Introduction

1 Introduction

1.1 Cell migration and cancer metastasis

Cell migration is an integral part in development and maintenance of the organism. Migration of cells can either be chemotactic, meaning the movement towards or against a chemical substance, or chemokinetic, which is not directed, but instead independs on a concentration gradient of the stimulating agent. Chemokines, growth factors, and extracellular matrix (ECM) molecules are potent inductors of cell movement.

Cell movement is an important process in embryogenesis, renewal of skin and intestines, and is critical for tissue repair and immune responses, but it also contributes to a variety of pathological processes, such as osteoporosis, vascular disease, rheumatoid arthritis, and multiple sclerosis. Moreover, it is strongly involved in cancer metastasis, which is considerably implicated in mortality of cancer patients (Coleman et al., 1998).

Metastasis is the migration of cancer cells from the original tumour site to distant organs, where they invade normal tissue and proliferate to form new tumours. Changes of cell-substrate and cell-cell adhesions enable cancer cells to displace themselves from the tumour to invade the circulatory system, which transfers them to secondary organs. Each cancer type metastasises to preferred sites. Breast cancer cells, for example, spread past the breast and axillary lymph nodes to other areas of the body. Among these, the most common region is bone, followed by lung and liver (Helbig et al., 2003; Liotta, 2001; Nicolson, 1993).

Metastasising proceeds in three major steps: departure of cells, penetration into the circulatory system, and establishment of new tumours in the target organ. Leaving the original tissue demands disengagement of adhesive junctions, meaning cell-cell interactions, and focal adhesions between cell and ECM. Typically, highly invasive cancer types are characterised by a decreased number of proteins, which form adhesive structures and keep cells integrated in their normal neighbourhood. Focal adhesions are mediated by integrins, membrane associated receptors, later described in this section. In cancer cells the expression profile and activation state of integrins are often modified, adapting their ligand binding properties (Fawcett and

Harris, 1992; Mizejewski, 1999). In primary breast cancer, for example, the integrin expression level is reduced (Bartolazzi et al., 1994; Pignatelli et al., 1991).
Penetration into blood or lymphatic vessels requires migration through the tissue, endothelial cells, and the basement membrane. As part of the ECM the basement membrane is located between endothelial cells and the surrounding tissue and is organised as a thin sheet built of a variety of proteins, such as laminin, fibronectin as well as type III and IV collagens. To overcome this barrier, metastatic cells secrete matrix metalloproteinases (MMPs), which degrade structure proteins of the basement membrane (Deryugina et al., 1997; Egeblad and Werb, 2002). The subsequent migratory process is presented further down. There exists only limited knowledge about the mechanism leading to the transition out of the blood vessel into the target organ. Selectins, transmembrane peptides of endothelial cells, probably interact with carbohydrate ligands on the cancer cell surface (Borsig et al., 2002; Fukuda et al., 2000; Sheen-Chen et al., 2004; Zetter, 1993). Finally, integrins mediate the attachment of metastasising cells to the vessel wall. The following intrusion process involves almost the reverse event that allows cancer cells to leave the primary organ. Metastatic cells reach the host tissue by proteolytic digestion of the basement membrane via MMPs and migration through the vessel wall. Upon arriving, the cancer cells proliferate to establish new tumours, often regulated by autocrine and paracrine growth factors, such as basic fibroblast growth factor (Halaban et al., 1988; Rodeck et al., 1991), interleukin-8 (IL-8) (Schadendorf et al., 1993), IL-6, macrophage colony stimulating factor, granulocyte macrophage colony stimulating factor, transforming growth factor -β 2 (TGF-β 2) (Pederson et al., 1999), insulin-like growth factor type 1 (IGF-1), and epidermal growth factor (EGF) (de Wit et al., 1992).

In all migratory cell types the process of cell movement occurs as a cyclic process that is composed of four stages: polarisation, extension, adhesion, and least traction and tail retraction (Lauffenburger and Horwitz, 1996; Sheetz et al., 1999) (Fig. 1).

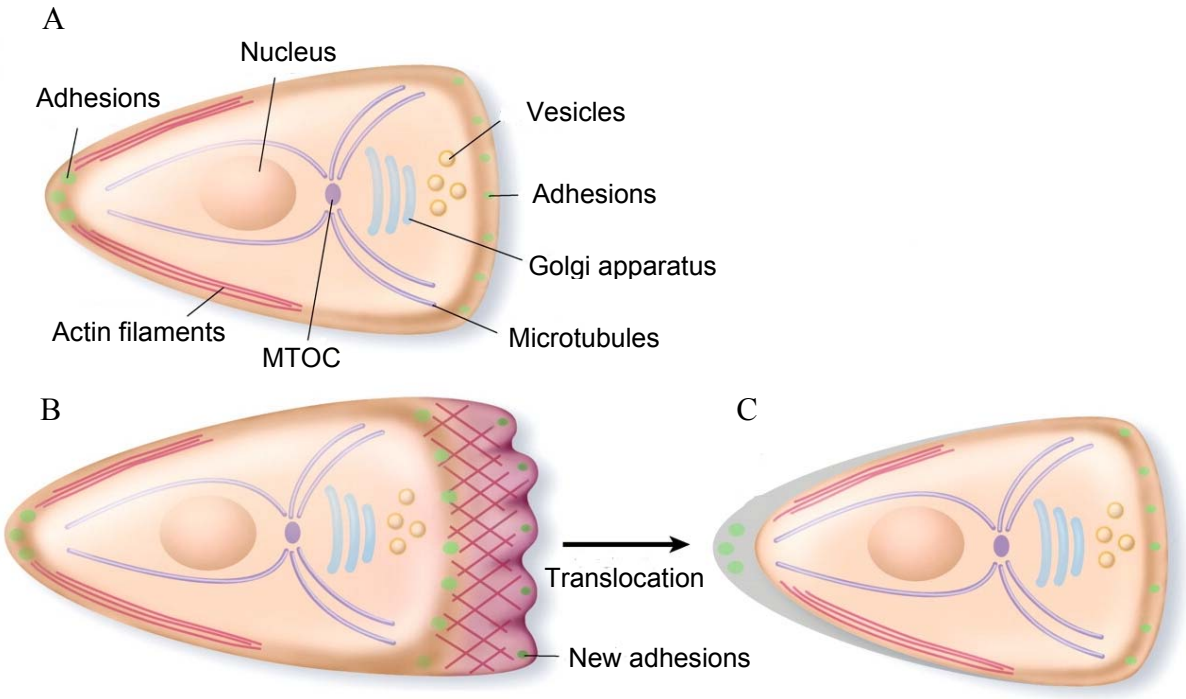

Fig. 1 Phases of cell migration. (A) In the first stage the cell undergoes a phase of polarisation realised by vesicle trafficking (enclosing membrane and other proteins) to the leading edge, microtubule organisation, and translocation of the microtubule organising centre (MTOC) and the Golgi apparatus towards the front of the nucleus. These processes demand the contribution of several proteins, including Rho-GTPases (guanosine triphosphatases), phosphatidyl-inositol-3-kinase (PI3K), integrins, and microtubule proteins (B) Reorganisation of the actin cytoskeleton allows the formation of protrusion extensions, which are stabilised by adhesions via integrin proteins. (C) Adhesions disassemble to form new protrusions at the cell front and to afford tail retraction at the cell rear. (modified according to Ridley et al., 2003)

The initial response of a cell to a migratory stimulus is polarisation and the extension of protrusions, wide lamellipodia or spike-like filopodia, usually driven by actin polymerisation. Proteins from the Rho family, small GTP-binding proteins (Rho-GTPases), such as Rac, Cdc42, and Rho, are considered as central regulators of protrusions involved in actin organisation and formation of lamellipodia and filopodia. Cdc42 regulates the polarity of eukaryotic cells and is active toward the front of migrating cells (Itoh et al., 2002). In slow moving cells it contributes to migration by localising the MTOC and Golgi apparatus in front of the nucleus to ensure providence of proteins and membrane material needed for protrusion extension (Etienne-Manneville and Hall, 2002; Rodriguez et al., 2003). Besides Rho-GTPases, phosphatidyl inositol 3-kinase (PI3K), integrins, microtubules, and the vesicular transport also contribute to the establishment and maintenance of cell polarity in response to an extracellular signal. Thus, PI3Ks accumulate to the leading edge of the cell downstream of a chemoattractant signal, whereas the corresponding

phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten) localises at the sides and the rear (Devreotes and Janetopoulos, 2003; Merlot and Firtel, 2003). Rac is involved in initiating and stabilising protrusions in a very complex manner by interacting with PI3Ks (Srinivasan et al., 2003; Welch et al., 2003), integrins (del Pozo et al., 2000; Kiosses et al., 2001; Schwartz and Shattil, 2000), and microtubules (Rodriguez et al., 2003). By suppressing each other's activity, Rho and Rac are mutually antagonistic (Evers et al., 2000), thereby suppressing Rho effects at the cell front and Rac induced formation of protrusions at the sites (Worthylake and Burridge, 2003; Xu et al., 2003).

