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The ErbB2 Signaling Network as a Target for Breast Cancer Therapy

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Abstract Overexpression of the ErbB2/Her2 receptor tyrosine kinase in breast cancers is associated with the most aggressive tumors. Experimental studies have revealed that ErbB2 shows many features of a therapeutic target: ErbB2 is able to confer many of the characteristics of a cancerous cell, including uncontrolled proliferation, resistance to apoptosis and increased motility; ErbB2 overexpression is specific to tumor cells; as a cell surface-associated protein, it is easily accessible to drugs and as a kinase it is amenable to targeted inhibition by small molecules. Recent clinical results demonstrate the efficacy of ErbB2-targeting therapy and promise an expanding use of ErbB2-targeting drugs for breast cancer treatment. However, as only a fraction of patients responds successfully to therapy and risks of recurrence are still high, further investigation is required for an improved understanding of the complex network of signaling pathways underlying ErbB2-driven cancer progression.

Keywords ErbB2/HER2/Neu · Breast cancer · Signaling · Cancer therapy · Trastuzumab

Abbreviations

ADCC	antibody dependent cellular toxicity
Cox	cyclooxygenase
EGF	epidermal growth factor
HAS	Her2-associated sequence
iNOS	nitric oxide synthase
NRG	neuregulin
PLCγ	phospholipase Cγ
TGF	transforming growth factor

> It is only the beginning, let us continue the fight (Anonymous slogan, May 1968, La Sorbonne, Paris).

Introduction

Receptor tyrosine kinases are major players in transduction of extracellular cues into intracellular signals that allow a cell to adjust to the environment. As such, they play key roles during embryonic development and homeostasis in the adult. Not surprisingly, aberrant expression of receptor tyrosine kinases leads to dramatic physiological consequences, as illustrated by malignant tumor formation. More than 15 years ago, seminal studies were reported that abnormal expression of ErbB2 receptor tyrosine kinase in breast tumors was linked to poor prognosis. Since then, countless investigations identified many effectors of ErbB2 and their roles in many fundamental cellular processes, such as cell division or cell motility. Because these data have suggested that ErbB2 could have a causal role in

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tumor formation, it was anticipated that inactivating ErbB2 might impede cancer progression in those patients with overexpressed ErbB2. Recent clinical reports, showing that indeed a humanized antibody targeting ErbB2 can dramatically improve outcomes of women with ErbB2-expressing tumors, are the culminating point of this success story of translational research. The very encouraging successes of ErbB2 therapy, as well as its limitations, call for further exploration of ErbB2 cellular physiology.

In this review, we summarize our understanding of ErbB2 signal transduction, emphasizing recent findings which reveal the ever increasing complexity of the ErbB2 signaling network and the multiplicity of ErbB2 modes of action. We also provide a snapshot of the rapidly evolving field of ErbB2-directed therapies, which shows both spectacular advances and hurdles yet to overcome.

Structure of ErbB2

ErbB2, also called HER2 or Neu, belongs to the ErbB family of receptor tyrosine kinases. This family also includes the epidermal growth factor receptor (EGFR)/ErbB1, the founding member of the family, ErbB3 and ErbB4. They are transmembrane proteins with a conserved domain organization: an extracellular EGF-related peptide-binding region composed of domains I to IV, a transmembrane helix, an intracellular region comprising a well conserved tyrosine kinase domain and a less conserved regulatory C-terminal tail. A number of EGF-related peptides have been shown to associate with and activate specific ErbB receptors: EGF, transforming growth factor (TGF) α and amphiregulin bind ErbB1, betacellulin, heparin-binding EGF and epiregulin bind both ErbB1 and ErbB4 and neuregulins (NRGs) associate with ErbB3 and ErbB4 (NRG1 and NRG2) or only with ErbB4 (NRG3 and NRG4) [1]. Ligand binding triggers ErbB receptor homo- and hetero-dimerization, a prerequisite for kinase activation and signal transduction. Recent structural studies [2] indicate that, in the absence of ligand, ErbB1, ErbB3 and ErbB4 are in a so-called tethered conformation, in which domain II interacts with domain IV (Fig. 1). Ligand binding to domain I and III induces a transition in the conformation from a tethered to an extended state, exposing a dimerization arm in domain II. This arm interacts with domain II of a nearby receptor to form ErbB homo- or hetero-dimers.

