Material and Methods

2 Materials and methods

2.1 Materials

2.1.1 Technical devices

Autoclave Tuttnauer 2540EVL	Tuttnauer, Breda, Netherlands
Bandelin Sonorex RK100	Bandelin electronics, Berlin
Chamber for agarose gels Agagel Standard	Biometra, Göttingen
Developing cassette	Kodak, München
Electrophoresis chamber Multigel Long	Biometra, Göttingen
Eppendorf Centrifuge 5415D	Eppendorf, Hamburg
Freezer Hera Freeze, -80 °C	Heraeus Instruments, Düsseldorf
Incubator BB 6220 (for cell culture)	Heraeus Instruments, Düsseldorf
Incubator Function line (for E. coli culture)	Heraeus Instruments, Düsseldorf
Lamin Air-Sterile Working Bench HB2472	Heraeus Instruments, Düsseldorf
Magnetic stirrer IKA Kombimag® RCT	Jahnke und Kunkel GmbH & Co. KG IKA Labortechnik, Staufen
Megafuge 1.0 R, max. 2800 rpm	Heraeus Sepatech, Osterode
Microplate reader Flashscan S12 + software WinFlash	Analytik Jena AG, Jena
Microscope Axiovert 135, phase contrast, inverted	Carl-Zeiss, Jena
Microscope Olympus BX41	Olympus, Hamburg
Multilabel counter Wallac Victor^2 1420 + software Wallac 1420 workstation	Perkin Elmer, Rodgau
Neubauer Improve 1 (0.0025 mm^2/0.1 mm)	VWR, Darmstadt
Nikon Digital still Camera DMX 1200 + ACT-1 software	Nikon, Düsseldorf
pH-Meter 766 Calimatec	Knick, Nürnberg
UV-Photometer Gene-Ray	Biometra, Göttingen
Pipetboy® plus	Integra Biosciences, Fernwald
Pipettes Eppendorf Reference®	Eppendorf Hamburg
Pipettes Eppendorf Research®	Eppendorf Hamburg
Pump Easyload Masterflex	Microgon, Laguna Hills, CA
Rocking Platform WT16	Biometra, Göttingen
Rotary shaker KS10 Swip + TH10	Edmund Bühler GmbH, Tübingen
Shaker IKA® MTS 2	Jahnke und Kunkel GmbH & Co. KG IKA Labortechnik, Staufen
Standard Power Pack P25	Biometra, Göttingen
Tank Arpege 40 (for liquid nitrogen)	Air Liquide, Berlin
Tankblot	Biometra, Göttingen
Thermoblock TB1	Biometra, Göttingen
Thermocycler T Gradient	Biometra, Göttingen
Tissue cell Scraper	TPP, Trasadingen, Switzerland
Transluminator BioDoc Analyze + software	Biometra, Göttingen
Vacuum Set Vacuboy®	Integra Biosciences, Fernwald
Vortex mixer REAX top	Heidolph Instruments, Schwabach

2.1.2 Consumptive materials

18 mm coverslips	Carl-Roth GmbH, Karlsruhe
Cell culture dish (10 cm)	TPP, Trasadingen, Switzerland
Cell culture inserts 24-well (8 µm pore size)	TPP, Trasadingen, Switzerland
Centrifugal tubes (15, 50 ml)	Sarstedt, Nümbrecht
Cryotube™ vials	Nunc, Wiesbaden
Cuvette, polystyrene, optical pathway, 10 mm	Sarstedt, Nümbrecht
Film Kodak X-OMAT, XAR-5	Sigma-Aldrich, Taufkirchen
Filters cellulose acetate (0.2 µm pore size)	Sartorius, Göttingen
Filter for sterilisation Filtropur S 0.2 µm	Sarstedt, Nümbrecht
Filter Tips Biosphere® 10, 100, 200, 1000	Sarstedt, Nümbrecht
Flasks, tissue culture, 75 cm^2	TPP, Trasadingen, Switzerland
Microscope slides	Menzel-Gläser, Braunschweig
Micro tube, safe-seal 0.5, 1, 2 ml	Sarstedt, Nümbrecht
Optiplate™-96	Packard, Ramsey, MN
Petri dishes Falcon™, 10 cm, bacteriological	BD Biosciences, Heidelberg
Pasteur pipettes	Carl-Roth GmbH, Karlsruhe
PCR tubes (1.5 ml)	Eppendorf, Hamburg
PCR tubes (0.2 ml)	Abgene, Hamburg
Pipette, serological, 2, 5, 10, 25 ml	Sarstedt, Nümbrecht
Pipette tips 10, 200, 1000 µl	Sarstedt, Nümbrecht
Qiafilter™ Plasmid Maxi Kit (25)	Qiagen, Hilden
QuickPrep™ mRNA Purification Kit	GE Healthcare, Munich
Syringe (0.9 x 40 mm, 20G x 11/2“)	B/Braun, Melsungen
Syringe (Omnifix® 0.5 x 16 mm, 25G x 5/8“)	B/Braun, Melsungen
Test plates, tissue culture 6, 12, 24 well	TPP, Trasadingen, Switzerland
Transfer membranes, polyvinyliden-difluoride (PVDF) immobilon	Millipore, Schwalbach
UV cuvettes Plastibrand, semi-micro	Brand, Wertheim

2.1.3 Chemicals and reagents

100 bp DNA ladder	New England Biolabs, Frankfurt/Main
17-β-estradiol (E2)	Fluka, Buchs, Switzerland
Acrylamid (Rotiphorese® Gel 40 (29:1)	Roth, Karlsruhe
β-Actin antibody, mouse-IgG, monoclonal	Santa Cruz, Heidelberg
Agarose	Sigma-Aldrich, Taufkirchen
Ammonium persulfate	Sigma-Aldrich, Taufkirchen
Ampicillin sodium salt	Sigma-Aldrich, Taufkirchen
Anti-goat IgG antibody, HRP-conjugated	Santa Cruz, Heidelberg
Anti-mouse IgG antibody, HRP-conjugated	Cell Signalling Technology, Beverly, MA
Anti-rabbit IgG antibody, HRP-conjugated	Cell Signalling Technology, Beverly, MA
Aprotinin	Sigma-Aldrich, Taufkirchen
β-Catenin antibody, mouse IgG, monoclonal	BD Transduction Laboratories, Heidelberg
β-Mercaptoethanol	Sigma-Aldrich, Taufkirchen

Boric acid	Sigma-Aldrich, Taufkirchen
Bovine serum albumin (BSA)	Sigma-Aldrich, Taufkirchen
BSA-conjugated estradiol (BSA-E2)	Sigma-Aldrich, Taufkirchen
Calcium chloride ($CaCl_2$)	Fluka, Buchs, Switzerland
Charcoal Norit® A	Serva, Heidelberg
Developer and replenisher, GBX	Sigma-Aldrich, Taufkirchen
Dextran	Sigma-Aldrich, Taufkirchen
dGTP, dCTP, dATP, dTTP (10 mM)	Abgene, Hamburg
Dharmafect1 transfection reagent	Dharmacon RNA Technologies, Lafayette, CO
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Taufkirchen
Dimethyl sulfoxide (99 %) (DMSO)	Sigma-Aldrich, Taufkirchen
Dithiothreitol (0.1 M) (DTT)	Invitrogen, Karlsruhe
Dithiothreitol (30x) (DTT)	New England Biolabs, Frankfurt/Main
Dry milk powder Sucofin	TSI, Zeven
Dual-Luciferase® Assay System	Promega, Mannheim
ERα antibody, rabbit-IgG, polyclonal	Santa Cruz, Heidelberg
Ethanol (for molecular biology)	Sigma-Aldrich, Taufkirchen
Ethanol (other use)	Sigma-Aldrich, Taufkirchen
Ethidium bromide	Sigma-Aldrich, Taufkirchen
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Taufkirchen
Ethyleneglycol-bis(2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA)	Sigma-Aldrich, Taufkirchen
Fetal calf serum (FCS)	Biochrom, Berlin
Fibronectin, human	Sigma-Aldrich, Taufkirchen
First strand buffer (5fold)	Invitrogen, Karlsruhe
FITC-conjugated anti-rabbit IgG antibody	Sigma-Aldrich, Taufkirchen
Fixer and replenisher, GBX	Sigma-Aldrich, Taufkirchen
Fugene6™	Roche Diagnostics, Mannheim
Geneticin sulfate	Sigma-Aldrich, Taufkirchen
Gentamycin sulfate	PAN Biotechnology, Aidenbach
Giemsa solution	Sigma-Aldrich, Taufkirchen
Glutardialdehyde	Merck, Darmstadt
Glycerol	Sigma-Aldrich, Taufkirchen
Glycine (for electrophoresis, 99 %)	Sigma-Aldrich, Taufkirchen
GPR30-antibody rabbit-IgG, polyclonal (Western blot)	MBL, Woburn, MA
GPR30-antibody, rabbit-IgG, polyclonal (Immunofluorescence)	Acris Antibodies, Hiddenhausen
GPR30-siRNA + control-siRNA	Dharmacon RNA Technologies, Lafayette, CO
Hydrochloric acid (HCl)	Sigma-Aldrich, Taufkirchen
ICI182,780 (Faslodex™)	Tocris Cookson, Avonmouth, UK
Igepal CA-630	Sigma-Aldrich, Taufkirchen
Isopropanol (for molecular biology, 99 %)	Sigma-Aldrich, Taufkirchen
Isopropanol (other use)	Sigma-Aldrich, Taufkirchen
Kanamycin	Sigma-Aldrich, Taufkirchen
LB-Broth	Sigma-Aldrich, Taufkirchen
Leupeptin	Sigma-Aldrich, Taufkirchen
L-Glutamine	Sigma-Aldrich, Taufkirchen

