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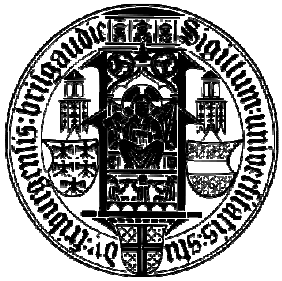

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Growth hormone (GH) modulation of Epidermal growth factor (EGF) signalling in human mammary epithelial cells

Dissertation presented to obtain the degree of Medical Doctor of the Albert-Ludwigs-Universität Freiburg i.Br.

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Contents

Abbreviations

AKT	Proteinkinase B
ATP	Adenosine triphosphate
BCL	B-cell lymphoma
BSA	Bovine serum albumin
BTC	Betacellulin
c-Cbl	Casitas B-lineage Lymphoma, an E3 ubiquitin protein ligase
CDK	Cyclin-dependent kinase
DAB	3,3'-diaminobenzidine
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ErbB	Gene symbol from the name of a viral oncogene to which these receptors are homologous: Erythroblastic Leukemia Viral Oncogene
ErbB1	EGFR
ERK1/2	Extracellular regulated MAP kinase
Gab 1	Grb2-associated binder 1
GH	Growth homone
GHR	Growth hormone receptor
Grb2	Growth factor receptor-bound protein 2
GSK-3 β	Glycogen synthase kinase-3 β
GTP	Guanosine triphosphat
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her-2	Human epidermal growth factor receptor 2
IGF-1	Insulin-like growth factor 1 receptor
IGF-1R	Insulin-like growth factor receptor
JAK	Januskinase
Mab	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase (MAPKK)
mTOR	Mammalian target of rapamycin
MVB	Multi-vesicular bodies
NSCLC	Non small cell lung cancer
PBS	Phosphate buffered saline
PDGF	Plateled derived growth factor
PDK	Phosphoinositide-dependent kinase
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein inhibitors of activated STAT
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B, AKT
PKC	Protein kinase C
PLC	Phosphoinositide phospholipase C

PMSF	Phenylmethylsulphonyl fluoride
PR	Progesterone receptor
PRL	Prolactin
PTEN	Phosphatase and tensin homologue
Raf	Proto-oncogene serine/threonine-protein kinase, guanosine exchange protein
Ras	Rapidly accelerated fibrosarcoma, a serin/threonine kinase
RB	Retinoblastoma
RTK	Receptor tyrosine kinases
SDS	Sodium dodecyl sulfate
Ser (S)	Serine
SHC	Src Homology 2 domain
SOCS	Suppressors of cytokine signalling
SOS:	Son of sevenless
Src	Tyrosine-protein kinase Src
SS	Somatostatin
STAT	Signal-transducer and activator of transcription protein
SWI/SNF	SWItch/Sucrose Non Fermentable
TEBS	Terminal end buds
TEMED	N, N, N ', N'- tetramethylethylenediamine
TGF	Transforming growth factor
Thr (T)	Threonine
TKI	Tyrosine kinase inhibitors
Tyr (Y)	Tyrosine

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Introduction

Epidermal Growth Factor (EGF) and ErbB ligands

A healthy extracellular environment promotes cell survival. One important extracellular signalling molecule which advances cell survival, cell growth, differentiation, motility and adhesion is the Epidermal growth factor (EGF) (Boonstra et al., 1995). Cells are more sensitive to apoptotic stimuli when EGF is absent in the extracellular surroundings (Jost et al., 2000).

EGF is a member of the EGF family which includes two major subfamilies: the EGF-like growth factors and the neuregulins. These growth factors include a disulfide-bonded three loop moiety of 53 amino acids which is called the EGF-like sequence. This sequence is necessary to interact with specific cell surface receptors (Stern et al., 2003; Troyer et al., 2001; Olayioye et al., 2000; Yarden et al., 2001; Frank et al., 2008) (Figure 1). Besides EGF, the EGF-like growth factors include the transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin and epiregulin (Frank et al., 2008).

EGF, amphiregulin (AR) and TGF-α bind specifically to the ErbB-1 receptors (Henson & Gibson, 2006; Frank, 2008). Betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR) bind ErbB-1 and ErbB4 (Olayioye et al., 2000). Neuregulins (NRGs) NRG-1 and NRG-2 bind ErbB-3 and ErbB-4 whereas NRG-3 and NRG-4 bind only to ErbB-4 (Harari et al., 1999; Carraway et al., 1997).

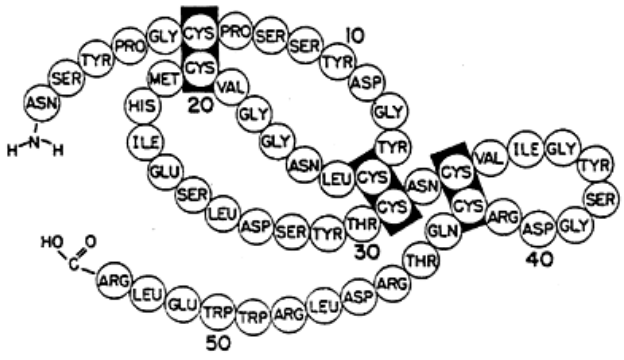


Fig. 1 The amino acid sequence of EGF with placement of disulfide bonds. The figure has been published in J. Biol. Chem. 1973, 248, p. 7670 (Cohen and Levi-Montalcini; 1973).

EGFR and the ErbB family

EGFR (ErbB-1) belongs to a receptor family, including ErbB-2 (also called Her-2 in humans and Neu in rodents), ErbB-3 and ErbB-4 receptors, which are activated by growth factors of the EGF family (Lin et al., 1984; Bargmann et al., 1986; Kraus et al., 1989; Plowman et al., 1993). Each of the receptors has an extracellular domain with cystein rich regions, a transmebrane domain and, except for ErbB-3, harbours an intrinsic tyrosine kinase activity in its cytoplasmic domain (Huang et al., 2003). The receptor tyrosine kinase (RTK) domain leads to phosphorylation and activation of multiple tyrosine residues within their intracellular domains (Boeri Erba et al. 2005, Wu et al., 2006) (Fig. 2).

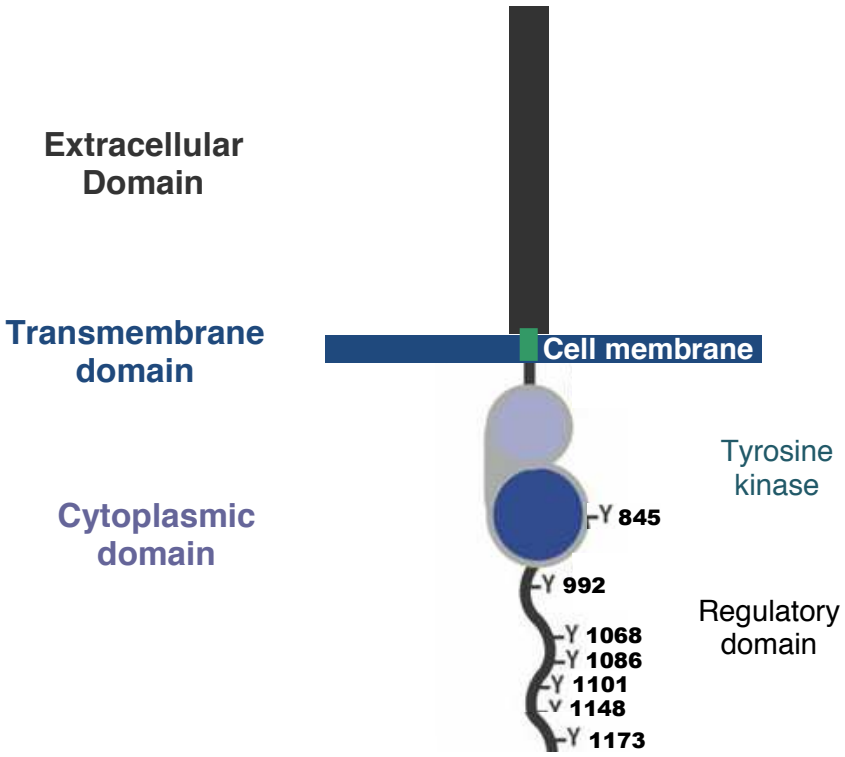


Fig. 2 Structure of the Epidermal growth factor receptor (EGFR). The model shows the three domains of the receptor. The model is adapted from Ferguson et al., 2008.

ErbB-1 and ErbB-4 are autonomous. After ligand binding, the receptors undergo dimerization, followed by generation of intracellular signals. ErbB-3 has no intrinsic tyrosine kinase activity and a specific ErbB2 ligand has not been discovered yet; therefore, they are considered as no autonomous. In addition to homodimers the following heterodimers can be formed: ErbB-1 – ErbB-2, ErbB-1- ErbB-3, ErbB-2- ErbB-3 and ErbB-3- ErbB-4 (Frank, 2008). ErbB-2 is the preferred heterodimer of other EGFR family members (Karunagaran et al., 1996; Graus-Porta et al., 1997, Huang et al., 2006) and plays a role in the potentiation of ErbB signalling (Graus-Porta et al., 1995; Olayioye et al., 2000). EGF induces tyrosine phosphorylation of ErbB-2 through heterodimerization with ErbB-1 (EGFR) (Goldmann et al., 1990; Wada et al., 1990). Simultaneous overexpression of ErbB-1 and ErbB-2 increases the amplitude and duration of the signal and affects synergistically malignant transformation of murine fibroblasts (Kokai et al., 1989; Kanuagaran et al., 1996).

EGF-induced phosphorylation of EGFR at its multiple tyrosine residues leads to recruitment of docking and signalling proteins such as Growth factor receptor-bound protein 2 (Grb-2), Src Homology 2 domain (SHC), Protein tyrosine phosphatases-1 (PTP-1), Posphoinositide phospholipase C-y (PLC-y) and Tyrosine-protein kinase Src (Src) resulting in activation of downstream signalling cascades as Ras/Raf/MEK/ERK (p44/p42 MAPK), PKC, PI3-kinase/AKT and the STAT pathways (Jorrisen et al., 2003; Schulze et al., 2005; Normanno et.al., 2006) (Fig. 3). Consequence of this downstream signalling is the regulation of gene expression and cell behaviour.

ErbB-receptors are not only activated by specific ligands, they can also be activated indirectly by Growth hormone (GH) or Prolactin (PRL) and other growth factors through the JAK-2/STAT pathway (Yamauchi et al. 1997, 1998, 2000). EGFR can also be phosphorylated at sites in the intracellular domain as serine and threonine residues (Li et al., 2008). In fibroblasts, receptor phosphorylation at mutations of serines 1046 or 1047 by Ca2+/calmodulin kinase II resulted in enhanced autophosphorylation (Feinmesser et al., 1999). GH and PRL can also control EGFR turnover by threonine phosphorylation, thus modulating EGFR signalling (Kim et al., 1999, Huang et al., 2003, 2004). There are not only interactions among the different ErbB-receptors, but also with

other receptor tyrosine kinases as the Insulin- like growth factor 1 receptor (IGF-1R) (Jin and Esteva, 2008).

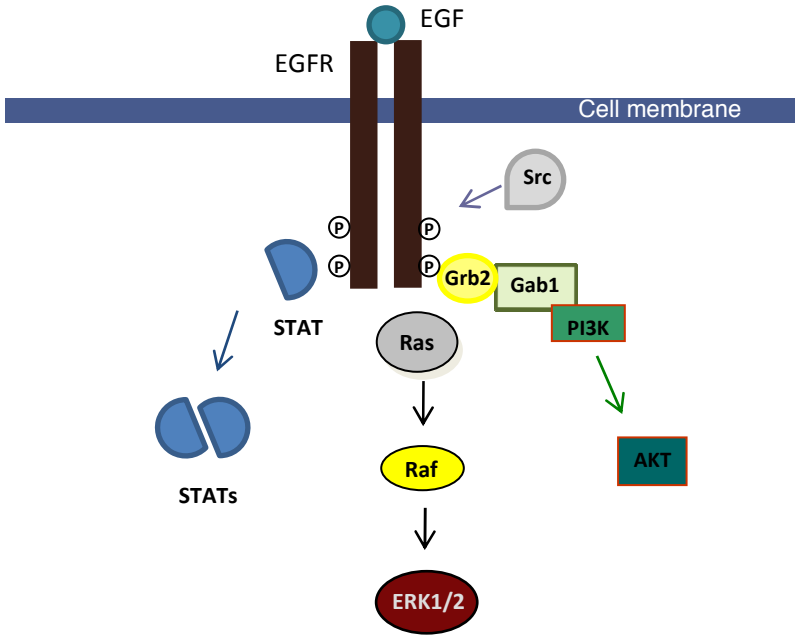


Fig. 3 Downstream signalling pathways of EGFR. Activation of EGFR leads to homo- or heterodimerization and phosphorylation of tyrosine residues. The mayor signalling pathways by EGFR receptors are Ras/ERK1/2, PI3K/AKT and STATs. STAT: Signal Transducers and Activators of Transcription Src: tyrosine-protein kinase Src, Ras: guanosine exchange protein, Raf: rapidly accelerated fibrosarcoma, a serin/threonine kinase ERK1/2: Extracellular signal-regulated kinase, Grb2: Growth factor receptor-bound protein 2, PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase, AKT: Protein kinase B.

EGFR feedback regulation

EGFR signalling is regulated via ligand-receptor dissociation (Karunagaran et al., 1996) or through phosphorylation, e.g. by Protein kinase C (PKC) (Stern, 2000). A consequence of ligand-induced receptor tyrosine activation is its down-regulation, also seen as negative feedback, to terminate or modulate signalling. The propensity of ligand-induced downregulation varies under the ErbB-receptors and ligands. EGFR activation upon EGF binding leads to internalisation and downregulation of the receptor, in contrast, transforming growth factor α (TNF-α) causes EGF-receptor recycling (Decker et al., 1990). ErbB-2 is less accessible to endocytic downregulation (Roepstor

et al., 2008) and also EGFR-ErbB-2 chimaera are slower internalised than EGFR (Sorkin et al., 1993). In case of ErbB-2 overexpression, as in cells of mammary neoplasia, ErbB-2 impairs EGF-induced EGFR-ErbB-2 downregulation compared to cells with low ErbB-2 level. Here, signalling of the EGFR is stabilized and augmented by ErbB-2 overexpression (Wang et al., 1999). ErbB-3 and ErbB-4 are also slower internalised than EGFR (Baulida et al., 1996; Sorkin and Goh, 2009).

After ligand- induced endocytosis, the EGF- receptor undergoes the endosomal pathway. EGF induces EGFR dimer internalization through clathrin-coated membranes (Carpenter and Cohen, 1976). In case of EGF absence, the receptor is recycled to the cell surface. In presence of EGF, the receptor advances from early to late endosomes (Frank, 2008) (Fig. 4). In transit for recycling or degradation, the EGF-EGFR-interaction is stable at the endosomal pH (French et al., 1995) and the internalized EGF-receptor still generates signals from the endosomes. Inside the endosome, molecules are sorted into small vesicles, which lead to the multivesicular appearance of late endosomes, therefore they are called multi-vesicular bodies (MVBs). These vesicles transfer ligand-loaded receptors from the plasma membrane to the pre-lysosomal compartment (Grimes et al., 1997; Avraham and Yarden, 2011). Fusion of the MVB membrane with the lysosomal membrane results in EGFR degradation (Levkowitz et al., 1999; Huang F. et al., 2006). Phosphorylated receptors are caught by growth-factor Receptor-bound protein 2 (GRB2) and c-Cbl, an ubiquitin ligase, is recruited for ubiquitination. C-cbl aims the receptors with ubiquitin-binding proteins to the MVB lumen, the pre-lysosomal compartment (Marmor and Yarden, 2004). Whereas the current consensus supposes that EGFR transfer from clathrin-coated pits to endosomes prolongs MAPK signalling while terminating PI3K-AKT signalling (Vieira et al., 1996; Fehrenbacher et al., 2009), it is also reported that it is exactly the other way around (Goh et al., 2010). Furthermore, EGFR is negatively regulated by suppressors of cytokine signaling-5 (SOCS5). SOCS also induce ubiquitination of the receptor (Nicholson et al., 2005).

As mentioned above, the receptors can also be recycled to the plasma membrane. Throughout the endocytic pathway and at the MVB, recycling is still possible. Receptors that should be recycled, stay in the outer MVB membrane and become not

sorted to internal vesicle. The sorting of EGFR is as well controlled by tyrosine kinase activity (Felder et al., 1990).

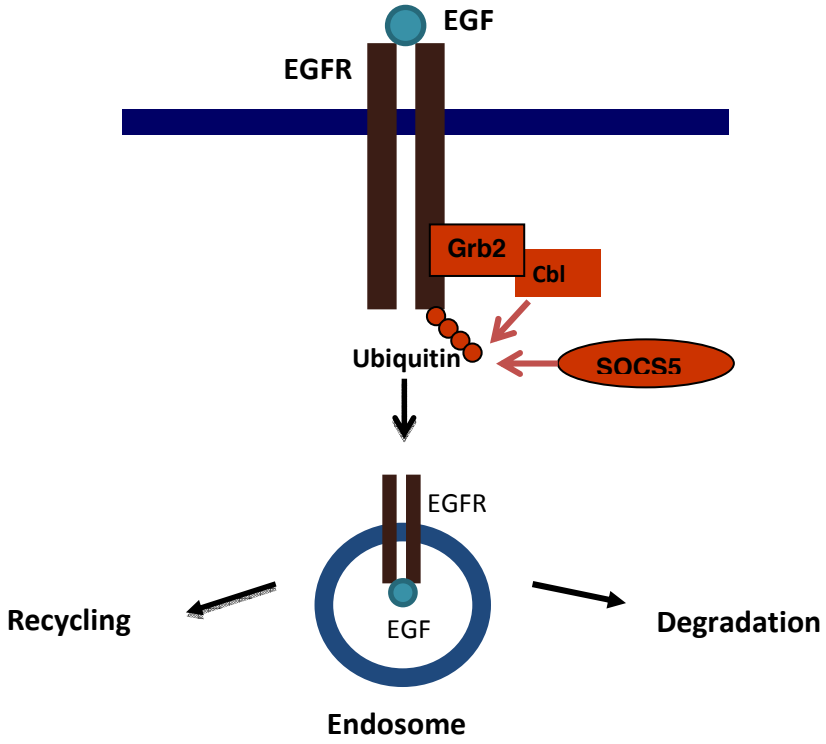


Fig. 4 Regulation of the EGF receptor. Ligand-loaded EGFR can be internalized through clathrin-coated membranes, resulting in receptor degradation or recycling to the cell membrane. SOCS5: suppressor of cytokine signaling, Grb2: growth factor receptor-bound protein 2, Cbl: Ubiquitin ligase

EGFR and breast cancer

A big amount of breast cancers occur relatively early in lifetime (less than 55 years) and breast cancer is the main reason for cancer mortality in women worldwide. There is a lifetime risk of approximately 8-10% of the disease in the industrial countries (http://www.rki.de/Krebs/DE/Content/Krebsarten/Brustkrebs/brustkrebs_node.html, 23.05.12;47h). Epidermal growth factor receptor (EGFR) is overexpressed in many types of cancer (Berger et al., 1988; Xie er al., 1997; Biscardi et al., 2000; Mendelsohn and Baselga, 2000, 2003; Stern, 2000; Huang et al., 2006) including breast (Verbeek et

al., 1998), lung (Suzuki et al., 2005), oesophageal (Itakura et al., 1994) and head and neck cancer (Sok et al., 2006). EGFR undergoes different alterations such as gene amplification, structural rearrangements and somatic mutations in human carcinomas. In addition, some types of tumours produce an excess of EGF that leads to an increased activation of EGFR (Henson and Gibson 2006). EGFR is not only expressed in cancer cells, but also in cells which are in the microenvironment around the tumour such as endothelial cells, inflammatory cells, pericytes, neutrophils, fibroblasts, T and B lymphocytes, natural killer cells, antigen presenting cells and components of the extracellular matrix. There is growing evidence that the tumour microenvironment plays an important role in tumour initiation and has a supportive role in cell proliferation. Angiogenesis stimulated by the tumour, is regulated via EGFR by growth factors such as EGF and TGF-α (Seshacharyulu et al., 2012**)**.

All four ErbB members are expressed in several tissue compartments of the adult mammary gland. EGF-receptor and ErbB-2 are preferable expressed in young females (Sebastian et al., 1998; Schroeder et al., 1998) whereas the expression of the Neuroregulin (NRG) receptors, ErbB-3 and ErbB-4, increases after maturity as well as Transforming growth factor α (TGF-α), Amphiregulin (AR) and Epidermal growth factor (EGF), which are essential for ductal morphogenesis (Bublil and Yarden, 2007). The first big postnatal step in breast development takes place in puberty; in form of elongation and branching of the mammary ducts. In mice, expression of a dominant-negative EGF-receptor impairs ductal morphogenesis (Xie et al., 1997), indicating its importance during puberty.

The ErbB family regulates epithelial differentiation and migration of different types of epithelia, including cells in the mammary gland. ErbB-driven cancer cells have the ability to invade blood and lymph vessels. In vitro experiments suggest that EGF-like growth factors, together with the Transforming growth factor-β (TGF-β) are the main inducers of epithelial-mesenchymal transition (EMT). EMT is a process in which cells switch morphologically from a polarized epithelial phenotype to a mensenchymal fibroblastoid phenotype. This includes the change of epithelial markers as E-cadherin and cytokeratins to mesenchymal markers as vimentin and fibronectin. Lower amount of E-cadherin is associated with metastatic breast cancer (Micalizzi and Ford, 2009).

Invasive growth and EMT are two important steps of embryonic morphogenesis and metastasis (Bublil and Yarden, 2007).

EGFR as well as ErbB-2 are relevant in pathogenesis of breast cancer (Walker et al., 1999; Fox et al., 1994). Expression of EGFR and/or ErbB-2 has been associated with increased proliferation, disease progression and a poor clinical outcome (Fox et al., 1994; Klijn et al., 1992; Alroy and Yarden, 1997). In breast carcinomas, presence of EGFR is associated with high tumour grade (Nicholson et al., 1989; Walker & Dearing, 1999) and reduced survival (Sainsbury et al., 1987). The expression of EGFR is inverse correlated with oestrogen receptor (ER) expression and associated with resistance to endocrine therapy (Walker & Dearing, 1999; Klijn et al, 1992, Morris, 2002). Although EGFR overexpression is found in all subtypes of breast cancer, it is overexpressed more often in triple-negative breast cancer, characterized by a lack of Estrogen receptor (ER), Progesterone receptor (PR), and Her-2 expression and also in inflammatory breast cancer, both particular aggressive phenotypes (Burness et al., 2010; Guerin et al., 1989). Hormone resistance plays an important role in breast cancer progression. It is supposed that hormone-resistant cells rely on EGFR for growth regulation, compared with hormone-sensitive cells, which principally rely on endocrine growth signals (Walker & Dearing, 1999; Morris, 2002). Because ErbB-2 is constitutively phosphorylated in some breast cancers, it has been assumed that transmodulation of ErbB-2 possibly happens due to EGFR signalling. Co-expression of both ErbB members, ErbB-1 and ErbB-2, was found in 10% to 36% of primary human breast carcinomas (Normanno et al., 2001).

