Cancer and Metastasis Reviews **24:** 47–69, 2005.

Biological significance of c-*erb*B family oncogenes in head and neck cancer

Susanne J. Rogers[1,2], Kevin J. Harrington[2,3], Peter Rhys-Evans[2], Pornchai O-Charoenrat[1,2,†] and Suzanne A. Eccles[1,*]

[1]*Tumour Biology and Metastasis Team, CRUK Centre for Cancer Therapeutics, McElwain Laboratories, Institute for Cancer Research, Cotswold Road, Sutton, Surrey SM2 5NG;* [2]*Head and Neck Unit, Royal Marsden Hospital, Fulham Rd, London SW3 6JJ;* [3]*Targeted Therapy Team, Section of Cell and Molecular Biology, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK*

*Key words: c-erb*B oncogenes, squamous cell carcinoma, head and neck cancer, metastasis, invasion, angiogenesis

Abstract

Squamous cell carcinoma of the head and neck (SCCHN) tends to run an aggressive course and the prognosis has remained virtually unchanged in recent decades. The development of novel therapeutic strategies to improve patient outcome centres on the biology of the disease, namely the pivotal c-*erb*B family of growth factor receptors. *c-erbB1* (or epidermal growth factor receptor, EGFR), is key to the pathogenesis of SCCHN and plays a central role in a complex network of downstream integrated signalling pathways. EGFR overexpression, detected in up to 90% of SCCHN, correlates with an increased risk of locoregional tumour relapse following primary therapy and relative resistance to treatment. The biological sequelae of erbB receptor activation are not simply cell proliferation, but also inhibition of apoptosis, enhanced migration, invasion, angiogenesis and metastasis: the 'hallmarks of cancer' [1]. As EGFR overexpression is associated with a poor clinical outcome in SCCHN, this receptor is attractive as a therapeutic target and the successful development of targeted therapies represents a paradigm shift in the medical approach to head and neck cancer. However, the extensive cross talk between signalling pathways, the multiple molecular aberrations and genetic plasticity in SCCHN all contribute to inherent and acquired resistance to both conventional and novel therapies. Understanding the cancer cell biology, in particular the significance of co-expression of c-erbB (and other) receptors, and the cell survival stimuli from (for example) activation of the phosphoinositide 3-kinase (PI3-kinase) cascade is fundamental to overcome current limitations in biologically targeted therapies.

1. Cancers of the head and neck

Squamous cell carcinoma of the head and neck (SCCHN) represents 90% of head and neck cancers. While the management of SCCHN has improved, there is no evidence to suggest that therapeutic advances have resulted in better outcome; five year survival is of the order of 50% overall (and 30–40% for advanced disease Stages III–IV), and there has been an increase in presentation of distant metastases. Clearly, a more sophisticated understanding of the pathogenesis of these tumours is needed to provide a framework for predicting outcome and for developing novel therapies. In some extreme cases metastases arise where the primary tumour cannot be detected (Figure 1) but the determinants of such aggressive behaviour are not understood. The ability to produce a molecular profile of a tumour which sheds light on its malignant potential would permit the individualisation and intensification of primary therapy where indicated. In addition, identification of key molecules involved in malignant progression will offer new targets for therapy.

[†]Present address: Division of Head, Neck & Breast Surgery, Department of Surgery, Siriraj Hospital Medical School, Bangkok 10700, Thailand.
[*]Corresponding author. E-mail: Sue.Eccles@icr.ac.uk

2. Expression of c-erbB receptors and ligands during SCCHN progression

C-*erb*B receptors of the transmembrane type I receptor tyrosine kinase family have four members: the epidermal growth factor receptor EGFR, c-*erb*B-2/*neu*/HER-2, c-*erb*B-3/HER-3 and c-*erb*B-4/HER-4. They consist of a large glycosylated extracellular ligand-binding domain, a hydrophobic trans-membrane component and an intracellular domain with tyrosine kinase activity. SCCHN is probably the best example of EGFR-driven oncogenesis as this is the pre-eminent signalling pathway responsible for the malignant features of the disease and overexpression has been shown to correlate with poor survival [2,3]. Their biology and role in tumour progression has recently been covered in several reviews [4–6]. Currently 12 major ligands with a shared EGF-like motif and affinity for the family of c-*erb*B receptors are known. The consequences of receptor dimerisation and activation (recently reviewed [7]) and subsequent intracellular signalling provide mechanistic explanations for much of the malignant behaviour of SCCHN [8].

2.1.1. Epidermal growth factor receptor/c-erbB1 (EGFR). EGFR is a 170 kDa protein and the archetypal founding member of this important receptor family [6]. Homology with v-*erb*B, the avian erythroblastosis viral gene product, led to recognition of EGFR's oncogenic properties and the human orthologue was cloned in 1984 [9]. EGFR is expressed at low levels on the surface of most normal cells except those of haematopoietic origin. The signalling pathway is normally tightly controlled and responsible for regulating physiological cellular processes such as epithelial tissue development and response to injury. EGFR is implicated in the development of various malignancies, being overexpressed in 30% of human solid tumours and up to 90% of SCCHN [2]. In contrast to certain tumour types where EGFR gene amplification or mutation is implicated, overexpression of the receptor, without gene amplification, appears to drive SCCHN.

Elevated levels of EGFR mRNA and protein, and of TGFα are present in normal mucosa several centimetres from a malignant lesion, [10] and TGFα upregulation is also detectable in pre-invasive lesions and mild dysplasia, [11], consistent with the theory of 'field cancerisation' due to exposure to environmental chemical carcinogens. Upregulation of EGFR is a significant early event in the progression from pre-invasive mucosal dysplasia to invasive SCCHN and is most marked in lesions displaying greater dysplasia [12]. The signal transcription and transduction protein 3 (STAT3) has been detected in areas of oral mucosa 'field change', alongside upregulated EGFR, implying an early role for STAT3 in SCCHN [13].

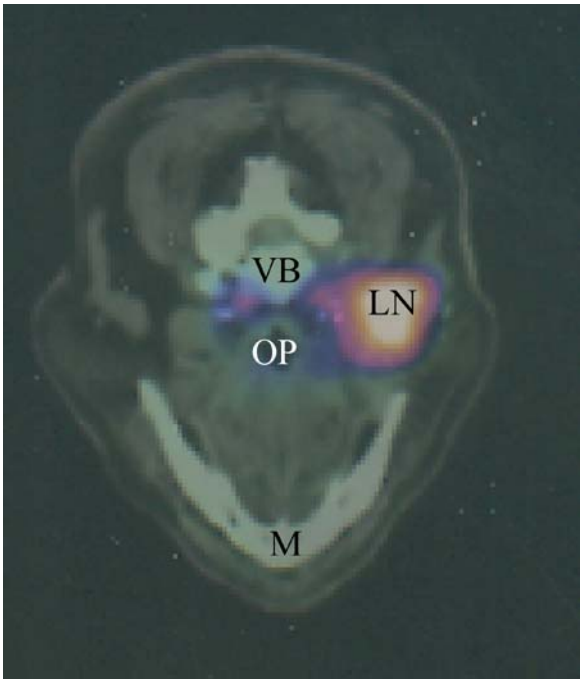

Figure 1. 18-Fluorodeoxyglucose Positron emission tomography (18F-FDG PET) scan fused with a diagnostic computerised tomography (CT) scan in a patient with squamous cell carcinoma of the head and neck. Intense 18F-FDG tracer uptake is seen in a lymph node (LN) in the left side of the neck. Reference structures include the oropharynx (OP), a vertebral body (VB) and the mandible (M). Despite the presence of this large metastatic tumour, extensive investigation failed to reveal a primary source in the mucosa of the upper aerodigestive tract.

Several studies have shown links between EGFR overexpression and SCCHN oncogenesis and progression [14–16]. In an experimental SCCHN xenograft model where highly metastatic sublines were isolated by *in vivo* selection from nodal metastases, EGFR was one of only 33 differentially expressed genes, showing a 2-fold upregulation [17]. However, not all studies show a positive correlation and in a recent small study of 23 patients with cancers of the tongue, neither EGFR nor c-*erb*B-2 expression was associated with lymph node status, invasion, recurrence or survival [18].

2.1.2. EGFR vIII mutant. Whereas the function of the extracellular domain (ECD) was previously thought to be solely ligand-binding, it has recently been discovered that EGFR can form homodimers in the absence of ligand and that the ECD can negatively regulate the intracellular tyrosine kinase in a ligand-independent fashion [19]. EGFRvIII is the most common of seven known variants of EGFR. Deletion of exons 2–7 of the EGFR gene results in a truncated extracellular domain and constitutive activation of the intracellular tyrosine kinase, which continuously triggers multiple downstream phosphorylation cascades. EGFRvIII can associate with and activate wild-type EGFR in the absence of ligand, but many anti-EGFR monoclonal antibodies are unable to bind to the aberrant extracellular domain to inhibit ligand-induced receptor activation. This particular mutation is common in tumour types such as glioblastoma multiforme and non-small cell lung cancer, but is found infrequently in head and neck cancer.

2.2. c-erbB2, c-erbB3 and c-erbB4

The c-erbB-2 receptor is a 185 kDa receptor-like phosphoglycoprotein but so far, no exogenous ligands have been identified which bind directly to it. When highly overexpressed it may spontaneously dimerise and autoactivate, but it is more frequently activated by heterodimerisation with other erbB receptors. Experimental studies suggest that erbB-2 serves as a favoured binding partner that that co-operates with other c-*erb*B receptor family members to enhance downstream signalling [20]. The contribution of erbB-2 expression to the pathogenesis of SCCHN is less well defined in comparison to other tumour types such as breast and ovarian cancer. However, erbB-2:erbB-3 heterodimers are potent inducers of the PI3-kinase pathway [21] as erbB-3 can bind directly to the PI3-kinase p85 subunit. Increasing expression of erbB-2 has been shown in parallel with acquisition of a more malignant phenotype in a series of oral carcinomas, which may imply a role in progression [16,22].

The erbB-3 protein distribution in tissues is distinctly different from that of EGFR and erbB-2. erbB-3 does not have an intrinsic tyrosine kinase activity but it can be transphosphorylated by both the EGFR and erbB-2. erbB-3 over-expression (but not amplification) has been found in SCCHN cell lines and in some cases related to malignant potential. There has been comparatively little investigation of erbB-3 and erbB-4 in clinical SCCHN, probably due to the low detection (∼10%) in immuno-histochemical surveys of clinical specimens [23]. However, in a study of developing verrucous carcinoma of the oral mucosa, erbB-3 expression increased with immunohistochemical staining for proliferating nuclear cell antigen (PCNA), a marker of cellular proliferation, suggesting a contribution to malignant transformation [24]. Xia et al. have indicated that expression of all four receptors is associated with shortened survival in patients with oral SCC, with the combination of EGFR, erbB-2 and erbB-3 (but not erbB-4) giving the greatest prognostic information [25].

We found that expression of erbB-4 *in vitro* tends to be higher in newly-derived rather than in well-established SCCHN cell lines [26] suggesting that it may be lost during continuous culture, and may have a more significant biological role than previously thought. Nevertheless, our clinical observations concurred with Xia in that expression of erbB-4 (unlike that of EGFR, erbB-2 and erbB-3) did not correlate with invasion, angiogenesis, metastasis or other aspects of tumour progression [27]. However, since co-expression of all receptors is common in SCCHN, it is likely that their relative levels introduce variability and/or integration in the type and duration of signalling networks and functional responses that are elicited.

2.3. erbB ligands

The erbB ligand family (including splice variants) consists of more than 30 members. Ligand binding induces receptor homo- or heterodimerization and subsequent auto- or cross-phosphorylation. This initiates a complex series of molecular events causing a spectrum of biological activities via simultaneous stimulation of multiple signal transduction pathways. SCCHN cells produce several erbB ligands that may enhance their proliferation through autocrine, paracrine and juxtacrine pathways [21] accounting for their relative independence of exogenous growth factors.

EGF is a potent mitogen, however its effects may depend on the cell context. In KB oral SCC cells,high levels of exogenous EGF led to cellular proliferation and the inhibition of apoptosis *in vitro*, whereas at low concentrations of EGF, apoptosis was inhibited without impact on cellular proliferation. Also, high levels of EGF downregulated EGFR expression, whereas the converse was true for low levels [28]. Very few cancers

have been shown to synthesise EGF. Studies in oral mucosae [29] have shown that EGF is mainly located in the underlying connective tissues and in stroma near the epithelium and the intensity of staining is increased with the degree of epithelial malignancy. Concomitant increased expression of EGFR in severe epithelial dysplasia and malignancy was also found. This suggests a paracrine epithelial-mesenchymal dependency in EGF secretion.

