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# Epidermal growth factor receptor family activation and intracellular signaling: implications for cancer therapeutics

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## Epidermal Growth Factor Receptor Family Activation and Intracellular Signaling: Implications for Cancer Therapeutics

A thesis presented

by

Judy M. Richman

to The Department of Biology

## In partial fulfillment of the requirements for the degree of Master of Science

in the field of

Biology

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by

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## ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology in the Graduate School of Arts and Sciences of Northeastern University, October, 2008

#### ABSTRACT

Epidermal growth factor receptor tyrosine kinase family members have distinct characteristics that specify their interactions and downstream signaling capabilities. In response to ligand, the receptors dimerize, resulting in activation of their kinase domains. Phosphorylation of specific C-terminal tyrosine residues specifies signal transduction pathways, resulting in mitogenic consequences for the cell. Epidermal growth factor receptors may translocate to the nucleus, initiating transcription.

When epidermal growth factor receptors are dysregulated, aberrant signaling may result in cancer. Some factors that promote dysregulation are receptor overexpression and defects in negative regulation. A greater understanding of cancer therapeutics targeted to these receptors has been gained through crystal structures that elucidate ligand binding sites and dimerization contact points. Monoclonal antibodies and tyrosine kinase inhibitors targeted to epidermal growth factor receptors are approved for clinical use in advanced cancers, but show limited efficacy. New therapeutics target abnormal epidermal growth factor receptor activity by terminating receptor translation, uncoupling receptor accessory molecules, and restoring lost negative regulatory molecules. Most existing therapeutics target EGFR or HER2, leaving HER3 and HER4 therapeutics open for development.

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## TABLE OF CONTENTS

Abstract		3
Acknowledgements		
Table of Contents		
List of Tables		
List of Figures		
List of Abbreviations		
Introduction		11
Chapter 1	ErbB Activation	14
Chapter 2	ErbB Structure	24
Chapter 3	ErbB Downstream Signaling Pathways	33
Chapter 4	Negative Regulation of ErbBs	41
Chapter 5	Nuclear Localization of ErbBs	52
Chapter 6	ErbB-Targeted Cancer Therapeutics	61
I.	Monoclonal Antibodies	62
II.	Tyrosine Kinase Inhibitors	79
III.	Additional Therapeutic Strategies	86
Tables		95
Figures		97
References		111

# LIST OF TABLES

Table 1. Functional characteristics of ErbB receptors	95
Table 2. Nuclear localization of ErbB receptors	96

## LIST OF FIGURES

Figure 1. Epidermal growth factor receptor structure	97
Figure 2. The epidermal growth factor receptor family	98
Figure 3. The biological role of ErbBs	99
Figure 4. EGFR activation events as cancer therapeutic targets	100
Figure 5. HER2 heterodimers correlate with mitogenicity in cell lines	101
Figure 6. HER2 regulatory molecule Hsp90 and Muc4 binding sites	102
Figure 7. Crystal structure of the EGFR dimer	103
Figure 8. EGFR dimer extracellular domain inhibition sites	104
Figure 9. Full length EGFR homodimer inhibition sites	105
Figure 10. ErbB heterodimer and homodimer combinations	106
Figure 11. ErbB downstream binding partners that couple to cell proliferation and survival pathways	107
Figure 12. ErbB negative regulatory molecules	108
Figure 13. Therapeutic interventions for the ErbB family	109
Figure 14. ErbB monoclonal antibody binding sites	110

8

# LIST OF ABBREVIATIONS

СНО	Chinese hamster ovary
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FAS	Fatty acid synthase
FRET	Forster resonance energy transfer
HER	Human epidermal growth factor receptor
Hsp90	Heat shock protein 90
IGF-1R	Insulin-like growth factor-1 receptor
IHC	Immunohistochemistry
Ιβ1	Nuclear transport receptor importin $\beta 1$
LRIG1	Leucine-rich repeats and
	immunoglobulin-like domains 1
MAb	Monoclonal antibody
МАРК	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-
	diphenyltetrazolium bromide
Nrdp1	Neuregulin receptor degradation protein-
	1
ΡLCγ	Phospholipase C-gamma
РТВ	Phosphotyrosine binding domain
PtdIns 3-kinase or PI3K	Phosphatidylinositol 3-kinase

PTEN	Phosphatase and tensin homolog
SH2	Src homology 2
STAT	Signal transducer and activator of
	transcription
TKI	Tyrosine kinase inhibitor

## I. Introduction

In this thesis, I will focus on epidermal growth factor receptor tyrosine kinase family interactions, downstream signaling by receptors, and negative regulation of receptors, with an emphasis on the role of these receptors in cancer. I will discuss cancer therapeutics targeted to the epidermal growth factor receptor family and probe their mechanisms of action, based on current knowledge of epidermal growth factor receptor family activation and signaling.

In 1975, epidermal growth factor (EGF) was found to bind to a cell surface receptor termed the epidermal growth factor receptor, abbreviated as EGFR (Carpenter et al., 1975). Three similar sequences to EGFR were discovered in the 1980's by screening cDNA libraries, and termed the human epidermal growth factor receptors: HER2, HER3, and HER4 (Gschwind et al., 2004). EGFR, HER2, HER3, and HER4 comprise the epidermal growth factor receptor family. Due to the prevalence of the term EGFR in the journal literature, I will use EGFR to refer to the human epidermal growth factor receptor 1, instead of HER1.

The epidermal growth factor receptor family members are transmembrane glycoproteins containing an extracellular domain, a transmembrane domain, and a cytoplasmic region containing the juxtamembrane region, the tyrosine kinase domain, and the C-terminal region with tyrosine phosphorylation sites (Figure 1) (Linggi and Carpenter, 2006). Epidermal growth factor receptors are usually found on epithelial cells, and have access to their growth factor ligands from nearby extracellular matrix (Normanno et al., 2005) or through the bloodstream (Ramsauer et al., 2003). The epidermal growth factor receptors play a central role in development. In worms and flies, a single epidermal growth factor receptor regulates specific events in organogenesis (Bublil and Yarden, 2007). Additional epidermal growth factor receptors in vertebrates allow for more combinatorial interactions to specify increased signal diversification (Marmor et al., 2004). Gene inactivation in mammals shows that EGFR is needed for normal skin, kidney, and GI tract (Bublil and Yarden, 2007). HER2, HER3 and HER4 are involved in heart and brain formation in mice (Zhou and Carpenter, 2002). All epidermal growth factor receptors are involved in mammary gland development (Bublil and Yarden, 2007). In adults, epidermal growth factor receptors are part of homeostatic systems that control proliferation and differentiation of epithelial tissues (Zhou and Carpenter, 2002).

The epidermal growth factor receptor family is referred to widely as the ErbB family (Figure 2) due to the similarity of the epidermal growth factor receptor protein sequence to that of an avian erythroblastosis viral oncogene v-ErbB. This finding suggested that mutations in the EGFR could transform it into an oncogene (Gschwind et al., 2004). Due to the fundamental role of ErbBs in controlling cellular processes, ErbB dysregulation can promote cancer (Figure 3). Overexpression and mutation of EGFR is found in cancers of the breast, head and neck, lung, brain (Marmor et al., 2004), and in advanced colorectal cancer (Toffoli et al., 2007). HER2 overexpression is associated with many cancer types, including breast, lung, ovarian, prostate, gastric, oral, kidney, pancreatic, cervical, and endometrial cancer (Rabindran, 2005) and (Kumar Pal and Pegram, 2006). HER3 is overexpressed in multiple types of

cancers and in almost one third of invasive breast carcinomas (Karamouzis et al., 2007). The role of HER4 in cancer is not clear cut. HER4 overexpression has been observed in some cancers (Zhou and Carpenter, 2002), but the presence of HER4 also correlates with reduced primary breast tumor progression and improved patient prognosis (Vidal et al., 2007).

## Chapter 1 ErbB Activation

In normal development, ErbB family member interactions specify development of many systems in the body. Investigation of the behavior of dysregulated ErbB family members due to overexpression or mutation has shown that their interactions promote cancer (Stern, 2003). The link between ErbBs and cancer was observed during study of the EGFR, the first ErbB to be discovered. Early research established that EGF ligand activation of EGFR kinase function results in increased glycolysis, enhanced cell proliferation, and cell morphological changes (Carpenter and Cohen, 1979). Overexpression of EGFR in the A431 epidermoid cancer cell line suggested that there might be a link between EGFR activation and the increased cell proliferation that occurs in cancer cells. Therefore, much research was done on the A431 cell line to probe the details of EGFR activity.

Experiments revealed that activated EGFR correlated with increased phosphorylation. In A431 epidermoid cancer cells stimulated with radiolabeled EGF, complexes of EGF and EGFR were identified. When radiolabeled ATP was added, enhanced phosphorylation of endogenous proteins was detected (Carpenter and Cohen, 1979). EGFR purified from A431 cells could phosphorylate tyrosine residues in response to EGF stimulation (Ushiro and Cohen, 1980). When EGFR was stimulated by EGF, EGFR itself became phosphorylated (Yarden and Schlessinger, 1987). Thus, it appeared that activated EGFR could both phosphorylate other proteins, and phosphorylate itself. It was thought that phosphorylation of EGFR could initiate intracellular signaling, resulting in mitogenic consequences for the cell. An understanding of the details of EGFR activation in a cancer cell line might suggest therapeutic interventions to suppress EGFR activity. It was hoped that halting EGFR activity would thwart the cell proliferation that leads to cancer progression. However, several decades of research elapsed before therapeutics targeting EGFR became available.

Activation of EGFR is correlated with its dimerization. A detailed understanding of dimerization is important because interfering with dimerization could be a cancer therapeutic strategy. EGFR exists predominantly as a dimer when stimulated. EGFR purified from A431 cells was incubated with radiolabeled EGF, cross-linked, and examined by autoradiography. EGFR was observed bound to EGF, with the majority present as dimers (Yarden and Schlessinger, 1987).

Much research has probed the structural details of receptor dimerization. The EGFR extracellular domain may be sufficient to promote dimerization. EGFR extracellular domain was incubated with radiolabeled EGF and cross-linked. When analyzed by autoradiography, dimers were present (Hurwitz et al., 1991). Subsequent research has shown that other parts of the receptor may be involved in dimerization, and continues to be an area where more data are needed. This knowledge could assist in designing therapeutics that inhibit dimerization contacts throughout the entire length of the receptor.

Stoichiometric details of the EGF interaction with EGFR within a dimer were elucidated using biophysical techniques to examine soluble EGFR extracellular

15

domains. The EGFR homodimer contained two EGF ligands and two EGFR extracellular domains. The initial reaction involved one EGF binding to one EGFR to form EGFR/EGF. Two EGFR/EGF molecules associated to form the dimer EGFR <sub>2</sub> EGF<sub>2</sub> (Lemmon et al., 1997). This result showed that two EGF ligands were present in the dimer. Determination of the location of the ligand binding sites would be valuable in designing therapeutics to block the binding. This understanding came from crystal structures which will be discussed in Chapter 2.

Dimerization may be important in restraining the potent biological activity of EGFR by requiring the receptor to find a partner prior to signal transduction. Furthermore, an activated receptor may not be able to phosphorylate itself directly. There is evidence that dimer partners transphosphorylate each other on particular Cterminal tyrosine residues. An EGFR dimer was created in which one of the dimer partners was an EGFR mutant that had a non-functional kinase domain, and therefore was unable to phosphorylate itself directly. However, this kinase negative mutant was able to be phosphorylated on the appropriate C-terminal tyrosines by its kinase-active EGFR dimer partner, indicating that transphosphorylation occurs (Honegger et al., 1989).

In summary, early EGFR research showed that EGFR activation depends on EGF binding, dimerization of EGFR, and EGFR kinase function, resulting in phosphorylation. These three events are targets for EGFR therapeutic interventions (Figure 4). After the three other ErbBs were discovered, it became apparent that EGFR could dimerize with other ErbBs, not only with itself. EGFR/HER2 dimers were found by treating the human breast carcinoma SKBR3 cell line with radiolabeled EGF and cross-linking reagent (Goldman et al., 1990). Elucidating the identity of ErbB dimers in SKBR3 cells is important in understanding the nature of ErbB signaling in breast cancer. Efforts to identify additional combinations of ErbBs characterized ErbB research in the mid 1990s. For example, Chinese hamster ovary (CHO) cells were transfected with HER2, HER3 and HER4 to create a triple ErbB line. After stimulation with ligand, the receptors were cross-linked, and immunoprecipitation revealed the existence of HER2/HER3 and HER2/HER4 dimers (Tzahar et al., 1996).

