Discussion

4 Discussion

4.1 Chemotactic properties of E2 in MCF-7 cells

Estrogens regulate cell proliferation, differentiation, and apoptosis and therefore they possess a central role not only in normal mammary gland cells, but also in breast cancer cells (Couse and Korach, 1999; Mangelsdorf et al., 1995). The motility and invasiveness of cancerous cells are important indications of their metastatic potential. In the present study an *in vitro* transwell migration assay, based on the chemokine induced movement of cells through a polycarbonate membrane, was used to determine the migratory potency of breast cancer cells.

In the literature it is controversially discussed whether, besides their impact on the cell cycle, estrogens also contribute to tumour progression by supporting cell motility and invasiveness leading to metastatic distribution. Several studies investigated the migratory potential of cells after treatment with estrogens, but the results vary considerably according to cell types. Comparable to the outcome of the present study, E2 has previously been recognised to affect cell migration in some model systems. Thus, it promotes invasiveness of endometrial cancer cells to the interstitium (Fujimoto et al., 1995) and the passage through the basement membrane (Fujimoto et al., 1996) as well as motility of endothelial cells (Razandi et al., 2000). Stimulation of MCF-7 cells with E2 enhances the ability of these cells to attach to a laminin substrate, to migrate towards laminin, and to pass through a reconstituted basement membrane, probably caused by an increase in laminin receptor expression (Albini et al., 1986). The small TFF1 protein (trefoil factor family 1), expressed and secreted in the mucosa of the gastrointestinal tract and by malignant ER positive breast epithelial cells in response to E2, stimulates the invasion and chemotaxis of MCF-7 cells (Prest et al., 2002). Furthermore, in recent experiments with estrogen treated MCF-7 cells Saji et al. observed an enhanced expression of histone deacetylase 6 (HDAC6) catalysing the deacetylation of α-tubulin and in turn increasing cell movement measured by a transwell migration assay (Saji et al., 2005). In these studies estrogens induced migration is associated with translational processes and *de novo* protein synthesis. The major difference to the presented results relies on the considerably longer period of stimulation with E2, which lasted several days.

In contrast to the chemotatic properties of E2, undirected hormone induced movement of MCF-7 cells has not been investigated so far. Compensation of the concentration gradient of E2 by adding the estrogen to the upper chamber allows the determination of chemokinetic effects. The results demonstrate that the role of chemokinesis in E2 induced migration is negligible.

In the following experiments, the role of ERs in mediation of E2 induced migration has been investigated. No movement was achieved with MDA-MB-231 cells, which represents an estrogen independent, highly metastatic breast cancer cell line. Evidently, migration towards E2 is associated with ER expression. This assumption could be substantiated via blocking ER signalling in MCF-7 cells by the pure antiestrogenic compound ICI182,780.

The cell migration process in general is extremely complex. The underlying cellular mechanisms of the motility inducing effect of E2 are cell type specific and remain to be completely elucidated. In endometrial cells E2 induced migration is preceded by the activation of Src and FAK signalling pathways as well as structural changes in the actin cytoskeleton (Acconcia et al., 2006). The motility and invasiveness of cancerous cells has been compared with the movement of cells into a healing wound. Indeed, several models clearly indicate that estrogens promote the healing process in skin by regulating inflammation, proliferation, and remodelling (Ashcroft and Ashworth, 2003; Calvin, 2000). Furthermore, they increase the closure of wounded confluent cultures of the MCF-7 cell line (Meng et al., 2000). It was proposed that the promoting effect of estrogens on wound healing is associated with a secretion of TGF-β by dermal fibroblasts (Ashcroft et al., 1997). Since treatment of normal human breast epithelial as well as MCF-7 cells with E2 does not alter or even inhibit secretion of TGF-β (Arrick et al., 1990; Malet et al., 2001), it can be excluded that autocrine and paracrine effects of newly synthesised chemokine accounts for the migratory property of E2 observed in the present study.

Frequently, activation of the ERK1/2-MAPK pathway is an integral event in the induction of cell motility. In breast cancer cell lines, among these MCF-7 cells, magnitude and duration of ERK1/2 activity has been shown to modulate cell migration (Krueger et al., 2001). Experiments with PD098,059, an inhibitor of the MAPK kinase MEK, responsible for the phosphorylation of ERK1/2, confirmed that MCF-7 cell migration in response to E2 depends on proper ERK1/2 activation. Even though Acconia et al. demonstrated that E2 triggers rapid ERK1/2, Src, and FAK

activation as well as remodelling of filamentous actin cytoskeleton, which is a crucial process in cell migration, the authors owe a concrete proof for a correlation between MAPK activation and cell migration (Acconcia et al., 2006).

4.2 Chemotactic properties of TGF-β in MCF-7 cells

One aspect of tumour progression promoted by estrogens is their ability to negatively modulate the expression or functional activity of tumour suppressors. TGF-β, which is secreted by almost all cells, has been identified as a prominent member of these opponents, as it potently reduces tumour cell proliferation of epithelial origin (Sun et al., 1994) and stimulates apoptosis of MCF-7 cells (Ciftci et al., 2003; Hishikawa et al., 1999). However, in later phases of tumorigenesis TGF-β also facilitates progression of cancer development, since it enhances the invasive properties of tumour cells, promotes angiogenesis, and suppresses the immune system (Miyazono et al., 2003; Wakefield and Roberts, 2002). Furthermore, a few studies suggest that TGF-β is implicated in antiestrogen resistance and independence of estrogens (Arteaga et al., 1993; Arteaga et al., 1999; Daly et al., 1990; Herman and Katzenellenbogen, 1994; Thompson et al., 1991).

In accordance with several studies, the presented results indicate a potent migratory response of ER dependent MCF-7 and ER independent MDA-MB-231 cells to TGF-β that may contribute to the invasive role of the cytokine (Muraoka-Cook et al., 2005; Muraoka et al., 2002; Tong et al., 2002). In this context, it should be considered that, MCF-7, compared with MDA-MB-231 cells, are characterised by a great quantity of adhesive junctions, which is reflected in lower basal migration rates.

The ability of TGF-β to increase motility of cells is mainly based on the induction of actin polymerisation and expression of ECM proteins and ECM degrading MMPs. TGF-β induces secretion of fibronectin and tenascin (Walker et al., 1994), which in turn lead to increased expression of MMP-9 in MDA-MB-231 cells (Kalembeyi et al., 2003). In MCF-10A breast cancer cells, TGF-β promotes MMP-2 and MMP-9 expression (Kim et al., 2004). While growth arrest of epithelial cells stimulated with TGF-β is well characterised and could be traced back to Smad signalling, the molecular mechanisms that lead to TGF-β migration are largely unknown. The involvement of Smad proteins, especially Smad3, in TGF-β induced chemotaxis has

been reported in a variety of cells (Ashcroft et al., 1999; Roberts et al., 2003; Tian et al., 2003). In agreement with these studies, also MCF-7 cells receive their migratory signal from TGF-β via a Smad dependent pathway, as they were unresponsive when the Co-Smad, Smad4, was abrogated by use of RNA interference technique. However, TGF-β did not loose its migratory property completely, which could be due to an incomplete repression of Smad4 expression. More likely other, Smad independent, pathways participate in TGF-β induced migration of MCF-7, such as activation of small Rho-GTPases, RhoA, Rac, and Cdc42, as well as MAPKs.