Transforming growth factor (TGF)-α mRNA and protein are frequently found in cancer tissues, and TGF-α activity has been detected in the urine of patients with disseminated SCCHN. The overexpression of TGF-α in SCCHN is frequently accompanied by elevated levels of EGFR [14], again suggesting the possibility of autocrine stimulation. Issing et al. observed that patients with SCCHN overexpressing EGFR and TGFα had a significantly shorter survival than patients overexpressing EGFR only [30] whereas Grandis et al. reported that both TGF-α and EGFR protein levels in primary cancers were each independently associated with adverse outcomes [31].

Our group has demonstrated that betacellulin (BTC), heparin-binding EGF, amphiregulin (AR) and heregulins (HRGs) also appear to be involved in the erbB mediated signalling which underlies SCCHN pathogenesis [32–34]. BTC has the ability to activate all four members of the erbB receptor family directly or via cross-induction, and seems to be especially potent at enhancing invasion and angiogenesis (see below).

The third class of ligands, HRGs, do not bind to EGFR, but bind directly to erbB-3 and erbB-4 and activate erbB-2 through formation of complexes with erbB-3 or erbB-4 [35]. HRG-β1 has been shown to be the most potent in terms of receptor tyrosine phosphorylation and activation of downstream signal elements such as mitogen-activated protein kinase (MAPK) [36] (Table 1).

Table 1. Binding specificity of ligands for different c-*erb*B receptors

Ligand	EGFR	c-erbB3	c-erbB4
EGF	+		
Heparin-binding EGF	+		+
TGFα	+		
Betacellulin (BTC)	+		+
Amphiregulin (AR)	+		+
Epiregulin (EPI)	+		+
Heregulins (HRG)		+	+

All of the known ligands are synthesized as glycosylated integral membrane proteins. Some can act in a juxtacrine fashion when still tethered to the cell membrane, but most require further processing. Matrix metalloproteinases (MMPs) and adamalysins (ADAMs) cleave the precursor ligands releasing them in their mature, active form [37]. Our group has found that matrix metalloproteinase inhibition can arrest SCCHN growth *in vitro* in cell lines overexpressing EGFR [38] but that this can be overcome by the addition of exogenous ligand, suggesting a mechanism based on the autocrine ligand processing described above. The upregulation of MMPs downstream of EGFR activation provides a mechanism by which an autocrine loop could be potentiated. In addition, we have shown that the addition of ligand to SCCHN cells *in vitro* upregulates expression both of the ligand itself and of the other ligand family members thus reinforcing positive feedback loops [32].

Overexpression of ligands in the tumour milieu not only stimulates tumour progression but may also have an impact on the efficacy of EGFR inhibitors, since they may compete with antibodies for receptor binding or increase the overall level of activation of the receptor population in a cell. When exogenous ligands were added to EGFR-overexpressing breast and gastric cancer cell lines (BT474 and MKN7), EGFR inhibition by antibodies and small molecules was attenuated by around 50%. Similarly, HRG could fully rescue erbB-3-overexpressing MKN7 cells from EGFR inhibition [39]. These data infer potential mitigating effects of elevated ligand levels on receptor inhibition, *in vivo* and in the clinical setting, which may not be seen *in vitro.*

The nett effect of erbB-mediated responses is complex and will depend on the relative frequency of receptors (which will impact on the probability of different dimerisation patterns), the availability of soluble and/or tethered autocrine and paracrine ligands and their affinity for the receptors displayed which will be integrated into the activation of downstream signalling matrices. The activation of each major pathway (e.g. MAP kinases, PI3-kinase) may in turn be modulated by stimuli received from different upstream activators be they other receptor tyrosine kinases, integrins, G-protein coupled receptors etc and "horizontal" cross talk/regulation between second messengers.

2.4. Receptor activation and cross-talk

Ligand binding to wild type EGFR may generate up to 10 types of homo- or heterodimeric complexes

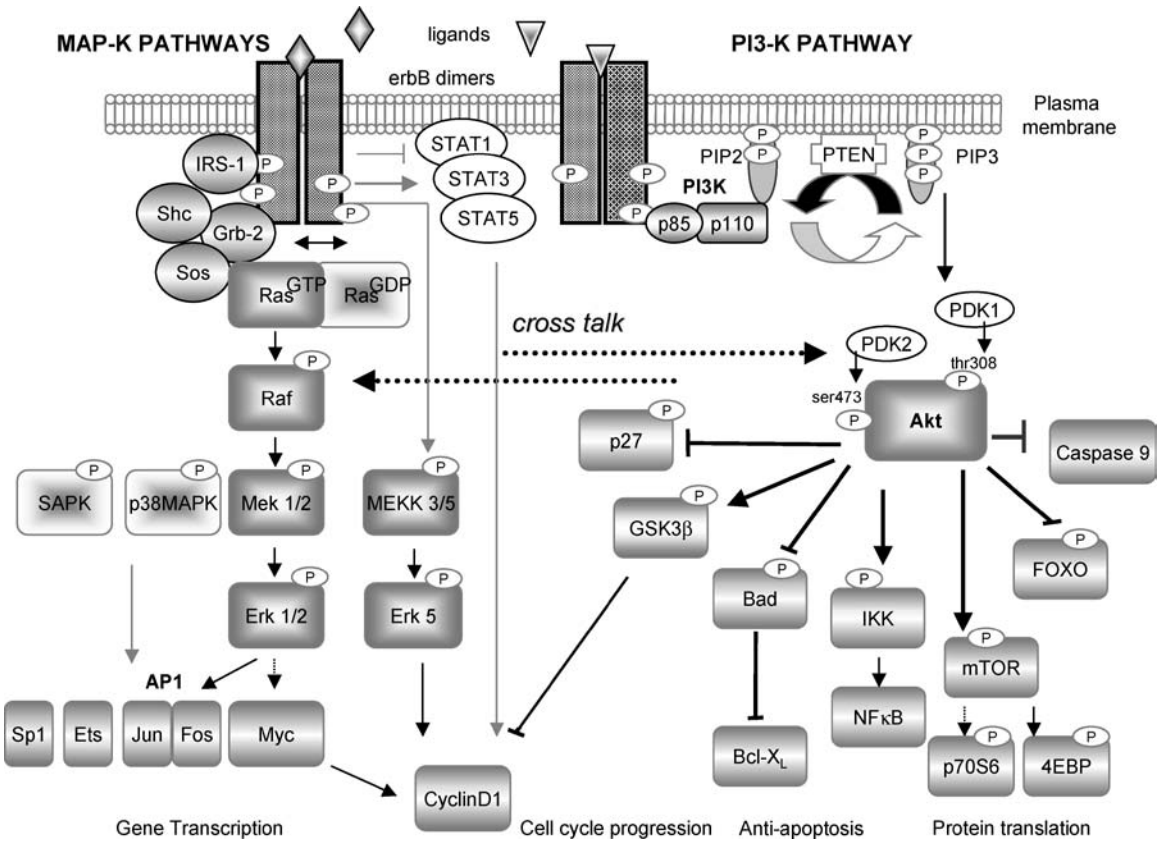

Figure 2. Major signalling pathways downstream of c-erbB receptors. The length and strength of signals and the resulting cellular responses depend upon the type and abundance of receptors (and hence frequency of particular homo-or heterodimers) the availability of autocrine or paracrine ligands or the presence of constitutively activated receptors.

resulting in conformational changes in the intracellular kinase domain, triggering autophosphorylation and activating its kinase function. Consequent to phosphorylation is the recruitment of protein signalling molecules, including growth factor receptor-bound protein 2 (Grb2), Shc and IRS-1 to the cell membrane, and the initiation of phosphorylation signalling cascades down to transcription factors in the nucleus. Cell signalling is complex and diverse with one signalling molecule able to trigger several cascades, predominantly the Ras-MAP kinase and PI3K-Akt pathways (Figure 2). In addition, abundant cross talk exists between the pathways both intracellularly and at the level of the extracellular receptor.

EGFR can be activated by other receptor tyrosine kinases (RTK) such as insulin-like growth factor receptor (IGF1-R), cell adhesion molecules (such as E-cadherin and integrins) and G-protein coupled receptors (GPCR) [40] (Figure 3). In the latter case, this appears to be primarily due to proteolytic cleavage of ligand precursors (see "proteolysis" below), but Src and Pyk tyrosine kinases may also mediate EGFR activation downstream of GPCR. This has led to the concept of "triple membrane passing signals" (TMPs) involving a sequence of three transmembrane signalling events: GPCR activation, MMP activation and finally EGFR activation [41] (Figure 4). The consequences of direct ligand binding and indirect GPCR transactivation may differ since only the former induced phosphorylation of phospholipase C (PLC)γ. GPCRs are also able to induce phosphorylation of EGFR and of the MAPK pathway by independent means [41]. Lysophosphatidic acid (LPA)-induced EGFR transactivation in SCCHN has been linked to enhanced cell proliferation and migration involving ADAM17 and AR [40].

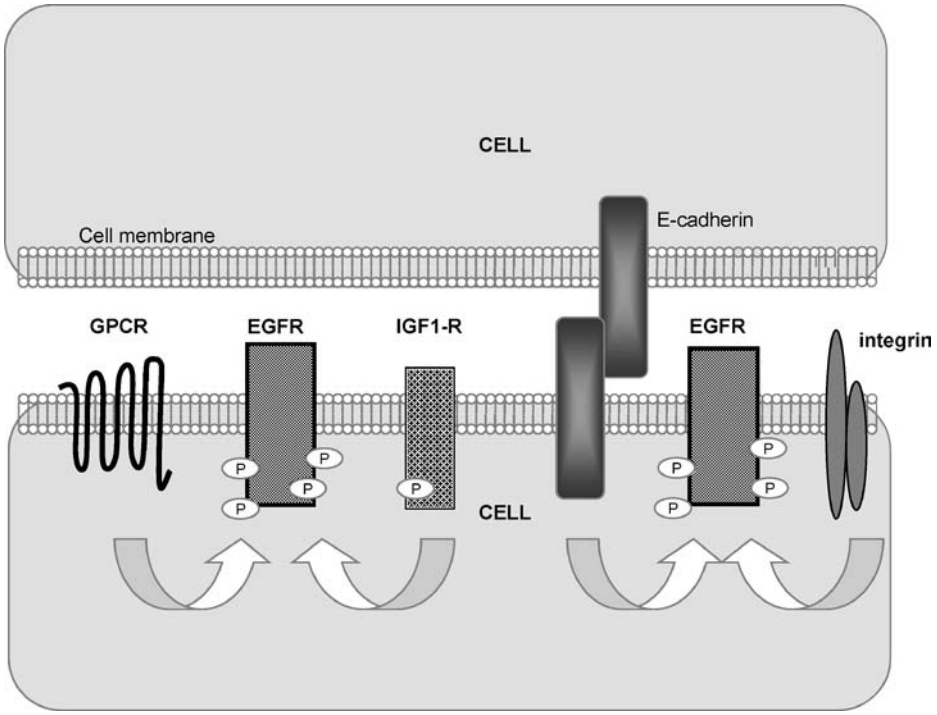

Figure 3. Transactivation of EGFR. EGFR (and other c-erbB receptors) can be activated (phosphorylated) by a number of cell membrane-spanning molecules including other RTKs (such as IGF1-R), GPCR, integrins and cadherins. Physical interactions can result in bi-directional co-operative signalling using a variety of mechanisms (see ref. [68]).

