

ADAM-mediated amphiregulin shedding and EGFR transactivation

S. Kasina*, P. A. Scherle‡, C. L. Hall* and J. A. Macoska*†

*Department of Urology, The University of Michigan, Ann Arbor, MI, †The Comprehensive Cancer Center, The University of Michigan, Ann Arbor, MI, and ‡Drug Discovery, Incyte Corporation, Wilmington, DE, USA

Received 8 October 2008; revision accepted 13 April 2009

Abstract

Introduction: The ectodomain shedding of epidermal growth factor receptor (EGFR) ligands, such as amphiregulin (AREG), by ADAMs (A Disintegrin And Metalloproteases) can be stimulated by G protein-coupled receptor (GPCR) agonists. Interactions between the CXCR4 GPCR and the CXCL12 chemokine have been shown to mediate gene transcription and cellular proliferation in non-transformed and transformed prostate epithelial cells, as well as motility/invasiveness in transformed cells.

Objectives: In this report, we investigated the ability of CXCL12 to stimulate amphiregulin ectodomain shedding in non-transformed and transformed prostate epithelial cells that respond proliferatively to sub-nanomolar levels of CXCL12 and amphiregulin.

Materials and Methods: Non-transformed N15C6 and transformed PC3 prostate epithelial cells were assessed for amphiregulin shedding, ADAM activation, Src phosphorylation and EGFR activation using ELISA, immunoblot, and immunoprecipitation techniques, and for proliferation using cell counting after stimulation with CXCL12 or vehicle.

Results: The results of these studies identify CXCL12 as a novel inducer of amphiregulin ectodomain shedding and show that both basal and CXCL12-mediated amphiregulin shedding are ADAM10- and Src kinase-dependent in non-transformed N15C6 cells. In contrast, amphiregulin shedding is not amplified subsequent to stimulation with exogenous CXCL12, and is not reduced subsequent

to metalloprotease- or Src kinase-inhibition, in highly aggressive PC3 prostate cancer cells. These data also show that CXCL12-mediated cellular proliferation requires EGFR transactivation in a Src- and ADAM-dependent manner in non-transformed prostate epithelial cells. However, these same mechanisms are dysfunctional in highly transformed prostate cancer cells, which secrete amphiregulin in an autocrine manner that cannot be repressed through metalloprotease- or Src kinase inhibition.

Conclusion: These findings show that non-transformed and transformed prostate epithelial cells may employ different mechanisms to activate EGFR ligands and thereby utilize the EGFR axis to promote cellular proliferation.

Introduction

Chemokines are soluble, low molecular weight (8–14 kDa) chemotactic cytokines that bind to their cognate G-protein coupled receptors (GPCRs) to elicit cell responses (1). They govern multiple aspects of host defence and inflammation such as haematopoiesis, leukocyte trafficking and angiogenesis (2). However, beside their classical role as leukocyte chemoattractants, chemokines have been shown to play important roles in tumour biology, specially with regard to angiogenesis, motility, cell invasiveness, and cell proliferation (3,4).

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a CXC-type chemokine that is constitutively expressed in a wide variety of tissues and binds to the seven-transmembrane GPCR, CXCR4. The CXCL12/CXCR4 axis is involved in angiogenesis, migration and invasiveness of cancer cells, and tumour-cell proliferation (reviewed by 5). Recently, our laboratory studies have shown that the CXCL12/CXCR4 axis mediates cell proliferation in both non-transformed and transformed prostate epithelial cells (6,7). Several cellular mechanisms could account for the observed CXCL12-mediated

Correspondence: J. A. Macoska, PhD, Department of Urology, The University of Michigan, 6217 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0944, USA. Tel.: (734) 647-8121; Fax (734) 647-9271; E-mail: jcoska@umich.edu

proliferative response. For example, CXCL12/CXCR4 interactions may activate molecular pathways known to stimulate cell proliferation. It has been shown by us and others that CXCL12/CXCR4 interactions can stimulate signalling through pro-proliferative Raf/MEK/ERK as well as PI3K/PTEN/Akt pathways, although the CXCL12-mediated proliferative response has been observed to be ERK-dependent in non-transformed prostate epithelial cells and ERK-independent in transformed prostate epithelial cells (7–9). These studies suggest that common CXCL12-mediated proliferative and transcriptional responses observed for prostate epithelial cells may, in fact, be governed by multiple signalling pathways.

A further mechanism that may account for the observed CXCL12-stimulated proliferative response is that CXCL12/CXCR4 interactions may transactivate other membrane-bound receptors, which then stimulate signalling pathways involved in cell proliferation. Several studies have shown that GPCRs (such as chemokine receptors, for example CXCR4) can cross-activate other receptors, including epidermal growth factor receptor (EGFR). Such cross-activation is thought to occur through a G $\beta\gamma$ -mediated mechanism (10–13). Activated EGFR, in turn, can activate (primarily) the Raf/MEK/ERK, PI3K/PTEN/Akt and JAK/STAT pathways, and thereby promote cell proliferation (14–17).

A third type of mechanism that may account for CXCL12-mediated cell proliferation is upregulation and/or activation of growth-factor type ligands (cytokines, EGF-type ligands, and more) that promote cell proliferation. GPCRs, like CXCR4, are known to activate Src, PKC and reactive oxygen species, which serve as second messengers of activated G-proteins and can catalyse activation of membrane-bound sheddases termed ADAMs (14,18,19). In particular, interactions between the CXC-type chemokine, IL-8, and GPCRs that recognize it, CXCR1 and CXCR2 have been shown to stimulate shedding of EGFR ligands, heparin-binding epidermal growth factor (HB-EGF) and amphiregulin, in gastric cancer cells, in an ADAM10-dependent manner (20). The study reported here raises the possibility that chemokines may stimulate shedding of potent, mitogenic EGF-type ligands, that this process may be mediated by ADAMs, and that EGF-type ligand/receptor interactions may contribute to the observed CXCL12/CXCR4-mediated proliferative response. To test this hypothesis, we examined whether CXCL12/CXCR4 interactions may mediate shedding of EGF-type ligands in non-transformed or transformed human prostate epithelial cells, whether this process is Src- and ADAM-dependent, and whether consequent EGFR transactivation contributes to the CXCL12/CXCR4 mediated proliferative response.

Materials and methods

Cell cultures

N15C6 and N1 cells were produced through immortalization of primary human prostate epithelial or stromal fibroblast cultures, respectively, by transduction with a recombinant retrovirus encoding the *E6* and *E7* genes of HPV-16, as described previously (21). Both N15C6 and N1 cells are non-transformed and grow continuously in culture, but do not form colonies in soft agar nor tumours in immunocompromised mice (22,23). N15C6 and N1 cells were maintained in 5% HIE media (Ham's F12; Mediatech Inc., Herndon, VI, USA) with 5% foetal bovine serum (Invitrogen, Carlsbad, CA), 5 $\mu\text{g}/\text{ml}$ insulin, 10 ng/ml EGF and 1 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma Chemical Co., St Louis, MO, USA), or in defined serum-free HI (SF) media supplemented with 5 mM ethanolamine (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich), 5 $\mu\text{g}/\mu\text{l}$ transferrin (Sigma Aldrich), 10 μM 3,3',5-triiodo-L-thyronine (Sigma Aldrich), 50 μM sodium selenite (Sigma Aldrich), 0.1% bovine serum albumin (BSA; JRH Biosciences, Lenexa, KS, USA), 0.05 mg/ml gentamicin (Invitrogen, Carlsbad, CA) and 0.5 $\mu\text{g}/\text{ml}$ fungizone (Cambrex Bioscience, Walkersville, MD, USA). Benign prostatic hyperplasia-1 (BPH-1) cells are SV40 Large T-immortalized, non-transformed prostate epithelial cells and were maintained in 10% RPMI media (24). Androgen-sensitive LNCaP and 22Rv1, and androgen-insensitive PC3 and DU145 transformed prostate epithelial cell lines were maintained in 10% RPMI media or SF RPMI (without phenol red) and antibiotics as described above (25–28). For amphiregulin shedding assays, N1 and N15C6 cells were maintained in SF HI media with 0.05% BSA, and BPH-1, LNCaP, 22Rv1, PC3 and DU145 cell lines were maintained in phenol red-free SF RPMI with 0.05% BSA.

Quantitative real-time PCR

All quantitative real-time assays were conducted as previously described using an Applied Biosystems 7900HT instrument and reagents (6,7). Cells were grown to 70% confluence in 60 mm dishes prior to RNA purification using Trizol reagent (Invitrogen, Carlsbad, CA). For all experiments, 1 μg of RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen). Resulting cDNA was diluted to the ratio of 1:100. Real-time PCR was performed using Assays on Demand (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Reactions within individual experiments were performed in triplicate, including no template controls and an endogenous control probe, RPLPO (ribosomal protein, large, PO), to assess template

concentration. Cycle numbers to threshold were calculated by subtracting averaged control from averaged experimental values, and fold gene expression was calculated by raising these values to log 2. 6-carboxy-fluorescein-conjugated, gene-specific assays were Hs00155832_m1 for AREG and Hs99999902_m1 for the control, RPLPO.