Imamichi et al. identified ERK, JNK, and RhoA to be substantially involved in TGF-β induced migration of MCF-7 cells. Activation of these pathways appears to be independent of ALK5 to a large extent, since inhibition of TβRI only slightly decreased activation of these effects. The authors further propose that, in contrast to the findings of the present study, these TGF-β processes occur independently of Smad4 signalling, since actin reorganisation and migration was not impaired in the Smad4 deficient breast cancer cell line MDA-MB-468 (Imamichi et al., 2005). Several studies provide further evidence for a contribution of MAPKs and GTPases in the migration promoting effects of TGF-β (Huang et al., 2004; Mulder, 2000; Wakefield and Roberts, 2002).

4.3 A role of estrogens in TGF-β induced signal transduction pathways

4.3.1 Inhibition of TGF-β induced migration of MCF-7 cells

Comparative experiments with hormone dependent and independent cell lines suggested that the expression level of ERs are inversely correlated with cell motility and that estrogens decrease the *in vitro* invasiveness and migration of breast and ovarian cancer cells (Garcia et al., 1992; Garcia et al., 1997; Platet et al., 2000). ERα transfected into MDA-MB-231 cells reduced matrigel invasiveness by threefold in the absence of hormone and by sevenfold in its presence (Platet et al., 2000). These studies investigated either passive motility or migration towards unspecific stimuli of fibroblast conditioned medium or FCS. A decreased risk of tumour invasion is in accordance with epidemiological studies about breast cancer in women under hormone replacement therapy .

On account of the tumour promoting properties of TGF-β and since a broad range of physiological processes are controlled by a cross-communication between estrogens and growth factors, it has been of great interest whether estrogens and ERs also exert antimetastatic action on TGF-β induced chemotaxis. The findings reveal a bimodal effect of estrogens on migration of MCF-7 cells. While a gradient of estrogens promotes targeted cell movement, which can be further enhanced by a simultaneous TGF-β stimulus, direct exposition of MCF-7 cells to estrogens resulted in a loss of responsiveness to the migratory stimulus of the growth factor. In contradiction to previously cited studies, this effect was specific for the cytokine, as the chemotactic ability of other serum contained chemoattractants was not affected. These effects were only seen with the activated form of ERs, since desensitisation of ERs with ICI182,780 almost completely abolished the repressive effect of estrogens, suggesting hormone receptors to interfere with TGF-β signalling.

A negative impact of estrogens has been reported for other growth factors and ECM proteins, as exemplified by the attenuation of female rat vascular smooth muscle cell migration induced by platelet-derived growth factor (PDGF), IGF-1, and fibronectin (Kolodgie et al., 1996). Similarly, it decreases PDGF mediated migration of porcine aortic endothelial cells, whereas motility of porcine smooth muscle cells is enhanced (Geraldes et al., 2002).

These findings indicate that estrogens may have a dual effect on tumour fate in ER positive breast cancers. On the one hand they demonstrably stimulate tumour growth, but on the other hand they might inhibit the development of metastasis from a solid tumour.

4.3.2 Inhibition of the Smad signal transduction pathway

As the migratory response of MCF-7 cells to TGF-β has been proved to depend on proper function of the Smad signalling cascade, it seems likely that estrogens interfere with TGF-β signalling on the level of Smad proteins. Consistently, sensitisation of MCF-7 cells by estrogens resulted in a functional inactivation of Smad proteins that is reflected in a loss of TGF-β induced Smad phosphorylation, Smad complex formation, and reporter gene activation.

Interplay between Smads and several intracellular proteins has been documented, which may result in both a functional activation and a repression of Smad signalling. It is of interest that members of the steroid receptor family can influence the TGF-β pathway positively or negatively to impact a variety of physiological or pathological processes. ARs were identified to directly bind to Smad3, which results in a functional activation (Kang et al., 2002), whereas this Smad protein represses the transcriptional activity of the steroid receptor (Hayes et al., 2001). In analogy, a fortified transcription was reported for the vitamin D receptor due to its interaction with Smad3 (Yanagisawa et al., 1999). On the contrary, a legation of Smad3 and a subsequent suppression of TGF-β signalling has been observed in the presence of activated glucocorticoid receptors (Song et al., 1999). In mesangial cells, E2 reverses TGF-β stimulated transcription of the gene, encoding the α_1-chain of collagen type IV, and type IV collagen synthesis on a transcriptional level, presumably via interaction with the transcription factor SP-1 (Silbiger et al., 1998). Moreover, novel molecular mechanisms have been reported whereby estrogens inhibit TGF-β function via direct physical and functional interactions between Smad3 (Matsuda et al., 2001) or Smad4 (Wu et al., 2003) and ERs with bidirectional regulation. It should also be mentioned that estrogens not only impair TGF-β responses, but also those of the related family of BMPs. Thus, BMP induced activation of Smad1 and the resulting transcription were repressed by E2 in breast cancer and mesangial cells (Yamamoto et al., 2002).

4.4 Mechanisms underlying the cross-talk between E2 and TGF-β induced pathways

4.4.1 Involvement of nuclear ERs

Estrogenic effects are mediated by various pathways, which are part of an extremely complex signalling network. Further experiments were performed with the objective to specify, which of the pathways are causally involved in the inhibition of TGF-β mediated chemotaxis of E2 sensitised MCF-7 cells. Toxic effects of E2 that might impair cell viability and therefore their efficiency of reacting to the TGF-β stimulus could be excluded by a cytotoxicity assay.

The lack of responsiveness of ER negative MDA-MB-231 versus MCF-7 cells, which are, in contrast, referred to as ER positive on account of the high expression levels of

nuclear ERs, rather directs attention to a receptor mediated effect. With respect to the time pattern of regulation, E2 induced genes can be subdivided into three groups: early stimulated genes (4 - 8 h), early and late stimulated genes (4 – 48 h), and late stimulated genes (24 – 48 h). In the majority of cases, gene expression by E2 is a middle- and long-term process. Time course experiments with U2OS human osteosarcoma cells revealed that, among the genes observed, 95 % of those induced by ERα and 84 % of those induced by ERβ belong to category two or three (Stossi et al., 2004). In consideration of the fact that inhibition of MCF-7 migration after 30 min of pre-treatment with E2 occurs to the same extent as after 19 h of incubation, it appears likely that gene expression is not involved.

