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# Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion

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**Signalling through G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTK) is involved in the regulation of essential cellular processes and its deregulation is associated with tumorigenesis *in vitro* and *in vivo*. We investigated pathophysiological processes that are regulated by GPCR pathways in human kidney and bladder cancer cell lines. Our results show that GPCR ligands induce tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) as well as downstream signalling events such as recruitment of the adapter protein Shc and activation of the mitogen-activated protein kinases (MAPK) ERK1/2, JNK and p38. Moreover, we report that the EGFR transactivation signal involves the EGFR ligands amphiregulin, HB-EGF and TGFα as well as the metalloproteinases ADAM 10, 15 and 17, depending on the cellular system. Finally, we demonstrate that EGFR transactivation is part of a regulatory system that modulates the migratory and invasive behaviour of kidney and bladder cancer cells. In conclusion, our findings demonstrate that metalloproteinase-mediated transactivation of the EGFR is a key mechanism of the cellular signalling network that promotes MAPK activation as well as tumour cell migration and invasion in response to a variety of physiologically relevant GPCR ligands, and therefore represents a novel target for cancer intervention strategies.**
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## Introduction

Bladder and kidney cancer belong to the most frequent tumour types of the urogenital tract. The risk to fall ill with any of these kinds of cancer is 2–3-fold higher in men than in women, and increases with age. There exists a strong link between lifestyle and the probability to acquire cancer. An important risk factor is cigarette smoke, but genetic disposition is of equal relevance. Although early diagnosis positively affects the disease outcome, the probability of relapse is high, especially for bladder cancer. Moreover, each passing year, the incidence of bladder cancer as well as death rate is rising. Furthermore, after progression to a later tumour stage, metastases are detected in 30–50% of the patients. Surgical removal of bladder, prostate and seminal vesicles is required if bladder tumours have invaded muscular layers (http://www.health.harvard.edu/medline/Men/N0402b.html). The main therapy for kidney cancer is the complete resection of the kidney including the adrenal gland and ureter (Vogelzang and Stadler, 1998). This means that the common treatment of bladder and kidney cancer results in complete loss of function of the affected organ and thus a severely impaired quality of life for the patient.

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

Overexpression of the EGFR, HER2/neu and EGF-like ligands has been shown to promote tumour growth in bladder and kidney cancer cells and, in addition, has been correlated with metastatic behaviour (Dempsey *et al*., 1997). EGF activates its receptor in a direct manner, whereas HER2 represents the preferred heterodimerization partner for other EGFR family members (Yarden, 2001). Constitutive receptor activation due to overexpression or autocrine growth factor stimulation as well as aberrant downstream signalling has been linked to the development of hyperproliferative diseases such as cancer (Gill *et al*., 1987). Moreover, the EGFR serves as a prognostic marker in several types of human cancer (Dong *et al*., 1999), and is used as a pharmacological target in experimental cancer therapy involving the inhibition of oncogenic signalling pathways by anti-EGFR antibodies and small compounds such as Iressa (Normanno *et al*., 2003; Ritter and Arteaga, 2003).

In addition, the EGFR has been shown to be activated by heterologous extracellular stimuli such as cell adhesion molecules, cytokine receptors, stress agents and GPCRs (Zwick *et al*., 1999). Recently, we and others have demonstrated the involvement of EGF-like precursor cleavage by a metalloproteinase activity in

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EGFR transactivation pathways (Prenzel et al., 1999; Uchiyama-Tanaka et al., 2001; Gschwind et al., 2002; McCole et al., 2002; Wallasch et al., 2002). Given the prominent role of the EGFR in the development and progression of kidney and bladder tumours, we investigated the pathophysiological function of triple-membrane-passing signal (TMPS) pathways in this type of cancer.

## Results

### *A metalloproteinase activity mediates tyrosine phosphorylation of the EGFR and Shc in human kidney and bladder carcinoma cell lines*

We and others have previously demonstrated that the EGFR functions as an integral element of mitogenic GPCR signals leading to the activation of the Ras/MAPKinase pathway and cell proliferation (Daub et al., 1996; reviewed in Wetzker and Bohmer, 2003). Mechanistically, EGFR signal transactivation by GPCR ligands can involve processing of transmembrane precursors of EGF-like ligands such as amphiregulin (AR) (Gschwind et al., 2003), TGFα (Pai et al., 2002) and HB-EGF (Prenzel et al., 1999) by ADAM family metalloproteinases. To investigate the functional role of EGFR transactivation pathways in neoplasia, we tested the responsiveness of kidney and bladder carcinoma cell lines to GPCR ligands or to the control ligand EGF (Table 1). The results showed that stimulation with the GPCR agonists angiotensin II, bombesin, bradykinin, carbachol, ET-1, LPA and thrombin leads to rapid tyrosine phosphorylation of endogenous EGFR in the kidney cancer cell lines Caki2, ACHN, HK2, A498 and A704, as well as in the bladder cancer cell lines SCABER, HT1376, TccSup and 5637. To examine if a metalloproteinase activity is involved, we analysed the effect of the metalloproteinase inhibitor batimastat (BB94) on tyrosine phosphorylation of the EGFR and the downstream adaptor protein Shc. Pretreatment of tumour cells with BB94 (10 $\mu$M) or the EGFR-specific tyrphostin AG1478 (25 nM) completely abolished the transactivation signal and subsequent Shc tyrosine phosphorylation. Direct stimulation of EGFR and Shc by EGF, however, was not affected by BB94 (Figure 1a, b). Together, these results demonstrate that one tumour cell line can be stimulated by a wide variety of physiologically important GPCR ligands. Moreover, the EGFR acts as a point of convergence for multiple GPCR signals via ADAM metalloproteinases in kidney and bladder carcinoma cells.

