Results

3 Results

3.1 Effect of E2 on migration of human breast cancer cells

Migration of tumour cells is an important event for their dissemination and the formation of metastases. Owing their ability to induce proliferation and antiapoptosis, estrogens are widely accepted to be risk factors for the development and progression of breast cancer. In the present study, it should be investigated, whether the tumour promoting properties of estrogens may also be attributed to enhanced cell movement and invasion. There exist several studies examining the chemoattracting properties of E2 in MCF-7 cells by means of wound closure assays, but the outcomes are to some extent inconsistent. Therefore, the migratory potency of estrogens was further characterised using a transwell assay system.

When the lower chambers were prepared with different concentrations of E2, enhanced migration of MCF-7 cells was observed in a dose dependent and saturable manner (Fig. 9). Migration already appeared at 10^{-11} M of E2, whereas a maximum effect, leading to an almost 7.5fold increased motility compared with control cells, occurred at 10^{-7} M of E2.

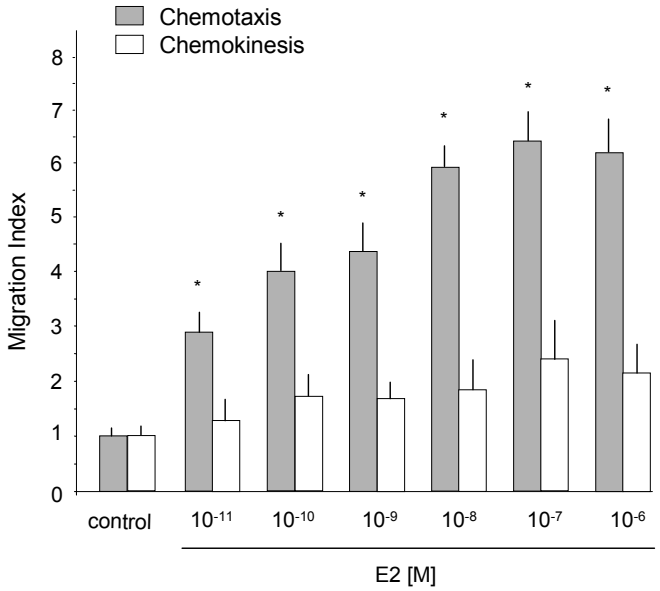

Fig. 9 Migration of MCF-7 cells in response to different concentrations of E2. Cell migration was measured in a transwell assay system as described in section 2.2.7. For determination of directed, chemotactic motility, the lower chamber was supplemented with control vehicle or E2 in the indicated concentrations (chemotaxis). Undirected, chemokinetic movement was measured by abolishing the concentration gradient by adding E2 to the upper and lower chamber in the indicated concentrations (chemokinesis). The data are expressed as migration index and are means ± SD of two determinations. The experiment was repeated at least two times with similar results (* $P \leq 0.05$).

Various chemokines not only induce directed cell movement according to a concentration gradient, but also undirected chemokinetic motion. To allow distinction between chemokinetic and chemotactic cell movement towards E2, equal concentrations of the estrogen were placed on both sites of the filter, thus eliminating the concentration gradient between cell suspension in the filter and the medium in the lower compartment of the chamber. Under these conditions almost no enhanced motility was seen, indicating chemotaxis, and not chemokinesis to be the major response of MCF-7 cells to an E2 stimulus (Fig. 9).

3.1.1 Involvement of ER signalling in E2 mediated chemotaxis

To investigate the dependency of E2 induced migration on the expression of ERs, the capacity of E2 to provoke chemotaxis was also assayed in the breast cancer cell line MDA-MB-231, which is considered ER negative. Indeed, E2 failed to enhance migration rates of MDA-MB-231 in comparison with MCF-7 cells significantly (Fig. 10).

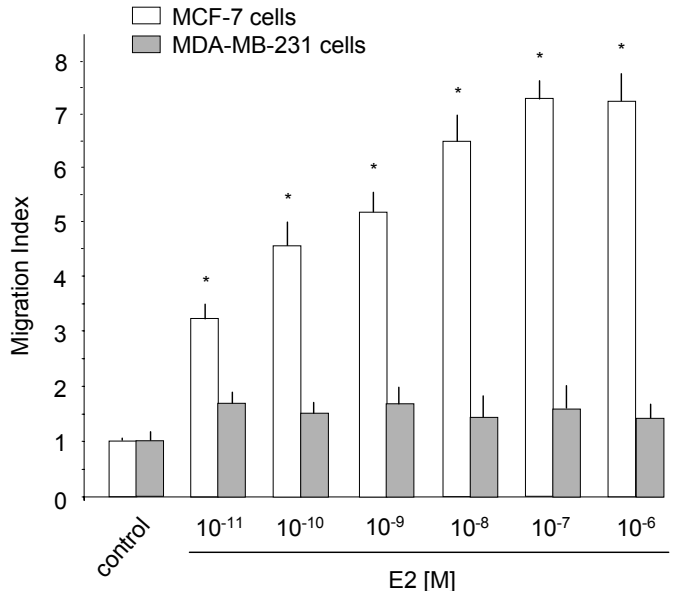

Fig. 10 Migration of ER positive and negative cells in response to a gradient of E2. Migration of MCF-7 and MDA-MB-231 cells was measured in a transwell assay system with the quoted concentrations of E2 in the lower chamber. The data shown represent the outcome of three independent experiments with comparable results (* $P \leq 0.05$). Each value was calculated as the average migratory index ± SD of double determinations.

To further investigate whether the migratory stimulus of E2 is mediated by ERs, chemotaxis of MCF-7 cells in response to E2 was measured in presence of the ER

antagonist ICI182,780. This pure antiestrogen is a steroidal ER down-regulator, which competitively binds to the receptor and provokes a rapid loss of ER protein (DeFriend et al., 1994; Diel et al., 1999; Dudley et al., 2000). Down-regulation of ERα protein after 19 h treatment with 10^{-6} M of the antihormone could successfully be demonstrated by Western blot analysis. In contrast, the amount of ERα remains unchanged when MCF-7 cells were incubated with ICI182,780 in nanomolar scale for 30 min (Fig. 11B).

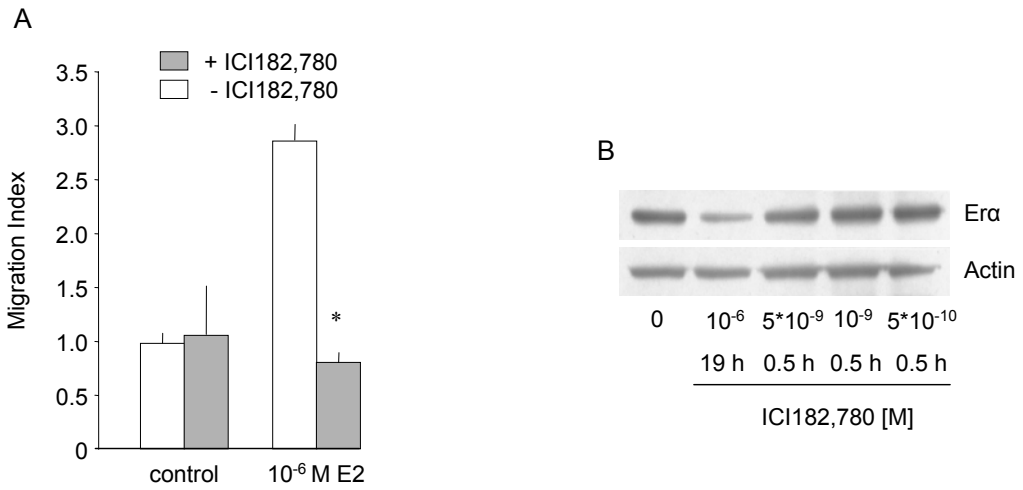

Fig. 11 Dependency of E2 induced chemotaxis of MCF-7 cells on ER functioning. (A) MCF-7 cells were pre-treated with control vehicle or 10^{-6} M ICI182,780 for 19 h and then migration towards 10^{-6} M E2 was measured in a transwell assay system. The represented data demonstrate the average migratory index of two determinations ± SD (* P ≤ 0.05). (B) MCF-7 cells were treated with ICI182,780 or vehicle in the indicated concentrations and times, and 20 µg of lysated protein were separated by SDS-PAGE. ERα expression was analysed by Western blot utilising ERα specific antibodies. Antibodies were stripped off and the membrane re-probed with an anti-Actin antibody. The results have been verified in at least three independent experiments

As presented in Fig. 11A, treatment with ICI182,780 completely inhibited the migratory response of MCF-7 cells to E2. In consideration of the results with ER negative cells, these findings indicate that the E2 mediated migration depends on functional ER expression.

3.1.2 Involvement of ERK 1/2 in E2 mediated chemotaxis

Estrogen signalling is often connected with or rather dependent on the activation of ERK1/2. Activation of ERK1/2 by E2 in MCF-7 cells has been proved so far in divers studies (Filardo et al., 2000; Imamichi et al., 2005; Migliaccio et al., 2002; Zivadinovic and Watson, 2005). In order to examine if ERK1/2 signalling is likewise involved in

the migratory response of MCF-7 cells towards E2, activation of ERK1/2 was avoided by use of PD098,059, a specific inhibitor of the ERK activator kinase MEK. In fact, E2 failed to induce chemotaxis of MCF-7 cells pre-incubated with PD098,059 (Fig. 12).

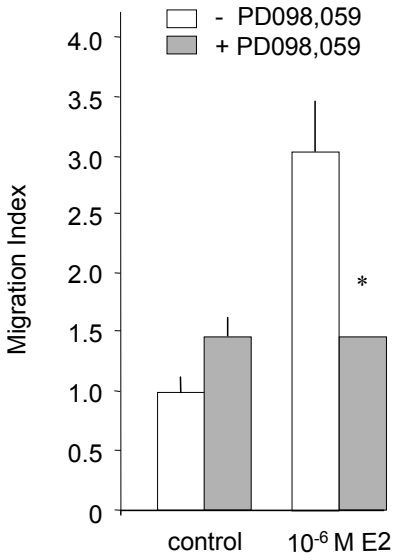

Fig. 12 Dependency of E2 induced chemotaxis of MCF-7 cells on ERK1/2 activation. Cells were pre-treated with control vehicle or 50 µM PD098,059 for 1.5 h and then migration towards 10^{-6} M E2 was measured in a transwell assay system. Each value represents the average migration index of duplicate determinations ± SD. The experiments have been performed at least three times with comparable results (* $P \leq 0.05$).

3.2 Effect of E2 on TGF-β induced pathways

3.2.1 Migration of MCF-7 and MDA-MB-231 cells

The chemotactic properties of TGF-β on a variety of cell types are well established and contribute to several physiological processes, such as wound healing and immunological responses. TGF-β is the most potent known inhibitor of cell growth in normal mammary and transformed cells of early tumour stages. In advanced breast carcinomas, however, cells are refractory to the antiproliferative effects of the chemokine and even secrete high amounts of TGF-β, which may promote tumour spread by enhancing cell migration and invasion.

The motility of MCF-7 and MDA-MB-231 cells towards different concentrations of TGF-β has been verified in this study utilising a transwell assay system. MCF-7 and MDA-MB-231 cell lines were found to be stimulated to migration by a concentration

gradient of TGF-β in a dose-dependent manner, revealing a maximum effect at concentrations of 1 to 2 ng/ml (Fig. 13).

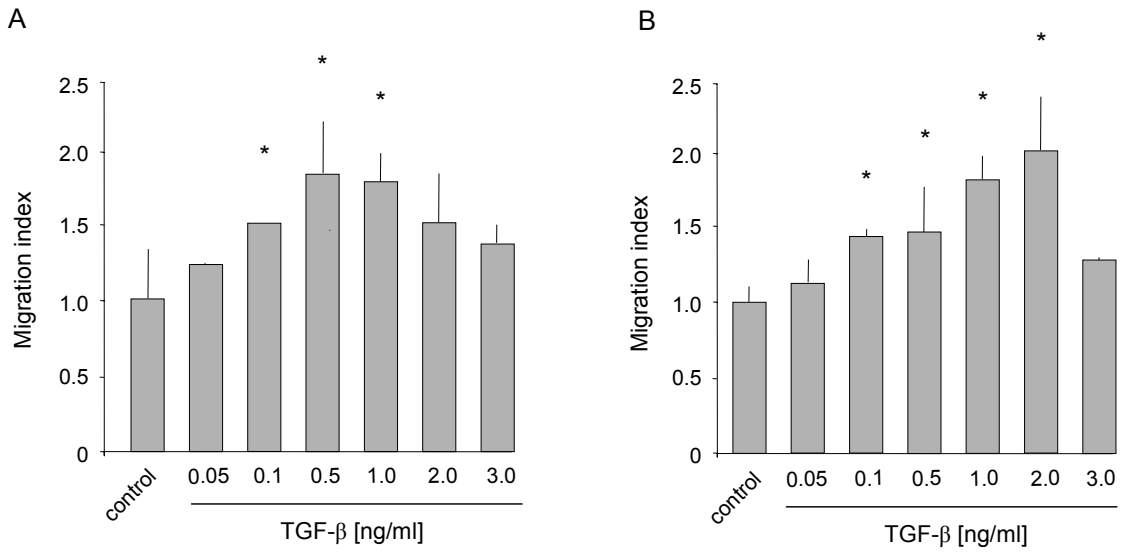

Fig. 13 Effect of TGF-β on chemotaxis of human breast cancer cells. Migration of MCF-7 (A) and MDA-MB-231 cells (B) in response to a gradient of TGF-β was measured in a transwell assay system as described. The lower chamber, containing serum-free medium, was supplemented with control vehicle or TGF-β in the indicated concentrations. After 5 h, migrated cells in the filter membranes were quantified. The data are expressed as means ± SD of two determinations. The experiment has been repeated at least two more times with similar results (*P ≤ 0.05).

