

# Interactions between estrogen and insulin-like growth factor signaling pathways in human breast tumor cells

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## Abstract

Estrogens and insulin-like growth factors (IGFs) act as mitogens promoting cell proliferation in normal breast tissue as well as in breast carcinomas. Both hormones have been shown to play a role in the development of breast cancer and were found to activate multiple signaling pathways leading to proliferation of human breast cancer cell lines *in vitro*. Originally, it was considered that these agents manifest their mitogenic actions through separate pathways, but a growing body of evidence suggests that the IGF- and estrogen-mediated signaling pathways are intertwined.  $17\beta$ -Estradiol (E2) has been shown to enhance IGF signaling at multiple levels. E2 treatment of breast cancer cells alters expression of nearly all of the IGF family members including IGF-I, IGF-II, IGF-binding proteins, IGF type I receptor (IGF-RI), and insulin receptor substrate 1. The ligand-bound estrogen receptor has been reported to bind to and to activate the IGF-RI directly. Vice versa, IGF signaling has been reported to enhance estrogen receptor activation in human breast cancer cells by inducing phosphorylation of the estrogen receptor. Finally, several groups have described synergistic effects of the combination of E2 and IGF-I on S phase entry in breast tumor cell lines. Here, we review recent, often contradictory, reports describing the effects of E2 and IGFs on the proliferation of breast tumor cells, with special emphasis on the synergistic effects of the two hormones.

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## Introduction

Breast cancer is the most common cancer in women worldwide and continues to be a major health problem (Harris *et al.* 1992, Henderson 1993, Garfinkel *et al.* 1994, Henson & Tarone 1994). Research in breast cancer now extends in many directions, from identification of genes that predispose to breast cancer, to cellular models for preneoplastic disease, to investigation of the tumor and its local environment, and to identification of environmental risk factors for the development of breast cancer.

For some tumors, like colorectal cancer, the most common sequence of genetic events and their timing, eventually leading to tumor formation, has been determined (Fearon & Vogelstein 1990). In contrast to colorectal cancer, pathological findings in breast cancer are highly variable (Beckmann *et al.* 1997). The evolution of breast cancer and the relationships of genetic predisposing factors with somatic changes are very complicated. Table 1 shows some of the genetic and hormonal factors that are involved in the development of breast cancer and progression of the disease. Sev-

**Table 1** Genetic and hormonal factors involved in the development and progression of breast cancer

	Gene, hormone, or growth factor	Reference
Predisposing genetic factors	BRCA1 and 2	Venkitaraman (2002)
	<i>p53</i>	Blackburn & Jerry (2002)
Hormones and growth factors	Estrogen, progesterone, and prolactin	Portier (2002)
	IGFs	Sachdev & Yee (2001)
	EGF	Biscardi <i>et al.</i> (2000)
	TGF $\beta$	Wakefield <i>et al.</i> (2001)

eral genetic alterations have been associated with an increased risk of breast cancer development. Mutations in two breast cancer susceptibility genes, BRCA 1 and 2 have been shown to predispose women to breast cancer, however mutations in these genes account for only 5% of breast

cancer cases. The factors responsible for the remaining 95% are still obscure (Venkitaraman 2002). A significant proportion of sporadic breast cancers show point mutations clustered within exons 5–8 of the *p53* gene (Blackburn & Jerry 2002).

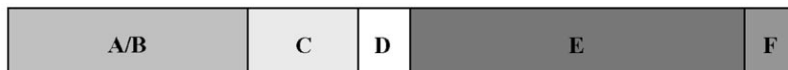
Other factors known to be involved in breast cancer development are hormones and growth factors. The finding that breast cancer is rare in males as well as in non-estrogenized women suggests that most, if not all, breast cancers originate as a hormone-dependent disease. Moreover, prolonged estrogen exposure, caused by early menarche (MacMahon *et al.* 1970), late menopause (Trichopoulos *et al.* 1972) or late age at first pregnancy (MacMahon *et al.* 1970) is associated with increased risk of developing breast cancer. Abundant evidence indicates that other hormones, like insulin-like growth factors (IGFs), epidermal growth factor (EGF), and transforming growth factor (TGF)- $\beta$  affect breast cancer growth as well (Biscardi *et al.* 2000, Sachdev & Yee 2001, Wakefield *et al.* 2001, Portier 2002). Studies from several laboratories have shown that IGF-I and IGF-II are potent mitogens for breast cancer cells (Ellis *et al.* 1996, Dufourmy *et al.* 1997), and may act synergistically with estrogen to stimulate cell proliferation (Van der Burg *et al.* 1988). When their actions are blocked, inhibition of tumor growth is observed (Osborne *et al.* 1990). This review will focus on the importance of estrogen and IGFs in the development of breast cancer, and of the signaling pathways activated by the two hormones, leading to cell proliferation of breast cancer cells. In particular, the mechanisms proposed for the observed synergistic actions of estrogens and IGF will be reviewed.

## Estrogen signaling in breast cancer

Clinical studies on estrogen deficiency syndromes in humans (Pertzelan *et al.* 1982) have implicated estrogen in the normal development of the breast. Definitive evidence for the importance of estrogen signaling in normal mammary gland development has been obtained from experiments in mice. First, castrated immature mice do not show ductal growth through the fat pad of the mammary gland, signifying that mammary ductal development is hormone dependent (Borellini & Oka 1989). Secondly, the mammary glands of ovariectomized mice are stimulated to grow by implanted estrogen pellets (Daniel *et al.* 1987), and implants of pure anti-estrogens inhibit mammary growth in intact mice (Silberstein *et al.* 1994). Finally, female estrogen receptor (ER) knockout mice develop mammary glands with only vestigial ducts present at the nipples (Korach 1994).

Epidemiological data strongly suggest an association between estrogens and the risk of breast cancer. The estrogen responsiveness of malignant breast epithelial cells has been demonstrated in culture and in tumor xenografts in nude mice (Shafie 1980). The primary physiological effects of estrogen are therefore major factors in both development and progression of the disease (Lupulescu 1993).

