ErbB receptor ligands bind to their respective receptors, initiating the formation of homo- or heterodimers. The intracellular kinase domain of one receptor trans-phosphorylates the intracellular tyrosine residues on the opposite receptor thereby activating downstream signalling pathways.

At a cellular level, ErbB receptor ligands control a number of processes, including cell cycle progression, proliferation, cell death, protein synthesis, metabolism and differentiation. Physiologically this results in regulation of wound healing, neonatal growth and development as well as the development of adult tissues. Alterations in ErbB receptor signalling can result in oncogenesis in response to increased proliferation and decreased cell death as well as up-regulation of processes required for cell metastasis, such as adhesion, migration, invasion and neo-angiogenesis.

1.1 ErbB receptors and their structure

The epidermal growth factor receptor (EGFR/HER1) and the other family members (c-ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4) are 160–190 kDa transmembrane (type 1) receptor tyrosine kinases. They each comprise extracellular ligand binding and cysteine-rich domains, a transmembrane region, a kinase domain and an intracellular C-terminal tail, which contains the multiple tyrosine phosphorylation sites that are required for regulating receptor activation (reviewed in Ferguson, 2008).

EGFR was the first member of the family to be identified; it is a 170 kDa glycoprotein (Carpenter, 1987). Co-purification of the receptor with its growth factor ligand (epidermal growth factor, EGF) was reported in 1979 (McKanna et al.,
1979), which followed the discovery of EGF in 1972 (Savage et al., 1972) and Stanley Cohen’s pioneering work showing that EGF bound to the surface of cells (Cohen et al., 1975; Carpenter et al., 1975, 1978). HER2 was characterised in the 1980s as a 185 kDa protein (Schechter et al., 1984) and has been shown to be highly homologous to EGFR (Coussens et al., 1985). There are proto-oncogenic and oncogenic forms of HER2 and these differ in sequence by a single amino acid substitution (Bargmann et al., 1986). HER2 is the preferred dimerisation partner of the other three family members and it is always available for dimerisation, as it largely exists in normal cells in a monomeric state (Weiner et al., 1989a).

ErbB3 and ErbB4 were identified as the third and fourth members of the EGFR/ErbB family in the late 1980s based on their sequence homology with EGFR (Plowman et al., 1990, 1993; Kraus et al., 1989). Much of the sequence is conserved between the family members, with the highest degree of homology between each receptor and EGFR being in the kinase domain.

Of the family, EGFR and ErbB4 are the only fully functional members. ErbB3 has minimal kinase activity so the formation of ErbB3 homodimers does not result in active signalling and, as yet, there has been no ligand identified for HER2.

1.2 ErbB ligands

The monomeric growth factor ligands in this peptide family are 45–60 amino acids and they contain six conserved cysteine residues, which are linked by three disulphide bonds. EGF was the first factor to be characterised over 40 years ago (Savage et al., 1972), followed eight years later by transforming growth factor-α (TGFα) (Roberts et al., 1980; Torado et al., 1980). Shortly following its initial discovery, EGF was shown to stimulate DNA synthesis and cell proliferation (Carpenter and Cohen, 1976).

In the 30 years since the discovery of TGFα, the family has grown to over 12 ligands that have different receptor binding preferences and therefore have the ability to regulate different cellular events (Figure 1.1).

EGF, TGFα, epigen and amphiregulin bind to EGFR; epiregulin and heparin binding EGF-like growth factor (HB-EGF) bind to EGFR and HER4; the neuregulins (1–6) have binding preferences for both HER3 and HER4, and betacellulin (BTC) binds to HER2, HER3 and HER4 (reviewed in Eccles, 2011). What is perhaps most striking is that despite appearing to have a functional ligand-binding domain, no ligand has yet been identified that binds HER2 with high affinity, although HER2 will bind with low affinity to the EGF family of ligands.

1.2.1 Ligand production

EGF family ligands are secreted but often require cleavage, unlike ligands for other receptor tyrosine kinases. The ligands are found tethered to the external
1.2 ERBB LIGANDS

NeuReGulins (NRG1-6)
BTC
EGF, TGF
Amphiregulin (AR)

Figure 1.1 Schematic representation of the ErbB receptors. All four receptors are depicted and the percentage sequence homology in each domain with the EGFR is indicated. The extracellular region of the receptor has four subdomains, two of which (1 and 3) are involved in ligand binding and two (2 and 4) are cysteine rich and are involved in mediating dimerization. Individual ligands have different binding affinities for specific receptors; note that ErbB2/HER2 does not have a ligand (indicated by ?) and that ErbB3 does not have an active kinase domain (indicated by X), possibly as a result of its reduced homology with EGFR.