The formation of extensions depends on ECM proteins, which support the formation of protrusion attachments to the ECM, termed adhesion plaques, at the front of the cell to provide stable traction. These attachments are realised by cell-surface located receptors, known as integrins (Mizejewski, 1999). Integrins are transmembrane receptors and composed of non-covalently associated transmembrane α and β chains. They possess specific affinity to various matrix proteins, such as laminin, vitronectin, fibronectin, different collagens, fibrinogen, and thrombospondin. Integrin receptors transmit force from the actin cytoskeleton to the ECM, thus establishing traction sites for migration (adhesion stage) (Felsenfeld et al., 1996; Felsenfeld et al., 1999), and they transfer information about the quality of the ECM to the cell interior, resulting in adequate cytoskeletal changes (Beningo et al., 2001; Galbraith et al., 2002; Lauffenburger and Horwitz, 1996). Ligand binding leads to integrin clustering and accumulation at the cell front (Kiosses et al., 2001), initiating the activation of Rho-GTPases, reorganisation of the cytoskeleton, modulation of adhesion-related phospholipid synthesis, and cell polarity during migration (Geiger et al., 2001). The affinity of these receptors can be modulated by the Rho-GTPase Rap1, protein kinase C (PKC) and Raf-1 kinase (Kinbara et al., 2003). Integrin-cytoskeleton interactions are mediated by the Src tyrosine kinase (Src), which is localised near integrin receptors at the cytoplasmatic site of the plasma membrane (Felsenfeld et al., 1999) (Felsenfeld et al., 1996). Following integrin mediated cell attachment, focal adhesion kinases (FAKs) are recruited to focal adhesions by anchor proteins, such as $p130^{Cas}$ (Cas), Crk, paxillin, and talin and cross-phosphorylate each other, creating a docking site for proteins of the Scr family. Additional phosphorylation of FAK by Scr causes recruitment of divers intracellular signalling proteins, which transfer the signal into the cell and initiate Src-dependent integrin-cytoskeleton cross-

talk, which is required for migration (Cary et al., 1998; Ilic et al., 1995; Klemke et al., 1998). Extracellular signals, activating Rho-GTPases Rac and Cdc42, may affect Src signalling and therefore modulate integrin-cytoskeleton interactions. The formation of focal adhesions is typical for non-migratory or slowly migrating cell types, such as MCF7 cells.

In the final stage (traction and tail retraction), the main cell body moves forward by loosening the linkages at the cell rear, promoting tail retraction along with the formation of new protrusions at the leading edge (Webb et al., 2002). Similar to the formation of attachments, adhesion disassembling is regulated by FAK-Src interaction with Cas and Crk via Rac depending pathways (Turner et al., 2001). Further downstream mediators of Rac and the extracellular regulated kinase 1 and 2 (ERK1/2) are also involved in adhesion turnover (Brahmbhatt and Klemke, 2003).

With respect to the migratory process *in vivo* there are still many unexplained issues. In different cell types, the details of migration processes vary depending on cell environment and nature. Fibroblasts, for example, are slowly migrating cells, in which the described migration stages proceed very distinct. Unlike fibroblasts, neutrophils migrate fast and seem to slide over the surface. Cancer cells are able to change their morphology and mode of migration according to environmental conditions (Friedl and Wolf, 2003; Knight et al., 2000).

1.2 Transforming growth factor β

1.2.1 The TGF-β superfamily

Transforming growth factor -β is the prototype of the TGF-β superfamily, which, in addition to the TGF-β isoforms, comprises activins (Act), inhibins, Anti-Mullerian hormone (AMH), growth differentiation factors, and bone morphogenetic proteins (BMPs). The expression levels, receptor affinity, and effects of the TGF-β isoforms differ tissue dependent (Centrella et al., 1994). In mammalian cells, three different TGF-β isoforms are expressed, termed TGF-β 1, 2, and 3. They exhibit similar intracellular functions, as they engage the same receptor system, but vary in the expression pattern (Attisano and Wrana, 1996). Normal breast tissues produce TGF-β 1 and 3 and fewer amounts of TGF-β 2 (Chakravarthy et al., 1999). The founding member of the family, TGF-β 1, is the most frequently expressed species in cell lines

and tissues of breast cancer (MacCallum et al., 1994) and the most potent isoform in inhibiting MCF-7 (Michigan cancer foundation -7) cell growth (Arrick et al., 1990).

Depending on cell type and context, TGF-β modulates a wide spectrum of cellular processes, among these proliferation, apoptosis, differentiation, and migration. It is further a critical regulator of several physiological events, like wound healing and embryogenesis and may also contribute to pathogenesis (de Caestecker et al., 2000; Derynck et al., 2001; Massague et al., 2000).

The activity of TGF-β is restrained by secretion as a latent, inert form and the release of the mature, biological active protein from these complexes (Koli et al., 2001). The majority of TGF-βs are synthesised in and released from platelets, but also from a number of other cell types, including normal and malignant breast epithelial cells. TGF-β is secreted as a biological inactive precursor protein complex, referred to as small latent TGF-β complex consisting of the mature 24 kDa TGF-β protein and an 80 kDa propeptide, called TGF-β latency associated protein (LAP), containing the signal sequence for secretion. Alternatively, this complex can be conjugated to the latent TGF-β binding protein (LTBP), forming the large latent TGF-β complex. LTBP serves for anchoring TGF-β in the ECM.

Under physiological conditions activation of latent TGF-β, a process, which plays a critical role in the biological activity of TGF-β, occurs extracellularly via enzymatic cleavage of the precursor protein to release the ligand at or near the cell surface, where it immediately binds to its receptors. TGF-β synthesis and secretion can be affected by mitogen activated kinases (MAPKs) and other growth factors, but also drugs like antiestrogens are able to initiate pathways leading to the release and activation of TGF-β (Buck et al., 2004; Zhu and Burgess, 2001). Thus, 4-hydroxytamoxifen (4-OHT) induces a 4-fold and 10-fold increase in TGF-β 1 and 2 secretion in MCF-7 and T47D cells, whereas no change has been detected with 17β-estradiol (E2) (Knabbe et al., 1987; Malet et al., 2001). Another study even demonstrated an inhibitory effect of E2 on TGF-β 2 and 3 secretion in T47D cells, but no effect of the antiestrogen on any of the TGF-β isoforms (Arrick et al., 1990). In contrast, the promoting effect of estrogens on cutaneous wound healing has been implicated in an increase in latent TGF-β 1 secretion from dermal fibroblasts (Ashcroft et al., 1997).

1.2.2 TGF-β receptors

All members of the TGF-β superfamily mediate their functions through specific transmembrane glycoprotein receptors with serine/threonin kinase activity (Massague, 1998). The TGF-β isoforms elicit their signal through three structural and functional different transmembrane receptors, type I TGF-β receptor (TβR-I), type II TGF-β receptor (TβR-II), and type III TGF-β receptor (TβR-III). These are further categorised as seven TβR-Is, referred to as activin receptor-like kinases 1-7 (ALK1-7) and five TβR-IIs, termed TβR-II, ActR-II, ActR-IIB, BMPR-II and AMHR-II (Kingsley, 1994). TGF-β 1, 2, and 3 signals are primarily mediated via ALK5 and TβR-II (Massague, 1998). TβR-III, also known as betaglycan, is a proteoglycan with a large extracellular domain that binds TGF-β with high affinity, but lacks a cytoplasmatic signalling domain (Lopez-Casillas et al., 1991; Wang et al., 1991). Expression of the TβR-III domain can regulate TGF-β signalling through presentation of the ligand to the TGF-β signalling complex.

In its active form, TGF-β binds to TβR-II, which functions as a receptor assembly factor, inducing complex formation with TβR-I. Upon autophosphorylation at serine and threonin residues of TβR-II, its kinase function is initiated, phosphorylating TβR-I at serines and threonins in a glycine and serine rich region (GS-domain). Thereby, the GS-domain translocates from the catalytic centre of TβR-I, thus activating its kinase function (Chen and Weinberg, 1995; Souchelnytskyi et al., 1996; Wieser et al., 1995; Wrana et al., 1994). Activated TβR-I recognises and phosphorylates specific receptor substrates, so-called Smad proteins.

1.2.3 Smad proteins and the Smad signal transduction pathway

The founding member of the Smad family is mothers against dpp (Mad), first identified in *Drosophila melanogaster* (Sekelsky et al., 1995). Mad homologues have been identified in *Caenorhabditis elegans* and termed Sma (small body size)-2, -3, and -4 (Savage et al., 1996). The name Smad was created as a combination of Sma and Mad, attributed when new homologues have been described in vertebrates shortly thereafter (Itoh et al., 2000).

The Smad family comprises eight isoforms of Smad proteins, which are divided into three subfamilies based on their structural and functional properties (Fig. 2A). R-

Smads (receptor regulated Smads) are direct substrates of the TβR-I kinase and include Smad1, 2, 3, 5, and 8. In general, ALK4, 5, and 7, mediating TGF-β and activin signals, phosphorylate Smads2 and 3, whereas Smads1, 5, and 8 are substrates of ALK1, 2, 3, and 6, corresponding to BMPs. Co-Smads (common mediator Smads) are associated in Smad signalling by formation of heteromeric complexes with phosphorlylated R-Smads. The only Co-Smad proved to be expressed in mammalians is Smad4, originally referred to as DPC4 ("deleted in pancreatic carcinoma locus 4") due to its role in pancreatic cancer (Hahn et al., 1996).

The third subgroup of Smad proteins contains Smad6 and 7 and is termed I-Smads (inhibitory Smads), since they antagonise receptor mediated phosphorylation of R-Smads by competing for binding of R-Smads to their respective receptors (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997).

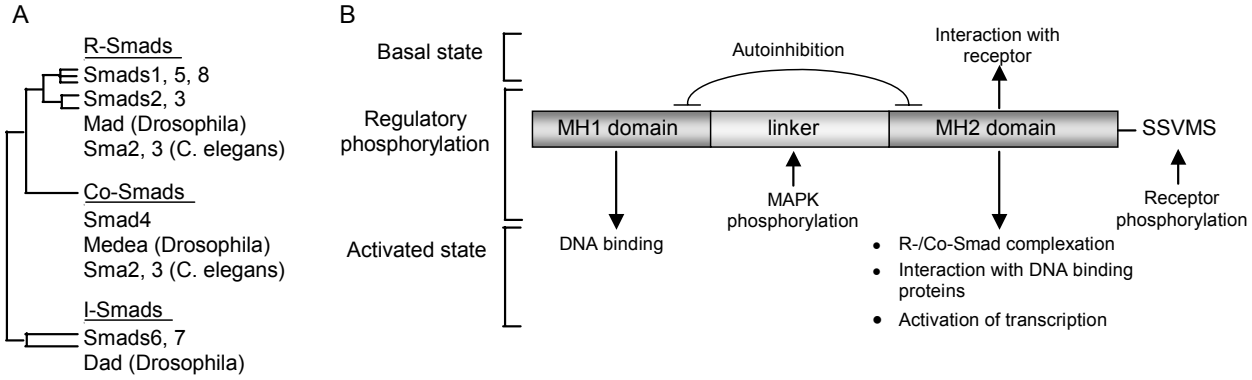

Fig. 2 The Smad family. (A) Phylogenetic tree of vertebrate (or otherwise indicated) Smads. (B) Domains of R-Smads, their phosphorylation sites, and functions. (modified according to Massague, 1998)

R- and Co-Smad proteins resemble each other in amino acid sequences at their highly conserved N- and C-termini, called MH1 and 2 (Mad homology 1 and 2) domains, respectively (Fig. 2B). The MH domains are separated by a linker region of variable length and sequence (Shi et al., 1997; Shi et al., 1998). In the basal state, the MH1 and MH2 domains inhibit each others function (Liu et al., 1996). In contrast to R- and Co-Smads, I-Smads lack the MH1-domain, which serves for DNA binding.