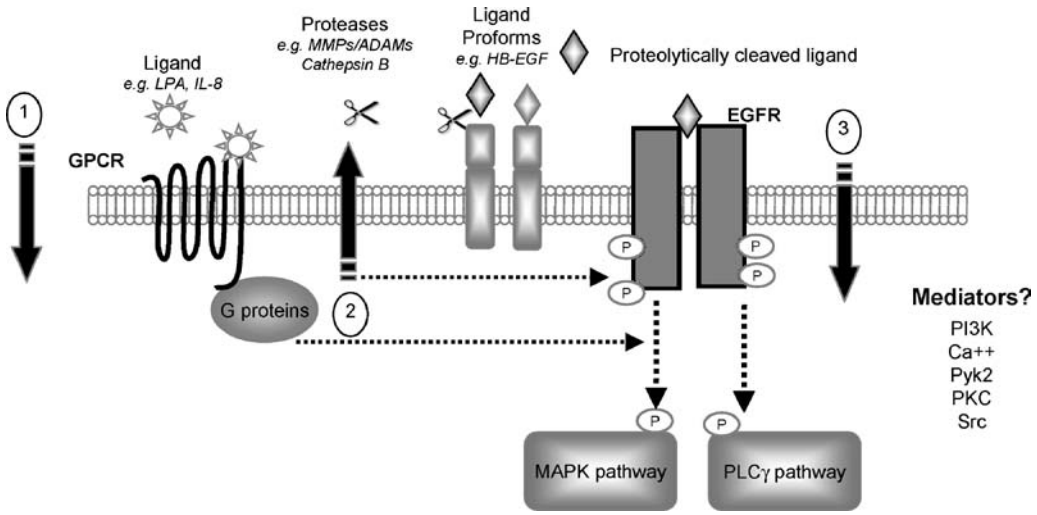

Figure 4. GPCR-mediated transactivation of EGFR. Many GPCR ligands including lysophosphatidic acid (LPA), thrombin and IL-8 (via CXCR-2) can activate EGFR. One major mechanism involves GPCR-mediated activation of proteases including MMPs, ADAMs (and cathepsin B at least in endothelial cells). These enzymes process pro-forms of EGFR ligands such as HB-EGF, TGFα and AR releasing mature ligands capable of directly activating EGFR. This has led to the "triple membrane-passing" signal hypothesis, indicated in the figure by numbered black arrows. In addition, GPCR can directly activate certain PLC isoforms and MAPK pathways, thus bypassing or amplifying ligand-mediated EGFR signalling ("multitrack" hypothesis)(see refs. [40,41]).

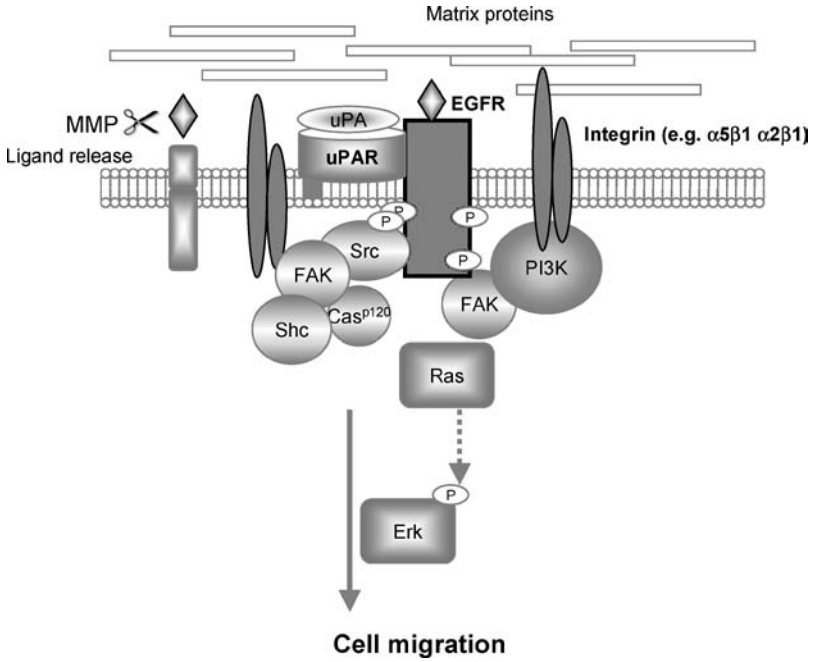

Figure 5. Integrin and uPAR-mediated transactivation of EGFR. uPAR (a GPI-linked receptor) binds the serine protease uPA which then becomes a membrane-associated ectoenzyme in the pericellular environment; however proteolytic activity is not required for EGFR transactivation. The two receptors physically interact and activate Erk, but this signalling favours cell migration and invasion whereas ligand-mediated EGFR activation preferentially potentiates mitosis. The mechanism is not fully understood, but is dependent on Src and MMP activity and has also been suggested to involve integrin $\alpha5\beta1$ and FAK. Other $\beta1$ integrins (notably $\alpha2\beta1$) can also associate with and activate EGFR (see refs. [42,68]). Ligation of integrins with ECM proteins is required for this activity.

Recently, a novel form of EGFR transactivation has been described involving the urokinase plasminogen activator receptor (uPAR) via a mechanism involving $\alpha5\beta1$ integrin, Src and MMPs (Figure 5). Interestingly, EGFR activation by uPAR stimuli mediated cell invasion, in contrast to EGF activation which favoured proliferation [42]. In addition, erbB oncogene activities can be modulated by their participation in multimeric signalling complexes in the cell membrane involving scaffold proteins, intracellular kinases, phosphatases, cytoskeletal proteins such as F-actin and adhesion molecules, but in each of the cases described, they represent a pivotal component in oncogenic signalling. Successful blockade of EGFR and erbB-2 has demonstrated proof of principle for these novel therapeutic targets, and helped to clarify their cellular roles. Downstream signalling cascades implicated in the various functional consequences of c-erbB activation are discussed in the relevant sections below.

3. The phenotypic effects of c-erbB receptor upregulation

It is clear that in addition to potentiation of cell survival and proliferation (perhaps classical roles for "growth factor receptors") erbB receptor activation results in pleiotropic effects on differentiation, metabolism, cytoskeletal organisation, motility, proteolysis and angiogenic potential. The means by which incoming environmental signals are sensed by the receptors and integrated into appropriate responses is far from elucidated, but several downstream signalling pathways, especially the PI3 kinase axis, play a pivotal role in these functions.

3.1. Cell proliferation

On activation of EGFR, Ras is recruited to the SH2 domain of the adaptor protein GRB2, located in the plasma membrane bound to Sos. Activated Ras

induces phosphorylation of Raf proteins, which signal downstream to the members of the MAPK family, MEK 1/2 and ERK 1/2. These serine/threonine kinases are components of the dominant signalling cascade downstream of EGFR, however additional MAPK cascades exist, namely the stress-activated protein kinase (SAPK) and p38 MAPK cascades. At the nuclear level, the Ras-Raf-MEK-ERK pathway leads to upregulation of the cell cycle protein cyclin D1, permitting cycle progression from the G1 checkpoint to S-phase and consequently DNA synthesis and mitosis (Figure 2).

An additional downstream phosphorylation cascade leading to upregulation of cyclin D1 and cell proliferation, is the Ras-independent activation of MEKK 3 and 5 leading to phosphorylation of erk-5 or BMK1 [43]. It has been demonstrated *in vitro* that EGF can activate erk-5 and that expression of a dominant-negative form of Erk-5 prevents cells from entering S-phase [44].

Other key proteins implicated in the pathogenesis of SCCHN are the STAT proteins. Binding of the STAT src-homology-2 (SH2) domains to activated EGFR leads to phosphorylation, dimerisation and nuclear translocation of these messenger proteins. In the nucleus they bind to promoter elements and regulate gene expression [45] to drive cell proliferation and inhibit apoptosis through effects on cyclin D1 and Bcl-xL. Of the seven known STAT proteins, STAT 1, −3 and −5 are the most widely implicated in cancer. The former functions as a tumour suppressor gene whereas STAT 3 and −5 behave as oncogenes [46].

Until 2003, STAT 5 had no known role in epithelial carcinogenesis. However, a study of 33 patients with SCCHN found consistently increased expression and phosphorylation of STAT 5b in tumour compared to normal epithelium [47]. In a xenograft model of squamous cell carcinoma, antisense blockade of STAT 5b expression inhibited tumour growth whereas abrogating STAT 5a function did not, showing differential functions of the two isoforms in oncogenesis. Although STAT3 expression can be inhibited by an EGFR antisense plasmid [13], STAT3 activation is not always dependent on EGFR overexpression [48] and under these circumstances, IL6 may play a role [49]. STATs are activated by numerous additional tyrosine kinases, including Src, Jnk and Bcr-Abl, whilst erbB-2, erbB-3 and erbB-4 do not potentiate STAT5 phosphorylation by EGF [50].

STAT1 has been demonstrated to exist in a constitutively active form in SCCHN, however blockade of its expression was not associated with tumour growth inhibition [51]. This is not surprising as STAT1 mediates the ability of interferon (IFN)γ to inhibit the growth of cultured cells and loss of STAT1 is associated with more rapid tumour growth *in vivo.* Also, unlike STAT 3 and −5, STAT1 is pro-apoptotic, as summarised by Battle [52] and negatively regulates angiogenesis, tumorigenicity and metastasis [53]. Tumours with high levels of EGFR frequently also express constitutively activated Src family kinases, which upregulate expression of Bcl-X_L, further potentiating cell survival and proliferation.

3.2. Inhibition of apoptosis

Cell death by p53-mediated apoptosis has evolved to prevent the survival of genetically aberrant cells, thus potentially controlling incipient tumour development. Malignant cells are adept at avoiding apoptosis, leading to the proliferation of mutation-bearing cells and further progression. One of the main mechanisms by which tumour cells circumvent apoptosis is via activation of the PI3-kinase pathway. PI3-kinase phosphorylates phosphatidylinositols (PtdIns) and catalyses the conversion of PtdIns 4,5 P2 (PIP2) to a lipid second messenger, PtdIns 3,4,5-P3 (PIP3) at the cell membrane [54]. PIP3 aids in recruitment and activation of signalling proteins implicated in cell survival, such as PKB/Akt [55] via PDKs (Figure 2). Other downstream targets of PI3 kinase include p70 S6 kinase, Rac and guanine exchange factors, thus endowing it with pleiotropic roles not only in mitogenesis and cell survival, but also in regulation of actin functions and cell motility (see below).

EGFR can lead to activation of PI3-kinase both directly and indirectly through Ras; other c-erbB family members can also activate PI3K directly or indirectly. Activation induces downstream amplification of phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates a range of cell signalling proteins, including thr^{308} on Akt, and a second (unidentified) kinase "PDK2" phosphorylates a second site, ser^{473}. Akt regulates cell survival by phosphorylating and sequestering downstream targets including the FOXO family of forkhead transcription factors (e.g. FKHRL1), the pro-apoptotic protein Bad, the protease caspase 9 and by activating the pro-survival transcriptional regulator protein nuclear factor-κB (NF-κB). Akt-mediated phosphorylation of FOXO also promotes

cyclin D1 expression and represses cell cycle inhibitors [54].

In addition, the PI3-kinase pathway can be indirectly activated through deletion of PTEN (phosphatase and tensin homologue), a tumour suppressor protein that dephosphorylates PIP3, thus negatively regulating the pathway [56] (Figure 2). Somatic PTEN mutations are found frequently in glioblastoma multiforme and in endometrial carcinoma, in particular. However in two studies in SCCHN which examined 28 tumours and cell lines [57] or 50 untreated tumours [58], no homozygous deletion of PTEN was detected. In a series of 81 oral cancers, inactivation of PTEN was more frequent in tumours demonstrating microsatellite instability but was still not common [59].

It appears therefore, that the contribution of activated PI3-kinase-Akt to the inhibition of apoptosis and rapid doubling time of SCCHN tumours is mediated primarily by upregulation of EGFR/c-erbB signalling via receptor overexpression rather than loss of PTEN. In the squamous cell line A431, experimental deletion of PTEN correlated with an increase in phosphorylated Akt and decreased response of EGFR to tyrosine kinase inhibition (using ZD 1839, Iressa), thus suggesting a mechanism of resistance to EGFR tyrosine kinase inhibitors for which there is a growing body of evidence [60,61].

Also, it has recently been found that gain of 3-4 copies of the *PIK3CA* gene which encodes a major catalytic subunit of PI3K (p110α) is a common occurrence in SCCHN, leading to higher levels of activity [62]. It remains to be seen whether this leads to increased sensitivity to upstream signals from RTK such as the erbB family, or a degree of innate resistance. Additional consequences of the PI3-kinase cascade are activation of mammalian target of rapamycin (mTOR) [63] which stimulates protein translation, and activation of endothelial nitric oxide (eNOS); both of which have been implicated in angiogenesis [64,65]

3.3. *Invasion*

With a few exceptions, such as placental trophoblasts, the ability of malignant cells to invade into surrounding tissue is arguably the key property that distinguishes them from normal cells. Indeed recent work comparing gene expression profiles of normal oral mucosa, primary and recurrent SCCHN tumour samples has identified expression signatures associated with invasion and metastasis only in the recurrent tumours [66]. Cancer cell invasion is a complex, multistep process involving active interactions between the invading cell, the extracellular matrix (ECM) and other stromal elements. HNSCC are characterised by local invasiveness and a propensity for dissemination to cervical lymph nodes. We and others have shown that activation of erbB receptors can induce many of the phenotypic traits associated with invasion, including loss of E-cadherin, acquisition of a motile phenotype and upregulation of a variety of proteolytic enzymes, described below.