Characterization of the properties of the individual ErbBs revealed differences in structure and function, influencing their ultimate signal transduction capabilities (Table 1). These differences impact how therapeutics need to be designed to inhibit the individual ErbBs. Unlike the other ErbBs, HER2 does not have a growth factor ligand. HER2 could not be tyrosine phosphorylated in response to a panel of ErbB ligands (Klapper et al., 1999). Furthermore, in a recent attempt to discover a HER2 ligand, a genome wide screen for EGF-like domains did not reveal any novel ErbB ligands (Kochupurakkal et al., 2005). Although a growth factor ligand has not been found for HER2, other molecules appear to play regulatory roles, such as the chaperone heat shock protein 90 (Hsp90) and the mucin Muc4. The lack of a HER2 ligand precludes the possibility of blocking ligand binding as a therapeutic strategy to prevent HER2 activation. However, blocking HER2 regulatory molecules may present a therapeutic option. A unique attribute of HER3 compared to the other ErbBs is its impaired kinase activity. This finding seemed to dampen interest in HER3 as a therapeutic target. HER3's weak kinase activity was observed in ErbB infected Sf9 insect cells. Sf9 cells were used in this experiment because they do not express endogenous ErbBs. Therefore, results could be attributed to the activity of the ErbB introduced into the cell line. Sf9 cells were infected either with baculovirus encoding EGFR or encoding bovine ErbB3. EGFR and ErbB3 immunoprecipitates were incubated with tyrosine substrates and radiolabeled ATP. Autoradiography analysis showed that ErbB3 had significantly less ability to phosphorylate tyrosine substrates than EGFR. Stimulation of ErbB3 with its ligand neuregulin did not increase the weak ErbB3 phosphorylation of tyrosine substrates. Therefore, the small amount of tyrosine phosphorylation of the substrates observed from the ErbB3 immunoprecipitate was attributed to the activity of an endogenous insect kinase, and not to ErbB3 (Guy et al., 1994).

It was hypothesized that ErbB3 has impaired tyrosine kinase activity due to a substitution in the highly conserved amino acids of the kinase domain. However, ErbB3 was still able to bind an ATP analog. The compromised kinase activity of HER3 suggests that HER3 cannot be activated within a HER3 homodimer. A model was proposed in which HER3 forms a heterodimer with EGFR or HER2 that can transphosphorylate HER3, leading to recruitment of intracellular signaling molecules to HER3 (Guy et al., 1994).

Data from multiple research groups working with ErbB transfected animal cell lines supported a model of HER2 cooperation with HER3. In a number of experiments, when HER2 was present with HER3, tyrosine phosphorylation of both receptors increased (Sliwkowski et al., 1994), (Pinkas-Kramarski et al., 1996a), and (Carraway et al., 1994). These results suggest that the way in which HER3 becomes activated is through HER2 transphosphorylation of HER3. However, the increased tyrosine phosphorylation of HER2 in the presumed HER2/HER3 heterodimers created another puzzle that has not been solved. Given the observed impaired HER3 kinase activity by Guy et al., how HER3 might transphosphorylate HER2 is not understood. Whether HER3 has absolutely no kinase activity is still questioned (Berger et al., 2004). Sliwkowski et al. speculated that HER3 may be a weak kinase, and in circumstances of high HER2 concentration could have the ability to transphosphorylate HER2.

The impaired kinase activity of HER3 may have suggested initially that its role in promoting cancer is secondary to other ErbBs. Perhaps this is why few therapeutic interventions exist that target HER3 directly. However, blocking HER3 signaling is likely to be important in treating cancer because the combination of HER3 with HER2 leads to the strongest mitogenic effect of any ErbB dimer combination when expressed in a cell line (Pinkas-Kramarski et al., 1996b). Supporting a role for HER3 in cancer, data show that the HER2/HER3 dimer pair is prevalent in HER2 overexpressing N87 gastric carcinoma cells (Tzahar et al., 1996), and the breast cancer SKBR3 and BT474 cell lines (Sergina et al., 2007). The

HER2/HER3 dimer requires further study because a complete understanding would aid in therapeutic design.

Another unique characteristic of HER2 is its potent mitogenicity. This was unexpected given its inability to bind growth factor ligand. When HER2 was present in a dimer with any other ErbB, there were especially strong biological consequences for cells (Figure 5). This finding from basic research is confirmed clinically, in that many cancers overexpress HER2 (Rabindran, 2005). The mitogenicity of HER2 suggests that this ErbB is an important cancer therapeutic target.

The presence of HER2 correlates with mitogenicity of growth factor ligand activated Ba/F3 mouse pro-B-lymphocyte cells. This cell line was useful in ErbB research because exogenous IL-3 is necessary for survival and proliferation of these cells. Therefore, if a ErbB transfected cell could survive without IL-3, this indicated that the ErbB was a survival factor. EGFR, HER2, HER3, and HER4 were transfected singly or in pairs into these cells, and growth factor ligand was added to induce tyrosine phosphorylation. Based on trypan blue staining, lines containing HER2 alone, or HER2 with EGFR, HER3, or HER4, survived in the absence of IL-3 (Riese et al., 1995).

In support of HER2's mitogenic effect, the HER2/HER4 dimer was more mitogenic than the HER4 homodimer. HER4 alone, or HER2 and HER4 together, were transfected into mouse hematopoietic 32D cells. This cell line was helpful in ErbB research because 32D cells do not express endogenous ErbBs. Therefore, results could be attributed to the transfected receptors, and not confounded with the contribution from endogenous ErbBs. Compared to HER4 cells, HER2/HER4 cells were mitogenic to more ErbB ligands, and at lower ligand concentrations, based on [<sup>3</sup>H] thymidine incorporation. In comparison to HER4 cells, HER2/HER4 cells exhibited increased adherence to the flask, increased surface markers of cell differentiation, and morphological changes (Wang et al., 1998).

The lack of ligand for HER2 prevents the strategy of blocking ligand binding to shut down HER2 activity. However, because HER2 has the regulatory molecules heat shock protein 90 (Hsp90) and Muc4, modulating their activity is an avenue for therapeutic intervention. The HER2 kinase domain associates with the Hsp90 chaperone (Figure 6) (Citri et al., 2004a). Hsp90 maintains the stability of HER2 in the cell membrane, and the loss of Hsp90 results in the proteasomal degradation of HER2. Hsp90 inhibitors have already been developed to disrupt the stabilization of HER2 in cancer cells (Citri et al., 2004b).

HER2 is also regulated by the mucin Muc4 in cancer cells. Muc4 is a potential target for a therapeutic. In epithelial tissues, mucins are large cell surface proteins that serve normally as a protective barrier. Muc4 was isolated originally as a transmembrane complex that associated with ErbB2 in the rat 13762 mammary adenocarcinoma cell line. It was thought that this interaction might be involved in producing the unusual features of these cells, such as highly stable microvilli and restricted cell surface mobility (Carraway et al., 1993). The Muc4 protein, termed ascites sialoglycoprotein-2 when it was first discovered, contained two EGF-like domains (Wu et al., 1994), suggesting that it might be a ligand for ErbB2.

Further investigation of Muc4 showed that it bound to the ErbB2 extracellular domain (Figure 6), but was not a conventional growth factor ligand. Deletion analysis showed that Muc4 could be immunoprecipitated with the ErbB2 extracellular domain, but not with the intracellular or transmembrane domains (Carraway et al., 1999). Muc4 expression correlated with increased activity of the mitogenic HER2/HER3 dimer pair in cancer cells, but required the neuregulin ligand. In A375 human melanoma cells, induction of Muc4 expression enhanced the ability of neuregulin to increase the tyrosine phosphorylation of HER2 and HER3, and increased the incorporation of [<sup>3</sup>H] thymidine into DNA (Carraway et al., 1999). However, this result could not be achieved with Muc4 activation only, but required the presence of neuregulin. Therefore, Muc4 has been classified as a HER2 regulatory molecule.

Muc4 may regulate HER2 activity by influencing its localization in the cell. In CACO-2 human colon carcinoma polarized epithelial cells, HER2 is found originally on the basolateral surface. When these cells were transfected with Muc4, immunofluorescence microscopy results showed that HER2 co-localized with Muc4 at the apical surface. HER2 that translocated with Muc4 was phosphorylated on tyrosine 1248 (Ramsauer et al., 2003). Thus, it appeared that Muc4 might be involved in transporting HER2, and activating HER2 in colon carcinoma cells.

More research is needed to understand how Muc4 regulates HER2 and HER3 in cancer cells because these interactions may have clinical significance. Muc4 was immunoprecipitated with HER2 in signet ring stomach carcinoma. When Muc4 expression was blocked with a mucin inhibitor, phosphorylation of HER3 was decreased (Yokoyama et al., 2007). These results suggest that the interaction of Muc4 with a HER2/HER3 dimer may be important in some tumors, and a potential point for therapeutic intervention.

## Chapter 2 ErbB Structure

Although much had been learned about ErbB interactions in the early studies, therapeutic development was hampered by the lack of receptor structural data. Due to the correlation of ErbB dimerization with receptor activity, the details of dimerization were important to elucidate. Crystal structures provided much sought after information about dimerization.

A crystal structure of a human EGFR extracellular domain fragment bound to human EGF showed that the mechanism of EGFR dimerization is mediated by receptor contacts, not ligand contacts. This was surprising, since no other growth factor receptor had exhibited receptor-mediated dimerization (Ogiso et al., 2002). This structure was important for therapeutic design in that the ligand binding site and dimerization site were identified for EGFR.

The extracellular portion of EGFR was characterized as containing four domains: I (residues 1-165), II (residues 166-309), III (residues 310-481), and IV (residues 482-618) (Ferguson et al., 2003). In the crystal structure containing EGFR extracellular domain bound to EGF, a 2:2 complex was formed in which the two EGF ligands were located on opposite sides of the receptor dimer. EGF bound to Domains I and III within each monomer. The two receptor molecules were bound through Domain II regions with hand-like structures that made contact in the dimer. The dimerization "arm" encompassed 20 amino acids protruding from Domain II with the seven residue "hand" at the tip of the arm making contact with the hand of the other dimer partner (Ogiso et al., 2002) (Figure 7). A similar crystal structure was obtained with a complex of truncated human EGFR extracellular domain and the ligand human transforming growth factor  $\alpha$ , indicating that EGFR binds different ligands using the same mechanism (Garrett et al., 2002). This suggests that a therapeutic that blocks ligand binding could be effective against multiple ligands.

The mechanism of EGFR dimerization was described based on observations from an EGFR crystal structure that exhibited incomplete ligand binding. The crystal structure was a monomer of the human EGFR extracellular domain, in which the low pH of the crystal allowed for weak binding of EGF to Domain I, but not to Domain III. EGFR was in an autoinhibited form in which Domain II was involved in extensive contacts with Domain IV. The Domain III ligand binding site was far away from EGF. Modeling predicted a substantial conformational change was required for EGF to bind to Domain III. In this extended configuration with bound EGF, the Domain II/IV interaction was disrupted, exposing the Domain II dimerization arm and specifying other contacts that allowed dimerization to occur. It was suggested that EGFR is in a dynamic state in which it is primarily in the autoinhibited form, but samples the extended configuration (Ferguson et al., 2003). These results suggest that a therapeutic could thwart dimerization by inhibiting EGF ligand binding to EGFR. This could be achieved by blocking the Domain I or III binding sites, or stabilizing the interaction between Domain II and Domain IV. A therapeutic could also be designed to inhibit dimerization by blocking the Domain II dimerization site (Figure 8).

Another study identified a Domain III contribution to EGFR dimerization, suggesting that blocking a specific residue in Domain III might also inhibit dimerization. Domain II and Domain III residues were mutated, and the effects on dimerization measured by biophysical techniques. Contacts C-terminal to the Domain II dimerization arm in EGFR were critical for dimerization to be achieved, and required the support of a residue in Domain III (Dawson et al., 2005).

In addition to dimerization of extracellular domains, transmembrane and cytoplasmic regions of EGFR may also dimerize, and these additional dimeric contacts may be necessary for the induction of kinase activity. Therefore, therapeutics that inhibit dimerization of transmembrane and cytoplasmic regions may also be an effective way to prevent signal transduction (Figure 9).