surface of the cell membrane in pro-forms and require proteolytic cleavage in order to be released. For many ErbB ligands this is carried out by the disintegrin and metalloproteinase, ADAM17 (Hinkle et al., 2004; Sahin et al., 2004 reviewed in Booth and Smith, 2007) via a process that is known as ectodomain shedding. In vivo evidence that ADAM17 acts upstream of EGFR also comes from knock-out mice. Both ADAM17−/− and EGFR−/− mice display aberrant developmental phenotypes (Wiesen et al., 1999; Jackson et al., 2003; Yamazaki et al., 2003) and EGFR activation only occurred when ADAM17 and amphiregulin were expressed (Sternlicht et al., 2005).

Once soluble, ligands can activate the receptors in paracrine, autocrine or endocrine fashions. This mechanism forms the basis of some types of signalling cross-talk (Chapter 9).

1.2.2 Effects of ligand binding to receptors

The extracellular domains of the receptors are responsible for ligand binding and facilitate most of the dimerisation events. Many of our insights into the
mechanisms of ligand binding and the events involved in receptor dimerisation have come from experimental mutations of the receptors (reviewed in Brennan et al., 2000; Ferguson, 2008). Once the ligand has bound to the receptor, an event that occurs with a 1:1 stoichiometry, a change occurs in the conformation of the receptor that facilitates downstream phosphorylation events and signalling transduction.

A number of groups have presented models for investigating ligand–receptor interactions. When the ligand binds to its receptor, a large domain rearrangement occurs that ultimately results in receptor dimerisation. Dimerisation itself is mediated in part, but not solely, by a dimerisation arm or loop that protrudes from the receptor due to the structural rearrangement that takes place upon ligand binding removing the arm from its intra-molecular tether (Garrett et al., 2002; Ogiso et al., 2002; Ferguson et al., 2003; Greenfield et al., 1989). Exposure of the dimerisation arm initiates the subsequent dimerisation of the receptors with an asymmetric interaction between the intracellular domains (Figure 1.2). In contrast with other signalling pathways (such as IGF, see Chapter 2) ErbB dimerisation involves direct interaction between the receptors, rather than via an association mediated through a divalent ligand that acts as a molecular ‘bridge’ (reviewed in Ferguson, 2008).

In addition, ligand binding also brings about additional conformational changes that are required for dimerisation including rotation of part of the receptor (Ogiso et al., 2002 and Ferguson et al., 2003). It is clear that the spatial arrangement of the receptors is important in order that additional contact points can be made at the extracellular interface between the two receptors undergoing dimerisation.
1.3 Downstream signalling molecules and events

Wilson’s hypothesis is supported by our biological knowledge. It has been evident for some time that the different receptors are capable of activating different downstream signalling cascades. When ErbB2/ErbB3 heterodimers form, the cytoplasmic tail of ErbB2 activates the Erk-MAPK pathway and ErbB3 activates PI3K-Akt (phosphatidylinositol 3-kinase, PI3K) signalling pathway (Alimandi...
These differences come about, in part, by the specificities of each of the tyrosine phosphorylation events on the C-terminal tail of the receptor. The variety of adapter molecules that can then potentially bind to, or dock with, each receptor is summarised in Table 1.2. It can be seen from the table that some signalling effector molecules can dock on all four receptors, potentially at multiple sites, whereas others only have specificity for one receptor or a single site on a limited number of receptors.

Once adapter molecules have bound to the activated receptors, a number of downstream signalling cascades can be induced (Figure 1.3). EGFR activation by EGF, TGFα, amphiregulin, heregulin and HB-EGF, and ErbB3 activation

