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ErbB2/HER2: Its Contribution to Basic Cancer Biology and the Development of Molecular Targeted Therapy

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1. Introduction

ErbB2, one of the receptor tyrosine kinase superfamily has attracted the attention of cancer researchers since its discovery. Thirty years ago, *ErbB2* was discovered as an oncogene that transforms NIH3T3 cells. The first decade of ErbB2 research revealed that it is a member of the ErbB receptor family and is deregulated in various types of human cancer. In the second decade, one significant discovery came from the crystallography with the rational theory that explains why no ligands specific for ErbB2 have been identified so far, and the other breakthrough came from the clinical field with the appearance of ErbB-targeted therapeutics. Today, cancer researchers strive to describe the elaborate signaling network of ErbB receptors by proteomic analysis, and our knowledge of their function, which is far from complete, is being applied to develop more efficient ErbB-targeted therapeutics for cancer patients.

We will begin the story of *ErbB2* with its discovery as an oncogene called *neu*. In the dawn of oncogene research, this gene, derived from a rat tumor, was classified as one of the most pivotal genes in human cancer, along with the oncogenes Ras and Myc and the tumor suppressor gene p53. This is because *ErbB2* is frequently amplified and overexpressed in certain human cancers, such as breast carcinoma. Similar to other oncogenes, subsequent research demonstrated its indispensable role in development. Now, our interest is whether ErbB2 acts on the same target proteins in cancer and in normal development.

The signaling networks downstream of ErbB receptors are complex because there are various ligands for each receptor, except ErbB2, and the composition of the ErbB dimer seems to define downstream signaling targets. For example, EGFR (epidermal growth factor receptor)-containing heterodimers prefer to stimulate the mitogen-activated protein kinase (MAPK) cascade, while ErbB3-containing dimers preferably activate phosphatidylinositol 3-kinase (PI3K). This characteristic is reflected in the difficulty in choosing the best therapeutics corresponding to each case in the clinic.

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Our passion for determining the function of ErbB2 in cancer has inspired us to develop experimental methods to reveal mechanisms of tumorigenesis in humans. One such method is the MCF10A morphogenesis assay, which will be useful for identifying new oncogenes.

2. Discovery of *ErbB2*

This section describes the history of the discovery of *ErbB2/HER2/neu*. The *ErbB2* gene was initially identified as an oncogene named *neu* in NIH3T3 cells. Soon after, several groups, including our laboratory revealed that this gene was the second member of the EGFR family. Initial studies showed that this gene was amplified in human cancer cell lines and tissues, indicating its importance in human cancer. This finding prompted the extensive studies of ErbB2 in human cancer. By the way, official NCBI gene symbol is ERBB2 (human), however, for simplicity, we will use one term "ErbB2" in this article.

2.1 *Neu*, an oncogene cloned from murine brain tumors

The origin of *ErbB2/HER2/neu* research can be traced back to the 1980s, when virtually all cancer researchers were hunting for novel oncogenes. Cancer researchers, using the tools for introducing foreign DNA into mammalian cells, were seeking genes that render normal cells cancerous, what we call "oncogenes." Scientists introduced genomic DNA that was isolated from mouse or human tumor cells into NIH3T3 mouse fibroblast cells (Shih et al., 1979) using the calcium-phosphate DNA precipitation technique (Graham & van der Eb, 1973) and examined their morphological changes, or their "transformation". This transformation assay described in detail in section 6 produced several fruitful discoveries. For us, the most significant was the discovery of *neu*.

The first report about *neu* came in 1981 when Shih et al. indicated that DNA prepared from rat neuro-/glioblastoma cell lines transformed NIH3T3 cells (Shih et al., 1981). In 1984, Schechter et al. demonstrated that several independently isolated transformed cells contained the same oncogene, based on resistance to inactivation by restriction enzyme cleavage, and thus named the oncogene "*neu*" (Schechter et al., 1984). They also showed that the *neu* gene encoded a protein of relative molecular mass 185,000 (p185), and it was related to EGFR serologically. This finding was consistent with the fact that *neu* showed significant similarity to v-*erbB*, a retroviral oncogene of avian erythroblastosis virus (Vennstrom & Bishop, 1982; Yamamoto et al., 1983), and was homologous with the cellular gene (c-*erbB*) encoding EGFR (Downward et al., 1984).

2.2 A second member of the ErbB family, *c-erbB2*/*HER2*

After the initial discoveries regarding *neu*, *neu* blossomed out into one of the most famous oncogenes. In 1985, Schechter et al. demonstrated that the homology between *neu* and *erbB* was limited to the region of the kinase domain of EGFR and that *neu* mapped to human chromosome 17, distinct from c-*erbB* on chromosome 7 (Schechter et al., 1985). Semba et al. identified the human v-*erbB*–related sequence as distinct from the EGFR gene and its amplification in the human adenocarcinoma of the salivary gland (Semba et al., 1985), and they named the gene c-*erbB2*, as a separate gene from c-*erbB1*, encoding EGFR. Soon after, several groups cloned this human version of the *neu* oncogene and designated it c-*erbB2* or *HER2* (Bargmann et al., 1986b; Coussens et al., 1985; Yamamoto et al., 1986), and cDNA clones of the *neu* oncogene itself were also isolated (Hung et al., 1986). Subsequent studies revealed that *ErbB2* encodes a 185-kDa transmembrane glycoprotein that is highly similar to

EGFR (Akiyama et al., 1986; Bargmann et al., 1986b). To date, there are four ErbB family receptors: EGFR (also ErbB1, HER1), ErbB2(HER2, Neu), ErbB3(HER3) (Kraus et al., 1989), and ErbB4(HER4) (Plowman et al., 1993). We will describe their structural similarities and differences in section 3.

In what ways does the *neu* oncogene cause murine neuro-/glioblastoma? In the case of the v-*erbB* oncogene, encoding the truncated form of EGFR, we could assume that its aberrant protein, which has no ligand binding domain, phosphorylates its substrates independently of ligand binding, resulting in hyperproliferation (Khazaie et al., 1988). The *neu* proto-oncogene, however, is activated by a single point mutation, V664E, in the transmembrane domain (Bargmann et al., 1986a), and not by gross rearrangements as seen in v-*erbB*. Does this single point mutation, which causes neuro-/glioblastoma in rats, also occur in humans? Interestingly, such a single point mutation of c-*erbB2* has never been observed in cancer patients. Instead, gene amplification and overexpression of its protein product are frequently observed in various types of human cancer, especially breast cancer (Slamon et al., 1989), as described in section 4.

3. Regulation of ErbB receptors and downstream signaling pathways

The ErbB receptors are closely related, single-chain glycoproteins. ErbB receptors are activated by binding to their specific ligands. Activated ErbB receptors then transmit signals to downstream signal transducers. In this section, we describe structural features of ErbB receptors and focus on recent observations of ErbB2 signal transduction pathways.

3.1 ErbB ligands
ErbB ligands act in a paracrine or autocrine fashion. Whereas paracrine ErbB ligands are derived from stromal cells, autocrine ErbB ligands are produced as transmembrane precursors that are subsequently cleaved by proteases to be released as soluble ligands when cells are stimulated (Hynes & Lane, 2005).

At least ten ErbB ligands are known (Figure 1) and are divided into three groups with respect to binding specificity. The first group includes EGF, amphiregulin (AR) and transforming growth factor-α (TGF-α), which bind specifically to ErbB1. The second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which show dual specificity, binding to both ErbB1 and ErbB4. The third group includes neuregulins (NRGs). They are composed of two subgroups. NRG1 and NRG2 bind specifically to both ErbB3 and ErbB4, whereas NRG3 and NRG4 bind specifically to only ErbB4 (Hynes & Lane, 2005).

None of these ErbB ligands bind and activate ErbB2. However, the mucin MUC4, a transmembrane glycoprotein, modulates the ErbB2 signaling pathway. MUC4 is composed of two subunits, ASGP-1, an O-glycosylated mucin subunit, and ASGP-2, an N-glycosylated transmembrane subunit. ASGP-2 possesses two EGF-like domains, EGF-1 and EGF-2, and interacts specifically with ErbB2, inducing its phosphorylation. MUC4 retains ErbB2 and ErbB3 on the cell membrane by suppressing their ligand-induced internalization. However, the mechanisms of ErbB2 signaling activation by MUC4 are largely unknown (Singh et al., 2007).