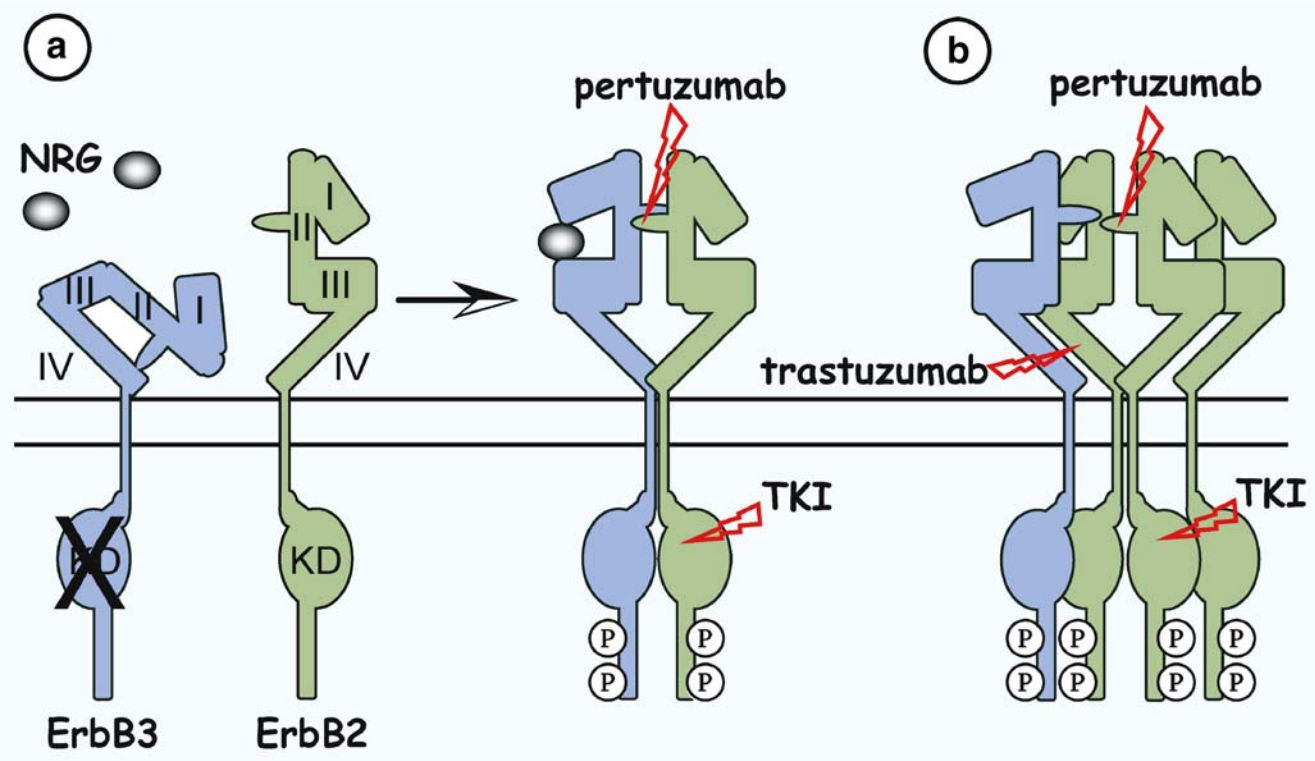

Figure 1 Modes of ErbB2 activation. a Ligand binding to ErbB receptors (neuregulin (NRG)-binding to ErbB3 is shown here) induces a transition from a tethered to an extended state, allowing the dimerization arm protruding from domain II to interact with domain II of another ErbB. In contrast, ErbB2 is constitutively present in the extended conformation. ErbB dimerization might also involve interactions between the respective transmembrane and kinase domains. b When overexpressed, ErbB2 can be activated in the absence of ligand. However, oncogenic signaling from overexpressed ErbB2 also involves other ErbB receptors such as ErbB3. The mechanism of ErbB3 recruitment to the receptor complex is not known. Sites of interaction of humanized antibodies trastuzumab and pertuzumab and tyrosine kinase inhibitors (TKI) are indicated. Trastuzumab interferes with overexpressed ErbB2 activity, while pertuzumab prevents both over-expressed and ligand-induced activity. The crossed kinase domain (KD) of ErbB3 indicates that it lacks kinase activity.

Interestingly there has been no convincing evidence for an ErbB2 extracellular ligand and the existence of an elusive ligand for ErbB2 has remained a latent question in the field. The resolution of ErbB2 structure might have provided a definitive answer to this issue. Indeed, analysis of ErbB2 extracellular domain has demonstrated that the structure of ErbB2 putative ligand-binding site prevents access to EGF-related peptides and that key residues for ligand binding are not conserved in ErbB2 [3]. Resolution of ErbB2 structure also revealed that ErbB2 constitutively adopts an extended state, exposing the dimerization arm in a ligand-independent manner (Fig. 1). The fact that ErbB2 appears to be constantly poised for interaction with other ErbB receptors provides a rationale to previous results showing that, despite the lack of an extracellular ligand, ErbB2 is the preferred heterodimerization partner for other ErbB receptors [4]. The ErbB2/ErbB3 dimer is the perfect illustration of ErbB2's pivotal role. Indeed, because of the substitution of critical residues in the kinase catalytic domain, ErbB3 essentially lacks kinase activity [5]. The association of kinase-dead ErbB3 with ligand-less ErbB2, however, makes a remarkably efficient signaling complex [6]. Another distinctive characteristic of ErbB2 is that overexpression results in activation of ErbB2 and downstream intracellular signaling, even in the absence of ligand. While one might speculate that ErbB2 auto-activated conformation could facilitate self-association in the absence of ligand, biophysical studies have failed to detect ErbB2 extracellular domain homodimers. *In vivo* dimer formation most probably involves other regions, including the transmembrane and kinase domains [7, 8]. Furthermore, signaling from overexpressed ErbB2 also involves other ErbB family members, as indicated by studies showing that ErbB3 is required for overexpressed ErbB2 biological activity [9]. However, the mechanism whereby ErbB3 is recruited to ErbB2 clusters remains unknown. Finally Muc4/sialomucin, a transmembrane glycoprotein complex, has been identified as an intramembrane ligand for ErbB2, capable of modulating its activity [10].

ErbB2-Dependent Intracellular Signaling Pathways

ErbB dimerization triggers tyrosine kinase activity, leading to phosphorylation of specific tyrosine residues within the C-terminal regulatory domain. These phosphotyrosines serve as docking sites for SH2 and PTB domain-containing signaling molecules: adaptors (such as Shc, Grb2, p85), enzymes (e.g., the SHP-2/PTP-2c tyrosine phosphatase) or STAT transcription factors [11]. ErbB2 molecular partners include Shc, Grb2, the Crk family of adaptor proteins, phospholipase Cγ (PLCγ) and the potential repressors of signaling CHK and Dok-2 [11]. Recruitment of these adaptors is the primary event of a signaling cascade which will instruct the cell to proliferate, differentiate, survive or migrate. For instance, ErbB2 is known to activate the Ras/Raf/Erk signaling cascade through the recruitment of Shc, Grb2 [12], which might in turn recruit the Gab2/SHP-2 complex [13] and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway via trans-phosphorylation of ErbB3 tyrosine residues [14], ultimately activating nuclear effectors, i.e., transcription factors and cell cycle regulators.