Liquid Nitrogen	Praxair, Düsseldorf
LumiGlo® reagent and peroxide	New England Biolabs, Beverly, MA
Luria Agar	Sigma-Aldrich, Taufkirchen
Magnesium chloride solution ($MgCl_2$, 25 mM)	Abgene, Hamburg
Manganese chloride ($MnCl_2$)	Fluka, Buchs, Switzerland
Medium, Dulbecco's modified Eagle's, Nutrient mixture F-12 HAM	Sigma-Aldrich, Taufkirchen
Medium, Eagle`s Minimal Essential	Sigma-Aldrich, Taufkirchen
Medium, McCoy`s 5A	Sigma-Aldrich, Taufkirchen
Methanol	Sigma-Aldrich, Taufkirchen
Molecular weight marker, prestained	Cell Signalling Technology, Beverly, MA
Mowiol	Calbiochem, Darmstadt
N, N, N', N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Taufkirchen
Oligo$(dt)_{12-18}$-primer	Invitrogen, Karlsruhe
OptiMEM® Gibco	Invitrogen, Karlsruhe
Ortho-Phosphoric acid (85 %) (H_3PO4)	Fluka, Buchs, Switzerland
Para-formaldehyde	VWR, Vienna, Austria
PD098,059 (2'-Amino-3'-methoxyflavone)	Sigma-Aldrich, Taufkirchen
Penicillin	Sigma Aldrich, Taufkirchen
Pepstatin	Sigma-Aldrich, Taufkirchen
Pertussis toxin (PTX)	Calbiochem, Darmstadt
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich, Taufkirchen
Phosphate buffered saline (w/o Ca^{2+}, Mg^{2+})	Sigma-Aldrich, Taufkirchen
Phospho-p44/42-MAPK antibody, mouse-IgG, monoclonal (T202/Y204)	Cell Signalling Technology, Beverly, MA
Phospho-Smad2 antibody, rabbit-IgG, monoclonal (Ser465/467)	Cell Signalling Technology, Beverly, MA
Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)	Sigma-Aldrich, Taufkirchen
Potassium chloride (KCl)	Sigma-Aldrich, Taufkirchen
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich, Taufkirchen
Potassium hydroxide (KOH)	Sigma-Aldrich, Taufkirchen
Primers	Roth, Karlsruhe
Protein G-plus Agarose	Merck, Darmstadt
Reaction buffer IV (10fold)	Abgene, Hamburg
Reverse Transcriptase Superscript™ II	Invitrogen, Karlsruhe
Ribonuclease inhibitor	Fermentas, St. Leon-Rot
Serva Blue G	Serva, Heidelberg
Smad1,2,3-antibody, mouse-IgG, monoclonal (H-2)	Santa Cruz, Heidelberg
Smad3-antibody, mouse-IgG, monoclonal (FL-425)	Santa Cruz, Heidelberg
Smad4-antibody, mouse-IgG, monoclonal (B-8)	Santa Cruz, Heidelberg
SOB Broth	Fluka, Buchs, Switzerland
Sodium chloride (NaCl)	Sigma-Aldrich, Taufkirchen
Sodium deoxycholic acid	Sigma-Aldrich, Taufkirchen
Sodium dodecyl sulfate (SDS)-sample buffer	New England Biolabs, Frankfurt/Main

Sodium dodecyl sulfate (98.5 %) (SDS)	Sigma-Aldrich, Taufkirchen
Sodium fluoride (NaF)	Sigma-Aldrich, Taufkirchen
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt
Sodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich, Taufkirchen
Sodium hydroxide (NaOH)	Sigma-Aldrich, Taufkirchen
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Taufkirchen
Sodium pyruvate	Sigma-Aldrich, Taufkirchen
Streptomycin	Sigma Aldrich
Tamoxifen-citrate (TAM-citrate)	Sigma-Aldrich, Taufkirchen
Transforming growth factor -β1 (TGF-β)	Sigma-Aldrich, Taufkirchen
Thermoprime plus Taq DNA polymerase	Abgene, Hamburg
Tris-base (99.9 %)	Sigma-Aldrich, Taufkirchen
Tris-HCl (99 %)	Sigma-Aldrich, Taufkirchen
Trypsin	Biochrom, Berlin
Tween 20	Sigma-Aldrich, Taufkirchen

2.2 Methods

2.2.1 Culture media and solutions

Eagle's minimum essential medium (EMEM)

9.7 g/l	EMEM (with 11 mg/l phenol red)
50 mg/l	Gentamycin
2.2 g/l	$NaHCO_3$
110 mg/l	Sodium pyruvate
2 mM	L-glutamine

McCoy's

9.7 g/l	McCoy's medium (with 11 mg/l phenol red)
50 mg/l	Gentamycin
2.2 g/l	$NaHCO_3$
110 mg/l	Sodium pyruvate
1.5 mM	L-glutamine

Dulbecco's modified Eagle's medium (DMEM)

9.7 g/l	DMEM (without phenol red)
100 U/ml	Penicillin
100 µg/ml	Streptomycin
0.5 %	Geneticin sulfate (80 mg/ml PBS)

3.7 g/l	$NaHCO_3$
110 mg/l	Sodium pyruvate
4 mM	L-glutamine

After preparation of EMEM, McCoy's medium, and DMEM the pH value was adjusted to 7.4 and the medium was sterilised by filtration through a cellulose acetate filter (pore size 0.2 µm) and by use of the Pump Easyload Masterflex.

2.2.2 Solutions for cell culture

Stop medium

10 %	FCS (fetal calf serum)
100 U/ml	Penicillin
100 µg/ml	Streptomycin
in	DMEM nutrient mixture F-12 HAM

Freezing medium (MCF-7, MDA-MB-231)

10 %	DMSO
10 %	FCS
100 U/ml	Penicillin
100 µg/ml	Streptomycin
in	DMEM nutrient mixture F-12 HAM

Freezing medium (MCF-7-2a)

10 %	DMSO
5 %	FCS
in	DMEM

Phosphate buffered saline (PBS)

0.2 g/l	KCl
8.0 g/l	NaCl
0.2 g/l	KH_2PO_4
1.44 g/l	Na_2HPO_4
in	Aqua bidest.

Trypsin-EDTA solution

1.67 g/ml	Trypsin
0.67 mg/ml	EDTA
in	PBS

Phenol red-free EMEM medium

9.7 g/l	EMEM (without phenol red)
50 mg/l	Gentamycin
2.2 g/l	$NaHCO_3$
110 mg/l	Sodium pyruvate
2 mM	L-glutamine

DMEM (for transfection)

2 mM	L-glutamine
in	DMEM nutrient mixture F-12 HAM

2.2.3 Charcoal treated FCS (ct-FCS)

To retain estrogens contained in culture media from distorting measurements, FCS was relieved from endogenous steroids by treatment with dextran coated charcoal. 10 g charcoal Norit® A was suspended in Tris-buffer (pH 7.4), stirred for 3-4 h, and kept over night at 4 °C. Non-moistened charcoal was aspirated, 100 mg dextran were added, stirred at room temperature for 20 min, distributed into two 50 ml tubes, and centrifugated for 10 min (3500 * g/4 °C). The supernatant was decanted and the first charcoal pellet was loaded with 500 ml FCS, which was inactivated before for 45 min at 56 °C in a water bath. The suspension was stirred for 3 h at 4 °C, centrifugated for 20 min (8000 * g/4 °C), and the supernatant was given to the second charcoal pellet. The procedure was repeated, the ct-FCS was sterilised by filtration through 0.2 µm membrane filters, and kept at –20 °C until use.

