Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

Transactivation of the EGFR Signal in Human Cancer Cells

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aus

Wissen

2004

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. Horst Domdey betreut

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, den 09.03.2004

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Dissertation eingereicht am 09.03.2004

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2. Gutachter	Prof. Dr. Horst Domdey
Mündliche Prüfung am	15.06.2004

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1 Introduction

One characteristic commonality to all organisms is the dynamic ability to coordinate complex physiological processes with environmental changes. The capability of cells to communicate with their environment is achieved through a number of pathways that receive and process signals. Signals can originate from the external environment ranging from soluble endocrine and paracrine factors to signalling molecules located on neighbouring cells, but also from different intracellular regions. Integration of external stimuli with internal signal transduction pathways is essential for the ability of cells to respond correctly to the environment in order to achieve an appropriate biological response. This type of information transfer is an important part of the cellular repertoire of regulatory mechanisms. During normal embryonic development and in adult life, signalling needs to be precisely coordinated and integrated at all times. Deregulated signal transmission is now recognized as a cause of many human diseases such as cancer and diabetes (Hanahan and Weinberg 2000; Shawver et al. 2002).

Receptors of the tyrosine kinase family play pivotal roles in the regulation of biological processes during development and in adulthood of multicellular organisms. Cellular signalling of these receptors is counterbalanced by protein tyrosine phosphates (PTPs) which therefore act as key regulatory components in directing and modulating signal transduction pathways. Defects in this highly complex regulatory system result in pathological disorders such as cancer.

G-protein coupled receptors (GPCRs) are integral membrane proteins that, in response to activation by extracellular stimuli, regulate intracellular second messenger levels via their coupling to heterotrimeric G-proteins (guanin-nucleotide binding protein). They are dynamically regulated via phosphorylation by G-protein coupled receptor kinases (GPKs) which desensitize the receptor after prolonged or repeated exposure to agonists. GPCRs represent the largest family of signal transduction molecules known and their dysfunction is responsible for numerous diseases.

1.1 Protein tyrosine kinases

Protein tyrosine kinases are important regulators of intracellular signal transduction pathways mediating aspects of multicellular communication and development in metazoans (Cohen

2002). These enzymes catalyze the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target proteins. Tyrosine kinases play an important role in the control of most fundamental cellular processes including the cell cycle, migration, metabolism and survival, as well as proliferation and differentiation. There are currently more than 90 known tyrosine kinase genes in the human genome; 58 encode transmembrane receptor tyrosine kinases (RTKs) distributed into 20 subfamilies based on their structural characteristics (Fig. 1), and 32 encode cytoplasmic non-receptor tyrosine kinases (NRTKs) distributed into 10 subfamilies.

1.1.1 Receptor tyrosine kinases (RTK)

RTKs are type I transmembrane proteins and contain an extracellular ligand-binding domain that is usually glycosylated (Hubbard and Till 2000). The structural diversity of RTK ectodomains is due to the presence of one or several copies of immunoglobulin-like domains, fibronectin type III-like domains, EGF-like domains, cysteine-rich domains or other domains (Fig. 1).

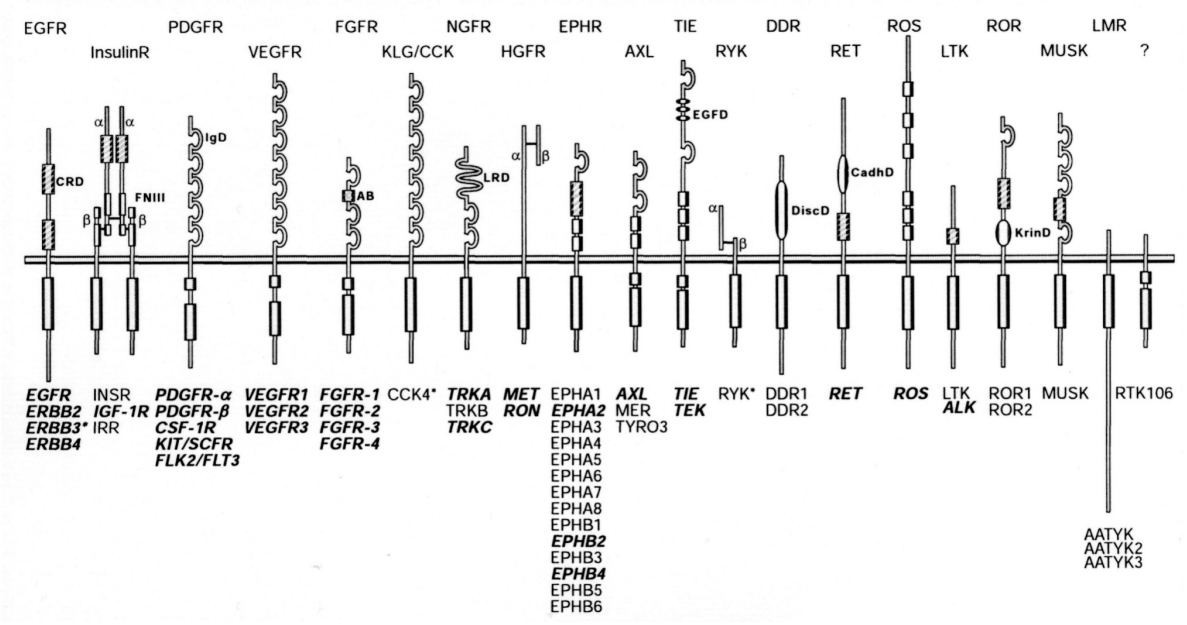


Figure 1. Subfamilies of receptor tyrosine kinases (Blume-Jensen and Hunter, 2001).

The ligand binding domain is connected to the cytoplasmic domain by a single transmembrane helix. The cytoplasmic domain contains a highly conserved protein tyrosine kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases.

The EGFR family consists of four RTKs: EGFR, HER2/neu for which no ligand has been described so far, HER3 which is kinase-inactive and HER4 (Ullrich and Schlessinger 1990). The EGFR was the first cell surface signalling protein and protooncogene product to be characterized by molecular genetic methods and exemplifies prototypical features of RTKs. The EGFR signalling module has been highly conserved throughout the course of evolution. The primordial signalling unit found in the nematode *Caenorhabditis elegans* consists of one receptor protein called LET-23 and a single EGF-like ligand known as LIN-3 (Yarden and Sliwkowski 2001). In this organism, the EGFR network plays a central developmental role. A single receptor and four ligands are present in insects such as *Drosophila melanogaster* and - moving further up the evolutionary ladder - four receptors and so far ten ligands have been identified in mammals.

1.1.2 EGF-like ligands

Several growth factors have been shown to directly activate the EGFR: EGF, transforming growth factor alpha (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BC), epiregulin (Epi) (Riese and Stern 1998), cripto (Salomon, Bianco et al. 1999) and epigen (Strachan, Murison et al. 2001). The various neuregulin (NRG) isoforms are ligands for HER3 and HER4.

All these molecules share a common motif of 30-50 amino acids in the active peptide, called the EGF structural unit which contains six conserved cysteine residues. These cysteins form three intramolecular disulfide bonds, thereby restraining the peptide in a tertiary structure containing three disulfide bonded loops.

EGF-like ligands are synthesized as transmembrane precursors which are subject to proteolytic cleavage at the cell surface to produce the soluble and diffusible growth factors (Massague and Pandiella 1993). Subsequently, the mature ligands activate RTKs of the EGFR family by autocrine or paracrine stimulation. In addition, several studies indicate that the membrane-anchored precursors may be biologically active via juxtracrine stimulation (Brachmann, Lindquist et al. 1989; Wong, Winchell et al. 1989).

1.1.3 Ligand-induced activation of receptor tyrosine kinases

Ligand-induced activation of receptor tyrosine kinases is mediated by intermolecular autophosphorylation of cytoplasmatic key tyrosine residues in the activation loop of the

catalytic tyrosine kinase domain (Schlessinger 2002). In the inactive state, the activation loop adopts a configuration preventing access to ATP and substrate. Upon tyrosine phosphorylation, the activation loop adopts an "open configuration" enabling access to ATP and substrate, thus resulting in enhanced tyrosine kinase activity.

Recent structural studies have revealed that receptor dimerization is mediated by receptor interactions in which a loop protruding from neighbouring receptors mediates receptor dimerization and activation (Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002). Dimerization of EGFR requires the binding of two molecules of monomeric EGF to two EGFR molecules in a 2:2 EGF:EGFR complex formed from stable intermediates of 1:1 EGF:EGFR complexes. Each EGF molecule is bound exclusively to a single EGFR molecule, and dimerization is mediated entirely by receptor-receptor interactions. The crystal structures are consistent with the "receptor-mediated" mechanism for dimerization (Lemmon, Bu et al. 1997), in which the binding of EGF to EGFR induces a conformational change that exposes a receptor-receptor interaction site in the extracellular domain, resulting in dimerization of two EGFR monomers only when EGF is bound. The dimerization loop-mediated mechanism of receptor dimerization may function as a key regulatory step to control the tyrosine kinase activity of the EGFR and other members of the family.

The presence of multiple ligands and receptors imparts the EGFR signalling network with an expanded repertoire of cellular responses, as the four receptors can potentially form ten distinct homo- and heterodimers that in turn are activated by different ligands (Olayioye, Neve et al. 2000). Because of the absence of a specific ligand for HER2, this RTK functions as the preferred heterodimeric partner of the other members of the EGFR family (Alroy and Yarden 1997), and provides an additional platform for recruitment of intracellular signalling pathways.

1.1.4 Cytoplasmic tyrosine kinases

There are ten known subfamilies of cytoplasmic, non-receptor tyrosine kinases (NRTKs): Src, Abl, Jak, Ack, Csk, Fak, Fes, Frk, Tec and Syk (Blume-Jensen and Hunter 2001). NRTKs lack receptor-like features such as an extracellular ligand-binding domain and a transmembrane-spanning region. Most NRTKs are localized in the cytoplasm, whereas some are anchored to the cell membrane through amino-terminal modifications, such as myristoylation or palmitoylation. In addition to a tyrosine kinase domain, NRTKs possess domains that mediate protein-protein, protein-lipid, and protein-DNA interactions. The most common theme in

NRTK regulation, as in RTK function, is tyrosine phosphorylation. In particular, phosphorylation of tyrosines in the activation loop of NRTKs leads to an increase in enzymatic activity. Activation loop phosphorylation occurs via *trans*-autophosphorylation or phosphorylation by a different number of other NRTKs (Hubbard and Till 2000). Phosphorylation of tyrosines outside of the activation loop can negatively regulate kinase activity.

The largest subfamily of NRTKs, with nine members, constitutes the Src family (Blume-Jensen and Hunter 2001). Src family members participate in a variety of signalling processes, including mitogenesis, T- and B-cell activation, and cytoskeleton remodelling. Multiple *in vivo* substrates have been described for Src and include the PDGFR and EGFR, the NRTK focal adhesion kinase Fak, the adapter protein p130Cas which is involved in integrin- and growth factor-mediated signalling and cortactin, an actin-binding protein important for the proper formation of cell matrix contact sites. Regulation of Src catalytic activity has been studied extensively. Src and its family members contain a myristoylated amino terminus, a stretch of positively-charged residues that interact with phospholipid head groups, a short region with low sequence homology, a SH3 domain, a SH2 domain, a tyrosine kinase domain, and a short carboxy-terminal tail. Src possesses two important regulatory tyrosine phosphorylation sites. Phosphorylation of Tyr-527 in the carboxy-terminal tail of Src by the NRTK Csk represses kinase activity. The importance of this phosphorylation site is clarified in v-Src, an oncogenic variant of Src that is a product of the Rous sarcoma virus. Owing to a carboxyterminal truncation, v-Src lacks the negative regulatory site Tyr-527 and is constitutively active, leading to uncontrolled growth of infected cells. A second regulatory phosphorylation site in Src is Tyr-416, an autophosphorylation site in the activation loop. Maximal stimulation of kinase activity occurs when Tyr-416 is phosphorylated. Src has also been implicated in several human carcinomas, including breast, lung and colon cancer.

1.1.5 Recruitment of downstream signalling molecules

Ligand-induced receptor dimerization and autophosphorylation of RTKs, as well as activation of NRTKs, generates phosphorylated tyrosine residues on target proteins that mediate the recruitment and activation of a variety of cytoplasmic signalling proteins (Hunter 2000). These signalling proteins are modular in nature and bring about interactions with other proteins, with phospholipids or with nucleic acids. Protein modules involved in cellular signalling processes

downstream of RTKs and other cell surface receptors range in size from 50 to 120 amino acids (Schlessinger 2000). SH2 domains bind specifically to distinct amino acid sequences defined by 1 to 6 residues C-terminal to the phosphotyrosine moiety, while PTB domains bind to phosphotyrosine residues within context of specific sequences 3 to 5 residues to its N-terminus. Certain PTB domains bind to nonphosphorylated peptide sequences, while others recognize both phosphotyrosine-containing and nonphosphorylated sequences equally well. SH3 domains bind specifically to the proline-rich sequence motif PXXP, while WW domains bind preferentially to another proline-rich motif PXPX. Pleckstrin homology (PH) domains comprise a large family of more than one hundred domains. While certain PH domains bind specifically to PtdIns(4,5)P_2, another subset of PH domains binds preferentially to the products of agonist-induced phosphoinositide-3-kinases (PI3-Ks). Finally, FYVE domains comprise another family of small protein modules that specifically recognize PtdIns-3-P. PDZ domains belong to another large family of independent protein modules that bind specifically to hydrophobic residues at the C termini of their target proteins. A large family of SH2 domain-containing proteins possess intrinsic enzymatic activities such as protein tyrosine kinase activity (Src-kinases), protein tyrosine phosphatase activity (SHP2), phospholipase C activity (PLCγ), or Ras-GAP activity. Another family of proteins exclusively contains SH2 or SH3 domains. These adaptor proteins (e.g. Grb2, Nck, Crk, Shc) utilize their SH2 and SH3 domains to mediate interactions that link different proteins involved in signal transduction. For example, the adaptor protein Grb2 links a variety of surface receptors to the Ras/mitogen-activated protein (MAP) kinase signalling cascade.

Agonist-induced membrane recruitment of signalling proteins stimulated by tyrosine phosphorylation is also mediated by a family of docking proteins which all contain in their N-termini a membrane targeting signal and in their C-termini a large region that contains multiple binding sites for the SH2 domains of signalling proteins. Docking proteins such as Gab1 become associated with the cell membrane by binding of its PH domain to PtdIns(3,4,5)P_3 in response to agonist-induced stimulation of PI3-K. In addition to the membrane targeting signal, most docking proteins contain specific domains such as PTB domains that are responsible for complex formation with a particular set of cell surface receptors. Because activated receptor tyrosine kinases selectively assemble and recruit signalling complexes every RTK is not only considered as a receptor with tyrosine kinase activity but also as a platform for the recognition and recruitment of a specific set of signalling proteins.

1.2 Mitogen-activated-protein-kinase (MAPK) pathways

The main signalling pathways linking activation of many cell surface receptors such as RTKs as well as GPCRs to the nucleus is via Ras (Schlessinger 2000), a small membrane-bound monomeric GTP-binding protein. Both biochemical and genetic studies have demonstrated that Ras is activated by the guanine nucleotide exchange factor Sos. The adaptor protein Grb2 plays an important role in this process by forming a complex with Sos via its SH3 domains. The Grb2/Sos complex is recruited to an activated RTK through binding of the Grb2 SH2 domain to specific phosphotyrosine sites of the receptor, thus translocating Sos to the plasma membrane where it is close to Ras and can stimulate exchange of GTP for GDP. Membrane recruitment of Sos can be also accomplished by binding of the Grb2/Sos complex to SHC, another adaptor protein that forms a complex with many receptors through its PTB domain. Alternatively, Grb2/Sos complexes can be recruited to the cell membrane by binding to membrane-linked docking proteins such as IRS1 or FRS2 which become tyrosine phosphorylated in response to activation of certain RTKs. Once in the active GTP-bound state, Ras interacts with several effector proteins such as Raf and PI3-K to stimulate numerous intracellular processes. Activated Raf stimulates MAPK kinase (MAPKK, MEK) by phosphorylating a key Ser residue in the activation loop. MAPKK then phosphorylates MAPK on Thr and Tyr residues in the activation-loop leading to its activation. Activated MAPK phosphorylates a variety of cytoplasmic and membrane linked substrates. In addition, MAPK is rapidly translocated into the nucleus where it phosphorylates and activates transcription factors. The signalling cassette composed of MAPKKK, MAPKK, and MAPK is highly conserved in evolution and plays an important role in the control of metabolic processes, cell cycle, cell migration and cell shape as well as in cell proliferation and differentiation (Hunter 2000).

The specificity of MAPK interactions and the effector molecules stimulated depends largely on the MAPK subtypes involved. In particular, extracellular signal-regulated kinases (ERK1/2)/MAPKs are primarily stimulated by growth factors and modulate cell growth and differentiation, whereas c-Jun N-terminal kinases (JNKs) and p38 MAPKs are most commonly activated by stress stimuli and are involved in cell growth, diffentiation, survival, apoptosis, and cytokine production (Marinissen and Gutkind 2001).

1.3 Protein kinase B/Akt

PKB/Akt is a serine/threonine kinase that exists in three isoforms in mammals (Akt 1, 2 and 3) which are structurally tightly related. They are composed of three functionally distinct regions: an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal hydrophobic motif (HM).

Protein kinase B is activated by the phosphoinositide 3-kinase (PI3-K) pathway. Generation of PIP_3 and PI(3, 4)P2 is necessary for the localization of PKB to the membrane surface. The activation takes place by multisite phosphorylation but the main site of phosphorylation is at Thr 308. The fully active multiphosphorylated Akt then dissociates from the plasma membrane and targets substrates located in the cytoplasm and nucleus. It causes the activation of genes involved in diverse cellular processes. Furthermore, Akt activation contributes to tumourigenesis and tumour metastasis as well as chemotherapeutic resistance.

1.4 G-protein-coupled receptors

G-protein coupled receptors (GPCRs) are the largest family of cell-surface receptors involved in the regulation of numerous physiological functions such as neurotransmission, photoreception, chemoreception, metabolism, growth and differentiation (Fukuhara, Chikumi et al. 2001). For signal transmission, GPCRs interact with heterotrimeric G proteins which are composed of an α-, β- and γ-subunit. GPCRs are also frequently referred to as heptahelical or serpentine receptors, because they contain a conserved structural motif consisting of seven α-helical membrane-spanning regions. Based on certain key sequences, GPCRs can be divided into three major subfamilies, receptors related to rhodopsin (type A), receptors related to the calcitonin receptor (type B), and receptors related to the metabotropic receptors (type C) (Gether and Kobilka 1998). All GPCRs have an extracellular N-terminal segment, seven transmembrane helices, which form the transmembrane core, three exoloops, three cytoloops, and a C-terminal segment. Each of the seven transmembrane helices is generally composed of 20-27 amino acids. On the other hand, N-terminal segments, loops, and C-terminal segments vary in size, an indication of their diverse structures and functions. Interestingly, there is a weak correlation between the N-terminal segment's length and ligand size, suggesting a role in ligand binding, in particular for large polypeptides and glycoprotein hormones. Domains which are critical for interaction with the G proteins have been localized to the second and third cytoplasmic loops and the C terminus (Ji, Grossmann et al. 1998).

The observation that muscarinic acetylcholine M_1, M_3 and M_5 receptors transform murine fibroblasts provides evidence that wild-type GPCRs can be tumourigenic when exposed to an excess of agonists (Marinissen and Gutkind 2001). Moreover, if mutated, GPCRs might be rendered transforming even in an agonist-independent fashion as shown, for example, for α_{1B}-adrenoceptors, thyroid-stimulating hormone receptors and leuteinizing hormone receptors. Although activating mutations are infrequent in GPCRs, these receptors often contribute to neoplasia when persistently stimulated by agonists released from tumours in an autocrine or paracrine fashion. The block of GPCR signalling effectively prevents tumour growth in animal models, which raises the possibility of developing novel agents that act on GPCRs for therapeutic intervention in cancer.

Sixteen distinct mammalian G-protein α–subunits have been cloned and are divided into four families based upon sequence similarity: α_s, which activates adenylyl cyclase, α_i, which inhibits adenylyl cyclase, α_q, which activates phospholipase C and α_{12} of unknown function. Similarly, eleven G-protein γ subunits and five G-protein β subunits have been identified (Gutkind 2000). Therefore, GPCRs are likely to represent the most diverse signal transduction systems in eukaryotic cells.

GTPase-deficient mutants of α_i, α_q, α_{12}, and α_{13} were found to display oncogenic properties when expressed in several cellular systems; and naturally occurring activated mutants of certain G-proteins were also identified in various disease states, including cancer.

GPCR activation causes a profound change in the transmembrane helices, which affects the conformation of intracellular loops and uncovers previously masked G-protein binding sites (Gutkind 2000). The GPCR-G protein interaction in turn promotes the release of guanosine diphosphate (GDP) bound to the G protein α subunit and its exchange for guanosine triphosphate (GTP) and causes a conformational change in three flexible "switch regions" of the Gα subunit, thus activating Gα and causing the dissociation and exposure of effector interaction sites in the βγ heterodimers.

Activated G-protein subunits then initiate intracellular signalling responses by acting on a variety of effector molecules (Gutkind 2000). These include adenylyl and guanylyl cyclases, phosphodiesterases, phospholipase A_2 (PLA_2), phospholipase C (PLC) and PI3-Ks, thereby activating or inhibiting the production of a variety of second messengers such as cAMP, cGMP, diacylglycerol, inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3], phosphatidyl inositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3], arachidonic acid and phosphatidic acid, in addition to

promoting increases in the intracellular concentration of Ca^{2+} and the opening or closing of a variety of ion channels.

A myriad of extracellular agonists have been demonstrated to act through GPCRs including biogenic amines, peptide and glycoprotein hormones, neuropeptides, serine proteases, neurotransmitters, eicosanoids and phospholipids such as sphingosine-1-phosphate and lysophosphatidic acid (LPA) (Ji, Grossmann et al. 1998).

LPA is an extracellular lipid mediator that has been implicated in the regulation of both, physiological and pathophysiological processes (Moolenaar, Kranenburg et al. 1997; Fang, Yu et al. 2000). LPA represents the major mitogenic activity in serum and numerous cellular responses to LPA have been documented including rapid cytoskeletal rearrangements (Gohla, Harhammer et al. 1998), stimulation of cell proliferation (van Corven, Groenink et al. 1989), suppression of apoptosis (Fang, Yu et al. 2000) and induction of tumour cell migration and invasion (Imamura, Horai et al. 1993; Fishman, Liu et al. 2001). LPA levels are elevated in plasma and ascites of ovarian cancer patients (Imamura, Horai et al. 1993; Xu, Gaudette et al. 1995; Xu, Shen et al. 1998; Fishman, Liu et al. 2001) and LPA is likely to play a prominent role in the pathology of other types of human cancer.

The cell-surface receptors for LPA and for the structurally related phospholipid sphingosine-1-phosphate (S1P) belong to the EDG (endothelial cell differentiation gene) subfamily of GPCRs (Pyne and Pyne 2000; Kranenburg and Moolenaar 2001). To date, four functional LPA receptors have been described (EDG2, EDG4 and EDG7) which couple to G_i, G_q and G_{12} subtypes of G proteins and show distinct properties in ligand specificity and activation of intracellular signalling pathways. According to the cellular context, LPA was shown to be involved in the modulation of adenylate cyclase, stimulation of phospholipase C (PLC) and subsequent Ca^{2+} mobilization, activation of the Ras/MAPK pathway, phosphorylation of the survival mediator Akt/protein kinase B (PKB) by PI3-K and transcriptional regulation of immediate-early genes (Moolenaar, Kranenburg et al. 1997; Moolenaar 1999; Pyne and Pyne 2000; Kranenburg and Moolenaar 2001).

1.5 EGFR signal transactivation

Various studies have revealed that cellular responses to GPCR agonists depend on the function of the EGFR in several cell systems, a phenomenon that was termed interreceptor cross-talk or

EGFR signal transactivation (Daub, Weiss et al. 1996; Luttrell, Ferguson et al. 1999; Zwick, Hackel et al. 1999; Marinissen and Gutkind 2001). The pioneer studies of H. Daub and colleagues have described a critical role of the EGFR in GPCR-induced mitogenesis of rat fibroblasts (Daub, Weiss et al. 1996). They have demonstrated that the EGFR and HER2/neu are rapidly tyrosine phosphorylated after stimulation of Rat-1 cells with the GPCR agonists endothelin-1 (ET-1), LPA or thrombin (Figure 2a). This transactivation of a receptor tyrosine kinase couples GPCR-ligand engagement to ERK activation, induction of *fos* gene expression and DNA synthesis, which are abrogated either by the selective EGFR inhibitor tyrphostin AG1478 or by expression of a dominant-negative EGFR mutant.

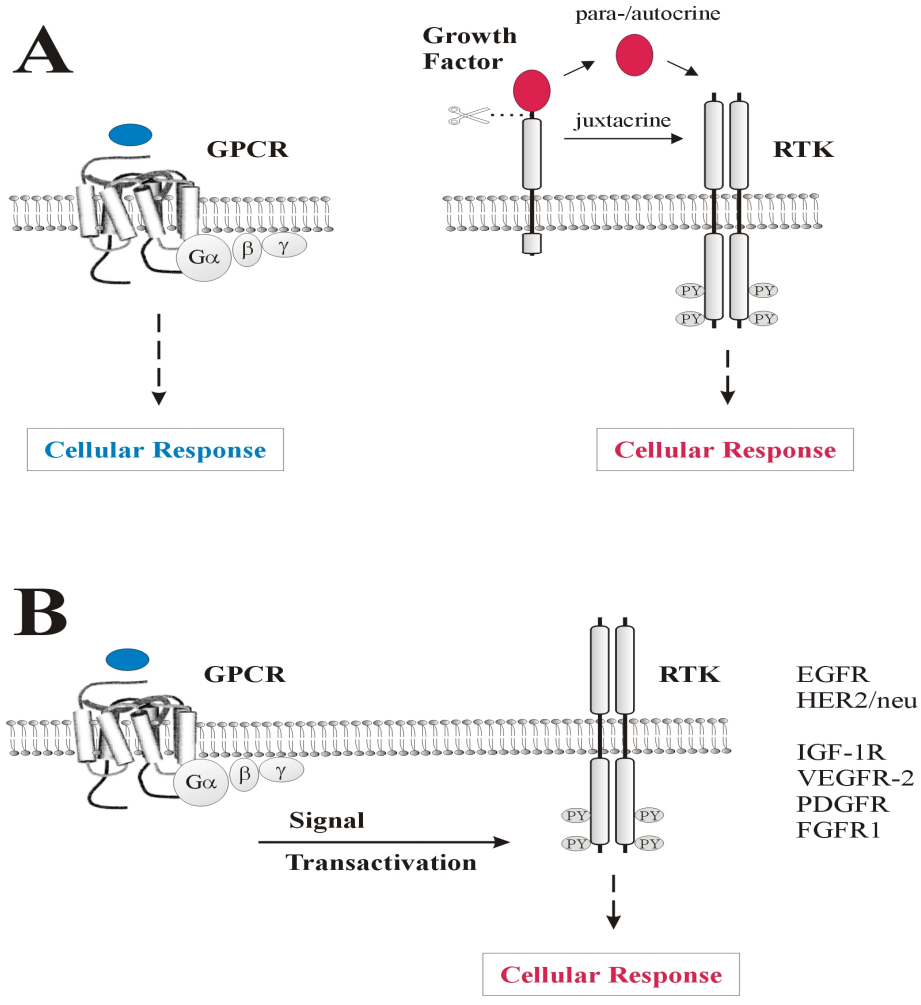


Figure 2. GPCR and RTK signaling systems. A) Individual pathways transmit signals along linear tracts resulting in regulation of discrete cell functions. B) RTK signal transactivation leads to RTK-characteristic cellular responses upon GPCR stimulation.

Further investigations have revealed that the GPCR-EGFR cross-talk mechanism is installed in a variety of other cell types such as human keratinocytes, primary mouse astrocytes, PC-12

cells and vascular smooth muscle cells (Daub, Wallasch et al. 1997; Zwick, Daub et al. 1997; Eguchi, Numaguchi et al. 1998) and established it as a widely relevant pathway towards the activation of the MAP kinase signal.