EGFR target therapies

There are small molecule tyrosine kinase inhibitors (TKIs) against EGFR like Gefitinib, Lapatinib and Erlotinib among others. Furthermore there are monoclonal antibodies (Mabs) against EGFR as Cetuximab and Panitumumab. These monoclonal antibodies were created to block the extracellular region of EGFR via ligand competitive inhibition. Through this mechanism receptor dimerization, auto-phosphorylation and downstream signalling are prevented and moreover, Mabs lead to EGFR degradation and prolonged downregulation (Seshacharyulu et al., 2012). For example, Cetuximab binds the ligand

and the receptor dimerization domains within the receptor and impairs signalling (Li S et al., 2005).

Small molecule inhibitors of EGFR, TKIs, are adenosine triphosphate (ATP) analogues and inhibit EGFR signalling by competing and binding to the ATP-binding sites in the tyrosine kinase domain of the receptors (Mendelsohn and Baselga, 2000, Seshacharyulu et al., 2012). Gefitinib (Iressa) binds selectively the ATP pocket in the catalytic domain and prevents consequently auto-phosphorylation and signalling of the receptor (Henson and Gibson, 2006). There are pure EGFR TKIs as well as combined EGFR and Her-2 TKIs. TKIs are not specific for EGFR and affect other kinases, too. In contrast MAbs are specific for EGFR.

It was demonstrated that Erlotinib could inhibit invasion, cell motility and reversed the mesenchymal phenotype to an epithelial phenotype in inflammatory breast cancer cells, a process dependent on the ERK1/2 pathway (Zhang D. et al., 2009). Cells treated with Erlotinib showed higher level of E-cadherin and lower level of vimentin than the control. Therefore, it was suggested that Erlotinib functions through inhibition of epithelial-mesenchymal transition (EMT) (Zhang D. et al, 2009).

Growth Hormone (GH)

Growth hormone (GH), also called somatotropin, was first isolated in 1944 (Li and Evans, 1944). It is composed of 191 amino acid residues which form a single chain with four helical regions and two disulfide bridges. The 22 kDa hormone is produced and secreted by the anterior pituitary gland in somatotropic cells. GH plays the main role in postnatal body growth, has an important metabolic function and modulates proliferation, differentiation, motility and apotosis. Its secretion is stimulated by Growth hormone releasing hormone (GHRH), secreted by the hypothalamus, and by ghrelin, synthesised in the stomach. Somatostatin (SS, also called somatotropin release inhibiting factor, SRIF) regulates GH negatively. This interplay leads to a pulsative pattern of GH release, with brief secretory episodes and clearance during the intervals (Hartman et al., 1993). In humans these pulses occur approximately every two and a half hours (Van den Berg et al., 1996). Release of GH from the anterior pituitary gland is stimulated by stress,

exercise, malnutrition and anorexia. In contrast hyperglycaemia, obesity and overeating inhibits its secretion (Hartman et al., 1993; Bartke et al., 2013; Cornford et al., 2011). Prepubertal, there is an elevation of GH levels, accounting to the huge growth in this period. Testosterone and estradiol stimulate release of GH (Bartke et al., 2013). During lifetime, GH secretion declines. It is supposed that the decline of GH levels contribute to produce certain changes that are associated with advanced age like increased obesity and less muscle mass (Giustina et al., 1998¸ Muniyappa et al., 2007) (Fig. 5).

GH induced endocrine stimulation of hepatic IGF-1 is one of the main manners of GH in growth stimulation (Le Roith et al., 2001). Circulating IGF-1 inhibits GH release by interaction with the hypothalamus, creating a classic feedback loop on peripheral GH action (Le Roith et al., 2001) (Fig. 5).

There is synthesis of GH at extrapituitary sites as well. GH secretion is localised in neuronal populations within the central nervous system, endothelial cells of blood vessel, fibroblasts, thymic epithelial cells, cells of the immune system including macrophages, B-cells, T-cells, natural killer-cells and epithelial cells of the mammary gland (Liu et. al, 1997; Harvey et al., 1997). Besides its endocrine function GH has autocrine and paracrine effects (Liu et al., 1997; Harvey et al., 1997; Le Roith et al., 2001).

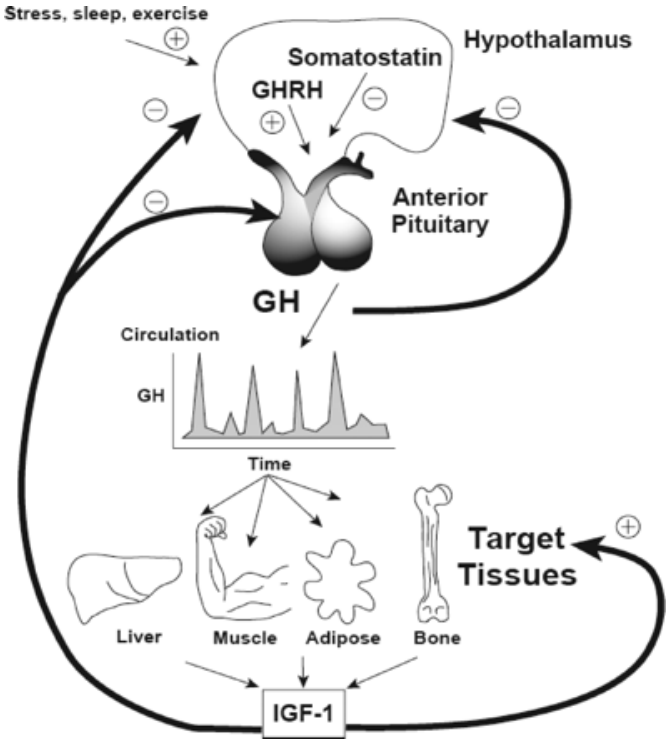


Fig. 5 The hypothalamus hormones GH releasing hormone (GHRH) and somatostatin control the circulation GH level. GHRH increases whereas somatostatin decreases GH levels. Peripheral GH binds to its receptor (GHR) on tissue as muscle, liver and bone where it provokes secretion of insulin-like factor 1 (IGF-1). IGF-1 creates a negative feedback loop to the hypothalamus/ GH release. (Redrawn from Kopenick et al, 2002).

Downstream signalling of GH

The Growth hormone receptor (GHR) belongs to the family of cytokine receptors, which are single transmembrane receptors. As all cytokine receptors, GHR has no tyrosine kinase activity. GH-receptors are present in various tissues like liver, muscle, kidney, heart, skin, thymus, adipose tissue, placenta, testis, ovary and mammary gland (Mertani et al., 1995). The GH molecule presents two receptor binding sites that allow interaction with two receptors at once. After ligand-binding, the receptor activates concomitantly JAK-2, a member of the Janus family of tyrosine kinases. Activated JAK-2 phosphorylates JAK-2 itself and the GH receptor (Bartke et al., 2013). In this manner GH activates pathways like the signal Transducers and activators of transcription (STATs), the Phospatidylinositol 3'-kinase (PI3K)/AKT and the Mitogen activated protein kinase (p44/p42 MAPK) ERK1/2 pathway, to name the main pathways of GH signalling (Zhu et al., 2001) (Fig. 6).

Concerning the STAT-pathways, GH has been reported to activate STAT-1 and STAT-3, but mainly STAT-5a and STAT-5b. STATs dissociate from the receptor-JAK-2-complex and translocate to the nucleus, binding to promoters of GH-regulated genes. The JAK-2/STAT pathway regulates GH signalling by a negative feedback loop, by stimulating the expression of suppressors of cytokine signalling (SOCS) protein. SOCS impede activation of the JAK/ STAT pathway and stimulate GH-receptor internalization (Bartke et al., 2013). STAT-5b regulates the transcription of the insulin-like growth factor 1 (IGF-1) gene (Zhu et al., 2001; Woelfle and Rotwein, 2004).

Human GH is also involved in cancer development and tumour progression. GH overexpression is associated with proliferative diseases in animals and humans (Raccurt et al., 2002). Acromegalic patients have an increased risk for developing cancer (Webb et al., 2002; Jenkins, 2004; Siegel and Tomer, 2005). On the other hand, people with a Growth hormone deficiency or GH-receptor mutation have not shown even one malignancy in a worldwide study, however, their first and second degree relatives had shown a 10- 24% incidence of cancer (Brooks and Waters, 2010). Mice overexpressing GH have also shown an increased incidence of hepatocellular carcinomas at advanced ages (Snibson 2002, Bartke 2003). Up-regulation of proliferative and anti-apoptotic cascades were shown in mice-overexpressing GH (Miquet et al. 2008). In this study the

main GH signalling pathway JAK-2/STAT5 was desensitized, assuming that the JAK-2/STAT-5 pathway is not involved in proliferative effects of overexpressed GH (Gónzalez et al., 2002, Miquet et al. 2004, 2005).

There is an important role of IGF-1 and its receptor (IGF-1R) in oncogenesis, too. An increased risk of breast, prostate and colorectal cancer in humans is linked to elevated serum levels of IGF-1 (Laban et. al, 2004; Baserga et. al, 2003).

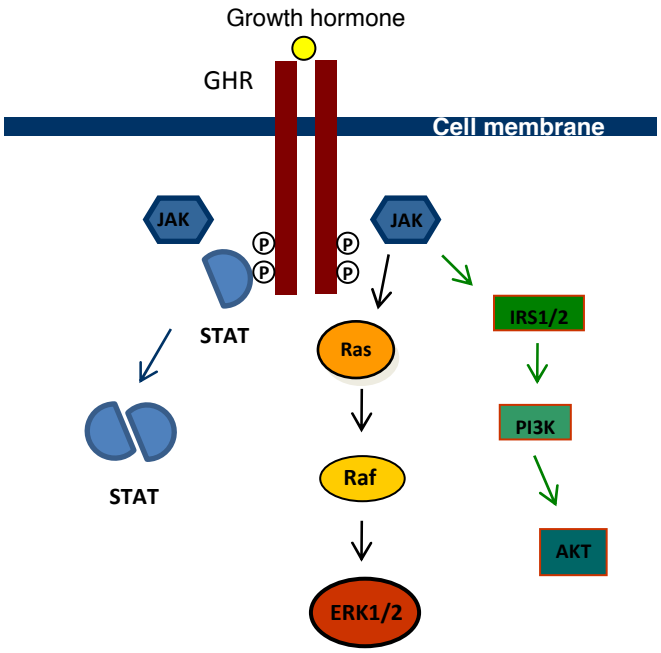


Fig. 6 Downstream signalling of GH by binding to its receptor. GH binding to the GHR induces conformational changes of the GHR, which activates JAK-2. The phosphorylated JAK-2 initiates a multitude of signalling cascades including major signalling pathways such as JAK/STAT, PI3K/AKT and MAPK pathways. STAT: Signal Transducers and Activators of Transcription, JAK: Janus kinase, Raf: rapidly accelerated fibrosarcoma, a serin/threonine kinase, Ras: guanosine exchange protein, ERK1/2: Extracellular signal-regulated kinase, IRS1/2: Insulin regulated substrate; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase, AKT: Protein kinase B.

GH and breast cancer

The mammary gland is one of the few organs with dramatic postnatal changes. GH is needed for the development of the mammary gland during puberty. Full mammary development, including lactogenesis, occurs only when GH and estrogen are present (Lyons et al., 1958; Kleinberg, 1997). GH interacts with the stroma and with the epithelial tissue, leading to ductal elongation and differentiation of ductal epithelium into highly proliferative terminal end buds (TEBS) (Kleinberg, 1997; Walden et al., 1998). GH-infusions lead to a fourfold augmentation of mammary glandular size in primates, although it is unknown if this results are induced by GH or IGF-1 (Ng et al., 1997; Laban et al., 2003). In 1952 a regression of metastatic mammary tumours was reported after hypophysectomy (Luft et al., 1952; Perry et al., 2008) which led to hypophysectomy as therapy for breast cancer in the 1950´s (Ray et al., 1962; Waters & Barkley, 2007). This was a first evidence that Growth hormone is involved in breast cancer pathogenesis.

Women with an increased birth weight have a higher risk for developing pre- and postmenopausal mammary carcinomas (Michels et al., 2006; Ahlgren et al., 2006). In a multiple cohort study, it is demonstrated that the biggest height category women (over 175cm) have an increased breast cancer risk of about 22% compared to the smallest category (less 160cm) (Gunnel et al., 2001). Twin cohort studies showed that genetic or shared environmental factors are improbably responsible for the association between height and mammary carcinomas (Lundquist et al., 2007; Perry et al., 2008). One parameter for this relation could be the GH/IGF-1 axis. It is the main player in somatic growth during childhood and needed for mammary gland development by regulating cell proliferation, differentiation and apoptosis (Kleinberg et al., 1997; Perry et al., 2008). Breast cancer patients show a higher serum level of GH or IGF-1 compared with controls (Peyrat et al., 1993).

In a human mammary epithelial cell line, autocrine GH increases proliferation, impairs apoptosis and is able to promote epithelial to mesenchymal transition (EMT) (Mukhina et al., 2004). The mammary gland development needs interactions between ovarian and pituitary hormones. Critical points occur in puberty, pregnancy, parturition, lactation and involution (Sternlicht et al., 2006; Kleinberg et al., 1997). In addition to estrogen,

Growth hormone is obligatory for mammary proliferation. In ovariectomized animals estrogen can save mammary development, but not in hypophysectomised ones, what implicates GH requirement for mammary development (Sternlicht et al., 2006; Kleinberg et al., 1997; Perry et al., 2008). As mentioned above, GH is not only produced from the pituitary gland, but also locally in the mammary gland, influencing mammary development, too (Mol et al., 1995; Raccourt et al., 2002). In humans, most expression of autocrine GH**,** GHR mRNA and GHR protein is found in the epithelial and myoepithelial ductal cells (Mol et al., 1995; Raccourt et al., 2002; Mertani et al., 1998). GHR-null mice show impaired mammary ductal development (Gallego et al., 2001). GH acts through induction of IGF-1 (Sternlicht et al., 2006; Kleinberg et al., 1997; Walden et al., 1998; Mukhina et al., 2006). Transgenic mice expressing a GH antagonist have lower level of IGF-1, are smaller in body size and show less mammary gland tumorigenesis than control (Pollak et al., 2001). Whereas transgenic mice overexpressing genes that encode GH or IGF-1 receptor agonists, show mammary gland epithelial cell hyperplasia and develop mammary neoplasia more frequently compared to controls (Laban et al., 2003).

Growth Hormone modulation of Epidermal Growth Factor signalling

As mentioned above, the ErbB-receptors can also be phosphorylated by GH and PRL, a protein related to GH (Yamauchi et al., 1997, 1998, 2000). GH was first described to induce Tyr1068 phosphorylation at EGFR in vitro and in vivo (Yamauchi et al., 1997, 1998). Furthermore, in murine preadipocytes the tyrosine residues Tyr845, Tyr992 and Tyr1173 were shown to be phosphorylated upon GH, too (Kim et al., 1999; Huang et al., 2003). Receptor transactivation upon GH is JAK-2 dependent and independent of EGFR kinase activity. GH-induced tyrosine phosphorylated EGFR binds Grb-2 and reinforces GH-induced ERK activation (Yamauchi et al., 1997). Additionally, GH causes ERK-mediated threonine phosphorylation of EGFR and ErbB-2 (Kim et al., 1999; Huang et al., 2004). That leads to delayed EGFR downregulation and enhances EGF-induced signalling (Huang et al., 2003. 2004, 2006, Li et al., 2008).

The expression of hepatic EGFR is regulated by GH. In hypophysectomized and partially GH-deficient mutant mice, the receptor expression is diminished. By

exogenous GH administration this expression deficit can be reversed (Jansson et al., 1988; Johansson et al., 1989). In transgenic mice overexpressing GH, EGFR levels are increased, whereas GH receptor-KO mice have diminished protein content (Miquet et al., 2008; González et al., 2010). In mice lacking the GH-receptor, consequently to less EGFR-expression, EGF-stimulated AKT, ERK1/2, STAT-3 and STAT-5 phosphorylation is diminished compared to normal mice (González et al., 2010). Transgenic mice overexpressing GH show, in spite of elevated EGFR content, less ligand-induced activation of ERK1/2 and AKT while STAT-3 and STAT-5 are desensitized (Gónzalez et al., 2010, Díaz et al., 2012). Recently it was shown that EGFR protein content is increased in normal rodent livers by a pulsatile plasma GH administration. But continuous GH administration decreased EGFR protein content and mRNA level (Díaz et al., 2014). Concordantly, EGF-induced downstream signalling as ERK1/2, AKT, STAT-3 and -5 increased by pulsatile and decreased by continuous GH administration (Díaz et al., 2014). ERK1/2, AKT and STATs protein content did not vary.

The Signal transducers and activators of transcription (STAT)

The Signal transducers and activators of transcription (STATs) are transduction proteins which are activated in the cytoplasm by ligand binding to transmembrane receptors. In humans, seven functionally and structurally related STAT proteins have been found (Stat-1, Stat-2, Stat-3, Stat-4, Stat-5a, Stat-5b and Stat-6). They all contain an oligomerization domain, a DNA binding domain and a Src homology 2 domain (SH2) (Quesnelle et al., 2007). In the mammary gland STAT-5a and STAT-5b are mostly expressed (Liu X et al., 1995; Kiu and Nicholson, 2012). STAT-5a and -5b are encoded by two linked genes and share over 90% identity with two related sequences (Azam et al., 1995) and differ only at their carboxy termini.

STATs can be activated by cytokines as GH. As mentioned above, the GHR has no tyrosine kinase activity and recruits members of the JAKs. Two of them get closed upon ligand binding to the corresponding receptor what allows trans-phosphorylation (Horvath and Darnell, 1997). Once connected to the receptor, JAKs phosphorylate STATs at the single tyrosine residue. STATs need to be phosphorylated at a tyrosine

residue near the C-terminus, to dimerize. Upstream of this tyrosine residue the Src homology (SH) 2 domain is located. SH2-domains recognize and bind phosphotyrosines at the transmembrane receptor (Horvath and Darnell, 1997). After phosphorylation, the dimerization of activated STATs is mediated through SH2-phosphotyrosyl peptide interactions (Shuai et al., 1994). After dimerization, the STAT dimer translocates to the nucleus and represses or activates transcription of target genes by binding to response elements of their promoters (Horvath and Danell, 1997) (Fig.7). Above all, GH activates STAT-5a and STAT-5b (Bartke et al., 2013). The activation of JAK/STAT stimulates proliferation, differentiation and cell migration, but also apoptosis. Thereby the JAK pathway influences haematopoiesis, immune development, mammary gland development and lactation as well as other processes (Rawlings et al., 2004).

Besides activation upon cytokine receptors, STATs can also be phosphorylated by non-cytokine receptors like receptor-tyrosine-kinases (RTKs) and G-protein-coupled receptors at their tyrosine residue (Schindler and Strehlow, 2000). Epidermal growth factor (EGF) and Plateled derived growth factor (PDGF) signal through RTKs. EGF can activate STAT- 1, -3 and -5 whereas PDGF activates STAT-1 and -3 (David et al., 1996; Vignais et al., 1996). In breast cancer cell lines, EGF-induced activation of STAT-5b needs overexpression of the Epidermal growth factor receptor (EGFR) as it is found in many cancers including breast cancer (Kloth et al., 2001). EGFR can activate STATs by direct binding through the SH2 domain or by EGFR-mediated activation of the protein tyrosine kinase Src (Src) (Fig.7). In case of direct STAT binding to EGFR, JAK is not necessary for activation (Quesnelle et al., 2007). In breast cancer cells ligand-induced EGFR activation of STAT-1, STAT-3 and -5 happens through Src kinases, independently of JAKs (Olayioye et al., 1999). But for maximal STAT activation JAKs are necessary (Quesnelle et al., 2007). Interestingly, the EGFR-induced STAT-5 phosphorylation via Src occurs at a different tyrosine site, at Y694, not at the JAK phosphorylated site Y699. Phosphorylation at Y694 leads to different nuclear localization and an impaired ability to bind DNA (Quesnelle et al., 2007).

Suppressors of cytokine signalling (SOCS), Protein inhibitors of activated STATs (PIAS) and Protein tyrosine phosphatases (PTPs) regulate the JAK/STAT pathway negatively (Greenhalgh and Hilton, 2001). The tyrosine phosphatases reverse the activity of JAKs. Activated STATs stimulate transcription of SOCS genes whereas

SOCS proteins bind phosphorylated JAKs and the cytokine receptor, hence limiting JAK signalling by creating a classical feedback loop. The PIAS proteins prevent STAT binding at the DNA by occupying activated STAT dimers (Rawlings et al., 2004).

As mentioned above, in mammary gland basically STAT-5a and -5b are expressed (Liu et al., 1995; Kiu and Nicholson, 2012). STAT-5a is needed for functional development of mammary tissue. The alveolar proliferation and functional differentiation are inhibited when STAT-5a is absent (Liu X et al., 1997, 1998). STAT-5a/ STAT-5b deficient mice are retarded in growth and have impaired development of the mammary gland (Cui et al., 2004; Kiu and Nicholson, 2012). STAT-1, STAT-3 and STAT-5 are constantly phosphorylated in solid tumours in breast, lung and head and neck cancers via increased levels of cytokines and cytokine receptors (Quintás-Cardama and Verstovsek, 2013).

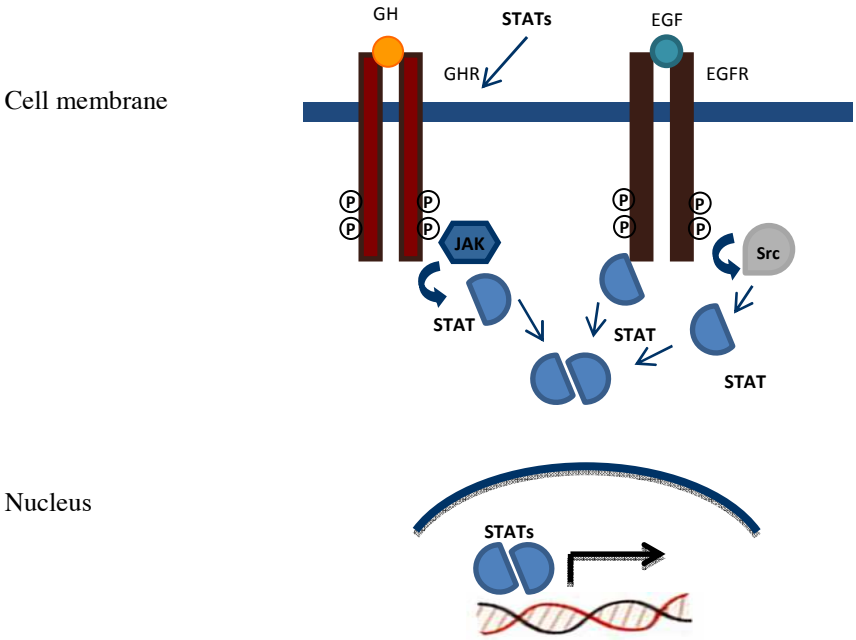


Fig. 7 The STAT signalling pathway upon ligand binding to GHR (left) and to EGFR (right). STATs can be activated through JAK via GH, via EGFR directly or via EGFR-mediated Src. Activated STATs are released from the receptor, dimerize, translocate to the nucleus and bind to promoters. GH: Growth hormone, JAK: Janus kinase; STAT: Signal transducers and activators of transcription, EGF: Epidermal growth factor, EGFR: EGF-receptor, Src: protein tyrosine kinase Src.

Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are enzymes that interconnect extracellular signals from the cell surface to the cell interior. They induce growth, proliferation, differentiation, migration and apoptosis. The mammalian MAP-kinases consist of cytoplasmic protein-serine/threonine kinases. MAPK are activated by mitogen-activated protein kinase kinases (MAPKKs), which themselves are activated through phosphorylation by mitogen-activated protein kinase kinase kinases (MAPKKKs). There are six known groups of MAPKs in mammals: Extracellular signal-regulated kinase (ERK) 1/2, ERK 3/4, ERK 5, ERK 7/8, Jun N-terminal kinase (JNK) 1/2/3 and p38 isoforms α, β, ɣ (ERK6)/ δ (Schaeffer and Weber, 1999; Kyrias and Avruch, 2001; Krens et al., 2006).

In some forms of human breast cancers, in many human breast cancer experimental models and approximately in one-third of all entities of human cancers the ERK1/2 pathway is deregulated and plays an important role in tumorogenicity (Santen et al., 2002; Dhillon et al., 2007). The serine/threonine kinases ERK1 and ERK2 are protein isoforms, which are 85% homologous, especially concerning the core regions that are involved in binding substrates (Boulton et al., 1990; Boulton et al., 1991). ERK1/2 cascades are activated via consecutive phosphorylations. Raf kinase (MAPKKK) phosphorylates MEK1/2 (MAPKK), which in turn phosphorylates and thus activates ERK1/2 (Fig. 8).

Among others, the ERK pathway is triggered by growth factors and cytokines. They activate the receptor in a ligand-mediated fashion and lead to guanosine triphosphat (GTP) loading of the Ras GTPase. Ras itself is able to trigger Raf kinases to the plasma membrane for subsequent activations. The ERK pathway is also activated by G-protein coupled receptors and non-nuclear activated steroid hormone receptors (Boonstra et al., 1995; Alessi et al., 1994; May et al., 2008; Whyte et al., 2009).

Activated ERK1/2 phosphorylates diverse cytoplasmic proteins or translocates from the cytoplasm to the nucleus and activates nuclear targets such as kinases, transcription factors and cytoskeletal proteins (Yoon and Seeger, 2006). ERK 1/2 regulates different processes like proliferation, differentiation, survival, migration, angiogenesis and

chromatin remodelling (Pearson et al., 2001). ERK1/2 determines these specific processes through different ways, which are not completely understood so far. Sustained, not transient ERK signalling stabilizes and promotes genes that are required for cell cycle entry as Cyclin D1. Signalling of Ras/ERK is associated with Cyclin D1 in cell cycle progression from G0/G1 to S phase and subsequent with retinoblastoma (Rb) phosphorylation (Sears et al., 2002; Lavoie et al., 1996; Torii et al., 2006). ERK activation needs to be sustained 2-3 hours before S phase onset (Yamaomoto et al., 2006).

ERK also represses the expression of antiproliferative genes (Yamamoto et al., 2006). Bim, a pro-apoptotic BH3-only domain protein of the BCL (B-cell lymphoma)-2 family is phosphorylated by ERK1/2. Bim drives lumen clearance through cell death in the terminal end buds during puberty (Reginato et al., 2005). Phosphorylated by ERK1/2 Bim degrades, thus leading to cell survival. (Ley et al., 2003; Harada et al., 2004). EGFR induced ERK1/2 activation regulates Bim negatively at a transcriptional level and also by phosphorylation (Reginato et al., 2005).

ERK activity is balanced by a negative feedback from members of the Sprouty family, Spred and Sef (Hacohen et al., 1998; Wakioka et al., 2001; Furthauer et al., 2002). Spred controls the duration of ERK activity (Hanafusa et al., 2002), whereas Sef inhibits nuclear translocation (Torii et al., 2004). Furthermore, the ERK1/2 pathway is positively and negatively controlled by scaffold proteins (McCubrey et al., 2006).

Dysregulated ERK1/2 signalling is involved in breast cancer. But it needs more than a dysregulation to cause cancer. Additional mutations or changes are required, for example in the expression of other genes as the ErbB-2 receptor, p53 or c-myc (Marampon et al., 2006; Torii et al., 2006). Hyperproliferation in breast cancer cell lines is often mediated by EGFR- or ErbB-2- dependent activation of ERK1/2 (Mc Cubrey et al., 2006). Growth hormone requires EGFR for ERK1/2 activation (Rodland et al., 2008). GH-induced-ERK1/2 dependent phosphorylation retards EGF-induced EGFR downregulation, thus potentiates EGF-induced signalling (Huang et al., 2006; Li et al., 2008).

The correlation between breast cancer and ERK1/2 activation level is unclear. Clinical studies led to contrary results. Node negative breast cancer patients and patients with relapse-free survival had lower ERK 1/2 activity than node-positive patients and

patients with relapse (Mueller et al., 2000). On the other hand, there are clinical results which link high ERK1/2 protein and high activated ERK1/2 levels to a good outcome (Berggvist et al., 2006; Milde-Langosch et al., 2005).

Growth hormone (GH) and Epidermal growth factor (EGF) synergize in ERK activation in murine preadipocytes at the level of Raf/MEK activation (Li et al., 2008). Crosstalk with other pathways as with the Phophoinositide-3-kinase (PI3K)/AKT enhance cell proliferation and prevent apoptosis (Mc Cubrey et al., 2006).

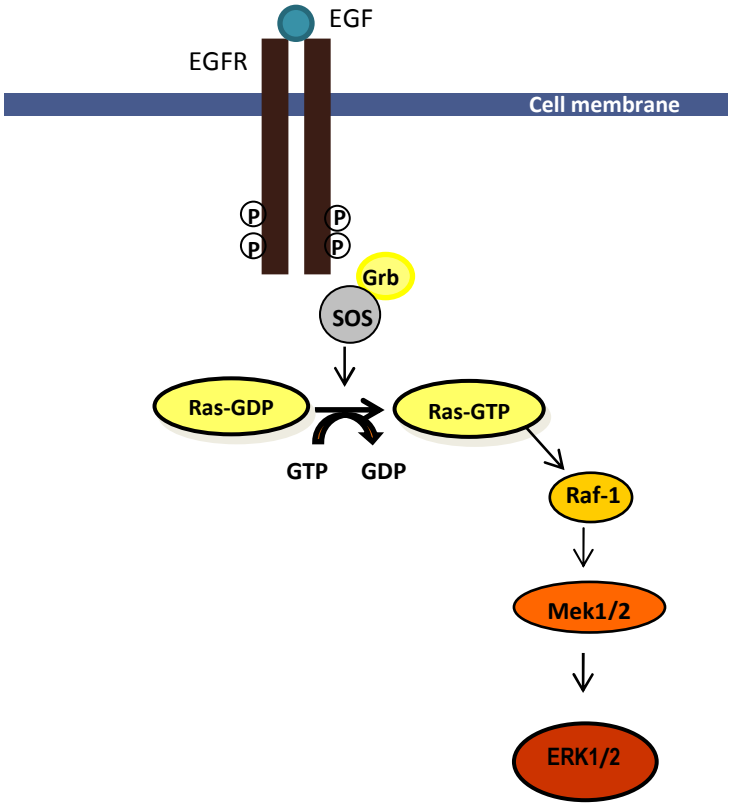


Fig. 8 The Ras/Raf/Mek/ERK pathway. Stimulation by a mitogen like EGF, the activated receptor recruits Ras guanine nucleotide-exchange factors, such as SOS through the adaptor protein Grb2, which generates Ras-GTP. Ras-GTP induces Raf-1, which activates Mek1/2 that phosphorylates ERK1/2. ERK1/2 itself translocates to the nucleus to activate target genes. Grb2: growth-factor-receptor-bound-2, SOS: son of sevenless , Ras: guanine nucleotide-exchange factor, Raf: proto-oncogene serine/threonine-protein kinase, Mek: Mitogen-activated protein kinase kinase (MAPKK), ERK: extracellular-signal-regulated kinase.

The Phosphatidylinositol-3-Kinase-AKT Pathway

Activated Phosphatidylinositol-3-kinases (PI3Ks) are able to phosphorylate inositol ring 3`OH-groups in inositol phospholipids, characterizing them as a lipid kinase family (Fruman et al., 1998). Class I Phosphatidylinositol-3-kinases are heterodimers, consisting of an adaptor subunit (p85) and a catalytic subunit (p110) (Fry MJ, 1994). Class I is divided into Class IA which adaptor subunit is a phosphoprotein substrate of many receptor tyrosine kinases such as insulin receptor, Insulin-like growth factor receptor and Epidermal growth factor family members. Class IB is activated by G-protein coupled receptors and oncogenes such as Ras. Activated PI3K converts the second messenger phosphatidylinositol-4,5-biphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3) in the membrane through phosphorylation of the 3`hydroxyl group. PIP3 activates downstream pathways like AKT and other proteins (Vivanco and Sawyers, 2002). GH activates PI3K through association of the p85 subunit and Insulin receptor substrate (IRS) 1, IRS-2 and JAK-2 (Zhu et al., 2001) or directly via JAK-2 (Birzniece et al., 2009). It is also reported that PI3K can bind directly to the phosphorylated tyrosine residues of the GH-receptor (Zhu et al., 2001).

Furthermore PI3K is activated through the phosphorylated EGFR which generates docking sites for the Growth factor receptor-bound protein 2 (Grb2), an adaptor protein that associates with Gab1 (Grb2-associated binder 1). This association leads to tyrosine phosphorylation of Gab1 (Gu et al., 2003). Phosphorylation at Tyr472 serves as binding site for the regulatory subunit of PI3K (p85) (Cunnick et al. 2001). When the regulatory subunit of PI3K binds to Gab1, PI3K becomes active and produces PIP_3 leading to the activation of AKT.

AKT is a main downstream kinase of PI3K and is also called protein kinase B (PKB). AKT encodes a 57kDa Ser/Thr kinase that has a pleckstrin homology (PH) domain, a central catalytic domain and a carboxy-terminal regulatory domain. The AKT family consists of three members (AKT-1, AKT-2, AKT-3). AKT-1 and -2 are ubiquitously expressed, AKT-3 is more limited in tissue distribution (Yang et al., 2003). However, generation of PIP3 recruits AKT by binding to its PH-domain, leading to AKT translocation to the plasma membrane where it can be phosphorylated and activated by Phosphoinositide-dependent kinase (PDK) 1, PDK2 and integrin-linked kinase (ILK)

(Fig. 9). AKT gets phosphorylated at its kinase domain at threonine 308 and at its regulatory domain at serine 473. Phosphorylation at Thr308 by PDK-1 activates AKT partially, but full activation requires phosphorylation at Ser473 (Osaki et al. 2004, Nicholson et al. 2002, Alessi et al. 1996). Both sites can be phosphorylated independently (Nicholson et al. 2002, Alessi et al. 1996).

Activation of AKT leads to cell survival, proliferation and growth. AKT acts as an anti-apoptotic signal by blocking the function of proapoptotic proteins. For example, the protein phosphorylates and consequently inhibits action of the BH3-only protein Bad, a pro-apoptotic member of the BCL-2 (B-cell lymphoma 2) family (Datta et al., 1997). Furthermore, AKT inhibits the expression of BH3-only proteins via interaction with transcription factors, such as FOXO and p53. Preventing the transcription of these target genes, which promote apoptosis or cell cycle arrest, leads to cell survival (Manning and Catley, 2007). The protease Caspase 9 is phosphorylated and therefore inhibited by AKT, too, what prevents its catalytic function of cell-death (Cardone et al., 1998).

AKT plays also a role as pro-survival factor by preventing Cyclin D1 from degradation. The cell cycle is regulated by cyclin-dependent kinase (CDK) complexes and CDK-inhibitors (CKIs) (see below). Cyclin D1 plays an important role in cell cycle progression in G1/S phase transition. AKT prevents Cyclin D1 degradation via regulation of the Glycogen synthase kinase-3 β (GSK3β). GSK3β regulates Cyclin D1 through phosphorylation, initiating its degradation by the proteasome. The kinase function of GSK3β is blocked when phosphorylated by AKT, giving Cyclin D1 the possibility to accumulate (Diehl et al., 1998).

One central regulator of cell growth is the serine/threonine kinase mTOR (the mammalian target of rapamycin). mTOR functions as a sensor, regulating protein synthesis based on the presence of nutrients and growth factor signalling. mTOR is a direct target of AKT and mTOR activity can be suppressed by wortmannin, a PI3K inihibitor (Vivanco and Sawyers, 2002). AKT is able to enhance protein synthesis of mTOR, whereas activated mTOR stimulates Cyclin D mRNA translation (Muise-Helmericks et al., 1998).

Negative regulation of the PI3K/AKT pathway is performed by PTEN (phosphatase and tensin homologue deleted on chromosome 10). PTEN is a PIP3 phosphatase, first isolated as tumour-suppressor gene in breast cancer and glioblastomas (Steck et al.,

1997; Li J et al., 1997). PTEN reverses the action of PI3K by PIP3 dephosphorylation, and hence regulates AKT activity (Wang et al., 2008).

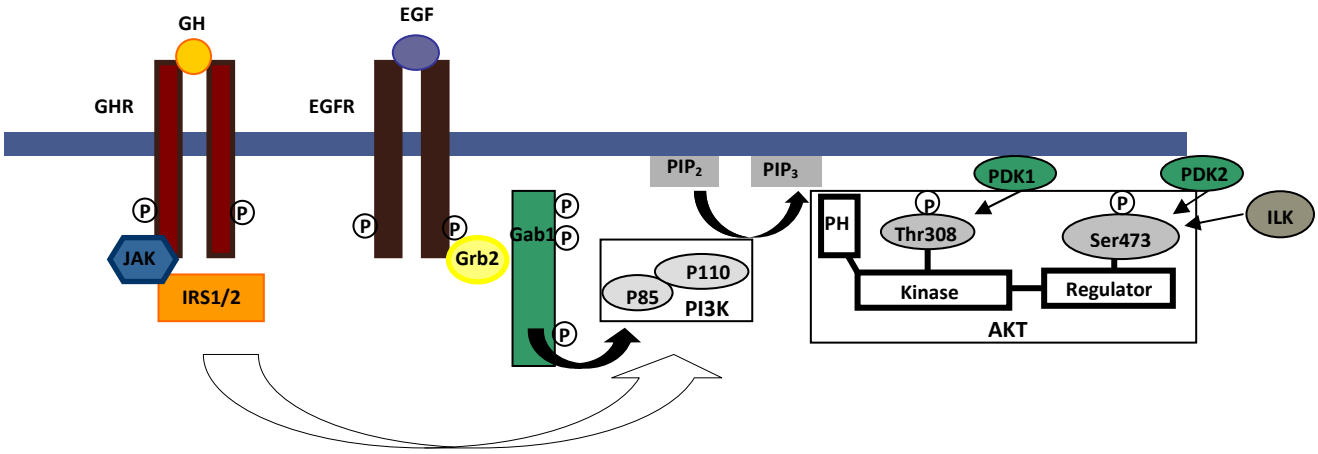


Fig. 9 Model of PI3K-AKT pathway, class I. Activation of PI3K through the GHR (left) or the EGFR (right). Activation stimulates the phosphatidylinositol 3-kinase (PI3K) containing a p85 and a p110 subunits. PI3K turns phosphatidylinositole-4,5 biphosphonate (PIP2) to phosphatidylinosisol 3,4,5 triphosphonate (PIP3). AKT interacts with PIP3 via its PH domain and translocates to the cell membrane. AKT gets phosphorylated at its kinase domain at Thr308 and its regulator domain Ser473 by phosphoinositol-dependent kinase (PDK) 1 and 2 and integrin-linked kinase (ILK).
GH: Growth hormone, GHR: GH-receptor, JAK: Janus kinase, IRS: Insulin receptor substrate, EGF: Epidermal growth factor, EGFR: EGF-receptor, Gab1: Grb2 associated binder-1, PI3K: phosphatidylinositol 3-kinase, PTEN: Phosphatase and tensin homologue deleted on chromosome ten, AKT: Protein kinase B, PDK: phosphoinositol-dependent kinase, ILK: integrin-linked kinase.

Cyclin D1 and the cell cycle

The cell cycle is divided into four sequential phases. It passes through gap 1 (G1), synthesis (S), gap 2 (G2) and mitosis (M) phases. Progress through the cell cycle is controlled by cyclin and cyclin-dependent kinase (CDK) complexes. Cyclin-dependent kinases require cyclins as regulatory subunit for kinase activity (Morgan et al., 1997; Liu et al., 2004). The protein level of cyclins changes throughout the cell cycle. Cyclins bind to the corresponding CDK and form complexes: Cyclin D- CDK4 and/or 6 complex for G1 progression, cyclin-E CDK2 for the G1/S transition, cyclin A-CDK-2

to pass synthesis and cyclin B-CDK1 to enter mitosis (Matsushime et al., 1991; C.J. Sherr,1994; Stamatkos et al., 2010) (Fig. 10).

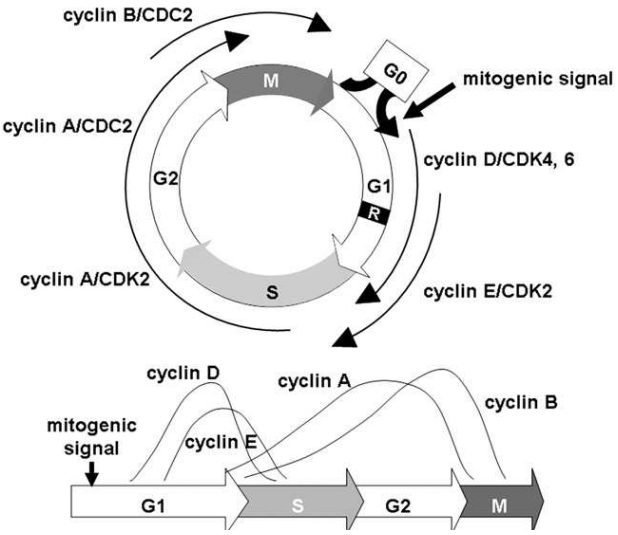


Fig. 10 Model of the mammalian cell cycle. Progress through the cell cycle contains gap 1 (G1), synthesis (S), gab 2 (G2) and mitosis (M) phases. Cyclins control together with cyclin-dependent kinases (CDK) the progression through the phases, respectively. Cyclins have a typical pattern of expression and degradation. R. restriction point. CDK; cyclin-dependent kinase. Redrawn from Takahashi-Yanaga et al., 2008.

Cyclin D1/CDK4/6 together with cyclin E/CDK2 phosphorylate and consequently inactivate the retinoblastoma protein (pRb). pRb functions as a gatekeeper of the G1 phase inhibiting the transition from G1 to S phase by repressing the transcription of genes needed for DNA synthesis. It binds E2F transcription factors and forms a repressor complex that contains proteins with intrinsic histone deacetylase activities (HDACs) and SWI/SNF (SWItch/Sucrose Non Fermentable) nucleosome remodeling complexes. pRb represses the E2F activity at the cyclin E promoter. After Cyclin D1/CDK4/6 phosphorylation of the pRb, the repression is destructed and unbound E2F can function as transcriptional factor. Among others, E2Fs induce the expression of cyclin E and A genes. Cyclin E binds CDK2 and as complex it completes pRb phosphorylation (Baldin et al., 1993; Hinds et al., 1992 ; Harbour JW et al., 1999; Zhang HS et al., 2000; Sherr CJ, 2000) (Fig. 11).

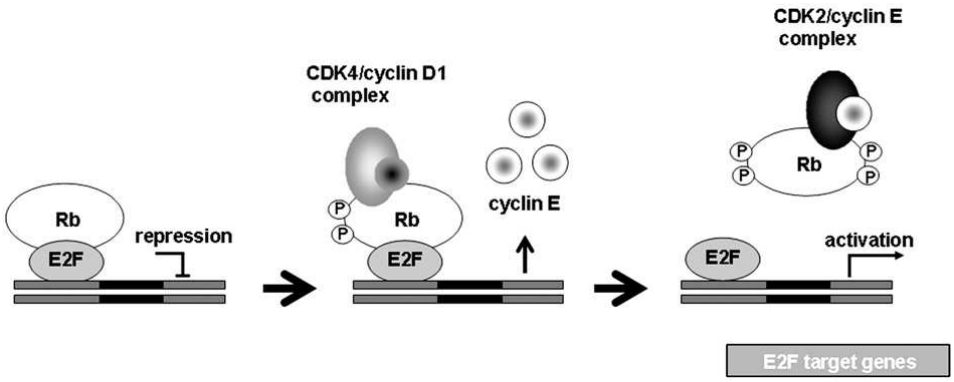


Fig. 11 In the cell cycle, G1 to S phase transition is regulated by retinoblastoma protein (Rb), which serves as a gatekeeper of G1 phase. Rb binds to E2F. Rb is partially phosphorylated by Cyclin D1/CDK4 or 6 resulting in the transcription of genes like cyclin E. Cyclin E binds to and activates CDK2, leading to complete phosphorylation of Rb and its release. E2F induces the expression of target genes needed for S-phase entry. CDK: cyclin-dependent kinase. Rb: Retinoblastoma protein, E2F: transcription factor. Redrawn from Takahashi-Yanaga et al., 2008.

Protein level of Cyclin D1 rises in early G1 and starts to accumulate until the G1/S-phase. In contrast to the CDKs, cyclin has a short half-life of approximately 30 minutes before degradation (Sherr, 1994). When cells once have progressed to S phase of the cell cycle, Cyclin D1 is exported from the nucleus to the cytoplasm leading to its proteolysis by a 26S proteasom.

Cyclin D1 expression is induced by growth factors, hormones and oncogenes including Ras (Albanese et al., 1995), ErbB-2 (Lee et al., 2000) and STATs (Matsumura et al., 1999; Brockman et al, 2002). EGF leads to p42/44 MAP kinases, which up-regulate the expression of c-fos and c-jun genes, whose products then heterodimerize and bild the transcription factor Activator protein 1 (AP-1). AP-1 binds to the promoter of Cyclin D1 (Shaulian & Karin, 2001). In addition Insulin like growth factor stimulates phosphatidylinositol-3 kinase which induces Cyclin D1 mRNA (Joyce et al., 1999). IGF-1 stabilises Cyclin D1 mRNA, suggesting that PI3K might stabilise Cyclin D1 mRNA (Dufourny et al., 2000).

Cyclin D is an important target of the STAT signalling pathways as enhancers of the Cyclin D1 promoter (Leslie et al., 2006; Matsumura et al., 1999; Brockman et al, 2002).

CDK inhibitory proteins, Cyclin-dependent Kinase Inhibitors CKIs, negatively regulate CDKs (Sherr and Roberts, 1999). In mammals the CDK-inhibitory protein/kinase Inhibitor protein (Cip/Kip) family is formed by three different proteins p21Cip1, p27Kip1 and p57Kip2.