In general, estrogens induce their physiological effects through an interaction with ERs, of which two isoforms ( $\alpha$  and  $\beta$ ) have been identified. The ERs are members of the steroid hormone–thyroid hormone–retinoic acid receptor superfamily of nuclear transcription factors (Tsai & O’Malley 1994, Beato *et al.* 1995, Beato & Klug 2000). Six



Domain	Domain function	Difference in $\alpha/\beta$ isoform
A/B	Constitutive transcription activation (AF-1; cell- and promoter-specific activity)	Domain with the biggest differences in sequence In ER $\alpha$ AF-1 activity much stronger
C	DNA binding NLS	–
D	Hinge:Transcriptional repression	–
E	Estrogen-dependent transcription activation (AF-2; cell- and promoter-specific activity) Hormone binding Co-factor binding dimerization	Some divergence in sequence No difference in AF-2 activities
F	?	–

**Figure 1** Schematic representation of the ER, showing the different domains and their function. NLS, nuclear localization signal.

conserved functional domains, A–F, are common structural features of this family of proteins (Fig. 1) (Gronemeyer 1991). The A/B region in the amino-terminal end of the protein shows the highest variability in sequence and size among the family members. This domain was shown to contain a constitutive, hormone-independent transcription activation function (AF-1) (Tora *et al.* 1989, Gronemeyer 1991). Domain C encompasses two zinc finger DNA-binding motifs responsible for recognition of the *cis*-acting hormone response element, and a nuclear localization signal (NLS). The D or hinge region may be involved in steroid-mediated transcriptional repression (Adler *et al.* 1988). The E domain, the most complex region, contains the hormone-binding site, the region required for stable dimerization of the receptor, and a second estrogen-inducible transcription activation function (AF-2) (Tora *et al.* 1989, Gronemeyer 1991). The function of the F region is presently unclear, a possible interaction with cell type-specific factors that regulate ER function has been proposed (Montano *et al.* 1995).

Although ER $\alpha$  was cloned over 10 years ago (Green *et al.* 1986), ER $\beta$  has been discovered only recently (Mosselman *et al.* 1996, Kuiper *et al.* 1996). ER $\alpha$  and ER $\beta$  diverged early during evolution (Kelley & Thackray, 1999), and differ mostly in the N-terminal A/B domain and to a lesser extent in the ligand-binding domain (E domain). These differences suggest that the two receptors could serve distinct actions. Indeed, their transcription activation domains, AF-1 and AF-2, display activities that are promoter and cell-type specific (Tora *et al.* 1989, McInerney *et al.* 1998, Cowley & Parker 1999). Cowley & Parker (1999) have shown that the AF-1 activity of ER $\beta$  is weak, compared with that of ER $\alpha$ , on estrogen-responsive promoters, whereas their AF-2 activities are similar.

In the classical model of estrogen action, estrogens bind the E region in the ER, thereby inducing an allosteric conformational change, which facilitates dimerization of the receptor. The receptor dimer then binds with high affinity to DNA, resulting in activation of transcription (Tsai & O'Malley 1994). Targets of classical estrogen action include many genes involved in proliferative signaling and cell cycle progression, i.e. cyclin D1, *c-fos*, *c-jun*, *c-myc* and *c-myb* (Loose-Mitchell *et al.* 1988, Weisz & Bresciani 1993, May & Westley 1995, Altucci *et al.* 1996). Other gene products, like p21<sup>*cip1/waf1*</sup>, p27<sup>*kip1*</sup>, and TGF $\beta$ , which prevent cell cycle progression, have been shown to be down-regulated by 17 $\beta$ -estradiol (E2) treatment of an epithelial breast cancer-derived ER-positive cell line (MCF-7) (May & Westley 1995, Foster *et al.* 2001).

Several co-activators (SRC-1, TIF2, AIB1, CBP and P/CAF) and co-repressors (N-CoR and SMART) have been shown to modulate the transcriptional activity of the ER. In the absence of ligand, the ER is unable to bind steroid receptor co-activators (SRCs) directly, as its leucine-rich co-activator interaction motif (in the E domain) is sterically

unavailable for interaction. Binding of estrogen to ER exposes AF-2 and allows recruitment of SRCs to the ER, leading to its activation. However, recently a hormone-independent binding of SRCs to the ER has been described by Zwijzen *et al.* (1997, 1998). They have shown that hormone-independent binding of cyclin D1 to the ER provides a leucine-rich interaction motif in the carboxy terminus of cyclin D1 for SRCs to bind to the cyclin D1/ER complex. The binding of SRCs to the motif in cyclin D1 has been shown to result in partial activation of the ER. When estrogen is added to this complex, it binds to the ER and thereby exposes a second leucine-rich binding motif inducing co-operative recruitment of SRCs to the complex. In this way, cyclin D1 and E2 can synergistically activate the ER (Zwijzen *et al.* 1997, 1998).

ER $\alpha$  and ER $\beta$  knockout mice have demonstrated strikingly different expression patterns and functions of the two ERs (Krege *et al.* 1998, Couse & Korach 1999). ER $\beta$  knockout mice show normal mammary gland development and lactation, whereas ER $\alpha$  knockout mice exhibit an estrogen-insensitive undeveloped mammary gland. ER $\beta$  thus does not seem to play an important role in proliferation of hormone-dependent breast cancer cells. ER $\beta$  expression is often down-regulated in breast cancer tissue when compared with expression in normal breast tissue (Iwao *et al.* 2000, Roger *et al.* 2001). Experiments in the human ER-negative MDA-MB-231 breast cancer cell line engineered to express functional ER $\beta$  have shown that ER $\beta$  expression inhibits proliferation, and decreases cell motility (Lazennec *et al.* 2001). This study has indicated that ER $\beta$  may even act as an inhibitor of breast cancer development, rather than inducing a mitogenic response like ER $\alpha$  (Ferguson & Davidson 1997). The expression of ER $\alpha$  is generally considered as a marker of breast cancer steroid hormone sensitivity (McGuire, 1978). The most widely used and highly successful group of drugs used in treatment of ER $\alpha$ -positive breast cancers are the anti-estrogens, which interfere with ligand-receptor binding (Early Breast Cancer Trialists Collaborative Group 1992, Kinsinger *et al.* 2002). The expression or function of ER $\alpha$  is often lost during progression of breast cancer (Clarke *et al.* 1989, Murphy & Watson 2002). Surprisingly, tumors lacking ER $\alpha$  expression are generally associated with poorer histological differentiation, higher growth fraction and worse clinical outcome than ER-positive cancers. This apparent paradox may be explained by the observation that ER $\alpha$  is only detected in tumor cells in a well-differentiated state. De-differentiated, fast growing carcinoma cells have often reached a hormone-independent state (reviewed in Lapidus *et al.* 1998).

In addition to the 'genomic' pathway of estrogen action, a growing number of reports have suggested that estrogens can act via additional signaling pathways in breast cancer cells. Numerous reports have linked E2 signaling to the

activation of cytoplasmic signaling cascades. An overview of the pathways, which have been described to be activated by E2, is presented in Table 2. Direct activation of the extracellular signal-regulated kinase (ERK)-1 and -2 cascade has been demonstrated by several laboratories. Migliaccio *et al.* (1996, 1998) and Castoria *et al.* (1999) have shown that the E2/ER complex binds and activates *c-src* in MCF-7 breast cancer cells. Activated *c-src* phosphorylates Shc, which then associates with the Grb2/mSos complex. Subsequently *c-raf1*, p21<sup>ras</sup> and ERK are activated. These authors have shown that activation of the ERK pathway is essential in the induction of cell proliferation by inhibiting this pathway with the MEK-inhibitor (MEK, MAP kinase kinase) PD98059. Furthermore, they have shown that the induction of cell cycle progression is not dependent on ER-induced transcriptional activity since a transcriptionally inactive mutant of ER is able to induce S phase entry. Improta-Brears *et al.* (1999) have confirmed the activation of the ERK pathway by E2. How-

ever, these investigators have not been able to detect any activation of *c-raf1* within the limits of their assay and have concluded that *c-raf1* is not an important component of E2-mediated ERK activation. Rather, they have demonstrated that addition of E2 to the culture medium of MCF-7 cells causes a release of intracellular calcium and that this leads to activation of ERK.