In addition to the classical genomic function of estrogens through nuclear ERs, these receptors have been implicated in various alternative processes, not involving transcriptional regulation of target genes. Thus, downstream target gene transcription by estrogens is interrupted when ERα cooperates with Smad4, identifying Smad4 as a corepressor for ERα (Wu et al., 2003). Smad4 and ERα form a complex, when the steroid receptor binds to the ERE within a target gene promoter of breast cancer cells. Mapping of the involved interaction motifs indicates that the AF-1 domain of ERα and the MH1 and linker domain of the Smad protein are essential for their interaction. Moreover, ERs have been described to act as transcriptional corepressors of Smad3. ERα as well as ERβ possess a binding motif for the MH2 domain of Smad3, mediating a physical interaction and repression of Smad3 reporter gene activity, whereas activation of ER responsive promoters is enhanced (Matsuda et al., 2001). The present study could provide supplementing data showing that repression of Smad activity already occurs on the level of protein phosphorylation and complex formation between R- and Co-Smads. In consistence with another study (Cherlet, 2002), coimmunoprecipitation experiments could not corroborate the assumption of a direct binding of Smad1, 2, or 3 proteins to ERα.

4.4.2 Involvement of membrane localised ERs

Non-transcriptional estrogenic pathways not only proceed through intracellular receptors. In fact, in recent years a considerable number of studies related rapid estrogen effects to membrane associated receptors, initiating divers signalling

cascades via MAPK, adenylyl cyclase, PI3K, and Akt activation as well as calcium mobilisation. The impact of E2 on the migratory response of MCF-7 cells to TGF-β is not altered when the permeation of the hormone through the plasma membrane is prevented by conjugation to BSA. This result leads to the assumption that these novel membrane located estrogen responsive receptors participate in the repression of TGF-β migration and additionally excludes non-transcriptional effects of intracellular ERs. The ability of estrogens to suppress cancer cell migration through a fibronectin coated membrane in a transwell assay has been referred to an expression of fibulin-1 and its attachment to fibronectin (Hayashido et al., 1998; Rochefort et al., 1998). Different from the results presented in this study, this effect required transcriptional activity of nuclear ERs and an E2 incubation time up to seven days.

It should be considered that, although E2, covalently linked to BSA, is a widely used means to study estrogen effects mediated through membrane receptors, controversy is kept in literature regarding its usability. In several studies, the use of these conjugates as surrogates for estrogens has been queried (De Goeij et al., 1986; Stevis et al., 1999). Doubts have been further supported by a study, proofing commercially available BSA-E2 to be contaminated by free steroid. In contrast to E2, BSA-E2 preparation relieved of free E2 by ultrafiltration exhibited no binding affinity for either ERα or ERβ in that study (Stevis et al., 1999). For the BSA-E2 used in the present study, the manufacturers indicated the content of non-conjugated E2 to be less than 2 %. In order to eliminate potential effects due to remaining traces of free hormone, the latter was treated with dextran coated charcoal. Measurement of ERE activation by charcoal treated BSA-E2 proved that the agent is devoid of free E2. Unconjugated E2 is demonstrably irrelevant for the repressive property of BSA-E2 on TGF-β migration. A larger number of studies, however, recommend the use of BSA-E2 (Berthois et al., 1986; Qiu et al., 2003; Razandi et al., 1999; Tassignon et al., 1997). Taguchi et al. demonstrated that BSA-E2 binds to purified estrogen receptors *in vitro* and to cell membranes of intact cells (Taguchi et al., 2004). In uterine artery endothelial cells E2 as well as BSA-E2 caused rapid phosphorylation of endothelial nitric oxide synthase (eNOS), and binding of FITC-tagged BSA-E2 provided evidence for the presence of ERs in the plasma membranes of these cells (Chen et al., 2004).

It is remarkable that, in contrast to unconjugated E2, BSA-E2 appears to be more effective in smaller concentrations. In the literature opposite opinions about the

efficiency of BSA-E2 result from different findings. On the one hand Chen et al. also found 10^{-8} M BSA-E2 to be more effective than 10^{-8} M E2 in total NO production (Chen et al., 2004). Since the large protein complex sterically prevents diffusion through the membrane, the whole applied amount of estradiol is available for receptor activation. This could result in a reduction of the effective concentration. On the other hand a lower affinity of BSA-E2 in binding to putative mERs in comparison to free E2 has been shown by other investigators (Morey et al., 1997; Pappas et al., 1995).

Until today mERs have been identified in divers cell types (Table 1). Several concepts about the nature of these receptors exist among investigators. Most studies refer to either receptors that are related to the classical nuclear types, ERα and ERβ, or to an orphan estrogen responsive receptor, GPR30. Reverse transcriptase PCR analysis confirmed the existence of GPR30 and ERα mRNA in MCF-7 cells, whereas no detectable amounts of transcripts could be measured in MDA-MB-231 cells.
Although the coexistence of non-related mERs in a single type of cell has not been proven yet, it should not be foreclosed, since in MCF-7 cells mERα (Pedram et al., 2006; Razandi et al., 2004; Zhang et al., 2002; Zivadinovic and Watson, 2005) and small amounts of mERβ (Pedram et al., 2006) and, according to other authors, GPR30 is expressed (Carmeci et al., 1997; Filardo, 2002; Filardo et al., 2000; Filardo et al., 2002; Sukocheva et al., 2006; Thomas et al., 2005). The expression profile for mERs in MCF-7 therefore may not allow drawing conclusions about their involvement in the interference with the TGF-β signalling cascade.

The majority of studies defining mERs reported that they are capable and dependent on activation of guanosine triphosphate (GTP)-binding proteins (G-proteins). In consistence, E2 failed to inhibit TGF-β induced migration and Smad activation in the presence of PTX. PTX is produced by the bacterium Bordetella pertussis and catalyses adenosinediphosphate (ADP) ribosylation of the α-subunit of Gi-proteins, which thereby retain their bound guanosine diphosphate (GDP) and lose the ability to regulate target molecules.
The family of G-proteins consists of various types, each specific for particular downstream targets. Stimulatory G-proteins (G_s-proteins) activate calcium channels