Since ligand binding appears necessary to induce conformational changes that permit extracellular domains to dimerize, the existence of unliganded dimers is unexpected. Analysis by single wavelength fluorescence cross-correlation spectroscopy and Forster resonance energy transfer (FRET) revealed that preformed unliganded EGFR homodimers, HER2 homodimers, or EGFR/HER2 heterodimers were present on the cell surface in transfected CHO cells (Liu et al., 2007a). Using FRET and image correlation microscopy, EGFR was found on the surface of A431 cells as clusters of unliganded oligomers (Clayton et al., 2007)

Unliganded dimers are likely to be mediated by transmembrane and cytoplasmic contacts. Transmembrane domains alone may have the capacity to dimerize. A study using FRET analysis of ErbB fluorescent labeled transmembrane domains revealed dimerization. A model of the EGFR extracellular domain was extended to include dimerized transmembrane domains. The extracellular domain dimeric structure was observed to be compatible with the transmembrane dimer (Duneau et al., 2007).

Full length EGFR could form an unliganded homodimer mediated by cytoplasmic contacts. EGFR dimer complexes were detected in EGFR transfected cells that had no EGF stimulation. A transfected EGFR-erythropoietin receptor chimeric molecule that contained a substituted Epo cytoplasmic domain showed that the cytoplasmic domain of EGFR was necessary for unliganded dimer formation. It was hypothesized that the biological significance of preformed EGFR dimers is to accelerate the rate of EGFR signal transduction by reducing the amount of time a ligand bound monomer would need to find a dimer partner (Yu et al., 2002). This suggests that cytoplasmic dimerization contacts are important to expedite receptor function.

In support of kinase interactions promoting dimerization, unliganded EGFR oligomers dispersed when A431 cells were subjected to a tyrosine kinase inhibitor (Clayton et al., 2007). Dimerized kinase domains were seen in a crystal structure. A crystal structure of the EGFR kinase domain complexed to an ATP analog revealed two forms of kinase dimers, with mostly hydrophobic interactions (Zhang et al., 2006).

Although it appears that transmembrane and cytoplasmic domain contacts occur in dimerized unliganded receptors, ligand binding to the extracellular domain is pivotal for signal transduction. Mutants of EGFR extracellular domain dimer interface residues transfected into CHO cells showed reduced phosphorylation of EGFR in response to EGF (Ogiso et al., 2002). Stimulation with EGF was necessary for tyrosine phosphorylation of unliganded EGFR homodimers (Yu et al., 2002).

In sum, these data suggest that blocking ligand binding and blocking dimerization of EGFR extracellular domains would have the most significant negative impact on signal transduction. However, prevention of transmembrane and cytoplasmic interactions may also prevent signal transduction. Current therapeutics focus on ErbB extracellular domains, and have not been designed for specific inhibition of transmembrane and cytoplasmic contacts.

Structures of HER3 and HER4 are similar to that of EGFR, suggesting that similar therapeutics could be designed to inhibit the activity of these three ErbBs. Unliganded crystal structures of HER3 and HER4 were similar to the autoinhibited EGFR crystal bound weakly to EGF. In an unliganded crystal of the HER3 extracellular domain, the dimerization arm from Domain II contacted the C-terminal of Domain IV, forming a pore. It was hypothesized that Domain II/IV contact in EGFR and HER3 may prevent Domains I and III from the proximity necessary to bind ligand (Cho and Leahy, 2002). A crystal structure of the unliganded HER4 extracellular domain also adopted a tethered conformation in which Domains II and IV contacted each other similarly to unliganded EGFR and HER3 (Bouyain et al., 2005). These structures suggest that linking Domains II and IV together is a strategy for prevention of ligand binding. However, a HER3 extracellular domain with Domains II and IV tethered showed high affinity for the neuregulin ligand in surface plasmon resonance assays. A constitutively locked conformation of HER3 was generated by adding a disulfide bridge into the tether region between Domains II and IV (Kani et al., 2005). The ability of ligand to bind to the locked conformation of HER3, if biologically relevant, suggests that HER3 may be more easily activated than EGFR. More data on the structure of HER3 is needed to clarify the nature of ligand binding to this receptor.

In contrast to the crystal structures of EGFR, HER3, and HER4, a structure of HER2 was strikingly different, possible explaining the extremely mitogenic character of HER2. A crystal structure of the human HER2 extracellular domain showed that Domains I and III formed a stable interface, similar to that of the EGFR crystal in which EGF was bound. Domains II and IV did not contact each other, and the Domain II binding loop was exposed constitutively. The fixed open structure of HER2 may explain why it does not require ligand to become active (Cho et al., 2003). Structural data showing the ability of HER2 to dimerize easily in an unregulated fashion further supports its importance as a therapeutic target.

Another human HER2 extracellular domain crystal reinforces the prevailing conclusion that HER2 has no ligand. Ligand binding to the HER2 extracellular domain may not be possible structurally. In the HER2 structure, Domains I and III make direct contact such that a EGF-like ligand would not have access to either domain binding site. Amino acid differences in Domain I and Domain III of HER2

29

compared to EGFR are probably responsible for the inability of HER2 to bind ligand (Garrett et al., 2003).

Since the characteristics of ErbB dimer partners affect their signaling capabilities, knowledge of dimer partner identity in tumors will be important in designing appropriate therapeutics. All ErbB heterodimer combinations, EGFR homodimers, and HER4 homodimers have been observed in transfected cells (Tzahar et al., 1996). The existence of HER2 and HER3 homodimers is uncertain, and is not supported by available crystal structures (Figure 10). Crystal structures of ErbB heterodimers are needed to determine if there are unique ligand binding sites or dimerization sites for these pairs which may be used as therapeutic targets.

Crystal structures do not show the presence of HER2 homodimers or HER3 homodimers. This finding has been mostly supported by biochemical data. HER2 extracellular domains did not form a homodimer in the Cho et al. (2003) crystal structure, possibly due to a difference in one residue at the dimer interface of HER2 compared to EGFR. HER2 homodimers were also not observed in the Garrett et al. (2003) crystal structure. It was hypothesized that this is because the HER2 Domain II dimerization arm and dimerization arm docking pocket are both electronegative, and repel each other. This electrostatic repulsion does not characterize EGFR homodimers.

In the unliganded HER3 crystal structure, no HER3 homodimers were reported (Cho and Leahy, 2002). However, a crystal structure of HER3 with bound ligand is needed to confirm this finding. Biochemical data from a HER3 chimera supports the lack of HER3 homodimers. A HER3 chimera with HER3 extracellular and transmembrane domains was fused to the EGFR cytoplasmic region to provide kinase activity, because HER3 has impaired kinase function. The HER3 chimera was transfected into an insect cell line that had no endogenous ErbBs, precluding heterodimerization of the transfected HER3. Immunoblotting did not reveal phosphorylation after the cells were stimulated with neuregulin (Berger et al., 2004).

In contrast to the HER2 and HER3 crystal structure data, other biochemical studies did show the existence of HER2 homodimers and HER3 homodimers. Full length HER2 homodimers were observed in solution by single wavelength fluorescence cross-correlation spectroscopy (Liu et al., 2007a). These results might be explained by the potential of HER2 transmembrane domains to dimerize. ErbB transmembrane homodimerization ability measured by FRET showed high affinity for HER2 homodimers. HER3 transmembrane homodimers were also present but were characterized by lower affinity (Duneau et al., 2007). Even if HER2 and HER3 homodimers are not present in normal cells, they may exist in cancers in which mutations or other supporting molecules allow these homodimers to form and possess signaling capability.

The possibility that ErbBs may interact with receptors outside their own family opens up a new area for research. In breast cancer cells, HER2 forms a dimer with a receptor tyrosine kinase from a different family. In SKBR3 breast cancer cells, data from immunoprecipitation experiments showed an association between HER2 and the insulin-like growth factor-1 receptor (IGF-1R), suggesting that the two receptors could form a heterodimer (Nahta et al., 2005) (Figure 10). It is not known whether this is a feature unique to these abnormal cells. Although the crystal structure of an IGF-1R extracellular domain shows similarities to the EGFR structure (Ward and Garrett, 2004), it is unclear how the two receptors could interact to form a dimer. Prevention of ErbB dimerization with receptors from other receptor tyrosine kinase families may be a new area for therapeutic intervention.

## Chapter 3 ErbB Downstream Signaling Pathways

ErbBs exert effects on cell cycle progression and survival pathways (Marmor et al., 2004). Receptor activation results in cytoplasmic signaling that conducts signal transduction to the nucleus (Mass, 2004), leading to gene transcription and resulting in cellular responses (Mendelsohn and Baselga, 2006). Ultimately, it is dysregulation of these downstream pathways that drives cancer progression. So far, discussion of therapeutic intervention sites has centered on prevention of activation of receptors at the cell surface by blocking ligand binding to extracellular domains, or by prevention of dimerization of extracellular domains. The possibility of interfering with dimerization of transmembrane and cytoplasmic receptor contacts has been mentioned. The inhibition of ErbB regulatory molecules may also be therapeutic targets. As will be discussed, the link between ErbB activation and downstream signaling molecules is another point for therapeutic intervention.

Since ErbB kinase activity drives intracellular signaling cascades, the mechanism by which the ErbB kinase domain phosphorylates C-terminal tyrosines is important to understand. Two ErbB kinase domains appear necessary to mediate phosphorylation. A crystal structure of the EGFR kinase domain complexed to an ATP analog showed an asymmetric kinase dimer in which the C-terminal lobe of one kinase domain interfaces with the N-terminal lobe of the other kinase domain. Mutations of this asymmetric kinase dimer interface disabled EGFR phosphorylation (Zhang et al., 2006). However, if the kinase domain of one receptor within a dimer requires the kinase domain of its dimer partner for activation, it is still unclear how EGFR, HER2, or HER4 could become activated when dimerized with the kinase-impaired HER3. Further information about kinase interactions between HER3 and the other ErbBs is needed through crystal structures. Disabling kinase interactions within a dimer could be a means of therapeutic intervention. Current therapeutics aimed at the kinase domain do not focus on disabling kinase domain interactions directly. These tyrosine kinase inhibitors (TKIs) shut down kinase activity by competing with ATP for the binding cleft (Rabindran, 2005).

Once the ErbB kinase domain is active, C-terminal tyrosines on the receptor are phosphorylated, attracting downstream molecules. The tyrosines that are phosphorylated vary depending on the dimer pair. Different patterns of tyrosine phosphorylation were found in HER4 transfected cells using phosphopeptide mapping, depending on the stimulating ligand. The different phosphorylation patterns correlated with different downstream signaling cascades that influenced biological activity of the cells (Sweeney et al., 2000). Knowledge of the identity of dimer pairs in tumor tissue, and the identity of the ligands that are stimulating these pairs, is important information in predicting which downstream pathways will be triggered in the tumor.

If ErbB phosphorylation is blocked, presumably mitogenic signals to the nucleus could be prevented. Researchers have probed the identities of the cytoplasmic molecules that bind to phosphorylated ErbB tyrosine residues (Figure 11). Prior to proteomics studies, the identity of some of the downstream proteins were uncovered by immunoprecipitation studies (Olayioye et al., 2000).

General conclusions were that most ErbB dimers could activate the mitogenactivated protein kinase (MAPK) pathway. In the MAPK pathway, ErbBs couple to adaptor proteins Grb2 and Shc that can activate Ras. Further downstream, MAPK is phosphorylated. MAPK translocates into the nucleus to activate transcription factor targets that promote mitogenicity (Zhou and Carpenter, 2002).

The MAPK pathway could be stimulated by multiple ErbB heterodimer combinations. ErbB heterodimers transfected into cells were stimulated with EGF or neuregulin. Increased MAPK activity was determined by in vitro kinase assay. The degree of activation and kinetics varied depending on the dimer pair (Pinkas-Kramarski et al., 1996b).

Consistent with other data showing that HER2 is very mitogenic, HER2 was able to increase downstream signaling through MAPK. HER2 resulted in increased MAPK pathway activation in T47D human breast cancer cells. Neuregulin stimulation increased MAPK activation, based on evaluation by in vitro kinase assay. The increased MAPK activation was due to HER2 because in T47D-5R cells that lack membrane HER2 due to expression of a monoclonal antibody to HER2 in the endoplasmic reticulum, MAPK activation did not increase (Karunagaran et al., 1996). This study suggested that increased signaling through MAPK, mediated by HER2, could be promoting proliferation of cancer cells. Therapeutics could be designed that
block the association of ErbBs with adaptor proteins that couple to the MAPK pathway.

HER3 mediates phosphatidylinositol 3-kinase (PI3K) activation. Alterations in the PI3K pathway are common in many cancers. PI3K recruits the Akt kinase which promotes cell survival by blocking apoptosis, and promotes cell cycle progression through multiple targets (Marmor et al., 2004).

