Characterisation of the Molecular Mechanisms of EGFR Signal Transactivation in Human Cancer

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Martinsried,

Stefan Hart

For my Mum and Dad

Contents

1. Introduction

No mammalian cell lives in isolation and therefore has to respond and coordinate a wide variety of extracellular signals which are necessary for multicellular organisms during embryonic development and adult life. This ability allows the organism to alter physiological processes due to changes of its environment. The adjustment of complex cell functions is achieved by the regulated information transfer along signalling pathways. It has become apparent, however, that these linear pathways are not free-standing entities but parts of larger networks. Communication pathways transduce and exchange information between different cells but also from different compartments within one cell. Neighboring cells often communicate by direct cell-cell contact, whereas distant cells use secreted signalling molecules, such as growth factors and hormones, which bind to cognate receptors at the target cells and ultimately induce distinct biological responses like cell proliferation, migration, differentiation or apoptosis. Deregulation of these signalling events was identified as a cause of many severe diseases e.g. cancer, diabetes, immune deficiencies and cardiovascular diseases (Hanahan and Weinberg, 2000; Schlessinger, 2000; Shawver et al., 2002).

The reversible phosphorylation has been identified as a central regulatory element of most signal transduction processes (Cohen, 2002). Phosphorylation by the action of a protein kinase and dephosphorylation catalyzed by protein phosphatases can reversibly modify protein function by increasing or decreasing its biological activity, by stabilization or targeting proteins for degradation, by alteration of the localization within the cell or by facilitating or disrupting protein-protein interactions. These findings are even more fascinating by the fact that one third of all mammalian proteins can be phosphorylated. The sequencing of the human genome project identified 518 putative protein kinase genes and 130 protein phosphatases (Blume-Jensen and Hunter, 2001b; Manning et al., 2002). According to their localization and their substrate specificity, both protein kinases and phosphatases can be subdivided into cellular and transmembrane molecules and into tyrosine or serine/threonin-specific kinases and phosphatases.

1.1. Receptor tyrosine kinase

A large family of cell surface receptors contains intrinsic protein tyrosine kinase activity. These receptor tyrosine kinases (RTKs) consist of a glycosylated extracellular domain, a single transmembrane portion and an intracellular domain with a protein kinase core catalyzing the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target

proteins (Hunter, 1998). Activation of receptor tyrosine kinases occurs through ligand-induced dimerisation, which juxtaposes the catalytic domains resulting in transphosphorylation initially in the catalytic domain and afterwards in regions lying outside the catalytic domain. The phosphotyrosines generated create docking sites for target proteins such as intracellular signal transducers containing phosphotyrosine interaction domains (Hunter, 2002; Schlessinger, 2000).

RTKs play a critical role in the regulation of various cellular processes including the cell cycle, migration, metabolism, survival, proliferation and differentiation. The human genome contains 90 tyrosine kinase genes of which 58 encode transmembrane receptor kinases (RTKs). RTKs are split up in 20 subfamilies depending on the structural motives of the extracellular ligand binding domain such as cystein rich domains, EGF-like (epidermal-growth factor-like) domains, immunoglobuline-like domains, cadherin-like domains and kringle-like domains among others (Figure 1).

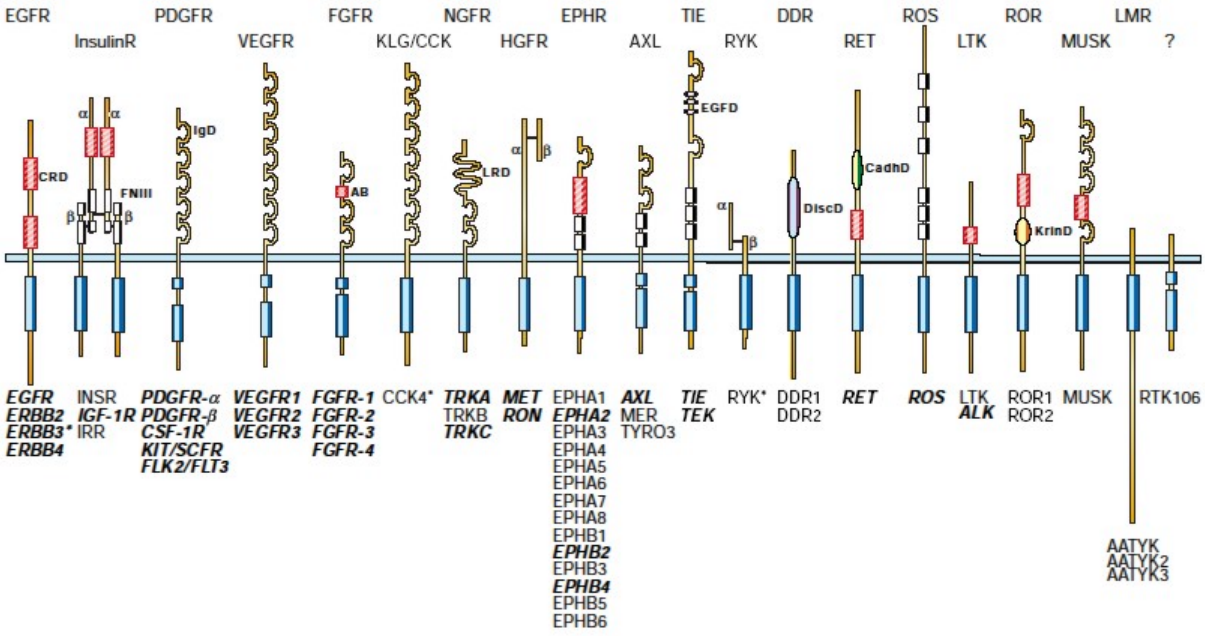


Figure 1 Subfamilies of human receptor tyrosine kinases. Abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols α and β denote distinct RTK subunits (Blume-Jensen and Hunter, 2001b).

1.1.1. The EGFR family

The epidermal growth factor receptor (EGFR) family which consists of four closely related receptor tyrosine kinases, the EGFR itself, HER2/neu, HER3 and HER4, has been recognised as a convergence point for diverse signal transduction pathways. Ligand binding induces the

formation of various homo- or heterodimers of EGFR family members subsequently triggering autophosphorylation of cytoplasmic tyrosine residues. These phosphorylated tyrosine residues represent docking sites for a variety of signal transducers, such as SHC, Grb2 and Gab1.

The EGFR is the most prominent RTK and was the first cell surface signalling protein to be identified by molecular genetic methods (Ullrich and Schlessinger, 1990).

Deregulation of this tightly controlled system of receptor–receptor interactions by overexpression, amplification or mutations of critical pathway elements and/or autocrine stimulation through aberrant growth factor loops is frequently linked to hyperproliferative diseases such as cancer.

Various investigations revealed a unique role of HER2, which has to be transphosphorylated through heterodimerisation with an other ligand-occupied EGFR family member for activation, as no ligand for HER2 has been described so far (Goldman et al., 1990). In addition, HER2 is the preferred dimerisation partner for EGFR, HER3 and HER4 and shows a high transforming activity (Beerli et al., 1995; Graus-Porta et al., 1997). On the other hand, HER3 possesses an impaired kinase activity due to point mutations within the catalytic domain.

1.1.2. EGF-like ligands and receptor activation

There are 8 known ligands that act as direct agonists for the EGFR: epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor alpha (TGFα), amphiregulin, betacellulin, epigen, epiregulin and cripto which are (except cripto) synthesized as transmembrane precursors and proteolytically cleaved by metalloproteases to release the mature growth factors (Massague and Pandiella, 1993; Riese and Stern, 1998; Salomon et al., 1999; Strachan et al., 2001)(Figure 2). Subsequently, the soluble ligands activate RTKs of the EGFR family by autocrine or paracrine stimulation. Furthermore, several studies indicate that EGF-like growth factor precursors may be also biologically active (Brachmann et al., 1989; Wong et al., 1989). In addition, various neuregulin (NRG) isoforms act as ligands for HER3 and HER4.

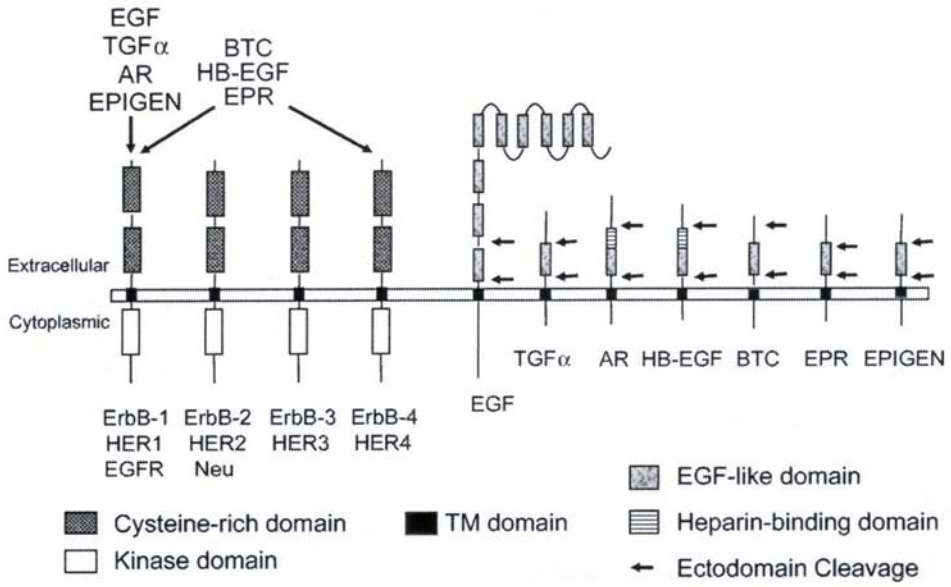


Figure 2 The EGFR family and EGF-like ligands binding to the EGFR (Harris et al., 2003). The neuregulins are not shown. The EGF-like ligands that are capable of stimulating the EGFR are depicted, including their domain structure. The characteristic feature of this family of growth factors is that they are synthesized as transmembrane proteins that have to be processed to become fully active (Jorissen et al., 2003).

Despite having distinct receptor binding specificity, all of these growth factors have an EGF-like domain as a common motif, which is defined by six conserved cystein residues forming three disulfide bridges that generate three looped regions, designated the A-, B-, and C-loop (Wingens et al., 2003).

New insights on the ligand-binding and receptor activation mechanisms were achieved by recent structural studies supporting the concept of ligand-induced conformational changes leading to receptor dimerisation rather than bridging of receptor monomers by ligand molecules (Garrett et al., 2002a; Jorissen et al., 2003; Ogiso et al., 2002; Schlessinger, 2002). After ligand-binding , a so-called “dimerisation-loop”, which has been found so far only in EGFR-family receptors, is responsible for the entirely receptor-mediated receptor dimerisation. Mutations or deletions in this loop completely prevent ligand-induced EGFR activation (Garrett et al., 2002a; Ogiso et al., 2002). Interestingly, HER2, which has no direct ligand, constitutively mimics an extended configuration with its dimerisation arm exposed (Cho et al., 2003; Garrett et al., 2002b).

1.1.3. Cytoplasmic tyrosine kinases

Many cell surface receptors depend on tyrosine kinase activity for their signalling properties, but lack tyrosine kinase function. In order to compensate the deficiency, they act through associated cytoplasmic tyrosine kinases which phosphorylate various target proteins. The 32

cytoplasmic tyrosine kinases, also called nonreceptor tyrosine kinases (NRTKs), are classified into 10 subfamilies based on kinase domain sequence: ABL, ACK, CSK, FAK, FES, FRK, JAK, SRC, TEC and SYK (Robinson et al., 2000).

Besides binding to cell surface receptors, NRTKs are localized at several subsellular sites including the nucleolus, cytoplasm, mitochondria, the endoplasmatic reticulum and the cell membrane through amino-terminal modifications, such as myristylation or palmitoylation (Hantschel and Superti-Furga, 2004). In addition to a tyrosine kinase activity, NRTKs contain domains mediating protein-protein, protein-lipid, and protein-DNA interactions (Hubbard and Till, 2000).

Currently, there are at least nine members of the largest subfamily of NRTK, the Src family : Src, Yes, Fyn, Fgr, Yrk, Lyn, Blk, Hck, and Lck. (Blume-Jensen and Hunter, 2001a). Src family members participate in a variety of signalling processes, including mitogenesis, T- and B-cell activation, and cytoskeleton restructuring. Furthermore, multiple substrates have been described for Src, such as PDGF and EGF receptors, the NRTK focal adhesion kinase (Fak), p130Cas, an adapter protein involved in integrin- and growth factor-mediated signalling (Hubbard and Till, 2000). Src has been implicated in several human carcinomas, including breast, lung, and colon cancer (Biscardi et al., 1999).

1.1.4. RTK downstream signalling and protein interaction domains

Ligand-induced RTK dimerisation and autophosphorylation activates specific intracellular signal transduction pathways, depending on the stimulus and the cellular context. To regulate many different cellular processes, most proteins involved in intracellular signalling contain modular protein domains, that specifically interact with other protein domains, lipids, and nucleic acids. In RTK signalling, among the different signalling domains, the most important are those which recognise the phosphorylated tyrosine itself (Schlessinger and Lemmon, 2003). Src homology 2 (SH2) domains are the most frequent interaction domains for phosphorylated tyrosines, which bind specifically to distinct amino acid sequences defined by 1-6 residues C-terminal to the phosphotyrosine moiety (Schlessinger, 2000). On the other hand, phosphotyrosine binding (PTB) domains are not restricted to bind phosphotyrosine residues, but also nonphosphorylated peptide sequences.

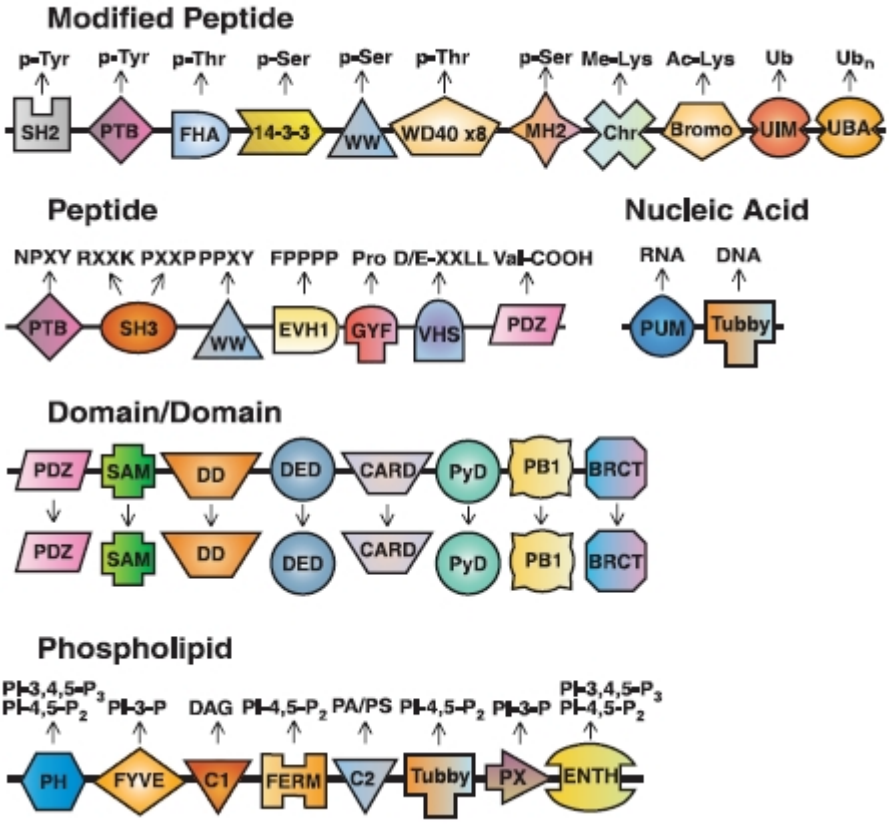


Figure 3 Modular interaction domains in signal transduction. Interaction domains bind proteins, phospholipids, or nucleic acid. A subset of such domains is illustrated and their general binding functions are indicated (Pawson and Nash, 2003).

Upon ligand-induced autophosphorylation of RTKs, SH2 domain-containing signalling proteins are recruited and activated, such as PLC-γ which binds to the autophosphorylated tail of the EGFR and translocates to the plasma membrane. Besides phosphotyrosine residues, WW and 14-3-3 domains bind to phosphoserine and phosphothreonine residues are recognised by FHA and WD40 domains. SH3 domains bind specifically to the proline-rich sequence motiv PXXP, pleckstrin homology (PH), phox homology (PHOX), FERM and FYVE domains bind to phosphoinositides. Figure 3 shows an overview of different interaction domains and their specific binding specificities.

Furthermore, a wide variety of proteins possesses besides their interaction domain an enzymatic activity. In addition to their SH2 domain, Src kinases have a protein kinase activity, SHP2 has a protein tyrosine phosphatase activity and PLC-γ a phospholipase C activity. Some signalling proteins exclusively consist of SH2 and SH3 domains, such as Grb2, Crk of SHC linking activated RTKs to downstream signalling events.

The ability of RTKs to recruit and activate a wide variety of adaptor proteins provides the cell with a powerful tool to regulate biological responses.

1.2. G protein-coupled receptors

With more than 1000 receptors, the cell surface superfamily of G-protein-coupled receptors (GPCRs) is the largest protein family representing up to 1% of the total genome of mammalians (Hermans, 2003). GPCRs are involved in diverse physiological functions including neurotransmission, photoreception, chemoreception, metabolism, growth and differentiation (Fukuhara et al., 2001).

Based on their highly conserved protein structure, GPCRs are also called seven-transmembrane domain receptors as seven α-helices cross the plasma membrane. The NH_2 terminus is exposed to the extracellular environment and is suggested to play a role in ligand binding. Both the C-terminus and the intracellular loops interact with intracellular signalling partners, such as G proteins, but also with a wide variety of proteins containing structural interacting domains including PDZ, SH3, or PTB domains (Bockaert et al., 2003). To add another layer of complexity, dimers or higher oligomers among identical GPCRs, close family members, or GPCRs from different families influence ligand binding, receptor activation, desensitization, trafficking and receptor signalling (Breitwieser, 2004).

1.2.1 Heterotrimeric G proteins

A common biochemical characteristic of GPCRs is their interaction and activation of downstream signalling cascades by G proteins, although some studies reported G protein independent signalling through some GPCRs (Bockaert and Pin, 1999; Hall et al., 1999).

G proteins are composed of an α-subunit interacting with a βγ subunit. Ligand binding to GPCRs alters the conformation of intracellular domains of the receptor and induces the association with heterotrimeric G proteins. This results in an exchange of GDP for GTP in the active site of the α-subunit, followed by the dissociation of the heterotrimeric complex. Both the Gα and Gβγ subunits activate effector molecules which can be membrane bound or cytoplasmic (Hermans, 2003).

The specific and complex signalling induced by GPCRs is due to at least 23 α-subunits derived from 17 different genes, 6 β- and 12 different γ-subunits. G proteins are generally referred to their Gα-subunits, which are subdivided on the basis of amino acid similarities in four distinct families, namely $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12}$ (Hermans, 2003; Pierce et al., 2001).

On the other hand, the number of downstream effectors is rather limited and many G proteins couple with the same intracellular effector. Effectors comprise adenylyl and guanyly cyclase, calcium channels, GTPase-activating proteins (GAPs), guanine-nucleotide exchange-factors

(GEFs), c-Src tyrosine kinase, phosphodiesterase and phospholipases. Thereby, the signalling cascades induced by activated G proteins influence the level of second messengers like cyclic AMP and cyclic GMP, diaglycerol, inositol (1,4,5)-triphosphate, phosphatidyl inositol (3,4,5)-triphosphate or calcium levels (Marinissen and Gutkind, 2001; Pierce et al., 2001).

1.2.2 Mitogenic GPCR Signalling

Many reports suggest GPCRs to induce mitogenic responses and to be involved in the regulation of pathologic proliferation. Potent mitogens such as acetylcholine, angiotensin, bombesin, bradykinin, endothelin-1, isoproterenol, LPA, neurotensin, prostaglandin or thrombin have been demonstrated to induce mitogenic responses in tissue culture systems (Daaka, 2004).

The discovery of the *mas* oncogene, which protein product displays a heptahelical structure, demonstrated a connection between GPCRs and cellular transformation (Young et al., 1986).

Furthermore, several oncogenes encode mutated forms of GPCRs or their associated G proteins. Activating mutation of the thyroid-stimulating hormone and luteinizing hormone receptor have been detected in adenoma of the thyroid and hyperplastic Leydig`s cells, respectively (Parma et al., 1993; Shenker et al., 1993). In addition, the genomes of various transforming viruses such as Kaposi`s sarcoma-associated herpesvirus contain sequences encoding constitutive active GPCRs which are shown to induce cancer in animal models (Montaner et al., 2003).

Activating mutants of Gα-subunits encoding transforming oncogenes such as *gsp, gip2* and *gep* are described (Landis et al., 1989; Lyons et al., 1990; Xu et al., 1993). Moreover, ectopically expressed GPCRs such as muscarinic acetylcholine M_1, M_2, and M_5 receptors were capable to transform fibroblasts dependent on agonists stimulation suggesting that endogenous GPCRs can be tumourigenic when exposed to an excess of locally produced or circulating agonists. LPA, a phospholipid, represents the major mitogenic activity in serum and is involved in rapid cytoskeletal rearrangements (Gohla et al., 1998), cell proliferation (van Corven et al., 1989), suppression of apoptosis (Fang et al., 2000) and tumour cell migration and invasion (Fishman et al., 2001; Imamura et al., 1993). Interestingly, the LPA concentration is elevated in the plasma of ovarian cancer patients (Xu et al., 1995). The cell surface receptors for LPA and the structurally related phospholipids sphingosine-1-phosphate (S1P) are members of the EDG (endothelial cell differentiation gene) subfamily of GPCRs (Kranenburg and Moolenaar, 2001) (Pyne and Pyne, 2000). Three LPA receptors (EDG2, EDG4 and EDG7) and four S1P receptors (EDG1, EDG3, EDG5 and EDG7) have been

described which couple to various subtypes of G proteins and show distinct properties in ligand specificity and activation of intracellular signalling pathways.

1.2.3. GPCR signalling in the central nervous system

Neuropeptides, including neurotensin, vasopressin, substance P, and bradykinin among many others are molecular messengers that regulate multiple functions in the central nervous system and in the periphery via G protein-coupled receptors. These peptides function as neurotransmitters and play a crucial role in smooth muscle contradiction, pain transmission, blood pressure and inflammation. In the central nervous system, they regulate food intake, body temperature and behavioural responses (Rozengurt, 2002).

In addition, neuropeptides can act directly as potent cellular growth factors for different cell types. Furthermore, many studies propose neuropeptides to be involved as autocrine/paracrine growth factors in human cancer (Rozengurt, 2002).

1.2.4. Cannabinoid receptors and cancer

Δ^9-Tetrahydrocannabinol (THC), the endogenous cannabinoid anandamide and synthetic cannabinoids like HU-210 and Win55,212-2 interact with specific G protein-coupled receptors. Two subtypes of the cannabinoid receptors, CB1 and CB2, have been cloned and characterised (Matsuda et al., 1990; Munro et al., 1993). The CB1 receptor, which is responsible for the well known psychotropic effects of cannabinoids, is highly expressed in the central nervous system but lower levels are also present in immune cells and peripheral tissues including testis, whereas the CB_2 receptor is predominantly expressed in immune cells (Parolaro et al., 2002; Pertwee, 1997; Pertwee and Ross, 2002). Both cannabinoid receptors are coupled to heterotrimeric $G_{i/o}$-proteins and activate the mitogen activated protein kinases (MAPK) ERK1/2 and p38 as well as the Akt/PKB survival pathway (Guzman et al., 2002; Piomelli, 2003).

Recent investigations propose that drugs activating the endogenous cannabinoid system might be used in cancer therapy to slow down or block cancer growth (Hall and Degenhardt, 2003). The endogenous cannabinoid anandamide (AEA) acts antiproliferative in MCF-7, EFM-19, T47D and DU145 cells (Guzman et al., 2002). Furthermore, the biological responses to cannabinoids critically depend on drug concentration and cellular context. High concentrations of cannabinoids are shown to block immune cells, whereas Derocq and coworkers demonstrated proliferation in human B-cells after cannabinoid stimulation at

nanomolar concentrations (Cabral and Dove Pettit, 1998; Derocq et al., 1995; Klein et al., 1998).

1.3. Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes and have been implicated in cell migration, invasion, proliferation, angiogenesis, cell differentiation and cell survival. MAPKs are serine/threonine protein kinases mediating the response of cells to extracellular stimuli to critical regulatory targets within the cell. At least four distinctly regulated groups of MAPKs are expressed in mammals, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38α/β/γ/δ) and ERK5 (Chang and Karin, 2001). A major function of MAPK pathways is the control of gene expression by either direct phosphorylation of transcription factors, but they can also target coactivators and corepressors (Yang et al., 2003).

The most prominent MAPK cascade is the activation of ERK1/2 by RTKs. Stimulation of RTKs leads to the recruitment of the adaptor protein Grb2 and association and activation of the RAS-GEF Sos, which subsequently activates membrane-associated Ras. Ras in turn induces the serine/threonine kinase activity of the MAPK kinase kinase (MAPKKK) Raf-1 which phosphorylates and activates the MAPK kinases 1/2 (MAPKK, MEK 1/2). Finally, MEK1/2 activate ERK1/2 by phosphorylation of threonine and tyrosine residues in the regulatory TEY-motif (Robinson and Cobb, 1997). Thereafter, ERK1/2 either translocate into the nucleus to regulate gene expression or effect cytoplasmic or membrane bound effectors, such as influencing transmembrane protein processing by phosphorylation of the intracellular domain of the metalloprotease ADAM17 (Diaz-Rodriguez et al., 2002; Fan and Derynck, 1999).

On the other hand, JNK-family MAPKs are also known as stress-activated kinases as their activation results as a response to environmental stress but also by radiation and growth factors. p38 becomes activated by hormones, GPCR agonists and cellular stress such as osmotic and heat shock or chemotherapeutic agents, but also by inflammatory cytokines in immune cells (Benhar et al., 2002; Chen et al., 2001).

Figure 4 shows the complexity and diversity of MAPK signalling, as each MAPKK can be activated by more than one MAPKKK.

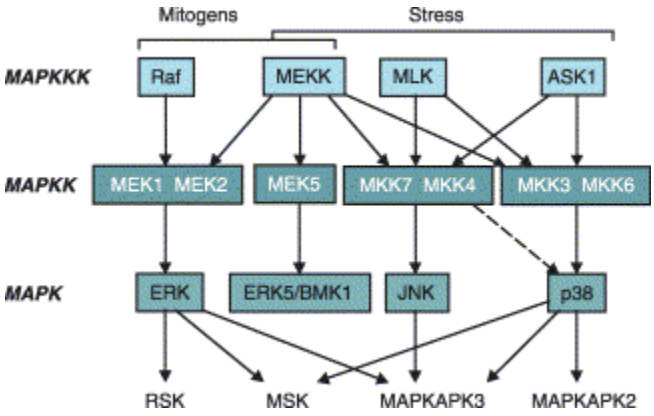


Figure 4 Organisation of mammalian MAPK cascades. MAPK cascades feature a core triple kinase module consisting of MAPKKKs, MAPKKs and MAPKs. There are a number of MAPKKK families and individual MAPKKKs are often components of more than one MAPK cascade. MAPKKs selectively target a particular MAPK. A potential exception is MKK4 which has been reported to activate p38 (dashed line) in addition to its major target JNK. MAPKs can phosphorylate transcriptional targets directly or this can occur via the indicated downstream protein kinases (Yang et al., 2003).

1.4. EGFR signal transactivation

In 1996, Daub and co-workers identified the EGFR as an essential element in GPCR-induced mitogenesis of rat fibroblasts (Daub et al., 1996). Upon treatement of cells with the G protein-coupled receptor agonists lysophosphatidic acid (LPA), endothelin-1 (ET-1) and thrombin, the EGFR and its relative HER2 were found to be rapidly tyrosine-phosphorylated. This transactivation of a receptor tyrosine kinase coupled GPCR-ligand engagement to extracellular regulated kinase (ERK)1/2 activation and induction of *c-fos* gene expression.

Further investigations revealed that GPCR-EGFR cross-talk pathways are established in a variety of other cell types such as human keratinocytes, primary mouse astrocytes, PC12 cells and vascular smooth muscle cells and established them as widely relevant towards the activation of the MAP kinase signal (Hart, 2003). In addition, preincubation of Cos-7 cells with pertussis toxin blocked LPA-induced EGFR signal transactivation, implicating the involvement of Gα subunits of the $G\alpha_{i/o}$ family of G proteins (Daub et al., 1997). Interstingly, EGFR transactivation was observed after agonist stimulation of ectopically expressed $G\alpha_q$-coupled bombesin (BombR) or $G\alpha_i$-coupled M_2 muscarinic acetylcholine receptor (M_2R) indicating that various Gα subunits of the G proteins are involved in transactivation of the EGFR depending on the stimulus and the cellular context.

Due to the rapid onset of GPCR-induced EGFR tyrosine phosphorylation and the fact that EGF-like ligands were not found in cell culture medium after G protein activation the transactivation mechanism was postulated to exclusively rely on intracellular elements (Daub et al., 1996; Zwick et al., 1999). Different cytoplasmic tyrosine kinases, Ser/Thr kinases and

second messengers have been discussed as potential mediators of the transactivation signal. However, several studies indicate that the EGFR transactivation mechanism is subject to different cell type-characteristic regulatory influences. For example, it was reported, that EGFR activation by G_q-coupled receptors involves Ca^{2+} in neuron-like PC12 cells, vascular smooth muscle cells, ovarian cancer cells and intestinal epithelial cells (Eguchi et al., 1998; Murasawa et al., 1998; Venkatakrishnan et al., 2000; Zwick et al., 1997).

In contradiction to a ligand-independent mechanism, Prenzel *et al.* reported that a chimeric RTK consisting of the ligand-binding domain of the EGFR and the transmembrane and intracellular portion of the PDGFR, was transactivated upon treatment of Rat-1 fibroblasts with GPCR ligands resulting in a PDGFR-characteristic intracellular signal (Prenzel et al., 1999). These findings suggested a critical function of the EGFR extracellular domain in the GPCR-EGFR cross-talk mechanism, as the PDGFR was not tyrosine phosphorylated upon treatment of Rat-1 cells with GPCR ligands. Furthermore, a system of co-cultured cell lines stably expressing either the M1R or the human EGFR resulted in intercellular EGFR transactivation after carbachol stimulation. In addition, LPA-, carbachol- or tetra-decanoyl-phorbol-13-acetate (TPA) - induced transactivation of the EGFR and tyrosine phosphorylation of SHC were completely abrogated by the diphtheria toxin mutant CRM197 that specifically blocks proHB-EGF function or the metalloprotease inhibitor batimastat (BB94) in Cos-7 and HEK 293 cells. Flow cytometric analysis directly confirmed cell-surface ectodomain shedding of proHB-EGF upon treatment with GPCR agonists or TPA.

These data established the concept of a "Triple-Membrane-Passing-Signal" (TMPS) mechanism of EGFR signal transactivation involving a metalloprotease activity and processing of the transmembrane EGF-like growth factor precursor proHB-EGF (Prenzel et al., 1999)(Figure 5).