and the adenylyl cyclase, thereby initiating the production of the small intracellular mediator cAMP and cAMP-dependent PKA, whereas inhibitory G-proteins (G_i-proteins) suppress the induction of the enzyme and activate potassium channels. G_q-protein signalling results in activation of PKC and in turn the inositol phospholipid pathway. Additional examples for G-protein family members are G_0, $G_{12/13}$, G_t, and G_{olf}. G-proteins are heterotrimeric structures composed of an α-, β-, and γ-subunit. Mostly, the stimulating or inhibiting influence of G-proteins on adenylyl cyclase depends on the α-subunit. G-proteins are attached to the cytoplasmic site of the cell membrane, where they functionally couple the receptors to enzymes or ion channels in the membrane. It should be considered that neither for membrane ERα and ERβ, nor for GPR30 a common opinion about the type of the associated G-protein exists. In addition to G_i-proteins, mERs have been associated with proteins of the G_q- and G_s-type. In Chinese hamster ovary cells, membrane ERα and ERβ activate $G\alpha_q$- and $G\alpha_s$-proteins (Razandi et al., 1999; Razandi et al., 2004), whereas in MCF-7 cells $G\alpha_q$- and $G\alpha_i$-proteins (Razandi et al., 2003) and in COS7 cells $G\alpha_i$-proteins are involved (Wyckoff et al., 2001). GPR30 causes ERK1/2 activation via EGFR trans-activation in a PTX-sensitive manner (Filardo et al., 2000; Maggiolini et al., 2004; Sukocheva et al., 2006; Sukocheva et al., 2003). This activation can be attenuated by a cAMP and CTX (cholera toxin) dependent inactivation of Raf-1, which is consistent with the activation of a stimulatory G-protein (Filardo et al., 2002; Thomas et al., 2005).

Principally, GPCRs interact with exclusively one type of G-protein at a definite time, but it is also known that some GPCRs are not consistent in their preference for a specific type of G-protein. Although coupled to G_s-proteins and adenylylcylase, the β_2-adreneric receptor raises MAPK activity. This contradiction has been implicated with distinct components for adenylylcyclase and MAPK activation, on the one hand the β/γ-subunit of a G_i-protein, stimulating the Ras to MAPK pathway and on the other hand the $G\alpha_s$-subunit, inhibiting MAPK activation via induction of cAMP and PKA (Crespo et al., 1995). The alternative use of G_i-proteins may probably be traced back to a feedback mechanism of activated PKA or their localisation in membrane caveolae (Daaka et al., 1997; Hasseldine et al., 2003; Kilts et al., 2000; Xiang et al., 2002).

Similar observations suggest a shift from a G_s- to a PTX-sensitive G_i-protein of adrenomedullin in ventricular myocytes (Mittra and Bourreau, 2006). Furthermore, an agonist concentration depending switch between G_q-, G_s-, and G_i-protein coupling is discussed, albeit controversially, for the guanotropin releasing hormone receptor in hypothalamic cells (Grosse et al., 2000; Kaiser et al., 1997; Krsmanovic et al., 2003). Cooperative signalling by multiple G-proteins has likewise been reported for HEK293T cells, where activation of the ERK1/2 cascade by the bradykinin B(2) receptor requires a cooperation of $G\alpha_i$- and G_q-signalling (Blaukat et al., 2000).

In consideration of the mechanistic diversity of ERK1/2 modulation, mERs may also be subdued to G-protein switching. It has been suggested that mERs resemble the β_2-adreneric receptor –like pathway of MAPK regulation, involving G_s/G_i-switching (Fig. 42) (Belcher et al., 2005; Filardo et al., 2002). However, this question has not been concretely investigated yet.

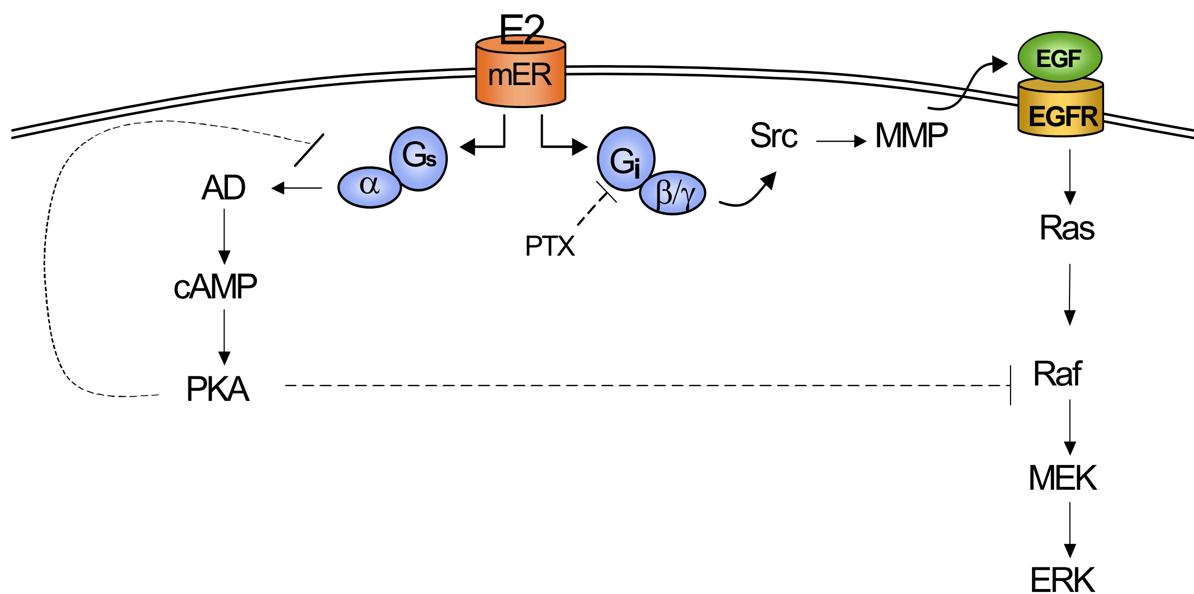

Fig. 42 Proposed mechanism of ERK1/2 regulation by mERs. Data from various investigators suggest that estrogens and antiestrogens, acting via $G\alpha_s$, stimulate adenylyl cyclase (AD), cAMP release, and protein kinase A (PKA), which in turn blocks the Raf-to-ERK pathway and triggers receptor coupling to G_i-proteins. Thereby, the β/γ-subunit induces the tyrosine kinase Src, which promotes matrix metalloproteinase (MMP) dependent cleavage of heparan bound EGF (HB-EGF) from the cell surface and thus the transactivation of EGF receptor (EGFR). EGFR enhances ERK1/2 activity via Ras, Raf, and the MAPK kinase MEK. (modified according to Belcher et al., 2005; Filardo, 2002)