### *EGFR transactivation by the GPCR agonist LPA involves the EGF-like ligands AR, HB-EGF and TGFα*

To identify EGF-like ligands involved in EGFR signal transactivation, we preincubated different kidney and bladder carcinoma cells with blocking antibodies against AR (20 $\mu$g/ml), HB-EGF (20 $\mu$g/ml) or TGFα (1 $\mu$g/ml). Preincubation of 5637 cells with the AR neutralizing antibody specifically reduced the LPA and bradykinin-induced transactivation signal, whereas blocking of TGFα could not inhibit EGFR transactivation (Figure 1c). The combination of AR and TGFα neutralizing antibody showed no cooperative effect (data not shown). Next, we confirmed the expression and cell surface localization of proAR in 5637 cells by flow cytometry using ectodomain-specific antibodies. Treatment of the cells with LPA and bradykinin resulted in rapid reduction in the cell surface content of endogenous proAR (Figure 1d).

Moreover, we found that HB-EGF is specifically involved in transactivation of the EGFR in the kidney carcinoma cell lines Caki2, ACHN and A498, while TGFα is required for the transactivation signal in TccSup bladder carcinoma cells in response to LPA (Table 2 and data not shown). These findings demonstrate that EGFR activation in response to the GPCR agonist LPA involves different EGFR ligands in 5637, ACHN, Caki2, A498 and TccSup cancer cells.

### *Inactivation of the EGFR or metalloproteinases interferes with GPCR-mediated MAPK activation*

Since recruitment of adapter proteins such as Shc is an essential step linking cell surface receptor stimulation to the activation of the Ras/MAPK pathway, we investigated the effects of GPCR agonists on activation of the MAPKs ERK1/2, JNK and p38. MAPK activation was monitored by immunoblotting cell lysates with activation state-specific antibodies. We found that ERK1/2

**Table 1** Activation of the EGFR by GPCR agonists in bladder and kidney carcinoma cell lines

| *Cell line* | *Tissue* | *AngII* | *Bomb* | *Bk* | *Carb* | *ET-1* | *LPA* | *Thr* |
|---|---|---|---|---|---|---|---|---|
| CaKi2 | Kidney | – | + | – | + | – | + | + |
| ACHN | Kidney | + | + | + | + | + | + | + |
| HK2 | Kidney | n.d. | + | + | + | + | + | + |
| A498 | Kidney | + | + | + | + | – | + | + |
| A704 | Kidney | n.d. | – | + | + | – | + | – |
| SCABER | Bladder | – | – | + | – | + | + | – |
| HT1376 | Bladder | – | – | + | + | + | + | + |
| TccSup | Bladder | – | – | + | – | + | + | – |
| 5637 | Bladder | – | – | + | – | + | + | – |

+ increased tyrosine phosphorylation of the EGFR monitored by Western blot analysis; – no detectable influence; n.d., not determined