Amphiregulin shedding assays

To assess basal levels of amphiregulin shedding, 300 000 cells each were plated in six-well plates in complete medium (5% HIEC for N1 and N15C6 and 10% RPMI for BPH-1, LNCaP, 22Rv1, PC3 and DU145) which was changed to SF HI for N1 or N15C6 cells, or to SF RPMI for BPH-1, LNCaP, 22Rv1, PC3, or DU145 cells for 24 h prior to assessment of basal amphiregulin shedding, which was determined by ELISA (R&D Systems, Minneapolis, MN) and normalized to pg/ml/million cells. Effects of exogenous CXCL12 on amphiregulin shedding were determined by treating serum-deprived cells with vehicle (0.1% BSA in phosphate-buffered solution (PBS)) and 10 pM CXCL12 for N15C6 cells or 100 pM CXCL12 for PC3 cells or 10 pM and 100 pM for both LNCaP and 22Rv1 cells, for 60 min, followed by replacement with fresh serum-free medium for 4 h. Conditioned medium was then harvested, concentrated and tested by ELISA to quantify amphiregulin (R&D Systems #262-AR-100, #MAB262, #BAF262). Effects of pan-metalloprotease inhibitors, *O*-phenanthroline (OPT), galardin (GM 6001), and tumour necrosis factor- α inhibitor-1 (TAPI-1), ADAM10-specific inhibitor, INCB008765 (29), Src family kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)-pyrimidine (PP1) and its negative control 4-amino-7-phenylpyrazol[3,4-*d*] pyrimidine, PP3, on CXCL12-stimulated amphiregulin shedding, were examined by treating serum-deprived cells with 500 μ M OPT (Sigma), 5 μ M GM6001 (Calbiochem, San Diego, CA), 2 μ M TAPI-1 (Calbiochem), 5 or 10 μ M INCB008765, 5 or 10 μ M of PP1 (Invitrogen, Carlsbad, CA), 10 μ M of PP3 (Calbiochem) or vehicle (0.1% methanol for OPT, 0.05% dimethyl sulphoxide (DMSO) for GM6001, TAPI-1, and PP3, 0.025% or 0.05% DMSO for INCB008765 and PP1, for 1 h, followed by treatment with 10 pM CXCL12 for N15C6 cells or 100 pM CXCL12 for PC3 cells. The conditioned medium was then harvested, concentrated and tested using ELISA to quantify amphiregulin presence, which was normalized as pg/ml/million cells.

Immunoblotting and protein analysis

Cells were lysed, proteins resolved by electrophoresis and electroblotting was carried out as described previ-

ously (6,7). Proteins were detected using antibodies against ADAM10 (Millipore, Billerica, MA, #AB19026), ADAM17 (R&D Systems, #AB930), phospho-Src Y418 (Invitrogen #44-660G), pan-Src family protein (Cell Signaling, Danvers, MA, #2110), pan anti-phosphotyrosine, clone 4G10 (Millipore, Billerica, MA, NY, USA), phospho-EGFR Y992 (Cell Signaling, #2235), EGFR (Cell Signaling, #2232), tubulin (Millipore, Billerica, MA, #DM1A) or actin (Santa Cruz, #sc-1615) in conjunction with an ECL detection system. Secondary antibodies including goat anti-rabbit (Cell Signaling, #7074), goat anti-mouse (Santa Cruz, CA, #SC-2005), and mouse anti-goat (Santa Cruz, #A3105) were used at 1:5000 concentration. Immunoblots shown are representative of replicate experiments. Densitometric quantification of immunoblot films was accomplished by scanning original films and converting .tiff files to greyscale. Images were inverted and mean band intensities were measured using IMAGEJ (<http://rsbweb.nih.gov/ij/index.html>). Mean intensity of adjacent background was also measured for each band and subtracted from band intensity. Densitometric data were averaged from replicate experiments (as indicated in the figures) and standard deviations were calculated for graphical depiction and statistical analysis.

Immunoprecipitation assay and ELISA for Src activity

The effect of Src family kinase inhibitor, PP1, and its negative control, PP3, on CXCL12-stimulated Src family protein activity in N15C6 cells, was examined by treating serum-deprived cells with vehicle (0.025% or 0.05% DMSO), 5 or 10 μ M of PP1 (Biosource), 10 μ M of PP3 for 1 h, followed by treatment with vehicle (0.1% BSA in PBS), or 10 pM CXCL12. Cell lysates were evaluated using ELISA for Src family protein phosphorylation at tyrosine residue Y418, as described by the manufacturer (Upstate, #14-468), with pSrcY418 quantified as units/ml medium/ μ g total protein (U/ml/ μ g). For immunoprecipitation assays, cells were lysed in an RIPA buffer as described previously (6,7). Cell lysates were centrifuged at 14 000 *g* for 10 min at 4 °C, and 200 μ g of total protein were exposed to 1:330 dilution of anti-Src family protein antibody overnight at 4 °C. Immunoprecipitates were immobilized using protein-G-sepharose beads for 2 h at 4 °C and were washed three times in the same buffer. Precipitated proteins were eluted in 40 μ l of 2x Laemmli sample buffer, boiled for 5 min and separated using 8% SDS-PAGE. Immunoblots were subjected to densitometric analysis as described previously and these data were averaged from replicate experiments (as indicated in the figures); standard deviations were calculated for graphical depiction and statistical analysis.

Proliferation assays

Cell proliferation was assessed in triplicate in 24-well plates and cells counted after 24 and 96 h incubation as described previously (6,7). To assess the effects of exogenous amphiregulin on cell proliferation, recombinant human amphiregulin (R&D Systems, #262-AR-100) was added at the desired concentration in 0.5 ml SF medium (or 0.5 ml SF media alone for control) to each well. To assess the effect of ADAM 10 specific inhibitor INCB008765, MEK1 inhibitor U0126, and EGFR inhibitor AG1478 (Tyrophostin) on cell proliferation, the cells were pre-treated with vehicle (DMSO, 0.025% for INCB008765 and 0.01% for U0126 and AG1478) or 5 μM INCB008765, 1 μM U0126, 250 nM AG1478, followed by supplementing medium with 10 ng/ml EGF, 10 μM CXCL12, or 100 μM amphiregulin. Cells were re-fed at 24 h growth and were counted at 24 and 96 h. Average cell numbers and standard deviations were calculated over three separate experiments for graphical depiction and statistical analysis.

Statistical analysis

Data from replicate experiments (as indicated in the figures) per study were averaged and standard deviations were calculated for graphical depiction and statistical analysis. Data were assessed using the *t*-test or analysis of variance, with $P < 0.05$ considered statistically significant.

Results

Autocrine secretion of amphiregulin by prostate epithelial cells

To begin the study on whether CXCL12 might activate EGF-type ligands to promote cell proliferation, we first established basal levels of EGF-type ligand secretion in non-transformed N1 prostate fibroblast cells, non-transformed N15C6 and BPH-1 prostate epithelial cells, and transformed LNCaP, 22Rv1, PC3 and DU145 prostate epithelial cell lines. Using sandwich ELISA of serum-free media conditioned by these cells for 24 h, we detected levels of amphiregulin to be between 100 and 1000 pg/ml/million cells for five – N1, N15C6, BPH-1, PC3 and DU145 – out of the seven cell lines tested. In contrast, only very low levels of amphiregulin protein shed into the media were detected for 22Rv1 (<10 pg/ml/million cells) or LNCaP (<1 pg/ml/million cells) cells (Fig. 1a). These results are consistent with those reported previously by Topping *et al.* (30), which showed that expression of amphiregulin transcript and

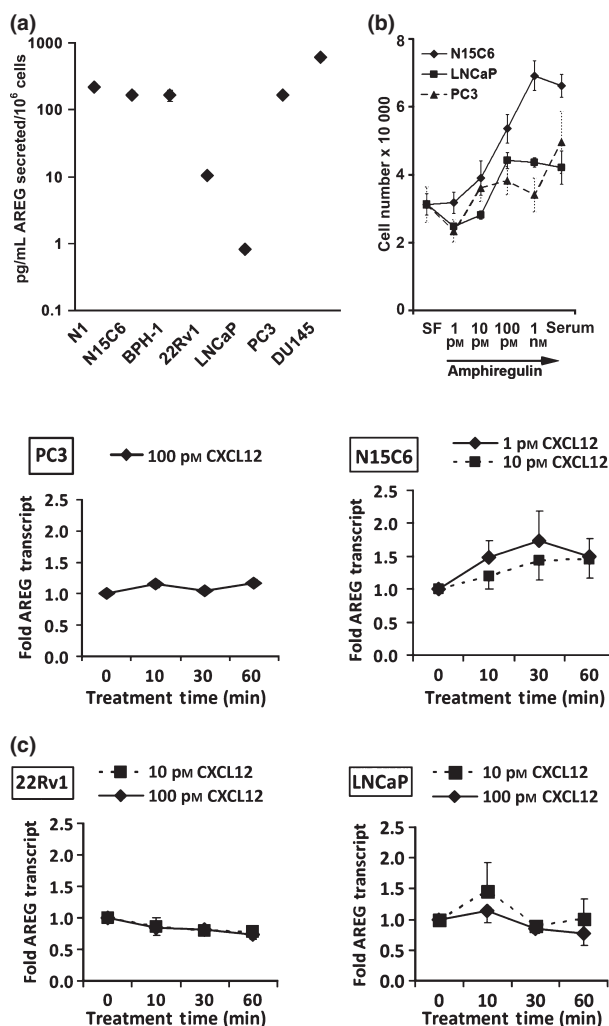


Figure 1. Profile of amphiregulin shedding, proliferative responses and gene transcription in prostate epithelial cells. (a) Basal protein levels (pg/ml/million cells) of amphiregulin (AREG) secreted after 12 h growth in serum-free media by N1 prostate stromal fibroblast or N15C6, BPH-1, 22Rv1, LNCaP, PC3 or DU145 prostate epithelial cells were determined using ELISA and shown plotted on a logarithmic axis (*y*-axis). Error bars reflecting deviations from the mean are plotted, but are not evident as they comprise <1% error over replicate measures. (b) Total cell number achieved by N15C6 (diamonds), LNCaP (squares) or PC3 (triangles) cells after 96 h growth in serum-free (SF) media supplemented with 1, 10 or 100 μM or 1 nM AREG, or complete media with serum (SERUM) normalized to growth in SF media alone is shown. Both N15C6 and LNCaP cells proliferated in response to high concentrations of CXCL12 to levels comparable with those achieved in complete media with serum. In contrast, PC3 cells did not proliferate in response to amphiregulin. Error bars reflect deviations from the mean for replicate measures. (c) PC3, N15C6, 22Rv1 and LNCaP prostate epithelial cells were treated with 1, 10 or 100 μM CXCL12 (as shown) and assessed for transcription of the AREG gene using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) at 0, 10, 30 and 60 min treatment. Transcript levels are normalized to those of an endogenous control transcript, RPLPO and are shown as fold amphiregulin transcript to levels detected at time 0 (normalized to 1-fold). Error bars reflect deviations from the mean for replicate experiments.

protein was increased 10- to 100-fold in androgen-insensitive PC3 compared to androgen-sensitive LNCaP cells. Detection of presence of EGF-type ligands using ELISA, for transforming growth factor (TGF)-alpha, HB-EGF and EGF shed into the media by the seven cell lines, showed only very low levels (<1 pg/ml/million cells) of these proteins (not shown).