4.4.3 Involvement of GPR30

Further migration experiments demonstrated that the intrinsically oppositional agents ICI182,780 and E2 may function in equal mode. Thus, 30 min pre-incubation with ICI182,780 reduced migration and Smad2 phosphorylation of MCF-7, but not MDA-MB-231 cells, in response to TGF-β. These discoveries are noticeable, given the fact that this compound has always been considered a pure antagonist of estrogen function. This finding is in consistence with other studies, which, for example, reveal that progesterone and its antihormone RU486 act via membrane localised receptors to initiate similar rapid responses (Ehring et al., 1998). Moreover, an inhibition of TGF-β mediated chemotaxis has also been achieved with TAM. It should be mentioned that the antimetastatic potential of antiestrogens has been examined, but appeared to differ depending on the type of antiestrogen and cells. The pure antiestrogen ICI182,780 was found to inhibit the migratory effect of TGF-β on MCF-7 cells in an *in vitro* wound model, whereas TAM even enhanced MCF-7 and MDA-MB-231 cell movement into a wounded area of the cell monolayer (Mathew et al., 1997; Thompson et al., 1988). These findings are partially supported by Tong et al., who proved pure antiestrogens capable of reversing the migration promoting effect of TGF-β in MCF-7, but not MDA-MB-231 cells. TAM also slightly decreased MCF-7 migration, but was not effective on MDA-MB-231 cells (Tong et al., 2002). In these studies, an inhibition of TGF-β induced migration only occurred when cells were treated with ER antagonists over a time period of three to four days. In contrast, other published data delineate that an almost coeval length of antiestrogen treatment of MCF-7 cells induces sequential activation of p38 MAPK, transcription of TGF-β2 and TβRII genes, and Smad dependent promoter activation, a function which the authors discussed to contribute to the antiproliferative effect of the drug (Buck et al., 2004). Such a long-time treatment, however, almost excludes a direct physical interaction, but instead argues for an unspecific action of antiestrogens on the physiological effects of TGF-β.

Since both E2 and ICI182,780 impaired TGF-β induced migration through a PTX-sensitive mechanism, a common causal mode of action should be considered. GPR30 has been proved to be liganded by ICI182,780 and TAM acting as agonists in an estrogen like manner (Thomas et al., 2005). By this means, ICI182,780 mimics estrogenic actions in a broad range of targets. Stimulation with the antiestrogen, for

example, causes GPR30 dependent cAMP release in membranes from SKBR3 cells (Thomas et al., 2005) and MDA-MB-231 cells transfected with GPR30 (Filardo et al., 2002), a rapid increase in ERK1/2 phosphorylation in these and the MCF-7 cell line (Filardo et al., 2000), and a release of calcium from intracellular stores in MCF-7 cells (Improta-Brears et al., 1999). These cellular responses appear, similar to the interruption of the TGF-β signalling cascade, within minutes of stimulation. Western blot analysis clearly proved that neither time nor dose of ICI182,780 application used in these experiments are sufficient to modify the ERα content, fortifying the theory of mERs as mediators for these effects. This result is consistent with several studies examining the efficiency of the antihormone, as exemplified by Long et al., who detected a degradation of ERα after immobilisation and accumulation in the nuclear matrix by ICI182,780 not until 60 min of exposure to the antiestrogen (Long and Nephew, 2006). Furthermore, no downregulation of ERα protein has been measured after 40 min treatment with the structurally related ICI187,634 (Bulayeva et al., 2004). Investigations regarding the concentration of ICI182,780 required to block the ER signalling pathway revealed that an inhibition of E2 induced over-expression of the PR in MCF-7 cells first appears at an antihormone concentration of 100 nM, whereas no effect could be measured at 1 and 10 nM of ICI182,780 (Etique et al., 2006).

Concrete evidences for GPR30 as a mediator of E2 induced interruption of TGF-β signalling could successfully be provided by transfection experiments. RNA interference targeting the GPR30 gene downregulated the protein expression at the plasma membrane of MCF-7 cells and prevented E2 dependent repression of TGF-β signalling. The GPR30 deficient MDA-MB-231 cell line appeared to be insensitive to the inhibitory influence of E2 on TGF-β mediated migration. Transfection of these cells with a plasmid encoding the GPR30 gene resulted in an over-expression of this protein in membrane fractions and created an estrogen responsive phenotype of this cell line, in which TGF-β induced chemotaxis and Smad phosphorylation are diminished in response to E2.

It must be stressed that, after long-term treatment with ICI182,780, E2 failed to reduce TGF-β migration in MCF-7 cells, suggesting a participation of ERα. Since nuclear effects could be excluded so far and G-protein mediated pathways have been proved to be involved, this, if at all, could be a matter of membrane located ERα (mERα). As assumed by Sukocheva et al., mERα and GPR30 might either

share a common function or this effect might be a concern of a cross-communication between these two receptors (Sukocheva et al., 2006). It remains to be examined if and how these receptors communicate. Furthermore, until today it has remained unproven that both receptors really coexist in general. It must be stressed that investigators differ fundamentally regarding their opinion about the identity of mERs, but on principle they agree in the proposal that mER is identical with either ERα or ERβ relocated to the membrane or GPR30. Until now all studies concerning this question detected either mERα/β or GPR30 in the MCF-7 cell line.

On the other hand GPR30 exhibits only a single binding site (Thomas et al., 2005), at which ICI182,780 may function competitively antagonistic, blocking the ligand binding domain for E2. Competitive bindings assays showed that ICI182,780 and TAM in high concentrations (from 10^{-8} M for TAM and 10^{-7}M for ICI182,780) are able to effectively displace tritium labelled E2 from SKBR3 plasma membrane preparations (Thomas et al., 2005). Measurement of ERK1/2 phosphorylation by ICI182,780 indicated that GPR30 mediated effects initiated by this agent are transient and decrease after 30 min of stimulation (Filardo et al., 2000). Moreover, ICI182,780 concentrations sufficient for the displacement of E2 from the receptor were unable to induce ERK1/2 activation. Long-term treatment with 10^{-6} M of the antiestrogen might therefore inhibit E2 association with the receptor, but simultaneously miss any stimulating influence on GPR30. Thereby, the process of E2 induced repression of the TGF-β pathway may totally be beyond the control of GPR30.

Permanent internalisation of GPR30 by ICI182,780 treatment is another mechanism, which could prevent activation through E2. Some steroid hormone receptors residing in the plasma membrane are capable to internalise upon ligand binding. The membrane located testosterone receptor (mAR), identified in the murine macrophage cell line IC-21, internalises after ligation with the androgen, but, in contrast to other GPCRs, independently of clathrin-coated vesicles and caveolae (Benten et al., 1999). The functioning of this receptor bears remarkable resemblance to the mER, identified in the present study, as exemplified by its inducability by BSA-conjugated ligand and the sensitivity to PTX. Most interestingly, these authors additionally discovered a G-protein dependent membrane steroid receptor responsive to free and conjugated E2 in this cell line. This mER is unrelated to nuclear ERα and ERβ and exhibits comparable internalisation properties like the mAR (Benten et al., 2001).

Furthermore, the so-called non-activated mER (na-mER) in goat uterine plasma membranes dissociates from the plasma membrane in response to estrogenic compounds (Sreeja and Thampan, 2004; Sreeja and Thampan, 2004). A great variety of GPCRs becomes sequestrated after activation, a process that may be crucial for regulation of responsiveness, activation of specific signalling factors, receptor downregulation, and recycling (Fan et al., 2001; Kohno et al., 2002; Mundell et al., 2006). Therefore, a similar process can be anticipated for GPR30. However, until now there exist no data about this issue.
Except from its localisation in the plasma membrane, GPR30 has been identified in the endoplasmatic reticulum of SKBR3 and COS7 cells transfected with GPR30 (Revankar et al., 2005). Another alternative source of GPR30 are mitochondrial membranes of MCF-7 and endothelial cells (Chen et al., 2004; Pedram et al., 2006). In consideration of the results with membrane impermeable E2, a role for such intracellular GPR30 sources in the communication between estrogen and TGF-β pathways can be excluded.