Several studies have reported the activation of the phosphatidylinositol 3-kinase (PI3-kinase) route by E2 in MCF-7 breast carcinoma cells. Castoria *et al.* (2001) have shown that E2 activates PI3-kinase signaling in addition to the ERK pathway. According to these authors, the activated ER forms a ternary complex with *c-src* and p85 $\alpha$  in an E2-dependent fashion. The formation of this ternary complex results in the activation of the PI3-kinase and of protein kinase B (PKB; Akt). Furthermore, they have shown that inhibition of the PI3-kinase pathway with the PI3 kinase inhibitor LY294002 inhibits E2-induced cyclin D1 upregulation and cell cycle

**Table 2** Non-genomic, mitogenic estrogen signaling in MCF-7 breast cancer cells: activation of cytoplasmic signaling cascades

Activated protein/ signaling cascade	Reference	Mechanism of activation	Other observations
ERK1 and 2	Migliaccio <i>et al.</i> (1996), Castonia <i>et al.</i> (1999)	The E2/ER complex binds to <i>c-SRC</i> . This results in the activation of the p21 <sup>ras</sup> /ERK pathway	
	Improta-Brears <i>et al.</i> (1999)	Mobilization of intracellular calcium	No activation of <i>c-raf-1</i> or hydrolysis of phosphatidyl-inositol was detected.
P13-kinase	Castoria <i>et al.</i> (2001)	ER $\alpha$ interacts with <i>src</i> and the p85 $\alpha$ subunit of P13-kinase and activates the kinase. Subsequently, Akt is activated.	Interaction is ligand independent.
	Sun <i>et al.</i> (2001)	ER $\alpha$ interacts with p85 $\alpha$ subunit of P13-kinase and activates the kinase. Subsequently, Akt2 is activated.	No evidence for an adaptor protein like <i>src</i> between ER and p85 $\alpha$ is shown. Interaction is ligand independent.
Akt1	Ahmad <i>et al.</i> (1999)	?	—
No direct activation of cytoplasmic signaling cascades	Lobenhofer <i>et al.</i> (2000)	—	Inhibition of ERK and P13-kinase pathways did result in a reduced mitogenic response. No phosphorylation or activation of either ERK or P13-kinase was detected directly after addition of E2 to the cells.
No activation of cytoplasmic signaling cascades	Caristi <i>et al.</i> (2001)	—	No activation of ERK, Jun-kinase, p38MAPK, or PKA could be detected after addition of E2 to the cells.
	Hamelers <i>et al.</i> (2002a)	—	No activation of PKB (Akt) or ERK was found up to 1 h after addition of E2 (activity measured in kinase assays).

P13-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; p38MAPK, p38 mitogen-activated protein kinase. Akt, homologue of v-akt (murine thymoma viral oncogene).

progression (Castoria *et al.* 2001). Sun *et al.* (2001) have found an interaction of ER with p85 $\alpha$  as well, which leads to the activation of PI3-kinase and Akt2 pathway. In contrast to Castoria *et al.* (2001) they have shown that the interaction of the ER and p85 $\alpha$  is ligand independent. The activation of PKB (Akt) has been confirmed by a report from Ahmad *et al.* (1999). The mechanisms by which E2 activates PKB have not been investigated. Activation of PKB by E2 has been shown to result in enhanced proliferation and survival of the MCF-7 cells (Ahmad *et al.* 1999).

Other reports have shown no direct activation of ERK and PI3-kinase signaling cascades by E2. Lobenhofer and colleagues (2000) have shown that treatment of MCF-7 cells with inhibitors of the PI3-kinase and the ERK pathway results in reduced cell cycle progression in E2-stimulated cells. However, they have not detected any phosphorylation or activation of either ERK or PI3-kinase after addition of E2 to the cells. They have concluded that either basal activity or delayed activation of these kinases is essential for E2-induced cell cycle progression. Caristi *et al.* (2001) and Hamelers *et al.* (2002a) have not detected any activation of cytoplasmic signaling cascades, in spite of close examination of the activity of PKA, p38MAPK, Jun-kinase (Caristi *et al.* 2001), ERK (Caristi *et al.* 2001, Hamelers *et al.* 2002a), and PKB (Hamelers *et al.* 2002a). Taken together, it remains unclear whether E2 is a physiological activator of cytoplasmic signaling cascades and, if so, what the contribution of this activation might be for the mitogenic effect of the hormone.