The SARA (Smad anchor for receptor activation) protein presents Smad2 and 3 to the activated TβR-I (Fig. 3) (Tsukazaki et al., 1998). Upon receptor mediated phosphorylation of the outermost C-terminal sequence of the R-Smads, the serine-

serine-valin-methionin-serine (SSVMS)-motif, the MH2 domain becomes accessible for and drives association with Co-Smads (Wu et al., 2000).

Controlled by specific signals within the R-Smad, the heterodimeric Smad complex translocates into the nucleus, where it induces transcriptional regulation of target genes. The MH1 domain of R- and Co-Smads, except for Smad2, possesses affinity for specific Smad binding elements (SBEs) of Smad responsive genes (Dennler et al., 1999; Dennler et al., 1998; Jonk et al., 1998; Shi et al., 1998; Yingling et al., 1997; Zawel et al., 1998). Optimal DNA binding is achieved in the CAGAC sequence of the promoter and is mediated by a β-hairpin structure that protrudes from the surface of the MH1 domain (Shi et al., 1998; Zawel et al., 1998). The most common splice variant of Smad2 lacks DNA binding activity due to an insert next to the β-hairpin structure (Shi et al., 1998). DNA binding of the Smad2-Smad4 complex is then mediated by the Smad4 MH1 domain, whereas regulation of transcription results from its opposed MH2 subunit (Liu et al., 1997).

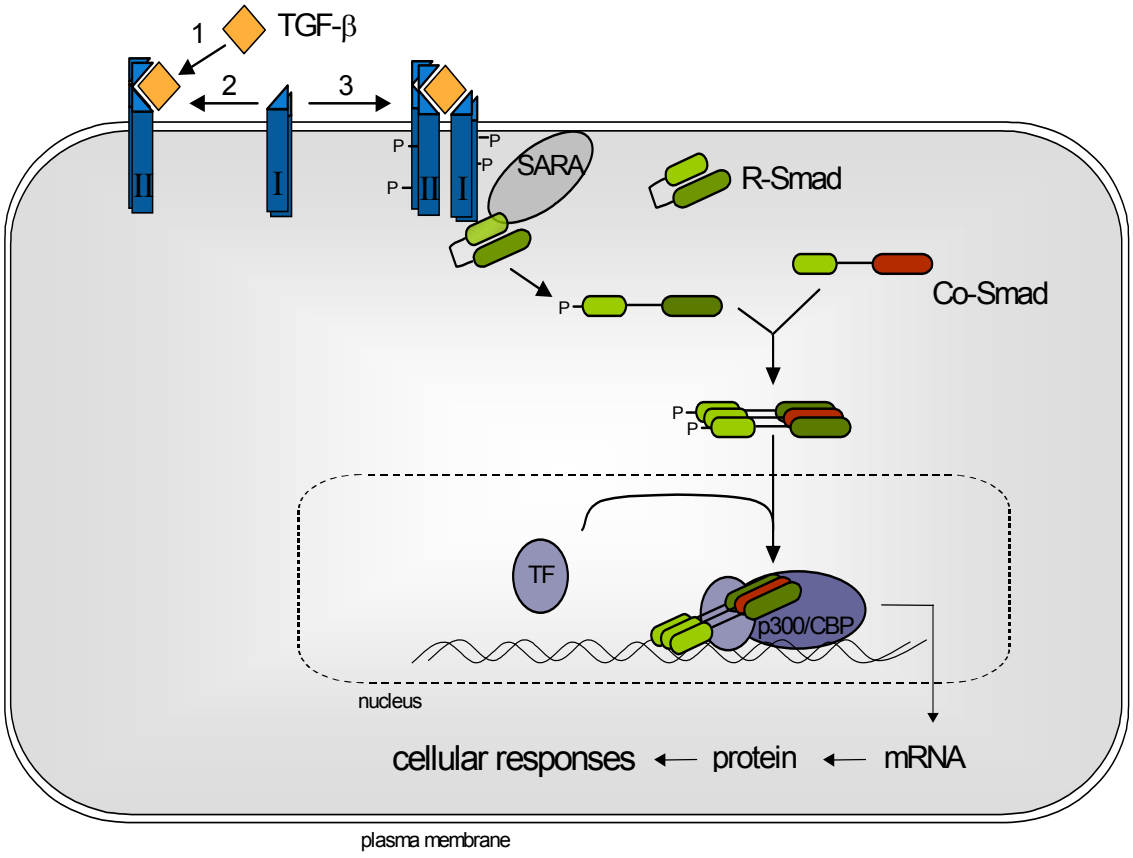

Fig. 3 The TGF-β signal transduction cascade. I: TβR-I; II: TβR-II; SARA: Smad anchor for receptor activation; p300/CBP: transcriptional coactivator; TF: transcription factor (modified according to Itoh et al., 2000)

Interaction of Smads with different DNA-binding partners facilitates selective binding and regulation of target genes. The forkhead activin signal transducer (FAST) protein was first identified to bind to activin response elements (ARE) derived from the *Xenopus Mix.2* promoter (Chen et al., 1996; Osada et al., 2000). The mammalian homologue associates with Smad2-Smad4 and Smad3-Smad4 complexes, resulting in efficient binding to target sequences (Chen et al., 1998).
The duration and intensity of TGF-β/Smad responses is tightly controlled. Phosphorylation of R-Smads leads to ubiquitination and subsequent degradation in proteasomes, a process that posttranslationally may regulate R-Smad levels (Lo and Massague, 1999). The activity of the Smad signalling pathway is further controlled by the I-Smads Smad6 and 7, which are expressed in response to TGF-β (Smad7) or BMP (Smad6), thus establishing an auto-inhibitory feedback system (Itoh et al., 2000; Massague, 2000).

Although there exists increasing evidence that TGF-β initiates signalling pathways downstream of receptor activation that are independent of Smad proteins, their connection with TGF-β receptor activation and gene regulation is quite ambiguous and cell type specific. Regulation of cellular functions by TGF-β can be a mean of MAPKs, c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK1/2 (Frey and Mulder, 1997; Hanafusa et al., 1999) and activation of small Rho-GTPases, RhoA, Rac, and Cdc42 (Bhowmick et al., 2001; Edlund et al., 2002). Furthermore, NF-κB (Ogawa et al., 2004) and the PI3K/Akt pathway (Bakin et al., 2000; Engel et al., 1999) have been implicated in Smad independent TGF-β signal transduction.

1.2.4 The role of TGF-β in human cancer

In many human disorders, alterations of the TGF-β signalling pathway could be demonstrated. In fibrotic diseases, a gain in TGF-β activity plays a central role (Fynan and Reiss, 1993), and an abnormal TGF-β signalling was also reported in inflammatory diseases (Kulkarni et al., 1993). Moreover, TGF-β has been implicated with tumourigenic processes. Studies on the role of TGF-β in cancerogenesis often focus on TGF-β 1, since it is most frequently upregulated in tumour cells (Derynck et al., 1987).

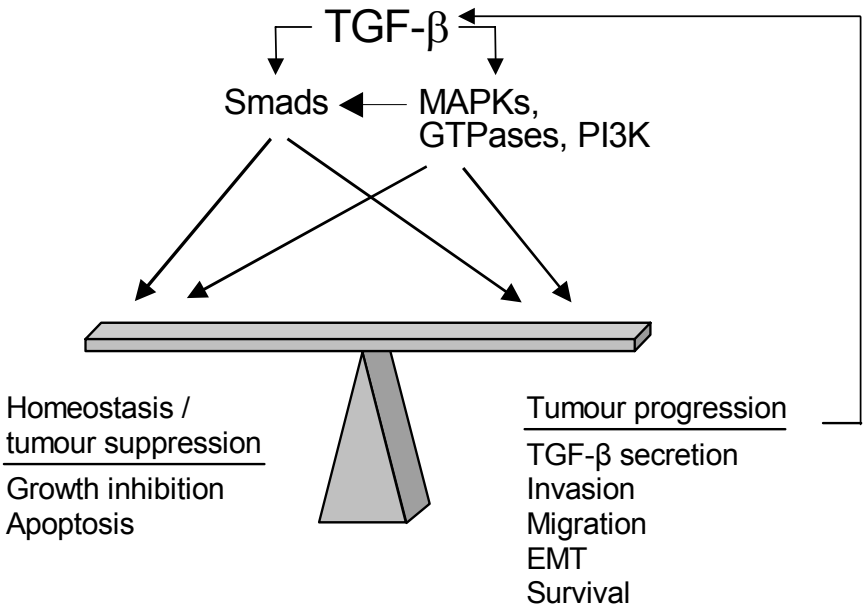

Fig. 4 The balance between cell homeostasis and tumour progression is shifted by activation of oncogenic pathways: Involvement of TGF-β signalling pathways. Tumour progression is driven by decreased sensitivity to the growth inhibiting properties of TGF-β and enhanced release of the chemokine. TGF-β induced cell migration and invasion as well as epithelial-to-mesenchymal transition (EMT) and prolonged survival of cells further amplify the course of tumour development. (modified according to Akhurst, 2002)

In contrast, TGF-β negatively regulates the growth of most normal and transformed epithelial cells, including those of the mammary gland, for example MCF-7 and MDA-MB-231 cells (Reiss and Barcellos-Hoff, 1997; Hosobuchi and Stampfer, 1989; Valverius et al., 1989). As TGF-β exerts positive as well as negative influences on tumour cells, it is considered as both promoter and suppressor of tumour progression (Fig. 4).