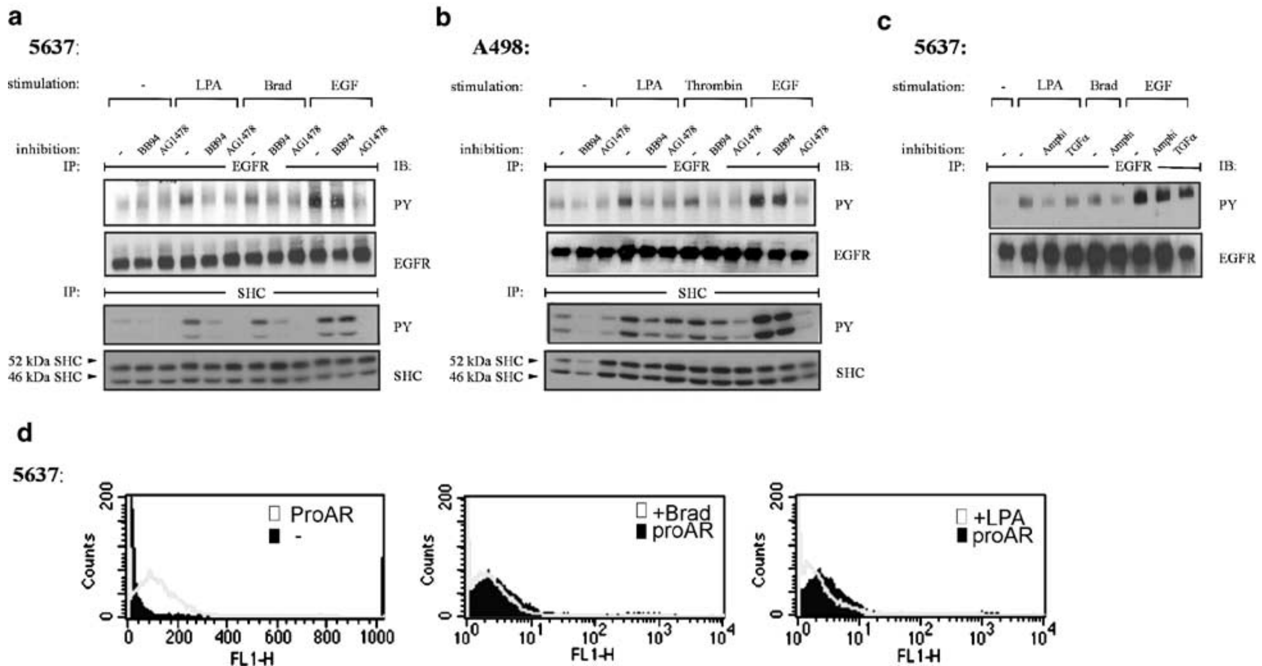

Figure 1 GPCR stimulation induces tyrosine phosphorylation of EGFR and Shc, which involves the EGFR, a metalloproteinase and AR. (**a**) Quiescent 5637 bladder carcinoma cells were preincubated with 250 nM AG1478 or 10 $\mu$M BB94 as indicated, and treated with 10 $\mu$M LPA, 5 $\mu$M bradykinin or 3 ng/ml EGF for 3 min. After immunoprecipitation with antibodies against EGFR or Shc, the proteins were immunoblotted with anti-PY antibody. Reprobing against the EGFR or Shc ensured precipitation of equal protein amounts. (**b**) Starved A498 kidney carcinoma cells were preincubated with DMSO as control, BB94 or AG1478 followed by a 3-min stimulation with 10 $\mu$M LPA, 2 U/ml thrombin or 3 ng/ml EGF. Cell lysates were immunoprecipitated with antibodies against EGFR or Shc, and analysed by immunoblotting with anti-PY antibody. Reprobing against EGFR or Shc showed loading of equal protein amounts. (**c**) Starved 5637 bladder carcinoma cells were preincubated with 20 $\mu$g/ml AR or 1 $\mu$g/ml TGF$\alpha$-blocking antibodies for 1 h and afterwards stimulated with 10 $\mu$M LPA, 5 $\mu$M bradykinin or 3 ng/ml EGF for 3 min. The EGFR was immunoprecipitated and immunoblotted against anti-PY antibody. (**d**) Flow-cytometric analysis of AR precursor expression. In all, 5637 cells were collected and stained for surface AR and analysed by flow cytometry. Control cells were labelled with FITC-conjugated secondary antibody alone. Stimulation with LPA as well as bradykinin for 20 min induces a proteolytic release of AR

**Table 2** EGFR transactivation by LPA leads to processing of precursors of different EGF-like ligands by different metalloproteinases of the ADAM family

| *Cell line* | *Tissue* | *GPCR ligand* | *metalloproteinase* | *EGF-like ligand* |
|---|---|---|---|---|
| CaKi2 | Kidney | LPA | ADAM 17 | HB-EGF |
| ACHN | Kidney | LPA | ADAM 10 | HB-EGF |
| A498 | Kidney | LPA | ADAM 17 | HB-EGF |
| TccSup | Bladder | LPA | ADAM 15 | TGF$\alpha$ |
| 5637 | Bladder | LPA | ADAM 15 | Amphiregulin |

were activated upon stimulation with the GPCR ligands LPA and ET-1 in TccSup cells (Figure 2a), and that preincubation with the metalloproteinase inhibitor batimastat or AG1478 specifically diminished GPCR-induced MAPK activation (Figure 2a).

To further quantify GPCR-triggered ERK stimulation, we measured endogenous ERK2 activity in an *in vitro* kinase assay using myelin basic protein (MBP) as a substrate. Treatment of HT1376 cells with LPA lead to a sixfold, with ET-1 to a twofold and with carbachol to a fourfold increase in ERK2 activity. Furthermore, we found that both BB94 and AG1478 almost completely blocked ERK activation by GPCR stimuli (Figure 2b).

Next, we assessed the effect of GPCR ligands on activation of the stress-responsive MAPKs JNK and p38 in TccSup cells. In time-course experiments, JNK and p38 activation was detectable 5 min after addition of LPA and reached its maximum after 10–15 min (Figure 2c). Immunoblotting of cell lysates with phospho-specific JNK and p38 antibodies revealed that phosphorylation of these MAPKs by LPA was also batimastat- and AG1478-sensitive, whereas the effects evoked by EGF were, as expected, metalloproteinase-independent (Figure 2d). Together, the data show that metalloproteinases and the EGFR are critically involved in the regulation of the MAPK signal by GPCR ligands in kidney and bladder tumour cells.

### *Metalloproteinases ADAM 10, 15 and 17 are involved in LPA-induced EGFR signal transactivation*

To identify the metalloproteinases which are involved in LPA-induced cleavage of EGF-like ligand precursors in kidney and bladder tumour cell lines, we investigated the effect of dominant-negative mutants of ADAM 10, 15 and 17 (MP10, 15, 17), which lack the pro- and metalloproteinase domain (Gschwind *et al*., 2003) on LPA-induced EGFR tyrosine phosphorylation. As shown in Figure 3a and Table 2, the transactivation

