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The epidermal growth factor receptor/Erb-B/HER family in normal and malignant breast biology

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ABSTRACT The EGFR/Erb-B receptor tyrosine kinases each play distinct and complementary roles in normal breast development. The four receptors form both homodimers and heterodimers in response to binding by ligands which show selectivity for one or more of the receptors (except Erb-B2). Together with the additional flexibility generated by the formation of different dimer pairs, these signalling networks play key roles in directing a variety of both autocrine and paracrine cellular responses. Complex two-way interactions between mammary epithelial cells and the surrounding stroma direct proliferation, duct formation, branching and terminal differentiation during puberty, pregnancy and lactation, with each receptor and ligand fulfilling distinct roles. Caricatures of the normal role of EGFR/Erb-B signalling resulting in aberrant cellular responses are seen in breast cancers, where over-expression and/or (less commonly) mutation of one or more of the receptors results in enhanced cell proliferation, motility, release of proteases and angiogenic factors. Given their importance in tumour progression and their links with resistance to chemotherapy and anti-endocrine therapy, Erb-B receptors (most notably Erb-B2) have been exploited as therapeutic targets. Monoclonal antibodies (e.g. trastuzumab, pertuzumab) and small molecule tyrosine kinase inhibitors (e.g. lapatinib, afatinib) have shown significant clinical responses in some breast cancer subtypes. Additional approaches include targeted toxins or drugs, peptide vaccines, immunRNase and chaperone inhibitors to deplete Erb-B2 protein levels. Greater understanding of the full spectrum of Erb-B-mediated signalling pathways and their misregulation in breast cancer will provide additional strategies to control malignant progression.

KEY WORDS: *EGFR, c-Erb-B2, c-Erb-B3, c-Erb-B4, cancer*

The EGFR/ERB-B/HER family

The epidermal growth factor receptor (EGFR) and its close relatives HER2/c-Erb-B2, Erb-B3 and Erb-B4 are type 1 transmembrane receptor tyrosine kinases (RTK) with key roles in embryonic development, tissue renewal/repair and cancer. A great deal has been learned about their structure, signalling pathways and aberrations linked to malignant transformation since the explosion of interest in this family in the 1980's.

Early discoveries

EGFR, a 170kDa glycoprotein, was the first member of the family to be identified as the receptor for a 'growth factor' (EGF) which regulated eyelid opening in mice, and as the binding partner for radiolabelled EGF on fibroblast cell membranes. Subsequently, EGFR was found to have kinase activity when stimulated with ligands and to be capable of phosphorylating tyrosine residues on both itself and downstream targets. Even before EGFR was identified, there were early indications that EGF (then termed epithelial growth factor, even though it is a mitogen for mesenchymal cells too) could be important in breast development as it stimulated growth of mouse mammary gland explants. The role of EGFR was later proved by showing impaired mammary gland development in mice harbouring EGFR mutations (Cohen, 1997).

Structure and function

Erb-B signalling comprises a complex network with an 'input' (ligand-receptor) layer, an intermediate signalling core processing

Abbreviations used in this paper: ADAM, a disintegrin and metalloproteinase; AREG, amphiregulin; BTC, betacellulin; EGF(R), epidermal growth factor (receptor); EREG, epiregulin; ER, oestrogen receptor; HB-EGF, heparin binding EGF; MEC, mammary epithelial cells; MMP, matrix metalloprotease; NRG, neuregulin(heregulin); RTK, receptor tyrosine kinase; (SM)TKI, (small molecule) tyrosine kinase inhibitor; TEB, terminal end bud; TGFα, transforming growth factor alpha; TN(BC), triple negative (breast cancer).

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level and an 'output' layer of transcriptional regulation and ultimately cellular responses (Citri and Yarden, 2006).

All Erb-B receptors consist of an extracellular domain which binds ligands (except in the case of Erb-B2), a transmembrane region and a cytoplasmic domain with kinase activity. Although there are 10 possible combinations of Erb-B dimers, not all are fully biologically active. Erb-B2 has no known ligands, but is the preferred partner of all family members, due to an intrinsically extended interaction loop rendering it constitutively available for dimerisation. Erb-B2 can stabilise EGFR in a conformation that potentiates dimerisation and phosphorylation in the absence of ligand and alters endocytosis and intracellular trafficking. Alternatively, at least partial transactivation of EGFR can be achieved by ligand-independent intracellular mechanisms, such as G protein-coupled receptor (GPCR) stimulation of Src or elevated calcium levels (Prenzel *et al.*, 2000). Finally, the receptors interact with, and are modulated by, steroid hormone receptors and co-receptors. Erb-B3 was generally accepted to be kinase dead due to the lack of several key functional residues including the catalytic base aspartate, but a recent paper suggests that it nevertheless retains the ability to transphosphorylate its own intracellular domain (Shi *et al.*, 2010). In any event, it can certainly form a very active signalling complex with all other EGFR RTK, especially Erb-B2. There is a high degree of homology in the kinase domain of the four receptors (59-81%) but more divergence in the C-terminal domains (only 11-25% identity). In addition to cross-talk between members of the EGFR/Erb-B family, there is evidence for significant interactions with other RTK such as c-MET and IGF-1R, and it is possible that such alternative signalling pathways are linked to resistance to targeted therapies (Jin and Esteva, 2008). Erb-B receptors also integrate signals from the extracellular microenvironment by forming macromolecular clusters with integrins and tetraspanins in specialised membrane microdomains (Alexi *et al.*, 2011)

Ligands

There are up to 13 recognised ligands of the EGFR family: EGF itself, heparin-binding (HB)-EGF, transforming growth factor (TGF)α, amphiregulin (AREG) epiregulin (EREG), epigen (EPG), betacellulin (BTC) and neuregulins (NRG) 1-6 (also known as heregulins), which have multiple splice variants. EGF and TGFα are the key EGFR binding ligands, BTC can bind and activate all receptors, and the NRGs have a preference for Erb-B3 and Erb-B4. All EGF family ligands exist as membrane-anchored precursors and are cleaved by metalloproteases (mainly ADAMs) resulting in ectodomain shedding and the release of soluble factors. The cleaved products, particularly of HB-EGF, have been implicated in transactivation of adjacent Erb-B receptors, and the remaining intracellular carboxy-terminal fragments may have additional intracellular signalling functions (Higashiyama *et al.*, 2008). The EGFR ligand shedding and subsequent receptor activation can be stimulated by many factors, including cytokines which bind G-protein couple receptors, activating PKC and MAPK signalling pathways (in the so-called triple membrane-passing signal mechanism) or via Wnt ligands binding Fzd receptors. Uncleaved, membrane-bound ligands can also stimulate adjacent cells via a juxtacrine mechanism which may be particularly important in epithelial-stromal communication. There is evidence that different ligands can promote specific patterns of EGFR phosphorylation and dictate the duration of signalling events and divergent cellular responses. For example, TGFα and AREG are more potent stimulators of motility and invasion than EGF. This is reportedly due to sustained activation of PLCγ and MAPK by the former ligands, whereas EGF promotes more rapid ubiquitination and degradation of EGFR.

Downstream signalling

Ligand binding induces conformational rearrangements of the receptors to expose the interaction loop, promoting association of both homodimers and heterodimers, followed by internalisation and/or phosphorylation events. The phosphorylated (activated) receptors act as docking points for a number of direct substrates and/or adaptor proteins. Systematic profiling of phosphotyrosine interaction sites has shown that the four receptors have specific patterns of binding partners, although each may be recruited to more than one site, albeit with different affinities or kinetics. For example, binding of Shc, Grb2 or PI3K to Erb-B2 is influenced by the mode of activation and the dimerisation partner (Schulze *et al.*, 2005). This degree of flexibility therefore allows differential responses to external stimuli in different microenvironmental contexts and the integration of stimuli into co-ordinated cellular functions.

STAT5 was identified as a direct binding partner of EGFR and Erb-B4, and this interaction is required in the breast during lactation (Schulze *et al.*, 2005). Interestingly, EGFR and Erb-B4, the only fully functional receptors (in contrast to Erb-B2 and Erb-B3) have the greatest number of interactors and probably fulfil similar functions in different cellular contexts in response to their preferred ligands (EGF family and NRGs respectively).