The tumour suppressing effects of TGF-β on early stage tumours are achieved by inducing growth arrest in the G1 phase of the cell cycle and by promoting apoptosis (Alexandrow and Moses, 1995; Hosobuchi and Stampfer, 1989; Reiss and Barcellos-Hoff, 1997; Valverius et al., 1989). During cancer development, the cells become less sensitive to TGF-β mediated growth arrest as a result of dysfunctional TGF-β receptors or Smad pathway components (Kalkhoven et al., 1995; Kim et al., 1996; Laiho et al., 1991). In line with this, the tumour promoting properties of the cytokine gain in importance (Fynan and Reiss, 1993).

Altered expression of TGF-β 1, 2, and 3 at the protein level appears not to be the major feature that leads to malignant developments of breast epithelial cells, raising the possibility that altered cellular responses to TGF-β may play a role in progression

of mammary carcinomas (McCune et al., 1992). As an example, the TGF-β signal transduction has been shown to be interrupted by mutations of TβR-II in gastrointestinal cancers (Markowitz and Roberts, 1996) or aberrant TGF-β receptor trafficking, reducing receptor expression at the cell surface in murine plasmacytomas and cutaneous T-cell lymphoma (Amoroso et al., 1998; Knaus et al., 1996). Other abnormal TGF-β functions in cancer can be traced back to mutations in Smad encoding genes. Disruptive mutations of *Smad4* often occurs in pancreatic cancer (Hahn et al., 1996), and inactivation of *Smad2* function has been demonstrated in colorectal and lung cancers (Eppert et al., 1996; Riggins et al., 1997). In early stage tumours, this loss of signalling components leads to increased DNA synthesis, enhancing the probability of cytogenetic changes that drive tumour progression.

More frequently, the TGF-β signalling network remains intact and perturbation of Smad signalling occurs via oncogenic proteins, e.g. the transcriptional corepressors Ski and Ski-related novel protein N (SnoN) (Xu et al., 2000) as well as MAPKs (Rhyu et al., 2005), which regulate TGF-β responses. It is widely assumed that Ras and receptor tyrosine kinases (RTKs), acting via ERK1/2, cause phosphorylation of R-Smads in the linker region between the MH1 and MH2 domain (Fig. 2B,) thereby abrogating the nuclear translocation of Smads (Calonge and Massague, 1999; Kretzschmar et al., 1997; Kretzschmar et al., 1999). Furthermore, MMP-2 and -3, transcriptionally activated by Ras and other effectors, promote tumour invasion and angiogenesis by proteolytic activation of latent TGF-β complexes in the ECM (Oft et al., 2002; Yu and Stamenkovic, 2000).

By autostimulation of the *TGFβ1* gene, late stage tumour cells even secrete large amounts of TGF-β (Dalal et al., 1993; Oft et al., 1998; Sargent et al., 1989; Steiner et al., 1994; Walker et al., 1994). Over-expression of TGF-β has been associated with estrogen independence and antiestrogen resistance in human breast cancer (Reiss and Barcellos-Hoff, 1997) and, moreover, contributes to tumour progression by induction of an EMT (Miettinen et al., 1994) and enhancement of tumour cell invasion and metastasis (Muraoka-Cook et al., 2005; Muraoka et al., 2002; Tong et al., 2002). By use of a dominant negative truncated mutant of TβR-II expressed in MDA-MB-231 cells, Yin et al. present evidence that TGF-β promotes breast cancer metastasis to bone (Yin et al., 1999). Stimulation of 4TI breast cancer cells with TGF-β caused conversion to the metastatic phenotype, but not growth inhibition. Moreover,

expression of a dominant negative mutant of TβR-II in these cells dramatically diminished the metastatic spread of 4TI (McEarchern et al., 2001).

Moreover, elevated TGF-β levels are proposed to drive clonal dissemination of tumour cells resistant to TGF-β mediated growth inhibition (Reiss and Barcellos-Hoff, 1997) (Fig. 4).

Owing to the tumour promoting effects of TGF-β, a large number of studies about cancer treatment exist, dealing with TGF-β antagonists. Application of TGF-β neutralising antibodies suppressed intraabdominal tumour and lung metastases in mice inoculated with MDA-MB-231 cells (Arteaga et al., 1993). In comparison, transgenic mice expressing an antagonist of the Fc:TβR-II fusion protein class revealed decreased mammary tumour cell motility and lung metastases (Yang et al., 2002) (Muraoka et al., 2002). In MDA-MB-231 cells the expression of a truncated soluble extracellular domain of TβR-III diminished the amount of TGF-β 1 and 2 in the conditioned medium and reduced the antiproliferative effect of this medium on mink lung epithelial cells CCL64 (Bandyopadhyay et al., 1999). Admittedly, there are concerns about these approaches due to the tumour suppressive effects of the chemokine (Akhurst, 2002; Akhurst and Derynck, 2001).

1.3 Estrogens

Referring to their respective position in biosynthesis, estrogens are termed E1 for estrone, E2 for 17β-estradiol (Fig. 7) and E3 for estriol, with E2 being the most potent, biological active form. They exert a broad range of effects on growth, differentiation, and function of several human tissues. Among these they participate in the regulation of male and female reproductive organs, such as the development of the mammary gland and secondary sexual characteristics (Muramatsu and Inoue, 2000). They further contribute to protective procedures in so-called non-target tissues, including the skeletal, cardiovascular, and nervous system (Farhat et al., 1996; Iafrati et al., 1997; Leonelli et al., 2006; Ozawa, 2005; Turner et al., 1994). Moreover, estrogens are known for their proliferative effects on estrogen sensitive tissues, thereby promoting tumourigenesis of the endometrium, ovaries, and breast (Deroo and Korach, 2006; Yager, 2000; Yue et al., 2003). The majority of mammary carcinomas are initially dependent on estrogens to support growth.

1.3.1 Synthesis, release, and activation of estrogens

In women, estrogens are predominantly synthesised in the ovaries and, to a minor extent, in adipose tissues, the adrenal cortex, skin fibroblasts, bone, brain, and smooth as well as skeletal muscle cells (Simpson, 2003). Small amounts of estrogens are formed in men in the testis and the adrenal cortex. Estrogen biosynthesis starts with the formation of androstenedione from cholesterol, which is then converted to E1 or E2, either directly or through testosterone. This transformation of the androgenic precursor molecules is catalysed by the enzyme aromatase (Nelson and Bulun, 2001). Excessive aromatase expression has been demonstrated in adipose fibroblasts surrounding a breast carcinoma which generates increased local estrogen concentrations (Zhou et al., 2001). In addition to systemically delivered estrogens, these locally formed steroids as well contribute to growth of the breast tissue.

There is increasing evidence that the levels of E2 clearly differ in normal and transformed mammary cells. Thus, in mammary carcinomas 50 to 100fold higher concentrations were measured than in nontransformed breast tissues of postmenopausal women, which is considered to be predominantly due to *in situ* synthesis (McNeill et al., 1986; Purohit et al., 2002).

Divers factors, like IL-6, IL-1β, tumour necrosis factor -α (TNF-α), IGF-1, and prostaglandin E_2 play an important role in regulating estrogen synthesis in breast tissues by modifying aromatase activity (Adams et al., 1991; Duncan et al., 1994; Purohit et al., 2002; Singh et al., 1992). The mode of influence of these factors on aromatase function depends on the cell type. In MCF-7 cells, for example, most cytokines, except for EGF, TGF-α, and TGF-β 2, have been found to preferentially promote conversion of E1 to E2 by estradiol-17β-hydroxysteroid dehydrogenase (Erbas and Lai, 2000; Singh et al., 1992).

1.3.2 Estrogen receptors and their functions

In general, estrogen effects are mediated by two distinct receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which are encoded by two discrete genes (Enmark et al., 1997). In the nervous and cardiovascular system as well as in bone and urogenital tract, both subtypes occur simultaneously, but with different extent. Other organs reveal a more distinct distribution of ERs. Thus, ERα is almost

exclusively expressed in liver, breast, and uterus, whereas only ERβ could be detected in the gastrointestinal tract (Gustafsson, 1999). It is widely accepted that ERα and ERβ account for dissimilar functions, according to their distribution and cellular context. Moreover, evidence is accumulating that in tissues where both receptors appear they reveal a different, oppositional mode of action. On account of its dominant occurrence in breast and uterus, regulation of reproductive processes is attributed to ERα. Due to its proliferative effect, ERα has been implicated in the development of breast cancer, while it has been proposed that in normal mammary gland ERβ exhibits protective properties by modulating effects of ERα on cell growth (Gustafsson, 1999). With respect to tumourigenesis in breast tissues, it is of interest that the expression ratio of ERα and ERβ in malign tissues is shifted in favour of ERα, which is suggested to be one step of transition from a normal into a malign state (Leygue et al., 1998; Speirs et al., 1999).

ERs belong to the nuclear receptor superfamily that comprises receptors for steroid and thyroid hormones, retinoids, vitamin D, peroxisome proliferator activated receptors, and orphan receptors, for which no ligand has been established yet. ERα and ERβ act as ligand inducible transcription factors of the class of steroid receptors. The schematic composition of ERs and the degree of homology between the two isoforms are presented in Fig. 5. Both receptors consist of six functional domains (A-F). The N-terminal A/B domain differs largely between the ER subtypes and contains the trans-activation function -1 (AF-1).