However, this simple picture, which has provided a satisfactory framework to investigate many characteristics of ErbB2 oncogenic ability, has been challenged by recent studies which reveal the complexity of ErbB2 signal transduction. Proteomic approaches suggested that ErbB2 signaling might involve many more adaptors and signaling molecules than previously described [15, 16]. And recent reports revealed that ErbB2 could use alternative signaling routes, by controlling the translation efficiency of pro-oncogenic mediators [17, 18] or bypassing cytoplasmic effectors altogether to shuttle to the nucleus [19] and act as a component of a transcription factor complex (Fig. 2).

System-Wide Analysis of ErbB2 Signaling

While ErbB signaling has been extensively studied in the past decade, recent developments in quantitative proteomics provide a fresh, unbiased perspective at the complex network of signaling pathways mobilized upon ErbB activation.

Systematic profiling of phospho-dependent interactions, through a quantitative proteomic approach combining differential isotope labeling and mass-spectrometry, has uncovered many novel potential phosphotyrosine docking sites within all four ErbB receptors [15]. The actual phosphorylation of these sites and recruitment of adapter molecules thereto in the context of an intact receptor remains to be verified. However, even though this study failed to identify some previously identified ErbB-binding proteins, it confirmed that ErbB2 signals mostly through Shc, ErbB3 through the p85 and ErbB4 via Grb2/Shc. This approach uncovered novel interactions, including Stat5 association to a binding module present in both EGFR and ErbB4 and, interestingly, the interaction of the PTP-2c tyrosine phosphatase with ErbB2 tyrosine residue 1023 (Tyr1023). This site was known to repress ErbB2 signaling and biological activity, but the mediator of this activity was not identified yet [20]. It is thus tempting to speculate that PTP2c is actually the Tyr1023-associated negative regulator of ErbB2 signaling. This observation illustrates the relevance of the novel interactors identified by the systematic proteomic approach. Another high-throughput study, investigating the interaction of all predicted PTB and SH2 domains in the human genome with the major phosphorylation sites of ErbB receptors revealed yet another layer of complexity in ErbB signaling [16]. Many novel interactions

were described with a wide range of affinities. Analysis of ErbB networks at different affinity thresholds revealed that, in contrast to ErbB3, EGFR and ErbB2 are markedly more promiscuous as threshold is lowered, i.e., the composition of the ErbB2 complex is largely dependent on the abundance of the receptor [16]. The fact that specific pathways might be turned on at high levels of active ErbB2 has implications for ErbB2 oncogenic potential, as ErbB2 is often aberrantly expressed in tumors. Again it will be important to confirm the physiological relevance of these observations.

A pioneer temporal quantitative proteomic analysis of EGFR-dependent signaling demonstrated the power of such global approaches for the understanding of receptor tyrosine kinase signal transduction at a systems level [21]. This study detected activation of at least 50 proteins that have been previously shown to be direct or indirect EGFR effectors, with activation profiles over time generally consistent with the inferred chronological sequence of events. The study also revealed effectors that have not been linked to EGFR signaling before, including regulators of small G proteins and kinases. Not the least surprising was the presence of several RNA-binding proteins in the EGF signaling complex. This observation might be linked to the newly described role for these proteins in cell spreading [22]. Interestingly, typical activation profiles allowed association of proteins of unknown function to specific biological processes. A similar analysis of ErbB2-associated signaling will undoubtedly reveal both similar, as well as specific features.

Unconventional ErbB2 Signaling: "Off the Beaten Path"

As transmembrane receptors, ErbBs are traditionally described to activate cytoplasmic signaling cascades, which ultimately lead to activation of nuclear transcription factor and gene transcription. Classification of EGFR effectors identified in the temporal analysis revealed that only one third of the proteins were linked to signal transduction to the nucleus, with another third being involved in cell adhesion and cytoskeleton remodeling. This observation indicates that EGFR signaling does not necessarily follow a linear path from the receptor to cytoplasmic intermediates to the nucleus.

Several studies have now demonstrated that ErbBs can also function within the nucleus. The various ErbBs, however, appear to use different modes of action. ErbB4 nuclear activity