Erb-B3 is activated primarily by NRG-1 and -2 and is a strong activator of the PI3 kinase pathway, having six binding sites for the p85 regulatory subunit. The PI3 kinase pathway is a pivotal point in cell signalling (mainly via AKT and mTOR) regulating cell size, metabolism, survival and proliferation. Negative regulation of pro-apoptotic and growth inhibitory pathways is mediated via FOXO transcription factors and GSK3β. There are additional links to promotion of motility via Rac and Rho, and angiogenesis via activation of HIF-1α.

In summary, the major signalling pathways activated by EGFR-Erb-B receptors are mediated by PI3 kinase, Ras-Raf (MAPK), JNK, PLCγ and result in a plethora of biological functions. Although initially termed "growth" factors, the ligands induce not only cell proliferation but also alter adhesion and motility and protect against apoptosis at the cellular level, and promote invasion and angiogenesis at the physiological level. Given that these signalling systems are critical in development, it is not surprising that their activation should result in multiple co-ordinated cell- and tissue-level responses in normal cells, but these are subverted by overexpression/misregulation in pathological processes such as cancer.

Roles in normal breast development

Much of our understanding of the functions of the Erb-B family in development has come from rodent models. EGFR is of course best known as the epidermal growth factor receptor and is primarily responsible for maintaining skin and squamous epithelia. The other three family members are important for the development and maintenance of the cardiovascular and nervous systems, but also play complementary roles in breast development. Most of the Erb-B family receptors and their ligands are expressed in the mammary gland at some time during development, maturation and involution (Fig. 1). The precise and co-ordinated roles of the receptors

and their ligands are to some extent confounded by spatial and temporal complexities, a degree of redundancy, and many layers of regulation. Nevertheless, those functions that have been elucidated have proved informative for the better understanding of consequences of their misregulation in the caricatures of cancer.

The mammary gland is an unusual organ in that most of its development occurs not in embryonic development, but at puberty, stimulated by steroid hormones. Interestingly, the rudimentary embryonic ductal branching, and all subsequent phases of epithelial differentiation are orchestrated by signals from the surrounding stroma; indeed mammary epithelial cells can be completely redirected (e.g. toward salivary gland morphology and function) by placing them in association with mesenchymal cells from different tissues.

The earliest phases of breast development are oestrogen independent. In adolescence, oestrogen and oestrogen receptor (ER)α induce the next stage of branching, and in the adult progesterone (PRG) plays a key role. The major contribution of Erb-B receptors is during puberty, pregnancy and lactation, when the steroid hormones upregulate production of many growth factors, including those of the EGF family.

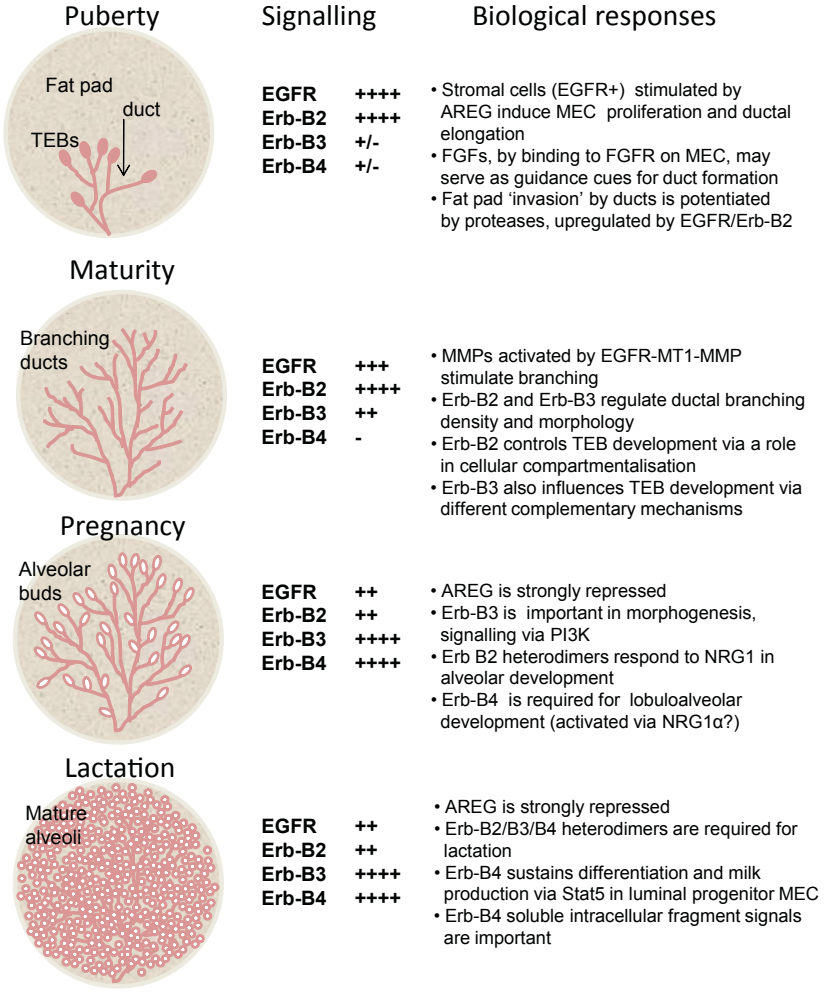

Fig. 1. Differential roles of EGFR/Erb-B receptors and ligands during normal mammary gland development and maturation. *The Figure illustrates the time- and differentiation-dependent expression of the four Erb-B receptors and key ligands at puberty and during adult breast maturation, pregnancy and lactation. Their contrasting roles in ductal outgrowth, terminal end-bud (TEB) development, alveolar maturation and lactation are shown, and differential expression in epithelial and stromal cells is highlighted.*

EGFR signalling and the role of proteolytic regulation of ligands

Both EGF and TGFα *in vitro* and under certain circumstances *in vivo* can stimulate growth of mammary epithelium and ductal differentiation. However, careful genetic knockdown, array and *in situ* hybridisation studies have identified epithelial AREG as the main (if not the only) physiologically relevant ligand *in vivo*. What is more, it is strongly induced by oestrogens. Mammary morphogenesis has been shown to require release of soluble AREG from ductal epithelial cells, its activation of EGFR on adjacent stromal cells, (particularly those immediately adjacent to developing ductal end buds) and then reciprocal inductive signals back to the epithelium (Sternlicht and Sunnarborg, 2008) (Fig. 2).

ADAM17 null mice show the same developmental defects as those lacking TGF-α, or HB-EGF, suggesting that this protease is primarily responsible for their processing and cleavage (Sahin *et al.*, 2004). Further evidence of its physiological importance in mammary development was provided by its appropriate localisation in the gland (although this was also the case for many other ADAMs). However, the reciprocal lack of its sole inhibitor (TIMP3) in TEBs suggests that ADAM17 would be active in areas of active ductal development.

The means by which EGFR-activated stromal cells induce mammary epithelial development has also been intensively studied but it still not fully elucidated. Given the multiplicity of cellular responses required (cell proliferation, stromal 'invasion', of ducts, terminal differentiation) it is likely that many simultaneous cellular responses are elicited. MT1-MMP (MMP14) is enriched in stroma surrounding TEBs and its activity, like that of ADAM17, would be enhanced by the local downregulation of TIMP3. MMP14 may stimulate ductal branching by activating MMP2 and degrading collagen 1. However it is membrane-bound and could only influence epithelial cells indirectly. Structural defects in TEBs in Erb-B2$^{-/-}$ mammary epithelium may contribute to the observed deficiency in ductal penetration into the fatpad, secondary to misregulation of matrix metalloproteinases, since branching morphogenesis requires MMP-2, and MMP-3 promotes secondary and tertiary branching. Another potential contributory mechanism is signalling via FGFR2 on mammary epithelial cells stimulated by stromal FGF10 providing both mitogenic stimuli and guidance cues for ductal development (Sternlicht and Sunnarborg, 2008) (Fig. 2).

Other Erb-B family members: additional roles in later mammary differentiation

AREG becomes strongly repressed during pregnancy and lactation and EGFR is not required for alveolar development. Erb-B2 in the epithelium is required for ductal outgrowth and TEB development and its functional partner is thought to be Erb-B3,

since knock-out of either gene results in similar abnormalities in these processes.