Similar to E2 induced migration, the effectiveness of the hormone in the repression of growth factor mediated migration increases with augmentation of dose. This effect can already be measured at physiological concentrations and achieves a maximum response at 10^{-6} M E2. In contrast, estrogen induced activation of the ERE is already saturated at 10^{-9} M E2. Based on the different maximal efficient concentrations of E2, distinct mechanisms for estrogen induced gene transcription in comparison to other physiological effects have been suggested by several authors. In the estrogen responsive rat cell line PR1, for example, the concentration of required E2 for proliferation is 1000fold higher than for prolactin gene transcription by E2 (Caporali et al., 2003). E2 binding studies with separated membranes of SKBR3 and HEK293 cells transfected with GPR30 determined a tenfold lower affinity than in Chinese hamster ovary cells transfected with ERα, leading to the presumption of a higher threshold concentration for GPR30 activation (Thomas et al., 2005). Maggiolini et al. gave evidence for the ability of 10^{-6} M E2 to induce the protooncogene *c-fos*, however, they owe to demonstrate the effectiveness of smaller concentrations (Maggiolini et al., 2004).

4.4.4 The importance of MAPK activation

It was further sought to identify, by which mechanisms GPR30 interferes with the TGF-β cascade. It has successfully been shown that E2 treatment interrupts TGF-β signalling on the level of Smad activation. The Smad signalling cascade is integrated within a complex network of other pathways. In consequence of these cross-talks, Smad signalling is alternatively regulated and may either be amplified, synergised, or antagonised.

A critical event in signalling via GPR30 is the regulation of ERK1/2 activity. GPR30 expressing MCF-7 cells rapidly activated ERK1/2 in response to estrogens and ICI182,780. Moreover, MDA-MB-231 cells, independent of endogenous GPR30, are insensitive to estrogens, but ERK1/2 activation by E2 could be established in these cells by over-expressing the receptor. The requirement of GPR30 in activation of ERK1/2 by estrogen and its antihormone is consistent with other studies, where E2 as well as ICI182,780 activate ERK-MAPK in several cell types, among these breast cancer cells independent of nuclear ERs. ERα/β negative breast cancer cells SKBR3 (Filardo et al., 2000; Maggiolini et al., 2004), ERα negative endometrial cancer cells HEC1A (Vivacqua et al., 2005), MCF-7 (Filardo et al., 2000; Filardo et al., 2002; Maggiolini et al., 2004), and ER positive Ishikawa cells (Vivacqua et al., 2005), all endogenously expressing GPR30, as well as MDA-MB-231 cells, transfected with a GPR30 expression vector (Filardo et al., 2000; Filardo et al., 2002), respond with an increase of ERK1/2 activity within minutes after stimulation with E2 or antiestrogens. Several studies suggest that estrogens binding to GPR30 activate MMPs to cleave HB-EGF from the cell surface (Filardo, 2002; Filardo et al., 2000; Prenzel et al., 1999), becoming available for the EGFR-to-ERK signalling pathway (Maggiolini et al., 2004) (Fig. 42). This process has been circumstantiated in MCF-7 cells by Sukocheva et al. as a criss-cross transactivation event involving three receptor systems. They suggest that GPR30, activated through E2, stimulates sphingosine kinase -1 (SphK1), which enhances intracellular sphingosine 1-phosphate (S1P) levels. S1P is ligand to the GPCR S1P-3, which induces MMP activation, release of HB-EGF, and in turn transactivation of EGFR (Sukocheva et al., 2006; Sukocheva et al., 2003).

Smads are a target of MAPK signalling in response to either TGF-β itself or other chemokines, for example via RTKs. In contrast to TGF-β and BMP mediated

phosphorylation of R-Smads, activation by ERKs occurs at specific serines in the linker region (Fig. 2B) that may abrogate the nuclear import of Smads in divers epithelial cell types (Calonge and Massague, 1999; Kretzschmar et al., 1997; Kretzschmar et al., 1999). The influence of MAPKs on Smad signal transduction pathways appears to be cell-type specific, since in other studies TGF-β stimulated ERK1/2, but not p38 MAPK, further enhances Smad2 and Smad3 phosphorylation and transcriptional activity in human mesangial cells (Hayashida et al., 2003), renal tubular epithelial cells (Rhyu et al., 2005), and a mink lung epithelial cell line (Funaba et al., 2002).

Application of the MEK inhibitor PD098,059 proved the participation of ERK1/2 in E2 induced inhibition of TGF-β migration. Hence, it is adjacent to assume that activation of ERK1/2 by MEK contributes to the downregulation of TGF-β signalling in MCF-7 cells, induced by E2, via interfering with the Smad signalling cascade. However, for this cell system there exist no data about Smad functioning in the presence of ERK1/2. Considering that ERK1/2 is supposed to inhibit the import of Smad complexes into the nucleus, it especially appears unclear, why the repression of TGF-β already occurs on the level of Smad phosphorylation and complex formation. Blocking the nuclear translocation of activated Smad complexes, would lead to an accumulation in the cytoplasm and could cause a negative feedback loop that prevents further phosphorylation of Smad proteins. Several forms of negative feedback mechanisms regulating intracellular TGF-β signals are known. Some of those are mediated by the inhibitory Smad7, which is expressed in response to TGF-β itself and competitively represses binding of Smad2 and 3 to the activated TβR complex (Nakao et al., 1997). The protein SMURF (Smad ubiquitin regulatory factor) provokes R-Smad degradation by proteasomes and induces the export of Smad7 from the nucleus and its recruitment to the TβR (Kavsak et al., 2000; Murakami et al., 2003). Smad7 is not constitutively active, but underlies regulation by transcription factors and proliferative signalling cascades. The ability of the EGF-to-ERK signalling cascade to induce Smad7 activity (Afrakhte et al., 1998) might be of particular importance for the regulation of R-Smad activity. It remains to be investigated whether the repressive input on Smad phosphorylation and complex formation is subject to a negative feedback loop and whether it is mediated by ERK1/2.

Admittedly, motility induced by TGF-β, in contrast to Smad phosphorylation and reporter gene activation, is not inhibited in the same ratio. While E2 sensitised cells almost completely failed to migrate towards TGF-β, the Smad signal transduction cascade is only partly decreased. Smad dependent chemotaxis of MCF-7 cells might require a threshold concentration of phosphorylated Smad proteins, which is not achieved in the presence of E2. Moreover, one could suggest additional proceedings to be affective on migratory events. One of these might be intracellular S1P sources, which increases, catalysed by SphK1, in response to GPR30 signalling and are capable to inhibit chemotactic motility of MCF-7 cells via a yet unknown mechanism (Wang et al., 1999).