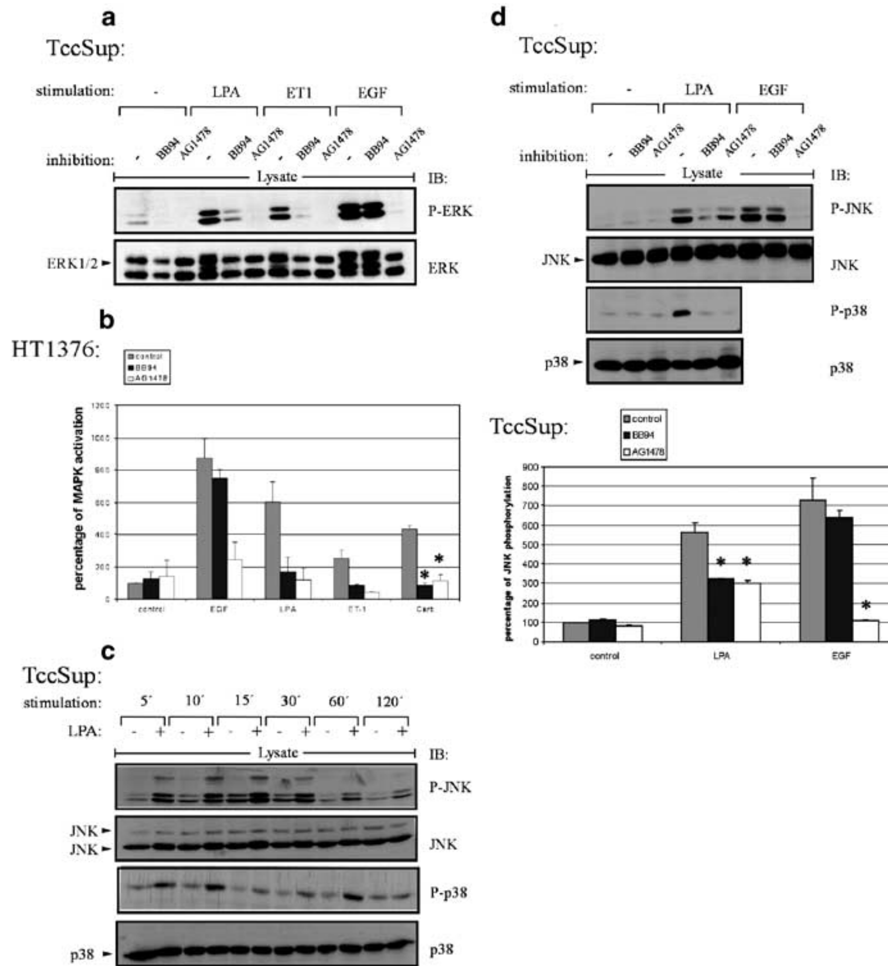

Figure 2 GPCR-mediated MAPK activation is BB94 and AG1478-sensitive. (a) Starved TccSup bladder carcinoma cells were pretreated with DMSO as vehicle, BB94 or AG1478, and then stimulated with 10 $\mu$M LPA, 100 nM ET-1 or 3 ng/ml EGF for 7 min. ERK activation was determined by immunoblotting with antiphospho-ERK antibody. (b) Quiescent HT1376 bladder cancer cells were preincubated with vehicle, BB94 or AG1478 and treated with 3 ng/ml EGF, 10 $\mu$M LPA, 100 nM ET-1 or 1 mM carbachol for 7 min. Cells were lysed and ERK activity was determined using MBP as a substrate. Phosphorylated MBP was visualized by autoradiography after gel electrophoresis. The filters were immunoblotted in parallel using anti-ERK2 antibody to verify the immunoprecipitation of equal amounts of ERK2 (data not shown). *$P$-value $<0.002$. (c) To evaluate the kinetics of JNK and p38 MAPK activation in TccSup bladder carcinoma cells, serum-starved cells were treated with LPA for the indicated periods of time. Crude lysate was immunoblotted with phospho-specific JNK or p38 antibody. To ensure loading of equal amounts of protein, the membrane was reprobed with anti-JNK or anti-p38 antibody. (d) Starved TccSup bladder carcinoma cells were preincubated with vehicle, BB94 or AG1478, and then stimulated with 10 $\mu$M LPA and 3 ng/ml EGF for 15 min. JNK activation was determined by immunoblotting with anti-phospho JNK antibody. Quiescent TccSup bladder carcinoma cells were pretreated with DMSO as control, BB94 or AG1478, followed by stimulation with 10 $\mu$M LPA for 15 min. Activation of MAPK was analysed by immunoblotting with anti-phospho p38 antibody. *$P$-value $<0.02$

signal was blocked by MP17 in Caki2 and A498 cells, by MP10 in ACHN cells and by MP15 in TccSup and 5637 cells.

To verify the specific requirement of ADAM 17/TACE for the EGFR transactivation pathway in A498 kidney cancer cells, we blocked the endogenous expression of TACE and ADAM 10 by RNA interference. In fact, gene silencing of TACE by siRNA specifically attenuated the LPA-induced transactivation signal in A498 cancer cells (Figure 3b).

Together, these results demonstrate that LPA is able to activate different ADAM proteases to process EGF-like ligand precursors in a cell system-specific manner.

*LPA-triggered cell motility requires both EGFR and a metalloproteinase activity*

One of the hallmark characteristics of cancer cells is enhanced motility. Therefore, we investigated the effect of LPA on tumour cell migration in a modified Boyden chamber assay in which cells migrate through a polycarbonate filter. We found that LPA stimulated the rate of A498 cell migration more than fivefold. This effect was specifically blocked by pretreatment of cells with BB94 or AG1478 (Figure 3c). These findings were further substantiated by analogous results obtained in Caki2, ACHN and TccSup cells (Table 3). Together, these data demonstrate that highly abundant GPCR