Erb-B3 appears when mammary glands mature and Erb-B4 is only expressed during pregnancy and lactation. In Erb-B3-/- mice, there was a decrease in the size of TEBs but increases in branch density and the number of TEBs. This was associated with an increase in apoptosis but no change in cell proliferation rates in TEBs. The major signalling pathway activated by Erb-B3 in this context seems to be PI3 kinase (Stern, 2008). *Nrg3* polymorphisms can also result in abnormalities in rodent mammary gland development, also likely via activation of Erb-B3. Thus Erb-B3 plays a key role in regulating morphogenesis of mammary epithelium (Fig. 1).

During pregnancy and lactation, Erb-B2, in association with its partners and in response to neuregulins, is required for alveolar differentiation and milk protein production. Erb-B4 is essential for lobuloalveolar development and for maintaining lactation reportedly via Stat5a. The ligand(s) responsible for activating Erb-B4 in these processes have not been finally elucidated, although NRG/HRG1α has been implicated. Most of Erb-B4's functions in the mammary gland seem to be mediated by a soluble intracellular fragment (4ICD). This fragment can localise in mitochondria and nuclei, eliciting different functional responses in cells. The 180 kDa membrane-bound Erb-B4 is cleaved by ADAM17, releasing a 120 kDa ligand-binding ectodomain and an 80kDa transmembrane peptide (m80) with kinase activity (Blobel *et al.*, 2009). The latter fragment is released from the membrane by presenilin-dependent γ-secretase cleavage. Cleavage can be stimulated by ligand binding (generally HRG, HB-EGF or BTC) or simply in response to Erb-B4 overexpression.

Nuclear 4ICD in secretory mammary epithelium signalling via Stat5a is thought to be the major driver of lactation since Stat5a transcriptionally regulates β-casein and whey acidic protein (WAP) promoters. The proposed mechanism is as follows: Erb-B4 when activated becomes phosphorylated at Y964, providing a docking site for Stat5a SH2 domains. The following regulated intra-membrane proteolysis (RIP) previously described results in liberation of the 4ICD-Stat5a complex and its translocation to the nucleus. It has been suggested that the 4ICD simply acts as a Stat5a chaperone, but may also serve as a regulator of transcription (Jones, 2008) or indeed have intrinsic independent transactivation activity. The 4ICD fragment also functions as a selective ERα co-activator since it regulates expression of PGR, SDF-1 and Erb-B4 itself. This involves ERα recruitment not to canonical ERE sites but to AP-1 sites in a complex with c-Jun (DeNardo *et al.*, 2007). In contrast, cytosolic 4ICD may have different functions, which may explain some of the apparently contradictory findings, especially in relation to breast cancer biology.

Mutations and mechanisms of activation

Deregulation of Erb-B signalling pathways has been described in many cancers, including breast, linked to a multiplicity of molecular mechanisms including overexpression due to gene amplification or epigenetic mechanisms, activating mutations of the receptors themselves or activation induced by autocrine/paracrine ligands. EGFR is frequently activated by heterodimerisation with other TKR, and also heterologous receptors such as GPCR via Src. Recently a novel mechanism of EGFR signalling has been suggested: EGF-induced translocation to the nucleus associated with p-Tyr-1068 and indirect binding to DNA via STAT3 enabling EGFR to act as a transcriptional regulator of genes such as cyclin D1 and iNOS - discussed in (Burness *et al.*, 2010).

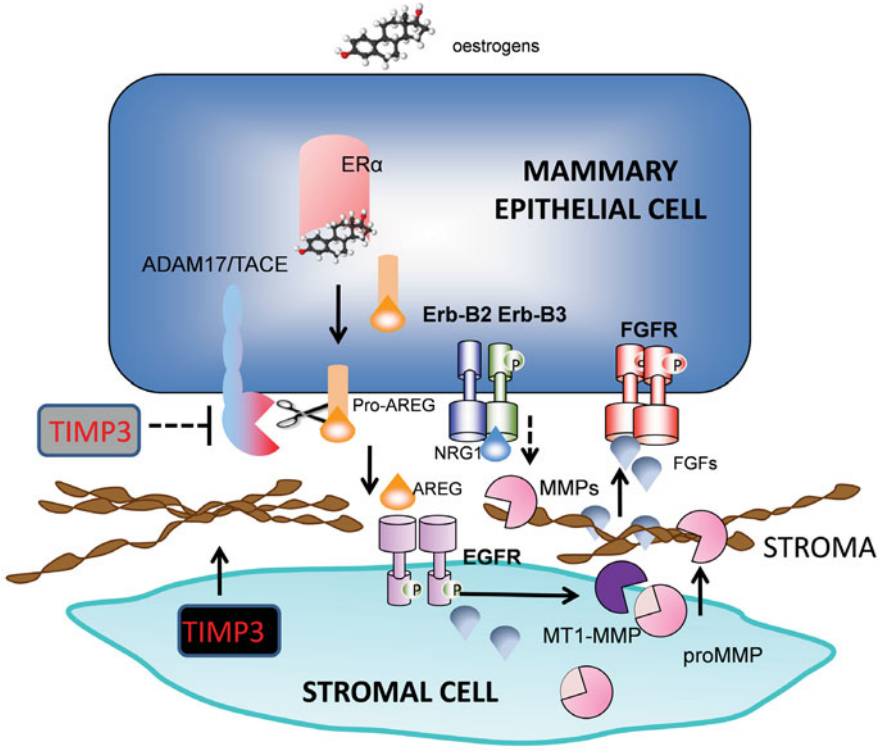

Fig. 2. EGFR/Erb-B signalling linked to epithelial cell-stromal cell interactions in ductal development in the mammary gland. *The Figure illustrates the reciprocal interactions between stromal cells and mammary epithelial cells (MEC) during normal development. ADAM sheddases, regulated by oestrogens, release ligands such as AREG which stimulate EGFR on neighbouring stromal cells. Additional proteases (e.g MMP2 activated by MT1-MMP) release growth factors which reciprocally stimulate MECs to proliferate. MMPs are also required for ductal 'invasion' into the fat pad. Adapted from (Sternlicht and Sunnarborg, 2008).*

Links to breast cancer

All of the four receptors are overexpressed to varying degrees in breast cancer, with their prominence being in rank order Erb-B2>EGFR>Erb-B3>Erb-B4. Many ligands, including NRG splice variants are also overexpressed, suggesting the possibility of autocrine signalling, although the combined measurement of EPG and NRG4 were the strongest predictors of relapse free interval and overall survival (McIntyre *et al.*, 2010).

EGFR

Many early reports of expression/overexpression of EGFR and its ligands in breast cancer and links to prognosis were contradictory as they were largely based on immunohistochemical data and did not necessarily address the activation state of the signalling pathway. More

recent gene expression analyses and also functional studies have clarified the key role that EGFR may play in specific breast cancer subsets (Foley *et al.*, 2010).

Mutations in EGFR are rare in breast cancers but it is amplified in some cases (e.g. metaplastic subtype) (Burness *et al.*, 2010) and is also highly expressed (with or without amplification) in basal breast cancers, a subset of triple negative breast cancers (TNBC; i.e. lacking ER, PGR or Erb-B2). TNBCs represent 10-17% of all breast cancers, are more common in certain non-Caucasian ethnic groups (e.g. those of African descent) and tend to occur at less than 50 years of age. These cancers are also generally of high grade and show distinct patterns of metastasis; notably visceral, liver and brain involvement leading to particularly poor prognosis (Dawson *et al.*, 2009). EGFR expression was found to be higher in patients with nodal or distant metastases than in those without (Sutton *et al.*, 2010). Also, TGFα and NRG2β and the proteases responsible for their cleavage and activation are frequently overexpressed in ER- cancers (suggesting autocrine signalling) whereas AREG is expressed in ER+ cancers and may rather promote paracrine activation *via* the stroma (Foley *et al.*, 2010).