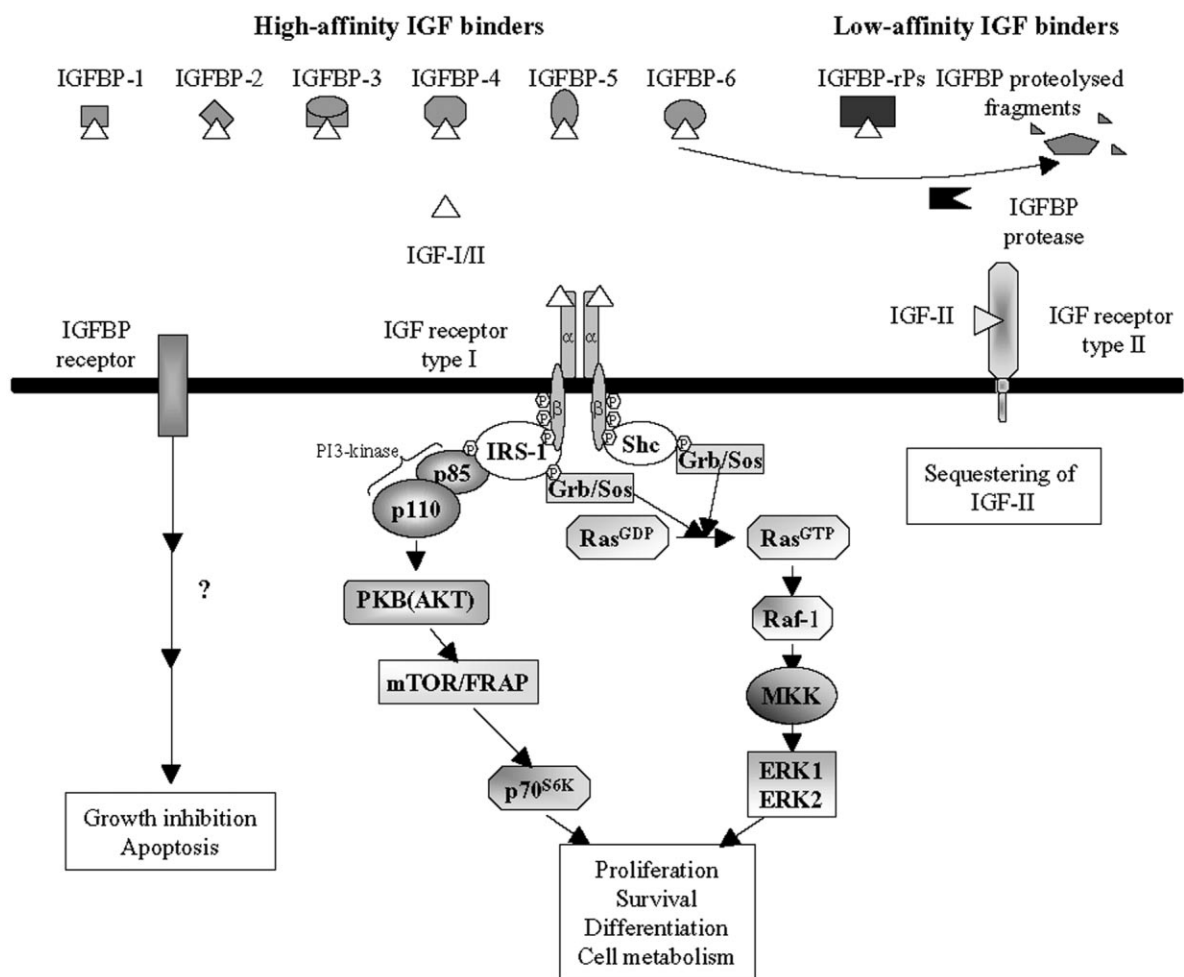
## The IGF system in breast cancer

The IGFs are a family of hormones with structural homologies to insulin (Rinderknecht & Humbel 1976a,b). IGFs were discovered during the search for factors that mediate the growth-stimulating effect of growth hormone (Salmon & Daughaday 1957). Further research revealed that two IGFs exist, IGF-I (70 amino acids long) and IGF-II (67 amino acids long) (Rinderknecht & Humbel 1978a,b). IGF-I and -II play pivotal roles in growth and development. IGF-II is most important during fetal development, although the circulating level of IGF-II in humans (in contrast to rodents) remains high postnatally. The levels of IGF-I are low during fetal development and rise gradually after birth, indicating that this growth factor is mainly involved in postnatal growth (reviewed in Steenbergh *et al.* 1997). In all stages of life, a clear pattern of tissue-specific expression of IGFs is observed. Hepatocytes are the main source of circulating IGFs but, in addition, numerous non-hepatic tissues are capable of IGF synthesis. Many cells have been identified as producing as well as responding to the IGFs, including fibroblasts, chondrocytes, osteoblasts, granulosa cells, and epithelial breast cells (D'Ercole *et al.* 1984, Adashi *et al.* 1985, Han *et al.* 1987).

In order to exert their action, IGFs bind to membrane-associated receptors and thus activate multiple cytoplasmic signaling cascades involved in the transduction of the mitogenic and anti-apoptotic signal to the nucleus of the cell. An overview of the IGF signaling system and the molecules modulating the actions of this system are depicted in Fig. 2. Two main types of IGF receptors have been described. The IGF type II receptor (IGF-RII) exclusively binds IGF-II. The IGF-RII, which is also the cation-independent mannose-6-phosphate receptor, is a 300 kDa protein with a large extracellular domain and a small intracellular domain. The IGF-RII does not have tyrosine kinase activity and its ability to transduce signals is not clear. It is thought that it is important for the sequestration of IGF-II, thus preventing the interaction of IGF-II with the IGF type I receptor (IGF-RI) (Ellis *et al.* 1996). The IGF-RII is also involved in targeting proteins to the lysosomes and may play a role in degradation of the extracellular matrix (reviewed in Sachdev & Yee 2001).

The IGF-RI binds both IGF-I and IGF-II, although IGF-I binding is stronger than IGF-II binding. Most of the physiological effects of the IGFs are mediated by the type I receptor (Leroith *et al.* 1995). The IGF-RI is expressed ubiquitously in human tissues with the exception of hepatocytes and T-lymphocytes (reviewed in Sachdev & Yee 2001). The IGF-RI is transcribed from a single gene as a precursor that is then processed into an  $\alpha$ - and a  $\beta$ -subunit (Ullrich *et al.* 1986). The functional receptor is heterotetrameric consisting of two  $\alpha$ -subunits and two  $\beta$ -subunits, which are linked by two disulfide bonds. The  $\alpha$ -subunits are extracellular and form the ligand-binding domain. The  $\beta$ -subunits, which contain a short extracellular and transmembrane segments and a larger intracellular segment, transmit the ligand-induced signal. Activation of the IGF-RI by ligand binding leads to a conformational change in the receptor, auto-phosphorylation of tyrosine residues in the intracellular  $\beta$ -subunits of the receptor and subsequent activation of downstream signaling molecules. The  $\beta$ -subunits are known to bind signaling adaptor proteins like insulin receptor substrate (IRS)-1 and Shc, linking the receptor to downstream signaling pathways like the PI3-kinase and the ERK pathway (reviewed in Surmacz *et al.* 1998).

The IGF-binding proteins (IGFBPs) play a modulating role by regulating receptor–ligand interactions in the IGF system. The IGFBP family comprises six proteins with high affinity for the IGFs (IGFBP) and several lower affinity IGFBP-related proteins (IGFBP-rP). The binding of IGFBPs to IGF-I and IGF-II inhibits the mitogenic effects of the IGFs by restricting binding of these ligands to their receptor. The IGFBPs also regulate IGF bioavailability by maintaining a circulating reservoir of IGFs, and by prolonging their half-life. In addition to the regulatory effects of IGFBPs on IGF signaling, there is increasing evidence for autonomous effects, independent of IGF, in a number of tissues. How these autonomous effects are mediated is not yet clear, although a putative receptor for IGFBP-3 and -5 has been



**Figure 2** General outline of the IGF system and its downstream effectors. IGFBP, IGF-binding protein; IGFBP-rP, IGFBP-related proteins. mTor, target of rapamycin; FRAP, FkBP-rapamycin associated protein; MKK, MAP kinase kinase.

described (reviewed in Hwa *et al.* 1999). A number of observations indicate that IGFBPs may be involved in regulation of apoptosis and growth inhibition (reviewed in Perks & Holly 2000). The expression of IGFBPs is regulated at the transcriptional level by steroid hormones, growth factors and cytokines. Post-translational modifications like phosphorylation, glycosylation and proteolytic cleavage have been shown to be involved in the regulation of IGFBP function (reviewed in Clemmons 1997).