3.3.1. Cell-cell and cell-matrix adhesion. The abnormal expression of three key molecular adhesion systems has been linked with nodal metastasis in SCCHN: E-cadherin, CD44 and Ep-CAM [67] and several may be associated with erbB activation (Figure 6). It is known that EGFR activation can cause internalisation and downregulation of the cell-cell adhesion molecule E-cadherin via phosphorylation of β catenin. Conversely, the assembly of calcium-dependent adherens junctions potentiates autophosphorylation of EGFR and MAPK activation [68].

CD44 isoforms form a direct link between the extracellular matrix and the cytoskeleton via external hyaluronic acid binding residues and intracellular ankyrin binding regions. The role of the standard (CD44s) and the "metastatic" (CD44v6) variant in SCCHN is controversial. However CD44 acts as a co-receptor for EGFR and erbB-2 and is involved in the MMP-7 processing of HB-EGF and also potentiates erbB-2:erbB-3 heterodimerisation in response to HRGs. CD44v3,8-10 also co-localises with erbB proteins and MMP-9 in invadopodia, potentiating cell migration and invasion [69].

EGFR activation can also alter expression of integrins, resulting in decreased homotypic cell aggregation and increased motility on ECM proteins [70]. Morphological and mitogenic responses are potentiated through interactions between cells and the extracellular matrix, primarily via integrins (Figure 6). Tumour cell adhesion to ECM proteins can be modulated by EGFR activation [71] and also by EGFR inhibitors. For example, Iressa was shown to reduce the ability of SCCHN cells to adhere to and migrate upon the ECM component fibronectin, downregulated several integrin components (α3, αv, β1, β4, β5, β6) and inhibited activation of focal adhesion kinase (FAK). The authors suggested that these effects contributed to the observed

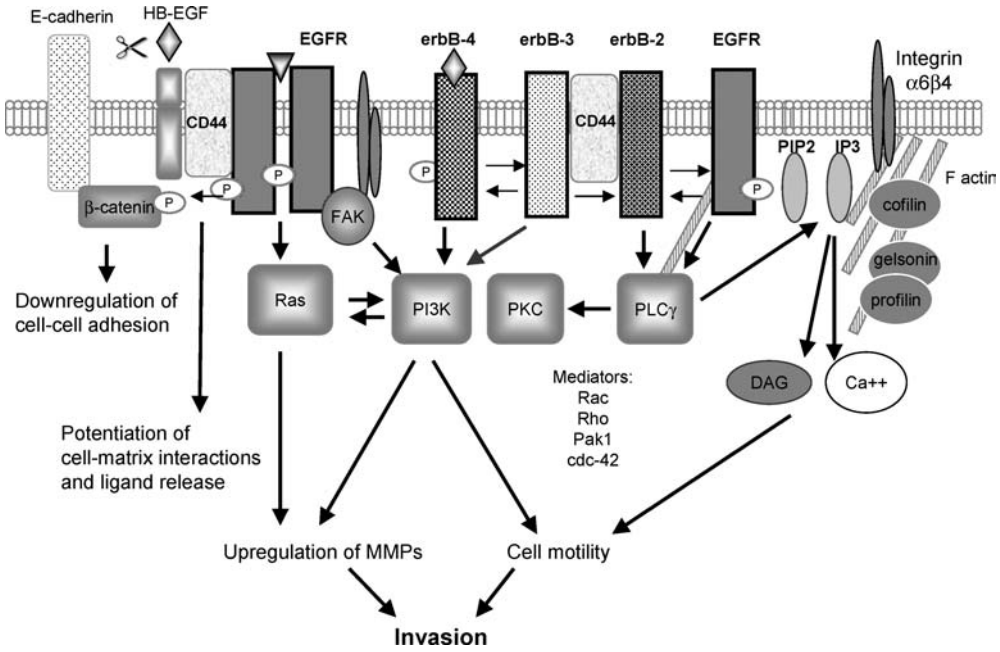

Figure 6. Co-operation between c-erbB receptors and other cell membrane components in potentiating cell migration and invasion. EGFR-mediated phosphorylation of β-catenin causes dissociation of cell-cell adhesions via internalisation of E-cadherin, the first step in acquisition of a motile phenotype. Also, phosphorylation of $\alpha 6\beta 4$ integrins induces its redistribution from hemidesmosomes to actin-rich motility structures and potentiation of cell migration on laminins. CD44 potentiates dimerisation of c-erbB-2-B3, participates in MMP-7 processing of HB-EGF and some variants co-localise with MMP-9 in invadopodia. Activation of PLCγ is involved in $\beta 4$ phosphorylation, regulation of PIP2 levels, release of diacylglycerol and Ca++ and mobilisation of actin-binding proteins such as cofilin etc. All of these processes result in alterations in cell plasticity and cytoskeletal deformability. The PI3 kinase pathway is also activated by multiple erbB receptors, and is linked to cell motility via Rac and Rho. Activation of the MAPK pathway is linked to transcriptional upregulation of metalloproteases which together with enhanced motility, give tumour cells invasive potential (see refs. [68,80]).

decreased invasion and nodal metastasis in an SCCHN xenograft model [72].

It is also clear that there is a reciprocal co-operation between several RTK (including EGFR and erbB-2) and integrins, since it appears that cell-matrix adhesion is necessary to implement receptor activation and growth factors are necessary to mediate cell adhesion and migration. Also, it has been reported that integrin ligation can activate RTKs in a ligand-independent fashion- eg $\alpha 2\beta 1$ activation of EGFR [68]. FAK is an important bivalent cytosolic tyrosine kinase that can physically link integrins and RTKs; however $\beta 1$ integrins seem to be able to transactivate EGFR in the absence of FAK via Src-dependent mechanisms [68].

3.3.2. Cell motility. One major rate-limiting, component of invasion and metastasis is tumour cell motility. EGFR signalling links to the cytoskeleton and there are several proteins downstream of EGFR that contribute to the metastatic potential of SCCHN. One of the first effects of EGFR activation in cells *in vitro* is the generation of a motile "scattered" phenotype as epithelial islands disintegrate and cells acquire a fibroblast-like appearance which can be reversed by anti-EGFR antibodies. This "motogenic" response is distinct from the "mitogenic" response to ligands, and EGFR mutants engineered to lack PLCγ binding motifs are competent for the latter but not the former. Although both PLCγ and MAPK activation are implicated in cell motility induced by EGFR ligands, MAPK alone is insufficient to induce this phenotype [73] although it may contribute by inducing focal adhesion disassembly [74].

Phospholipase Cγ associates with EGFR and erbB-2, but not erbB-3 and erbB-4 [4] and has been particularly implicated with SCCHN migration and invasion. PLCγ activation was inhibited by an EGFR TK inhibitor, PD153035 or blocking antibody C225. Invasion of SCCHN cells through Matrigel (but not cell proliferation) was significantly reduced by the PLCγ

inhibitor U73122 and antisense oligonucleotides. In addition, levels of activated PLCγ1 were increased in SCCHN compared with normal adjacent mucosa [75]. PLCγ hydrolyses PIP2 to release IP3 and diacylglycerol, resulting in the release of Ca^{++} from intracellular stores and activation of the serine- threonine kinase PKC, which may attenuate EGFR mitogenic signalling and perhaps shift cell responses towards migration. It has also been linked to mobilization of the actin-modifying proteins cofilin, profilin and gelsonin [76] (Figure 6). Both PLCγ and Shc (activated by EGFR) associate directly with the actin cytoskeleton, as does EGFR via the ABD domain. This domain contains Tyr992, the activated form of which binds PLCγ, Shc and Src, providing an integrated signalling system which modulates cell detachment from the substratum, actin dynamics, cell shape, deformability and motility [77].

Enhanced phosphorylation of $\alpha 6\beta 4$ integrin phosphorylation by EGFR (mediated via Fyn and PLCγ) leads to its loss from hemidesmosomes and their subsequent disassembly and redistribution of the integrin in actin-rich structures where it mediates cell motility [78] (Figure 6). It seems that the integrin's laminin-binding adhesive interactions may not be required for this function, since erbB-2, interacting with an adhesion-defective truncated $\alpha 6\beta 4$, results in enhanced invasion via a PI3 kinase-mediated mechanism [79].

Together, these experiments suggest that PLC $\gamma 1$ mediates key aspects of invasion and metastasis downstream of EGFR [80] especially in SCCHN. Other contributors to tumour cell migration include the small GTP-binding proteins of the Rho subfamily [81] and PI3 kinase through activation of Ras and Rac [82].

Tumour cells with high levels of EGFR usually express constitutively activated Src family kinases, which not only upregulate Bcl-X_L (contributing to cell survival and proliferation) but are also implicated in cell motility and invasion. Ligand activation of receptor tyrosine kinases (including the c-erbB oncogenes) activate small GTPases cdc42 and Rac1 which are involved in the formation of lamellipodia and membrane ruffles via the serine-threonine kinase Pak1. This protein is also highly expressed in invasive SCCHN cancers. Recent studies have shown that EGFR inhibitors (such as Iressa or siRNA) can inhibit ligand induced cytoskeletal remodelling, Src and Pak1 activation and *in vitro* invasion of SCCHN cells [83] indicating that this signalling pathway is potentially critically important in tumour progression.

ErbB-2 has been identified on microvilli and plasma membrane protrusions in adenocarcinomas cells stimulated by chemotactic factors [84] but its role in SCC may be minor compared with EGFR. However *in vitro* migration/invasion of SCCHN cell lines induced by BTC and HRGβ1 (and to a lesser extent EGF) can be inhibited by anti-erbB-2 antibodies [33]. ErbB-4 can also potentiate cell motility when activated via HB-EGF, in this case apparently via PI3 kinase activation [85].

3.3.3. Pleiotropic effects of proteases. Invasion and migration are dependent on two key cellular functions: cell motility and proteolysis, both of which are linked to erbB receptor activity [86]. The initial step in invasion is proteolysis by enzymes including MMPS. MMPs are zinc- and calcium-dependent endopeptidases frequently upregulated in cancers. A complex interaction of multiple MMPs is required to degrade the ECM, and the gelatinases MMP-2 and MMP-9 have been shown to digest collagenase IV, a key component of basement membranes. Detachment from this membrane is a key step in tumour cell migration and the dissolution of extracellular matrix at secondary sites enables tumour cells to become established as metastases.

The first study of MMPs in HNSCC *in vivo* was reported by Polette et al. Using *in situ* hybridisation, they showed that tumour cells and adjacent stromal cells (such as fibroblasts, macrophages) frequently expressed MMP-1 and MMP-10 along disrupted basement membrane [87]. MMP-11 expression was detected in stromal cells immediately surrounding invasive cancer cells and was suggested to define a particularly aggressive phenotype [88]. MMP-3 was also localised primarily in the advancing front of cancers and was found to correlate with invasion and metastasis in oral SCCs [3]. MMP-2 and MMP-9 have been shown to be highly expressed and strongly correlated with the malignant phenotype and lymph node metastasis in SCCHN [89].

Several studies have shown that MMP-2 may be derived not from the tumour cells, but from the surrounding stroma; in contrast, MMP-9 is expressed by malignant keratinocytes and localized at the tumour/stroma interface. MMP-13 is detected in the majority of SCCHN tissues but not in normal skin or oral mucosa [90]. The finding that expression of some MMPs is higher in stromal cells suggests that tumour cells are capable of inducing and utilizing host tissue MMPs and support an active role of the stroma in HNSCC invasion.