2.2.4 Cultivation of cell lines

2.2.4.1 Cultivation

MCF-7 as well as MDA-MB-231 cell lines were purchased from ATCC (American Type Culture Collection, Rockville, MD). MCF-7-2a cells stably transfected with the $ERE_{wt\ luc}$ plasmid are derived from the MCF-7 cell line (Hafner et al., 1996). MCF-7, MDA-MB-231, and MCF-7-2a cells were maintained as monolayer cultures in EMEM with 10 % FCS (MCF-7), McCoy's 5A medium with 5 % FCS (MDA-MB-231), and DMEM with 5 % FCS (MCF-7-2a). All cell lines used in this study grew in cell culture flasks with a growth area of 75 cm^2 at 37 °C in a steamed atmosphere with 5 % CO_2.

2.2.4.2 Subculture of cell lines

When reaching a density of 70 % (MCF-7) or 80 % (MDA-MB-231), usually after 5 to 7 days of culture, or two times a week for MCF-7-2a, the cells were detached and a new subculture was established. Therefore, the cell layers were washed with PBS and incubated with 1.5 ml 0.25 %/0.2 % Trypsin-EDTA solution at 37 °C, 5 % CO_2 until all cells were detached from the culture flasks. The enzyme reaction was finished by addition of 3.5 ml Stop medium. Cells were homogenised using a serological pipette and transferred to 50 ml centrifugal tubes. After centrifugation (220 * g/4 °C) for 5 min the cells were resuspended with growth medium, counted (Neubauer Improve 1), and seeded in 10 ml growth medium at a density of $5*10^5$ of MCF-7 or $2*10^5$ of MDA-MB-231. MCF-7-2a suspension was dispensed in a ratio of 1:20 in 10 ml growth medium and incubated at 37 °C, 5 % CO_2. After 24 h of cultivation not attached cells were removed and fresh medium was supplied.

2.2.4.3 Cryopreservation and resuscitation of frozen cells

Cells from one culture flask were brought into suspension with Trypsin-EDTA solution as described above. The culture was centrifuged (220 * g/4 °C/5 min), resuspended in 2 ml freezing medium and transferred into cryoprotective tubes. The tubes were wrapped in paper towels and aluminium foil to decelerate the freezing process. Frozen ampules from -80 °C freezer were transferred to a liquid nitrogen storage vessel.

Ampules of cells were collected from liquid nitrogen container and placed in a water bath at 37 °C until thawed. The cells were pipetted in pre-warmed growth medium to dilute out the DMSO and centrifugated for 5 min (220 * g/4 °C). The pelleted cells were resuspended in to 10 ml growth medium, transferred into culture flasks, and incubated at 37 °C, 5 % CO_2.

2.2.5 Preparation of test substances

E2 and ICI182,780 were solved in 96 % ethanol to make a stock solution with a concentration of $5*10^{-3}$ M for E2 and 10^{-2} M for ICI182,780. The E2 stock solution was freshly prepared directly before use, whereas the ICI182,780 solution could be stored at 4 °C. Both substances were further diluted with 96 % ethanol according to the requirements of the assay. TAM-citrate was diluted in aqua bidest. (sterile filtered: Filtropur S 0.2 µm) to obtain a 0.5 mM stock solution.

TGF-β was solved in PBS supplemented with 0.1 % BSA (bovine serum albumin) and 4 mM HCl (sterile filtered) to a concentration of 1 mg/ml, aliquotated, and stored at -80 °C. This stock solution was further diluted in 0.4 % BSA in PBS (sterile filtered) directly before use.

Lyophilised PTX was solved in sterile filtered aqua bidest. to a concentration of 100 µg/ml and stored at 4 °C. A 25 mM stock solution of PD098,059 in sterile filtered DMSO was prepared, aliquotated, and stored at –20 °C. In order to prevent precipitation of the inhibitor, the stock solution was diluted to 10 mM in DMSO immediately before test start.

The required final concentrations of all test substances were produced by dilution in the test medium. Control determinations contained solvents in the appropriate concentrations, but not exceeding a concentration of 0.5 % ethanol (E2, ICI182,780),1 % aqua bidest. (TAM-citrate, PTX), 0.5 % DMSO (PD098,059) and 2 % 0.4 % BSA in PBS (TGF-β).

2.2.6 Purification of BSA-conjugated E2 with dextran-coated charcoal

According to the manufacturer, the content of free estradiol in the BSA-conjugated E2 is supposed not to exceed 2 %. In order to remove remaining traces of non-

complexed steroid, the conjugate was treated with dextran-coated charcoal, which was prepared as subsequently described.

Charcoal Norit® A (400 mg) was suspended in 50 mM Tris-buffer (pH 7.4) to a final concentration of 8 % (m/v), stirred for 3-4 h, and kept over night at 4 °C. Non-moistened charcoal was aspirated, 40 mg (final concentration 0.8 %, m/v) dextran were added, and stirred at room temperature for 20 min. BSA-E2 (5 mg) was diluted in 50 mM Tris-buffer (pH 8.5) to a final E2 concentration of $1.33*10^{-5}$ M. The dextran-charcoal suspension was centrifugated for 10 min (3500 * g/4 °C). The resulting charcoal pellet was resuspended in the whole volume of BSA-E2 solution and stirred over night at 4 °C. On the next day the suspension was centrifuged for 20 min (8000 * g/4 °C) and the supernatant containing BSA-E2 was kept for further use.

For sensitisation of cells, 752 µl purified BSA-E2 was added to 10 ml growth medium reaching a final estradiol concentration of 10^{-6} M. The cultures were incubated for 30 min at 37 °C/5 % CO_2.

2.2.7 Migration assay

For detection of the chemoattracting properties of test substances, a transwell system was utilised. The main components are cell culture filter inserts establishing two distinct chambers separated by a polycarbonate membrane with 8 µm pore width. In order to enhance migration rates, the filter membranes were covered with 70 µl of 3 µg/ml fibronectin (diluted in aqua bidest.), an adhesive protein in the ECM, and incubated for 60 min at 37 °C/5 % CO_2. Thereafter, the solution was aspirated and the inserts were left upside down to air dry.

$2*10^6$ MCF-7 and $1*10^6$ MDA-MB-231 cells were seeded in 10 cm-culture dishes until 70-80 % confluence. A 24-well plate (lower chamber) was charged with 500 µl/well basic medium (without FCS supplementation) containing TGF-β, E2, serum or solvent were given and finally the inserts.

According to the experiment, the cultures were treated with E2, BSA-E2, ICI182,780, TAM-citrate, PD098,059 or PTX. After the particular incubation time, the cells were detached and 500 µl of cell suspension containing $1*10^6$ cells were given into the inserts (upper chamber). For determination of chemokinetic movement, the stimulating substances were additionally applied to the filter inserts thus abrogating the concentration gradient between upper and lower chamber. After 5 h of incubation

at 37 °C/5 % CO_2 all media were removed and the cells fixed with 96 % ice-cold ethanol for 2 min at –20 °C. The ethanol was aspirated and the cells on the upper side of the filter were removed with a Q-tip, whereas migrated cells were stained with 500 µl Giemsa solution (0.04 %, m/v) for 30 min at 37 °C. The dye was removed and, when almost dried, the upper side of the insert membrane was wiped off with a Q-tip. Migrated cells in and under the membranes could be observed under an inverted phase contrast microscope. For each determination 10 randomly chosen fields were counted and averaged.

To permit a comparison of different experiments, the counted migration rates were referred to those of non-stimulated controls, which were defined to have a migration index of one. Migration indices were calculated as follows:

$$Migration\ index = \frac{V_{test}}{V_{contr}}$$

V_{test} median values of migrated cells (stimulated)

V_{contr} median values of migrated non-stimulated cells

2.2.8 Cell viability assay

100 µl cell suspension containing $1*10^4$ MCF-7 or $5*10^3$ MDA-MB-231 cells were seeded in 96-well plates und allowed to attach for 3 days. For each time point and for determination of the initial cell biomass (C_0) one separate plate is demanded.

E2 was solved in 96 % ethanol and the stock solution was diluted with solvent to 1000fold of concentration required for the test. These solutions were diluted in the adequate medium to reach the final test concentration. 200 µl of the test or vehicle solutions were given into 6 wells of the 96-well plate. At this time the plate for the determination of the initial cell biomass is stopped and kept in PBS at 4°C.