In the breast, IGF-I is expressed in stromal cells adjacent to normal epithelial cells. IGF-II is not expressed in the normal breast and, thus far, the role for IGF-II in normal breast development is not clear (Paik 1992). However, many reports have identified IGFs as key regulators of growth in human breast cancer. IGF-I, IGF-II, and insulin (at supraphysiological concentrations) have been reported to stimulate growth of breast cancer cell lines (Van der Burg *et al.* 1988, Osborne *et al.* 1990, Musgrove & Sutherland 1993, Dufourny *et al.* 2000,

Bartucci *et al.* 2001). In addition, breast tumors have been shown to overexpress many components of the IGF system (reviewed in Lee *et al.* 1998) and the role of IGF signaling in breast tumors has been confirmed by different strategies interfering with receptor function. These studies have shown inhibition of proliferation of breast cancer cell lines *in vitro* and inhibition of growth of tumor xenografts in nude mice (reviewed in Lee *et al.* 1998, Surmacz *et al.* 1998). If the IGFs play a critical role in the development of human breast cancer, their expression should be a predictor of the prognosis of the disease. Unfortunately, due to the complexity of the system, measurement of a single component of the system may not give a meaningful result, and analysis of a number of the key components will need to be performed.

A number of studies have shown that circulating levels of IGF-I are elevated in breast cancer patients compared with age-matched controls (Peyrat *et al.* 1993), and that either removal of the tumor or treatment with anti-estrogens lowers

circulating IGF-I levels (reviewed in Pollak *et al.* 1992). It is tempting to speculate that endocrine IGF-I may be responsible for tumor growth. However, there are no direct data supporting this hypothesis. In breast cancer patients with significantly higher circulating IGF-I levels, no correlation has been found with several prognostic values such as ER expression or nodal status (Barni *et al.* 1994). Measurement of IGF-I levels in advanced stage breast cancer patients has not revealed any correlation with short-term survival (Bhatavdekar *et al.* 1994).

Only a few studies have directly examined the expression of IGFs in breast tumors. In these studies, IGF-I and -II expression in breast tumor tissue has been measured either by enzyme-linked immunosorbent assay on cytosolic fractions of the tumor (Yu *et al.* 1996), or by immunohistochemistry on tumor tissue sections (Toropainen *et al.* 1995*a,b*). Yu *et al.* (1996) have demonstrated that IGF-I expression does not correlate with other prognostic markers, whereas the data of Toropainen *et al.* (1995*a*) suggest that IGF-I expression is related to malignant histopathological features in breast cancer. Toropainen *et al.* (1995*a*) have further shown that expression of IGF-I has independent prognostic significance in the early phases of the breast cancer. Both groups (Toropainen *et al.* 1995*b*, Yu *et al.* 1996) have found that the expression of IGF-II in the tumor was weakly inversely related with poor prognostic features like tumor grade, high percentage of cells in S phase, and aneuploidy.

IGF-RI is often found to be overexpressed in breast carcinomas with respect to its status in normal breast epithelium or in benign tumors (reviewed in Surmacz *et al.* 1998), although the IGF-RI gene copy is rarely amplified (Berns *et al.* 1992). Expression of the IGF-RI has been shown to correlate with a better prognosis both in relapse-free survival and overall survival of breast cancer patients (Papa *et al.* 1993). The positive prognosis for patients in whom the tumor expresses high levels of IGF-RI is in conflict with the majority of *in vitro* studies supporting a role for the receptor in transformation and malignancy. Some studies (Arteaga 1992, Brunner *et al.* 1993) have demonstrated that a blockade of the IGF-RI with an anti-IGF-RI antibody inhibits the proliferation of breast tumor cell lines *in vitro* and the growth of tumor xenografts in nude mice. The reason for the favorable prognostic value of the IGF-RI expression by breast tumors may be similar to that for ER $\alpha$  expression. Also here, expression of the receptor may reflect well-differentiated tumors that still require IGF for proliferation (Beckmann *et al.* 1997), and are thus sensitive to anti-IGF therapy.

In ER-positive breast cancer cell lines, one of the major effectors of the activated IGF-RI is IRS-1 (Surmacz 2000). In ER-negative cell lines, IRS-2 instead of IRS-1 seems to be the major effector of the IGF-RI (reviewed in Sachdev & Yee 2001). Both IRS-1 and IRS-2 become phosphorylated upon binding to the activated IGF-RI, and serve to connect the receptor to multiple downstream pathways (reviewed in Surmacz *et al.* 1998). Although both ERK and PI3-kinase

pathways are activated following IGF-I stimulation of the ER-positive MCF-7 breast cancer cell line, only the PI3-kinase cascade signal is required for the transduction of the mitogenic signal. Activation of the PI3-kinase pathway leads to elevation of cyclin D1 levels and subsequent cell cycle progression (Dufourny *et al.* 1997). IRS-1 is overexpressed in some primary breast tumors, and from studies in breast tumor cell lines it has become clear that overexpression of IRS-1 makes the cells estrogen independent (Surmacz & Burgaud 1995). Moreover, it has been shown that high levels of IRS-1 in tumors correlate with a higher recurrence rate (Rocha *et al.* 1997). This suggests that IRS-1-mediated signaling in breast cancer cells could be involved in the development and progression of breast cancer.

The expression and activity of IGF-BPs is regulated by a complex system in mammary epithelium involving steroid hormones (estrogen and progesterone) (Figuerola *et al.* 1993) and polypeptide hormone regulators (TGF $\beta$ ) (Oh *et al.* 1995). Clinical data presented to date have provided ambiguous evidence as to whether the IGF-BPs, in particular IGFBP-3, predict a good or bad prognosis for breast cancer. One study has indicated that increased IGFBP-3 levels correlate with a number of features that predict an unfavorable outcome, e.g. larger tumor size and aneuploidy (Rocha *et al.* 1996). However, a second study examining disease recurrence or disease-free survival has found no significant correlation with IGFBP-3 expression levels (Rocha *et al.* 1997). In addition, recent studies have indicated that a low IGFBP-3 concentration in combination with a high IGF-I level in the blood circulation predicts a high risk of breast cancer development (Hankinson *et al.* 1998). This observation reinforces the notion that the balance in the expression of proteins of the IGF system may be an important factor in the prediction of cancer risk and prognosis.

### Cross-talk between the IGF and E2 signaling pathway

Thus far, only the effects of single hormones on the regulation of breast cancer growth have been discussed. The idea that a single growth factor or hormone determines cell growth has been proven false in virtually every system examined. Over the last decade, accumulating evidence for interactions between estrogen and IGF signaling pathways has been reported. Four distinct ways of interaction can be distinguished, which will be discussed below. The first type of interaction has been documented by a number of reports showing long-term regulation of expression of proteins of the IGF system by E2. The second type of interaction is a more rapid one, indicating direct activation of the IGF-RI by the liganded ER. A third type of interaction between IGF and E2 signaling pathways has been described in several reports showing transcriptional activation of the ER by IGF signaling. Finally, several groups have reported synergistic

effects of the two hormones on the expression and activation of cell cycle components, leading to enhanced proliferation rates.