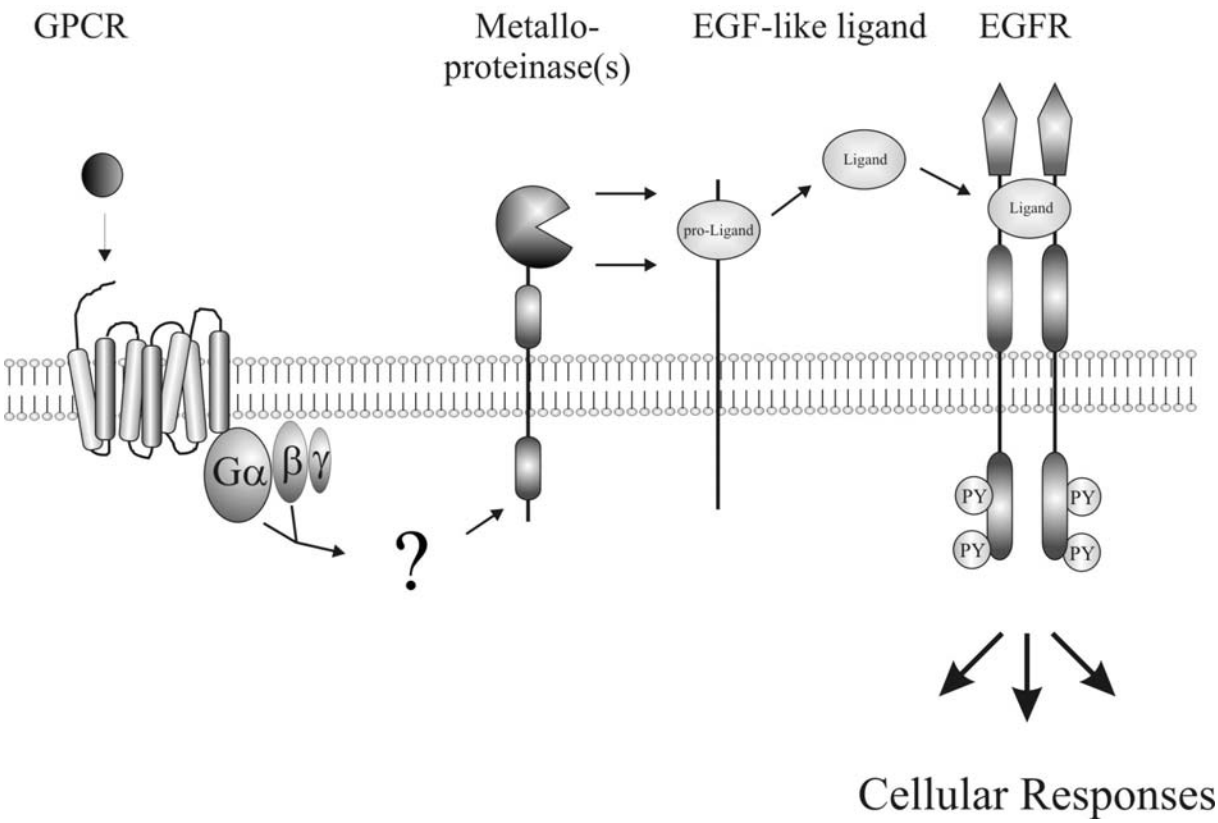


Figure 5 Triple-Membrane-Passing Signal Mechanism (TMPS) of EGFR transactivation. GPCR-induced and metalloprotease-mediated cleavage of proEGF-like growth factor ligands leads to transactivation of the EGFR. Three membrane passages are enclosed in this mechanistic model for ligand-dependent interreceptor communication and couples GPCR activation to EGFR characteristic downstream signalling pathways.

Since the initial discovery of this mechanism, many studies demonstrate the broad relevance of this signalling mechanism within a variety of cellular systems and, importantly, the involvement in the development and progression of pathophysiological processes. In this regard, growing evidence indicates transmembrane metalloproteases as the key enzymes of growth factor precursor shedding.

The metalloprotease inhibitor batimastat (BB94) was shown to inhibit bombesin- and TPA-induced transactivation of the EGFR in PC-3 human prostate cancer cells and to reduce high constitutive levels of EGFR tyrosine phosphorylation in unstarved PC3 (Prenzel et al., 1999) and SCC25 squamous cell carcinoma cells (Gschwind et al., 2002). Furthermore, Dong and colleagues reported that BB94 reduced cell proliferation and cell migration of an human mammary epithelial cell line by interfering with the release of EGFR ligands (Dong et al., 1999). BB94 also inhibited proliferation of colon and breast cancer cell lines which were known to depend on autocrine signalling through the EGFR. Very recently, Gschwind *et al.* showed that LPA-induced proliferation and motility of head and neck cancer cells involves metalloprotease-dependent transactivation of the EGFR (Gschwind et al., 2002). Moreover, EGFR function was reported to be critical for GPCR stimulated mitogenic signalling in

several other cancer cell lines including U-373 MG glioblastoma cells (Castagliuolo et al., 2000) and SK-OV-3 ovarian cancer cells (Venkatakrishnan et al., 2000).

In addition to EGFR transactivation, other RTKs such as the insulin-like growth factor receptor (IGF-1R), hepatocyte growth factor receptor (Met-R), vascular endothelial cell growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and the Fibroblast growth factor receptor (FGFR)-1 have been shown to be activated by GPCR agonists (Belcheva et al., 2001; Endo et al., 2002; Herrlich et al., 1998; Pai et al., 2003; Weiss et al., 1997).

1.5. Metalloproteases

Proteases of the zinc protease superfamily are classified according to the primary structure of their catalytic sites and include carboxypeptidase, DD carboxypeptidase, gluzincin, inuzincin and metzincin subgroups (Hooper, 1994). The metzincins are further subdivided in serralysins, astacins, matrixins and adamalysins. Metalloproteases are fundamental in biological processes like cell proliferation, differentiation, remodelling of the extracellular matrix (ECM) and cell migration. These events are based on actions of metalloproteases, such as degradation of the ECM so that founder cells can move across tissue into nearby stroma, proteolytic cleavage of growth factors allows availability to cells not in direct physical contact and receptor cleavage to margin migratory signalling.

For the hydrolytic processing of substrates, the active centre of zinc metalloproteases contains water ions and zinc which is coordinated by three conserved histidine residues and a downstream methionine. The methionine constitutes a Met turn motiv that loops around to face the consensus HexxHxxGxxH site. The glutamic acid is believed to transfer hydrogen atoms and to polarize the 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 containing a terminal prodomain which keeps the metalloprotease site inactive by a cysteine switch (Becker et al., 1995). The processing and activation of metalloproteases by furin and other proprotein convertases (PCs) is proposed to occur at the trans-Golgi-network (Anders et al., 2001; Kang et al., 2002).

1.5.1. ADAMs

Metalloproteases of the ADAM (a disintegrin and a metalloprotease domain) family consists of a N-terminal signal sequence, followed by a prodomain, metalloprotease and disintegrin

domain, a cysteine-rich region, a single transmembrane domain and a cyplasmic domain of variable length (Figure 6). They contain features of both proteinases and cell surface adhesion molecules.

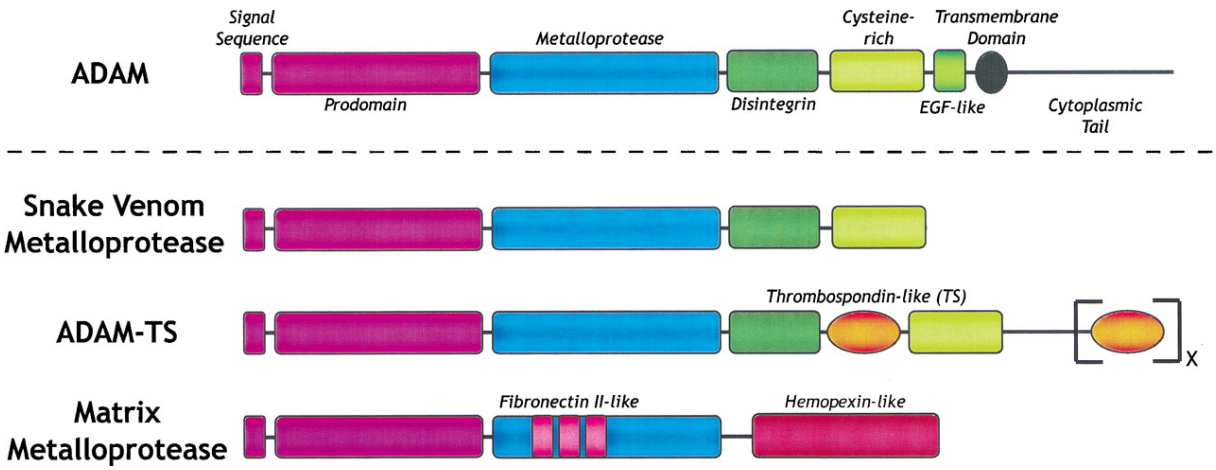


Figure 6 The domain structure of ADAM proteins and related metalloproteases. (Seals and Courtneidge, 2003)

19 different ADAM genes are known in humans, of which 12 show proteolytic activity (Kheradmand and Werb, 2002). Proteases of the ADAM family have been implicated in the control of membrane fusion, cytokine, RTK and growth factor shedding, cell migration and biological processes like muscle development, fertilization, cell fate determination and pathologies such as inflammation and cancer (Kheradmand and Werb, 2002; Schlondorff and Blobel, 1999).

The disintegrin domain of ADAMs allows the interaction with integrin receptors, but not all ADAM proteases have the RGD sequence in their disintegrin loop (Blobel and White, 1992). In contrast, the cystein-rich and EGF-like domain are not well characterised, but it is suggested that the cystein rich domain supplements the binding capacity of the disintegrin domain. Cancer cells are shown to interact with cell surface heparin-sulfate proteoglycans in an *in vitro* binding assay with the cystein-rich domain of ADAM12 serving as a ligand for the cell-adhesion molecule syndecan (Iba et al., 2000; Iba et al., 1999).

The cytoplasmic tails of proteases of the ADAM family vary both in length and in the protein sequence and contain motifs like PxxP binding sites for SH3 domain-containing proteins or potential phosphorylation sites for serine-threonine and/or tyrosine kinases. Many studies postulate that the cytoplasmic tail is involved in maturation and regulation of metalloprotease activity.

Tumour necrosis factor-α convertase (TACE/ADAM17) is the best characterised metalloprotease and was identified as the protease responsible for the release of the inflammatory cytokine tumour necrosis factor (TNF)-α from its membrane-bound precursor proTNFα (Black et al., 1997; Moss et al., 1997). Besides TNFα, ADAM17 mediates cleavage of diverse integral membrane proteins like L-selectin, p75 TNF receptor (Peschon et al., 1998), fractalkine (Garton et al., 2001), MUC1 (Thathiah et al., 2003), β-amyliod precursor protein (βAPP) (Buxbaum et al., 1998), p55 TNFR, interleukin-1 receptor (IL-1R) II (Reddy et al., 2000), erbB4/HER4 (Rio et al., 2000), the Notch1 receptor (Brou et al., 2000), IL-6R (Althoff et al., 2000), growth hormone-binding protein (Zhang et al., 2000), and cellular prion protein (Vincent et al., 2001). Studies using fibroblasts derived from ADAM17 knock-out mice implicated ADAM17 in the release of TGFα and other EGF-like ligands as well as the constitutive availability of these growth factors (Peschon et al., 1998; Sunnarborg et al., 2002). Mice with *tace*$^{\Delta Zn/\Delta Zn}$ null mutation die at birth with phenotypic defects, including failure of eyelid fusion, hair and skin defects, and abnormalities of lung development (Shi et al., 2003). The epithelial defects observed in *tace*$^{\Delta Zn/\Delta Zn}$ fetuses are similar to those reported in mice lacking the epidermal growth factor receptor (EGFR) (Peschon et al., 1998). Abrogation of TACE`s function in mice leads in perinatal lethality which is not seen in EGFR or TGFα deficient mice, indicating that TACE has additional substrates required for the development of some important organs that are necessary for survival (Shi et al., 2003).

1.5.2. MMPs and TIMPS

Matrix metalloproteases (MMPs) belong to the matrixins and are involved in degradation and remodelling of the extracellular matrix, development, wound healing and in the pathology of hyperproliferative diseases such as arthritis and cancer (Chang and Werb, 2001; Seals and Courtneidge, 2003). Furthermore substrates of MMPs are EGF-like growth factors such as HB-EGF and therefore suggested to be involved in EGFR signal transactivation (Roelle et al., 2003; Suzuki et al., 1997; Yu et al., 2002). The proteolytic activities of MMPs are controlled during activation from their precursors and inhibition by the endogenous inhibitors α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs). TIMPs are the major endogenous regulators of MMPs, but also of ADAM activities in the tissue, and four homologous TIMPs have been identified to date (Crocker et al., 2004)(Table 1). Different reports show that TIMPs inhibit cell invasion *in vitro*, tumourigenesis, metastasis *in vivo*, and angiogenesis (Gomez et al., 1997) .

Table 1: Characteristics of the mammalian TIMP protein family on inhibition of metalloproteases of the MMP and ADAM family (Crocker *et. al.*)

	Extracellular localization	*Proteases inhibited*
TIMP-1	Secreted	All but MT1-MMP
		ADAM10
TIMP-2	Secreted	Inhibits all MMPs tested
TIMP-3	Membrane	MMP-1,-2,-3,-7,-9,-14
		ADAM10,-12S,-17
		ADAM-TS4,-TS5
TIMP-4	Secreted	MMP-1,-2,-3,-7,9

1.7 Aim of this study

Aim of this study was to investigate the pathophysiological role of EGFR signal transactivation in human cancer and to elucidate the underlying molecular mechanisms governing this prototypical cell surface receptor cross-communication pathway.

In order to address these questions, both, tumour and non tumour cell lines and primary cells were supposed to be analysed for metalloprotease-dependent EGFR signal transactivation and thereof dependent downstream signalling pathways. The relevance of this signalling mechanism was going to be addressed by using a diverse array of GPCR ligands ranging from well-known mitogens such as LPA to neurotransmitters. Within this context, biological responses dependent on the GPCR-induced signal transactivation of the EGFR were to be investigated, including cell proliferation and migration.

Of special interest was the identification of the metalloproteases involved in this cross-talk mechanism. Therefore, appropriate model systems were to be selected to allow detailed investigations focussing on the elucidation of the metalloproteases involved in this signalling mechanism. Moreover, the EGF-like ligand-metalloprotease combination involved in this signalling pathway in different types of human cancer was going to be investigated. The focus of this study was to be human breast cancer cell lines.

There is increasing interest in cannabinoids as a treatment option in cancer therapy. Since cannabinoids act through GPCRs, this study was going to address the potential consequences of cannabinoid-induced EGFR signal transactivation in human cancer.

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
Collagenase (Type III)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
Diphtheria toxin CRM mutant	List Biological Lab., CA, USA
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
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
Leupeptin	Sigma, Taufkirchen
Lipofectamine®	Invitogen, Karlruhe
Lysozyme	Sigma, Taufkirchen
LY-294,002	Alexis, Grünberg
Marimastat	Sugen Inc., CA, USA
MBP (Myelin basic protein)	Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen
MOPS (3-Morpholinopropanesulfonic acid)	Biomol, Haub
N,N-Dimethylsphingosine	Sigma, Taufkirchen
Oligofectamine®	Invitrogen, Karlruhe
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
pNPP (p-Nitrophenyl phosphate)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
PD98059	Alexis, Grünberg
PEG (Polyethylene glycol) 4000, 6000	Serva, Heidelberg
Ponceau S	Sigma, Taufkirchen
PP2	Calbiochem, Bad Soden
PTX (Pertussis toxin)	List, Campbell, USA

Salmon sperm DNA	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sphingosine-1-phosphat, D-erythro	Biomol, PA, USA
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

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
[α-^{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
Dowex AG1-X8	Bio-Rad, München
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm Dynatech,	Denkendorf
Poly Prep® Chromatography columns	Bio-Rad, München
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

ACEA (Arachidonyl-2`-chloroethylamide)	Tocris, Bristol, UK
Amphiregulin	R&D Systems
HB-EGF	R&D Systems
BML-190 (1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1-[2-(4-morpholinyl)-2-oxoethyl]-1*H*-indole)	Tocris, Bristol, UK
Bradykinin	Calbiochem
EGF (murine)	Toyoba, Japan
HU-210	Tocris, Bristol, UK
(Arg^8)-Vasopressin	Bachem, Weil am Rhein
WIN 55,212-2 mesylate	Tocris, Bristol, UK

All other growth factors and ligands were purchased from Sigma.

2.1.6. Media and buffers

Medium for *E.coli*

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.

2.1.7 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.

RPMI 1640 medium supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate.

Complete medium for primary epithelial cells:
Dulbecco's modified eagle medium (DMEM) with 4.5 mg/mL glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids, 5µg/mL insulin, 10ng/mL EGF, 10% heat-inactivated FCS

(All are Gibco reagents, except insulin and EGF are from Sigma)
Freeze medium: 90% heat-inactivated FCS, 10% DMSO.

2.1.8. Stock solutions for 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
Denhardt (100x)	2.0 % Polyvinylpyrollidon 2.0 % Ficoll 2.0 % BSA
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
Tris-Glycine-SDS (10x)	248.0 mM Tris/HCl pH 7.5 1918.0 mM Glycine 1.0 % SDS

2.1.9. Bacterial strains, cell lines and antibodies

2.1.9.1. E.coli strains

E. coli	Description	Origin/ Reference
DH5αF'	F'/endA1 hsd17 (rk-mk-),supE44, recA1, gyrA (Nal), thi-1, (lacZYA-argF)	Genentech, San Francisco, USA
CJ236	dut-, ung-, thi-, relA-	(Kunkel, 1985)

2.1.9.2. Cell lines

Cell Line	Description	Origin/ Reference
1321N1	Human astrocytoma	Roche Bioscience
5637	Human bladder carcinoma	ATCC HTB-9
A498	Human kidney carcinoma	
Cos-7	African green monkey, SV40-transformed kidney fibroblasts	Genentech
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 ectropic and amphotropic retroviruses, based on HEK-293	Nolan, Stanford
MDA-MB-231	Human mammary carcinoma	ATCC HTB-26

MCF7	Human breast adenocarcinoma metastatic to brain	ATCC HTB-22
NCI-H292	Human lung epidermoid carcinoma	ATCC CRL-1848
Rat-1	Rat fibroblasts	Genentech
SCC9	Human squamous cell carcinoma of the tongue	ATCC CRL-1629
T98G	Human glioblastoma	Roche Bioscience
U1240	Human glioblastoma	Sugen
U373-MG	Human glioblastoma	DKFZ

ATCC, American Type Culture Collection, Manassas, USA
DKFZ, Deutsches Krebsforschungszentrum, Heidelberg

2.1.9.3 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; recognises 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; recognises phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M.
P-p38	Rabbit, polyclonal; recognises phospho-p38 (Thr-180/Tyr-182) MAPK	NEB
P-Akt/PKB	Rabbit, polyclonal; recognises phospho-Akt (Ser-473)	NEB
ADAM10	Rabbit, polyclonal/ AA 732-748 of human ADAM10	Chemicon, Hofheim
ADAM15	Rabbit, polyclonal/ AA 189-208 of human ADAM15	This study
ADAM17/TACE	Rabbit, polyclonal/ AA 807-823 of human ADAM17	Chemicon, Hofheim
ADAM17/TACE	Mouse, monoclonal/ AA 215-477 of human ADAM17	This study
Amphiregulin	Goat, polyclonal/ recombinant, human Amphiregulin	R&D Systems, Wiesbaden
HB-EGF	Goat, polyclonal/ recombinant, human HB-EGF	R&D Systems, Wiesbaden
ERK2	(C-14) Rabbit, polyclonal/ peptide at C-terminus	Santa Cruz ERK2

	of rat ERK2	
(K-23)	Rabbit, polyclonal/ peptide from sub-domain XI of rat ERK2	Santa Cruz
HA	Mouse, monoclonal; recognises the influenza hemagglutinin epitope	Babco, California, USA
VSV (P5D4)	Mouse, monoclonal; recognises an epitope of eleven AA derived from the vesicular stomatits virus glycoprotein VSV-G	Roche, Mannheim,

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 and FITC-conjugated goat anti-mouse secondary antibodies for flow cytometry were obtained from Sigma.

2.1.10. Plasmids and oligonucleotides

2.1.10.1 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

2.1.10.2 Constructs

Vector	Description	Reference
pcDNA3-HA-ERK2	cDNA of ERK2 in pcDNA3, HA-Tag	(Daub *et al*., 1997)
pcDNA3-M1R	cDNA of human M1R in pcDNA3	(Daub *et al*., 1997)

pLXSN-M1R	cDNA of human M1R in pLXSN	(Prenzel *et al.*, 1999)
pcDNA3-proHB-EGF-VSV	cDNA of human proHB-EGF in pcDNA3	(Prenzel *et al.*, 1999)
pLXSN-ESK-Timp-1-VSV	cDNA of human Timp-1 in pLXSN-ESK C-terminal VSV-tag	A. Gschwind
pLXSN-ESK-Timp-3-VSV	cDNA of human Timp-3 in pLXSN-ESK C-terminal VSV-tag	This study
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	This study
pLXSN-ESK-Δ(Pro-MP)--hADAM12-HA	cDNA of ADAM12 lacking the prodomain and metalloprotease domain in pLXSN-ESK; HA-tag	This study
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	This study
pcDNA3-hADAM17/TACE-HA	cDNA of human ADAM17 pcDNA3; C-terminal HA-tag	This study
pLXSN-ESK-hADAM17/TACE-HA	cDNA of human ADAM17 in pLXSN-ESK; C-terminal HA-tag	A. Gschwind
pcDNA3-Δ(Pro-MP)--hADAM17/TACE-HA	cDNA of ADAM17 lacking the prodomain and metalloprotease domain Δ(AA18-473) in pcDNA3; C-terminal HA-tag	A. Gschwind
pLXSN-ESK-Δ(Pro-MP)--hADAM17/TACE-HA	cDNA of ADAM17 lacking the prodomain and metalloprotease domain in pLXSN-ESK; C-terminal HA-tag	A. Gschwind

2.1.10.3 Important oligonucleotides

Sequence (description)	Name
ACAGAATTCGCCACCATGACCCCTTGGCTCGGGC (cloning of human Timp-1 cDNA; forward primer)	Timp3/1
ACATCTAGATCCTCCGGGGTCTGTGGCATTGATGATGC (cloning of human Timp-1 cDNA; reverse primer)	Timp3/2
AGAGATATCGCCACCGCCGGCGACGATGGCAGCG (cloning of human ADAM12 cDNA; forward primer)	ADAM12/1
GCATCTAGAGGAGGACTTAATATAGGCGGTGTGGGTG (cloning of human ADAM12 cDNA; reverse primer)	ADAM12/2
ACAGGATCCGCCACCATGCGGCTGGCGCTGCTCTG (cloning of human ADAM15 cDNA; forward primer)	ADAM15/1
GCATCTAGAGGAGGAGAGGTAGAGCGAGGACACT (cloning of human ADAM15 cDNA; reverse primer)	ADAM15/2
ACAGAATCCGCCACCATGAGGCAGTCTCTCCTATCC (cloning of human ADAM17 cDNA; forward primer)	ADAM17/1
GCATCTAGATCCTCCGCACTCTGTTTCTTTGCTGTC (cloning of human ADAM17 cDNA; reverse primer)	ADAM17/2
CCATAAGCTCACCCCTCGTGCGGCCGCGGCCTCGCA GGGCGCGAG (cloning of ΔPro-MP-ADAM12 lacking the prodomain and metalloprotease domain; creation of NotI-site between signal peptide and prodomain)	ΔPro-MP-12/1
GAAAGACTCCCTGACTTCTGCGGCCGCCGGCAGGTT AAACAGGCA (cloning of ΔPro-MP-ADAM12 lacking the prodomain and metalloprotease domain; creation of NotI-site between the metalloprotease and the disintegrin domain)	ΔPro-MP-12/2
CTGCTGCTCCTCAGTGCCGATATCACCTATATTTGG GAGCGG (cloning of ΔPro-MP-ADAM15 lacking the prodomain and metalloprotease domain; creation of EcoRV-site between signal peptide and prodomain)	ΔPro-MP-15/1

TCCGCAGAAAGCAGCCATGATATCAGGGGGTAGGCT AGGCAG (cloning of ΔPro-MP-ADAM15 lacking the prodomain and metalloprotease domain; creation of EcoRV-site between the metalloprotease and the disintegrin domain)		ΔPro-MP-15/2

2.1.10.4 siRNA-Oligonucleotides

siRNA	Description/Sequence	Reference
gl2	directed against firefly luciferase CGUACGCGGAAUACUUCGAdTdT	This study
ADAM9	AAUCACUGUGGAGACAUUUGCdTdT AAACUUCCAGUGUGUAGAUGCdTdT	This study
ADAM10	AAUGAAGAGGGACACUUCCCUdTdT AAGUUGCCUCCUCCUAAACCAdTdT	This study
ADAM12	AACCUCGCUGCAAAGAAUGUGdTdT AAGACCUUGATACGACUGCUGdTdT	This study
ADAM15	AACUCCAUCUGUUCUCCUGACdTdT AAAUUGCCAGCUGCGCCCGUCdTdT	This study
ADAM17	AAGUUUGCUUGGCACACCUUdTdT AAGUAAGGCCCAGGAGUGUUdTdT AACAUAGAGCCACUUUGGAGAdTdT	This study
proAR	AACCACAAAUACCUGGCUATAdTdT AAAUCCAUGUAAUGCAGAAdTdT	This study
proHB-EGF	AAGUGAAGUUGGGCAUGACUAdTdT AAUACAAGGACUUCUGCAUCCdTdT	This study
proTGFα	AAAACACUGUGAGUGGUGCCGdTdT AAGAAGCAGGCCAUCACCGCCdTdT	This study

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 manufacturers´ recommendations.

2.3. Enzymatic manipulation of DNA

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 was adjusted to the specific application according to the manufacturers´recommendations.

2.3.1.2. Dephosphorylation of DNA 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.

2.3.1.3. 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.

2.3.1.4. 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.

2.3.1.5. 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).

2.3.2. Introduction of plasmid DNA into *E.coli* cells

2.3.2.1. Preparation of competent cells

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.

2.3.2.2. Transformation of competent cells

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.

2.3.3. Oligonucleotide-directed mutagenesis

A DNA sequence can be specifically altered by synthesizing the desired sequence change within an oligonucleotide, and then converting this into a biologically active circular DNA strand by using the oligonucleotide to prime *in vitro* synthesis on a single-stranded circular template. This protocol (Kunkel, 1985; Messing, 1983) uses a DNA template containing a small number of uracil residues in place of thymine. Use of the uracil-containing template allows rapid and efficient recovery of mutants.

2.3.3.1. Preparation of uracil-containing, single-stranded DNA template

CJ236 bacteria were transformed with the DNA of interest (typically pcDNA3 constructs). 2 mL 2xYT-medium were inoculated with several colonies of transformed CJ236 at 37°C until the early log-phase was reached. Cultures were infected with $2x10_7$ M13K07 phages/mL (Amersham) and incubated for further 1.5 h. Next, kanamycin was added (70 µg/mL final concentration) and the culture was incubated with vigorous shaking at 37 °C overnight. Cells were pelletet twice by centrifugation (13000 rpm, 5 min) to clear the supernatant. Phage was then precipitated by adding 200 µL 2.5 M NaCl/ 20% PEG 6000 and incubation for 15 min at room temperature. Precipitated phage was collected by centrifugation. The phage sediment was resuspended in 100 µL TE10/0.1 buffer and subjected to phenol extraction/ ethanol precipitation in order to purify the single-stranded phage DNA. Quality and concentration of DNA was determined spectrophotometrically at 260 nm. For visual examination and documentation an aliquot of the single-stranded DNA was run on a 1% agarose gel.

2.3.3.2. Primer extension

The uracil-containing DNA was used as a template in oligonucleotide-directed mutagenesis experiments: 200 ng single-stranded template DNA, 2-3 pmol phosphorylated

oligonucleotide, 1 µL 10x hybridization buffer (20 mM Tris/HCl pH 7,4, 2 mM $MgCl_2$, 50 mM NaCl) in a total volume of 10 µL were incubated for 2 min at 90°C and allowed to cool to room temperature. To the hybridization mixture 1 µL 10x synthesis buffer (5 mM dNTPmix, 100 mM Tris/HCl pH 7.5, 50 mM $MgCl_2$, 20 mM DTT), 5 U T4-DNA Ligase (1 µL), 1 µg T4-Gen 32 Protein (0.5 µL) and 3 U T4-DNA Polymerase (1 µL) were added. The reaction was incubated for 5 min on ice, 5 min at 25 °C and finally for 90 min at 37°C. The reaction was stopped by adding 66 µL TE. 100 ng of double-stranded DNA product were used for transformation of E. coli. Resulting clones were chosen randomly for isolation of plasmid DNA which was analysed by sequencing.

2.3.4. 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).

2.3.5. 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 analysed on a 310-Genetic Analyzer (ABI Prism).

2.3.6. cDNA array hybridization

Filters spottet with genes of interest (cloned into pBluescript SKII+) were a gernerous gift from J. Ruhe, cDNA probes of the various cell lines were from T. Knyazeva and generated according to standard molecular biology methods. Labeling of 3– 5 µL of cDNA was performed with the Megaprime kit (Amersham) in the presence of 50 µCi of [α-^{32}P]dATP. The prehybridization solution was replaced from filters by the hybridization solution containing 5x SSC, 0.5% (v/v) SDS, 100 µg/mL baker yeast tRNA (Roche), and the labeled cDNA probe (2–5 x 10^6 cpm/mL) and incubated at 68°C for 16 h. Filters were washed under stringent conditions. A phosphorimager system (Fuji BAS 1000; Fuji) was used to quantify the hybridization signals. Average values for each slot were calculated using the formula: A = (AB - B) x 100/B; [A, final volume; AB, intensity of each slot signal (pixel/mm^2); B, background (pixel/mm^2)].

2.3.7. RT-PCR analysis

Specific silencing of targeted genes was confirmed by RT-PCR analysis. RNA isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) was reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim, Germany). PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) were used for PCR amplification. Primers (Sigma Ark, Steinheim, Germany) were ADAM9, 5'-AGT GCA GAG GAC TTT GAG AA-3´ and 5'-TGC CGT TGT AGC AAT AGG CT-3´, ADAM10, 5'-TTG CTC ACG AAG TTG GAC AT-3' and 5'-TTT CCC AGG TTT CAG TTT GC-3', ADAM12, 5'-CAG TTT CAC GGA AAC CCA CT-3´ and 5'-GAC CAG AAC ACG TGC TGA GA-3´, ADAM15, 5'-GGC TGG CAG TGT CGT CCT ACC AGA GGG-3' and 5'-GGT GCA CCC AGC TGC AGT TCA GCT CAG TCC-3', ADAM17, 5'-CGC ATT CTC AAG TCT CCA CA-3´ and 5'-TAT TTC CCT CCC TGG TCC TC-3´, proAR, 5'-TGG TGC TGT CGC TCT TGA TA-3´ and 5'-GCC AGG TAT TTG TGG TTC GT-3´; proHB-EGF, 5'-TTA TCC TCC AAG CCA CAA GC-3´ and 5'-TGA CCA GCA GAC AGA CAG ATG-3´; proTGFα, 5'-TGT TCG CTC TGG GTA TTG TG-3´ and 5'-ACT GTT TCT GAG TGG CAG CA-3´. PCR products were subjected to electrophoresis on a 2.5% agarose gel and DNA was visualized by ethidium bromide staining.

2.4. Methods in mammalian cell culture

2.4.1. General cell culture techniques

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 mycoplasma contamination using a bisbenzimidestaining kit (Sigma). Before seeding cells were counted with a Coulter Counter (Coulter Electronics). SCC9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) : Ham´s F12 medium (1:1) containing 400 ng/mL hydrocortisone and 10% FCS. U373MG and NCI-H292 were cultured in Eagle´s Minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and 10% FCS. MDA-MB-231, MCF7, U373MG were cultured in RPMI 1640 supplemented with 2 mM L-

glutamine and 10% FCS. HEK-293, Cos-7, Rat-1, 1321N1, T98G were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate and 10% FCS

2.4.2. Transfection of cultured cell lines

2.4.2.1. Transfection of cells with calcium phosphate

SCC9 cells or 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 of SCC9 cells was typically about 50% as determined by LacZ staining after transfection of a LacZ-containing expression plasmid. For transfection of Phoenix cells HBS was used instead of BBS.

2.4.2.2. Transfection of Cos-7 cells using lipofectamine®

Cos-7 cells were transiently transfected using Lipofectamine® (Gibco-BRL) essentially as described (Daub *et al*., 1997). For transfections in 6-well dishes, 1.0 mL of serum-free medium containing 10 µL of Lipofectamine and 1.5 µg of total plasmid DNA per well were used. After 4 h the transfection mixture was supplemented with an equal volume of medium containing 20% FCS and, 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis.

2.4.2.3. RNA interference

Transfection of 21-nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting endogenous genes was carried out using Oligofectamine (Invitrogen) and 4.2 µg siRNA duplex per 6-well plate as previously described (Elbashir et al., 2001).
24 h after transfection, cells were serum-starved and assayed 3 d after transfection. Highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest.

2.4.3. Primary epithelium cell cultures

After sacrifice of the mouse (day 14-15 of pregnancy), it was dunk entirely in isopropanol for 5 seconds, glands #4 were isolated and the lymph nodes were removed. First, the glands were set in PBS in culture dish on ice, then placed on an inverted 100mm dish cover under the sterile hood and 300 µL of complete medium with collagenase (1mg/mL) was added. The glands were minced with scalpel blades to an estimated fragment size of 1.0-2.0mm^3 and then were added to 20 mL of complete medium with collagenase in 50 mL conical tube and were incubated for overnight (37°C, 5% CO_2).
Next day, the tissue was dissociated with a 10 mL pipette until no tissue clumps were observed. Centrifugation was carried out at 100g for 10 minutes, and the top fat layer and the liquid phase was discarded. The pellet was resuspended and dissociated in 5 mL of complete medium (w/o collagenase, from this point on). After centrifugation at 100g for 10 min the supernatant was removed, the pellet was resuspended in 5-10 mL of complete medium with a 19 gauge needle (twice) and centrifuged again at 100g for 10 min. Finally, the resuspended pellet was plated in complete medium on a cell culture dish (one gland per 100mm dish), followed by 48 h of incubation (37°C, 5 % CO_2). Before stimulation, the cells were serum-starved for 24 h.