The reaction was stopped after 72, 125, 150, 170, and 240 h for MCF-7 and after 48, 72, 96, 118, and 167 h for MDA-MB-231 by aspirating of media. For fixation of cells, 100 µl glutardialdeyde solution (500 µl glutardialdehyde in 12.5 ml PBS, pH 7.4) were added and removed after 20 to 30 min. After a washing step with 180 µl PBS cells were stained with 100 µl 0.02 % crystal violet for 30 min. The plates were washed twice with water and left in washing solution for 15 min. The water was removed carefully by rapping on a paper towel. Remaining dye was eluted in 180 µl 70 %

ethanol and the plates were slightly shaken for 3 to 4 h. Absorption measurement was carried out in a microplate reader at 590 nm.

The median absorption values of the appropiate wells were determined. The relative percentage growth inhibition is expressed as T/C_{corr}, which was calculated by referring the absorbance value of cells treated with E2 to those treated with vehicle only according to the following equation.

$$T / C_{corr}[\%] = \frac{(T^* - C_0)}{(C^* - C_0)} \cdot 100$$

T*: average absorption value of the test substance

C_0: average absorption value of the initial cell mass

C*: average absorption value of solvent blank value

The results are generally interpreted as follows:

$T/C_{corr} > 80$ %	no antiproliferative effect
$T/C_{corr} < 80$ %	antiproliferative effect
$T/C_{corr} < 20$ %	cytostatic effect
$T/C_{corr} < 0$ %	cytocidic effect.

2.2.9 Preparation of membrane protein enriched cell fractions

Lysis buffer

20 mM	Tris-HCl
5 mM	$MgCl_2$
1 mM	EDTA
0.6 mM	EGTA

freshly supplemented with protease and phosphatase inhibitors:

50 mM	NaF
1 mM	PMSF
1 mM	Na_3VO_4
1 µg/ml	Leupeptin
1 µg/ml	Aprotinin
1 µg/ml	Pepstatin

On account of the small amount of GPR30 protein in the cells, the detection of the receptor was performed in isolated membrane fractions. The method described in the following chapter was used to accumulate membrane proteins from whole cell lysates. MCF-7 and MDA-MB-231 cells were grown and transfected in 75 cm^2 cell culture flasks (one per sample) as described in chapters 2.2.14 and 2.2.17. The cultures were washed with 10 ml PBS and 1 ml pre-chilled lysis buffer, which has been supplemented with protease and phosphatase inhibitors immediately before use, was added and the cells incubated for at least 30 min. Cells were harvested by a cell scraper. The destruction of cells was supported by triple 10 sec sonification in a water bath followed by passing through 20- and 30-gauge syringes, ten times each. Cell shred was removed by centrifugation at 400 * g, 4 °C for 5 min. The cold membrane fraction was separated from the soluble fraction by centrifugating the supernatant at 30.000 * g, 4 °C for 60 min. 50 µl lysis buffer were added to the pellet and homogenised with a pipette. Protein content was determined by Bradford staining (see chapter 2.2.10.4) and the probes stored at –80 °C.

2.2.10 Protein detection by immunoprecipitation, SDS-polyacrylamide gelelectrophoresis (SDS-PAGE), and Western blot

2.2.10.1 Solutions for SDS-PAGE and Western blot

Tris-buffered solution (TBS buffer, 10x)

12.14 g/l	Tris-HCl
87.66 g/l	NaCl
in	Aqua bidest.

For TBST 0.1 % Tween 20 was added and pH was adjusted to 8.

Blocking buffer

5 %	low fat dry milk power Sucofin
in	TBST

Blotting buffer (10x)

144 g/l	Glycine

30 g/l	Tris-base
in	Aqua bidest.

Running buffer (10x, pH 8.3)

144 g/l	Glycine
30.2 g/l	Tris-base
10 g/l	SDS
in	Aqua bidest.

Collecting gel buffer (pH 6.8)

60 g/l	Tris-HCl
in	Aqua bidest.

Separating gel buffer (pH 8.8)

224.8 g/l	Tris-base
in	Aqua bidest.

SDS-polyacrylamid gel (separating gel, 10 %)

5.4 ml	Aqua bidest.
1.2 ml	SDS (1 % in aqua bidest.)
2.4 ml	Separating gel buffer
10 µl	TEMED (N, N, N', N'-Tetramethylethylenediamine)
60 µl	Ammonium persulfate (0.1 g/ml in aqua bidest.)
3.0 ml	Acrylamid Rotiphorese® Gel 40

Collecting gel (5 %)

2.3 ml	Aqua bidest.
0.4 ml	SDS (1 % in aqua bidest.)
0.8 ml	Collecting gel buffer
4 µl	TEMED
20 µl	Ammonium persulfate
0.5 ml	Acrylamid Rotiphorese® Gel 40

Strip buffer

50 ml	10x Tris-buffer (pH 6.8, 500 mM)
100 ml	SDS (10 % in aqua bidest.)
3.5 m	β-mercaptoethanol
ad	500 ml aqua bidest.

Radioimmunoprecipitationassay buffer (RIPA)

1 %	Igepal CA-630
0.5 %	Sodium deoxycholate
0.1 %	SDS
1 mM	EDTA
in	PBS without Ca^{2+}, Mg^{2+}

Immediately before cell lysis RIPA-buffer was supplemented with protease and phosphatase inhibitors:

50 mM	NaF
1 mM	PMSF
1 mM	Na_3VO_4
1 µg/ml	Aprotinin
1 µg/ml	Leupeptin
1 µg/ml	Pepstatin

For activation of the Na_3VO_4 stock solution pH was adjusted to 10. The yellowish solution was boiled until it turned colourless and after cooling to room temperature the pH was readjusted to 10. The procedure was repeated until pH stabilised at 10.

2.2.10.2 Stimulation of cells

For detection of phosphorylated Smad2 and ERK1/2 proteins MCF-7 ($3*10^5$/well) or MDA-MB-231 ($1.2*10^5$/well) were seeded in 6-well plates and cultivated for 24 h. For immunoprecipitation experiments $1.4*10^6$ MCF-7 and $9*10^5$ MDA-MB-231 were seeded in 10 cm culture dishes and cultivated for three days. ERK1/2 phosphorylation required a triple washing with PBS and incubation in serum- and phenol red-free medium for 3 days to suppress basal phosphorylation levels of negative control values. For detection of Smad2 phosphorylation, cultures were kept in medium with 0.5 % ct-FCS 24 h prior to stimulation. Cells were pre-treated with

inhibitors and/or E2 or ICI182,780 followed by stimulation with TGF-β. Negative controls were treated with the appropriate solvents (PBS with 0.4 % BSA for TGF-β, 96 % ethanol for E2 and ICI182,780, DMSO for PD098,059 and aqua bidest. for PTX and TAM-citrate).

2.2.10.3 Cell lysis and immunoprecipitation

Aspirating the solutions and duplicate washing with ice-cold PBS without calcium and magnesium finished the stimulation. 150 µl/well (6-well test plate) or 500 µl/well (10 cm culture dish) RIPA buffer containing protease and phosphatase inhibitors were added and the plates shaken at 4 °C for 30 min until cell lysis could be recognised under an inverted microscope. Then cells were harvested on ice by a cell scraper, transferred into eppendorf tubes. After centrifugation (22.000 * g/4 °C/30 min), the protein content was determined by Bradford staining (see chapter 2.2.10.4). The lysates could be stored at –80 °C for several days.

Lysate proteins (15 – 20 µg) were diluted in a volume ratio of 2:1 with threefold concentrated sample buffer, supplemented with DTT (200 mM), and denaturated by boiling at 95 °C for 5 min. The specimen were either directly applied for SDS-PAGE or store at –80 °C.

Detection of complex formation between ERα and Smad proteins or R-Smad and Co-Smads among themselves was worked out by immunoprecipitation experiments. After stimulation with TGF-β and/or E2 cells were harvested in RIPA buffer. 1800 µg of lysate protein were immunoprecipitated overnight at 4 °C with 6 µg anti-Smad1,2,3 antibody, followed by a precipitation with 40 µl protein G plus agarose at 4 °C for 90 min under gentle shaking on a rocking platform WT16. After centrifugation the pellets were washed four times with 1 ml complete RIPA buffer and the immunoprecipitates were eluted and denaturated by boiling for 5 min in 60 µl threefold concentrated SDS sample buffer with 200 mM DTT. Precipitated proteins were further processed by SDS-PAGE and Western blot using an anti-ERα or anti-Smad4 antibody (20 ml, 1:1000 in aqua bidest.) for detection.