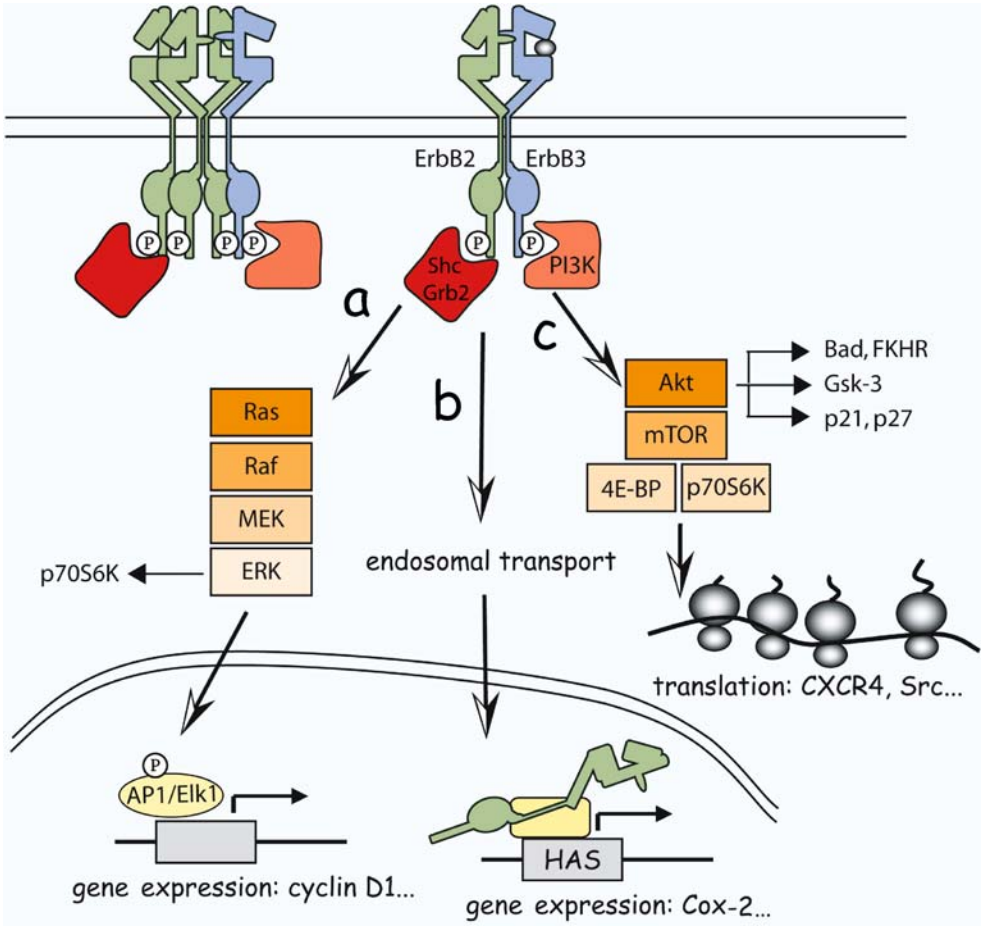

Figure 2 ErbB2-induced intracellular signaling pathways. ErbB2 can signal through various routes: upon ErbB2 activation, signaling molecules are recruited to phosphorylated tyrosine residues triggering intracellular signaling cascades such as the Ras/Erk and PI3K/Akt pathways. These pathways lead in turn to activation of a specific set of transcription factors and specific gene expression (a). Alternatively, ErbB2 was shown to translocate to the nucleus and associate with specific sequences within the promoter region of pro-oncogenic genes to regulate their expression (b). Finally, ErbB2 was demonstrated to control expression of specific proteins at the level of translation, via the Akt/ mTOR pathway (c).

involves proteolytic cleavage of the intracellular domain, nuclear co-translocation with the Stat5A transcription factor and binding at target promoters [23]. In contrast, EGFR and ErbB2 translocation to the nucleus doesn't require proteolytic cleavage. EGFR was demonstrated to form a transcriptional complex together with Stat3 leading to activation of the inducible nitric oxide synthase (iNOS) gene [24]. Through a systematic search for genomic material associated with ErbB2, Wang et al. demonstrated that nuclear ErbB2 binds to several gene promoters, including the promoter of cyclooxygenase (Cox-2), a known contributor to tumor development, both in cell lines and primary breast tumors [19]. Moreover nuclear expression of ErbB2 was significantly correlated with Cox-2 expression [19]. While the C-terminal domain of ErbB2 was shown to possess an intrinsic transactivation function, a co-factor allowing ErbB2 DNA binding was not yet identified. The contribution of nuclear ErbB2 to the tumorigenic process clearly deserves further investigation.

In addition to regulating the expression of specific genes, ErbB2 activity was also shown to control expression of pro-oncogenic proteins at the translational level. For instance, ErbB2-activated breast cancer cells show high metastatic potential and increased activity of the cytoplasmic tyrosine kinase Src. Increased Src activity contributes to ErbB2-driven metastasis. In fact, it was shown that ErbB2 does not affect *src* mRNA, but promotes both Src synthesis and stability [17]. Control of the expression of the CXCR4 chemokine receptor was also shown to occur at the translational level [18]. CXCR4 plays a critical role in mediating the homing of tumor cells to metastatic organs; release of the CXCR4 ligand, SDF1α, attracts CXCR4-expressing breast cancer cells to organs such as lung, bone and liver [25]. Interestingly, CXCR4 appears to be a critical effector of ErbB2-induced lung metastasis [18]. Translational regulation of both Src and CXCR4 involves the PI3K/Akt/mTOR-dependent pathway. In addition, ErbB2-induced signaling pathways are known to control the translation machinery. The Ras/Erk pathway regulates the expression and phosphorylation of the eukaryotic initiation factor eiF-4E, and, most importantly, the PI3K pathway triggers the mTOR-mediated activation of p70S6 kinase and inactivation of 4E-BP1, respectively, activator and inhibitor of translational initiation [26]. Finally, a microarray analysis of actively translated mRNA in a Ras and Akt-dependent cellular model revealed that these pathways affect recruitment of specific mRNAs to ribosomes more than transcription. Upon signaling blockade, major changes in actively translated mRNA (encoding proteins involved in cell cycle progression and cell motility) occurred before any transcriptional changes could be detected [27]. Thus, control of protein production by ErbB2 signaling could also provide a highly specific and rapid control of pro-oncogenic gene expression. While inhibition of the mammalian target of rapamycin (mTOR) pathway has proven efficient in reducing tumor size in transgenic mice bearing the activated *ErbB2* gene [28], the exact contribution of translational effects to ErbB2-driven tumor formation and maintenance still needs to be evaluated.

The understanding of the many routes followed by ErbB2 intracellular signals and the identification of the critical mediators of ErbB2 tumorigenic potential are required for the rational design of efficient therapeutics.

Role of ErbB2 in Development

Even though there seems to be a great deal of overlap between the major signaling pathways induced by the respective ErbB receptors, gene targeting studies indicate that specific ErbBs have distinct, non-redundant functions. This is probably due to the time- and tissue-specific patterns of ErbB receptor and ErbB ligand expression, as well as specific signaling of individual ErbBs.

ErbB2, in particular, plays a vital role during development as evidenced by the dramatic phenotype resulting from the *ErbB2* gene knock-out. Mice impaired for ErbB2 expression die in utero because of improper formation of cardiac trabeculae [29]. While myocardial expression of an *erbB2* transgene rescues the embryonic lethality [30], mice die at birth due to loss of motor neurons and defects in Schwann cell development. Tissue specific knock-out mouse models and dominant negative ErbB2-expressing transgenic mice demonstrate the critical function of ErbB2 in multiple organs: ErbB2 is required for terminal differentiation of oligodendrocytes and myelin formation in the central [31] and peripheral nervous system [30], establishment of radial glia in cerebral cortex [32], muscle spindle maintenance [33], ductal development of the mammary gland [34] and prevention of cardiomyopathy in the adult heart [30].

Cellular Responses to ErbB2

A wealth of experimental data has demonstrated the multiple facets of the cellular response to ErbB2, which altogether contribute to its function during development and tumorigenesis.