Only few structural and functional data have been obtained for AF-1. The activity of AF-1 is ligand-independent, weak constitutive (Berry et al., 1990; Kumar et al., 1987), and highly cell-type specific (Katzenellenbogen et al., 1996). The partial agonistic effect of SERMs, like TAM and RAL, have been attributed to the activity of AF-1 (Berry et al., 1990). Either AF can initiate transcriptional activation. In most cases, however, a synergistic effect between AF-1 and AF-2 can be observed (Pham et al., 1992; Tzukerman et al., 1994).

The DNA-binding domain lies in the highly conserved C-region of ERs, in which ERα and ERβ share the highest homology. It contains eight cysteine molecules forming two zinc fingers, which account for the recognition of specific DNA-sequences in estrogen responsive genes, referred to as estrogen response elements (EREs), and for receptor dimerisation (Evans, 1988; Freedman, 1992).

The ligand binding site (LBD) resides in the E-domain, which additionally functions in hetero- and homodimerisation of ERs and in interaction with coactivators and corepressors. Although ERα and ERβ share low sequence homology in the LBD, the three-dimensional structure of these domains is similar.

Domains D and F are of variable size and not conserved. The D-domain is considered as a linker between the DNA binding domain (DBD) and the LBD, whereas the F domain is an extension region of the LBD. The latter is not very conserved among vertebrates and it is supposed to play a role in distinguishing between agonists and antagonists in ERα (Montano et al., 1995).

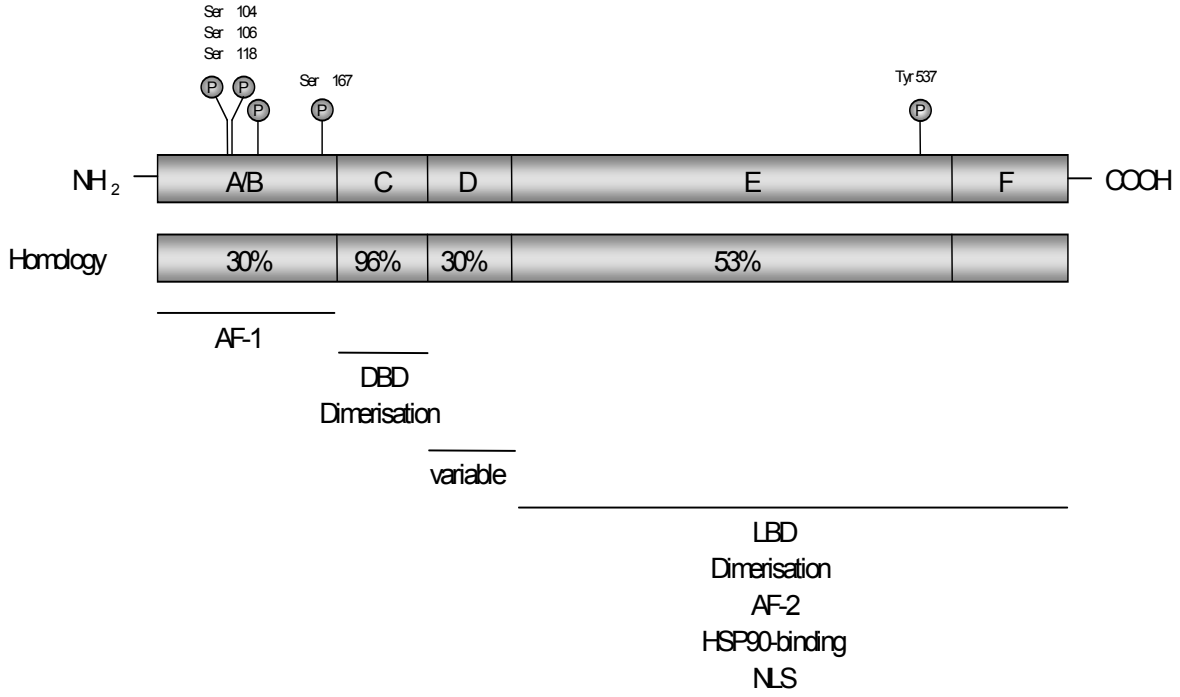

Fig. 5 Functional domains of ERs, percentage of identity between ERα and ERβ, and phosphorylation sites. DBD: DNA-binding domain; LBD: ligand binding domain; AF-1/2: trans-activation functions -1 and -2; HSP90: heat shock protein 90; NLS: nuclear localisation signal.

1.3.3 Receptor transformation and gene activation

Ligands, among these E2, have to pass the plasma membrane and arrive in the nucleus either by passive diffusion through pores in the nuclear membrane or by active transport mediated by a nuclear localisation signal (NLS) in the E-domain (Guiochon-Mantel et al., 1996) (Fig. 5).

Fig. 6 represents the process of transcriptional activation via nuclear ERs. In its physiological inactive form, the ER is associated with a heat shock protein 90

(HSP90) interfering with the DNA binding function (Renoir et al., 1986; Sanchez et al., 1987). Ligand binding causes dissociation of HSP90 from the receptor, thereby facilitating interaction with the DNA (Htun et al., 1999). Full transcriptional activity of the receptor is achieved by ligand induced phosphorylation at serine 118 or, to a minor extent, at serine 104 and 106, resulting in conformational changes of the receptor and exposing of the dimerisation function. ERα and β can both homodimerise and, less frequently, heterodimerise. Conformational changes of the ER provokes repositioning of helix 12 in the LBD, thus exposing amino acids that are critical for binding to specific coactivator proteins.

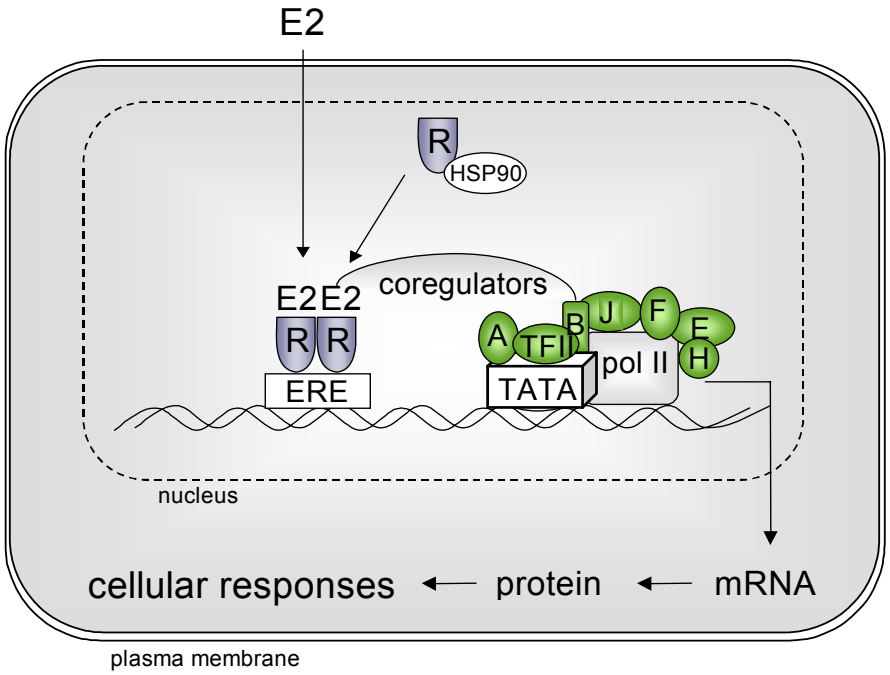

Fig. 6 Transactivation of nuclear estrogen receptors (ERs). R: ERα or β; E2: 17β-estradiol; TATA: base pair sequence 5'-TATAAA-3' in the promoter region (TATA-box); ERE: estrogen response element; HSP90: heat shock protein 90; pol II: RNA-polymerase II; TFII: transcription factors of the TFII-family; A/B/E/F/H/J: transcription factors of RNA-polymerase II. (modified according to Katzenellenbogen et al., 1997; Tsai and O'Malley, 1994)

The dimeric receptor binds to the ERE with high affinity (Denton et al., 1992) and activates specific gene expression (Klein-Hitpass et al., 1986; Seiler-Tuyns et al., 1986). The transcription factor TFIID, composed of the TATA-binding protein (TBP) and TBP associated factors, binds to the so-called TATA-box in the promoter, a sequence 25-30 base pairs upstream of the transcription initiation point (Greenblatt, 1991). Further coordination of transcription factors of the TFII-family (TFIIB, F) with the AF-2 domain of the ER and the RNA-polymerase II establishes a stable

transcription preinitiation complex. Finally, RNA-polymerase II initiates the transcription of mRNA (Buratowski, 1994; Tsai and O'Malley, 1994).

Several coregulator proteins, coactivators and corepressors, have been identified that are recruited by the ligand occupied receptor and mediate between the receptor and transcription factors of the preinitiation complex, thereby enhancing or repressing the transcriptional process (Horwitz et al., 1996).