2.2.10.4 Determination of protein concentration by Bradford staining

Measurement of protein concentration in the cell lysates was performed by the Bradford dye assay (Bradford, 1976). It is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acidic conditions, the dye is most stable as a doubly-protonated red form and upon binding to proteins it shifts to an unprotonated blue form.

Bradford solution (5x)

125 mg	Serva Blue G
125 ml	Ethanol (96 %)
250 ml	H_3PO_4 (85 %)
125 ml	Aqua bidest.

The dye was diluted 1:5 with aqua bidest. immediately before use.
A standard curve was established using a BSA stock solution (50 µg/100 µl aqua bidest.). Detection of lysate protein concentration was performed by dilution of lysates in aqua bidest and addition of diluted Bradford dye. The specimens were prepared as follows (Table 2):

Table 2 Scheme for protein detection by Bradford staining

BSA stock solution [µl]	BSA con-centration [µg]	RIPA buffer [µl]	Aqua bidest. [µl]	Bradford solution [ml]
0	0	5	95	1
5	2.5	5	90	1
10	5.0	5	85	1
15	7.5	5	80	1
20	10.0	5	75	1
cell lysate [µl]		5	95	1

Absorption was measured photometrically at 595 nm. To determine the protein concentrations of the original samples a linear regression of the data from BSA standard was conducted and evaluated according to the following equation:

$$A_{St} = x \cdot \mu gBSA$$

$$protein[\mu g / 100 \mu l] = \frac{A}{x} \cdot 20$$

A_{St}: BSA standard absorption at 595 nm

A: sample absorption at 595 nm

2.2.10.5 SDS-PAGE

Molecular weights:

ERα	64 kDa
Actin	41 kDa
Smad2	58 kDa
Smad3	62 kDa
Smad4	65 kDa
ERK1 (p44)	44 kDa
ERK2 (p42)	42 kDa
GPR30	38 kDa

SDS-PAGE was run on a 10 % polyacrylamid separating gel and a 5 % polyacrylamid collecting gel. Two glass plates (one notched, one including two side spacers) were assembled using clamps and grease and stand upright using clamps as stage. The ingredients for the separating gel were mixed in the order shown above. 9.5 ml were poured between the glass plates and overlayed with 1 ml isopropanol to ensure a flat surface and to exclude air. After setting of the lower gel layer isopropanol was decanted, the collecting gel was mixed as listed before, and poured onto the top of the separating gel. Immediately the comb was inserted. When dried two gel sandwiches were set into each electrophoresis chamber. Running buffer was diluted with aqua bidest. and filled in both top and bottom tanks. The combs were removed and the collecting gel was loaded with either the whole specimen volume or with 25 µl of immunoprecipitates.

Separation was implemented at 35 mA in the collecting gel and 55 mA in the separating gel.

2.2.10.6 Western blot

Separated proteins were transferred from the gel to PVDF membranes in a wet tank blot system. After the gel has finished running, the front glass plate and the collecting gel were carefully removed and the remaining separating gel was incubated in blotting buffer for 15 min. The membranes were moistened with methanol (15 sec), washed with aqua bidest. for 2 min and kept in blotting buffer for 5 min. Membranes were laid across the separating gel, making sure that air bubbles between gel and membrane are excluded and sandwiched in the blotting cassette outward sheltered by pre-wetted filter paper and cassette sponges. Each blot sandwich contained not exceeding four membranes. The stack was placed in a tank blot container filled with blotting buffer, which was diluted before with aqua bidest., and run over night at 100 mA.

On the next day the blots were gently shaken for 1 h in 50 ml blocking buffer at 37 °C on a rocking platform in order to saturate unspecific binding sites. Since GPR30 antibody could only be obtained in small amounts, these blots were cut narrowly above and beneath the 38 kDa panel to keep the required volume of antibody solution small. After three 5 min-washing steps with TBST the target proteins were marked by probing with the primary antibody and gently rocked for 2 h at room temperature (Smad4, phospho-ERK1/2, ER α) or over night at 4 °C (phospho-Smad2, GPR30). Phospho-Smad2, Smad4, GPR30, and ER α antibodies were diluted 1:1000 either in aqua bidest. (20 ml per membrane) or in 0.4 % BSA in PBS for GPR30 (2 ml per membrane). Phospho-ERK1/2 antibody was diluted 1:2000 in aqua bidest. (20 ml per membrane). The blots were washed 3 times for 5 min in TBST. The primary antibody for phospho-Smad2, Smad4 and phospho-ERK1/2 were saved for at least one additional blot procedure. The appropriate horse radish peroxidase (HRP) conjugated secondary antibodies were diluted in blocking buffer, added to the blots and incubated for 1 h at room temperature.

After the usual washing steps detection of chemiluminescence was performed using the LumiGlo®-Kit containing luminol reagent and peroxydase. The blots were shaken for 1 min in a pool of luminol solution (peroxidase and luminol reagent diluted each in a 1:10 ratio in aqua bidest.), put between two transparency sheets and inserted into the exposition cassette. The membranes were covered with an X-ray film; the exposure time was adjusted according to the requirements of the antibody and the

actual test. Light exposure of the X-ray film was subsequently visualised by developer and fixer solutions.

For detection of total Smad2, ERK1/2, β-Catenin, and Actin protein the antibodies were removed from the blots. Therefore, the membranes were stripped in 50 ml pre-heated stripping buffer (50 °C) for 20 min. After triple 10 min washing with TBST the membranes were blocked and reprobed with anti-Smad2, anti-ERK1/2, β-Catenin, or anti-Actin antibodies (1:2000 in 20 ml aqua bidest.) and incubated for 2 h at room temperature. The procedure was continued as described above.

2.2.11 Analysis of mRNA transcription

2.2.11.1 Solutions

DEPC treated water

Treatment with DEPC is the most common method to remove RNAases from solutions. 1ml of 0.1 % DEPC was added to 1000 ml aqua bidest., mixed thoroughly, and let set at room temperature for 1 h. Then the water was sterilised by autoclaving and cooled to room temperature prior to use.

Stock solution for cDNA synthesis

4 µl	5fold first strand buffer
2 µl	0.1 M DTT
1 µl	dNTP-Mix (10 mM solutions of dATP, dTTP, dGTP, and dCTP)
1 µl	RNAsin™ (Ribonuclease inhibitor)
1 µl	SuperscriptTM Reverse Transkriptase (200 units/µl) added immediately before use or 1 µl DEPC water for negative controls respectively

Stock solution for PCR

5 µl	10fold reaction buffer IV
3 µl	25 mM $MgCl_2$
2 µl	dNTP-Mix (10 mM solutions of dATP, dTTP, dGTP, and dCTP)
0.25 µl	Thermoprime plus Taq DNA polymerase (5 units/µl)
32.75 µl	DEPC water

Agarose gel

3 g (3 %) or 0.75 g (0.75 %) agarose
boiled in 100 ml TBE-buffer (pH 8)

Tris - Boric acid - EDTA (TBE) buffer (5x)

5.4 g/l	Tris-base
27.5 g/l	Boric acid
2.92 g/l	EDTA
in	Aqua bidest.

2.2.11.2 Isolation of mRNA

Expression of GPR30 and ERα mRNA was examined by **R**everse-**T**ranscriptase **P**olymerase **C**hain **R**eaction (RT-PCR). For that purpose mRNA was isolated from MCF-7 and MDA-MB-231 cells by means of the QuickPrep™ Micro-mRNA Isolation Kit according to the manufacturers instructions.

The kit was pre-warmed to room temperature. Cells from two 75 cm^2-culture flasks were detached by trypsin digestion as depicted in chapter 2.2.4, transferred to 1.5 ml PCR tubes, and centrifugated. The pelleted cells were lysed by vortexing thoroughly in 400 µl RNAse inhibitor containing guanidinium isothiocyanate buffer (extraction buffer) until complete homogenisation. Remaining proteins were precipitated by addition of 800 µl elution buffer (10 mM Tris pH 7.5, 1 mM EDTA). The tubes were vortexed and centrifugated at 22.000 * g for 1 min. 1 ml of oligo(dt) coated cellulose (25 mg/ml) was transferred into a 1.5 ml PCR tube and centrifugated. The supernatant was discarded and topped up with the homogenised cell suspension. The tube was gently shaken by hand for 3 min and the polyA containing mRNA adsorbed to oligo(dt) cellulose was centrifugated at 22.000 * g for 10 sec. Then the mRNA containing pellets were washed five times with high salt buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.5 M NaCl) and three times with low salt buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.1 M NaCl) each time with 1 min shaking by hand. A microspin column tip was opened, stacked in a 1.5 ml PCR tube, and the oligo(dt) cellulose/mRNA pellets, re-suspended in 200 µl low salt buffer, were applied to the column. The column was centrifugated and the tube was emptied followed by triple

washing with 500 µl low salt buffer. After transferring the column into a new 1.5 ml PCR tube the mRNA was eluted by applying 400 µl elution buffer (pre-warmed to 65 °C) and centrifugation for 10 sec.