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Signaling effector</th>
<th>Adaptor docking site^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Shc</td>
<td>Y703 Y974 Y1086 Y1148 Y1173</td>
</tr>
<tr>
<td>STAT5</td>
<td></td>
<td>Y954 Y974</td>
</tr>
<tr>
<td>Crk</td>
<td></td>
<td>Y954 Y974</td>
</tr>
<tr>
<td>PTP-2c</td>
<td></td>
<td>Y974 Y992</td>
</tr>
<tr>
<td>Src</td>
<td></td>
<td>Y974</td>
</tr>
<tr>
<td>PLC gamma</td>
<td></td>
<td>Y992 Y1173</td>
</tr>
<tr>
<td>Cbl</td>
<td></td>
<td>Y1045</td>
</tr>
<tr>
<td>Grb2</td>
<td></td>
<td>Y1068 Y1086 Y1101 Y1148 Y1173</td>
</tr>
<tr>
<td>SHP1</td>
<td></td>
<td>Y1173</td>
</tr>
<tr>
<td>HER2</td>
<td>Shc</td>
<td>Y735 Y1005 Y1196 YY1222 Y1248</td>
</tr>
<tr>
<td>SH3BGRL</td>
<td></td>
<td>Y923 Y1196</td>
</tr>
<tr>
<td>PTP-2c</td>
<td></td>
<td>Y1023</td>
</tr>
<tr>
<td>Grb2</td>
<td></td>
<td>Y1139</td>
</tr>
<tr>
<td>HER3</td>
<td>p85 PI3-K</td>
<td>Y1054 Y1197 Y1222 Y1276 Y1289</td>
</tr>
<tr>
<td>Grb2</td>
<td></td>
<td>Y1199 Y1262</td>
</tr>
<tr>
<td>Shc</td>
<td></td>
<td>Y1328</td>
</tr>
<tr>
<td>HER4</td>
<td>Shc</td>
<td>Y733 Y1188 Y1258 Y1284</td>
</tr>
<tr>
<td>PLC gamma</td>
<td></td>
<td>Y875</td>
</tr>
<tr>
<td>STAT5</td>
<td></td>
<td>Y984</td>
</tr>
<tr>
<td>PTP-2c</td>
<td></td>
<td>Y984</td>
</tr>
<tr>
<td>Crk</td>
<td></td>
<td>Y1022 Y1150</td>
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<tr>
<td>STAT1</td>
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<tr>
<td>Abl</td>
<td></td>
<td>Y1056 Y1081 Y1150 Y1162 Y1188 Y1242</td>
</tr>
<tr>
<td>Grb2</td>
<td></td>
<td>Y1162 Y1188 Y1202 Y1208 Y1221 Y1242 Y1268</td>
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<tr>
<td>Cbl</td>
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<td>Syk</td>
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<td>Y1150 Y1202</td>
</tr>
<tr>
<td>Ras A1</td>
<td></td>
<td>Y1150</td>
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<tr>
<td>Vav2</td>
<td></td>
<td>Y1162</td>
</tr>
<tr>
<td>Nck</td>
<td></td>
<td>Y1268</td>
</tr>
</tbody>
</table>

^a There are numerous potential phosphor-tyrosine (Y) docking sites for signalling effectors (adapted from Wilson et al., 2009). Note the number of putative Grb2 docking sites on both EGFR and HER4, and Shc sites on EGFR, HER2 and HER4, although Grb and Shc potentially dock on four receptors. This contrasts with Abl, which docks exclusively on HER4 and p85 PI3-K that is almost exclusive to HER3.
via neuregulins leads to the activation of phosphatidylinositol 3-kinases (PI3K), resulting in phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce phosphatidylinositol 3,4,5-triphosphate (PIP$_3$). Akt then translocates to the membrane, and the conformational change that is produced when this happens allows Akt to be activated through phosphorylation of its active sites (threonine 308 and serine 473) by phosphoinositide-dependent protein kinase 1 (PDK1) and the mTOR complex 2 (mTORC2) (Sarbassov et al., 2005). Akt activation results in phosphorylation of key downstream targets such as NF-$\kappa$B, FOXO family members and mTOR complex 1 (mTORC1 – Chapter 5) (reviewed in Vivanco and Sawyers, 2002). At a cellular level, apoptosis is inhibited and cell proliferation and growth are induced in response to Akt activation (reviewed in Freudlsperger et al., 2011).

Regulation of Akt activation occurs thorough the actions of PTEN (phosphate and tensin homolog deleted on chromosome 10) which antagonises PI3K activity by de-phosphorylating PIP$_3$ (reviewed in Cully et al., 2006; Carnero et al., 2008).

Docking proteins such as Grb2 and Shc are also capable of binding to phosphorylated residues on the cytoplasmic tail of the receptor (Schulze et al., 2005). This ultimately results in formation of a Grb2/SOS complex initiating removal of GDP
from a Ras family member, and activation by substitution with GTP. Ras then activates Raf, which subsequently activates the mitogen-activated-protein-kinase (MAPK) cascade. The outcome of MAPK cascade signaling is increased transcription, as a result of transcriptional activator activation (e.g. myc) (Chuang and Ng, 1994) and increased translation due to phosphorylation of the 40S ribosomal protein S6 kinase (Shahbazian et al., 2006). Interestingly S6 kinase isoforms are also targets of mTOR activation (Chapter 5).