Cell Proliferation

Overexpression of ErbB2 leads to cell transformation, a phenomenon largely dependent on increased cell proliferation. The molecular mechanism implies the disruption of cell cycle checkpoints. In particular, regulators of the G1/S transition are under the control of ErbB2-dependent path-

ways, including the Ras/Erk, p38MAPK and PI3K pathways. Consequently, activation of ErbB2 promotes the expression of various D-type cyclins and regulates the activity of the $p21^{cip1/WAF1}$ and $p27^{kip1}$ cyclin-dependent kinase (CDK) inhibitors, by controlling their expression levels, phosphorylation and nuclear localization [35, 36].

Cell Survival

ErbB2 transforming ability also involves prevention of programmed cell death. Because PI3K/Akt signaling is an important pathway in the context of cell survival, it is often considered the main pathway mediating ErbB receptors anti-apoptotic activity. Akt can act by directly controlling components of the apoptotic machinery. For instance, Akt-mediated phosphorylation prevents association of the pro-apoptotic Bcl family member BAD with Bcl-x_L, allowing Bcl-x_L to promote survival. Similarly, Akt could inhibit several components of the apoptosome, such as caspase-9 or APF-1. Akt also affects pro-apoptotic molecules indirectly; phosphorylation of Forkhead transcriptional regulators prevents their nuclear localization and, as a consequence, transcription of a number of pro-apoptotic genes (see [37] for complete review). Interestingly, ErbB2 was also shown to prevent tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway [38]. The critical role of ErbB2 anti-apoptotic signaling during development is illustrated in an elegant *in vitro* model of mammary morphogenesis. When grown within a reconstituted extracellular matrix, non-transformed breast cancer cell lines form polarized, growth-arrested, lumen-containing spheroids, reminiscent of the mammary alveolae [39]. Lumen formation results from controlled death of the inner cells, a phenomenon which is prevented by ErbB2. As cyclin D1 overexpression is capable of preventing cavitation only in the presence of the anti-apoptotic molecule, Bcl2 [39], these data clearly suggest that ErbB2 confers both mitogenic and survival signals.

Cell Motility

Examination of ErbB2 knock-out mice rescued for ErbB2 cardiac expression suggested a failure of pre-migratory Schwann cells to migrate away from the dorsal root ganglia and onto the axons [40]. ErbB2 also regulates breast cancer cell motility *in vitro* [41] and metastasis in various models [42, 43].

Cell motility is a complex multi-step process which implicates major morphogenetic events and timely and spatially regulated changes in cell adhesion [44]. One of the earliest events consists of formation of membrane extensions, fillopodia and lamellipodia, filled with a dense meshwork of actin filaments. The molecular mechanism underlying polymerization of this meshwork is dependent on consecutive activation of the small GTPases Rac1/Cdc42, and the WAVE/Scar and WASP nucleation-promoting factors, allowing Arp2/3-mediated assembly of new actin filaments. ErbB signaling can influence these events in many ways. For instance, PI3K was shown to activate Rac1 and Cdc2, while PLCγ mediates activation of calcium-dependent nucleating protein [45]. Interestingly, components of the Arp2/3 complex were found to associate with EGFR with a very specific activity-dependent chronological pattern [21]. Finally, ErbB2 was also shown to affect focal adhesions via diverse mechanisms [46, 47].

Recently a novel effector of ErbB2, Memo (mediator of ErbB2-driven motility), was shown to be involved in ErbB2-dependent migration of breast tumor cells [48]. In contrast to other signaling molecules recruited to ErbB2 (Shc, PLCγ, Crk), Memo doesn't play a major role in early events linked to actin cytoskeleton remodeling and lamellipodia formation. In fact, data indicate that Memo controls microtubule outgrowth toward the cell cortex. The molecular mechanism is still under investigation.

Finally ErbB2 also controls cell migration indirectly, via regulated expression of motogenic genes at the translational [17, 18] or transcriptional levels [49, 50]. Thus, ErbB2 regulates cell motility through an intricate network of signaling pathways, the complexity of which is only emerging. The observation that ErbB2 cooperates with signaling emanating from factors such as hepatocyte growth factor [51] and TGFβ [52, 53] to promote an invasive phenotype adds yet another layer of intricacy to the picture.

While increased proliferation, survival and increased motility are certainly critical for embryonic development, they are also some of the features characterizing cancer cells. Since ErbB2 is also known to promote expression of pro-invasive proteases [54, 55] and pro-angiogenic factors [56], it appears that ErbB2 can contribute several distinct capabilities required to complete tumorigenesis [57].

ErbB2 as a Major Therapeutic Target in Breast Cancer

ErbB2 protein was shown to be overexpressed in a broad panel of human tumors, including breast [58], but also ovary, lung, stomach, and bladder cancers [36]. Such an overexpression is essentially due to gene amplification, which seems to be the major cause of ErbB2 pathway deregulation in human cancers. Of note, mutations in the kinase domain of ErbB2 were recently identified in a small subset of non-small cell lung cancer [59], and subsequently in other tumor types including head and neck, ovarian, brain and gastric cancers, even though with a low prevalence. The functional consequences of these mutations are still under investigation.

Importantly, experimental approaches, using inhibitors of ErbB2 activity, including antagonistic antibodies, small

molecules kinase inhibitors or inducers of ErbB2 degradation, have shown that blockade of ErbB2 signaling leads to reversal of most ErbB2 tumorigenic features [36], identifying it as an exciting potential therapeutic target.

ErbB2 as a Prognostic Parameter and a Predictive Factor for Therapeutic Response to Conventional Anticancer Agents

Although ErbB2 overexpression/amplification was shown in various human cancers, tight and independent correlation with prognosis was only absolutely demonstrated in breast cancer. ErbB2 is amplified and/or overexpressed in approximately 25% of human breast tumors [58] and is associated with increased tumor aggressiveness, increased rates of recurrence, and increased mortality [60, 61].