3.2.2 TGF-β induced chemotaxis of E2 sensitised MCF-7 cells

Since it has been shown that not only TGF-β but also E2 exhibit migratory properties in MCF-7 cells, it was of note if this migration could be further enhanced by simultaneous stimulation. When maximal effective concentrations of both TGF-β and E2 were coevally placed in the lower chamber, the migration rates were further enhanced indicating a synergistic action of these substances (Fig. 14).

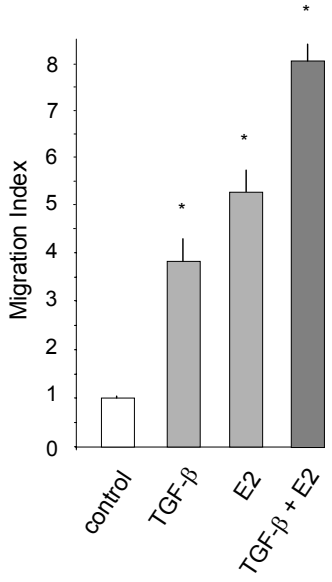

Fig. 14 Migration of MCF-7 cells in response to a simultaneous gradient of E2 and TGF-β. Migration was measured in a transwell assay system. The lower chamber was supplemented with either control vehicle, 10^{-6} M E2, 1 ng/ml TGF-β, or a combination of E2 and TGF-β. Each value represents the average migration index ± SD from duplicate wells. Experiments have been repeated at least three times with similar results (* $P \leq 0.05$).

Recently it has been shown that there is a broad cross-talk between ERs and growth factor signal transduction pathways, for example with RTKs, but also serine/threonine kinases. Thus, a bidirectional interplay of the steroid receptors with the TGF-β signal transduction cascade has been indicated, resulting in an enhanced transcriptional activity of estrogen responsive genes, whereas Smad promoter activation was diminished (Matsuda et al., 2001). In the following section, the interaction of estrogens and TGF-β is specified. More precisely, as TGF-β has been proven to be a potent migratory stimulus for MCF-7 cells, the modulatory influence of E2 on TGF-β mediated chemotaxis has been examined.

Although E2 itself is a powerful inductor of MCF-7 cell motility, when these cells were sensitised to E2 by pre-incubation over night and addition to the upper chamber, the ability of TGF-β to stimulate migration was dose-dependently reduced (Fig. 15). The inhibitory effect was first visible at a concentration of 10^{-8} M of E2, increasing to a most effective dose of 10^{-6} M. When cells were exposed to 10^{-6} M E2, the chemotactic potency of the cytokine was reduced by more than 60 %.

To ensure that E2 does not antagonise chemotaxis in general, but is a specific inhibitor of TGF-β mediated migration, chemotaxis of MCF-7 cells has also been measured in response to serum. FCS acts as a non-specific migratory stimulus

mainly due to its content of divers chemokines. MCF-7 cells were pre-incubated with different concentrations of E2, and subsequently cell movement towards 10 % serum was measured. Serum strongly enhanced migration of these cells. Fig. 15 illustrates TGF-β versus serum induced migration. The unspecific migratory response to FCS was not affected by any concentration of E2, indicating a specific inhibitory role of E2 on TGF-β induced pathways. It should be noted that the migratory index of E2 treated cells that were not exposed to a gradient of TGF-β or serum remained on basal levels.

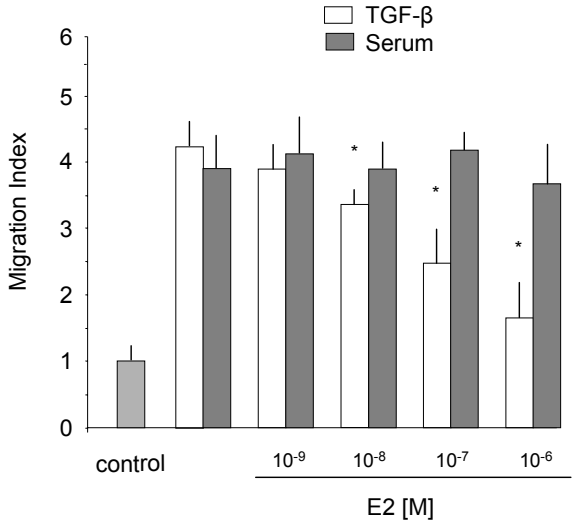

Fig. 15 Serum induced migration in comparison to TGF-β migration of E2 sensitised MCF-7 cells. Cells were incubated with the indicated concentrations of E2 or ethanol (control) over night and migration in response to 1 ng/ml TGF-β or 10 % FCS was measured in a transwell assay system. Control cells remained unstimulated. Data are means of migration index ± SD of two wells. All results have been confirmed in three independent experiments with comparable results (* $P \leq 0.05$).

In addition to estrogen induced transcriptional regulation of target genes, which requires several hours (genomic, chronic effects), large sets of estrogenic events occur within seconds or minutes (non-genomic, acute effects). As presented in Fig. **16** long-term pre-incubation with E2 and its presence in the assay is not necessary, but rather sensitisation with the estrogen can only be performed for 30 min before stimulation with TGF-β without affecting its inhibitory potency. For that reason, in the following assays pre-incubation was carried out over a time period of 30 min.

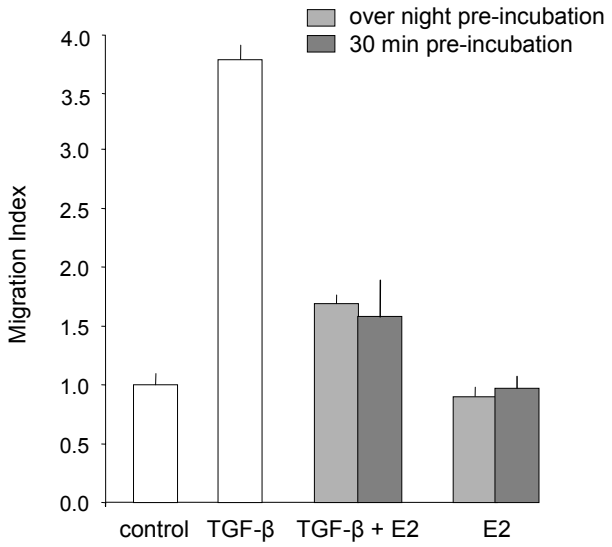

Fig. 16 The effectiveness of different incubation periods of E2 in the down-regulation of TGF-β mediated chemotaxis. MCF-7 cells were pre-treated with 10^{-6} M E2 for 19 h (over night) or 30 min or with ethanol (control). The potency of 1 ng/ml TGF-β to induce chemotactic movement has been examined in a transwell assay system three times with similar results. Each value results from two determinations ± SD.

3.2.3 Influence of E2 on viability of human breast cancer cells

In order to exclude cytotoxic effects of E2 having a bearing on TGF-β migration, the influence of E2 on the survival of hormone dependent MCF-7 and hormone independent MDA-MB-231 cells has been determined by crystal violet staining according to Gillies et al. (Gillies et al., 1986). This colorimetric method serves for the detection of the cell biomass, which corresponds to the cell number (Spruss et al., 1991).

As shown in Fig. 17A, in MCF-7 cells no cytotoxic action was observed over a time period of 250 h in a concentration range up to $5*10^{-6}$ M. In a concentration of 10^{-5} M E2 acts in an antiproliferative manner on this cell line already after 72 h. In contrast, MDA-MB-231 cells appeared to be more sensitive to E2. Nevertheless, no effect on the growth of these cells was detected for E2 in concentrations of up to 10^{-6} M over 50 h of incubation. An antiproliferative effect was first visible after 72 h of incubation with 10^{-6} M E2. In higher concentrations ($5*10^{-6}$ and 10^{-5} M) E2 even exerts cytostatic action after an incubation period of 72 h (Fig. 17B). In conclusion, E2 did not impair the viability of MCF-7 and MDA-MB-231 cells in the concentrations and time periods used in this study. Cytotoxic effects could therefore not be esteemed as cause for the impact of the estrogen cell motility induced by TGF-β.

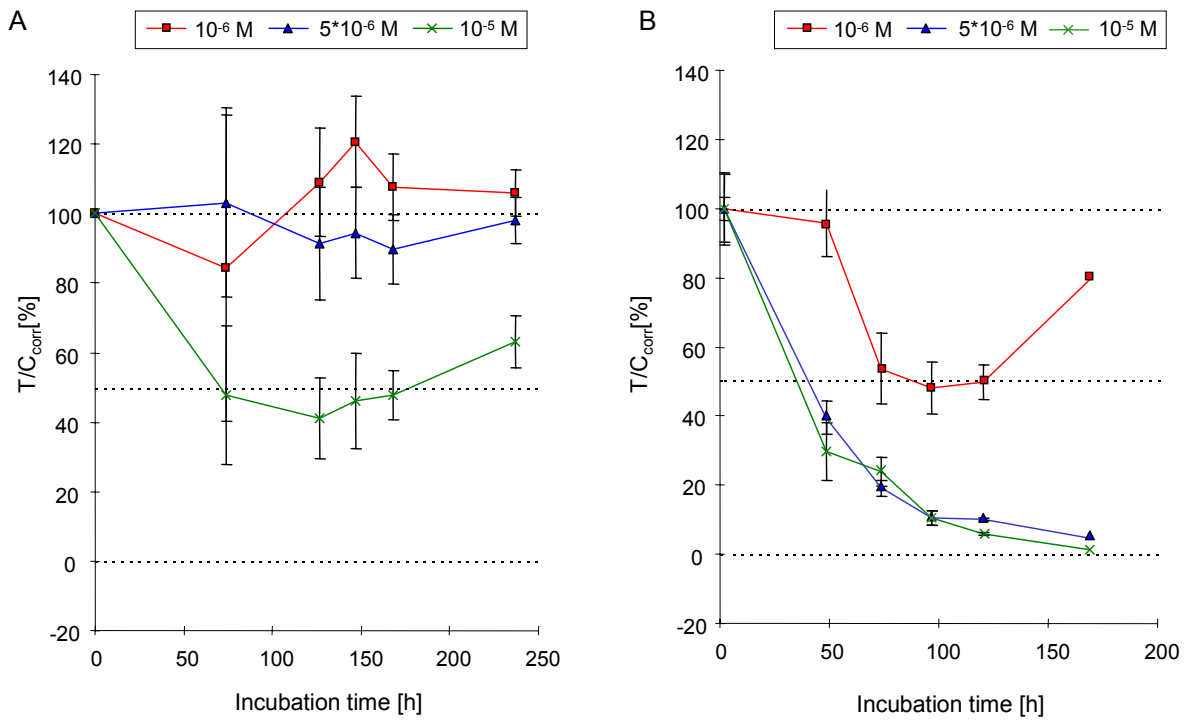

Fig. 17 Influence of E2 on the viability of human breast cancer cells. MCF-7 (A) and MDA-MB-231 (B) cells were treated with vehicle control or $1*10^{-6}$ to $1*10^{-5}$ M E2. After the indicated incubation times, reactions were stopped and the surviving cells stained with crystal violet as depicted. T/C_{corr} was calculated due to six determinations ± SD. Comparable results have been obtained in three independent experiments.

3.2.4 Smad signalling in TGF-β induced migration of MCF-7 cells

The following sections are engaged in the clarification of the mechanisms underlying the inhibitory effect of E2 on TGF-β induced migration. The TGF-β signalling pathway is well characterised and Smad proteins are the main mediators of TGF-β signal transduction. Therefore, the repressive influence of E2 may presumably occur on the basis of Smad activation. Phosphorylation of Smad2 and Smad3 has been identified in a variety of cells as a crucial event to initiate TGF-β mediated chemotaxis (Ashcroft et al., 1999; Roberts et al., 2003; Tian et al., 2003). Nevertheless, it was not known whether in MCF-7 cells Smad proteins contribute to their migratory properties in response to the cytokine. This question was subject to the following experiment.