The amount of isolated mRNA was quantified by measurement of absorption at 260 and 280 nm. A UV-cuvette was incubated with concentrated HCl and methanol (1:1) to destroy RNAses and washed with elution buffer before the entire mRNA solution was poured in. Absorption was measured at 260 and 280 nm against the equal volume of elution buffer with a Photometer Uvikon 922.

The following equation demonstrates how RNA concentration was determined on the basis of UV-absorption.

$$c(RNA)\ [\mu g/ml] = 40 * Abs_{260}$$

Abs_{260}: absorption at 260 nm

Abs_{280}: absorption at 280 nm

High RNA purity is guaranteed when an Abs_{260}/Abs_{280} ratio of about 2 is obtained. $5*10^6$ MCF-7 and $5*10^6$ to $1*10^7$ MDA-MB-231 cells yielded 7 to 12 µg mRNA of adequate purity.

After UV-measurement the RNA solution was removed from the cuvette and transferred to a 1.5 ml PCR tube. 10 µl glycogen solution (5-10 mg/ml in DEPC water) plus 40 µl potassium acetate solution (2.5 M, pH5) were added and the mRNA was precipitated with 1 ml 96 % ethanol (for molecular biology, pre-cooled to –20 °C) for 30 min at –20 °C. After centrifugation (28.000 * g/4 °C/5 min) mRNA probes were either further processed or stored at –80 °C.

2.2.11.3 Synthesis of copy DNA (cDNA)

The mRNA probes were either defrosted or immediately centrifugated (28.000 * g/4 °C/30 min). Afterwards, the mRNA pellet was dissolved in DEPC water to a final concentration of 50 ng/µl assuming the yield of mRNA after precipitation to be 50 %.

The enzyme used to copy cDNA from an mRNA template is reverse transcriptase, an RNA dependent DNA polymerase. As with other polymerases for synthesis of single stranded DNA, a short double-stranded sequence is needed at the 3’ end of the

mRNA that acts as a start point for the polymerase reaction. This is provided by a short synthetic oligonucleotide (oligo(dT)) primer complementary to the poly(A) tail found at the 3' end of most eukaryotic mRNAs. Therefore, 500 ng mRNA and 1 µl (1 pmol) oligo(dt)-primer were incubated at 80 °C for 3 min and at 37 °C for further 10 min. 9 µl of freshly prepared stock solution for cDNA synthesis were added and reverse transcription took place by incubation at 37 °C for 90 min. After addition of 20 µl 0.4 M NaOH the probes were incubated at 37 °C for 10 min and after addition of with 20 µl Tris-HCl frozen at –20 °C. DNA probes could be kept that way for several months.

2.2.11.4 Amplification of DNA by PCR

PCR technique allows amplification of more than 10^7 copies of a target DNA sequence and was realised by means of Taq-Polymerase. 2 µl of cDNA template corresponding to 50 ng mRNA were supplemented with 5 µl primer mixture (2.5 µl of forward and 2.5 µl of reverse primer solution each 10 pmol/µl; see Table 3) and completed with 43 µl stock solution for PCR. No signal was supposed to be detected in negative probes prepared without Taq polymerase to exclude contaminations with genomic DNA.

Table 3 Primer sequences

	Forward Primer	**Reverse Primer**	**Product size [bp]**
ERα	5´- TCC AAC TGC ATT TCC TTT CC -3`	5´- TTG. GAA CAT GGC AGC ATT TA -3`	201
GPR30	5´- AGT CGG ATG TGA GGT TCA G -3`	5´- TCT GTG TGA GGA GTG CAA G -3´	240
GAPDH	5'- ATG CAA CGG ATT TGG TCG TAT -3'	5'- TCT CGC TCC TGG AAG ATG GTG -3'	221

PCR was performed in a Thermocycler T Gradient. Reaction was initiated by DNA denaturation at 94 °C for 1 min, afterwards 35 cycles were run as described in Table 4.

Table 4 Cycles of polymerase chain reaction

	35 cycles
Template denaturation	1 min 94 °C
Primer annealing	1 min 55 °C
Extension of annealed primers by DNA polymerase reaction	1 min 72 °C

A final polymerisation step for 2 min at 72 °C served for the completion of fragmental DNA strands. Finally, the reaction was finished by a 1 min cooling step to 4 °C.
The amplified DNA fragments were separated in a 3 % agarose gel, stained with ethidium bromide solution (10 µl of 10 mg/ml solution in 200 ml aqua bidest.) for 1 h, observed at 254 nm on a UV-light box (Transluminator Biometra Ti5), and evaluated with the BioDoc Analyze 2.0 software.

2.2.12 Cultivation of Escherichia coli (E. coli) bacteria

2.2.12.1 Agar plate culture

Table 5 Plasmid Specifications

Plasmid	**Basic vector**	**Selecting antibiotic**	**Antibiotic concentration [µg/ml]**
Renilla	pRL-CMV	Ampicillin	100
3ARE-luc (Smad2 promoter)	pGL2-Basic	Ampicillin	100
MykFast (transcription factor)	CS2	Ampicillin	100
SBE_4-luc (Smad3 promoter)	pGL3-Basic	Ampicillin	100
GPR30	pBK-CMV	Kanamycin	50

Luria Broth (LB) agar plates

40 g Luria agar
in 1 l Aqua bidest.
sterilised by autoclaving

The autoclaved agar-agar solution was supplemented with the appropriate antibiotic (see Table 5) after cooling down to 55 °C to select transformed E. coli. Control plates were produced to determine the general viability of the cells using antibiotic free agar. The solutions were poured into sterile petri dishes and when hardened stored in the dark at 4 °C. A liquid or glycerol culture of E. coli was dispersed in a grid-shaped pattern using a sterile metal loop (sterilised by flaming). The plates were incubated over night at 37 °C and stored at 4 °C until use.

2.2.12.2 Liquid culture

LB medium

25 g LB Broth
in 1 l Aqua bidest.
sterilised by autoclaving

A single colony was picked by tapping a sterile metal loop on the surface of the agar plate, transferred into 3 ml antibiotic containing LB medium in a 50 ml centrifugal tube, and incubated for 8 h at 37 °C with intense shaking on a rotary shaker. 250 ml selective LB medium were inoculated with 400 µl starter culture and cultured at 37 °C for 12 to 16 h with vigorous shaking until an optical density of 0.6 is achieved.

2.2.12.3 Cryopreservation and resuscitation of E. coli bacteria

In a cryoprotective tube 1 ml of a liquid culture was mixed with 1 ml 80 % glycerol and frozen at –80 °C. For long-time storage the tubes were transferred to a liquid nitrogen container.
Dissemination on agar plates by means a metal loop reactivated the cells, thawed at room temperature.

2.2.13 Amplification of plasmid DNA

2.2.13.1 Production of competent E. coli cells

SOB medium

25.5 g SOB Broth diluted in 950 ml aqua bidest.

10 ml 50 mM KCl
pH was adjusted to 7 with 5 N NaOH
ad 1 l with aqua bidest.
sterilised by autoclaving

TB buffer

10 mM Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)
15 mM $CaCl_2$
250 mM KCl
pH was adjusted to 6.7 with KOH
55 mM $MnCl_2$ added
sterilised by filtration (0.2 µm pore size)

To enable E. coli cells to take up circular vector DNA they have to be competent, which was implemented chemically.
250 ml SOB medium in a 500 ml flask was inoculated with a single colony of the E. coli strain NW8. The bacteria grewn over night at 18 °C on a rotary shaker until the optical density measured at 600 nm (UV-Photometer Gene-Ray) reached 0.6. The flask was placed on ice for 10 min and then spun for 20 min at 4 °C and 40.000 * g. The cells were resuspended in 80 ml ice-cold TB buffer and supplemented with sterile DMSO to a final concentration of 7 %. After 10 min on ice the probes were aliquoted in pre-cooled batches and frozen immediately at –80 °C. The next day, the tubes were transferred to liquid nitrogen.