A number of reports have demonstrated that various extracellular stimuli, unrelated to EGF-like ligands and GPCR agonists can also activate the EGF receptor (Zwick, Hackel et al. 1999). These diverse stimuli include agonists for cytokine receptors (prolactin, growth hormone), adhesion receptors (integrins), membrane-depolarizing agents (KCl) and environmental stress factors (ultraviolet and gamma irradiation, oxidants, heat shock, hyperosmotic shock). In addition to the EGFR, other RTKs have been shown to be activated by GPCR ligands (Fig. 2B). For example, in primary rat smooth muscle cells the insulin-like growth factor receptor (IGF-1R) phosphorylation is induced by thrombin (Weiss, Daub et al. 1997) while the VEGFR-2 is transactivated by S1P in human umbilical vein endothelial cells (HUVECs) (Endo, Nagashima et al. 2002). Moreover, it was reported that LPA induces PDGFR tyrosine phosphorylation in L cells (Herrlich, Daub et al. 1998) and that opioid receptor agonists transactivate the fibroblast growth factor receptor (FGFR)-1 in rat C6 glioma cells that lack the EGFR (Belcheva, Haas et al. 2002) suggesting that transactivation of distinct RTKs can contribute to GPCR signalling in a cell-type-specific manner.

Subsequent work provided evidence for widespread use of EGFR signal transactivation by diverse GPCRs and the capacity of different G-proteins to generate the necessary connections (Table 1). Interestingly, LPA-induced transactivation of the EGFR in COS-7 cells was attenuated by pertussis toxin (PTX) which inactivates Gα subunits of the $G_{i/o}$ family of G proteins. In contrast, thrombin stimulated EGFR tyrosine phosphorylation and downstream signalling was not affected (Daub, Wallasch et al. 1997). Furthermore, agonist stimulation of ectopically expressed G_q-coupled bombesin (BombR) or G_i-coupled M_2 muscarinic acetylcholine receptor (M_2R) triggered EGFR transactivation followed by tyrosine phosphorylation of SHC and formation of SHC-Grb2 complexes. These results demonstrated that EGFR transactivation occurs via both PTX-insensitive and -sensitive pathways and that the EGFR mediates MAP kinase activation by G_q- and G_i-coupled receptors in COS-7 cells. More recent studies showed that $G\alpha_{13}$ subunits mediate LPA-induced actin polymerization and actin stress fiber formation in Swiss 3T3 cells and mouse fibroblasts via EGFR transactivation (Gohla, Harhammer et al. 1998; Gohla, Offermanns et al. 1999).

GPCR ligand	G protein	Cell line or tissue	Cellular response	Reference
Endothelin-1, LPA, thrombin		Rat-1	ERK activation, FOS transcription	Daub et al., 1996
Bradykinin	G_q	PC-12	ERK activation	Zwick et al., 1997
Bombesin, Carbachol, LPA	G_q, G_i	COS-7	ERK activation	Daub et al., 1997
Isoproterenol		COS-7	ERK activation	Maudsley et al., 2000
Angiotensin II	G_q	vascular smooth muscle	ERK activation	Eguchi et al., 1998
Thrombin, LPA		HaCaT	ERK activation	Daub et al., 1997
Thrombin		primary astrocytes	ERK activation	Daub et al., 1997
Thrombin		vascular smooth muscle	ERK activation, migration	Kalmes et al., 2000
Carbachol	G_q	HEK 293	Modulation of Kv1.2 ion channel activity	Tsai et al., 1997
Carbachol	G_q	T84	ERK activation, inhibition of Cl^- secretion	Keely et al., 1998
LPA	G_{13}	Swiss 3T3	stress fiber formation	Gohla et al., 1998
LPA		HeLa	ERK activation	Cunnick et al., 1998
LPA		NIH 3T3	MKK1/2 activation, DNA synthesis	Cunnick et al., 1998
Bombesin		PC3	EGFR tyrosine phosphorylation	Prenzel et al., 1999
Substance P	G_i	U-373 MG	ERK activation, DNA synthesis	Castagliuolo et al., 2000
Interleukin-8	G_q	SK-OV-3	ERK activation, morphology changes	Venkatakrishnan et al., 2000

Table 1: Cross-talk between GPCRs and the EGFR (Gschwind et al., 2001)

In summary, G_i-, G_q- as well as G_{13}-coupled receptors have been reported to transactivate the EGFR after agonist stimulation in diverse cell systems, whereas up to now there is no data available concerning an analogous function of G_s-coupled receptors.

Several studies indicate that the EGFR transactivation mechanism is subject to different cell type-characteristic regulatory influences. In PC-12, vascular smooth muscle cells and intestinal epithelial cells intracellular Ca^{2+} concentration has been demonstrated to be a critical parameter in G_q-coupled receptor-mediated EGFR transactivation (Zwick, Daub et al. 1997; Eguchi, Numaguchi et al. 1998; Murasawa, Mori et al. 1998; Soltoff 1998; Iwasaki, Eguchi et al. 1999). Activation of the Ser/Thr protein kinase C (PKC) was shown to be required for G_q-coupled receptors to induce EGFR transactivation in cell lines such as HEK-293 and PC-12 cells (Tsai, Morielli et al. 1997; Soltoff 1998; Grosse, Roelle et al. 2000).

Besides the function of PKC in GPCR-mediated EGFR transactivation, Matsubara and co-workers reported Ca^{2+}/calmodulin-dependent receptor activation in Ang II-stimulated cardiac fibroblasts (Murasawa, Mori et al. 1998). Similarly in PC-12 cells, Zwick and colleagues (Zwick, Wallasch et al. 1999) demonstrated the involvement of a Ca^{2+}-calmodulin-dependent kinase II (CaMK II) activity in K^+- but not bradykinin-induced EGFR signal transactivation. The role of another Ca^{2+}-dependent kinase, PYK2, in the transmission of mitogenic signals is controversial. While several reports suggested a role of this tyrosine kinase in G_q-mediated EGFR tyrosine phosphorylation in PC-12 (Soltoff, 1998) and intestinal epithelial cells,

respectively (Keely, Calandrella et al. 2000), Zwick *et al*. reported Ca^{2+}-dependent, but PYK2-independent EGFR transactivation in response to bradykinin in PC-12 cells (Zwick, Wallasch et al. 1999).

Furthermore, tyrosine phosphorylated Src is often found in association with the EGFR (Luttrell, Ferguson et al. 1999) or with PYK2 (Soltoff 1998; Keely, Calandrella et al. 2000) upon stimulation of G_q-coupled receptors and has therefore been proposed to function as a mediator of EGFR transactivation. Since other reports have demonstrated Src-independent EGFR transactivation, but Src-dependent SHC tyrosine phosphorylation and ERK activation (Daub, Wallasch et al. 1997; Adomeit, Graness et al. 1999; Slack 2000) it seems likely that Src is recruited by the transactivated EGFR and thereby contributes to activation of the Ras signalling pathway.

Due to the rapid kinetics of EGFR signal transactivation and the fact that release of EGFR ligands was not detectable after GPCR stimulation, the mechanism of EGFR transactivation was proposed not to involve the interaction of the EGFR with a ligand. Hence, EGFR activation by GPCR agonists was assumed to exclusively rely on intracellular elements such as Ca^{2+}, PKC and Src (Carpenter 1999).

Very recently, a new mechanistic concept of strictly ligand-dependent EGFR transactivation by GPCRs has been presented and summarizes experimental data obtained from Rat-1, COS-7 and HEK-293 cells (Prenzel, Zwick et al. 1999). The GPCR ligands LPA, carbachol and bombesin were shown to induce the proteolytic processing of the transmembrane proHB-EGF precursor to yield the mature ligand. Blocking of this process either with the metalloprotease inhibitor batimastat or the HB-EGF antagonistic diphtheria toxin mutant CRM197 completely abrogated GPCR-induced EGFR transactivation and SHC tyrosine phosphorylation. The so called triple-membrane-passing signal (TMPS) model includes the G protein-mediated activation of a metalloprotease via an unknown mechanism (Gschwind, Zwick et al. 2001). The TMPS mechanism also allows the transactivation of EGFRs on neighbouring cells but only over short distances and under participation of the heparan sulfate proteoglycan matrix which in retrospect explains the failure of Daub and colleagues (Daub, Weiss et al. 1996) to detect EGF like activity in conditioned medium of GPCR-ligand-stimulated Rat1 cell cultures. In this context, growing evidence points to transmembrane metalloproteases as the key enzymes of growth factor precursor shedding.

1.6 Metalloproteases

Metalloproteases are important in many aspects of biology, ranging from cell proliferation, differentiation and remodelling of the extracellular matrix (ECM) to vascularization and cell migration. These events occur several times during organogenesis in both normal development and during tumour progression. Mechanisms of metalloprotease action underlying these events include the proteolytic cleavage of growth factors so that they can become available to cells not in direct physical contact, degradation of the ECM so that founder cells can move across tissues into nearby stroma and regulated receptor cleavage to terminate migratory signalling. Most of these processes require a delicate balance between the functions of matrix metalloproteases (MMPs) or metalloprotease-disintegrins (ADAMs) and natural tissue inhibitors of metalloproteases (TIMPs).

Metalloproteases are generally characterized by a catalytically indispensable zinc ion in their active site. Many of these enzymes contain a conserved HEXXH (X is any amino acid residue) consensus sequence (Hooper 1994). Due to the presence of an extended zinc-binding motif, HEXXHXXGXXH and a methionine-containing turn of similar conformation close to the active site, the astacins, the serralysins, the MMPs and the adamalysins (ADAMs) are grouped into the metzincin superfamily of metalloproteases (Bode, Gomis-Ruth et al. 1993). The three histidines of the extended HEXXH sequence serve as ligands to the zinc, whereas the glutamic acid is believed to transfer hydrogen atoms and to polarize a zinc-bound water molecule for nucleophilic attack on the scissile peptide bond of bound substrate (Stocker and Bode 1995). Many metalloproteases are synthesized as inactive precursors in which the prodomain is responsible for maintaining latency of the protease via a cysteine switch mechanism: In particular, the free sulfhydryl group a cysteine residue in the prodomain provides a forth coordination site keeping the protease inactive until the prodomain is removed (Bode, Gomis-Ruth et al. 1993). Besides its role as an inhibitor of the protease domain, the prodomain appears to be important for the proper maturation and intracellular transport of metalloproteases. Although prodomain removal is probably a prerequisite for protease activity, this processing appears to be mediated constitutively by a furin-type proprotein convertase in the trans-Golgi network.

1.6.1 ADAMs

Metalloprotease-disintegrins are transmembrane glycoproteins that play roles in cell-cell interaction and in the processing of the ectodomains of proteins (Wolfsberg, Primakoff et al. 1995). They combine features of both cell surface adhesion molecules and proteinases and are characterized by a conserved domain structure consisting of N-terminal signal sequence followed by a prodomain, metalloprotease and disintegrin domains, a cysteine-rich region and finally a transmembrane domain and cytoplasmic tail (Fig. 3). Thus family members are referred to as ADAM (a disintegrin and metalloprotease domain) or as MDCs (metalloprotease, disintegrin, cysteine-rich proteins).

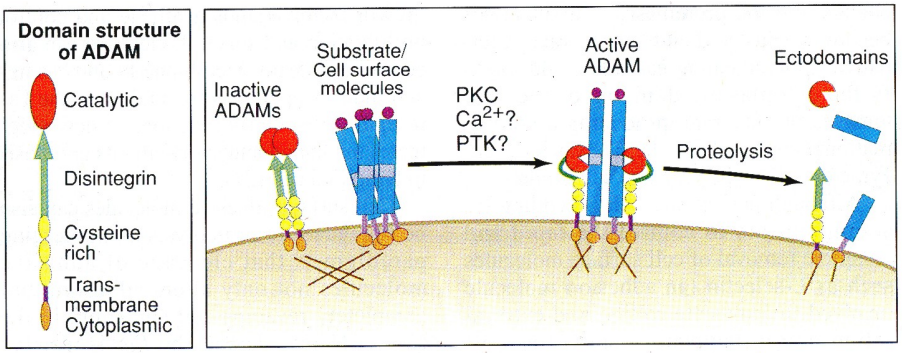


Figure 3. Structure of ADAM family metalloproteases and their involvement in cell surface ectodomain shedding of multiple substrates (PTK, protein tyrosine kinase; PKC, protein kinase C) (Werb and Yan, 1998).

More than 30 ADAM cDNA sequences have been identified to date in organisms ranging from *S. pombe* to humans (Primakoff and Myles 2000). Interestingly, although all ADAMs have a relatively well-conserved metalloprotease domain, only 15 of those identified contain the zinc-binding catalytic-site consensus sequence (HEXXH). Thus, only half of the known ADAMs is predicted to be catalytically active, whereas the others most likely lack metalloprotease activity. ADAMs have been implicated in diverse processes, including sperm-egg binding and fusion, myoblast fusion, protein-ectodomain processing or shedding of cytokines, cytokine receptors, adhesion proteins and other extracellular protein domains (Schlondorff and Blobel 1999). The regulation of ADAM metalloprotease activity after prodomain removal is only poorly understood. Processing of membrane proteins by ADAMs requires both the membrane-anchored enzyme and its substrate to be present in cis on the

same cell, probably anchored in distinct domains of the plasma membrane through cytoskeletal interactions (Fig. 3).

Upon cell activation, for example by PKC agonists, increases in cytoplasmic Ca^{2+} levels or tyrosine kinase stimulation, the attachments change and the proteinases and substrates become co-clustered and can interact. Alternatively, the signalling cascade could modify the cytoplasmic domains of the proteinases or substrate, producing a conformational change that either activates the enzyme or makes the cleavage site available (Schlondorff and Blobel 1999).

For most processing reactions there appears to be a constitutive level of ectodomain shedding. Processing is necessary to make paracrine growth and survival factors available including EGF-like ligands allowing for the consistent supply of EGFR agonists. The first and best-characterised "sheddase" is TACE (tumour necrosis factor alpha converting enzyme, ADAM17) (Black, Rauch et al. 1997; Moss, Jin et al. 1997). Besides TNFα, TACE mediates cleavage of several other unrelated membrane proteins, such as TGFα, L-selectin, p75 TNFR and HER4 (Black 2002). Surprisingly, mice lacking functional TACE display multiple defects in epithelial cell maturation and organization in multiple organs such as the eye, hair and skin. This phenotype is similar in animals engineered to lack the EGFR (Peschon, Slack et al. 1998). In addition, targeted disruption of the TACE genes causes a much more severe phenotype than knock-out of TGFα alone, suggesting the involvement of TACE not only in proTGFα shedding, but also in the membrane cleavage of other EGF-like ligand precursors.

1.6.2 The Matrix Metalloproteinases (MMPs)

MMPs, which are closely related to the ADAM family of metalloproteases, play a central role in the timely breakdown of virtually any component of the extracellular matrix (ECM) (Shapiro 1998). Matrix remodelling is essential for embryonic development, morphogenesis, reproduction, and tissue resorption.

MMPs were historically divided into collagenases, gelatinases, stromelysins and matrilysins on the basis of their specificity for ECM components. However, a sequential numbering system for the more than 20 known human MMPs has been adapted, and the MMPs are now grouped according to their structure (Nagase and Woessner 1999). There are eight distinct classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs). All MMPs are synthesized as prepro-enzymes and secreted as inactive pro-MMPs in most cases.

The prodomain has a conserved unique PRCG (V/N)PD sequence. The cysteine within this sequence coordinates the catalytic zinc to maintain the latency of pro-MMPs. The catalytic domains of MMPs have an additional structural zinc ion and 2-3 calcium ions, which are required for the stability and the expression of enzymatic activity. The gelatinases MMP-2 and MMP-9 have three repeats of fibronectin-type II domain inserted in the catalytic domain.
These repeats interact with collagens and gelatins. Most of the MMPs are activated outside the cell by other activated MMPs or furin-like serine proteases. Endogenous inhibitors such as α-macroglobulins, and TIMPs tightly control the proteolytic activities of MMPs. The expression of many MMPs is transcriptionally regulated by growth factors, hormones, cytokines and cellular transformation (Brinckerhoff and Matrisian 2002).

1.7 Molecular oncology and aberrant signalling in cancer

In non-transformed cells cell division, survival and death are in balance promoting homeostasis. The products of oncogenes and tumour suppressor genes however interact in overlapping pathways and dysfunction leads to cancer. Tumourigenesis is a multistep process involving genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg 2000). The genomes of tumour cells are altered at multiple sites, having suffered disruption through lesions as subtle as point mutations and as obvious as changes in chromosome complement (Blume-Jensen and Hunter 2001). Furthermore, cancer is the most common genetic disease: one in three people in the western world develop cancer, and one in five die from it. Therefore, it is an important task to elucidate the mechanisms behind transformation of normal cells to cancer cells and conversion of normal tissue into malignant tumours. It is a general phenomenon both in normal and transformed cells that signalling pathways are not freestanding entities but parts of larger signalling networks.
Bladder and kidney cancer belong to the most frequent tumour types of the urogenital tract. The risk to fall ill with any of these kinds of cancer is two to three-fold higher in men than in women and increases with age. A strong link exists between lifestyle and the probability to acquire cancer. An important risk factor is cigarette smoke, but genetic disposition is of equal relevance. Although early diagnosis positively affects disease outcome the probability of relapse is high especially for bladder cancer. Moreover, each year the incidence of bladder cancer as well as the death rate caused by this form of cancer is rising. Furthermore, after progression to a later tumour stage metastases are detected in 30-50% of the patients. Surgical

removal of bladder, prostate and seminal vesicles is required if bladder tumours have invaded muscular layers. The main therapy for kidney cancer is the complete resection of the kidney including the adrenal gland and ureter (Vogelzang and Stadler 1998). This means that the common treatment of bladder and kidney cancer results in complete loss of function of the affected organ and thus a severely impaired quality of life for the patient.

Hence, there is a strong need to identify novel intervention targets and to design patient-tailored therapies for prevention and treatment of these tumours. In order to reverse or at least contain tumour spreading it is important to understand the molecular mechanisms underlying cancer development and progression.

1.8 Cancer cell characteristics

Observations of human cancers and animal models argue that tumour development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells.

The vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth and which are now recognized as the six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. It is assumed that these six capabilities are shared in common by most and perhaps all types of human tumours (Hanahan and Weinberg 2000).

Cell growth is controlled by proto-oncogenes and tumour-suppressor genes such as growth factors and growth factor receptors, intracellular signal transduction proteins, transcription factors, cell-cycle control proteins and DNA-repair proteins. The cell cycle is regulated by cyclic variations in the concentration of cyclins, which control checkpoints. G1/S transition of the cell cycle is controlled by the G1 checkpoint. The transcription factor E2F is necessary to transcribe proteins essential for the S-phase, normally it is inhibited by Rb. Growth factors cause an increased cyclin D1 expression, which forms a complex with cdk2/4 and phosphorylates Rb and therefore releases E2F. In cancer, virus-encoded activators of growth factor receptors act as oncoproteins and activating mutations or overexpression of growth factor receptors transform cells by constant dimerization of RTKs and constitutive activation

of signal transduction pathways. This leads to a disruption of the cell cycle and escape from growth constraints.

During development and differentiation newly forming tissues are sculped by a proper regulation of cell proliferation and programmed cell death. A balance between both is further important in the maintenance of cell and tissue homeostasis. For tumour progression, cancer cells need to evade apoptosis. One of the most important genes in this process is Bcl-2 which is regulated by growth factors and signal transduction pathways. Moreover, in 1997 a novel anti-apoptosis gene called Survivin was detected which is prominently expressed in transformed cells and in most common human cancers (Ambrosini, Adida et al. 1997).

Benign tumours grow locally restricted, but the normal organization of the affected tissue gradually becomes disrupted by the increasing number of dividing cells. As the dividing cancer cells extend through the basement membrane into the surrounding stroma, malignancy begins. Invasion stimulatory pathways depend on the activation of trimeric G-proteins, PI3-K and the Rac and Rho family of small GTPases. Proteolysis plays a role for the breakdown of extracellular matrix as well as cleavage of pro-invasive fragments from cell surface glycoproteins.

As the dividing cancer cells extend into blood vessels tumours undergo metastasis by spreading from the primary site to more distant sites in the body making cancer much more difficult to cure.

1.9 Aim of the study

The EGFR is part of signalling networks that are activated by heterologous stimuli. Most importantly, agonists for GPCRs, which comprise the largest family of cell-surface receptors, have been recognized as potent inducers of EGFR signalling activity.

Previous studies established the EGFR as an essential element in GPCR mitogenic signalling in a variety of cell systems including COS-7, HaCaT, PC-12 and HEK-293 cells (Carpenter 1999; Zwick, Hackel et al. 1999; Gschwind, Zwick et al. 2001).

Recent experimental data supports the view of a strictly ligand-dependent mechanism of EGFR signal transactivation involving proteolytic processing of transmembrane EGFR-ligand precursors to yield the mature ligand (Prenzel, Zwick et al. 1999). Inhibition of this process with the metalloprotease inhibitor batimastat completely abrogated GPCR-induced EGFR

transactivation. These experimental findings led to the establishment of the Triple-Membrane-Passing-Signalling (TMPS) pathway model of EGFR signal transactivation.

On the basis of these findings and the fact that deregulation of both GPCR and EGFR signalling systems has been recognized as a major cause of hyperproliferative diseases, the aim of this study was to investigate the molecular mechanisms and the pathophysiological significance of EGFR signal transactivation in kidney and bladder cancer. Overexpression of the EGFR, HER2/neu and EGF-like ligands has been shown to promote tumour growth in bladder and kidney cancer cells and in addition has been correlated with metastatic behaviour (Dempsey, Meise et al. 1997). Moreover, the EGFR serves as a prognostic marker in these types of cancer. Given the prominent role of the EGFR in the development and progression of kidney and bladder tumours we have chosen cells derived from these types of cancer as experimental model systems to investigate the significance of GPCR ligands in cancer progression.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3´-triphosphate)	Pharmacia, Freiburg
Batimastat	British Biotech, Oxford, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Fibronectin	Calbiochem, Bad Soden
GF-109203X	LC Laboratories, Grünberg
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine	Gibco, Eggenstein
Lipofectamine®	Gibco, Eggenstein
MBP	(Myelin basic protein) Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen
MOPS (3-Morpholinopropanesulfonic acid)	Biomol, Haub
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
PP1	Calbiochem, Bad Soden
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Aldrich, Steinheim
Scintillation cocktail (Rotiszint®ecoplus)	Roth, Karlsruhe
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg

Wortmannin	Sigma, Taufkirchen

All other chemicals were purchased from Merck (Darmstadt).

2.1.2 Enzymes

Alkaline Phosphatase	Roche, Mannheim
Restriction Endonucleases	Pharmacia, Freiburg
	Roche, Mannheim
	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
T7-DNA Polymerase	Pharmacia, Freiburg
Taq-DNA Polymerase	Roche, Mannheim
	Takara, Japan
Trypsin	Gibco, Eggenstein

2.1.3 Radiochemicals

[γ-^{32}P] ATP	>5000 Ci/mmol
[a-^{33}P] dATP	2500 Ci/mmol

All radiochemicals were obtained from PerkinElmer Life Sciences, Köln.

2.1.4 "Kits" and other materials

Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Pharmacia, Freiburg
Protein G-Sepharose	Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Random-Primed DNA Labeling Kit	Pharmacia, Freiburg
Sephadex G-50 (DNA Quality)	Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Transwells	Corning, New York, USA
Whatman 3MM	Whatman, USA

2.1.5 Growth factors and ligands

Anisomycin	Calbiochem
Amphiregulin	R&D Systems
Bradykinin	Calbiochem
EGF (murine)	Toyoba, Japan

All other growth factors and ligands were purchased from Sigma.

2.1.6 Media and buffers

2.1.6.1 Media for E. coli bacteria

LB-Medium	1.0 % Tryptone
	0.5 % Yeast Extract
	1.0 % NaCl
	pH 7.2
2xYT-Medium	1.6 % Tryptone
	1.0 % Yeast Extract
	1.0 % NaCl
	pH 7.2

When necessary the following antibiotics were added to the media after autoclavation:

Ampicillin	100 µg/mL
Kanamycin	100 µg/mL
Chloramphenicol	30 µg/mL

LB-plates additionally contained 1.5% Agar.

2.1.6.2 Cell culture media

All cell culture media and additives were from Gibco (Eggenstein), fetal calf serum (FCS) was purchased from Sigma.
Dulbecco's modified eagle medium (DMEM) with 4.5 mg/mL glucose, 2 mM L-glutamine, 1 mM sodium pyruvate.
Eagle´s minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate.
Nutrient mixture F12 (HAM) with L-glutamine.
MEM alpha medium
Freeze medium: 90% heat-inactivated FCS, 10% DMSO.

2.1.7 Stock solutions and buffers

BBS (2x)	50 mM BES
	280 mM NaCl
	1.5 mM Na_2HPO_4
	pH 6.96 (NaOH)
HBS (2x)	46 mM HEPES pH 7.5
	274 mM NaCl
	1.5 mM Na_2HPO_4

	pH 7.0
DNA loading buffer (6x)	0.25 % Bromphenol blue 0.25 % Xylencyanol 30.0 % Glycerol 100.0 mM EDTA pH 8.0
Laemmli buffer (2x)	187.5 mM Tris/HCl pH 6.8 6.0 % SDS 30.0 % Glycerol 0.01 % Bromphenol blue 5.0 % ß-Mercaptoethanol
NET (1x)	150.0 mM NaCl 5 mM EDTA 50 mM Tris 0.05 % Triton X-100 pH 7.4 (HCl)
PBS	13.7 mM NaCl 2.7 mM KCl 80.9 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.4 (HCl)
SD-Transblot	50.0 mM Tris/HCl pH 7.5 40.0 mM Glycine 20.0 % Methanol 0.004 % SDS
"Strip" buffer	62.5 mM Tris/HCl pH 6.8 2.0 % SDS 100 mM ß-Mercaptoethanol
SSC (20x)	3.0 M NaCl 0.3 M Sodium citrate
TAE (10x)	400 mM Tris/Acetate 10 mM EDTA pH 8.0 (Acetic acid)
TE10/0.1	10.0 mM Tris/HCl pH 8.0 0.1 mM EDTA pH 8.0 0.2
Tris-Glycine-SDS (10x)	248.0 mM Tris/HCl pH 7.5 1918.0 mM Glycine 1.0 % SDS

2.1.8 Bacteria strains (E. coli)

E. coli	**Description**	**Origin/ Reference**
DH5aF'	F'/endA1 hsd17 (rk-mk-),supE44,recA1, gyrA (Nal), thi-1, (lacZYA-argF)	Genentech, San Francisco, USA

2.1.9 Cell lines

Cell Line	**Description**	**Origin/ Reference**
CaKi2	Human kidney carcinoma cell line	ATCC
ACHN	Human kidney carcinoma cell line	ATCC CRL-1611
HK2	Human kidney carcinoma cell line	ATCC
A498	Human kidney carcinoma cell line	SUGEN
A704	Human kidney carcinoma cell line	ATCC CRL-7911
SCABER	Human bladder carcinoma cell line	ATCC HTB-3
HT1376	Human bladder carcinoma cell line	DSMZ
TccSup	Human bladder carcinoma cell line	ATCC HTB-5
5637	Human bladder carcinoma cell line	ATCC HTB-9
HEK-293 T	Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC CRL-1573
Phoenix E, A	Retrovirus producer cell lines for the generation of helper free ecotropic and amphotropic retroviruses based on HEK-293	Nolan, Stanford

All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and grown as recommended by the supplier.

2.1.10 Antibodies

The following antibodies were used in immunoprecipitation experiments, as primary antibodies in immunoblot analysis or for staining of cell surface proteins in FACS analysis.