Smad2 and Smad3 signalling was interrupted by means of small interfering RNA (siRNA) for the common partner Smad4, which complexes Smad2 and Smad3 subsequently to their activation by TGF-β. In a transwell assay system, the migratory property of TGF-β and the unspecific stimulus of serum on Smad4 deficient MCF-7 cells was measured. Control determinations were performed with cells treated with

transfection reagent only or a non-targeting control siRNA to assess cytotoxic effects of either agent.

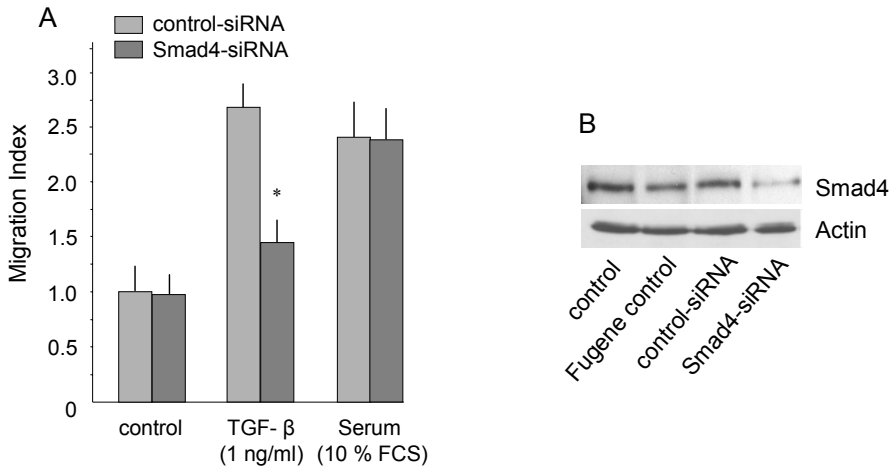

Fig. 18 TGF-β induced migration of MCF-7 cells after abrogation of Smad signalling. To down-regulate Smad4 protein levels and therefore to interrupt Smad2 and Smad3 signalling, cells were seeded in 10 cm dishes or six-well plates, respectively, and transfected with control- or Smad4-siRNA oligonucleotides for 48 h. (A) The chemotactic migration of the transfected MCF-7 cells in response to a gradient of 1 ng/ml TGF-β or 10 % FCS was measured in a transwell assay system. Each value represents the average migration index ± SD from duplicate wells. (B) To reveal the reduction of Smad4 expression by siRNA treatment, 15 µg of lysate protein were electrophoresed and immunoblotted with an anti-Smad4 (upper panel) or an anti-Actin (lower panel) antibody. All siRNA experiments have been confirmed in three independent tests, revealing similar results (* P ≤ 0.05).

As presented in Fig. 18B, treatment of MCF-7 with Smad4-siRNA resulted in a significant reduction of Smad4 expression determined by Western blot using Smad4 specific antibodies. Furthermore, siRNA treated cells were tested for their ability to be attracted by a TGF-β stimulus. Fig. 18A shows that migration towards TGF-β was drastically reduced when Smad4 was abrogated, clearly indicating an involvement of Smad activation in the property of TGF-β to induce migration of MCF-7 cells. In contrast, serum induced migration was not diminished in Smad4 deficient MCF-7 cells, indicating the specific role of Smad signalling in TGF-β induced migration of these cells.

3.2.5 Phosphorylation of Smad proteins

It could successfully be shown that Smad signalling contributes to the migratory properties of MCF-7 cells in response to a concentration gradient of TGF-β. Smad2 phosphorylation by TGF-β was confirmed in MCF-7 and MDA-MB-231 cells by

Western blot analysis using phospho-Smad2 specific antibodies. The assembly of R-Smad proteins 2 and 3 with the Co-Smad4 is a pivotal event in nuclear translocation and transcriptional regulation of target genes by TGF-β. Smad complex formation was examined by immunoprecipitation experiments using an anti-Smad1,2,3 antibody for precipitation and an anti-Smad4 antibody for detection in the following Western blot.

As expected, in both cell lines, TGF-β induced a dose dependent phosphorylation of Smad2 and complexation of R-Smads and Co-Smads, whereas total protein contents were not affected. In MCF-7 cells the maximal Smad2 phosphorylation and Smad complex formation occurred at a concentration of 1 ng/ml of TGF-β, whereas in MDA-MB-231 cells the strongest response already appeared at concentrations between 0.5 and 1 ng/ml (Fig. 19).

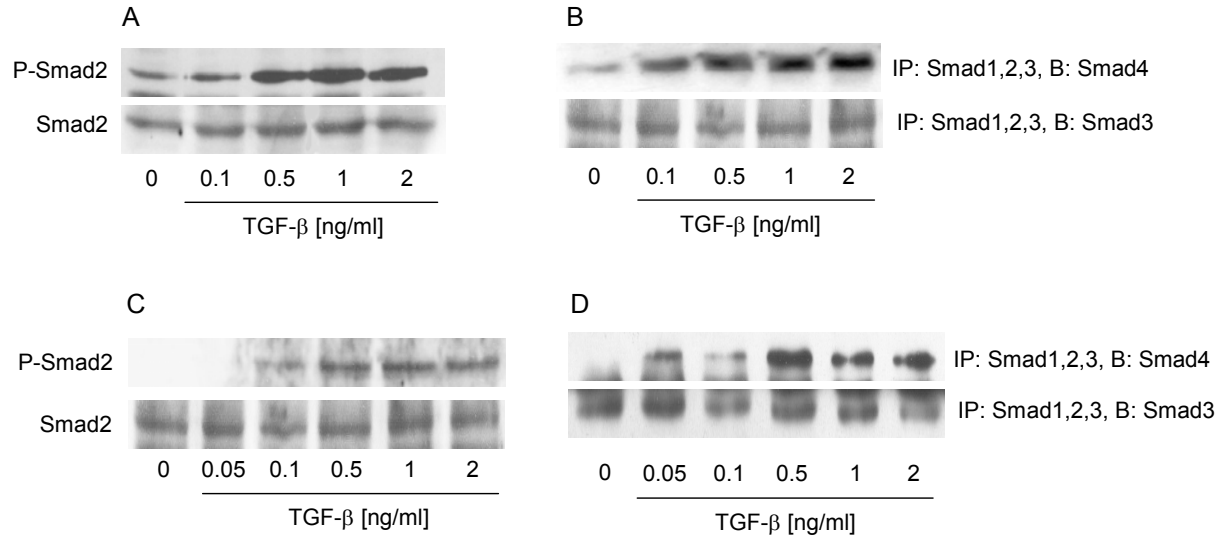

Fig. 19 Phosphorylation of Smad2 and Smad2,3/Smad4 complex formation by TGF-β in human breast cancer cells . MCF-7 (A, B) and MDA-MB-231 cells (C, D) were stimulated for 30 min with the indicated concentrations of TGF-β. For measurement of Smad complexation, Smad1,2,3 proteins were immunoprecipitated and Western blot analysis was performed with an anti-Smad4 (upperpanel) or an anti-Smad3 antibody (lower panel) (B, D). 15 µg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 (upper panel) or anti-Smad2 antibodies (lower panel) (A, C). The measurements have been repeated two more times in independent experiments with similar results.

In order to determine the most effective stimulation period, MCF-7 and MDA-MB-231 cells were incubated with 1 ng/ml TGF-β for 10 to 90 min. This treatment resulted in a transient phosphorylation with a maximum after 30 to 60 min for Smad2 phosphorylation and 30 min for the Smad complex formation. For MDA-MB-231 cells, similar Smad2 phosphorylation kinetics were found, as a strong activation appeared at a TGF-β exposure time of 30 to 60 min. Comparable to MCF-7, the strongest

complexation rate could be measured after 30 min, but decreased more rapidly with only weak residual stimulation after 60 min (Fig. 20).

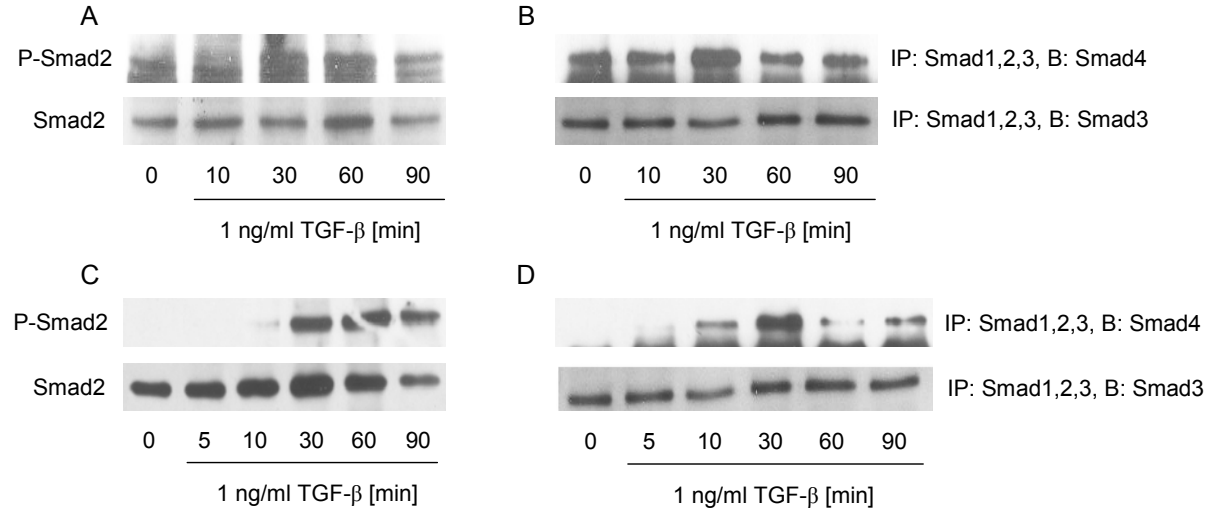

Fig. 20 Time dependent phosphorylation of Smad2 and Smad2,3/Smad4 complex formation by TGF-β in human breast cancer cells. MCF-7 (A, B) and MDA-MB-231 cells (C, D) were stimulated with 1 ng/ml TGF-β for the indicated times. For measurement of Smad complexation, Smad1,2,3 proteins were immunoprecipitated and Western blot analysis was performed with an anti-Smad4 antibody (B, D). 15 µg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 (upper panel) or anti-Smad2 antibodies (lower panel) (A, C). Similar findings have been achieved in three independent experiments.

For the purpose of simultaneously detecting both activating as well as inhibiting effects of substances on Smad phosphorylation, stimulation with TGF-β was performed in further experiments with a concentration of 0.5 ng/ml for 30 min, which was able to induce half maximal activation.

3.2.6 Influence of E2 on the activation of the Smad signalling pathway

It has been documented that members of the steroid receptor family are able to influence the TGF-β pathway positively or negatively to impact a variety of physiological or pathological processes. Among these, androgen receptors (ARs) (Hayes et al., 2001; Kang et al., 2002), the vitamin D3 receptor (VDR) (Yanagisawa et al., 1999), the glucocorticoid receptor (GR) (Song et al., 1999), and finally also ERs (Matsuda et al., 2001) have been described to act as transcriptional modulators of Smad proteins.

In this study, Smad proteins have been identified as essential signalling molecules in TGF-β mediated chemotaxis of MCF-7 cells. Thus, it seems likely that ERs interfere with TGF-β signalling on the level of Smad proteins. In order to go further into that question, Smad2 phosphorylation and the formation of a functional Smad2,3/Smad4 complex was measured after stimulation with TGF-β in cells sensitised with E2. For that purpose, MCF-7 cells were treated with E2 or ethanol, respectively, and stimulated afterwards with 0.5 ng/ml TGF-β for another 30 min. Cells lysates were either immunoprecipitated with an anti-Smad1,2,3 antibody or remained untreated. Detection of complexed Smad2,3/Smad4 in the precipitates or phosphorylated Smad2 in whole cell lysates was performed by Western blot analysis using an anti-Smad4 antibody or anti-phospho-Smad2 antibody, respectively.

As presented in Fig. 21, phosphorylation of Smad2 by TGF-β was reduced by E2 compared with cells treated with vehicle only, whereas total Smad2 protein levels remained unaffected. In consistence, analogous results were obtained for measurement of complex formation, since TGF-β induced assembly of R- and Co-Smads was also reduced in E2 sensitised cells. Comparable to the outcome of the migration experiments, in both assays an E2 concentration of 10^{-6} M appeared to be by far more potent than 10^{-8} M.

It should be mentioned that without TGF-β stimulation the Smad2 phosphorylation and Smad2,3/Smad4 complexation in E2 sensitised cells remained on basic levels, comparable to non-stimulated control cells.