Amphiregulin-stimulated prostate epithelial cell proliferative response

To understand whether basal levels of amphiregulin detected in the previous experiments might be sufficient to induce proliferative responses in prostate epithelial cells, representative cell lines – non-transformed N15C6 cells, androgen-sensitive transformed LNCaP cells and androgen-insensitive transformed PC3 cells – were grown in serum-free media supplemented with escalating doses of amphiregulin. As seen in Fig. 1b, N15C6 cells exhibited a robust proliferative response to amphiregulin over a concentration range of 10 pM (~100 pg/ml) to 1 nM (~10 ng/ml). The observation reported in this study was that N15C6 cells secrete ~100 pg/ml/million cells of amphiregulin under basal conditions. Figure 1a suggests that these cells may respond proliferatively to basal, as well as higher, levels of amphiregulin. PC3 cells proliferated modestly in response to exogenously administered amphiregulin over a concentration range of 10–100 pM (~100–1000 pg/ml) (Fig. 1b). This concentration range included that observed under conditions of basal secretion by PC3 cells (~170 pg/ml/million cells). LNCaP cells responded proliferatively to exogenous administration of amphiregulin in the 100 pM (~1 ng/ml) to 1 nM (~10 ng/ml) range, well above the low (<10 pg/ml) level of amphiregulin secreted by these cells under basal conditions. Therefore, unlike N15C6 or PC3 cells, LNCaP cells failed to secrete endogenously sufficient levels of amphiregulin under basal conditions, to induce cell proliferation.

CXCL12 does not stimulate amphiregulin gene expression

It has been shown previously by our team that low, picomolar levels of CXCL12 stimulate both proliferative and robust and complex transcriptional responses in both non-transformed N15C6 and transformed LNCaP prostate epithelial cells (7). As other CXC-type chemokines, notably IL-8, had been shown to stimulate shedding of EGF-type ligands (20), it was examined whether shedding of amphiregulin (EGF-type ligand most commonly detected in media of the cell lines tested), might be stimulated in response to treatment with concentrations of CXCL12 shown to promote proliferative responses in these cells. The first set of experiments examined whether stimulation with exogenous CXCL12

might be associated with transcriptional activation of the amphiregulin gene. As shown in Fig. 1c, none of the four cell lines tested – non-transformed N15C6 cells, androgen-sensitive transformed LNCaP or 22Rv1 cells, or androgen-insensitive transformed PC3 cells – demonstrated significant transcription of the amphiregulin gene in response to concentrations of CXCL12 associated with cell proliferation (1 or 10 pM CXCL12 for N15C6 cells, or 10 or 100 pM CXCL12 for PC3, LNCaP or 22Rv1 cells). These data suggested that increased levels of amphiregulin expression as a result of transcriptional activation of the amphiregulin gene were not part of the mechanism involved in CXCL12-mediated proliferative responses.

CXCL12 stimulated amphiregulin shedding in N15C6, but not PC3, prostate epithelial cells

GPCR/ligand interactions, including those involving CXC-type chemokine receptors and ligands, can stimulate proteolytic processing and functional activation of EGFR ligands (14,18–20). Therefore, we examined whether CXCL12 could stimulate shedding of amphiregulin in prostate epithelial cell lines. As shown in Fig. 2a, neither LNCaP nor 22Rv1 cells shed amphiregulin into the media in response to treatment with 10 or 100 pM CXCL12. However, N15C6 cells treated with 10 pM CXCL12 demonstrated approximately 1.3× higher levels of shed amphiregulin than untreated cells. This concentration of CXCL12 has been shown to promote the proliferation of N15C6 cells (6,7). In contrast, PC3 cells did not shed additional amphiregulin in response to treatment with 50 pM CXCL12 (Fig. 2b) or 100 pM CXCL12 (not shown).

CXCL12 stimulates ADAM activation in N15C6, but not PC3, cells

The preceding studies showed that CXCL12 did not stimulate amphiregulin gene expression (Fig. 1c), but did stimulate shedding of the ectodomain of amphiregulin (Fig. 2b) in N15C6 cells. As recently reviewed by Ohtsu *et al.* (19), ectodomains of EGF-type ligands, including amphiregulin, EGF, epiregulin, HB-EGF and TGF-alpha, are shed through activities of multiple ADAM-type proteases, including ADAM9, 10, 12, 15, 17 and 19. Amphiregulin shedding is dependent on ADAM17 activation in mouse embryonic cells (31), whereas chemokine-mediated amphiregulin shedding is dependent on ADAM10 activation (19,20). Thus, next we examined whether CXCL12/CXCR4 interactions could stimulate activation of ADAM 10 and/or ADAM17 and whether this mechanism was associated with amphiregulin ectodomain shedding and the observed cell proliferative response. These experiments focused on CXCL12-mediated ADAM acti-

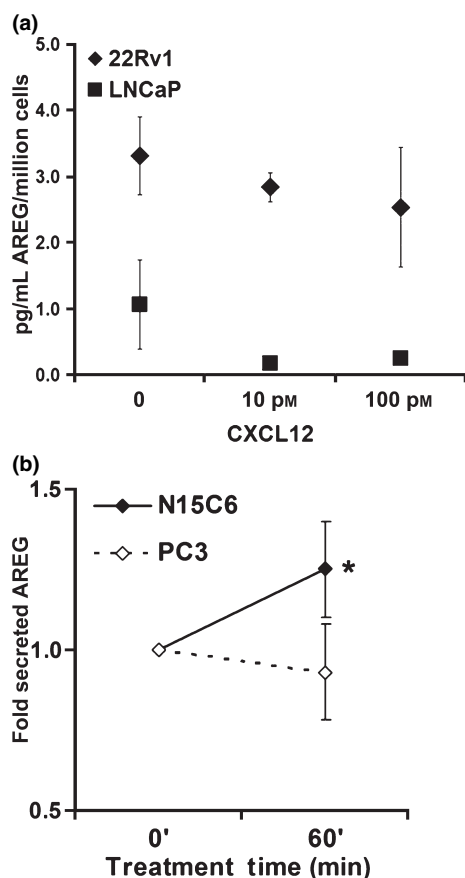


Figure 2. Amphiregulin ectodomain shedding in response to CXCL12. (a) 22Rv1 or LNCaP cells were treated for 60 min with vehicle (0.1% BSA in PBS), 10 pM CXCL12, or 100 pM CXCL12 as shown, their media replaced with fresh serum-free media and the cells grown for 4 h. Media were collected, shed amphiregulin levels were determined using ELISA, and then normalized to pg/ml per million cells. Neither 22Rv1 nor LNCaP shed amphiregulin in response to CXCL12. Error bars reflect deviations from the mean for replicate measures. (b) N15C6 or PC3 were treated for 60 min with vehicle (0.1% BSA in PBS), 10 pM CXCL12 (N15C6) or 100 pM CXCL12 (PC3) and processed as described in (a). Amphiregulin secretion is indicated as fold protein secreted compared with time 0 (which is normalized to 1-fold). N15C6, but not PC3, demonstrated significant ($P < 0.05$, indicated by *) secretion of amphiregulin in response to CXCL12. Error bars reflect deviations from the mean for replicate measures.

vation in N15C6 and PC3 cells, as both these cell lines exhibit high basal levels of shed amphiregulin (Fig. 1a), but only N15C6 demonstrated additional amphiregulin shedding upon treatment with CXCL12 (Fig. 2b).

ADAM proteins can exist as pro-form zymogens that do not exert sheddase activities until they themselves are proteolytically cleaved and activated (29,32). Cleavage of the pro-forms of ADAM10 (100 kDa) and ADAM17 (120 kDa) and appearance of the active forms of these proteins (at 60 and 80 kDa, respectively) may be observed through immunoblot analysis. Immunoblot analysis of

protein lysates prepared from N15C6 cells grown in SF HI media supplemented with 10 pM CXCL12 for 0, 10, 30 or 60 min demonstrated a 2.5-fold increase in the ratio of active:pro-form ADAM10. Accumulation of pro-form ADAM10 remained unchanged subsequent to CXCL12 stimulation. In contrast, N15C6 cells did not demonstrate accumulation of pro- or active forms of ADAM17 (Fig. 3a,b). These data showed that CXCL12 selectively stimulated ADAM10 activation in N15C6 cells. Similar experiments conducted with PC3 cells grown in SF RPMI media supplemented with 100 pM CXCL12 for 0, 10, 30 or 60 min did not demonstrate either activation or accumulation of the pro-forms of ADAM10 or ADAM17 in these cells (Fig. 3c,d). Low-level constitutive activation of both ADAM10 and ADAM17 was also evident for both N15C6 and PC3 cells grown in serum-free media lacking EGF-ligands. Taken together, these data show that CXCL12 stimulates rapid and robust activation of ADAM10, but not ADAM17, in N15C6 cells.