In addition to its prognostic impact, it has been suggested that ErbB2 expression may also predict response to conventional chemotherapy. At the preclinical level, studies have proven controversial. On one hand, some have found that ErbB2 overexpression induced paclitaxel and docetaxel resistance in model cell lines. This effect could be due to ErbB2-mediated $p34^{Cdc2}$-Tyr15 phosphorylation and p21 (Cip1) up-regulation, impeding activation of $p34^{Cdc2}$ and induction of apoptosis upon taxane treatment, this process being reversed by trastuzumab [62]. On the other hand, others failed to identify significant alteration in the sensitivity to a broad range of drugs [63], including taxanes, suggesting that ErbB2 alone is not sufficient to modify responses to chemotherapeutic drugs. At the clinical level, the same uncertainty exists, likely due to the retrospective nature of data available. Some studies in the adjuvant setting have suggested that ErbB2-overexpressing tumors may be associated with resistance to a cyclophosphamide/methotrexate/FU (CMF) regimen, but with sensitivity to anthracycline-containing regimens [64]. Nevertheless, data from a recently published randomized trial in the adjuvant setting did not detect any significant differences in the benefit derived from the drug between ErbB2 positive and negative tumors [65].

Data also suggest that ErbB2 overexpression may predict response to endocrine therapy [66]. In particular, it seems that ErbB2-overexpressing tumor cells might grow in an estrogen-depleted condition and be resistant to selective estrogen receptor modulators, such as tamoxifen, relative to ErbB2 negative cells, and it has been hypothesized that ErbB2 may stimulate estrogenic (agonist) proliferative effects of tamoxifen. Again, clinical studies have yielded conflicting results in both adjuvant and metastatic settings, but various studies have suggested a diminished efficacy of hormonal therapy in ErbB2 positive tumors [67]. Interestingly, prospective studies comparing tamoxifen to aromatase inhibitors in the neoadjuvant setting have suggested that this latter class of drug could be more active in ErbB2 positive patients [68].

ErbB2 Drives a Novel Molecular Entity in Breast Cancer

From a more general view, it appears increasingly clear that breast cancer is a clinically heterogeneous disease, the complexity of which relates to the different molecular abnormalities driving the tumor phenotype. Recently, DNA microarray technology revealed a novel molecular classification of breast cancer, based on gene expression profiles [69]. Thus, luminal, basal-like, normal-like, and ErbB2 positive subgroups were isolated on the basis of distinct gene expression profiles and were shown to have different prognoses [70] and possibly distinct therapeutic responses [71]. The basal-like (mostly estrogen receptor negative) and ErbB2 (mostly ErbB2 amplified and estrogen receptor negative) subgroups had the shortest relapse-free and overall survival, whereas the luminal-type (estrogen receptor-positive) tumors had a more favorable clinical outcome. Therefore, ErbB2 positive tumors can be individualized within the same distinct subgroup, suggesting that they represent a particular clinical and biological entity within breast cancer. Recently, 36 genes and expressed sequence tags were identified that were differentially expressed in tumors and cell lines with ErbB2 protein overexpression [72]. Interestingly, while some of the genes in the signature reported in this study were known to be associated with ErbB2 and cancer, others constitute new associations and could represent novel ErbB2 regulators or effectors.

ErbB2 Targeting and Trastuzumab

Trastuzumab (Herceptin®, Roche) is a recombinant humanized monoclonal antibody directed against the extracellular domain of ErbB2. The murine complementary-determining regions involved in epitope targeting has been incorporated into the framework of a human IgG1, leading to a 95% humanized antibody. Thus, trastuzumab targets the ErbB2 protein with high affinity (K_d = 0.1 nM), the potential for immunogenicity is decreased, and the potential for recruiting immune effector mechanisms is increased. Although several molecular and cellular effects have been observed *in vitro*, the mechanisms by which trastuzumab induces regression of ErbB2-overexpressing tumors are incompletely defined. Two models, which are not mutually exclusive, are proposed to explain how trastuzumab may function:

- the "signaling" hypothesis involves ErbB2 internalization and degradation [73] leading to disruption of the receptor dimerization and PI3K signaling and ultimately apoptosis. Cells treated with trastuzumab may also undergo arrest during the G_1 phase of the cell cycle via regulation of $p27^{kip1}$ [36]. Additional mechanisms of trastuzumab may include suppression of angiogenesis via induction of antiangiogenic factors

and repression of proangiogenic factors, and inhibition of ErbB2 proteolytic cleavage/shedding [73]. However, while ErbB2 down-modulation has long been considered the primary event of trastuzumab inhibitory effect, some studies examining ErbB2 localization and trafficking suggest that trastuzumab does not actually down-modulate ErbB2 [74]. In fact, it is not known whether any of the events observed *in vitro* actually occur in tumors. Core biopsies obtained during pilot preoperative trastuzumab studies indicate that, while the antibody induced tumor regression, expression of membrane ErbB2 and cell cycle biomarkers were unchanged in the tumors [75, 76]. Interestingly, a transient induction of apoptosis was observed in one study; whether apoptosis is responsible for the cytocidal effect is still controversial [76, 77].