3.2 ErbB receptors
3.2.1 Structures of ErbB receptors and their conformational changes on ligand binding
Each ErbB receptor comprises five functional domains, the extracellular domain for ligand binding (~620 residues), the α-helical transmembrane segment (~23 residues), and
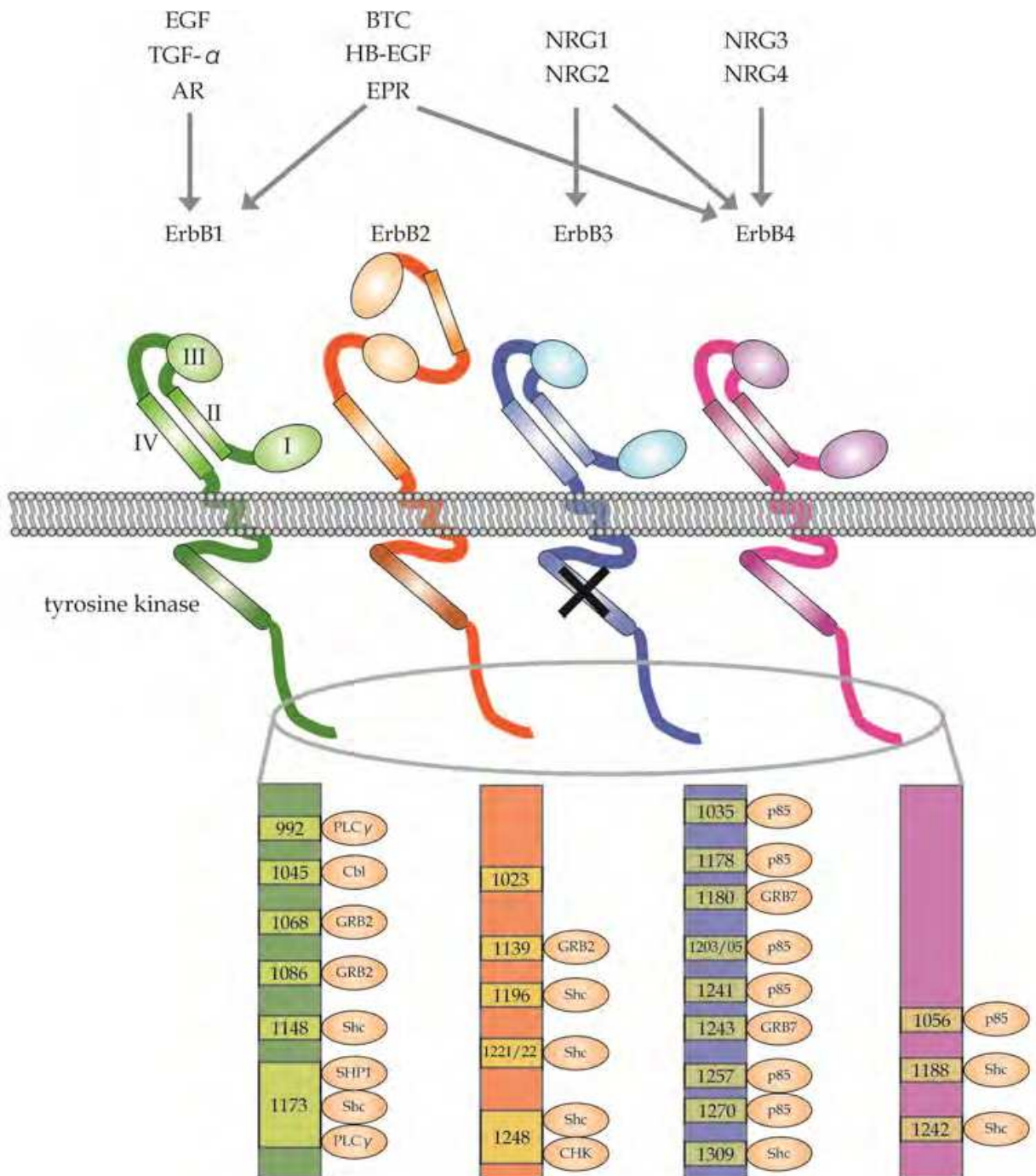
Fig. 1. ErbB ligands and receptors.
ErbB ligands, which act in an autocrine or paracrine fashion, are divided into three groups based on binding specificity. None of the ErbB ligands binds to ErbB2. However, MUC4 modulates ErbB2 signaling activity. Ligand binding to the extracellular domain of ErbB receptors changes the receptor's conformation and promotes homo- and heterodimerization. Its dimerization induces the tyrosine kinase activity of the intracellular domain, resulting in the cross-phosphorylation of specific tyrosine residues, which recruits and activates specific downstream signaling proteins. The intracellular domain of each ErbB receptor possesses unique docking sites for downstream signaling proteins, which consist of tyrosine residues and the surrounding amino acids (Baselga & Swain, 2009; Hynes & Lane, 2005; Olayioye et

al., 2000). ErbB1 has docking sites for growth factor receptor-bound 2 (GRB2) and Src homology 2-containing (Shc), which activate the MAPK and PI3K–Akt pathways though Ras activation, although it has no direct docking site for PI3K (Hynes & Lane, 2005). ErbB1 has a docking site for Cbl, an E3 ubiquitin ligase. ErbB1 dimerization induced by ligand binding induces receptor internalization into endosomes, which is followed by recycling. In the endosome, Cbl directly binds to tyrosine residues of ErbB1 and undergoes ubiquitylation, resulting in its degradation in lysosomes (Citri & Yarden, 2006). ErbB2 functions as the preferred partner of other ErbB receptors and possesses the strongest kinase activity. ErbB3 possesses six p85 docking sites, which effectively activate the PI3K–Akt pathway, although it lacks tyrosine kinase activity (Moasser, 2007). ErbB1, ErbB2 and ErbB3 are implicated in the progression of cancer. However, ErbB4 is associated with the inhibition of cell proliferation, although it has docking sites for p85 and Shc (Baselga & Swain, 2009).

a juxtamembranedomain (~40 residues), an intracellular tyrosine kinase domain (~260 residues), and a C-terminal regulatory region (~232residues) (Burgess et al., 2003). Intriguingly, the structures of ErbB2 and ErbB3 are unique among the ErbB family. ErbB2 is an orphan receptor, but it always has a ligand-activated conformation (Garrett et al., 2003), while ErbB3 has impaired intrinsic tyrosine kinase activity (P.M. Guy et al., 1994).
The ErbB family is conserved during evolution. Although we will not discuss the issue in detail, the biological significance of EGFR in the physiological state lies in its involvement in epithelial development in mammals (Miettinen et al., 1995; Sibilia & Wagner, 1995; Threadgill et al., 1995). EGFR has similar functions in invertebrates. For instance, both *Caenorhabditis elegans* and *Drosophila melanogaster* have a single EGFR homolog (Aroian et al., 1990; Livneh et al., 1985). EGFR regulates vulva development in *Caenorhabditis elegans* (Moghal & Sternberg, 2003) and the development of various organs in *Drosophila* (Shilo, 2003). In *Caenorhabditis elegans*, in parallel with the simplicity of the ErbB family of receptors, there are only one ligand for the receptors (Yarden & Sliwkowski, 2001), while humans have ten. The versatility of ErbB receptors and their ligands in mammals, which evolved from a simple cascade important for development in invertebrates, can endow signaling networks with not only robustness but also vulnerability, represented by tumorigenesis, a collapse of the regulatory circuit. The evolutional conservation of tumor-promoting ability of EGFR is well known, for instance, from the melanoma model of *Xiphophorus* (Gomez et al., 2004).
The extracellular domain consists of domains I–IV. Domain II promotes receptor dimerization. In the absence of ligand binding, ErbB exists in a tethered conformation in which intramolecular interaction between domains II and IV blocks the function of dimerization domain II. Ligand binding to ErbB receptors changes the tethered conformation into the extended conformation, which exposes domain II, allowing them to undergo homo- and heterodimerization (Hynes & Lane, 2005). However, ErbB3 lacks the ability to homodimerize (Baselga & Swain, 2009). ErbB2 has a unique structure of the ligand-activated conformation and is the most preferred partner for other ligand-bound ErbB receptors. This is because the structure of the ErbB2 extracellular domain originally resembles the extended conformation that exhibits no interaction between domain II and domain IV and exposes the dimerization domain II (Hynes & Lane, 2005). The intracellular domain possesses the protein tyrosine kinase activity and unique docking sites for specific downstream signaling proteins, which consist of tyrosine residues and surrounding amino acid side chains (Hynes & Lane, 2005). ErbB2 possesses the strongest tyrosine kinase activity among the ErbB receptors (Moasser, 2007). However, ErbB3 lacks the tyrosine kinase activity because it is unable to bind to ATP (Baselga & Swain, 2009).

3.2.2 HSP90–ErbB2 complex

Heat shock proteins (HSPs), a large family of highly conserved molecular chaperone proteins, mediate the conformational maturation and folding of target proteins. HSP90 protects the ErbB system from damage. Whereas other ErbB receptors are HSP90-independent, ErbB2 and several downstream signaling proteins are stabilized by HSP90. A ternary complex of HSP90, ErbB2 and a co-chaperone, CDC37, stabilizes ErbB2 at the cell membrane (Baselga & Swain, 2009; Citri & Yarden, 2006) . Inhibition of HSP90 function by specific drugs results in ubiquitylation and proteasomal degradation of ErbB2 and its downstream signaling proteins (Baselga & Swain, 2009). HSP90 also controls ErbB2 signaling activity. Binding of the complex of HSP90 and CDC37 to the tyrosine kinase domain of ErbB2 suppresses its tyrosine kinase activity and heterodimerization with other ligand-bound ErbB receptors.

3.3 Signaling pathways activated by ErbB receptors

Ligand-binding to the extracellular domain of ErbB receptors changes the tethered conformation into the extended conformation, inducing homo- and heterodimerization. Only ErbB2 is able to dimerize without ligand-binding. ErbB receptor dimerization results in phosphorylation on specific tyrosine residues of the intracellular domain. These phosphorylated tyrosine residues and surrounding amino acid side chains allow the recruitment and activation of downstream signaling proteins, which initiate multiple signaling pathways (Baselga & Swain, 2009).

The combination of ligands and heterodimer partners determines which downstream signaling proteins are recruited and which signaling pathways are activated. The heterodimer of ErbB2–ErbB3 is the most active in ErbB downstream signaling (Baselga & Swain, 2009).

Two main signaling pathways activated by ErbB receptors are the MAPK and the PI3K-Akt pathways. Other important ErbB signaling proteins are the signal transducers and activators of transcription (STATs) and the Src tyrosine kinase (Hynes & Lane, 2005). Although ErbB receptors are largely known as receptor tyrosine kinases (RTKs), recent studies indicate they can localize to the nucleus and act as transcription factors (S.-C. Wang et al., 2004).

3.3.1 MAPK pathway

GRB2, an adaptor protein, binds to ErbB receptors either indirectly through Shc or directly to the phosphorylated tyrosine residues. The GRB2–son of sevenless (Sos) complex, with or without Shc, recruits Ras and activates the MAPK pathway, inducing cell proliferation, migration, differentiation and angiogenesis (Baselga & Swain, 2009). Recent analysis using MCF10A cells showed that Shc is required for the inhibition of apoptosis and for paclitaxel resistance (see also section 6 for MCF10A system).