CXCL12-stimulated amphiregulin shedding is metalloprotease-dependent

The previous experiments suggested that CXCL12 stimulated amphiregulin shedding and robust ADAM10 activation in N15C6 cells, but not PC3 cells. However, as recently reviewed by Sanderson *et al.* (33), ectodomain shedding and activation of EGF-type ligands may be mediated through the activities of any of a family of matrix metalloproteases (MMPs). MMPs comprise an enzyme family that requires a zinc ion at their active site for catalytic activity and include 'classical' MMPs, membrane-bound MMPs (MT-MMPs), ADAMs and ADAMTS (a disintegrin and metalloprotease with thrombospondin motif). To determine whether metalloprotease activity consistent with MMP family activation was required for CXCL12-stimulated amphiregulin shedding, N15C6 cells were treated with 10 pM CXCL12 in the presence of pan-metalloprotease inhibitor, OPT, or solvent vehicle, 0.1% methanol. The results of these experiments confirmed accumulation of active 60 kDa form of ADAM10 to levels approximately 3-fold higher in N15C6 cells treated for 60 min with CXCL12 compared to untreated cells. Moreover, cells pre-treated with 0.5 mM OPT followed by 60 min treatment with 10 pM CXCL12 failed to accumulate 60 kDa active form of ADAM10 (Fig. 4a-c). The results were paralleled in experiments examining amphiregulin shedding under the same conditions, which showed that N15C6 cells pre-treated with the solvent vehicle 0.1% methanol shed amphiregulin into the media subsequent to treatment with CXCL12 for 60 min. In contrast, pre-treatment with OPT not only inhibited CXCL12-mediated amphiregulin shedding, but reduced the level of

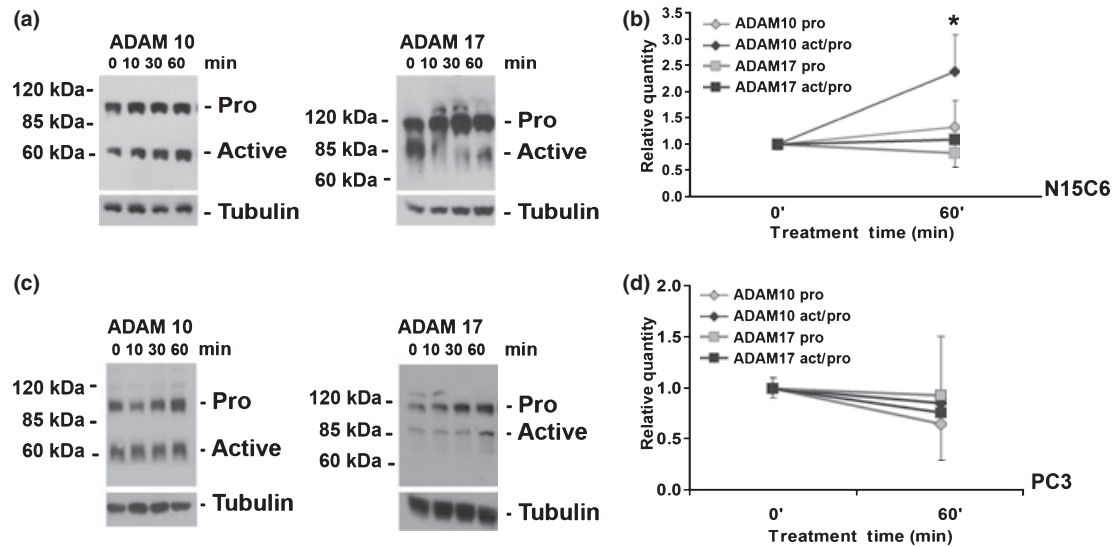


Figure 3. CXCL12 stimulates ADAM activation in prostate cancer cells. (a) Immunoblot analysis of protein lysates prepared from N15C6 cells grown in SF HI media supplemented with 10 pM CXCL12 for 0 or 60 min. The active and pro-forms of ADAM 10 migrated at molecular weights of approximately a 60 kDa form and 100 kDa and for ADAM17 an 80 kDa form and 120 kDa respectively. (b) Densitometric analysis of replicate immunoblots similar to that shown in (a). ADAM activation is indicated as the ratio of active/pro-form normalized to 1-fold at time 0. Treatment with CXCL12 increased the ratio of active:pro-form ADAM10 by 2.5-fold ($P < 0.05$, indicated by *). Error bars reflect deviations from the mean for replicate measures. (c) Immunoblot analysis of protein lysates prepared from PC3 cells grown in SF RPMI media supplemented with 100 pM CXCL12 for 0 or 60 min did not demonstrate accumulation of either the pro- or active forms of ADAM10 or ADAM 17. (d) Densitometric analysis of replicate immunoblots similar to that shown in (c) demonstrated a lack of accumulation of either the pro- or active forms of ADAM10 or ADAM 17 in PC3 cells treated with CXCL12. ADAM activation is indicated as the ratio of active/pro-form normalized to 1-fold at time 0. Error bars reflect deviations from the mean for replicate measures.

amphiregulin shed into the media below basal levels (Fig. 4e). The results of these experiments implied that metalloprotease activity, particularly that of activated ADAM10, was required, and likely mediated, CXCL12-stimulated amphiregulin shedding by N15C6 cells.

Similar experiments conducted in PC3 cells showed that accumulation of activated ADAM10 was similar in cells pre-treated with either OPT or vehicle, then treated with 100 pM CXCL12 (Fig. 4b,d). Moreover, accumulation of shed amphiregulin remained unaffected in PC3 cells under the same conditions (Fig. 4f). The results were consistent with those reported earlier that CXCL12 did not stimulate amphiregulin shedding above basal levels and did not appreciably activate ADAM10 in PC3 cells. Moreover, inhibition of metalloprotease activity did not repress either ADAM10 activation nor amphiregulin shedding in these cells.

CXCL12-mediated cell proliferation and amphiregulin shedding are ADAM10-dependent

The results reported in this study suggest that metalloprotease activity was required for CXCL12-mediated amphiregulin ectodomain shedding in N15C6 cells and that this activity was associated with ADAM10 activation

(Fig. 3,4). To assess whether CXCL12-mediated ADAM10 activation contributed to the observed CXCL12-stimulated proliferative response, N15C6 cells were grown for 96 h in serum-free media in the presence of DMSO (vehicle), 5 μ M INCB008765 + vehicle, 10 pM CXCL12 + vehicle or 5 μ M INCB008765 + 10 pM CXCL12 + vehicle. INCB008765 selectively inhibits activity of ADAM10 ($IC_{50} = 97 \mu$ M), but not that of ADAM9, ADAM17, ADAM33 or the MMPs 1, 2, 3, 7, 9, 12, 14 and 15 and has been shown to inhibit ectodomain shedding of multiple EGF-type ligands including amphiregulin (29). As shown in Fig. 5a, CXCL12-mediated proliferative response observed for N15C6 cells was abrogated when the cells were grown in media supplemented with the ADAM10-specific inhibitor, INCB008765. Further studies were then pursued to determine whether ADAM10 was also required for CXCL12-mediated amphiregulin shedding. For these experiments, N15C6 cells were treated with 10 pM CXCL12 after pre-treatment with the solvent vehicle 0.05% DMSO or with one of three metalloprotease inhibitors: 10 μ M INCB008765, 2 μ M TAPI-1 or 5 μ M GM6001 (all dissolved in 0.05% DMSO). The results here show that N15C6 cells pre-treated with vehicle and stimulated with CXCL12 shed amphiregulin into the media, consistent with previously acquired data.