HER3 contains seven of the binding site motifs for the p85 subunit Src homology 2 (SH2) domain of PI3K. In contrast, EGFR lacks this motif. In A431 epidermoid cancer cells stimulated with EGF, there was increased PI3K *in vitro* kinase assay activity from immunoprecipitation of HER3, but not from immunoprecipitation of EGFR or HER2 (Soltoff et al., 1994). The association of HER3 with PI3K was also observed in ErbB transfected fibroblast cells. Examination of HER3 and EGFR immunoprecipitations showed more p85 associated with HER3 than EGFR (Fedi et al., 1994). Evidence that HER3 couples to the PI3K pathway more strongly than other ErbBs suggests an explanation for HER3's role in producing the mitogenic qualities of the HER2/HER3 dimer. Therapeutics could be designed that block the association of HER3 to PI3K.

A proteomics study probed the identity of EGFR C-terminal tyrosine phosphorylation sites. Targeting therapeutics to block known tyrosine phosphorylation sites could be a strategy to prevent recruitment of cytoplasmic signaling molecules. The technique qPACE (quantitative proteomic assessment of very early signaling events) was used to examine EGFR phosphorylation sites in human cervical carcinoma HeLa cells. After EGF stimulation, cell lysates were immunoprecipitated with anti-p-tyrosine. The retrieved phosphopeptides were separated by SDS-PAGE and examined by mass spectrometry. Three sites of EGFR showed increased phosphorylation within five seconds of EGF stimulation: Tyr1092, Tyr1172, and Tyr1197. Tyrosines 1172 and 1197 are known to bind Shc, an activator of the MAPK pathway (Dengjel et al., 2007).

Dengjel et al. (2007) also identified a downstream binding partner of EGFR. The investigators found that phospholipase C-gamma (PLC $\gamma$ ) activation correlated with EGFR activation. PLC $\gamma$  induces calcium release which activates protein kinase C (Marmor et al., 2004), a transcription factor (Yarden and Sliwkowski, 2001). Phosphorylation of specific PLC $\gamma$  tyrosines occurred within one minute of EGF stimulation of EGFR, suggesting that EGFR provides a swift link to the PLC $\gamma$ pathway. Blocking the interaction of EGFR with PLC $\gamma$  could be a therapeutic intervention point.

In an effort to determine the phosphorylated tyrosine binding partners for all the ErbBs, a proteomics screen was performed using bait peptides representing all 89 ErbB cytosolic tyrosine residues. Bait peptides with phosphorylated tyrosines were immobilized on streptavidin beads and incubated with human cervical carcinoma HeLa cell lysate. After elution, bait peptides were precipitated out; proteins were then trypsinized and analyzed by MS. HER3 had a large number of binding sites for PI3K. EGFR and HER2 did not bind PI3K. HER2 had few interaction partners (usually Shc). EGFR and HER4 had multiple tyrosines that bound Grb2, or Grb2 and Shc, coupling to the MAPK pathway. Signal transducer and activator of transcription (STAT5) was identified as a direct interaction partner of specific tyrosines on EGFR and HER4 (Schulze et al., 2005). STAT5 is a transcription factor that promotes cell proliferation (Marmor et al., 2004).

The Schulze et al. study was useful in confirming results from studies in the 1990s that used biochemical methods, such as the HER3 connection to PI3K, and the connection of the ErbBs with the MAPK pathway. This study was also useful in identifying STAT5 as a putative downstream player, opening up a new area for research, and therapeutic intervention. However, in contrast to the Dengjel et al. experiment, PLC $\gamma$  was not identified as an interaction partner of EGFR. The authors suggested that binding of some molecules may be specified by the conformational attributes of receptor dimers, but not by the peptides which were used in the assay.

Another recent proteomics study cast the net wider to look at ErbB interactions using a larger pool of cytoplasmic molecules. Bait peptides were generated representing 33 ErbB phosphorylated tyrosine sites that had been experimentally verified in the literature. These were incubated with microarrays of all Src homology 2 and phosphotyrosine binding domain-containing proteins in the human genome. Most of the previously reported interactions were detected, and novel interactions were found (Jones et al., 2006).

EGFR, HER2, and HER3 all bound to PLCγ, PI3K, and Shc. No interactions with STAT were found. Some novel findings were that all ErbBs bound to Abl, and HER2 and HER3 bound to CrkL. Abl and CrkL are involved in promoting leukemia,

and the investigators found that CrkL becomes phosphorylated in many cancer cell lines after stimulation with ErbB ligands. The HER2 phosphotyrosines could bind to twice as many proteins as the other ErbBs. HER4 bound few proteins, but the investigators commented that this may have been a function of insufficient HER4 tyrosine sites included in the assay (Jones et al., 2006).

Results from proteomic studies were sometimes contradictory, such as the finding that PLC $\gamma$  was not an interaction partner of EGFR by Schulze et al. (2005), and the finding that PLC $\gamma$  was a partner of multiple ErbBs by Jones et al. (2006). HER2 had few partners according to Schulze et al., but had the greatest number of partners in the Jones et al. experiment. Conditions in the two experiments were quite different. In the Jones et al. study, there was a larger pool of downstream molecules on the microarrays with a smaller number of bait phosphotyrosines, compared to a smaller pool of downstream molecules in a specific cell lysate with a larger number of bait phosphotyrosines in the Schulze et al. study. Biological relevance of results from peptide studies is unclear since the conformation of phosphorylated tyrosine sites may differ *in vivo* compared to synthetic bait peptides. More data are needed to map all the ErbB tyrosine phosphorylation sites, and the molecules that are recruited to them.

It is possible that ErbB phosphorylated tyrosines in cancer cells may have different kinetics of binding to cytoplasmic signaling partners, or even different binding partners than in normal cells due to mutations in receptors that affect the tyrosine phosphorylation pattern. The tyrosine phosphorylation pattern of the C- terminal tail of wild type EGFR is different from that of a constitutively active EGFR lung cancer mutant that has somatic mutations in its tyrosine kinase domain. Based on modeling studies, phosphorylation of Tyr1068 increased, while that of Tyr1173 decreased for the mutant compared to wild type EGFR (Liu et al., 2007b). More data are needed to understand the significance of this difference in phosphorylation pattern. An understanding of ErbB mutant connections to cytoplasmic signaling molecules in tumor cells may be useful information in determining therapeutic strategies to inhibit these connections in patients.

## Chapter 4 Negative Regulation of ErbBs

In recent years, signal transduction research has shifted to study of feedback regulation. This is in contrast to the preceding research on forward-signaling activated by external stimuli leading to signaling cascades (Amit et al., 2007). Receptor activation initiates signaling, but also triggers processes that repress signaling through negative feedback loops (Rubin et al., 2005). Factors that influence the length of stimulation of an ErbB signaling cascade such as negative regulatory mechanisms play a role in determining the kinetics of receptor degradation and recycling known as downregulation (Levkowitz et al., 1998). Negative feedback is of interest clinically because these proteins are often imbalanced in cancer (Amit et al., 2007).

Early ErbB research had established that radiolabeled membrane bound EGFR/EGF complexes are internalized and reach lysosomes (Carpenter and Cohen, 1979). This process may be viewed as a mode of receptor regulation, existing to turn off EGFR activity. Defects in this process could result in constitutive EGFR activity, leading to sustained mitogenic consequences for the cell, and ultimately cancer. Existing research on EGFR downregulation suggests therapeutic interventions that restore downregulation pathways that may be lost in cancer. Currently, a small and diverse group of negative regulators of ErbBs are known (Figure 12).

Levkowitz et al. (1998) reported that Cbl negatively regulates EGFR. Extensive study of Cbl by these investigators culminated in a pre-clinical study that established Cbl as a potential therapeutic molecule. Using an *in vitro* ubiquitination system with purified EGFR from A431 epidermoid cancer cells, Cbl was found to perform the role of an E3 ubiquitin ligase by transferring a ubiquitin molecule from a specific E2 ubiquitin-conjugating enzyme to EGFR. The ubiquitinated EGFR was degraded when it was incubated with a proteasome (Levkowitz et al., 1999). This result suggests that Cbl may play a role in the degradation of EGFR in cancer cells.

Immunoblotting data showed Cbl blocked tyrosine phosphorylation of EGFR after EGF stimulation in cells co-transfected with Cbl and EGFR (Levkowitz et al., 1998). Cbl expression correlated with reduced activity from the MAPK mitogenic pathway. When cells transfected with EGFR and Cbl were stimulated with EGF, immunoblotting showed that MAPK was not phosphorylated (Waterman et al., 1999). Cbl may promote EGFR endocytosis. In Cbl transfected cells, immunoblotting with antibody to EGFR revealed that there was a loss of EGFR. Viewed by immunofluorescence microscopy, EGFR associated with Cbl in intracellular vesicles after EGF stimulation. An association of EGFR and Cbl was found when EGFR was immunoprecipitated from the purified endosomal fraction, followed by immunoblot with anti-Cbl (Levkowitz et al., 1998).

Further work revealed that Cbl binds to the cytoplasmic region of EGFR. EGFR mutants were transfected singly with Cbl into cells and stimulated with EGF to isolate the interaction sites between Cbl and EGFR based on their physical association, and resulting downregulation of EGFR. The tyrosine kinase domain, and the C-terminal of EGFR were needed to interact with Cbl (Levkowitz et al., 1998). Cellular expression of EGFR C-terminal tyrosine mutants showed that EGFR phosphorylated tyrosine 1045 was part of the Cbl docking site (Levkowitz et al., 1999).

Levkowitz et al. (2000) found that Cbl also associated with HER2. The investigators made sure they were looking at only HER2 interactions by transfecting HER2 into ErbB-negative 32D cells. The cells were treated with monoclonal antibody to induce HER2 homodimers. HER2 obtained by immunoprecipitation with anti-ptyrosine could be immunoblotted with antibody to Cbl.

Cbl has clinical relevance in ErbB2 expressing tumor cells. A mutated form of ErbB2 termed Rat Neu was used in the experiments because it had been linked with cancer in these animals. Rat Neu is derived from a carcinogen-induced neuro/glioblastoma, and is constitutively active due to a transmembrane domain point mutation. Transfection of Cbl and Neu into cells correlated with increased ubiquitination of Neu and downregulation of Neu, followed by decreased MAPK activity. B104 rat neuro/glioblastoma tumor cells expressing endogenous Neu were infected with Cbl retrovirus and injected into athymic mice. Higher levels of Cbl, measured by immunoblotting, correlated with smaller tumor volume (Levkowitz et al., 2000). The results showed Cbl had the capability of downregulating Neu, reducing downstream signaling from Neu, and decreasing tumor volume, establishing Cbl as a potential therapeutic molecule.

Although Cbl negatively regulates EGFR and Neu/ErbB2, an attempt to find the association of Cbl with HER3 failed. Immunofluorescence microscopy data showed that HER3 transfected into CHO cells did not associate with Cbl in intracellular vesicles after neuregulin ligand treatment. A radiolabeled ligand binding assay showed that neuregulin treatment of HER3 and Cbl co-transfected cells did not result in HER3 downregulation (Levkowitz et al., 1998).

Recently, Cbl was found to associate only with EGFR, and no other ErbB, in a proteomic assay analyzing phosphorylated tyrosine bait peptides from all four ErbBs incubated with HeLa cell lysate (Schulze et al., 2005). Given the evidence, the ability of ErbBs other than EGFR to recruit Cbl is unclear, although it appears that Cbl can downregulate rat Neu, a mutated form of ErbB2. Currently, there is little data on the role of Cbl in human tumors. Investigation of Cbl as a therapeutic remains in the pre-clinical stage.

Research has uncovered molecules other than Cbl that negatively regulate EGFR. The soluble extracellular matrix proteoglycan decorin binds to EGFR and promotes EGFR internalization and degradation by endocytosis. Decorin is downregulated in transformed cells and in multiple carcinomas, suggesting that its restoration may be of therapeutic benefit.

Immunoblot data from A431 epidermoid cancer cells treated with decorin and a cross linking reagent showed that decorin was cross-linked to EGFR dimers. Unlike Cbl, decorin appeared to compete with EGF, based on data showing a reduction in dimer formation when cells were exposed to both EGF and decorin. Immunofluorescence microscopy data revealed that after decorin exposure, EGFR colocalized with decorin in intracellular vesicles. Antibody staining of endosomal compartments suggested that decorin targets EGFR to the late endosome and presumably to lysosomal degradation. Use of a tyrosine kinase inhibitor showed that internalization of EGFR induced by decorin did not depend on EGFR kinase activity. This is in contrast to Cbl, which bound to an EGFR phosphorylation site (Zhu et al., 2005). Since decorin is known to be downregulated in cancers, is soluble, acts extracellularly, and promotes receptor degradation, it seems like a good candidate for further study as a therapeutic.