3.3.2 PI3K–Akt pathway

PI3K, a heterodimer composed of a p85 regulatory subunit and p110 catalytic subunit, is activated by at least two ErbB-related pathways. In the first pathway, p85 directly binds to phosphorylated tyrosine residues, triggering the activation of the p110 catalytic subunit. In the second pathway, GRB2 binds to ErbB either indirectly though Shc or directly to phosphorylated tyrosine residues and activates Ras, which also triggers the activation of

p110 (Baselga & Swain, 2009; Cully et al., 2006). Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits Akt and phosphatidylinositol-dependent kinase 1 (PDK1) and activates PDK1. PDK1 phosphorylates and activates Akt. The tumor-suppressor phosphatase with tensin homology (PTEN) dephosphorylates PIP3 into PIP2 and inhibits PI3K–Akt pathway activation (Cully et al., 2006).

Activated Akt phosphorylates many target proteins associated with cell survival, proliferation (increased cell number), and growth (increased cell size). In addition, Akt promotes angiogenesis through vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) (Vivanco & Sawyers, 2002).

3.3.2.1 Survival

Activated Akt directly phosphorylates several target proteins to suppress apoptosis. BAD, a pro-apoptotic member of the BCL2 family, antagonizes the survival protein BCL-X$_L$ to promote cell death. Activated Akt phosphorylates BAD, which prevents it from interacting with BCL-X$_L$, allowing BCL-X$_L$ to function as an anti-apoptotic protein. Caspase-9, a member of the cysteine-dependent aspartyl-specific protease family, cleaves and activates pro-caspase-3, resulting in apoptosis. Activated Akt phosphorylates caspase-9 and inhibits its catalytic activity. Forkhead box 1 (FOXO1), a member of the forkhead family of transcription factors, activates several pro-apoptotic proteins, including BIM and FAS ligand. Activated Akt phosphorylates FOXO1 and prevents its nuclear translocation. Nuclear factor κB (NF-κB), a transcription factor, is constantly inhibited by IκB. Activated Akt phosphorylates IκB kinase (IKK), which degrades IκB, allowing NF-κB to translocate to the nucleus and activate its target genes. Murine double minute (MDM2), a p53-binding protein, mediates proteasomal degradation of p53. Activated Akt phosphorylates and activates MDM2 (Vivanco & Sawyers, 2002).

3.3.2.2 Proliferation

Activated Akt directly phosphorylates several target proteins to regulate cell cycle control. Glycogen synthase kinase-3β (GSK-3β) phosphorylates cyclin D1 which mediates G1/S phase transition to induce its proteasomal degradation. Activated Akt directly phosphorylates and inhibits GSK-3β, which allows cyclin D1 to accumulate (Vivanco & Sawyers, 2002). Activated Akt phosphorylates and inhibits p27, a CDK inhibitor (CKI), promoting cell cycle entry. In addition, p27 expression is regulated by the transcription factor FOXO3A. FOXO3A activates p27 and BIM expression, and inhibit cyclin D1 expression. Activated Akt phosphorylates FOXO3A, which promotes its translocation from the nucleus. Activated Akt also modulates p21 activity by affecting its phosphorylation, presumably through other kinases (Cully et al., 2006).

3.3.2.3 Growth

Activated Akt directly phosphorylates tuberous sclerosis 2 (TSC2) to affect cell growth. TSC2 heterodimerizes with TSC1 to promote the GTPase activity of the Ras homolog enriched in brain (RHEB). Activated Akt phosphorylates TSC2 and inhibits the ability of the TSC1–TSC2 complex to act as RHEB-GTPase activating protein (RHEB-GAP), which allows GTP-bound RHEB to accumulate. Active GTP-bound RHEB promotes the kinase activity of the mammalian target of rapamycin (mTOR), a key regulator of cell growth. Activated mTOR, regulatory associated protein of TOR (raptor) and G-protein β-subunit-like (GβL)

complex phosphorylates S6 kinase (S6K) and the eukaryotic translation-initiatin factor 4E (EIF4E)-inhibitory binding protein (4E-BP) to modulate the mRNA translation and protein synthesis (Cully et al., 2006).

3.3.3 ErB2–ErbB3 heterodimer and PI3K–Akt pathway

Several lines of evidence indicate that the heterodimer ErbB2–ErbB3 is the most important oncogenic signaling associated with the activation of the PI3K–Akt pathway. ErbB2, which lacks p85-binding sites, possesses strong kinase activity, and ErbB3, which lacks tyrosine kinase activity, possesses six p85-binding sites. Akt is frequently activated in ErbB2-overexpressing tumors, as well as in tumors generated in mouse mammary tumor virus (MMTV)-*neu* transgenic mice. Cell transformation by overexpressed ErbB2 *in vitro* is associated with increased ErbB3 phosphorylation and activation of the PI3K–Akt pathway. These results suggest the transactivation of ErbB3, and the PI3K–Akt pathway is strongly associated with the tumorigenic function of overexpressed ErbB2 (Moasser, 2007).
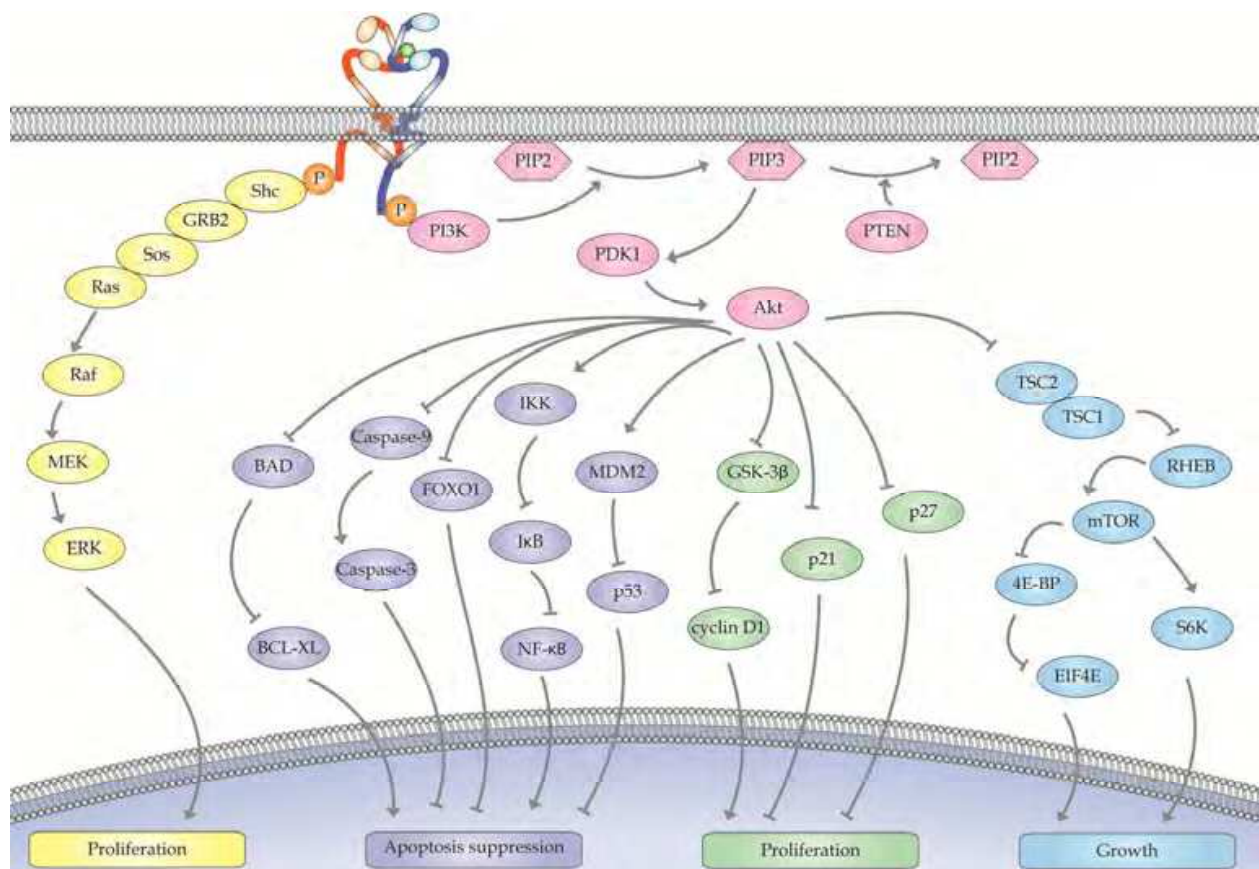

Fig. 2. Downstream signaling pathways of ErbB2–ErbB3 heterodimer.

The MAPK pathway and PI3K–Akt pathway are the two main pathways activated by ErbB receptors. GRB2–Sos complex, with or without Shc, recruits Ras and activates MAPK signaling, inducing cell proliferation, migration, differentiation and angiogenesis. PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. p110 is activated by p85 directly binding to phosphorylated tyrosine residues or indirectly binding though the GRB2–Sos–Ras complex, with or without Shc, to phosphorylated tyrosine residues within RTKs. Activated PI3K phosphorylates PIP2 into PIP3, which recruits and

activates PDK1. PDK1 phosphorylates and activates PI3K. PTEN dephosphorylates PIP3 into PIP2. Activated Akt phosphorylates many downstream proteins, inducing cell survival, proliferation, growth, and angiogenesis (Baselga & Swain, 2009; Cully et al., 2006; Vivanco & Sawyers, 2002). The heterodimer ErbB2–ErbB3 is the most oncogenic signaling activator of the PI3K–Akt pathway because ErbB2 possesses strong kinase activity, and ErbB3 possesses six p85-binding sites (Moasser, 2007).