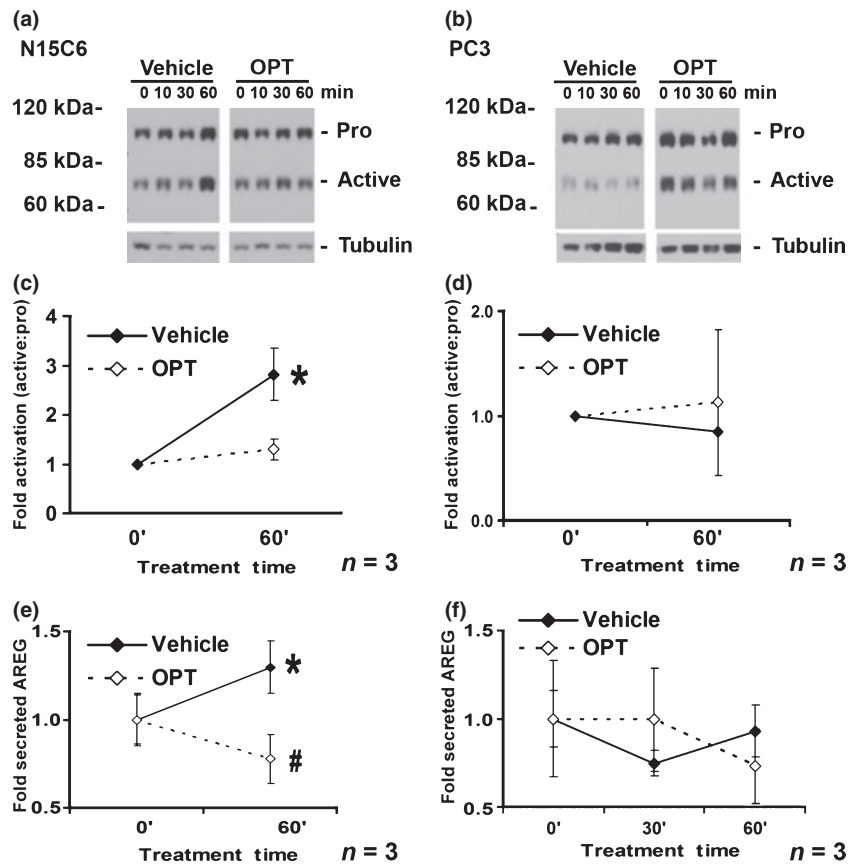


Figure 4. CXCL12-mediated ADAM activation and amphiregulin shedding are metalloprotease-dependent. (a, b) Immunoblot analysis demonstrating that N15C6 cells (a) pre-treated with the solvent vehicle, 0.1% methanol, then treated with 10 pM CXCL12 rapidly activated ADAM10, which was abrogated by pre-treatment with the pan-metalloprotease inhibitor, o-phenanthroline (OPT). In contrast, PC3 cells (b) pre-treated with the solvent vehicle, 0.1% methanol, then treated with 10 pM CXCL12 did not demonstrate ADAM10 activation. Pre-treatment with OPT did not dampen basal ADAM10 activation in these cells. (c, d) Densitometric analysis of replicate immunoblots similar to that shown in (a) and (b) N15C6 cells (c) treated with CXCL12 for 60 min demonstrated significant ($P < 0.05$, *) accumulation of the active form of ADAM10 to levels approximately 3-fold higher after pre-treatment with vehicle compared with OPT-treated cells. PC3 cells (d) treated with CXCL12 did not demonstrate differences in accumulation of the active form of ADAM10 after pre-treatment with either vehicle or OPT. ADAM10 activation is indicated as the ratio of active ADAM10/pro-form ADAM10 normalized to 1-fold at time 0. ADAM. The graph plots data were averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures. (e, f) N15C6 cells (e), but not PC3 cells (f) treated for 60 min with CXCL12 shed significantly ($P < 0.05$, indicated by *) higher levels of amphiregulin after pre-treatment with vehicle (0.1% BSA in PBS) compared with OPT pre-treated cells. Moreover, amphiregulin shedding was reduced to below basal levels in OPT pre-treated N15C6 cells ($P < 0.05$, indicated by #) but not in OPT pre-treated PC3 cells. Amphiregulin secretion is indicated as fold protein secreted compared with time 0 (which is normalized to 1-fold). The graphs plot data averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures.

However, amphiregulin shedding in response to CXCL12 was abrogated in N15C6 cells pre-treated with the selective ADAM10 inhibitor, INCB008765 (Fig. 5b). Interestingly, pre-treatment with the ADAM- and MMP-inhibitor TAPI-1, as well as the MMP inhibitor, GM6001, also prevented CXCL12-mediated amphiregulin shedding. Taken together, these experiments suggest that the CXCL12-mediated proliferative response in N15C6 cells was ADAM10-dependent. These studies also showed that CXCL12-mediated ADAM10 activation was required for amphiregulin shedding in these cells and suggested that MMP activation may be a component of this mechanism.

Src activation is required for CXCL12-mediated ADAM10 activation and amphiregulin shedding

CXCR4, the primary receptor for CXCL12, is a GPCR. GPCRs are known to activate Src, which, in turn, has been shown to activate other membrane-bound molecules through ADAM-dependent mechanisms. In particular, GPCR-coupled mechanisms have been shown to activate both ADAM10 and ADAM17 (19). To understand the mechanism through which CXCL12/CXCR4 interactions activate ADAM10, which, in turn, mediates amphiregulin shedding, the potential role of CXCL12/CXCR4-medi-

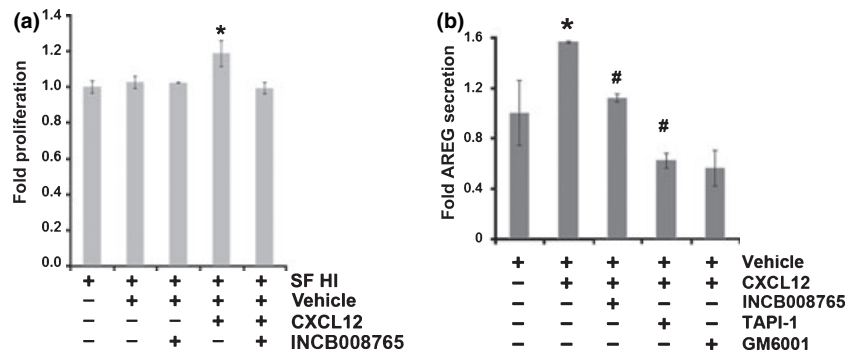


Figure 5. CXCL12-mediated amphiregulin shedding and cell proliferation are ADAM10-dependent. (a) N15C6 cells were grown for 96 h in serum-free (SF) media either non-supplemented or supplemented with vehicle, vehicle + 10 pM CXCL12 or vehicle + 10 pM CXCL12 + 5 μ M INCB008765 (as shown). Cell proliferation is indicated as the ratio of the total cell number under each supplemented condition (numerator) to non-supplemented cell population growth (SF, which is set = 1). CXCL12-treated cells proliferated to levels significantly above those achieved in either SF or SF + Vehicle ($P < 0.05$, indicated by *) except when grown in media supplemented with the ADAM10-specific inhibitor, INCB008765. Graph plot data are averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures. (b) N15C6 cells were pre-treated with vehicle or vehicle + 10 μ M INCB008765, 10 μ M TAPI-1 or 1 μ M GM6001 for 60 min, and then stimulated with 10 pM CXCL12 for 4 h. The media were collected and shed amphiregulin levels were determined using ELISA. Amphiregulin secretion is indicated as the ratio of the protein detected in media from cells supplemented with 10 pM CXCL12 to that from non-supplemented cells (which is normalized to 1-fold). N15C6 cells demonstrated significant ($P < 0.05$, indicated by *) secretion of amphiregulin in response to CXCL12, but this response was significantly diminished ($P < 0.05$, indicated by #) in cells pre-treated with INCB008765, TAPI-1 or GM6001. Error bars reflect deviations from the mean for replicate measures.

ated activation of Src was examined. For these experiments, N15C6 cells grown for 16 h in serum-free media were pre-treated for 60 min with either vehicle (DMSO) or vehicle + 10 μ M of Src family tyrosine kinase inhibitor, PP1, supplemented with 10 pM CXCL12 and grown for an additional 10, 30 or 60 min. Analysis of Src and ADAM10 activation by immunoblot analysis showed that N15C6 cells demonstrate significant and concomitant accumulation of both phosphorylated Src (pSrc Tyr418) and 60 kDa active form of ADAM10, in response to CXCL12 (Fig. 6a). However, pre-treatment with the Src family kinase inhibitor, PP1, prevented both Src phosphorylation and ADAM10 activation consequent to CXCL12 stimulation (Fig. 6a). Immunoprecipitation of Src protein from similarly treated N15C6 cells also demonstrated Src phosphorylation in response to CXCL12, which was abrogated by pre-treatment with PP1 (Fig. 6b,c). Notably, constitutive phosphorylation of Src within the activation loop, indicative of Src kinase catalytic activity (34,35), is almost undetectable using conventional Western blotting in N15C6 cells, but is clearly detectable and abundant in PC3 cells (Fig. 6b). Using ELISA-based assay for measuring CXCL12-mediated Src phosphorylation that quantified accumulation of pSrcY418, we showed that N15C6 cells significantly ($P < 0.05$) accumulated pSrc in response to CXCL12 and that this response was prevented by pre-treatment with Src family kinase inhibitor PP1, but not by the inactive analogue PP3 (Fig. 6d). Together, these assays demonstrate that N15C6 cells phosphorylate

Src-family kinases that activate ADAM10 in response to CXCL12 and that these responses can be abrogated by Src family kinase inhibitor, PP1.

CXCL12-treated N15C6 or PC3 cells were further examined to determine whether CXCL12-mediated Src activation was required for amphiregulin shedding. N15C6 or PC3 cells were cultured for 16 hrs incubation in serum-free media followed by pre-treatment with PP1 or vehicle for 60 min, then with CXCL12 for an additional 60 min. Sandwich ELISA assessment of conditioned media harvested from the cells demonstrated significant accumulation of amphiregulin in the media of N15C6 cells in response to CXCL12, which was abrogated by pre-treatment with Src family kinase inhibitor, PP1. Neither CXCL12 treatment nor PP1 pre-treatment affected amphiregulin shedding by PC3 cells (Fig. 6e). Moreover, pre-treatment of N15C6 cells with inactive PP1 analogue, PP3, failed to diminish amphiregulin shedding significantly in response to CXCL12 stimulation (Fig. 6f).

Taken together, the data described above suggest a mechanism whereby CXCL12/CXCR4 interactions activate Src, which subsequently activates ADAM10, which mediates amphiregulin cleavage and shedding and promotes a proliferative response in non-transformed N15C6 cells. Conversely, amphiregulin shedding, ADAM10 activation and Src phosphorylation occur constitutively through mechanisms that are unresponsive to chemical inhibitors in highly transformed PC3 cells.