Antibody	**Description/ Immunogen**	**Origin/ Reference**
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, Lake Placid
EGFR	Sheep, polyclonal/ part of cytoplasmic domain of the human EGFR	UBI
EGFR (108.1)	Mouse, monoclonal/ ectodomain of the human EGFR	(Daub et al., 1997)
HER2/neu	Rabbit, polyclonal/ C-terminal peptide of human HER2/neu	(Daub et al., 1996)
Akt1/2	Rabbit, polyclonal/ AA 345-480 of human Akt1	Santa Cruz, USA
SHC	Mouse, monoclonal	Santa Cruz
SHC	Rabbit, polyclonal/ 220 AA at C-terminus of human SHC	(Daub et al., 1997)
Gab1	Rabbit, polyclonal/ AA 23-189 of human Gab1	(Daub et al., 1997)

P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M.
P-p38	Rabbit, polyclonal; recognizes phospho-p38 (Thr-180/Tyr-182) MAPK	NEB
P-Akt/PKB	Rabbit, polyclonal; recognizes phospho-Akt (Ser-473)	NEB
HB-EGF	Goat, polyclonal/ recombinant, human HB-EGF	R&D Systems, Wiesbaden
AR	Goat, polyclonal/ recombinant, human AR	R&D Systems
TGFα	Mouse, monoclonal/ recombinant, human TGFα	Oncogene, Bad Soden
ERK2 (C-14)	Rabbit, polyclonal/ peptide at C-terminus of rat ERK2	Santa Cruz
ERK2 (K-23)	Rabbit, polyclonal/ peptide from sub-domain XI of rat ERK2	Santa Cruz
Pan-ERK	Mouse monoclonal/ AA 219-358 of human ERK2	Transduction Lab.
HA	Mouse, monoclonal; recognizes the influenza hemagglutinin epitope	Babco, California, USA
VSV (P5D4)	Mouse, monoclonal; recognizes an epitope of eleven AA derived from the vesicular stomatits virus glycoprotein VSV-G	Roche, Mannheim
Cyclin D1	Mouse, IgM, monoclonal, corresponding to amino acids 1-200	Transduction Lab
p38 (C-20)	Rabbit, polyclonal/ peptide at C-terminus of murine p38	Santa Cruz

For western blot secondary antibodies conjugated with horseradish peroxidase (HRP) were utilized.

Antibody	**Dilution**	**Origin**
Goat anti-mouse	1 : 10,000	Sigma
Goat anti-sheep	1 : 25,000	Dianova, Hamburg
Goat anti-rabbit	1 : 25,000	BioRad, München

The FITC-conjugated rabbit anti-goat secondary antibody for flow cytometry was obtained from Sigma.

2.1.11 Plasmids and oligonucleotides

2.1.11.1 Primary vectors

Vector	**Description**	**Origin/ Reference**
pcDNA3	Mammalian expression vector, Amp_r, CMV promotor, BGH poly A, high copy number plasmid	Invitrogen, USA

pLXSN	Expression vector for retroviral gene transfer, Amp_r, Neo_r, ori from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 promotor	Clontech, Palo Alto, USA
pLXSN-ESK	Modified pLXSN vector with multipe cloning site from pBluescript	J. Ruhe
pRK5	Expression vector, Amp_r, CMV Promoter, SV 40 poly A, high copy number plasmid	Genentech

2.1.11.2 Constructs

Vector	**Description**	**Reference**
pcDNA3-hADAM10-HA	cDNA of human ADAM10 in pcDNA3; C-terminal HA-tag	A. Gschwind
pLXSN-ESK-Δ(Pro-MP)--hADAM10-HA	cDNA of ADAM10 lacking the prodomain and metalloprotease domain Δ(AA19-455); pLXSN-ESK; HA-tag	A. Gschwind
pcDNA3-Δ(Pro-MP)--hADAM12-HA	cDNA of ADAM12 lacking the prodomain and metalloprotease domain Δ(AA29-416); in pcDNA3; C-terminal HA-tag	S. Hart
pLXSN-ESK-Δ(Pro-MP)--hADAM12-HA	cDNA of ADAM12 lacking the prodomain and metalloprotease domain in pLXSN-ESK; HA-tag	S. Hart
pLXSN-ESK-Δ(Pro-MP)--hADAM15-HA	cDNA of ADAM15 lacking the prodomain and metalloprotease domain Δ(AA29-419) in pLXSN-ESK; C-terminal HA-tag	S. Hart
pcDNA3-mADAM17/TACE	cDNA of murine TACE in pcDNA3	(Black et al., 1997)
pLXSN-ESK-mADAM17/TACE	cDNA of murine TACE in pLXSN-ESK	A. Gschwind
pcDNA3-hADAM17/TACE-HA	cDNA of human TACE pcDNA3; C-terminal HA-tag	A. Gschwind
pLXSN-ESK-hADAM17/TACE-HA	cDNA of human TACE pLXSN-ESK; C-terminal HA-tag	A. Gschwind
pcDNA3-Δ(Pro-MP)-	cDNA of TACE lacking the	A. Gschwind

-hADAM17/TACE-HA	-hADAM17/TACE-HA prodomain and metalloprotease domain Δ(AA18-473) in pcDNA3; C-terminal HA-tag	
pLXSN-ESK-Δ(Pro-MP)--hADAM17/TACE-HA	cDNA of TACE lacking the prodomain and metalloprotease domain in pLXSN-ESK; C-terminal HA-tag	A. Gschwind

2.2 Methods in molecular biology

2.2.1 Plasmid preparation for analytical purpose

Small amounts of plasmid DNA were prepared as described previously (Lee and Rasheed 1990).

2.2.2 Plasmid preparation in preparative scale

For transfection experiments of mammalian cells DNA of high quality was prepared using Qiagen Maxi-Kits (Qiagen, Hilden) according to the manufacturer's recommendations.

2.2.3 Enzymatic manipulation of DNA

2.2.3.1 Digestion of DNA samples with restriction endonucleases

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction were adjusted to the specific application according to the manufacturer´s recommendations.

2.2.3.2 Dephosphorylation of 5'-termini with calf intestine alkaline phosphatase (CIAP)

Dephosphorylation of 5´-termini of vector DNA in order to prevent self-ligation of vector termini. CIP catalyzes the hydrolysis of 5´-phosphate residues from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. The dephosphorylated products possess 5´-hydroxyl termini.

For dephosphorylation 1-20 picomoles of DNA termini were dissolved in 44 µL deionized water, 5 µL 10x reaction buffer (500 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.5) and 1 µL CIP (1 U/µL). The reaction was incubated 30 min at 37°C and stopped by heating at 85°C for 15 minutes.

2.2.3.3 DNA insert ligation into vector DNA

T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA. T4 DNA Ligase thereby joins doublestranded DNA with cohesive or blunt termini.

In a total volume of 10 µL the digested, dephosphorylated and purified vector DNA (200 ng), the foreign DNA to be inserted, 1 µL 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7,5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 1 µL T4 DNA Ligase (2 U for sticky ends and 4 U for blunt ends) were mixed. The reaction was incubated at 15°C overnight. T4 DNA Ligase was inactivated by heating the reaction mixture at 65°C for 10 minutes. The resulting ligation reaction mixture was directly used for bacterial transformation.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25 kb DNA fragments. 0.6-2%, horizontal agarose gels with 1x TAE electrophoresis buffer were used for separation. The voltage was set typically to 1-10 V/cm of gel. Gels were stained by covering the gel in a dilute solution of ethidium bromide (0.5 µg/mL in water) and gently agitating for 30 min and destained by shaking in water for an additional 30 min.

2.2.5 Isolation of DNA fragments using low melting temperature agarose gels

Following preparative gel electrophoresis using low melting temperature agarose, the gel slice containing the band of interest was removed from the gel. This agarose slice was then melted and subjected to isolation using the QIAquick Gel Extraction Kit (Qiagen).

2.2.6 Introduction of plasmid DNA into E.coli cells

2.2.6.1 Preparation of competent E. coli bacteria

Competent cells were made according to the procedure described before (Chung and Miller 1988). For long-term storage competent cells were directly frozen at –70°C. Transformation frequency ranged between 10^6 and 10^7 colonies/µg DNA.

2.2.6.2 Transformation of competent E. coli bacteria

100 µL competent cells were added to 10 µL ligation mix and 20 µL 5x KCM (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) in 70 µL H_2O and incubated on ice for 20 min. Upon incubation at room temperature for 10 min 1 mL LB medium was added and incubated 45 min at 37°C with mild shaking to allow expression of the antibiotic resistance gene. Transformants were selected on appropriate plates.

2.2.7 Enzymatic amplification of DNA by polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA (Mullis and Faloona 1987). A multitude of applications have been developed including direct cloning from cDNA, *in vitro* mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents and analysis of allelic sequence variations. For long and accurate cDNA amplification LATaq ™ polymerase (TaKaRa) was used:

0.5 µL template cDNA
2 µL "sense" oligonucleotide, 10 pmol/µL
2 µL "antisense" oligonucleotide, 10 pmol/µL
5 µL 10x LA PCR buffer II (w/o $MgCl_2$)
5 µL $MgCl_2$, 25 mM
8 µL dNTP-Mix, 2.5 mM each
0.5 µL LA-Taq™ (5 U/µL)
ad 50 µL H_2O

PCR reactions were performed in a automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to the specific application:

first denaturation:	3 min	94°C
amplification 25-30 cycles:	1 min	94°C (denaturation)
	1 min	58°C (hybridization)
	1 min/ kb product	72°C (extension)
last extension:	7 min	72°C

10 µL from each reaction were electrophoresed on an agarose gel appropriate for the PCR product size expected. PCR products were subjected to isolation using the PCR purification kit (Qiagen).

2.2.8 DNA sequencing

DNA sequencing was performed according to the "Big Dye Terminator Cycle Sequencing Protocol" (ABI). The following mix was subjected to a sequencing-PCR run:

	0.5 µg	DNA of interest
	10 pmol	oligonucleotide
	4 µL	Terminator Ready Reaction Mix
	ad 20 µL	H_2O
25 cycles:	30 sec	94°C
	15 sec	45-60°C
	4 min	60°C

The sequencing products were purified by sodium acetate/ EtOH precipitation, dissolved in 20 µL template suppression reagent, denatured for 2 min at 90°C and analyzed on a 310-Genetic Analyzer (ABI Prism).

2.3 Methods in mammalian cell culture

2.3.1 General cell culture techniques

Kidney and bladder cancer cell lines were grown in a humidified 93% air, 7% CO_2 incubator (Heraeus, B5060 Ek/CO_2) at 37°C and routinely assayed for mykoplasma contamination using a bisbenzimide staining kit (Sigma). Before seeding cells were counted with a Coulter Counter (Coulter Electronics). Cells were cultured in the medium recommended by the manufacturer.

2.3.2 Transfection of cultured cell lines

2.3.2.1 Transfection of cells with calcium phosphate

HEK-293 cells in six-well dishes were transfected transiently at about 70% confluency with a total of 2 µg DNA by using a modified calcium phosphate precipitation method as described previously (Chen and Okayama 1987). In this protocol, a calcium phosphate-DNA complex is formed gradually in the medium during incubation with cells.
The transfection mix of DNA and $CaCl_2$ in water was prepared as follows:

Dish	**6-well**	**6 cm**	**10 cm**
Area	10 cm^2	21 cm^2	57 cm^2
Volume of medium	1 mL	2 mL	4 mL
DNA in H_2O_{bidest}	2 µg in 90 µL	5 µg in 180 µL	10 µg in 360 µL
2.5 M $CaCl_2$	10 µL	20 µL	40 µL
2 x BBS (pH 6.96)	100 µL	200 µL	400 µL
Total volume	200 µL	400 µL	800 µL

To initiate the precipitation reaction the adequate volume of 2x BBS was added and mixed by vortexing. The reaction was incubated for 10 min at room temperature before being added to each well. Plates were placed in a humidified container at 3% CO_2 overnight. One day following transfection, cells were serum-starved for 24 hours in standard cell culture medium without FCS. Transfection efficiency was determined by LacZ staining after transfection of a LacZ-containing expression plasmid. For transfection of Phoenix cells HBS was used instead of BBS.

2.3.2.2 RNA interference

Transfection of 21 nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO) for targeting endogenous genes was carried out using Lipofectamine (Invitrogen) and 4,2 µg of siRNA duplex per 6-well plate as previously described (Elbashir, Harborth et al. 2001). Transfected A498 cells were serum starved and assayed 4 days after transfection. Sequences of siRNAs used have been described before (Gschwind, Hart et al. 2003). Specific silencing of targeted genes was confirmed by western blot (TACE) and RT-PCR analysis (data not shown).

2.3.3 Retroviral gene transfer in cell lines

The pLXSN (Clontech, Palo Alto, CA) constructs encoding wildtype and dominant negative ADAMs lacking the pro- and metalloproteinase domain) have been described before (Gschwind, Hart et al. 2003). All protease constructs included a C-terminal HA tag, detectable with an anti-HA monoclonal antibody (Babco, Richmond, CA). The amphotropic packaging cell line Phoenix was transfected with pLXSN retroviral expression plasmids by the calcium phosphate/ chloroquine method as described previously (Kinsella and Nolan 1996). 24 h after transfection the viral supernatant was collected and used to infect subconfluent kidney and bladder cancer cells (5x 10^4 cells/6-well plate).
Retroviral supernatant was then replaced with fresh medium. 2d following infection, target protein expression was monitored by western blot. Polyclonal ACHN kidney cancer and TccSup bladder cancer cell lines stably expressing dominant-negative ADAMs were generated by growing retrovirally infected cells in medium containing G418 (1g/ml) for 2 weeks.

2.4 Protein analytical methods

2.4.1 Lysis of eucaryotic cells with Triton X100

Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated in the figure legends. Cells were washed with cold PBS and then lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin. Lysates were precleared by centrifugation at 13000 rpm for 10 min at 4°C.

2.4.2 Determination of protein concentration in cell lysates

The „Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturer´s recommendations.

2.4.3 Immunprecipitation and *in vitro* association with fusion proteins

An equal volume of HNTG buffer was added to the precleared cell lysates that had been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20 µL of protein A-Sepharose for 4 h at 4°C. Alternatively, lysates were subjected to *in vitro* associations with either 3 µg of GST-Grb2 (Daub, Wallasch et al. 1997) or 2 µg of GST as control pre-bound to 30 µL of gluthathione-agarose beads. Precipitates were washed three times with 0.5 mL of HNTG buffer, suspended in 2× SDS sample buffer, boiled for 3 min, and subjected to SDS-PAGE.

2.4.4 SDS-polyacrylamide-gelelectrophoresis

SDS-PAGE was conducted as described previously (Sambrook 1990). The following proteins were used as molecular weight standards:

Protein	**MW (kD)**	**Protein**	**MW (kD)**
Myosin	205.0	Ovalbumin	42.7
ß-Galaktosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

2.4.5 Transfer of proteins on nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade 1982) for 2 h at 0.8 mA/cm^2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer proteins were stained with Ponceau S (2 g/l in 2% TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

2.4.6 Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25% gelatin for at least 4 h. The membrane was then probed with primary antibody (typically overnight at 4°C). Antibodies were diluted 1:500 to 1:2000 in NET, 0.25% gelatin. The membrane was washed 3x 20 min in 1x NET, 0.25% gelatin, incubated for 1 h with secondary antibody and washed again as before. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films or a digital camera unit. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 h at 50°C. Stripped membranes were blocked and reprobed with different primary antibody to confirm equal protein loading.

2.5 Biochemical and cell biological assays

2.5.1 Stimulation of cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 48 h bladder and kidney cancer cells were treated with inhibitors and agonists as indicated in the figure legends, washed with cold PBS and then lysed for 10 min on ice

2.5.2 ERK1/2 and AKT/PKB phosphorylation

For determination of ERK1/2 and Akt phosphorylation, approximately 20 µg of whole cell lysate protein/lane was resolved by SDS-PAGE and immunoblotted using rabbit polyclonal phospho-specific ERK/MAPK antibody. Akt phosphorylation was detected by protein immunoblotting using rabbit polyclonal anti-phospho-Akt antibody. Quantitation of ERK1/2 was performed using the Luminescent Image Analyis System (Fuji). After quantitation of ERK1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-ERK1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

2.5.3 ERK/MAPK activity

Endogenous ERK2 was immunoprecipitated from lysates obtained from six-well dishes using 0.4 µg of anti-ERK2 antibody. Precipitates were washed three times with HNTG buffer, and washed once with kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 µM sodium orthovanadate). Kinase reactions were performed in 30 µL of kinase buffer supplemented with 0.5 mg/mL myelin basic protein, 50 µM ATP and 1 µCi of [γ-^{32}P]ATP for 10 min at room temperature. Reactions were stopped by addition of 30 µL of Laemmli buffer and subjected to gel electrophoresis on 15% gels. Labeled MBP was quantitated using a Phosphoimager (Fuji).

2.5.4 Flow cytometric analysis of cell surface proteins

Was performed as described before (Prenzel, Zwick et al. 1999). In brief, cells were seeded, grown for 20 h and in some cases retrovirally infected as indicated. Upon serum-starvation for 24 h cells were treated with inhibitors and growth factors as indicated. After collection, cells were stained with ectodomain-specific antibodies against HB-EGF, TGFα or AR for 45 min. After washing with PBS, cells were incubated with FITC-conjugated secondary antibodies for 15 min and washed again with PBS. Cells were analysed on a Becton Dickinson FACScalibur flow cytometer.

2.5.5 Incorporation of ^{3}H-thymidine into DNA

Kidney or bladder cancer cells were seeded into 12-well plates (60000 cells per well). Upon serum deprivation for 48 h, cells were subjected to preincubation with inhibitors before ligand treatment. After 18 h incubation, cells were pulse-labelled with ^{3}H thymidine (1 µCi/mL) for 4 h, and thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid-scintillation counting.

2.5.6 Distribution of cell cycle phases

Rat-1 fibroblasts and TccSup bladder cancer cells were seeded into 6-Well plates ($1x10^5$ cells per well). Upon serum deprivation for 18 h or 24 h cells were subjected to 20 min preincubation with DMSO, BB94 or AG1478 and treated with LPA, endothelin I, thrombin and EGF. Apoptosis was induced by adding doxorubicin (1 µg/mL) or anti-CD95/FasL (50 µg/mL). After the indicated time periods cells were collected and incubated in hypotonic buffer containing 0,1 % sodium acetate, 0,1 % Triton X-100 and 20 µg/mL propidiumiodide for 2 h at 4°C. Samples were analysed with a Becton Dickinson FACScalibur flow cytometer.

2.5.7 *In vitro* wound closure

The assay was performed as previously described (Fishman, Liu et al. 2001) with some modifications. Confluent monolayers of kidney and bladder cancer cells were wounded with a

uniform scratch, the medium was removed and cells were washed twice with PBS. Medium without FCS was added and cells were subjected to 20 min preincubation with either DMSO (control), 250 nM AG1478 or 10 µM batimastat before ligand treatment. Cells were permitted to migrate into the area of clearing for 24 h. Wound closure was monitored by visual examination using a Zeiss microscope.

2.5.8 Migration and invasion

Cell migration assays were performed using a modified Boyden chamber (Sieuwerts, Klijn et al. 1997). Serum free medium containing LPA as a chemoattractant was added to the lower well of a Boyden chamber. A polycarbonate filter (6.5 mm in diameter, 8 µm pore size) was placed over the lower well of the Boyden chamber and was secured with a gasket. $1x10^5$ cells in exponential growth were harvested and then preincubated with the inhibitor for 20 min and added to the upper well of the chamber in serum free medium. The chambers were incubated for 6 h in a humified 7% CO_2, 37°C incubator. Finally, the cells that had migrated to the lower surface of the membrane were stained with crystal violet and counted under the microscope.

Analysis of cell motility of clonal A498 kidney cancer cell lines stably expressing dominant-negative ADAM 17 or wildtype ADAM 17 was performed in 24-transwell dishes. Cells were permitted to migrate for 24h. Cells that had migrated to the lower surface were fixed with methanol and stained with crystal violet. The stained cells were solubilized in 10 % acetic acid, and the absorbance at 570 nm was measured in a micro-plate reader. Experiments done with several, individual clones showed similar results.

Cell invasion assays were also performed in modified Boyden chambers containing a polycarbonate filter coated with Matrigel on the upper surface (Sieuwerts, Klijn et al. 1997). As described above the chemoattractant was added to the lower well and $1x10^6$ cells were preincubated with the inhibitor and then added to the upper well. The chambers were incubated overnight. Finally cells were wiped from the upper surface with a cotton tip swab and the cells on the other side were stained and counted under the microscope.

2.6 Statistical analysis

Student's *t*-test was used to compare data between two groups. Values are expressed as mean ± standard deviation (s. d.) of at least triplicate samples. $P < 0.05$ was considered statistically significant.

3 Results

3.1 A variety of GPCR agonists stimulate EGFR tyrosine phosphorylation in kidney and bladder carcinoma cell lines

Signalling through G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) is involved in the regulation of essential cellular processes and its deregulation is associated with tumourigenesis *in vitro* and *in vivo*. Previously, it was demonstrated that the EGFR functions as an integral element of mitogenic GPCR signals in non-transformed cell lines such as Rat-1 fibroblasts (Daub, Weiss et al. 1996; Wetzker and Bohmer 2003). The GPCR-EGFR crosstalk was demonstrated to involve a triple membrane passing signalling (TMPS) mechanism in COS7, Rat-1 and PC3 cells (Prenzel, Zwick et al. 1999).

So far little is known about the function of EGFR transactivation in pathophysiological processes such as cancer. Therefore, the responsiveness of the EGFR to prominent GPCR agonists was evaluated in a variety of kidney and bladder carcinoma cell lines.

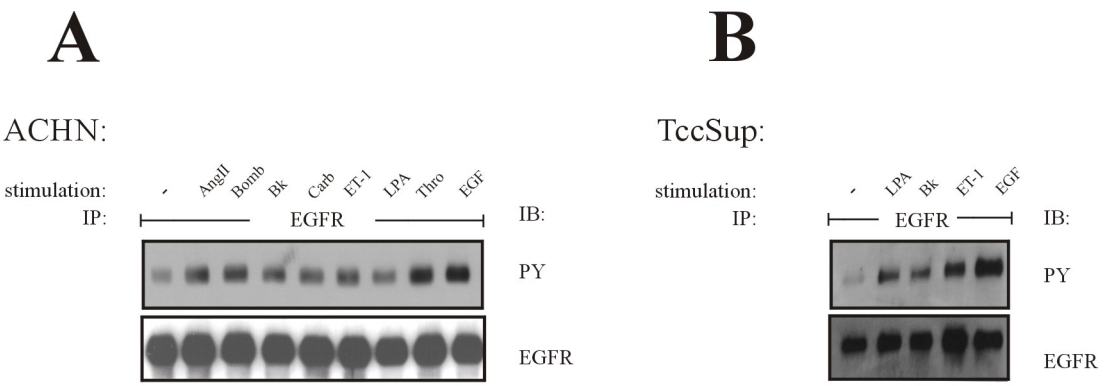


Figure 4. A variety of GPCR ligands stimulate the EGFR in kidney and bladder carcinoma cell lines

ACHN kidney cancer cells were starved for 48 h, TccSup bladder cancer cells for 24h and treated with various GPCR ligands (1 µM angiotensin II, 200 nM bombesin, 5 µM bradykinin, 1 mM carbachol, 100 nM ET-1, 10 µM LPA, 1U/ml thrombin and 3ng/µL EGF) for 3 min. After immunoprecipitation with antibodies against EGFR proteins were immunoblotted with anti-PY antibody. Reprobing against the EGFR ensured immunoprecipitation of equal protein amounts.

As shown in Figure 4, angiotensin II (AngII), bombesin (Bomb), bradykinin (Bk), LPA, endothelin-1 (ET-1), carbachol (Carb) and thrombin (Thr) rapidly induced EGFR activation in

ACHN kidney cancer cells. In addition, it was found that in TccSup bladder cancer cells LPA, bradykinin and ET-1 potently stimulated tyrosine phosphorylation of the EGFR.

Table 2 gives an overview of GPCR-EGFR cross talk pathways in a variety of other kidney and bladder cancer cell lines. The results indicated that stimulation with the GPCR agonists angiotensin II (1 µM), bombesin (200 nM), bradykinin (5 µM), carbachol (1 mM), endothelin-1 (100 nM), LPA (10 µM) and thrombin (1 U/mL) at physiological concentrations leads to rapid tyrosine phosphorylation of endogenous EGFR in the kidney cancer cell lines Caki2, ACHN, HK2, A498 and A704 as well as in the bladder cancer cell lines SCABER, HT1376, TccSup and 5637. Most importantly, all cell lines were responsive to at least three different GPCR ligands.

Cell line	tissue	AngII	Bomb	Bk	Carb	ET-1	LPA	Thr
CaKi2	kidney	-	+	-	+	-	+	+
ACHN	kidney	+	+	+	+	+	+	+
HK2	kidney	n.d.	+	+	+	+	+	+
A498	kidney	+	+	+	+	-	+	+
A704	kidney	n.d.	-	+	+	-	+	-
SCABER	bladder	-	-	+	-	+	+	-
HT1376	bladder	-	-	+	+	+	+	+
TccSup	bladder	-	-	+	-	+	+	-
5637	bladder	-	-	+	-	+	+	-

Table 2 ***Activation of the EGFR by GPCR agonists in bladder and kidney carcinoma cell lines.***
+ increased tyrosine phosphorylation of the EGFR monitored by western blot analysis; - no detectable influence; n.d. not determined

Furthermore, all cell lines tested showed EGFR transactivation by LPA and in all except one cell line (CaKi2) the EGFR was activated upon stimulation with bradykinin suggesting that LPA and bradykinin are potent and prominent EGFR activators in cell lines derived from urogenital cancers. Furthermore, carbachol and ET-1 stimulated EGFR tyrosine phosphorylation in all kidney cancer and bladder cancer cell lines, respectively. Together, these data demonstrate that a variety of GPCR ligands activate the EGFR in human urogenital tumour cell lines suggesting that the EGFR functions as a point of convergence for multiple, physiological relevant GPCR stimuli. The GPCR agonists bombesin, carbachol, thrombin, and LPA appear to be especially potent activators of the EGFR transactivation signal in

human kidney cancer cells, whereas bradykinin, ET-1, and LPA are the preferred GPCR stimuli leading to EGFR activation in bladder carcinoma cells.

3.2 EGFR transactivation by the GPCR agonists LPA and angiotensin II involves distinct EGF-like ligands and ADAM metalloproteinases in kidney and bladder carcinoma cell lines

Given that mitogenic EGFR transactivation pathways are broadly established in a variety of transformed (see 3.1.1) and non-transformed cell systems (Gschwind, Zwick et al. 2001) it was hypothesized that the cross-talk between GPCRs and the EGFR involves a metalloproteinase and EGF-like growth factor dependent mechanism in kidney and bladder cancer cells.

TccSup:

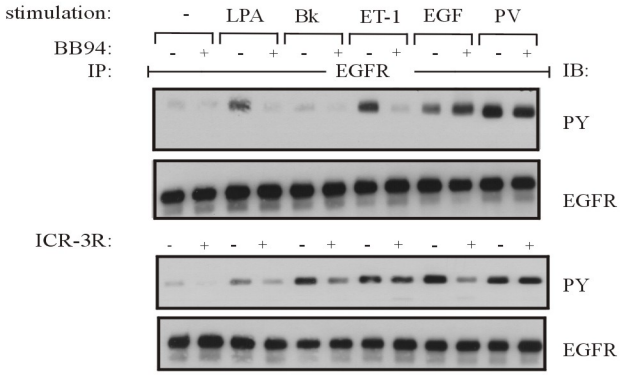


Figure 5. Transactivation of the EGFR in TccSup bladder cancer cells is dependent on a metalloproteinase and EGF-like ligands
Quiescent TccSup cells were pretreated with batimastat for 20 min or the monoclonal anti-EGFR antibody ICR-3R for 1 h. Afterwards cells were stimulated with angiotensin II, endothelin I, LPA, bradykinin, EGF or the tyrosine phosphatase inhibitor pervanadate for 3 min. Cell lysates were immunoprecipitated with anti-EGFR antibodies and proteins were immunoblotted with anti-PY antibody.

To test this hypothesis, the effect of the broad spectrum metalloproteinase inhibitor batimastat (BB94) on the EGFR transactivation signal was investigated. As shown in Figure 5, pre-incubation of cells with BB94 abrogated the EGFR transactivation signal in TccSup cancer cells whereas EGFR stimulation with the tyrosine phosphatase inhibitor pervanadate or with EGF was not affected. Moreover, pre-treatment of TccSup cancer cells with the monoclonal anti-EGFR antibody ICR-3R, which blocks binding of EGF-like ligands to the EGFR ecto-domain (Mateo, Moreno et al. 1997), specifically abolished GPCR- and EGF-induced tyrosine

phosphorylation of the EGFR (Figure 5). Together, these results indicated that both, a metalloproteinase activity and the extracellular ligand-binding domain of the EGFR are involved in EGFR activation by GPCR stimuli and suggested the GPCR-EGFR signal transmission to occur through an EGF-like ligand-dependent mechanism in TccSup bladder carcinoma cells.