2.4.4. Generation of monoclonal antibodies against ADAM17

Monoclonal antibodies (Mabs) were raised against the metalloprotease-domain of human TACE /ADAM17 (AA 215-477). Recombinant protein expressed in CHO cells was used for immunization of BALB/c mice (MPI of Biochemisty, Tierhaus and department of Prof. Nigg, general guidelines for the generation of hybridoma cells (J.H. Peters, 1985). Purification of Mabs with T-Gel™ Adsorbent from Pierce, Rockford, IL, USA).

2.5. Protein analytical methods

2.5.1. Lysis of cells with triton X-100

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.

2.5.2. Determination of protein concentration in cell lysates

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

2.5.3. Immunoprecipitation 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 *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.

2.5.4. SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

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	

Because of the small size of pro-HB-EGF and the processed form of HB-EGF, the tricine SDS-PAGE system was used as described (Schagger and von Jagow, 1987).

2.5.5. 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/cm2 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.

2.5.6. 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). 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 reproped with different primary antibody to confirm equal protein loading.

2.6. Biochemical and cell biological assays

2.6.1. 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 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. In some cases cells were transfected 24 h after seeding and serum-starved two days following transfection before being stimulated as indicated above.

2.6.2. ERK1/2 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.

2.6.3. ERK/MAPK activity

HA-ERK2 or endogenous ERK2 were immunoprecipitated from lysates obtained from sixwell dishes using 0.5 µg of anti-HA antibody or 0.4 µg of anti-ERK2 antibody, respectively. 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).

2.6.4. Flow cytometric analysis of cell surface proteins

Was performed as described before (Prenzel *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.

2.6.5. Incorporation of ^{3}H-thymidine into DNA

U373-MG cells were seeded into 12-well plates (1.5 x 10^4 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.

2.6.5. Migration

MDA-MB-231 cells in exponential growth were harvested, washed and resuspended in standard medium without FCS. Cells were preincubated with either DMSO (control) or the inhibitors for 20 min. 2x10^5 cells were seeded into polycarbonate membrane inserts (6.5 mm diameter and 8 µm pore size) in 24-transwell dishes. The lower chamber was filled with standard medium without FCS containing the chemoattractant. Cells were permitted to migrate for 6 h.

Following incubation, nonmigrated cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed and stained with crystal violet. The evaluation of migrated cells was performed by counting the cells using a microscope.
Analysis of cell motility of SCC9 cells was performed as described before (Gschwind et al., 2002) using a modified Boyden chamber. 24 h after transfection with siRNAs SCC9 cells were seeded into polycarbonate membrane inserts (6.5 mm diameter and 8 µm pore size) in 24-transwell dishes at 1 x 10^5 cells/well in the presence or absence of agonist. The lower chamber was filled with standard medium without FCS containing 10 µg/mL fibronectin as chemoattractant. Cells were permitted to migrate for 36 h. Following incubation, nonmigrated cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed and stained with crystal violet. The stained cells were solubilized in 10% acetic acid, absorbance at 570 nm was measured in a micro-plate reader.

2.6.5. MTT assay

In a 96-well flat bottom plate (Nunc, Naperville, Ill.) approximately 2,000 cells/100 µl of cell suspension were seeded. Upon serum-starvation for 24 h cells were incubated with inhibitors and growth factors as indicated for another 24h. MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide; thiazolyl blue, SIGMA, St. Louis, MO) was added to each well to a final concentration of 1 mg/mL MTT. Plates were incubated in the presence of MTT for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which is solubilized (DMSO, acidic acid, SDS) and absorbance was read at 570 nm on an micro-plate reader.

2.6.6. Apoptosis Assay

NCI-H292 lung cancer cells were seeded and grown for 20 h. Upon serum-starvation for 24 h cells were treated with Δ^9-Tetrahydrocannabinol as indicated for 6h. Cells were collected in assay buffer (1% sodium citrate, 0.1% Triton X-100) containing propidium iodine (PI) and incubated at 4°C for 3 h. Nuclear DNA staining was analysed on a Becton Dickinson FACScalibur flow cytometer.

2.7. 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. GPCR agonists induce EGFR signal transactivation and downstream signalling events

3.1.1. GPCR agonists stimulate EGFR tyrosine phosphorylation via a metalloprotease-dependent pathway

The EGFR represents both key regulator of normal cellular development as well as a critical player in hyperproliferative diseases such as cancer (Zwick et al., 2001; Zwick et al., 1999). To address the question whether the EGFR provides a convergence point for GPCR-induced signalling in transformed and non-transformed cells within a metalloprotease-dependent EGFR signal transactivation pathway, we stimulated different cell lines and primary cells with GPCR agonists such as lysophospholipids, thrombin and neurotransmitters and investigated EGFR phosphorylation depending on metalloprotease activity by preincubation with the metalloprotease inhibitor batimastat (BB94). As shown in Figure 7, GPCR stimulation induces metalloprotease-dependent phosphorylation of the EGFR in various cancer cell lines, but also in primary mammary epithelial cells (Figure 7, representative data shown for SCC9, MDA-MB-231, MCF7 and mammary epithelial cells).

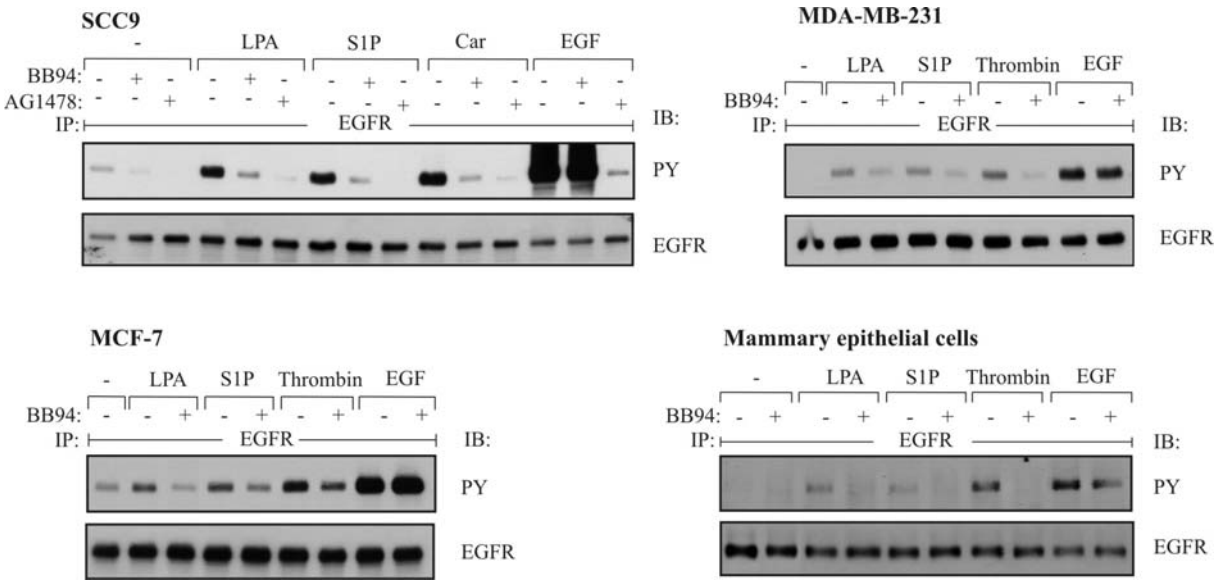


Figure 7 Different GPCR agonists stimulate metalloprotease-dependent EGFR phosphorylation in cancer cell lines and primary mammary epithelial cells. Following serum starvation for 48 h SCC9, MDA-MB-231 MCF7 and mammary epithelial cells were incubated with BB94 (5 μM, 20 min) or AG1478 (250 nM, 20 min) and subsequently stimulated with GPCR agonists as indicated. After lysis, EGFR was immunoprecipitated (IP), transferred to nitrocellulose and tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with anti-phosphotyrosine (PY) antibody. The same membranes were stripped and reprobed for total EGFR contend.

To extend the data obtained for Figure 7, we investigated the capacity of various GPCR agonists to induce the TMPS pathway in a broader panel of non-tumour and tumour cell lines (Table 2).

Table 2 *Metalloprotease-dependent tyrosine phosphorylation of EGFR by GPCR agonists*

Cell line	*Tissue*	*LPA*	*S1P*	*Thrombin*	*Carbachol*	*Bombesin*	*Et-1*
SCC9	squamous	+	+	+	+	+	+
MDA-MB-231	breast	+	+	+	-	n.d.	n.d.
MCF7	breast	+	+	+	n.d.	n.d.	n.d.
A498	kidney	+	+	+	+	+	+
1321N1	astrocytoma	+	+	+	+	n.d.	n.d.
T98G	glioblastoma	+	+	+	+	+	+
U1240	astrocytoma	+	+	+	n.d.	n.d.	n.d.
Rat-1	fibroblasts	+	+	+	n.d.	n.d.	+
Cos-7	kidney	+	+	+	-	n.d.	nd.
primary mec	breast	+	+	+	n.d.	n.d.	n.d.

+, Transactivation of the EGFR by immunoblot analysis
-, Transactivation of the EGFR not detected
n.d., not determined

The data compiled in Table 2 clearly demonstrates that metalloprotease-dependent GPCR-EGFR cross-talk is established in various tumour and non-tumour cell lines and also in primary mammary epithelial cells.

Furthermore we examined the capability of neurotransmitters to transactivate the EGFR in astrocytoma and glioblastoma cell lines in a metalloprotease-dependent manner. The astrocytoma cell line 1321N1 and the glioblastoma cell lines U1240, T98G and U373-MG were preincubated with BB94 followed by stimulation with different neurotransmitters and detection of EGFR tyrosine phosphorylation.

Table 3 Neurotransmitters induce EGFR signal transactivation in astrocytoma and neuroblastoma cells

Cell line / *neurotransmitter*	1321N1	U1240	T98G	U373-MG
Apelin13	+	-	n.d.	-
Endomorphin	+	-	n.d.	-
Nociceptin	+	n.d.	-	-
Neurotensin	-	+	+	-
Prolactin releasing peptide	+	+	-	-
Motilin	+	+	-	-
Substance P	+	+	+	+
Vasopressin	+	+	n.d.	-
Urotensin II	+	-	n.d.	+
Bradykinin	+	+	n.d.	-

+, Transactivation of the EGFR by immunoblot analysis
-, Transactivation of the EGFR not detected
n.d., not determined

As shown in Table 3, various neurotransmitters are capable to induce metalloprotease-dependent tyrosine-phosphorylation of the EGFR in astrocytoma and glioblastoma cell lines.

3.1.2. EGFR signal transactivation-induced downstream signalling

The finding that GPCR ligands induce metalloprotease-dependent EGFR signal transactivation in various cancer cell lines raised the question whether GPCR agonist treatment of these cells leads to EGFR specific signalling events. One key downstream event of the mitogenic EGFR signalling cascade is the tyrosine phosphorylation of adaptor proteins (Prenzel et al., 1999). Therefore we analysed the tyrosine phosphorylation content of the adaptor protein SHC, which links RTKs to the ERK/MAPK pathway. Figure 8 demonstrates that treating SCC9 and MDA-MB-231 cells with LPA, S1P, carbachol and thrombin resulted in tyrosine phosphorylation of SHC. Furthermore, pre-treatment with batimastat or AG1478 completely blocked GPCR-induced tyrosine phosphorylation of SHC.

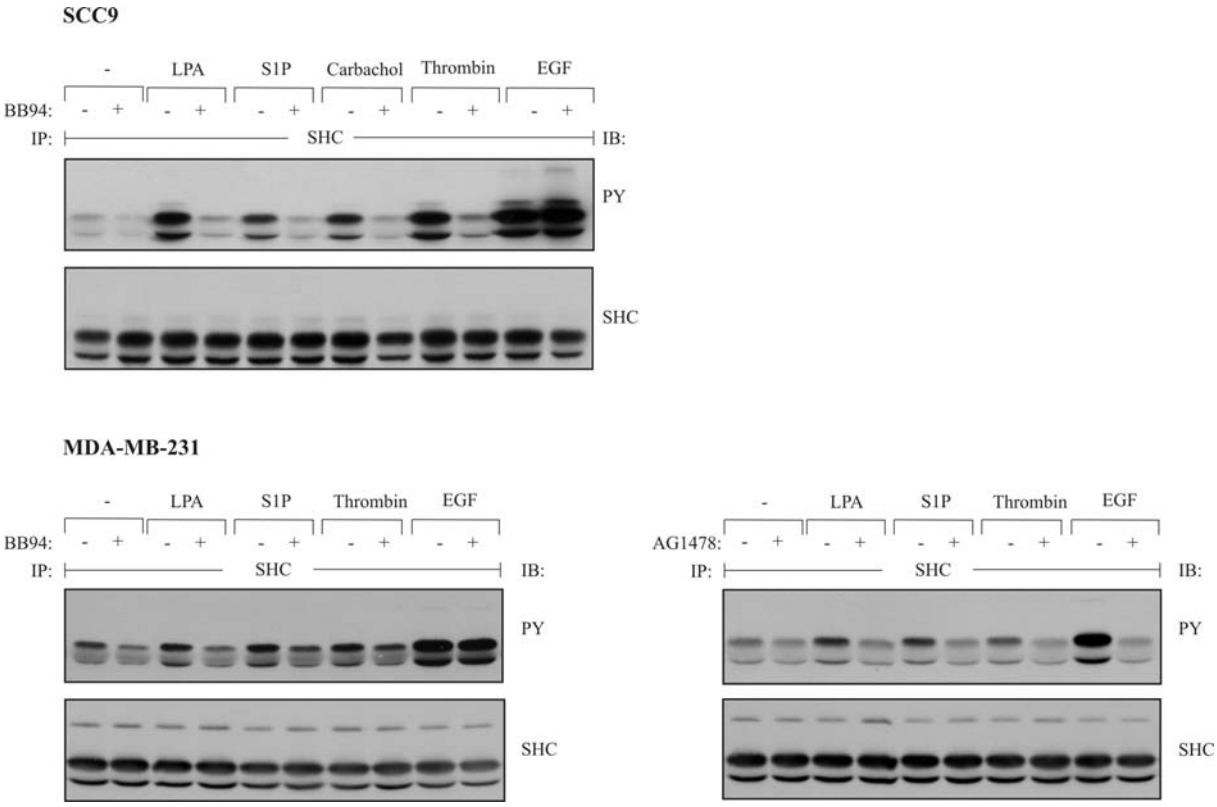


Figure 8 GPCR agonist-induced phosphorylation of SHC is metalloprotease-dependent. SCC9 and MDA-MB-231 cells were serum-starved for 48 h, preincubated with BB94 (5 μM, 20 min) or AG1478 (250 nM, 20 min) and stimulated as indicated. SHC was immunoprecipitated from total lysates, after SDS-PAGE transferred to nitrocellulose and the membrane was probed for total SHC protein, stripped and reprobed for phosphotyrosine content.

Activation of the serine/threonine kinase ERK1/2 is so far the best characterised EGFR downstream signalling pathway and serves as a paradigm for signal transmission from cell surface receptors to the nucleus. Furthermore, activation of the MAPKs ERK1/2 is a key step in the regulation of cellular responses such as cell proliferation, differentiation or anti-apoptosis (Chang and Karin, 2001). Therefore, the effect of GPCR agonists on MAPK activation in SCC9, MDA-MB-231 and primary mammary epithelial cells was investigated by using phosphospecific antibodies to detect the phosphorylation state of the MAPKs ERK1/2.

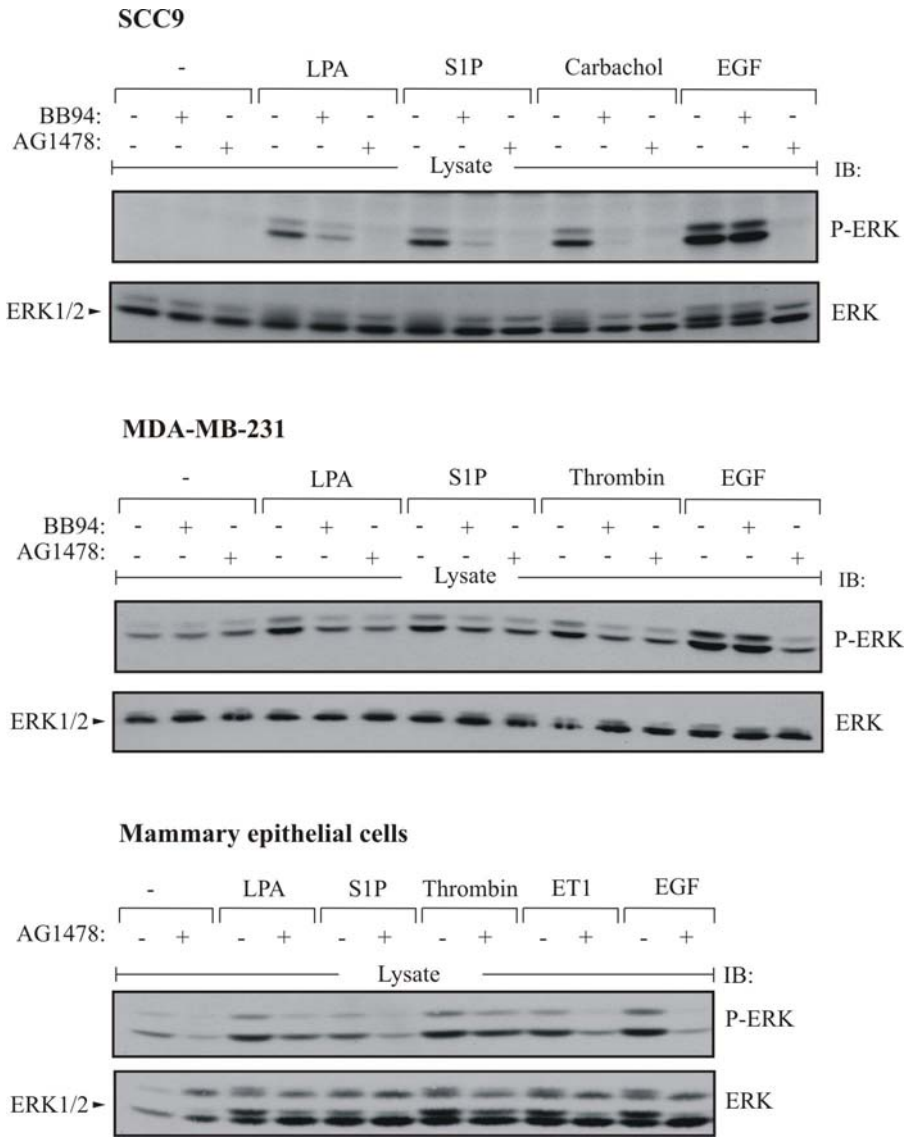


Figure 9 Activation of ERK1/2 in response to GPCR agonist treatment depends on metalloprotease- and EGFR kinase activity. SCC9, MDA-MB-231 and primary mammary epithelial cells were serum-starved for 48 h, incubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min) and stimulated as indicated for 10 min. After lysis, activated ERK1/2 was detected by immunoblotting of total lysates with anti-phospho-ERK (P-ERK) antibody, followed by reprobing of the same filters with anti-ERK (ERK) antibody.

Moreover, as shown in Figure 10, metalloprotease- and EGFR-dependent ERK2 activation in response to GPCR agonist treatment could be observed in the breast cancer cell line MCF7 and in the astrocytoma cell line 1321N1 using an ERK2 kinase assay. While in MCF7 GPCR agonist-induced ERK2 activation was completely blocked by both the metalloprotease inhibitor BB94 and the selective EGFR kinase inhibitor AG1478, in 1321N1 both inhibitors blocked ERK2 activation only by 50%.

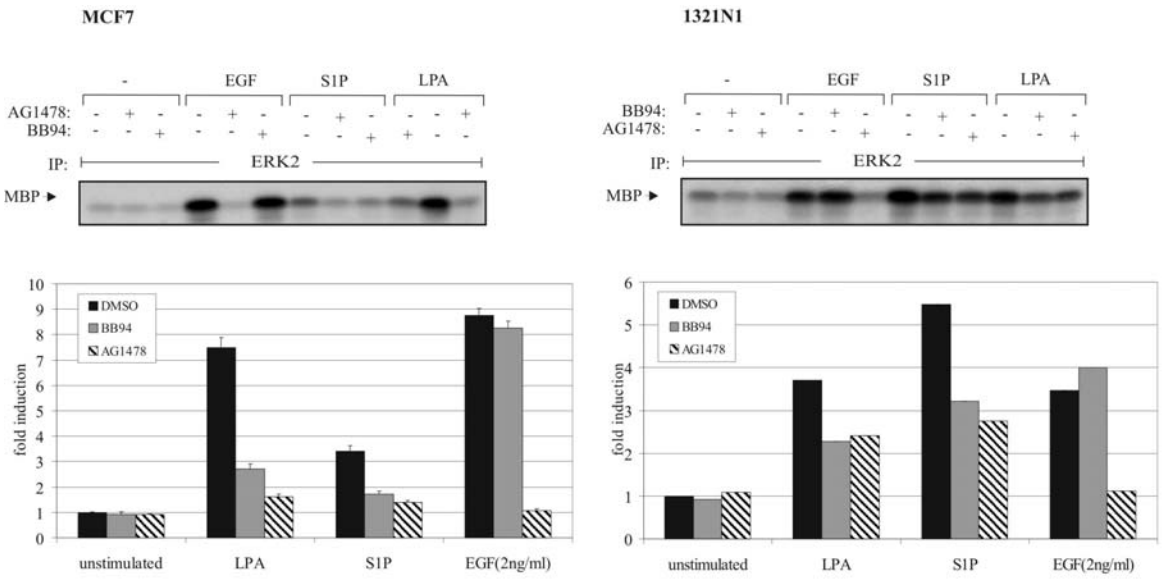


Figure 10 MAPK activity assay of ERK2 in MCF7 and 1321N1 cells in response to GPCR agonist treatment. Quiescent MCF7 and 1321N1 cells were preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min) and stimulated as indicated for 10 min. ERK2 was immunoprecipitated and a radioactive kinase assay performed as described (materials and methods). Quantitative analysis of labeled MBP from three independent experiments (mean ±s.d.) using a Phosphoimager (Fuji).

In addition to mitogenic ERK signalling, GPCR agonists were shown to act as survival factors by activating both the ERK/MAPK pathway and the PI-3K-dependent phosphorylation of the survival mediator Akt/PKB in diverse cell types (Fang et al., 2002; Sautin et al., 2001). Therefore, we addressed the question whether GPCR agonist stimulation induces phosphorylation of Akt/PKB in our cell systems and whether this depends on EGFR signal transactivation. Interestingly, activation of Akt/PKB in SCC9 but not in MDA-MB-231 cells was blocked by the selective EGFR inhibitor AG1478 and by the metalloprotease inhibitor BB94 (Figure 11).

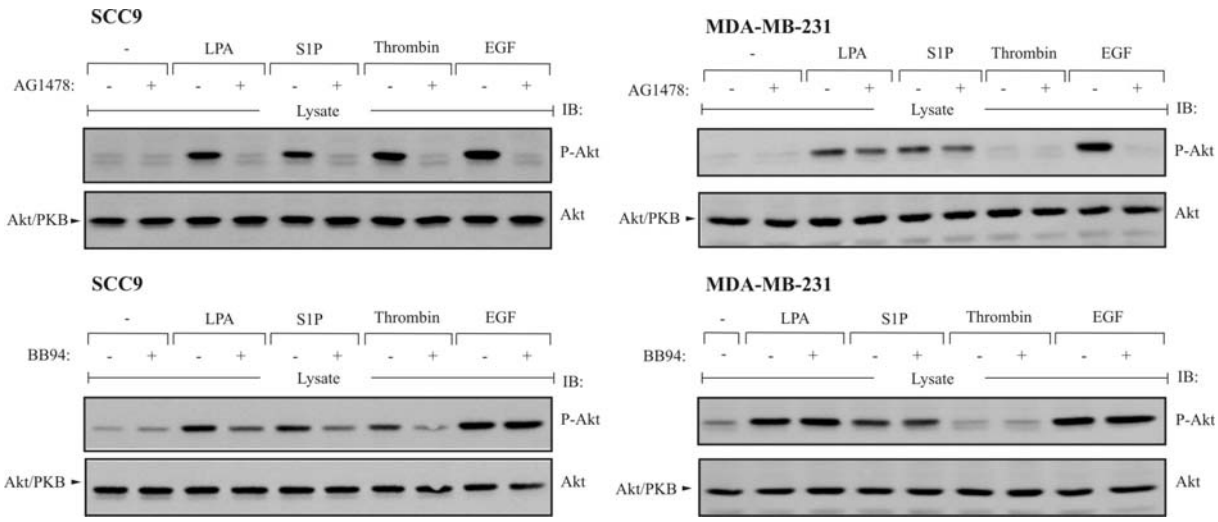


Figure 11 Akt/PKB phosphorylation after GPCR stimulation is metalloprotease- and EGFR-dependent. Quiescent SCC9 and MDA-MB-231 cells were pretreated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min) and stimulated as indicated for 10 min. After lysis, activated Akt/PKB was detected by immunoblotting of total lysates with anti-phospho-Akt/PKB (P-Akt) antibody, followed by reprobing of the same filters with anti-Akt/PKB (Akt) antibody.

Furthermore, in MDA-MB-231cells lysophospholipid induced EGFR and Akt activation was also blocked by preincubation with pertussis toxin, which specifically inhibits $G\alpha_{i/o}$ subunits. In addition, the sphingosine-kinase inhibitor N,N-Dimethylsphingosine (DMS) and the PI3K inhibitor LY-294,002 also blocked Akt activation following lysophospholipid stimulation. In contrast, EGFR signal transactivation was unaffected by pre-treatment of the cells with DMS (Figure 12).

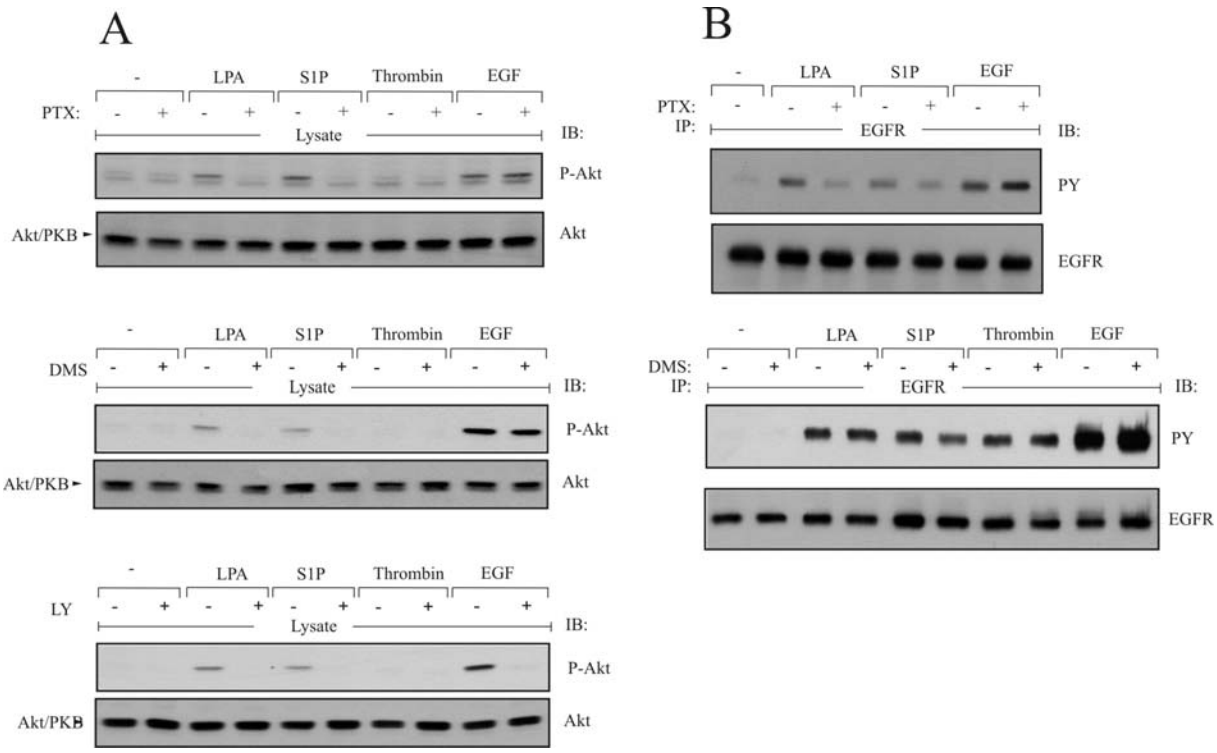


Figure 12 Akt/PKB phosphorylation after GPCR stimulation is pertussis-toxin, SPHK and PI3K-dependent in MDA-MB-231 cells. (A) Quiescent cells were pretreated with PTX (150 ng/mL , 20 min) or DMS (1 μM, 20 min) and stimulated as indicated for 10 min. After lysis, activated Akt/PKB was detected by immunoblotting of total lysates with anti-phospho-Akt/PKB (P-Akt) antibody, followed by reprobing of the same filters with anti-Akt/PKB (Akt) antibody. (B) Quiescent cells were pretreated with PTX (150 ng/mL , 20 min) or DMS (1 μM, 20 min), stimulated as indicated for 3 min, and EGFR tyrosine phosphorylation contend was determined.

3.2. GPCR agonist-induced EGFR signal transactivation and downstream signalling events depend on AR or HB-EGF function

Depending on the cellular context, different proEGF-like ligands like proAR, proHB-EGF and proTGFα are involved in GPCR-EGFR crosstalk (Asakura et al., 2002; Izumi et al., 1998; Schafer et al., 2004; Yan et al., 2002). As shown in Figure 13, predominantly proAR, proHB-

EGF and proTGFα are expressed in the cell systems tested. To investigate which ligand is involved in the EGFR signal transactivation pathway following GPCR stimulation in squamous cell carcinoma cells the endogenous expression of proAR, proHB-EGF and proTGF-α was silenced by small interfering RNA (siRNA) in SCC9 cells. Efficient and specific knockdown of target gene expression was monitored by RT–PCR (Figure 14) confirming that gene silencing occurred by mRNA degradation. Concomitantly, the effect of siRNAs on the EGFR transactivation signal was examined. As shown in Figure 14, siRNA to proAR completely blocked GPCR-induced EGFR tyrosine phosphorylation. siRNAs to proHB-EGF and proTGF-α, however, did not significantly alter the transactivation signal, demonstrating a specific requirement for proAR.

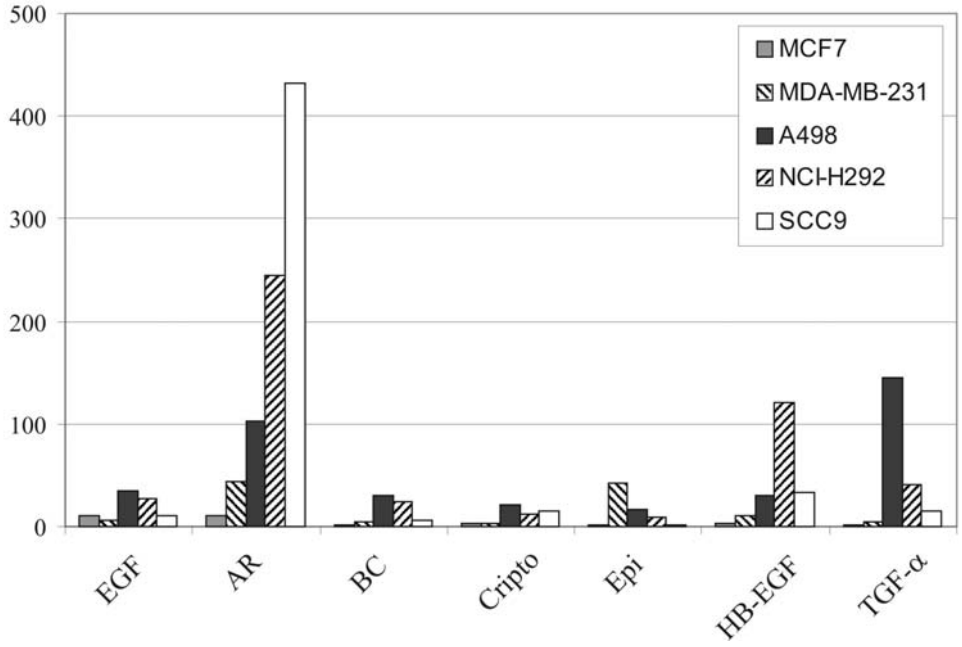


Figure 13 Expression of EGF-like growth factors by cDNA macro array analysis. cDNA synthesized from MCF7, MDA-MB-231, A498, NCI-H292 and SCC9 cells was labelled with α[^{33}P]dATP and hybridized on array filters as described under materials and methods. Data represent relative hybridization signals for individual genes.