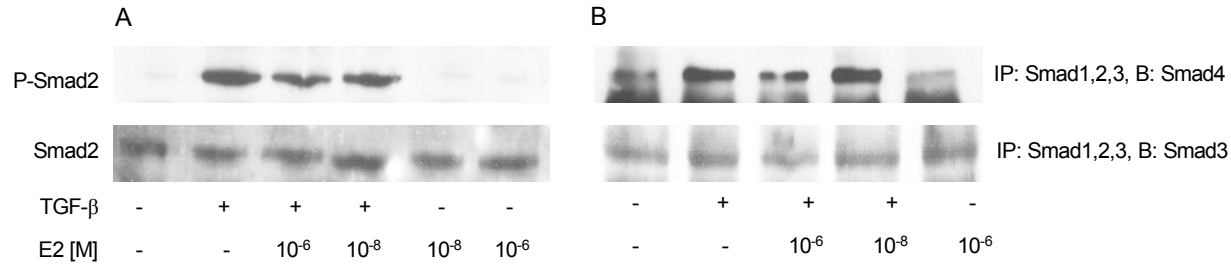

Fig. 21 Influence of E2 on TGF-β induced Smad2 phosphorylation and Smad complex formation in MCF-7 cells. Cells were incubated with 10^{-6} M E2 for 30 min and then stimulated for 30 min with 0.5 ng/ml TGF-β. (A) For measurement of Smad2 phosphorylation, 15 µg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 (upper panel) or anti-Smad2 antibodies (lower panel). (B) Smad complexation was determined by immunoprecipitation of Smad1,2,3 proteins. Western blot analysis was performed with an anti-Smad4 antibody. Three independent experiments produced comparable results.

Furthermore, pre-incubation with E2 was carried out for different time periods. Hormone induced inhibition of Smad2 phosphorylation was first visible after 5 to 15 min incubation time. The most efficient effect appeared after 30 min pre-incubation, as shown in Fig. 22.

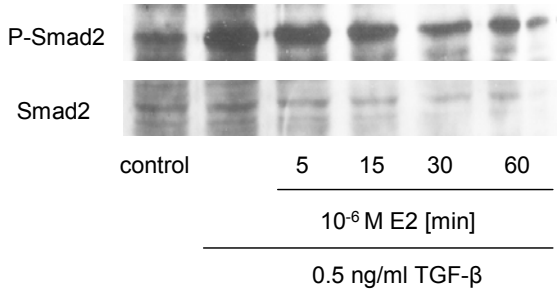

Fig. 22 Time course of the repression of TGF-β induced Smad2 phosphorylation in E2 sensitised MCF-7 cells. Cells were incubated with vehicle (control) or 10^{-6} M E2 for the indicated time periods and then stimulated for 30 min with vehicle or 0.5 ng/ml TGF-β. 15 µg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 (upper panel) or anti-Smad2 antibodies (lower panel). Three independent experiments revealed similar findings.

3.2.7 Activation of Smad responsive promoters by TGF-β

Subsequent to complex formation of Smad4 with the phosphorylated R-Smads, the activated Smad complex translocates into the nucleus, thus initiating the modulation of transcriptional activities of Smad dependent genes. To affirm the ability of TGF-β to activate Smad2 and Smad3 responsive genes, dual luciferase reporter gene experiments were performed.

MCF-7 and MDA-MB-231 cells were transfected with Smad3 or Smad2 responsive promoters with inducible Firefly luciferase activity. The SBE_4-promoter, used in this study, has been proven to be activated by Smad3 only (Nagarajan and Chen, 2000). Transcriptional activity of Smad2 was measured by means of the 3ARE-luc enhancer sequence that requires additional transfection of the MykFast protein as transcriptional coregulator. In this system promoter activation by phosphorylated Smad2 has been proven to be multiple higher than by Smad3 (Nagarajan and Chen, 2000). Simultaneously, the cells were cotransfected with the Renilla luciferase expression vector pRL-CMV. In contrast to Firefly luciferase, this enzyme is constitutively transcribed and therefore used to display the transfection efficacy. After transfection, the cells were stimulated with TGF-β for 24 h. Binding of activated Smad complexes to the appropriate promoter sequence initiates expression of Firefly

luciferase, which can be quantified by application of LARII substrate. After measurement, the Firefly signal is quenched and the Renilla luciferase reaction is initiated by the appropriate substrate. The relative Firefly luciferase activity resulted from referring the absolute Firefly luciferase signal to that of the Renilla luciferase.

As expected, stimulation of MCF-7 and MDA-MB-231 cells with TGF-β resulted in an activation of Smad3 as well as Smad2 promoters in a dose dependent manner, appearing as an increase in Firefly luciferase activity relatively to Renilla luciferase (Fig. 23). In both cell lines, the maximal activation of Smad2 as well as Smad3 promoters occurred at a concentration of 2 ng/ml of TGF-β, with the relative activation levels of MDA-MB-231 cells being substantially higher than of MCF-7 cells.

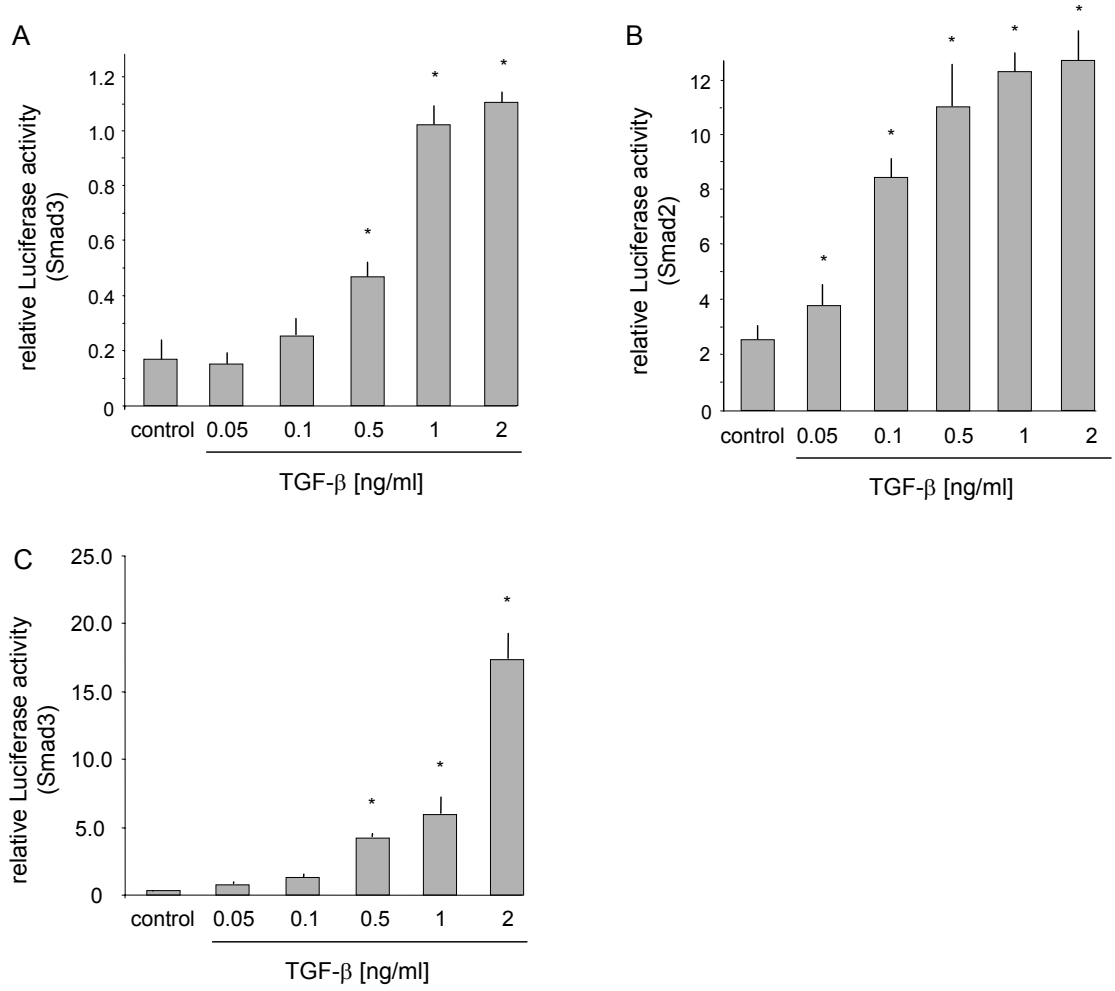

Fig. 23 TGF-β induced activation of Smad3- and Smad2 responsive promoters. MCF-7 (A, B) and MDA-MB-231 cells (C) were transfected with luciferase coupled promoter constructs responsive to Smad3 (A, C) or Smad2 in combination with the transcription factor MykFast (B). After 24 h, cells were incubated with TGF-β in the indicated concentrations for further 24 h. Luciferase activities after application of the appropriate substrate were measured in a luminometer. The data are expressed as means of counts derived from Firefly luciferase reaction corrected by Renilla luciferase activity ± SD from triplicate wells. All experiments have been repeated two more times in independent experiments, achieving comparable results (* $P \leq 0.05$).

3.2.8 Transcriptional activity of Smads in hormone sensitised cells

To elucidate whether signalling events downstream of Smad phosphorylation and Smad complex formation are also influenced by E2, the ability of TGF-β to activate Smad2 and Smad3 responsive genes was examined in E2 treated cells. Therefore, MCF-7 cells, transiently transfected with plasmids including Smad2 and Smad3 responsive promoters, were sensitised with different concentrations of E2 and subsequently stimulated with TGF-β. Luminescence, resulting from Firefly luciferase expression, was measured and normalised against the Renilla luciferase signal.

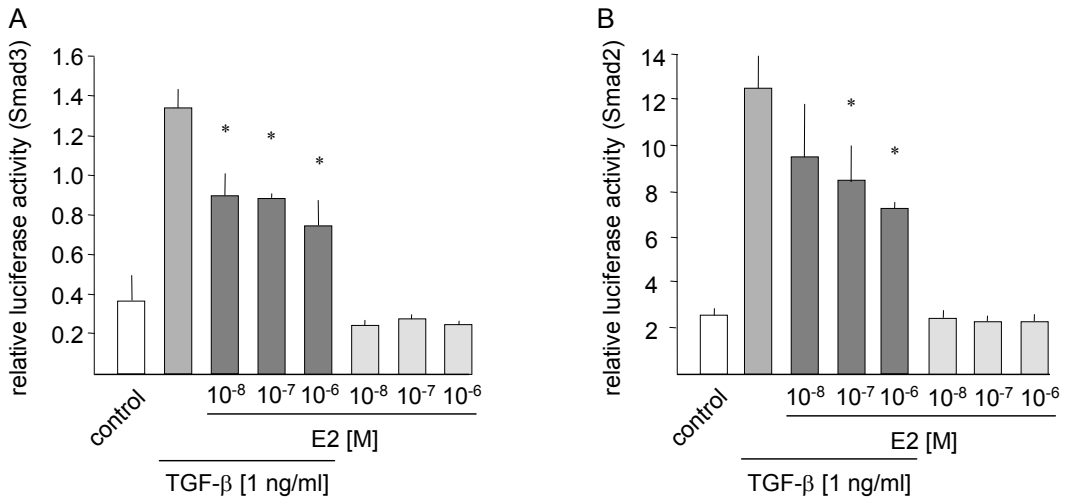

Fig. 24 Influence of E2 sensitisation on TGF-β induced activation of Smad3 and Smad2 responsive promoters. MCF-7 cells were transfected with luciferase coupled promoter constructs responsive to Smad3 (A) or Smad2 (B). After 24 h, cells were incubated with vehicle (control) or E2 in the indicated concentrations for 30 min and treated with vehicle or 1 ng/ml TGF-β for further 24 h. Luciferase activities after application of the appropriate substrate were measured in a luminometer. Each value represents the average number of counts from Firefly luciferase reaction corrected by Renilla luciferase activity ± SD from triplicate wells. Comparable findings have been achieved in three independent experiments (* $P \leq 0.05$).

When MCF-7 were sensitised with E2, TGF-β induced Smad promoter activation was significantly reduced compared with untreated control cells (Fig. 24). A significant reduction of the Smad2 as well as of the Smad3 dependent luciferase activities by about 50 % occurred at a concentration of 10^{-6} M of E2. Comparable to Smad phosphorylation determined by Western blot, E2 itself did not influence neither Smad3 nor Smad2 promoters.

3.3 The estrogen-to-TGF-β cross-talk: Involvement of nuclear ERs

In the following, it was of interest, which cellular processes mediate the inhibitory effects of E2. Since activation of nuclear ERs is essential in a multitude of estrogenic events, it was investigated whether the counteracting effect of E2 on TGF-β mediated chemotaxis is dependent on ER expression. MDA-MB-231 cells, considered as a hormone independent cell system, and MCF-7 cells, as positive control, were sensitised with E2 and migration rates towards TGF-β were measured. As presented in Fig. 25A, no significant reduction of cell movement was detected in pre-treated MDA-MB-231 cells compared to the MCF-7 cell line.

Signalling of nuclear ERs was interrupted in MCF-7 cells by over night treatment with ICI182,780. The so prepared cells were exposed to E2 and TGF-β migration was measured. In agreement with the last-presented result, inhibition of the steroid receptor significantly reversed the repression of TGF-β induced migration by E2 (Fig. **25**B), indicating an interaction of activated ERs with the TGF-β signalling cascade. As shown in Fig. 25B, this long-term treatment with ICI182,780 did not impact directed migration towards TGF-β in general.