Previous studies have demonstrated that autocrine growth factor stimulation is a common mechanism of RTK deregulation in human cancer cells (Zwick, Bange et al. 2002) often mediated through the ERK/MAPK pathway (Uchiyama-Tanaka, Matsubara et al. 2001). In consequence, autocrine EGFR has been demonstrated to contribute to sustained mitogenic activity of cancer cells (Murphy, Cluck et al. 2001).

To assess the potential involvement of autocrine processing of EGF-like ligands in basal EGFR activity in kidney and bladder cancer cells the effect of metalloprotease inhibition on basal EGFR tyrosine phosphorylation levels in HT1376 cells was investigated. In time course experiments it was found that BB94 treatment of cells that were grown in full medium containing 10% FCS resulted in a steady decrease of the EGFR tyrosine phosphorylation content reaching almost complete inhibition after 120 min (Figure 6).

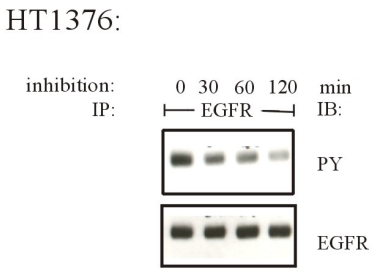


Figure 6. Treatment with batimastat decreases basal tyrosine phosphorylation of the EGFR
Unstarved HT1376 bladder cancer cells were incubated with BB94 for 30, 60 and 120 min. After immunoprecipitation against the EGFR proteins were immunoblotted with anti-PY antibody. Reprobing with EGFR antibodies ensures equal protein amounts.

This could be a hint for autocrine activation of the EGFR by shedding of precursors of EGF-like ligands via the TMPS pathway. The results of this experiment demonstrated that a metalloprotease activity mediates the high basal EGFR tyrosine phosphorylation levels in HT1376 cells presumably through cleavage of EGF-like growth factor precursors.

As demonstrated before (Fig. 5), GPCR-EGFR signal transmission occurs to require a metalloprotease activity in kidney and bladder carcinoma cells.

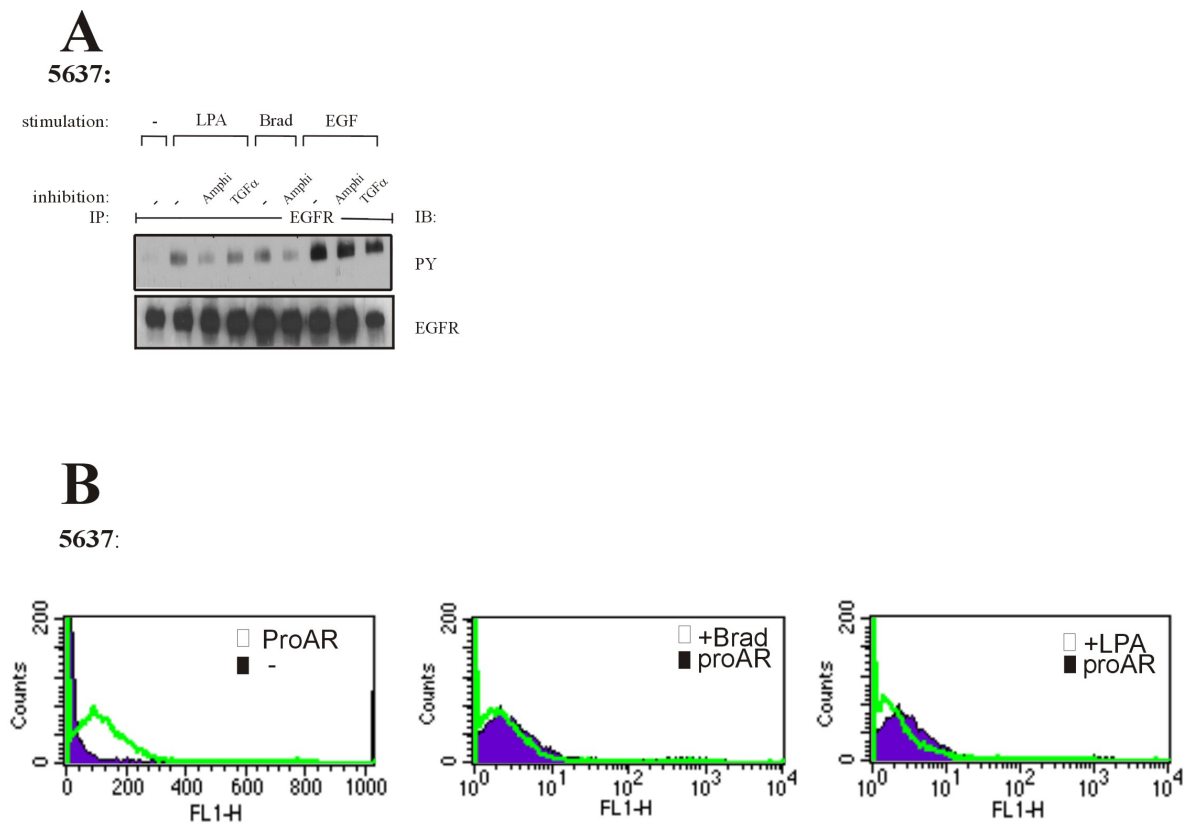


Figure 7. EGFR transactivation by the GPCR agonist LPA involves the EGF-like ligand amphiregulin in 5637 bladdercancer cells

a) Starved 5637 bladder carcinoma cells were preincubated with 20 µg/mL amphiregulin or 1 µg/mL TGFα blocking antibodies for one hour and afterwards stimulated with 10 µM LPA, 5 µM bradykinin or 3 ng/mL EGF for 3 min. The EGFR was immunoprecipitated and immunoblotted against anti-PY antibody.

b) Flow cytometric analysis of amphiregulin precursor expression. 5637 cells were collected and stained for surface AR and analysed by flow cytometry. Control cells were labelled with FITC-conjugated secondary antibody alone. Stimulation with LPA as well as bradykinin for 20 min induces a proteolytic release of AR.

To identify the EGF-like growth factors which are involved in the EGFR signal transactivation pathway different kidney and bladder carcinoma cells were pre-incubated with blocking antibodies against amphiregulin (20 µg/mL), HB-EGF (20 µg/mL) or TGFα (1µg/mL). Pre-incubation of 5637 cells with the amphiregulin (AR) neutralizing antibody markedly and specifically inhibited the LPA and bradykinin-induced transactivation signal whereas blocking of TGFα did not inhibit EGFR transactivation. To further substantiate these data the expression and cell surface localization of proAR in 5637 cells was determined by flow cytometry using ectodomain-specific antibodies. It was found that treatment of the cells with LPA and bradykinin resulted in rapid reduction in the cell surface content of endogenous proAR (Figure 7).

In summary, these findings demonstrate that EGFR activation in response to GPCR agonists involves amphiregulin in 5637 bladder cancer cells.

To identify EGF-like growth factors that are involved in the EGFR transactivation pathway in TccSup and ACHN cell lines cells were pre-incubated with blocking antibodies against amphiregulin (20 µg/mL), TGFα (1 µg/mL) or HB-EGF (20 µg/mL). Pre-incubation with amphiregulin or TGFα neutralizing antibodies attenuated the LPA-induced transactivation signal in TccSup cells whereas direct stimulation of the EGFR by EGF was not affected (Figure 8). In ACHN cells EGFR stimulation by angiotensin II was completely inhibited by the HB-EGF neutralizing antibody, pre-treatment with amphiregulin or TGFα blocking antibodies however showed no effect. These findings demonstrated that EGFR activation in response to GPCR agonists involves both TGFα and amphiregulin in TccSup bladder cancer and HB-EGF in ACHN kidney cancer cells.

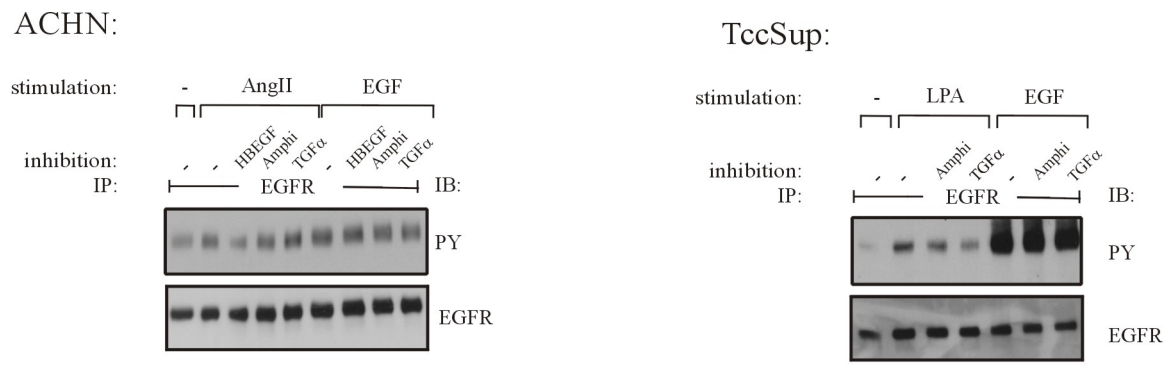


Figure 8. GPCR-induced EGFR transactivation is dependent on shedding of EGF-like ligands

Effect of neutralizing antibodies on the transactivation signal. Quiescent TccSup or ACHN cells were pre-incubated with 20 µg/mL amphiregulin, 20 µg/mL HB-EGF or 1 µg/mL TGFα blocking antibodies for 1 h and stimulated with LPA, angiotensin II or EGF for 3 min. The EGFR was immunoprecipitated and immunoblotted with anti-PY antibody.

To identify the metalloproteinases which are involved in angiotensin II as well as LPA-induced cleavage of EGF-like ligand precursors in the kidney cancer cell line ACHN and bladder cancer cell line TccSup the effect of dominant-negative mutants of ADAM 10, 12, 15 and 17 (ΔMP10, 12, 15, 17), which lack the pro- and metalloproteinase domain (Gschwind, Hart et al. 2003) on EGFR transactivation was investigated. As shown in Figure 9, the transactivation signal was blocked by ΔMP17 in ACHN cells upon stimulation with Ang II, and by ΔMP15 in TccSup cells upon stimulation with LPA.

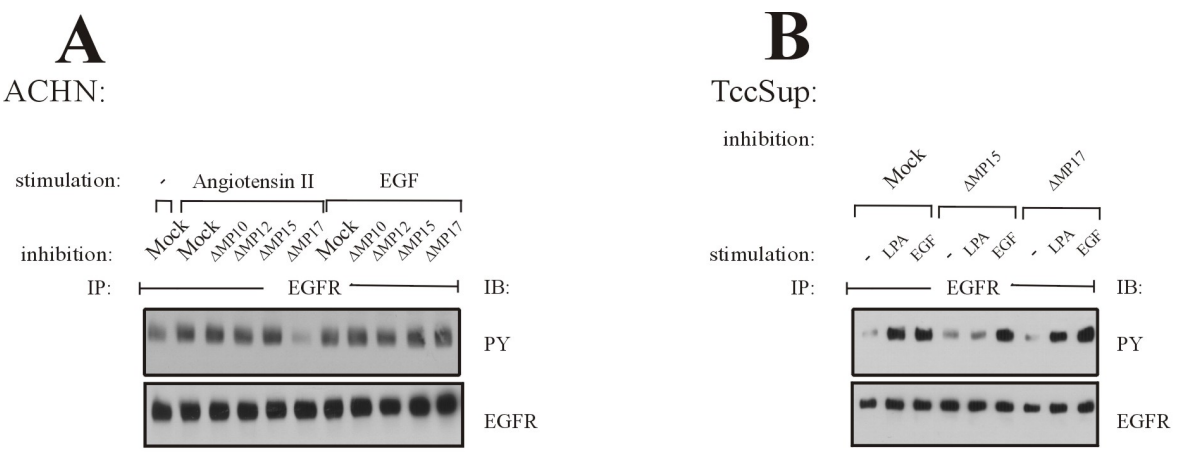


Figure 9. GPCR induced EGFR transactivation is dependent on ADAM 15 or ADAM 17 in ACHN kidney and TccSup bladder cancer cells

ACHN as well as TccSup cell lines were infected with pLXSN constructs of a dominant negative mutant of ADAM 10, 12, 15 and 17. Starved cells were stimulated with LPA, Angiotensin II or EGF for 3 min and tyrosine-phosphorylated EGFR was detected by immunoblotting.

To further substantiate the requirement for ADAM 17 in GPCR-induced ectodomain cleavage of proHB-EGF, the effect of angiotensin II on the cell surface content of endogenous proHB-EGF was investigated by flow cytometry (Figure 10).

Ectodomain shedding of proHB-EGF in cells infected with pLXSN was compared to those stably expressing the dominant negative mutant of ADAM 15 and ADAM 17. Treatment of the cells with angiotensin II resulted in rapid reduction of the cell surface content of endogenous proHB-EGF. In contrast, angiotensin II-induced proHB-EGF shedding was markly inhibited in ACHN cells stably expressing dominant negative ADAM 17 (ΔMP17). ΔMP15 however showed no effect.

Together, these findings demonstrate a role of ADAM 17 in angiotensin II-triggered cleavage of proHB-EGF and EGFR transactivation as well as identify ADAM 15 as a critical sheddase in LPA-induced EGFR activation in TccSup bladder carcinoma cells.

ACHN:

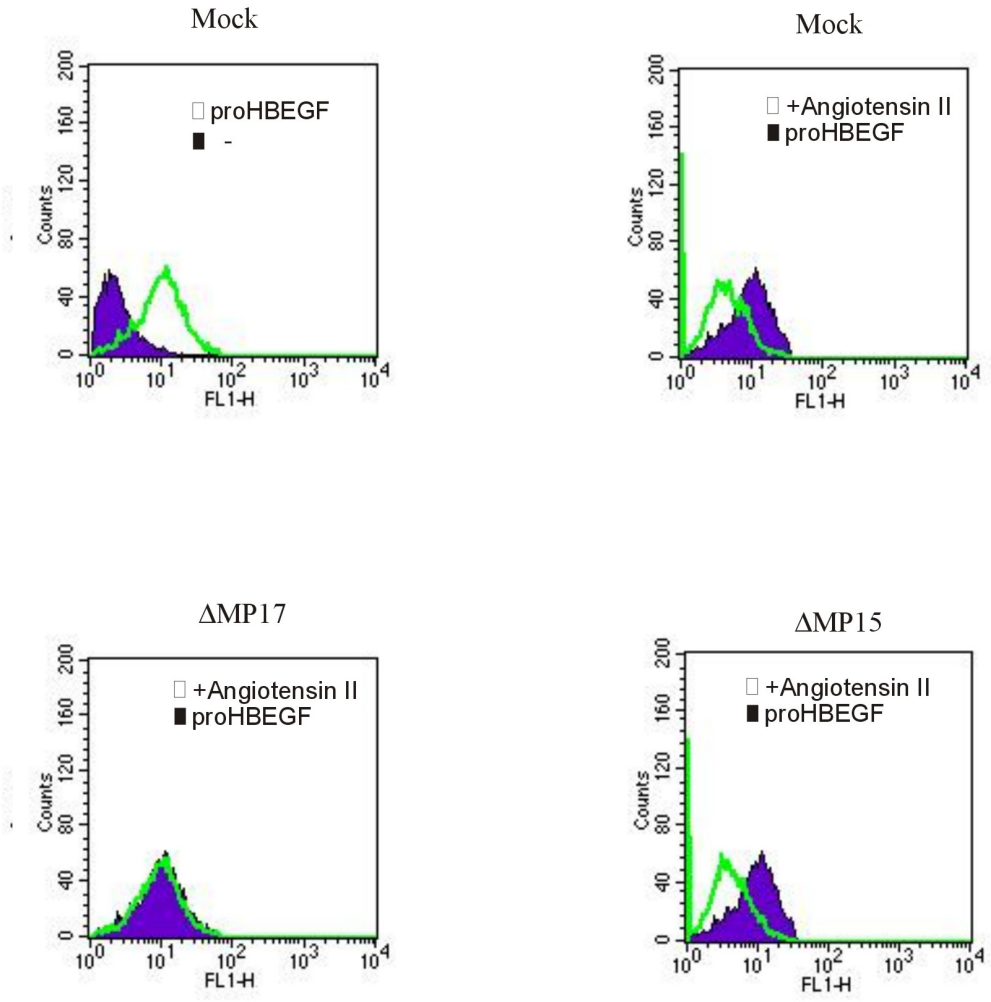


Figure 10. Expression of a ADAM 17 mutant partly inhibits HBEGF processing upon AngII stimulation

Flow cytometric analysis of HBEGF precursor expression. ACHN cells stably infected with pLXSN as well as pLXSN constructs of dominant negative mutants of ADAM 17 and ADAM 15 were collected and stained for surface HBEGF and analysed by flow cytometry. Control cells were labelled with FITC-conjugated secondary antibody alone. Stimulation with angiotensin II for 20 min induces a proteolytic release of HBEGF in Mock cells as well as in cells containing the ADAM15 mutant. Pro HBEGF shedding is reduced in cells expressing the ADAM 17 mutant.

As shown above (Figure 9, 10), the specific requirement of metalloproteinases for EGFR transactivation was demonstrated by generating cell lines expressing dominant negative mutants of ADAMs in bladder and kidney cancer lines.

To verify the data a small interfering RNA (siRNA) was utilized to inhibit endogenous gene expression of individual ADAMs. The specific requirement of ADAM17/TACE for the EGFR transactivation pathway in A498 kidney cancer cells was investigated by blocking the endogenous expression of TACE and ADAM10 by RNA interference.

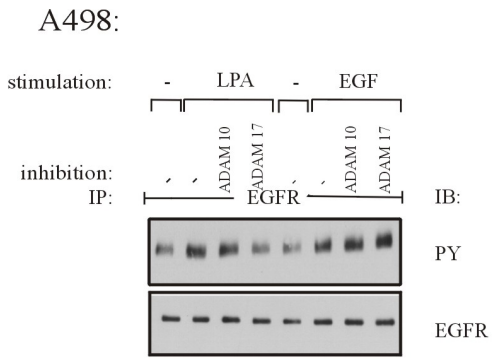


Figure 11. EGFR transactivation by LPA in A498 cells can be blocked by TACE siRNA
TACE siRNA inhibits EGFR signal transmission by GPCR agonists in A498 kidney cancer cells. A498 cells were transfected with ADAM 10 and ADAM 17 siRNA and stimulated with LPA or EGF. Subsequently activation of the EGFR was determined. TACE siRNA blocks endogenous TACE expression. A498 cells were transfected with ADAM 12 or TACE siRNA. Gene expression was analysed by immunoblot with polyclonal anti-TACE antibody.

In fact, gene silencing of TACE by siRNA specifically attenuated the LPA-induced transactivation signal in A498 cancer cells, whereas inhibition of ADAM 10 expression by siRNA showed no effect (Figure 11). These results confirmed the specific requirement of TACE for LPA-triggered EGFR transactivation in A498 kidney cancer cells.

Table 3 gives a systematic overview of the signalling components involved in the LPA-triggered TMPS pathways in kidney and bladder cancer cells. In particular, HB-EGF is specifically involved in transactivation of the EGFR in the kidney carcinoma cell lines Caki2, ACHN and A498 while TGFα and amphiregulin are required for the transactivation signal by LPA in TccSup and 5637 bladder carcinoma cells, respectively. These findings demonstrate that EGFR activation in response to the GPCR agonist LPA involves different EGFR ligands in 5637, ACHN, Caki2, A498 and TccSup cancer cells.

Cell line	tissue	GPCR ligand	metalloproteinase	EGF-like ligand
CaKi2	kidney	LPA	ADAM 17	HB-EGF
ACHN	kidney	LPA	ADAM 10	HB-EGF
A498	kidney	LPA	ADAM 17	HB-EGF
TccSup	bladder	LPA	ADAM 15	TGFα
5637	bladder	LPA	ADAM 15	Amphiregulin

Table 3 ***EGFR transactivation by LPA leads to processing of precursors of different EGF-like ligands by different metalloproteinases of the ADAM family***

Furthermore, the transactivation signal was blocked by ΔMP17 in Caki2 and A498 kidney cancer cells, by ΔMP10 in ACHN kidney cancer cells and by ΔMP15 in TccSup and 5637 bladder cancer cells.

In summary, the results demonstrate that in these cancer indications the metalloproteinases ADAM 10, 15 and 17 are required for cleavage and release of the transmembrane EGF-like ligand precursors pro-amphiregulin, proHB-EGF and proTGFα depending on the cellular system. All kidney cancer cell lines have proHB-EGF as the EGF-like ligand in common which is processed by ADAM 17 in Caki2 and A498 cells and by ADAM 10 in ACHN cells. In bladder cancer cells ADAM 15 is involved which cleaves proTGFα in TccSup and pro-amphiregulin in 5637 cells.

3.3 Transactivation of Her2 is dependent on a metalloproteinase function and EGFR tyrosine kinase activity

The EGFR and the oncoprotein HER2/neu regulate cell proliferation and in addition the responsiveness of a variety of cell types to pro-apoptotic stimuli such as serum-deprivation, death receptor activation and cytotoxic drugs. Previously, HER2/neu has been reported to be transactivated by GPCRs in Rat-1 fibroblasts (Daub, Weiss et al. 1996) and to be expressed at high levels in aggressive bladder tumours (Miyamoto, Kubota et al. 2000). Therefore, the question was raised if besides the EGFR, HER2/neu can be activated in response to LPA in TccSup bladder cancer cells.

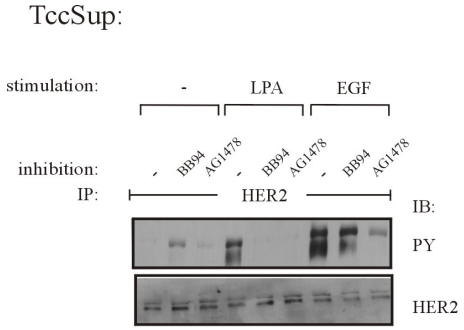


Figure 12. Stimulation of TccSup bladder cancer cells with LPA leads to EGFR- and metalloproteinase dependent activation of HER2
TccSup bladder cancer cells were starved for 24 h and preincubated with BB94 and AG1478 for 20 min. After immunoprecipitation with antibodies against HER2 proteins were immunoblotted with anti-PY antibody.

Figure 12 demonstrates that LPA stimulation resulted in tyrosine phosphorylation of HER2/neu in TccSup bladder carcinoma cells and that HER2/neu transactivation was sensitive to batimastat. In addition, tyrosine phosphorylation of HER2/neu following LPA or EGF treatment was abolished by the EGFR inhibitor AG1478 suggesting that HER2/neu is trans-phosphorylated by the EGFR.

Together, a metalloproteinase as well as the EGFR activity are critical for LPA-induced HER2/neu signal transactivation in TccSup bladder cancer cells.

3.4 EGFR association and tyrosine phosphorylation of Shc and Gab1 upon treatment with GPCR ligands are metalloproteinase dependent

A critical step in the transduction of the mitogenic signal from GPCRs to the EGFR and finally to the Ras/MAPK pathway is the association and tyrosine phosphorylation of adapter proteins such as Shc and Gab1 (Daub, Wallasch et al. 1997).

As shown in Figure 13, stimulation of bladder carcinoma cells with LPA, Bradykinin and EGF resulted in tyrosine phosphorylation of Shc. Furthermore, in co-immunoprecipitation experiments a tyrosine phosphorylated band of 170kDa was identified as the EGFR. Pre-treatment of the cells with batimastat (10 µM) or AG1478 (25 nM) inhibited the LPA-triggered tyrosine phosphorylation of Shc (Figure 13 a). In the bladder carcinoma cell line TccSup LPA as well as endothelin-1 activated the adaptor protein Gab1. Gab1 phosphorylation was diminished by addition of BB94 or AG1478 (Figure13 b).

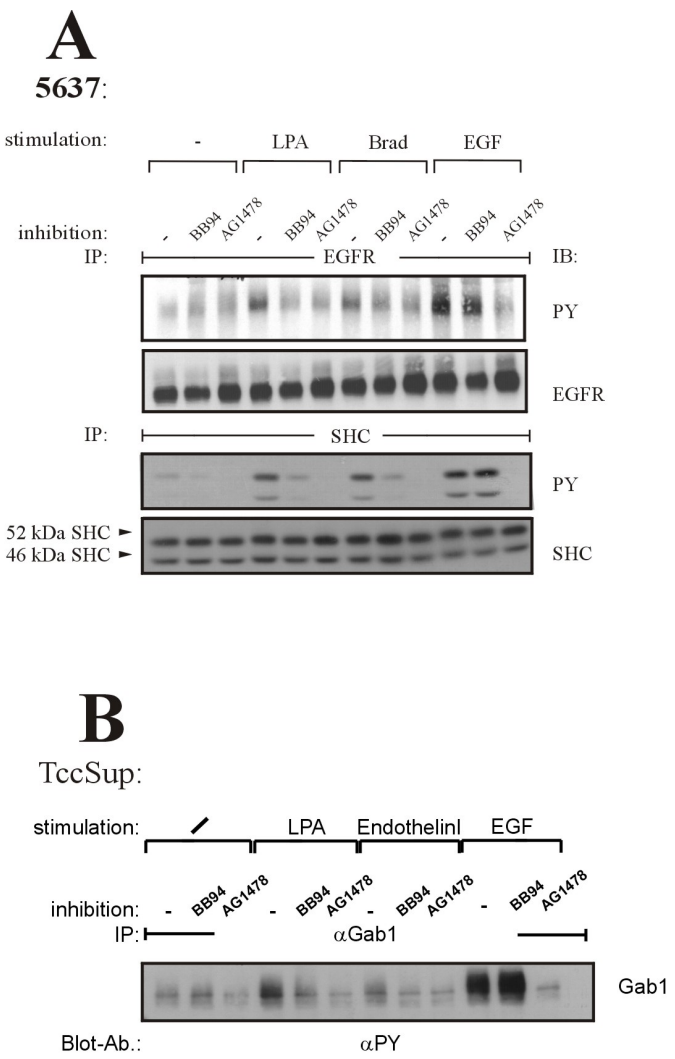


Figure 13. GPCR stimulation induces tyrosine phosphorylation of Shc and Gab1 which involves the EGFR and a metalloproteinase

a)Quiescent 5637 bladder carcinoma cells were preincubated with 250 nM AG1478 or 10 µM BB94 as indicated and treated with 10 µM LPA, 5 µM bradykinin or 3 ng/mL EGF for 3 min. After immunoprecipitation with antibodies against EGFR or Shc proteins were immunoblotted with anti-PY antibody. Reprobing against the EGFR or Shc ensured precipitation of equal protein amounts.

b)Starved TccSup bladder cancer cells were treated with AG1478 and BB94 and stimulated with LPA, ET-1 and EGF for 3 min. Lysate of the cells was immunoprecipitated with Gab1 antibody and immunoblotted with anti-PY antibody.

These data suggested that stimulation of TccSup and 5637 bladder cancer cells with GPCR agonists leads to membrane recruitment and activation of adapter proteins such as Shc and Gab1 by the EGFR, signalling events that require metalloproteinase activity.

3.5 GPCR-mediated activation of MAPK, Akt/PKB and cyclin D1 expression are batimastat and AG1478- sensitive

Since recruitment of adapter proteins such as Shc and Gab1 is an essential step linking cell surface receptor stimulation to activation of the Ras/MAPK pathway, the effects of GPCR agonists on activation of the MAPKs ERK1/2, JNK and p38 was investigated.