Objective

Growth hormone is one of the main players in postnatal growth, stimulates cell proliferation and differentiation. There are physiological situations in which GH levels are elevated in humans as in puberty (Bartke et al., 2013). But the permanent elevation of GH is associated with cancer development. Acromegalic patients, who are always exposed to high GH levels, have an increased risk to develop cancer (Webb et al., 2002; Jenkins, 2004; Siegel and Tomer, 2005). Otherwise, in people with a deficiency of GH or a GHR mutation, no malignancy could be observed in a worldwide study (Brooks and Waters, 2010). GH is required for the maturation of the mammary gland (Lyons et al., 1958; Kleinberg, 1997), but is also involved in the process of breast cancer development. In the 1950´s, it was reported on patients with mammary tumours who had a regression of metastases after hypophysectomy (Luft et al., 1952). This was the first evidence of GH involvement in breast cancer progression. Transgenic mice overexpressing genes that encode GH or IGF-1 receptor agonists, show mammary gland epithelial cell hyperplasia and more frequent mammary neoplasia than their normal siblings (Laban et al., 2003). Another important protein which is involved in cell survival and differentiation is EGF and its receptor, the EGFR (Boonstra et al., 1995). The overexpression of EGFR is involved in different types of cancer, including breast cancer (Xie er al., 1997; Berger et al., 1988; Verbeek et al., 1998; Biscardi et al., 2000; Mendelsohn and Baselga, 2000, 2003). EGFR expression is associated with high tumour grade and reduced survival (Sainsbury et al., 1987; Nicholson et al., 1989; Walker & Dearing, 1999). Especially aggressive phenotypes, as triple-negative breast cancer show EGFR overexpression (Burness et al., 2010; Guerin et al., 1989).

GH and EGF advance cell survival, cell growth and differentiation and elevated acivity is involved in cancer development. There is evidence that this two factors influence each other. GH leads to tyrosine phosphorylation of the EGFR in mice livers and in cell culture (Yamauchi et al., 1997). GH also induces 1068Y, 845Y, 992Y and 1173Y phosphorylation in EGFR in mouse preadipocytes (Kim et al., 1999; Huang et al., 2003). It is reported that GH prevents EGF-induced EGFR degradation, action that would be mediated by the MEK-ERK1/2 pathway (Huang et al., 2003). In hypophysectomized or GH-deficient mutant mice, EGFR expression is diminished in liver tissue (Jannson et al.,

1988; González et al., 2010) and GH-treatment reverses this downregulation of receptors (Jannson et al., 1988). Moreover, EGFR expression is increased in the liver of transgenic mice overexpresing GH (Miquet et al., 2008; González et al., 2010)

EGF-induced phosphorylation of EGFR at its tyrosine residues results in downstream signalling cascades as the mitogen activated protein kinase Ras/Raf/MEK/ERK (p44/p42 MAPK), the PI3K/AKT and the STAT-pathways (Jorrisen et al., 2003; Schulze et al., 2005; Normanno et.al., 2006). These pathways are involved in cell survival and growth. Hyperproliferation in breast cancer cell lines is often mediated by EGFR- or ErbB-2-dependent activation of ERK1/2 (Mc Cubrey et al., 2006). Growth hormone requires EGFR for ERK1/2 activation (Rodland et al., 2008). The same pathways are involved in GH-signalling. Via JAK-2, GH activates mainly STAT-5a and -5b, the PI3K/AKT and the ERK1/2-pathways (Zhu et al., 2001). Via GH/GHR, STAT-5b regulates the transcription of the IGF-1 gene, a main way through which GH stimulates growth (Zhu et al., 2001; Woelfle and Rotwein, 2004). The dysregulation of the ERK1/2-pathway plays an important role in tumorogenicity (Santen et al., 2002; Dhillon et al., 2007). ERK and AKT function as pro-survival factors by stabilizing Cyclin D1 (Lavoie et al., 1996; Diehl et al., 1998; Torii et al., 2006).

These three downstream pathways of GH and EGF, ERK1/2, AKT and STAT5, that regulate gene expression and cell behaviour, were examined in this thesis.

The objective of this study was to examine direct and long-term influence between GH and EGF in breast cancer cell lines, considering the relevance of GH and EGF in mammary gland tumorigenesis and reported crosstalk of these growth factors in other cell types. For this purpose, two experimental conditions were used. First, concomitant treatment of GH and EGF was examined to analyse short-term effects, especially inhibition or activation of proteins of the growth factor pathways as ERK1/2, AKT and STAT-5. Furthermore, 24 hours GH-pre-treatment before acute EGF stimulation was analysed to examine if long-term GH stimulation has a modulatory role over EGF-induced signalling in breast cancer cells. Two different breast cancer cells lines were used. A ER and PR positive cell line, MCF-7 and a hormone-independent cell line MDA-MB-231, which is triple negative for ER, PR and Her-2, (http://www.lgcstandards-atcc.org/products/all/HTB-26.aspx?geo_country=de#generalinformation, 23.05.2014, 13:09h, Tate et al., 2012).

In order to correlate signal transduction pathway activation with pro-mitogenic actions of these growth factors, cell viability was examined under concomitant treatment of GH and EGF and under GH-pre-treatment before EGF stimulation to analyze if GH-pre-treatment modulates EGF proliferative actions over breast cancer cells.

Materials and methods

Reagents

Recombinant human GH was obtained from Biosidus, Argentina. Recombinant human EGF was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). 3,5cm wells and clear 96-well plates were acquired from Corning Costar, Fisher Scientific, USA. Polyvinylidenedifluoride (PVDF) membranes, Amersham ECL-Prime Western Blot Detectant Reagents (ECL-Plus, ECL-Prime) were purchased from Amersham Biosciences). Hyperfilm were purchased from GE Healthcare. Secondary antibodies conjugated with horseradish peroxidase and antibodies anti-Cyclin D1 anti-STAT5 were purchased from Santa Cruz Biotechnology Laboratories. Antibody anti-phospho-STAT5a/b Tyr694/696 was from Upstate Laboratories. Antibodies anti-phospho-AKT Ser473, anti-AKT pan, anti-p44/42 MAP kinase (ERK1/2), anti-phospho-p44/42 MAPkinase Thr202/Tyr204 (pERK1/2), anti-EGFR were from Cell Signaling Technology Inc. Recombinant human EGF, Trizma base, HEPES, Tween 20, Triton X-100, sodium dodecyl sulfate (SDS), glycine, ammonium persulphate, aprotinin, phenylmethylsulphonyl fluoride (PMSF), sodium ortho-vanadate, 2-mercaptoethanol, Kodak X-Omat XAR-5 films, molecular weight markers and BSA-fraction V were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA.). Mini Protean apparatus for SDS-polyacrylamide electrophoresis, miniature transfer apparatus, acrylamide, bis-acrylamide and N, N, N', N'- tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, California, USA). BCA protein assay kit was obtained from Thermo Scientific, Pierce Protein Research Products. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay from Promega, Madison, WI, USA.

Cell culture

The human breast cancer cell line, MCF-7 and MDA-MB-231 human breast cancer cell line were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were maintained in Dulbecco's Modified Essential Medium (DMEM)

supplemented with 10% fetal bovine serum (FBS), 50 mg/ml gentamicin sulfate (Invitrogen, Life technology) and 2 mM L-glutamine (Invitrogen, Life technology). Cells were cultured in 75 cm^2 culture flasks at 37ºC in a humidified atmosphere of 5% CO_2.

MCF-7 (Michigan Cancer Foundation-7) cells derive from a human epithelial cell line isolated in 1970 from the breast-tissue of a 69-year old Caucasion woman, Frances Mallon. The women suffered from a malignant adenocarcinoma. MCF-7 cells originate from pleural effusion, where tumour tissue was taken. These cells are hormone dependent and have the ability to process estrogen via estrogen receptors (ER) in the cell cytoplasm. MCF-7 cells are also capable to perform domes in vitro and express insulin-like growth factor binding proteins 2,4 and 5. By treatment with anti-estrogens insulin-like growth factor binding proteins can be modulated, resulting in cell growth reduction. Growth of MCF-7 cells can be inhibited by tumour necrosis factor alpha (TNF alpha). MCF-7 cells have a population doubling time of 29 hrs (http://www.mcf7.com/, 23.5.2014, 09:58h; http://www.lgcstandards-atcc.org/Products/All/HTB-22.aspx, 23.5.2014, 10:05h).

The MDA-MB-231 cell line is an epithelial estrogen-receptor negative breast cancer cell line, which derives from an adenocarcinoma of the mammary gland of a 51-year old Caucasian women. Cells were taken from pleural effusion. (http://www.lgcstandards-atcc.org/products/all/HTB-26.aspx?geo_country=de#generalinformation, 23.05.2014, 11:08). MDA-MB-231 cells overexpress the Epidermal Growth Factor Receptor (EGFR) and are resistant to the small molecule EGFR inhibitor (TKIs) Erlotinib (Bartholomeusz et al., 2011). They also express the transforming growth factor alpha (TGF alpha) receptor. MDA-MB-231 cells have a population doubling time of 38 hrs (http://www.lgcstandards-atcc.org/products/all/HTB-26.aspx?geo_country=de#generalinformation, 23.05.2014, 11:08).

Cell culture treatment "Concomitant treatment with GH and EGF"

To analyze GH and EGF signalling, cells were seeded in clear 3,5cm wells (Corning Costar, Fisher Scientific, USA) at a density of 350.000 cells/plate and incubated for 24

hours at 37ºC in a humidified atmosphere of 5% CO_2 to allow cell attachment. Cells were incubated again for 24 hours preserved in DMEM without serum. Serum-starved cells were stimulated with GH (1 μg/ml), hEGF (100 ng/ml) or both for 15 minutes, 30 minutes and 120 minutes or vehicle (as control) (Table 1). Stimulations were terminated by washing cells with cold phosphate-buffered saline (PBS) and dishes were kept at -80ºC until cell solubilization to prepare cells extracts.

Control	-		
GH	15min	30min	120min
EGF	15min	30min	120min
GH+EGF	15min	30min	120min

Table 1 Experimental setting of cells for concomitant treatment with GH and EGF

Cell culture treatment "GH-pre-treatment effects over EGF signalling"

For the investigation of GH pretreatment effects over EGF signalling, cells were seeded in clear 3,5cm wells (Corning Costar, Fisher Scientific, USA) at a density of 350.000 cells/well and incubated for 24 hours at 37ºC in a humidified atmosphere of 5% CO_2 to allow cell attachment. To synchronize, cells were incubated again 24 hours maintained in DMEM without serum. Serum-starved cells were treated with human GH (1 μg/ml) or vehicle and incubated for 24 hours again. Afterwards, cells were stimulated with human EGF (100ng/ml) or vehicle (as controls) for time-periods of 15 minutes, 30 minutes and 120 minutes as specified in each experiment (table 2). Stimulations were terminated by washing the cells with cold phosphate-buffered saline (PBS) and dishes were kept at -80ºC until cell solubilization to prepare cells extracts.

24hours pre-treatment	Acute EGF stimulation			
No pre-treatment	-	15min	30min	2hs
GH-pre-treatment	-	15min	30min	2hs

Table 2 Experimental setting of cells for GH-pre-treatment effects over EGF signalling

Preparation of cell extracts and immunoblotting

Cells were homogenized in buffer composed of 1% v/v Triton, 0.1 M Hepes, 0.1 M sodium pyrophosphate, 0.1 M sodium fluoride, 0.01 M EDTA, 0.01 M sodium vanadate, 0.002 M PMSF, and 0.035 trypsin inhibitory units/ml aprotinin (pH 7.0) at 4ºC. After centrifugation at 15,000 x g for 40 minutes at 4ºC, the detergent extracts (supernatant) were measured by the BCA protein assay kit to determine protein concentration. An aliquot of solubilized cells was diluted in Laemmli Sample Buffer, boiled for 5 minutes and stored at -20ºC until electrophoresis.

Samples were subjected to electrophoresis in SDS-polyacrylamide gels using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Equal amount of total protein was loaded in each lane. Electrotransference of proteins from gel to PVDF membranes was performed for 1 h at 100 V (constant) using the Bio-Rad miniature transfer apparatus in 0.025 mol/l Tris, 0,192 mol/l glycine, and 20% v/v methanol, pH 8.3. To reduce non-specific antibody binding, membranes were incubated 1,5 h at room temperature in T-TBS buffer (0.01 mol/l Tris–HCl, 0.150 mol/l NaCl, and 0.02% v/v Tween 20, pH 7.6), containing 3% w/v BSA. The membranes were incubated overnight at 4 ºC with the primary antibodies. After washing with T-TBS, membranes were incubated with a secondary antibody conjugated with HRP for 1 h at room temperature and washed with T-TBS. Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL-Plus, ECL-Prime, Amersham Biosciences) using preflashed Kodak XAR film (Eastman 222 Kodak). Band intensities were quantified using Gel-Pro Analyzer 4.0 software

(Media Cybernetics, Silver Spring, MD, USA). To reprobe with other antibodies, the membranes were washed with T-TBS for several times for 3-5 hours while shaking.

Non-Radioactive Cell Proliferation Assay

Cells were seeded in clear 96-well plates (Corning Costar, Fisher Scientific, USA) at a density of 20,000 cells/well maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 mg/ml gentamicin sulfate (Invitrogen, Life technology) and 2 mM L-glutamine (Invitrogen, Life technology). Cells were further incubated at 37ºC for 24 in a 5% CO_2 atmosphere for 24 hours. Then medium was changed to DMEM without serum and the cells incubated again for 24 hours. Cells received the following treatment for 48 hours: I) vehicle, II) 1 μg/ml GH (cc), III) 100 ng/ml EGF (cc) or IV) both cellular mediators, additionally V) cell were incubated with GH for 24 hours and afterwards medium was changed to medium with human EGF. Cells were incubated for another 24 hours (Table 3).

I	Control (vehicle)	
II	GH 48hs	
III	EGF 48hs	
IV	GH+EGF 48hs	
V	GH 24hs	+ EGF 24hs

Table 3 Experimental setting of cells treated for Non-Radioactive Cell Proliferation Assay

After incubation viability was evaluated using the CellTiter 96® AQueous Non-radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). Values were expressed in terms of percent of untreated control cells. The CellTiter 96® AQ_{ueous}

Assay is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490nm can be measured directly from 96-well assay. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture (Product information, http://www.promega.de/resources/protocols/technical-bulletins/0/celltiter-96-aqueous-nonradioactive-cell-proliferation-assay-protocol/; 9/2013).

Densitometric and statistical analyse

Immunoblots were scanned using a high-resolution scanner. Band intensities were quantified using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Results are presented as mean +- S.E.M. of the number of samples indicated. Statistical analyses were performed by ANOVA followed by the Newman–Keuls multiple comparison test using the GraphPad Prism 4 statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Student's t-test was used when the values of two groups were analyzed. Data were considered significantly different if $p<0.05$.

Results

1. Concomitant treatment with GH and EGF in MCF-7 cells

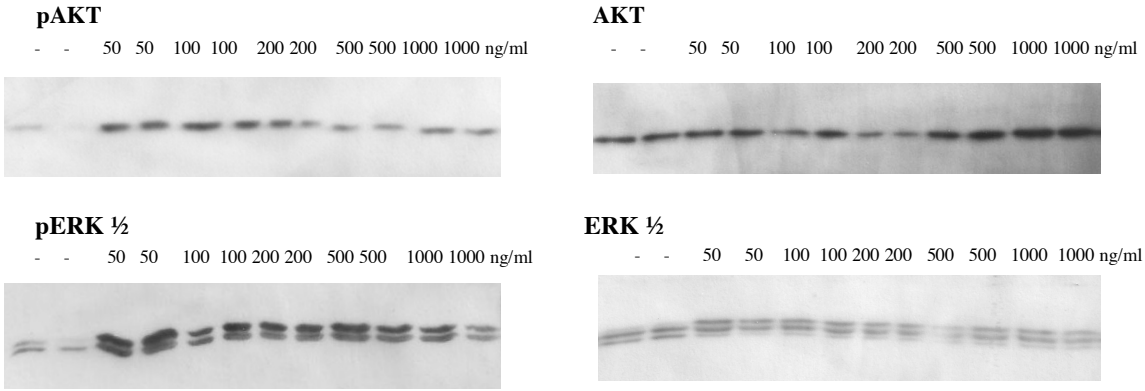
The MCF-7 human breast cancer cell line was used to examine GH and EGF action. First, cell response to each stimulus was tested. In addition, the appropriate dose and stimulation period with GH and EGF was determined. For this purpose, serum-starved cells were treated with 0-1000 ng/ml EGF for 15 min (Figure 12a). Extracted proteins were resolved and immunoblotted with anti-AKT; anti-pAKT, anti-ERK1/2 and anti-pERK1/2 antibodies. Phosphorylation of AKT and ERK1/2 were detectable with EGF doses from 50 to 100 ng/ml. As from a dose of 500 ng/ml onwards, AKT-activation decreased. Subsequently, serum-starved cells were treated with 100 ng/ml EGF for 0–60 min (Figure 12b). Detectable phosphorylation of AKT and ERK1/2 by EGF was observed at 5 minutes after stimulation, transient phosphorylation gradually diminished at 30 minutes after induction. STAT5 phosphorylation was also assayed, but phosphorylation of the transcription factor was not evidence at any time point.

To examine dose effects of GH over MCF-7 cells, serum-starved cells were treated with 0-10 μg/ml GH for 15 minutes. Activation of signalling mediators, involved in the main pathways induced by GH -AKT, ERK1/2 and STAT5- were detectable with doses of 0,5 μg/ml GH (Figure 12c). Subsequent, cells were treated with GH (1 μg/ml) for different time periods from 0 to 60 minutes (Figure 12d). Activation of AKT was evidenced after 15 minutes, ERK1/2 and STAT-5 are phosphorylated after 10 minutes of GH stimulation. While GH-induced AKT and ERK1/2 phosphorylation diminished after 30 minutes, STAT-5 phosphorylation showed a different kinetic. Its phophorylation levels persist 1 hour after GH stimulation.

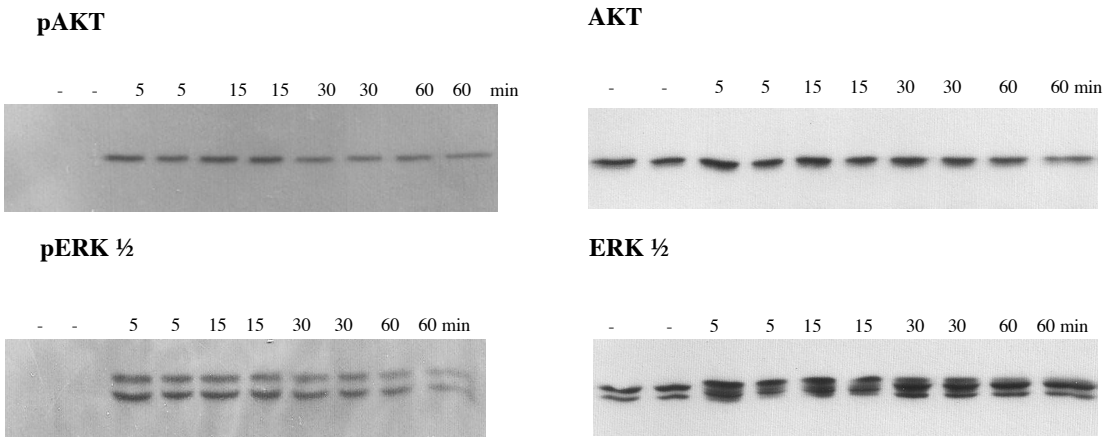
To summarize, detectable stimulation of both hormones, EGF and GH, was observed. This data illustrate MCF-7 breast cancer cell line responsiveness to EGF and GH, making it an attractive system to study crosstalk between EGF and GH signalling pathways.

Figure 12

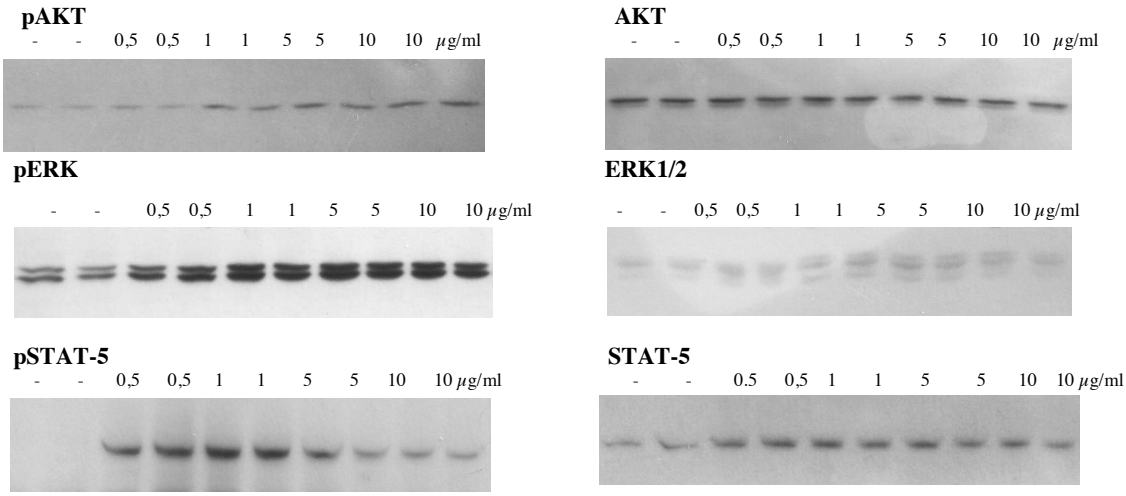
a) Dose effects of EGF on MCF-7 cells (15 minutes stimulation)



b) Time course effects of EGF on MCF-7 cells (by treatment with 100ng/ml)



c) Dose effects of GH on MCF-7 cells (15 minutes stimulation) in μg/ml



d) Time course effects of GH on MCF-7 cells (by treatment with 100ng/ml)

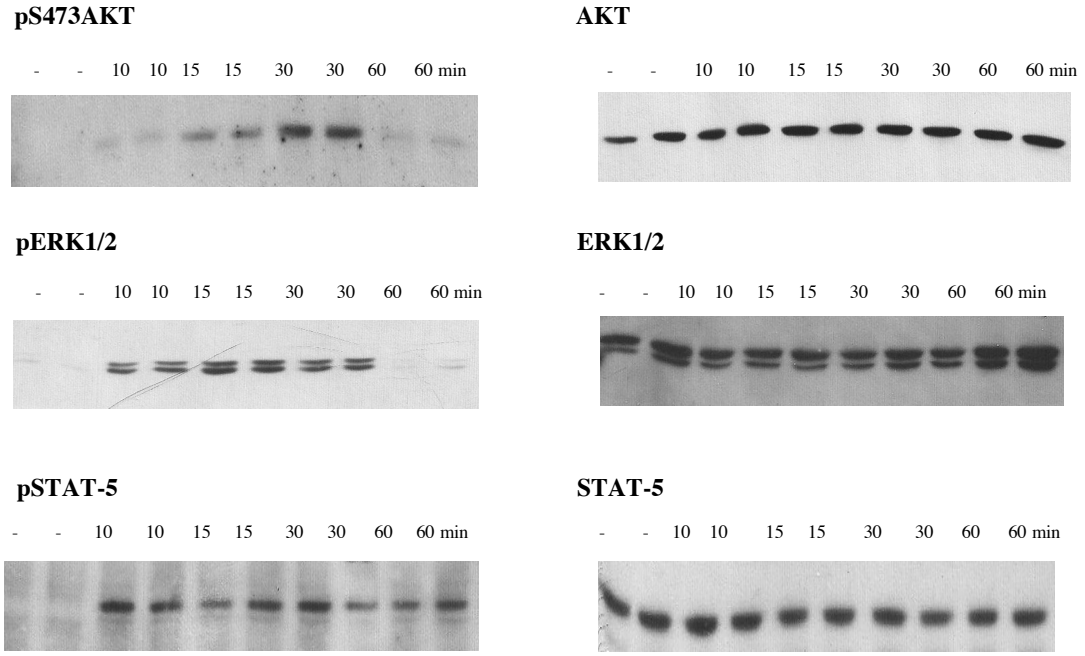


Fig. 12 MCF-7 cells response to EGF and GH. To analyse EGF-signalling, serum starved- cells were stimulated with 0- 1000 ng/ml EGF for 15 minutes **(a)** or 100 ng/ml EGF for 0–60 min **(b)**. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis with anti-AKT; anti-pAKT, anti-ERK1/2 and anti-pERK1/2 antibodies, respectively. In order to evaluate GH responsiveness serum starved- cells were stimulated with 0-10 μg/ml GH for 15 minutes **(c)** or 1 μg/ml GH for 0–60 min **(d)**. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis with anti-AKT; anti-pAKT, anti-ERK1/2, anti-pERK1/2, anti-STAT-5 and anti-pSTAT-5 antibodies, respectively. Representative immunolots are shown.