A human transmembrane protein called leucine-rich repeats and immunoglobulin-like domains 1(LRIG1) is related to decorin, and to a Drosophila protein kekkon-1, known to be a negative regulator of EGFR that inhibits EGF binding. It is unclear whether LRIG proteins are tumor suppressors, or if they could be harnessed as therapeutics. They are downregulated in some carcinomas but upregulated in others (Hedman and Henriksson, 2007). Recent data from epidermoid and prostate cancer cell lines suggests that LRIG1 reduces downstream signaling from EGFR.

Soluble LRIG1 extracellular domain reduced cell proliferation in the high EGFR expressing A431 epidermoid carcinoma and MDA-468 breast carcinoma cells, but not in cells that do not express EGFR. When A431 cells were incubated with LRIG1 prior to EGF stimulation, immunoblotting showed LRIG1 reduced tyrosine phosphorylation of EGFR and MAPK, but there was no loss of EGFR. When A431 cells were incubated with LRIG1 and examined by immunofluorescence microscopy, LRIG1 and EGFR co-localized at the cell surface and did not move into the cytoplasm. In response to LRIG1, immunoblots showed that EGFR was not phosphorylated on tyrosine 1045 (the Cbl docking site), suggesting that LRIG1 may prevent Cbl-mediated downregulation of EGFR. The mechanism of action of LRIG1 is not known. It may prevent EGFR dimerization by blocking EGF binding, as suggested for decorin (Goldoni et al., 2007).

When LRIG1 was expressed in prostate cancer cells, there was decreased EGFR activity. In PC3 prostate cancer cells stimulated with EGF, induction of LRIG1 expression resulted in increased ubiquitination of EGFR and decreased tyrosine phosphorylation of EGFR. LRIG1 expression prevented the cells from entering S phase, based on flow cytometry analysis (Laederich et al., 2004).

Recently, a connection was found between activated EGFR and neurofibromatosis type 2 tumor suppressor mutations. Neurofibromatosis type 2 mutations are associated with intractable cancers. Neurofibromatosis type 2 is found at the cell membrane and is involved in linking membrane proteins to the actin cytoskeleton. Immunoblotting with anti-p-EGFR showed that mouse epithelial neurofibromatosis type 2 gene deletion mutant cell lines maintained high levels of phosphorylated EGFR. The cells did not undergo contact-dependent inhibition of proliferation. Wild type neurofibromatosis type 2 could act as a negative regulator of EGFR when it was restored to the cells. Rescue with wild type neurofibromatosis type 2 resulted in decreased phosphorylation of EGFR and contact-dependent inhibition (Curto et al., 2007).

In EGF-stimulated neurofibromatosis type 2 deletion mutant cells that are highly proliferative, EGFR interacted with downstream signaling molecules and, surprisingly, with Cbl. Immunofluorescence microscopy studies revealed that EGFR was internalized in neurofibromatosis type 2-negative cells (Curto et al., 2007). It was surprising that EGFR interaction with Cbl, and internalization of EGFR were found in these mitogenic cells. Previous work by other investigators had shown that Cbl binding to EGFR led to reduced EGFR activity (Levkowitz et al., 1998) and (Waterman et al., 1999). A model was suggested in which neurofibromatosis type 2 physically restrains EGFR from binding to its downstream targets, from binding to Cbl, and from internalization (Curto et al., 2007). This study established neurofibromatosis type 2 as a candidate for cancer therapy. It also suggested that the original view of Cbl as a terminator of EGFR signaling might be too narrow.

Since there is little evidence that Cbl acts as a negative regulator of ErbBs other than EGFR, a search was conducted for similar molecules that might regulate the other ErbBs. Cao et al. (2007) suggested that there are defects in negative regulation in HER3 overexpressing tumors. While HER3 mRNA is not overexpressed in tumors, HER3 protein is overexpressed. This might indicate that normal levels of HER3 protein are produced in tumors, but HER3 is not cleared away effectively.

A putative ubiquitin ligase termed neuregulin receptor degradation protein-1 (Nrdp1) was discovered as a negative regulator of HER3 and HER4. A yeast twohybrid screen using the intracellular region of bovine ErbB3 as bait to screen a human brain cDNA library revealed that the neuregulin receptor degradation protein-1 interacted with the ErbB3 intracellular domain. Cells were infected with the four ErbB family members and Nrdp1. Immunoprecipitation of each ErbB followed by immunoblotting for Nrdp1 showed Nrdp-1 associated with HER3 and HER4, but not with HER2 or EGFR (Diamonti et al., 2002).

Cells transfected with ErbBs and Nrdp1 were examined by immunoblotting with antibodies to ErbBs. Reduced levels of HER3 and HER4, but not of HER2 or EGFR, were found in the presence of Nrdp1. Immunofluorescence microscopy data from transfected cells showed that Nrdp1 altered the cellular location of HER3 from the cell surface to perinuclear compartments. It was proposed that because Nrdp1 is a RING finger-containing protein like Cbl, it may act as a ubiquitin ligase to reduce HER3 and HER4 levels by targeting them for degradation (Diamonti et al., 2002).

Since Nrdp1 levels are often decreased in HER3 overexpressing human tumors, restoration of Nrdp1 in cancer cells could be of therapeutic value. In human breast cancer MCF7 cells, Nrdp1 could downregulate HER3. After neuregulin stimulation of these cells, immunoblotting showed increased Nrdp1 levels, increased ubiquitination of HER3, and a loss of HER3 (Cao et al., 2007).

There is some evidence that LRIG1 may also regulate the other ErbBs, in addition to EGFR. Cells were transfected with full length human LRIG1 and ErbBs. After growth factor stimulation, all of the ErbBs could be immunoblotted from immunoprecipitated LRIG1. In transfected cells analyzed by immunofluorescence, all of the ErbBs co-localized with LRIG1 at the cell periphery and in perinuclear compartments (Laederich et al., 2004). If LRIG1 acts as a negative regulator of all the ErbBs, it could be useful in turning off the activity of all the ErbBs simultaneously.

Doherty et al. (1999) reported that herstatin is a negative regulator of HER2. Herstatin has been tested as a therapeutic in pre-clinical studies. Herstatin was discovered as an alternative HER2 transcript that retains intron 8. It is a secreted 68 kD protein, and is expressed as half of the HER2 extracellular domain (subdomains I and II) with an additional C-terminal sequence of 79 amino acids encoded by intron 8. When HER2 transfected cells were incubated with radiolabeled herstatin and crosslinker, herstatin was found bound to immunoprecipitated cell surface HER2. Herstatin treatment reduced HER2 dimer levels in these cells. Recent data confirmed the interaction of herstatin with HER2, and revealed biological consequences for cells in response to herstatin. When cells transfected with herstatin and HER2 were analyzed by immunofluorescence microscopy, herstatin and HER2 appeared to co-localize in the cytoplasm. The presence of herstatin correlated with decreased HER2 at the cell membrane. MTT-based cell proliferation assay showed that herstatin reduced cell proliferation (Hu et al., 2006). Since herstatin is a HER2 extracellular domain fragment, it would be interesting to study whether herstatin may bind to other ErbB extracellular domains, possibly creating non-functional heterodimers. Investigation of herstatin in cancer cell lines is needed to elucidate whether herstatin is lost.

Although negative regulators such as Cbl, decorin, and Nrdp1 appear to achieve ErbB degradation by promoting ErbB internalization, downstream signaling may continue after receptor internalization. Some data have shown that EGFR may associate with downstream signaling molecules throughout the endocytic process. Immunofluorescence data showed that after 10 minutes of EGF stimulation, EGFR and the phosphorylated mitogenic factor p-MAPK co-localized in early endosomes of cells. After 30 minutes of EGF stimulation, they were found co-localized in late endosomes (Kim et al., 2007). After EGF stimulation, tyrosine phosphorylated EGFR, and the MAPK pathway adaptor proteins Grb2 and Shc were found by immunoanalysis in membranes from rat liver endosomes (Balbis et al., 2007).

Enhanced activation of downstream signaling was observed during endocytosis of rat ErbB4 in neurons. Immunoblotting experiments using rat hippocampal neurons stimulated with neuregulin showed increased p-MAPK and p-Akt in immunoblots during endocytosis, compared to when endocytosis was inhibited (Liu et al., 2007c).

Downstream signaling from endosomes seems to contradict the results of previous studies showing that endocytosis reduced tyrosine phosphorylation of ErbBs (Levkowitz et al., 1998) and their downstream partners (Waterman et al., 1999), reduced cell proliferation (Goldoni et al., 2007), and prevented cell cycle progression (Laederich et al., 2004). Further understanding of the signaling capacity of ErbBs from endosomes will assist in determining the conditions in which endocytosis leads to receptor degradation, or to enhancement of downstream signaling. A third function of endocytosis may be to transport ErbBs to the nucleus, and will be discussed in the next chapter.

The discovery of negative regulatory molecules for ErbBs shows that the receptors require active removal from the cell membrane. In cancer cells that lack functional negative regulatory molecules, therapeutics that target ErbB activation may

be insufficient. Despite inhibiting receptor activity through blocking ligand binding, dimerization, and tyrosine kinase activity, the receptor would still remain on the cell membrane if negative regulatory molecules are not functioning. However, restoration of intracellular negative regulatory molecules in tumor cells awaits sophisticated drug delivery systems that can transport large molecules into the cell, or gene therapy.

## Chapter 5 Nuclear Localization of ErbBs

Since the 1990s, EGFR has been observed in the nucleus of cells in many cancer types. However, most research continued to focus on EGFR activation of intracellular signaling cascades (Lin et al., 2001), possibly due to lack of techniques to study direct interactions between EGFR and DNA. If EGFR and the other ErbBs can function as transcription factors, therapeutics that terminate ErbB signaling would need to suppress pathways by which ErbBs could travel to the nucleus, and prevent ErbB interaction with nuclear processes that produce mitogenic responses (Table 2).

Due to the possibility that nuclear ErbBs play a role in cancer, the University of Texas M.D. Anderson Cancer Center has been an active site for research on this subject. Based on analysis of previous studies, investigators hypothesized that EGFR is localized to the nucleus in tissues that are highly proliferative. EGFR was found in the nucleus in normal proliferative tissues and human cancer tissue by immunohistochemistry (IHC), but not in non-proliferative normal tissue controls. In experiments with cancer cells, highly activated EGFR appeared quickly in the nucleus of cancer cells after EGF stimulation. The experiments suggested that EGFR might be able to activate cyclin D1, a cell cycle progession factor that drives cell proliferation (Lin et al., 2001).

EGF stimulation of EGFR-overexpressing A431 epidermoid cancer and MDA-MB-468 breast carcinoma cells resulted in highly tyrosine phosphorylated EGFR in nuclear extracts. In the breast carcinoma cells, radiolabeled EGF that was bound to EGFR with a membrane-impermeable cross-linker was found in the nucleus within five minutes (Lin et al., 2001). This result suggests that the EGFR found in the nucleus came from the cell membrane, since the cross-linker could not cross the cell membrane by itself.

A search for a DNA binding site for EGFR in A431 epidermoid cancer cell lysate revealed an AT-rich minimal consensus sequence. A reporter construct containing the AT-rich minimal consensus sequence transfected into cancer cells could be activated by EGF. The cyclin D1 gene was found to contain two AT-rich minimal consensus sequences. Transfected cyclin D1 reporter construct could be activated by EGF in MDA-MB-468 breast carcinoma cells. Supporting an interaction between the cyclin D1 gene and EGFR, after EGF stimulation, complexes of EGFR with cyclin D1 gene were found in anti-EGFR immunoprecipitations from A431 nuclear extracts (Lin et al., 2001).

Researchers are investigating how EGFR might travel to the nucleus from the cell membrane. A cancer therapeutic could be designed to block this process. A series of experiments established that EGFR has a putative nuclear localization sequence, EGFR interacts with a nuclear transport receptor, and endocytosis of EGFR assists its progression to the nucleus. The nuclear transport receptor importin  $\beta$ 1 (I $\beta$ 1) was found to associate with EGFR in EGF stimulated A431 epidermoid cancer cells, based on immunoprecipitation with anti-EGFR followed by immunoblot with anti-I $\beta$ 1. Electron microscopy and immunofluorescence microscopy showed co-localization of EGFR and I $\beta$ 1 near nuclear membranes, and in the nucleus of MDA-MB-468 breast carcinoma cells treated with EGF. A putative nuclear localization

sequence on EGFR promoted the association of EGFR and I\u03c41, based on results showing an EGFR nuclear localization sequence mutant transfected into breast cancer cells reduced the association between EGFR and I\u03c41 (Lo et al., 2006).