3.3.4 Inhibition of PI3K–Akt signaling though PTEN by trastuzumab

Nagata et al. suggested the potency of trastuzumab (a humanized anti-ErbB2 monoclonal antibody, Herceptin®) is dependent on the ability to inhibit PI3K–Akt signaling pathway though activation of PTEN. PTEN dephosphorylates PIP3 into PIP2 and inhibits PI3K–Akt pathway activation. Overexpression of ErbB2 induces dimerization, resulting in phosphorylation on specific tyrosine residues of the intracellular domain. These phosphorylated tyrosine residues and surrounding amino acid side chains allow the recruitment and activation of downstream signaling proteins, including PI3K and Src. Activated PI3K activates PI3K–Akt pathway associated with cell survival, proliferation, growth and angiogenesis. Phosphorylated Src becomes activated and phosphorylates PTEN on tyrosine residues within the PTEN C2 domain, preventing PTEN from localizing to the cell membrane and dephosphorylating PI3K, and this also activates PI3K–Akt signaling. Treatment with trastuzumab keeps Src from binding ErbB2, leading to the dephosphorylation and inactivation of Src. PTEN is released from phosphorylation of Src and is localized to the cell membrane, which allows PTEN to antagonize PI3K function and negatively regulate the PI3K–Akt signaling pathway. However, Nagata et al. did not explore the mechanism that trastuzumab treatment rapidly keeps Src from ErbB2 (Crowder et al., 2004; Nagata et al., 2004).

3.3.5 The role of Src in the signaling pathway activated by ErbB2

Src, a non-receptor tyrosine kinase, is a critical component of multiple signaling pathways, leading to proliferation, survival, metastasis and angiogenesis. Several results indicate a functional and physical interaction between ErbB2 and Src. Src and ErbB receptors are overexpressed in ~70% of breast tumors (Ishizawar & Parsons, 2004). Src and Yes are activated in mammary tumors of MMTV-*neu* transgenic mice. Activation of Src is also observed in mammary epithelial cells transformed by ErbB2, but not by H-Ras, indicating that Src is a downstream signaling protein of ErbB2. Src directly interacts with the kinase domain of ErbB2. ErbB2 activates Src function by stabilizing Src and promoting increased Src expression or by directly phosphorylating Src in its SH2 domain. Src promotes ErbB2–ErbB3 dimerization, resulting in activation of their signaling activity. Src also phosphorylates the activation loop of the tyrosine kinase domain of ErbB2, which increases its kinase activity (Moasser, 2007).

3.3.6 The role of Cyclin D1 in ErbB2–induced tumorigenesis

Cyclin D1 is the key protein in ErbB2-induced tumorigenesis. ErbB2 overexpression in breast epithelial cells shows a short G1 phase and early S phase entry, mediated by the up-regulation of cyclin D1, cyclin E, and Cdk6 expression and enhanced degradation and relocalization of p21, leading to hyperproliferation (Timms et al., 2002). Cyclin D1-deficient mice show resistance to breast cancers induced by the *neu* oncogene, whereas they remain

fully sensitive to other oncogenic pathways, such as c-myc or Wnt1 (Yu et al., 2001). Ectopic expression of *neu* or Wnt oncogene in the mammary glands of mice deficient in Cdk4, the partner of cyclin D1, shows Cdk4 expression is required for efficient *neu*-induced tumorigenesis, whereas it is not required for Wnt-induced tumorigenesis (Reddy, 2005). p16, a CKI, blocks Cdk4 and Cdk6 activity, and the MMTV-p16 transgene blocks ErbB2-induced tumorigenesis. These results suggest the importance of cyclin D1 in ErbB2-induced tumorigenesis and cell cycle control (Yang et al., 2004). Furthermore, cyclin D1 kinase activity is required for the self-renewal of mammary stem and progenitor cells that are targets of MMTV-ErbB2 tumorigenesis (Jeselsohn et al., 2010).

3.3.7 Cooperation of ErbB2 and α6β4 integrin to promote breast cancers

Integrins, heterodimeric cell surface proteins mediate adhesion between cells and the extracellular matrix (ECM) and also regulate signaling pathways. Guo et al. suggested β4 integrin amplifies ErbB2 signaling, promoting mammary tumorigenesis, by studying a targeted deletion of the β4 signaling domain in MMTV-*neu* mice. A complex of ErbB2, Src, and β4 integrin induces phosphorylation of the signaling domain of β4 integrin and the P loop kinase domain of ErbB2, which enhances ErbB2 kinase activity through activation of Src. Cooperative signaling by ErbB2 and α6β4 integrins activates Jun and STAT3. JNK-mediated phosphorylation of Jun promotes oncogenic hyperproliferation. STAT3 promotes the loss of epithelial adhesion and acquisition of an invasive phenotype (W. Guo et al., 2006; Muthuswamy, 2006).

3.3.8 The role of ErbB2 as a nuclear tyrosine kinase receptor

Although ErbB receptors have been considered strictly plasma membrane receptors, recent studies suggest that they can translocate to the nucleus and act as transcription factors. Full-length ErbB1 and ErbB3 translocate to the nucleus. Nuclear ErbB1 physically interacts with STAT3, leading to the transcriptional activation of inducible nitric oxide synthase (iNOS). An intracellular fragment of ErbB4 translocates to the nucleus. First, the extracellular domain of ErbB4 is cleaved by ADAM17/TACE. Second, the transmembrane domain is cleaved by γ-secretase, allowing the cytoplasmic fragment of ErbB4 to translocate to the nucleus (Citri & Yarden, 2006; S.-C. Wang et al., 2004). The study of Wang et al. suggested that ErbB2 acts as a nuclear tyrosine kinase receptor. Full-length ErbB2 is also localized in the nucleus in both cultured cells and primary tumor tissues, where it is recruited to several gene promoters. For example, ErbB2 is recruited to the cyclooxygenase-2 (COX-2) promoter and activates its transcription. The increased expression of COX-2 is associated with angiogenesis, invasiveness and anti-apoptotic effects (S.-C. Wang et al., 2004). Because it lacks a DNA-binding domain, however, ErbB2 may interact with other nuclear factors to indirectly bind to these promoters.

3.3.9 Mass spectrometry–based quantitative proteomics of ErbB2 signaling networks

Recent technological advances in mass spectrometry (MS) have enabled us to understand the whole signaling networks in combination of biological and mathematical analyses. Pioneering research investigated EGFR signaling with MS-based quantitative proteomics (Blagoev et al., 2004). In this report, three cell populations were labeled by stable isotopes using amino acids in a cell culture technique called SILAC, and each population was

stimulated by EGF for a variable length of time. Subsequently, tyrosine-phosphorylated proteins and associated proteins were purified by anti-phosphotyrosine antibodies and then quantified by MS. A large number of signaling proteins were identified, and the dynamics of their activation were revealed. Following this study, the ErbB2 signaling pathway was studied by similar methods (Bose, 2006). Three groups of cell lysates (ErbB2-overexpressing cells, cells transfected with empty vector, ErbB2-overexpressing cells with EGFR and the ErbB2 selective tyrosine kinase inhibitor PD168393) were affinity-purified. Phosphoproteins were separated by SDS-PAGE and subjected to liquid chromatography–tandem MS. In their study, 462 proteins were identified and quantified. There were four major patterns of dynamics in protein phosphorylation. For example, the adaptor protein Dok1 showed increased phosphorylation in ErbB2-overexpressing cells and decreased phosphorylation when treated with PD168393. Another adaptor protein, Fyn-binding protein (Fyb), showed increased phosphorylation in ErbB2-overexpressing cells, but under PD168393 treatment, there was no significant change in phosphorylation. Focal adhesion kinase (FAK) showed decreased phosphorylation in ErbB2-overexpressing cells, whereas Grb2 showed no significant changes in phosphorylation. Overall, 198 proteins showed remarkable (>1.5-fold) increases in phosphorylation, and 81 proteins showed remarkable (<0.66-fold) decreases.

Another report examined phosphorylation in the ErbB2 signaling pathway with MS (Wolf-Yadlin et al., 2006). In this report, ErbB2-overexpressing cells were stimulated by EGF or NRG. From this analysis, they identified 332 phosphorylated peptides from 175 proteins. Among these peptides, 289 were singly phosphorylated, 42 were doubly phosphorylated, and one was triply phosphorylated. Altogether, 20 phosphorylation sites were identified on EGFR, HER2, and HER3. These results show that EGF stimulation of ErbB2-overexpressing cells activates multiple signaling pathways to induce migration, while HRG stimulation of these cells leads to the amplification of a specific subset of proteins in the migratory signal pathway. Most of these novel phosphorylations have not been studied in detail, but these studies have definitely improved our understanding of ErbB2 signal transduction networks. Besides phosphorylation, EGF-induced ubiquitination network also has been studied (Argenzio et al., 2011).

4. ErbB2 in human cancer

ErbB2 amplification and overexpression have been reported in several human cancers, including breast, ovarian, lung and other cancers (Baselga & Swain, 2009; Santarius et al., 2010). In contrast, recent sequence analysis revealed that point mutation and internal deletion of *ErbB2* were rare (http://www.sanger.ac.uk/). ErbB2 overexpression is associated with poor prognosis in most cancers (Baselga & Swain, 2009; Santarius et al., 2010). ErbB2 is thus used as a tumor marker as well as a target for cancer therapy. ErbB2-targeted therapy uses the humanized monoclonal antibody trastuzumab or the ErbB kinase inhibitor lapatinib, pertuzumab, trastuzumab-DM1, or ertumaxomab (see section 5). However, resistance to trastuzumab is a critical issue for this therapy. As described above in section 3.3, PTEN and Src are the key modulators of trastuzumab resistance. PTEN function has been positively correlated with the clinical effect of trastuzumab (Baselga & Swain, 2009). A recent report showed that targeting Src in combination with trastuzumab sensitized trastuzumab-resistant cells to trastuzumab and eliminated trastuzumab-resistant tumors *in vivo* (Zhang et al., 2011). This section summarizes the current findings on *ErbB2* amplification and prognosis in each type of cancer (Table 1).

4.1 Breast cancer

In the initial study by Slamon et al., 28% of 189 breast tumors showed evidence of *ErbB2* gene amplification (Slamon et al., 1989). In other reports, amplification rates vary from 18 to 40% of breast cancers (Baselga & Swain, 2009; Santarius et al., 2010). ErbB2 is overexpressed in most amplified cases and in some non-amplified cases as well. *ErbB2* amplification and overexpression are associated with poor prognosis, namely overall survival and time to relapse (Baselga & Swain, 2009; Santarius et al., 2010; Sircoulomb et al., 2010). As described in section 6.2, various types of breast cancer models with enforced expression of ErbB2 have been developed. Intriguingly, the growth of both primary mammary tumors and pulmonary metastases depends on the continuous expression of ErbB2 (Moody et al., 2002).