Cell proliferation effects of GH, EGF or both in MCF-7 cells

Cell proliferation was examined after Growth hormone and Epidermal growth factor treatment. After synchronising, cells were treated with vehicle, GH, EGF or both concomitantly during 48 hours. Aferwards, cell viability was analyzed by a Non-Radioactive Cell proliferation Assay. Cell viability increased approximately 21% under GH stimulation and 25% after incubation with EGF with respect to control ($p<0,05$) (Fig.13b). Co-treatment with GH and EGF for 48 hours produced a 34% increase in cell proliferation in respect of control. Therefore co-stimulation caused 9-13% more cell growth than each hormone by itself ($p<0,05$).

To evaluate if effects of concomitant treatment with both growth factors on cell proliferation relied on additive effects of both hormones, experimental values were

compared with the summation of each separate stimulus (Fig. 13b). Calculated addition of GH and EGF effects over cells proliferation surpasses experimentally obtained proliferation rates. Calculated values showed a 51% increase over control levels, whereas co-treatment with GH and EGF showed a 34% increase in cell viability. Cell viability was significantly decreased by co-treatment ($p<0,05$).

There seems to be an interrelationship between EGF and GH proliferative effects. Each hormone given separately nearly showed the same stimulation of cell growth, but concomitant treatment decreased cell viability compared to calculation, suggesting that these growth factors interfere with each other.

Figure 13

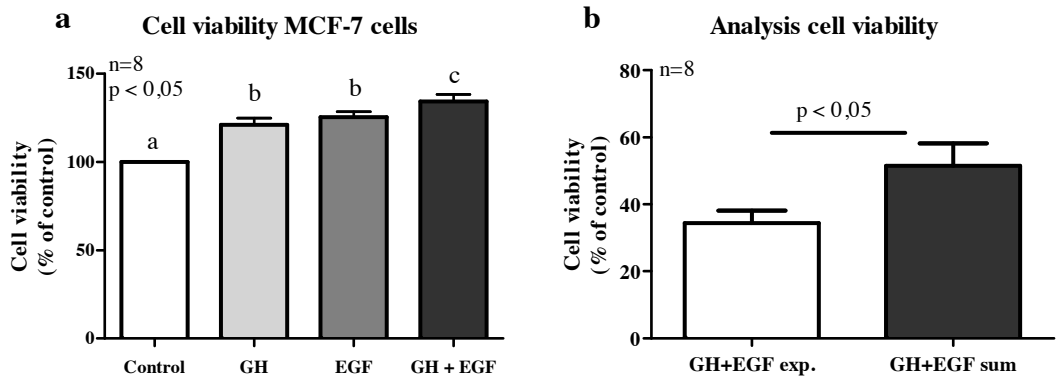


Fig. 13 Comparison of cell viability induced by GH, EGF or GH plus EGF measured by a Non-Radioactive Cell proliferation Assay. **(a)** MCF-7 cells were seeded in a 96 multiwell- plate with 20.000 cells per well. Serum-starved cells were stimulated with vehicle (control), 1 μg/ml Growth hormone (GH), 100ng/ml, Epidermal growth factor (EGF) or costimulated with GH and EGF (G+E) for 48 hours. After incubation cell viability was evaluated by using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay and read by 490nm absorbance. Pooled data from 8 such experiments, each repeated five times are shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at $p<0,05$. **(b)** Comparison of the level of cell viability induced by GH and EGF co-stimulation (G+E exp.) with the sum of that induced separately by GH and EGF (GH+EGF sum). Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Statistical analysis was performed by Student's t test. Significant difference at $p<0,05$.

Signalling under concomitant treatment with GH and EGF in MCF-7 cells

EGF-induced phosphorylation of EGFR at its multiple tyrosine residues leads to activation of downstream signalling cascades as the mitogen activated protein kinase Ras/Raf/MEK/ERK (p44/p42 MAPK), the phospatidylinositol 3'- kinase (PI3K)/AKT and the the signal transducers and activators of transcription (STAT) pathways (Jorrisen et al., 2003; Schulze et al., 2005; Normanno et.al., 2006). GH also activates the STATs, PI3K/AKT and ERK1/2 pathway (Le Roith et al., 1997; Zhu et al., 2001). To examine crosstalk between EGF and GH, cells were treated as follows: with vehicle, GH or EGF alone or with both growth factors for 15, 30 or 120 minutes before detergent extraction. First ERK1/2 protein content and phosphorylation were appraised (Figure 14). ERK1/2 protein level kept alike by all treatments. As expected, GH as well as EGF caused robust ERK1/2 activation 15 minutes after stimulation. Concomitant treatment with GH and EGF did cause more potent activation of ERK1/2 at 15 minutes, but the activation rate was not significantly different from GH or EGF induced activation of this kinase. ERK1/2 phosphorylation after 30 and 120 minutes treatment was comparable to control, in every case. Phosphorylation levels produced by GH and EGF concomitant treatment were compared with calculated phosphorylation rates, determined as the addition of EGF and GH-induced phosphorylation levels (Fig. 14c). Concomitant treatment with GH and EGF during 15 minutes resulted in ERK1/2 desensitization; however, such effect was not evidenced when cells were stimulated for 30 or 120 minutes. To assess if low activation at these time points depended only on poor blotting signal, ERK1/2 activation was analysed exclusively at these time points. In accordance with results obtained at 15 minutes stimulation, a significant decrease in ERK1/2 phosphorylation was evidenced after 120 minutes stimulation (Fig. 14d). Co-stimulation with GH and EGF showed significantly less phosphorylation than theoretically expected by additive effects of EGF plus GH alone at 120 minutes ($p<0,007$) (Fig. 14e). Concomitant treatment with GH and EGF during 15 and 120 minutes resulted in ERK1/2 desensitization.

Figure 14

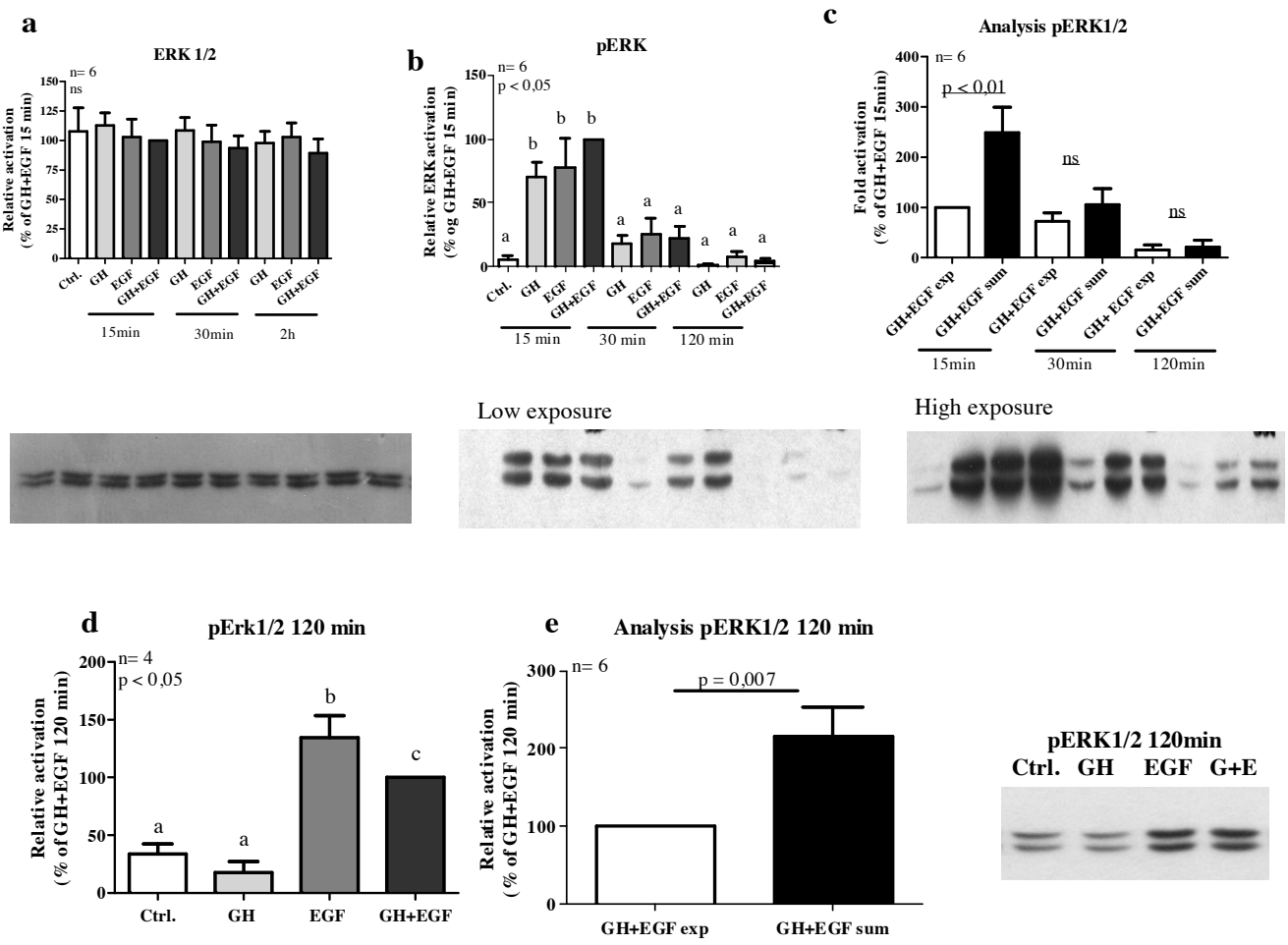


Fig. 14 Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 15, 30 or 120 minutes before detergent extraction. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 15 minutes (G+E 15'). **(a)** ERK protein content in MCF-7 cells. Representative result of immunoblots with anti-ERK is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference has been shown at p< 0,05 (ns). **(b)** ERK1/2 phosphorylation in MCF-7 cells. Representative result of immunoblots with anti-pERK1/2 is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,05. **(c)** Comparison of the level of ERK activation induced by GH and EGF co-stimulation (GH+EGF exp.) with the sum of that induced separately by GH and EGF (GH+EGF sum). Each value was compared at 15, 30 and 120 minutes, respectively. GH+EGF exp 15min was considered as 100%. Statistical analysis was performed by Student t- test. Different letters denote significant difference at p<0,05. **(d)** ERK activity, stimulated by GH alone (GH) , EGF alone (EGF) or co-treatment at 120 minutes (GH+EGF) is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,05. **(e)** Comparison of the level of ERK activation induced by GH and EGF co-stimulation (GH+EGF exp) with the sum of that induced by GH alone plus by EGF alone (GH+EGF sum) at 120 minutes. Statistical analysis was performed by Student´s t-test. Significant difference has been shown at p=0,007.

It is reported, that GH modulates EGF-induced PI3K/AKT-pathway in different tissues as in mice liver (Díaz et al., 2012). Extracted proteins were resolved and sequentially immunoblotted with anti-AKT and anti- pAKT antibodies. AKT protein level did not change among treatments (Figure 15a). GH and EGF caused robust activation of AKT. Most activation was seen after 15 minutes of stimulation. EGF-induced phosphorylation of AKT stayed almost equal at 30 minutes, whereas AKT-phosphorylation by GH diminished significantly at 30 minutes ($p<0,05$). Both growth factors given together did not significantly decrease AKT activity at 30 minutes compared to GH+EGF at 15 minutes. At 120 minutes, AKT phosphorylation level diminished under EGF- as well as under GH-treatment. AKT phosphorylation by concomitant treatment (GH+EGF) was increased at all time points with respect to GH+EGF 15min (Fig. 15b). GH and EGF activation were calculated at each time point to determine activity-increase by GH and EGF co-stimulation. At 15, 30 and 120 minutes the augmentation just reflects the calculation of EGF plus GH (Fig. 15c).

Induction of Cyclin D1, required for cell cycle G1/S transition, was examined as well. Protein level of Cyclin D1 rises in early G1 and starts to accumulate until the G1/S-phase (Sherr, 1994). Cells were incubated with GH, EGF or both for two hours before detergent extraction. Induction of Cyclin D1 was detected by western blotting of cell extracts with anti-Cyclin D1-antibody (Figure 16). Both, GH and EGF, caused more Cyclin D1 expression than basal. EGF provoked more than GH, but results were not significantly different. Interestingly, each stimulus alone caused more Cyclin D1 expression than concomitant stimulation. Induction was significantly reduced with respect to calculation ($p<0,01$). Concomitant treatment with GH and EGF results in attenuation of the induction theoretically expected from additive effects. Less induction of Cyclin D1 correlates with the desensitisation of ERK1/2 and reduced cell proliferation.

Figure 15

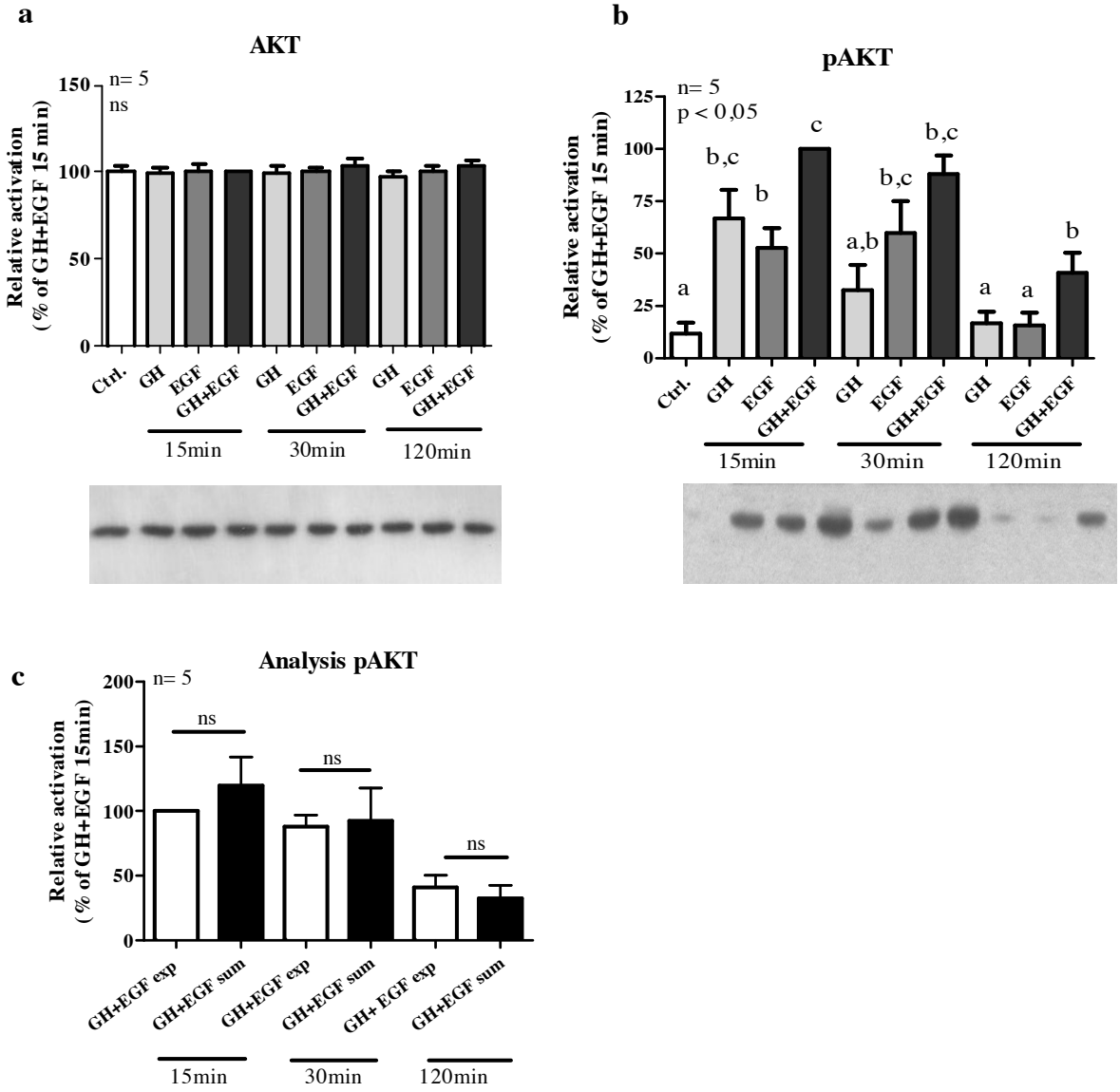


Fig. 15 Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 15, 30 minutes or 120 minutes before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). **(a)** AKT protein content in MCF-7 cells. Representative result of immunoblots with anti-AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at p< 0,05 (ns). **(b)** AKT phosphorylation at Ser473 in MCF-7 cells. Representative result of immunoblots with anti-pS473AKT is shown. Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 15 minutes (GH+EGF 15min). Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at P<0,05. **(c)** Comparison of the level of AKT activation induced by GH and EGF co-stimulation (GH+EGF exp.) with the sum of that induced separately by GH and EGF (GH+EGF sum). Each value was compared at 15, 30 minutes or 120 minutes, respectively. GH+EGF 15min exp. was considered as 100%. Statistical analysis was performed by Student's t test. No significant difference has been shown at p<0,05 (ns).

Figure 16

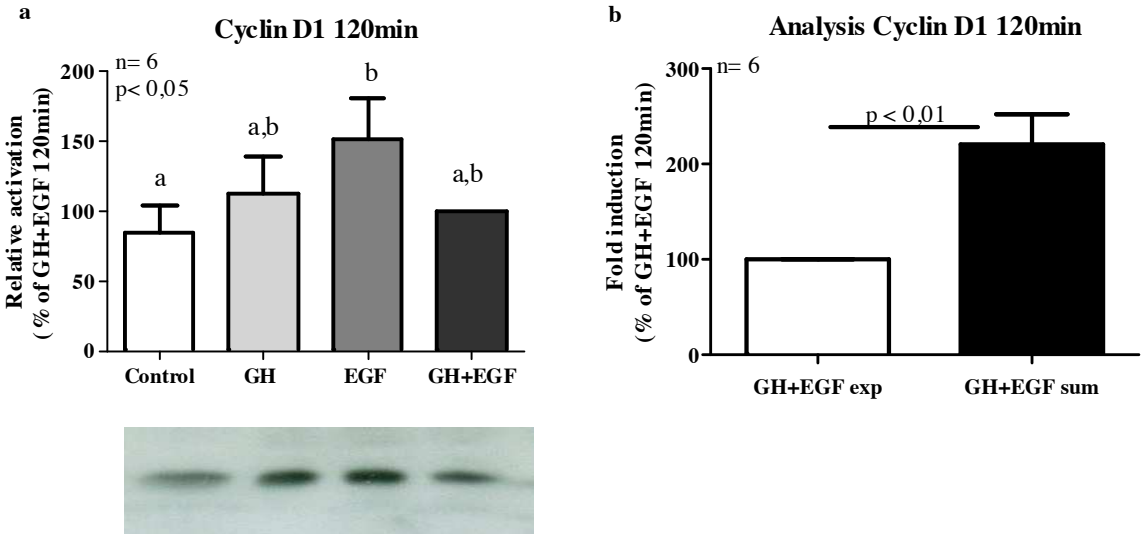


Fig. 16 (a) Cyclin D1protein content in MCF-7 cells. Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (G+E) before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis. Representative result of immunoblots with anti-Cyclin D1 is shown. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 120 minutes (GH+EGF120min). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at $p < 0,05$. **(b)** Comparison of the level of Cyclin D1 activation induced by GH and EGF costimulation for 120 minutes (G+E exp.) with the sum of that induced separately by GH and EGF (GH+EGF sum). GH+EGF 120min exp. was considered as 100%. Statistical analysis was performed by Student's t test. Significant difference at $p<0,01$.

Extracted proteins were also immunoblotted with anti-EGFR to examine Epidermal growth factor receptor (EGFR) protein content (Figure 17). EGF induces EGFR dimer internalization through clathrin-coated membranes (Carpenter and Cohen, 1976). The receptor internalization is reflected by its less protein content at 120 minutes. Internalisation under GH treatment seems to diminish, EGFR protein content depleted 20% compared to 15 minutes of co-stimulation. By EGF, its receptor showed less protein content or therefore more internalisation at 120 minutes. Interestingly there was no diminution of EGFR protein content under concomitant treatment with both hormones. However these data are not significant. Less internalisation by concomitant treatment correlates with the desensitisation of ERK1/2, less induction of Cyclin D1 and less cell proliferation.

Figure 17

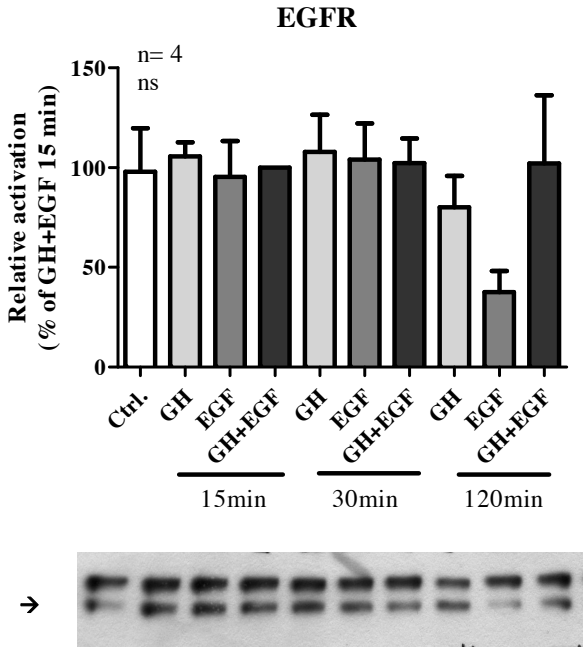


Fig. 17 EGFR protein content in MCF-7 cells. Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 15, 30 or 120 minutes before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis . Representative result of immunoblots with anti-EGFR is shown. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 15 minutes (GH+EGF 15min). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference has been shown at p<0,05 (ns).