2.2.13.2 Transformation of competent E. coli with plasmid DNA

For cells that were made competent chemically the heat shock procedure is convenient. One tube of competent cells was thawed on ice and the required number of empty PCR microcentrifuge tubes was pre-chilled. 1 µg of plasmid DNA was provided in the pre-chilled tubes, completed by 50 µl of the E. coli suspension, and gently mixed. The specimen was incubated on ice for 30 min, heat shocked for 2 min at 42 °C, and immediately placed on ice for 2 min. 500 µl of pre-warmed (37 °C) LB medium was added and incubated at 37 °C for 60 min. The bacteria suspension was plated on agar plates containing the appropriate solution of selecting antibiotic (Table

5) and incubated overnight at 37 °C. For control of cell viability, one agar plate without antibiotic was prepared. To ensure the integrity of the antibiotic, non-transformed cells were disseminated on selective plates (with antibiotic).

2.2.13.3 Plasmid purification

Tris – EDTA (TE) buffer

10 mM Tris-HCl
1 mM EDTA
in Aqua bidest.
pH was adjusted to 8

Purification of plasmid DNA was achieved by use of the Qiafilter™ Plasmid Purification Maxi Kit. All of the following mentioned chemicals and materials are provided in the kit. This procedure is based on a modified alkaline lysis, followed by binding of plasmid DNA to an anion exchange resin under appropriate low-salt and pH conditions.

The cell suspension from a liquid culture in selective LB medium was transferred to 50 ml centrifugal tubes and harvested by centrifugation at 8000 * g and 4 °C for 30 min. The pellets were thoroughly resuspended in 5 ml buffer P1 (resuspension buffer: 50 mM Tris pH 8, 10 mM EDTA) supplemented with RNase A (100 µg/ml). The cell suspensions of two tubes were combined. 10 ml buffer P2 (lysis buffer: 200 mM NaOH, 1 % SDS) was added, mixed by inverting the tube 4 to 6 times, and incubated at room temperature for 5 min. Cell lysis reaction was finished by addition of 10 ml pre-chilled buffer P3 (neutralisation buffer: 3 M potassium-acetate, pH 5), which simultaneously caused complexation of denaturated proteins, chromosomal DNA and cell relicts. Inverting the tube 4 to 6 times performed mixing. To remove the complexes, the lysates were poured into the barrel of the Qiafilter cartridge and incubated for 10 min at room temperature. The cell lysates were filtered by use of a plunger into a Qiagen-tip which has been equilibrated before by applying 10 ml QBT-buffer (equilibration buffer: 750 mM NaCl, 50 mM MOPS pH 7, 15 % isopropanol, 0.15 % Triton® X-100). After the clear lysates have been adsorbed to the resin, the Qiagen-tip was washed twice with QC-buffer (washing buffer: 1 M NaCl, 50 mM MOPS pH 5, 15 % isopropanol). This procedure provides a further purification step,

as only plasmid DNA binds to the anion exchange column of the Qiagen-tip, whereas vitiations remain in the liquid phase. 15 ml QF-buffer (elution buffer: 1.25 M NaCl, 50 mM Tris pH 8.5, 15 % isopropanol) were used to elute the DNA from the resin. DNA was precipitated with 10.5 ml isopropanol (for molecular biology). After centrifugation (8000 * g/1 h/4 °C) the pellet was desalted with 5 ml 70 % ethanol and centrifugated (8000 * g/1 h/4 °C). The air-dried pellet was dissolved in 200-300 µl TE buffer.
To determine the yield of plasmid DNA, UV-absorption was measured at 260 and 280 nm (Photometer Uvikon 922) and calculated according to the following equation:

$$c\ (DNA)[\mu g/ml] = 50 * d * A_{260}$$

d: dilution factor
A_{260}: absorption at 260 nm
A_{280}: absorption at 280 nm

The applied procedure provided an A_{260}/A_{280} ratio of 1.7 to 2.0 indicating sufficient purity. The purity of the plasmid DNA was further checked by gelelectrophoresis on a 0.75 % agarose gel.

2.2.14 Transfection of MDA-MB-231 cells with the GPR30 plasmid

The GPR30 expression plasmid was kindly provided by R. Weigel (University of Iowa, Iowa, USA) (Carmeci et al., 1997) and plasmid amplification was carried out as described in chapter 2.2.13.
Table 6 represents all specifications concerning Western blot, migration assay, and immunofluoescence analysis for cell dissemination and transfection. MDA-MB-231 cells were seeded 24 h prior to transfection. Transfection master mix was prepared starting with dilution of transfection reagent (Fugene6^{TM}) in tenfold volume of McCoy's medium without serum and incubated at room temperature for 5 min. GPR30 plasmid was added and incubated for 45 min at room temperature. Finally, the appropriate volume of transfection master mix was provided drop by drop to the growth medium. After 48 h the cultures were applied to the respective assay.

The transfection efficiency was controlled by immunofluorescence microscopy (see chapter 2.2.19) and Western blot technique of membrane fractions (see chapters 2.2.9 and 2.2.10) using an anti-GPR30-antibody.

Table 6 Specifications for transfection of MDA-MB-231 with the GPR30 plasmid

	Western blot	**Migration assay**	**Immunofluorescence analysis**
Seeded cells per well	$1.6*10^5$ (6-well-plate)	$1.2*10^6$ (10-cm-dish)	$3*10^4$ (12-well-plate)
Fugene6™ [µl per well]	3.75	30	1.5
GPR30 plasmid [µg/well]	1.25	10	0.5
Growth medium [ml/well]	1	10	0.5

2.2.15 Measurement of Smad2 and Smad3 promoter activation

Activation of Smad2 and Smad3 dependent promoters was measured by reporter gene assays, which are commonly used in cell biology to study gene expression and other cellular events coupled to gene expression, such as receptor activity, intracellular signal transduction, mRNA processing, protein folding, and protein-protein interactions. All Smad promoters as well as the MykFast plasmid were kindly provided by Dr. Anita Roberts (National Cancer Institute, Bethesda, MA).

Besides the Smad binding domain the Smad3-specific SBE_4-luc and Smad2-specific 3ARE-luc promoter contain the reporter gene *luc* of the firefly *Photinus pyralis* coding for the Firefly luciferase, a monomeric 61 kDa protein that is transcribed upon promoter activation and catalyses luciferin oxidation using ATP-Mg^{2+} as a cosubstrate. Renilla luciferase was used as a co-reporter for normalisation of experimental variations such as differences in transfection efficiencies. This monomeric 36 kDa protein is originally descended from sea pansy (*Renilla reniformis*) and catalyses coelenterate-luciferin (coelenterazine) oxidation to produce light. In contrast to Firefly luciferase it is constitutively expressed in successfully transfected cells.

MCF-7 and MDA-MB-231 cells ($9*10^4$ cells/well) were seeded in a 24-well-plate 24 h prior to transfection. Growth medium was exchanged and the transfection master mix

was prepared starting with 50 µl/well Optimem medium, 2 µl/µg plasmid DNA of Fugene6™, and the appropriate volume of plasmid DNA were added. Cells were cotransfected with 0.5 µg/well SBE_4-luc or 3ARE-luc each with 25 ng/well Renilla. Initiation of 3ARE-luc dependent transcription requires additional transfection with the transcription factor MykFAST-1 (0.5 µg/well). The transfection master mix was gently shaken, incubated at room temperature for at least 15 min, and the respective volume of this solution was applied drop by drop to the cultures.

After 24 h media were replaced and cells were treated with TGF-β and/or E2 in the indicated concentrations. 24 h later cells were washed with PBS and harvested with 100 µl per well of passive lysis buffer, shaken for 30 min, and assayed for luciferase activity using the Dual-Luciferase® Assay System according to the manufacturers instructions. 20 µl aliquots of lysate were transferred to an opaque polystyrene microplate (Optiplate™-96). The Firefly luciferase expression was measured by adding 50 µl of Luciferase Assay Reagent II (LAR II). After recording the luminescence, 50 µl of Stop & Glo® reagent was added to quench the Firefly luciferase reaction and simultaneously activate the Renilla luciferase reaction. Firefly and Renilla luciferase activities were measured using a Multilabel counter Wallac Victor^2 1420. The luminometer software Wallac Workstation allowed for direct capture of the data stream over the 10-second integration period of each luciferase reaction after a 2-second delay time. Firefly-luciferase activity of each well was corrected using the corresponding Renilla-luciferase activity.