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) cascade also regulate cell survival. Members of the JAK/STAT pathway also interact with activated receptors to initiate signalling. As their name suggests, the outcome of signalling results in an increase in transcription, especially of target genes whose protein products are involved in increased proliferation and decreased cell death responses.

### 1.4 Signalling regulation

#### 1.4.1 Regulation of phosphorylation events

**Receptor conformation**

In normal cells, the activation of signalling cascades is tightly regulated. The relative levels, as well as the combinations of receptors and growth factors that are available will govern specificity of signalling. The result will be that certain pathways will be activated to a higher or lesser extent than the alternatives based on the nature of receptor dimerisations that occur, and by the conformational changes that result from ligand binding, only allowing specific adaptor proteins to have accessibility to the cytoplasmic tail of the activated receptors (see Section 1.2.2).

**Action of phosphatases**

Control of signalling, where receptors and/or growth factor levels are not limiting, can also occur within the cell by families of lipid phosphatases or serine/threonine phosphoprotein phosphatases. PTEN is a well reviewed example and inhibits PI3-K/Akt signalling by de-phosphorylating PIP$_2$ and PIP$_3$ (reviewed in Zhang and Claret, 2012). Given that amongst its many roles PI3K activation results in increased proliferation, cell survival and motility, it is not surprising that PTEN functions as a tumour suppressor protein, inhibiting these actions. It is even less surprising then that PTEN is commonly lost in many tumour types (reviewed in Sansal and Sellers 2004), and individuals with syndromes involving germline mutations in PTEN (e.g. Cowden’s Syndrome) are at increased risk of breast tumour development (Liaw et al., 1997).

The 145 kDa protein, SHIP, specifically hydrolys PIP$_3$ and is ubiquitously expressed in differentiated cells (reviewed in Zhang and Claret, 2012). As an
antagonist of haematopoietic cell proliferation, SHIP is also classed as a tumour suppressor and deletion, especially in conjunction with PTEN, results in the formation of highly aggressive lymphomas (Helgason et al., 1998; Miletic et al., 2010).

Two members of the serine/threonine protein phosphatase family, PP1 and PP2, account for the vast majority of cellular phosphatase activity, despite being present in the cell in low amounts (Lin et al., 1998). This family of proteins exist as heterotrimeric holoenzymes and the range of regulatory subunits that are available increase both the number of phosphoproteins that exist and allow for specificity of the de-phosphorylation reaction (reviewed in Zhang and Claret, 2012). The biological outcome of such specific phosphatases is that distinct pathways such as MAPK cascade, Wnt signalling and PI3K activity can be regulated (reviewed in Mumby, 2007).

1.4.2 Internalisation of receptors

It has been known for over three decades that receptors internalise and our knowledge of EGFR internalisation stems from early work by Stanley Cohen (Carpenter and Cohen, 1976). After internalisation the EGF/EGFR complex is degraded. In normal cells without over-expression of EGFR, this process takes a few hours, however when the receptor is over-expressed the half-life of internalisation increases (reviewed in Sorkin and Goh, 2008). Under normal physiological conditions, the majority of ErbB receptors are in the cell membrane and are internalised into endosomes at a constant rate as the membrane recycles. Inactive receptors are then recycled back to the cell membrane (Wiley, 2003). The constitutive rate of recycling is higher than internalisation, meaning that receptors will mainly be localised at the cell membrane, and this is enhanced by over-expression of receptors as both internalisation and degradation are saturable processes (reviewed in Sorkin and Goh, 2008).

Binding of a ligand to a receptor increases the rate of receptor internalisation via clathrin-coated pits. Here, the receptors are ‘pinched’ off the cell membrane and internalised into endosomes (reviewed in Sorkin, 2004). Once internalised, receptors are carried in budding vesicles, sorted and then trafficked to alternate cellular localisations including the Golgi, ER and nucleus as well as the mitochondria (reviewed in Wang and Huang, 2012). A number of groups have reported localisation of ErbBs in the nucleus (reviewed in Wang et al., 2010a). Regulation of trafficking is carried out by Rab GTPases, and different Rab proteins are required for specific trafficking ‘routes’ (Maxfield and McGraw 2004; Grant and Donaldson, 2009; Rink et al., 2005; Ceresa and Bahr, 2006).