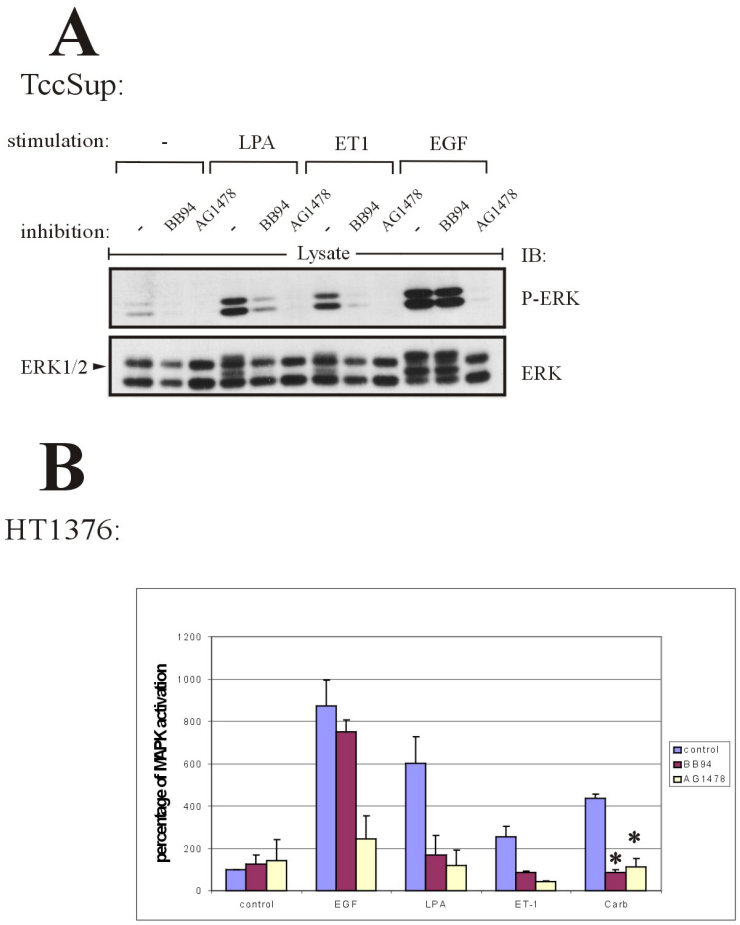


Figure 14. GPCR-mediated ERK activation is BB94 and AG1478-sensitive
a) Starved TccSup bladder carcinoma cells were pretreated with DMSO as vehicle, BB94 or AG1478 and then stimulated with 10 µM LPA, 100 nM ET-1 or 3 ng/mL EGF for 7 min. ERK activation was determined by immunoblotting with anti-phospho ERK antibody.
b) Quiescent HT1376 bladder cancer cells were preincubated with vehicle, BB94 or AG1478 and treated with 3 ng/mL EGF, 10 µM LPA, 100 nM ET-1 or 1 mM carbachol for 7 min. Cells were lysed and ERK activity was determined using MBP as a substrate. Phosphorylated MBP was visualized by autoradiography after gel electrophoresis. The filters were immunoblotted in parallel using anti-ERK2 antibody to verify immunoprecipitation of equal amounts of ERK2.
∗ p value< 0,002

MAPK activation was monitored by immunoblotting cell lysates with activation state-specific antibodies. It was found that ERK1/2 were activated upon stimulation with the GPCR ligands LPA and ET-1 in TccSup cells and that pre-incubation with the metalloproteinase inhibitor batimastat or AG1478 specifically diminished GPCR-induced MAPK activation (Figure 14 a).

To further quantify GPCR-triggered ERK stimulation endogenous ERK2 activity was measured in an *in vitro* kinase assay using myelin basic protein (MBP) as a substrate. Treatment of HT1376 cells with LPA lead to a six-fold, with ET-1 to a two-fold and with carbachol to a four-fold increase in ERK2 activity (Figure 14 b).

Moreover, the results demonstrated that both, BB94 and AG1478 almost completely blocked ERK activation by GPCR stimuli.

Next, the effect of GPCR ligands on activation of the stress-responsive MAPKs JNK and p38 was assessed in TccSup cells. To evaluate the kinetics of stress kinase activation time-course experiments were performed (Figure 15). JNK and p38 activation was detectable 5 min after addition of LPA and reached its maximum after 10-15 min.

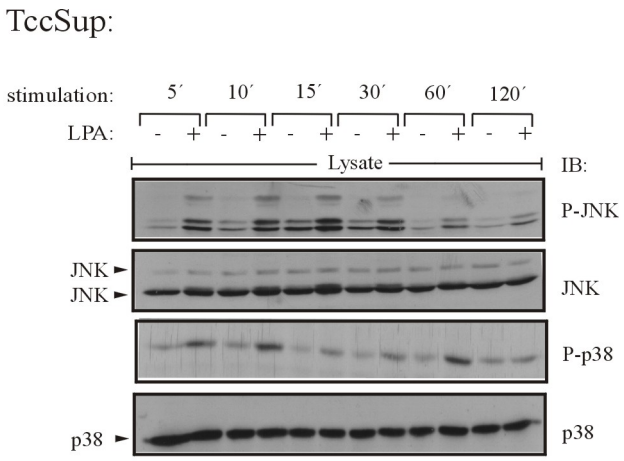


Figure 15. Kinetics of JNK and p38 activation
To evaluate the kinetics of JNK and p38 MAPK activation in TccSup bladder carcinoma cells serum starved cells were treated with LPA for the indicated periods of time. Crude lysate was immunoblotted with phospho-specific JNK or p38 antibody. To ensure loading of equal amounts of protein the membrane was reprobed with anti-JNK or anti-p38 antibody.

To determine if a metalloproteinase as well as the EGFR are involved in activation of the stress kinases TccSup bladder cancer cells were treated with BB94 and AG1478 (Figure 16). Immunoblotting of cell lysates with phospho-specific JNK and p38 antibodies revealed that phosphorylation of these MAPKs by LPA was also batimastat- and AG1478-sensitive

whereas the effects evoked by EGF were metalloproteinase-independent as expected. Additionally, signals were quantified using a Fuji LAS 1000 CCD camera and the "Image Gauge" program.

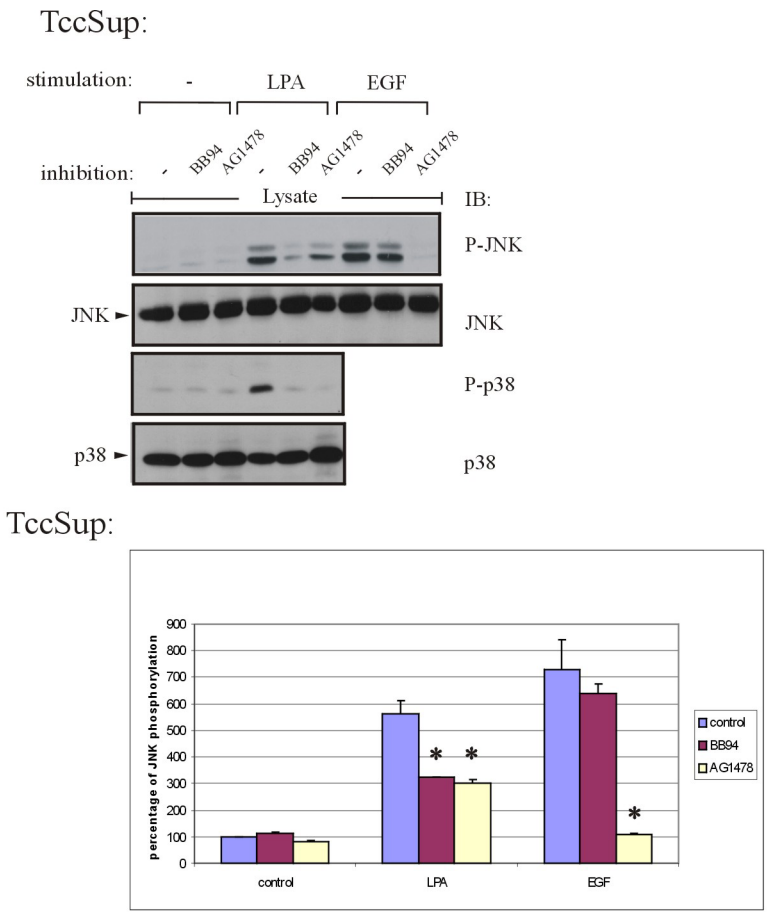


Figure 16. GPCR-mediated JNK and p38 activation is BB94 and AG1478-sensitive

Starved TccSup bladder carcinoma cells were preincubated with vehicle, BB94 or AG1478 and then stimulated with 10 µM LPA and 3 ng/mL EGF for 15 min. JNK activation was determined by immunoblotting with anti-phospho JNK antibody.
Quiescent TccSup bladder carcinoma cells were pretreated with DMSO as control, BB94 or AG1478 followed by stimulation with 10 µM LPA for 15 min. Activation of MAPK was analysed by immunoblotting with anti-phospho p38 antibody.
∗ p value < 0,02

Together, the data showed that metalloproteinases and the EGFR are critically involved in the regulation of the MAPK signal by GPCR ligands in kidney and bladder tumour cells.

It is well established that the EGFR and its agonists play a critical role in initiating signalling events that control cell cycle progression, and mitogenesis (Lui and Grandis 2002).

Recently, EGFR activity was shown to be required for GPCR-induced cell cycle progression by promoting accumulation of cyclin D1 in mid-late G1 phase in non-transformed cells (Santiskulvong, Sinnett-Smith et al. 2001). Therefore, it was interesting to investigate whether EGFR transactivation is involved in GPCR-induced cyclin D1 expression. Furthermore, the potential role of metalloproteinases was evaluated by treatment with batimastat (Figure 16).

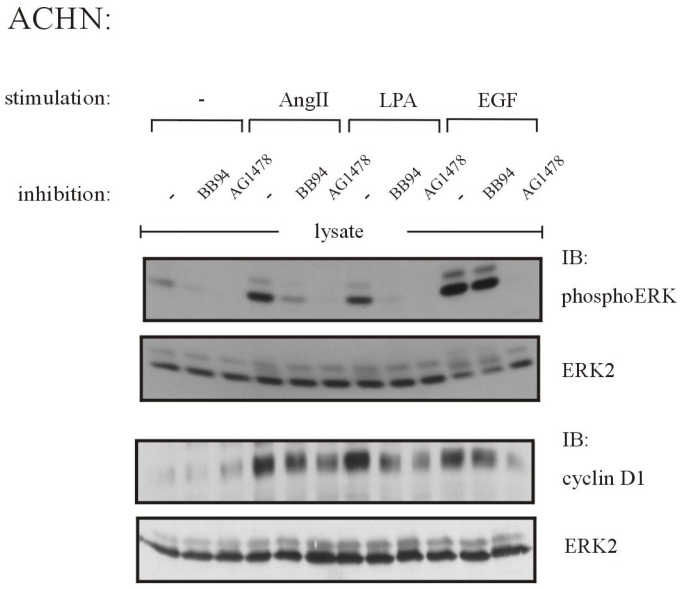


Figure 17. EGFR transactivation byAngII and LPA leads to an increased cyclin D1 expression

Starved ACHN kidney carcinoma cells were preincubated with DMSO, BB94 or AG1478 and then stimulated with angiotensin II, LPA and EGF for 7 min or for 8 h, respectively crude lysates were immunoblotted with phospho-specific ERK antibody or cyclin D1 antibody.

The results demonstrated that stimulation with angiotensin II and LPA leads to accumulation of cyclin D1 in ACHN kidney cancer cells which is sensitive to BB94 and AG1478.

Downstream of the EGFR and HER2/neu, PI3-K and Akt/PKB are critical elements of survival pathways activated by growth factors, cytokines and integrins (Madrid, Wang et al. 2000). In analogy to MAPK signalling the PI3-K/Akt pathway regulates fundamental cellular responses such as proliferation, apoptosis, cell motility and adhesion. Furthermore, in T cells it was shown that PI3-K also links GPCR stimulation to Akt activation (Sasaki, Irie-Sasaki et al. 2000). Therefore, the possible involvement of EGFR transactivation pathways in GPCR-induced activation of the cell survival regulator Akt was investigated.

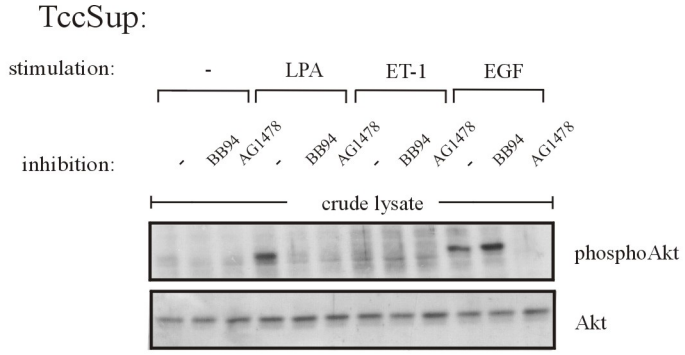


Figure 18. Stimulation with GPCR ligands leads to Akt activation

Following preincubation with DMSO as control, BB94 or AG1478 for 20 min starved TccSup bladder cancer cells were stimulated with LPA, endothelin I or EGF. Akt activation was determined by immunoblotting with anti-phospho Akt antibody. Reblotting against Akt confirmed loading of equal protein amounts.

Akt/PKB activity was determined by immunoblotting TccSup cell lysates with phospho-specific antibodies (Figure 18). Indeed, Akt/PKB was strongly activated upon stimulation with the GPCR ligand LPA in an EGFR- and metalloproteinase-dependent manner in TccSup bladder cancer cells whereas stimulation with endothelin-1 showed no effect on Akt phosphorylation.

3.6 MAPK activation by LPA is dependent on src-kinase whereas Akt/PKB activation by LPA is dependent on src-kinase as well as PI3-kinase

In non-transformed cells it was reported previously that inhibition of phosphoinositide 3-kinase and src-kinase affects LPA-induced signalling downstream of the EGFR (Daub, Wallasch et al. 1997). Furthermore, in rat adrenal pheochromocytome PC12 cells it was demonstrated that ERK phosphorylation induced by LPA is mediated by an EGFR and PKC-dependent mechanism (Kim, Park et al. 2000). Moreover, src-kinase was shown to be responsible for metastatic spread of bladder carcinoma cells (Boyer, Bourgeois et al. 2002) and inhibition of PKC was shown to block invasion in bladder cancer cell lines (Schwartz, Redwood et al. 1990).

It was therefore conceivable, that the three kinases src, PKC and PI3-K play a role in LPA-induced EGFR transactivation in human bladder carcinoma cells. In the cell line TccSup it was shown that inhibition of PKC with GF-109203X does not affect EGFR tyrosine

phosphorylation or activation of downstream signalling proteins induced by stimulation with LPA or EGF. Akt activation by treatment with LPA and EGF was blocked by pre-incubation with the PI3-K inhibitor Wortmannin as well as the src-kinase inhibitor PP1. Activation of the MAPK ERK1/2 in response to LPA and EGF was only diminished after treatment with PP1, treatment with Wortmannin however showed no effect. In addition, EGFR tyrosine-phosphorylation triggered by LPA was partly inhibited by Wortmannin and PP1. EGFR activation after direct stimulation with EGF was not influenced, as expected (Figure 19).

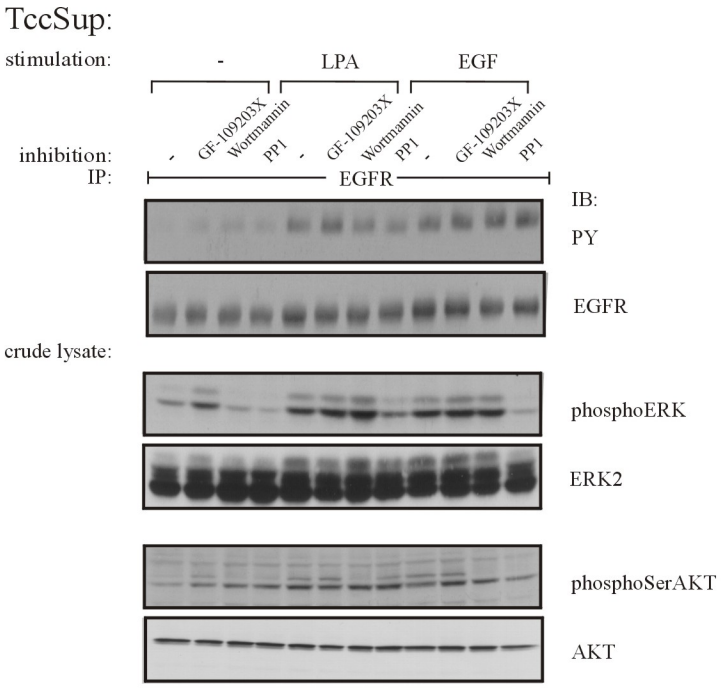


Figure 19. GPCR stimulation induces activation of the EGFR and downstream signalling molecules which is sensitive to BB94, AG1478, PP1 and Wortmannin

Quiescent TccSup cells were preincubated with the PKC inhibitor GF-109203X (25 nM), the PI3Kinase inhibitor Wortmannin (100 nM) and the Src kinase inhibitor PP1 (10 µM) for 20 min. Cells were then stimulated with LPA or EGF for 5 min. Cell lysates were either immunoprecipitated with anti-EGFR antibodies and immunoblotted against PY or were immunoblotted with phospho-specific ERK or AKT antibodies.

In summary, ERK/MAPK stimulation by LPA involves src-kinase signalling whereas Akt activation is dependent on both, src as well as PI3-K in the bladder cancer cell line TccSup. Furthermore, PKC seems to play no role in EGFR transactivation in this cell line.

3.7 GPCR ligands induce mitogenic signalling and cell cycle progression in Rat-1 fibroblasts via metalloproteinase-dependent EGFR transactivation

Expertise about molecular mechanisms of cellular signalling is essential for the understanding of biological and pathogenic processes in organisms. Signal transduction cascades involving the EGFR have a important function in the regulation of cellular proliferation, differentiation, motility and survival as well as development. Beyond the EGFR is expressed in a number of tumours and its expression correlates with tumour progression, resistance to chemotherapy and a poor prognosis (Yano, Kondo et al. 2003). In analogy GPCRs initiate the transmission of multiple cellular signals leading to a wide variety of physiological and pathophysiological effects.

Knowledge of the role of EGFR and GPCRs in normal tissues will promote the understanding of their task in pathogenesis.

It has previously been demonstrated in Rat-1 fibroblasts that GPCR signals lead to activation of the ERK/MAPK cascade and cellular proliferation through EGFR signal transactivation (Daub, Weiss et al. 1996; Santiskulvong, Sinnett-Smith et al. 2001). To assess if a metalloproteinase activity is involved Rat-1 fibroblasts were treated with the metalloproteinase inhibitor batimastat (BB94) prior to stimulation with GPCR ligands. The results showed that tyrosine phosphorylation of the EGFR, the downstream adapter protein SHC and the MAPK ERK1/2 in response to LPA, thrombin, bradykinin and ET-1 was abolished by BB94 as well as the specific EGFR inhibitor AG1478 (Figure 20a).

Next, the effect of these inhibitors on GPCR-induced cell cycle progression was investigated by flow cytometric analysis. In quiescent Rat-1 cells BB94 and AG1478 completely abolished S-phase progression in response to LPA, ET-1 or thrombin. S-phase entry by direct EGF stimulation, however, remained unaffected by BB94 treatment. For further quantification of mitogenic signalling in response to GPCR ligands, the rate of DNA synthesis was measured by an ^{3}H-thymidine incorporation assay. Both, BB94 and AG1478 completely inhibited DNA synthesis induced by various GPCR agonists (Figure 20b).

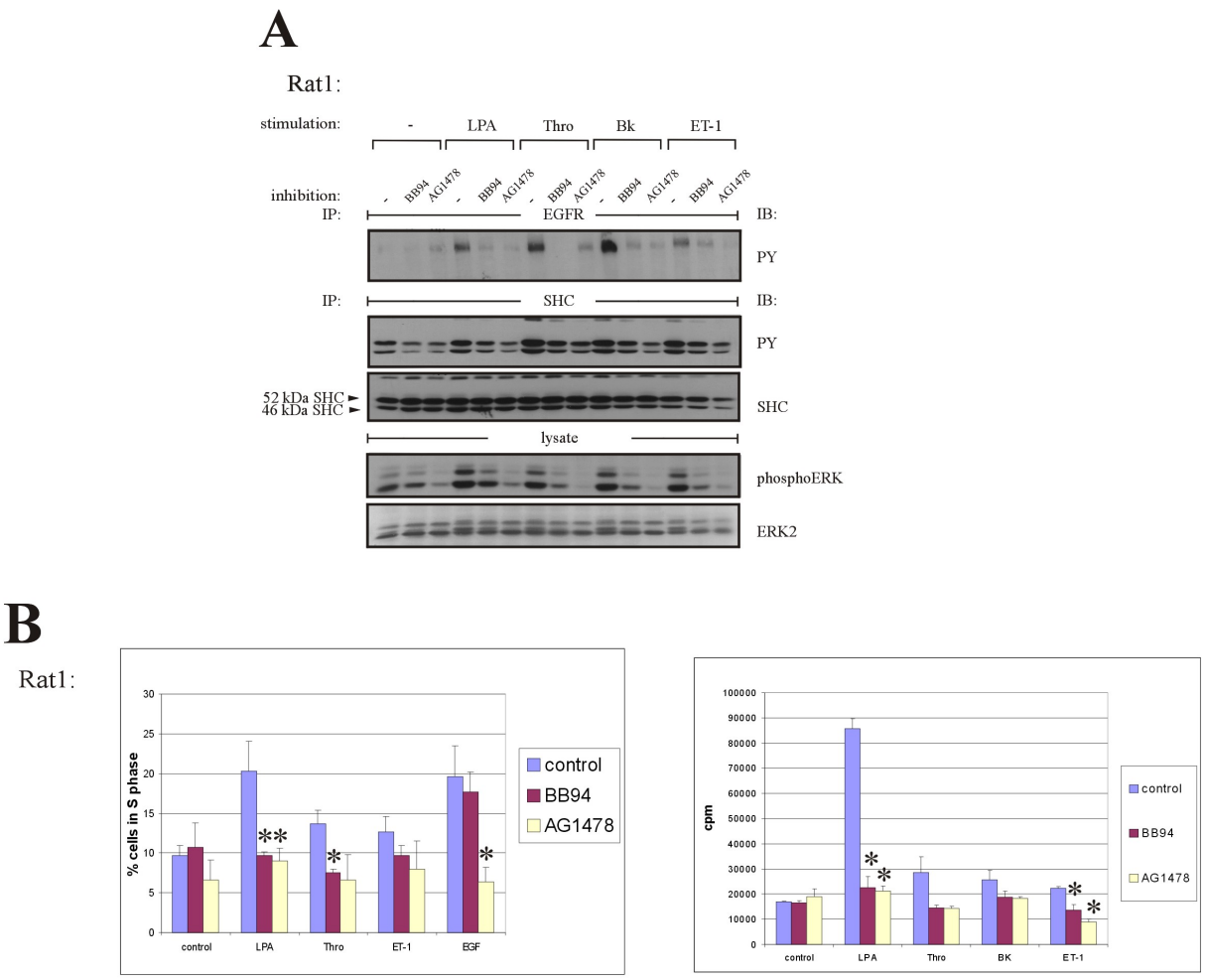


Figure 20. GPCR ligands induce mitogenic signalling and cell cycle progression in Rat1 fibroblasts via metalloproteinase-dependent EGFR transactivation

a) Rat1 cells were starved for 18 h. After inhibition with BB94 or AG1478 cells were treated with 10 µM LPA, 2U/mL thrombin, 5 µM bradykinin or 100 nM endothelin I for 3 min. After immunoprecipitation with antibodies against EGFR or SHC proteins were immunoblotted with anti-PY antibody. Reprobing of the same filters with EGFR or SHC antibodies ensured precipitation of equal protein amounts. To determine ERK activation cells were stimulated for 7 min with the indicated ligands and proteins were immunoblotted with anti-phospho ERK antibody and with anti-ERK2 antibody as a control.

b) To evaluate the effect of EGFR- and metalloproteinase-inhibition on S-phase progression quiescent Rat1 cells were preincubated with DMSO as vehicle, batimastat (5 µM) or AG1478 (250 nM) for 20 min and stimulated with endothelin I, thrombin or EGF for 24 h. Cells were harvested and analysed by flow cytometry. ∗ p value < 0,05
Cell proliferation was also determined by ^{3}H-thymidine incorporation into DNA. Starved Rat1 cells were preincubated with DMSO, BB94 or AG1478 for 20 min and stimulated with LPA, thrombin, bradykinin or endothelin I for 18 h. Cells were pulse-labelled with ^{3}H-thymidine and thymidine incorporation was measured by liquid-scintillation counting. ∗p value < 0,025

Together, these data indicated the critical involvement of a metalloproteinase activity in transactivation of the EGFR, downstream SHC adapter protein recruitment, activation of the

ERK/MAPK pathway as well as cell proliferation induced by the GPCR agonists LPA, thrombin, bradykinin and ET-1 in Rat-1 fibroblasts.

3.8 Angiotensin II induced EGFR transactivation and DNA synthesis in ACHN kidney cancer cells is dependent on the EGFR and ADAM 17

Cross communication of GPCR and EGFR has been implicated in the regulation of cell proliferation in non-transformed cells and abnormal cellular growth is an important hallmark of cancer. Both receptor types are crucial regulators of cell proliferation in normal as well as in cancer cells. A recent report by Schuttert et al. demonstrated that the GPCR ligand angiotensin II increases cell proliferation in human renal fibroblasts (Schuttert, Liu et al. 2003).

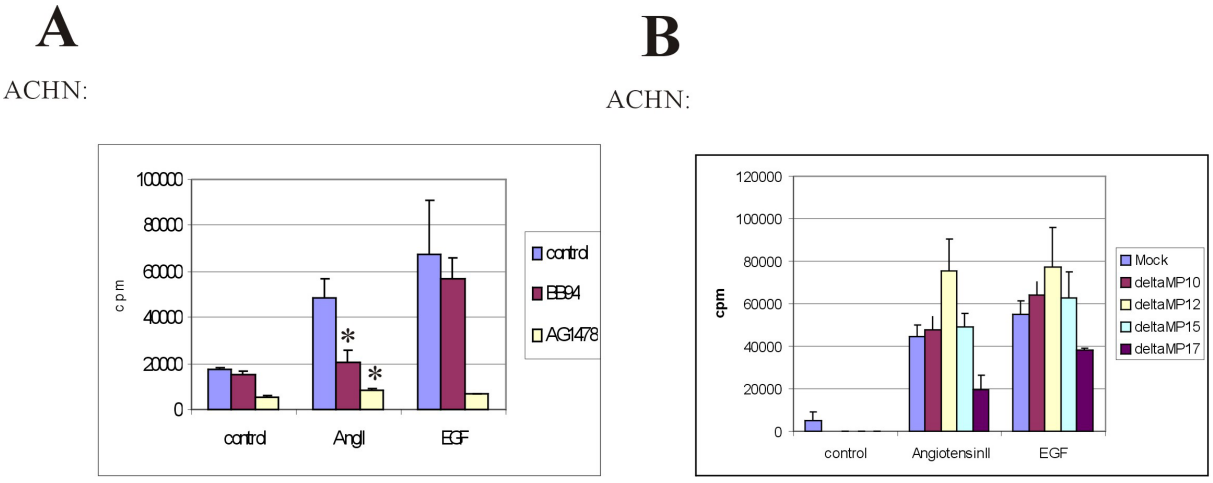


Figure 21. Batimastat, AG1478 and a dominant negative mutant of ADAM 17 inhibit GPCR-induced DNA synthesis in ACHN kidney cancer cells

a) The effect of EGFR transactivation on cell proliferation was determined by ^{3}H-thymidine incorporation into DNA. Starved ACHN cells were preincubated with DMSO, BB94 or AG1478 for 20 min and stimulated with angiotensin II and EGF for 18 h. Cells were pulse-labelled with ^{3}H-thymidine and thymidine incorporation was measured by liquid-scintillation counting. ∗ p value < 0,02

b) ACHN kidney cancer cells stably infected with pLXSN constructs of dominant negative mutants of ADAM 10, 12, 15 and 17 were starved for 48 h and stimulated with angiotensin II or EGF for 18 h. Incorporation of ^{3}H-thymidine into the cells was measured by liquid-scintillation counting.

Moreover, in the kidney cancer cell line ACHN it was shown that stimulation with angiotensin II leads to activation of the EGFR and the mitogenic Ras/MAPK pathway and further to accumulation of the cell cycle regulator cyclin D1 (Figure 17). Here, the effect of angiotensin II on proliferation of ACHN cells was investigated.

Cell cycle progression in response to the GPCR ligand angiotensin II was measured by a ^{3}H – thymidine incorporation assay. The results showed enhanced DNA synthesis in response to angiotensin II that was specifically blocked by the metalloproteinase inhibitor BB94 and AG1478 (Figure 21a).