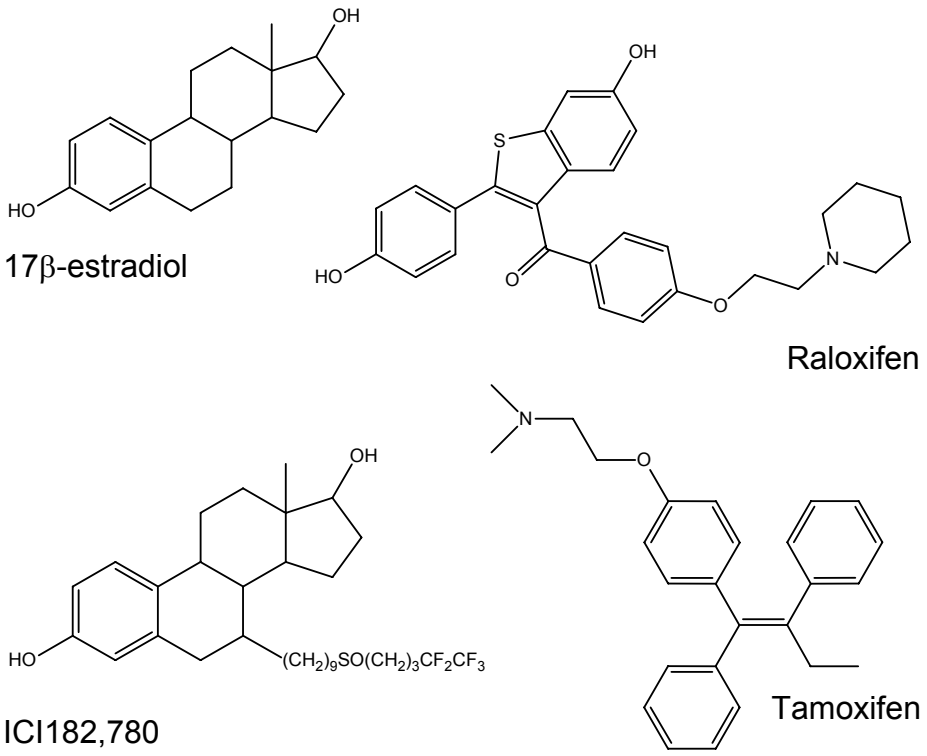

Fig. 7 Structures of estrogenic and antiestrogenic compounds.

The effects of estrogens can be antagonised by antiestrogenic compounds (Fig. 7). Tamoxifen (TAM) and Raloxifen (RAL) belong to the group of selective estrogen receptor modulators (SERMs). They are characterised as partial agonists depending on tissue and response examined. Due to the antagonistic effect on mammary tissues, SERMs are applied in the treatment of metastasised mammary carcinoma and as adjuvant to surgery of primary breast tumours, whereas in other tissues, like bone, adipose tissue, and endometrium, (Gottardis et al., 1988) an agonistic effect has been reported, which limits the clinical efficacy. In contrast, ICI182,780 (7α-[9-(4,4,5,5,5-pentafluoro-pentylsulphinyl)nonyl]estra-1,3,5(10)-triene-3,17β-diol) was de-

signed to be devoid of estrogenic activity and is therefore referred to as pure antagonist (Howell et al., 2000; Wakeling and Bowler, 1988; Wakeling and Bowler, 1988). ICI182,780, marketed under the trade name Faslodex^{TM} (Howell et al., 2000), is applied for treating estrogen receptor dependent tumours and is a more potent inhibitor of MCF-7 cell growth than TAM (Wakeling and Bowler, 1992).

In general, it is assumed that antiestrogens antagonise estrogen action by competing with estrogens for receptor binding. The detailed mechanism of antihormone functioning, however, especially of partial ER antagonists, is complex and not totally elucidated yet. TAM and RAL may prevent the repositioning of helix 12, which is critical for transcriptional activation by ERs (Osborne et al., 2000). It was further suggested that SERMs repress ER functioning by promoting the association of the receptor with specific transcriptional corepressors (Osborne et al., 2000). The specific response of cells to SERMs may depend on the balance between activator and repressors in the cell. Even in case of ICI182,780, different mechanisms are discussed. In contrast to SERMs, this antihormone blocks transcriptional activation originating at both AF-1 and AF-2 domains (Wakeling, 1995). The drug may impair receptor dimerisation, but most importantly it induces ER degradation. Thus, in divers studies it was concluded that ICI182,780 competitively binds to the ER and triggers a rapid degradation of the receptor and decreased nuclear localisation (DeFriend et al., 1994; Diel et al., 1999; Dudley et al., 2000; Howell et al., 2000; Pink and Jordan, 1996). Other investigators assume that ICI182,780 blocks the nucleocytoplasmic shuttling of the ER by inhibiting its translocation into the nucleus (Dauvois et al., 1993). Some of these ideas have been combined in another study, where ICI182,780 induces interactions of ERα with the cytokeranines CK8 and CK18 in the nuclear matrix, thereby inhibiting proper nuclear import and instead, bringing the receptor in close proximity to proteasomes, where it is degraded by the ubiquitin pathway (Long and Nephew, 2006). The effects of ICI182,780 on conformation and stability of the ER isoforms appear to be distinct. Most studies solely proof a downregulation of ERα. By facing both subtypes, some investigators have even suggested that the agent fails to inhibit ERβ (Oliveira et al., 2003; Pace et al., 1997; Van Den Bemd et al., 1999).

1.3.4 Alternative estrogen pathways

In addition to hormone mediated regulation of the ER, it is now well accepted that ER signalling is intertwined with a set of cellular mediators. Not all estrogenic effects can be explained by direct transcriptional regulation of target genes through ERs. With respect to estrogenic effects, two mechanisms are distinguished: genomic, also referred to as chronic effects, and non-genomic, so-called acute effects (Fig. 8).

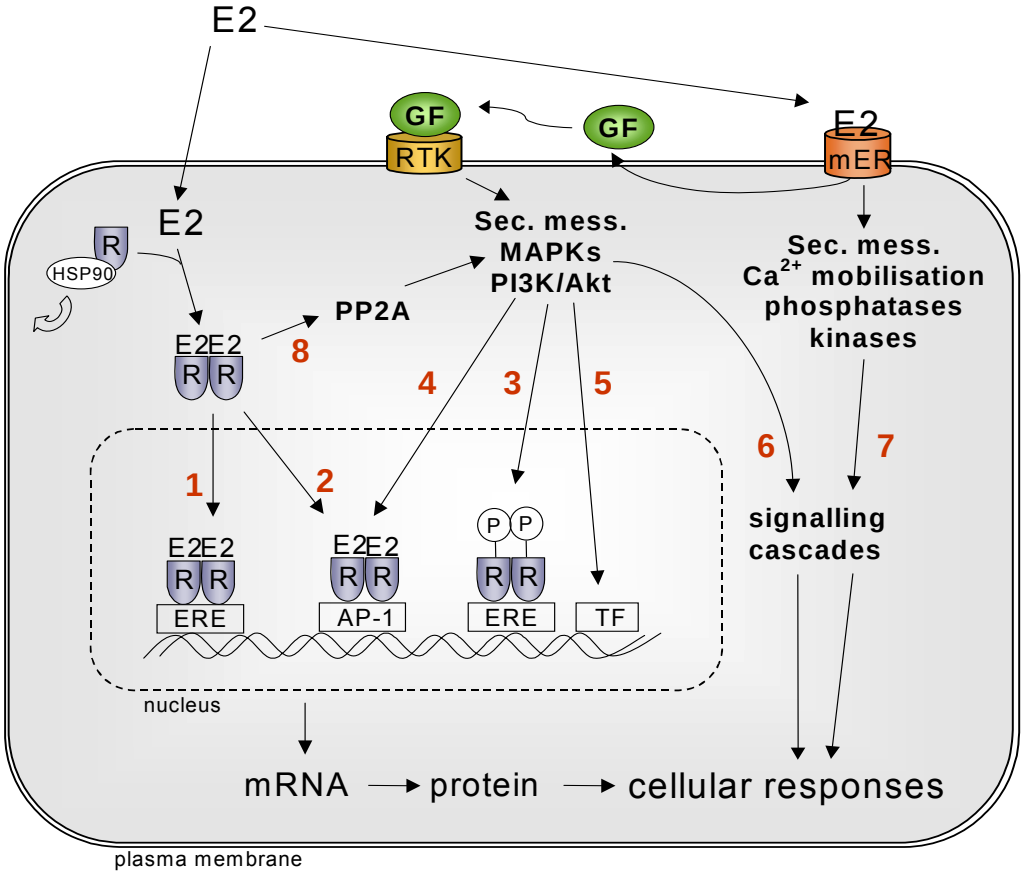

Fig. 8 Interconnection of genomic and non-genomic estrogen effects with MAPK pathways.

Transcriptional activation of target genes by E2 (genomic effect) occurs via
(1) the classical mechanism of nuclear ERs (R) at estrogen response elements (EREs) or
(2) ERE-independently via recruitment of transcription factors to AP-1 (activator protein-1) or SP-1 by nuclear ERs.

Growth factor (GF) binding to receptor tyrosine kinases (RTKs) inducing an activation of MAPKs and PI3K may provoke transcriptional regulation
(3) ligand independently via phosphorylation of nuclear ERs,
(4) ERE-independently at AP-1 or SP-1 binding sites, or
(5) independently of nuclear ERs by recruitment of transcription factors (TF).

Non-genomic effects appear via membrane estrogen responsive receptors by
(6) induction of signalling cascades through MAPKs or PI3K/Akt or
(7) direct activation of enzymes, second messengers (sec. mess.), or Ca^{2+}-mobilisation, or via nuclear ERs by
(8) interaction with divers intracellular targets (PP2A: protein phosphatase 2A).

Many classes of steroid hormones, among these estrogens, induce rapid (within seconds and minutes) activation of signalling molecules and signal transduction pathways. These events appear to proceed independently of the classical pathway involving ERE activation, but rather through an interplay between intracellular ERs with divers signalling molecules, including protein phosphatase 2A (PP2A) (Belcher et al., 2005), or a novel class of ERs localised at the plasma membrane. The specific response to estrogens is dependent on the biological status of the concerning cell or tissue. The following sections provide a general overview of the divers and complex field of estrogenic pathways.

1.3.4.1 Cross-talk with growth factors: Ligand independent ER activation

Several studies established the potential of non-estrogenic substances to activate ERs and target gene expression in the absence of ligand, among these extracellular signals, such as EGF (Curtis et al., 1996; Ignar-Trowbridge et al., 1995; Marquez et al., 2001), IGF-1 (Ignar-Trowbridge et al., 1996; Lee et al., 1997; Thorne and Lee, 2003), TGF-α (Ignar-Trowbridge et al., 1996), and heregulin (Pietras et al., 1995) as well as intracellular signal molecules like 8-bromo-cyclic adenosine monophosphate, an activator of protein kinase A (PKA) (Smith, 1998). This trans-activation of ER is mediated by downstream targets of RTKs, predominantly MAPKs and PI3K (Kato et al., 1995; Martin et al., 2000; Simoncini et al., 2000).