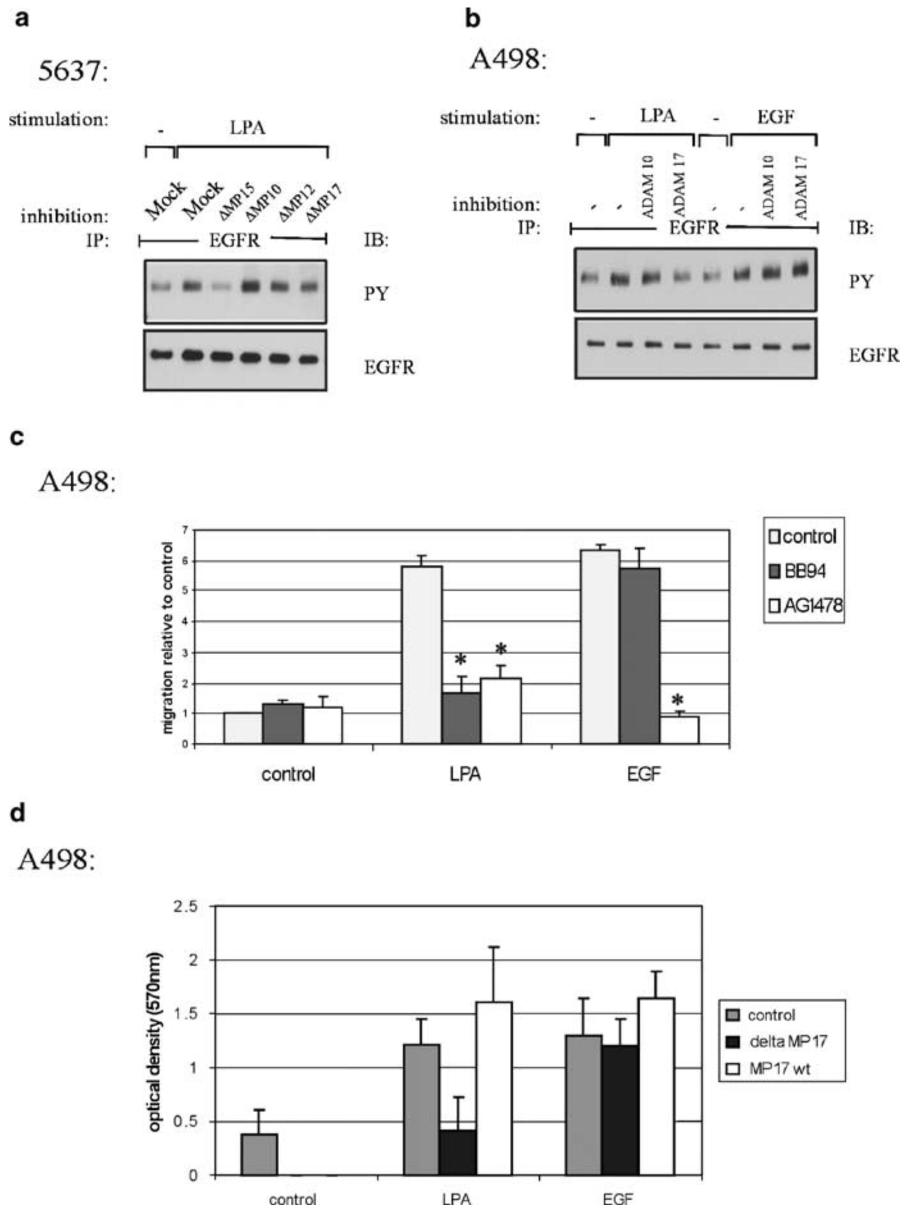


**Figure 3** LPA promotes cell migration, which is inhibited by BB94, AG1478 and a dominant-negative mutant of ADAM 17. (**a**) 5637 bladder cancer cell lines were infected with pLXSN constructs of a dominant-negative mutant of ADAM 10, 12, 15 and 17. The starved cells were stimulated with LPA for 3 min and tyrosine-phosphorylated EGFR was detected by immunoblotting. (**b**) TACE siRNA inhibits EGFR signal transmission by GPCR agonists in A498 kidney cancer cells. A498 cells were transfected with ADAM 10 and 17 siRNA and stimulated with LPA or EGF. Subsequently, activation of the EGFR was determined. TACE siRNA blocks endogenous TACE expression. A498 cells were transfected with ADAM 12 or TACE siRNA. Gene expression was analysed by immunoblotting with polyclonal anti-TACE antibody. (**c**) Quiescent A498 kidney carcinoma cells were added on top of a polycarbonate filter and permitted to migrate through the membrane in response to LPA added to the lower chamber. After 6 h, the migrated cells were fixed, stained and counted. Experiments were performed in triplicates, three fields from each well were counted and data are expressed as the average of five independent experiments. **P*-value $<0.003$. (**d**) Cell motility of A498 kidney cancer cells in response to LPA depends on TACE. A498 cells stably infected with a dominant-negative mutant and the wild type of ADAM 17 were treated with LPA or EGF and analysed in a transwell migration assay

ligands such as LPA promote the motility of kidney and bladder cancer cells by an EGFR- and metalloproteinase-dependent mechanism.

To evaluate whether ADAM 17 is involved in the regulation of LPA-promoted cell motility of kidney cancer cells, we analysed chemotactic migration of A498 cells which stably express either wild-type ADAM 17 or a dominant-negative ADAM 17 mutant (Gschwind *et al*., 2003). We found that expression of dominant-negative ADAM 17 completely prevented migration of A498 cells in response to LPA, whereas expression of the wild-type ADAM 17 slightly enhanced the migration rate of LPA-stimulated A498 cancer cells (Figure 3d). Together, our data demonstrate a critical role for TACE

**Table 3** EGFR transactivation by LPA promotes migration and invasion in kidney and bladder carcinoma cell lines

| *Cell line* | *Tissue* | *EGFR transactivation by LPA* | *migration* | *invasion* |
|---|---|---|---|---|
| CaKi2 | Kidney | + | + | + |
| ACHN | Kidney | + | + | + |
| HK2 | Kidney | + | n.d. | n.d. |
| A498 | Kidney | + | + | + |
| A704 | Kidney | + | n.d. | n.d. |
| SCABER | Bladder | + | – | n.d. |
| HT1376 | Bladder | + | – | n.d. |
| TccSup | Bladder | + | + | + |
| 5637 | Bladder | + | + | – |

+ increased tyrosine phosphorylation of the EGFR monitored by Western blot analysis; – no detectable influence; n.d., not determined