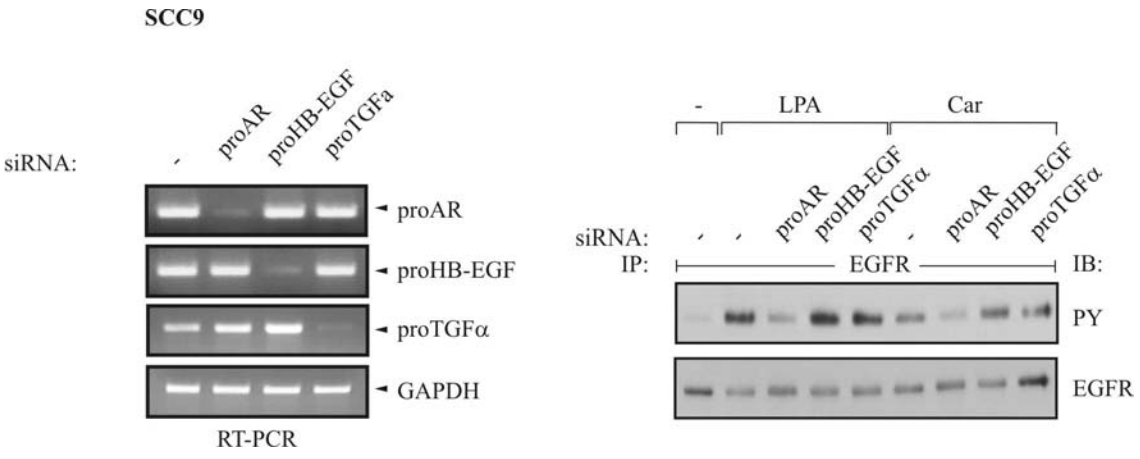


Figure 14 Effect of proAR siRNA on GPCR-induced EGFR activation. Blockade of EGF-like growth factor precursor expression by RNA interference (RNAi). SCC9 cells were transfected with siRNA for proAR, proHB-EGF or proTGF-α, cultured for 2 days and analysed for gene expression by RT–PCR as indicated or stimulated with LPA or carbachol and assayed for EGFR tyrosine phosphorylation content.

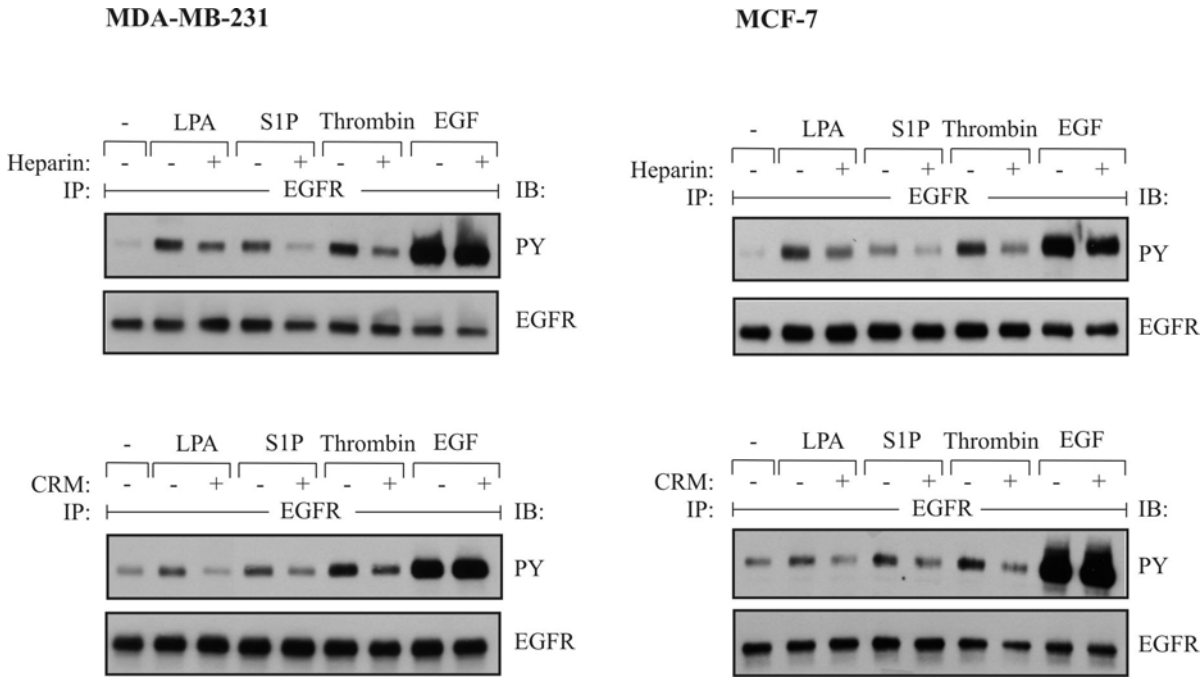


Figure 15 Effect of blockade of HB-EGF function in breast cancer cells on GPCR-induced EGFR activation. Quiescent MDA-MB-231 cells were preincubated with heparin (100 ng/mL, 20 min) or the diphtheria toxin mutant CRM197 (10 μg/mL, 20 min), and stimulated for 3 minutes with LPA (10 μM), S1P (1 μM) and thrombin (1 U/mL). Following immunoprecipitation of cell extracts with anti-EGFR antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and re-probed with anti-EGFR antibody.

Next, we investigated the effect of heparin in breast cancer cell lines, which abrogates both proAR and proHB-EGF function. Heparin was able to abrogate EGFR phosphorylation after GPCR agonist treatment of MDA-MB-231 and MCF7 cells (Figure 15).

Furthermore, the ability of the diphtheria toxin mutant CRM197, a specific inhibitor of proHB-EGF function, to block EGFR phosphorylation in response to GPCR agonists indicates that proHB-EGF mediates EGFR transactivation (Figure 15).

3.3. EGFR signal transactivation is mediated by ADAM metalloproteases

3.2.1. ADAM17 is required for EGFR signal transactivation after lysophospholipid and carbachol stimulation.

Recent *in vitro* and *in vivo* investigations underscored the role of the metalloprotease-disintegrin TACE/ADAM17 in shedding of EGF-like growth factor like proAR (Peschon et al., 1998; Sunnarborg et al., 2002). As shown in Figure 16 and Figure 17, ADAM17 and other

ADAM family members which have already been implicated in EGF-like ligand cleavage, but also members of the MMP-family are widely expressed in the cell systems investigated on the mRNA and protein level (Asakura et al., 2002; Izumi et al., 1998; Yan et al., 2002).

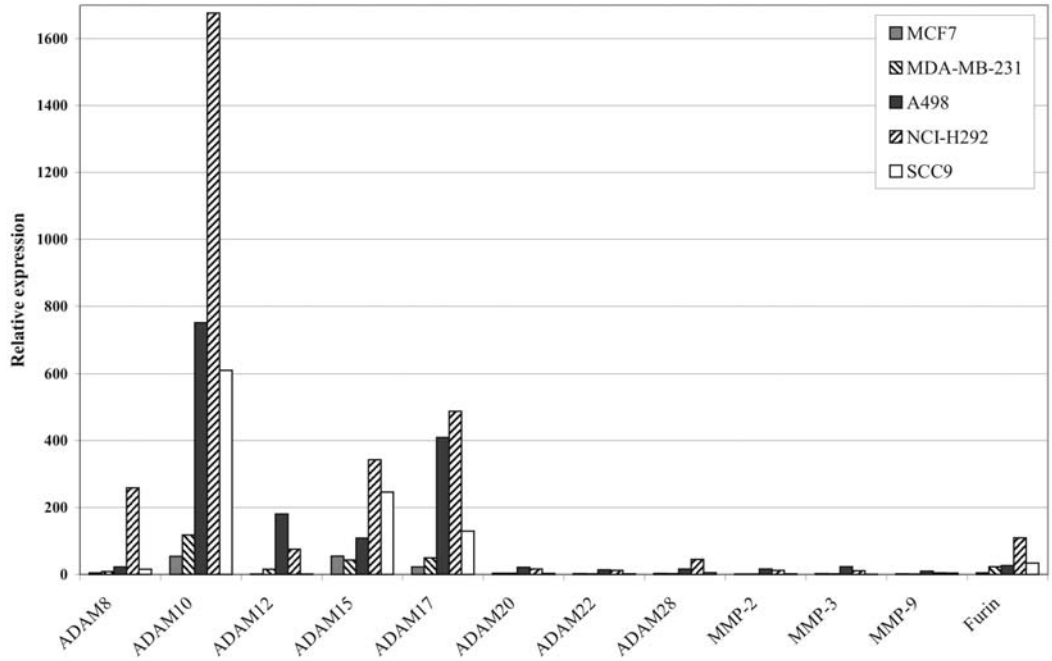


Figure 16 Expression of ADAM17 and other metalloproteases by cDNA macro-array analysis. cDNA synthesized from MCF7, MDA-MB-231, A498, NCI-H292 and SCC9 cells was labelled with α[^{33}P]dATP and hybridized on array filters as described under materials and methods. Data represent relative hybridization signals for individual genes.

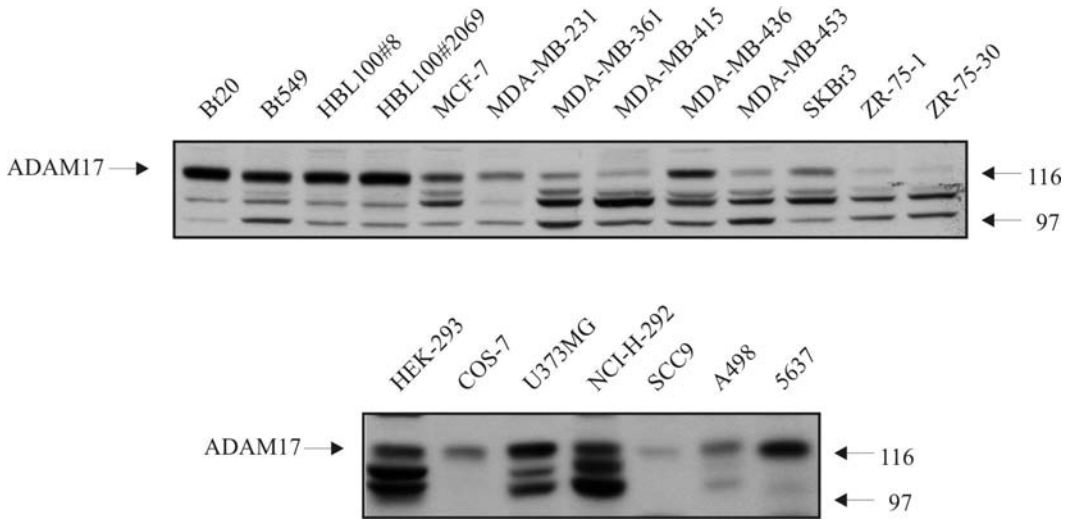


Figure 17 Expression of ADAM17 in breast cancer cell lines and other cancer and non cancer cell lines by immunoblot analysis. Total lysate was immunoblotted with polyclonal anti-ADAM17 antibody.

ADAM17 activity was shown to be blocked by the tissue inhibitor of metalloproteases-3 (TIMP-3) but not by TIMP-1 *in vitro* (Amour et al., 1998). To investigate whether ADAM17 is involved in the EGFR signal transactivation we tested the effect of TIMP-1 and TIMP-3 in

GPCR-EGFR cross-talk. Indeed, ectopic expression of VSV-tagged TIMP-3 but not TIMP-1 inhibited GPCR-induced EGFR tyrosine phosphorylation in SCC9 cells (Figure 18). These results indicate ADAM17 to be the sheddase participating in the GPCR-EGFR cross-communication.

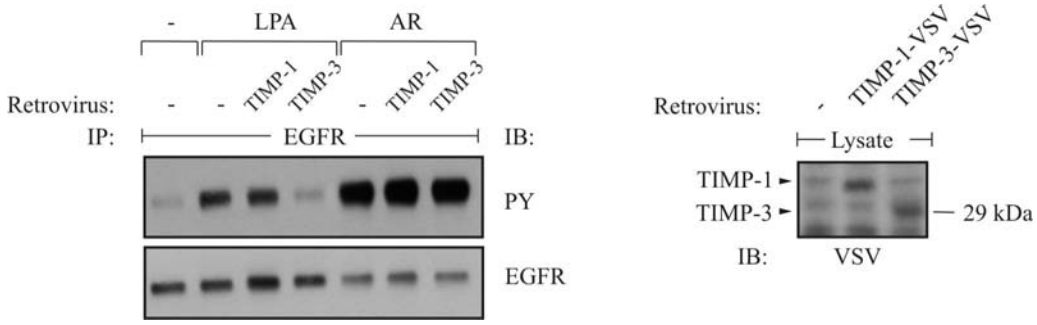


Figure 18 Inhibition of EGFR signal transactivation by TIMP-3 but not by TIMP-1. SCC9 cells were retrovirally infected with human Timp-1 or Timp-3 expression constructs. After 48 h of serum starvation, cells were stimulated as indicated and EGFR tyrosine-phosphorylation and TIMP expression was determined by immunoblot analysis.

To further substantiate this finding, we used the RNA interference technique to knock down the endogenous expression of the proteases ADAM10, -12, -15 and ADAM17. Figure 19 shows the specific and efficient inhibition of target gene expression by the siRNAs against ADAM12, -15 and ADAM17 by RT-PCR analysis and the inhibition of ADAM 10, -15 and ADAM17 protein levels by Western blot analysis. The siRNA against ADAM10 has been described previously (Fischer, 2004). The knockdown of ADAM17 protein was still observed 144 h after transfection (Figure 19C). Interestingly, siRNA-directed inhibition of ADAM17 resulted in the accumulation of proAR at the cell surface of SCC9 cells (Figure 20), supporting the view that ADAM17 is involved in basal proAR ectodomain shedding.

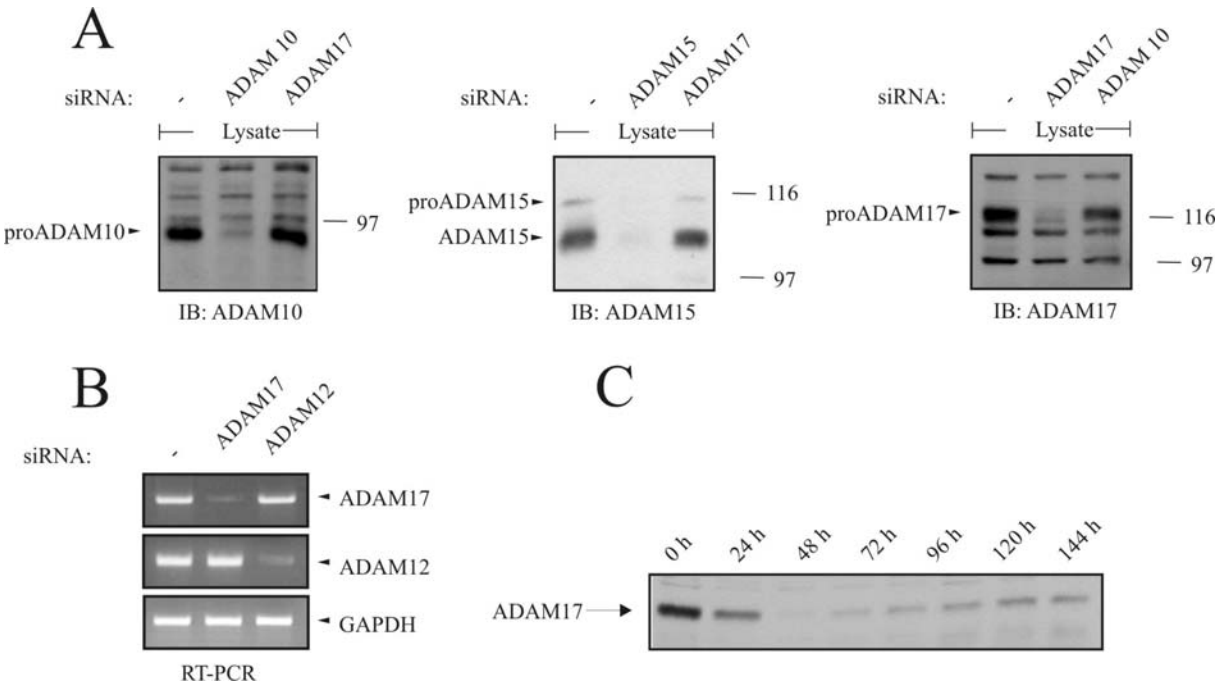


Figure 19 Knockdown of endogenous ADAM expression on mRNA and protein level. SCC9 cells were transfected with siRNA raised against the respective ADAM proteases. Gene expression was analysed by immunoblot analysis (A) or RT-PCR (B). (C) SCC9 cells were transfected with ADAM17 siRNA, lysed at different time points after transfection as indicated. ADAM17 expression was analysed by immunoblot analysis.

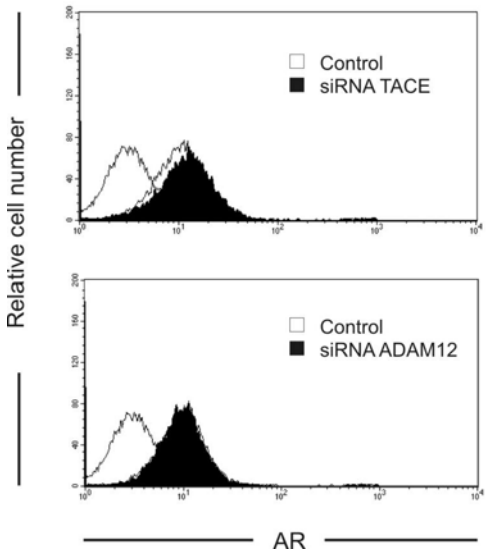


Figure 20 Knockdown of ADAM17 results in accumulation of proAR at the cell surface. siRNA transfected SCC9 cells were analysed for AR cell surface content by FACS.

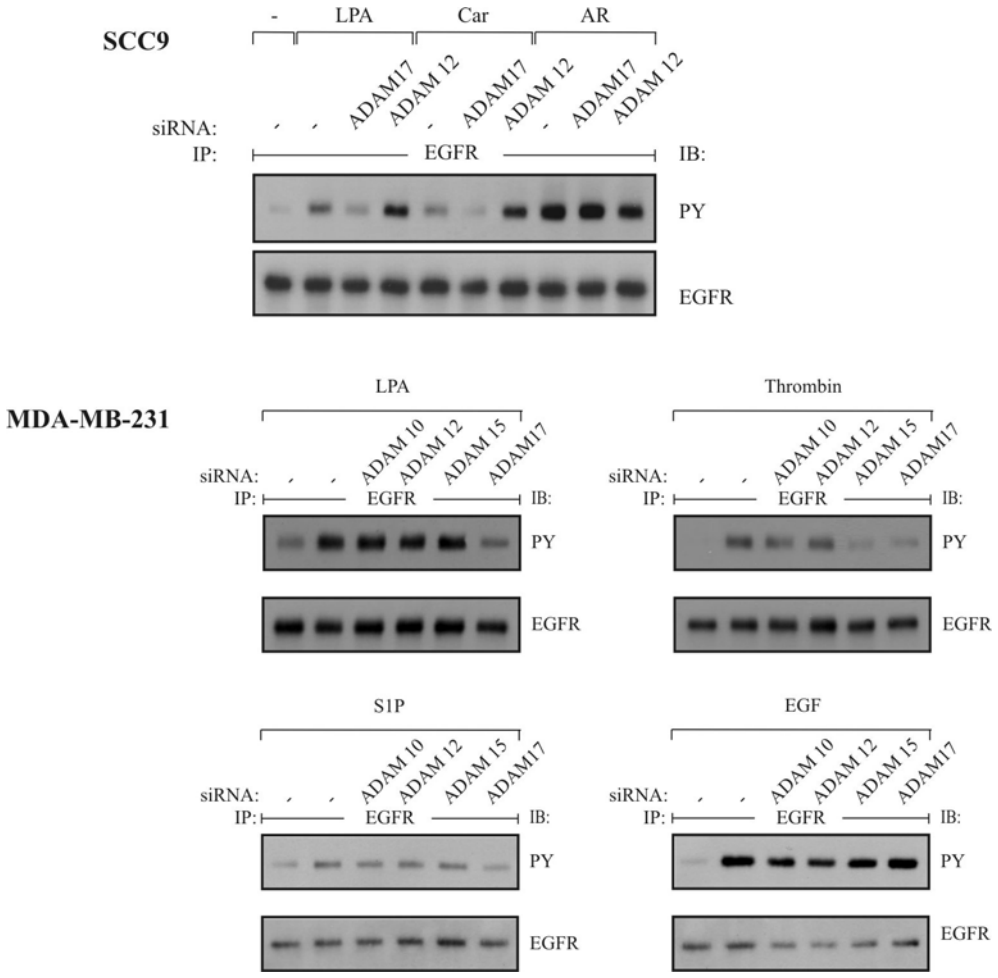


Figure 21 ADAM17 siRNA inhibits GPCR-induced EGFR signal transactivation. SCC9 und MDA-MB-231 cells were transfected with siRNAs and stimulated with agonists as indicated. Following immunoprecipitation of cell extracts with anti-EGFR antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and re-probed with anti-EGFR antibody.

Transient transfection of these siRNAs raised against the individual proteases followed by analysis of the phosphotyrosine content of the immunoprecipitated EGFR revealed that ADAM17 is mediating EGFR activation in response to lysophospholipids and carbachol in SCC9 and MDA-MB-231 cells (Figure 21). On the other hand, thrombin-induced EGFR signal transactivation in breast cancer cells was only partially inhibited by ADAM17 knockdown but also by the downregulation of ADAM 15 expression.

In addition, ADAM17 siRNA specifically suppressed GPCR-induced SHC, ERK/MAPK and Akt/PKB activation in SCC9 cells (Figure 22) without significantly altering the signalling events evoked by direct AR stimulation.

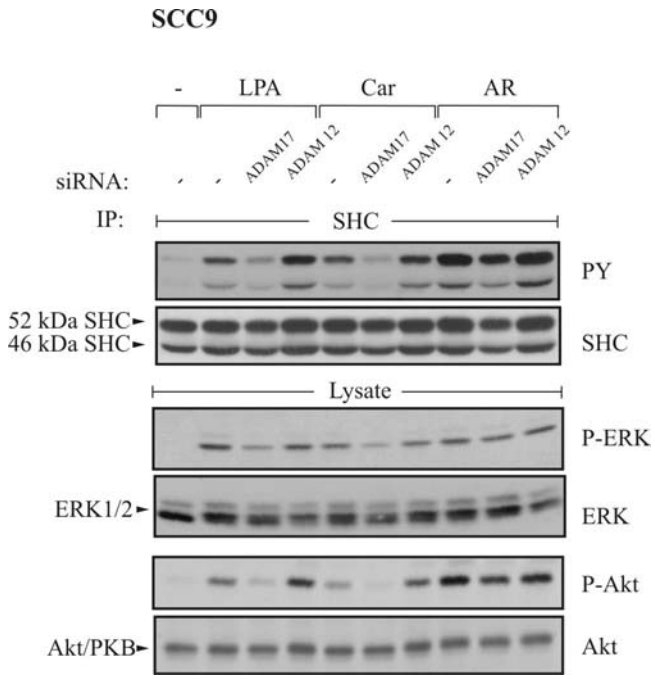


Figure 22 EGFR signal transmission upon GPCR activation requires ADAM17. SCC9 cells were transfected with siRNAs and stimulated with agonists as indicated. Following immunoprecipitation of cell extracts with anti-SHC-antibody, proteins were immunoblotted with SHC-antibody and re-probed with anti-phosphotyrosine antibody. Phosphorylated ERK1/2 and Akt were detected by immunoblotting total lysates with anti-phospho-ERK or anti-phospho-Akt antibody, respectively. The same filters were re-probed with anti-ERK or anti-Akt antibody, respectively.

Blocking antibodies against ADAM17 serve as an additional approach to confirm the involvement of the protease in the TMPS mechanism in the investigated cellular system. Therefore we raised monoclonal antibodies (MAbs) against the metalloprotease-domain of ADAM17 and tested the MAbs to immunoprecipitate endogenous as well as ectopically expressed ADAM17 of cell lysates. As shown in Figure 23 the monoclonal antibodies 432-2, 400-1, 343-3 and 432-7 specifically immunoprecipitate the mature form of endogenous or ectopically expressed ADAM17, whereas the α-HA antibody immunoprecipitates predominantly the pro-form of ectopically expressed ADAM17.

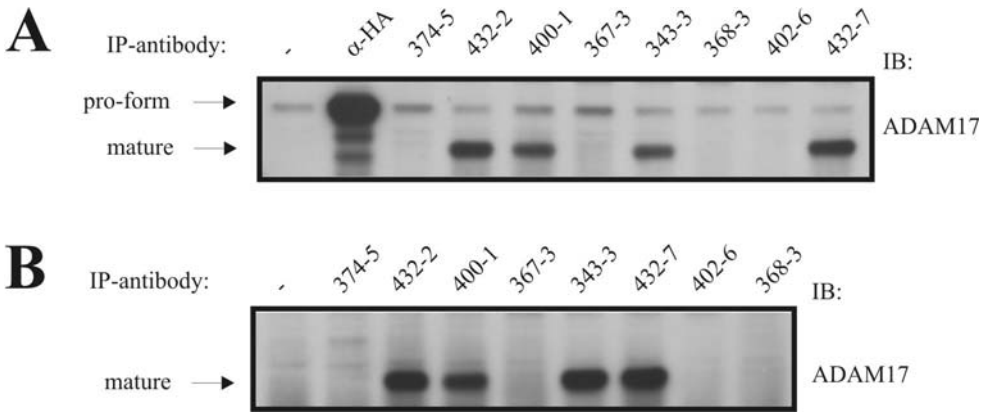


Figure 23 Immunoprecipitation of mature ADAM17 protein by monoclonal antibodies raised against the metalloprotease-domain. (A) HEK-293 cells transiently expressing ADAM17-HA or (B) SCC9 cells were serum-starved for 24 h and lysed with TritonX-100 lysis buffer containing 5 μM BB94 as metalloprotease inhibitor. 200 μg of crude lysate was used for immunoprecipitation with 5 μg contol IgG (monoclonal α-HA antibody) or 5 μg monoclonal α-ADAM17 antibody. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membrane. Immunoprecipitated ADAM17 protein was analysed by immunoblotting with polyclonal ADAM17 antibody.

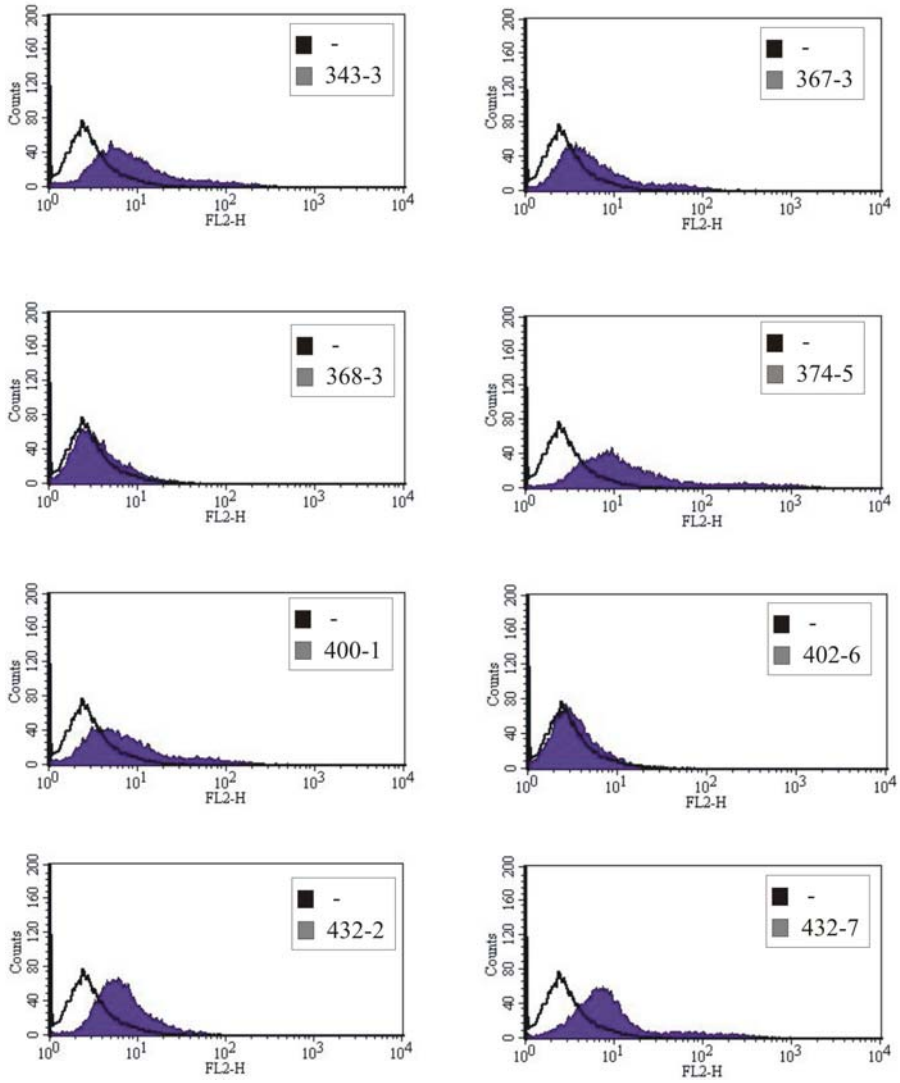


Figure 24 Flow cytometric analysis of ADAM17-binding of monoclonal antibodies. SCC9-cells were seeded and grown for 24 h. After collection, cells were stained with monoclonal ADAM17 antibodies raised against the metalloprotease domain of ADAM17 for 45 min. After washing with phosphate-buffered saline (PBS), cells were incubated with PE-conjugated secondary antibodies for 45 min and washed again with PBS. Cells were analysed on a Becton Dickinson FACScalibur flow cytometer.

Furthermore, monoclonal antibodies were tested for their ability to stain ADAM17 at the cell surface of living cells. MAbs 402-6 and 368-3 showed no cell surface staining, whereas 367-3 showed a weak and 343-3, 374-5, 400-1, 432-2 and 432-7 showed a strong cell surface staining of endogenous ADAM17 (Figure 24). Finally, we examined the effect of monoclonal ADAM17 antibodies on EGFR tyrosine phosphorylation by LPA in the squamous cell

carcinoma cell line SCC9. The results show that pre-treatment with MAbs 374-5, 432-2, 400-1 and 367-3 inhibited the EGFR signal transactivation induced by LPA, whereas direct stimulation of the EGFR with EGF was not affected by pre-treatment with the Mabs (Figure 25).