2. Concomitant treatment with GH and EGF in MDA-231 cells

To analyse GH and EGF action in another breast cancer cell line, the hormone-independent, EGFR- overexpressing human breast cancer cell line MDA-231 was used. First, cell response was tested to each stimulus (Fig. 18). Serum-starved cells were treated with 1 μg/ml GH or 100 ng/ml EGF for 0-30 minutes. For comparison purposes, the same GH and EGF doses used for studies in the MCF-7 cell line were used to analyze growth factor effects over the MDA-231 cell line. Extracted proteins were resolved and immunoblotted with anti-AKT; anti-pAKT, anti-ERK1/2, anti-pERK1/2, anti-STAT-5 and anti-pSTAT-5 antibodies. No difference in protein content was found for AKT, ERK and STAT under stimulation and control. There was robust activation of AKT at 5 minutes of EGF stimulation, but GH-induced phosphorylation was not detectable at any time point. EGF-induced ERK activity was also noticeable at 5 minutes, whereas GH-induced ERK1/2 phosphorylation increased only slightly at 30 minutes. Phosphorylation of STAT-5 was hardly promoted by GH or EGF.

Figure 18

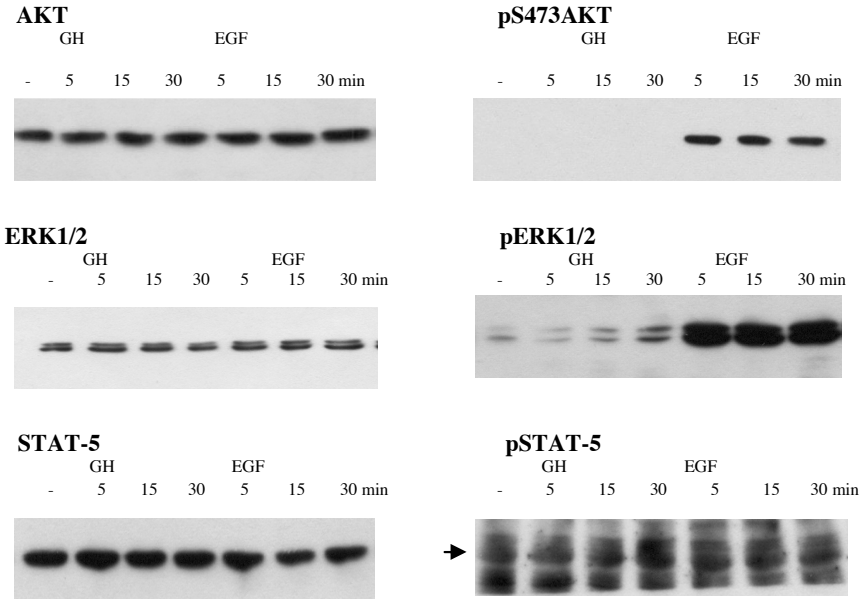


Fig. 18 MDA-231 cells response to EGF and GH. To analyse GH- and EGF-signalling, serum starved-cells were stimulated with 100μg/ml GH (first three pannels) or 100 ng/ml EGF (last three pannels) for 0–30 minutes (5, 15 and 30min). Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis with anti-AKT; anti-pAKT, anti-ERK1/2, anti-pERK1/2, anti-STAT-5 and anti-pSTAT-5 antibodies, respectively. Representative immunobots are shown.

Cell proliferation effects of GH, EGF or both in MDA-231 cells

The cell viability of MDA-231 cells was analysed as well. Serum-starved cells were treated with vehicle, GH, EGF or concomitant with both growth factors for 48 hours. After incubation, cells were analysed by a Non-Radioactive Cell proliferation Assay.

GH and EGF induced approximately 50% more cell viability than control (p<0,001). Co-treatment with GH and EGF led to nearly the same augmentation of proliferation in MDA-231 cells (p<0,001) (Fig. 19a).

Experimentally obtained values for concomitant treatment were compared with the sum of individual effects of the growth factors (Fig. 19b). Concomitant treatment provoked 50% less cell viability than expected by the summation of GH and EGF effects (p<0,0001) (Fig. 19b). Concordantly with the results for co-treatment with GH and EGF in MCF-7 cells, there seems to be a desensibilizing influence between the two mitogens. Concomitant treatment decreased cell viability compared to calculation, suggesting that these hormones interfere with each other.

Figure 19

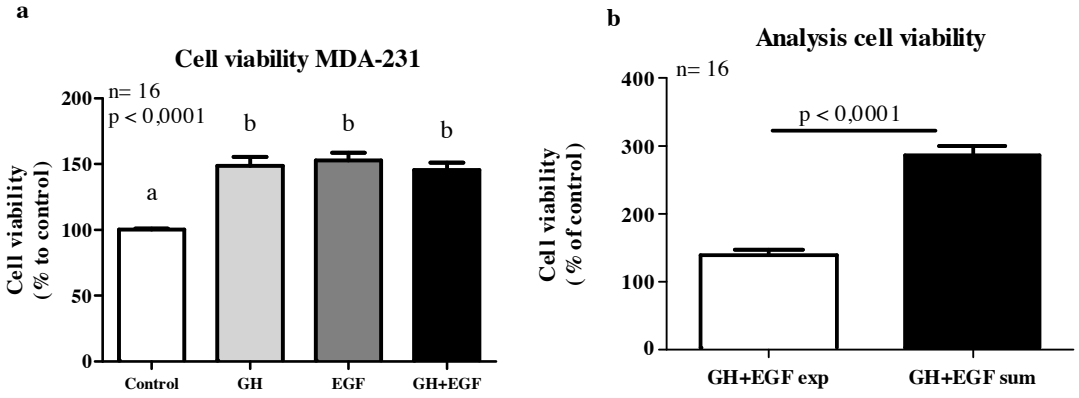


Fig. 19 Comparison of cell viability induced by GH, EGF or GH plus EGF, measured by a Non-Radioactive Cell proliferation Assay. (a) MDA-231 cells were seeded in a 96 multiwell- plate with 20.000 cells per well. Serum-starved cells were stimulated with vehicle (control), 1 μg/ml Human Growth hormone (GH), 100 ng/ml Epidermal growth factor (EGF) or costimulated with GH and EGF (GH+EGF) for 48 hours. After incubation cell viability was evaluated using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay and read by 490nm absorbance. Pooled data from 16 such experiments, each repeated five times are shown. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denotes significant difference at p < 0,0001. **(b)** Comparison of the level of cell viability induced by GH and EGF co-stimulation (GH+EGF exp.) with the sum of that induced by GH alone plus EGF alone (referred as GH+EGF sum). Statistical analysis was performed by t-test student. Significant difference at p<0,0001.

Signalling under concomitant treatment with GH and EGF in MDA-231 cells

ERK1/2 protein content as well as ERK1/2 phosphorylation was analysed after GH, EGF and concomitant stimulation with both growth factor by western blotting of solubilized MDA-231 cells (Figure 20). ERK1/2 protein content did not vary among treatments. GH did not induce ERK1/2 phosphorylation compared to control. EGF-induced ERK1/2 activation at 15 minutes was as high as co-treatment of GH and EGF for 15 minutes. Most activity was observed after 30 minutes EGF stimulation.

Figure 20

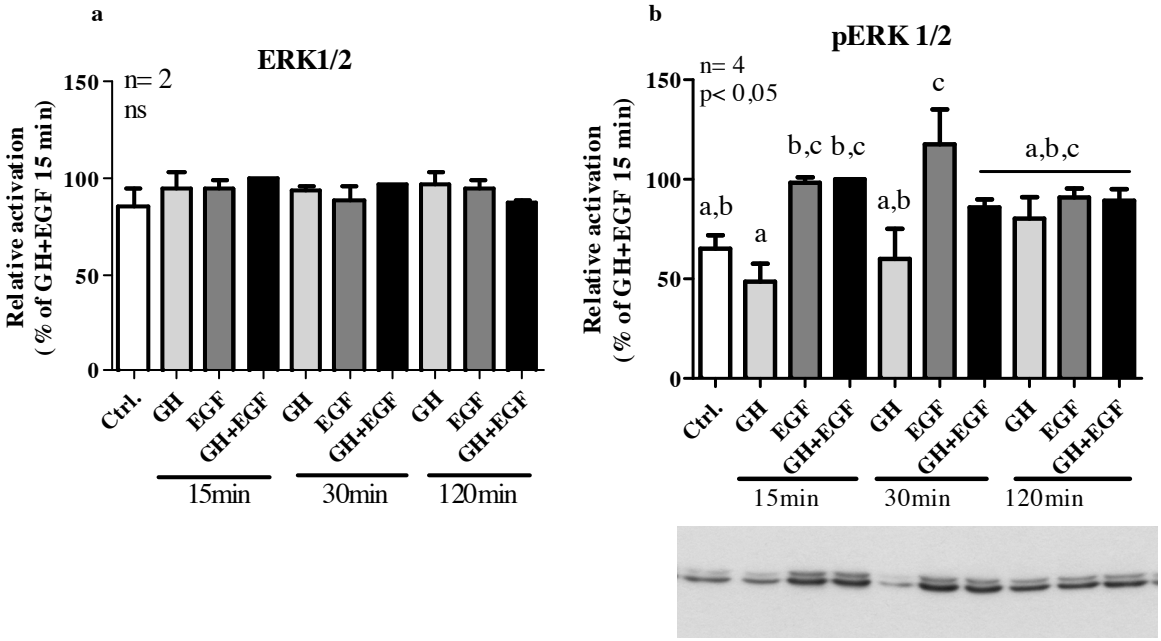


Fig. 20 Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 15, 30 minutes or 120 minutes before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis. .Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 15 minutes (GH+EGF 15min). **(a)** ERK1/2 protein content in MDA-231 cells. Representative result of immunoblots with anti-ERK1/2 is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference has been shown at p< 0,05 (ns). **(b)** ERK1/2 phosphorylation in MDA-231cells. Representative result of immunoblots with anti-pERK1/2 is shownStatistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,05

AKT protein content and phosphorylation at Ser473 was also examined. Protein content remained constant under vehicle (Figure 21a). GH-induced phosphorylation of AKT was not detectable at any time point (Fig. 21b). EGF alone caused robust AKT activation at 15 minutes, which diminished significantly under co-treatment with GH ($p<0,05$). EGF-induced AKT phosphorylation at 30 minutes was less, diminished also under co-treatment, but not significantly. At 120 minutes no AKT activity was observed nor after EGF stimulus neither after incubation with GH.

Figure 21

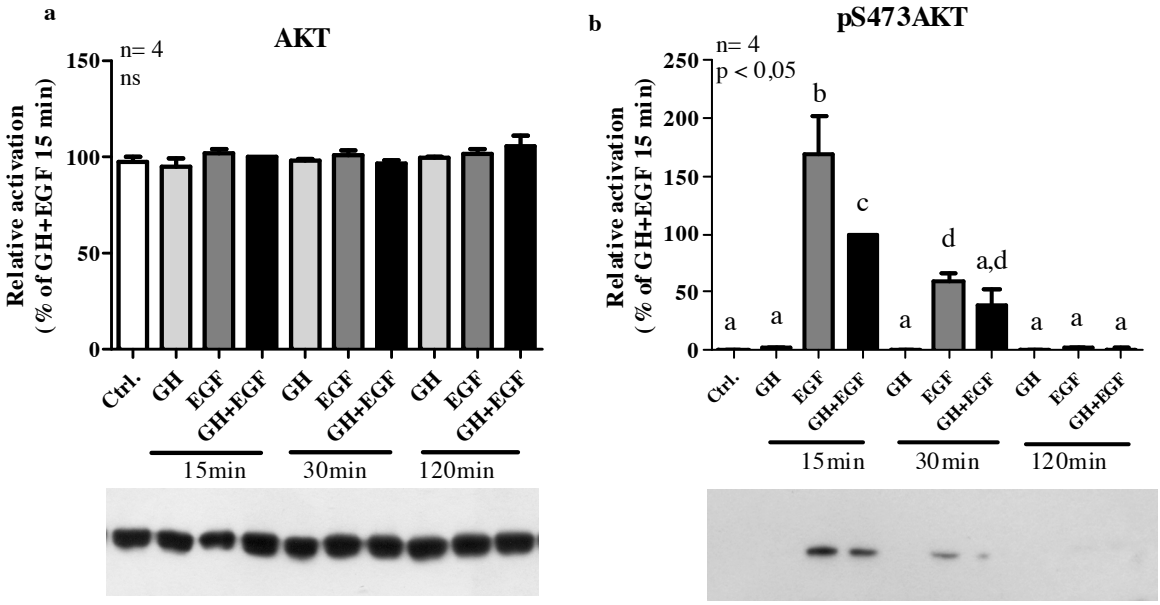


Fig. 21 Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 15, 30 minutes or 120 minutes before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis. Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 15 minutes (GH+EGF 15min). Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). **(a)** AKT protein content in MDA-231cells. Representative result of immunoblots with anti-pSer473AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at $p<0,05$ (ns). **(b)** AKT phosphorylation at S473 in MDA-231cells. Representative result of immunoblots with anti-pS473AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at $p<0,05$.

Protein content and activity of STAT-5 was analysed as well. However, no crosstalk between GH and EGF signalling was evidenced (data not shown).

Cyclin D1 was induced by GH, EGF and concomitant treatment for 120 minutes (Figure 22a). Induction was significantly increased ($p<0,05$) compared to control (data not shown, statistical analysis was performed by Student's t test) but not when compared to GH+EGF 120 minutes (Fig. 22a). Experimental values were compared to calculation of each stimulus. Cyclin D1 was more than 50% less induced by concomitant treatment with GH and EGF compared to the theoretical expected value (Figure 22b) ($p=0,016$).

Figure 22

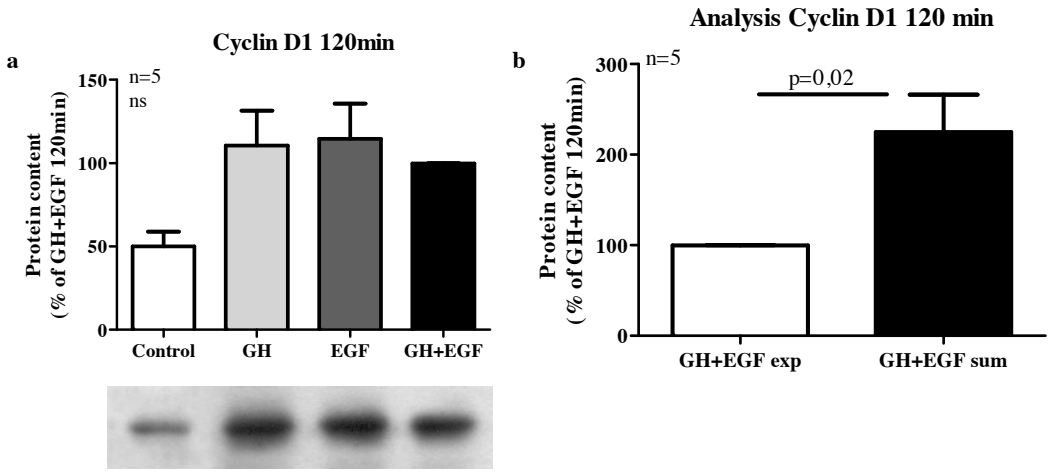


Fig. 22 Cyclin D1 protein content in MDA-231cells. Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (G+E) before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis . Representative result of immunoblots with anti-Cyclin D1 is shown. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 120 minutes (GH+EGF120min). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at $p<0,05$. **(b)** Comparison of the level of Cyclin D1 activation induced by GH and EGF co-stimulation for 120 minutes (G+E exp.) with the sum of that induced separately by GH and EGF (GH+EGF sum). GH+EGF 120min exp. was considered as 100%. Statistical analysis was performed by Student's t test. No significant difference has been shown at $p<0,05$ (ns).

Furthermore, GH and EGF signalling over EGFR protein content was analysed (Fig. 23a). There was no significantly difference among stimulation or vehicle. As expected, receptor internalisation was not detectable by GH-treatment at 120 minutes. But there

was also less EGF-induced receptor internalisation in MDA-231 cells compared to that observed in MCF-7 cells. When EGFR protein content was analysed exclusively at 120 minutes (Fig. 23b) it was observed that EGFR protein content kept alike under GH treatment while EGF as well as by co-treatment induced EGFR down-regulation, possibly reflecting receptor internalisation. However, data was not significant. Therefore, reduced proliferation, induction of Cyclin D1 and less activation of AKT by GH and EGF co-treatment does not correlate with decreased EGFR downregulation as occurred for MCF-7 breast cancer cells. However, GH seems to desensibilize EGF signalling by a different mechanism.

Figure 23

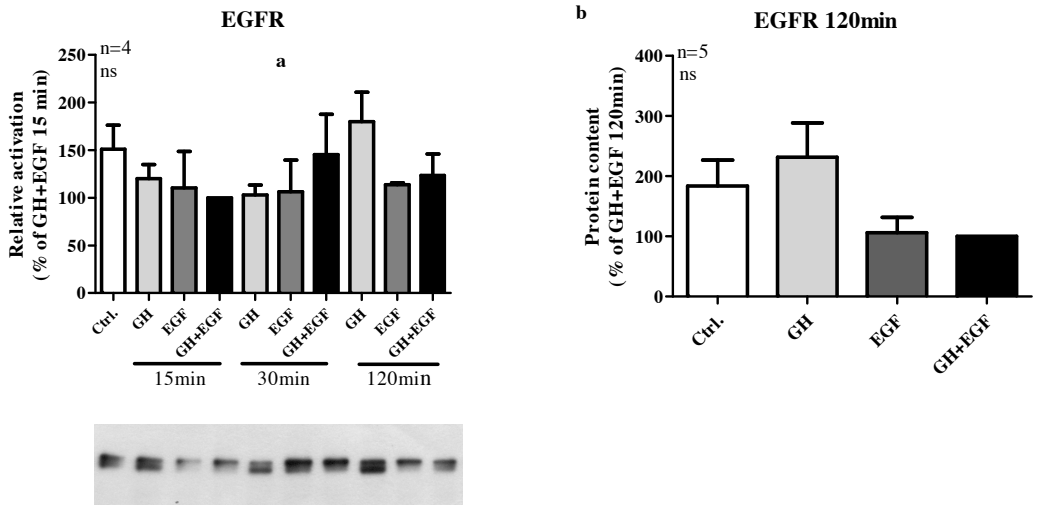


Fig. 23 Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 15, 30 minutes or 120 minutes before detergent extraction. Equal amounts of solubilized cell proteins were separeted by SDS-PAGE and subjected to immunoanalysis. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Quantification was expressed as percentage, considering 100% the result for cells costimulated with GH and EGF for 15 minutes (GH+EGF 15min). **(a)** EGFR protein content in MDA-231 cells. Representative result of immunoblots with anti-EGFR is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at p<0,05 (ns). **(b)** Receptor internalisation at 120min. Serum-starved cells were stimulated with vehicle (Ctrl.), 1 μg/ml GH, 100 ng/ml EGF or costimulated with GH and EGF (GH+EGF) for 120 minutes before detergent extraction. Representative result of immunoblots with anti-EGFR is shown. Quantification was expressed as percentage, considering 100% the result for 120 minutes GH plus EGF stimulation. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference has been shown at p<0,05 (ns).

3. Effects of GH-pre-treatment over EGF-signaling in MCF-7 cells

Effects of GH-pre-treatment on EGF-induced cell proliferation

To analyze if GH-pre-treatment modulates EGF proliferative actions over breast cancer cells, MCF-7 cells were assayed as follows: in presence of 1 μg/ml GH or vehicle during 24 hs. Afterwards, GH-pretreated and non-GH treated cells were further divided in two groups: a group that received 100 ng/ml EGF stimulation and another incubated in presence of vehicle.

As expected, there was more cell viability under treatment with growth stimulus than control. The viability increased approximately 15% under GH and EGF treatment compared to control (Fig. 24) ($p<0,05$). The cell proliferation augmented under pre-treatment with GH and subsequent EGF stimulation about 27% compared to control ($p<0,05$) (Fig. 24); however, the increment was not significantly different from GH or EGF induced proliferation.

Figure 24

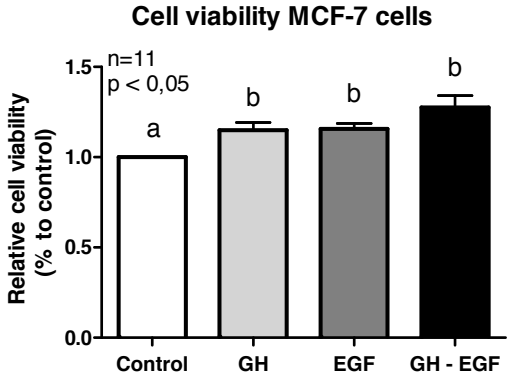


Fig. 24 Cell viability induced by GH, EGF or GH-pre-treatment with subsequent EGF stimulation, measured by a Non-Radioactive Cell proliferation Assay. MCF-7 cells were seeded in a 96 multiwell-plate with 20.000 cells per well. Serum-starved cells were stimulated with vehicle (control), 1 μg/ml Human Growth hormone (GH) or 100 ng/ml Epidermal growth factor (EGF) for 24 hours. After incubation cells treated with GH were stimulated with 100 ng/ml Epidermal growth factor (GH – EGF) or vehicle for 24 hs. Then cell viability was evaluated using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay and read by 490nm absorbance. Pooled data from 11 such experiments, each repeated five times are shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at $p<0,05$.

EGF signalling under GH-pre-treatment in MCF-7 cells

To examine GH effects over EGF signalling in MCF-7 cells, serum-starved cells were treated with vehicle or 1 μg/ml GH. After 24 hours of incubation, cells were stimulated with human EGF (100ng/μg) or vehicle (as control) for different time-periods: 15, 30 and 120 minutes before detergent extraction. A study over the impact of GH on the cell surface binding of EGF showed that GH-pre-treatment for 10 minutes led to less 125I-EGF binding in an ERK-dependent manner (Huang et al., 2004). ERK1/2 protein content and phosphorylation was analysed by western blotting with a specific antibody (Fig. 25). As expected, acute EGF stimulation caused substantial ERK1/2 phosphorylation (Figure 25b). Most ERK1/2 phosphorylation was found at 15 minutes after acute EGF stimulation, kept nearly at this level at 30 minutes and diminished at 120 minutes. Pure Growth hormone stimulation for 24 hours showed no enhanced ERK1/2 phosphorylation compared to control. No significant difference was observed among cells, pre- or non-treated with GH and acute EGF stimulation at any time points.

Figure 25

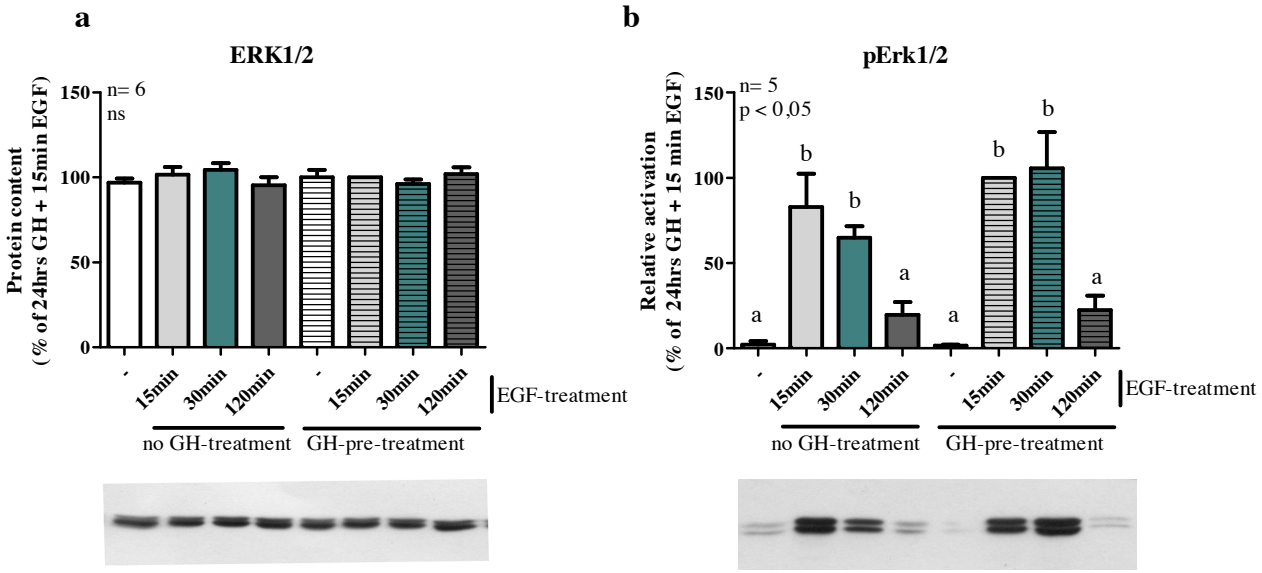


Fig. 25 Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis . Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. **(a)** ERK protein content in MCF-7 cells. Representative result of immunoblots with anti-ERK1/2 is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at p<0,05 (ns). **(b)** ERK1/2 phosphorylation in MCF-7 cells. Representative result of immunoblots with anti-pERK1/2 is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,05.