TNBC is particularly prevalent in women carrying a *BRCA1* mutation and EGFR overexpression is found in 67% of *BRCA1* related cancers vs ~ 18% of sporadic cancers. Using human mammary epithelial cell (hMEC) cultures it was shown that even partial suppression of BRCA1 function (using RNAi) could induce EGFR expression and an increase in EGFR+ cancer stem-like cells, suggesting that this receptor could provide a growth advantage at early stages of transformation. Treating (MMTV-Cre) $BRCA1^{flox/flox}$ $p53^{+/-}$ transgenic mice with the EGFR inhibitor erlotinib significantly increased the latency period before mammary tumours developed, although this was limited to ER- and not ER+ subtypes (Burga *et al.*, 2011). These data suggest that early intervention with EGFR inhibitors could be beneficial to *BRCA1* mutation carriers in a preventive setting. However, in established cancers, clinical trials to date have not shown major responses in unselected patient populations (Burness *et al.*, 2010).

Inflammatory breast cancer is particularly aggressive, with the majority of patients having disease in their lymph nodes and over one third with distant metastases at the time of diagnosis. It is characterised by loss of ER, activation of NF-κB, overexpression of RhoC GTPase (resulting in a highly angiogenic phenotype) and a hyperactivated MAPK signalling path which has been linked to overexpression of EGFR and Erb-B2 (Van Laere *et al.*, 2007).

EGFR has also been implicated as a key player in the mitogenic and motogenic effects mediated by the HGF-c-MET signalling axis in breast cancer. HGF and/or c-MET expression increase with tumour progression and each is independently associated with poor prognosis. Cross-talk between these RTK has been identified in several tumour types, with HGF able to transactivate EGFR and conversely EGFR ligands activating c-MET via intracellular signalling pathways. EGFR inhibitors have been shown to attenuate HGF-mediated proliferation, motility and invasion of several breast cancer cell lines *in vitro*.

Erb-B2

Neu, the rat homologue of Erb-B2, was first identified in a chemically-induced neuroblastoma and shown to be similar to a retroviral oncogene (v-Erb-B) related to EGFR. Multiple subsequent studies identified Erb-B2/HER2 in 20-30% of breast cancers, where it is associated with poor prognosis, although it is an early event occurring in over half of *in situ* carcinomas. Interestingly, an Erb-B2 subtype clearly emerged from a genetic analysis of breast cancer which identified five molecular signatures with distinct biological properties (Sorlie, 2004). Breast cancers may express between 25-50 copies of the *ERB-B* gene resulting in up to 2 million receptors per cell. This differential provides a relatively tumour-selective therapeutic target, as levels are absent or low in most normal adult tissues. One exception is the heart, which may explain some of the cardiomyopathies seen with Erb-B targeted therapies, particularly when they were administered with anthracyclines, which is no longer a recommended combination (Procter *et al.*, 2010).

Erb-B2-positive cancers have some unique biological properties, including increased sensitivity to doxorubicin (perhaps because the *HER2* gene is co-amplified with topisomerase 2, the target of doxorubicin) and relative refractoriness to anti-endocrine agents (partly due to an inverse relationship between Erb-B2 and ERα expression levels). It has been suggested that Erb-B2+ breast cancers may be especially prone to post-surgical recurrences due to their proliferative responses to growth factors in wound fluid, and that this could be prevented by trastuzumab. However, only a subset of Erb-B2+ breast cancers responds to trastuzumab, suggesting additional levels of complexity. Recently, more detailed analyses have revealed not only genetic heterogeneity within Erb-B2+ tumours, but also significant epigenetic influences. Those that express high levels of hypoxia-regulated genes show characteristics of basal cancers, and those without behaved more like luminal cancers, showing that even oncogenic drivers such as Erb-B2 are susceptible to modulation by the host microenvironment (Gatza *et al.*, 2011).

Erb-B3

In mouse mammary carcinoma models induced by PyVMT or mutated or overexpressed *neu,* Erb-B3 is frequently activated and found in association with Erb-B2, again attesting to the effective association of this specific dimerisation partnership. These tumours are inhibited by the EGFR inhibitor gefitinib and the dual inhibitor lapatinib: response has been associated with inhibition of Erb-B3 and AKT phosphorylation. In contrast resistance has been linked with a *de novo* point mutation in Erb-B2. In a panel of six Erb-B2-overexpressing human tumour cell lines, Erb-B3 knockdown by RNAi was as effective as Erb-B2 knockdown at inhibiting proliferation *in vitro*, and xenograft tumour growth *in vivo,* whereas EGFR expression was dispensable. Preferential phosphorylation of Erb-B3 was also seen in Erb-B2+ human breast cancers, suggesting a pivotal role for Erb-B3 in Erb-B2-driven tumours (Stern, 2008).

Erb-B3 is overexpressed in around 10% of breast cancers, and its common association with Erb-B2 makes its specific role difficult to determine. Since it has no intrinsic kinase activity, it must function solely as a key dimerisation partner, although it can become phosphorylated by other receptors. The phosphorylated form strongly activates the PI3 kinase pathway as it contains multiple binding sites for the p85 regulatory subunit. In some studies Erb-B3 expression seemed to correlate with ER positivity and was associated with longer overall survival. There is a stronger association with poor prognosis when the gene is amplified (suggesting active genetic selection), although a role for co-amplified genes cannot be excluded. There are thought to be important but poorly understood roles for nuclear localization and secreted isoforms such as p85 soluble Erb-B3, akin to the p95Erb-B2 ECD. The

disparate results reported could also be related to the subcellular localisation of Erb-B3 and its activation status, which in turn are regulated by ligand availability.

Erb-B4

There are contradictory data on the role of Erb-B4 in breast cancer since both positive and negative associations with prognosis have been described; also the full-length and cleaved (4ICD) splice variants may have different functions (Sundvall *et al.*, 2008). It is reportedly associated with luminal A breast cancer subtypes (which have a better prognosis than other groups) perhaps linked to its role in differentiation. It is also generally associated with positive ER status and hence has been predicted to be oestrogen regulated. In support of this is the fact that its promoter contains three possible oestrogen response element half-sites and oestrogen recruits ERα to one of these sites.

Inhibition of Erb-B4 expression can inhibit the proliferation of ER+ breast carcinoma cell lines, suggesting a growth promoting effect that is ERα-dependent and reliant upon cross-talk between these two signalling pathways. Other Erb-B family members also show reciprocal interactions, with mechanisms that include phosphorylation of ERα or its co-activators, linked to ERα-induced upregulation of Erb-B ligands and a fostering of autocrine signalling loops. However, the Erb-B4 mechanism involving interactions between a cell surface and a nuclear receptor is unique.

Paradoxically, stimulation of ER+ breast cancer cells with Erb-B4 ligand, however, can result in cell death, even though it is also dependent on proteolytic release of 4ICD. It has been proposed that, since this activity is independent of nuclear localisation, it may be the result of 4ICD activity in the cytosol. 4ICD contains motifs similar to pro-apoptotic BH3 proteins and localises in mitochondria and the endoplasmic reticulum; also its anti-tumour activity is attenuated by the caspase inhibitor zVAD, suggesting that it is a *bone fide* apoptosis inducer. In support of this function, an association between cytosolic expression of 4ICD and apoptotic cells was noted in human breast cancers (Jones, 2008). A unifying hypothesis to explain these disparate functions has been proposed whereby in early stages of breast cancer development, ligand (mainly HRGα) activation of Erb-B4 generates nuclear 4ICD. ER+ cells have a growth advantage and a 4ICD-dependent autocrine loop develops, shifting from a Stat5 co-activator of differentiation to an ERα co-activator driving proliferation. At later stages both ERα and Erb-B4 may be lost, perhaps being redundant in the face of additional even stronger oncogenic drivers (Jones, 2008). These intriguing possibilities (and their therapeutic implications) are currently being actively investigated.

These varying associations between the Erb-B receptors and breast cancer biology are interesting as they may echo their contrasting roles in mammary gland development: promoting cell proliferation/'invasion' of ductal epithelial cells into the mammary fat in the case of EGFR/Erb-B2/3 and a function in epithelial differentiation for Erb-B4 (Stern, 2008). It may be that subsets of precursor cells, representative of a specific stage of breast development become trapped in that phenotype, unresponsive to normal regulatory cues.