To investigate whether EGFR is transported to the nucleus via endocytosis, the dynamin protein known to be involved in endocytosis was mutated. Cells transfected with a dynamin mutant were stimulated with EGF, resulting in an 80% reduction in nuclear EGFR levels, based on immunoblot results. A similar result was seen in A431 epidermoid cancer cells treated with an endocytosis inhibitor. Colocalization of EGFR with an early endosome marker was observed by electron microscopy in the cytoplasm and nucleus of EGF stimulated MDA-MB-468 breast carcinoma cells (Lo et al., 2006), suggesting that EGFR had undergone endocytosis prior to appearing in the nucleus.

Similar studies were carried out for HER2, focusing on breast cancer, at The University of Texas M.D. Anderson Cancer Center. First, many experiments were done to determine if HER2 is present in cancer cell nuclei. Then, in breast cancer cell nuclei, HER2 was found to associate with the cyclooxygenase enzyme (COX-2) gene. COX-2 enzyme catalyzes the production of prostaglandins that promote inflammation, and is overexpressed in many human cancer types (Xi et al., 2005).

Multiple biochemical techniques showed the presence of HER2 in the nucleus of cancer cells. Nuclear expression of full length HER2 was detected in HER2overexpressing BT474 human breast cancer cells by immunofluorescence microscopy. Nuclear expression of HER2 was observed by electron microscopy in MCF-7 breast cancer cells transfected with HER2. HER2 was detected by immunoblot in the nuclear fraction of the HER-2 overexpressing breast cancer SKBR3 cell line. IHC staining revealed nuclear HER2 in paraffin tissue from multiple cancer types (Wang et al., 2004).

Genomic targets of HER2 were identified by chromatin immunoprecipitation. HER2-DNA complexes were immunoprecipitated with anti-HER2 from BT-474 breast cancer cell nuclei. The HER2-associated DNA was sequenced, revealing several targets including COX-2. COX-2 was chosen for further study due to its overexpression in many human cancer types. The specific sequence fragment in the COX-2 gene that associated with HER2 was connected to a luciferase reporter system and transfected with HER2 into fibroblast cells. Reporter activity resulted, suggesting that HER2 has transactivation activity *in vivo*. Data from chromatin immunoprecipitation analysis showed HER2 associated with the COX-2 promoter in HER2-overexpressing fresh breast cancer tumor tissue (Wang et al., 2004).

Experiments similar to those by Lo et al. (2006) were carried out with HER2, identifying an interaction between HER2 and the I $\beta$ 1 nuclear transport receptor. Therefore, HER2 and EGFR seem to proceed to the nucleus by a similar route. HER2 associated with I $\beta$ 1 in breast cancer cell line cytoplasmic and nuclear lysates. When cells were treated with I $\beta$ 1 siRNA, HER2 expression in the nuclear fraction was reduced, suggesting that I $\beta$ 1may assist in HER2 nuclear import. A putative nuclear localization sequence in the HER2 transmembrane domain appeared to mediate the interaction with I $\beta$ 1. More evidence for HER2 entry into the nucleus was gathered

from immunoprecipitation experiments showing HER2 associated with nuclear pore protein 358 in breast cancer cells. Co-localization of HER2, I $\beta$ 1, and nuclear pore protein 358 close to the nuclear envelope was observed by electron microscopy (Giri et al., 2005).

HER2 may travel to the nucleus by endocytosis, similarly to EGFR. In HER2 transfected breast cancer cells, cell surface HER2 was traced by biotin labeling and found to appear within one minute in the nucleus. Immunoprecipitation and immunoblot studies showed that the endocytic proteins adaptin and clathrin associated with HER2, suggesting HER2 may travel to the nucleus by endocytosis. Electron microscopy showed that Iβ1 and HER2 co-localized in the endosome wall. Transfection of a mutant of the known endocytic protein dynamin resulted in the loss of nuclear expression of HER2 (Giri et al., 2005).

A model was proposed by Giri et al. in which HER2 is internalized by endocytosis, allowing its nuclear localization sequence to interact with Iβ1 which guides it to the nucleus via nuclear pore protein 358. This model identifies several intervention points at which HER2 could be prevented from traveling to the nucleus. However, no therapeutics have yet been designed to intervene in this process. The use of endocytosis as a path to the nucleus for EGFR and HER2 suggests caution in using therapeutics that promote endocytosis as a means to degrade ErbBs.

While data are not available regarding nuclear HER3 from the University of Texas M.D. Anderson Cancer Center, other studies have shown HER3 in the nucleus of cancer cell lines. In MCF-7 breast cancer cells, immunoprecipitation with antibody to HER3 extracellular domain followed by immunoblotting with antibody to HER3 intracellular domain showed the presence of full length HER3 in both nuclear and cytoplasmic fractions (Offterdinger et al., 2002). Immunoblotting of nuclear fractions of prostate cancer cell lines such as LNCaP confirmed the presence of HER3 in the nucleus (Koumakpayi et al., 2006).

Details of HER3 translocation to the nucleus have not been determined. However, HER3 contains a putative nuclear localization sequence. A HER3 Cterminal putative nuclear localization sequence fused to green fluorescent protein and transfected into human mammary epithelial cells concentrated in the nucleus. Transfection of this putative nuclear localization sequence fused to the exclusively cytoplasmic protein chicken pyruvate kinase resulted in translocation of the kinase to the nucleus (Offterdinger et al., 2002).

Nuclear HER3 has clinical significance in prostate cancer. IHC staining of paraffin tissue samples from cancerous prostate showed full length HER3 in the nucleus, particularly in patients with advanced disease. In contrast, normal tissues rarely exhibited nuclear staining of HER3 (Koumakpayi et al., 2006). This study is useful in establishing that nuclear HER3 has diagnostic significance as a marker for poor prognosis.

Unlike HER2 and HER3, which were found as full length receptors in the nucleus, HER4 was found in a cleaved form in the nucleus. HER4 is unique among the ErbBs in that it exists in two isoforms (JM-a and JM-b) generated by alternate splicing, containing different sequences in the extracellular juxtamembrane region

(Schlessinger and Lemmon, 2006). Neuregulin binding to HER4 JM-a isoform resulted in ectodomain cleavage of HER4, releasing a 120 kD fragment into extracellular medium, and leaving behind a membrane associated 80 kD fragment that eventually finds its way to the nucleus (Carpenter, 2003).

A protease cleaves the 80 kD transmembrane portion of HER4 remaining after release of HER4 extracellular domain. The 80 kD transmembrane portion is freed as a soluble fragment termed s80. The generation of s80 seems to be controlled by the growth factor ligand neuregulin. After neuregulin stimulation, s80 could be detected by immunoblotting with antibody to HER4 cytoplasmic domain in HER4 transfected cells. Transfected HER4 cytoplasmic domain fused to green fluorescent protein was visualized in the cell nucleus. Since s80 is a cytoplasmic fragment of HER4, these data suggested that s80 is found in the nucleus (Ni et al., 2001).

Investigation of the activity of s80 showed it to be an active kinase dimer. Immunoprecipitation of s80 followed by immunoblot with anti-p-tyrosine showed that s80 was heavily tyrosine phosphorylated. s80 could phosphorylate an exogenous substrate in an in vitro assay, suggesting that s80 is an active tyrosine kinase. Lysate incubated with a cross-linking agent revealed the presence of s80 homodimers (Linggi et al., 2006). Whether s80 may phosphorylate substrates in the nucleus remains to be determined.

There is some evidence that nuclear HER4 affects transcriptional activity. The C-terminal of HER4 showed weak transcriptional activity using a GAL4 transactivation system (Ni et al., 2001). Nuclear HER4 may have transcriptional activity during embryonic brain development. Immunoprecipitation of HER4 from neuregulin stimulated rat neuronal precursor cells showed that HER4 associated with a transcriptional corepressor. Immunostaining showed that the HER4/corepressor complex translocated to the nucleus. In normal development, astrocytic differentiation is induced in rat neuronal precursor cells by transcriptional activation of the glial fibrillary acid protein promoter. However, when s80 was transfected into rat neuronal precursor cells, and the cells were stimulated with neuregulin, glial fibrillary acid protein promoter activity was reduced. It was hypothesized that s80 represses transcription of glial genes that cue differentiation into astrocytes (Sardi et al., 2006). Investigation of possible nuclear HER4 transcriptional activity in cancer cells is needed.

Many of the studies described reveal nuclear ErbBs in cancer cell lines and tumor tissues. It remains to be determined whether nuclear ErbBs are predominantly found in dysregulated cells, or if they could be serving a normal physiological purpose. The importance of growth factor ligands in regulating ErbB nuclear localization, phosphorylation status in the nucleus, and transcriptional activity will require additional investigation. Carpenter (2003) suggested that phosphorylated nuclear ErbBs may have enzymatic activity that could serve to modify nuclear proteins. The significance of cleaved forms of receptor versus full length receptor in the nucleus remains to be addressed. How an intact receptor is transported from the cell membrane to the nucleus is unclear. Endocytosis appears to be a possible mechanism for EGFR and HER2 (Lo et al., 2006), and (Giri et al., 2005). A possible intervention point for a therapeutic could be to prevent the receptor from taking an endocytic pathway to the nucleus.

The possibility that nuclear ErbBs regulate gene transcription is emerging. neuregulin stimulation resulted in HER4 repression of the glial fibrillary acid protein promoter in rat neuronal precursor cells (Sardi et al., 2006). EGF stimulation may result in EGFR activation of transfected cyclin D1 reporter construct in breast cancer cells (Lin et al., 2001). HER2 may activate the COX-2 promoter when transfected into fibroblast cells (Wang et al., 2004). Another point of therapeutic intervention might be to block ErbB activation of nuclear targets.

## Chapter 6 ErbB-Targeted Cancer Therapeutics

Examination of the basic biology of ErbBs has suggested many intervention points for therapeutics to combat cancer by reduction or termination of ErbB signaling (Figure 13). Some existing interventions target ErbB activation. MAb therapeutics may block ligand binding to the ErbB extracellular domain to prevent the conformational changes that promote receptor dimerization. Other MAbs, or a natural compound TNQ, may block dimerization sites in the extracellular domain, preventing the interfacing of two ErbBs that enables transphosphorylation of their C-terminal tyrosine residues. Blockade of ErbB dimerization with receptors from other receptor tyrosine kinase families such as the IGF-1R may be a new area for therapeutic intervention. To block activation, HER2 interaction with regulatory molecules could be targeted, such as Muc4 that binds to the HER2 extracellular domain, and the Hsp90 chaperone that binds to the HER2 kinase domain.

The link between ErbB activation and downstream signaling molecules is another point for therapeutic intervention. One intervention might be to block ErbB phosphorylation by disabling kinase interactions within a dimer. Targeting therapeutics to block phosphorylation of specific tyrosine sites could prevent recruitment of cytoplasmic signaling molecules. Therapeutics could be designed that prevent ErbBs from associating with adaptor proteins that couple to cancer-promoting pathways. Moving further downstream, restoring downregulatory molecules that might be lost in cancer, such as Cbl, decorin, neurofibromatosis type 2, Nrdp1, LRIG1, and herstatin, may reduce ErbB signaling. Since high levels of nuclear ErbBs have been shown to correlate with reduced cancer patient survival, blocking the nuclear transport of ErbBs may have potential therapeutic value (Lo and Hung, 2006). EGFR and HER2 could be prevented from taking an endocytic pathway to the nucleus. HER4 could be prevented from traveling to the nucleus by blocking its cleavage into s80. Another point of therapeutic intervention may be to inhibit ErbB transactivation of nuclear targets.

Despite all the possible routes to turn off ErbB signaling, the only ErbBtargeted therapeutics FDA approved for clinical use are MAbs that bind to ErbB extracellular domains and tyrosine kinase inhibitors (TKIs) that bind to ErbB cytoplasmic kinase domains (Zhang et al., 2007a). There are many therapeutics in development. Therapeutics that aim to sidestep some of the clinical difficulties with MAbs are peptide mimics of MAbs, and RNA aptamers. Toxin-conjugated ErbB ligands are used to bring a toxin into the cancer cell. Antisense RNA oligonucleotides and RNA interference reduce translation of ErbBs, preventing their expression at the protein level. Metalloprotease inhibitors disable cleavage of HER2 extracellular domain. Metalloprotease inhibitors also prevent cleavage of ErbB ligands, precluding ligand activation of receptors. Hsp90 chaperone inhibitors prevent the binding of ATP to Hsp90, blocking Hsp90's ability to stabilize HER2. Novel therapeutics continue to be inspired by increasing knowledge of ErbB structure and signaling.