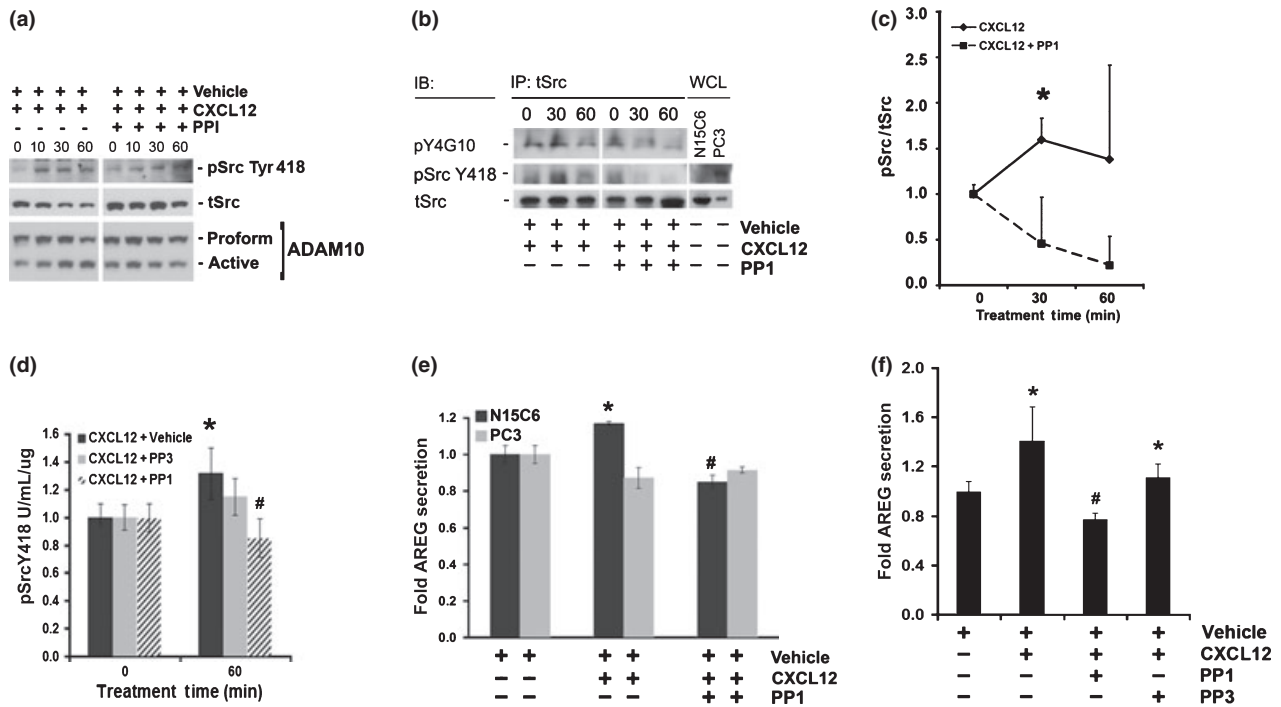


Figure 6. CXCL12-mediated amphiregulin shedding is Src-dependent. (a) Immunoblot analysis demonstrating that N15C6 cells pre-treated with vehicle (0.025% DMSO) for 60 min, then treated with 10 μ M CXCL12 rapidly accumulate phosphorylated Src (pSrc Tyr 418) as well as the active form of ADAM10, whereas cells pre-treated with 10 μ M of the Src family kinase inhibitor, PP1, neither accumulate pSrc Tyr 418 nor the active form of ADAM10. (b) Cell lysates prepared from N15C6 cells treated as described in (a) were subjected to immunoprecipitation (IP) using an antibody against total Src (tSrc). Immunoprecipitated proteins were electrophoresed and immunoblotted against antibodies that recognize phosphotyrosine residues (pY4G10), pSrcY418, or tSrc. Whole-cell lysates (WCL) from 100 μ g untreated N15C6 or 25 μ g untreated PC3 cells are included as negative and positive controls respectively. The immunoblot shown is representative of replicate experiments. (c) Densitometric analysis of replicate immunoblots similar to that shown (b) demonstrates significant and concomitant accumulation of phosphorylated Src (pSrc Tyr418) ($P < 0.05$, indicated by *) in N15C6 cells pre-treated for 60 min with vehicle, then treated with 10 μ M CXCL12 for 1 h. Cells pre-treated with PP1 failed to accumulate pSrc in response to CXCL12. Src activation is indicated as the ratio of pSrc/tSrc normalized to 1-fold at time 0. Graph plot data have been averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures. (d) Assessment and quantification of CXCL12-mediated Src activation in N15C6 using sandwich ELISA to pSrcY418. One million N15C6 cells grown in serum-free media for 16 h were pre-treated with vehicle (0.025% DMSO) or vehicle + 5 μ M PP1 or +5 μ M PP3 for 60 min, followed by stimulation with 10 μ M CXCL12 for 60 min. pSrcY418 accumulated to significantly higher levels ($P < 0.05$, indicated by *) in response to CXCL12 in vehicle-pre-treated (black bars) and PP3-pre-treated (grey bars), but not in PP1-pre-treated (striped bars), cells. Graph plot data are averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures. (e) Conditioned media harvested from N15C6 cells (black bars) or PC3 cells (grey bars) plated at 300 000 cells each in triplicate was evaluated after 16 h incubation in serum-free media followed by pre-treatment with PP1 or vehicle for 60 min, and then with CXCL12 (10 μ M for N15C6 cells, 100 μ M for PC3 cells) for an additional 60 min. Sandwich ELISA detected quantified significant ($P < 0.05$, indicated by *) accumulation of amphiregulin in the media of N15C6 cells in response to CXCL12, which was significantly ($P < 0.05$, indicated by #) abrogated by pre-treatment with the Src family kinase inhibitor, PP1. Neither CXCL12 treatment nor PP1 pre-treatment affected amphiregulin shedding by PC3 cells. The graph plots data are averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures. (f) Conditioned media harvested from N15C6 cells plated at 300 000 cells each in triplicate was evaluated after 16 h incubation in serum-free media followed by pre-treatment with vehicle, PP1 or PP3 for 60 min, and then with 10 μ M CXCL12 for an additional 60 min. Sandwich ELISA detected significant ($P < 0.05$, indicated by *) accumulation of amphiregulin in response to CXCL12 in vehicle- and PP3-, but not in PP1-, pre-treated cells, which was significantly ($P < 0.05$, indicated by #) abrogated by pre-treatment with the Src family kinase inhibitor, PP1. Neither CXCL12 treatment nor PP1 pre-treatment affected amphiregulin shedding by PC3 cells. Graph plot data are averaged over replicate experiments as indicated. Error bars reflect deviations from the mean for replicate measures.

CXCL12-mediated proliferative response requires EGFR transactivation

The observations reported in the present study, that CXCL12/CXCR4 interactions stimulate amphiregulin shedding through a Src- and ADAM10-dependent mecha-

nism, has suggested that this mechanism might promote transactivation of EGFR and that this transactivation might in turn, mediate CXCL12/CXCR4-stimulated proliferative responses. To investigate this, N15C6 cells were monitored for phosphorylation of EGFR subsequent to treatment with CXCL12. Treatment of these cells with

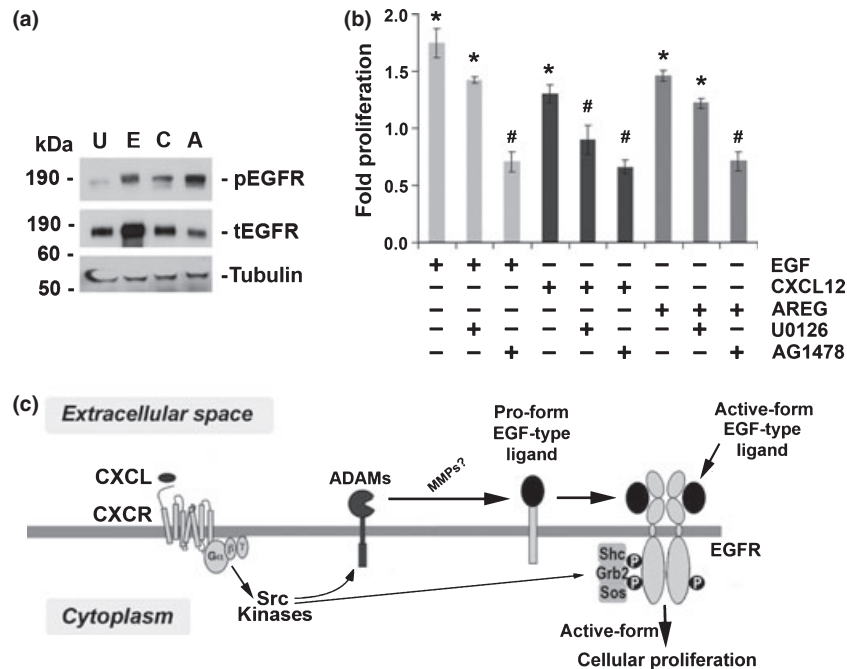


Figure 7. CXCL12-mediated proliferative responses require EGFR transactivation. (a) N15C6 cells were treated with vehicle (U), 10 ng/ml EGF (E), 10 pM CXCL12 (C) or 100 pM amphiregulin (A) for 60 min, protein lysates purified, electrophoresed and immunoblotted against antibodies specific for phospho-EGFR (Y992), total EGFR or tubulin. Robust phosphorylation of EGFR is evident for cells treated with EGF, CXCL12 or amphiregulin. (b) N15C6 cells were grown for 96 h in SF media supplemented with 10 ng/ml EGF, 10 pM CXCL12 or 100 pM amphiregulin containing vehicle (0.01% DMSO) or vehicle + 1 μM U0126 or +250 nM AG1478. Proliferation at levels significantly above those achieved in SF media alone (normalized to 1-fold, not shown) was evident for cells treated with CXCL12, amphiregulin or EGF (*, $P < 0.05$). Cells co-treated with the EGFR inhibitor, AG1478, failed to proliferate in response (#, $P < 0.05$) to EGF, CXCL12 or amphiregulin. However, co-treatment with the MEK inhibitor, U0126, suppressed only the CXCL12-mediated (#, $P < 0.05$), but not EGF- or amphiregulin-mediated, proliferative response. (c) This schematic drawing demonstrates potential mechanism(s) for CXCL12-mediated cellular proliferation. Consistent with the data provided in this study, CXCL12/CXCR4-mediated Src activation and subsequent ADAM10 (and, possibly, MMP) activation are required for amphiregulin ectodomain shedding. The same mechanisms may also activate EGFR in a ligand-dependent (amphiregulin shedding) or ligand-independent (Src activation) manner. In either scenario, EGFR transactivation is required for CXCL12-mediated proliferation of non-transformed N15C6 prostate epithelial cells. Modified from Prenzel *et al.*, *Endocr.Relat. Cancer* 8: 11–31, 2001. Society for Endocrinology (2009). Reproduced by permission.