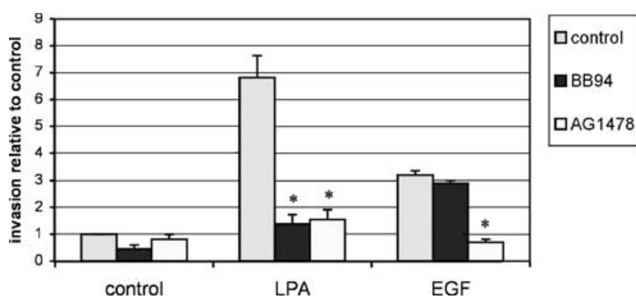


**Figure 4** Effect of BB94 and AG1478 on LPA-induced tumour cell invasion. Quiescent CaKi2 kidney carcinoma cells were plated on top of a matrigel-coated polycarbonate membrane. Cell invasion in response to LPA was analysed after 18 h, as described in Figure 3c. *$P$-value $<0.002$

in the regulation of cell motility of A498 kidney cancer cells in response to LPA.

### *Enhanced cancer cell invasion after stimulation with LPA through EGFR transactivation*

Penetration of the extracellular matrix and the basement membrane by cancer cells is a key step of tumour dissemination and invasion. Therefore, we evaluated the invasive capacity of cancer cells relating to EGFR transactivation by an *in vitro* invasion assay. The number of cells that penetrated Matrigel as an artificial basement membrane was determined. We found that stimulation of Caki2 kidney cancer cells with LPA resulted in a sevenfold increase in the invasion rate and that this effect was abolished by treatment with batimastat and AG1478 (Figure 4). Similar results were obtained in ACHN and A498 kidney cancer cells and in TccSup bladder cancer cells (Table 3).

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

## Discussion

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

Here, we found that a variety of potent mitogenic GPCR agonists such as LPA, thrombin, bombesin, bradykinin or angiotensin II induce EGFR transactivation in kidney and bladder cancer (Figure 1, Table 1).

Our results also demonstrate that, in these cancer indications, the metalloproteinases ADAM 10, 15 and 17 are required for cleavage and release of the transmembrane EGF-like ligand precursors proamphiregulin, proHB-EGF and proTGFα, depending on the cellular system (Table 2). In all the three kidney cancer cell lines tested, LPA stimulation of the EGFR is dependent on HB-EGF; while in Caki2 as well as in A498 cells, ADAM 17 is involved, in ACHN cells ADAM 10 is activated. Our data are consistent with previous studies demonstrating that EGFR transactivation by bombesin involves ADAM 10-dependent cleavage of HB-EGF in COS-7 and PC-3 cells (Yan *et al*., 2002), while ADAM 17 has been implicated in proteolytic cleavage of several EGF family members (TGFα, AR and HB-EGF) in murine fibroblasts (Sunnarborg *et al*., 2002; Gschwind *et al*., 2003).

Our study also provides experimental evidence for a novel role of the metalloproteinase disintegrin ADAM 15 in growth factor precursor cleavage, since we demonstrate its requirement for LPA-induced EGFR transactivation in the bladder carcinoma cell lines 5637 and TccSup (Table 2; Figure 3a). As a further variation of the mechanism, ADAM 15 induces shedding of two different EGF-like ligands in different cancer cell lines: AR in 5637 and TGFα in TccSup cells (Table 2).

Another important result of this study is that crosstalk between mitogenic LPA receptors and the EGFR leading to Shc and Gab1 recruitment and MAPK activation was detected in all cell lines tested (Figures 1a, b and 2) and that inhibition of EGFR or metalloproteinase activity by small chemical compounds blocked GPCR-triggered EGFR tyrosine phosphorylation and downstream signalling events (Figure 2).

Other reports have previously shown that LPA enhances wound closure and invasion in ovarian cancer cells (Xu *et al*., 1995) and to promote proliferation in kidney cancer cells (Levine *et al*., 1997). On the other hand, Ishikawa *et al*. (1989) demonstrated that EGF induces anchorage-independent growth and invasion of bladder cancer cells. Here, we provide evidence that both LPA and EGF promote cell migration and invasion in kidney and bladder carcinoma cells via the EGFR (Figures 3 and 4) and that the LPA-induced cellular responses require a metalloproteinase activity. Here, we also identify ADAM 17 as the metalloproteinase being required for cell migration in response to LPA of the kidney cancer cell line A498. Our results thereby further expand recently published data demonstrating that ADAM 17 cleavage of pro-AR regulates LPA-induced motility of squamous carcinoma cells (Gschwind *et al*., 2003).

In summary, this study describes an important pathophysiological function of EGFR transactivation by TMPS pathways in urogenital cancer. Elements of TMPS pathways therefore represent potential intervention targets for the treatment of kidney and bladder tumours.

## Materials and methods

### *Reagents and antibodies*

AG1478 was obtained from Alexis Biochemicals. Bradykinin was from Calbiochem. Batimastat (BB94) was kindly provided by Dr K. Maskos, Martinsried. LPA, angiotensin II, bombesin, ET-1, thrombin, EGF and all other chemicals were purchased from Sigma. The antibodies used were sheep polyclonal anti-EGFR antibody and mouse monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, UBI), rabbit polyclonal anti-ERK2 antibody, rabbit polyclonal anti-p38 antibody and rabbit polyclonal anti-JNK antibody (Santa Cruz), rabbit polyclonal anti-phospho-p44/42 MAPK antibody, rabbit polyclonal anti-phospho-p38 MAPK antibody and rabbit polyclonal anti-phospho-JNK MAPK antibody (New England Biolabs, NEB), mouse monoclonal anti-Shc antibody (BD Transduction Laboratories). Secondary HRP-conjugated antibodies were goat anti-mouse antibody (Sigma), donkey anti-sheep antibody (Dianova) and goat anti-rabbit antibody (BioRad). Goat anti-human AR, goat anti-human HB-EGF and goat anti-human TGFα-blocking antibodies were obtained from R&D systems.