Effects of GH and EGF on AKT signalling were also examined and analysed by western blotting. AKT protein content remained constant under all treatments (Fig. 26a). EGF-induced phosphorylation of AKT at Ser473 provoked robust AKT signalling at 15 and 30 minutes and decreased at 120 minutes (Fig. 26b). Basal phosphorylation at Ser473 was not different between non-treated and GH-pretreated cells. Moreover, GH-pre-treatment did not significantly change EGF-induced AKT phosphorylation at any time point.

Figure 26

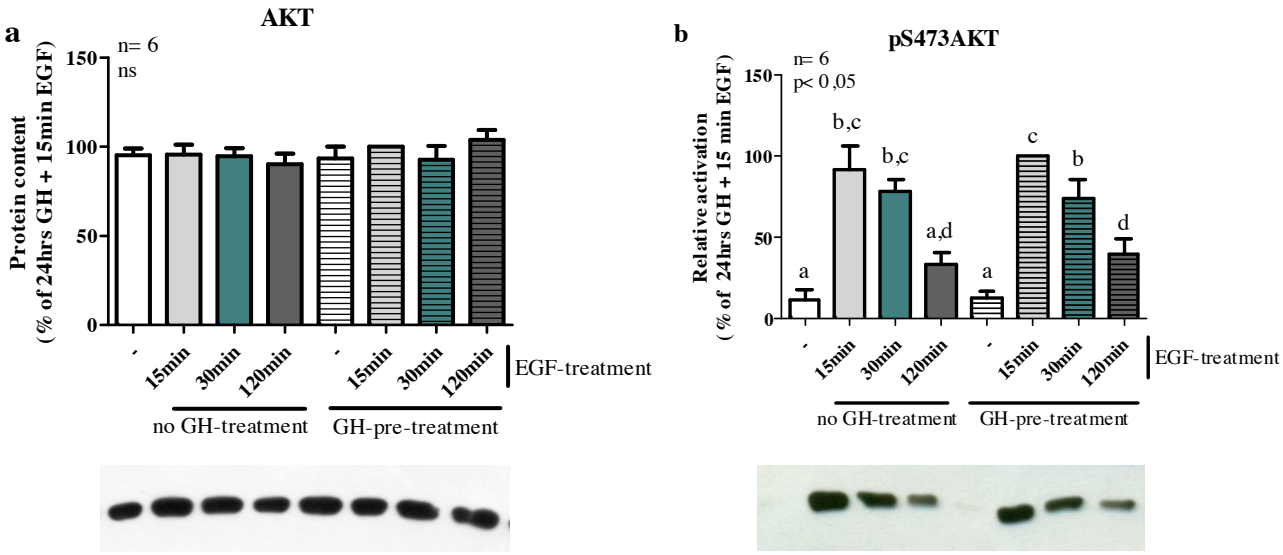


Fig. 26 Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis . Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. **(a)** AKT protein content in MCF-7 cells. Representative result of immunoblots with anti-AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at p< 0,05 (ns). **(b)** AKT phosphorylation at S473 in MCF-7 cells. Representative result of immunoblots with anti-pS473AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,05.

To examine the effects over a cell proliferation mediator, induction of Cyclin D1 was analysed (Figure 27). EGF stimulation of non-GH-pre-treated cancer cells did not significantly induce Cyclin D1 expression compared to control. GH-pre-treatment for 24 hours did not enhance EGF-induced expression at 15 or 30 minutes, but at 120 minutes pre-treatment enhanced Cyclin D1 induction significantly.

Subsequently, EGFR protein content was detected by western blotting of cells extracts with anti-EGFR antibody. No significant difference in protein content was observed between non- and GH-pre-treated cells (data not shown).

Figure 27

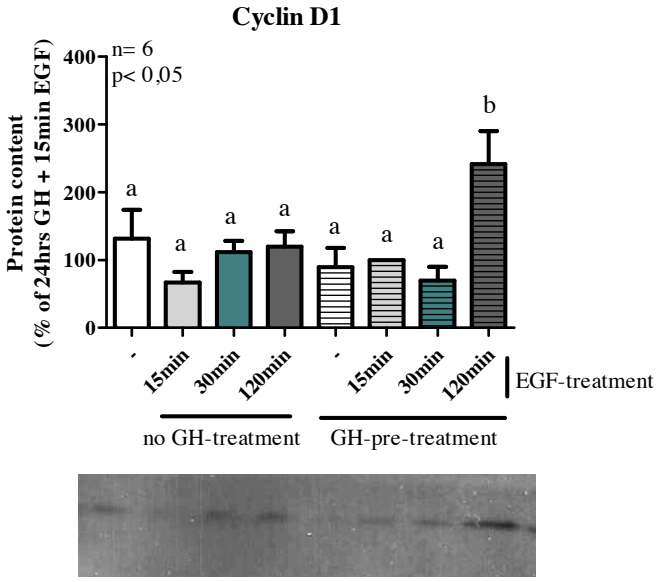


Fig. 27 Cyclin D1 protein content in MCF-7 cells. Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis. Cyclin D1 protein content in MCF-7 cells. Representative result of immunoblots with anti-Cyclin D1 is shown. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. * denotes significant difference at p<0,05.

4. Effects of GH-pre-treatment over EGF-signaling in MDA-231 cells

Effects of GH-pre-treatment on EGF-induced cell proliferation

To analyze if GH-pre-treatment modulates EGF mitogenic actions in another breast cancer cells, MDA-231 cells were assayed as follows: cells were incubated in presence of 1 μg/ml GH or vehicle during 24 hours and GH-pre-treated and non-GH- treated cells were further divided in two groups: a group that received EGF (100 ng/ml) and another, incubated in presence of vehicle. Cells were analyzed by a Non-Radioactive Cell proliferation Assay. Cell proliferation showed a 50% increase over control by treatment with GH and EGF (p<0,0001). Interestingly, EGF-induced cell viability significantly diminished when cells were pretreated with GH (p<0,0001) (Fig.28). GH-pre-treatment for 24 hours seems to downregulate EGF-induced cell proliferation.

Figure 28

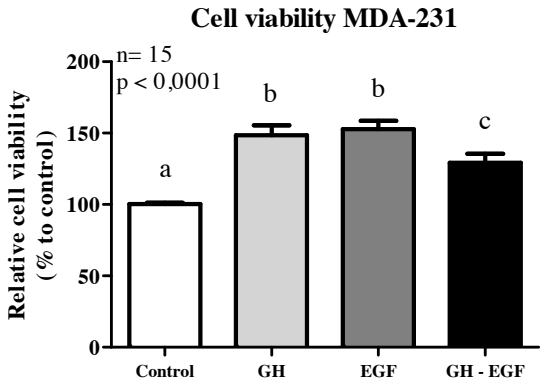


Fig. 28 Comparison of cell viability induced by GH, EGF or GH plus EGF, measured by a Non-Radioactive Cell proliferation Assay. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). **(a)** MDA-231 cells were seeded in a 96 multiwell- plate with 20.000 cells per well. Serum-starved cells were stimulated with vehicle (control), 1 μg/ml Human Growth hormone (GH), 100 ng/ml Epidermal growth factor (EGF) or costimulated with GH and EGF (G+E) for 48 hours. After incubation cell number viability was evaluated using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay and read by 490nm absorbance. Pooled data from 16 such experiments, each repeated five times are shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,0001.

EGF signalling under GH-pre-treatment in MDA-231 cells

To examine GH effects over EGF signalling in MDA-231 cells, serum-starved cells were treated with vehicle or 1 μg/ml GH for 24 hours. After incubation, cells were stimulated with human EGF (100ng/μg) or vehicle (as control) for different time-periods: 15, 30 and 120 minutes before detergent extraction. ERK1/2 protein content and activation was analysed by western blotting with a specific antibody (Figure 29). As expected, acute stimulation with EGF caused substantial ERK1/2 activation. Most ERK1/2 activity was found at 15 minutes (Figure 29a). ERK activity after 15 minutes acute EGF stimulation is significantly increased compared to all other stimulations and time points (p<0,05). Therefore, GH-pretreated cells showed significantly less acute ERK1/2 phosphorylation than non-treated cells at 15 minutes EGF stimulation.

Figure 29

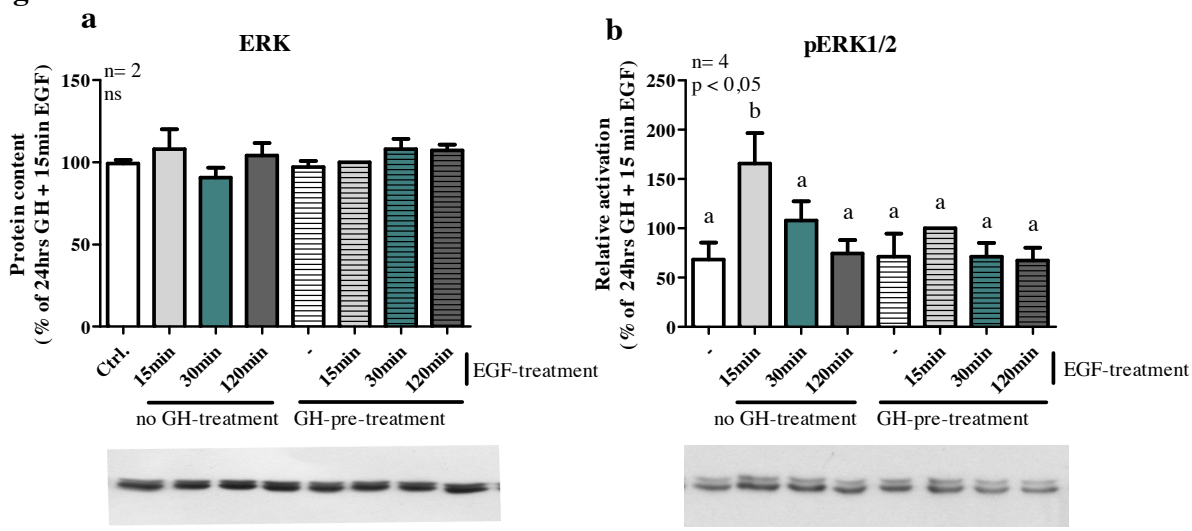


Fig. 29 Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at p<0,05 (ns). **(a)** ERK protein content in MDA-231 cells. Representative result of immunoblots with anti.ERK1/2 is shown. **(b)** ERK1/2 phosphorylation in MDA-231 cells. Representative result of immunoblots with anti-pERK1/2 is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at p<0,05.

Next, AKT protein content and phosphorylation at Ser473 were analysed. AKT protein content did not differ among treatments (Figure 30a). Concordantly to ERK1/2 activation, most AKT phosphorylation was found in non-GH-treated, EGF-stimulated cells after 15 minutes (Figure 30b). This robust activity is significantly increased compared to all other stimulations and time points ($p<0,01$). AKT showed twice as much phosphorylation level in non-treated-cells compared to GH-pretreated cells after 15 minutes EGF stimulation ($p<0,01$).

Figure 30

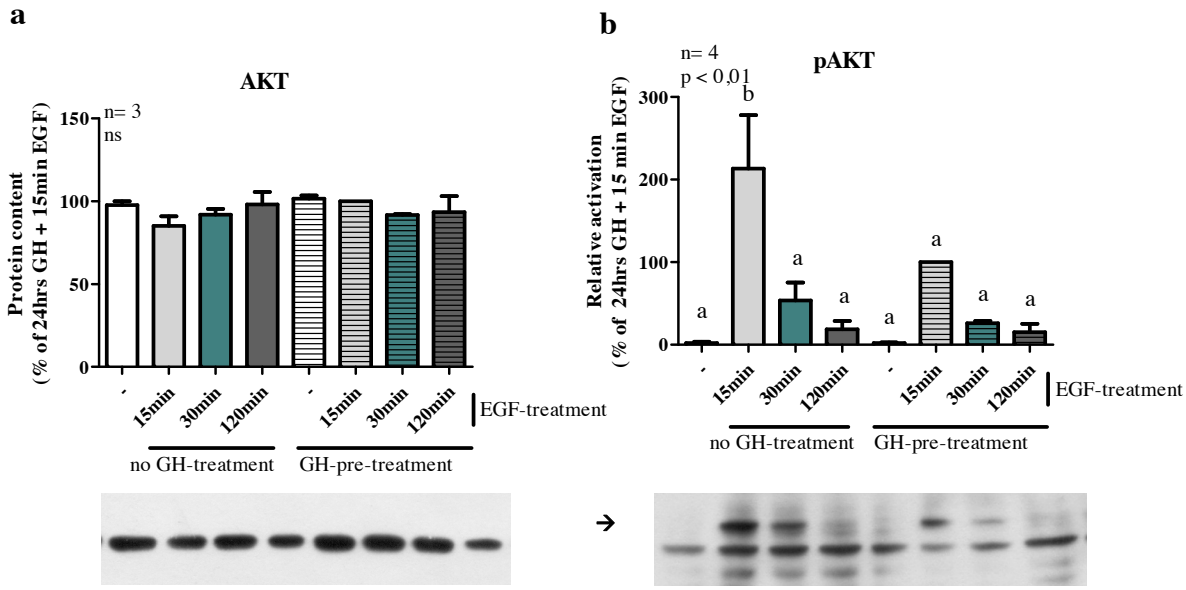


Fig. 30 Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. **(a)** AKT protein content in MDA-231 cells. . Representative result of immunoblots with anti-AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference at $p<0,05$ (ns). **(b)** AKT phosphorylation at S473 in MDA-231 cells. Representative result of immunoblots with anti-pS473AKT is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. Different letters denote significant difference at $p<0,01$.

Afterwards, Cyclin D1 induction was evaluated to investigate if effects over ERK1/2 and AKT correlated with effects over Cyclin D1 induction. This protein increased 120 minutes after EGF stimulation, but not significantly (Figure 31). No considerable difference in Cyclin D1 expression was observed between non-treated and GH-pre-treated cells.

Figure 31

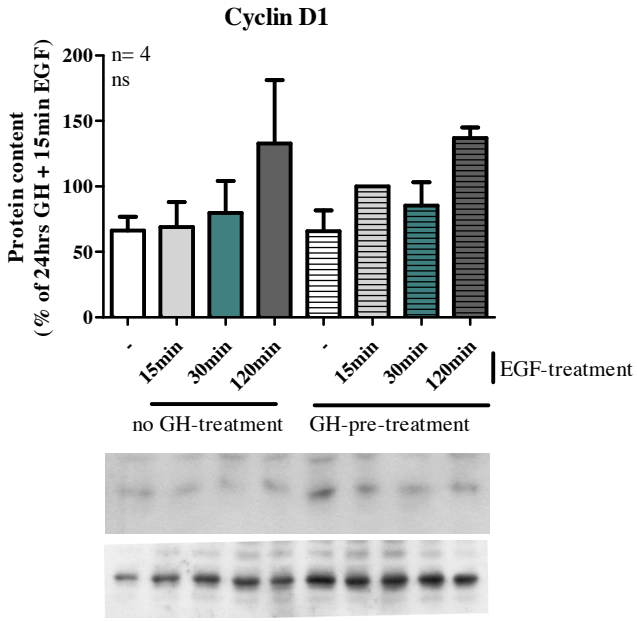


Fig. 31 S Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. Cyclin D1 protein content in MDA-231 cells. Representative result of immunoblots with anti-Cyclin D1 is shown. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference has been shown at $p<0,05$.

EGFR revealed almost the same protein content under all treatments (Figure 32). As mentioned above, EGFR protein content should decrease 120 minutes after EGF stimulation due to EGFR internalisation (Frank, 2008). In accordance, EGFR decreased

120 minutes after stimulation, but not significantly. Interestingly, EGFR did not diminish when cells were pretreated with GH for 24 hours. Summarising, GH-pre-treatment seems to attenuate ERK1/2 and AKT phosphorylation, what coincidences with less cell viability in pretreated cells, but expression of Cyclin D1 did not vary. EGFR internalisation would be decreased by GH-pre-treatment.

Figure 32

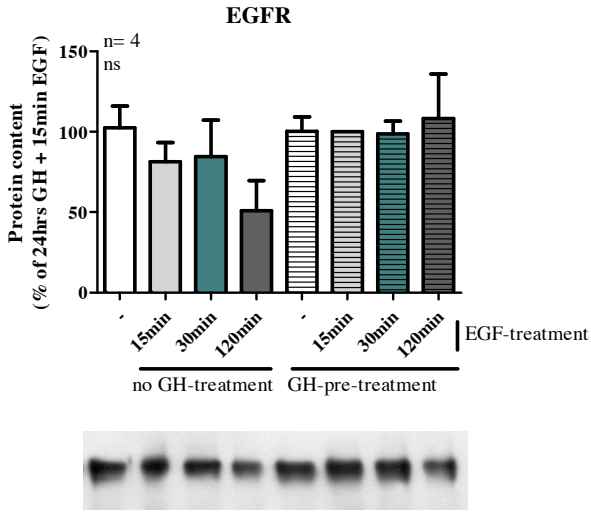


Fig. 32 EGFR protein content in MDA-231 cells. Serum-starved cells were stimulated with vehicle (no GH-treatment) or 1 μg/ml GH for 24 hours (GH-pre-treatment). After incubation cells were stimulated with vehicle (-) or 100 ng/ml EGF for 15, 30 or 120 minutes (EGF treatment), respectively. Equal amounts of solubilized cell proteins were separated by SDS-PAGE and subjected to immunoanalysis. Representative result of immunoblots with anti-EGFR is shown. Data are expressed as the mean±S.E.M. of the indicated number of subsets (n). Pretreatment with GH for 24 hours and 15 minutes EGF stimulation (15min EGF-treatment and GH-pre-treatment) was considered as 100%. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. No significant difference has been shown at $p<0,05$ (ns).

Discussion

Crosstalk between GH and EGF signalling pathways has been described; moreover, EGFR expression has been demonstrated to be regulated by GH (Jansson et al., 1988; Johansson et al., 1989; Miquet et al., 2008; González et al., 2010). GH caused tyrosine phosphorylation of the EGFR and subsequent MAPK signalling in mice livers and cell culture (Yamauchi et al., 1997). GH-induced EGFR phosphorylation required JAK-2 but not EGFR kinase activity. EGFR phosphorylation of 1086Y was associated with Grb-2, a main mediator of the ERK-pathway in response to GH (Yamauchi et al., 1997). GH induced EGFR phosphorylation at residues 1068Y, 845Y, 992Y and 1173Y in mouse preadipocytes (Kim et al., 1999; Huang et al., 2003). However, in livers of normal and transgenic mice overexpressing GH, no EGFR phosphorylation after acute GH stimulation was detected (Gónzalez et al., 2010).

It was reported that EGF-induced EGFR degradation, receptor redistribution from the cell surface to intracellular compartments and downregulation were prevented by GH-pre-treatment (Huang et al., 2003). These GH effects were prevented by MEK1 inhibition. Loss of EGF-induced EGFR intracellular distribution by GH, goes along with increased EGF-induced signalling as ERK-phosphorylation (Huang et al., 2003).

PTP101 is a monoclonal antibody that detects proteins phosphorylated at serine/threonine residues, consensus sites for proline-directed protein kinases, such as the ERKs. GH is able to induce PTP101-reactive EGFR phosphorylation, supposing that GH induces EGFR phosphorylation at ERK consensus sites (Huang et al., 2003). Pre-treatment with GH diminished acute and transient 125-I-EGF binding at preadipocytes cell surface due to less binding affinity (Huang et al., 2004). The decrease in 125-I-EGF binding in presence of GH was temporally accompanied by PTP101-reactive EGFR phosphorylation and GH-induced ERK activation and could be prevented by the ERK-inhibitor PD98059, suggesting that this GH effect is related to GH-induced ERK activation, too (Huang et al., 2004). That suggests, GH can modulate EGF binding kinetics and EGFR´s post-binding signalling in an ERK-dependent manner (Huang et al., 2004).

Research about GH and EGF co-treatment over ErbB-2 led to contrarily results. Cyclin D1 induction and DNA synthesis decreased compared to EGF administration alone. GH-induced threonine phosohorylation of ErbB-2 damped EGF-induced tyrosine kinase activity in 3T3-F442A fibroblasts (Kim et al., 1999).

As mentioned above, GH regulates the expression of EGFR in mice liver. In hypophysectomized or GH-deficient mutant mice, receptor expression was diminished and GH administration reversed the loss of receptors (Jannson et al., 1988). Whereas in transgenic mice overexpressing GH, the EGFR content was increased; however, EGF-induced signalling via its receptor was diminished (González et al., 2010; Díaz et al. 2012). In those transgenic mice the proteins content were diversely modified. AKT and ERK were increased compared to normal siblings, but whereas the phosphorylation level of AKT was also increased, the phosphorylation of ERK1/2 was not and STAT-3 and STAT-5 activation were inhibited. Anyway, EGF-induced phosphorylation of AKT and ERK1/2 was decreased in GH overexpressing mice probably due to less EGFR response (González et al., 2010).

EGFR is overexpressed in different types of breast cancer and as mentioned before, particularly in aggressive breast cancer types as triple negative or inflammatory breast cancer (Burness et al., 2010; Guerin et al., 1989).

The objective of this study was to examine direct and long-term influence between GH and EGF signalling and mitogenic effects in breast cancer cell lines. For this purpose, two different breast cancer cells lines were used. A hormone-dependent, ER and PR positive cell line, MCF-7, and a hormone-independent cell line, overexpressing EGFR, the MDA-231. To examine direct interactions between GH and EGF, concomitant treatment with both growth factors was explored. To investigate if GH has modulatory effects over EGF-signalling, cells were 24 hours pre-treated with GH, before they received an acute stimulation with EGF.

Treatment with GH, EGF or concomitantly both

Cell viability of MCF-7 cells under treatment with GH or EGF or concomitantly with both mitogens for 48 hours was examined. Each stimulus by itself increased cell viability compared to control and concomitant treatment caused more proliferation

compared to each stimulus by itself. Theoretically expected values from additive effects of each stimulus were compared to the real experimental increase by co-treatment. Interestingly, co-treatment induced a cell proliferation rate significantly smaller than the expected (Fig. 13). EGF and GH seem to influence each other by impairing cell proliferation when administered together.