PI3K activation has been linked to microtubule-associated vesicle trafficking affecting both receptor turn-over and transport to alternative cellular localisations. The p85 domain has a wide range of binding partners that play central roles in receptor internalisation and vesicle formation (reviewed Mellor et al., 2012).
The evolutionary conserved family of snx-BAR proteins induce deformation of the cell membrane resulting in tubule formation and ultimately endosome production. They regulate sorting in the maturing endosome, where receptors are either degraded or recycled back to the cell membrane (reviewed in van Weering et al., 2010).

Whilst it was initially assumed that receptor internalisation served to terminate signalling, more recent data suggest, at least for EGFR, that endocytosis can be a requirement for full activation of signalling. In some cellular circumstances increased internalisation of EGFR/HER2 complexes was associated with increased proliferation and cell invasion (Gao et al., 2012). It is also becoming clear that the requirements for endocytosis of receptors may differ depending on the stimuli (Grandal et al., 2012).

1.5 Dysregulation of signalling in cancer

Dysregulation of ErbB receptor expression and signalling and has been identified in a number of different cancer types, including tumours from breast, ovary, brain, prostate, GI tract, lung and head and neck (reviewed in Burden and Yarden, 1997; Hynes and Stern, 1994). There are a number of mechanisms through which increased signalling would occur such as mutations, increased receptor, ligand and adaptor protein expression, as well as altered cellular localisation of signalling components.

1.5.1 Receptor over-expression

One of the most well described gene amplifications is that of \textit{ErbB2}, which was first reported in the mid-1980s (Ullrich et al., 1984; King et al., 1985). In high-grade ductal and inflammatory breast cancer the \textit{ErbB2} gene is amplified and over-expressed which contrasts with benign lesions where \textit{ErbB2} is expressed at low levels (Allred, 1992; Gusterson, 1998, reviewed in Freudenberg, et al., 2009).

Overall the \textit{ErbB2} gene is amplified in 25–30\% of breast cancers (Slamon et al., 1989), as well as some ovarian, stomach and aggressive uterine tumours, and the elevated HER2 protein that is produced as a result is crucial to driving tumour cell proliferation and migration. The differences in expression levels between benign lesions and higher-grade tumours, along with our knowledge of the cellular events that are regulated by HER2, suggest that HER2 over-expression occurs during disease development. There is also evidence that HER2 increases the metastatic potential of cells that have not been fully transformed, indicating that \textit{ErbB2} amplification and over-expression is a driving force in breast cancer progression (reviewed in Freudenberg et al., 2009).

Increased levels of HER2 will mean that, as the preferred heterodimerisation partner for all the receptors, there will possibly be an increase in heterodimerisation
rather than homodimerisation, resulting in increased levels of signalling without the need for elevated ligand levels. Given that the dimerisation arm is constitutively exposed in HER2, high levels of HER2 protein could also result in increased formation of HER2:HER2 homodimers that are constitutively active, even in the absence of ligand. HER2/ErbB2 expression on its own is not sufficient to cause cellular transformation of normal cells and co-expression with ErbB3 is required for pre-neoplastic transformation (Vaught et al., 2012). However in an already partially transformed cell, increased HER2 homodimers could certainly promote proliferation and evasion of apoptosis (reviewed in Freudenberg et al., 2009).

EGFR is over-expressed, with or without gene amplification, in a wide variety of epithelial tumours (reviewed in Arteaga, 2002). In ovarian cancer, at least 70% of tumours are reported to over-express EGFR (Kohler et al., 1989), and there is a relationship between EGFR expression and decreased survival in both ovarian and cervical cancers (Psyrri et al., 2005a; Perez-Regadera et al., 2011). However not all ErbB receptors, when elevated, are linked to increased tumour formation. There are reports that in breast cancers ErbB4 over-expression is linked to improved prognosis and increased tumour suppression, as well as a correlation with the positive prognostic indicator, the oestrogen receptor (Fujiwara et al., 2012; reviewed Wang et al., 2010a). However, ErbB4 has also been shown to be oncogenic (reviewed in Burgess, 2008). The relative levels of other family members are likely to be important in governing both ErbB3 and ErbB4 function, as ErbB1 or HER2 are required for ErbB3 and ErbB4 mediated transformation and oncogenesis (Mill et al., 2011; Gilbertson et al., 1998; Zhang et al., 1996). Histopathological analysis of all ErbBs may therefore be required, especially for heterogeneous epithelial tumours.