### *Cell culture, plasmids and retroviral infections and generation of stable A498 cell lines*

Bladder carcinoma cell lines except SCABER were obtained from the German Collection of Microorganisms and Tissue Culture (DSMZ); all other carcinoma cell lines were purchased from the American Type Culture Collection (ATCC) and cultured as recommended.

The pLXSN (Clontech, Palo Alto, CA, USA) constructs encoding wild-type and dominant-negative ADAMs lacking the pro- and metalloproteinase domain have been described before (Gschwind *et al*., 2003). All protease constructs included a C-terminal HA tag, detectable with an anti-HA monoclonal antibody (Babco, Richmond, CA, USA). The amphotropic packaging cell line Phoenix was transfected with pLXSN retroviral expression plasmids by the calcium phosphate/chloroquine method, as described previously (Kinsella and Nolan, 1996). At 24 h after transfection, the viral supernatant was collected and used to infect subconfluent kidney and bladder cancer cells ($5 \times 10^4$ cells/six-well plate). Clonal A498 kidney cancer cell lines stably expressing dominant-negative ADAM 17 or wild-type ADAM 17 were generated by growing retrovirally infected cells in medium containing G418 (1g/ml) for 2 weeks.

### *Cell lysis, immunoprecipitation and immunoblotting*

Prior to lysis, cells grown to 80% confluency were serum starved for 48 h, treated with inhibitors or DMSO as a vehicle for 20 min and agonists for 3 min unless otherwise stated, and lysed for 10 min on ice in HNTG 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 phenylmethylsulphonyl fluoride and 10 μg/ml aprotinin. Lysates were precleared by centrifugation at 13 000 r.p.m. for 10 min at 4°C. Supernatants were diluted with an equal volume of HNTG buffer and subsequently immunoprecipitated using the respective antibodies and 30 μl of protein A-sepharose for 4 h at 4°C. Precipitates were washed three times with 0.5 ml of HNTG buffer, suspended in SDS sample buffer and subjected to gel electrophoresis on 7.5 or 10% gels. Following SDS–PAGE, proteins were transferred to a nitrocellulose membrane and immunoblotted with the respective antibodies. Signals were

developed by an enhanced chemiluminescence detection system (ECL, Amersham). Before reprobing, filters were stripped with a solution containing 2% SDS at 65°C for 1 h. Additionally, signals were quantified using a Fuji LAS 1000 CCD camera and the 'Image Gauge' program.

*MAPK assay (Daub* et al.*, 1996; Gerthoffer* et al.*, 1996)*

After treatment with inhibitors and ligands as indicated, endogenous ERK2 was immunoprecipitated from cell lysates. Precipitates were washed three times with HNTG buffer and subsequently once with kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 $\mu$M sodium orthovanadate). Kinase reactions were performed in 30 $\mu$l kinase buffer supplemented with 0.5 mg/ml MBP, 50 $\mu$M ATP and 1 $\mu$Ci [$\gamma$-32P] ATP for 10 min at room temperature. The reaction was stopped by addition of 30 $\mu$l Laemmli buffer (20% glycerin, 3% SDS, 10 mM EDTA pH 8, 0.05% Bromphenol blue) and analysed by SDS–PAGE (15% gels). Labelled MBP was visualized by autoradiography and quantified using a Phosphoimager (Fuji).

*RNA interference*

Transfection of 21 nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting endogenous genes was carried out using Lipofectamine (Invitrogen) and 4.2 $\mu$g of siRNA duplex per six-well plate, as previously described (Elbashir *et al*., 2001). The transfected A498 cells were serum starved and assayed 4 days after transfection. Sequences of siRNAs used have been described before (Gschwind *et al*., 2003). Specific silencing of targeted genes was confirmed by Western blot (TACE) and RT–PCR analysis (data not shown).

*Flow-cytometric analysis*

Fluorescence-activated cell sorting (FACS) analysis was performed as described before (Prenzel *et al*., 1999). In brief, the cells were seeded at 70 000 cells/six wells, grown for 24 h and serum starved for 24 h. After treatment with the growth factors as indicated, cells were collected and stained with ectodomain-specific antibodies against AR (R&D Systems) for 1 h. After washing with phosphate-buffered saline (PBS), cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 15 min and washed again with PBS. Cells were analysed on a Becton Dickinson FACScalibur flow cytometer.

*Migration assay*

Cell migration assays were performed using a modified Boyden chamber (Sieuwerts *et al*., 1997). Serum-free medium containing LPA as a chemoattractant was added to the lower well of a Boyden chamber. A polycarbonate filter (6.5 mm in diameter, 8 $\mu$m pore size) was placed over the lower well of the Boyden chamber and was secured with a gasket. In all, $1 \times 10^5$ cells in exponential growth were harvested and then preincubated with the inhibitor for 20 min, and then added to the upper well of the chamber in serum-free medium. The chambers were incubated for 6 h in a humified 7% $CO_2$, 37°C incubator. Finally, the cells that had migrated to the lower surface of the membrane were stained with crystal violet and counted under the microscope.

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

*Invasion assay*

Cell invasion assays were also performed in modified Boyden chambers containing a polycarbonate filter coated with Matrigel on the upper surface (Sieuwerts *et al*., 1997). As described above, the chemoattractant was added to the lower well and $1 \times 10^6$ cells were preincubated with the inhibitor and then added to the upper well. The chambers were incubated overnight. Finally, cells were wiped from the upper surface with a cotton tip swab and the cells on the other side were stained and counted under the microscope.