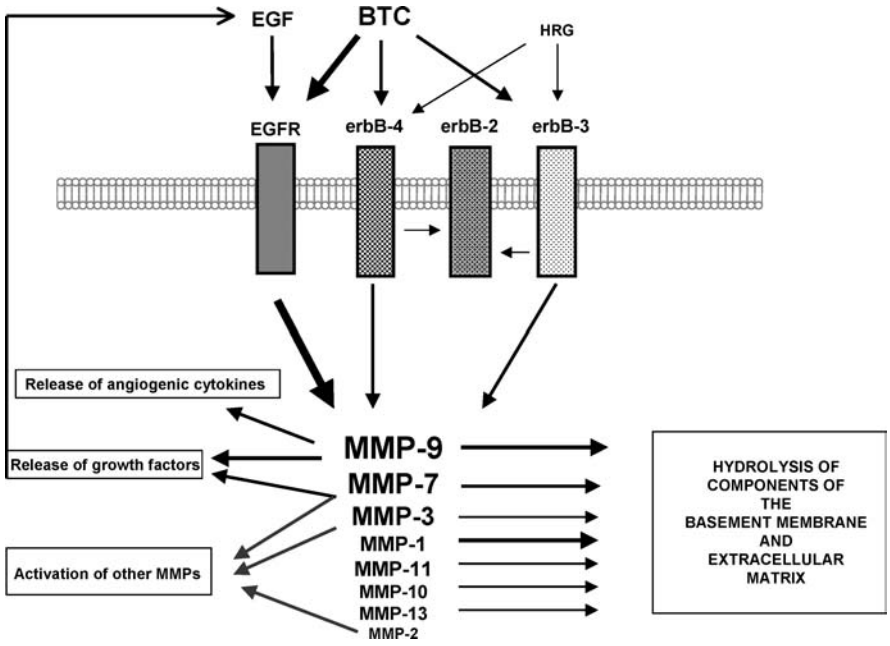

Figure 7. Differential activation of MMPs by c-erbB ligands and receptors in SCCHN. The size of the font and thickness of the arrows indicates the potency of the ligand or pathway. MMPs potentiate diverse functions in addition to their "classical" role in matrix degradation. These include processing and release of ligands resulting in autocrine activation of c-erbB receptors; processing and release of angiogenic and lymphangiogenic cytokines (VEGF-A, VEGF-C, bFGF) and activation of other MMPs resulting in proteolytic cascades.

We found that activation of EGFR (and to a lesser extent erbB-3 and erbB-4) was able to upregulate all of the key epithelial MMPs implicated above, i.e. MMP-3, 7,9,11,1,10 and 13, although the most dramatic increase seen was that of MMP-9 (gelatinase B) [33] (Figure 7). Levels of MMP-9 mRNA, protein and proteolytic activity were significantly increased by several erbB ligands with varying potencies (BTC>EGF>HRG) and the degree of upregulation correlated with the ability of SCCHN cells to invade through Matrigel *in vitro* [33,34]. In these studies, there was little or no effect on the levels of MMP-2 (gelatinase A), although in adenocarcinomas overexpression of erbB2 is associated with upregulation of MMP-2 and -9 [91]. In a panel of SCCHN cell lines EGFR levels correlated positively with MMP-9 expression and invasive potential and negatively with TIMP-1 expression [32]. MMP-9 (strongly) and MMP-2, MMP-7 and MMP-11 (weakly) correlated with lymph node involvement in a study of 54 SCCHN patients [92]. We also found that an anti-EGFR antibody (ICR62) was able to inhibit both MMP production and SCCHN invasion but an anti-c-erbB2 antibody (ICR12) had no effect on MMP levels, but could inhibit cell migration [32]. These data show how erbB receptors may differentially, but co-operatively enhance tumour cell invasion.

SCCHN *in vitro* invasion was also abrogated significantly, but not completely, by an anti-MMP-9 antibody. To determine if the residual invasive potential might be mediated by other MMPs, a broad-spectrum hydroxamate MMP inhibitor (marimastat) was tested. In addition to inhibiting invasion as expected, there was an anti-proliferative effect in some cell lines, notably those overexpressing EGFR. The effects were reversible, and could be prevented by the addition of exogenous erbB ligands. As discussed above, all EGFR ligands are subjected to proteolytic cleavage of the ectodomain to yield soluble growth factors. Metalloproteinases including MMPs and the related ADAMs have been implicated; for example, MMP-3 has been shown to release active HB-EGF. ADAM 10 has been implicated as the main "sheddase" of EGF and BTC, ADAM 12 in release of HB-EGF, ADAM 17 in processing epiregulin, TGFα and pro-amphiregulin in SCCHN [93]. In addition, EGFR transactivation involves metalloprotease-mediated ligand shedding, since GPCR stimulation can

lead to activation of ADAMs and release of HB-EGF. The signalling pathways mediating these processes remain to be elucidated [40].

Addition of MMP inhibitors to SCCHN *in vitro* inhibited the release of autocrine TGF-α, BTC and HRG-β1 into the supernatant [38]. Thus MMPs induced by EGFR activation (and to a lesser extent erbB-3 and erbB-4) may promote cell invasion through ECM and basement membranes and may also contribute to a positive feedback loop via release of active autocrine ligands. In some experimental systems extravasation is not a rate-limiting step in establishing metastases, and also lymphatic vessels may present less of a physical barrier than capillaries since they have a discontinuous basement membrane. Thus the classical role of MMPs in assisting tumour cell escape and dissemination through proteolysis of structural proteins is only one aspect of their roles in invasion [94] and angiogenesis.

The signalling pathways implicated in MMP upregulation are primarily the MAP kinases, since selective inhibitors can prevent erbB ligand induced proteolytic activity. Sustained ERK/MAPK activation acting through Fos (and thus AP-1) seems to be generally associated with MMP-9 transcriptional activation (Figures 2 and 6), whereas the roles of JNK and p38MAPK (and indeed PI3-kinase) seem to be cell type-dependent [8,94,95].

The tissue inhibitors of matrix metalloproteinases (TIMPs) are a family of proteins that negatively regulate matrix metalloproteinase activity, in addition to playing a role in cell regulation and apoptosis and the differential regulation of angiogenic and inflammatory responses. The balance between levels of TIMPs and MMPs determine the extent of local proteolysis, although TIMPs should be seen as modulators rather than inhibitors, since they serve to localise proteolysis to the tips of invadopodia, for example, and TIMP-2 also induces the focal activation of MMP-2 via MT1-MMP. The consequences of elevated levels of TIMP mRNA may therefore appear conflicting, correlating both with increased malignancy [96] and a protective effect [97]. Inhibition of EGFR with mAb ICR62 in some SCCHN cell lines is accompanied by upregulation of TIMP-1 *in vitro*, suggesting that the negative regulatory effects of TIMP-1 are suppressed in EGFR-overexpressing SCCHN, which may contribute to the observed propensity for local invasion [32]. However, as TIMPs are often secreted by stromal cells, the consequences of inhibition *in vitro* may differ substantially from effects which may occur *in vivo*.

3.4. Neoangiogenesis

The establishment of new vasculature by a tumour is essential for its continued expansion and dissemination. Neoangiogenesis and its potential for therapeutic intervention have recently been extensively reviewed [98,99]. Histological examination of human tumour specimens has confirmed that increased vascularity is a common feature of SCCHN. However, the results of studies associating microvessel density and various clinicopathological parameters and/or outcome are inconclusive [100].

The major inducers of neoangiogenesis are the vascular endothelial growth factors (VEGFs) although fibroblast growth factors, angiopoetin 1, interleukins and other growth factors also contribute. VEGFs are highly potent angiogenic agents acting to increase blood vessel permeability, endothelial cell growth, proliferation, migration, and differentiation. The VEGF-A gene gives rise to six isoforms as a result of alternative splicing which bind to and activate two major tyrosine kinase receptors: VEGFR-1/Flt-1 and VEGFR-2/KDR, albeit with varying potency and differential effects on endothelial cell functions. The related placental growth factor (PlGF) binds to VEGFR1. Increased expression of VEGF-A has been demonstrated in SCCHN cell lines, xenografts [101] and clinical specimens [102]. However, the clinical relevance of VEGF-A expression is not clear, and it may be that VEGF receptor levels are also important. Other angiogenic cytokines such as basic fibroblastic growth factor (bFGF) and interleukin-8 (IL8) are also commonly produced by SCCHN cells, and may contribute to the overall pathology.

Other VEGF family members include VEGF-B which is transcribed as a mature protein with 167 (VEGF-B_{167}) and 186 (VEGF-B_{186}) amino acid residues; these ligands bind only to VEGF-R1. VEGF-C is suggested to be a selective lymphangiogenic factor, which induces proliferation of lymphatic endothelial cells and lymphatic vessels [103] (see below). VEGF-C and VEGF-D bind to and activate both VEGFR-2 and also, (unlike VEGF-A and VEGF–B isoforms) VEGFR-3/Flt-4. VEGFR-1 and VEGFR-2 are expressed on vascular endothelium whereas VEGFR-3

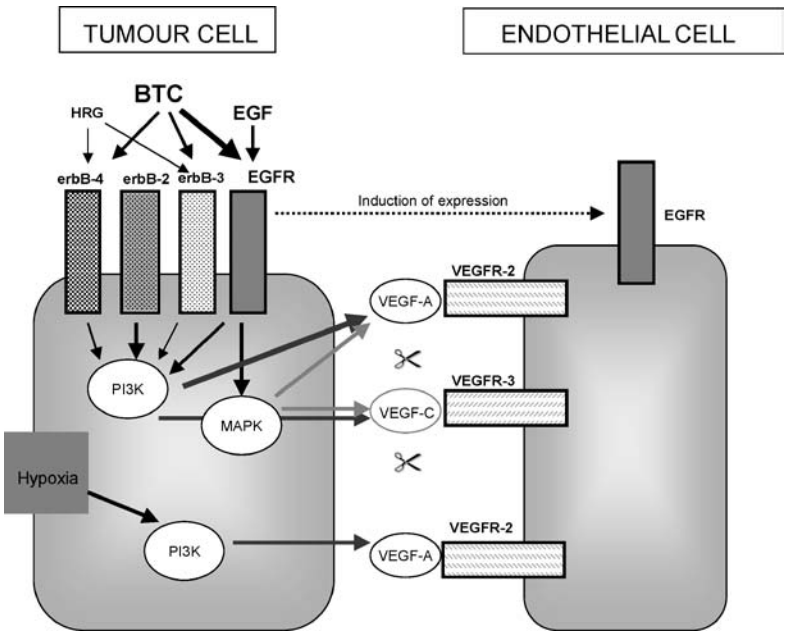

Figure 8. Pathways of induction of angiogenic cytokines. Activation of several c-erbB receptors has been linked to transcriptional activation of VEGF-A and/or VEGF-C. The potency of the ligand or signalling pathway is indicated by the size of the font and thickness of the arrows. Hypoxia potently induces VEGF-A via PI3 kinase, and c-erbB receptor activation results in enhanced production of both VEGF-A and VEGF-C in a MAPK and PI3K dependent manner. VEGF-A binds primarily to VEGFR-2 on vascular endothelial cells, and VEGF-C binds primarily to VEGFR-3 which is present on lymphatic endothelia and can also be expressed on tumour neovasculature. Similar pathways of activation also induce MMPs which have been implicated in processing and release of VEGFs, further potentiating angiogenesis and lymphangiogenesis. Tumours which overexpress EGFR can also apparently induce EGFR expression on adjacent endothelial cells.

is exclusively confined to the lymphatic endothelium in normal adult tissues. However, interestingly, VEGFR-3 can also be found in tumour endothelia [104] possibly due to the re-expression of a less differentiated phenotype.

VEGF-A is readily upregulated by hypoxia (a common feature in SCCHN) via stabilization of hypoxia-inducible factor (HIF)-1 transcription factor activity [105]. The next most potent inducers of VEGF-A are BTC, EGF and TGF-α [106]. These act via signalling pathways downstream of EGFR and other erbB receptors (Figure 8) involving the Sp1, AP-1 and AP-2 transcription binding sites in the promoters of the VEGF-A and VEGF-C genes (and also many MMP genes). This upregulation can occur through both HIF-1-dependent and -independent pathways; the former being mediated via PI3 kinase. VEGF-A transcription in SCCHN is also regulated by ERK 1/2 [107] PI3 kinase [108] and by STAT3 as a final common pathway [109]. This role of STAT3 in angiogenesis may partially explain its significant contribution to the pathogenesis of SCCHN.

If activation of EGFR (and other erbB family members) results in upregulation of angiogenesis, then inhibition of these receptors or downstream signalling pathways should reverse the effects, and this has been shown to be the case using a variety of biological and pharmacological agents. Treatment of the A431 SCC cell line with an antagonistic EGFR mAb (C225) down-regulates VEGF-A expression both *in vitro* and *in vivo* [108]. We found that stimulation of SCCHN cells *in vitro* with erbB ligands led to significant upregulation of all major isoforms of VEGF-A and VEGF-C with the same relative potency as induction of MMPs (BTC>EGF>HRG). These effects were reversed by both anti-EGFR and anti-c-*erb*B-2 mAbs [111].