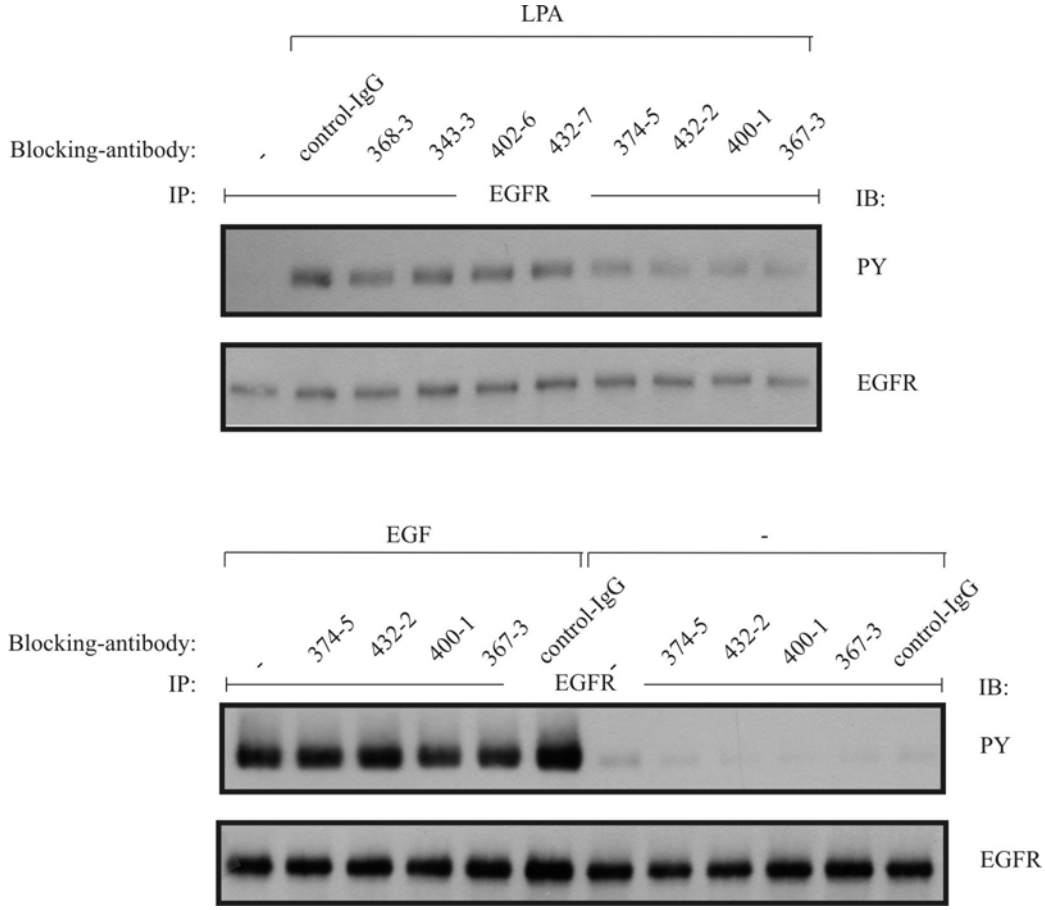


Figure 25 EGFR signal transactivation requires ADAM17 activity. Serum-starved SCC9 cells were preincubated for 30 minutes with 5 µg control IgG (monoclonal α-HA antibody) or 5 µg monoclonal ADAM17 antibody as indicated and treated with LPA (10 µM) or EGF (2 ng/mL) for 3 min. After lysis, EGFR was immunoprecipitated (IP) using anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with anti-phosphotyrosine (αPY) antibody, followed by reprobing of the same filter with anti-EGFR antibody.

3.2.2. Thrombin activates EGFR signal transactivation via ADAM10, -15 and/or ADAM17 depending on the cellular context.

Both ADAM15 and ADAM17 knockdown by siRNA interference blocked thrombin-induced EGFR transactivation by 50% each in breast cancer cells. To further confirm these findings, dominant negative mutants, which lack the pro- and metalloprotease domain (Solomon et al., 1999) were ectopically expressed in MDA-MB-231 and MCF7 cells (Figure 26). These results show that stimulation with one GPCR agonist activates two different ADAM proteases mediating EGFR transactivation by processing the same EGF-like growth factor HB-EGF. On

the other hand, expression of dominant negative ADAM 15 or knockdown by siRNA did not interfere with the lysophospholipid-induced EGFR tyrosine-phosphorylation.

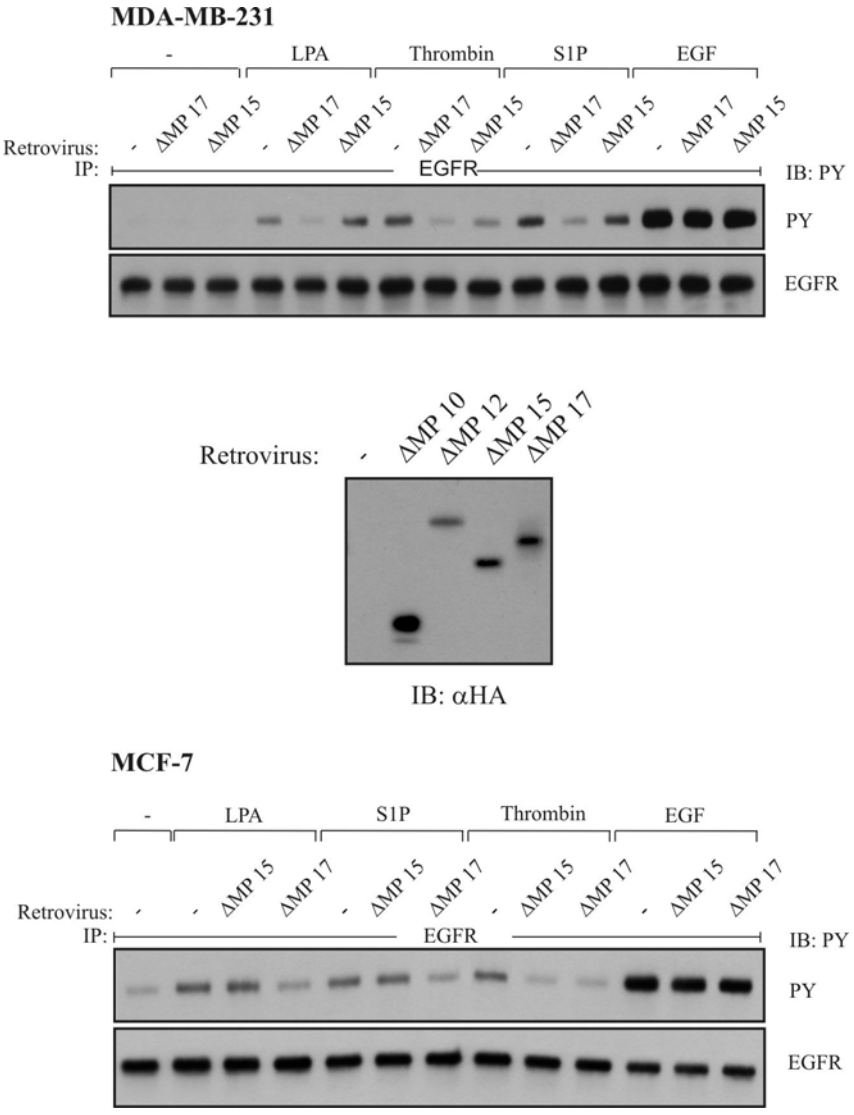


Figure 26 Dominant-negative ADAM constructs suppress GPCR-induced EGFR signal transactivation. MDA-MB-231 and MCF7 cells were retrovirally transfected with dominant-negative ADAM15 (ΔMP 15) and ADAM17 (ΔMP 17) constructs and stimulated as indicated. Following immunoprecipitation of cell extracts with anti-EGFR antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and re-probed with anti-EGFR antibody.

Furthermore, siRNA interference technique in the kidney cancer cell line A498 showed that thrombin-induced EGFR signal transactivation solely depends on ADAM10 activity, whereas ADAM17 mediated LPA-induced TMPS pathway (Figure 27) .

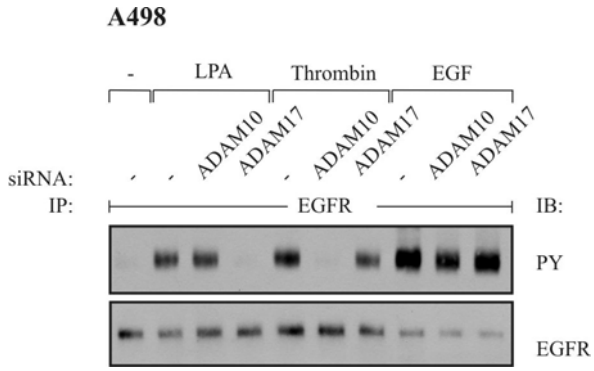


Figure 27 Specific activation of a single ADAM proteases after GPCR agonist treatment. A498 cells were transfected with siRNAs and stimulated with agonists as indicated. Following immunoprecipitation of cell extracts with anti-EGFR antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and re-probed with anti-EGFR antibody.

Table 4 *Depending on the cellular context and the GPCR agonists distinct ADAM proteases and EGF-like growth factors are mediating EGFR signal transactivation*

Zelllinie	*Ligand*	*ADAM9*	*ADAM10*	*ADAM12*	*ADAM15*	*ADAM17*	*HB-EGF*	*AR*
Cos7	LPA	30%	/	/	/	60%	95%	/
	Carbachol	/	/	/	/	90%	95%	/
	Thrombin	/	/	/	50%	50%	95%	/
A498	LPA	/	/	/	/	80%	80%	/
	Carbachol	/	/	/	/	90%	90%	/
	Thrombin	/	90%	/	/	20%	100%	/
	Bradykinin	/	/	/	/	50%	n.d	/
Caki II	LPA	/	/	/	/	80%	80%	/
	Thrombin	/	90%	/	/	20%	90%	/
	AngiotensinII	/	/	/	/	50%	n.d	/
SCC9	LPA	/	/	/	/	80%	/	70%
	S1P	/	/	/	/	85%	/	70%
	Carbachol	/	/	/	/	95%	/	80%
	Thrombin	/	/	/	/	80%	70%	/
NCI-H292	LPA	/	/	/	/	80%	/	/
	S1P	/	/	/	/	85%	/	/
	Thrombin	/	/	/	90%	/	/	/
MDA-MB-231	LPA	/	/	/	/	95%	90%	/
	S1P	/	/	/	/	60%	90%	/
	Thrombin	/	/	/	50%	50%	90%	/
MCF7	LPA	/	/	/	/	80%	70%	/
	S1P	/	/	/	/	70%	70%	/
	Thrombin	/	/	/	50%	50%	90%	/

3.4. GPCR-induced EGFR signal transactivation is involved in cell motility

In addition, having established a role for metalloprotease-dependent EGFR signal transactivation in ERK1/2 activation, we examined whether the TMPS pathway is capable of inducing chemotactic migration of head and neck cancer cells towards fibronectin *in vitro*.

LPA induced chemotactic migration of SCC9 cells, which was completely blocked by knockdown of AR or ADAM17 (Figure 28).

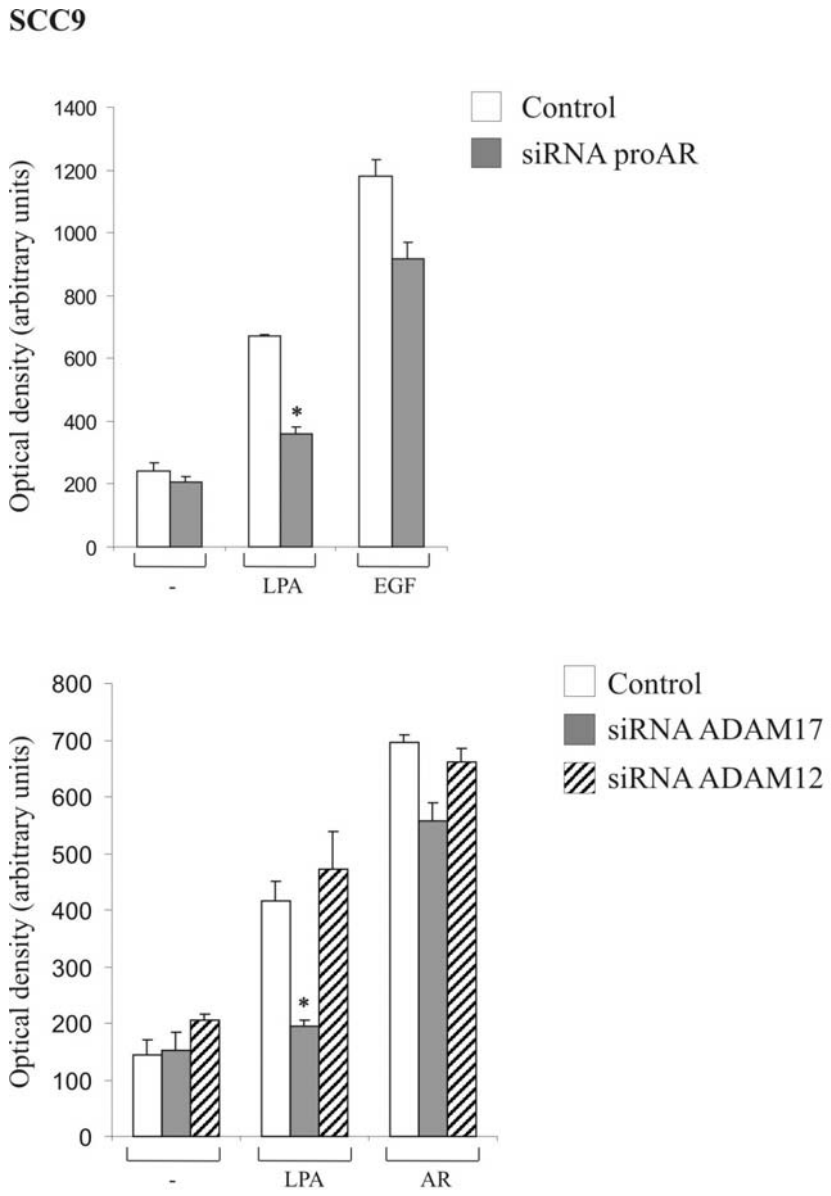


Figure 28 Amphiregulin and ADAM17 siRNA inhibit EGFR cell migration induced by GPCR agonists. siRNA-transfected SCC9 cells were seeded into membrane inserts of transwell dishes in the presence or absence of ligands (LPA, 10 μg/mL; EGF, 10 ng/mL; AR, 10 ng/mL) and were permitted to migrate for 36 h toward fibronectin as a chemoattractant. Nonmigrated cells were removed from the upper surface of the membrane, while migrated cells were fixed and stained with crystal violet. Absorbance of solubilized cells was measured with a micro plate reader. Each bar is the average of quadruplicate values (mean ± SD). *$p < 0.001$ for control siRNA + LPA versus siRNA transfected + LPA.

Chemotactic responses induced by EGF were reported in the highly metastatic human breast cancer cell line MDA-MB-231 (Sturge et al., 2002). We therefore assumed that GPCR stimulation followed by EGFR signal transactivation might influence the migratory behaviour of breast cancer cells.

We investigated the effect of lysophospholipids and thrombin on the chemotactic motility of breast cancer cells in a modified Boyden chamber assay. Both lysophospholipids, LPA and S1P, and EGF but not thrombin were able to promote chemotactic migration as a chemoattractant (Figure 29). Moreover, inhibition of the EGFR kinase, the metalloprotease activity or HB-EGF function abolished lysophospholipid and EGF induced migration of MDA-MB-231 cells (Figure 29).

In addition, we evaluated the effect of ADAM17 siRNA and proHB-EGF siRNA on the migratory behaviour of MDA-MB-231 cells. Downregulation of ADAM17 protein efficiently blocked S1P-induced chemotactic migration, while EGF-induced migration was also slightly reduced by ADAM17 siRNA. On the other hand, knockdown of proHB-EGF completely blocked migration of MDA-MB-231 after S1P treatment (Figure 30).

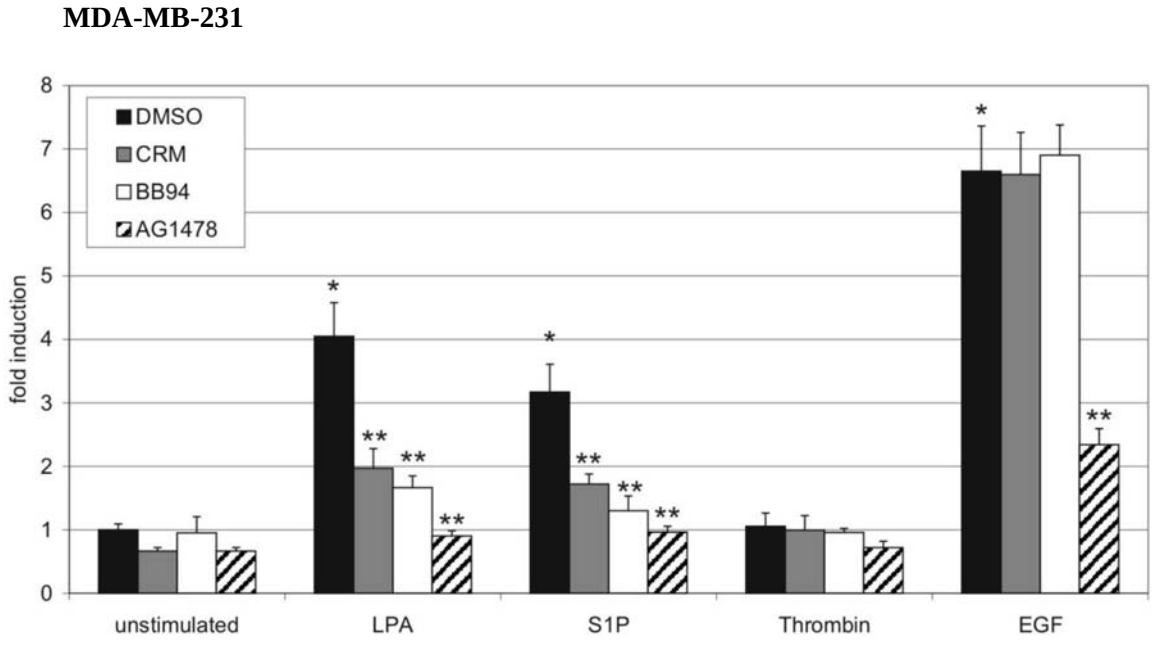


Figure 29 EGFR signal transactivation-induced chemotactic migration in MDA-MB-231 cells. MDA-MB-231 cells were preincubated with AG1478 (250 nM, 20 min), BB94 (5 μM, 20 min) or the diphtheria toxin mutant CRM197 (10 μg/mL , 20 min). Cells were seeded into membrane inserts of transwell dishes and were permitted to migrate for 6 h towards ligands as chemoattractants (LPA, 10 μM; S1P, 1 μM; Thrombin, 1 U/mL; EGF, 2 ng/mL). Nonmigrated cells were removed from the upper surface of the membrane, while migrated cells were fixed and stained with crystal violet. Migrated cells were optically counted under the microscope. Each bar is the average of quadruplicate values (mean ± SD.). *, $p < 0.01$ for the difference between control versus LPA, S1P and EGF. **, $p < 0.005$ for the difference between stimulation versus inhibitors.

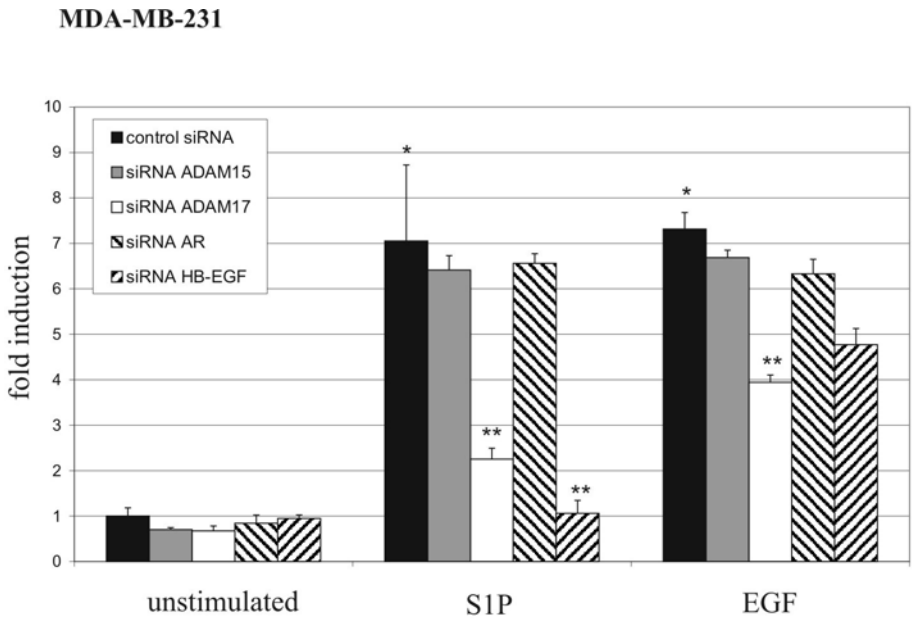


Figure 30 EGFR signal transactivation-induced chemotactic migration in MDA-MB-231 cells. siRNA transfected MDA-MB-231 cells were seeded into membrane inserts of transwell dishes and were permitted to migrate for 6 h towards ligands as chemoattractants (S1P, 1 μM; Thrombin, 1 U/mL; EGF, 2 ng/mL) and analysed as described in Figure 29. Each bar is the average of quadruplicate values (mean ± SD.). *, $p < 0.01$ for the difference between control versus S1P and EGF. **, $p < 0.005$ for the difference between stimulation versus inhibitors.

3.5 Cannabinoid-induced EGFR signal transactivation

3.5.1 EGFR signal transactivation upon cannabinoid treatment in human carcinoma cells depends on metalloprotease activity

In order to address the question whether cannabinoids lead to transactivation of the EGFR in human cancer cell lines, we treated NCI-H292 (lung cancer), SCC9 (squamous cell carcinoma), 5637 (bladder carcinoma), 1321N1 (astrocytoma) and A498 (kidney cancer) cells with the synthetic cannabinoids Win55,212-2 and HU210, the endogenous cannabinoid anandamide (AEA) and the naturally occurring Δ^9-Tetrahydrocannabinol (THC). Resulting EGFR tyrosine phosphorylation levels were monitored by immunoblot analysis. As shown in Figure 31, cannabinoids rapidly induced EGFR activation within 3 minutes. Preincubation of the cells with the metalloprotease inhibitor batimastat or the EGFR kinase specific inhibitor AG1478 prevented EGFR tyrosine phosphorylation in response to cannabinoid stimulation (Figure 31).

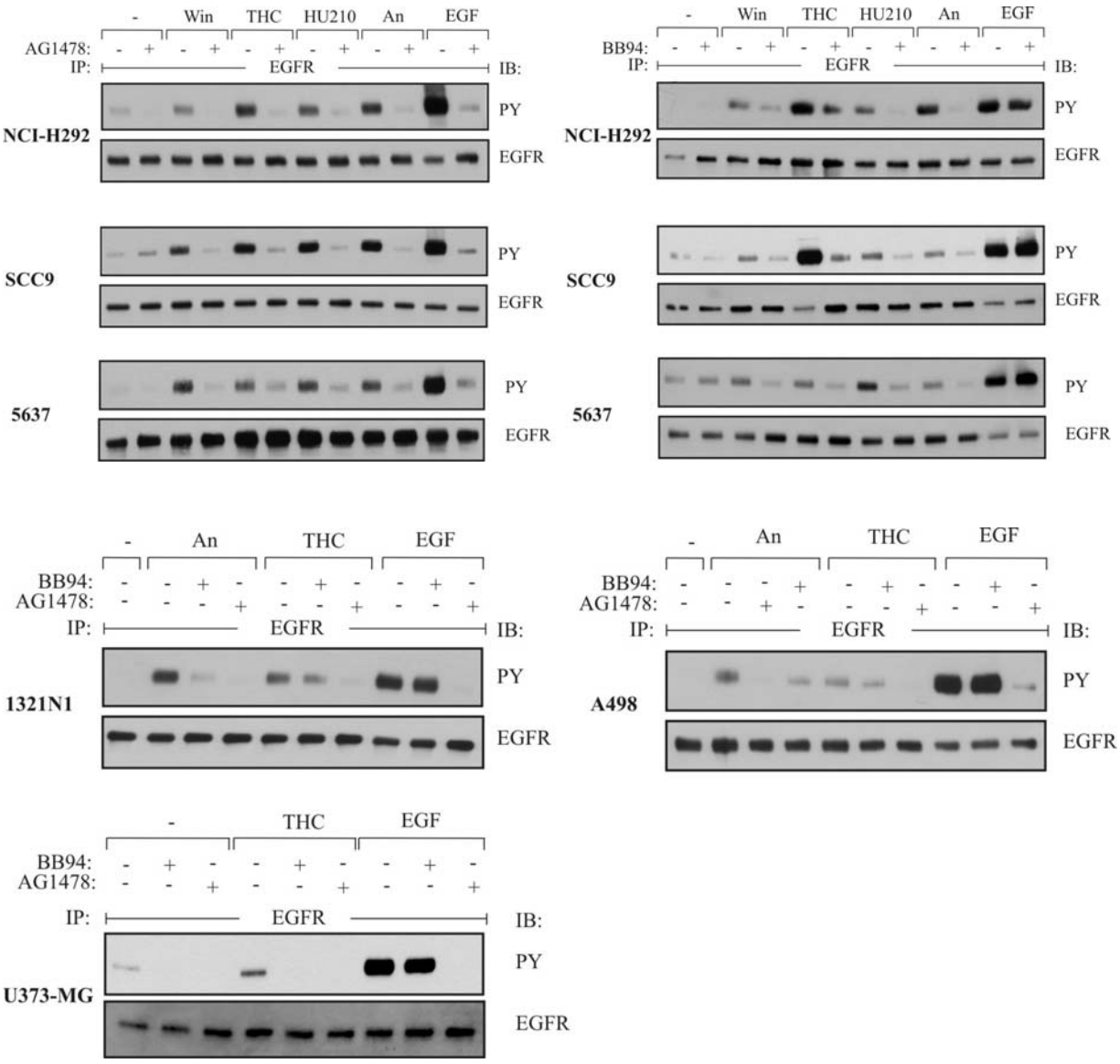


Figure 31 Cannabinoid-induced EGFR signal transactivation requires EGFR tyrosine kinase activity and a metalloprotease activity. Serum-starved NCI-H292, SCC9, 5637, 1321N1, U373-MG and A498 cells were treated with EGFR-specific tyrphostin AG1478 (250 nM, 20 min), the metalloprotease inhibitor BB94 (5 μM, 20 min) or vehicle (DMSO) and were treated with cannabinoids (Win55,212-2 (10 μM), THC (1 μM), HU210 (50 nM), AEA (10 μM)) and EGF (5 ng/mL) for 3 min. After lysis, EGFR was immunoprecipitated (IP) using anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with anti-phosphotyrosine (αPY) antibody, followed by reprobing of the same filter with anti-EGFR antibody.

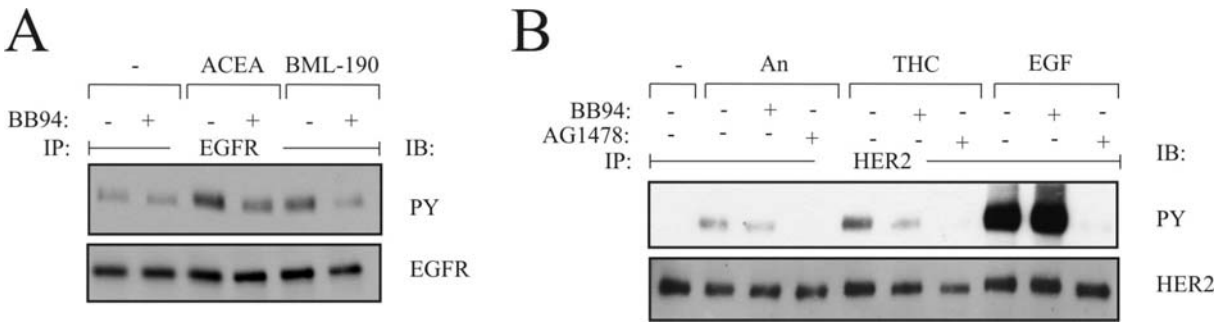


Figure 32 CB1 and CB2 receptor transactivate the EGFR and HER2. (A) Serum-starved NCI-H292 cells were preincubated with BB94 (5 μM, 20 min) and stimulated with ACEA (200 nM) or BML-190 (5 μM) for 3 min and analysed as described in Figure 31. (B) Serum-starved NCI-H292 cells were preincubated with BB94 (5 μM, 20 min), tyrphostin AG1478 (250 nM, 20 min) or vehicle (DMSO) and stimulated with AEA (10 μM) or THC (1 μM) for 3 min. After lysis, HER2 was immunoprecipitated and assayed for HER2 tyrosine phosphorylation content.

Stimulation of NCI-H292 cells with receptor subtype specific agonists ACEA and BML-190 for CB1 and CB2 receptor respectively, demonstrated that both cannabinoid receptors are capable of transactivating the EGFR (Figure 32A). Expression of both, the CB1 and the CB2 receptor, was detected by cDNA macroarray and Northern blot analysis in all five cancer cell lines (data not shown). Interestingly the EGFR relative HER2/neu, which serves as a prognostic marker in many different cancer types, was likewise activated in response to cannabinoid stimulation (Figure 32B). Both AEA- and THC-induced tyrosine phosphorylation of HER2/neu in NCI-H292 cells depended on metalloprotease and EGFR activity. Therefore, phosphorylation of HER2/neu appears to result from EGFR transphosphorylation. Taken together, these experiments demonstrate that cannabinoids rapidly induce EGFR and HER2/neu signal transactivation in a metalloprotease-dependent manner in different human cancer cell lines.

In order to assess whether the EGFR links cannabinoids to ERK1/2 activation, we first analysed the tyrosine phosphorylation content of the adaptor protein SHC. Figure 33 demonstrates that treating cells with Win55,212-2 , THC, HU210 and AEA resulted in EGFR-dependent tyrosine phosphorylation of SHC.

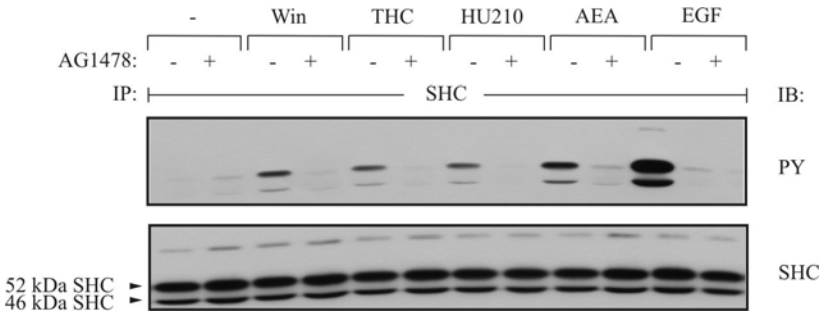


Figure 33 Cannabinoid-induced SHC tyrosine phosphorylation is EGFR- and metalloprotease-dependent. Serum-starved SCC9 cells were treated as described in Figure 31A. Precipitated SHC was immunoblotted with αPY antibody followed by reprobing of the same filters with anti-SHC antibody**.**

We further analysed ERK1/2 activation by immunoblot using activation state-specific MAPK antibodies. As shown in Figure 34A, treating NCI-H292 and SCC9 cells with cannabinoids potently activated ERK1/2 to a similar extent as EGF (5 ng/mL) stimulation. Preincubation with AG1478 or BB94 completely abrogated cannabinoid-induced MAPK activation

indicating that in these human carcinoma cells cannabinoid-induced ERK1/2 activation completely depends on EGFR signal transactivation (Figure 34A).