2.2.16 Determination of ERE-activation

Activation of the ERE of estrogen responsive genes was measured in a luciferase assay using the human breast cancer cell line MCF-7-2a cells stably transfected with the $ERE_{wt\ luc}$ plasmid (Hafner et al., 1996). $ERE_{wt\ luc}$ contains the ERE as enhancer sequence and the reporter gene *luc* encoding the enzyme luciferase. Binding of agonists to the LBD of the ER results in receptor dimerisation and complexation to the plasmid ERE leading to luciferase expression, which correlates with the agonistic effect.

One week before test start MCF-7-2a culture medium was exchanged by DMEM with 5 % ct-FCS. For the test $1*10^4$ cells/100 µl were disseminated in 96-well plates. An

equal number of additional plates were prepared for measurement of cell viability by crystal violet staining.

The test substances and E2 as positive control were diluted stepwise in a separate 96-well plate in DMEM plus 5 % ct-FCS to achieve a concentration range of 10^{-11} to 10^{-5} M. Culture media were aspirated and the wells were filled with 180 µl DMEM plus 5 % ct-FCS. 20 µl of test substances or solvent were added performing the last dilution step directly in the well (final concentration: 10^{-12} to 10^{-6} M). The plates for measurement of cell viability were treated according to the sample plates. Incubation was performed for 18 h at 37 °C/ 5 % CO_2.

After aspiration of media, 30 µl/well cell lysis reagent was applied and the plates incubated under vigorous shaking for 30-60 min at room temperature. 2 sec after addition of 30 µl/well Luciferase assay reagent luciferase activity was measured over 10 sec in a Multilabel counter Wallac Victor^2 1420. Detected light emission is assigned as RLU ("relative light units"). Cells stimulated with 10^{-9} M E2 were used as positive control and account for 100 % ERE-activation. Cell viability was examined by crystal violet staining as described in chapter 2.2.8 and each value was referred to the amount of surviving cells.

The ERE-activation was calculated according to the following equation:

$$ERE - activation = \frac{RLU_{sample}}{A_{sample}} - \frac{RLU_{vehicle}}{A_{vehicle}}$$

The percentage ERE-activation was determined by referring to the ERE-activation value of 10^{-9} M E2.

$$ERE - activation[\%] = \frac{ERE - activation \cdot 100}{\frac{RLU_{control}}{A_{control}} - \frac{RLU_{contrveh}}{A_{contrveh}}}$$

RLU_{sample}: luciferase activity of sample

$RLU_{vehicle}$: luciferase activity of vehicle control

$RLU_{control}$: luciferase activity of positive control

$RLU_{contrveh}$: luciferase activity of positive control vehicle

A_{sample}: Absorption value of crystal violet staining of sample

$A_{vehicle}$: Absorption value of crystal violet staining of vehicle control

$A_{control}$ Absorption value of crystal violet staining of positive control
$A_{contrveh}$ Absorption value of crystal violet staining of positive control vehicle

2.2.17 Abrogation of GPR30 by siRNA in MCF-7 cells

Gene silencing was performed using sequence specific GPR30-siRNA. Small interfering RNA (siRNA) is an evolutionary conserved mechanism to regulate gene expression in response to double stranded RNA (dsRNA), but is also used as a tool in molecular biology. RNases cleave dsRNA into small interfering sequences, which are incorporated into a RNA induced silencing complex (RISC). RISC selects complementary mRNA and simultaneously exhibits endonuclease activity to cleave the target mRNA sequence.

MCF-7 cells were disseminated in DMEM for transfection and grown for 24 h (for transfection specifications see Table 7). Cells were transfected with 100 nM of GPR30- or non-targeting-siRNA (control-siRNA) according to the manufacturer`s protocol using Dharmafect1 as transfection reagent. A 2 µM solution of siRNA or control-siRNA was prepared by dilution in siRNA buffer. In separate tubes, 2 µM siRNA or control-siRNA (Tube 1) and the appropriate volume of Dharmafect1 were added to DMEM for transfection and incubated at room temperature for 5 min. The contents of tube 1 and 2 were combined as transfection mix, gently blended by pipetting, and incubated at room temperature for 45 min. The final respective volume was obtained by diluting the transfection mix with DMEM for transfection with 10 % FCS. The culture medium was removed and the transfection mix was applied to the cells. To minimise toxic effects, after 24 h the transfection mix was exchanged by DMEM for transfection containing 10 % FCS. The silencing efficiency was detected by immunofluorescence microscopy (see chapter 2.2.19) and Western technique of membrane fractions (see chapters 2.2.9 and 2.2.10) using anti-GPR30-antibodies. After 48 h of transfection, the cells were sensitised with E2 and chemotaxis or Smad2 phosphorylation in response to TGF-β were detected.

Table 7 Specifications for transfection of MCF-7 with the GPR30-siRNA

	Western blot	**Migration assay**	**Immunofluorescence analysis**
Seeded cells per well	$3*10^5$ (6-well-plate)	$1.5*10^6$ (10-cm-dish)	$3*10^4$ (12-well-plate)
Tube 1 [µl/well]			
2 µM siRNA	50	250	25
DMEM for transfection	50	250	25
Tube 2 [µl/well]			
Dharmafect 1	1	5	0.5
DMEM for transfection	100	500	50
Final volume [ml]	1	5	0.5

2.2.18 Abrogation of Smad4 protein by siRNA

Smad4 expression was interrupted by siRNA technique. In 10 cm^2 culture dishes $6*10^5$ MCF-7 cells were seeded in growth medium and cultured for two days. Down-regulation of Smad4 protein was verified by Western blot analysis. Therefore $1.5*10^5$ cells/well were cultivated in growth medium in a 6-well plate for three days. Medium was exchanged by DMEM for transfection with 10 % FCS and the cells were transfected according to the manufacturer's protocols with minor variations.

SiRNA or non-targeting control-siRNA was diluted 1:5 in DMEM for transfection. In a second tube an equal volume of Fugene6^{TM} was blended in a tenfold volume of DMEM for transfection and gently mixed. Both solutions were incubated at room temperature for 5 min. The siRNA solution was added to the transfection reagent. After further incubation for 20 min, the appropriate volume of the transfection mix was pipetted drop by drop to the cultures to a final siRNA concentration of 50 nM. The cells were then incubated at 37 °C/5 % CO_2 for 72 h.

2.2.19 Immunofluorescence microscopy

MCF-7 or MDA-MB-231 cells (each $3*10^4$) were seeded on 18 mm-coverslips and transfected with GPR30 expression vector or siRNA as described before (see chapters 2.2.14 and 2.2.17). Cells were fixed with 100 µl/well 3.7 %

paraformaldehyde for 3 min and carefully washed three times with PBS whereat two drops of 1 M glycerine (pH 8.5) was added to the second washing buffer to quench the residual paraformaldehyde cross-linking activity. 1 ml/well 1 % BSA in PBS was applied for 50 min to block unspecific binding of antibody. The blocking solution was aspirated and the cells were washed with three changes of PBS. The cells were incubated with 100 µl/well of primary antibody against GPR30 for 30 min, diluted to a final concentration of 2 µg/ml in 0.4 % BSA in PBS. After two washing steps the procedure was continued in the dark where the secondary anti-rabbit antibody, conjugated to fluorescein isothiocyanate (FITC), was diluted to 10 µg/ml in 0.4 % BSA in PBS. The cells were incubated with 100 µl of antibody solution for 30 min and washed with PBS for three times. A little volume of PBS was gently squirted at the edge of each coverslip, which were then raised up by use of a 10 µl pipette and tweezers. Excess PBS was dried off on a paper towel and the coverslips were mounted on glass slides by placing cell side down on a 5 µl drop of Mowiol (see chapter 2.2.19.1). The samples were allowed to dry for at least 30 min and observed utilising the microscope Olympus BX41 with accessory equipment for fluorescence analysis. Images were taken by means of the Nikon Digital still Camera DMX 1200 and edited by ACT-1 software.

2.2.19.1 Preparation of Mowiol

6 g	Glycerol
2.4 g	Mowiol
6 ml	Aqua bidest.
12 ml	Tris-buffer (pH 8.5)

The ingredients were merged and blended for half a day on a shaker. The mixture was precipitated for 2 h and afterwards incubated at 50 °C for 10 min. After centrifugation at 22.000 * g for 15 min the supernatant was frozen at –20 °C until use.

2.2.20 Statistical Analysis

All data are presented as arithmetic averages. Standard deviation (± SD) was determined as measure of the statistical spread.

After ensuring that the prerequisite of normal distribution of data is given (Shapiro-Wilk-test), the F-test was used to determine if, regarding their variances, two samples were significantly different. The level of significance of differences between means was subsequently analysed with the T-test. The required level of significance was defined to be 5 % ($p \leq 0.05$).