Antisense inhibition of VEGF in a human SCCHN cell line resulted in a 20-fold reduction in VEGF secretion and 50% inhibition of endothelial cell migration, but there was no effect on *in vivo* tumorigenicity [112], possibly because murine host angiogenic factors were able to compensate. Nevertheless, there is compelling evidence that anti-EGFR and erbB-2 mAbs and kinase

inhibitors can inhibit angiogenesis *in vivo* (putatively via downregulation of angiogenic cytokines) although the relative contribution to therapeutic responses is unclear. Interestingly, it has been shown that endothelial cells in the neovasculature of tumours over-expressing EGF or TGFα themselves express activated EGFR, potentially rendering them especially sensitive to direct inhibition by EGFR antagonists [113].

These data strongly implicate erbB receptors as critically important in stimulating angiogenesis of capillary and lymphatic vessels in HNSCC (perhaps preferentially the tumour vessels which express both VEGFR-2 and VEGFR-3) and may also increase vessel permeability and tumour cell escape.

Most angiogenic factors (including VEGFs and bFGFs), like the EGFR ligands require proteolytic processing to develop their full activity. This may include release from sequestration in the ECM and/or stepwise proteolytic cleavage to produce forms with enhanced binding capacity to receptors. For instance, only fully processed VEGF-C can bind to VEGFR-3 and VEGFR-2 [114]. MMP-9 has been implicated as a key mediator of the "angiogenic switch" in transgenic tumour models [115]. Matrix metalloproteinases release active VEGF and bFGF from the stroma as well as acting on the endothelial basement membrane to facilitate capillary sprout formation [116].

3.5. Lymphangiogenesis

The head and neck region has a very rich lymphatic drainage, containing 300 of the body's 800 lymph nodes [117] and the development of cervical lymphadenopathy is a frequent pattern of treatment failure in SCCHN (Figure 1). Tumour spread may be via existing lymphatics or by the formation of new channels stimulated by VEGF-C [118] and possibly VEGF-D [119]. These ligands and their receptor VEGFR-3 (Flt 4) have been extensively reviewed [120].

Relatively few studies have examined VEGF-C and -D expression in SCCHN, although Saaristo et al. [121] reported that VEGF-C was evident in nasopharyngeal tumour cell islands with VEGFR-3 on adjacent angiogenic vessels. Our own studies have shown that enhanced expression of VEGF-A (isoforms 121 and 165) and VEGF-C in HNSCC had predictive value for the presence of cervical nodal metastases [111,122]. Subsequent work by others confirmed VEGF-C and VEGFR-3 mRNA and protein expression in a series of SCCHN specimens, with more VEGF-C positive vessels in larger tumours, implying that inhibition of these lymphangiogenic pathways may be an alternative therapeutic option. Interestingly, high expression of VEGF-D in our studies seemed to be associated with good prognosis, and although reports of its correlation with disease progression vary, this inverse relationship (compared with VEGF-C) has also been noted in other cancers such as lung and colon. This suggests that further work is required to investigate the roles of these two lymphangiogenic cytokines, as their functions may not be equivalent [122].

4. Response to therapy

Several pieces of experimental evidence and clinical observations suggest that the level of expression of erbB oncogenes (especially EGFR) may be associated with resistance to both chemotherapy and radiotherapy. This will not be discussed in detail here, except briefly to discuss possible mechanisms. Recent observations have shown a clear inverse correlation between EGFR density on SCCHN carcinoma cells and radiosensitivity [123] and transfection of EGFR confers cellular resistance to irradiation [124].

Several hypotheses have been proposed to explain these observations. Radiation can indiscriminately activate all erbB receptors, resulting in autophosphorylation patterns indistinguishable from those induced by ligands, and repeated exposures increase EGFR expression. These effects may contribute to the accelerated tumour re-population observed in EGFR-overexpressing tumour cells due to activation of cell survival, mitogenic and DNA repair pathways (and possibly also angiogenesis). EGFR activation seems to show a biphasic response with a short primary phase followed by a more protracted secondary phase which may be mediated by release of intrinsic TGFα [125]. The primary phase seems to be common to all erbB (and other receptor tyrosine kinases) suggesting a common mechanism. One suggestion is that this receptor activation is due to disablement of inhibitory phosphatases, possibly by interaction with radiation-induced reactive nitrogen species (reviewed in [126]).

The consequence of radiation–induced receptor phosphorylation is the activation of downstream signalling molecules. The pattern depends on the type and relative abundance of the c-erbB family members and

other RTKs which may synergistically activate MAPK and PI3-kinase-Akt pathways, culminating in cytoprotective and proliferative responses. These can be abrogated either by inhibition of EGFR or certain downstream components of the pathways.

Akt is emerging as a critically important cell survival promoter, as discussed previously. Many studies have also implicated activated Ras and/or Raf in radioresistance; however, further investigations have implicated not the direct downstream targets Mek-Erk or p38 MAPK but the PI3-kinase pathway in these effects. Transfection of constitutively active Akt can induce radioresistance independently of upstream RTKs, and inhibition of the PI3-kinase pathway more effectively reverses radioresistance than does MAPK inhibition [124,127].

The significance of these findings is that the activation status of the PI3 kinase pathway may be a major determinant of response to radiotherapy in SCCHN, and indeed there is good evidence for this (reviewed in [127]). In a series of SCCHN patients treated with chemoradiotherapy (carboplatin-paclitaxel induction followed by radiation) patients with cancers that overexpressed EGFR had a higher probability of local relapse, but a stronger correlation emerged when phospho-Akt was measured. *In vitro* studies with SCCHN cell lines suggested that the major pathway was EGFR-H-Ras-PI3K-Akt, although in cells expressing other members of the erbB family (or indeed other RTKs such as IGF1-R), direct activation of PI3-kinase-Akt may occur. Yet other cancers may be resistant to therapy by virtue of the presence of mutated or overexpressed H-Ras, PI3K or Akt, although these are less common in SCCHN. The potential mediators downstream of Akt have not been fully elucidated, and both NF-κB and RhoB have been implicated as effectors.

It will be important to determine accurately the precise pathway responsible for resistance in individual cancer patients if combined therapy is to be used (eg. EGFR, Ras or PI3K inhibitors, as has been suggested) to overcome this problem. Alternatively, inhibitors of a final common pathway or a pivotal point in the signalling network should be effective as these will be independent of the upstream activator, be it overexpression of the erbB oncogenes, loss of suppressors such as PTEN, or mutant second messengers.

In addition, other mechanisms may be in operation in resistance to therapy since transcriptional responses are induced which result in enhanced DNA repair. Radiation induces activation of the transcription factors CREB, STAT3, EGR and ETS in an EGFR-MAPK dependent manner [128]. Proteins that are regulated by these factors and have been implicated as effectors in EGFR-MAPK mediated radiation resistance include PCNA and DNA repair enzymes ERCC1 and XRCC1; also EGFR can physically interact with the repair enzyme DNA-PK [126].

Both anti-EGFR antibodies and small molecule kinase inhibitors such as Iressa and Tarceva have been shown to enhance sensitivity of tumour cells to radiation and some cytotoxic agents *in vitro* and *in vivo*. Shintani et al. [129] showed that the EGFR inhibitor Iressa reduced the levels of DNA-PK, Ku70 and Ku86 in the nucleus, thereby inhibiting repair of radiation-induced DNA double strand breaks and potentiating radiosensitivity. Holsinger et al. found that PK1166, a novel inhibitor of EGFR kinase activity, potentiated the effects of paclitaxel in an orthotopic xenograft model of oral cancer, with evidence of inhibition of Akt activation and increased levels of apoptosis [130]. Similar results have been observed following therapy with anti-EGFR or anti-erbB-2 antibodies. Also, since radiation tends to induce a G_2M arrest, and kinase inhibitors such as Iressa induce a G_1 cell cycle arrest, these effects may be additive or synergistic. In some, but not all cell types, apoptosis is enhanced by combined treatments.

Interestingly, in some *in vivo* studies, in addition to the expected enhancement of cell cycle arrest and/or apoptosis in combined radiotherapy/EGFR arms, it was proposed that a significant component of response was due to inhibition of angiogenesis [131]. EGFR antisense oligonucleotides in combination with docetaxel in a SCCHN xenograft model potentiated the effects of chemotherapy with evidence of more pronounced decreases in levels of phosphorylated STAT3 and Akt in the combined therapy group, and a concomitant decrease in VEGF [132].

Other observations are also in support of an angiogenic component in determining sensitivity/resistance of cells to anti-EGFR therapy. Kerbel's group generated a panel of SCC cells resistant to anti-EGFR mAb C225 *in vivo* which did not express this phenotype *in vitro*. A common feature in the resistant cells was enhanced expression of VEGF; its contributory role was confirmed by showing that transfection of the VEGF gene conferred resistance to SCC cells *in vivo*. However, the resistant cells also overexpressed cyclin D1 and Bcl-X_L, which potentiate cell survival and proliferation under 3D, anchorage-independent conditions [133].

5. Conclusions

Taken together, the evidence suggests that co-operative signalling via erbB receptors can regulate many key processes of angiogenesis and invasion in HNSCC. Ligands binding to EGFR, erbB-3 and erbB-4 affect cell-cell adhesion via downregulation of E-cadherin and desmosomal proteins, and alter the tumour cell's relationship with the matrix microenvironment via changes in integrin expression. Cell motility is enhanced, and MMPs upregulated by erbB signalling can release tumour cell and endothelial cell growth factors/chemotactic factors and potentiate invasion by proteolysis of ECM and basement membranes. erbB activation also upregulates VEGF-A and VEGF-C expression, further stimulating proliferation of vascular and lymphatic endothelial cells, and increasing vessel permeability. The enhanced angiogenic activity could sustain growth of the primary tumour, potentiate dissemination and also support the establishment of micrometastases.

Although most of these relationships have been established *in vitro* and in preclinical models, correlative observations in clinical material suggest that they may also be operative in SCCHN. These key contributors to invasion, angiogenesis and metastasis would therefore provide ideal targets for therapeutic intervention, and by aiming at the master switches of the erbB oncogene proteins (which are accessible at the cell membrane) it may be possible simultaneously to inhibit many different aspects of the malignant phenotype. However, if alternative downstream escape mechanisms are in operation (such as independent activation of the PI3-kinase survival pathway) then we will need to consider combinatorial therapy, or aim to identify "pivotal points" of convergent signalling for therapeutic intervention.

6. Key unanswered questions

- As EGFR is the dominant receptor tyrosine kinase in this disease, the roles of the other erbB receptors have not been evaluated in depth but are likely to contribute to oncogenesis via transactivation networks and thus they merit further study.
- The identification of pharmacogenetic indicators of patient sensitivity and pharmacodynamic markers of response to anti-EGFR agents is of paramount importance in the advancement of novel therapies.
- The mechanisms that mediate resistance to erbB inhibitors remain poorly understood and their elucidation would allow more effective inhibitor combinations. It is known that there is extensive cross talk between pathways, but it needs to be defined whether there is one predominant "escape route" following failure of EGFR inhibition or whether the response to receptor blockade varies between tumours.
- It appears that multiple target inhibition will be necessary to achieve better tumour control, but whether this should be at several levels within the EGFR cascade (e.g. inhibition of EGFR plus Mek or Erk) or across signalling pathways, (e.g. EGFR plus ErbB-3 or IGF-1R, VEGFR-2 or PI3-kinase) remains to be determined.

Acknowledgments

We would like to thank Dr Carol Sawyer for her critical review of the manuscript. The authors' work was supported by Cancer Research UK [CUK] grant number C309/A2187, The Royal Marsden Head and Neck Charitable Trust and Siriraj Hospital, Medical School, Bangkok.