Ligands and downstream signalling pathways in breast cancer

Given the importance of the ADAM17-AREG/TGFα-EGFR axis in normal mammary development, it is not surprising that all of these elements – and those that they regulate such as additional proteases and growth factor signalling pathways - are misregulated in breast cancers. ADAM17, AREG and TGFα are frequently upregulated, with co-expression of TGFα and EGFR being associated with particularly poor prognosis. Antisense suppression of AREG reduced the tumorigenicity of immortalised human mammary epithelial cells and prevented EGFR becoming activated in response to exogenous ligands (Ma *et al.*, 2010).

In an interesting series of experiments with human mammary epithelial cells at different stages of transformation from immortalised – premaligant - tumorigenic, it was shown that progression was associated with upregulation of AREG and TGFα, rendering them independent of exogenous EGF. In 3D cultures, reversion to a non-malignant phenotype was achieved by inhibitors of proteolytic activity or EGFR, suggesting an autocrine MMP/ADAM-dependent EGFR activation pathway. Similar effects were achieved by ADAM17 siRNA (Kenny and Bissell, 2007), suggesting that this protease may be a good therapeutic target in EGFR-dependent breast cancers.

Angiogenesis, invasion and metastasis

All solid tumours require the ability to co-opt host vasculature and/or stimulate *de novo* angiogenesis in order to grow progressively. The newly formed vasculature is leaky and provides a ready conduit for haematogenous dissemination. One of the major angiogenic growth factors is VEGF, which is upregulated not only by hypoxia, but also via the Erb-B oncogenes, likely via the PI3K-AKT signalling pathway. Erb-B2 and EGFR overexpression tends to correlate with increased levels of VEGF A (and also lymphangiogenic cytokines VEGF C and D) and in some cases with higher microvessel density.

Hypoxia itself is a known adverse prognostic indicator, and many HIF-responsive genes (such as MMPs, CXCR4, c-MET, LOX) are implicated in angiogenesis and metastasis. In the case of the G-protein-coupled chemokine receptors such as CXCR4, and its ligand CXCL12/SDF-1α, their expression and activation has also been linked to site-selective metastasis. Erb-B2 and CXCR4 levels tend to correlate in breast cancers, and the former is reported to enhance CXCR4 expression and to inhibit ligand-induced degradation. Interestingly, inhibition of CXCR4 expression suppressed Erb-B2-mediated malignant potential suggesting a mechanistic linkage between the two signalling axes.

In order to metastasise, cells must detach from underlying extracellular matrix (ECM), acquire the ability to survive under these anchorage independent conditions, and demonstrate the ability to invade surrounding tissues and basement membranes. Invasion requires a motile phenotype and, in most cases, is potentiated by proteolytic activity, although protease-independent 'amoeboid' motility has also been described. An early manifestation of breast cancer is the ability of transformed but premalignant cells to proliferate within the ductal lumen, away from their natural ECM niches. Detachment usually induces downregulation of EGFR (and cell death) but this can be overcome by Erb-B2, which stabilises EGFR and β1 integrin via Erk-Sprouty2 signalling and a lowered affinity of the heterodimers for c-Cbl, which normally promotes EGFR trafficking to lysosomes (Grassian *et al.*, 2011).

Erb-B2 and EGFR are also recognised as activating key signal-

ling pathways promoting cell motility; indeed EGFR ligands are very potent chemotactic factors, stimulating rapid migration along concentration gradients. AIB1/SRC3 is associated with breast invasion and metastasis. A splice variant (SRC-3Δ4) promotes NRG-Erb-B2 mediated motility, co-operating with Erb-B2 to induce progression of DCIS to invasive ductal carcinoma. Erb-B2 reportedly selectively promotes MDA-MB-468 migration mediated by EGF via phosphorylation of tyr^{1248} and a transient activation of PLCγ. Others have implicated Tyr^{1227} and Shc-Memo signalling in response to HRG in T47D cells, although in both cases PLCγ was an important contributor. HRGβ1-Erb-B3 signalling also significantly enhances metastasis via PI3 kinase signalling, since mutation of its six YXXM PI3K p85-binding domains inhibited breast carcinoma cell motility, invasion, vascular intravasation and invasion (Smirnova *et al.*, 2011).

Overall, EGFR/Erb-B signalling has been linked to all aspects of metastasis: stimulation of angiogenesis, alterations in cell-cell and cell-matrix adhesion, upregulation of proteases and other key molecules (CXCR4, CD44, specific integrins), vessel intravasation/extravasation and organ-selective colonisation (Eccles, 2001) (Fig. 3). There is now a growing appreciation that bone-marrow-derived mesenchymal stem cells (MSC) are recruited to the stroma of developing tumours and also contribute to formation of the 'premetastatic niche'. Stimulation of MSC with tumour cell-derived TGFα simulated release of angiogenic factors and induced breast carcinoma cell migration; another example of mutual Erb-mediated tumour-host interactions involved in tumour progression (De Luca *et al.*, 2011).

Dissemination to lymph nodes is a key factor in breast cancer staging, but increasingly identification of disseminated tumour cells (DTC) in the blood and in bone marrow aspirates is also being linked to poor prognosis. These cells frequently overexpress EGFR and Erb-B2 (even when the primary tumour is negative)(Braun *et al.*, 2001). Such observations have led to the acceptance that dissemination may be a much earlier event than originally appreciated (Eccles and Paon, 2005) and that the Erb-B proteins, amongst others, may significantly contribute to tumour cell escape, motility and dissemination.

Organotropism and site selectivity of metastasis

The commonest site of breast cancer metastasis is the bone, and there is a well-described 'vicious cycle' involving both tumour and host cells that perpetuates tumour cell colonisation, metastasis development and bone destruction (Guise, 2010). Although there is reportedly no clear association between high levels of specific Erb-B receptors (or their ligands) in bone metastases – and indeed Erb-B2 seems to be under-represented – there is good evidence of a role for ligand-activated proteases in the bone microenvironment (Foley *et al.*, 2010).

Seminal work by Massague's group deciphered the genetic determinants underlying key phases of metastasis in the MDA-MB-231 breast carcinoma model: escape from the primary tumour, extravasation and growth at secondary sites. Sublines with tropism for specific sites (bone, lung, brain) were isolated and their key characteristics analysed. Osteoclast activity in the bone microenvironment is regulated by a balance between RANKL and osteoprotegerin (OPG). The presence of breast cancer cells upsets this balance, leading to a net increase in bone destruction via os-