Amplification of the *ErbB2* gene correlates with enhanced ErbB2 expression. One study of the transcriptional regulation of *ErbB2* demonstrated that the X-linked FOXP3, which is a member of the forkhead/winged helix transcription factor family, represses transcription of *ErbB2* (Zuo et al., 2007). X-linked tumor suppressor genes are relevant to tumorigenesis because several of such genes are subject to X inactivation, leading to the realization of Knudson's two-hit hypothesis. According to that report, FOXP3 binds to its consensus sequence in the 5' promoter of the *ErbB2* gene and works as a transcriptional repressor. Moreover, somatic mutation of *FOXP3* was found in some breast cancer samples, and there was an inverse correlation between FOXP3 expression and that of ERBB2 among the samples, which is consistent with FOXP3's transcriptional activity. Finally, exogenous expression of FOXP3 inhibited the growth and tumorigenicity of various cancer cell lines. Recently, microRNAs (miRNAs) have attracted cancer researchers' attention because the relationships between deregulation of some miRNAs and tumorigenesis have been extensively reported. As expected, microarray analysis demonstrated that the expression level of each miRNA varied between types of cancer (Mattie et al., 2006). Interestingly, *miR-125b* and its homolog *miR-125a* were both downregulated in ErbB2-positive breast cancer. A subsequent study on the function of *miR-125* revealed that *miR-125* targeted the 3'-UTRs of *ErbB2* and *ErbB3*, and the overexpression of *miR-125* inhibited anchorage-dependent growth of SKBR3 and MCF10A cells. Interestingly, the migration and invasion of SKBR3 cells, which are derived from human ErbB2-positive breast cancer cells, was also inhibited by these miRNAs.

These results indicate that not only the amplification of the *ErbB2* gene but also regulation at the transcriptional level contributes to the overexpression of the gene, leading to human breast cancer.

4.2 Ovarian cancer

The initial study on ErbB2 in ovarian cancer was also conducted by Slamon et al., demonstrating that *ErbB2* is a reliable indicator of prognosis (Slamon et al., 1989). Among 73 ovarian cancers, 50 (68%) had staining similar to that of normal ovarian epithelium, while 23 (32%) showed strong staining (Berchuck et al., 1990). The prognosis of the 23 patients with ErbB2 overexpression was poorer than that of the 50 patients with normal ErbB2 expression (Berchuck et al., 1990). In ovarian cancer, therefore, ErbB2 is a predictor of prognosis. Recently, Zheng et al. showed that human immortalized ovarian epithelial cells with ErbB2 expression developed into papillary carcinoma in mice when injected intraperitoneally (Zheng et al., 2010).

cancer	amplification	mutation	prognosis	treatment	reference
breast cancer	18-40%	-	poor	trastuzumab, pertuzumab(phase III), trastuzumab-DM1(phase III), ertumaxomab(phase II), lapatinib	(Baselga & Swain, 2009; Santarius et al., 2010; Sircoulomb et al., 2010)
ovarian cancer	20%	-	poor	pertuzumab(phase II)	(Baselga & Swain, 2009; Berchuck et al., 1990; Santarius et al., 2010)
lung cancer	rare	10%	negative	lapatinib(phase II)	(Baselga & Swain, 2009; Hirsch et al., 2002; Santarius et al., 2010)
gastric cancer	10-30%	-	not clear	Trastuzumab(phase III), lapatinib(phase III)	(Baselga & Swain, 2009)
endometrial cancer	15-35%	-	poor	-	(Santarius et al., 2010)
oesophageal carcinoma	20%	-	poor	-	(Santarius et al., 2010)
bladder cancer	5-15%	-	poor	-	(Santarius et al., 2010)
medullo-blastoma	13%	-	poor	-	(Santarius et al., 2010)
glioma	-	-	poor	-	(Santarius et al., 2010)

Table 1. ErbB2 aberrations in human cancer.

4.3 Lung cancer

Ten percent of the adenocarcinoma subtype of lung cancer and a small proportion of non–small cell lung tumors show ErbB2-activating mutations in the kinase domain (Hirsch et al., 2002; Santarius et al., 2010). One important ErbB2 mutation is a G776 insertion in exon 20. *ErbB2* amplifications are rare in lung cancers (Hirsch et al., 2002). For example, among 238 non–small cell lung tumors, 39 (16%), including 35% of the adenocarcinomas, 20% of the large cell carcinomas and only 1% of the squamous cell carcinomas, were 2+ or 3+ overexpressed by immunohistochemistry using the HercepTest (S.E. Wang et al., 2006), whereas 3+ overexpression was rare (4%) (S.E. Wang et al., 2006). Inducible expression of ErbB2 mutations in lung epithelial cells causes invasive adenocarcinoma in mice (Perera et al., 2009). In contrast to EGFR, *ErbB2* mutations are not associated with prognosis (Tomizawa et al., 2011). In addition, one patient with the *ErbB2* mutation YVMA776–779ins responded to trastuzumab plus vinorelbine after failure of platinum-based chemotherapy and gefitinib.

4.4 Other cancers

10-30% of gastric cancers, 10–35% of endometrial cancers, 20% of Barrett's esophageal carcinomas, and 13% of medulloblastomas show *ErbB2* amplification and overexpression (Santarius et al., 2010). For example, in a panel of nine gastric cell lines, two of which

showed *ErbB2* amplification and overexpression, were more sensitive to trastuzumab than cells without *ErbB2* amplification. In endometrial cancer and esophageal carcinomas, *ErbB2* amplifications are associated with poor prognosis.

5. ErbB2 as a therapeutic target

5.1 Antibody-based agents

Accumulating evidence demonstrating the significance of ErbB2 in human cancer has prompted researchers and pharmaceutical companies to develop ErbB2-targeting cancer therapies. Monoclonal antibody therapy has finally demonstrated significant benefits for patients with cancer and has been established as a standard of care. Antibodies can inhibit tumor growth by several mechanisms (Hansel et al., 2010). In contrast to other ErbB receptors, ErbB2 has no known ligand. Therefore, antibodies targeting ErbB2 suppress ErbB2 signaling by inhibiting receptor homo- or hetero-dimerization and/or internalization (Chen et al., 2003). Alternatively, tumor-bound antibodies can initiate an immunological assault against the cancer by activating antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).

An important issue in the use of antibodies for therapy is the immunogenicity of the antibodies; antibodies derived from animals are easily recognized as foreign and cause strong immune responses (Khazaeli et al., 1994). This issue has been overcome by the generation of chimeric, humanized and fully human monoclonal antibodies, with a reduction in potentially immunogenic mouse components (Lonberg, 2005). The advances in antibody engineering have led to monoclonal antibodies with marked successes in the clinic (Adams & Weiner, 2005).

Trastuzumab, a humanized monoclonal antibody directed against the extracellular juxtamembrane (domain IV) of ErbB2, has had a major impact on the breast cancer therapy. In patients with breast cancer that overexpresses ErbB2, trastuzumab has anti-tumor activity and improves outcome and survival in combination with chemotherapy in patients with metastatic breast cancer (Slamon et al., 2001). Trastuzumab represents the standard of care in the adjuvant treatment of ErbB2-overexpressing breast cancers. The mechanisms through which trastuzumab exerts its effects are likely to include ADCC (Clynes et al., 2000), the inhibition of receptor dimerization and cleavage of the ErbB2 extracellular domain (Hudis, 2007).

However, the majority of patients with ErbB2-overexpressing tumors do not respond to trastuzumab or develop acquired resistance within a year. Several molecular mechanisms that could contribute to the development of trastuzumab resistance have been reported (Nahta & Esteva, 2006). In addition to alterations in ErbB2, other members of the ErbB family are thought to play roles in trastuzumab resistance by signaling downstream to the PI3K and MAPK pathways, bypassing trastuzumab's ErbB2-based inhibition of these pathways (Kruser & Wheeler, 2010). Loss of functionally of PTEN and mutations that activate PI3K are possible mechanisms of resistance to trastuzumab (see also 3.3.4 and Berns et al., 2007). Decreased interaction between trastuzumab and its target receptor ErbB2 due to steric hindrance of ErbB2 by MUC4 may prevent the actions of trastuzumab (Nagy et al., 2005). Because blocking ErbB2 to inhibit cancer cell growth can be circumvented through alternative signaling pathways, the combination of different agents that target molecules that contribute to anti-ErbB2 resistance may be a promising tool in the treatment of ErbB2-positive breast cancer.

Several other monoclonal antibodies that target ErbB2 are being tested in the clinic. Pertuzumab (developed by Genentech) is a humanized monoclonal antibody that binds to extracellular domain II of ErbB2, which is distinct from the binding site of trastuzumab. It sterically inhibits dimerization of ErbB2 with other ErbB proteins (Agus et al., 2002) and with IGF-1R (Nahta et al., 2005) and blocks the downstream signaling pathways of these dimers. The fact that pertuzumab and trastuzumab recognize different sites causes distinct downstream effects, and the combination of pertuzumab with trastuzumab synergistically inhibits the survival of breast tumor cells (Nahta et al., 2004).

Monoclonal antibodies are generally well tolerated in humans and are now established as targeted therapies. However, administration of mAbs carries the risk of immune and innate reactions (Presta, 2006). ErbB2-targeted therapies have been associated with cardiotoxicity because they can interrupt ErbB2 signaling, which is important for receptor signaling in the heart (Force et al., 2007). Fortunately, symptomatic cardiac toxicity frequently improves when trastuzumab therapy has been stopped (Romond et al., 2005).