1.5.2 Activating mutations

Oncogenic HER2 is largely found homodimerised rather than in the ‘normal’ monomeric state (Weiner et al., 1989b) suggesting that spontaneous ligand-independent dimerisation is occurring in tumours when HER2 is over-expressed (reviewed in Brennan et al., 2000). The amino acid substitution whereby glutamic acid is substituted with valine means that a negative charge on the transmembrane region of the receptor is introduced (Bargmann et al., 1986). Changing the charge on the receptor presumably impacts on the receptor conformation, thereby promoting the formation of homodimers, which is required for the tyrosine kinase activity of the receptor (Weiner and coworkers, 1989a and b).

A subset of extracellular EGFR mutations in glioblastomas enhance receptor activation by destabilising the inactive conformation of the receptor (Ferguson, 2008). In non-small cell lung carcinomas (NSCLC) mutations in components of the EGFR pathway are largely mutually exclusive. That is, in the vast majority of cell lines studied, only one member of the pathway harboured a mutation (Gandhi et al., 2009). These studies in cultured cell lines are also
supported by observations from clinical surgical samples (Shigamtsu et al., 2005), suggesting that multiple EGFR pathway mutations are not required to promote tumourigenesis but that single mutations may be sufficient. Interestingly, but not surprisingly in the light of Iressa failure (reviewed in Blagosklonny and Darzynkiewicz, 2003), the component of the pathway that was mutated had a bearing on whether the cells responded to anti-EGFR therapy or were intrinsically resistant to EGFR inhibition (Gandhi et al., 2009).

A number of mutations in the intracellular domains of EGFR, such as in-frame deletions in the catalytic pocket and the L834R missense mutation, result in both increased kinase activity and coupling to Akt and STAT5 phosphorylation (Zhang et al., 2006; Sordella et al., 2004).

### 1.5.3 Altered cellular localisation

EGFR has been identified in the nucleus of a number of different tumour cells, including breast cancer, ovarian cancer, and oropharyngeal and esophageal squamous cell carcinomas. Localisation to the nucleus appears to be correlated with poorer patient outcomes (Psyrri et al., 2005b; Lo et al., 2005b; Hoshino et al., 2007; Xia et al., 2009), and this is possibly due to its association with STAT3 in the nucleus where it can lead to transcriptional up-regulation of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) (Lo et al., 2005a; Lo et al., 2010). Elevated expression of iNOS and subsequent increase in nitric oxide (NO) is common in many tumours (Cianchi et al., 2003; Vakkala et al., 2000). Increased COX-2 expression is associated with inflammation in a number of tumour types, including breast cancer where it is associated with poorer patient outcomes (Subbaramaiah et al., 2012; Karray-Chouayek et al., 2011). The fact that HER2 can also activate COX-2 gene expression (Wang et al., 2010b) presumably contributes to the poor prognosis that has previously been associated with HER2 positive breast cancers.

Following internalisation and sorting, EGFR can localise to the mitochondria where it may modulate the function of cytochrome c oxidase subunit II and mitochondrial functions that are dependent on this enzyme, including regulation of apoptosis (reviewed in Wang and Huang, 2012).

One of the truncated variants of HER2 (p95HER2) can be found in the nucleus and the combination of expression and nuclear localisation is associated with local metastasis and decreased prognosis for breast cancer patients (reviewed in Arribas, 2011).

In contrast nuclear ErbB4 is associated with improved patient outcomes, especially for those treated with tamoxifen.

### 1.5.4 Changes in ligand levels

One of the simplest ways from a biological perspective for signalling to increase is via increased expression of the relevant ligands. As long as the ligand-binding
sites on the receptors are not saturated an increase in ligand concentration is likely to result in increased receptor occupancy and a subsequent increase in activation.

A number of ErbB ligands, especially those that bind to EGFR, are associated with tumour development and progression. TGF\(\alpha\), amphiregulin and HB-EGF are all associated with poorer patient prognosis or resistance to chemotherapeutic agents. In breast cancers, TGF\(\alpha\) and EGFR expression are associated with a clinically aggressive tumour subset and EGF expression is linked to poor prognosis (Castellani et al., 1994; Mizukami et al., 1991); in NSCLC serum levels of TGF\(\alpha\) and amphiregulin correlate with aggressiveness and poor responses to therapy (Ishikawa et al., 2005). In EGFR positive lung adenocarcinomas, patients with high EGF and TGF\(\alpha\) levels had shorter survival times than those with no EGF or TGF\(\alpha\) (Tateishi et al., 1990).