10 ng/ml EGF, 10 pM CXCL12 or 100 pM amphiregulin for 60 min resulted in abundant phosphorylation of the Y992 residue of EGFR (Fig. 7a). Prolonged treatment of N15C6 cells with 10 ng/ml EGF, 10 pM CXCL12 or 100 pM amphiregulin over 96 h promoted proliferation of these cells to levels 1.5- to 2.0-fold higher than achieved in serum-free media (Fig. 7b). However, proliferation of N15C6 cells grown for 96 h in serum-free media supplemented with 250 nM of the EGFR tyrosine kinase inhibitor, AG1478, and either 10 ng/ml EGF, 10 pM CXCL12, or 100 pM amphiregulin was significantly suppressed. These results show that, like EGF- and amphiregulin-, CXCL12-mediated cell proliferation required activation of EGFR. N15C6 cells grown for 96 h in serum-free media supplemented with 1 μM of the MEK/ERK inhibitor, U0126, and either EGF or amphiregulin, but not CXCL12, proliferated to levels significantly above those observed for population growth in serum-free media (Fig. 7b). Together, these results show that CXCL12, like

EGF and amphiregulin, must transactivate EGFR ligands to stimulate cell proliferation. These results also confirm our earlier findings that CXCL12-stimulated cell proliferation is MEK/ERK-dependent in N15C6 cells (7).

Discussion

Previous studies have shown that stimulation of the CXCL12/CXCR4 axis promotes intracellular signalling events through the Ras/MEK/ERK and P13K/Akt pathways that culminate in diverse cellular responses including proliferation (7). However, it has remained unclear whether the CXCL12/CXCR4 axis stimulated cell proliferation independently or through transactivation of other receptors, notably EGFR, that similarly activate these pathways (14–16). Therefore, the present study was intended to determine whether the CXCL12/CXCR4 axis mediated cell proliferation in an EGFR-dependent or -independent manner.

Initial studies examining CXCL12-mediated ectodomain shedding of EGFR ligands as a potential mechanism for CXCL12/CXCR4-mediated EGFR transactivation established that prostate stromal fibroblasts, as well as the majority of non-transformed and transformed prostate epithelial cells tested, constitutively shed the amphiregulin ectodomain. However, none of these cells shed significant levels of other EGFR ligands. Ectodomain shedding of amphiregulin comprises an important regulatory mechanism because it generates functional soluble molecules through multiple mechanisms, to provide important proliferative and survival advantages (17,33–39). Autocrine secretion of amphiregulin by pre-malignant and malignant prostate epithelial cells has been reported previously (40). Topping *et al.* (41) detected significantly higher levels of amphiregulin ($P < 0.05$) in prostate tissue obtained from patients with BPH treated for 3 months with finasteride, which inhibits conversion of testosterone into its active form dihydrotestosterone, compared with similar tissues from placebo-treated patients. This group also reported that castrated mice bearing CWR22 human prostate tumour xenografts demonstrated significantly higher levels of amphiregulin transcript and secreted protein compared to non-castrated mice (42). This finding is consistent with that reported here, as prostate epithelial cells (N15C6, BPH-1, PC3 and DU145) lacking expression of the androgen receptor, secreted the highest detectable levels of amphiregulin, whereas cells that express the androgen receptor (LNCaP and 22Rv1) secreted very low levels of amphiregulin. Although it is unlikely that endogenous amphiregulin shedding is strictly related to androgen receptor status in non-transformed nor transformed prostate epithelial cells, these data do suggest that expression of EGF-type ligands may provide an autocrine survival mechanism that enables prostate cells to persist and multiply in the absence of functional androgen receptor.

Ectodomain shedding of EGF-type ligands, including amphiregulin, EGF, epiregulin, HB-EGF and TGF- α , is accomplished through cleavage by multiple ADAMs including ADAM9, 10, 12, 15, 17 and 19 (18,19,32,43,44). Our studies also detected active forms of both ADAM10 and ADAM17 using immunoblot analysis on N15C6 and PC3 cells grown in serum-free media. Moreover, CXCL12-stimulated amphiregulin shedding was shown to require metalloprotease activity, as inhibition of this activity using the pan-metalloprotease inhibitor OPT, the ADAM- and MMP-inhibitor TAPI-1 or the ADAM10-specific inhibitor INCB008765, prevented CXCL12-mediated amphiregulin shedding in N15C6 cells. The observation that TAPI-1 inhibited CXCL12-stimulated amphiregulin secretion was consistent with a general role for ADAMs and/or MMPs in this process. Twin observations that CXCL12 stimulated ADAM10 activation over basal levels and that pre-treatment

with ADAM10-specific inhibitor, INCB008765, was sufficient to abrogate both CXCL12-mediated amphiregulin shedding and proliferation in these cells and indicated that CXCL12-mediated proliferation in N15C6 cells was predominantly ADAM10-dependent. This finding is consistent with that observed for another member of the CXC-type chemokine family, CXCL8 (IL-8), which has been shown to mediate amphiregulin shedding through activation of ADAM10 (19,20). The same study also demonstrated that knockdown of ADAM10, but not that of ADAM12 or ADAM17, suppressed CXCL8-induced amphiregulin shedding and CXCL8-mediated EGFR phosphorylation in KATOIII gastric cancer cells (20). However, the additional finding reported in the present study that MMP inhibitor, GM6001, also inhibits CXCL12-stimulated amphiregulin shedding implies that MMPs may play a role in this process too. Although not definitive and requiring a more exhaustive evaluation of MMP family members, these studies provide evidence that amphiregulin shedding may be modulated by CXC-type chemokines, including CXCL8 and CXCL12, in human tumour cells, through activation of ADAM10 and potentially through the further activation of selected MMPs. These data are consistent with a model of basal-level autocrine amphiregulin shedding mediated by constitutively activated ADAM10 and ADAM17 in non-transformed and transformed prostate epithelial cells. It should be noted, however, that pre-treatment with OPT did not diminish basal- nor CXCL12-stimulated ADAM10 activation nor amphiregulin secretion in transformed PC3 prostate epithelial cells. Taken together, these data suggest that activation of the metalloprotease activity of ADAM10 is required for CXCL12-mediated amphiregulin shedding and proliferation in non-transformed N15C6, but not in transformed PC3, prostate epithelial cells.

As noted above, the major cognate receptor for CXCL12 is CXCR4, which is G-protein-coupled. Upon activation, the G-protein heterotrimeric protein associated with such GPCRs dissociates into the $G\alpha$ and $G\beta\gamma$ heterodimer. $G\beta\gamma$ can activate several downstream effectors including Src family kinases and PKC (12,13). These kinases can in turn, activate molecules with metalloprotease activity, resulting in HB-EGF and amphiregulin shedding (14,45). In particular, GPCR-coupled mechanisms have been shown to activate both ADAM10 and ADAM17 (19). The results reported in this study show that inhibition of CXCL12-mediated Src family kinase activation prohibited CXCL12-mediated ADAM10 activation, amphiregulin secretion and cell proliferation. Furthermore, inhibition of Src family kinase activation also reduced amphiregulin shedding below basal levels in N15C6 cells. In contrast, inhibition of Src family kinase activity had no effect on basal amphiregulin shedding in PC3 cells. These data suggest that both basal and

CXCL12-mediated amphiregulin shedding are Src family kinase-dependent in non-transformed N15C6 cells, but not in transformed, highly aggressive PC3 cells. Moreover, these data suggest that Src family kinase activation is required for ADAM10 activation as part of a mechanism that promotes CXCL12-mediated amphiregulin shedding and cell proliferation in N15C6 cells.

The final studies presented here show that EGFR is phosphorylated equivalently in response to treatment with EGF, amphiregulin or CXCL12. Moreover, cell proliferation stimulated by these molecules is equivalently suppressed upon treatment of the cells with the EGFR inhibitor, AG1478. The observation that inhibition of signalling along the MEK/ERK pathway reduced cell proliferation significantly for CXCL12-treated cells, but not for EGF- or amphiregulin-treated cells corresponds to earlier studies from our laboratory demonstrating that CXCL12-mediated proliferative responses are MEK/ERK-dependent in N15C6 cells (7). Taken together, these experiments demonstrate not only that EGFR is transactivated through the CXCL12/CXCR4 axis but also that this transactivation is required for CXCL12/CXCR4-mediated proliferative response. Moreover, the observation that Src-dependent ADAM10 activation is required for both CXCL12-mediated amphiregulin shedding and cell proliferation in N15C6 cells is consistent with ligand-dependent transactivation of EGFR in these cells (46).

In summary, the data reported in this study and summarized schematically in Fig. 7c (modified with permission from 14 and 45) identify CXCL12 as a novel inducer of amphiregulin shedding and show that both basal and CXCL12-mediated amphiregulin shedding are predominantly ADAM10- and Src family kinase-dependent in non-transformed N15C6 cells. Moreover, activation of these molecules is coupled to CXCL12-mediated ligand-dependent EGFR transactivation and proliferation of these cells. In contrast, amphiregulin shedding is not amplified subsequent to stimulation with exogenous CXCL12 and is not reduced subsequent to metalloprotease- or Src family kinase inhibition, in highly aggressive PC3 prostate cancer cells. These findings indicate that, although autocrine and CXCL12-inducible secretions of amphiregulin are regulated predominantly through ADAM- and Src family kinase-mediated mechanisms in non-transformed prostate epithelial cells, these same mechanisms may be dysfunctional in highly transformed prostate cancer cells. The importance of these findings is that they show that non-transformed and transformed prostate epithelial cells may utilize different mechanisms to utilize the EGFR axis to promote cell proliferation. Further work defining the affects of other components of the cell phenotype, for example, androgen receptor status, competitive responses to other cytokines and chemokines, expression profiles of MMP family members, and how

these interact with the CXCL12/CXCR4 axis to govern amphiregulin shedding in non-transformed and transformed prostate epithelial cells, are clearly warranted.