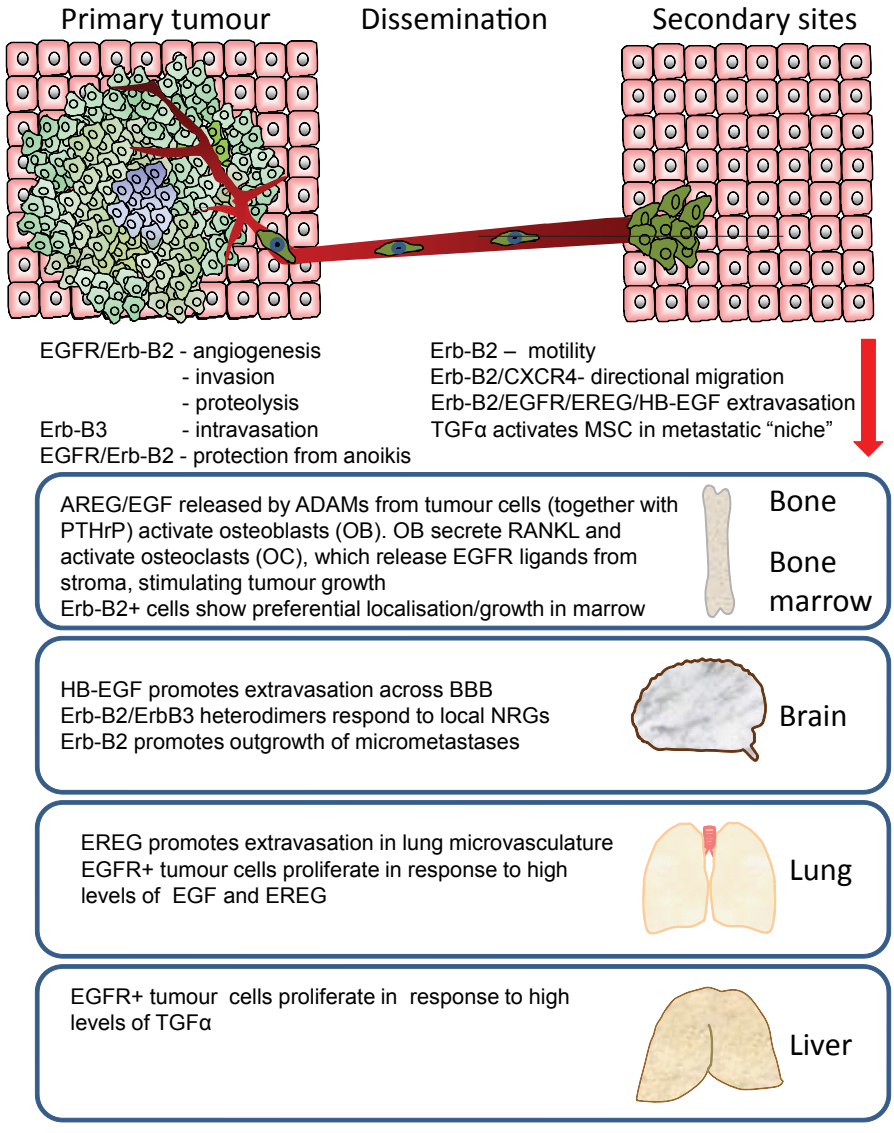

Fig. 3. Role of EGFR/Erb-B receptors and ligands in metastasis and organotropism. *Most if not all of the Erb-B family have been linked to an increased probability of breast cancer metastasis and, in some cases to site-selective growth. At the primary tumour site, EGFR and Erb-B2 in particular regulate factors that promote angiogenesis and invasion. Protection from anoikis and motility contribute to the ability of tumour cells to survive in the circulation, and specific ligands promote extravasation through the specialised microvasculature of different organs. There is limited involvement in the early generation of 'pre-metastatic niches', but finally, tumour cells expressing EGFR or Erb-B receptors respond to local growth factors to generate overt metastases. OB = osteoblasts, OC= osteoclasts.*

teoclasts, and release of sequestered growth factors which in turn stimulate growth and invasion of the tumour (Kang *et al.*, 2003).

Ligands such as EGF or AREG shed by tumour cells activate EGFR-expressing osteoblasts to secrete less OPG; simultaneously autocrine stimulation releases PTHrP from tumour cells to the same end, and the osteoblasts in turn release EGFR ligands and perpetuate the cycle of monocyte-derived osteoclast activation via RANKL or MCP and thus bone destruction (Foley *et al.*, 2010). PTHrP has been recognised as one of the "metastasis virulence factors" within a bone metastasis gene signature. It transcriptionally regulates AREG and to a lesser extent TGFα and HB-EGF, and may also increase ligand shedding via ADAM17. MMP1 and ADAMTS-1, additional members of the 11-gene signature, also increase AREG shedding and bone metastasis. Although MDA-MB-231 represents an ERα negative breast cancer, since AREG is regulated by oestrogen, this pathway could be of more general significance in breast cancer. However, it remains to be seen whether EGFR inhibitors will have an impact, alone or in combination, against bone metastasis.

A degree of organotypic metastasis selection is determined by the ability of tumour cells to extravasate through the phenotypically-distinct vasculature within specific organs. The bone marrow and liver vasculature is fenestrated and does not provide a significant barrier to tumour cell colonisation; however the lung endothelium has tight junctions and the blood-brain barrier (BBB) is even further specialised. EREG, together with COX-2, MMP-1 and ANGPTL4 has been linked to an enhanced capacity of breast tumour cells to extravasate in the lungs since together they can compromise the integrity of the pulmonary microvasculature. In contrast, HB-EGF, COX2 and specifically the α2,6-sialyltransferase ST6GALNAC5 were strongly associated with breast cancer brain metastases (Bos *et al.*, 2009) (Fig. 3).

The predilection of breast cancers expressing Erb-B oncogenes to metastasise to the brain may also be due to the fact that their cognate ligands (NRGs) are neural growth factors. It has been shown experimentally that Erb-B2 overexpression increases the outgrowth of breast cancer cells in the brain, rather than the initial seeding efficiency and formation of micrometastases (Palmieri *et al.*, 2007). The co-association of CXCR4 and Erb-B2 may be linked to visceral metastases, and similarly cells overexpressing EGFR could respond to the high levels of ligands such as TGFα and EREG in liver and lung (Eccles and Welch, 2007) (Fig. 3).

Therapy targeting the EGFR/Erb-B family

EGFR and Erb-B2 have been the main receptors considered as targets for immunotherapeutic approaches in breast cancer, mainly via antibody-based therapies, but also in active immunisation and gene therapy protocols, as well as ligand-targeted toxin and antisense/RNAi approaches and anti-Erb-B2 vaccines (Ladjemi *et al.*, 2010) (Table1; Fig. 4).

A novel means of inhibiting Erb-B expression and function is via HSP90 chaperone inhibitors such as 17-AAG and NVP-AUY922. HSP90 levels correlate with poor prognosis in breast cancer and Erb-B2 is a particularly sensitive 'client' protein, being highly dependent on HSP90 for its correct folding and cellular localisation. HSP72, a related chaperone, has also been shown to be essential for Erb-B2-driven oncogenesis in transgenic mouse models by

TABLE 1

EXAMPLES OF THERAPIES TARGETING THE EGFR/ERB-B FAMILY

Agent	Type	Target(s)	Comments
Trastuzumab (Herceptin) and T-DM1	Humanised IgG1 monoclonal antibody	Erb-B2 juxtamembrane region (domain IV)	Approved for Erb-B2+ MBC and node+ early stage disease. Also conjugated to DM1, (maytansine toxin) for targeted delivery. Phase III. Active in trastuzumab-resistant cells
Pertuzumab (Omnitarg)	Fully humanised IgG1 monoclonal antibody	Erb-B2 dimerisation domain (II)	Inhibits dimerisation with EGFR and Erb-B3. Phase III breast cancer
Cetuximab (Erbitux)	Chimaeric IgG1 monoclonal antibody	EGFR ECD	Little activity in EGFR+ breast cancer
Panitumumab (Vectibix)	Fully human IgG2 monoclonal antibody	EGFR ECD	Little activity in EGFR+ breast cancer
Ertumaxomab	Bispecific monoclonal antibody	Erb-B2 and FcγRI/III	Promotes ADCC via T cells. Phase II breast cancer
Gefitinib (Iressa)	Reversible TKI (quinazoline)	EGFR kinase domain	Limited activity in breast cancer
Erlotinib (Tarceva)	Reversible TKI (quinazoline)	EGFR kinase domain	Limited activity in breast cancer
Lapatinib (Tykerb)	Reversible TKI 4-anilinoquinine	EGFR/Erb-B2 kinase domain	Response linked primarily to Erb-B2 overexpression.
Neratinib (HKI-272)	Irreversible TKI	Pan-Erb-B kinase domain	Active in cells with EGFR and Erb-B2 mutations. Phase I/II + temsirolimus in Erb-B2+ or TNBC
Afatinib (BIBW-2992)	Irreversible TKI	EGFR/Erb-B2 kinase domain	Active in trastuzumab-resistant breast cancer. Plans to trial in inflammatory BC and in several combinations
Canertinib (CI-1033)	Irreversible TKI	Pan-Erb-B kinase domain	Phase II results poor in lung and ovarian cancer
AEE788	Reversible TKI	EGFR, Erb-B2 VEGFR	Added benefit with letrozole in preclinical breast cancer models. Phase I/II in other cancer types
BMS-599626 (AC480)	Reversible TKI	EGFR/Erb-B2 kinase domain	Inhibits EGFR-ErbB2 heterodimers. Phase I
Arry-334543	Reversible TKI	EGFR/Erb-B2/B4 kinase domain	Phase II in breast cancer
MM-111	Bispecific fusion protein	Blocks Erb-B3 ligand binding	Targets ErbB2-B3 heterodimers
Tanespimycin (17-AAG)	Ansamycin	HSP90 chaperones	Targets ErbB2, AKT, VEGFR, ERα. Phase III breast
Retaspimycin (IPI-504)	Ansamycin	HSP90 chaperones	Targets ErbB2, AKT, VEGFR ERα, Phase II breast
NVP-AUY922	Isoxazole resorcinol	HSP90 chaperones	Targets ErbB2, AKT, VEGFR, ERα. Phase I/II breast
BIIB 021	Purine scaffold	HSP90 chaperones	Phase II in ER+ MBC + Exemestane