Upon binding of growth factors to their membrane associated receptors with tyrosine kinase activity, these are subject to autophosphorylation on tyrosine residues and thereby achieve the ability to activate several signalling pathways. Among these the MAPK signalling cascade, for example activated by the EGF receptor, plays a central role. It includes the recruitment of Ras interacting with MAPK kinase kinases, like Raf-1, that activate MAPK kinases, Mek1 and 2, which then phosphorylate MAPKs, such as ERK1/2. Activated MAPKs may mimic genomic estrogen effects by interaction with multiple target proteins, for instance the transcription factors c-Jun or ATF-2 (activating transcription factor) (Lewis et al., 2005; Monno et al., 2000).

Ligand autonomous, growth factor driven activation of ERs appears to be dependent on phosphorylation of serine residues in the AF-1 domain, for example serine 118 of human ERα (Bunone et al., 1996; El-Tanani and Green, 1997; Kato et al., 1995). By this means ERs may interact with the coactivator p68 RNA helicase and activate

target gene transcription (Kato, 2001). Another phosphorylation site for MAPKs is situated at tyrosine 537 in the LBD of the ER, which appears not to be addressed by an E2 stimulus (Arnold et al., 1995). Amino acid substitution experiments revealed the importance of tyrosine 537 for ER function, implying that this amino acid is a target for alternative ER activation (White et al., 1997). Following ligand independent phosphorylation of ERs, the coactivator SRC-1 (steroid receptor coactivator -1) is recruited, which is essential for the transcriptional activity of ERα and β (Onate et al., 1995).

In addition to the ERK1/2 signalling pathway, responsive to divers growth factors, mitogens, and G-protein coupled receptors (GPCRs), there exist two more MAPK pathways. Stress activated protein kinase/JNK (SAPK/JNK) and p38 MAPK are activated in response to stress (UV and γ radiation, heat shock, osmotic stress, and reactive oxygen species) as well as GPCR agonists, inflammatory cytokines, and growth factors. They are mediators of differentiation, inflammatory, apoptotic, and proliferative events. Although MAPK pathways can be activated by a complex set of effects, to date only growth factor induced MAPK signalling has been proved to modify ER functioning.

It has been demonstrated that growth factors (EGF, IFG-1, TGF-α) participate in the regulation of uterine biology by interacting with estrogens (Klotz et al., 2002; Nelson et al., 1991). Moreover, in MCF-7 cells these chemokines mimic estrogen actions by inducing expression of estrogen responsive genes, like the progesterone receptor (PR) (Aronica and Katzenellenbogen, 1991; Katzenellenbogen and Norman, 1990). In contrast, TGF-β 1 treatment of MCF-7 cells results in a decline of ER protein expression, but does not affect ER activity (Stoica et al., 1997). A cross-talk between IGF-1 and estrogens with bi-directional regulation plays a decisive role in mammary gland development, but it is also implicated in cancerous processes and tumour progression in breast tissues (Thorne and Lee, 2003). Increasing evidence exists that the resistance of tumours to endocrine treatment is often associated with certain growth factor and cellular kinase pathways (Rau et al., 2005; Schiff et al., 2004).

It was further reported that, apart from its autocrine and paracrine effects on tumour cell growth, the TGF-β signal transduction pathway communicates with estrogens, and it was supposed that alterations in this cross-talk contribute to breast tumour development. Several experiments demonstrated that estrogens inhibit TGF-β

(Matsuda et al., 2001) and BMP (Yamamoto et al., 2002) signalling to specific reporter plasmids and gene expression, while ER mediated transcriptional activation was enhanced by these chemokines (Matsuda et al., 2001).

1.3.4.2 ERE-independent genomic actions of E2

A couple of studies revealed that agonist bound ERα may induce gene transcription without ERE involvement by recruitment of activator protein-1 (AP-1) to special DNA sequences, a pathway which has been implicated in the agonistic effect of SERMs (Kushner et al., 2000; Paech et al., 1997; Webb et al., 1995). Thus, expression of IGF-1 (Umayahara et al., 1994) and collagenase (Webb et al., 1992) appears via interaction of ERα with the transcription factors c-Fos and c-Jun at AP-1 binding sites. Furthermore, ER may also interact with the transcription factor SP1 forming a transcriptionally active ERα/Sp1-protein complex. In ER positive breast carcinoma cell lines this complex confers ERE independend estrogen responsiveness to the oncogene *c-myc* (Dubik and Shiu, 1992), the *c-fos* gene (Duan et al., 1998), the *retinoic acid receptor* α gene (Rishi et al., 1995), and the *cathepsin D* gene (Krishnan et al., 1994).

1.3.4.3 Estrogen signalling via receptors at the plasma membrane

In bone, breast, and the vascular and nervous system estrogens stimulate multiple second messenger signal transduction cascades as well as kinase and phosphatase activation and calcium mobilisation within seconds or minutes. These effects may contribute to gene transcription in a manner as described in section 1.3.4.1 and 1.3.4.2, but more frequently to non-genomic cellular responses. These steroid actions arise at estrogen responsive receptors localised at the plasma membrane (mERs). Estrogen binding to the surface of endometrial cells had been reported in 1977 in the first place (Pietras and Szego, 1977), but was dismissed by the majority of investigators as a contamination of the preparated membranes with ERα. Only in recent years, more accepted evidences for the existence and physiological significance of mERs have been provided. Although the presence of mERs has been proved, until today divers ideas exist about their nature, giving reason to a lot of

controversy in literature. Different identifications of putative membrane estrogen receptors, their incidences, and functional characteristics are listed in Table 1.

Table 1 Putative membrane estrogen receptors, their distributions, and defining characteristics (modified according to Hasbi et al., 2005).

Name	Distribution	Characteristics	References
pER	mouse liver, skeleton, neurons, vasculature, retina, breast carcinoma, endometrium, prostate	serine phosphatase activity, on cell surface or nuclear membrane	1
ER-X	mouse brain neocortex, uterus	MAPK/ERK activation	2, 3
ERα, ERβ	breast tumour cell line MCF-7, rat pituitary tumour cell line GH3/B6/F10, endothelial cells, monkey kidney cells COS1, mitochondria of MCF-7 and endothelial cells	identical genetic origin like nuclear ERα/β, EGFR trans-activation, ERK, adenylyl-cyclase, PI3K, and G_i-, G_s-, or G_q-protein activation, localised in caveolae	4 - 14
GPR30	heart, breast, lung, CNS, vascular endothelium, leucocytes, breast tumour cell lines MCF-7 and SKBR3, human keratinocytes	EGFR trans-activation and MAPK/ERK activation, adenylylcyclase, PKA, PKC, Src tyrosine kinase, and NO synthase activation, mobilisation of intracellular Ca^{2+}, *c-fos* expression, G-protein activation	15 - 25
unidentified	chinese hamster ovary cells CHO-K1, monkey kidney cells COS7, rat2 fibroblasts	MAPK activation	26
unidentified	isolated endometrial cells	binds E2	27
unidentified	mouse cerebral cortical explants	MAPK/ERK and B-Raf activation	28
unidentified	Guinea pig hypothalamic GABA, POMC, and dopamine neurons	modulation of γ-opioid and $GABA_B$ neurotransmission, PKC, G_q-protein activation	29, 30
unidentified	Guinea pig hypothalamic neurons	attenuation of γ-opioid antagonist activation of inward rectifying K^+ conductance, involvement of PKA	31
unidentified	neuronal cell line GT1-7	modulation of acetylcholine induced Ca^{2+} mobilisation	32
unidentified	pancreatic beta cells	activated by xenoestrogens and E2, G-protein activation	33

unidentified	mouse macrophages IC-21	G-protein activation, internalisation upon agonist binding independently of caveolae and clathrin-coated vesicles	34
na-ER	goat uterine plasma membrane	internalisation depedent on clathrin-coated vesicles	35

1 (Rao, 1998); **2** (Toran-Allerand et al., 2002); **3** (Toran-Allerand, 2004); **4** (Norfleet et al., 1999); **5** (Pappas et al., 1995); **6** (Razandi et al., 1999); **7** (Razandi et al., 2004); **8** (Pedram et al., 2006); **9** (Chambliss et al., 2002); **10** (Zhang et al., 2002); **11** (Campbell et al., 2002); **12** (Zivadinovic et al., 2005); **13** (Wyckoff et al., 2001); **14** (Chen et al., 2004); **15** (Sukocheva et al., 2006); **16** (Filardo and Thomas, 2005); **17** (Filardo et al., 2002); **18** (Filardo, 2002); **19** (Filardo et al., 2000); **20** (Carmeci et al., 1997); **21** (Kanda and Watanabe, 2003); **22** (Thomas et al., 2005); **23** (Revankar et al., 2005); **24** (Ylikomi et al., 2004); **25** (Maggiolini et al., 2004); **26** (Nethrapalli et al., 2005); **27** (Pietras and Szego, 1977); **28** (Singh et al., 2000); **29** (Qiu et al., 2003); **30** (Kelly et al., 2003); **31** (Lagrange et al., 1997); **32** (Morales et al., 2003); **33** (Nadal et al., 2000); **34** (Benten et al., 2001); **35** (Sreeja and Thampan, 2004)

GABA: γ-amino butyric acid; PKA: protein kinase A; PKC: protein kinase C; NO: nitric oxide; POMC: proopiomelanocortin; CNS: central nervous system

In consideration of the multitude of oppositional opinions emerging in literature, it appears that different types of mERs exist and are coexpressed, for example in MCF-7 cells. Several investigators favour the idea that the mER is related to the nuclear receptors ERα and ERβ (Morales et al., 2003; Norfleet et al., 1999; Pappas et al., 1995; Wyckoff et al., 2001). Most of these reports refer to an mER with homology to ERα, but there are also indices for an mER analogous to ERβ (Chambliss et al., 2002; Pedram et al., 2006; Razandi et al., 1999; Razandi et al., 2004). Only recently, even in the mitochondria of MCF-7 and endothelial cells high affinity estrogen receptors compatible with ERα and ERβ have been identified (Chen et al., 2004; Pedram et al., 2006). Membrane localisation and function of ERα has been referred to the NLS in the E-domain and the DBD (Chambliss et al., 2005) as well as serine 522 (Razandi et al., 2003) and cysteine 447 (Acconcia et al., 2005) residues of the receptor. Moreover, E2 activates a classical growth factor signalling pathway inducing interactions between the A/B domain of ERα with the adapter protein Shc (Src-homology and collagen-homology). These interactions may contribute to E2 induced formation of membrane ruffles and pseudopodia as well as the accumulation of ERα at the membrane (Song et al., 2002).