References

1. Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 100: 57–70, 2000
2. Dassonville O, Formento JL, Francoual M, Ramaioli A, Santini J, Schneider M, Demard F, Milano G: Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. J Clin Oncol 11: 1873–1878, 1993
3. Kusukawa J, Harada H, Shima I, Sasaguri Y, Kameyama T, Morimatsu M: The significance of epidermal growth factor receptor and matrix metalloproteinase 3 in squamous cell carcinoma of the oral cavity. Oral Oncol 32: 217–221, 1996
4. Klapper LN, Kirschbaum MH, Sela M, Yarden Y: Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. Adv Cancer Res 77: 25–79, 2000
5. Holbro T, Civenni G, Hynes NE: The ErbB receptors and their role in cancer progression. Exp Cell Res 284: 99–110, 2003
6. Barnes CJ, Kumar R: Epidermal growth factor receptor family tyrosine kinases as signal integrators and therapeutic targets. Cancer Met Rev 22: 301–307, 2003
7. Schlessinger J: Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell 110: 669–722, 2002

8. O-charoenrat P, Rhys-Evans P, Modjtahedi H, Eccles SA: The role of c-erbB receptors and ligands in head and neck squamous cell carcinoma. Oral Oncol 38: 627–650, 2002
9. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307: 521–527, 1984
10. Grandis JR, Tweardy DJ: Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 53: 3579–3584, 1993
11. Shin DM, Ro JY, Hong WK, Hittelman WN: Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis. Cancer Res 54: 3153–3159, 1994
12. Grandis JR, Tweardy DJ, Melhem MF: Asynchronous modulation of transforming growth factor alpha and epidermal growth factor receptor protein expression in progression of premalignant lesions to head and neck squamous cell carcinoma. Clin Cancer Res 4: 13–20, 1998
13. Grandis JR, Zeng Q, Drenning SD: Epidermal growth factor receptor-mediated stat3 signaling blocks apoptosis in head and neck cancer. Laryngoscope 110: 868–874, 2000
14. Ibrahim SO, Vasstrand EN, Liavaag PG, Johannessen AC, Lillehaug JR: Expression of c-erbB proto-oncogene family members in squamous cell carcinoma of the head and neck. Anticancer Res 17: 4539–4546, 1997
15. Werkmeister R, Brandt B, Joos U: Clinical relevance of erbB-1 and -2 oncogenes in oral carcinomas. Oral Oncol 36: 100–105, 2000
16. Wilkman TS, Heitanen JH, Malstrom MJ, Kontiinen YT: Immunohistochemical analysis of the oncoprotein c-erbB-2 expression in oral benign and malignant lesions. Int J Oral Maxillofac Surg 27: 209–212, 1998
17. Chen Z, Zhang K, Zhang X, Yuan XH, Yuan Z, Jin L, Xiong M: Comparison of gene expression between metastatic derivatives and their poorly metastatic parental cells implicates crucial tumor-environment interaction in metastasis of head and neck squamous cell carcinoma. Clin Exp Metastasis 20: 335–342, 2003
18. Ulanovski D, Stern Y, Roizman P, Shpitzer T, Popovtzer A, Feinmesser R: Expression of EGFR and c-erbB-2 as prognostic factors in cancer of the tongue. Oral Oncol 40: 532–537, 2004
19. Zhu HJ, Iaria J, Orchard S, Walker F, Burgess AW: Epidermal growth factor receptor: Association of extracellular domain negatively regulates intracellular kinase activation in the absence of ligand. Growth Factors, 21: 15–30, 2003
20. Graus-Porta D, Beerli RR, Daly JM, Hynes NE: ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 16: 647–655, 1997
21. Hellyer NJ, Kim MS, Koland JG: Heregulin-dependent activation of phosphoinositide 3-kinase and Akt via the ErbB2/ErbB3 co-receptor. J Biol Chem 276: 42153–42161, 2001
22. Hou L, Shi D, Tu SM, Zhang HZ, Hung MC, Ling D: Oral cancer progression and c-erbB-2/neu proto-oncogene expression. Cancer Lett 65: 215–220,1992
23. Bei R, Pompa G, Vitolo D, Moriconi E, Ciocci L, Quaranta M, Frati L, Kraus MH, Muraro R: Co-localization of multiple ErbB receptors in stratified epithelium of oral squamous cell carcinoma. J Pathol 195: 343–348, 2001
24. Sakurai K, Urade M, Takahashi Y, Takahashi Y, Kishimoto H, Noguchi K, Yasoshima H, Kubota A: Increased expression of c-erbB-3 protein and proliferating cell nuclear antigen during development of verruccous carcinoma of the oral mucosa. Cancer 89: 2597–2605, 2000
25. Xia W, Lau Y-K, Zhang H-Z, Xiao FY, Johnston DA, Liu AR, Li L, Katz RL, Hung MC: Combination of EGFR, HER-2/neu and -3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. Clin Cancer Res 5: 4164–4174, 1999
26. O-charoenrat P, Rhys-Evans P, Eccles S: Characterisation of ten newly-derived human head and neck squamous carcinoma cell lines with special reference to c-erbB proto-oncogene expression. Anticancer Res 21: 1953–1964, 2001
27. O-charoenrat P, Rhys-Evans PH, Archer DJ, Eccles SA: c-*erb*B receptors in squamous cell carcinomas of the head and neck: Clinical significance and correlation with matrix metalloproteinases and vascular endothelial growth factors. Oral Oncol 38: 73–80, 2002
28. Kamer AR, Sacks PG, Vladutiu A, Liebow C: EGF mediates multiple signals: dependence on the conditions. Int J Mol Med 13: 143–147, 2004
29. Shirasuna K, Hayashido Y, Sugiyama M, Yoshioka H, Matsuya T: Immunohistochemical localization of epidermal growth factor (EGF) and EGF receptor in human oral mucosa and its malignancy. Virchows Arch A Pathol Anat Histopathol 418: 349–353, 1991
30. Issing WJ, Liebich C, Wustrow TP, Ullrich A: Coexpression of epidermal growth factor receptor and TGF-alpha and survival in upper aerodigestive tract cancer. Anticancer Res 16: 283–288, 1996

31. Grandis JR, Melhem MF, Gooding WE, Day R, Holst VA, Wagener MM, Drenning SD, Tweardy DJ: Levels of TGFα and EGFR protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst 90: 824–832, 1998
32. O-charoenrat P, Rhys-Evans P, Modjtahedi H, Court W, Box G, Eccles S: Overexpression of epidermal growth factor receptor in human head and neck squamous carcinoma cell lines correlates with matrix metalloproteinase-9 expression and *in vitro* invasion. Int J Cancer 86: 307–317, 2000
33. O-charoenrat P, Rhys-Evans P, Court W, Box G, Eccles SA: Differential modulation of proliferation, matrix metalloproteinase expression and invasion of human head and neck squamous carcinoma cells by c-erbB ligands. Clin Exp Metastasis 17: 631–639, 1999
34. O-charoenrat P, Modjtahedi H, Rhys-Evans P, Court W, Box GM, Eccles SA: Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. Cancer Res 60: 1121–1128, 2000
35. Karunagaran D, Tzahar E, Beerli RR, Chen X, Graus-Porta D, Ratzkin BJ, Seger R, Hynes NE, Yarden Y: ErbB2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. MBO J 15: 254–264, 1996
36. Weiss FU, Wallasch C, Campiglio M, Issing W, Ullrich A: Distinct characteristics of heregulin signals mediated by HER3 or HER4. J Cell Physiol 173: 187–195, 1997
37. Massague J, Pandiella A: Membrane-anchored growth factors. Annu Rev Biochem 62: 515–541, 1993
38. O-charoenrat P, Rhys-Evans, P, Eccles S: A synthetic matrix metalloproteinase inhibitor prevents squamous carcinoma cell proliferation by interfering with epidermal growth factor receptor autocrine loops. Int J Cancer 100: 527–533, 2002
39. Motoyama AB, Hynes NE, Lane HA: The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides. Cancer Res 62: 3151–3158, 2002
40. Fischer OM, Hart S, Gschwind A, Ullrich A: EGFR signal transactivation in cancer cells. Biochem Soc Trans 31: 1203–1208, 2003
41. Wetzker R, Bohmer FD: Transactivation joins multiple tracks to the ERK/MAPK cascade. Nat Rev Molec Cell Biol 4: 651–657, 2003
42. Guerrero J, Santibanez JF, Gonzalez A, Martinez G: EGF receptor transactivation by urokinase receptor stimulus through a mechanism involving Src and matrix metalloproteinases. Exp Cell Res 292: 201–208, 2004
43. Kato Y, Chao TH, Hayashi M, Tapping RI, Lee JD: Role of BMK1 in regulation of growth factor-induced cellular responses. Immunol Res 21: 233–237, 2000
44. Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD: Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature 395: 713–716, 1998
45. Zhong Z, Wen Z, Darnell JE: Stat3: A STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 264: 95–98, 1994
46. Bromberg J, Darnell JE: The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19: 2468–2473, 2000
47. Xi S, Zhang Q, Gooding WE, Smithgall TE, Grandis JR: Constitutive activation of Stat5b contributes to carcinogenesis *in vivo*. Cancer Res 63: 6763–6771, 2003
48. Kijima T, Niwa H, Steinman RA, Drenning SD, Gooding WE, Wentzel AL, Xi S, Grandis JR: STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth *in vivo*. Cell Growth Differ 13: 355–362, 2002
49. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS: Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. Cancer Res 63: 2948–2956, 2003
50. Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE: ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J Biol Chem 274: 17209–17218, 1999
51. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS, Tweardy DJ: Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth *In vitro*. J Clin Invest 102: 1385–1392, 1998
52. Battle TE, Frank DA: The role of STATs in apoptosis. Curr Mol Med 2: 381–392, 2002
53. Huang S, Bucana CD, Arsdall M, Fidler IJ: Stat1 negatively regulates angiogenesis, tumorigenicity and metastasis of tumor cells. Oncogene 21: 2504–2412, 2002
54. Luo J, Manning BD, Cantley LC: Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 4: 257–262, 2003
55. Vivanco I, Sawyers CL: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489–501, 2002
56. Cantley LC, Neel BG: New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96: 4240–4245,1999
57. Chen Q, Samaranayake LP, Zhou H, Xiao L: Homozygous deletion of the PTEN tumor-suppressor gene is not

a feature in oral squamous cell carcinoma. Oral Oncol 36: 95–99, 2000
58. Mavros A, Hahn M, Wieland I, Koy S, Koufaki ON, Strelocke K, Koch R, Haroske G, Schackert HK, Eckelt U: Infrequent genetic alterations of the tumor suppressor gene PTEN/MMAC1 in squamous cell carcinoma of the oral cavity. J Oral Pathol Med 31: 270–276, 2002
59. Shin KH, Kim JM, Rho KS, Park KH, Oh JE, Min BM: Inactivation of the PTEN gene by mutation, exonic deletion, and loss of transcript in human oral squamous cell carcinomas. Int J Oncol 21: 997–1001, 2002
60. She Q-B, Solit D, Basso A, Moasser M: Resistance to gefitinib in PTEN-null-HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacological modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signalling. Clin Cancer Res 9: 4340–4346, 2003
61. Bianco R, Shin I, Ritter CA, Yakes FM, Basso A, Rosen N, Tsurutani J, Dennis PA, Mills GB, Arteaga CL: Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. Oncogene 22: 2812–2822, 2003
62. Worsham MJ, Pals G, Schouten JP, van Spaendonk RML, Concus A, Carey TE, Benninger MS: Delineating genetic pathways of disease progression in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 129: 702–708, 2003
63. Manning BD, Cantley LC: United at last: The tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signalling. Biochem Soc Trans 31: 573–578, 2003
64. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC: Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399: 597–601,1999
65. Guba M, Von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch KW, Geissler EK: Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: Involvement of vascular endothelial growth factor. Nature Med 8: 128–135, 2002
66. Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker SE, Pambuccian SE, Ondrey FG, Adams GL, Gaffney PM: Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res 64: 55–63, 2004
67. Rodrigo JP, Suarez C, Ferlito A, Davaney KO, Petruzzelli J, Rinaldo A: Potential molecular prognostic markers for lymph node metastasis in head and neck squamous cell carcinoma. Acta Otolaryngol 123: 100–105, 2003
68. Comoglio PM, Boccaccio C, Trusolino L: Interactions between growth factor receptors and adhesion molecules: Breaking the rules. Curr Opin Cell Biol 15: 565–571, 2003
69. Ponta H, Sherman L, Herrlich PA: CD44: From adhesion molecules to signalling regulators. Nat Rev Molec Cell Biol 4: 33–45, 2003
70. Shiozaki H, Kadowaki T, Doki Y, Inoue M, Tamura S, Oka H, Iwazawa T, Matsui S, Shimaya K, Takeichi M: Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. Br J Cancer 71: 250–258, 1995
71. Genersch E, Schneider DW, Sauer G, Khazaie K, Schuppan D, Lichtner RB: Prevention of EGF-modulated adhesion of tumor cells to matrix proteins by specific EGF receptor inhibition. Int J Cancer 75: 205–209, 1998
72. Shintani S, Li C, Mihara M, Nakashiro K, Hamakawa H: Gefitinib (Iressa) an epidermal growth factor receptor tyrosine kinase inhibitor, mediates the inhibition of lymph node metastasis in oral cancer cells. Cancer Lett 201: 149–155, 2003
73. Chen P, Xie H, Sekar MC, Gupta, Wells A: Epidermal growth factor receptor-mediated cell motility: Phospholipase C activity is required, but mitogen activated protein kinase activity is not sufficient for induced cell movement. J Cell Biol 127: 847–857 1994
74. Xie H, Pallero MA, Gupta K Chang P, Ware MF, Witke W, Kwiatkowski DJ, Lauffenberger DA, Murphy-Ullrich JE, Wells A: EGF receptor regulation of cell motility: EGF disassembly of focal adhesions independently of the motility-associated PLC gamma signalling pathway. J Cell Sci 111: 615–624, 1998
75. Thomas SM, Coppelli FM, Wells A, Gooding WE, Song J, Kassis J, Drenning SD, Grandis JR: Epidermal growth factor receptor-stimulated activation of phospholipase C gamma-1 promotes invasion of head and neck squamous cell carcinoma. Cancer Res 63: 5629–5635, 2003
76. Chen P, Murphy-Ullrich JE, Wells A: A role for gelsonin in actuating epidermal growth factor receptor-mediated cell motility. J Cell Biol 137: 481–492, 1996
77. Tang J, Gross DJ: Regulated EGF receptor binding to F-actin modulates receptor phosphorylation. Biochem Biophys Res Commun 312: 930–936, 2003
78. Marriotti A, Kedeshian PA, Dans M, Curatola AM, Gagnoux-Palacios L, Giancotti FG: EGF-R signalling through Fyn kinase disrupts the function of integrin $\alpha 6\beta 4$ at hemidesmosomes: Role in epithelial cell migration and carcinoma invasion. J Cell Biol 155: 447–458, 2001
79. Gambaletta D, Marchetti A, Benedetti L, Mercurio AM, Sacchi A, Falconi R: Co-operative signalling between