regulating senescence signalling pathways (Meng *et al.*, 2011). Other key client proteins are also important in breast tumour development (e.g. ERα) and progression, for example: AKT in cell survival and resistance to multiple agents; VEGF receptors in angiogenesis/lymphangiogenesis; FAK, Src and MET in invasion - to name but a few. HSP90 inhibitors induce depletion and proteasomal degradation of Erb-B2 and other client proteins *in vitro* and *in vivo,* resulting in potent antitumour and antiangiogenic activity in preclinical tumour models (Eccles *et al.*, 2008), most notably in ERα+/Erb-B2+ BT474 xenografts. Recent clinical trial data in MBC where patients had progressed on trastuzumab are very promising (Modi *et al.*, 2011). In preclinical and clinical studies, trastuzumab labelled with positron-emitting isotopes has also been used to monitor responses as described later in this review.

Inhibiting ligand binding/dimerisation

The anti-EGFR antibody cetuximab has been tested in combination with a variety of standard chemotherapeutic agents in breast cancer but with little benefit and sometimes unacceptable skin toxicity. Cetuximab is a chimeric IgG1, whereas panitumumab is a fully human IgG2 anti-EGFR monoclonal antibody which has been less frequently evaluated in breast cancer (Burness *et al.*, 2010).

Trastuzumab, a humanised anti-Erb-B2 monoclonal antibody targeting the juxtamembrane region of the extracellular domain, has been more successful in clinical trials, particularly in combination with standard chemotherapy and in the adjuvant setting (Goel *et al.*, 2011). This sensitisation to chemotherapy may involve downregulation of Mcl-1, an antiapoptotic protein and/or activation of PTEN which dephosphorylates AKT (a key survival signal) thereby promoting cell death. Trastuzumab is reportedly most active in tumours driven by Erb-B2 homodimers and is also effective in combination with antiendocrine therapies in ER+ tumours. Given the many key cellular functions activated downstream of Erb-B signalling, it is not surprising that trastuzumab (and other Erb-B-targeted therapies) also inhibit angiogenesis, which could contribute indirectly to tumour responses. Combinations of trastuzumab with bevacizumab, an antibody targeting the major angiogenic cytokine (VEGF) are also being trialled.

In addition to direct inhibition of Erb-B2 function, e.g. by promoting its internalisation and degradation, trastuzumab can promote antibody-directed cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells. Interestingly (but unfortunately) an increase in the incidence of brain metastases has been observed in patients treated with trastuzumab. This may reflect both the fact that Erb-B2 positive tumours have a predilection for colonising the brain and because antibodies fail to cross the BBB effectively.

Pertuzumab is another humanised antibody that inhibits Erb-B2 heterodimerisation with other family members by binding to the dimerisation loop of the former (i.e a different site from trastuzumab). It has shown some promise in Erb-B2+ breast and ovarian cancer patients and is also being evaluated in combination with trastuzumab and chemotherapy (CLEOPATRA trial) (Baselga and Swain, 2010). In general, Erb-B2 targeted therapies are only effective in cancers with gene amplification, and sensitive assays are needed to determine those eligible (e.g. HercepTest or Oncotype Dx). Antibodies to Erb-B2 have also been employed to measure expression levels in tumours and also to monitor responses to therapy using positron emission tomography (PET) or Erb-B2-targeted nanoparticles in MRI approaches, since they report non-invasively on the level of membrane-exposed receptor (Capala and Bouchelouche, 2010). Similarly, ELISA or dot-blot assays can be performed on plasma samples to monitor the levels of Erb-B2 ECD and also reportedly correlate with tumour levels in several studies.

Advances in antibody-based therapies include the targeting of

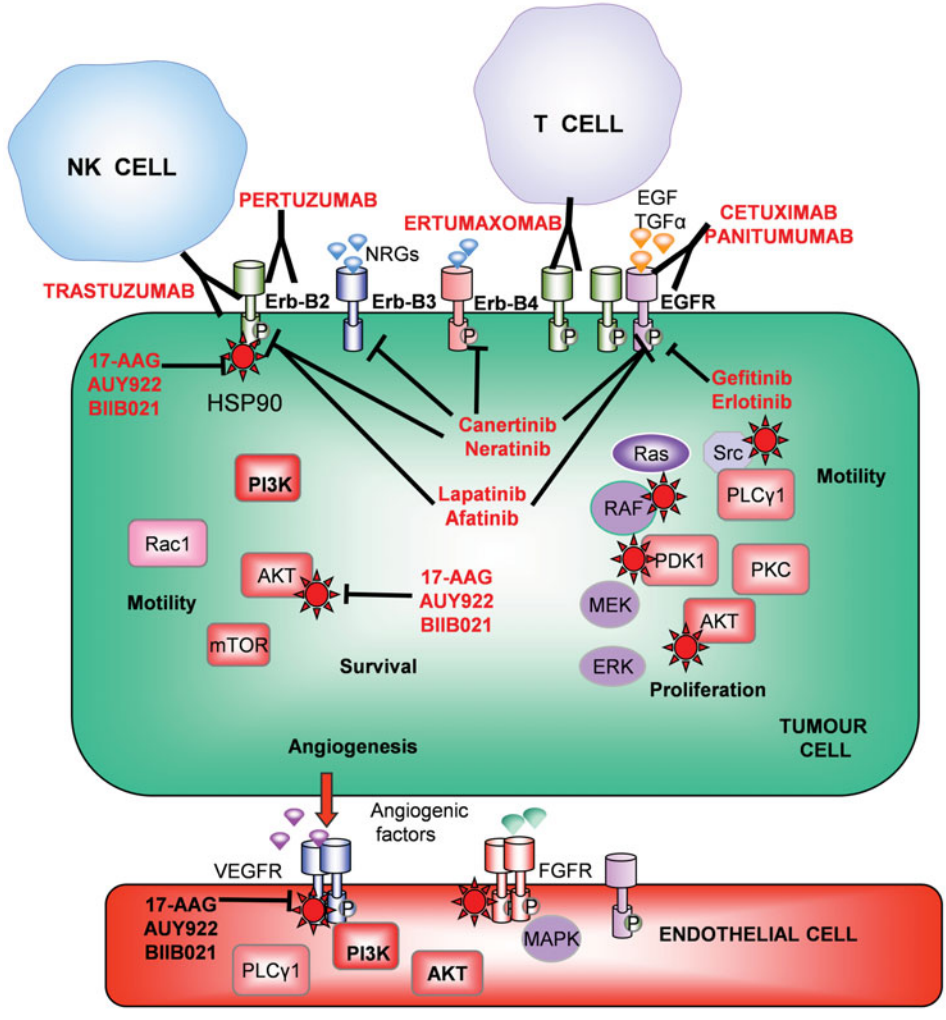

Fig. 4. Key EGFR/Erb-B signalling pathways and sites of action of therapeutic agents. *The figure illustrates monoclonal antibodies, small molecule kinase inhibitors and other agents (e.g. chaperone inhibitors) and their mechanisms of action in breast cancer cells and the associated vasculature.*

prodrug activating enzymes or nanoparticles carrying cytotoxic payloads (Colombo *et al.*, 2010). Recently, complexes of a compact human anti-Erb-B2 antibody and cytotoxic human pancreatic RNase have shown promise *in vitro* and *in vivo* in a rodent breast tumour model (Borriello *et al.*, 2011). In experimental systems, it has been shown that using combinations of anti-Erb-B2 antibodies recognising different epitopes are more effective at inhibiting ligand-mediated invasion than single agents.

Inhibiting kinase activity

Several reversible and irreversible SMTKI with varying degrees of selectivity have been developed. These small molecule agents compete with ATP binding in the kinase domain of the receptor and inhibit downstream signalling. The most well known are the EGFR selective inhibitors erlotinib and gefitinib, the dual EGFR/Erb-B2 inhibitor lapatinib and the more recently developed irreversible inhibitor afatinib.