## I. Monoclonal Antibodies

Currently, the two FDA approved MAbs to ErbBs are cetuximab (anti-EGFR MAb) and trastuzumab (anti-HER2 MAb). Additional MAbs are in development,

62

such as pertuzumab, which also binds to HER2. There are no MAbs available against HER3 or HER4 (Figure 14). This is surprising, given the known involvement of HER3 and HER4 in promoting downstream signaling that has mitogenic consequences for cells.

MAbs that bind to the EGFR extracellular domain appeared promising in theory as cancer therapeutics in that they might inhibit ligand binding, preventing EGFR activation. Cetuximab is a FDA approved MAb that binds to the EGFR extracellular domain (Cohenuram and Saif, 2007). Cetuximab interacts specifically with Domain III of EGFR, one of the domains responsible for ligand binding (Mendelsohn and Baselga, 2006). Cetuximab is approved for use in cancers that overexpress EGFR, including advanced colorectal cancer refractory to chemotherapy, and head and neck cancer (Toffoli et al., 2007). Although cetuximab is FDA approved, there is still only a low response rate (patients whose tumors decreased in size) of 23% to cetuximab plus chemotherapy in colorectal cancer (Mendelsohn and Baselga, 2006). While data support that cetuximab inhibits EGF binding, reduces EGFR phosphorylation, promotes EGFR internalization, and invokes antibody-dependent cellular cytotoxicity that results in tumor cell death, use of cetuximab as a therapeutic for advanced cancer patients has been mostly unsuccessful.

Cetuximab may compete with EGF for binding to EGFR, blocking receptor activation. Supporting this hypothesis, there was a reduction in EGFR phosphorylation in response to cetuximab in A431 epidermoid cancer cells that overexpress EGFR (Diaz Miqueli et al., 2007). However, the ultimate role of MAbs in terminating EGFR signaling may be due to EGFR downregulation. In response to MAb binding to EGFR, EGFR is internalized. KB human epidermoid carcinoma cells were treated with anti-EGFR MAbs and then evaluated for the remaining number of surface binding sites with radiolabeled EGF. MAbs induced slow internalization of EGFR (Friedman et al., 2005).

The investigators probed whether MAbs acted like EGF in inducing Cblmediated ubiquitylation of EGFR to promote EGFR internalization. Results showed that Cbl was not the mechanism used by MAbs to induce EGFR internalization. Anti-EGFR MAb treatment of cells transfected with EGFR, Cbl, and ubiquitin did not result in ubiquitylation of EGFR. Cells expressing a mutant form of EGFR that cannot recruit Cbl still displayed EGFR internalization when treated with MAbs (Friedman et al., 2005).

How MAbs cause EGFR to internalize is not known. An experimental result that may have clinical applications is that using multiple anti-EGFR MAbs to different epitopes on EGFR increased the amount and rate of EGFR removal from the cell surface. The investigators hypothesized that the larger the lattice formed by MAbs with receptor, the more effectively and swiftly receptor degradation occurs (Friedman et al., 2005). Research is ongoing to determine whether use of multiple MAbs for cancer treatment is more successful than a single MAb.

Beneficial effects of EGFR MAbs may be due to immune responses, in addition to preventing activation of EGFR and promoting its internalization. In tumor cell lines, the effects of cetuximab appeared to be mediated by antibody-dependent cellular cytotoxicity in which natural killer cells are recruited to the Fc region of MAbs, resulting in cell death. A431 and other EGFR-expressing cancer cell lines were analyzed for antibody-dependent cellular cytotoxicity in a cytotoxicity assay in which cells were incubated with cetuximab, and lymphocytes were added. There was more antibody-dependent cellular cytotoxicity activity in cells with high levels of bound cetuximab (Kimura et al., 2007). Since polymorphisms influence the affinity of immune cells for the Fc receptor of therapeutic MAbs, it was suggested that this may account for some interpatient variability in MAb treatment efficacy when antibody-dependent cellular cytotoxicity is the mechanism for killing of tumor cells (Dassonville et al., 2007).

The anti-HER2 MAb trastuzumab (Herceptin<sup>TM</sup>) was FDA approved in 1998 for metastatic breast cancer patients with HER2 overexpression (Kumar Pal and Pegram, 2006). As anticipated from the mitogenic nature of HER2 in cell lines, and crystal structure data predicting its ability to dimerize easily, HER2 overexpression is associated with many cancer types, including breast, lung, ovarian, prostate, gastric, oral, kidney, pancreatic, cervical, and endometrial cancer (Rabindran, 2005) and (Kumar Pal and Pegram, 2006). HER2 gene amplification occurs in approximately 20% of all breast cancers (Rabindran, 2005), but rarely in other cancers (Gross et al., 2004). This suggests that HER2 overexpression in most cancers occurs at the protein level, possibly due to defects in downregulation.

Unlike EGFR, HER2 has no known ligand. Therefore, trastuzumab does not work by blocking ligand binding sites. A crystal structure of the human HER2

65

extracellular domain complexed with the Fab fragment of trastuzumab showed that trastuzumab binds to HER2 on the C-terminal of Domain IV (Cho et al., 2003). Domain IV does not seem to play a pivotal role in dimerization, therefore trastuzumab may not block extracellular domain dimerization. Domain IV is the closest extracellular domain to the transmembrane portion of the receptor. It is possible that trastuzumab interference with transmembrane domain dimer contacts is effective in disabling HER2. Proposed mechanisms of action for trastuzumab include the following: inhibition of HER2 extracellular domain cleavage, antibody-dependent cellular cytotoxicity immune effects leading to cell death, internalization and degradation of HER2, reduction of Src binding to HER2, induction of inactive oligomers, and downregulation of the fatty acid synthase (FAS) gene.

Although trastuzumab is FDA approved, the effectiveness of trastuzumab in advanced cancer patients is limited. In HER2-overexpressing metastatic breast cancer patients, response rates to trastuzumab as a single agent were 12-34% for a median of nine months. Adding chemotherapy improved the response. From a Phase III trial (HERA) with patients who were HER2-positive by IHC, disease-free survival rate after 2 years was analyzed. The survival rate was increased when trastuzumab was given for one year as a single agent after adjuvant chemotherapy: 86% compared to 77% in the control group. Increased disease-free survival was seen in Phase III trials in which trastuzumab was added to chemotherapeutic agents. Investigators stress the need to determine the optimal timing for treatment with trastuzumab, as well as finding a predictive marker for selecting those who will respond to trastuzumab (Suzuki and Toi, 2007).

High serum levels of cleaved HER2 extracellular domain are associated with poor clinical response in patients with advanced breast cancer. One proposed mechanism for trastuzumab efficacy is that trastuzumab inhibits cleavage of HER2 extracellular domain. Molina et al. (2001) hypothesized that trastuzumab blocks shedding of HER2 extracellular domain by binding near the HER2 cleavage site at the transmembrane domain. Proteolytic cleavage of HER2 results in soluble HER2 extracellular domain and a p95 membrane-bound fragment. Cleavage of HER2 may be harmful in that p95 bound to the membrane is capable of enhanced kinase activity. In medium from HER-2 overexpressing BT-474 and SKBR3 breast cancer cells treated with trastuzumab, immunoblotting with antibody to the HER2 N-terminal showed there was a significant reduction in HER2 extracellular domain in the medium, suggesting that trastuzumab inhibited cleavage of HER2 extracellular domain.

A study found that antibody-dependent cellular cytotoxicity is the mechanism for trastuzumab efficacy rather than downregulation of HER2. Early stage breast cancer patients with HER2 positive tumors received trastuzumab prior to surgery. Approximately half of the patients responded to trastuzumab therapy. HER2-positive IHC staining from tumors removed after trastuzumab treatment was equivalent to staining in the pretreatment biopsies, suggesting that HER2 was not downregulated as a result of trastuzumab treatment. The number of proliferating cells was equivalent in tumors removed after trastuzumab treatment compared to pretreatment biopsies. However, responders showed increased peripheral blood mononuclear cell (PBMC) cytotoxic activity. Patient PBMCs were incubated with radiolabeled high HER2expressing breast cancer cells to determine the degree of cell lysis (Gennari et al., 2004). Although antibody-dependent cellular cytotoxicity is increased in patients who respond to trastuzumab, patients with advanced cancer are immunosuppressed, potentially interfering with this mechanism (Nahta and Esteva, 2007).

A biochemical study supported the conclusion from the Gennari et al. clinical data that trastuzumab does not promote HER2 downregulation. In the HER-2 overexpressing SKBR3 breast cancer cell line, trastuzumab did not downregulate surface HER2. Cells incubated with trastuzumab and analyzed by flow cytometry revealed only a small decline in surface HER2 levels over a 72 hour period. Trastuzumab and HER2 recycled back to the cell surface after endocytosis. After fluorescent-conjugated trastuzumab was incubated with cells, flow cytometry analysis showed initial rapid uptake of trastuzumab, with 85% of the trastuzumab recycled back to the cell surface within 30 minutes. Electron microscopy confirmed the presence of trastuzumab and HER2 in early endosomes and recycling tubulovesicular membranes (Austin et al., 2004). Although data from other researchers showed that the proliferation of SKBR3 cells was inhibited by trastuzumab, the investigators suggested that the mechanism is not by downregulation of HER2. The antibodydependent cellular cytotoxicity mechanism was ruled out because it is unable to account for growth inhibition of a cell line in vitro.

A study by Cuello et al. (2001) suggested that HER2 could be downregulated by trastuzumab in SKBR3 cells if the cells were treated for lengthy periods with trastuzumab. Cells treated with trastuzumab for short incubations showed no decrease in HER2 levels when examined by immunoblotting with anti-HER2. However, after 96 hours of trastuzumab treatment, HER2 was no longer visible. The PI3K downstream pathway, but not the MAPK pathway, was compromised in the cells that underwent HER2 downregulation. Immunoblotting with antibodies to p-Akt and p-MAPK revealed that only Akt activity was decreased. This suggests that in SKBR3 cells, downregulating HER2 is important in blocking the PI3K pathway, but has little effect on the MAPK pathway.

A novel hypothesis for the anti-tumor activity of trastuzumab involves the interaction of HER2 with the non-receptor Src tyrosine kinase. This interaction was uncovered only after many experiments and accompanying inferences. Treatment of SKBR3 cells with trastuzumab for only one hour resulted in the reduction of p-Akt (Nagata et al., 2004). Akt is recruited by PI3K after PI3K binds to an activated ErbB. Akt promotes cell survival by blocking apoptosis, and promotes cell cycle progression (Marmor et al., 2004). The swift reduction of p-Akt in response to trastuzumab could not be attributed to downregulation of HER2 because lengthy treatment for many hours with trastuzumab was necessary to induce downregulation of HER2 (Nagata et al., 2004).

Therefore, Nagata et al. looked in detail at molecules that regulate Akt for fast responses to trastuzumab. The tumor suppressor phosphatase and tensin homolog

69

(PTEN), known to negatively regulate Akt, proved to have dramatically increased activity within 20 minutes of cell exposure to trastuzumab. PTEN translocated from the cytoplasm to the cell membrane, culminating in Akt dephosphorylation. Since Src was known to regulate PTEN, Src activity was studied in response to trastuzumab. In response to trastuzumab, Src had less ability to phosphorylate PTEN, resulting in greater PTEN activity. This reduction in Src kinase activity was traced back to HER2. Data showed that trastuzumab reduced Src binding to HER2, resulting in inhibition of Src kinase activity. The results suggest that MAbs that bind close to transmembrane domains of ErbBs may be effective in blocking ErbB interactions with downstream molecules.

A recent explanation for trastuzumab efficacy involves trastuzumab induction of HER2 tetramers, resulting in conformational changes that inhibit tyrosine phosphorylation. ErbBs typically form dimers. However, formation of tetramers containing EGFR and HER2 is a common feature of cancer cell lines. Furuuchi et al. (2007) hypothesized that two ligand activated ErbB dimers interact through a flexible region of the dimer interface of Domain II to form a tetramer. Treating SKBR3 cells with trastuzumab increased the number of tetrameric complexes, and tyrosine phosphorylation decreased in tetramers compared to dimers.