To identify the metalloproteinases which are involved in angiotensin II-induced cell proliferation of ACHN kidney cancer cells, the effect of dominant negative mutants of ADAM 10, 12, 15 and 17 (ΔMP 10, 12, 15, 17) which lack the pro- and metalloproteinase domain on the incorporation of ^{3}H-thymidine was investigated. The results showed that dnTACE specifically inhibited angiotensin II induced cell cycle progression in ACHN kidney carcinoma cells (Figure 21b).

Together, these data demonstrated that stimulation with angiotensin II leads to a about four-fold increasing in thymidine incorporation which is diminished by blocking ADAM 17 or the EGFR.

3.9 LPA prevents apoptosis induced by serum-starvation or doxorubicin treatment in a BB94 and AG1478 -sensitive manner in Rat-1 fibroblasts

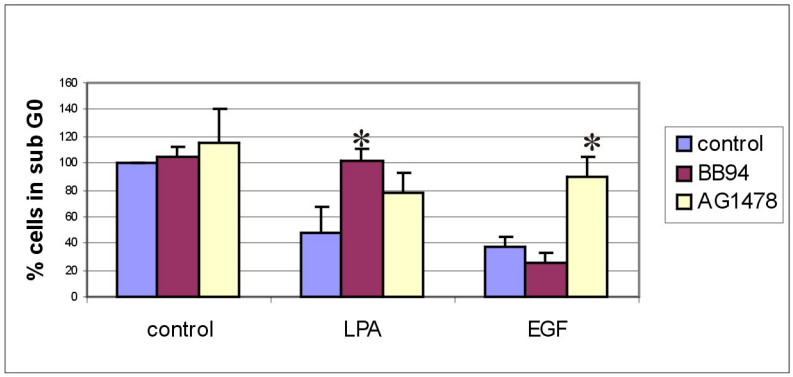
Besides deregulated cell proliferation, suppression of apoptosis is another critical characteristic of cancer cells (Evan and Vousden 2001). The EGFR and the oncoprotein HER2/neu have been demonstrated to regulate the responsiveness of a variety of cell types to pro-apoptotic stimuli such as serum-deprivation, death receptor activation and cytotoxic drugs. In Rat 1 fibroblasts EGFR transactivation by various GPCR ligands resulted in activation of the survival promoting proteins Akt and ERK1/2. Therefore, it was interesting to elucidate if EGFR transactivation has an anti-apoptotic effect in fibroblasts.

As shown in Figure 22a , apoptosis of Rat-1 cells induced by starvation was blocked in the presence of LPA or EGF. The anti-apoptotic effect of LPA was reversed by AG1478 or BB94, suggesting the critical involvement of metalloproteinases and the EGFR.

It was previously shown that activation of growth factor pathways enhance mechanisms of drug resistance in chemotherapy (Dickstein, Valverius et al. 1993). Therefore, the involvement of EGFR transactivation in survival of Rat-1 fibroblasts treated with doxorubicin, a prominent drug used in chemotherapy, was investigated. It was found that LPA prevented doxorubicin-induced apoptosis of Rat-1 fibroblasts in a batimastat and AG1478-sensitive fashion (Figure 22b).

A

Rat1:



B

Rat1:

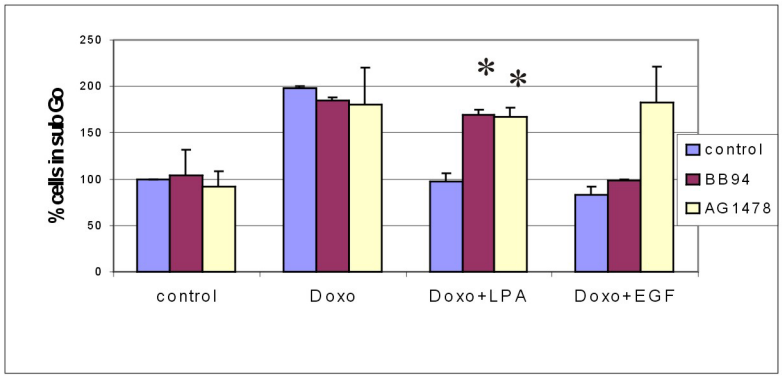


Figure 22. LPA prevents apoptosis in Rat1 cells induced by serum-starvation and doxorubicin in a BB94 and AG1478 -sensitive manner

a) To determine the effect of AG1478 and BB94 on apoptosis induced by serum deprivation quiescent Rat1 cells were preincubated with DMSO as vehicle, batimastat (5 µM) or AG1478 (250 nM) for 20 min. Next, the GPCR ligands LPA, endothelin I and thrombin or EGF were added to the medium for 48 h. Cells were harvested and analysed by flow cytometry. ∗ p value < 0,02

b) Effect of BB94, AG1478 and growth factors on doxorubicin-induced apoptosis. Rat1 cells were starved for 18 h, preincubated with vehicle, BB94 or AG1478 for 20 min, stimulated with LPA for 1 h and then treated with Doxorubicin (1µg/mL). After 12 h the percentage of apoptotic cells was determined by FACS analysis. ∗ p value < 0,002

In conclusion, the GPCR agonist LPA prevents apoptosis induced by serum starvation and the chemotherapeutic drug doxorubicin in Rat-1 fibroblasts through the EGFR and metalloproteinases .

3.10 LPA prevents apoptosis induced by anti-CD95/FasL in a BB94 and AG1478- sensitive manner in TccSup bladder carcinoma cells

Interestingly, a recent report provided evidence that EGF stimulation protects breast adeno-carcinoma cells from Fas-induced apoptosis by an Akt/PKB-mediated pathway (Gibson, Tu et al. 1999).

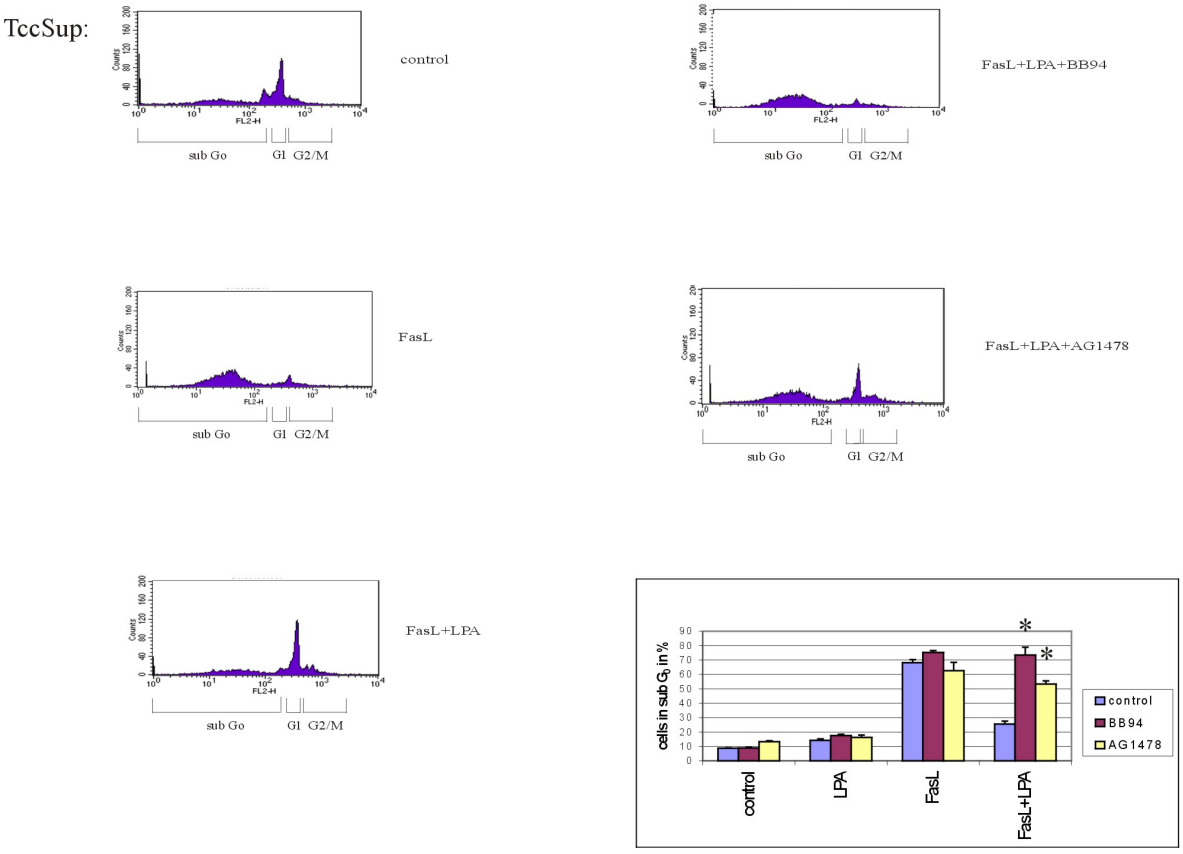


Figure 23. LPA prevents apoptosis in TccSup bladder cancer cells induced by anti-CD95/FasLigand in a BB94 and AG1478 sensitive manner
Quiescent TccSup bladder cancer cells were preincubated with vehicle, BB94 or AG1478, stimulated with LPA and then treated with anti-CD95/FasLigand (50µg/mL). After 24h the relative amount of apoptotic cells was determined by FACS analysis. ∗ p value < 0,004

In the bladder cancer cell line TccSup it was demonstrated above (Figure 18) that direct stimulation with EGF as well as EGFR transactivation by LPA leads to Akt activation**.** Hence, the involvement of EGFR signal transactivation in the regulation of survival of TccSup cells was investigated. To induce apoptosis in serum-starved TccSup bladder cancer cells a monoclonal anti-Fas CD95 antibody was used. The results of the experiment shown in Figure 23 revealed that LPA effectively prevented CD-95-induced apoptosis of TccSup cells in a BB94- and AG1478-sensitive manner.

In summary, these data showed that in addition to apoptosis induced by serum starvation and chemotherapeutic agents, also apoptosis triggered by anti-CD95/FasL can be reversed by incubation with LPA in an EGFR and metalloproteinase- dependent manner.

3.11 LPA promotes EGFR- and Metalloprotease-dependent cell motility

One of the hallmark characteristics of cancer cells is enhanced motility. An *in vitro* cell monolayer wound closure assay can serve as a model for cell migration. For EGF and EGF-like ligands a wound closure promoting function due to enhanced ERK/MAPK activation has been described previously (Draper, Komurasaki et al. 2003).

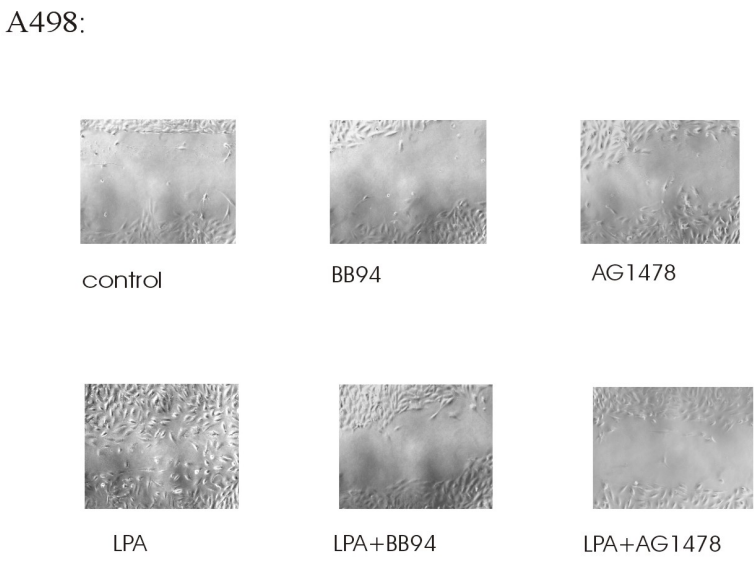


Figure 24. LPA promotes wound closure which can be inhibited by BB94 and AG1478
A498 kidney carcinoma cells show enhanced wound closure after stimulation with LPA. The effect can be blocked by treatment with BB94 or AG1478. Confluent monolayers were starved for 24h and scraped with a plastic tip. Wound closure was observed with a microscope 24h after stimulation.

For all tested kidney and bladder carcinoma cell lines (see table 4/Figure 14) it was demonstrated that stimulation with the GPCR agonist LPA or EGF leads to EGFR and ERK activation. Therefore, the effect of the GPCR ligand LPA on tumour migration in the presence or absence of specific EGFR inhibitors was investigated in an *in vitro* wounding assay. In A498 kidney cancer cells the results showed that LPA enhanced the rate of wound closure and incubation with the inhibitors BB94 and AG1478 abolished the effect.

In conclusion, these data demonstrated that LPA-induced wound closure of A498 cell monolayers involes both, EGFR function and metalloprotease activity.

3.12 LPA promotes cell migration which is inhibited by BB94, AG1478 and a dominant negative mutant of ADAM 17

To further verify these data, the effect of LPA on cell motility of urogenital cancer cell lines was evaluated in a quantitative Boyden Chamber assay that is generally accepted as a method for assessing *in vitro* tumour cell migration. It was found that LPA stimulated the rate of A498 cell migration more than five-fold. The effect was specifically blocked by pre-treatment of cells with BB94 or AG1478. These findings were further substantiated by analogous results obtained in Caki2, ACHN, TccSup cells.

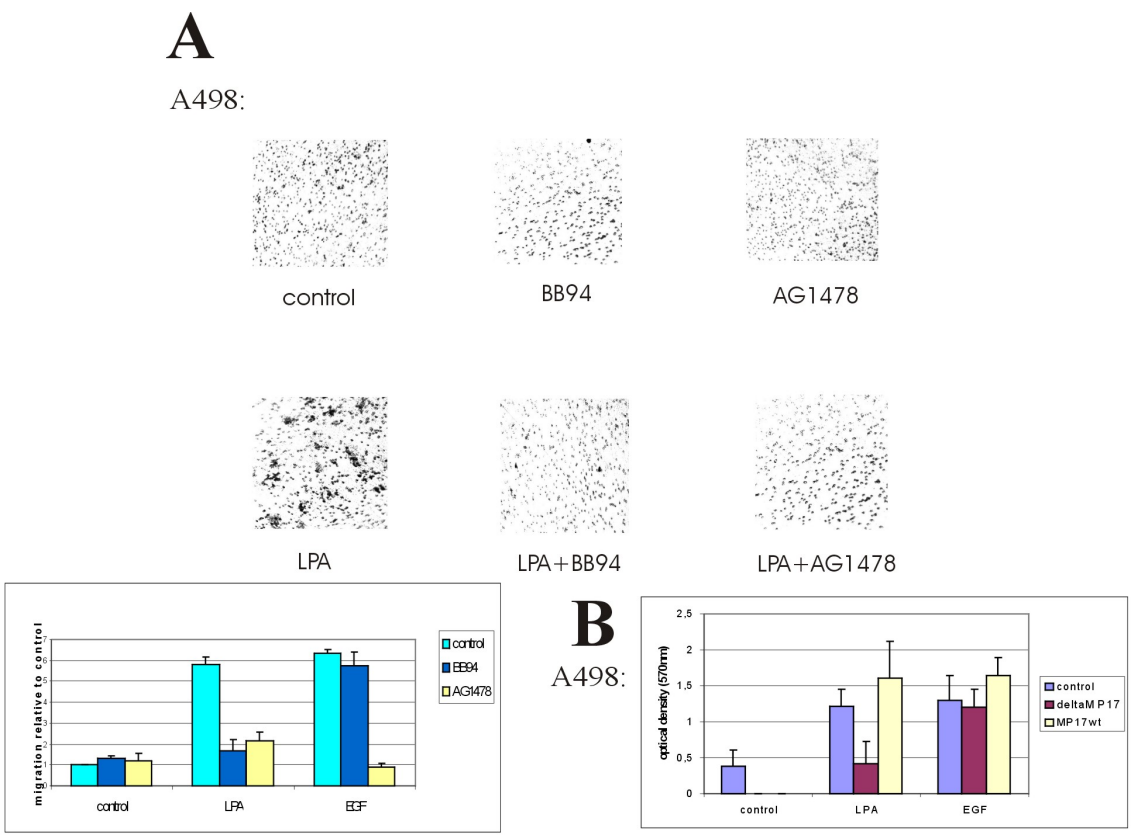


Figure 25 LPA promotes cell migration which is inhibited by BB94, AG1478 and a dominant-negative mutant of ADAM 17

a) Quiescent A498 kidney carcinoma cells were added on top of a polycarbonate filter and migrated through the membrane in response to LPA added to the lower chamber. After 6 hours migrated cells were fixed, stained and counted. Each condition was performed in triplicate, 3 fields from each well were counted and data is an average of 5 experiments.

b) Cell motility of A498 kidney cancer cells in response to LPA depends on TACE. A498 cells stably infected with a dominant-negative mutant and the wildtype of ADAM 17 were treated with LPA or EGF and analysed in a transwell migration assay.

As shown above (Fig 11), EGFR transactivation by LPA is blocked by a dominant negative mutant of ADAM17/TACE as well as TACE siRNA in A498 kidney carcinoma cells. To

evaluate whether ADAM 17 is involved in the regulation of LPA-promoted cell motility of kidney cancer cells the chemotactic migration of A498 cells which stably express either wildtype ADAM 17 or a dominant-negative ADAM 17 mutant was analysed. It was found that expression of dominant-negative ADAM 17 completely prevented migration of A498 cells in response to LPA whereas expression of the wildtype ADAM 17 minimally enhanced the migration rate of LPA stimulated A498 cancer cells (Figure 25).

Together, these data demonstrated that highly abundant GPCR ligands such as LPA promote motility of kidney and bladder cancer cells by an EGFR and metalloproteinase-dependent mechanism. Moreover, a critical role for TACE in the regulation of cell motility of A498 kidney cancer cells in response to LPA was established.

3.13 LPA promotes cell invasion dependent on the EGFR and a metalloproteinase

Penetration of the extracellular matrix and the basement membrane by cancer cells is a key step of tumour dissemination and invasion. For bladder cancer cell lines it was demonstrated previously that stimulation with EGF results in increased matrigel invasion (Kanno, Nonomura et al. 1998) whereas in ovarian cancer cells LPA was found to be a potent inducer of invasion (Fishman, Liu et al. 2001).

Therefore, the invasive capacity of cancer cells relating to EGFR transactivation was evaluated by an *in vitro* invasion assay. Kidney and bladder carcinoma cell lines which show migration in response to GPCR/EGFR cross talk were chosen as a model system. The number of cells which penetrated Matrigel as an artificial basement membrane was determined. Stimulation of Caki2 kidney cancer cells with LPA was found to result in a seven-fold increase in the invasion rate and this effect was abolished by treatment with batimastat and AG1478. Similar results were obtained in ACHN and A498 kidney cancer cells and in TccSup bladder cancer cells.

CaKi2

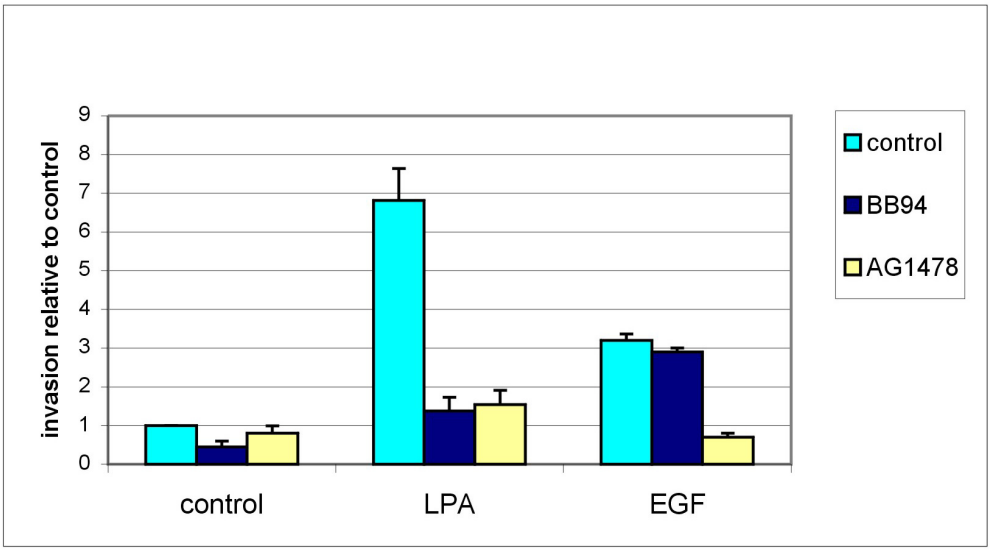


Figure 26. Effect of BB94 and AG1478 on LPA-induced tumour cell invasion
Quiescent CaKi2 kidney carcinoma cells were plated on top of a matrigel-coated polycarbonate membrane. Cell invasion in response to LPA was analysed after 18 h.
∗ p value < 0,002

Taken together these findings demonstrated that LPA promotes migration and invasion in a variety of kidney and bladder carcinoma cell lines via the EGFR and highlights the importance of metalloproteinases in the regulation of cancer cell invasion by GPCR signals. In addition, TMPS pathways appear to be of broad mechanistic significance for promoting invasiveness of tumours of the kidney and bladder.

Table 4 gives an overview over cell migration and invasion of the tested kidney and bladder carcinoma cells in dependence on EGFR transactivation by LPA.

cell line	tissue	EGFR transactivation by LPA	migration	invasion
CaKi2	kidney	+	+	+
ACHN	kidney	+	+	+
HK2	kidney	+	n.d.	n.d.
A498	kidney	+	+	+
A704	kidney	+	n.d.	n.d.
SCABER	bladder	+	-	n.d.
HT1376	bladder	+	-	n.d.
TccSup	bladder	+	+	+
5637	bladder	+	+	-

Table 4 EGFR transactivation by LPA promotes migration and invasion in kidney and bladder carcinoma cell lines
+ increased tyrosine phosphorylation of the EGFR monitored by western blot analysis; - no detectable influence; n.d. not determined

For the kidney cancer cell lines CaKi2, ACHN and A498 and the bladder cancer cell line TccSup cell migration as well as -invasion in response to LPA treatment was observed. Stimulation of 5637 bladder cancer cells with this GPCR agonist resulted only in cell migration, invasion through the matrigel matrix could not be detected.

Biological effect	**Number of tested cell lines showing effect**	**Number of tested cell lines**	**Sensitive to BB94**	**Sensitive to AG1478**
invasion	3	5	yes	yes
migration	5	7	yes	yes
anti-apoptosis	1	7	yes	yes
proliferation	1	8	yes	yes

Table 5. Tested kidney and bladder cancer cell lines showing an biological effect in response to LPA or angiotensin II

In all tested kidney cancer cell lines LPA treatment promotes migration as well as invasion, whereas 50 % of the cell lines derived from bladder tumours showed migration and 25 % show invasion in response to LPA stimulation. In addition to migration and invasion, treatment with LPA leads to anti-apoptosis in the bladder cancer cell line TccSup. The kidney cancer cell line ACHN shows proliferation upon stimulation with angiotensin II.
Sensitivity of the signalling to the metalloproteinase inhibitor BB94 and the EGFR inhibitor G1478 indicates the involvement of the TMPS-pathway.

In summary, the data highlight the importance of cross communication between GPCRs and the EGFR for the invasivity of bladder and kidney cancer. Moreover they show a function of EGFR transactivation in the regulation of proliferation and survival of kidney and bladder cancer cells.

4 Discussion

Cross talk between different members of receptor families has become a well-established concept in signal transduction. Signalling networks are elementary in the control of a high diversity of physiological processes. G-protein coupled receptors as well as receptor tyrosine kinases constitute prominent families of cell-surface proteins regulating the responsiveness of cells to environmental signals. The EGFR and its relatives mediate the biological signal of EGF as well as other related peptide growth factors and therefore comprise one of the biologically most pluripotent systems of autocrine/paracrine signalling. Deregulation of both GPCR and EGFR signalling systems has been linked to the etiology of hyperproliferative diseases. Therefore, it was hypothesized that GPCR-mediated EGFR activation might promote critical cancer cell characteristics such as tumour cell proliferation, anti-apoptosis and cell motility. A special interest existed in EGFR signalling in urological malignancies because EGFR activity which is indicative for the malignant potential of many solid cancers is also elevated in this tumour type in comparison to normal tissue (Ghanem, Van Der Kwast et al. 2001). High-grade, invasive bladder tumours often show genetic aberrations including overexpression of HER2/neu and EGFR associated with an increased frequency of progression to an advanced tumour stage and poor survival. Low-grade bladder tumours are non-invasive, but more than 70% of patients will have at least one recurrence after initial treatment (Van Brussel and Mickisch 1999). Overexpression of EGFR is correlated with a higher recurrence rate and higher tumour progression. Furthermore, in bladder cancer as well as in kidney cancer EGFR expression serves as a prognostic marker for clinical outcome and has been correlated with metastasis (Bue, Wester et al. 1998). Overexpression of EGF, TGFα, amphiregulin, and HB-EGF promotes transformation and proliferation by autocrine mechanisms (Ruck, Jakobson et al. 1994; Ruck and Paulie 1997). Furthermore, in bladder tumours EGF stimulation leads to enhanced cell motility which provides progression from superficial to invasive forms of the disease (Gildea, Harding et al. 2002). In several carcinomas including renal carcinomas overexpression of TGFα and/or amphiregulin has been observed and a direct function for EGF-like molecules in development and progression in transitional cell carcinomas has been described. For example, it was shown that amphiregulin triggers proliferation and TGFα induces invasion in cell lines derived from invasive transitional cancer tissue (De Boer, Houtsmuller et al. 1997). Little is known, however, about the function of GPCRs relating to bladder and kidney cancer with modest

EGFR levels although human transitional carcinoma cells express various GPCRs and display an enhanced migration rate in response to LPA and thrombin (Lummen, Virchow et al. 1997).

4.1 Treatment of Rat-1 fibroblasts and kidney and bladder carcinoma cells with GPCR agonists requires a metalloproteinase activity and the extracellular ligand-binding domain of the EGFR

The data presented here provide evidence that a variety of potent mitogenic GPCR agonists such as angiotensin II, bombesin, bradykinin, carbachol, endothelin-1, LPA, and thrombin induce EGFR transactivation in kidney and bladder cancer (Figure 4, Table 2).

The kidney peptide hormone Angiotensin II is important for the regulation of vasoconstriction and glomerular hemodynamics (Arima 2003). In vascular smooth muscle cells angiotensin II was shown to induce tyrosine phosphorylation of the EGFR via calcium (Eguchi, Numaguchi et al. 1998) In addition, the results of this study demonstrated that the kidney cancer cell lines ACHN and A498 show EGFR activation upon stimulation with Ang II.

The GPCR agonist bombesin has been shown to be highly expressed and secreted by neuroendocrine cells in prostate cancer. Moreover, EGFR transactivation upon bombesin treatment was described in this system (Xiao, Qu et al. 2003). RT-PCR experiments revealed the expression of bombesin receptors in other systems such as human renal cell carcinoma but not in surrounding normal kidney tissue. Furthermore, expression of the neuropeptide receptor was verified in the kidney cancer cell lines A498, Caki1, Caki2 and ACHN (Pansky, De Weerth et al. 2000). Expression in the bladder could only be detected during embryonal development and disappeared at birth (Battey, Wada et al. 1994). The data presented in this study here show EGFR transactivation by bombesin in the kidney cancer cell lines Caki2, ACHN, HK2 and A498 whereas none of the tested bladder cancer cell lines showed any effect upon bombesin treatment (Table 2).

Metalloproteinase-dependent EGFR transactivation pathways were also described for other ligands including LPA, endothelin-1, thrombin, and carbachol in COS-7, PC-3 and Rat-1 cells (Prenzel, Zwick et al. 1999). A majority of the investigated kidney and bladder cancer cell lines show cross talk between receptors of these GPCR ligands and the EGFR. Additionally, the peptide hormone bradykinin which normally participates in inflammatory and vascular regulation (Prado, Taylor et al. 2002) also leads to EGFR transactivation in nearly all tested

cell lines. An important result of this study is that crosstalk between mitogenic LPA receptors and the EGFR was detected in all cell lines tested.

Together, these experimental findings suggest that each tumour cell line can be stimulated by a wide variety of physiologically important GPCR ligands depending on the repertoire of signalling molecules. Moreover, the EGFR acts as a point of convergence for multiple GPCR signals in kidney and bladder carcinoma cells.