Gefitinib and erlotinib have shown little if any single agent activity in breast cancer, even in triple negative cases which often have high levels of EGFR. Several trials are underway where EGFR TKI are used in combination with other cytotoxic or molecularly targeted agents.

Lapatinib binds the ATP-binding pocket of both EGFR and Erb-B2, preventing autophosphorylation, potentially limiting 'escape' or rapid development of resistance to monotherapies. Unlike trastuzumab, activation of the PI3K pathway does not seem to impair its efficacy. It has been used alone and in combination with both cytotoxic and molecularly targeted agents. Combination with trastuzumab improved median progression-free survival and reduced the risk of disease progression in a phase III trial of heavily pre-treated patients and it has also been approved for use in those who have failed trastuzumab. Most responses have been seen in Erb-B2-positive patients, with little or no benefit in patients with triple negative or EGFR-positive cancers (Burness *et al.*, 2010). Neratinib is another orally active agent which irreversibly inhibits the kinase activity of EGFR, Erb-B2 and Erb-B4 which has shown promise in previously untreated and also trastuzumab-resistant breast cancers (Colombo *et al.*, 2010). It is also being trialled in combination with paclitaxel, capecitebine and vinorelbine.

Links between Erb-B signalling and resistance to therapy

EGFR activation has been linked to resistance to the anti-endocrine agent tamoxifen, possibly via phosphorylation of AIB1, an ERα co-activator which can result in mitogenic (rather than inhibitory) activity. Phosphorylation of ERα (which can be achieved by several upstream kinases including Erb-B2) can also lead to oestrogen-independent transcription of ERα targets, also resulting in resistance to tamoxifen. There is cross talk between ERα, c-Src, EGFR, and STAT5b in ER+ breast cancer cells and increased EGFR and c-Src signaling is associated with tamoxifen resistance. Constitutively active STAT5b increased DNA synthesis and conferred tamoxifen resistance in ER+ human breast carcinoma cell lines. Cells exposed to a pure anti-oestrogen (fulvestrant) acquired a resistant phenotype which included elevated expression and phosphorylation of EGFR and Erb-B2, together with TGFα and AREG expression. More recently, fulvestrant has also been shown to upregulate Erb-B3 and/or Erb-B4 and to sensitise cells to NRGβ1 (Hutcheson *et al.*, 2011). It is possible, therefore, that mitogenic autocrine signalling via any or all of the Erb-B receptors may subsume the role of oestrogen during breast cancer progression.

EGFR activation stimulates signalling via AKT and ERK, and has been linked to multidrug resistance via upregulation of MRP-1,3,5 and 7. Erb-B3 (and also Erb-B4) has also been linked to resistance to anti-endocrine therapy (Sutherland, 2011) and to paclitaxel, reportedly by upregulating survivin via the PI3K-AKT-mTOR pathway. Amphiregulin expression has been associated with resistance to cisplatin in MCF7 cells and in patients via enhanced EGFR, AKT and ERK1 activation. EGFR and Erb-B2 activation may also be involved with resistance to radiotherapy, since both are upregulated/activated following exposure to ionising radiation.

Several mechanisms of acquired resistance to EGFR/Erb-B2 targeted therapies have been described, including subversion of trastuzumab binding to Erb-B2 by shed p95 ECD fragments (Arribas *et al.*, 2011); a switch to alternative signaling via other Erb-B family members (notably Erb-B3), alternative RTK such as c-MET, IGF1R or EphA2, and finally activated downstream signalling elements (e.g. PI3 kinase-AKT-FOX1A) for example via loss of PTEN or by *PIK3CA* mutations. (Garrett and Arteaga, 2011). In the early days, the development of host antibodies against the therapeutic antibody could neutralise their activity upon repeat administration. However this has now largely been overcome by the development of humanised (or fully human) therapeutic antibodies.

In addition, there is evidence of primary resistance (or lack of sensitivity) in many patients, in spite of the fact that their tumours overexpress the target receptor, although the reasons for this – which are likely to be many and various – are less well understood. One mechanism may involve negative feedback loops in cases where trastuzumab fails to abolish Erb-B2 phosphorylation. This may occur due to trans-phosphorylation by other Erb-B receptors, following an AKT-dependent ADAM17-mediated release of Erb-B ligands (Gijsen *et al.*, 2010). Also, c-SRC has recently been identified as a common mediator of multiple trastuzumab resistance pathways in both acquired and *de novo* trastuzumab-resistant cells, involving dephosphorylation by PTEN. Increased c-SRC activation conferred resistance in breast cancer cells and correlated with trastuzumab refractoriness in patients. Targeting c-SRC re-sensitized cells to trastuzumab and eliminated trastuzumab-resistant tumours *in vivo*, suggesting a potential clinical application of this strategy (Zhang *et al.*, 2011).

Molecular mechanisms of resistance to small molecule inhibitors generally differ from those linked to refractoriness to antibody therapy. In the former case, mutations in the kinase domain of EGFR emerge which reduce the binding affinity SMTKI, whereas in the latter case, as described above, downregulation of the primary molecular target, a shift to non-inhibitable dimers (such as Erb-B2-IGF1R) or a short-circuit of the downstream signalling pathways is more common. Other reported resistance mechanisms include a mutation in exon 21 of the *HER2* gene that encodes a protein with reduced affinity for trastuzumab and upregulation of MUC4, a membrane-bound glycoprotein which binds Erb-B2 and can compete with trastuzumab binding.

Future strategies will need to include rational combinations of therapeutic agents to overcome (or pre-empt) resistance. Research laboratories developing new targeted agents are deliberately generating cells with acquired resistance in order to understand future likely escape mechanisms of cancers in the clinic. These

cells are then subjected to microarray or to proteomic analysis to uncover the mechanisms of resistance. If this reveals a mutated target (as in the case of EGFR), then screens can be performed to identify new compounds which can bind the altered conformation; alternatively, 'synthetic lethal' screens of either known therapeutic agents or siRNA libraries can be used to identify new vulnerabilities (Achilles' heels) in the resistant cells which may be exploitable as targets. One good example is the multikinase inhibitor dasatinib, which shows synergistic anti-proliferative activity with trastuzumab in Erb-B2 expressing BT474 human breast carcinoma cells both *in vitro* and *in vivo* (Seoane *et al.*, 2010). Since one of the kinase targets of dasatinib is c-SRC, this protocol may also help to prevent development of resistance as outlined in the previous section.

Cancer stem-like cells (CSC)

Cells which express one or more supposed markers of stem cells (e.g. $CD44^+/CD24^-$ $ALDH1^+$ and with their phenotypic characteristics (self-renewal capacity, anchorage-independent growth, pluripotency) have been isolated from mammary glands and breast cancers. It has been proposed that such cells may represent the true seeds of cancer, contributing to drug resistance (due to expression of drug efflux pumps and low proliferative rate) and relapse. Normal breast stem cells show a 'basal' phenotype, hence breast CSC may be endocrine resistant because they express low levels of ERα. Ligand-induced EGFR signalling is required to support the clonogenicity of CSC derived from cancers and DCIS in soft agar, and overexpression of Erb-B2 in MEC increases the proportion of cells with CSC properties. Normal breast epithelial stem cells are highly dependent on EGFR and other RTK, raising the possibility that their increased expression in endocrine-resistant breast cancers reflects an increased proportion of CSCs selected for by endocrine therapies. Several studies show that breast CSCs are ERα+/EGFR+/Erb-B2+, supporting this view (O'Brien *et al.*, 2011). These observations suggest that Erb-B2 and/or EGFR inhibitors could target the CSC population, and indeed one small study with the dual inhibitor lapatinib suggested that it reduced the frequency of CD44+/CD24- cells in breast cancer biopsies (Schmidt, 2008). Further studies are required to establish whether stem-like cells, perhaps shed early in cancer progression, may remain dormant and refractory to therapy until reactivated, when they may contribute to treatment relapse and metastasis. There is no doubt that we will need to pay more attention to the genotypic phenotypic properties of pre-existing micrometastases if we are ever to provide robust and sustained control of disseminated breast cancer.

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