and act as transcription factors. There is evidence that there exists a cross-talk between the MAPK pathway and TGF-β signaling; however, modulation of Smad activity by ERK1/2 is cell type-dependent (Kretzschmar et al., 1999; Massague, 2003). In our work with MCF-7 cells, we see that blocking MAPK activity abrogates the ability of E2 and ICI 182,780 to inhibit Smad-activation and TGF-β-induced migration. But it should be mentioned that ERK1/2 activation in response to E2 and ICI 182,780 occurred at lower concentrations than inhibition of TGF-β-mediated migration. This discrepancy may be the result of different experimental procedures because chemotactic activity has been measured over a time period of 5 h. Indeed, several reports indicate that Smads are phosphorylated by MAPK, such as phosphorylation of the linker region leading to an inactivation of Smad (Matsuura et al., 2005). Our data clearly indicate that GPR30 inhibits the phosphorylation of Smad proteins via ERK1/2 activation. This is consistent with several studies pointing out that GPR30 influences MAPK activity (Vivacqua et al., 2006). It has been demonstrated that in breast cancer cells estrogen acts via GPR30 to promote rapid transactivation of the EGF receptor to the MAPK pathway through the release of heparin-binding EGF (Filardo and Thomas, 2005). Moreover, it has been shown in MDA-MB-231 cells, which lack the orphan receptor, that ERK1/2 phosphorylation occurred only after transfection with GPR30 plasmids (Filardo et al., 2000). But it should be considered that an opposing effect of GPR30 on ERK activity through stimulation of adenylyl cyclase has been reported, suggesting that GPR30 acts to balance ERK1/2 activity (Filardo et al., 2002).

Our studies may also have profound implications for the treatment of breast cancer because the antiestrogen tamoxifen or its active metabolite 4-hydroxytamoxifen is used as a first-line treatment for ER-positive metastatic breast cancer. It is well established that a majority of patients with breast cancer benefit from treatment with the antiestrogen, but almost 50% of patients eventually show resistance to tamoxifen. Although a variety of studies have aimed to elucidate the reason of this resistance, the molecular mechanism has not been well defined (Clarke et al., 2001; Ring and Dowsett, 2004). The suppression of TGF-β signaling has been identified as crucial for the development of breast cancer resistance (Yoo et al., 2008). Because our results indicate that activation of GPR30 induces suppression of TGF-β signaling and moreover that tamoxifen is an agonist at this receptor, it would be of great interest to further elucidate whether an association between tamoxifen resistance and GPR30 expression exists.

When all the data are taken together, this is the first study in which GPR30 was identified as the essential receptor responsible for the inhibitory effect of estrogens and antiestrogens on TGF-β signaling and function. With respect to the divergent role of TGF-β as a tumor suppressor and a tumor promoter in late stages of breast cancer, the role of GPR30 on tumor growth and its invasiveness remain to be clarified.

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