## E2-regulated expression components of the IGF-I system

The long-term effects of E2 on the IGF system have been studied extensively. E2 appears to interact with the IGF system and signaling route at several points. The expression of IGF-RI, IRS-1, and IGF-BPs have been shown to be upregulated by E2 (Huynh *et al.* 1996, Lee *et al.* 1999, Molloy *et al.* 2000, Perks & Holly 2000). A number of reports show that E2 enhances the synthesis of both IGFs in malignant breast epithelial cell lines. Huff *et al.* (1988) and Cohen *et al.* (1990) report IGF-I mRNA and protein expression in MCF-7 cells, which is upregulated by E2, insulin, TGF $\alpha$ , and EGF. Other groups have reported that MCF-7 cells do not express IGF-I (Yee *et al.* 1989, Van der Burg *et al.* 1990, Gebauer *et al.* 1998). Thus far, no explanation for the inconsistency in the experimental results on IGF-I expression has been found, and it remains unclear whether breast cancer cells secrete IGF-I.

Osborne *et al.* (1989) have detected expression of IGF-II in T47D and MCF-7 cells, with highest expression of IGF-II in T47D. However, Van der Burg *et al.* (1990) and Gebauer *et al.* (1998) failed to detect IGF-II secretion by MCF-7 breast carcinoma cells. The discrepancies in the results on IGF-II expression in breast cancer cell lines may be explained by the observation that IGF-II expression in MCF-7 cells could only be detected in late passage subclones of MCF-7. In early passages (up to passage 40) of the MCF-7 cell line, Yee *et al.* (1988) did not detect any IGF-II mRNA in an RNase protection assay.

## Rapid effects of E2 on the IGF signaling pathway

An unexpected link between the estrogen and IGF signaling pathways has been demonstrated in a study by Kahlert *et al.* (2000). They transfected ER $\alpha$  or ER $\beta$  in COS7 and HEK293 cells, which both express IGF-RI. In the presence of ER $\alpha$ , but not ER $\beta$ , E2 rapidly induced phosphorylation of the IGF-RI and ERK-1 and -2. Furthermore, upon stimulation with E2, ER $\alpha$ , but not ER $\beta$ , bound rapidly to the IGF-RI in COS7. The interaction of the ER $\alpha$  and the IGF-RI is also observed in L6 cells, which express ER $\alpha$  and IGF-RI endogenously. Control experiments in the IGF-RI-deficient fibroblast cell line R- showed that only ER $\alpha$  binds to the transfected IGF-RI after stimulation with E2. Overexpression of dominant negative mitogen-activated protein kinase kinase inhibits this effect. Finally, these authors demonstrate that ER $\alpha$ , but not ER $\beta$ , is required to induce the activation of the

estrogen receptor-responsive luciferase reporter (ERE-LUC) in IGF-I-stimulated cells. Taken together, these data have demonstrated that ligand-bound ER is required for rapid activation of the IGF-RI signaling cascade. If the activation also occurs in human breast cancer cells, treatment of MCF-7 cells with E2 would be expected to lead to a strong activation of the IGF-RI/PI3-kinase pathway. However, in the MCF-7S model system used in our laboratory, we have not been able to demonstrate the interaction of the ER $\alpha$  and IGF-RI, or activation of the ERK and PI3-kinase pathways in E2-stimulated MCF-7S cells (Hamelers *et al.* 2002a).

## Rapid effects of IGF on the activation of the E2 receptor (ER)

IGFs have been implicated in the regulation of the activity of the ER by a number of observations. Several studies have demonstrated that IGFs as well as other growth factors activate the (unliganded) ER (Kato *et al.* 1995, Lee *et al.* 1997, Ram *et al.* 1998, Stoica *et al.* 2000, Campbell *et al.* 2001). Co-administration of E2 and growth factors to cells has been shown to result in an additive effect on the expression of endogenous estrogen-regulated genes (Stoica *et al.* 2000).

Kato *et al.* (1995) have shown that the human ER $\alpha$  is phosphorylated by ERK on Ser<sup>118</sup> within 15 min after the addition of EGF and IGF-I to the cells *in vitro*. The phosphorylation of this serine is required for full activity of the ER $\alpha$  AF-1 domain. Overexpression of active ERK kinase (MEK) or of the active p21<sup>ras</sup>, both of which activate ERK, enhances estrogen-induced transcriptional activity of the wild-type ER $\alpha$ , but not of a mutant ER $\alpha$  with an alanine in place of Ser<sup>118</sup> (Kato *et al.* 1995). More recently, Endoh *et al.* (1999) have identified a coactivator associating with the human ER $\alpha$  AF-1 in an ERK-mediated, phosphorylation-dependent manner. They have shown that the interaction of the p68 coactivator with the A/B domain is essential for the full activation of the human ER $\alpha$  AF-1.

PI3-kinase, which is activated by IGF-I and other growth factors, has been shown to phosphorylate the ER $\alpha$  within 1 h after addition of the activating stimulus. Active PI3-kinase increases the transcription-stimulating activity of both the AF-1 and AF-2 domains of ER $\alpha$ , whereas active PKB only increases the activity of AF-1. Phosphatase and tensin homologue PTEN, the phosphatase responsible for inactivation of PI3-kinase, and a catalytically inactive PKB decreased PI3-kinase-induced AF-1 activity, suggesting that PI3-kinase utilizes PKB-dependent and PKB-independent pathways in activating ER $\alpha$ . The consensus PKB phosphorylation site at Ser<sup>167</sup> of ER $\alpha$  is required for activation by PKB. In addition, LY294002, a specific inhibitor of the PI3-kinase/PKB pathway, reduced phosphorylation of ER $\alpha$  *in vivo* (Campbell *et al.* 2001).

### The combined mutual reinforcing effects of IGF-I and E2 on cell cycle regulation

Both IGF-I and E2 have been shown to synergistically stimulate proliferation of various tissues and cultured cells, including breast tumor cells (Van der Burg *et al.* 1988, Cardona-Gomez *et al.* 2001). Several groups have tried to identify the pathways involved in the induction of a mitogenic response of breast cancer cells upon addition of the combination of E2 and submitogenic amounts of IGF-I. Thus far, two different models have been put forward to explain the synergy of IGF-I and E2. The first model, schematically depicted in Fig. 3, is based on the combined results of two laboratories (Dupont *et al.* 2000, Lai *et al.* 2001). In these experiments, MCF-7 cells were synchronized by anti-estrogen (ICI 182780) treatment. Both laboratories have shown that treatment of MCF-7 cells with E2 and IGF-I induces changes in the expression of cell cycle components, leading to activation of the cyclin E/cyclin-dependent kinase inhibitor (CDK)-2.