For several steroids, unconventional membrane located forms of receptors have been found, as for instance for the androgen receptor (AR) (Benten et al., 1999; Lieberherr and Grosse, 1994) and the PR (Zhu et al., 2003; Zhu et al., 2003). In

recent years, however, several rapid estrogen mediated effects could be traced back to membrane associated estrogen responsive receptors (Rambo and Szego, 1983). These are unrelated to their intracellular counterparts, but instead exert characteristics of GPCRs. In the course of these discoveries, attention was directed to an orphan member of GPCRs, GPR30, which revealed facilities of an estrogen responsive receptor (Thomas et al., 2005). Regarding its structural sequence homology to receptors for angiotensins, interleukins, and a variety of chemokines, it had been suggested to be a receptor for peptide ligands (Carmeci et al., 1997; Takada et al., 1997). However, this assumption has been disproved (Feng and Gregor, 1997; Owman et al., 1996). Characteristically for members of the large superfamily of GPCRs, it consists of seven membrane spanning helices and transduces extracellular stimuli into intracellular signals through interaction of its cytosolic domain with heteromeric G-proteins.

GPR30 exhibits a widespread expression pattern with abundant levels not only in breast cancer, but also in placental, bone, brain, ovarian, prostate, vascular epithelial, and hepatic tissues. A large number of investigators proved that GPR30 accounts for a substantial set of rapid cellular responses to estrogens. Since most of GPR30 expressing tissues are considered to be ER positive, it is adjacent to assume that their estrogenic responses are partially mediated by GPR30. GPR30 demonstrably regulates the phosphorylation state of ERK1/2 (Filardo et al., 2000; Filardo et al., 2002; Sukocheva et al., 2006), induces mobilisation of intracellular calcium (Improta-Brears et al., 1999; Revankar et al., 2005), cAMP (cyclic adenosin monophosphate) release (Filardo et al., 2002), and synthesis of PI3K (Revankar et al., 2005). In the course of mER signalling, a trans-activation of EGFR, IGF-1 receptor, and human epithelial growth factor receptor type 2/neu (Her-2/neu) occurs, leading to the activation of their downstream mediators ERK1/2 and PI3K/Akt (Filardo, 2002; Kahlert et al., 2000; Stoica et al., 2003). By this means, estrogens may induce cell proliferation without involvement of gene transcription. Moreover, ERK1/2 activation by E2 in MCF-7 cells also partially occurs by secretion of heregulin, which activates the HER-2/neu receptor, PKC δ, and the Ras pathway (Keshamouni et al., 2002). As depicted in chapter 1.3.4.1 activated MAPKs and PI3K may phosphorylate certain residues of nuclear ERs promoting their transcriptional activity or stability.

A growing body of evidence suggests that signalling through mERs is interconnected with an activation of G-proteins (Filardo et al., 2002; Razandi et al., 1999; Thomas et al., 2005; Wyckoff et al., 2001). Not only the GPCR GPR30, but also membrane localised ERα and β have been demonstrated to be dependent on G-protein activation (Razandi et al., 1999; Razandi et al., 2004; Wyckoff et al., 2001). It was suggested that mERs exist as a cytoplasmatic pool embedded into caveolae similar to some growth factor receptors (Razandi et al., 2002). In a large signalling complex, mER assembles with G-proteins, RTKs (EGFR, IGF-1 receptor), and non-receptor RTKs, like Src.

Activation of target genes via mERs also appears independently of nuclear ERs. As an example, PI3K and Akt, activated through mER, demonstrably induce transcriptional regulation of 250 genes, thus affecting multiple aspects of cellular functions (Pedram et al., 2002). In addition to the ERE-independent genomic functions of estrogens as described in section 1.3.4.2, activation of the *c-fos* gene may be mediated through interaction of MAPKs and PI3K with transcription factors (Duan et al., 2001; Duan et al., 2002).

Several non-transcriptional effects of mERs have been reported, primarily via posttranslational modification of phosphatases or kinases. Indicative of this is the down-regulation of MAPK phosphatase 1 activity by E2 resulting in a rapid increase of ERK activity in breast cancer cells (Razandi et al., 2004). Rapid estrogen effects include calcium flux in several cell types (Armen and Gay, 2000; Kelly et al., 1977; Mermelstein et al., 1996). It was further presented that rapid mobilisation of intracellular calcium precedes MAPK activation in MCF-7 cells (Improta-Brears et al., 1999) and the release of nitric oxide (NO) in bovine aortic endothelial (BAECs) (Goetz et al., 1999) and uterine artery endothelial cells (UAECs) (Chen et al., 2004) by E2, an effect which has been implicated with vasodilatory processes.

1.4 Breast cancer cells

Breast cancer is the most common malignancy among women in the western world and the second leading cause of cancer deaths in woman today, only exceeded by lung cancer. MCF-7 and MDA-MB-231 cells are cell lines of epithelial origin from mammary carcinomas. MCF-7 cells were established in 1970 from the pleural effusion of a 69-year old Caucasian woman whose metastatic mammary adenocarcinoma was medicated with radio and hormone therapy for three years (Soule et al., 1973). This well-characterised cell line expresses ERα in large scale and moderate levels of ERβ (Watanabe et al., 1997) (Fuqua et al., 1999; Vladusic et al., 2000), but also receptors for progesterones and androgens (Horwitz et al., 1975). Due to its high hormone receptor content, it is referred to as hormone dependent.

Similar to MCF-7 cells, the MDA-MB-231 cell line was isolated from a malign pleural effusion of a 51-year old Caucasian woman with metastasising breast carcinoma after a three week chemotherapeutic treatment in 1974 (Cailleau et al., 1974). On the basis of the low expression levels of hormone receptors - small amounts of ERβ and no ERα - MDA-MB-231 cells are considered as hormone independent (Fuqua et al., 1999; Vladusic et al., 2000). By virtue of these substantial differences in the expression profile for ERs, these cell lines are often used as model systems for *in vitro* studies of hormone functions.

Furthermore, they often apply for cell invasion assays due to their markedly varying migratory potential. While MCF-7 cells are poorly invasive, MDA-MB-231 cells reveal high migration rates, even in the absence of chemotactic stimuli. The reason for this difference is complex. High activation and receptor expression levels for the serine protease urokinase plasminogen activator (uPA), a ECM degrading protein, in MDA-MB-231 cells is causally involved in cancer invasion and metastasis and is a prognostic marker for breast cancer patients (Duffy, 2002; Holst-Hansen et al., 1996). Migration not only depends on traction on the ECM, but, moreover, migrating cells must overcome contact inhibition that blocks cell motility upon formation of cell-cell contacts, called adherens junctions. Multiprotein complexes assembled around cadherins, for instance e-cadherin, established these linkages. Cadherins are transmembrane proteins that hook into cadherins of adjacent cells and interact with

cytoplasmic proteins, such as α-, β-, and γ-catenins that transmit the adhesion signal and connect the adherens junctions with the cytoskeleton.

The migratory behaviour of MDA-MB-231 and MCF-7 cells is reflected in their e-cadherin expression status with high protein levels in poorly migrating MCF-7 cells (Corn et al., 2000; Nakamura et al., 2003) and almost no detectable expression in highly invasive MDA-MB-231 cells (Wong and Gumbiner, 2003).

Disruption of the e-cadherin/catenin complex, usually by interruption of e-cadherin expression, has been shown to be correlated with breast tumour invasion, metastasis, and proliferation (Bukholm et al., 1998; De Leeuw et al., 1997; Hunt et al., 1997; Kowalski et al., 2003; Meng et al., 2000; Siitonen et al., 1996).

1.5 Goals of this study

Estrogens are considered as risk factors for tumourigenesis, especially in tissues that respond to sex steroids with receptor mediated proliferation, namely breast epithelium and the endometrium. TGF-β acts as a potent inhibitor of tumour progression, owing to an induction of growth arrest and apoptosis. However, TGF-β concomitantly functions as a tumour promoter. The balance between tumour promoting and suppressing effects may be destabilised in late stage tumours when the sensibility of cells towards the antiproliferative and proapoptotic effects of the chemokine declines during tumour progression. Concomitantly, its migratory properties gain in importance, contributing to the metastatic spread of cancer cells.

Interactions between estrogens and growth factors, especially IGF-1 and EGF, play a crucial role in growth and development, but also in pathological processes and have been characterised extensively. Similar correlations may be expected for TGF-β. Nevertheless, there exist only few concrete findings regarding this question. The ability of cancer cells to migrate to a chemotactic stimulus is considered to be indicative of their metastatic potential. In this study, the migratory potential of TGF-β and E2 on mammary carcinoma cells has been characterised more closely.

Indications exist for a negative regulation of TGF-β function by estrogens, which might exert protective effects in advanced tumours. The influence of estrogens on TGF-β induced processes has been investigated with particular regard to the migratory ability of hormone dependent and independent breast cancer cells. Moreover, the cellular mechanisms underlying this cross-talk have been of particular interest.