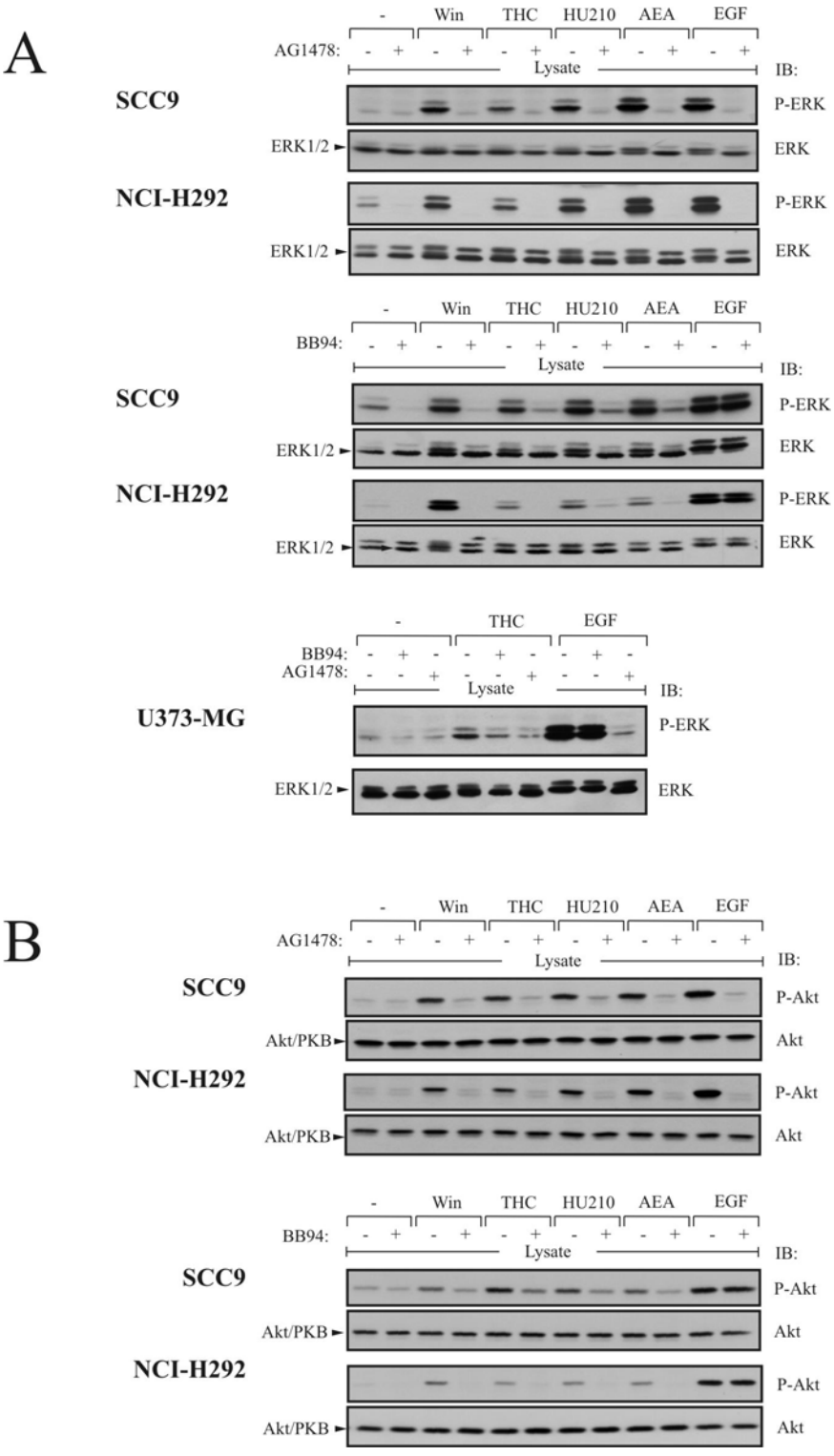


Figure 34 Cannabinoid-induced activation of ERK1/2 and Akt/PKB pathways is EGFR- and metalloprotease-dependent. (A) Serum-starved SCC9 cells and NCI-H292 cells were preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min), respectively, and stimulated for 7 min as indicated. Phosphorylated ERK1/2 was detected by immunoblotting with phospho-specific ERK1/2 antibody. The same filters were reprobed with anti-ERK antibody. (B) Serum-starved SCC9 cells and NCI-H292 cells were

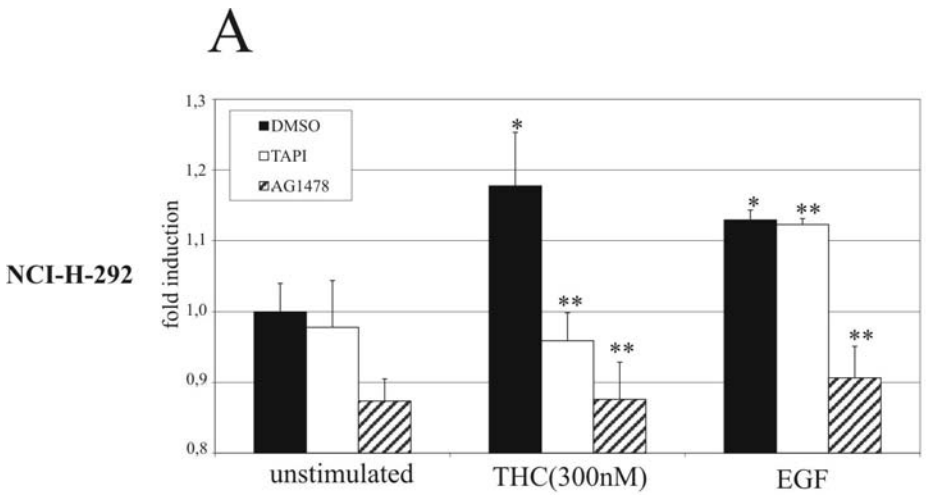
preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min), respectively, and stimulated for 7 min as indicated. Activated Akt/PKB was detected by immunoblotting with phospho-specific Akt/PKB antibody. The same filters were reprobed with anti-Akt/PKB antibody.

In addition to ERK stimulation, a variety of GPCR agonists were shown to activate the survival mediator Akt/PKB (Fischer et al., 2003; Gschwind et al., 2003). Since cannabinoids were known to activate Akt/PKB in astrocytoma cells (Galve-Roperh et al., 2002), we addressed the question whether cannabinoids activate Akt/PKB activation in human cancer cells and whether this depends on the EGFR. Indeed, activation of Akt/PKB by all four cannabinoids was completely blocked by the selective EGFR inhibitor AG1478 and by the metalloprotease inhibitor BB94 (Figure 34B).

Taken together, tyrosine phosphorylation of SHC, activation of the Akt/PKB and the ERK/MAPK pathway after cannabinoid treatment of different human cancer cell lines critically depend on metalloprotease-mediated EGFR signal transactivation.

3.5.2 EGFR mediates cannabinoid-induced proliferation.

Since ERK1/2 are generally known to mediate proliferation in a variety of cells, we addressed the question whether cannabinoids induce proliferation of cancer cells (Guzman et al., 2002). To determine the proliferation rate in response to THC stimulation, the turnover of MTT and ^{3}H-thymidine incorporation in DNA was measured.



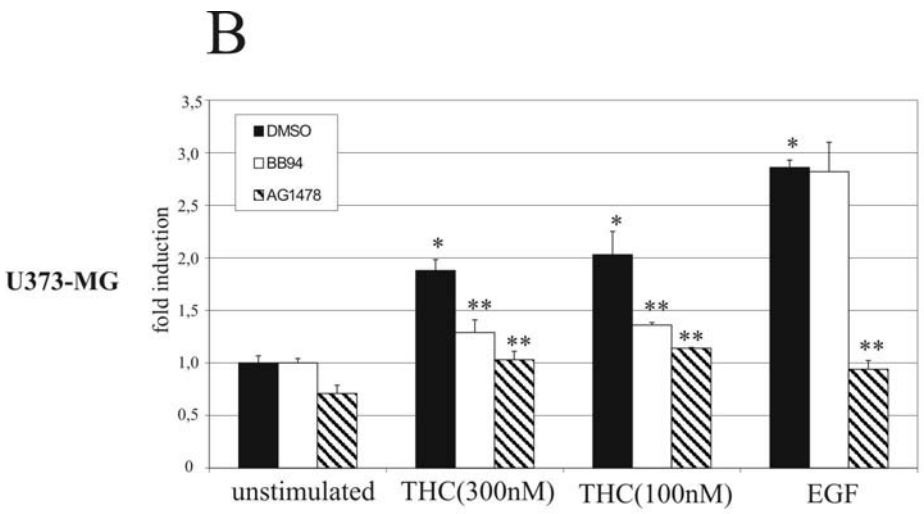


Figure 35 THC-induced proliferation of NCI-H292 and U373-MG cells depends on EGFR transactivation. (A) MTT-Assay. Serum-starved NCI-H292 cells were treated with AG1478 (250 nM) and the metalloprotease inhibitor TAPI (5 μM) and incubated for 24 h in the presence or absence of ligands (THC 300 nM, EGF 5 ng/mL). *bars*, ± SD of OD at 570 nm of three independent experiments. *, $p < 0.01$ for the difference between DMSO *versus* THC and EGF; **, $p < 0.01$ for the difference between agonists *versus* inhibitors + agonists.(B) ^{3}H-thymidine incorporation in DNA. Serum-starved U373-cells were pretreated with AG1478 (250 nM) or the metalloprotease inhibitor BB94 (5 μM) and incubated in the presence or absence of ligands (THC 300 nM/100 nM, EGF 5 ng/mL) for 18 h. Cells were then pulse-labelled with ^{3}H-thymidine and thymidine incorporation was measured by liquid-scintillation counting. ± SD of four independent experiments. *, $p < 0.01$ for the difference between DMSO *versus* THC and EGF; **, $p < 0.01$ for the difference between agonists *versus* inhibitors + agonists

As shown in Figure 35A, THC (300 nM) was similar potent as EGF (5 ng/mL) to increase MTT-turnover in NCI-H-292 cells. To investigate the involvement of the EGFR signal transactivation pathway for cannabinoid-induced cell proliferation, we preincubated the cells with AG1478 and the metalloprotease inhibitor TAPI. The proliferative effect of THC was abolished in the presence of either AG1478 or TAPI. Furtheremore, the glioblastoma cells U373-MG displayed a significant enhanced rate of DNA synthesis triggered by 300 nM and also by 100 nM THC (Figure 35B). Batimastat and the EGFR-specific inhibitor tyrphostin AG1478 decreased DNA synthesis by LPA to basal level.

In contrast to these observations, several earlier studies reported induction of apoptosis upon treatment of cells with higher concentrations of THC. Therefore, we tested whether and in particular what concentrations of THC would be able to trigger cell death of NCI-H292 cells. As shown in Figure 36, micromolar concentrations of THC were able to induce apoptosis, while sub-micromolar concentrations did not affect cell survival.

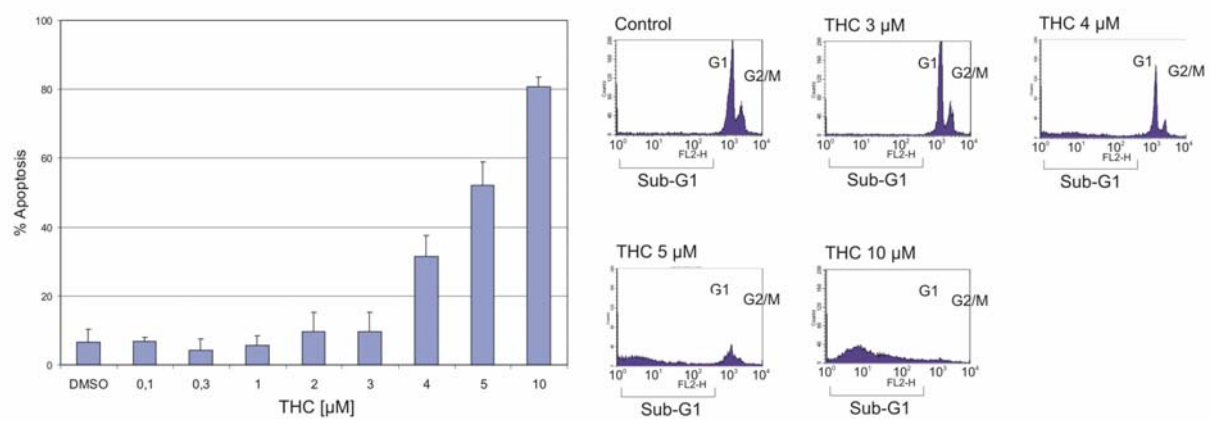


Figure 36 Micomolar concentrations of THC induce apoptosis rate. Serum-starved NCI-H292 cells were treated with different concentrations of THC as indicated for 6 h. After collection of the cells in assay buffer, nuclei were stained with PI and analysed by flow cytometric analysis. Quantification of experiments performed in quadruplicate; *bars*, ± SD.

These results demonstrate that physiological relevant concentrations of THC promote cell proliferation of human carcinoma cells and identify EGFR signal transactivation as the underlying molecular mechanism.

3.5.3 TACE/ADAM17 mediates cannabinoid-induced EGFR activation.

Our observations clearly showed that cannabinoid receptors transactivate the EGFR in a wide variety of human cancer cell lines involving metalloprotease activity (Figure 31). We have previously shown the critical involvement of the metalloprotease ADAM17 in proAR shedding after GPCR stimulation (Gschwind et al., 2003). Therefore we raised the question whether ADAM17 is required for cannabinoid-induced EGFR signal transactivation. We blocked endogenous ADAM17 expression using a siRNA approach. ADAM17 mRNA and protein was effectively and specifically reduced by transfecting siRNAs into NCI-H292 cells (Figure 37A/B). Inhibition of ADAM17 expression but not of the related ADAM12 completely suppressed cannabinoid-induced EGFR tyrosine phosphorylation in NCI-H292 and SCC9 cells (Figure 37C). Likewise, the cannabinoid-induced phosphorylation and activation of ERK1/2 and Akt/PKB was abolished in the absence of ADAM17 but was unaffected by the downregulation of ADAM12 (Figure 37D). As expected, suppression of neither protease had an affect on signalling events induced by direct EGF stimulation of the EGFR.

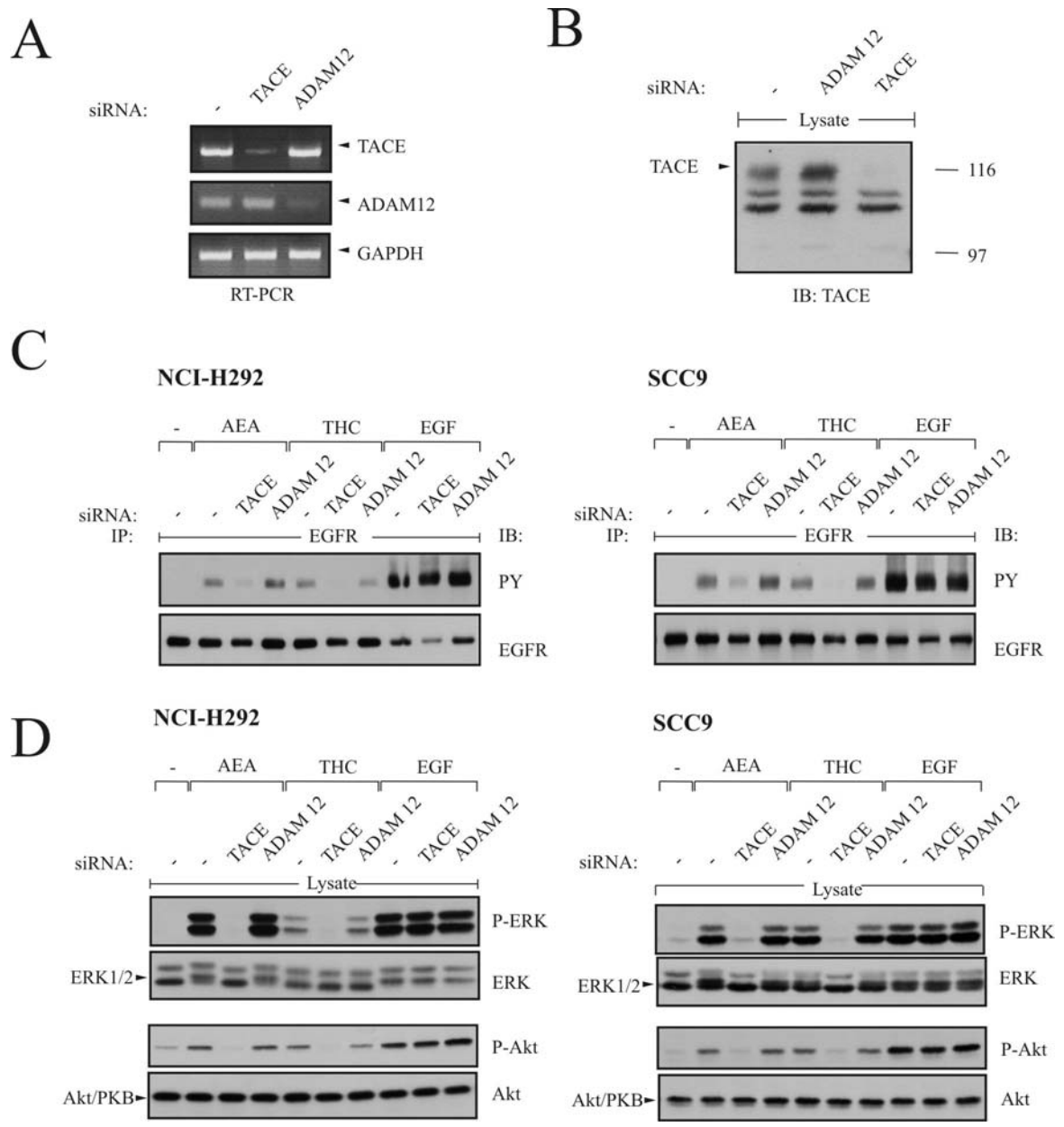


Figure 37 ADAM17 mediates cannabinoid-induced EGFR transactivation, ERK1/2 and Akt/PKB activation. (A/B) NCI-H292 cells were transfected with ADAM17 or ADAM12 siRNA. Gene expression was analysed by RT-PCR (A) or immunoblotting with ADAM17 antibody (B). (C) NCI-H292 cells and SCC9 cells were transfected with siRNAs raised against ADAM17 and ADAM12, serum-starved, stimulated for 3 min with THC (1 μM), AEA (10 μM) and EGF (5 ng/mL) and assayed for EGFR tyrosine phosphorylation content. (D) NCI-H292 cells and SCC9 cells were transfected with siRNAs and stimulated for 7 min with agonists as indicated. Phosphorylated ERK1/2 and activated Akt/PKB were detected by immunoblotting with phospho-specific ERK1/2 and Akt/PKB antibodies, respectively. The same filters were reprobed with anti-ERK1/2 antibody and anti-Akt/PKB, respectively

3.5.4 Ectodomain shedding of proHB-EGF and proAR mediates cannabinoid-induced EGFR activation.

Among the different EGF-like precursors proAR, proHB-EGF and proTGFα are predominantly expressed in NCI-H292 and SCC9 cells as indicated by cDNA macroarray analysis (Figure 13). To investigate which ligand is involved in the EGFR signal

transactivation pathway following cannabinoid stimulation, we transiently transfected siRNAs and efficient and specific silencing of the endogenous expression of proAR, proHB-EGF and proTGFα was monitored by RT-PCR (Figure 38). AEA- and THC-induced tyrosine phosphorylation of the EGFR in NCI-H-292 cells solely depend on proHB-EGF (Figure 38B). The ability of the diphtheria toxin mutant CRM197, a specific inhibitor of proHB-EGF function, to block EGFR phosphorylation in response to THC stimulation substantiated this observation (Figure 38C).

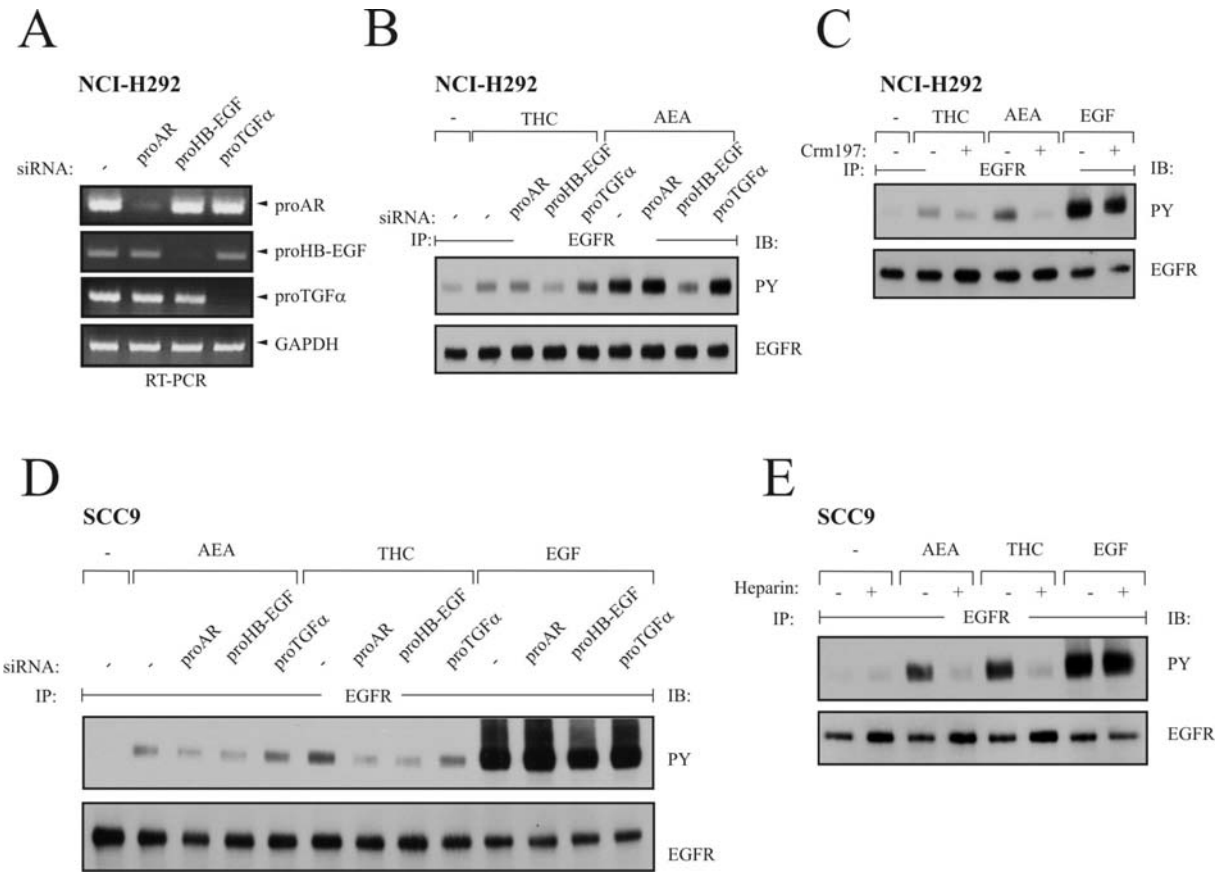


Figure 38 Cannabinoid-induced EGFR signal transactivation is mediated by proAR and/or proHB-EGF. (A) NCI-H292 cells were transfected with siRNAs raised against proAR, proHB-EGF and proTGFα. Gene expression was analysed by RT-PCR. (B) NCI-H292 cells were transfected with siRNAs as indicated, stimulated with THC (1 μM) and AEA (10 μM) and assayed for EGFR tyrosine phosphorylation. (C) Serum-starved NCI-H292 cells were preincubated with CRM197 (10 μg/mL, 20 min), stimulated with THC (1 μM) and AEA (10 μM) for 3 min and assayed for EGFR tyrosine phosphorylation. (D) SCC9 cells were transfected with siRNAs raised against proAR, proHB-EGF and proTGFα, stimulated with THC (1 μM) and AEA (10 μM) for 3 min and assayed for EGFR tyrosine phosphorylation content. (E) Serum-starved SCC9 cells were preincubated with Heparin (100 ng/mL, 15 min), stimulated with THC (1 μM) and AEA (10 μM) and assayed for EGFR tyrosine phosphorylation.

In contrast, in SCC9 cells cannabinoid-induced transactivation of the EGFR required proAR as well as proHB-EGF expression (Figure 38D). Suppression of either ligand resulted in partial reduction of cannabinoid-induced EGFR phosphorylation, whereas proTGFα

inhibition did not affect EGFR phosphorylation at all. Furthermore, preincubation with heparin, which abrogates both proAR and proHB-EGF function, also interfered with cannabinoid receptor-EGFR cross-talk (Figure 38E).

Together these results show that cannabinoid-induced EGFR signal transactivation is mediated by specific proteolytic processing of the two heparin-binding EGFR ligands, proAR and proHB-EGF, by one and the same zinc-dependent metalloprotease ADAM17.

4. Discussion

Aberrant signalling of RTKs is critically involved in the development and progression of hyperproliferative diseases such as cancer (Blume-Jensen and Hunter, 2001a). Constitutive activation of RTKs originates by gene amplification, overexpression, activating mutations or autocrine stimulation of the receptors by growth factors (Zwick et al., 2001).

In addition deregulated GPCR signalling by autocrine production of GPCR agonists or activating mutations has been frequently associated with different types of human cancer (Marinissen and Gutkind, 2001; Moody et al., 2003). In consideration of the pathophysiological significance of both heptahelical receptor-mediated and direct EGFR signalling, this study investigates the role and the molecular mechanisms of EGFR activation in response to GPCR agonists in human tumour cells.

4.1. GPCR-induced EGFR signal transactivation in human cancer cells requires a metalloprotease activity

Previous publications implicated the EGFR in the molecular pathology of many tumour types including NSCLC, breast cancer, kidney cancer glioblastoma and astrocytoma (Konecny et al., 2003; Lage et al., 2003; Mischel and Cloughesy, 2003; Sridhar et al., 2003). Moreover GPCR agonist signalling loops have been demonstrated in various types of cancer (Fischer et al., 2003; Moody, 1996; Whitehead et al., 2001).

Cross-communication between GPCRs and the EGFR was originally proposed as a ligand-independent, intracellular pathway. This notion was supported by the absence of detectable soluble EGF-like growth factors in the cell culture medium and the fast kinetics of EGFR tyrosine phosphorylation in response to GPCR agonists. However, the finding that a chimeric receptor, consisting of the ligand binding domain of the EGFR and the PDGF receptor`s transmembrane and intracellular part can be transactivated supported the notion that the EGFR extracellular domain is involved in the GPCR-EGFR cross-talk (Prenzel et al., 1999) The data presented in this study provide evidence for the functional relevance of GPCR stimulation and EGFR downstream signalling pathways employing metalloprotease-dependent EGFR signal transactivation. Stimulation with GPCR ligands induces the rapid tyrosine phosphorylation of the EGFR in different tumour types but also in non-tumour cells and primary mammary epithelial cells (Figure 7, Table 2), depending on the cellular system and the stimuli investigated. These data are in agreement with studies which demonstrate that EGFR signal transactivation in Cos-7, HEK-293 and Rat-1 cells critically depends on cell

surface shedding of the EGF-like growth factor HB-EGF through an unknown metalloprotease which is sensitive to the inhibitor batimastat (Prenzel et al., 1999).

The experimental finding that LPA, S1P and thrombin are able to induce GPCR-EGFR cross communication in all cell lines examined suggests that EGFR signal transactivation plays an important role in transformed and non-transformed cells. Mammary epithelial cells reveal only a weak GPCR-induced stimulation of EGFR phosphorylation which can be attributed to a very low EGFR expression level. LPA was previously described to induce EGFR tyrosine phosphorylation in several model systems including Rat-1 (Daub et al., 1996), HEK-293 (Della Rocca et al., 1999), PC-12 (Kim et al., 2000), Swiss 3T3 (Gohla et al., 1998), HaCaT and Cos-7 (Daub et al., 1997). Three LPA receptors which show differences in G protein-coupling are known. EGFR signal transactivation was strongly inhibited by PTX (Figure 12B) in MDA-MB-231 cells suggesting that predominantly PTX-sensitive G protein subunits of the $G\alpha_{i/o}$ family mediate EGFR transactivation following LPA treatment in this cell type. The finding that many different GPCR agonists induce the transactivation signal (Table 2) suggests the EGFR as a central integrator of signalling by diverse GPCR ligands. Furthermore, cancer cell lines derived from the neuronal system are responsive to a broad spectrum of neurotransmitters to mediate metalloprotease-dependent EGFR tyrosine phosphorylation (Table 3). On the other hand, growth promoting effects of neuropeptides in cancer cells which are described for breast cancer, colon cancer, prostate and pancreatic cancer, ovarian and cervical carcinoma (Rozengurt, 2002), were not observed in cancer cell lines derived from the neuronal system (unpublished observation).

Besides the EGFR itself, GPCR agonist treatment induces EGFR characteristic downstream signalling cascades. Metalloprotease-dependent phosphorylation of the adaptor protein SHC occurs in response to GPCR stimulation (Figure 8), which is linked to activation of the MAPKs ERK1/2 (Figure 9, Figure 10). The role of the EGFR as a transducer of GPCR stimulation to these MAPKs has been demonstrated in a variety of other cell systems, including Rat-1 (Daub et al., 1996), Cos-7 (Daub et al., 1997; Prenzel et al., 1999), PC-12 (Kim et al., 2002), HEK-293 cells (Della Rocca et al., 1999) and head and neck squamous cell carcinoma (Gschwind et al., 2002). These experimental data indicate that the EGFR is instrumental in transducing mitogenic signals in response to GPCR agonist stimulation in a variety of human cancer cell lines. Besides EGFR dependent MAPK activation by GPCR agonists, studies with batimastat indicated the involvement of a metalloprotease activity in the activation of ERK (Figure 9, Figure 10). Previous reports demonstrated a ligand dependent ERK activation in vascular smooth muscle cells (Eguchi et al., 2001; Kalmes et al., 2000).

Interestingly, depending on the cellular context, activation of the survival mediator Akt/PKB was dependent or independent of EGFR signal transactivation. Akt/PKB activation in the squamous cell carcinoma cell line SCC9 was completely dependent on metalloprotease and EGFR activity. On the other hand, pre-treatment of the breast carcinoma cells MDA-MB-231 with batimastat or AG1478 did not interfere with Akt/PKB activation (Figure 12). Preincubation of MDA-MB-231 cells with PTX blocked both, EGFR and Akt/PKB phosphorylation suggesting the involvement of PTX-sensitive G_{α}-subunits in both signalling pathways (Figure 12).

Importantly, N,N- Dimethylsphingosine, a specific inhibitor of SPHK was able to block GPCR-induced PI3K-dependent Akt/PKB phosphorylation without interfering with EGFR signal transactivation (Figure 12), indicating that SPHK is a downstream effector of the EDG-receptors independent of EGFR kinase activity in MDA-MB-231 cells. Activation of SPHK by GPCRs such as the estrogen receptor (ER) or muscarinic acetylcholine receptors (mAChRs) leading to ERK1/2 or Ca^{2+} signalling, respectively, was noted before (Meyer zu Heringdorf et al., 1998; Sukocheva et al., 2003). Recently, SPHK was shown to be phosphorylated and activated by MAPK, which was not seen in our cellular system by preincubation of the cells with PD98059 (unpublished observation).

4.2. ProAR and/or proHB-EGF shedding is a prerequisite to EGFR activation by GPCR agonists in various human carcinoma cells

Although different observations suggested a ligand-independent intracellular signalling mechanism (Daub et al., 1997; Eguchi et al., 1998; Tsai et al., 1997), Prenzel and colleagues were the first to show the metalloprotease-mediated processing of the EGF-like ligand proHB-EGF in Cos-7 cells and therefore a ligand-dependent mechanism in EGFR signal transactivation (Prenzel et al., 1999). Our findings in the breast cancer cell lines MDA-MB-231 and MCF7 are in agreement with the previous findings, that HB-EGF function is required for GPCR agonist-induced EGFR signal transactivation. Blocking of HB-EGF using the diphtheria toxin mutant CRM197 or heparin abrogated GPCR-stimulated EGFR phosphorylation (Figure 15).

Interestingly, even though SCC9 cells express proAR and proTGFα in addition to proHB-EGF (Figure 13) and some functional redundancy within the EGFR ligand family in developmental processes (Luetteke et al., 1999), LPA and Carbachol selectively induce EGFR signal transactivation via proAR shedding. Downregulation of proAR mRNA level using a

siRNA approach totally blocked GPCR-induced tyrosine phosphorylation of the EGFR (Figure 14). In these experiments, the potential involvement of proHB-EGF and proTGFα was excluded. ProAR-shedding by a metalloprotease was previously shown by Brown *et. al.* after stimulation with non-physiological stimuli such as TPA, pervanadate or calcium ionophore (Brown et al., 2001). This is the first report proposing proAR processing induced by GPCR agonists.

Cross-communication between GPCRs and the EGFR can not only be mediated by proHB-EGF or proAR. Carbachol treatment of T84 cells and prostaglandin-E2 stimulation of gastric epithelial cells leads to shedding of proTGFα by an unknown metalloprotease and subsequently EGFR transactivation and downstream ERK1/2 activation (McCole et al., 2002; Pai et al., 2002).

4.3. Specific activation of distinct metalloproteases of the ADAM family in response to GPCR stimulation

Metalloprotease-mediated ectodomain shedding of growth factor precursors *in vivo* is as yet only poorly understood. A growing body of evidence suggests the involvement of several members of the ADAM family of zinc-dependent proteases in the processing of EGF-like precursors (Werb and Yan, 1998). ADAM9 was implicated in the shedding of proHB-EGF by TPA in Vero-H cells (Izumi et al., 1998), while LPA-induced proHB-EGF cleavage in the same cell system is independent of ADAM9 (Umata et al., 2001). The severe phenotype of mice lacking ADAM17 suggests an essential role for this metalloprotease in processing of proTGFα and possibly other EGFR ligands in normal development. Furthermore, absence of functional ADAM17 results in impaired basal cleavage of proAR and proHB-EGF in murine fibroblasts (Merlos-Suarez et al., 2001; Sunnarborg et al., 2002). ADAM17 and other members of the ADAM family are widely expressed in the cell lines tested for EGFR transactivation on the mRNA (Figure 16) and protein level (Figure 17). Here we demonstrate that ADAM17 is the sheddase of proAR after LPA, S1P and Carbachol in SCC9 cells (Table 4). Recently, Lemjabbar et. al found ADAM17 to be the responsible sheddase of proAR in the lung cancer cell line NCI-H-292 after incubation of the cells with tabacco smoke (Lemjabbar et al., 2003). Most importantly, in SCC9 cells thrombin-induced activation of ADAM17 leads to proteolytic cleavage of proHB-EGF instead of proAR (Table 4). These data can be explained by preclustering of the different GPCRs and ADAM17 in lipid rafts, as different pools of ADAM17 have to be activated which specifically cleave two different EGFR ligands within one cell line depending on the GPCR agonist. Although recent reports implicate

ADAM 10 (Lemjabbar and Basbaum, 2002; Yan et al., 2002) and ADAM12 (Asakura et al., 2002) in proHB-EGF-dependent EGFR signal transactivation in MDA-MB-231 cells the involvement of these metalloproteases was excluded (Figure 21). Interestingly, in the kidney carcinoma cells A498 and Caki II LPA specifically induced proHB-EGF-dependent EGFR signal transactivation via ADAM17, whereas thrombin stimulation of the same cells specifically activates ADAM10-induced proHB-EGF processing. Moreover, the finding that in the breast cancer cell lines MCF7 and MDA-MB-231 both ADAM15 and ADAM17 were involved in HB-EGF-mediated EGFR signal transactivation after thrombin stimulation, wheras LPA and S1P stimulation soley depended on ADAM17 activity, added an additional layer of complexity. Taken together, the molecular details of the TMPS mechanism seem to diverge on different levels, namely besides the GPCR agonists, the metalloprotease and the EGFR ligand (Figure 39). Within this context, one GPCR ligand is specifically activating two ADAM proteases within one cell line, leading to the processing of the same EGF-like ligand proHB-EGF (Figure 26).