The next generation of mAbs currently under development incorporates additional beneficial modifications, such as alterations in glycosylation and sequences that enhance ADCC (Lazar et al., 2006) or modifications in size and antigen-binding affinity that increase the ability of the mAb to penetrate solid tumors. Recombinant technology allows extensive modifications to be made to the structures of antibodies, including the production of recombinant antibody fragments. One advantage to this is that they are smaller and penetrate tissues and tumors more rapidly and deeply than whole antibodies (Jain, 1990). Bispecific and trispecific antibodies that target ErbB2 are also under investigation. These antibodies are designed to interact with other key proteins to facilitate, for example, the binding of a single antibody to all of the target antigens, to draw the immune cell into close proximity with the tumor cell that overexpresses ErbB2, with consequent recruitment of cytotoxic T cells to the T cell–antibody–tumor cell complex and immunological destruction of the tumor cell (Kiewe et al., 2006). Multifunctional mAbs will achieve more selective and effective cancer therapies.

5.2 Chemical compounds

In addition to antibodies targeting the extracellular domain of ErbR2, small-molecule tyrosine kinase inhibitors that directly inhibit the tyrosine kinase activity of ErbB2 have been developed. Among them, lapatinib (Tykerb®) is a dual tyrosine kinase inhibitor that targets both ErbB2 and EGFR. Lapatinib was developed as a safe and orally effective drug for the treatment of EGFR- or/and ErbB2-overexpressing breast cancer (Reid et al., 2007). It binds the ATP-binding pocket of the EGFR/ErbB2 protein kinase domain, preventing self-phosphorylation and the subsequent activation of the signaling cascade, leading to an increase in apoptosis and a decrease in cellular proliferation. In comparison to other tyrosine kinase inhibitors in clinical trials (for example, gefitinib and erlotinib), the interaction of lapatinib with EGFR and ErbB2 is reversible, but dissociation is much slower, allowing for prolonged downregulation of receptor tyrosine phosphorylation in tumor cells. Lapatinib in combination with trastuzumab exhibits a synergistic effect in ErbB2-overexpressing breast cancer cells, and it also has activity against trastuzumab-resistant cells (Konecny et al., 2006). Furthermore, due to the small size of lapatinib compared to antibodies, it can penetrate the blood–brain barrier and act against central nervous system (CNS) metastases. Moreover, clinical trials have demonstrated that lapatinib is a safe drug with no serious or symptomatic cardiotoxicity (Cameron & Stein, 2008).

5.3 Innovative materials for future drugs

Antibodies can also be used as a targeting device for other cytotoxic systems. The overexpression of ErbB2 on tumor cells and the accessibility of the extracellular domain of ErbB2 make it an ideal target for the targeted delivery of anti-tumor drugs as well as imaging agents (Colombo et al., 2010). Cytotoxic drugs, radioisotopes, toxins, enzymes or nano-scaled drug carriers can be conjugated with the antibody (Wu & Senter, 2005).

One successful application of such a complex is trastuzumab–DM1 (T–DM1), which consists of the trastuzumab antibody conjugated to the potent antimicrotubule drug DM1, a maytansine derivative. After binding to ErbB2, T–DM1 is internalized, and DM1 is subsequently released into the cell, thus delivering chemotherapy directly to cells that overexpress ErbB2. T-DM1 has antitumor activity in trastuzumab-sensitive and trastuzumab-resistant ErbB2-overexpressing breast cancer (Lewis Phillips et al., 2008).

Antibody-conjugated nano-scaled drug carriers offer major improvements in the therapeutic index of anticancer agents through the site-specific, efficient delivery of agents, which reduces side effects. Moreover, they can avoid multi-drug resistance (Peer et al., 2007). Many materials, such as liposomes, micelles, polymeric and metal nanoparticles, solid lipid particles, dendrimers and quantum dots, are used as nanocarriers. The material properties of each nanocarrier have been developed to enhance delivery to the tumor. These nano-scaled systems can be used to deliver small-molecule drugs as well as nucleic acid drugs. For example, anti-ErbB2 antibody fragment–conjugated, PEG-stabilized immunoliposomes display long-term circulation and selective delivery of the encapsulated drug into ErbB2-overexpressing cancer (Park et al., 2002). These anti-ErbB2 delivery systems greatly increase the therapeutic index by enhancing anti-tumor efficacy and reducing systemic toxicity. In part, this superior activity is attributed to the ability of the immunoliposomes to deliver their load inside the target cells via receptor-mediated endocytosis. The initial target accumulation could be achieved by passive targeting via the enhanced permeability and retention (EPR) effect (Maeda et al., 2009). Although antibody targeting is regarded as a promising strategy, some groups have reported that antibody targeting did not increase tumor localization but instead increased internalization (Kirpotin et al., 2006). Many pharmaceutical agents need to be delivered intracellularly to exert their therapeutic action in the cytoplasm or on individual organelles.

However, a problem with intracellular delivery is that any molecule/particle entering the cell via the endocytic pathway can become trapped in the endosome and eventually be degraded in the lysosome. As a result, only a small fraction of unaffected substance appears in the cytoplasm. Thus, even if efficient cellular uptake via endocytosis is observed, the delivery of intact agents is compromised by insufficient endosomal escape and subsequent lysosomal degradation (Lee et al., 2008). Improvement against lysosomal degradation is an important issue to be resolved.

6. Experimental methods to assesscellular transformation and tumorigenesis

This chapter focuses on representative experimental methods that have contributed to the studies of ErbB2 function, which include classical transformation assays using NIH3T3 cells, three-dimentional(3D) culture of MCF10A, transgenic and knock-in mice and recent techniques with non-germline genetically engineered mouse models.

6.1 Cell culture–based analysis

To analyze ErbB2 function, the transformation assay is the first choice because *ErbB2* was identified as an oncogene, as discussed above. We will review the classical transformation assay and introduce a novel, improved method we developed.

Transformation assays are usually carried out with NIH3T3 cells (Todaro & Green, 1963). The term "transformation assay" classically includes three representative assays: focus formation, colony formation, and tumor formation. Although they differ in detectable phenotype, the principle behind them is simple; if candidate genes introduced into NIH3T3 cells are oncogenic, they can confer cancerous phenotypes, such as the loss of contact inhibition, anchorage-independent growth, and tumorigenicity to the NIH3T3 cells.

The focus formation assay detects one of these oncogenic abilities, to cause the loss of contact inhibition. Contact inhibition is a phenotype usually present in NIH3T3 cells; NIH3T3 cells proliferate until they form a monolayer of cells. If oncogenes are introduced, then some of the cells might become transformed into cancer cells. Transformation can be quantified by the appearance of "foci" of transformants (Figure 3).

The colony formation assay detects another ability, to cause anchorage-independent growth. Anchorage-independent growth is a phenotype virtually all cancer cells have (Cifone & Fidler, 1980); cancer cells can multiply without attachment to the extracellular matrix or solid substrate, such as the bottom of the Petri dish. A transformant that can proliferate in soft agar in suspension forms a "colony", that is, amass of descendants from a single transformant. Thus, transformation activity can be quantified by the number of colonies formed.

The tumor formation assay detects the ability to generate tumors *in vivo*. In this assay, transformants are injected into a syngeneic host or immunocompromised mice, such as nude (*nu/nu*) mice, to examine whether they are able to grow and form tumors in their animal hosts.

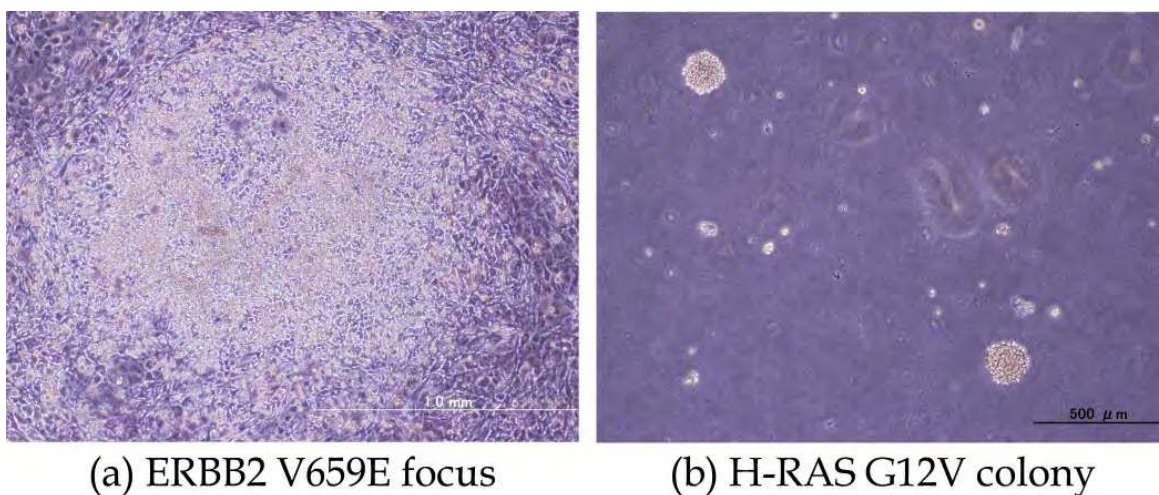(a) ERBB2 V659E focus   (b) H-RAS G12V colony

Fig. 3. Transformation assay with NIH3T3. (a)ERBB2 V659E has transformation activity. (b)H-RAS G12V, a mutant frequently observed in human cancer can also transform NIH3T3.