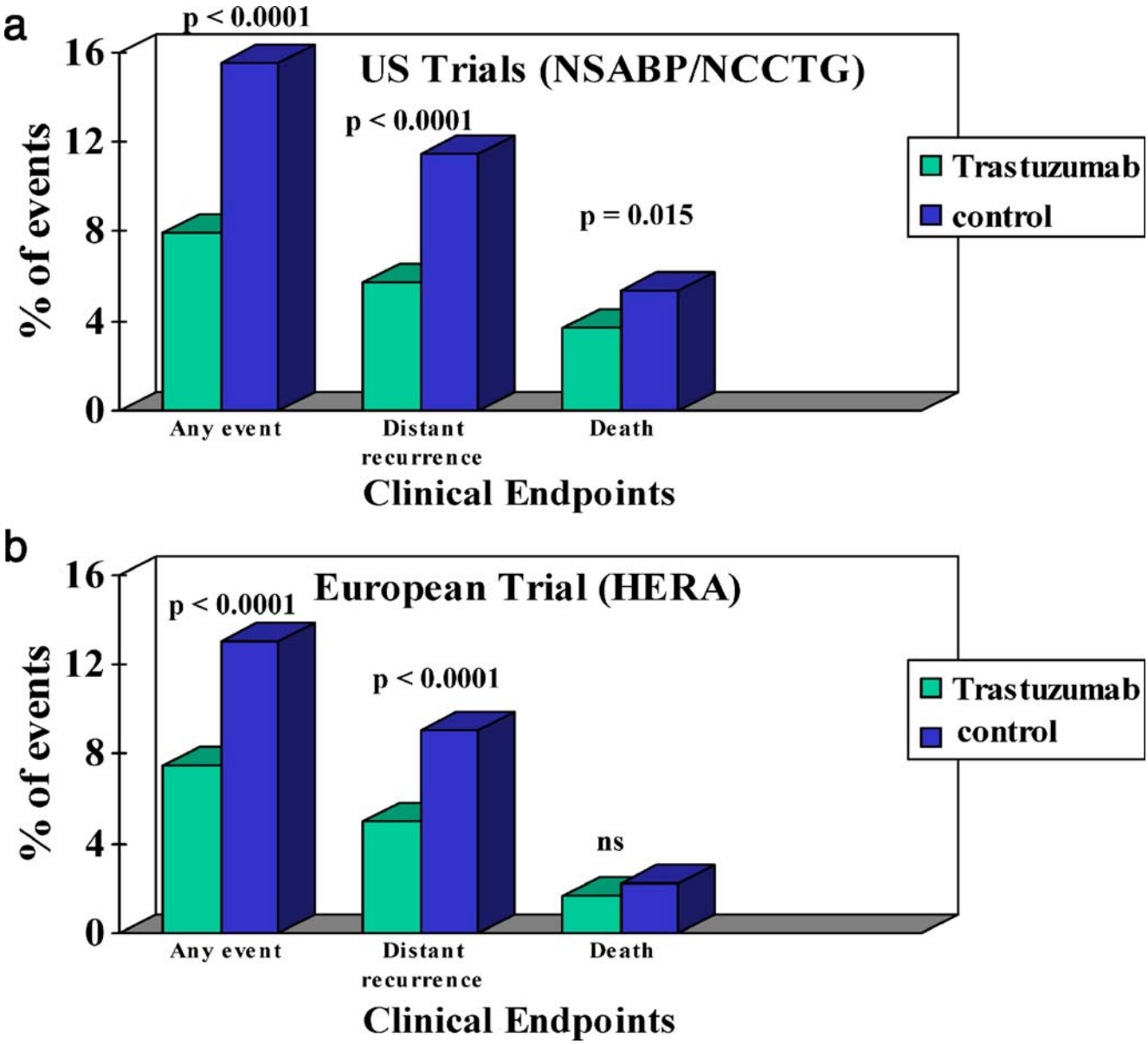

Figure 3 Adjuvant trastuzumab in ErbB2 overexpressing early breast cancer : impact on main clinical endpoints in recently released US (a) and European (b) trials [82, 83]. In US trials (combined results of the National Surgical Adjuvant Breast and Bowel Project trial [B-31] and the North Central Cancer Treatment Group trial [N9831]), hazard ratio for any event, distant recurrence and death were 0.48 (95 percent confidence interval, 0.39 to 0.59; $p < 0.0001$), 0.47 (95 percent confidence interval, 0.37 to 0.61; $p < 0.0001$) and 0.67 (95 percent confidence interval, 0.48 to 0.93; $p = 0.015$), respectively. In European trials (Herceptin Adjuvant [HERA] randomized trials), hazard ratio for any event, distant recurrence and death were 0.54 (95 percent confidence interval, 0.43 to 0.67; $p < 0.0001$), 0.49 (95 percent confidence interval, 0.38 to 0.63; $p < 0.0001$) and 0.76 (95 percent confidence interval, 0.47–1.23 $p = 0.26$, not significant (*ns*)), respectively. Event is defined as local or regional recurrence, distant recurrence, contralateral breast cancer, other second primary cancer or death with no evidence of disease.

– the "immunological" hypothesis favors the role of antibody dependent cellular cytotoxicity (ADCC) resulting from activation of Fc receptors on immune effector cells [78], the key argument being that knock-out mice lacking inhibitory FcγRII receptors showed increased ADCC, while mice lacking activating FcγRIII receptors or treated with antibodies unable to bind FcγRIII showed a marked attenuation of anti-tumor activity in xenografts models. To support the same concept, a pilot study on a very limited number of patients with early breast cancer receiving trastuzumab as single agent, shows no down-modulation of ErbB2 and no changes in proliferation in tumors, but a strong infiltration by lymphoid cells was observed in all cases. Patients with partial or complete remission were found to have a higher *in situ* infiltration of leukocytes and a higher capability to mediate *in vitro* ADCC activity [75].

Trastuzumab and ErbB2-Positive Breast Cancer: From a Standard in Advanced/Metastatic Breast Cancer to a Historical Breakthrough in the Adjuvant Setting

In a pivotal randomized clinical trial including first-line treated metastatic breast cancer patients overexpressing ErbB2, Slamon et al. [79] showed that combining trastuzumab with either adriamycin/cyclophosphamide or single-agent paclitaxel, both standard drugs in this setting, produced a longer time to progression, higher response rates, and improved survival rates compared with chemotherapy alone. Subsequent studies have shown that these results apply only to significant ErbB2 overexpression/amplification [80]. Importantly, trastuzumab is associated with an increased risk of cardiac dysfunction, which is greatest in patients receiving concurrent anthracyclines [81]. If the molecular determinants of trastuzumab cardiotoxicity are not clearly understood, they have been linked to its role in normal heart physiology: loss of cardiac ErbB2 can cause dilated cardiomyopathy in adult mice [30].