Cell migration is an extremely complex event and in MCF-7 probably not exclusively dependent on proper course of the Smad signalling cascade. Apparently, basal levels of phosphoryated MAPK are generally capable to reduce cell movement, since migration of unstimulated control cells slightly increased in the presence of PD098,059. Nevertheless, this is probably quite irrelevant for the repression of TGF-β migration, since E2 induced MAPK was unable to inhibit MCF-7 motility towards unspecific chemoattracting constituents of FCS.

It should be considered that, in contrast to Smad phosphorylation that is measured after an incubation time of only 30 min, cell migration is determined after 5 h. During this time, signalling cascades, like ERK1/2, rapidly initiated by GPR30 after binding of ligand, have been proceeding and might have induced genomic or non-genomic downstream targets. Thereby, migratory influences initiated by remaining Smads would be compensated. In consistence with the argumentation above, the GPR30 mediated activation of MAPK by E2 might affect migration beyond an interruption of nuclear translocation of activated Smad complexes.

It could be proved that ERK1/2 is initiated by E2 and its antihormone and substantially interferes with the estrogen provoked repression of TGF-β signalling. MAPK activation by E2 and ICI182,780 is dose dependent, but in concentrations, which appear to be most efficient in inhibiting TGF-β migration, the activity is already considerably declined. Therefore, it should be examined, how ERK1/2 activity is altered in cells costimulated with E2 and TGF-β. Both TGF-β (Imamichi et al., 2005) and E2 (Filardo et al., 2000) induce a transient activation of ERK-MAPK with a maximum effect after 5 to 15 min of stimulation. As could be expected, this peak of

ERK activity coincided with the first appearance of a reduction of TGF-β induced Smad2 phosphorylation. Although for both agents ERK1/2 phosphorylation has been shown to return to almost basal levels after 30 min, it is striking that, after that time, solely in cells costimulated with 10^{-6} M E2 and TGF-β the enzyme activity is enhanced in a synergistic manner. Obviously, estrogen and TGF-β cross-communicate to augment common signalling pathways. In consistence with previously presented results, this peak in ERK activity is reflected in an even more effective suppression of Smad2 and migration after 30 min of exposure to E2 than after shorter time. This finding further indicates that for the most effective inhibition of TGF-β dependent pathways, Smad phosphorylation has to occur simultaneously with MAPK activation, since preceding ERK1/2 stimulation by 10^{-9} M E2 could not be further enhanced by TGF-β and only poorly diminished migration towards the chemokine.

Fig. 43 shows a compilation of signalling events that might be involved in the repression of TGF-β induced migration by E2.

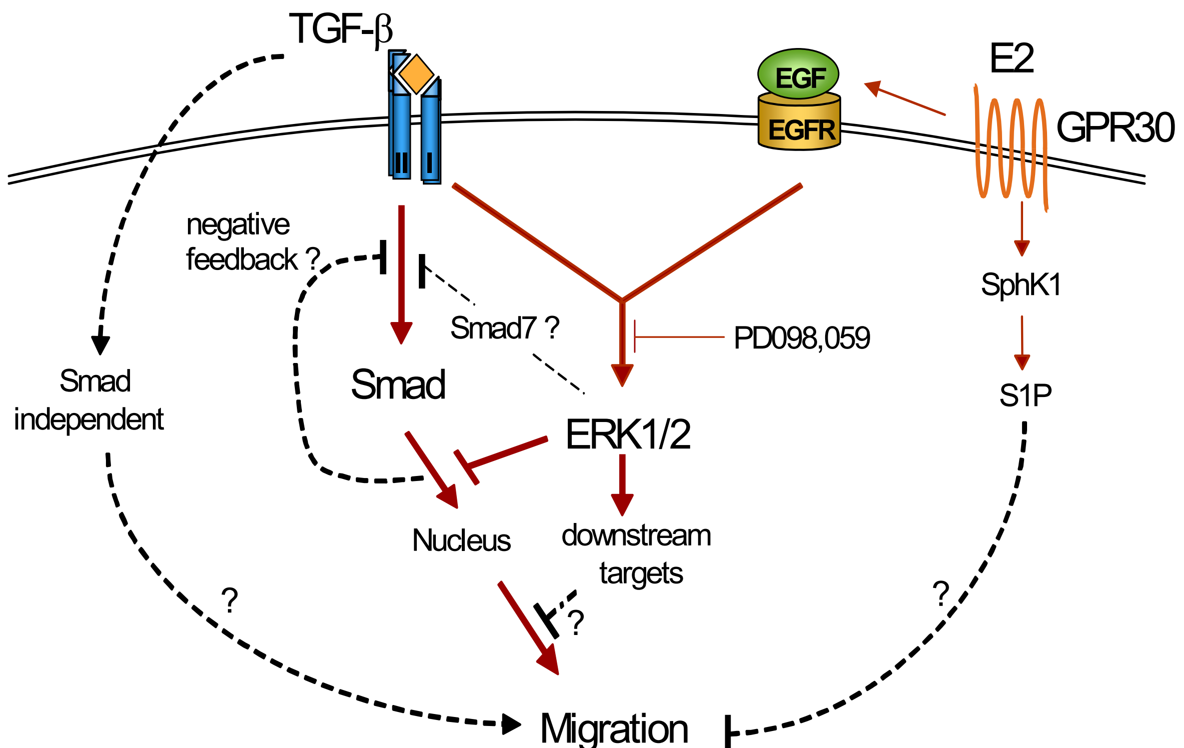

Fig. 43 Feasible correlations of signalling events that might be involved in the suppression of the TGF-β signal transduction pathway in E2 sensitised cells. SphK1: sphingosine kinase- 1; S1P: sphingosine 1-phosphate.

4.5 The physiological relevance of the cross-talk of TGF-β and estrogen signalling pathways

In their role as mediators of development and normal functioning of reproductive organs, estrogens are often involved in a mutual agonistic interaction with divers growth factors. The biological function of this phenomenon still awaits final clarification. During puberty, for example, the cross-communication with IGF-1 regulates growth and the development of gender specific body attributes. It may also facilitate ER activation under low estrogen conditions, for instance in males, or the amplification of growth factor signalling and mitogenesis. Thereby, each pathway may be dependent on the other for complete proceeding. Estrogens are further capable of regulating the expression of several growth factors and their receptors. Therefore, it is possible that the cross-talk of the steroid hormone with growth factors may establish a positive feedback system that promotes a stronger or extended response.