Rat *neu* transforms NIH3T3 cells, and the transformants are recognized as foci, which led to the discovery of *neu* itself (Schechter et al., 1984). Overexpression of wild-type ErbB2, as seen in human cancers, also transforms NIH3T3 cells (Di Fiore et al., 1987; Hudziak et al., 1987) by the colony formation and tumor formation assays. In contrast to human *ErbB2*, the

rat *neu* proto-oncogene *ErbB2* does not transform cells when only expressed in NIH3T3 cells (Hung et al., 1986), but it transforms them when coexpressed with EGFR (Kokai et al., 1989). About ten years after its discovery, the *neu* oncogene was found not to transform the NIH3T3-7d cell line, which is devoid of detectable ErbB family members (Cohen et al., 1996). *ErbB2* itself probably does not have enough oncogenic activity to achieve the transformation of regular NIH3T3 cells but can transform them in the presence of EGFR. Our data also indicate that ErbB2 itself does not transform NIH3T3 cells (M.S., unpublished data), in contrast to DiFiore et al. (Di Fiore et al., 1987; Hudziak et al., 1987). This discrepancy probably results from the variations in the NIH3T3 cells used in these assays, as they have been passaged dozens of times since their early establishment. To detect a novel gene that transforms NIH3T3 cells in harmony with *ErbB2*, we established *ErbB2*-expressing NIH3T3 cells and introduced genes that are overexpressed in breast cancer using a retroviral expression vector. In fact, we demonstrated that one gene transforms NIH3T3 cells only in the presence of ErbB2 (M.S., unpublished data). This phenomenon seems to resemble the tumorigenesis of ErbB2-positive breast cancer *in vitro*.

Breast cancer can progress step by step along a continuum of changes from a normal phenotype to a malignant disease. The human mammary gland consists of multiple ducts and lobes (Vargo-Gogola & Rosen, 2007). Lobes are composed of multiple acinal structures with hollow lumens surrounded by layers of luminal epithelial cells, outer myoepithelial cells, and basement membranes. However, this well-organized structure is disrupted at early premalignant stages of breast cancer, such as ductal carcinoma *in situ* (DCIS). DCIS is the most common type of non-invasive breast cancer in women. DCIS can progress to malignant invasive breast cancer (IBC) and, subsequently, metastatic cancer (Espina & Liotta, 2010). The genetic events that cause progression to malignant stages are only partially understood.

A 3D culture of MCF10A cells within Matrigel, which are derived from the Englebreth-Holm Swarm (EHS) tumor (Kleinman et al., 1982), is an excellent *in vitro* model for understanding the biological processes and signaling pathways responsible for tissue morphogenesis *in vivo* and the disruption of epithelial architecture at early stages of tumorigenesis (Debnath & Brugge, 2005). MCF10A cells are a spontaneously immortalized but non-transformed human breast epithelial cell line (Soule et al., 1990). Following a reported method (Debnath, 2003b), MCF10A cells in Matrigel form acinar structures characterized by a hollow lumen surrounded by polarized, growth-arrested luminal epithelial cells, as shown in Figure 4. 3D cultures can provide a physiologically relevant context to the *in vivo* breast microenvironment, while monolayer cultures on plastic involve an environment that is considerably different from the *in vivo* environment (Vargo-Gogola & Rosen, 2007; Yamada & Cukierman, 2007). Certainly, both primary cultures of human tumor tissues and mouse models of cancer are practical to study carcinoma formation; however, they are relatively difficult to handle for understanding the biochemical and molecular biological pathways involved in the early stages of oncogenesis (Debnath, 2003b).

Madin-Darby canine kidney (MDCK) cells represent another widely used 3D culture model. When MDCK cells are embedded within collagen gels as single cells, they form polarized cysts with a central hollow lumen (Figure 5). During morphogenesis, intracellular vesicles containing apical membrane components are delivered to the cell surface between closely apposed cells, and membranes are separated, leading to the generation of several small lumens (Andrew & Ewald, 2010; Bryant & Mostov, 2008). Fusion of the lumens into a single large lumen subsequently occurs, and this phase requires apoptosis of luminal cells to clear
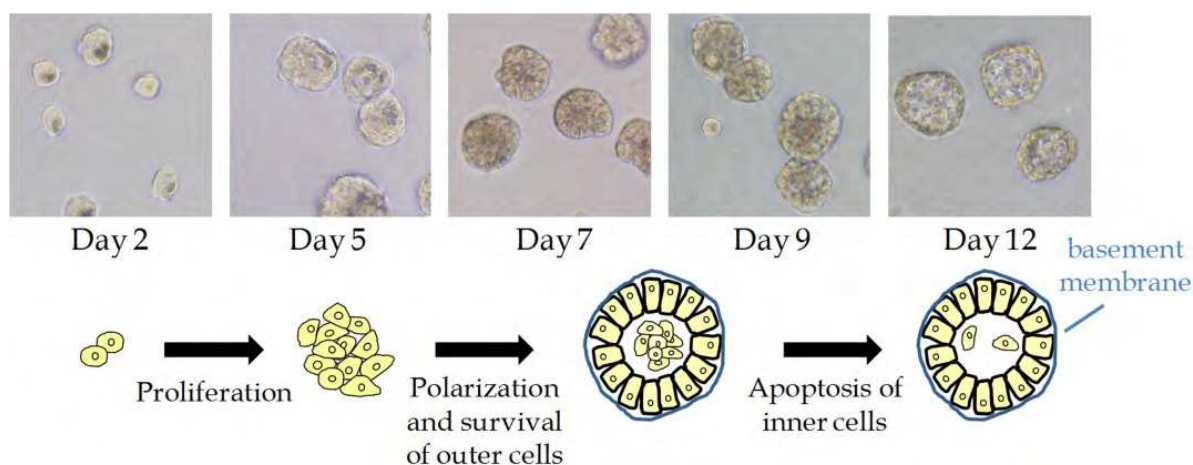
Fig. 4. MCF10A acinal structures constructed within Matrigel.
(1) Single cells embedded in Matrigel proliferate and form cell clusters. (2) At day 5–7, cells
are distinguished between matrix-attached outer cells and matrix-deprived inner cells
within each acinus. (3) After their fate has been determined, outer epithelial cells acquire
apical-to-basal polarity and receive survival signals. (4) At day 8, however, non-polarized
inner cells start to die via apoptosis. (5) Finally, replicated mammary acini with luminal
spaces are constructed at day 14.

the lumen (Andrew & Ewald, 2010; Martin-Belmonte et al., 2008). Given that laminin is
essential for initiating polarization mediated by the interaction between integrins and the ECM
in the MDCK system, MDCK cells grown within the laminin-rich ECM, as within Matrigel, can
polarize more efficiently than within collagen gels. The cells polarize and form clear lumens
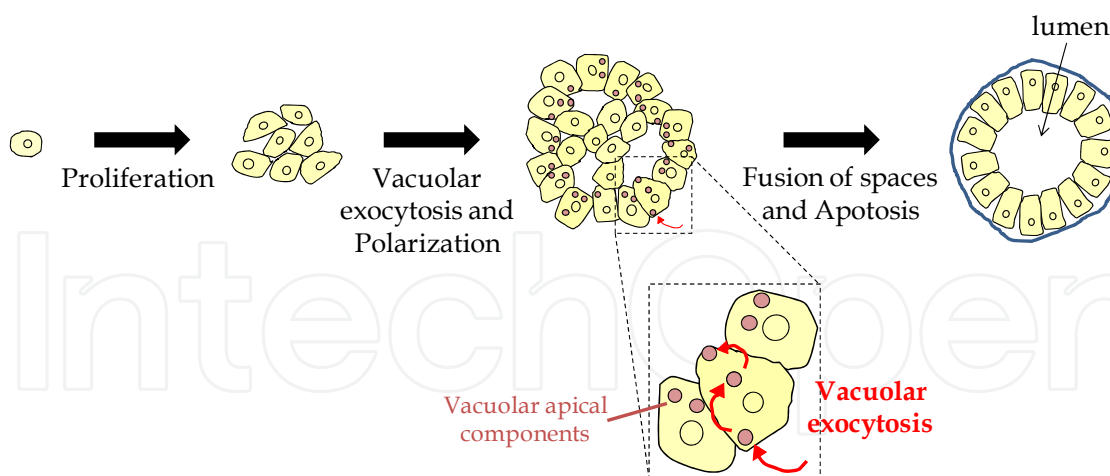much faster, without the requirement of apoptosis (Martin-Belmonte et al., 2008).

Fig. 5. Lumen formation by MDCK cells through vacuolar exocytosis of apical proteins.
(1) Single cells embedded in collagen gel or Matrigel proliferate to form groups of cells. (2)
Intracellular vesicles containing apical membrane components are delivered to regions
between cells and create luminal spaces via the fusion of vesicles and the plasma membrane.
The surrounding cells now exhibit apical–basal polarity. (3) Several small lumens fuse with
each other and generate one large lumen. Under the condition where cells are cultured
within the laminin-lacking ECM, inner cell death by apoptosis is essential to clear the lumen
(Andrew & Ewald, 2010; Bryant & Mostov, 2008).

Using the MCF10A 3D culture system, previous studies have revealed the effects of several oncogenes and viral oncoproteins on the process of acini formation (Debnath & Brugge, 2005; Shaw et al., 2004). For example, inactivation of the retinoblastoma protein (Rb) by HPV E7 facilitates proliferation (Debnath et al., 2002). Co-overexpression of CSF1-R and CSF1, both of which are elevated in mammary tumors, induces inner cell survival and loss of cell–cell adhesion, as well as hyperproliferation (Wrobel, 2004). Activated Akt leads to large, distorted, and filled-lumen structures (Debnath, 2003a). Each phenotype in these studies results from the biological activities of the introduced gene, suggesting that specific biological processes and pathways are modulated by the oncogene. Furthermore, these morphologies in 3D culture resemble the histological changes observed in human tumors *in vivo* (Debnath & Brugge, 2005).

Recently, the MCF10A 3D culture has been used to search for novel candidate oncogenes, such as the Yes kinase–associated protein (YAP) gene. *YAP* is included in the chromosome 11q22 amplicon that frequently appears in human tumors. Overexpression of YAP induces an anti-apoptotic and invasive morphology (Overholtzer, 2006). Further analysis has identified tumorigenic functions of generally non-tumorigenic proteins. For example, a study of MCF10A 3D structures suggested that not only oncogenes but also antioxidants could facilitate malignancy of carcinoma. In mammary acini, centrally located cells lack glucose transport and produce reactive oxygen species (ROS). Antioxidants could antagonize such metabolic stresses and promote the survival of cells detaching from the ECM by rescuing ATP production through fatty acid oxidation (FAO) (Schafer et al., 2009). Additionally, overexpression of a glycosyltransferase, N-acetylglucosaminyltransferase V (GnT-V) induces the disarrangement of mammary acinar morphogenesis, including increased cell proliferation, filled lumens, and disrupted polarity. Thus, altered expression of GnT-V, which catalyzes posttranslational modification, affects early stages of breast carcinogenesis (H.B. Guo et al., 2010).