Lai *et al.* (2001) have shown that addition of IGF-I does not re-initiate cell cycle progression. This is in marked contrast to cells synchronized by serum starvation, in which the addition of 20 ng/ml IGF-I has been shown to induce cell cycle progression and cell proliferation (Dufourny *et al.*

1997). Furthermore, Lai and colleagues (2001) have shown that in MCF-7 cells synchronized with the ICI compound, IGF-I increases cyclin D1, cyclin E, and p21<sup>cip1/waf1</sup> gene expression and induces the formation of active CDK4 complexes. However, addition of IGF-I results in only minor increases in cyclin E–CDK2 activity. Treatment with E2 results in a larger increase in cyclin D1 gene expression than with IGF-I, and markedly decreases p21<sup>cip1/waf1</sup> expression, with a concurrent increase in CDK4 and CDK2 activity and subsequent synchronous entry of cells into S phase. Co-administration of IGF-I and E2 induces synergistic stimulation of S phase entry coincident with synergistic activation of cyclin E–CDK2 complexes lacking p21<sup>cip1/waf1</sup>. To determine if the ability of E2 to deplete p21<sup>cip1/waf1</sup> was central to these effects, cells stimulated with insulin and E2 were infected with recombinant adenovirus expressing p21<sup>cip1/waf1</sup>. Induction of p21<sup>cip1/waf1</sup> to levels equivalent to those following treatment with IGF-I alone markedly inhibits the synergism of E2 and IGF-I on S phase entry.

Dupont *et al.* (2000) have shown that MCF-7 cells synchronized with ICI 182780 are stimulated to proliferate by both IGF-I and E2 individually, and the addition of the combination of the mitogens resulted in a synergistic response.

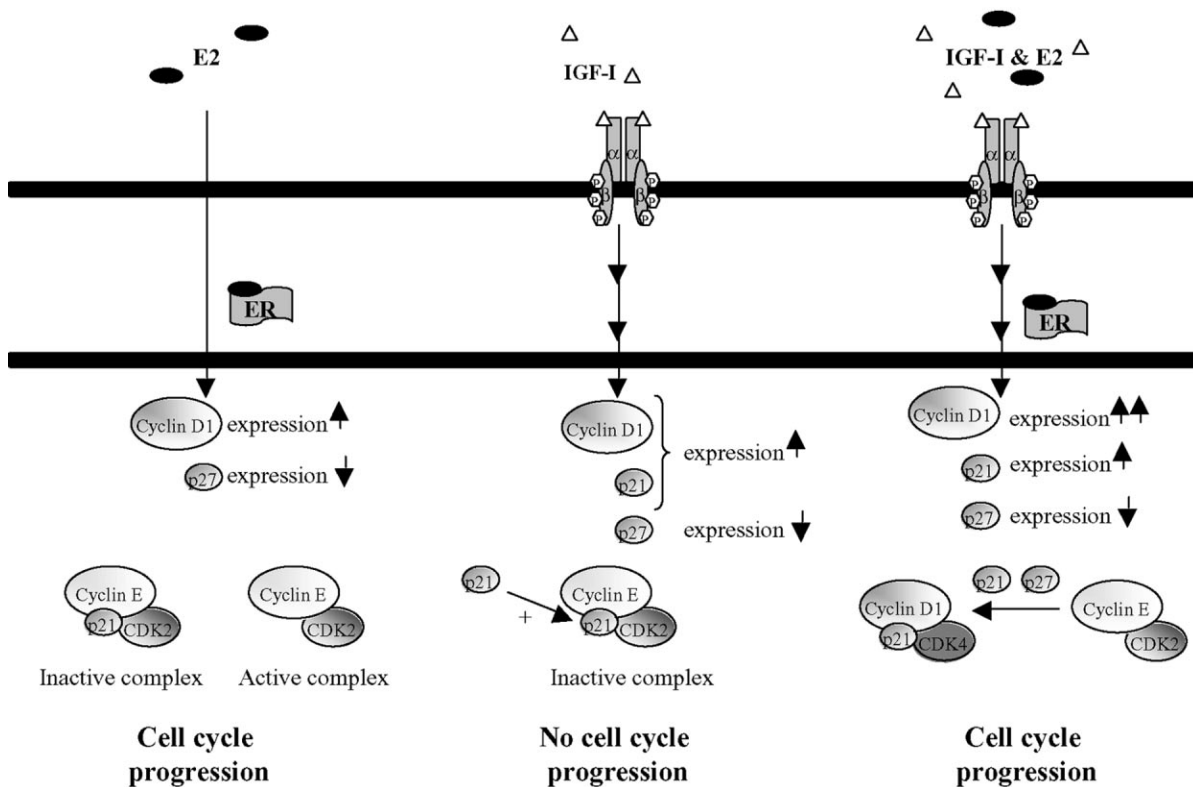


Figure 3 Model for the synergism of IGF-I and E2 in ICI 182780-synchronized cells.

They demonstrated that E2 is also able to potentiate the IGF-I effect on the expression of cyclin D1 and cyclin E and on the phosphorylation of retinoblastoma protein. Stimulation with IGF-I increases the protein level of p21<sup>cip1/waf1</sup> and the transcriptional activity of the p21<sup>cip1/waf1</sup> promoter, whereas it reduces the protein level of p27<sup>kip1</sup>, without affecting p27<sup>kip1</sup> promoter activity. E2 does not affect the expression of p21<sup>cip1/waf1</sup>, but it decreases the protein level of p27<sup>kip1</sup> and the p27<sup>kip1</sup> promoter activity. The decreased expression of p27<sup>kip1</sup> is accompanied by a decrease of p27<sup>kip1</sup>-associated CDK2. This suggests that IGF-I and E2 regulate the activity of CDK2 by regulating the expression of both p21<sup>cip1/waf1</sup> and p27<sup>kip1</sup>.

Taken together, these data suggest that, in MCF-7 cells, two distinct actions induced by IGF-I and E2 may be responsible for synergistic stimulation of cell proliferation of ICI 182780-synchronized MCF-7 cells by the two hormones. The first action involves sequestration of the cyclin-dependent kinase inhibitors (CKI) p21<sup>waf1/cip1</sup> and to a lesser extent p27<sup>kip1</sup> in E2-induced cyclin D1/CDK4 complexes, and the second involves inhibition of p21<sup>waf1/cip1</sup> gene transcription. Both actions lead to formation of active cyclin E/CDK2 complexes lacking CKIs.

Based on our work in MCF-7S cells, we have proposed a different model for the synergistic effect of E2 and IGF-I in breast cancer (Hamelers *et al.* 2002a,b). In contrast to experiments on which the first model is based, our MCF-7S cells were synchronized by serum deprivation. IGF-I, in a concentration of 20 ng/ml, is a potent mitogen in synchronized MCF-7S cells (Dufourny *et al.* 1997). A tenfold lower concentration (i.e. 2 ng/ml) is by itself unable to trigger the mitogenic response (Hamelers *et al.* 2002a). We have demonstrated that E2, in concentrations ranging from 0.1 to 100 nM, does not induce cell cycle progression in quiescent MCF-7S cells. However, the combination of submitogenic amounts of IGF-I (2 ng/ml) and E2 (1 nM) synergistically induces cell cycle progression and proliferation. We have found that both E2 and mitogenic amounts of IGF-I induce cyclin D1 expression, whereas submitogenic amounts of IGF-I do not significantly elevate cyclin D1 levels. IGF-I, but not E2, is able to activate PI3-kinase, which leads to inhibition of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) activity. Here, a *per se* non-mitogenic amount of IGF-I suffices. Inhibition of GSK3 $\beta$  triggers nuclear accumulation of the cyclin D1, but only if cyclin D1 levels are strongly induced concomitantly. After cyclin D1 accumulates in the nucleus, activation of the cyclin D1-CDK4 complex and subsequent cell cycle progression is observed.