A lifelong cyclical fluctuation of the steroid hormone leading to repetitive stimulation of the breast tissue is associated with an increased risk of developing a mammary carcinoma. The fate of cancerogenic tissues may similarly be subject to the complex interplay of genomic and non-genomic estrogen pathways with growth factors and other kinases. Thus, the synergistic functioning of estrogen and IGF-1 is also a critical element in progression of mammary carcinomas (Thorne and Lee, 2003). Moreover, overexpression of HER-2/neu is discussed as a fundamental event in developing resistance against endocrine therapy and is often correlated with a bad prognosis (Osborne et al., 2005; Pietras et al., 1995; Schiff et al., 2004). Since growth factors are regulators of proliferation and apoptosis, this cross-talk might impair the antiproliferative effect of therapies using antiestrogens or, to a minor extent, aromatase inhibitors. But concomitantly, it might imply novel therapeutic targets against disorders, which are based on excessive growth factor-steroid action.

On the other hand an estrogen-to-growth factor cross-talk in many instances underlies the protective role of the steroid in non-reproductive target tissues. As an example, the inhibition of TGF-β induced collagen IV synthesis in vascular smooth muscle and mesangial cells by estrogens has been associated with a decreased risk of artherosclerosis (Matsuda et al., 2001) and glomerulosclerosis (Lei et al., 1998; Silbiger et al., 1998) in female gender. The low risk of women to develop coronary

artery disease before menopause is further attributed to an interplay with growth factors exerting positive effects on the lipid metabolism. Furthermore, E2 reverses TGF-β 1 induced apoptosis of mesangial cells, which may have protective effects on the course of chronic renal disease in women (Negulescu et al., 2002).

Recent studies have confirmed early observations demonstrating that, in addition to their genomic function as transcription factors, estrogens induce rapid and transient effects originating at receptors in the plasma membrane or in the cytoplasm by direct activation of various signal transduction components. It has been well documented that non-genomic pathways are implicated in estrogen induced proliferation (Castoria et al., 1999) and survival (Razandi et al., 2000) of breast cancer cells. The discovery of ERs unrelated to the nuclear form provides a new model to investigate structural requirements for estrogen binding and receptor activation.

The identification of GPR30 as an estrogen responsive membrane receptor provoked investigations on its role in the physiology and pathology of breast, prostate, placenta, ovarian, neural, and vascular tissues. Rapid non-genomic estrogenic effects in ER negative, non-reproductive tissues have often been due to signalling through GPR30, which is therefore discussed as a potential target for therapeutic interventions by a new class of SERMs. The discovery that antiestrogens are capable to mimic estrogenic effects via GPR30, have raised considerable doubts about the application of antiestrogens in medical treatment of breast cancer. In this context and with respect to the variety of estrogenic functions, the application of aromatase inhibitors constitutes an advantage over the classic antiestrogen therapy. Moreover, they might offer a means for the treatment of ER negative carcinomas.

However, it should be considered that GPR30 might also exert protective properties. Advanced tumours have become resistant against the antiproliferative function of TGF-β, which hence may facilitate metastatic distribution of the tumour by stimulation of cell migration and invasion. The present study uncovers an anticarcinogenic role for GPR30 in late stage tumours, in which the receptor is the essential mediator for a cross-talk between TGF-β and estrogens, impairing the metastatic potential of the chemokine. Moreover, it has been demonstrated that GPR30 expression correlates with and is a prerequisite for progestin induced growth inhibition in MCF-7 cells (Ahola et al., 2002; Ahola et al., 2002).

4.6 Outlook

The presented results indicate that GPR30 might contribute to a mechanism, by which estrogens use the ERK-MAPK pathway to adjust TGF-β signals, partly via intervention in the Smad pathway, with protective consequences in advanced metastasising tumours. Experiments with membrane impermeable E2 indicate that this effect can be referred to membrane associated receptors, only. However, several authors suggest that ERα and β exist in plasma membranes of MCF-7 cells. Thus, it remains to be finally clarified, if classical ERs have a bearing on this effect. A novel GPR30 specific agonist, G-1, provides a means to investigate processes, which are exclusively mediated by this orphan receptor (Bologa et al., 2006).

The importance of GPR30 for the inhibition of TGF-β signal transduction in E2 sensitised cells has further been proved by its overexpression in the MDA-MB-231 cell line, which is considered hormone independent. SKBR3 represents an ER negative breast cancer cell line, which endogenously expresses GPR30 (Filardo, 2002; Filardo et al., 2000; Filardo et al., 2002) and therefore may serve as a more physiological model system, in which to examine GPR30 responses.

The influence of E2, signalling via GPR30, on MAPK is not one-sided, but it rather acts to balance ERK1/2 activity in MCF-7 cells. If the conversion from the CTX dependent suppression of the EGFR-to-MAPK cascade through cAMP production to the rapid, PTX sensitive activation of ERK1/2 via trans-activation of EGFR is due to a switch between Gs- und Gi-protein coupling of the receptor, remains to be investigated. The conditions under which such a switch of GPR30 in G-protein coupling would be initiated, is of great interest, since it can be predicted that it would reverse the effect of E2 on TGF-β induced migration.

TGF-β is an inductor of EMT, which is implicated in the dissemination of cancer cells from the primary tumour site and the dedifferentiation of cells that leads to malignant carcinomas. In this context, an additional protective role of estrogens acting via GPR30 might be assumed. With respect to the role of TGF-β as a tumour suppressor, it remains to be clarified whether, in non-transformed cells or in early stage tumours, GPR30 is able to promote development and progression of cancer by reversing antiproliferative and pro-apoptotic abilities of TGF-β.

In addition to breast cancer cells, GPR30 is widely expressed in normal, non-reproductive tissues, among these the central nervous and cardiovascular system, bone, and brain. In these tissues, the function of estrogen-TGF-β interactions under physiological and especially pathological conditions could be a target of further investigations. GPR30 is moderately expressed in human keratinocytes. The skin appears to be an end-organ target for estrogens, which contribute to the maintaining of homeostasis in this tissue. Strikingly, when compared with TGF-β, estrogens acting via GPR30, exert opposing effects in keratinocytes, such as stimulation of cell growth (Kanda and Watanabe, 2004) and prevention of apoptosis induced by reactive oxygen species (Kanda and Watanabe, 2003). So far it is unknown if this is due to a direct interference with TGF-β signalling by the hormone. Estrogens enhance the rate of cutaneous wound healing, which has been explained by a secretion of latent TGF-β by dermal fibroblasts (Ashcroft and Ashworth, 2003; Ashcroft et al., 1997), but it is further of interest whether estrogens acting via GPR30 may also affect wound healing by disruption of TGF-β signalling.

Interactions between estrogen and RTK signal transduction pathways are in most cases bi-directional and also TGF-β has been shown to enhance the transcriptional activity of the steroid (Matsuda et al., 2001). The physiological and, most probably, the pathological significance of this effect still require clarification.