1.5.5 Changes in adaptor protein levels

Over-expression or re-localisation of novel adapter proteins can enhance ErbB signalling. For example, Brk/PTK6 is physiologically expressed in a limited number of normal epithelial tissues where it plays a role in regulating differentiation and appears to have a more nuclear localisation. However, during carcinogenesis Brk is found to be cytoplasmic and associated with ErbB receptors where its role is believed to be oncogenic (Aubele et al., 2010; Derry et al., 2003; Petro et al., 2004; reviewed in Harvey and Burmi, 2011).

The effects are more striking in the breast, as Brk is not found in normal mammary tissue (Barker et al., 1997; Llor et al., 1999) but is over-expressed in breast carcinomas with higher mRNA and protein levels correlating with increased tumour grade (Ostrander et al., 2007; Chakraborty et al., 2008; Harvey et al., 2009). Brk augments the mitogenic effects of EGF (Kamalati et al., 1996) and this may well be a result of increasing recruitment of p85 PI3K to ErbB3 (Kamalati et al., 2000).

Brk and other ErbB signalling effector proteins are also implicated in other signalling pathways such as IGF (Irie et al., 2010) (see Chapter 2), suggesting that cross-talk plays a crucial role in signalling regulation (see Chapter 9).

1.6 Therapeutic opportunities

Some breast cancers can express up to 50 copies of the ErbB2 gene (reviewed in Sørlie, 2004) making HER2 an attractive tumour target, especially given the low expression of HER2 in most adult tissues.

The Greene laboratory were the first to show that targeting a protein involved in the cellular transformation process for degradation could reduce the malignant phenotype of the cells by blocking downstream signalling. Strikingly, this effect was observed both in vivo, as well as in in vitro studies (Drebin et al., 1985, 1986).
Patients with HER2 positive early stage breast cancer or hyperplasia that has yet to become disseminated or fully transformed should benefit most from HER2 targeted therapies, as the additional changes required for full transformation that are mediated by HER2 have yet to occur and will therefore be prevented. Comparison of clinical trial data from patients with HER2 positive breast cancer that had been surgically removed showed that HER2-targeting improved patient outcomes (Romond et al., 2005). Patients with late stage breast cancer also benefit from HER2 targeted therapy and the guidelines for the HER2 screening of breast cancer patients were updated by the American Society for Clinical Oncology (ASCO) in 1998 and further optimised in 2007 (ASCO, 1998, 2007).

1.6.1 Current strategies

There are a number of therapeutic strategies that can be considered for suppressing ErbB signalling. In addition to blocking receptor function through the use of monoclonal antibodies, or kinase activity with small molecule inhibitors, inhibiting the function of downstream signalling components and molecules involved in stabilising receptor conformation could also be considered.

Monoclonal antibodies

Trastuzumab (Herceptin) and Pertuzamab (Perjeta) are the most commonly used monoclonal antibodies directed against HER2 in the clinic. They both prevent HER2 dimerisation but, as they are targeted towards different extra cellular domains of HER2, the nature of the dimer affected by each antibody is different. Trastuzumab prevents HER2 homodimerisation, whereas Pertuzamab is more effective against HER2 heterodimerisation with HER3 or EGFR (reviewed in Eccles, 2011). Patients with advanced metastatic breast cancer that had acquired resistance to Herceptin responded to a combination of Herceptin and Pertuzamab (Gelmon et al., 2008). As well as providing benefit to patients by overcoming the adaptive features of Herceptin resistance, these data provide an insight into the mechanisms of acquired resistance and the contribution of signalling cross-talk to therapeutic responses.

Given that only a subset of HER2 positive breast cancer patients reportedly responded to Trastuzumab (Nahta et al., 2006), data from the Arteaga laboratory suggest measuring the levels of HER2 homo- and hetero-dimers may be important in stratifying HER2 positive patients for antibody therapy (Ghosh et al., 2011).

Kinase inhibitors

A number of different kinase inhibitors have been developed. Some, such as erlotinib and gefitinib, inhibit EGFR (Zhang et al., 2007) whilst others, such as lapatinib, target both EGFR and HER2 (Rusnak et al., 2001). In breast and in head and neck tumour cells, both MAPK and AKT pathways are inhibited in response to lapatinib treatment resulting in cell death (Xia et al., 2002).
Lapatinib has good clinical efficacy in HER2 positive cancers that have become refractory to treatment with other targeted antibody based agents such as Trastazumab (Olson et al., 2012; Hirano et al., 2012), or in combination with trastazumab to achieve complete HER2 blockade (Wang et al., 2011).

Although early pre-clinical studies with gefitinib were promising, Iressa™ became the first drug to be withdrawn following accelerated approval by the American FDA due to its lack of clinical efficacy (FDA, 2004; Frantz, 2005). This highlights the issues faced with kinase inhibition and the importance of relevant pre-clinical studies.