An unsolved question is the regulation and the specificity of sheddase activity. ERK has been shown to bind and phosphorylate the cytoplasmic tail of ADAM17 at threonine 735 in response to TPA stimulation (Diaz-Rodriguez et al., 2002), but GPCR-induced EGFR tyrosine phosphorylation is not blocked by preincubation with MEK inhibitor in HNSCC and breast cancer cells (unpublished observation). Part of the regulation was attributed to the specifity of the ADAMs for their targets. Experiments with chimeras of TGFα and APP propose that the secondary structure of the juxtamembrane region is the important recognition element for the sheddase (Arribas et al., 1997).

4.4. The migratory behaviour of HNSCC and breast cancer cells depends on components of the TMPS pathway

Various studies supported the notion that the EGFR acts as a central integrator of diverse GPCR signals (Carpenter, 1999; Gschwind et al., 2001). Here we present data indicating that the GPCR-induced EGFR signal transactivation appears to be a driving force for cell migration in HNSCC and breast cancer cells. Within this signalling context, both ADAM17 and AR function are critically involved in the migratory behaviour of SCC9 cells after LPA stimulation (Figure 28) . Recently, AR was shown to be a potent inducer of cellular invasion by activation of the EGFR in kidney and colonic cancer cells (Rodrigues et al., 2003).

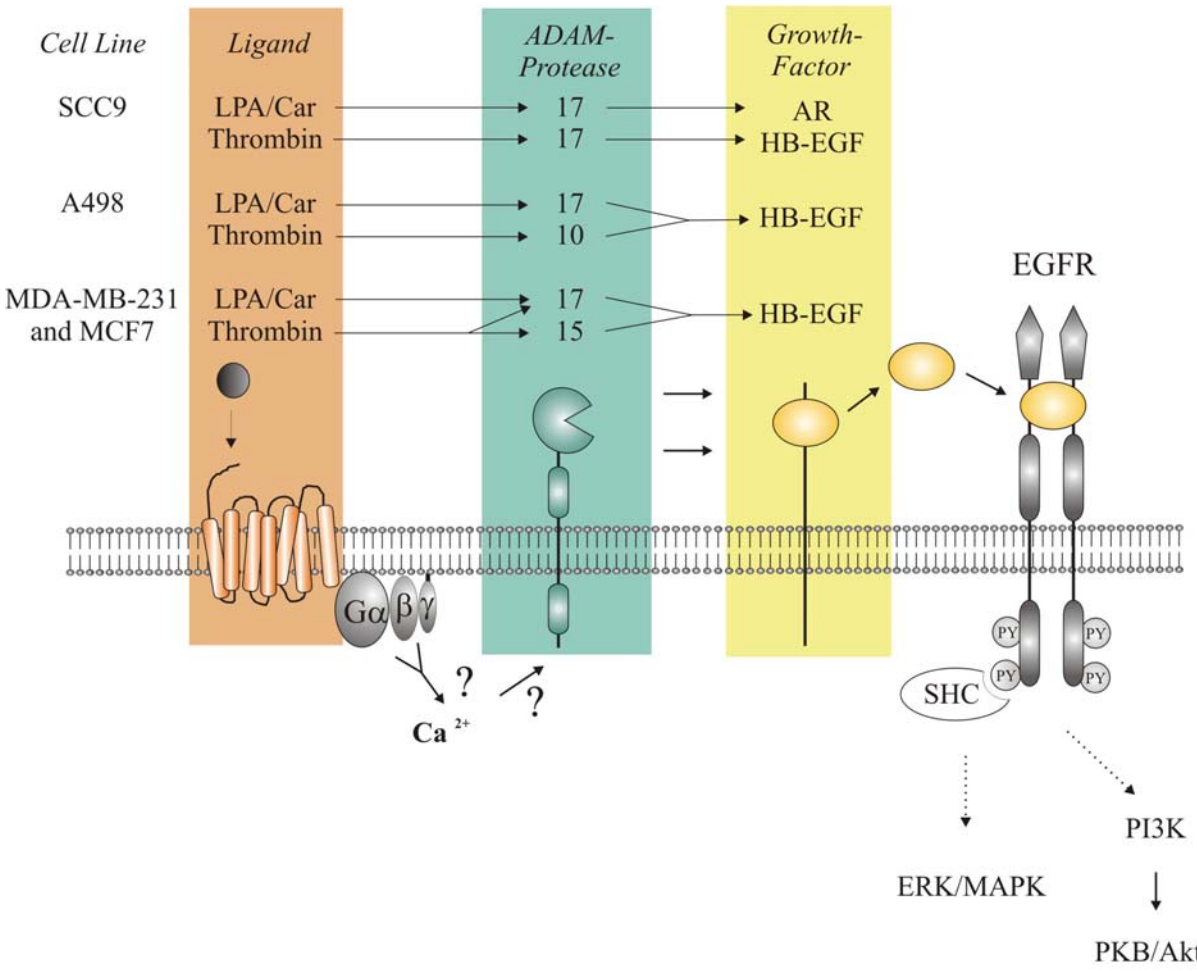


Figure 39 Critical elements of TMPS mechanism of EGFR signal transactivation in human cancer cells. Specific activation of ADAM family members by GPCR agonists results in cell surface ectodomain cleavage of proAR or proEGF, depending on the cellular context. Upon release of the mature EGF-like growth factor the EGFR is activated leading to an EGFR-characteristic intracellular signal.

Furthermore, the extracellular matrix metalloproteases (MMPs) which are believed to play a key role in tumour cell invasion and metastasis, are secreted in malignant mesothelioma cells after AR stimulation (Liu and Klominek, 2003). AR-mediated EGFR activation also leads to a proliferative response in SCC9 cells (Gschwind et al., 2003).

Price *et al.* demonstrated that the breast cancer cell line MDA-MB-231 react with a potent chemotactic response but not with a proliferative response to EGF. Our results in MDA-MB-231 cells demonstrating that EGFR signal transactivation by EDG-receptor agonists via HB-EGF leads to chemotaxis but not proliferation (unpublished data), are in agreement with these data (Figure 29, Figure 30). Furthermore, migration was blocked by inhibition of ADAM17 as well as HB-EGF function. In contrast, thrombin did not induce chemotaxis in MDA-MB-231 cells even though it was able to activate the EGFR. This finding could be explained by the fact that thrombin did not induce Akt phosphorylation, but it was noted before, that migration of MDA-MB-231 cells is PI3K-dependent (Price et al., 1999). Taken together, GPCR agonist-

induced chemotaxis of MDA-MB-231 cells is regulated by EGFR independent and dependent signalling pathways. The interaction of the TMPS mechanism mediated by ADAM17 and proHB-EGF with the EGFR-independent activation of PI3K after lysophospholipid stimulation is necessary for migration of breast cancer cells (Figure 40).

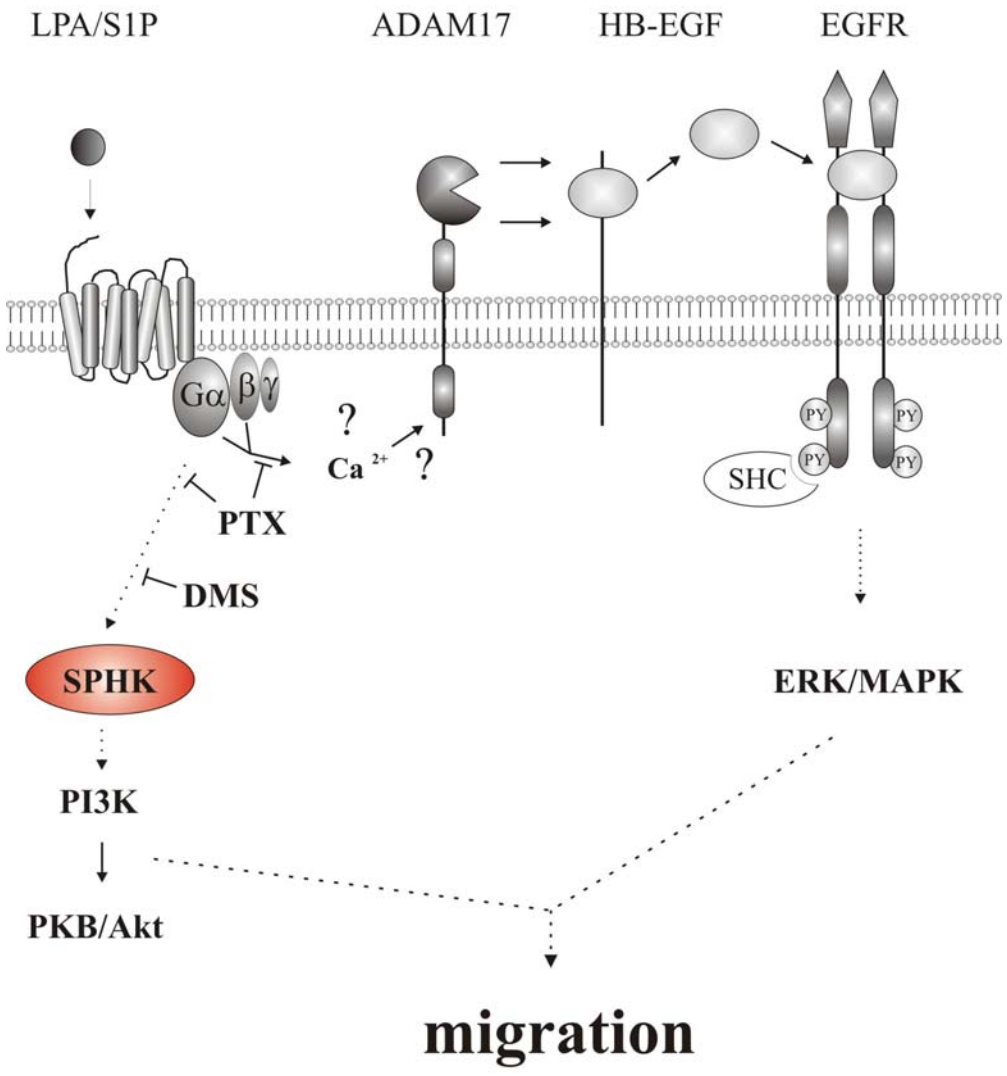


Figure 40 GPCR agonist-induced chemotaxis of MDA-MB-231 cells is regulated by EGFR independent and dependent signalling pathways. The interaction of the TMPS mechanism mediated by ADAM17 and proHB-EGF together with the EGFR-independent activation of PI3K after lysophospholipid stimulation is necessary for migration of breast cancer cells.

To sum up, the results of this study indicate that, depending on the cellular system, treatment of a wide variety of human cancer cells with GPCR agonists leads to the rapid and specific cleavage of a distinct EGF-like growth factor such as proAR or proHB-EGF at the cell surface by proteases of the ADAM family. Furthermore, EGF-like growth factor shedding leads to activation of the EGFR, subsequent SHC adaptor protein recruitment and downstream

activation of ERK1/2. Interestingly, depending on the cellular system, Akt/PKB phosphorylation upon GPCR agonist stimulation occurs by EGFR-dependent and -independent pathways. Finally, this Triple-Membrane-Passing signal (TMPS) mechanism of EGFR signal transactivation provides a molecular explanation of how GPCR ligands regulate chemotaxis in HNSCC and breast cancer cells.

4.5. Cannabinoids induce EGFR signal transactivation

4.5.1. EGFR signal transactivation links cannabinoid receptors to MAPK activation

Cannabinoids, the active components of marijuana and their endogenous counterparts were reported as useful analgetic agents to accompany primary cancer treatment by preventing nausea, vomiting and pain and by stimulating appetite. Moreover, they have been demonstrated to inhibit cell growth and induce apoptosis in tumour cells.

Binding of cannabinoids to their cognate receptors has been shown to enhance the activity of the MAPKs ERK1/2. The activation of the MAPK pathway in glioma cells upon cannabinoid treatment was suggested to involve the activation of Raf1 by increased ceramide levels (Galve-Roperh et al., 2000). However, our results identified EGFR signal transactivation as the key mechanism linking cannabinoid receptors to MAPK signalling cascades in a wide variety of human cancer cell lines. Activation of ERK1/2 by four different cannabinoids coincides with the phosphorylation of the EGFR and was blocked by a specific inhibitor of the EGFR, AG1478 (Figure 34). Although the ability of AG1478 to block cannabinoid-induced MAPK activation was noted before in U373-MG cells by Galve-Roperh *et al.*, they excluded the existence of cannabinoid receptor-mediated EGFR transactivation due to the inability to detect tyrosine phosphorylated EGFR, probably due to lack of sensitivity of detection (Galve-Roperh et al., 2002).

Interestingly, in addition to ERK1/2 activation, Akt/PKB phosphorylation was detected after cannabinoid treatment in an EGFR-dependent manner (Figure 34). Such a parallel contiguous activation of ERK1/2 and of Akt/PKB was observed before, for example in glioblastoma cells treated with cannabinoid receptor agonists and was suggested to protect astrocytes from ceramide-induced apoptosis in a dose- and time-dependent manner (Galve-Roperh et al., 2000; Gomez Del Pulgar et al., 2002).

Our experimental finding that cannabinoid-induced EGFR crosstalk is established in a variety of human cancer cell lines (Figure 31) implicates the EGFR as a central integrator of

cannabinoid signalling. The cross-communication between GPCRs and the EGFR involves the proteolytic processing of different membrane-spanning proEGF-like growth factor ligands like proAR, proHB-EGF and proTGFα by a zinc-dependent metalloprotease like ADAM10, ADAM12 and ADAM17, depending on the cellular context (Fischer et al., 2003; Gschwind et al., 2003). In human cancer cell lines, we demonstrate that ADAM17 mediates transactivation of the EGFR after cannabinoid stimulation via proteolytic processing of proHB-EGF and/or proAmphiregulin (Figure 37; Figure 38; Figure 41). Abrogation of either ADAM17 or the respective proEGF-like growth factor function completely blocked cannabinoid-induced EGFR tyrosine phosphorylation and subsequent activation of the mitogenic ERK pathway and the pro-survival Akt/PKB pathway (Figure 37; Figure 38). Furthermore, this is the first study demonstrating a ADAM17 and HB-EGF dependent EGFR signal transactivation after GPCR stimulation. Interestingly, knockout experiments by Jackson *et al.* show that newborn mice lacking ADAM17, HB-EGF and the EGFR have similar defective valvulogenesis and suggest EGFR activation by ADAM17-processed proHB-EGF (Jackson et al., 2003). Moreover, our data substantiate the concept that depending on the cell type and the stimulated GPCR, different ADAM proteases and proEGF-like growth factor ligands are capable to activate the EGFR (Fischer et al., 2003).

4.5.2. Cannabinoids induce cancer cell proliferation via ADAM17-mediated transactivation of the EGFR

Cannabinoids were shown to induce apoptosis in cells of the neuronal system including neurons, astrocytes, human grade IV astrocytoma, glioma C6, astrocytoma U373-MG, neuroblastoma N18 TG2 and pheochromocytoma PC12 cells and to inhibit proliferation of MCF7, EFM-19, T47D and DU145 cells (Guzman et al., 2001; Guzman et al., 2002). Based on these findings and their analgesic properties, cannabinoids were suggested as useful drugs to support cancer therapy. Here we show that various cannabinoids potently induce mitogenic kinase signalling in different cancer cell lines. Moreover, we demonstrate in contrast to other studies which used cannabinoids such as Δ^9-Tetrahydrocannabinol (THC) at micromolar concentrations, that nanomolar concentrations of THC induces proliferation of cancer cells (Figure 35). Importantly, the here used concentration of THC is more likely to reflect the therapeutically relevant situation detected in serum after drug treatment (Brenneisen et al., 1996; Consroe et al., 1991; Heishman et al., 1997; Heishman et al., 1990; Huestis et al., 1992).

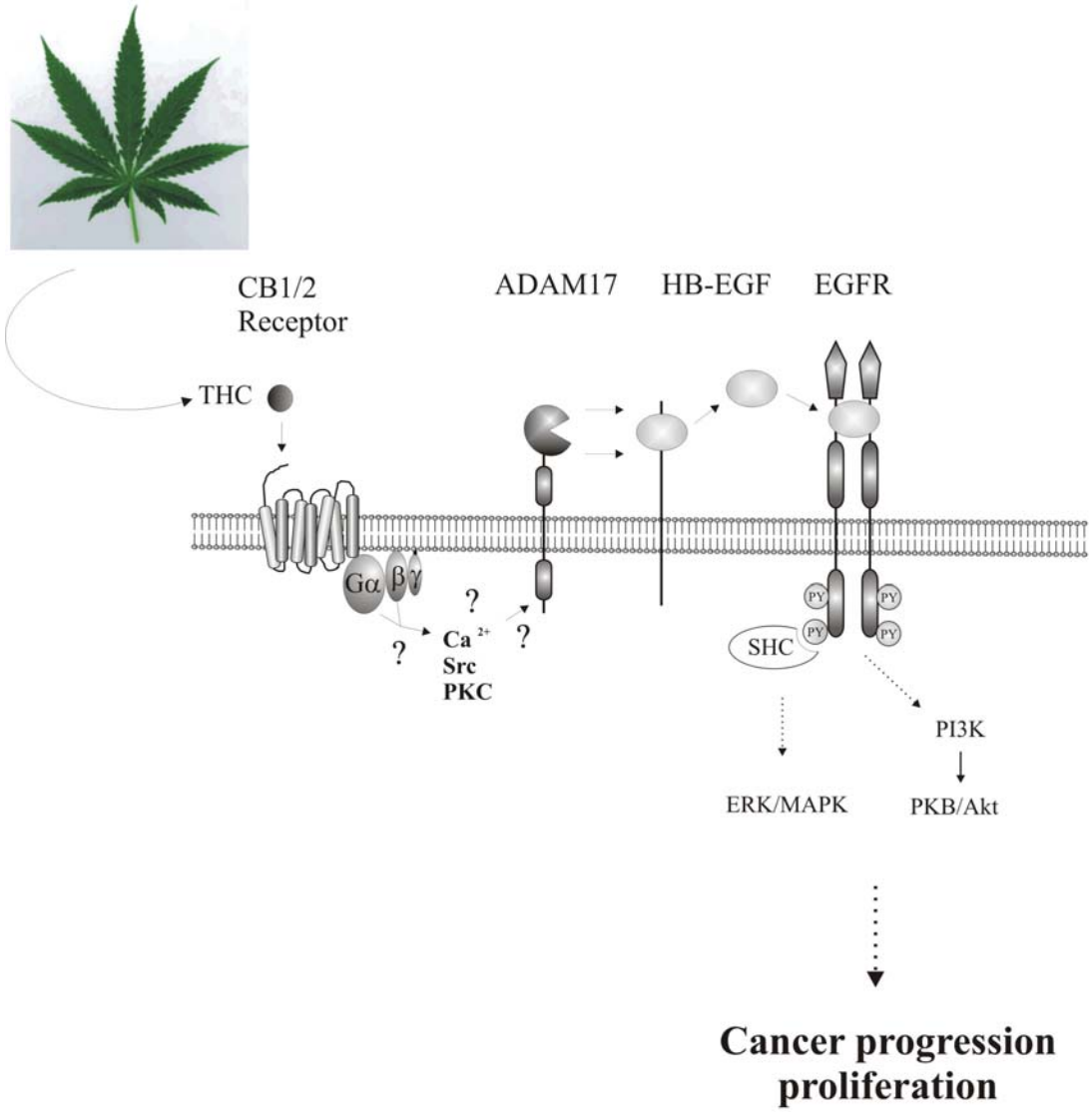


Figure 41 Model of the cannabinoid induced EGFR signal transactivation. Cannabinoids such as anandamide or Δ^9-Tetrahydrocannabinol bind to the cannabinoid receptor and induce the activate ADAM17 leading to specific processing of proHB-EGF, release of the mature growth factor and subsequent EGFR stimulation. Depending on this mechanistic concept are cellular responses such as cancer cell proliferation.

Cannabis-based drugs are in phase three clinical trials for treating pain associated with cancer. Furthermore, Δ^9-Tetrahydrocannabinol is currently used to treat nausea of cancer patients undergoing extensive chemotherapy (Guzman, 2003; Hall and Degenhardt, 2003; Walsh et al., 2003). In contrast, Grand and coworkers presented recently a case study of acute pancreatitis induced by cannabis smoking indicating that cannabinoids may be a risk factor for pancreatic cancer (Grant and Gandhi, 2004). Smoking of THC is the most effective route of delivery, as THC is rapidly absorbed after inhalation and the effects become fully apparent within minutes. Pharmacological activity of smoked THC depends on depth and length of inhalation. Maximum serum concentrations up to 267 ng/mL (850 nM) are measured after smoking THC (Heishman et al., 1997; Huestis et al., 1992), whereas maximum serum

concentrations of oral or rectal administered THC or its derivatives as a drug are lower (35-350 nM) (Brenneisen et al., 1996; Consroe et al., 1991; Sticht, 1988). Here we observed a proliferative response of glioblastoma and lung cancer cells at concentrations of 100-300 nM THC while THC at micromolar concentrations induced cell death in agreement with previous observations with neuronal cell types and immune cells (Figure 35; Figure 36) (Chan et al., 1998; Guzman et al., 2002; McKallip et al., 2002a; McKallip et al., 2002b). These findings indicate that the biological responses to cannabinoids critically depend on drug concentration and cellular context. Taken together, these results have to be taken into account when considering therapeutic applications of cannabinoids. The risk in the medical use of THC or cannabis for the treatment of patients with established tumours is the further acceleration of tumour growth due to the proliferative potential of cannabinoids.

4.6. Perspectives

Given the significance of the EGFR signal transactivation mechanism in pathophysiological disorders future studies will have to focus on both, mechanistic details linking GPCRs and metalloproteases, and on the molecular entities determining the protease´s specificity towards the EGF-like ligand in order to find key therapeutic intervention points.

To identify mechanistic elements that link GPCRs to the respective metalloprotease-ligand pair, one strategy is the investigation of intracellular binding partners of the metalloproteases using a conventional yeast two-hybrid system or a membrane based yeast two-hybrid method, which allows the identification of transmembrane binding partners. An important issue is to identify regulatory mechanisms like cytoplasmic phosphorylation of the metalloprotease which can be involved in the induction of the shedding event. Within this context, also a role for the recently described GPCR oligomerisation has to be taken into account, thereby excluding an intracellular signalling partner.

Moreover, future studies will be necessary to determine the substrate specificity of the metalloproteases. Signal specificity may be achieved by localizing the elements of the TMPS to discrete areas of the cell membrane such as lipid rafts which would readily explain the results of this work showing that in SCC9 cells ADAM17 activation leads to proAR shedding in response to LPA and carbachol, but to proHB-EGF shedding after thrombin treatment. In addition, preclustering could also elucidate the selective cleavage of a distinct EGF-like ligand, while other EGF-related growth factors are present at the cell surface. Interestingly, the β_2-adrenergic receptor was immunoprecipitated with the “transactivated” EGFR in Cos-7 cells, suggesting the formation of a macromolecular signalling complex which is likely to

contain other elements of the TMPS pathway (Maudsley et al., 2000). Further studies using experimental techniques like FRET (Fluorescence resonance energy transfer) or BRET (Bioluminescence resonance energy transfer) will be necessary to examine this theory.
Specific inhibition of single components of the TMPS using low molecular weight inhibitors or blocking antibodies will help to gain new insights into the physiological role of this pathway and will help to provide novel therapeutic targets for disease intervention.

5. Summary

In a variety of established tumour cell lines, but also in primary mammary epithelial cells metalloprotease-dependent transactivation of the EGFR, and EGFR characteristic downstream signalling events were observed in response to stimulation with physiological concentrations of GPCR agonists such as the mitogens LPA and S1P as well as therapeutically relevant concentrations of cannabinoids.

Moreover, this study reveals ADAM17 and HB-EGF as the main effectors of this mechanism in most of the cancer cell lines investigated. However, depending on the cellular context and GPCR agonist, various different members of the ADAM family are selectively recruited for specific ectodomain shedding of proAR and/or proHB-EGF and subsequent EGFR activation.

Furthermore, biological responses induced by LPA or S1P such as migration in breast cancer and HNSCC cells, depend on ADAM17 and proHB-EGF/proAR function, respectively, suggesting that highly abundant GPCR ligands may play a role in tumour development and progression. Moreover, EGFR signal transactivation could be identified as the mechanistic link between cannabinoid receptors and the activation of mitogen activated protein kinases (MAPK) ERK1/2 as well as pro-survival Akt/PKB signalling. Depending on the cellular context, cannabinoid-induced signal cross-communication was mediated by shedding of proAmphiregulin and/or proHB-EGF by ADAM17. Most importantly, our data show that concentrations of THC comparable to those detected in the serum of patients after THC administration accelerate proliferation of cancer cells instead of apoptosis and thereby may contribute to cancer progression in patients.

5. Zusammenfassung

Im Rahmen dieser Arbeit wurde gezeigt, dass in verschiedenen etablierten Tumorzelllinien, aber auch in primären Brustepithelzellen sowohl physiologische Konzentrationen von GPCR-Liganden, wie z.B. den Mitogenen LPA und S1P, als auch therapeutische Konzentrationen von Cannabinoiden zur metalloproteaseabhängigen Aktivierung des EGFRs führen. Abhängig von diesem Mechanismus konnte die Aktivierung der mitogenen Ras/MAPK-Kaskade als auch des antiapoptotischen Akt/PKB Signalweges beobachtet werden.

Untersuchungen mit Hilfe der siRNA-Technik oder dominant-negativen Konstrukten identifizierten ADAM17 sowie den EGFR-Liganden HB-EGF als wichtigste Komponenten dieses Signalweges. Abhängig vom Zellsystem konnte aber auch eine Beteiligung anderer Mitglieder der ADAM Familie sowie des EGFR-Liganden Amphiregulin nachgewiesen werden.

Weiterhin konnte in dieser Arbeit gezeigt werden, dass die durch LPA und S1P induzierten biologische Prozesse, wie z.B. die Migration in Brustkrebs- oder HNSCC-Zellen, vollständig von der Aktivität von ADAM17 und HB-EGF/AR abhängig waren. Außerdem konnte die ADAM17- und HB-EGF/AR-vermittelte EGFR-Transaktivierung als Bindeglied zwischen Cannabinoid-Rezeptoren und MAPK- und Akt-Aktivierung sowie erhöhter Zellproliferation identifiziert werden.

Die Ergebnisse dieser Arbeit unterstreichen die Rolle der EGFR Signaltransaktivierung und dadurch induzierter biologischer Antworten wie Zellmigration oder –proliferation in Tumorzellen, und sollten darüber hinaus zu einer Neubewertung der Krebstherapie mit Cannabinoiden führen.

6. References

Althoff, K., Reddy, P., Voltz, N., Rose-John, S. and Mullberg, J. (2000) Shedding of interleukin-6 receptor and tumor necrosis factor alpha. Contribution of the stalk sequence to the cleavage pattern of transmembrane proteins. *Eur J Biochem*, **267**, 2624-2631.

Amour, A., Slocombe, P.M., Webster, A., Butler, M., Knight, C.G., Smith, B.J., Stephens, P.E., Shelley, C., Hutton, M., Knauper, V., Docherty, A.J. and Murphy, G. (1998) TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett*, **435**, 39-44.

Anders, A., Gilbert, S., Garten, W., Postina, R. and Fahrenholz, F. (2001) Regulation of the alpha-secretase ADAM10 by its prodomain and proprotein convertases. *Faseb J*, **15**, 1837-1839.

Arribas, J., Lopez-Casillas, F. and Massague, J. (1997) Role of the juxtamembrane domains of the transforming growth factor-alpha precursor and the beta-amyloid precursor protein in regulated ectodomain shedding. *J Biol Chem*, **272**, 17160-17165.

Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H., Asanuma, H., Sanada, S., Matsumura, Y., Takeda, H., Beppu, S., Tada, M., Hori, M. and Higashiyama, S. (2002) Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med*, **8**, 35-40.

Becker, J.W., Marcy, A.I., Rokosz, L.L., Axel, M.G., Burbaum, J.J., Fitzgerald, P.M., Cameron, P.M., Esser, C.K., Hagmann, W.K., Hermes, J.D. and et al. (1995) Stromelysin-1: three-dimensional structure of the inhibited catalytic domain and of the C-truncated proenzyme. *Protein Sci*, **4**, 1966-1976.

Beerli, R.R., Graus-Porta, D., Woods-Cook, K., Chen, X., Yarden, Y. and Hynes, N.E. (1995) Neu differentiation factor activation of ErbB-3 and ErbB-4 is cell specific and displays a differential requirement for ErbB-2. *Mol Cell Biol*, **15**, 6496-6505.

Belcheva, M.M., Szucs, M., Wang, D., Sadee, W. and Coscia, C.J. (2001) mu-Opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation. *J Biol Chem*, **276**, 33847-33853.

Benhar, M., Engelberg, D. and Levitzki, A. (2002) ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep*, **3**, 420-425.

Biscardi, J.S., Tice, D.A. and Parsons, S.J. (1999) c-Src, receptor tyrosine kinases, and human cancer. *Adv Cancer Res*, **76**, 61-119.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. and Cerretti, D.P. (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature*, **385**, 729-733.

Blobel, C.P. and White, J.M. (1992) Structure, function and evolutionary relationship of proteins containing a disintegrin domain. *Curr Opin Cell Biol*, **4**, 760-765.

Blume-Jensen, P. and Hunter, T. (2001a) Oncogenic kinase signalling. *Nature*, **411**, 355-365.

Blume-Jensen, P. and Hunter, T. (2001b) Oncogenic kinase signalling. *Nature*, **411**, 355-365.

Bockaert, J., Marin, P., Dumuis, A. and Fagni, L. (2003) The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. *FEBS Lett*, **546**, 65-72.

Bockaert, J. and Pin, J.P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *Embo J*, **18**, 1723-1729.

Brachmann, R., Lindquist, P.B., Nagashima, M., Kohr, W., Lipari, T., Napier, M. and Derynck, R. (1989) Transmembrane TGF-alpha precursors activate EGF/TGF-alpha receptors. *Cell*, **56**, 691-700.

Breitwieser, G.E. (2004) G protein-coupled receptor oligomerization: implications for G protein activation and cell signaling. *Circ Res*, **94**, 17-27.

Brenneisen, R., Egli, A., Elsohly, M.A., Henn, V. and Spiess, Y. (1996) The effect of orally and rectally administered delta 9-tetrahydrocannabinol on spasticity: a pilot study with 2 patients. *Int J Clin Pharmacol Ther*, **34**, 446-452.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R.A. and Israel, A. (2000) A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell*, **5**, 207-216.

Brown, C.L., Coffey, R.J. and Dempsey, P.J. (2001) The proamphiregulin cytoplasmic domain is required for basolateral sorting, but is not essential for constitutive or stimulus-induced processing in polarized Madin-Darby canine kidney cells. *J Biol Chem*, **276**, 29538-29549.

Buxbaum, J.D., Liu, K.N., Luo, Y., Slack, J.L., Stocking, K.L., Peschon, J.J., Johnson, R.S., Castner, B.J., Cerretti, D.P. and Black, R.A. (1998) Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. *J Biol Chem*, **273**, 27765-27767.

Cabral, G.A. and Dove Pettit, D.A. (1998) Drugs and immunity: cannabinoids and their role in decreased resistance to infectious disease. *J Neuroimmunol*, **83**, 116-123.

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

Chan, G.C., Hinds, T.R., Impey, S. and Storm, D.R. (1998) Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol. *J Neurosci*, **18**, 5322-5332.

Chang, C. and Werb, Z. (2001) The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol*, **11**, S37-43.

Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature*, **410**, 37-40.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C. and Cobb, M.H. (2001) MAP kinases. *Chem Rev*, **101**, 2449-2476.