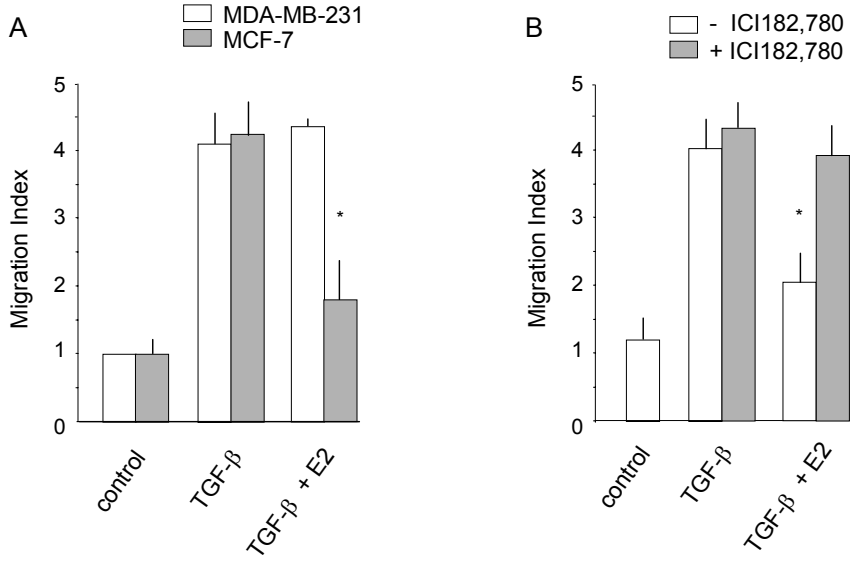

Fig. 25 Involvement of ERs in the inhibitory effect of E2 on TGF-β migration. (A) The potency of TGF-β (2 ng/ml) to enhance chemotaxis was measured in MDA-MB-231 cells treated with 10^{-6} M E2 for 30 min. (B) ER signalling was blocked in MCF-7 cells by pre-incubation with ICI182,780 (10^{-6} M) for 19 h. Cells were sensitised with 10^{-6} M E2 for 30 min and then migration to a gradient of TGF-β (1 ng/ml) was examined in a transwell assay system. Each value represents the average migratory index ± SD from duplicate wells. The experiments have been performed three times with comparable results (* $P \leq 0.05$).

To further elucidate whether ERs are involved in the observed effects of E2 on cellular events mediated by TGF-β, the influence of the hormone on activation of the Smad signalling cascade was investigated in ER negative cells. Therefore, TGF-β stimulated Smad2 phosphorylation and Smad3 promoter activation were measured in E2 sensitised MDA-MB-231 cells. In accordance with the results from chemotaxis assays, in MDA-MB-231 cells no significant reduction of Smad phosphorylation (Fig. 26A) and TGF-β induced reporter gene activity (Fig. 26B) by E2 were observed.

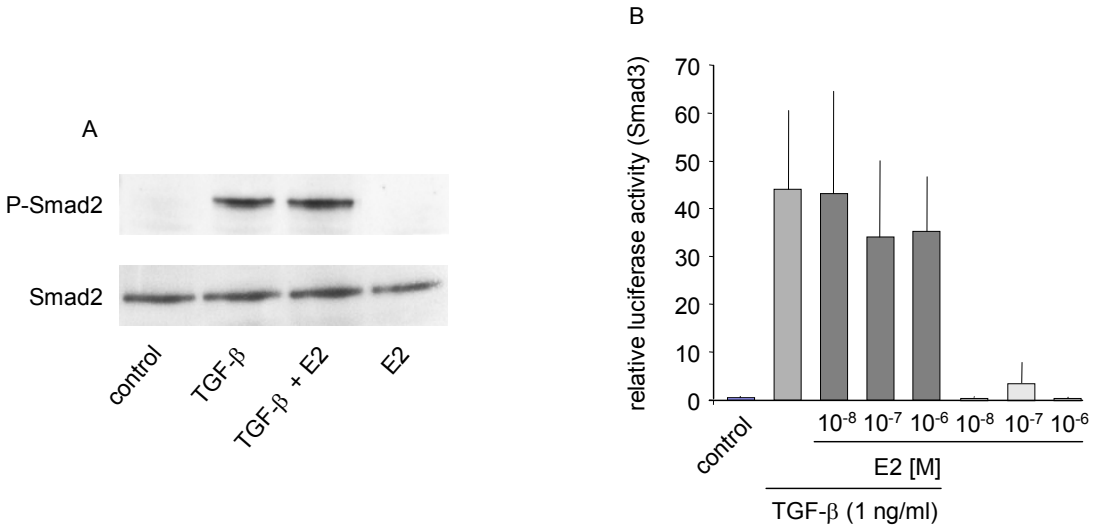

Fig. 26 Influence of E2 on TGF-β induced Smad2 phosphorylation and Smad promoter activation in MDA-MB-231 cells. (A) Cells were incubated with 10^{-6} M E2 for 30 min and then stimulated for 30 min with 0.5 ng/ml TGF-β. 15 µg of lysate protein were electrophoresed and immunoblotted with anti-phospho-Smad2 (upper panel) or anti-Smad2 antibodies (lower panel). (B) Cells were transfected with luciferase coupled, Smad3 dependent promoters. After 24 h, cells were incubated with vehicle (control) or E2 in the indicated concentrations for 30 min and treated with vehicle or 1 ng/ml TGF-β for further 24 h. Luciferase activity was determined as described. The data are expressed as means of counts from Firefly luciferase reaction referred to Renilla luciferase activity ± SD from triplicate wells. Three independent experiments revealed the same tendency in results.

ERs have been described to act as transcriptional co-repressors of Smad3. Thus, ERα as well as ERβ possess a binding motif for Smad3, mediating a physical interaction and repression of Smad3 activity when both ER and Smads are in their activated state (Matsuda et al., 2001). In order to investigate whether this mechanism can also be considered for the findings presented in this study, MCF-7 cells were stimulated with TGF-β and E2 or remained untreated. Smad proteins were immunoprecipitated from cell lysates and the presence of ERα in the precipitates was examined.

Fig. 27 demonstrates that ER binding to Smad1,2,3 of stimulated cells did not exceed that of non-stimulated cells (control). Thus, interruption of the Smad signalling pathway by direct physical binding to ERα appears to be improbable.

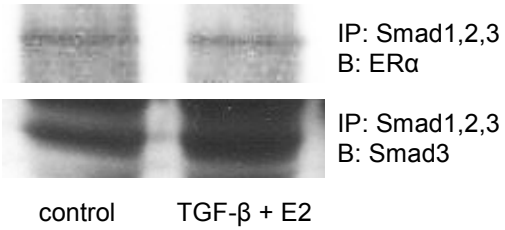

Fig. 27 Measurement of physical interaction between Smad proteins and ERα in MCF-7 cells. Cells were sensitised with 10^{-6} M E2 for 30 min and stimulated with 1 ng/ml TGF-β for further 30 min. Control cells were treated with vehicles, respectively. Smad proteins were immunoprecipitated from whole cell lysates with an anti-Smad1,2,3 antibody, separated by SDS-PAGE and blotted onto a PVDF-membrane. Complexes of ERα and Smad proteins were detected by incubation with an anti-ERα antibody. In order to verify consistent protein levels in the precipitates, membranes were stripped and re-probed with an anti-Smad1,2,3 antibody. Three independent experiments revealed similar results.

3.4 Involvement of non-genomic pathways

3.4.1 Expression of estrogen responsive receptors in breast cancer cells

The classical signal transduction pathway of estrogens via the nuclear ERs α and β, transcriptionally regulating genes at ERE binding sites, is well established. Rapid, mostly non-genomic estrogenic effects have been traced back to plasma membrane localised receptors, among which GPR30 has been identified as one very probable candidate known to activate several signalling pathways after binding of E2 (Filardo et al., 2000; Filardo et al., 2002; Thomas et al., 2005). To examine which of these ERs participate in the inhibitory impact of E2 on TGF-β pathways, the expression of ERα and GPR30 in human breast cancer cell lines has been measured by semi-quantitative PCR experiments (Fig. 28).

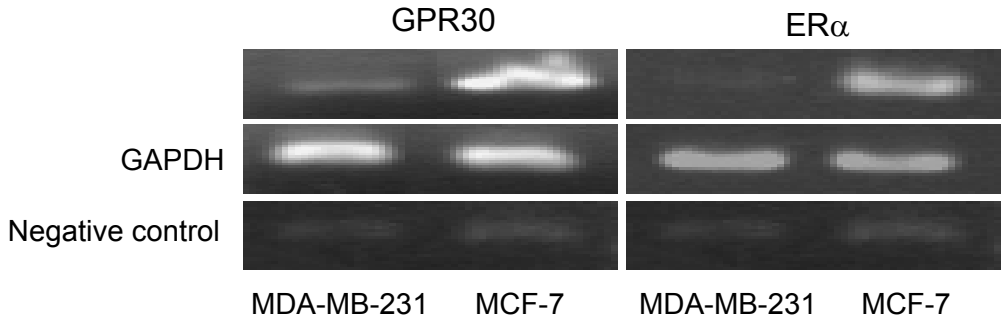

Fig. 28 Expression of GPR30 (upper left panel) and ERα (upper right panel) in human breast cancer cells measured by RT-PCR. Isolated mRNAs of MDA-MB-231 and MCF-7 cells were either transferred into cDNA by reverse transcriptase or the enzyme was omitted for negative controls (lower panels). The expression of ubiquitously expressed glycerine aldehyde phosphate dehydrogenase (GAPDH) was used as reference and is represented by middle panels. This experiment has beenperformed three times with similar outcomes.

It could successfully be confirmed that MCF-7 cells express high levels of ERα, whereas MDA-MB-231 cells are ERα negative. The expression of GPR30 in human breast cancer cells has been examined by Carmeci and coworkers by Northern blot analysis (Carmeci et al., 1997). Their findings could be verified as high endogenous expression levels of GPR30-mRNA were found in MCF-7, but not MDA-MB-231 cells, whereas both cell lines contained equal amounts of ubiquitously expressed glycerine aldehyde phosphate dehydrogenase (GAPDH) mRNA.

3.4.2 Effect of membrane impermeable E2 on TGF-β mediated migration

To examine whether membrane associated ERs are involved in the repression of TGF-β signalling by E2, MCF-7 cells were pre-incubated with E2 covalently linked to plasma membrane impermeable BSA (BSA-E2) (Taguchi et al., 2004). Afterwards migration towards TGF-β was measured in a transwell assay system. According to the manufacturer, this product contains non-complexed E2 not exceeding 2 %. In order to remove potentially remaining traces of free steroid, BSA-E2 was treated with dextran-coated charcoal as depicted in section 2.2.6.

Although by this means diffusion of E2 into the cytoplasm and activation of intracellular ERs is prevented, leaving only extracellular binding, the repression of TGF-β mediated migration could be preserved. Incubation of MCF-7 cells with BSA-E2 resulted in an inhibition of TGF-β induced migration by 60 % at an hormone content of 10^{-6} M and 80 % at 10^{-8} M E2 (Fig. 29). As reported for E2, BSA-

complexed E2, too, did not change migration of unstimulated cells. This finding leads to the presumption that ERs located in the plasma membrane contribute to the inhibition of TGF-β activated pathways by estrogens.

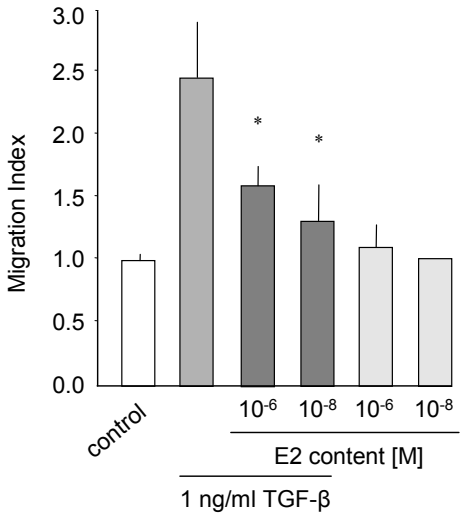

Fig. 29 Influence of BSA-conjugated E2 on TGF-β induced migration of MCF-7 cells. Cells were treated with BSA-E2 containing E2 in the indicated concentrations for 30 min and migration towards 1 ng/ml TGF-β was measured in a transwell assay system. Each value arises from two determinations ± SD. Similar results have been obtained in three independent experiments (* $P \leq 0.05$).

A luciferase assay served as means to exclude agonistic effects of BSA-E2 on intracellular ERs. The MCF-7-2a cell line is stably transfected with the $ERE_{wtc\ luc}$ plasmid including specific DNA sequences to which both ERα and ERβ bind with high affinity (ERE), and the reporter gene *luc* containing directions for the assembling of the luciferase enzyme. Ligand binding to the ERs results in allosteric changes in the receptor leading to the dissociation of heat-shock proteins, dimerisation, and binding to the ERE of the plasmid, thereby facilitating the generation of luciferase protein, which correlates with the estrogenic efficacy of the ligand (Biberger and von Angerer, 1998; Hafner et al., 1996).