ERBB2 amplification in breast cancer is correlated with poor prognosis due to increased metastasis and resistance to chemotherapy. This gene is overexpressed in 50–60% of DCIS (Lu et al., 2009; Nofech-Mozes et al., 2005). Muthuswamy et al. showed that ErbB2-induced cells generate aberrant acini in 3D culture *in vitro*. To trigger homodimerization and activate ErbB2 signaling, they constructed chimeric receptors, as shown in Figure 6(a) (Muthuswamy et al., 1999; Muthuswamy et al., 2001). Treatment with AP1510 induced receptor homodimerization, leading to multi-acinar structures with filled lumens but no invasive phenotype. Therefore, additional genetic events as "second hits" may be required for invasion (Seton-Rogers, 2004). Those features are reminiscent of ErbB2-overexpressing DCIS *in vivo* (Muthuswamy et al., 2001). A recent analysis of ErbB2-mediated transformation indicated the significance of Tyr 1201 phosphorylation for the disruption of apical–basal polarity of MCF10A cells (Lucs et al., 2010).

Our laboratory uses a full-length *ErbB2* mutant, V659E, as a model of the constitutively activated ErbB2 receptor. It has higher intrinsic kinase activity and increased ability to induce transformation compared to wild-type ErbB2. This receptor can form a heterodimer with the other ErbB receptor family members as well as a homodimer, whereas chimeric receptors only form homodimers (Figure 6(b)). Thus our system may mimic physiological condition *in vivo* (A.D., unpublished data). We control the induction timing of ErbB2VE using the reverse tetracycline (Tet)-controlled transcriptional activator system, called "Tet-on". Induction of ErbB2VE leads to unregulated proliferation and filling of luminal spaces, whose structures are similar to "multi-acini" reported in a previous study (Muthuswamy et al., 2001).
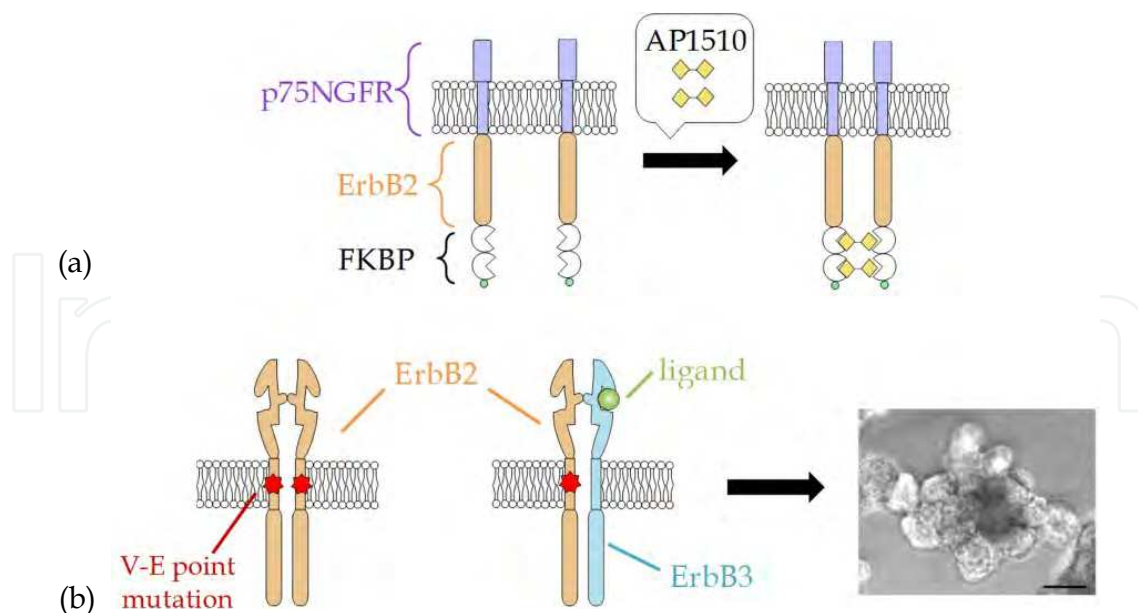
Fig. 6. Disruption of acinal structures due to ErbB2 signaling activation.
(a) Chimeric ErbB2 receptors induce homodimerization mediated by AP1510. MCF10A acini were treated with AP1510 at day 10 and cultured in Matrigel until day 20, resulting in the aberrant structure composed of multiple acini with filled lumens (Muthuswamy et al., 2001; Seton-Rogers, 2004). (b) In our laboratory, Tet-responsive ErbB2VE was expressed by treatment with Dox, forming both homo- and hetero-dimers. ErbB2VE activation from day 4 induces hyperproliferation and exhibits multi-acinar structures at day 12. This structure is similar to the phenotype previously reported (Muthuswamy et al., 2001). The scale bar represents 50 μm.

There is no evidence for how DCIS, which is a non-invasive and premalignant lesion, acquires invasive behavior. Given that DCIS with *ErbB2* amplification or overexpression frequently progresses to IBC, it is important to identify genes that collaborate with ErbB2 and induce tumor progression, including invasion and metastasis. Several investigators have been successful in detecting such factors using the MCF10A system. For example, either transforming growth factorβ (TGFβ) or prostate-derived Ets factor (PDEF) dramatically alters MCF10A spheroid-like acinal structures into protrusive cords invading the ECM in cooperation with ErbB2 (Gunawardane, 2005; Seton-Rogers, 2004). Additionally, co-overexpression of ErbB2 and 14-3-3γ, which shows increased expression in the early stages of breast cancer, results in larger acinar size, filled lumen, the gain of invasiveness, and disordered basal membrane protein laminin (Lu et al., 2009).

6.2 Animal models

Genetically engineered mouse (GEM) models have contributed broadly to the field of cancer research. First, we will review GEM models of breast cancer as a representative cancer related to the deregulation of ErbB2. The earliest model was generated by introducing *neu* oncogenes under the transcriptional control of the mouse mammary tumor virus (MMTV) LTR (Bouchard et al., 1989; C.T. Guy et al., 1992; Muller et al., 1988). MMTV LTR drives the expression of neu specifically in mammary gland intargeted mice. Tissue-specific expression is also achieved by using the whey acidic protein (WAP) promoter (Piechocki et al., 2003). The next useful development for GEM models was the Cre-loxP system (Wagner et al.,

1997). Cre-loxP models have genetic elements flanked by loxP sites that are processed by Cre recombinase, and its utility is widely known. Although *neu* under the strong viral LTR has questionable relevance to human breast cancers, mammary-specific expression of *neu* at endogenous levels had been desired. Thus, knock-in mice generated by interbreeding transgenic mice that have the *neu* promoter flanked by loxP sites with mice expressing Cre under the MMTV LTR (Andrechek et al., 2000) were developed, providing a valuable murine cancer model. *Neu* under the physiological promoter does not cause rapid tumor progression as seen in transgenic mice harboring MMTV-driven *neu*. However, they show amplification of the recombinant *neu* allele, as observed in human cancers (Andrechek et al., 2000). Furthermore, microarray profiling revealed that tumors arise in the knock-in mice, which demonstrated increased expression of GRB7 (Andrechek et al., 2003), as seen in human breast cancers.

More recently, several non-germline GEM models have been developed (Heyer et al., 2010). For example, generating chimeric mice is a novel way to increase the physiological relevance to human cancer, where cancer cells are surrounded by normal tissue, and it is also relatively cost-efficient. As in other models, chimeric mice carrying ErbB2 V659E in the lung, develop lung adenocarcinomas (Zhou et al., 2010). The necessity and desire to reproduce human cancers in mice will lead to more efficient translational research toward cancer therapies in the future.

7. Conclusion

We reviewed the history of the discovery of *ErbB2* with the recent progress on ErbB2 function. Thirty years have passed since the *neu* oncogene was discovered, and extensive research on ErbB proteins has been carried out since then. As long as tumor cell growth depends on ErbB2 expression, it is a rational target for therapy, and efforts will be made to develop more effective drugs than trastuzumab. In basic as well as clinical ErbB2 research, studies of cancer stem cells and the cells of origin (Visvader, 2011) of the ErbB2-induced tumors should be carried out. ErbB2 expression increases the population of stem/progenitor cells (Korkaya et al., 2008). Two distinct mammary progenitors have been identified, one of which is a target of MMTV-ErbB2 tumorigenesis and requires cyclin D1 kinase activity for its self-renewal (Jeselsohn et al., 2010). This suggests a combinational therapy with trastuzumab and cyclin D1 inhibitors may be more effective at treating ErbB2-mediated tumorigenesis than trastuzumab alone. The recent notion of non-oncogene addiction also needs to be considered (Luo et al., 2009). Identification of such genes will enable us to develop more effective targeted therapies as well as to understand the mechanism of ErbB2-mediated tumorigenesis itself.

8. Acknowledgement

9. References

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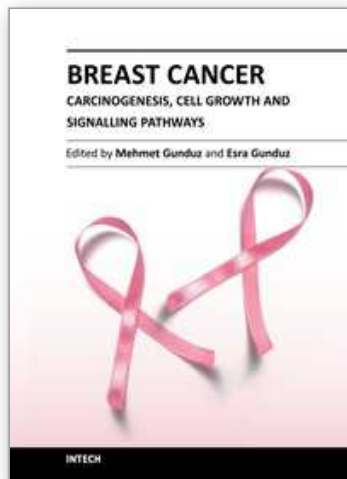
Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways

Edited by Prof. Mehmet Gunduz

Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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