The next logical step after demonstration of efficacy in the metastatic setting was to evaluate incorporation of trastuzumab treatment in the management of primary breast cancer. Four large multicenter trials were thus designed to test the role of trastuzumab as adjuvant therapy after surgical treatment of primary breast cancer. The results of three of these trials enrolling more than 6,000 patients in Europe and North America were recently reported [82, 83]. They included patients with early breast cancer, lymph node positive or high-risk lymph node negative, and ErbB2 overexpressed/amplified. Patients were treated by surgical removal, standard adjuvant chemotherapy (anthracyclines and/or taxanes), radiotherapy and hormonal therapy when appropriate, and were randomized between the association of standard treatment to trastuzumab during 1 year or no further treatment. Very impressively, trastuzumab regimens were associated to a dramatic reduction (~50%) in the risk of recurrence, either local or distant. In spite of a short follow-up (between 1 and 2.5 years), an advantage in overall survival was already significant in one of the trials. As illustrated in Fig. 3, the impact of trastuzumab on principal clinical endpoints seems to reach a magnitude better than observed in any clinical trials dealing with solid tumors. As anticipated from previous trials, a significant excess of cardiac dysfunction was observed in the trastuzumab arm. Overall, 2.9 to 7.1% of patients receiving trastuzumab experienced a certain level of cardiac toxicity, compared to 0 to 2.2% in the control group. While only longer follow-up will tell actual long term consequences of this toxicity and how it may impact morbidity and overall survival, strategies that limit emergence of cardiac toxicity are already under investigation [84].

Thus, trastuzumab, already a standard in the management of ErbB2-positive advanced and/or metastatic breast cancer, has the potential to dramatically increase outcomes among women with ErbB2-positive early breast cancer.

ErbB2 Targeting: Unresolved Questions and Future Directions

Importantly, when considering its use as single agent, only 30 to 40% of ErbB2 overexpressing metastatic breast cancer respond to trastuzumab [85]. However, molecular mechanisms regulating sensitivity and resistance to trastuzumab are still poorly known. Among potential mechanisms, activation of cell survival pathways, notably the PI3-kinase pathway, has been proposed to play a significant role in regulating trastuzumab activity. For instance, insulin-like

Table 1 ErbB2-targeting drugs in development.

Approach and specificity	Drug	Phase of development
Anti ErbB2 monoclonal antibodies		
ErbB2 specific	Trastuzumab (Herceptin®, Roche)	Registered
	Pertuzumab (Omnitarg®, Roche)	Phase II
ErbB2 inhibitors (small molecules)		
ErbB2 specific	CP-654,577 (Pfizer)	Phase I
ErbB2/EGFR	GW572016 Lapatinib (Glaxo-Smith-Kline)	Phase II/III
	AEE788* (Novartis)	Phase I/II
	CI-1033 Canertinib (Pfizer)	Phase II
ErbB2/EGFR/ErbB4 (pan ErbB inhibitors)	HKI-272 (Wyeth)	Phase I/II

growth factor receptor, the engagement of which activates PI3K/Akt, was shown to significantly impair trastuzumab efficacy in cellular models [86]. It was also shown that trastuzumab increases membrane localization and activity of the PTEN lipid phosphatase [87], a negative regulator of the PI3K pathway. In this preclinical model, PTEN inactivation led to trastuzumab resistance. Importantly, a correlation was found between PTEN expression in tumor tissue and response to trastuzumab-based treatment. These data provides strong rationale to examine whether regulators of the PI3K pathway are potential predictive factors for trastuzumab efficacy. Eventually, combination therapy targeting ErbB2 and PI3K (e.g., mTOR inhibitors [28]) might overcome resistance in selected patients.

Cells may also compensate for the blockade of overexpressed ErbB2 by activating ErbB2 through ligand-activated heterodimers formation, involving other ErbB family members [88]. This mechanism could be addressed by the use of inhibitors targeting multiple ErbBs or by preventing ErbB2 heterodimerization. In this context, small molecule kinase inhibitors may become very promising [89]. These compounds which target specifically ErbB2, ErbB2 and EGFR, or ErbB2/EGFR/ErbB4 (pan ErbB inhibitors) are under preclinical or clinical evaluation (see Table 1). Results of these studies will prove very interesting, as small molecules, in contrast to trastuzumab, lack ADCC activity, but show a clear effect on ErbB2 signaling. Blockade of the antibody binding site by Muc4 was also suggested as a possible cause for trastumab resistance [90]. To prevent ErbB2 dimerization, pertuzumab (Omnitarg®), a novel ErbB2-directed antibody, could prove useful. Contrary to trastuzumab, which binds a region not involved in dimerization, pertuzumab was recently shown to sterically hinder the ErbB2 dimerization motif, resulting in blockade of ErbB2 dimerization with other ErbB receptors, thus inhibiting both overexpression- and ligand-activated signaling (Fig. 2). Interestingly, pertuzumab inhibits growth of tumors cells with low levels of ErbB2 expression in xenograft models, raising the hypothesis that it could have a broader use than trastuzumab [91], provided a responsive population can be defined.

Finally, it is tempting to speculate that these different approaches might be synergistic when used in combination, since they target ErbB2 with distinct mechanisms of action.

Conclusions

When imatinib (gleevec®) initiated the era of highly effective targeted therapeutics in chronic myelogenous leukemia, it was a controversial issue whether or not this kind of approach could be effective in much more complex tumor phenotypes, such as solid tumors. According to recent clinical results, the answer is clearly yes: trastuzumab is probably going to change the natural history of ErbB2-overexpressing breast cancer, erasing at least partially the otherwise inherent aggressiveness of this disease and its resulting poor clinical outcome. However, major challenges are still ahead, as it will be important to optimize trastuzumab clinical use, to understand the mechanisms of resistance and define new populations that might benefit from ErbB2-directed therapies. A better understanding of ErbB2 signaling network could provide predictors of the response to therapy, markers for monitoring this response and new ways for targeting ErbB2 signaling. Concomitant efforts of basic and clinical research must therefore be continued to complete this exemplary tale of translational research.

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