*Statistical analysis*

Student's *t*-test was used to compare the data. Values are expressed as mean $\pm$ s.d. of at least triplicate samples. $P < 0.05$ was considered as statistically significant.

**Abbreviations**

DMSO, dimethylsulphoxide; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; JNK, c-jun N-terminal kinase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kiase; RTK, receptor tyrosine kinase.

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**References**

Bue P, Wester K, Sjostrom A, Holmberg A, Nilsson S, Carlsson J, Westlin JE, Busch C and Malmstrom PU. (1998). *Int. J. Cancer*, **76,** 189–193.

Daub H, Weiss FU, Wallasch C and Ullrich A. (1996). *Nature*, **379,** 557–560.

De Boer WI, Houtsmuller AB, Izadifar V, Muscatelli-Groux B, Van der Kwast TH and Chopin DK. (1997). *Int. J. Cancer*, **71,** 284–291.

Dempsey PJ, Meise KS, Yoshitake Y, Nishikawa K and Coffey RJ. (1997). *J. Cell Biol.*, **138,** 747–758.

Dong J, Opresko LK, Dempsey PJ, Lauffenburger DA, Coffey RJ and Wiley HS. (1999). *Proc. Natl. Acad. Sci. USA*, **96,** 6235–6240.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T. (2001). *Nature*, **411,** 494–498.

Gerthoffer WT, Yamboliev IA, Shearer M, Pohl J, Haynes R, Dang S, Sato K and Sellers JR. (1996). *J. Physiol.*, **495,** 597–609.

Ghanem MA, Van Der Kwast TH, Den Hollander JC, Sudaryo MK, Mathoera RB, Van den Heuvel MM,

Noordzij MA, Nijman RJ and van Steenbrugge GJ. (2001). *Cancer*, **92,** 3120–3129.
Gildea JJ, Harding MA, Seraj MJ, Gulding KM and Theodorescu D. (2002). *Cancer Res.*, **62,** 982–985.
Gill GN, Bertics PJ and Santon JB. (1987). *Mol. Cell. Endocrinol.*, **51,** 169–186.
Gschwind A, Hart S, Fischer OM and Ullrich A. (2003). *EMBO J.*, **22,** 2411–2421.
Gschwind A, Prenzel N and Ullrich A. (2002). *Cancer Res.*, **62,** 6329–6336.
Ishikawa J, Maeda S, Sugiyama T, Nishimura R, Mizoguchi A and Kamidono S. (1989). *Int. J. Cancer*, **44,** 1000–1004.
Kinsella TM and Nolan GP. (1996). *Hum. Gene Ther.*, **7,** 1405–1413.
Levine JS, Koh JS, Triaca V and Lieberthal W. (1997). *Am. J. Physiol.*, **273,** F575–F585.
Lummen G, Virchow S, Rumenapp U, Schmidt M, Wieland T, Otto T, Rubben H and Jakobs KH. (1997). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 769–776.
McCole DF, Keely SJ, Coffey RJ and Barrett KE. (2002). *J. Biol. Chem.*, **277,** 42603–42612.
Normanno N, Bianco C, De Luca A, Maiello MR and Salomon DS. (2003). *Endocr. Relat. Cancer*, **10,** 1–21.
Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D and Tarnawski AS. (2002). *Nat. Med.*, **8,** 289–293.
Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C and Ullrich A. (1999). *Nature*, **402,** 884–888.
Ritter CA and Arteaga CL. (2003). *Semin. Oncol.*, **30,** 3–11.
Ruck A and Paulie S. (1997). *Anticancer Res.*, **17,** 1925–1931.
Ruck A and Paulie S. (1998). *Anticancer Res.*, **18,** 1447–1452.
Sieuwerts AM, Klijn JG and Foekens JA. (1997). *Clin. Exp. Metastasis*, **15,** 53–62.
Sunnarborg SW, Hinkle CL, Stevenson M, Russell WE, Raska CS, Peschon JJ, Castner BJ, Gerhart MJ, Paxton RJ, Black RA and Lee DC. (2002). *J. Biol. Chem.*, **277,** 12838–12845.
Uchiyama-Tanaka Y, Matsubara H, Nozawa Y, Murasawa S, Mori Y, Kosaki A, Maruyama K, Masaki H, Shibasaki Y, Fujiyama S, Nose A, Iba O, Hasagawa T, Tateishi E, Higashiyama S and Iwasaka T. (2001). *Kidney Int.*, **60,** 2153–2163.
Van Brussel JP and Mickisch GH. (1999). *BJU Int.*, **83,** 902–908, quiz 908-9.
Vogelzang NJ and Stadler WM. (1998). *Lancet*, **352,** 1691–1696.
Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H and Ullrich A. (2002). *Biochem. Biophys. Res. Commun.*, **295,** 695–701.
Wetzker R and Bohmer FD. (2003). *Nat. Rev. Mol. Cell Biol.*, **4,** 651–657.
Xu Y, Gaudette DC, Boynton JD, Frankel A, Fang XJ, Sharma A, Hurteau J, Casey G, Goodbody A and Mellors A. (1995). *Clin. Cancer Res.*, **1,** 1223–1232.
Yan Y, Shirakabe K and Werb Z. (2002). *J. Cell Biol.*, **158,** 221–226.
Yarden Y. (2001). *Oncology*, **61,** 1–13.
Zwick E, Hackel PO, Prenzel N and Ullrich A. (1999). *Trends Pharmacol. Sci.*, **20,** 408–412.