The cells were incubated with BSA-E2 or uncomplexed E2 in a concentration range of 10^{-12} to 10^{-6} M. After cell lysis, light emission induced by the enzymatic conversion of the luciferase assay reagent was detected. Signal variations due to fluctuating amounts of viable cells were considered by crystal violet staining. As can be seen in Fig. 30, compared to BSA-E2 a significant ERE activation by E2 already appeared at a concentration of 10^{-11} M, achieving maximum levels from 10^{-9} M to 10^{-7} M. In contrast, BSA-E2 demonstrably failed to induce ERE activation at any concentration.

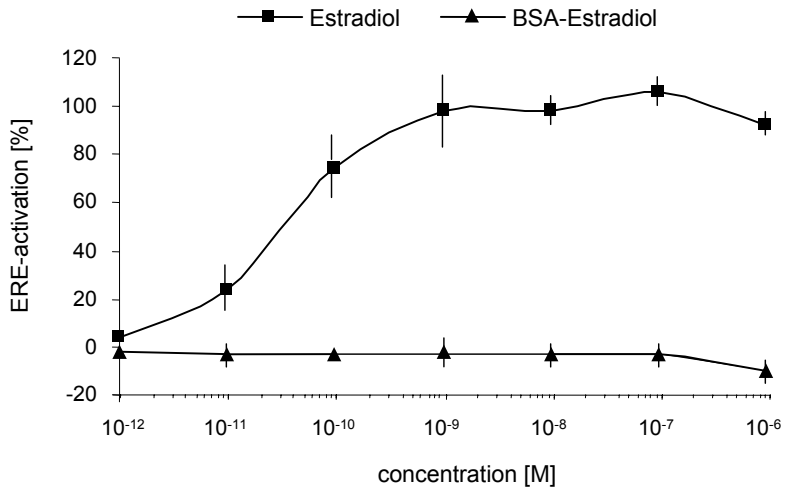

Fig. 30 Transcriptional activity of BSA-E2. MCF-7-2a cells stably transfected with $ERE_{wt\ luc}$, coupled to luciferase activity, were cultured in steroid deprived medium in 96-well plates. Stimulation was performed with E2 and BSA-E2 in the indicated concentrations, vehicle or 10^{-9} M E2 as positive control for 18 h. Then cells were lysed and luciferase activation was measured after addition of luciferase assay reagent. All values are referred to the amount of surviving cells determined by crystal violet staining in additional, equally treated cell cultures. The data are expressed as the median, percentage luciferase activity from six determinations minus luciferase activity of vehicle controls ± SD. In three independently performed experiments similar results have been obtained.

3.4.3 Involvement of G-protein coupled receptors

Evidence could be given so far that the blockade of TGF-β signalling by E2 is initiated at the plasma membrane. A growing body of studies proved that signalling through mERs relies on the activation of G-proteins (Razandi et al., 1999; Thomas et al., 2005; Wyckoff et al., 2001). To investigate if G-protein depending signalling pathways contribute to the modulatory effect of E2 on TGF-β migration, MCF-7 cells were pre-treated with PTX, an inhibitor of the alpha subunit of G_i-proteins, before sensitisation with the hormone. Afterwards, migration and Smad2 phosphorylation in response to a TGF-β stimulus were examined. Fig. 31 demonstrates that this treatment effectively prevented both the repression of TGF-β induced migration and Smad2 phosphorylation after E2 treatment, indicating G_i-proteins to be a prerequisite for estrogen signalling leading to inhibited migration.

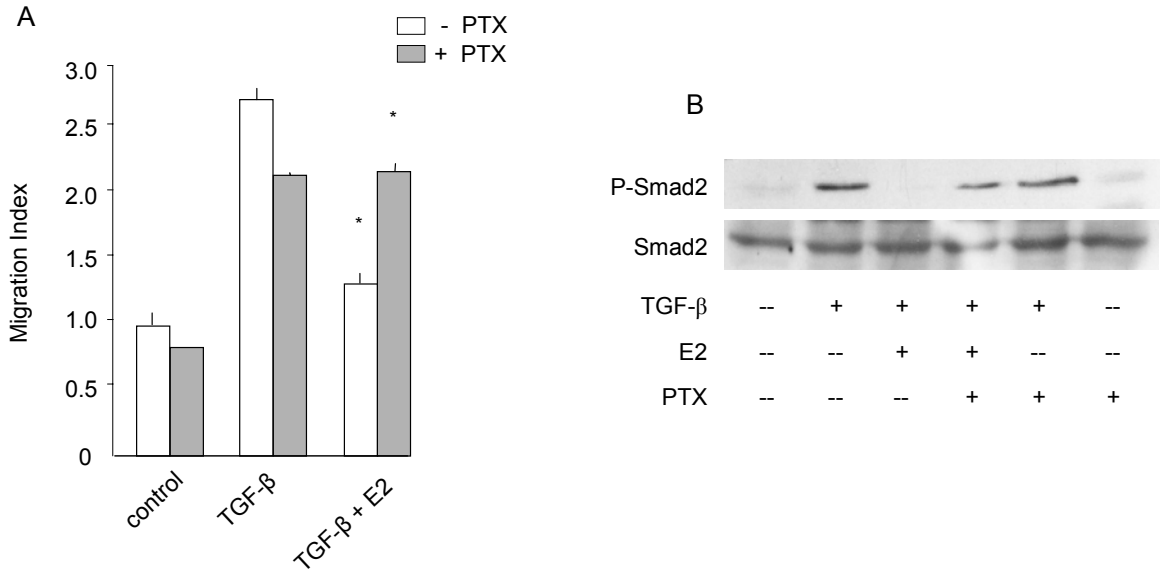

Fig. 31 Involvement of G_i-proteins in the repression of TGF-β signalling by E2. MCF-7 cells were treated with vehicle or 100 ng/ml pertussis toxin (PTX) for 3 h followed by incubation with 10^{-6} M E2 for 30 min. (A) The migratory response to 1 ng/ml TGF-β was measured in a transwell assay system. Each value demonstrates the median migratory index ± SD from duplicate wells (* $P \leq 0.05$). (B) Cells were stimulated with 0.5 ng/ml TGF-β, and Western blot analysis was performed from whole cell lysates. Smad2 activation was measured using an anti-phospho-Smad2 antibody (upper panel) and total Smad2 expression by an anti-Smad2 antibody. These experiments have been repeated two more times, thereby revealing comparable results.

3.4.4 TGF-β responses of MCF-7 cells after antiestrogen treatment

Although the first mention of specific binding sites for estrogens in the plasma membrane of endometrial cells already happened in 1977 (Pietras and Szego, 1977), the nature of membrane localised ERs still causes a lot of controversy in recent literature. A great number of investigators characterised GPR30 as a receptor with the properties revealed in this study. It is localised at the plasma membrane and rapidly initiates a variety of signalling cascades via differentiated activation of G-protein subunits α and β/γ.

Interestingly, in a great number of studies antiestrogens, namely ICI182,780 and TAM, were identified to interact with GPR30 in an agonistic manner (Filardo et al., 2000; Filardo et al., 2002; Thomas et al., 2005). As depicted previously, inhibition of nuclear ERs by an over night incubation with ICI182,780 reverses the repression of TGF-β induced migration by E2 without influencing TGF-β migration in general. Quite the contrary effect appeared, when MCF-7 cells were treated with ICI182,780 for only 30 min. Such short-time treatment with low doses of the antiestrogen has been

proved to be sufficient for the activation of GPR30 (Filardo et al., 2000; Filardo et al., 2002; Thomas et al., 2005), whereas the content of ERα protein remained unaffected (Fig. 11B).

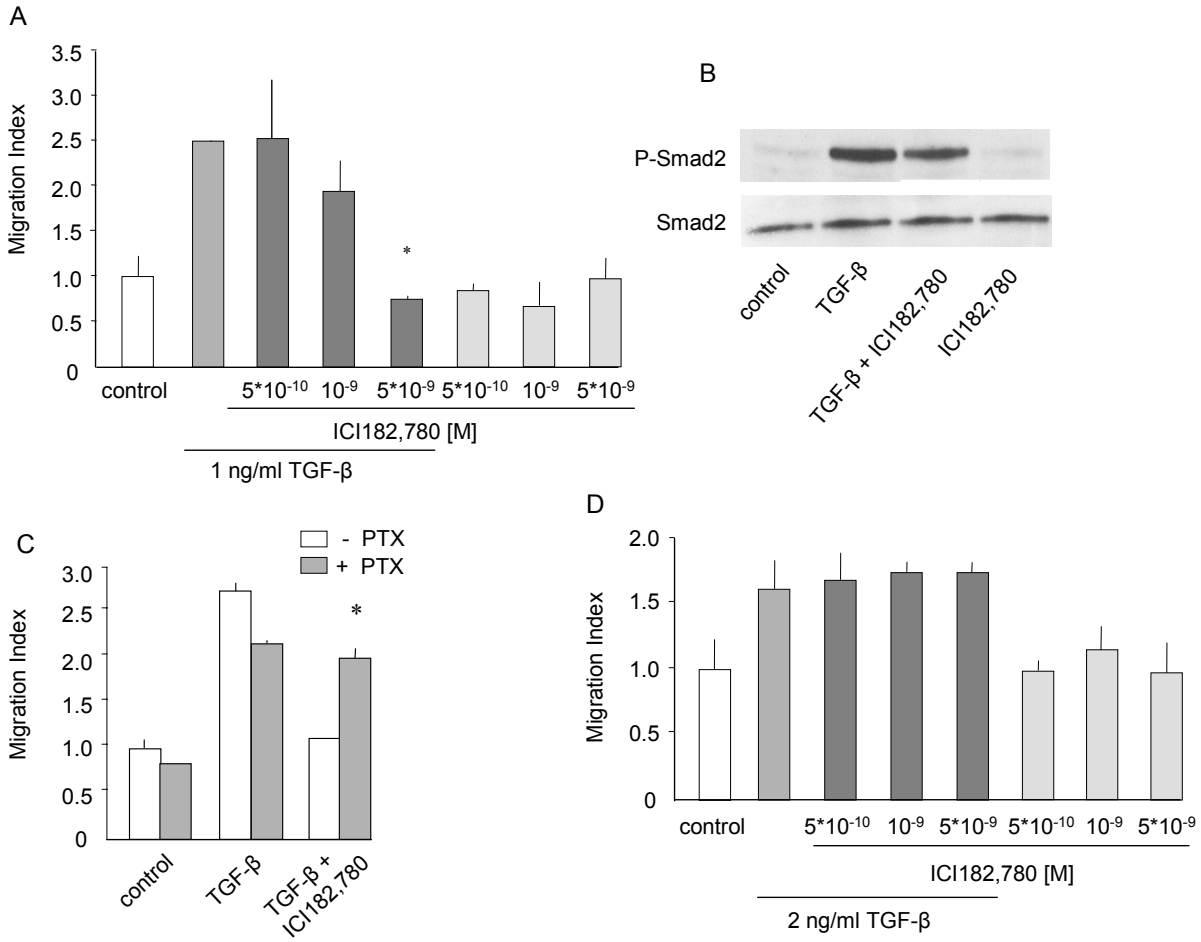

Fig. 32 Effect of short-time sensitisation of breast cancer cells with ICI182,780 on TGF-β signalling. (C) For inhibition of G_i-proteins, MCF-7 cells were incubated with 100 ng/ml PTX for 3 h. MCF-7 (A, B, C) and MDA-MB-231(D) cells were incubated for 30 min with control vehicle or ICI182,780 in the indicated concentrations. (A, C, D) Chemotaxis in response to 1 ng/ml TGF-β was measured in a transwell assay system. Each value represents the average migratory index ± SD from duplicate wells (* $P \leq 0.05$). (B) For detection of Smad2 phosphorylation, MCF-7 cells were incubated with 10^{-9} M of the antiestrogen, stimulated with 0.5 ng/ml TGF-β, each for 30 min, and Western blot analysis using anti-phospho-Smad2 or anti-Smad2 antibodies was performed. All experiments have been performed three times with similar results.

In that case, ICI182,780 displayed similar effects like E2. Thus, TGF-β mediated migration was significantly and dose-dependently suppressed in cells treated with the antiestrogen (Fig. 32A). A significant effect was first detectable at a concentration of 10^{-9} M and complete suppression of migration was achieved at $5*10^{-9}$ M of the agent. ICI182,780 treated MCF-7 cells exhibited no significant migration without a TGF-β

stimulus. As further presented, migration towards a TGF-β stimulus remained unaffected in the ERα and GPR30 deficient MDA-MB-231 cell line (Fig. 32C).

A comparable effect of ICI182,780 was also revealed in the activation of TGF-β dependent pathways, as Smad2 phosphorylation was repressed in an extent comparable to E2 (Fig. 32B). In contradiction to published data (Buck et al., 2004), exclusive treatment with ICI182,780 did not cause a measurable Smad2 phosphorylation in MCF-7 cells.