Acknowledgements

This study was supported by National Institutes of Health awards from the George M. O'Brien Center for Urologic Research at the University of Michigan 1 P50 DK065313 (J.A.M.), NIH/NIDDK 1 R01 DK081841 (J.A.M.), The University of Michigan Prostate SPORE 5P50 CA068568 (J.A.M.), The American Urological Association Foundation (S.K.), and funds awarded by Domino's Pizza, L.L.C. (J.A.M.).

References

- 1 Rot A, von Andrian UH (2004) Chemokines in innate and adaptive host defense: basic chemokine grammar for immune cells. *Annu. Rev. Immunol.* **22**, 891–928.
- 2 Baggiolini M, Dewald B, Moser B (1997) Human chemokines: an update. *Annu. Rev. Immunol.* **15**, 675–705.
- 3 Balkwill F (2004a) Cancer and the chemokine network. *Nat. Rev. Cancer* **4**, 540–550.
- 4 Burger JA, Kipps TJ (2006a) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* **107**, 1761–1767. Epub 2005 Nov 3.
- 5 Balkwill F (2004b) The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin. Cancer Biol.* **14**, 171–179.
- 6 Begley L, Monteleon C, Shah RB, Macdonald JW, Macoska JA (2005) CXCL12 overexpression and secretion by aging fibroblasts enhance human prostate epithelial proliferation in vitro. *Aging Cell* **4**, 291–298.
- 7 Begley LA, MacDonald JW, Day ML, Macoska JA (2007) CXCL12 activates a robust transcriptional response in human prostate epithelial cells. *J. Biol. Chem.* **282**, 26767–26774. Epub 2007 Jul 12.
- 8 Wang J, Wang J, Sun Y, Song W, Nor JE, Wang CY *et al.* (2005) Diverse signaling pathways through the SDF-1/CXCR4 chemokine axis in prostate cancer cell lines leads to altered patterns of cytokine secretion and angiogenesis. *Cell. Signal.* **17**, 1578–1592.
- 9 Zhao D, Li XP, Gao M, Zhao C, Wang JL, Wei LH (2006) Stromal cell-derived factor 1alpha stimulates human endometrial carcinoma cell growth through the activation of both extracellular signal-regulated kinase 1/2 and Akt. *Gynecol. Oncol.* **103**, 932–937. Epub 2006 Aug 1.
- 10 Marchese A, Benovic JL (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J. Biol. Chem.* **276**, 45509–45512.
- 11 Holland JD, Kochetkova M, Akekawatchai C, Dottore M, Lopez A, McColl SR (2006) Differential functional activation of chemokine receptor CXCR4 is mediated by G proteins in breast cancer cells. *Cancer Res.* **66**, 4117–4124. PMID: 16618732.
- 12 Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. *Nat. Rev. Cancer* **7**, 79–94.
- 13 Ye RD (2001) Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J. Leukoc. Biol.* **70**, 839–848.
- 14 Prenzel N, Fischer OM, 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**, 11–31.
- 15 Hart S, Fischer OM, Prenzel N, Zwick-Wallasch E, Schneider M, Hennighausen L *et al.* (2005) GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR-independent pathways. *Biol. Chem.* **386**, 845–855.
 - 16 Henson ES, Gibson SB (2006) Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. *Cell. Signal.* **18**, 2089–2097. Epub 2006 May 24.
 - 17 Blobel CP (2005) ADAMs: key components in EGFR signaling and development. *Nat. Rev. Mol. Cell Biol.* **6**, 32–43.
 - 18 Schäfer B, Marg B, Gschwind A, Ullrich A (2004) Distinct ADAM metalloproteinases regulate G protein-coupled receptor-induced cell proliferation and survival. *J. Biol. Chem.* **279**, 47929–47938.
 - 19 Ohtsu H, Dempsey PJ, Eguchi S (2006) ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am. J. Physiol. Cell Physiol.* **291**, C1–C10.
 - 20 Tanida S, Joh T, Itoh K, Kataoka H, Sasaki M, Ohara H *et al.* (2004) The mechanism of cleavage of EGFR ligands induced by inflammatory cytokines in gastric cancer cells. *Gastroenterology* **127**, 559–569.
 - 21 Schwab TS, Stewart T, Lehr J, Pienta KJ, Rhim JS, Macoska JA (2000) Phenotypic characterization of immortalized normal and primary tumor-derived human prostate epithelial cell cultures. *Prostate* **44**, 164–171.
 - 22 Macoska JA, Paris P, Collins C, Andaya A, Beheshti B, Chaib H *et al.* (2004) Evolution of 8p loss in transformed human prostate epithelial cells. *Cancer Genet. Cytogenet.* **154**, 36–43.
 - 23 Begley L, Keeney D, Beheshti B, Squire JA, Kant R, Chaib H *et al.* (2006) Concordant copy number and transcriptional activity of genes mapping to derivative chromosomes 8 during cellular immortalization in vitro. *Genes Chromosomes Cancer* **45**, 136–146.
 - 24 Hayward SW, Dahiya R, Cunha GR, Bartek J, Deshpande N, Narayan P (1995) Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell. Dev. Biol. Anim.* **31**, 14–24.
 - 25 Horoszewicz JS, Leong SS, Chu TM, Wajzman ZL, Friedman M, Papsidero L *et al.* (1980) The LNCaP cell line – a new model for studies on human prostatic carcinoma. *Prog. Clin. Biol. Res.* **37**, 115–132.
 - 26 Sramkoski RM, Pretlow TG II, Giaconia JM, Pretlow TP, Schwartz S, Sy MS *et al.* (1999) A new human prostate carcinoma cell line, 22Rv1. *In Vitro Cell. Dev. Biol. Anim.* **35**, 403–409.
 - 27 Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW (1979) Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest. Urol.* **17**, 16–23.
 - 28 Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF (1978) Isolation of a human prostate carcinoma cell line (DU 145). *Int. J. Cancer* **21**, 274–281.
 - 29 Zhou BB, Peyton M, He B, Liu C, Girard L, Caudler E *et al.* (2006) Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. *Cancer Cell* **10**, 39–50.
 - 30 Tørring N, Jørgensen PE, Sørensen BS, Nexø E (2000) Increased expression of heparin binding EGF (HB-EGF), amphiregulin, TGF alpha and epiregulin in androgen-independent prostate cancer cell lines. *Anticancer Res.* **20**, 91–95.
 - 31 Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J *et al.* (2004) Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J. Cell Biol.* **164**, 769–779.
 - 32 Maretzky T, Scholz F, Köten B, Proksch E, Saftig P, Reiss K (2008) ADAM10-mediated E-cadherin release is regulated by proinflammatory cytokines and modulates keratinocyte cohesion in eczematous dermatitis. *J. Invest. Dermatol.* **128**, 1737–1746. Epub 2008 Jan 17.
 - 33 Sanderson MP, Dempsey PJ, Dunbar AJ (2006) Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. *Growth Factors* **24**, 121–136.
 - 34 Roskoski R Jr (2004) The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem. Biophys. Res. Commun.* **319**, 1–11.
 - 35 Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13298–13302. Epub 2003 Oct 30.
 - 36 Singh AB, Harris RC (2005) Autocrine, paracrine and juxtacrine signaling by EGFR ligands. *Cell. Signal.* **17**, 1183–1193.
 - 37 Willmarth NE, Ethier SP (2006) Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. *J. Biol. Chem.* **281**, 37728–37737. Epub 2006 Oct 10.
 - 38 Castillo J, Erroba E, Perugorria MJ, Santamaria M, Lee DC, Prieto J *et al.* (2006) Amphiregulin contributes to the transformed phenotype of human hepatocellular carcinoma cells. *Cancer Res.* **66**, 6129–6138.
 - 39 Desbois-Mouthon C, Cacheux W, Blivet-Van Eggelpoël MJ, Barbu V, Fartoux L, Poupon R *et al.* (2006) Impact of IGF-1R/EGFR cross-talks on hepatoma cell sensitivity to gefitinib. *Int. J. Cancer* **119**, 2557–2566.
 - 40 Bostwick DG, Qian J, Maihle NJ (2004) Amphiregulin expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 93 cases. *Prostate* **58**, 164–168.
 - 41 Tørring N, Møller-Ernst Jensen K, Lund L, Nielsen JE, Djurhuus JC, Poulsen SS *et al.* (2002) Possible autocrine loop of the epidermal growth factor system in patients with benign prostatic hyperplasia treated with finasteride: a placebo-controlled randomized study. *BJU Int.* **89**, 583–590.
 - 42 Tørring N, Hansen FD, Sørensen BS, Ørntoft TF, Nexø E (2005) Increase in amphiregulin and epiregulin in prostate cancer xenograft after androgen deprivation-impact of specific HER1 inhibition. *Prostate* **64**, 1–8.
 - 43 Horiuchi K, Le Gall S, Schulte M, Yamaguchi T, Reiss K, Murphy G *et al.* (2007) Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx. *Mol. Biol. Cell* **18**, 176–188.
 - 44 Fridman JS, Caulder E, Hansbury M, Liu X, Yang G, Wang Q *et al.* (2007) Selective inhibition of ADAM metalloproteinases as a novel approach for modulating ErbB pathways in cancer. *Clin. Cancer Res.* **13**, 1892–1902.
 - 45 Kansra S, Stoll SW, Johnson JL, Elder JT (2005) Src family kinase inhibitors block amphiregulin-mediated autocrine ErbB signaling in normal human keratinocytes. *Mol. Pharmacol.* **67**, 1145–1157. Epub 2004 Dec 22.
 - 46 Bhola NE, Grandis JR (2008) Crosstalk between G-protein coupled receptors and epidermal growth factor receptor in cancer. *Front Biosci.* **13**, 1857–1865.