Cho, H.S., Mason, K., Ramyar, K.X., Stanley, A.M., Gabelli, S.B., Denney, D.W., Jr. and Leahy, D.J. (2003) Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature*, **421**, 756-760.

Cohen, P. (2002) The origins of protein phosphorylation. *Nat Cell Biol*, **4**, E127-130.

Consroe, P., Kennedy, K. and Schram, K. (1991) Assay of plasma cannabidiol by capillary gas chromatography/ion trap mass spectroscopy following high-dose repeated daily oral administration in humans. *Pharmacol Biochem Behav*, **40**, 517-522.

Crocker, S.J., Pagenstecher, A. and Campbell, I.L. (2004) The TIMPs tango with MMPs and more in the central nervous system. *J Neurosci Res*, **75**, 1-11.

Daaka, Y. (2004) G proteins in cancer: the prostate cancer paradigm. *Sci STKE*, **2004**, re2.

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) Signal characteristics of G protein-transactivated EGF receptor. *Embo J*, **16**, 7032-7044.

Daub, H., Weiss, F.U., Wallasch, C. and Ullrich, A. (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature*, **379**, 557-560.

Della Rocca, G.J., Maudsley, S., Daaka, Y., Lefkowitz, R.J. and Luttrell, L.M. (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. *J Biol Chem*, **274**, 13978-13984.

Derocq, J.M., Segui, M., Marchand, J., Le Fur, G. and Casellas, P. (1995) Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett*, **369**, 177-182.

Diaz-Rodriguez, E., Montero, J.C., Esparis-Ogando, A., Yuste, L. and Pandiella, A. (2002) Extracellular Signal-regulated Kinase Phosphorylates Tumor Necrosis Factor alpha-converting Enzyme at Threonine 735: A Potential Role in Regulated Shedding. *Mol Biol Cell*, **13**, 2031-2044.

Eguchi, S., Dempsey, P.J., Frank, G.D., Motley, E.D. and Inagami, T. (2001) Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *J Biol Chem*, **276**, 7957-7962.

Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E.D., Kawakatsu, H., Owada, K.M., Hirata, Y., Marumo, F. and Inagami, T. (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**, 8890-8896.

Endo, A., Nagashima, K., Kurose, H., Mochizuki, S., Matsuda, M. and Mochizuki, N. (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**, 23747-23754.

Fan, H. and Derynck, R. (1999) Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *Embo J*, **18**, 6962-6972.

Fang, X., Yu, S., LaPushin, R., Lu, Y., Furui, T., Penn, L.Z., Stokoe, D., Erickson, J.R., Bast, R.C., Jr. and Mills, G.B. (2000) Lysophosphatidic acid prevents apoptosis in fibroblasts via G(i)-protein-mediated activation of mitogen-activated protein kinase. *Biochem J*, **352**, 135-143.

Fang, X., Yu, S., Tanyi, J.L., Lu, Y., Woodgett, J.R. and Mills, G.B. (2002) Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol Cell Biol*, **22**, 2099-2110.

Fischer, O.M., Hart, S., Gschwind, A. and Ullrich, A. (2003) EGFR Signal Transactivation in Cancer Cells. *Biochem Society Transactions*, **31**, In press.

Fishman, D.A., Liu, Y., Ellerbroek, S.M. and Stack, M.S. (2001) Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. *Cancer Res*, **61**, 3194-3199.

Fukuhara, S., Chikumi, H. and Gutkind, J.S. (2001) RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? *Oncogene*, **20**, 1661-1668.

Galve-Roperh, I., Rueda, D., Gomez del Pulgar, T., Velasco, G. and Guzman, M. (2002) Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor. *Mol Pharmacol*, **62**, 1385-1392.

Galve-Roperh, I., Sanchez, C., Cortes, M.L., del Pulgar, T.G., Izquierdo, M. and Guzman, M. (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med*, **6**, 313-319.

Garrett, T., McKern, N., Lou, M., Elleman, T., Adams, T., Lovrecz, G., Zhu, H., Walker, F., Frenkel, M., Hoyne, P., Jorissen, R., Nice, E., Burgess, A. and Ward, C. (2002a) Crystal Structure of a Truncated Epidermal Growth Factor Receptor Extracellular Domain Bound to Transforming Growth Factor alpha. *Cell*, **110**, 763.

Garrett, T.P., McKern, N.M., Lou, M., Elleman, T.C., Adams, T.E., Lovrecz, G.O., Zhu, H.J., Walker, F., Frenkel, M.J., Hoyne, P.A., Jorissen, R.N., Nice, E.C., Burgess, A.W. and

Ward, C.W. (2002b) Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell*, **110**, 763-773.

Garton, K.J., Gough, P.J., Blobel, C.P., Murphy, G., Greaves, D.R., Dempsey, P.J. and Raines, E.W. (2001) TACE (ADAM17) mediates the cleavage and shedding of Fractalkine (CX3CL1). *J Biol Chem*, **8**, 8.

Gohla, A., Harhammer, R. and Schultz, G. (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**, 4653-4659.

Goldman, R., Levy, R.B., Peles, E. and Yarden, Y. (1990) Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry*, **29**, 11024-11028.

Gomez, D.E., Alonso, D.F., Yoshiji, H. and Thorgeirsson, U.P. (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol*, **74**, 111-122.

Gomez Del Pulgar, T., De Ceballos, M.L., Guzman, M. and Velasco, G. (2002) Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem*, **277**, 36527-36533.

Grant, P. and Gandhi, P. (2004) A case of cannabis-induced pancreatitis. *Jop*, **5**, 41-43.

Graus-Porta, D., Beerli, R.R., Daly, J.M. and Hynes, N.E. (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *Embo J*, **16**, 1647-1655.

Gschwind, A., Hart, S., Fischer, O.M. and Ullrich, A. (2003) TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *Embo J*, **22**, 2411-2421.

Gschwind, A., Prenzel, N. and Ullrich, A. (2002) Lysophosphatidic Acid-induced Squamous Cell Carcinoma Cell Proliferation and Motility Involves Epidermal Growth Factor Receptor Signal Transactivation. *Cancer Res*, **62**, 6329-6336.

Gschwind, A., Zwick, E., Prenzel, N., Leserer, M. and Ullrich, A. (2001) Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene*, **20**, 1594-1600.

Guzman, M. (2003) Cannabinoids: potential anticancer agents. *Nat Rev Cancer*, **3**, 745-755.

Guzman, M., Sanchez, C. and Galve-Roperh, I. (2001) Control of the cell survival/death decision by cannabinoids. *J Mol Med*, **78**, 613-625.

Guzman, M., Sanchez, C. and Galve-Roperh, I. (2002) Cannabinoids and cell fate. *Pharmacol Ther*, **95**, 175-184.

Hall, R.A., Premont, R.T. and Lefkowitz, R.J. (1999) Heptahelical receptor signaling: beyond the G protein paradigm. *J Cell Biol*, **145**, 927-932.

Hall, W. and Degenhardt, L. (2003) Medical marijuana initiatives : are they justified? How successful are they likely to be? *CNS Drugs*, **17**, 689-697.

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

Hantschel, O. and Superti-Furga, G. (2004) Regulation of the c-Abl and Bcr-Abl tyrosine kinases. *Nat Rev Mol Cell Biol*, **5**, 33-44.

Hart, S., Gschwind, A. Roidl, A. and Ullrich, A. (2003) EPIDERMAL GROWTH FACTOR RECEPTOR SIGNAL TRANSACTIVATION. *NATO SCIENCE SERIES: II: Mathematics, Physics and Chemistry*, **129**, 93-103.

Heishman, S.J., Arasteh, K. and Stitzer, M.L. (1997) Comparative effects of alcohol and marijuana on mood, memory, and performance. *Pharmacol Biochem Behav*, **58**, 93-101.

Heishman, S.J., Huestis, M.A., Henningfield, J.E. and Cone, E.J. (1990) Acute and residual effects of marijuana: profiles of plasma THC levels, physiological, subjective, and performance measures. *Pharmacol Biochem Behav*, **37**, 561-565.

Hermans, E. (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol Ther*, **99**, 25-44.

Herrlich, A., Daub, H., Knebel, A., Herrlich, P., Ullrich, A., Schultz, G. and Gudermann, T. (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**, 8985-8990.

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

Hubbard, S.R. and Till, J.H. (2000) Protein tyrosine kinase structure and function. *Annu Rev Biochem*, **69**, 373-398.

Huestis, M.A., Sampson, A.H., Holicky, B.J., Henningfield, J.E. and Cone, E.J. (1992) Characterization of the absorption phase of marijuana smoking. *Clin Pharmacol Ther*, **52**, 31-41.

Hunter, T. (1998) The role of tyrosine phosphorylation in cell growth and disease. *Harvey Lect*, **94**, 81-119.

Hunter, T. (2002) Tyrosine phosphorylation in cell signaling and disease. *Keio J Med*, **51**, 61-71.

Iba, K., Albrechtsen, R., Gilpin, B., Frohlich, C., Loechel, F., Zolkiewska, A., Ishiguro, K., Kojima, T., Liu, W., Langford, J.K., Sanderson, R.D., Brakebusch, C., Fassler, R. and Wewer, U.M. (2000) The cysteine-rich domain of human ADAM 12 supports cell adhesion through syndecans and triggers signaling events that lead to beta1 integrin-dependent cell spreading. *J Cell Biol*, **149**, 1143-1156.

Iba, K., Albrechtsen, R., Gilpin, B.J., Loechel, F. and Wewer, U.M. (1999) Cysteine-rich domain of human ADAM 12 (meltrin alpha) supports tumor cell adhesion. *Am J Pathol*, **154**, 1489-1501.

Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M. and Akedo, H. (1993) Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. *Biochem Biophys Res Commun*, **193**, 497-503.

Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S. and Mekada, E. (1998) A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *Embo J*, **17**, 7260-7272.

J.H. Peters, H.B.a.M.S. (1985) *Monoclonale Antikörper-Herstellung und Charakterisierung*. Springer-Verlag, Berlin Heidelberg New York Tokio.

Jackson, L.F., Qiu, T.H., Sunnarborg, S.W., Chang, A., Zhang, C., Patterson, C. and Lee, D.C. (2003) Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *Embo J*, **22**, 2704-2716.

Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W. and Burgess, A.W. (2003) Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res*, **284**, 31-53.

Kalmes, A., Vesti, B.R., Daum, G., Abraham, J.A. and Clowes, A.W. (2000) Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. *Circ Res*, **87**, 92-98.

Kang, T., Park, H.I., Suh, Y., Zhao, Y.G., Tschesche, H. and Sang, Q.X. (2002) Autolytic processing at Glu586-Ser587 within the cysteine-rich domain of human adamalysin 19/disintegrin-metalloproteinase 19 is necessary for its proteolytic activity. *J Biol Chem*, **277**, 48514-48522.

Kheradmand, F. and Werb, Z. (2002) Shedding light on sheddases: role in growth and development. *Bioessays*, **24**, 8-12.

Kim, J., Eckhart, A.D., Eguchi, S. and Koch, W.J. (2002) beta -Adrenergic Receptor-mediated DNA Synthesis in Cardiac Fibroblasts Is Dependent on Transactivation of the Epidermal Growth Factor Receptor and Subsequent Activation of Extracellular Signal-regulated Kinases. *J Biol Chem*, **277**, 32116-32123.

Kim, S.N., Park, J.G., Lee, E.B., Kim, S.S. and Yoo, Y.S. (2000) Characterization of epidermal growth factor receptor function in lysophosphatidic acid signaling in PC12 cells. *J Cell Biochem*, **76**, 386-393.

Klein, T.W., Newton, C. and Friedman, H. (1998) Cannabinoid receptors and immunity. *Immunol Today*, **19**, 373-381.

Konecny, G.E., Wilson, C.A. and Slamon, D.J. (2003) Is there a role for epidermal growth factor receptor inhibitors in breast cancer prevention? *J Natl Cancer Inst*, **95**, 1813-1815.

Kranenburg, O. and Moolenaar, W.H. (2001) Ras-MAP kinase signaling by lysophosphatidic acid and other G protein-coupled receptor agonists. *Oncogene*, **20**, 1540-1546.

Lage, A., Crombet, T. and Gonzalez, G. (2003) Targeting epidermal growth factor receptor signaling: early results and future trends in oncology. *Ann Med*, **35**, 327-336.

Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R. and Vallar, L. (1989) GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature*, **340**, 692-696.

Lemjabbar, H. and Basbaum, C. (2002) Platelet-activating factor receptor and ADAM10 mediate responses to Staphylococcus aureus in epithelial cells. *Nat Med*, **8**, 41-46.

Lemjabbar, H., Li, D., Gallup, M., Sidhu, S., Drori, E. and Basbaum, C. (2003) Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor alpha-converting enzyme and amphiregulin. *J Biol Chem*, **278**, 26202-26207.

Liu, Z. and Klominek, J. (2003) Regulation of matrix metalloprotease activity in malignant mesothelioma cell lines by growth factors. *Thorax*, **58**, 198-203.

Luetteke, N.C., Qiu, T.H., Fenton, S.E., Troyer, K.L., Riedel, R.F., Chang, A. and Lee, D.C. (1999) Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development*, **126**, 2739-2750.

Lyons, J., Landis, C.A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q.Y., Clark, O.H., Kawasaki, E., Bourne, H.R. and et al. (1990) Two G protein oncogenes in human endocrine tumors. *Science*, **249**, 655-659.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science*, **298**, 1912-1934.

Marinissen, M.J. and Gutkind, J.S. (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci*, **22**, 368-376.

Massague, J. and Pandiella, A. (1993) Membrane-anchored growth factors. *Annu Rev Biochem*, **62**, 515-541.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C. and Bonner, T.I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, **346**, 561-564.

Maudsley, S., Pierce, K.L., Zamah, A.M., Miller, W.E., Ahn, S., Daaka, Y., Lefkowitz, R.J. and Luttrell, L.M. (2000) The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *J Biol Chem*, **275**, 9572-9580.

McCole, D.F., Keely, S.J., Coffey, R.J. and Barrett, K.E. (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*, **28**, 28.

McKallip, R.J., Lombard, C., Fisher, M., Martin, B.R., Ryu, S., Grant, S., Nagarkatti, P.S. and Nagarkatti, M. (2002a) Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood*, **100**, 627-634.

McKallip, R.J., Lombard, C., Martin, B.R., Nagarkatti, M. and Nagarkatti, P.S. (2002b) Delta(9)-tetrahydrocannabinol-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression in vitro and in vivo. *J Pharmacol Exp Ther*, **302**, 451-465.

Merlos-Suarez, A., Ruiz-Paz, S., Baselga, J. and Arribas, J. (2001) Metalloprotease-dependent protransforming growth factor-alpha ectodomain shedding in the absence of tumor necrosis factor-alpha-converting enzyme. *J Biol Chem*, **276**, 48510-48517.

Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K.T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K.H. and van Koppen, C.J. (1998) Sphingosine kinase-mediated Ca2+ signalling by G-protein-coupled receptors. *Embo J*, **17**, 2830-2837.

Mischel, P.S. and Cloughesy, T.F. (2003) Targeted molecular therapy of GBM. *Brain Pathol*, **13**, 52-61.

Montaner, S., Sodhi, A., Molinolo, A., Bugge, T.H., Sawai, E.T., He, Y., Li, Y., Ray, P.E. and Gutkind, J.S. (2003) Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. *Cancer Cell*, **3**, 23-36.

Moody, T.W. (1996) Peptides and growth factors in non-small cell lung cancer. *Peptides*, **17**, 545-555.

Moody, T.W., Chan, D., Fahrenkrug, J. and Jensen, R.T. (2003) Neuropeptides as autocrine growth factors in cancer cells. *Curr Pharm Des*, **9**, 495-509.

Moss, M.L., Jin, S.L., Milla, M.E., Bickett, D.M., Burkhart, W., Carter, H.L., Chen, W.J., Clay, W.C., Didsbury, J.R., Hassler, D., Hoffman, C.R., Kost, T.A., Lambert, M.H., Leesnitzer, M.A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L.K., Schoenen, F., Seaton, T., Su, J.L., Becherer, J.D. and et al. (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature*, **385**, 733-736.

Munro, S., Thomas, K.L. and Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, **365**, 61-65.

Murasawa, S., Mori, Y., Nozawa, Y., Gotoh, N., Shibuya, M., Masaki, H., Maruyama, K., Tsutsumi, Y., Moriguchi, Y., Shibazaki, Y., Tanaka, Y., Iwasaka, T., Inada, M. and Matsubara, H. (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**, 1338-1348.

O-Charoenrat, P., Rhys-Evans, P., Modjtahedi, H., Court, W., Box, G. and Eccles, S. (2000) Overexpression of epidermal growth factor receptor in human head and neck squamous carcinoma cell lines correlates with matrix metalloproteinase-9 expression and in vitro invasion. *Int J Cancer*, **86**, 307-317.

Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M. and Yokoyama, S. (2002) Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell*, **110**, 775.

Pai, R., Nakamura, T., Moon, W.S. and Tarnawski, A.S. (2003) Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *Faseb J*, **17**, 1640-1647.

Pai, R., Soreghan, B., Szabo, I.L., Pavelka, M., Baatar, D. and Tarnawski, A.S. (2002) Prostaglandin E2 transactivates EGF receptor: A novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med*, **8**, 289-293.

Parma, J., Duprez, L., Van Sande, J., Cochaux, P., Gervy, C., Mockel, J., Dumont, J. and Vassart, G. (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature*, **365**, 649-651.

Parolaro, D., Massi, P., Rubino, T. and Monti, E. (2002) Endocannabinoids in the immune system and cancer. *Prostaglandins Leukot Essent Fatty Acids*, **66**, 319-332.

Pawson, T. and Nash, P. (2003) Assembly of cell regulatory systems through protein interaction domains. *Science*, **300**, 445-452.

Pertwee, R.G. (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther*, **74**, 129-180.

Pertwee, R.G. and Ross, R.A. (2002) Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids*, **66**, 101-121.

Peschon, J.J., Slack, J.L., Reddy, P., Stocking, K.L., Sunnarborg, S.W., Lee, D.C., Russell, W.E., Castner, B.J., Johnson, R.S., Fitzner, J.N., Boyce, R.W., Nelson, N., Kozlosky, C.J., Wolfson, M.F., Rauch, C.T., Cerretti, D.P., Paxton, R.J., March, C.J. and Black, R.A. (1998) An essential role for ectodomain shedding in mammalian development. *Science*, **282**, 1281-1284.

Pierce, K.L., Luttrell, L.M. and Lefkowitz, R.J. (2001) New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene*, **20**, 1532-1539.

Piomelli, D. (2003) The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci*, **4**, 873-884.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*, **402**, 884-888.

Price, J.T., Tiganis, T., Agarwal, A., Djakiew, D. and Thompson, E.W. (1999) Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3'-kinase and phospholipase C-dependent mechanism. *Cancer Res*, **59**, 5475-5478.

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

Reddy, P., Slack, J.L., Davis, R., Cerretti, D.P., Kozlosky, C.J., Blanton, R.A., Shows, D., Peschon, J.J. and Black, R.A. (2000) Functional analysis of the domain structure of tumor necrosis factor-alpha converting enzyme. *J Biol Chem*, **275**, 14608-14614.

Riese, D.J., 2nd and Stern, D.F. (1998) Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays*, **20**, 41-48.

Rio, C., Buxbaum, J.D., Peschon, J.J. and Corfas, G. (2000) Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. *J Biol Chem*, **275**, 10379-10387.

Robinson, D.R., Wu, Y.M. and Lin, S.F. (2000) The protein tyrosine kinase family of the human genome. *Oncogene*, **19**, 5548-5557.

Robinson, M.J. and Cobb, M.H. (1997) Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol*, **9**, 180-186.

Rodrigues, S., Attoub, S., Nguyen, Q.D., Bruyneel, E., Rodrigue, C.M., Westley, B.R., May, F.E., Thim, L., Mareel, M., Emami, S. and Gespach, C. (2003) Selective abrogation of the proinvasive activity of the trefoil peptides pS2 and spasmolytic polypeptide by disruption of the EGF receptor signaling pathways in kidney and colonic cancer cells. *Oncogene*, **22**, 4488-4497.

Rozengurt, E. (2002) Neuropeptides as growth factors for normal and cancerous cells. *Trends Endocrinol Metab*, **13**, 128-134.

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

Sautin, Y.Y., Crawford, J.M. and Svetlov, S.I. (2001) Enhancement of survival by LPA via Erk1/Erk2 and PI 3-kinase/Akt pathways in a murine hepatocyte cell line. *Am J Physiol Cell Physiol*, **281**, C2010-2019.

Schafer, 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**, 991-999.

Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell*, **103**, 211-225.

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

Schlessinger, J. and Lemmon, M.A. (2003) SH2 and PTB domains in tyrosine kinase signaling. *Sci STKE*, **2003**, RE12.

Schlondorff, J. and Blobel, C.P. (1999) Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding. *J Cell Sci*, **112**, 3603-3617.

Seals, D.F. and Courtneidge, S.A. (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev*, **17**, 7-30.

Shawver, L.K., Slamon, D. and Ullrich, A. (2002) Smart drugs: tyrosine kinase inhibitors in cancer therapy. *Cancer Cell*, **1**, 117-123.

Shenker, A., Laue, L., Kosugi, S., Merendino, J.J., Jr., Minegishi, T. and Cutler, G.B., Jr. (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature*, **365**, 652-654.

Shi, W., Chen, H., Sun, J., Buckley, S., Zhao, J., Anderson, K.D., Williams, R.G. and Warburton, D. (2003) TACE is required for fetal murine cardiac development and modeling. *Dev Biol*, **261**, 371-380.

Solomon, K.A., Pesti, N., Wu, G. and Newton, R.C. (1999) Cutting edge: a dominant negative form of TNF-alpha converting enzyme inhibits proTNF and TNFRII secretion. *J Immunol*, **163**, 4105-4108.

Sridhar, S.S., Seymour, L. and Shepherd, F.A. (2003) Inhibitors of epidermal-growth-factor receptors: a review of clinical research with a focus on non-small-cell lung cancer. *Lancet Oncol*, **4**, 397-406.

Sticht, G.K., H. (ed.). (1988) *Cannabis im Strassenverkehr*. Gustav Fischer Verlag, Stuttgart.

Stocker, W. and Bode, W. (1995) Structural features of a superfamily of zinc-endopeptidases: the metzincins. *Curr Opin Struct Biol*, **5**, 383-390.

Strachan, L., Murison, J.G., Prestidge, R.L., Sleeman, M.A., Watson, J.D. and Kumble, K.D. (2001) Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. *J Biol Chem*, **276**, 18265-18271.

Sturge, J., Hamelin, J. and Jones, G.E. (2002) N-WASP activation by a beta1-integrin-dependent mechanism supports PI3K-independent chemotaxis stimulated by urokinase-type plasminogen activator. *J Cell Sci*, **115**, 699-711.

Sukocheva, O.A., Wang, L., Albanese, N., Pitson, S.M., Vadas, M.A. and Xia, P. (2003) Sphingosine kinase transmits estrogen signaling in human breast cancer cells. *Mol Endocrinol*, **17**, 2002-2012.

Sunnarborg, S.W., Hinkle, C.L., Stevenson, M., Russell, W.E., Raska, C.S., Peschon, J.J., Castner, B.J., Gerhart, M.J., Paxton, R.J., Black, R.A. and Lee, D.C. (2002) Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J Biol Chem*, **31**.

Suzuki, M., Raab, G., Moses, M.A., Fernandez, C.A. and Klagsbrun, M. (1997) Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. *J Biol Chem*, **272**, 31730-31737.

Thathiah, A., Blobel, C.P. and Carson, D.D. (2003) Tumor necrosis factor-alpha converting enzyme/ADAM 17 mediates MUC1 shedding. *J Biol Chem*, **278**, 3386-3394.

Tsai, W., Morielli, A.D. and Peralta, E.G. (1997) The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *Embo J*, **16**, 4597-4605.

Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**, 203-212.

Umata, T., Hirata, M., Takahashi, T., Ryu, F., Shida, S., Takahashi, Y., Tsuneoka, M., Miura, Y., Masuda, M., Horiguchi, Y. and Mekada, E. (2001) A dual signaling cascade that regulates the ectodomain shedding of heparin-binding epidermal growth factor-like growth factor. *J Biol Chem*, **276**, 30475-30482.

van Corven, E.J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W.H. (1989) Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell*, **59**, 45-54.

Venkatakrishnan, G., Salgia, R. and Groopman, J.E. (2000) Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. *J Biol Chem*, **275**, 6868-6875.

Vincent, B., Paitel, E., Saftig, P., Frobert, Y., Hartmann, D., De Strooper, B., Grassi, J., Lopez-Perez, E. and Checler, F. (2001) The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein. *J Biol Chem*, **276**, 37743-37746.

Walsh, D., Nelson, K.A. and Mahmoud, F.A. (2003) Established and potential therapeutic applications of cannabinoids in oncology. *Support Care Cancer*, **11**, 137-143.

Weiss, F.U., Daub, H. and Ullrich, A. (1997) Novel mechanisms of RTK signal generation. *Curr Opin Genet Dev*, **7**, 80-86.

Werb, Z. and Yan, Y. (1998) A cellular striptease act. *Science*, **282**, 1279-1280.

Whitehead, I.P., Zohn, I.E. and Der, C.J. (2001) Rho GTPase-dependent transformation by G protein-coupled receptors. *Oncogene*, **20**, 1547-1555.

Wingens, M., Walma, T., van Ingen, H., Stortelers, C., van Leeuwen, J.E., van Zoelen, E.J. and Vuister, G.W. (2003) Structural analysis of an epidermal growth factor/transforming growth factor-alpha chimera with unique ErbB binding specificity. *J Biol Chem*, **278**, 39114-39123.

Wong, S.T., Winchell, L.F., McCune, B.K., Earp, H.S., Teixido, J., Massague, J., Herman, B. and Lee, D.C. (1989) The TGF-alpha precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell*, **56**, 495-506.

Xu, N., Bradley, L., Ambdukar, I. and Gutkind, J.S. (1993) A mutant alpha subunit of G12 potentiates the eicosanoid pathway and is highly oncogenic in NIH 3T3 cells. *Proc Natl Acad Sci U S A*, **90**, 6741-6745.

Xu, Y., Gaudette, D.C., Boynton, J.D., Frankel, A., Fang, X.J., Sharma, A., Hurteau, J., Casey, G., Goodbody, A., Mellors, A. and et al. (1995) Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. *Clin Cancer Res*, **1**, 1223-1232.

Yan, Y., Shirakabe, K. and Werb, Z. (2002) The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. *J Cell Biol*, **158**, 221-226.

Yang, S.H., Sharrocks, A.D. and Whitmarsh, A.J. (2003) Transcriptional regulation by the MAP kinase signaling cascades. *Gene*, **320**, 3-21.

Young, D., Waitches, G., Birchmeier, C., Fasano, O. and Wigler, M. (1986) Isolation and characterization of a new cellular oncogene encoding a protein with multiple potential transmembrane domains. *Cell*, **45**, 711-719.

Yu, W.H., Woessner, J.F., Jr., McNeish, J.D. and Stamenkovic, I. (2002) CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. *Genes Dev*, **16**, 307-323.

Zhang, Y., Jiang, J., Black, R.A., Baumann, G. and Frank, S.J. (2000) Tumor necrosis factor-alpha converting enzyme (TACE) is a growth hormone binding protein (GHBP) sheddase: the metalloprotease TACE/ADAM-17 is critical for (PMA-induced) GH receptor proteolysis and GHBP generation. *Endocrinology*, **141**, 4342-4348.

Zwick, E., Bange, J. and Ullrich, A. (2001) Receptor tyrosine kinase signalling as a target for cancer intervention strategies. *Endocr Relat Cancer*, **8**, 161-173.

Zwick, E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maly, K. and Ullrich, A. (1997) Critical role of calcium- dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling. *J Biol Chem*, **272**, 24767-24770.

Zwick, E., Hackel, P.O., Prenzel, N. and Ullrich, A. (1999) The EGF receptor as central transducer of heterologous signalling systems. *Trends Pharmacol Sci*, **20**, 408-412.

AA	Amino acid
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
CB	Cannabinoid receptor
cDNA	Complementary DNA
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
ECM	Extracellular matrix
EDG	Endothelial cell differentiation gene
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
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
HGF	Hepatocyte Growth Factor
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
NRG	Neuregulin
Nox	NADPH oxidase
OD	Optical density
p.a.	Per analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylenglycole
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
PNPP	p-Nitrophenyl-phosphate
PTP	Protein tyrosine phosphatase
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
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SAPK	Stress-activated protein kinase

S. D.	Standard deviation
SDS	Natriumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gel elektrophoresis
Sec	Second
SH2, 3	domain Src homology 2, 3 domain
SHP-2	SH2-containing PTP-2
Sos	Son of sevenless
SPHK	Sphingosine-Kinase
Src	Homologue to v-src (sarcoma viral oncogene)
TACE	TNFα-converting enzyme
TCA	Trichloroacetic acid
TCF	T cell factor
TGFα	Transforming growth factor alpha
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
THC	Δ^9-Tetrahydrocannabinol
Timp	Tissue inhibitor of metalloproteases
TMPS	Triple-Membrane-Passing signal
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
VEGFR	Vascular endothelial growth factor receptor
Vol	Volume
Wt	Wild type

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Publications:

Hart, S., Fischer, O. M., and Ullrich, A. (2004). "Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor." Cancer Res., *64:* 1943-1950.

Fischer, O. M., Hart, S., Gschwind, A., Prenzel, N., Ullrich, A. (2004). "Stress Signalling in Tumour Cells is Mediated by ADAM Proteases and HB-EGF." MCB in Press.

Fischer, O. M., Hart, S., Ullrich, A. (2004). "Dissecting the EGFR Signal Transactivation Pathway." Methods Mol. Biol. in Press

Hart*, S., Gschwind*, A., Fischer, O. M., Ullrich, A. (2003). "TACE cleavage of pro-amphiregulin regulates GPCR-induced proliferation and motility of cancer cells." EMBO J. **22**(10): 2411-21.
* cofirst author

Hart, S., Gschwind, A. Roidl, A. and Ullrich, A.(2003). „Epidermal Growth Factor Receptor Signal Transactivation." Nato Science Series: II: Mathematics, Physics and Chemistry, *129:* 93-103.

Fischer, O. M., Streit, S., Hart, S., Ullrich, A. (2003). "Beyond Herceptin and Gleevec." Curr. Opin. Chem. Biol. **7**(4): 490-5.

Fischer O.M., Hart S., Gschwind A., Ullrich A. (2003). "EGFR Signal Transactivation in Human Cancer Cells." Biochem. Soc. Transact. 31(6): 1203-8.

Prenzel, N., Fischer, O. M., Streit, S., Hart, S., Ullrich, A. (2001). "The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification." Endocr. Relat. Cancer **8**(1): 11-31.

Hart, S., Fischer, O.M., Prenzel, N., Zwick-Wallasch, E., Ullrich. A. "GPCR induced migration in breast cancer cells depends on both, EGFR signal transactivation and EGFR independent pathways." Manuscript in preparation.

Patents:

EP 03003935.8 Gschwind, A., Hart, S. and Ullrich, A. "EGF Receptor Signal Transactivation in Squamous Cell Carcinoma Requires Proamphiregulin Cleavage by TACE/ADAM17." *Submitted*

Talks / Poster Presentations

MPI Junior Award 2004, Martinsried, 2004, Oral Presentation
"TACE-mediated proamphiregulin shedding regulates GPCR-induced proliferation and motility of squamous carcinoma cells"

International Workshop on Cytokine Signaling in Development and Oncogenesis, Port Isabel, Virginia, USA, Oral Presentation
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National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA, 2004, Oral Presentation
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FEBS Young Scientist Meeting, Brussels,2003, Poster Presentation, Hart, S., Gschwind, A. and Ullrich, A. "TACE-mediated proamphiregulin shedding regulates GPCR-induced proliferation and motility of squamous carcinoma cells"

FEBS Meeting 2003, Brussels, Oral Presentation
"Stress-Induced Ligand-Dependent EGFR Activation in Human Carcinoma Cells"

NATO/FEBS Advanced Study Institute, Spetses, 2002, Poster presentation, Hart, S., Gschwind, A., Leserer, M. and Ullrich, A. "Metalloprotease Mediated Epidermal Growth Factor Receptor Signal Transactivation"

Herbsttagung der Gesellschaft für Biochemie und Molekularbiologie, München, 2000, Poster presentation, Gschwind, A., Prenzel, N., Zwick, E., Gensler, M., Hart, S. and Ullrich, A. „Epidermal Growth Factor Receptor Transactivation by G Protein-Coupled Receptors Requires Metalloprotease-Mediated Growth Factor Precursor Cleavage.