It was further examined if TAM, a selective estrogen receptor modulator, exhibits similar effects on TGF-β mediated chemotaxis on breast cancer cells. Fig. 33 demonstrates that, comparable to ICI182,780, the migration rates of MCF-7 cells were dose-dependently repressed by a short time treatment with TAM.

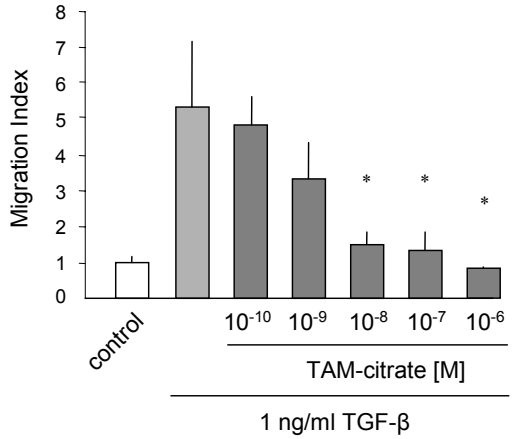

Fig. 33 Effect of short-term sensitisation of MCF-7 cells with TAM on TGF-β migration. Cells were incubated for 30 min with vehicle (control) or TAM-citrate in the indicated concentrations. Chemotaxis in response to 1 ng/ml TGF-β was measured in a transwell assay system. Each value represents the average migratory index ± SD from duplicate wells. Similar results have been achieved from three independent experiments (* $P \leq 0.05$).

3.4.5 The role of GPR30 in the repression of TGF-β migration in E2 treated cells

Several properties of the mechanism underlying the inhibitory effect of E2 on TGF-β induced signalling events could be revealed so far. In this section, the identity of the involved estrogen receptors shall be specified. Although the inhibitory influence of E2 on TGF-β migration could be prevented by an over night incubation with ICI128,780, an exclusive involvement of genomic pathways via ERα and β is not probable, as the hormone effect already appears after 30 min of pre-incubation and, moreover, the

same short-time effect on TGF-β pathways could be documented for ICI182,780 itself.

One probable candidate for the receptor in question is GPR30, since it possesses all required properties, namely membrane association, coupling to G_i-proteins, and responsiveness to both E2 and ICI182,780. Furthermore, GPR30 expression could be proved in MCF-7, but not in MDA-MB-231 cells, which were not responsive to E2 in TGF-β migration assays.

To concretely determine whether E2 caused inhibition of TGF-β migration is associated with signalling via GPR30 in MCF-7 cells, GPR30 expression was suppressed by specific small interfering RNA (siRNA). Immunofluorescence microscopy and Western blot analysis of membrane enriched fractions using GPR30-specific antibodies verified the reduction of its expression after transfection with GPR30-siRNA, whereas GPR30 protein levels of MCF-7 cells treated with a non-coding control-siRNA remained unaffected (Fig. 34A, B). In order to verify the consistence of protein application, all antibodies were removed and the membrane re-assessed for the existence of membrane associated β-Catenin protein. All samples revealed identical β-Catenin expression.

After treatment of transfected cells with E2, motility towards TGF-β and Smad2 phosphorylation were measured. As represented in Fig. 35, E2 failed to inhibit TGF-β induced migration and Smad2 phosphorylation in GPR30 deficient MCF-7 cells, whereas in cells treated with control-siRNA, E2 did not loose its inhibitory properties.

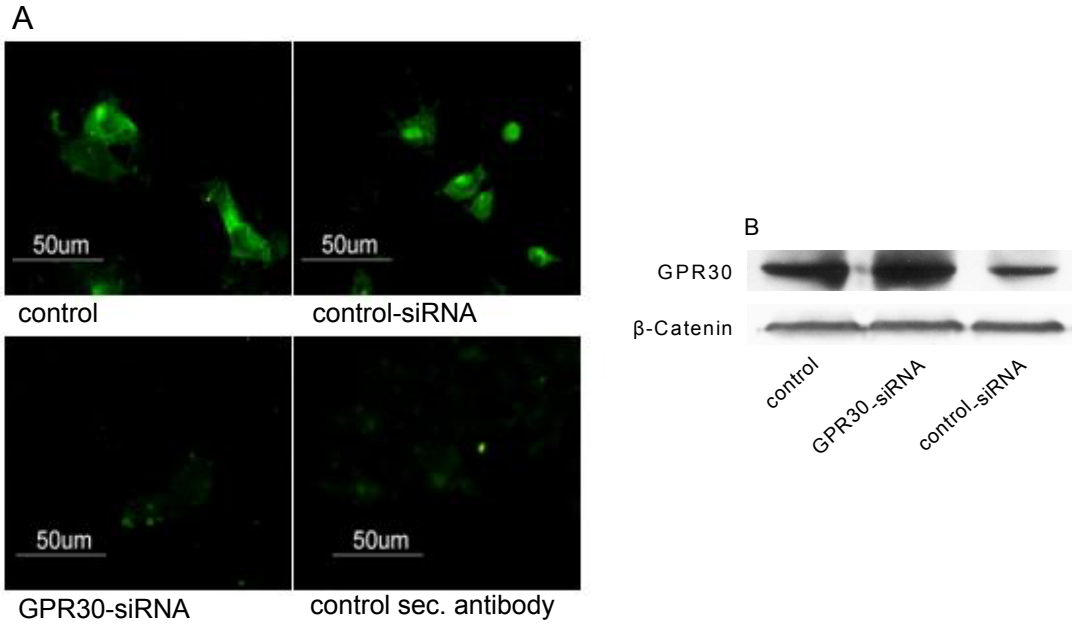

Fig. 34 Detection of GPR30 expression after transfection of MCF-7 cells with GPR30-siRNA. (A) Cells were seeded on 18 mm-coverslips and, when attached, transfected with GPR30-siRNA, non-specific siRNA (control-siRNA), or transfection reagent only (control) for 3 days. Cells were fixed with 3.7 % para-formaldehyde, blocked with 1 % BSA in PBS, and incubated with a rabbit-anti-GPR30 antibody followed by an anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) or secondary antibody only (control sec. antibody). The coverslips were transferred to an object slide and images were taken on the Olympus BX41. (B) MCF-7 cells were transfected with GPR30-siRNA, non-specific siRNA (control-siRNA), or transfection reagent only (control) for 3 days. Membrane enriched fractions were prepared, and 20 µg lysate proteins were separated by SDS-PAGE. Western blot analysis was performed with an anti-GPR30 antibody (upper panel). In order to verify that each sample contains equal amounts of membrane protein the blots were stripped and re-probed with an anti-β-Catenin antibody (lower panel). The data are representative for three independent experiments.

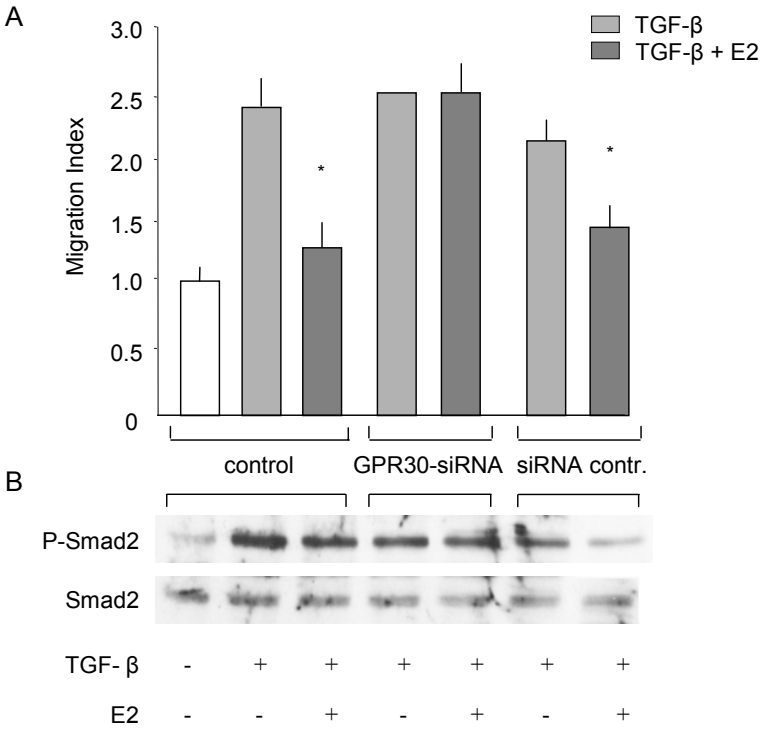

Fig. 35 Involvement of GPR30 in the repression of TGF-β migration and Smad2 phosphorylation in E2 sensitised MCF-7 cells. Cells were transfected with GPR30-siRNA, non-specific siRNA (siRNA contr.), or transfection reagent only (control) for 3 days. (A) After incubation with 10^{-6} M E2 or vehicle, cells were stimulated with 1 ng/ml TGF-β to determine migration in a transwell assay system. The data represent the average migration index of two determinations ± SD (* P ≤ 0.05). (B) Smad2 phosphorylation was determined by Western blot of 15 µg lysate protein after sensitisation with 10^{-6} M E2 and stimulation with 0.5 ng/ml TGF-β. Comparable results have been obtained in three independent tests.

These results indicate proper GPR30 functioning to be a prerequisite for the estrogen effects, observed in this study. This assumption could be further corroborated in the following experiments. GPR30 negative MDA-MB-231 cells were forced to overexpress GPR30 by transfection with the GPR30-cDNA inserted into the pBK-

CMV vector. The expression status after transfection was verified by immunofluorescence microscopy and Western blot as described above. Fig. **36**A and B show a significant increase in protein expression in GPR30 transfected versus non-transfected MDA-MB-231 cells. Supporting the basic outcome of the experiments with GPR30-siRNA transfected MCF-7 cells, the inhibitory properties of E2 on chemotaxis and Smad2 phosphorylation induced by TGF-β could be established in MDA-MB-231 cells by expression of GPR30 (Fig. 37). However, in MDA-MB-231 cells treated with transfection reagent only, no significant effect of E2 was measured.

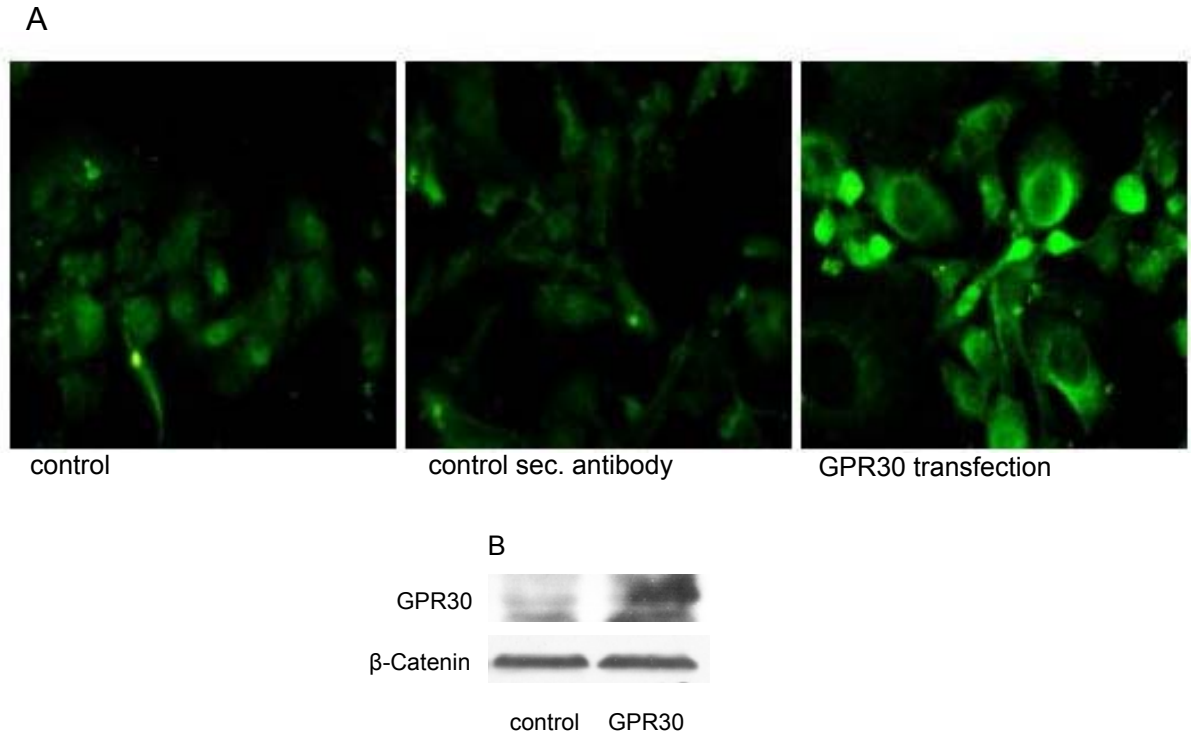

Fig. 36 Detection of GPR30 expression after transfection of MDA-MB-231 cells with a GPR30 expression plasmid. (A) Cells were seeded on 18 mm-coverslips and, when attached, transfected with GPR30 plasmid (GPR30 transfection) or transfection reagent only (control) for 2 days. Cells were fixed with 3.7 % para-formaldehyde, blocked with 1 % BSA in PBS, and incubated with a rabbit-anti-GPR30 antibody followed by a FITC conjugated anti-rabbit antibody or secondary antibody only (control sec. antibody). The coverslips were transferred to an object slide and images were taken on the Olympus BX41. (B) Cells were transfected with GPR30 plasmid DNA (GPR30) or transfection reagent only (control) for 48 h. Membrane fractions were prepared, and 20 µg lysate proteins were separated by SDS-PAGE. Western blot was performed with an anti-GPR30 antibody (upper panel). The blots were stripped and re-probed with an anti-β-Catenin antibody (lower panel).