Previously it was demonstrated that the EGFR functions as an integral element of mitogenic GPCR signals involving an intracellular mechanism (Daub, Weiss et al. 1996). Later Prenzel and colleagues demonstrated that EGFR transactivation in COS-7, HEK-293, and Rat-1 cells is dependent on a metalloproteinase which is sensitive to the broad spectrum inhibitor batimastat (BB94) (Prenzel, Zwick et al. 1999). To examine if a metalloproteinase activity is involved in GPCR-EGFR signal transmission in kidney and bladder carcinoma cells the effect of BB94 on tyrosine phosphorylation of the EGFR was analysed. It could be shown that pre-treatment of tumour cells with BB94 or the EGFR-specific tyrphostin AG1478 completely abolished the transactivation signal (Figure 5). The experimental observation that the antibody ICR-3R prevents GPCR-induced EGFR tyrosine phosphorylation (Figure 5) demonstrates that the extracellular portion of the EGFR is required for transactivation by GPCR ligands and these data are further consistent with the TMPS concept of EGFR signal transactivation.

The phenomenon of autocrine processing of EGFR ligands which can be blocked by metalloproteinase inhibitors has been previously described in HNSCC cells (P, Rhys-Evans et al. 2002). Furthermore, in human neuroendocrine tumours autocrine phosphorylation of the somatostatin receptor, a member of the GPCR family, has been recorded (Liu, Reubi et al. 2003). In kidney and bladder cancer cells basal tyrosine phosphorylation of the EGFR was diminished by incubation with batimastat. These results suggest that autocrine activation of the EGFR involves a metalloproteinase (Figure 6).

The precise signalling mechanisms governing autocrine growth factor stimulation however remain to be elucidated further. It is currently also only poorly understood if autocrine GPCR stimulation leads to activation of the EGFR *in vivo*.

4.2 EGFR transactivation involves the EGF-like ligands amphiregulin, HB-EGF and TGFα and the metalloproteinases ADAM 10, 15 and 17 in kidney and bladder cancer cells

The results shown in Figures 5 and 13 demonstrated that EGFR transactivation upon stimulation with GPCR ligand can be inhibited by small chemical compounds such as BB94 and AG1478 and additionally by a monoclonal antibody which blocks ligand binding to the ecto-domain of the EGFR. To identify the shedding activity induced upon GPCR ligand treatment which results in release of EGF-like ligands, cells were infected with dominant-negative mutants of ADAM 10, 12, 15 and 17 (Figure 9). The results showed that EGFR transactivation by LPA involves ADAM 17 in Caki2 and A498 kidney cancer cells, ADAM 10 in the kidney cancer cell line ACHN and ADAM 15 in the bladder cancer cell lines TccSup and 5637. Furthermore, stimulation with angiotensin II leads to ADAM 17 - dependent EGFR activation in ACHN kidney cancer cells. To verify the data obtained from studies employing dn ADAM mutants (Figure 9), the endogenous expression of prominent ADAM proteases was abolished by small interfering RNA (siRNA). Remarkably, the ADAM 17-dependent EGFR activation upon LPA stimulation could be confirmed in A498 cells by using ADAM17 siRNAs (Figure 11).

These data are consistent with previous studies demonstrating that EGFR transactivation by bombesin involves ADAM 10 activation in COS-7 and PC-3 cells (Yan, Shirakabe et al. 2002), while ADAM 17 has been implicated in proteolytic cleavage of several EGF family members in murine fibroblasts (Sunnarborg, Hinkle et al. 2002). *In vivo* studies showed that TACE is required for shedding of proTGFα, activation of the EGFR and tumour development in nude mice (Borrell-Pages, Rojo et al. 2003). A possible function of ADAM 15 in EGFR signalling was unknown so far.

In order to identify EGF-like ligands that are involved in the EGFR signal transactivation pathway blocking antibodies were applied (Figure 8). The three tested kidney cancer cells lines Caki2, ACHN and A498 showed processing of HB-EGF precursors upon LPA or angiotensin II treatment, whereas the bladder cancer cell line TccSup showed release of TGFα and 5637 showed release of amphiregulin (Table 3). To substantiate these findings further ectodomain cleavage of pro-amphiregulin upon LPA stimulation in 5637 cells and of HB-EGF upon angiotensin II stimulation in ACHN cells was demonstrated (Figure 7, Figure 10). Furthermore, the requirement of ADAM 17 for angiotensin II-triggered cleavage of proHB-EGF was shown in ACHN cells (Figure 10).

Together, this study provides experimental evidence for HB-EGF cleavage by the metalloproteinases ADAM 10 and 17. As a further variation of the TMPS mechanism, ADAM 15 was found to induce shedding of two different EGF-like ligands in different cancer cell lines: amphiregulin in 5637 and TGFα in TccSup cells (Figure 7/Table 3).

In analogy to these findings, mechanisms have been reported in which HB-EGF-dependent transactivation of the EGFR is mediated by ADAM 10 in lung epithelial cells (Lemjabbar and Basbaum 2002) and COS-7 cells (Yan, Shirakabe et al. 2002) or by ADAM 12 in cardiomyocytes (Asakura, Kitakaze et al. 2002). HB-EGF processing by TACE was described to be required for EGFR activation in mice (Jackson, Qiu et al. 2003). Processing of pro-amphiregulin as well as proTGFα was reported by ADAM 17 (Borrell-Pages, Rojo et al. 2003; Gschwind, Hart et al. 2003). The data presented here establish a novel role of the metalloproteinase disintegrin ADAM 15 in growth factor precursor cleavage, since this protease is required for LPA-induced EGFR transactivation in the bladder cancer cell lines 5637 and TccSup.

4.3 Transactivation of the EGFR involves HER2, SHC, Gab1, Akt, MAPK, Src, PI3-K and cyclin D1

An important result of this study is that LPA treatment of TccSup bladder cancer cells leads to tyrosine phosphorylation of the EGFR and additionally to transactivation of the oncoprotein HER2 (Figure 12). These findings confirm the previous observations in Rat-1 fibroblasts (Daub, Weiss et al. 1996) and further expand the significance of the TMPS pathway. A critical role of HER2/EGFR heterodimers in the etiology of bladder cancer has been suggested by the finding that HER2 is expressed at high levels in neoplastic epithelium of tumours when compared with normal tissue (Kruger, Weitsch et al. 2002). The findings that EGFR as well as HER2 activation upon LPA stimulation can completely be abolished by batimastat and AG1478 treatment provides evidence that GPCRs act as upstream regulators of the EGFR and HER2 signal. It remains to be investigated however if other receptors of the ERB-family like HER3 and HER4 also feature cross talk with GPCRs.

Another important result of this study is that cross communication between mitogenic GPCRs and the EGFR leads to recruitment of the adapter proteins Shc and Gab1 and that inhibition of the EGFR or metalloproteinases by small chemical compounds such as

BB94 and AG1478 block GPCR-triggered EGFR tyrosine phosporylation and downstream signalling events (Figure 13).

Investigation of the role of EGFR transactivation in GPCR-induced mitogenic signalling showed that inhibition of EGFR or metalloproteinase function also blocked the activation of Akt and the MAPKs ERK1/2, JNK and p38 (Figure 14, Figure 15, Figure 16, Figure 18). These findings are in accordance with recent observations by Daub and colleagues that GPCR mitogenic signals involve EGFR signal transactivation and the adapter proteins SHC and Grb2 via the Ras/MAP kinase pathway (Daub, Weiss et al. 1996). A previous study by Dent et al. has shown that cellular stress signals activate the MAPKs p38 and JNK through the EGFR (Dent, Yacoub et al. 2003). Morever, this study further demonstrated that also GPCR agonists lead to activation of these stress kinases. Consistent with the data presented here the PI3-K/PDK1/Akt signalling pathway has been decribed to act in a parallel fashion to the Ras/MAPK pathway downstream of the EGFR and is associated with development of breast cancer as well as its resistance to treatment with cytotoxic drugs (Navolanic, Steelman et al. 2003).

Further downstream in the mitogenic signalling pathway stimulation with LPA and angiotensin II results in accumulation of the cell cycle regulator cyclin D1 in the kidney cancer cell line ACHN which is dependent on the EGFR and a metalloproteinase (Figure 17). Analogous to these results it has been published that LPA increases the level of cyclin D1 in ovarian cancer cells (Hu, Albanese et al. 2003), whereas in CHO cells it was demonstrated that angiotensin II induces cyclin D1 upregulation though PI3-K and the Ras/MAPK pathway (Guillemot, Levy et al. 2001). In vascular smooth muscle cells it was described recently that amphiregulin-induced EGFR phosphorylation leads to upregulation of cyclin D1 (Shin, Lee et al. 2003). In analogy to the data presented here, it was reported that the potent mitogen HB-EGF increases Ras/MAPK activity followed by cyclin D1 expression (Moriuchi, Hirono et al. 2001). A novel aspect in the regulation of cyclin D1 levels is provided by this current study demonstrating the requirement of a metalloproteinase activity. Furthermore, it was previously shown that amplification of the cyclin D1 gene occurs frequently in conjunction with amplification of the EGFR gene in non-small-cell lung cancer (Reissmann, Koga et al. 1999) and that cyclin D1 overexpression in human pancreatic tumour cells is dependent on the mitogenic effect of EGFR signalling (Poch, Gansauge et al. 2001).

Further investigations utilizing pharmacological inhibitors of PI3-K and src-kinase activity suggested that these kinases act both up- as well as downstream of the EGFR to regulate EGFR tyrosine phosphorylation as well as downstream signalling. Moreover, it was

demonstrated here that ERK1/2 phosphorylation upon LPA treatment is only dependent on src whereas src as well as PI3-K are involved in Akt activation (Figure 19). The connection of EGFR signalling to the PI3-K/Akt pathway is well characterized (Wang, Quan et al. 2003), In addition, in endothelial cells it was shown that this survival pathway can also be activated by autocrine secretion of TGFα (Vinals and Pouyssegur 2001). Furthermore, consistent with the data presented here it was reported recently that LPA induces EGFR transactivation via PI3-K (Casas-Gonzalez, Ruiz-Martinez et al. 2003). The conjunction to the responsible metalloproteinase remains to be investigated.

Similar to the findings of this experimental studies, src-kinase was described to mediate GPCR/EGFR cross communication (Guerrero, Santibanez et al. 2004) and to regulate downstream signalling via ERK1/2 and PI3-K/Akt (Das, Mahabeleshwar et al. 2004). Furthermore, it could be demonstrated that transactivation of the EGFR by carbachol is mediated by metalloproteinase-dependent release of TGFα and src activation (McCole, Keely et al. 2002). Recently, another publication demonstrated that the cytoplasmic domain of ADAM 15 contains proline-rich sequences that mediate interactions with src-like kinases in hematopoietic cells (Poghosyan, Robbins et al. 2002). Whether src-ADAM 15 interactions and src-dependent tyrosine phosphorylation of ADAM 15 is critical for mediating the transactivation signal in TccSup cells remains to be elucidated.

4.4 Regulation of proliferation and survival of kidney and bladder cancer cells requires EGFR function and a metalloproteinase activity

While investigating mitogenic signalling by EGFR transactivation it was shown that the GPCR agonists LPA, thrombin, bradykinin and ET-1 induce DNA synthesis and S-phase cell cycle progression in Rat-1 fibroblasts (Figure 20), whereas stimulation of ACHN kidney cancer cells with angiotensin II causes cell proliferation, involving the metalloproteinase ADAM 17 (Figure 21).

In agreement with the findings here, it was previously shown that the EGFR is required for bombesin and bradykinin induced cell cycle progression in Rat-1 and Swiss 3T3 cells (Santiskulvong, Sinnett-Smith et al. 2001). The results shown here thereby further expand the data published by demonstrating EGFR transactivation and cell proliferation upon stimulation with further GPCR agonists such as LPA, thrombin and ET-1 (Daub, Weiss et al. 1996) and indicate the involvement of a metalloproteinase in Rat-1 fibroblasts. Prenzel et al. could demonstrate that EGFR transactivation in Rat-1 cells is mediated by HB-EGF (Prenzel, Zwick

et al. 1999). It remains to identify the metalloproteinase and other signalling molecules involved in the regulation of this biological process.

The kidney carcinoma cell line ACHN features mitogenic signalling via the EGFR, the Ras/MAPK pathway and the cell cycle regulator cyclin D1 upon angiotensin II treatment. These findings are consistent with results which show that proliferation of ACHN cells is dependent on the EGFR (Ciardiello, Caputo et al. 1998). The renin-angiotensin-aldosterone system plays an integral role maintaining vascular tone and optimal salt and water homeostasis in the healthy kidney, but overactivity can result in pathologically consequences (Brewster, Setaro et al. 2003). In normal kidney cells it was shown that Ang II does not only act as a vasoactive peptide but also regulates the synthesis of cytokines and chemokines which play a role in cell growth, inflammation and fibrosis (Ruiz-Ortega, Ruperez et al. 2002). In breast cancer cells it was demonstrated that Ang II induces cell proliferation via ERK1/2, PKC and the EGFR (Greco, Muscella et al. 2003) and in vascular smooth muscle cells it was suggested that angiotensin II triggers cell proliferation mediated by src-kinase and the Shc/Grb2/ERK signalling pathway (Sayeski and Ali 2003). *In vivo* studies showed that angiotensin II stimulation induces DNA synthesis in blood vessels by activation of cyclin D1 and cdk4 (Diep, El Mabrouk et al. 2001). In analogy to the current results, HB-EGF was implicated as a mediator of angiotensin II-induced growth promotion in human prostate stromal cells (Lin and Freeman 2003). In contrast to the observation presented here, ADAM 12 instead of ADAM 17 has been determined as a regulator of HB-EGF shedding upon AngII stimulation (Mori, Tanaka et al. 2003).

To upgrade these data it would be interesting to identify further signalling molecules which are important in this pathway leading to cell proliferation.

A further important aspect of the current findings is that, in addition to the proliferative responses, EGFR signal transactivation plays a direct role in the regulation of cell survival. The growth promoting signalling events in Rat-1 fibroblasts and TccSup bladder cancer cells are accompanied by phosphorylation of the survival mediator Akt/PKB downstream of the EGFR. Activation of Akt has recently been shown to suppress apoptosis of Rat-1 fibroblasts that had been detached from the extracellular matrix and to promote progression of quiescent cells into the S phase of the cell cycle (Mirza, Kohn et al. 2000). Furthermore, it was found that LPA treatment drastically increased survival of fibroblasts after serum deprivation or doxorubicin treatment in an EGFR- and ADAM-dependent manner (Figure 22). In analogy to the observations here, LPA was shown to cause resistance of cancer cells to chemotherapy in a recent study (Tanyi, Morris et al. 2003).

Another report demonstrated that GPCR ligands protect human lymphoblastoma cells from apoptosis triggered by CD95 antibodies via unknown mechanisms (Goetzl, Kong et al. 1999). In this study it was observed that death receptor-mediated apoptosis in TccSup bladder carcinoma cells was effectively prevented by LPA co-stimulation (Figure 23). Death receptor pathways are of pathophysiological significance since 75 % of transitional cell carcinoma (TCC) cells display apoptosis upon activation of the Fas/Fas-ligand pathway (Yu, Hsieh et al. 2003). Again, the anti-apoptotic effect of LPA on TccSup cells was dependent on the EGFR and metalloproteinase activity substantiating the importance of TMPS pathways in the regulation of cell death-versus-survival decisions.

These findings highlight the importance of EGFR signal transactivation in cancer cell proliferation and survival and strongly support a role of ADAM metalloproteinases as determinants of cancer progression.

4.5 Motility of kidney and bladder cancer cells is regulated by the EGFR and a metalloproteinase

Increased tumour cell motility is an essential feature of the malignant potential of tumours. Previous reports have shown that LPA enhances *in vitro* wound closure and invasion in ovarian cancer cells (Xu, Gaudette et al. 1995). On the other hand, Ishikawa and colleagues demonstrated that EGF induces anchorage-independent growth and invasion of bladder cancer cells (Ishikawa, Maeda et al. 1989). The current data provide evidence that both, LPA and EGF promote *in vitro* wound closure, cell migration and invasion in kidney and bladder carcinoma cells via the EGFR and that the LPA-induced cellular responses require a metalloproteinase activity (Figure 24, Figure 25, Figure 26).

Wound healing is a complex process featuring cell growth and motility. Previously was observed that LPA stimulates closure of wounded monolayers of human endothelial cells (Lee, Goetzl et al. 2000). *In vivo* data of bladder wound healing have shown to be mediated by the EGFR and HER2 via processing of EGF, TGFα or amphiregulin (Bindels, van der Kwast et al. 2002). In analogy to the current data, it was shown that LPA promotes wound closure of kidney and bladder cancer cells involving the EGFR, and a metalloproteinase shedding EGF-like ligands. Since heregulin-alpha has been demonstrated to play a role in keratinocyte wound healing (Schelfhout, Coene et al. 2002) it is of high relevance to further investigate the function of HER2, HER3 and HER4 in wound closure of kidney and bladder

cancer cells. In addition, metalloproteinase-dependent EGF-like ligand shedding has been reported to mediate EGFR transactivation and migration of vascular smooth muscle cells (Eguchi, Frank et al. 2003) whereas in colon cancer EGFR transactivation by Prostaglandin E2 regulates cell migration and invasion (Buchanan, Wang et al. 2003). Furthermore, it was suggested that LPA is a critical factor regulating motility of pancreatic cancer cells (Yamada, Sato et al. 2003) and the invasion of ovarian cancer cells (Fishman, Liu et al. 2001). Other studies indicated the involvement of HB-EGF contributing to migration of prostate cancer cells (Madarame, Higashiyama et al. 2003) and the modulation of invasion of metastatic breast cancer cells by amphiregulin (Kondapaka, Fridman et al. 1997). In analogy to the results presented here the metalloproteinase ADAM 17/TACE was identified as a key element of GPCR/EGFR cross talk pathways promoting cancer cell motility (Gschwind, Hart et al. 2003). The involvement of ADAM 15 in the migration of mesangial cells was demonstrated recently (Martin, Eynstone et al. 2002).

Collectively, these data suggest an important function of EGFR transactivation by TMPS pathways concerning cell motility in urogenital cancer. Elements of TMPS pathways therefore represent potential intervention targets for the treatment of kidney and bladder tumours.

4.6 Perspectives

The current data disclose the high complexity of cross talk mechanisms linking GPCR stimulation with activation of the EGFR. Signalling from LPA receptors to the EGFR requires processing of proHB-EGF by ADAM 17 in CaKi2 and A498 kidney cancer cells and ADAM 10 in ACHN cells while treatment with the GPCR ligand angiotensin II involves ADAM 17 mediated processing of HB-EGF in ACHN cells. In contrast, in bladder cancer cells stimulation with LPA leads to ADAM 15 activation which controls proTGFα shedding in TccSup cells and pro-amphiregulin shedding in 5637 cells.

In HNSCC cells LPA treatment leads to TACE dependent cleavage of pro-amphiregulin (Gschwind, Hart et al. 2003) whereas in the cardiovascular system stimulation with the GPCR ligand angiotensin II results in HB-EGF shedding by MMP-7 (Hao, Du et al. 2004). In nude mice TACE is required for processing of TGFα and subsequent EGFR activation (Borrell-Pages, Rojo et al. 2003). Yan et al. have discovered that ADAM 10 promotes HB-EGF release in COS 7 and PC-3 cells (Yan, Shirakabe et al. 2002).

It remains to investigate the regulation of the signalling mechanism and elucidate the factors leading to the choice of a distinct metalloproteinase processing a distinct EGF-like ligand upon treatment with a particular GPCR agonist.

Furthermore, EGFR transactivation pathways are the key to the regulation of diverse biological processes. Most of the tested kidney and bladder cancer cells showed cellular migration and invasion as a response to GPCR/EGFR cross communication and only a few showed cell proliferation and anti-apoptosis as a biological outcome of EGFR transactivation.

Recent publications reported on diverse biological functions of EGFR transactivation pathways depending on the cellular system, the stimulus and the repertoire of signalling molecules. In HNSCC cells for example, LPA receptor-EGFR cross talk results in migration and proliferation (Gschwind, Hart et al. 2003), whereas in endothelial cells EGFR-mediated migration is triggered by IL-8 (Schraufstatter, Trieu et al. 2003) and in prostate cancer cells stimulation with bombesin leads to migration involving the EGFR (Madarame, Higashiyama et al. 2003). In the cardiovasculary system adrenoceptor or Ang II receptor activation promotes vasoconstriction (Hao, Du et al. 2004) and further the EGFR plays a critical role in vascular remodelling triggered by Ang II (Eguchi, Frank et al. 2003). Collectively, these data show that EGFR signal transactivation pathways regulate major characteristics of neoplasia and cardiovascular diseases.

Concerning EGFR transactivation hardly any *in vivo* data have been published so far. Interestingly the phenotype of EGFR knockout mice exhibits a disorganized hair follicle phenotype and systemic disease resulting in death before three weeks and is similar to the phenotype of TACE knockout mice (Hansen, Alexander et al. 1997). Furthermore, it was demonstrated that TACE regulates TGFalpha ligand availability *in vivo* (Peschon, Slack et al. 1998). Further studies suggested an even broader role of this metalloprotease in cell surface ectodomain cleavage of other EGF-like precursors in cultured murine fibroblasts (Sunnarborg, Hinkle et al. 2002).

HB-EGF knockout mice which mostly died early had enlarged, dysfunctional hearts and poorly differentiated lungs (Jackson, Qiu et al. 2003). The ADAM 10-deficient mouse dies within 9.5 days of embryogenesis displaying multiple defects of the central nervous system, the somites and the cardiovascular system (Hartmann, de Strooper et al. 2002). Triple null mice lacking EGF, amphiregulin, and TGFα were growth retarded and showed intestinal defects (Troyer, Luetteke et al. 2001). Mice deficient for three LPA receptors show dysmorphism of the head, semilethality due to defective suckling behaviour and generation of

a small fraction of pups with frontal haematoma (Contos, Ishii et al. 2002) whereas AT-1 knockout mice revealed a severe heart failure (Harada, Sugaya et al. 1999).

These results suggest a role of EGFR, LPA, angiotensinII, HB-EGF, amphiregulin, TGFα, ADAM 10 and TACE for the embryonic development of organs in mice. An important issue of further studies will be the determination of the relevance of the TMPS-pathway for normal development and pathophysiology thereby paving the way for the validation of novel targets for the pharmaceutical intervention in anti-tumour therapy.

5 Summary

Taken together the main findings of this study are:

1. Stimulation of kidney and bladder carcinoma cells with the GPCR ligands angiotensin II, bombesin, bradykinin, carbachol, endothelin-1, LPA and thrombin results in rapid tyrosine phosphorylation of the EGFR and the oncoprotein HER2/neu as well as induces critical RTK downstream signalling events such as recruitment of the adapter proteins Shc and Gab1 and activation of the mitogen-activated protein kinases (MAPK) ERK1/2, JNK and p38.
2. The EGFR transactivation signal is blocked by pre-incubation of cells with the metalloprotease inhibitor batimastat and the EGFR blocking antibody ICR-3R suggesting that the GPCR-EGFR signal transmission involves an EGF-like ligand-dependent mechanism in fibroblasts, kidney- and bladder carcinoma cells.
3. By utilizing specific neutralizing antibodies against individual growth factors and by employing dominant negative ADAM mutants as well as siRNA technology the EGFR ligands amphiregulin, HB-EGF, and TGFα as well as the metalloproteinases ADAM 10, 15 and 17 were identified as mediators of TMPS pathways.
4. GPCR-induced activation of ADAMs results in discrete cellular responses modulating the migratory and invasive behaviour of kidney and bladder cancer cells.
5. GPCR agonists promote activation of the Ras/MAPK pathway, DNA synthesis and cell cycle progression via the EGFR in fibroblasts and ACHN kidney cancer cells.
6. EGFR transactivation pathways regulate activation of the survival mediator Akt/PKB and the susceptibility of fibroblasts and TccSup bladder carcinoma cells to pro-apoptotic signals such as serum-deprivation, death receptor stimulation and the chemotherapeutic drug doxorubicin.

Together, these results demonstrate that distinct combinations of growth factor precursors and ADAMs regulate GPCR-EGFR cross talk pathways in different urogenital cancer cell lines promoting cell migration, invasion, proliferation and anti-apoptosis. Therefore, elements of the TMPS pathway represent novel, promising targets for cancer intervention strategies.

6 References

Adomeit, A., A. Graness, et al. (1999). "Bradykinin B(2) receptor-mediated mitogen-activated protein kinase activation in COS-7 cells requires dual signaling via both protein kinase C pathway and epidermal growth factor receptor transactivation." Mol Cell Biol **19**(8): 5289-97.

Alroy, I. and Y. Yarden (1997). "The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions." FEBS Lett **410**(1): 83-6.

Ambrosini, G., C. Adida, et al. (1997). "A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma." Nat Med **3**(8): 917-21.

Arima, S. (2003). "Role of angiotensin II and endogenous vasodilators in the control of glomerular hemodynamics." Clin Exp Nephrol **7**(3): 172-8.

Asakura, M., M. Kitakaze, et al. (2002). "Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy." Nat Med **8**(1): 35-40.

Battey, J., E. Wada, et al. (1994). "Bombesin receptor gene expression during mammalian development." Ann N Y Acad Sci **739**: 244-52.

Belcheva, M. M., P. D. Haas, et al. (2002). "The Fibroblast Growth Factor Receptor Is at the Site of Convergence between &mgr;-Opioid Receptor and Growth Factor Signaling Pathways in Rat C6 Glioma Cells." J Pharmacol Exp Ther **303**(3): 909-918.

Bindels, E. M., T. H. van der Kwast, et al. (2002). "Functions of epidermal growth factor-like growth factors during human urothelial reepithelialization in vitro and the role of erbB2." Urol Res **30**(4): 240-7.

Black, R. A. (2002). "Tumor necrosis factor-alpha converting enzyme." Int J Biochem Cell Biol **34**(1): 1-5.

Black, R. A., C. T. Rauch, et al. (1997). "A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells." Nature **385**(6618): 729-33.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**(6835): 355-65.

Bode, W., F. X. Gomis-Ruth, et al. (1993). "Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'." FEBS Lett **331**(1-2): 134-40.

Borrell-Pages, M., F. Rojo, et al. (2003). "TACE is required for the activation of the EGFR by TGF-alpha in tumors." Embo J **22**(5): 1114-24.

Boyer, B., Y. Bourgeois, et al. (2002). "Src kinase contributes to the metastatic spread of carcinoma cells." Oncogene **21**(15): 2347-56.

Brachmann, R., P. B. Lindquist, et al. (1989). "Transmembrane TGF-alpha precursors activate EGF/TGF-alpha receptors." Cell **56**(4): 691-700.

Brewster, U. C., J. F. Setaro, et al. (2003). "The renin-angiotensin-aldosterone system: cardiorenal effects and implications for renal and cardiovascular disease states." Am J Med Sci **326**(1): 15-24.

Brinckerhoff, C. E. and L. M. Matrisian (2002). "Matrix metalloproteinases: a tail of a frog that became a prince." Nat Rev Mol Cell Biol **3**(3): 207-14.

Buchanan, F. G., D. Wang, et al. (2003). "Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor." J Biol Chem **278**(37): 35451-7.

Bue, P., K. Wester, et al. (1998). "Expression of epidermal growth factor receptor in urinary bladder cancer metastases." Int J Cancer **76**(2): 189-93.

Carpenter, G. (1999). "Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways." J Cell Biol **146**(4): 697-702.

Casas-Gonzalez, P., A. Ruiz-Martinez, et al. (2003). "Lysophosphatidic acid induces alpha1B-adrenergic receptor phosphorylation through G beta gamma, phosphoinositide 3-kinase, protein kinase C and epidermal growth factor receptor transactivation." Biochim Biophys Acta **1633**(2): 75-83.

Chen, C. and H. Okayama (1987). "High-efficiency transformation of mammalian cells by plasmid DNA." Mol Cell Biol **7**(8): 2745-52.

Chung, C. T. and R. H. Miller (1988). "A rapid and convenient method for the preparation and storage of competent bacterial cells." Nucleic Acids Res **16**(8): 3580.

Ciardiello, F., R. Caputo, et al. (1998). "Cooperative inhibition of renal cancer growth by anti-epidermal growth factor receptor antibody and protein kinase A antisense oligonucleotide." J Natl Cancer Inst **90**(14): 1087-94.

Cohen, P. (2002). "Protein kinases--the major drug targets of the twenty-first century?" Nat Rev Drug Discov **1**(4): 309-15.

Contos, J. J., I. Ishii, et al. (2002). "Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2)." Mol Cell Biol **22**(19): 6921-9.