$\alpha 6\beta 4$ integrin and erbB-2 receptor is required to promote phosphatidylinositol 3-kinase dependent invasion. J Biol Chem 275: 10604–10610, 2000
80. Wells A, Grandis JR: PhospholipaseC-$\gamma 1$ in tumour progression. Clin Exp Metastasis 20: 285–290, 2003
81. Ridley AJ, Hall A: The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70: 389–399, 1992
82. Kundra V, Escobedo JA, Kazlauskas A, Kim HK, Rhee SG, Williams LT, Zetter BR: Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. Nature 367: 474–476, 1994
83. Yang Z, Bagheri-Yarmand R, Wang R-A, Adam L, Papadimitrakopoulou VV, Clayman GL, El-Naggar A, Lotan R, Barnes CJ, Hong WK, Kumar R: The epidermal growth factor tyrosine kinase inhibitor ZD1839 (Iressa) suppresses c-Src and Pak1 pathways and invasiveness of human cancer cells. Clin Cancer Res 10: 658–667, 2004
84. Eccles SA: Cell biology of lymphatic metastasis: The potential role of c-erbB oncogene signalling. Recent Res Cancer Res 157: 41–54, 2000
85. Elenius K, Paul S, Allison G, Sun J, Klagsbrun M: Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. EMBO J 16: 1268–1278 1997
86. Eccles S: Parallels in invasion and angiogenesis provide pivotal points for therapeutic intervention in cancer. Int J Dev Biol (in press) 2004
87. Polette M, Clavel C, Muller D, Abecassis J, Binninger I, Birembaut P: Detection of mRNAs encoding collagenase I and stromelysin 2 in carcinoma of head and neck by in situ hybridisation. Invasion Metastasis 11: 76–83, 1991
88. Muller D, Wolf C, Abecassis J, Millon R, Engelmann A, Bronner G, Rouyer N, Rio MC, Eber M, Methlin G: Increased stromelysin 3 gene expression is associated with increased local invasiveness in head and neck squamous cell carcinomas. Cancer Res 53: 165–169, 1993
89. Kawamata H, Nakashiro K, Uchida D, Harada K, Yoshida H, Sato M: Possible contribution of active MMP2 to lymph-node metastasis and secreted cathepsin L to bone invasion of newly established human oral-squamous-cancer cell lines. Int J Cancer 70: 20–27, 1997
90. Johansson N, Airola K, Grenman R, Kariniemi AL, Saarialho-Kere U, Kahari VM: Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. Am J Pathol 151: 499–508, 1997
91. Yu D, Hamada J, Zhand H, Nicolson GL, Hung MC: Mechanisms of c-erbB2/neu oncogene-induced metastasis and repression of metastatic properties by adenovirus 5 E1A gene products. Oncogene 7: 2263–2270, 1992
92. O-charoenrat P, Rhys-Evans P, Eccles SA: Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 127: 813–820, 2001
93. Sahin U, Weskamp G, Kelly K, Zhou H-M, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP: Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol 164: 769–779, 2004
94. McCawley LJ, Matrisian LM: Matrix metalloproteinases: Multifunctional contributors to tumor progression. Molec Med Today 6: 149–156, 2000
95. O-charoenrat P, Wongkajornsilp A, Rhys-Evans P, Eccles SA: Signaling pathways required for matrix metalloproteinase-9 induction by betacellulin in head and neck squamous carcinoma cells. Int J Cancer (in press) 2004
96. Grignon DJ, Sakr W, Toth M, Ravery V, Angulo J, Shamsa F, Pontes JE, Crissman JC, Fridman R: High levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) expression are associated with poor outcome in invasive bladder cancer. Cancer Res 56: 1654–1659, 1996
97. Rhee JS, Diaz R, Korets L, Hodgson JG, Coussens LM: TIMP-1 alters susceptibility to carcinogenesis. Cancer Res 64: 952–961, 2004
98. Bergers G, Benjamin LE: Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3: 401–410, 2003
99. Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. Nat Med 9: 669–676, 2003
100. Homer JJ, Greenman J, Stafford ND: Angiogenesis in head and neck squamous cell carcinoma. Clin. Otolaryngol 25: 169–180, 2000
101. Petruzzelli GJ, Benefield J, Taitz AD, Fowler S, Kalkanis J, Scobercea S, West D, Young MR: Heparin-binding growth factor(s) derived from head and neck squamous cell carcinomas induce endothelial cell proliferation. Head Neck 19: 576–582, 1997
102. Smith BD, Smith GL, Carter D, Sasaki CT, Haffty BG: Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. J Clin Oncol 18: 2046–2052, 2000
103. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15: 290–298, 1996
104. Partanen TA, Alitalo K, Miettinen M: Lack of lymphatic vascular specificity of endothelial growth factor receptor 3 in 185 vascular tumors. Cancer 86: 2406–2412, 1999

105. Beasley N, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, Harris AL: Hypoxia-inducible factors HIF1α and HIF2α in head and neck cancer. Cancer Res 62: 2493–2497, 2002
106. Gille J, Swerlick RA, Caughman SW: Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. EMBO J 16: 750–759, 1997
107. Bancroft CC, Chen Z, Yeh J, Sunwoo JB, Yeh NT, Jackson S, Jackson C, Van Waes C: Effects of pharmacological antagonists of epidermal growth factor receptor, PI3K and MEK signal kinases on NF-κB and AP-1 activation and IL-8 and VEGF expression in human head and neck squamous carcinoma cell lines. Int J Cancer 99: 538–548, 2002
108. Brader S, O-Charoenrat P, Court W, Box G, Workman P, Eccles S: Signalling pathways in afferent and efferent arms of angiogenesis induced by activation of c-erbB receptors. Proc Am Assoc Cancer Res 43:(abstract #168) 33, 2002
109. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, Heller R, Ellis LM, Karras J, Bromberg J, Pardoll D, Jove R, Yu H: Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. Oncogene 21: 2000–2008, 2002
110. Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, Kerbel RS: Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: Angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 151: 1523–1530, 1997
111. O-charoenrat P, Rhys-Evans P, Modjtahedi H, Eccles SA: Vascular endothelial growth factor family members are differentially regulated by c-*erb*B signaling in head and neck squamous carcinoma cells. Clin Exp Metastasis 18: 155–161, 2000
112. Nakashima T, Hudson JM, Clayman GL: Antisense inhibition of vascular endothelial growth factor in human head and neck squamous cell carcinoma. Head Neck 22: 483–488, 2000
113. Baker CH, Kedar D, McCarty MF, Tsan R, Weber KL, Bucana CD, Fidler IJ: Blockade of epidermal growth factor receptor signalling on tumor cells and tumor-associated endothelial cells for therapy of human carcinomas. Am J Pathol 161: 929–938, 2002
114. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K: Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 16: 3898–3911, 1997
115. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nature Cell Biol 2: 737–744, 2000
116. Kalluri R: Basement membranes: Structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3: 422–433, 2003
117. Van Trappen PO, Pepper MS: Lymphatic dissemination of tumour cells and the formation of micrometastases. Lancet Oncol 3: 44–52, 2002
118. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K: Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 61: 1786–1790, 2001
119. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG: VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 7: 186–191, 2001
120. Alitalo K, Carmeliet P: Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell 1: 219–227, 2002
121. Saaristo A, Partanen TA, Arola J, Jussila L, Hytonen M, Makitie A, Vento S, Kaipainen A, Malmberg H, Alitalo K: Vascular endothelial growth factor-C and its receptor VEGFR-3 in the nasal mucosa and in nasopharyngeal tumors. Am J Pathol 157: 7–14, 2000
122. O-charoenrat, P Rhys-Evans P, Eccles, SA: Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. Cancer 92: 556–568, 2001
123. Ang KK, Berkey BA, Tu X, Zhang HZ, Katz R, Hammond EH, Fu KK, Milas L: Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma Cancer Res 62: 7350–7356, 2002
124. Liang K, Ang KK, Milas L, Hunter N, Fan Z: The epidermal growth factor receptor mediates radioresistance. Int J Radiation Oncol Biol Phys 57: 246–254, 2003
125. Dent P, Reardon DB, Park JS, Bowers G, Logsdon C, Valerie K, Schmidt-Ullrich RK: Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen activated protein kinase pathway in carcinoma cells, leading to increased cell proliferation and protection from radiation–induced cell death. Mol Biol Cell 10: 2493–2506, 1999
126. Schmidt-Ullrich RK, Contessa JN, Lammering G, Amorino G, Lin P-S: ErbB receptor tyrosine kinases and cellular radiation responses. Oncogene 22: 5855–5865, 2003
127. McKenna WG, Muschel RJ: Targeting tumor cells by enhancing radiation sensitivity. Genes Chromosomes Cancer 38: 330–338, 2003

128. Amorino GP, Hamilton VM Valerie K, Dent P, Lammering G, Schmidt-Ullrich RK: Epidermal growth factor receptor dependence of radiation-induced transcription factor activation in human breast cancer cells. Mol Biol Cell 13: 2233–2244, 2002
129. Shintani S, Li C, Mihara M, Terakado N, Yano J, Nakashiro K, Hamakawa H: Enhancement of tumor radioresponse by combined treatment with gefitinib (Iressa, ZD1839) and epidermal growth factor receptor tyrosine kinase inhibitor, is accompanied by inhibition of DNA damage repair and cell growth in oral cancer. Int J Cancer 107: 1 030–1037, 2003
130. Holsinger FC, Doan DD, Jasser SA, Swan EA, Greenberg JS, Schiff BA, Bekele BN, Younes MN, Bucana CD, Fidler IJ, Myers JN: Epidermal growth factor receptor blockade potentiates apoptosis mediated by paclitaxel and leads to prolonged survival in a murine model of oral cancer. Clin Cancer Res 9: 3183–3189, 2003
131. Huang SM, Li J, Armstrong EA, Harari PM: Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1939 (Iressa). Cancer Res 62: 4300–4306, 2002
132. Niwa H, Wentzel AL, Li M, Gooding WE, Lui V, Grandis JR: Antitumor effects of epidermal growth factor receptor antisense oligonucleotides in combination with docetaxel in squamous cell carcinoma of the head and neck. Clin Cancer Res 9: 5028–5035, 2003
133. Viloria-Petit AM, Kerbel RS: Acquired resistance to EGFR inhibitors: Mechanisms and prevention strategies. Int J Radiation Oncol Biol Phys 58: 914–926, 2004