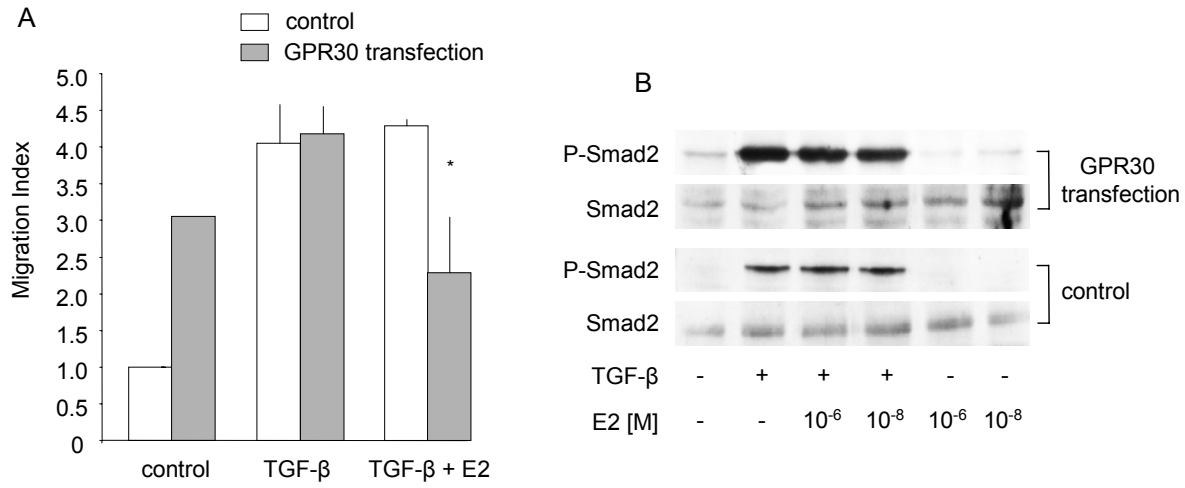

Fig. 37 TGF-β migration and Smad2 phosphorylation in GPR30 positive MDA-MB-231 cells sensitised with E2. Cells were transfected with a GPR30 expression plasmid or transfection reagent only (control). After 2 days, cells were incubated with 10^{-6} M or with the indicated concentrations of E2 or vehicle. (A) Measurement of cell migration towards 1 ng/ml of TGF-β was performed in a transwell assay system. The migratory index is due to two determinations ± SD (* $P \leq 0.05$). (B) For Smad2 phosphorylation, cells were stimulated with 0.5 ng/ml TGF-β, 15 µg lysated proteins were electrophoresed, and Western blot analysis using an anti-phospho-Smad2 or anti-Smad2 antibody was carried out. These experiments have been repeated two more times with similar results.

3.4.6 Participation of MAPK signalling in the down-regulation of TGF-β responses by E2

A broad range of studies exists, demonstrating that estrogen and MAPK signalling are bidirectionally cross-linked. The underlying mechanisms, however, are very complex and not completely clarified yet. On the one hand, the Raf-to-ERK pathway is rapidly activated in response to E2, which has been proven for MCF-7 cells in divers studies (Filardo et al., 2000; Imamichi et al., 2005; Migliaccio et al., 2002; Zivadinovic and Watson, 2005). On the other hand, activation of ERK1/2 by RTKs after growth factor binding is accompanied by transcriptional regulation of estrogen responsive genes via phosphorylation of ERα in its AF-1 domain (El-Tanani and Green, 1997; Kato et al., 1995) or by direct recruitment of transcription factors (Duan et al., 1998; Dubik and Shiu, 1992; Umayahara et al., 1994).

As estrogen induced chemotaxis is demonstrably dependent on the activation of ERK1/2, an involvement of MAPK signalling in the desensitisation of MCF-7 cells for the migratory stimulus of TGF-β can be assumed. In agreement with divers publications (Filardo et al., 2000; Imamichi et al., 2005; Migliaccio et al., 2002; Zivadinovic and Watson, 2005), Fig. 38 shows a concentration dependent

phosphorylation of ERK1/2 after stimulation with E2 and ICI182,780 in the MCF-7 cell line. Both steroids were most effective at low concentrations with a maximum activity at 10^{-8} M of E2 and $5*10^{-11}$ M of ICI182,780.

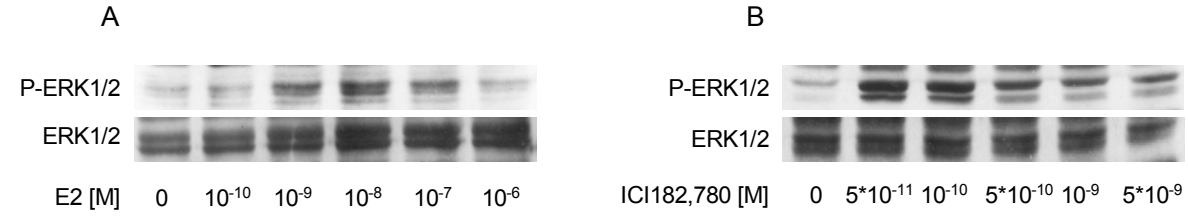

Fig. 38 Detection of ERK1/2 phosphorylation by E2 and ICI182,780 in MCF-7 cells. Cells were stimulated for 10 min with control vehicle, E2, or ICI182,780 in the indicated concentrations, and after preparation of electrophoresis and Western blot, activated and total ERK1/2 proteins were detected with an anti-phospho-p44/42 antibody or an anti-p44/42 antibody, respectively. Three independent experiments revealed comparable results.

It has further been shown in this study that GPR30 is a key player in the processes leading to E2 induced inhibition of MCF-7 migration in response to TGF-β. Divers studies revealed the role of ERK1/2 activation as a crucial event in signalling pathways initiated via GPR30 (Levin, 2003; Zivadinovic and Watson, 2005), and Filardo et al. demonstrated the requirement of GPR30 for ERK1/2 phosphorylation by E2 and ICI182,780 (Filardo et al., 2000). Consistently, in MDA-MB-231 cells overexpressing GPR30, ERK1/2 activation by E2 could be measured in comparison to non-transfected cells, proving the functional capability of the exogenous receptor (Fig. 39).

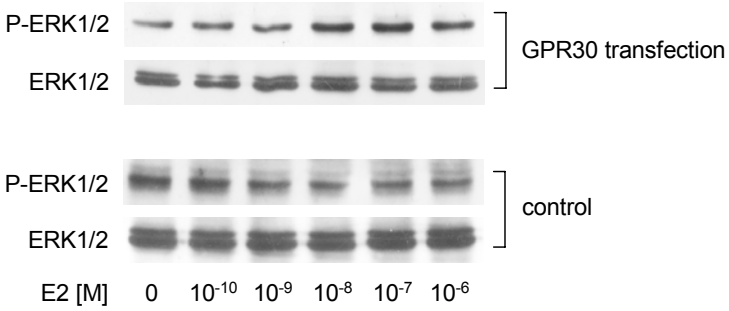

Fig. 39 Detection of ERK1/2 phosphorylation by E2 in GPR30 transfected versus original MDA-MB-231 cells. Cells were transfected with GPR30 plasmid or transfection reagent only (control) for 2 days and stimulated for 10 min with control vehicle or E2 in the indicated concentrations. Electrophoresis and Western blot were performed and phosphorylated as well as total ERK1/2 proteins were detected with an anti-phospho-p44/42 antibody or an anti-p44/42 antibody, respectively. Similar results have been obtained in two more experiments.

To determine if ERK1/2 phosphorylation is essential for the repression of TGF-β migration by E2, MCF-7 cells were pre-incubated with PD098,059. Western blot analysis proved the capability of PD098,059 to prevent ERK1/2 phosphorylation by E2 (Fig. 40B). As presented in Fig. 40A, treatment with the inhibitor completely impeded the down-regulation of TGF-β induced migration by E2.

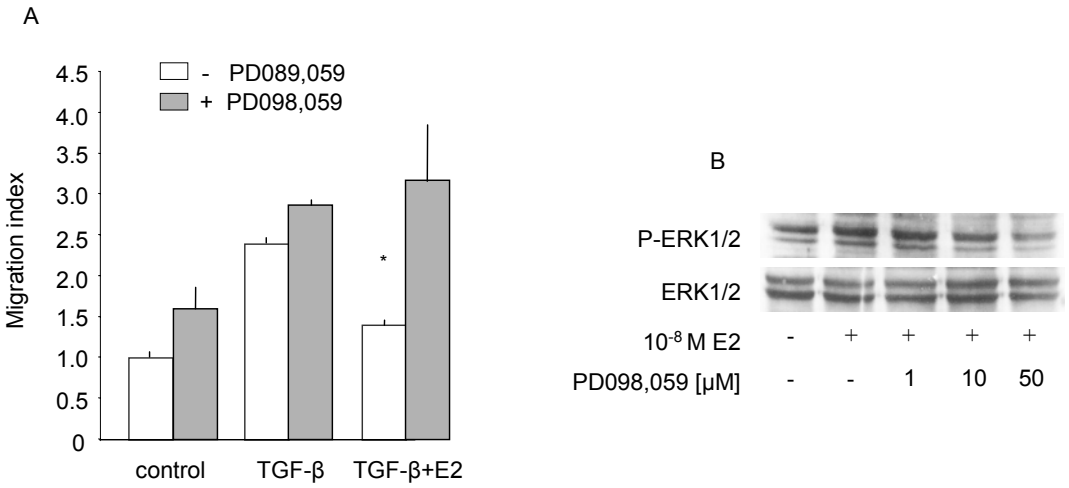

Fig. 40 Involvement of ERK1/2 phosphorylation in the inhibitory effect of E2 on TGF-β induced chemotaxis of MCF-7 cells. (A) Cells were pre-treated with 50 µM PD098,059 or vehicle for 90 min and then sensitised with 10^{-6} M E2 for 30 min. The migratory response towards TGF-β was measured in a transwell assay system and is expressed as the median migratory index ± SD of two wells (* $P \leq 0.05$). (B) To verify the efficacy of PD098,059 to prevent ERK1/2 phosphorylation, cells were treated for 90 min with the MAPK kinase inhibitor in the indicated concentrations and activated as well as total ERK1/2 were measured by Western blot utilising an anti-phospho-p44/42 antibody or an anti-p44/42 antibody. The experiments have been repeated at least two times with similar results.

These results demonstrate that ERK1/2 activation is a critical event in the inhibition of TGF-β induced migration by E2. To clarify more concretely which role MAPKs inhere in the disruption of TGF-β signalling, it was further tested, how ERK1/2 phosphorylation is affected by a costimulation with TGF-β and E2. For that purpose MCF-7 cells were sensitised with 10^{-6} M E2, the concentration that was most effective in repression of TGF-β migration, or 10^{-9} M E2, which had almost no inhibitory effect (see Fig. 15), followed by a stimulation with TGF-β. Although single doses of 10^{-6} M E2 and TGF-β induced only weak ERK1/2 phosphorylation, sensitisation with 10^{-6} M E2 and subsequent stimulation with TGF-β caused a strong ERK1/2 activation, which appeared to be more than additive (Fig. 41). In contrast, the intense ERK1/2 phosphorylation achieved by 10^{-9} M E2 could not be further enhanced by TGF-β.

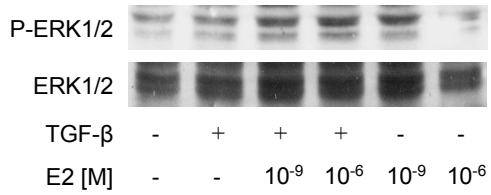

Fig. 41 Detection of ERK1/2 phosphorylation after stimulation with TGF-β in E2 sensitised MCF-7 cells. After 30 min pre-treatment with control vehicle or E2 in the indicated concentrations, cells were stimulated for 30 min with vehicle or 0.5 ng/ml TGF-β. SDS-PAGE and Western blot were performed and phosphorylated as well as total ERK1/2 proteins were detected by use of an anti-phospho-p44/42 antibody or an anti-p44/42 antibody, respectively. Two more experiments achieved comparable results.