Das, R., G. H. Mahabeleshwar, et al. (2004). "Osteopontin induces AP-1-mediated secretion of urokinase type plasminogen activator through c-Src dependent EGF receptor transactivation in breast cancer cells." J Biol Chem **2**: 2.

Daub, H., C. Wallasch, et al. (1997). "Signal characteristics of G protein-transactivated EGF receptor." Embo J **16**(23): 7032-44.

Daub, H., F. U. Weiss, et al. (1996). "Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors." Nature **379**(6565): 557-60.

De Boer, W. I., A. B. Houtsmuller, et al. (1997). "Expression and functions of EGF, FGF and TGFbeta-growth-factor family members and their receptors in invasive human transitional-cell-carcinoma cells." Int J Cancer **71**(2): 284-91.

Dempsey, P. J., K. S. Meise, et al. (1997). "Apical enrichment of human EGF precursor in Madin-Darby canine kidney cells involves preferential basolateral ectodomain cleavage sensitive to a metalloprotease inhibitor." J Cell Biol **138**(4): 747-58.

Dent, P., A. Yacoub, et al. (2003). "Stress and radiation-induced activation of multiple intracellular signaling pathways." Radiat Res **159**(3): 283-300.

Dickstein, B., E. M. Valverius, et al. (1993). "Increased epidermal growth factor receptor in an estrogen-responsive, adriamycin-resistant MCF-7 cell line." J Cell Physiol **157**(1): 110-8.

Diep, Q. N., M. El Mabrouk, et al. (2001). "Expression of cell cycle proteins in blood vessels of angiotensin II-infused rats: role of AT(1) receptors." Hypertension **37**(2 Part 2): 604-8.

Draper, B. K., T. Komurasaki, et al. (2003). "Epiregulin is more potent than EGF or TGFalpha in promoting in vitro wound closure due to enhanced ERK/MAPK activation." J Cell Biochem **89**(6): 1126-37.

Eguchi, S., G. D. Frank, et al. (2003). "Metalloprotease-dependent ErbB ligand shedding in mediating EGFR transactivation and vascular remodelling." Biochem Soc Trans **31**(Pt 6): 1198-202.

Eguchi, S., K. Numaguchi, et al. (1998). "Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells." J Biol Chem **273**(15): 8890-6.

Elbashir, S. M., J. Harborth, et al. (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-8.

Endo, A., K. Nagashima, et al. (2002). "Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells via vascular endothelial growth factor receptor and CrkII." J Biol Chem **277**(26): 23747-54.

Evan, G. I. and K. H. Vousden (2001). "Proliferation, cell cycle and apoptosis in cancer." Nature **411**(6835): 342-8.

Fang, X., S. Yu, et al. (2000). "Lysophosphatidic acid prevents apoptosis in fibroblasts via G(i)-protein-mediated activation of mitogen-activated protein kinase." Biochem J **352**(Pt 1): 135-43.

Faure, M., T. A. Voyno-Yasenetskaya, et al. (1994). "cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells." J Biol Chem **269**(11): 7851-4.

Fishman, D. A., Y. Liu, et al. (2001). "Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells." Cancer Res **61**(7): 3194-9.

Fukuhara, S., H. Chikumi, et al. (2001). "RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho?" Oncogene **20**(13): 1661-8.

Garrett, T., N. McKern, et al. (2002). "Crystal Structure of a Truncated Epidermal Growth Factor Receptor Extracellular Domain Bound to Transforming Growth Factor alpha." Cell **110**(6): 763.

Gershoni, J. M. and G. E. Palade (1982). "Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter." Anal Biochem **124**(2): 396-405.

Gether, U. and B. K. Kobilka (1998). "G protein-coupled receptors. II. Mechanism of agonist activation." J Biol Chem **273**(29): 17979-82.

Ghanem, M. A., T. H. Van Der Kwast, et al. (2001). "Expression and prognostic value of epidermal growth factor receptor, transforming growth factor-alpha, and c-erb B-2 in nephroblastoma." Cancer **92**(12): 3120-9.

Gibson, S., S. Tu, et al. (1999). "Epidermal growth factor protects epithelial cells against Fas-induced apoptosis. Requirement for Akt activation." J Biol Chem **274**(25): 17612-8.

Gildea, J. J., M. A. Harding, et al. (2002). "The role of Ral A in epidermal growth factor receptor-regulated cell motility." Cancer Res **62**(4): 982-5.

Goetzl, E. J., Y. Kong, et al. (1999). "Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax." J Immunol **162**(4): 2049-56.

Gohla, A., R. Harhammer, et al. (1998). "The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho." J Biol Chem **273**(8): 4653-9.

Gohla, A., S. Offermanns, et al. (1999). "Differential involvement of Galpha12 and Galpha13 in receptor-mediated stress fiber formation." J Biol Chem **274**(25): 17901-7.

Greco, S., A. Muscella, et al. (2003). "Angiotensin II activates extracellular signal regulated kinases via protein kinase C and epidermal growth factor receptor in breast cancer cells." J Cell Physiol **196**(2): 370-7.

Grosse, R., S. Roelle, et al. (2000). "Epidermal growth factor receptor tyrosine kinase mediates Ras activation by gonadotropin-releasing hormone." J Biol Chem **275**(16): 12251-60.

Gschwind, A., S. Hart, et al. (2003). "TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells." Embo J **22**(10): 2411-21.

Gschwind, A., E. Zwick, et al. (2001). "Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission." Oncogene **20**(13): 1594-600.

Guerrero, J., J. F. Santibanez, et al. (2004). "EGF receptor transactivation by urokinase receptor stimulus through a mechanism involving Src and matrix metalloproteinases." Exp Cell Res **292**(1): 201-208.

Guillemot, L., A. Levy, et al. (2001). "Angiotensin II-induced transcriptional activation of the cyclin D1 gene is mediated by Egr-1 in CHO-AT(1A) cells." J Biol Chem **276**(42): 39394-403.

Gutkind, J. S. (2000). "Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors." Sci STKE **2000**(40): RE1.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hansen, L. A., N. Alexander, et al. (1997). "Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development." Am J Pathol **150**(6): 1959-75.

Hao, L., M. Du, et al. (2004). "Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway." Circ Res **94**(1): 68-76.

Harada, K., T. Sugaya, et al. (1999). "Angiotensin II type 1A receptor knockout mice display less left ventricular remodeling and improved survival after myocardial infarction." Circulation **100**(20): 2093-9.

Hartmann, D., B. de Strooper, et al. (2002). "The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts." Hum Mol Genet **11**(21): 2615-24.

Herrlich, A., H. Daub, et al. (1998). "Ligand-independent activation of platelet-derived growth factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells." Proc Natl Acad Sci U S A **95**(15): 8985-90.

Hooper, N. M. (1994). "Families of zinc metalloproteases." FEBS Lett **354**(1): 1-6.

Hu, Y. L., C. Albanese, et al. (2003). "Dual mechanisms for lysophosphatidic acid stimulation of human ovarian carcinoma cells." J Natl Cancer Inst **95**(10): 733-40.

Hubbard, S. R. and J. H. Till (2000). "Protein tyrosine kinase structure and function." Annu Rev Biochem **69**: 373-98.

Hunter, T. (2000). "Signaling--2000 and beyond." Cell **100**(1): 113-27.

Imamura, F., T. Horai, et al. (1993). "Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D." Biochem Biophys Res Commun **193**(2): 497-503.

Ishikawa, J., S. Maeda, et al. (1989). "EGF stimulates anchorage-independent growth of a human bladder carcinoma cell line (KU1) with an amplified and over-expressed EGF receptor gene." Int J Cancer **44**(6): 1000-4.

Iwasaki, H., S. Eguchi, et al. (1999). "Endothelin-mediated vascular growth requires p42/p44 mitogen-activated protein kinase and p70 S6 kinase cascades via transactivation of epidermal growth factor receptor." Endocrinology **140**(10): 4659-68.

Jackson, L. F., T. H. Qiu, et al. (2003). "Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling." Embo J **22**(11): 2704-16.

Ji, T. H., M. Grossmann, et al. (1998). "G protein-coupled receptors. I. Diversity of receptor-ligand interactions." J Biol Chem **273**(28): 17299-302.

Kanno, N., N. Nonomura, et al. (1998). "Effects of epidermal growth factor on the invasion activity of the bladder cancer cell line." J Urol **159**(2): 586-90.

Keely, S. J., S. O. Calandrella, et al. (2000). "Carbachol-stimulated transactivation of epidermal growth factor receptor and mitogen-activated protein kinase in T(84) cells is mediated by intracellular ca(2+), PYK-2, and p60(src)." J Biol Chem **275**(17): 12619-25.

Kim, S. N., J. G. Park, et al. (2000). "Characterization of epidermal growth factor receptor function in lysophosphatidic acid signaling in PC12 cells." J Cell Biochem **76**(3): 386-93.

Kinsella, T. M. and G. P. Nolan (1996). "Episomal vectors rapidly and stably produce high-titer recombinant retrovirus." Hum Gene Ther **7**(12): 1405-13.

Kondapaka, S. B., R. Fridman, et al. (1997). "Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells." Int J Cancer **70**(6): 722-6.

Kranenburg, O. and W. H. Moolenaar (2001). "Ras-MAP kinase signaling by lysophosphatidic acid and other G protein-coupled receptor agonists." Oncogene **20**(13): 1540-6.

Kruger, S., G. Weitsch, et al. (2002). "HER2 overexpression in muscle-invasive urothelial carcinoma of the bladder: prognostic implications." Int J Cancer **102**(5): 514-8.

Lee, H., E. J. Goetzl, et al. (2000). "Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing." Am J Physiol Cell Physiol **278**(3): C612-8.

Lee, S. Y. and S. Rasheed (1990). "A simple procedure for maximum yield of high-quality plasmid DNA." Biotechniques **9**(6): 676-9.

Lemjabbar, H. and C. Basbaum (2002). "Platelet-activating factor receptor and ADAM10 mediate responses to Staphylococcus aureus in epithelial cells." Nat Med **8**(1): 41-6.

Lemmon, M. A., Z. Bu, et al. (1997). "Two EGF molecules contribute additively to stabilization of the EGFR dimer." Embo J **16**(2): 281-94.

Lin, J. and M. R. Freeman (2003). "Transactivation of ErbB1 and ErbB2 receptors by angiotensin II in normal human prostate stromal cells." Prostate **54**(1): 1-7.

Liu, Q., J. C. Reubi, et al. (2003). "In vivo phosphorylation of the somatostatin 2A receptor in human tumors." J Clin Endocrinol Metab **88**(12): 6073-9.

Lui, V. W. and J. R. Grandis (2002). "EGFR-mediated cell cycle regulation." Anticancer Res **22**(1A): 1-11.

Lummen, G., S. Virchow, et al. (1997). "Identification of G protein-coupled receptors potently stimulating migration of human transitional-cell carcinoma cells." Naunyn Schmiedebergs Arch Pharmacol **356**(6): 769-76.

Luttrell, L. M., S. S. Ferguson, et al. (1999). "Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes." Science **283**(5402): 655-61.

Madarame, J., S. Higashiyama, et al. (2003). "Transactivation of epidermal growth factor receptor after heparin-binding epidermal growth factor-like growth factor shedding in the migration of prostate cancer cells promoted by bombesin." Prostate **57**(3): 187-95.

Madrid, L. V., C. Y. Wang, et al. (2000). "Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB." Mol Cell Biol **20**(5): 1626-38.

Marinissen, M. J. and J. S. Gutkind (2001). "G-protein-coupled receptors and signaling networks: emerging paradigms." Trends Pharmacol Sci **22**(7): 368-76.

Martin, J., L. V. Eynstone, et al. (2002). "The role of ADAM 15 in glomerular mesangial cell migration." J Biol Chem **277**(37): 33683-9.

Massague, J. and A. Pandiella (1993). "Membrane-anchored growth factors." Annu Rev Biochem **62**: 515-41.

Mateo, C., E. Moreno, et al. (1997). "Humanization of a mouse monoclonal antibody that blocks the epidermal growth factor receptor: recovery of antagonistic activity." Immunotechnology **3**(1): 71-81.

McCole, D. F., S. J. Keely, et al. (2002). "Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor-alpha." J Biol Chem **277**(45): 42603-12.

Mirza, A. M., A. D. Kohn, et al. (2000). "Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB." Cell Growth Differ **11**(6): 279-92.

Miyamoto, H., Y. Kubota, et al. (2000). "C-ERBB-2 gene amplification as a prognostic marker in human bladder cancer." Urology **55**(5): 679-83.

Moolenaar, W. H. (1999). "Bioactive lysophospholipids and their G protein-coupled receptors." Exp Cell Res **253**(1): 230-8.

Moolenaar, W. H., O. Kranenburg, et al. (1997). "Lysophosphatidic acid: G-protein signalling and cellular responses." Curr Opin Cell Biol **9**(2): 168-73.

Mori, S., M. Tanaka, et al. (2003). "PACSIN3 binds ADAM12/meltrin alpha and up-regulates ectodomain shedding of heparin-binding epidermal growth factor-like growth factor." J Biol Chem **278**(46): 46029-34.

Moriuchi, A., S. Hirono, et al. (2001). "Additive and inhibitory effects of simultaneous treatment with growth factors on DNA synthesis through MAPK pathway and G1 cyclins in rat hepatocytes." Biochem Biophys Res Commun **280**(1): 368-73.

Moss, M. L., S. L. Jin, et al. (1997). "Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha." Nature **385**(6618): 733-6.

Mullis, K. B. and F. A. Faloona (1987). "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction." Methods Enzymol **155**: 335-50.

Murasawa, S., Y. Mori, et al. (1998). "Angiotensin II type 1 receptor-induced extracellular signal-regulated protein kinase activation is mediated by Ca2+/calmodulin-dependent transactivation of epidermal growth factor receptor." Circ Res **82**(12): 1338-48.

Murphy, L. O., M. W. Cluck, et al. (2001). "Pancreatic cancer cells require an EGF receptor-mediated autocrine pathway for proliferation in serum-free conditions." Br J Cancer **84**(7): 926-35.

Nagase, H. and J. F. Woessner, Jr. (1999). "Matrix metalloproteinases." J Biol Chem **274**(31): 21491-4.

Navolanic, P. M., L. S. Steelman, et al. (2003). "EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review)." Int J Oncol **22**(2): 237-52.

Ogiso, H., R. Ishitani, et al. (2002). "Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains." Cell **110**(6): 775.

Olayioye, M. A., R. M. Neve, et al. (2000). "The ErbB signaling network: receptor heterodimerization in development and cancer." Embo J **19**(13): 3159-67.

P, O. C., P. Rhys-Evans, et al. (2002). "A synthetic matrix metalloproteinase inhibitor prevents squamous carcinoma cell proliferation by interfering with epidermal growth factor receptor autocrine loops." Int J Cancer **100**(5): 527-33.

Pansky, A., A. De Weerth, et al. (2000). "Gastrin releasing peptide-preferring bombesin receptors mediate growth of human renal cell carcinoma." J Am Soc Nephrol **11**(8): 1409-18.

Peschon, J. J., J. L. Slack, et al. (1998). "An essential role for ectodomain shedding in mammalian development." Science **282**(5392): 1281-4.

Poch, B., F. Gansauge, et al. (2001). "Epidermal growth factor induces cyclin D1 in human pancreatic carcinoma: evidence for a cyclin D1-dependent cell cycle progression." Pancreas **23**(3): 280-7.

Poghosyan, Z., S. M. Robbins, et al. (2002). "Phosphorylation-dependent interactions between ADAM15 cytoplasmic domain and Src family protein-tyrosine kinases." J Biol Chem **277**(7): 4999-5007.

Prado, G. N., L. Taylor, et al. (2002). "Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B2 and B1 receptors." J Cell Physiol **193**(3): 275-86.

Prenzel, N., E. Zwick, et al. (1999). "EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF." Nature **402**(6764): 884-8.

Primakoff, P. and D. G. Myles (2000). "The ADAM gene family: surface proteins with adhesion and protease activity." Trends Genet **16**(2): 83-7.

Pyne, S. and N. J. Pyne (2000). "Sphingosine 1-phosphate signalling in mammalian cells." Biochem J **349**(Pt 2): 385-402.

Reissmann, P. T., H. Koga, et al. (1999). "Amplification and overexpression of the cyclin D1 and epidermal growth factor receptor genes in non-small-cell lung cancer. Lung Cancer Study Group." J Cancer Res Clin Oncol **125**(2): 61-70.

Riese, D. J., 2nd and D. F. Stern (1998). "Specificity within the EGF family/ErbB receptor family signaling network." Bioessays **20**(1): 41-8.

Ruck, A., E. Jakobson, et al. (1994). "Adaptation of human bladder carcinoma cell lines to serum-free growth. Evidence for autocrine growth stimulation." Anticancer Res **14**(1A): 55-60.

Ruck, A. and S. Paulie (1997). "The epidermal growth factor receptor is involved in autocrine growth of human bladder carcinoma cell lines." Anticancer Res **17**(3C): 1925-31.

Ruiz-Ortega, M., M. Ruperez, et al. (2002). "Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney." Kidney Int Suppl(82): 12-22.

Salomon, D. S., C. Bianco, et al. (1999). "Cripto: a novel epidermal growth factor (EGF)-related peptide in mammary gland development and neoplasia." Bioessays **21**(1): 61-70.

Sambrook, J., Fritsch, E.F. und Maniatis, T. (1990). "Molecular cloning: a laboratory manual." Cold Spring Habor Laboratory Press, New York, USA.

Santiskulvong, C., J. Sinnett-Smith, et al. (2001). "EGF receptor function is required in late G(1) for cell cycle progression induced by bombesin and bradykinin." Am J Physiol Cell Physiol **281**(3): C886-98.

Sasaki, T., J. Irie-Sasaki, et al. (2000). "Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration." Science **287**(5455): 1040-6.

Sayeski, P. P. and M. S. Ali (2003). "The critical role of c-Src and the Shc/Grb2/ERK2 signaling pathway in angiotensin II-dependent VSMC proliferation." Exp Cell Res **287**(2): 339-49.

Schelfhout, V. R., E. D. Coene, et al. (2002). "The role of heregulin-alpha as a motility factor and amphiregulin as a growth factor in wound healing." J Pathol **198**(4): 523-533.

Schlessinger, J. (2000). "Cell signaling by receptor tyrosine kinases." Cell **103**(2): 211-25.

Schlessinger, J. (2002). "Ligand-induced, receptor-mediated dimerization and activation of EGF receptor." Cell **110**(6): 669.

Schlondorff, J. and C. P. Blobel (1999). "Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding." J Cell Sci **112**(Pt 21): 3603-17.

Schraufstatter, I. U., K. Trieu, et al. (2003). "IL-8-mediated cell migration in endothelial cells depends on cathepsin B activity and transactivation of the epidermal growth factor receptor." J Immunol **171**(12): 6714-22.

Schuttert, J. B., M. H. Liu, et al. (2003). "Human renal fibroblasts derived from normal and fibrotic kidneys show differences in increase of extracellular matrix synthesis and cell proliferation upon angiotensin II exposure." Pflugers Arch **5**: 5.

Schwartz, G. K., S. M. Redwood, et al. (1990). "Inhibition of invasion of invasive human bladder carcinoma cells by protein kinase C inhibitor staurosporine." J Natl Cancer Inst **82**(22): 1753-6.

Shapiro, S. D. (1998). "Matrix metalloproteinase degradation of extracellular matrix: biological consequences." Curr Opin Cell Biol **10**(5): 602-8.

Shawver, L. K., D. Slamon, et al. (2002). "Smart drugs: tyrosine kinase inhibitors in cancer therapy." Cancer Cell **1**(2): 117-23.

Shin, H. S., H. J. Lee, et al. (2003). "Betacellulin and amphiregulin induce upregulation of cyclin D1 and DNA synthesis activity through differential signaling pathways in vascular smooth muscle cells." Circ Res **93**(4): 302-10.

Sieuwerts, A. M., J. G. Klijn, et al. (1997). "Assessment of the invasive potential of human gynecological tumor cell lines with the in vitro Boyden chamber assay: influences of the ability of cells to migrate through the filter membrane." Clin Exp Metastasis **15**(1): 53-62.

Slack, B. E. (2000). "The m3 muscarinic acetylcholine receptor is coupled to mitogen-activated protein kinase via protein kinase C and epidermal growth factor receptor kinase." Biochem J **348**(Pt 2): 381-7.

Soltoff, S. P. (1998). "Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P2Y2 receptor. Phorbol ester or [Ca2+]i elevation can substitute for receptor activation." J Biol Chem **273**(36): 23110-7.

Stocker, W. and W. Bode (1995). "Structural features of a superfamily of zinc-endopeptidases: the metzincins." Curr Opin Struct Biol **5**(3): 383-90.

Strachan, L., J. G. Murison, et al. (2001). "Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily." J Biol Chem **276**(21): 18265-71.

Sunnarborg, S. W., C. L. Hinkle, et al. (2002). "Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability." J Biol Chem **277**(15): 12838-45.

Tanyi, J. L., A. J. Morris, et al. (2003). "The human lipid phosphate phosphatase-3 decreases the growth, survival, and tumorigenesis of ovarian cancer cells: validation of the lysophosphatidic acid signaling cascade as a target for therapy in ovarian cancer." Cancer Res **63**(5): 1073-82.

Troyer, K. L., N. C. Luetteke, et al. (2001). "Growth retardation, duodenal lesions, and aberrant ileum architecture in triple null mice lacking EGF, amphiregulin, and TGF-alpha." Gastroenterology **121**(1): 68-78.

Tsai, W., A. D. Morielli, et al. (1997). "The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity." Embo J **16**(15): 4597-605.

Uchiyama-Tanaka, Y., H. Matsubara, et al. (2001). "Angiotensin II signaling and HB-EGF shedding via metalloproteinase in glomerular mesangial cells." Kidney Int **60**(6): 2153-63.

Ullrich, A. and J. Schlessinger (1990). "Signal transduction by receptors with tyrosine kinase activity." Cell **61**(2): 203-12.

Van Brussel, J. P. and G. H. Mickisch (1999). "Prognostic factors in renal cell and bladder cancer." BJU Int **83**(8): 902-8; quiz 908-9.

Van Corven, E. J., A. Groenink, et al. (1989). "Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins." Cell **59**(1): 45-54.

Vinals, F. and J. Pouyssegur (2001). "Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling." Mol Cell Biol **21**(21): 7218-30.

Vogelzang, N. J. and W. M. Stadler (1998). "Kidney cancer." Lancet **352**(9141): 1691-6.

Wang, H. Q., T. Quan, et al. (2003). "Epidermal growth factor receptor-dependent, NF-kappaB-independent activation of the phosphatidylinositol 3-kinase/Akt pathway inhibits ultraviolet irradiation-induced caspases-3, -8, and -9 in human keratinocytes." J Biol Chem **278**(46): 45737-45.

Weiss, F. U., H. Daub, et al. (1997). "Novel mechanisms of RTK signal generation." Curr Opin Genet Dev **7**(1): 80-6.

Wetzker, R. and F. D. Bohmer (2003). "Transactivation joins multiple tracks to the ERK/MAPK cascade." Nat Rev Mol Cell Biol **4**(8): 651-7.

Wolfsberg, T. G., P. Primakoff, et al. (1995). "ADAM, a novel family of membrane proteins containing A Disintegrin And Metalloprotease domain: multipotential functions in cell-cell and cell-matrix interactions." J Cell Biol **131**(2): 275-8.

Wong, S. T., L. F. Winchell, et al. (1989). "The TGF-alpha precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction." Cell **56**(3): 495-506.

Xiao, D., X. Qu, et al. (2003). "Activation of extracellular signal-regulated kinase mediates bombesin-induced mitogenic responses in prostate cancer cells." Cell Signal **15**(10): 945-53.

Xu, Y., D. C. Gaudette, et al. (1995). "Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients." Clin Cancer Res **1**(10): 1223-32.

Xu, Y., Z. Shen, et al. (1998). "Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers." Jama **280**(8): 719-23.

Yamada, T., K. Sato, et al. (2003). "Lysophosphatidic acid in malignant ascites stimulates motility of human pancreatic cancer cells through LPA1." J Biol Chem **3**: 3.

Yan, Y., K. Shirakabe, et al. (2002). "The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors." J Cell Biol **158**(2): 221-6.

Yano, S., K. Kondo, et al. (2003). "Distribution and function of EGFR in human tissue and the effect of EGFR tyrosine kinase inhibition." Anticancer Res **23**(5A): 3639-50.

Yarden, Y. and M. X. Sliwkowski (2001). "Untangling the ErbB signalling network." Nat Rev Mol Cell Biol **2**(2): 127-37.

Yu, D. S., D. S. Hsieh, et al. (2003). "Synergism of Fas-mediated apoptosis and tumor necrosis factor on adriamycin cytotoxicity to transitional cell carcinoma." Urol Int **70**(3): 161-6.

Zwick, E., J. Bange, et al. (2002). "Receptor tyrosine kinases as targets for anticancer drugs." Trends Mol Med **8**(1): 17-23.

Zwick, E., H. Daub, et al. (1997). "Critical role of calcium- dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling." J Biol Chem **272**(40): 24767-70.

Zwick, E., P. O. Hackel, et al. (1999). "The EGF receptor as central transducer of heterologous signalling systems." Trends Pharmacol Sci **20**(10): 408-12.

Zwick, E., C. Wallasch, et al. (1999). "Distinct calcium-dependent pathways of epidermal growth factor receptor transactivation and PYK2 tyrosine phosphorylation in PC12 cells." J Biol Chem **274**(30): 20989-96.

7 Abbrevations

Ab	Antibody
ADAM	A disintegrin and metalloprotease domain
Amp_r	Ampicilline resistence
APS	Ammoniumpersulfate
AR	Amphiregulin
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
cAMP	Cyclic adenosinmonophosphate
Ca^{2+}	Calcium Ions
CaM Kinase	Ca^{2+}-calmodulin-dependent kinase
c-fos	Cellular homologue to v-fos (FBJ murine osteosarcoma viral oncogene)
c-jun	Cellular homologue to v-jun (avian sarcoma virus 17 oncogene)
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DN	Dominant negative
DMSO	Dimethylsulfoxide
DNA	Desoxyribonukleic acid
dsDNA	Dooble-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethlendiamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fig	Figure
g	Gramm
Gab1	Grb2-associated binder-1
Gab2	Grb2-associated binder-2
GDP	Guanosindiphosphate
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor binding protein 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin
HB-EGF	Heparin-binding EGF-like growth factor
H_2O_{bidest}	Twice-destilled, deionised Water
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-

	Ethansulfonic acid
HER	Human EGFR-related
HNSCC	Head and neck squamous cell carcinoma
Ig	Immunglobulin
IP	Immunoprecipitation
IP_3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl-ß-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
l	Liter
LPA	Lysophosphatydic acid
µ	Micro
m	Milli
M	Molar
MAP	Mitogen-activated protein
MAPK	MAP kinase
MBP	Myelin basic protein
MEK	MAPK/ERK Kinase
min	Minute
MMP	Matrix metalloprotease
n	Nano
OD	Optical density
p.a.	Per analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylenglycole
PI 3-Kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
PNPP	p-Nitrophenyl-phosphate
PTX	Pertussis toxin
PY	Phospho-tyrosine
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
S. D.	Standard deviation
SDS	Natriumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Sek.	Second
SH2, 3	domain Src homology 2, 3 domain
SHP-2	SH2-containing PTP-2

Sos	Son of sevenless
Src	Homologue to v-src (sarcoma viral oncogene)
TACE	TNFα-converting enzyme
TCA	Trichloroacetic acid
TGFα	Transforming growth factor alpha
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
TNFα	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethan
Tween 20	Polyoxyethylensorbitanmonolaureate
U	Enzymatic activity unit
O/N	Overnight
UV	Ultraviolett
V	Volt
Vol	Volume
Wt	Wild type

Curriculum Vitae

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Publications arising from this work

Schäfer, B., Gschwind, A. and Ullrich A. (2004) Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene. 23(4):991-999*

Schäfer, B., Gschwind, A. and Ullrich A. (2004) Distinct ADAM metalloproteinases regulate G-protein coupled receptor-induced cell proliferation and survival. *JBC. (submitted)*

Patent Application

EP 02005452, Prof. A. Ullrich, B. Schäfer, O. Fischer, Andreas Gschwind, M. Leserer. Use of EGFR transactivation inhibitors in human cancer