Destabilising receptor conformations

Many cellular proteins are highly dependent on chaperone proteins for correct folding and cellular localisation. Inhibition of chaperone function would destabilise the conformation of the client protein and target it for proteosomal degradation. HER2 is no exception; it is highly dependent on the chaperone function of Hsp90 for correct folding (Xu et al., 2001 reviewed Buchner, 1999). Inhibition of Hsp90 results in degradation of HER2 both in vitro and in vivo and convincing antitumour effects have been observed in HER-positive mouse xenograft models (Eccles et al., 2008).

Targeting Hsp90 in HER2 over-expressing tumours could prove to be extremely effective as a number of downstream signalling effectors such as PDK1 and Brk/PTK6 are also reported to be Hsp90 client proteins (Fujita et al., 2002; Kang et al., 2012). In addition, as EGFR is also stabilised by Hsp90 (Ahsan 2012), inhibiting Hsp90 could antagonise EGFR, which would limit the ‘options’ for compensatory signalling via other members of the ErbB family in HER2/EGFR positive tumours.

Downstream signalling effectors

Downstream of Akt is mTOR. Blocking mTOR signalling is therapeutically very attractive as a number of pathways merge at this signalling ‘hub’ (Chapters 5 and 9). mTOR inhibitors show antitumour activity in a wide range of different tumour models and some of these are discussed in Chapter 5.

Therapeutic resistance

Signalling cross-talk plays a major role in the development of resistance to targeted anticancer therapies (see Chapter 9).

However, altered cellular localisation of receptors has been gaining more attention in recent years. EGFR has been shown to translocate to the mitochondria following tyrosine kinase inhibition, suggesting that mitochondrial localisation of receptors may play a role in therapeutic responses and resistance (Cao et al., 2011).

Nuclear EGFR has been reported as applying a role in acquired resistance to the anti-EGFR antibody, cetuximab (Li et al., 2009). Both cetuximab and gefitinib inhibit radiation-induced EGFR nuclear transport and sensitises cells to the effects of radiation, suggesting that EGFR may contribute to radio resistance (Dittmann
et al., 2005; Bailey et al., 2007). Lapatinib also inhibits the nuclear translocation of EGFR and HER2 sensitising cancer cells to 5-fluorouracil (Kim et al., 2009). These studies are part of a growing body of evidence suggesting that nuclear EGFR may be associated with therapeutic resistance to conventional therapies, and the development of kinase inhibitors and monoclonal antibodies are providing the tools to investigate this further.

1.6.2 Future strategies

Therapeutic combinations and alternative treatments

Using combinations of ErbB targeted therapies with the more conventional anti-cancer drugs is not straightforward.

HER2 positive cancers can be more resistant to some but not all chemotherapy agents (reviewed in Eccles, 2011). Conversely, topoisomerase 2 is co-amplified with the ErbB2 gene making HER2 positive cancers more sensitive to the topoisomerase inhibitor doxorubicin (reviewed in Bonnefoi, 2011). An obvious combination for treatment would therefore be an HER2-targeted therapy alongside doxorubicin; however, as both approaches result in cardiomyopathies, this strategy is no longer advisable (Procter et al., 2010).

As the scientific knowledge of signalling cross-talk improves alongside greater understanding of the cellular mechanisms that contribute to therapeutic resistance to EGFR- or HER2-targeted therapies, combinatorial approaches to signalling inhibition will no doubt predominate in clinical practice (Chapter 9).

As biochemical technology moves forward it is possible that, in the future, disrupting protein–protein interactions could offer therapeutic benefit by targeting very specific aspects of signalling.

Disease prevention

The Greene laboratory showed that anti-HER2 antibodies can prevent tumours from forming in transgenic mice models. Transgenic mice that over-expressed the activated neu oncogene (rat oncogenic HER2) were treated with HER-targeted antibodies at 20 weeks of age, which was before tumours had formed. The study showed that there was a decrease in tumour incidence that was dose-dependent, and mice that remained tumour free appeared to remain protected against tumour development for life (Katsumata et al., 1995). These data suggest that HER2 antibodies could be used to vaccinate against the formation of HER positive tumours.

Other pre-clinical studies are also suggesting that DNA-based vaccines may have benefit in treating HER positive cancers (Whittington et al., 2008). More recent Phase I/II clinical trials have shown efficacy of peptide-based HER2 vaccines in the treatment of cancer with only limited toxicity (reviewed in Baxevanis et al., 2012).


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