This first evidence about crosstalk between GH and EGF encouraged us to analyse which pathways are involved in impairing cell proliferation. It is known that the ERK-pathway is induced by GH and EGF (Boonstra et al., 1995; Zhu et al., 2001) and GH requires EGFR for ERK1/2 activation in human mammary epithelial cells (HMEC) (Rodland et al., 2008). Whereas no interference could be observed in AKT signalling, concomitant treatment with GH and EGF resulted in significant ERK1/2 desensitization. In MCF-7 cells, GH and EGF induced ERK1/2 phosphorylation, but simultaneous treatment caused less ERK1/2 activity than theoretically expected from additive effects. Concomitant administration seems to desensitise the ERK1/2 pathway at 15 minutes, but also after 120 minutes stimulation (Fig.14b-e). Previous research described that GH and EGF synergized in preadipocytes in an ERK-dependent manner (Huang et al., 2003), but in contrast, data of this study indicates that GH in combination with EGF diminishes ERK1/2 signalling in breast cancer cells. One of the other main pathways of GH and EGF, the PI3K-AKT-pathway, was not affected due to GH and EGF concomitant treatment in MCF-7 cells (Fig. 15a-c).

To analyse which are the effects over MCF-7 cell cycle promotion of the concomitant treatment with GH and EGF, Cyclin D1 expression was proved by Western blotting. Expression of Cyclin D1 rises in early G1 during the cell cycle (Sherr, 1994). Induction of Cyclin D1 was caused by GH and EGF treatment. Interestingly, each stimulus alone provoked more Cyclin D1 induction than both together (Fig. 16). Convenient with the results for reduced cell viability, co-treatment led to significantly less Cyclin D1 induction. Furthermore, the diminished ERK1/2 activation by GH and EGF co-treatment could be the reason for less Cyclin D1 expression.

EGF-induced receptor tyrosine activation leads to EGF-receptor dimer internalisation and down-regulation (Carpenter and Cohen, 1976). In this study, EGFR protein content diminished at 120 minutes after EGF stimulation, reflecting its internalisation. By GH treatment and co-treatment with GH, the protein content did not diminish (Fig. 17).

There is evidence that GH alone does not lead to EGF-receptor internalisation and that EGF-induced EGFR degradation is prevented by GH in an ERK-dependent manner (Huang et al., 2003). But loss of EGF-induced EGFR intracellular distribution by GH was accompanied with increased EGF-induced signalling as ERK activation (Huang et al., 2003). Impaired endocytotic downregulatiom of EGFR is commonly associated with cancer and can lead to uncontrolled signalling (Grandal and Madshus, 2008; Roepstorff et al.,2008). Data from this study suggest that GH extenuates EGFR internalisation but also ERK1/2-signalling.

Cell viability of MDA-231 cells was analysed under the same experimental conditions. Each individual stimulus increased robustly cell proliferation compared to control. Co-treatment with GH and EGF augmented cell viability, but compared to summation there was intensely less proliferation than awaited. Concordantly with results for MCF-7 cells, GH and EGF co-treatment led to a significant decrease of cell viability (Fig. 19). These results are interesting, because there are other findings saying that MDA-231 cells are only minimal (Decker, 1988) to unresponsive to EGF (Mueller et al., 1994) in respect to cell proliferation. One study demonstrated that EGF-induced growth rate in MDA-231 cells is dependent on administered EGF concentration. Low doses of 10ng/ml EGF exhibit an increased growth rate which decreased by higher EGF concentrations (Zhang et al., 2012). The dissimilar response to different EGF concentrations could explain the various findings about EGF responsiveness of MDA-231 cells. In this study, MDA-231 cells responded to EGF (100ng/ml) with a moderate increase of cell viability.
EGF and GH seem to interfere with each other, influencing cell proliferation in both cell lines. Also in MDA-231 cells, shared pathways by GH and EGF were analysed to find out in which pathway this interaction could take place. ERK1/2 phosphorylation was not induced by GH compared to control in MDA-231 cells. EGF induced ERK1/2 robustly as well as concomitant treatment. No significant desensibilisation by co-treatment could be observed as it was found for MCF-7 cells (Fig. 20b).
Analogously to ERK1/2, AKT phosphorylation was not induced by GH at any time point. EGF alone caused robust AKT activation at 15 minutes, which diminished significantly under co-treatment with GH, also at 30 minutes GH co-treatment, but not significantly. At 120 minutes no AKT activity was observed neither after EGF stimulus

nor co-treatment with GH (Fig. 21b). However, simultaneous administration led to less AKT induction than EGF by itself. GH seems to desensibilize EGF-induced AKT.

The third pathway, shared by GH and EGF STAT-5 was analysed, too. There was no difference in STAT-5 activity among GH and EGF signalling. The fact that STAT-5 was also induced by EGF could be due to EGFR-overexpression in MDA-231 cells. It is described that EGF requires EGFR overexpression for STAT-5 activation (Kloth et al., 2002).

Cyclin D1 was induced by all treatments, but concomitant treatment led to less Cyclin D1 induction (Fig. 22). More than 50% less Cyclin D1 induction by concomitant treatment was observed compared to theoretical expected value. It is reported that EGF induces EGFR internalisation also in MDA-231 cells (Decker 1988). In this study, EGF-induced EGFR internalisation was slight in MDA-231 cells after 120 minutes and not induced by GH (Fig. 23). GH did not seem to influence EGF-induced receptor internalisation. However, data was not significant.

Less Cyclin D1 induction and less cell viability by concomitant treatment correlate with the results in the MCF-7 cell line. Unlike in MCF-7 cells, no desensitisation of ERK1/2 could be observed, but instead a desensitisation of AKT signalling. EGFR receptor internalisation was not impaired by GH-co-treatment. Therefore, the reduced proliferation, less induction of Cyclin D1 and AKT by GH and EGF co-treatment does not correlate with decreased EGFR downregulation as occurred for MCF-7 breast cancer cells. However, GH seems to desensibilize EGF signalling in another way.

GH and PRL induced serine/threonine EGFR phosphorylation required ERK activation in CHO-GHR cells (Li et al., 2008). In a prostate cancer cell line, EGF-induced EGFR threonine phosphorylation potentized receptor tyrosine phosphorylation and enhanced EGFR endocytotic downregulation. These effects could be impaired by blockage of ERK, not AKT (Gan et al., 2010). In this study, GH did not phosphorylate ERK1/2 in MDA-231 cells, what could be the reason for no attenuation over EGFR downregulation as happened in MCF-7 cells.

Nevertheless, AKT phosphorylation is bated by GH-co-treatment. It is really interesting that GH did not induce AKT phosphorylation but reduced EGF-induced AKT signalling. Less AKT phosphorylation could be the reason for less Cyclin D1 induction and cell viability. AKT is an important protein for the cell cycle regulation and prevents Cyclin

D1 degradation by GSK3β phosphorylation (Diehl et al., 1998) which modulates cyclin-dependent kinase inhibitors p21/waf1/cip1 and p27/Kip2 (Lawlor et al., 2001). To learn more about this process, it would be interesting to further investigate if less AKT activation by GH and EGF alters one of its downstream targets in MDA-231 cells.

As mentioned above, GH attenuates EGFR signalling in different ways. GH might be dampening EGF binding affinity by its receptor, therefore reducing breast cancer cells response to the growth factor.

In conclusion, GH and EGF co-treatment results in desensitization of mitogenic signals respect to the effects of each growth factor *per se*; however, the mechanism implied seems to be different depending on the cell lines. In MCF-7 cells, the prevented downregulation of EGFR could be the reason for less ERK1/2 phosphorylation, leading to less Cyclin D1 induction and therefore less cell viability in MCF-7 cells. However, the EGFR downregulation in MDA-231 cells is not affected by co-treatment with GH. MDA-231 cells overexpress EGFR; while EGF induces its internalisation, its downregulation might be regulated differently. Moreover, desensitization would be related with effects over binding affinity of the EGFR or induction of molecules involved in the termination of the signal.

GH-pre-treatment and acute EGF stimulation

To analyse if GH has effects over EGF-induced cell proliferation, MCF-7 cells were pre-treated 24 hours with GH before stimulation with EGF. EGF-induced cell proliferation in those cells was compared with non-pre-treated cells. All treatments led to increased cell proliferation compared to control. GH-pre-treatment did not significantly alter cell proliferation when compared to individual stimuli (Fig. 24). Inconclusively with this result, Cyclin D1 expression was significantly enhanced by GH-pre-treatment and 120 minutes EGF stimulation (Fig. 27).

There are findings for chondrocytes, treated with Gonad releasing hormone (GnRH), in which GH promoted proliferation over EGFR signalling in an ERK-dependent manner. In those chondrocytes EGF expression and subsequent EGF-induced EGFR signalling was associated with GH-induced cell growth after GH treatment (Pan et al., 2011).

ERK1/2 and AKT phosphorylation were induced by acute EGF stimulation in MCF-7 cells, but not altered through GH exposure for 24 hours. In respect to the EGFR, no significant difference in EGF-receptor internalisation between non- and pre-treated cells could be observed (Fig. 25). In previous research on preadipocytes, it is reported that GH-pre-treatment diminished 125-I-EGF receptor binding transiently at the cell surface in an ERK-dependent manner, due to less affinity (Huang et al., 2004). In this study, GH-pre-treatment did not affect MCF-7 cell viability, EGF signalling or EGFR internalisation but enhanced Cyclin D1 expression. This issue keeps inconclusive: Why did an increased Cyclin D1 induction not lead to more cell viability? And which pathway led to the rise of Cyclin D1 expression? Moreover, these unanswered issues reveal the complexity of GH and EGF interactions, their dependence on the cell type and stimulation protocol.

Contrary results were observed in MDA-231 cells. Cell viability was also increased by GH and EGF treatment compared to control, but diminished substantially after GH-pre-treatment, suggesting that GH pre-treatment for 24 hours downsizes EGF-induced cell proliferation (Fig. 28). To investigate a responsible pathway, ERK1/2, AKT and STAT-5 were analysed.

In non-treated cells, ERK1/2 phosphorylation was significantly increased after 15 minutes EGF administration compared to control. Acute EGF treatment did not induce ERK1/2 activation when cells were pre-treated with GH (Fig. 29b). GH seems to alter EGF-induced ERK1/2 activity in MDA-231 cells. In agreement with data for ERK1/2, AKT phosphorylation was also mainly induced by EGF and significantly impaired after pre-treatment with GH (Fig. 30b). GH administration seems to prevent EGF-signalling through both pathways.

In spite of the altered cell viability after GH-pre-treatment, Cyclin D1 expression did not differ significantly between treatments (Fig. 31). EGF-induced EGFR decreased at 120 minutes reflecting its internalisation; in pre-treated cells, receptor internalisation was less observed, however, data was not significantly (Fig. 32). Previous research implied less internalisation due to GH protection. But these findings were accompanied by more EGF signalling and ERK1/2 activity (Huang et al., 2003). In this study, GH-

pre-treatment seems to lessen EGFR internalisation, but also ERK1/2 and AKT phosphorylation.

Interestingly, there are contrary results for MCF-7 and MDA-231 cells. Whereas GH-pre-treatment did not significantly affect EGF-signalling in MCF-7 cells, pre-treatment altered EGFR and ERK1/2 signalling in MDA-231 cells and attenuated AKT phosphorylation. The two breast cancer cell lines differ in EGFR expression. Possibly long-term GH administration influences ERK-signalling only in MDA-231 cells due to EGFR overexpression. As mentioned above, mice overexpressing GH had an increased EGFR content. In these mice ERK1/2 and AKT content was also increased but their phosphorylation upon EGF-stimulation was diminished (González et al., 2010; Diaz et al., 2012). Similarly, the EGF-induced phosphorylation levels of ERK1/2 and AKT were decreased due to long-term GH administration in MDA-231 cells, overexpressing EGFR. The protein content did not vary due to pre-treatment, in any case.

However, transgenic mice overexpressing GH are exposed to high GH levels for their whole lifetime, whereas cells in this study were exposed to GH only for 24 hours. That could explain why the attenuation was only observed at the phosphorylation level, but did not change AKT or ERK1/2 protein content.

As mentioned above, EGF-induced EGFR threonine phosphorylation led to more potent receptor tyrosine phosphorylation and enhanced EGFR downregulation in a prostate cancer cell line. These effects could be impaired by blockage of ERK, not AKT (Gan et al., 2010). In this study, concomitant treatment with GH and EGF in MCF-7 cells and pre-treatment with GH in MDA-231 cells led to less ERK1/2 activation and also less EGFR internalisation. One possible explanation could be that GH interferes in threonine phosphorylation of the EGFR or in ERK1/2 phosphorylation, leading to less receptor internalisation. In preadipocytes, GH causes ERK-mediated threonine phosphorylation of EGFR (Kim et al., 1999; Huang et al., 2004), leading to delayed EGFR downregulation and enhanced EGF-induced signalling (Huang et al., 2003. 2004, 2006, Li et al., 2008).

Prolactin (PRL) is a hormone, as GH mainly produced in the anterior pituitary gland, which blocked EGF-induced EGFR and Grb2 association with subsequent MAPK/Ras activation in mammary cells (Johnson et al., 1996). EGF-induced DNA synthesis

decreased when PRL was present in the normal murine mammary epithelial cell line (NMuMG) (Fenton and Sheffield, 1994) while in the breast cancer cell line T47D, PRL induced ERK1/2 mediated phosphorylation of EGFR, which resulted in increased EGF signalling (Huang et al., 2006). GH also seems to influence different cell lines in diverse ways as it is found in this thesis.

A short-term influence between GH and EGF over cell viability and signalling could be observed in both breast cancer cell lines after co-treatment, whereas long-term modification due to GH-pre-treatment was only detected in MDA-231 cells, overexpressing EGFR. Interestingly both GH and EGF play a role in developing breast cancer. EGFR is frequently overexpressed in mammary neoplasia (Verbeek et al., 1998) and breast cancer patients show a higher serum level of GH (Peyrat et al., 1993). Nevertheless, in this study GH attenuated EGF-signalling in an ERK1/2-dependent manner and also AKT-dependent in the hormone-independent breast cancer cell line. About the association between breast cancer and ERK1/2 activation different predications can be found, linking high protein activity to poor (Mueller et al., 2000) or good outcome (Berggvist et al., 2006; Milde-Langosch et al., 2005). The ERK-pathway seems to be manifold involved in breast cancer. Considering that the EGFR is associated with high tumour grade and poor clinical outcome (Alroy and Yarden, 1997) and GH seems to attenuate EGFR downregulation and signalling, it would be interesting to understand in which breast cancer subtypes this modulation is present, as it could enable the development of more effective and selective therapies.

Conclusion

Crosstalk between Growth hormone and Epidermal growth factor as well as GH modulation over EGF-induced ERK1/2, AKT and STAT-5 signalling were analyzed in the hormone-dependent cell line MCF-7 and the hormone-independent cell line MDA-231. Previous studies have demonstrated that interaction between these two growth factors exists and that GH regulates the expression of EGFR. Considering the relevance of breast cancer and the evidence that GH and EGF/EGFR signalling are involved in mammary gland tumorigenesis and taking into account that GH and EGF share many signalling pathways, the aim of this study was to analyse crosstalk between GH and EGF in signalling and proliferation. Furthermore, a modulatory role of GH over EGF-signalling was examined. In consideration of previous results about crosstalk between GH and EGF, the hypothesis of this study was that co-treatment and GH-pre-treatment would result in an exacerbation of EGF signalling, leading to augmented cell proliferation, while no GH co- or pre-treatment would decrease EGF signalling.

The two cell lines were detected to be valuable for the investigation of GH and EGF signalling as they responded to both stimuli. In MCF-7 cells, phosphorylation of AKT and ERK1/2 were detectable after GH and EGF treatment, but STAT-5 phosphorylation by EGF was not evidence at any time point. In MDA-231 cells, EGF induced AKT and ERK1/2 phosphorylation, whereas GH did not induce AKT or ERK activation. STAT-5 was lowly induced by GH but not by EGF.

GH and EGF signalling results in cell survival and proliferation in both cell lines. Interestingly, both mitogens that by themselves promote cell proliferation, interfered with each other, leading to significantly less cell viability. Whereas co-treatment had an effect on both cell lines, 24 hours GH-pre-treatment had only a consequence over MDA-231 cells. Data suggest that GH modulates EGF-signalling by preventing EGF-induced proliferation.

These evidences about crosstalk between GH and EGF encouraged to analyse which pathways are involved in impairing cell proliferation. The protein content and phosphorylation levels of ERK1/2, AKT and STAT-5 were analysed to consider if variances in these pathways could have lead to the alteration in cell viability. In MCF-7

cells, the ERK1/2 pathway was desensitised by co-treatment, whereas in MDA-231 cells GH-pre-treatment led to a desensibilisation of ERK1/2. Furthermore, the phosphorylation level of EGF-induced AKT was decreased by co- and pre-treatment in MDA-231, but not in MCF-7 cells. Interestingly, simple GH-treatment did not induce AKT phosphorylation in MDA-231 cells, but GH seems to attenuate EGF-induced AKT activation.

The cell viability was measured by an assay that notices bioreduction and hence the quantity of metabolically active cells, equal to the number of living cells. To examine, if the altered cell viability was due to less proliferation or less metabolism of the cells, Cyclin D1 induction, an important protein of cell cycle transition was analysed. Convenient with the results for cell viability, co-treatment led to significantly less Cyclin D1 induction in both cell lines. But results from pre-treatment in respect to Cyclin D1 were inconclusive.

Impaired ERK1/2 signalling could be a reason for the reduced cell viability. Sustained ERK signalling stabilizes and promotes genes that are required for cell cycle entry as Cyclin D1 (Torii et al., 2006). The expression of c-fos and c-jun genes is up-regulated through EGF-induced ERK1/2, whose products heterodimerize, build AP-1 and bind to the promoter of Cyclin D1 (Shaulian & Karin, 2001).

Whereas in other cell types GH and EGF synergize in ERK signalling, pre-treatment in MDA-231 cells and co-treatment in the MCF-7 cell line impaired ERK1/2 phosphorylation. Moreover, ERK1/2 represses the expression of antiproliferative genes (Yamamoto et al., 2006), what amplifies the possibilities how the impaired kinase could lead to less cell proliferation.

Also the diminished phosphorylation of AKT in MDA-231 cells could have influenced the cell proliferation as the proteinkinase leads to cell survival and proliferation and is also involved in Cyclin D1 stabilisation (Dufourny et al., 2000). AKT plays also a role as pro-survival factor by preventing Cyclin D1 from degradation (Diehl et al., 1998) and is able to enhance protein synthesis of mTOR that itself stimulates Cyclin D mRNA translation (Muise-Helmericks et al., 1998). An alteration in Cyclin D1 would be a probable explanation of less cell viability. But more investigation is needed to analyse if proapoptotic proteins which are prevented by AKT, are altered due to GH treatment.

Interestingly, the AKT-pathway was only altered in MDA-231 cells. AKT seems to play another role in hormone-independent breast cancer than in hormone-dependent. It is reported that AKT is inversely correlated with PR and patients who are resistance to endocrine therapy showed more expression of pAKT and had a worse outcome among endocrine-treated patients (Tokunaga et al., 2006). Furthermore, there is evidence that GH diminished EGF-induced AKT phosphorylation in mice, overexpressing GH (Díaz et al., 2013).

GH and EGF are able to signal via the EGFR. The receptor is one possible place where the interaction between GH and EGF could have occured. On these grounds EGFR protein content was analysed.

The expected receptor internalisation after EGF-treatment was less found after co-treatment with GH in MCF-7 cells. But in MDA-231 cells, simultaneous treatment with GH did not affect EGF-induced receptor internalisation. Pre-treatment seems to impair EGFR internalisation, but all data about the EGFR was not significant and further investigations are necessary. Usually, impaired EGFR internalisation is associated with increased signalling. This data proposes less signalling in combination with less receptor internalisation. It would be interesting for further investigation if this interaction between GH and EGF can be found in more breast cancer cell types. That could suggest new therapeutic strategies in signalling modulation of mammary cancer cells.

In summary, this study evidenced crosstalk between GH and EGF in two different breast cancer cell lines.

Long-term modification over cell viability and the ERK1/2 and AKT pathways was only detected in MDA-231 cells. In consideration of results from GH and EGF concomitant treatment, the interaction between GH and EGF in MCF-7 cells could be transient abrogated by GH-pre-treatment. Co-treatment with GH and EGF diminished cell viability in MCF-7 and MDA-231 cells. Furthermore, a direct influence between GH and EGF signalling resulted in less ERK phosphorylation in MCF-7 cells, whereas in MDA-231 cells less AKT phosphorylation could be observed. The attenuation of the EGFR-downregulation due to GH is a potential reason but keeps to be elucidated.

Summary

Der Epidermale WachstumsfAKTor (EGF) ist ein Schlüsselprotein für das Überleben von Zellen und ihrer Proliferation. Darüber hinaus ist er an der malignen Entartung verschiedener Zelltypen beteiligt. Neben spezifischer AKTivierung durch EGF wird der Epidermale WachstumsfAKTor-Rezeptor (EGFR) durch verschiedene WachstumsfAKToren transAKTiviert, u. a. durch das Wachstumshormon (GH). Zwischen GH und EGF, die beide an der Regulierung der Zellproliferation beteiligt sind, wurden InterAKTionen in verschiedenen Zelltypen festgestellt, die zu verstärkter Stimulierung von Signalkaskaden und zur Progression von Malignität führten. Die große Bedeutung beider Proteine sowie des EGFR in der Pathologie des Brustkrebses führten zu Untersuchungen über mögliche modulierende InterAKTionen zwischen beiden Mitogenen, die zur Malignitätsentstehung beitragen könnten. Die Untersuchungen wurden in der hormonabhängigen Brustkrebszelllinie MCF-7 und der hormonunabhängigen Brustkrebszelllinie MDA-231 durchgeführt. Neben der Zellproliferation wurden Mediatoren der Hauptsignalkaskaden von GH und EGF detektiert, ERK1/2, AKT sowie STAT-5. Darüber hinaus wurde eine Modulierung des EGFR untersucht.

In MCF-7 Zellen konnte gezeigt werden, dass die Proliferation durch gemeinsame Gabe von GH und EGF gemildert wurde. Es kam zu einer geringeren Induktion von Cyclin D1 und geringerer Phosphorylierung von ERK1/2 im Vergleich zur Applikation nur eines der beiden WachstumsfAKToren. Auch in den hormonunabhängigen MDA-231 Zellen wurde durch die gemeinsame Gabe von GH und EGF eine Abnahme der Proliferation sowie der Phosphorylierung von AKT beobachtet. Zur Exploration des Einflusses einer längerfristigen Stimulation mit GH auf die Zellkommunikation von EGF, wurden beide Zelllinien vor EGF Stimulation über 24 Stunden mit GH vorbehandelt. In MDA-231 Zellen zeigten sich durch die Vorbehandlung mit GH eine Abschwächung der Zellproliferation sowie eine Verringerung der EGF-induzierten Phosphorylierung von ERK1/2 und AKT. Wir schließen daraus, dass auch in diesen Brustkrebszellen eine InterAKTion zwischen EGF und GH stattfindet. Die gleichzeitige Gabe von GH und EGF sowie die Vorbehandlung mit GH führten zur

Desensibilisierung mitogener Signale. Die zugrunde liegenden Mechanismen scheinen abhängig der jeweiligen Zelllinie zu sein.

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