Based on these findings, we propose the model depicted in Fig. 4. Mitogenic amounts of IGF-I are proficient in inducing cyclin D1 and in inactivating GSK3 $\beta$ . E2 may substitute for IGF-I in its ability to enhance cyclin D1 levels. Since a much lower concentration of IGF-I is sufficient to suppress GSK3 $\beta$  activity, even a submitogenic amount of

IGF-I in synergy with E2 is now able to trigger cell cycle progression in MCF-7S cells.

This model predicts that IGF-I may be replaced by an inhibitor of GSK3 $\beta$ . Recently, we have demonstrated that E2 in the presence of LiCl, a well-documented inhibitor of GSK3 $\beta$ , induces nuclear accumulation of cyclin D1, CDK2 activation, hyperphosphorylation of retinoblastoma protein and DNA synthesis, without the need of co-administration of IGF-I (Hamelers *et al.* 2002b). This confirms that IGF-I-induced GSK3 $\beta$  inactivation is an essential step in the regulation of subcellular localization of cyclin D1 during G1 phase.

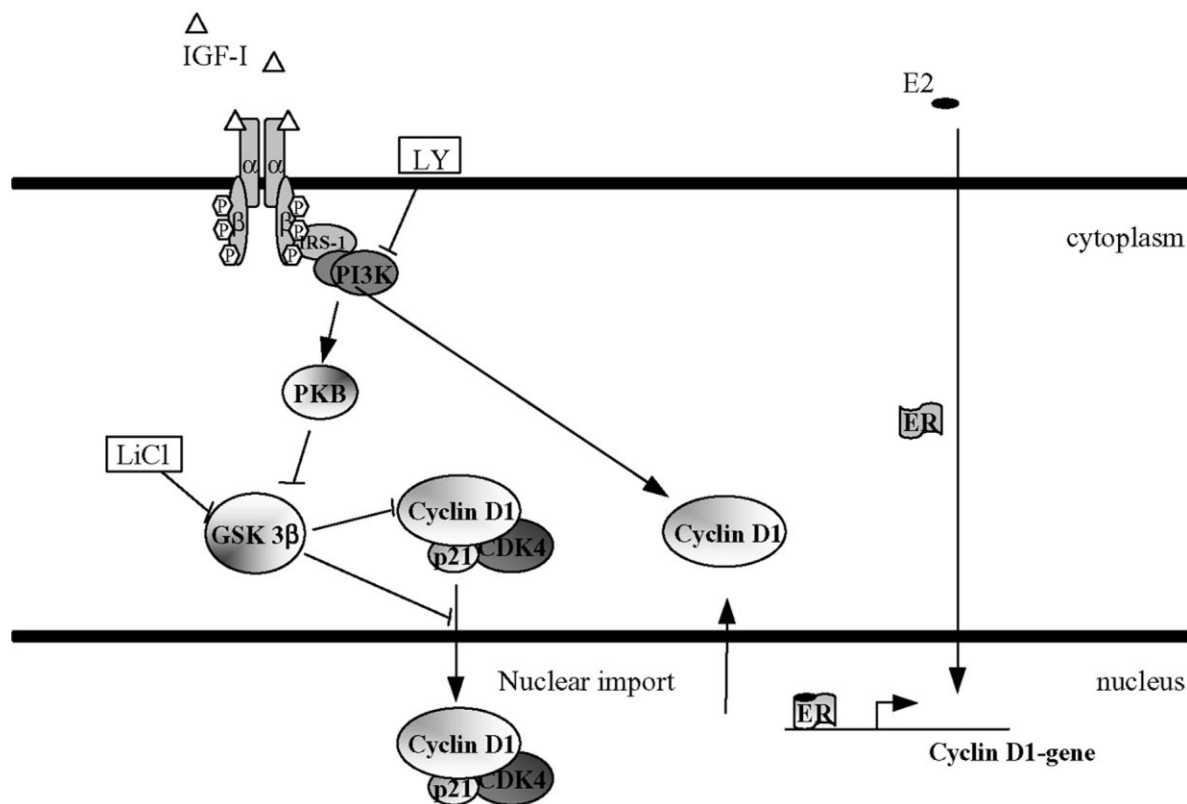
According to this model, E2 cannot by itself induce proliferation of MCF-7 breast cancer cells without the additional IGF-I signal. This holds true for MCF-7S, but other MCF-7 laboratory strains seemingly proliferate upon E2 stimulation. However, IGF signaling still seems to be required since E2-induced cell proliferation of these strains is blocked by preincubation with the IGF-RI blocking antibody,  $\alpha$ IR3 (author's unpublished observation) and by an inhibitor of the PI3-kinase pathway, LY 294002 (author's unpublished data). Moreover, E2 responsiveness could be transferred by incubating E2-unresponsive cells in conditioned medium of E2-sensitive cells. This suggests that E2-sensitive MCF-7 strains secrete a factor which supplies the required IGF-RI-dependent signal by an autocrine mechanism.

## Conclusion

In this review we have examined the mechanisms by which E2 and IGFs regulate breast cancer cell proliferation. E2, via activation of the ER, enhances the expression of growth-related genes, one of the most noteworthy being cyclin D1. In addition, E2 may regulate breast cancer cell growth via activation of signal transduction cascades, like the PI3-kinase and ERK pathways. However, the reports on the activation of non-genomic pathways by E2 are contradictory. More research is needed to establish whether E2 is a physiological activator of cytoplasmic signaling cascades and, if so, what the contribution of this activation might be to the mitogenic effect of the hormone.

IGF has been shown to induce proliferation in breast cancer cells as well. The IGF signal is transduced by the activation of the tyrosine kinase receptor IGF-RI. Subsequently, the ERK and the PI3-kinase pathway are activated, although only the activation of the PI3-kinase pathway has been shown to be essential in mediating the growth signal.

At lower, submitogenic concentrations, IGF is still able to trigger the proliferative signal in breast cancer cells in concert with E2. If one of the receptors is blocked, e.g. the ER by the anti-estrogen ICI 182{.}780 or the IGF-RI by the antibody  $\alpha$ IR3, the proliferative response is suppressed. This may well indicate the need for both hormonal signals to induce breast cancer cell growth when E2 and IGF are



**Figure 4** Model for the synergism of IGF-I and E2 in serum-starved cells. PI3K, PI3-kinase. LY, LY 294002.

present at physiological concentrations. As a logical consequence, it may be of great advantage to consider a combinational therapy of anti-IGF and anti-E2 treatment for breast cancer. This combination may provide a more effective way to treat breast tumors and may even prevent tumor progression.

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