Relevant to therapeutic applications, mixtures of MAbs to different epitopes on HER2 enhanced the formation of these less active tetrameric complexes (Furuuchi et al., 2007). This result was reminiscent of EGFR data in which multiple anti-EGFR MAbs to different epitopes on EGFR increased the amount and rate of EGFR removal from the cell surface (Friedman et al., 2005). These data support the conclusion that using multiple MAbs to different epitopes on HER2 or EGFR may be a worthwhile strategy for use in cancer patients. Supporting a link between use of multiple MAbs against HER2 and reduction in downstream signaling, after treatment of breast cancer cells with mixtures of MAbs to HER2, immunoblotting revealed that the mitogenic downstream molecule MAPK showed less phosphorylation (Friedman et al., 2005).

Specific genes downregulated by trastuzumab have been identified. This is an important step in identifying the genes regulated by HER2 that play a role in tumorigenesis. Further research is needed in this area since genes regulated by HER2 have not been well characterized. The investigators were particularly interested in how the FAS gene was downregulated by trastuzumab, because FAS is often overexpressed in breast and other cancers (Kumar-Sinha et al., 2003). FAS converts carbohydrates to fatty acids, providing proliferating cells with fatty acid for membrane phospholipid or other functions (Weng et al., 2007).

Human mammary epithelial cells that overexpress HER2 were examined by cDNA microarray to determine the genes regulated by HER2. These genes were narrowed to those that were downregulated by trastuzumab, including FAS. When a FAS promoter-reporter construct was transfected into the cells, increased reporter activity was observed, suggesting that HER2 mediates transcriptional regulation of FAS. Inhibition of FAS increased apoptosis of the cells. Increased PI3K activity that could be inhibited by trastuzumab was observed in the cells. The investigators hypothesized that trastuzumab may result in FAS inhibition by reducing HER2
activation of PI3K (Kumar-Sinha et al., 2003). This experiment suggests that therapeutics that block the HER2 activation of the PI3K pathway could prevent gene transcription that has mitogenic consequences for the cell.

Due to the low response rate to trastuzumab in metastatic breast cancer patients, there has been much interest in understanding what causes this *de novo* trastuzumab resistance (patients who do not respond). Furthermore, trastuzumab treatment-induced resistance occurs in most initial metastatic breast cancer patient responders within one year (Nahta and Esteva, 2007). Mechanisms of trastuzumab resistance are varied. Resistance may be due to HER2 interactions with non-ErbB tyrosine kinase receptors, such as IGF-1R. Resistance may occur due to the loss of tumor suppressors that regulate the same downstream pathways as HER2, such as PTEN. Resistance has been traced to a cleaved population of HER2 that has lost its extracellular domains and cannot bind trastuzumab.

In trastuzumab-resistant SKBR3 breast cancer cells, HER2 dimerizes with IGF-1R, a receptor tyrosine kinase from a different family. In SKBR3 trastuzumabresistant cells, but not in SKBR3 parental cells, HER2 was found associated with IGF-1R in IGF-1R immunoprecipitations. IGF-1 stimulates IGF-1R tyrosine kinase activity resulting in transphosphorylation of HER2. Since trastuzumab does not seem able to prevent the activation of HER2 by IGF-1R, use of IGF-1R targeted therapeutics in conjunction with trastuzumab was suggested for trastuzumab-resistant tumors (Nahta et al., 2005). PTEN status is important in assessing a patient's chance of responding to trastuzumab. One of the problems in HER2-overexpressing breast cancer cells is a cascade in which HER2 activation of Src leads to a loss of PTEN activity that culminates in constitutive Akt activity (Nagata et al., 2004). Loss of the tumor suppressor PTEN has been reported in nearly 50% of breast cancers, and in other cancers. Breast carcinomas analyzed by IHC revealed that patients with low expression of PTEN in their tumors had a significantly lower response rate to trastuzumab than those with PTEN positive tumors. Without PTEN, trastuzumab is unable to stop Akt activation. A lesson from PTEN is that therapeutics targeting only the ErbBs may not be sufficient if other molecular aberrations are present in the tumor.

It was hypothesized that patients who have cleaved HER2 are resistant to trastuzumab because HER2 without its extracellular domain is unable to bind trastuzumab. Mouse tumor xenografts derived from breast cancer cells transfected with full length HER2 responded to trastuzumab. However, tumor xenografts from cells expressing only the membrane bound fragment of HER2 without extracellular domains were trastuzumab resistant (Scaltriti et al., 2007). These data suggest that establishing the form of HER2 in a tumor is important in determining whether MAb treatment will be effective.

Another MAb has been developed against HER2 which binds to a different epitope on HER2 extracellular domains than trastuzumab. The MAb pertuzumab (Omnitarg<sup>TM,</sup> 2C4) binds to Domain II of HER2 in the crystal structure. This is in contrast to trastuzumab which binds to Domain IV. Modeling predicts pertuzumab binding will prevent HER2 from dimerizing with other ErbBs (Franklin et al., 2004). A drawback of pertuzumab is that it does not inhibit cleavage of HER2 extracellular domain, probably because it recognizes an epitope on HER2 that does not block the cleavage site (Molina et al., 2001). Due to the importance of dimerization for ErbB activation, pertuzumab is hoped to be effective in blocking the activation of HER2 and its dimer partners. Probably because the effects of pertuzumab are not limited to HER2 but include reduction of signaling from HER2 dimer partners, pertuzumab has shown promise in tumors that do not overexpress HER2. Pre-clinical studies with pertuzumab have validated the therapeutic strategy of blocking ErbB dimerization sites with MAbs.

In a comparison of pertuzumab and trastuzumab treatment of neuregulinstimulated SKBR3 and MCF-7 breast cancer cells, only pertuzumab blocked the association of HER2 with HER3, based on immunoprecipitation data. The effectiveness of pertuzumab in MCF-7 cells was significant because these cells contain low levels of HER2 (Karunagaran et al., 1996), and therefore are not expected to respond to trastuzumab. Since MCF-7 cells contain moderate levels of HER3 (Levkowitz et al., 1996), pertuzumab may function by disrupting the dimerization of HER2 and HER3. In MCF-7 cells, pertuzumab reduced the tyrosine phosphorylation of HER2 and the level of Akt activation. MAPK phosphorylation was inhibited by pertuzumab, but only slightly by trastuzumab (Agus et al., 2002). Pertuzumab was able to significantly reduce tumor growth in human androgen-independent prostate cancer xenografts that do not overexpress HER2 or respond to trastuzumab. The mechanism of action for pertuzumab in these tumors is unclear. Antibody-dependent cellular cytotoxicity could be ruled out as the pertuzumab mechanism of action. A pertuzumab Fab engineered without the Fc region (therefore unable to recruit natural killer cells) blocked tumor growth as well as the intact antibody. A unique feature of this study was that the investigators chose to analyze the level of ErbB ligands in the xenografts. The tumors showed high expression of the EGFR ligands transforming growth factor  $\alpha$ , HB-EGF, and EGF, measured by PCR analysis (Agus et al., 2002). If EGFR undergoes enhanced stimulation by overexpressed ligands in these tumors, it is possible that pertuzumab disruption of EGFR/HER2 dimers could be significant in reduction of signaling. The results suggest that the contribution of ErbB ligand overexpression to tumorigenesis should be further investigated as a possible therapeutic target.

Due to the non-overlapping functions of trastuzumab and pertuzumab, they have been tried in combination and found to produce a synergistic result. Trypan blue exclusion assay data showed that in HER2-overexpressing BT474 breast cancer cells treated with each MAb singly, there was little cell death, while treatment with both MAbs resulted in a loss of up to 60% of cells. The cell death appeared to occur by apoptosis, based on staining for markers of apoptosis (Nahta et al., 2004).

The mechanism of action for pertuzumab in the BT474 breast cancer cells appeared to be disruption of dimers, determined by immunoprecipitation with antiHER2 followed by immunoblotting for EGFR and HER3. These dimers were disrupted more completely if trastuzumab was also present. Akt phosphorylation was inhibited by the combined MAb treatment. The reason for apoptosis may be due to reduced signaling from the Akt cell survival pathway. In contrast, p-MAPK was not reduced, suggesting that either some ErbB signaling continues through ErbB dimer interactions not involving HER2, or through non-ErbB pathways (Nahta et al., 2004).

Due to the necessity for intravenous dosing of MAbs (Toffoli et al., 2007), difficulties with large-scale production of MAbs, limited penetration of certain tissues by MAbs, and potential for immunological side effects, there has been interest in producing MAb substitutes such as peptide mimics (Park et al., 2000), and RNA aptamers. However, a drawback of these MAb substitutes would be the lack of stimulation of the antibody-dependent cellular cytotoxicity immune response.

An anti-HER2 peptide was developed that binds to the same Domain IV epitope as trastuzumab, but is characterized by hydrophobic contacts not seen in trastuzumab binding. Anti-HER2 peptide was generated from the complementaritydetermining CDR3 loop of the heavy chain of trastuzumab. MTT assay analysis showed that anti-HER2 peptide inhibited cell proliferation similarly to trastuzumab in fibroblast cells transfected with HER2 or in SKBR-3 breast cancer cells that overexpress HER2. Anti-HER2 peptide inhibited progression of human tumor mouse xenografts with the eventual regrowth of the tumor, as seen with trastuzumab (Park et al., 2000). Recently, a strategy of fusing anti-HER2 peptide to streptavidin produced a molecule termed anti-HER2 peptide/streptavidin that had increased binding affinity for HER2 compared to anti-HER2 peptide. Each molecule of streptavidin is linked to one molecule of anti-HER2 peptide. This construct associates as a tetramer such that four copies of anti-HER2 peptide occur in each anti-HER2 peptide/streptavidin molecule. Anti-HER2 peptide/streptavidin showed better inhibition of tumor growth than anti-HER2 peptide. The incorporation of streptavidin into this anti-HER2 peptide provides the additional possibility of binding a biotinylated cytotoxin to the streptavidin (Masuda et al., 2006). This multifaceted therapeutic (containing the properties of a MAb and a cytotoxin) might have a more potent effect on tumor cells than a MAb or peptidomimetic molecule alone.

RNA aptamers have been developed as substitutes for MAbs. Advantages of RNA aptamers over MAbs are similar to those of peptide mimics and include ease of preparation, lack of induction of host immune responses, and smaller size. Investigators chose to develop aptamers against HER3, an ErbB that does not have an existing therapeutic. RNA aptamers that bind to the HER3 extracellular domain were identified by gel mobility-shift assays. One of the aptamers reduced HER2 phosphorylation and inhibited cell proliferation. This aptamer bound to a different site on HER3 than the HER3 ligand neuregulin, but the location of the binding site is not known (Chen et al., 2003).

The anti-HER3 aptamer was used to treat breast cancer cells stimulated with neuregulin. Immunoprecipitation with anti-HER2 followed by immunoblotting with

77

anti-p-tyrosine revealed inhibition of HER2 phosphorylation. This result suggests that in response to the aptamer, HER3 was prevented from interacting with HER2. Data from tetrazolium bromide-based assay showed the aptamer also inhibited cell proliferation by 50% in response to neuregulin. Further work to characterize the binding site of the aptamer may shed more light on its mechanism of action. If further study continues to show that the aptamer is effective, it may inspire more research into anti-HER3 therapeutics, an area that is largely unexplored.

Another approach to blocking ErbB extracellular domain activity involves a natural compound that inhibits dimerization. Theanaphthoquinone (TNQ), a polyphenol compound generated during black tea fermentation, is a natural compound that was found to inhibit dimerization of EGFR and HER2 in breast cancer cells. These data were obtained by immunoprecipitation with anti-HER2 followed by immunoblot with anti-EGFR in breast cancer cells pretreated with TNQ prior to EGF stimulation. Phosphorylation of EGFR and HER2 was also inhibited (Weng et al., 2007).

Detailed information was obtained about the downstream pathways disrupted in response to TNQ. In breast cancer cells stimulated with EGF, TNQ pre-treatment inhibited phosphorylation of MAPK and Akt, inhibited nuclear translocation of steroid regulatory element binding proteins known to stimulate FAS gene expression, and reduced FAS protein levels. TNQ induced cell death in breast cancer cells, based on flow cytometry analysis. The investigators hypothesized that TNQ may prevent EGF binding, but the mechanism whereby TNQ blocks EGFR dimerization with HER2 is not known (Weng et al., 2007). This study supports the prevention of ErbB dimerization as an effective strategy to block receptor signaling.

A different approach to therapeutics targeting the extracellular domain takes advantage of ligand binding, by using ligand to carry a toxin into the cell. EGF is used as a carrier to transport a diphtheria toxin into EGFR-overexpressing cells. Early research established that diphtheria toxin-EGF fusion protein resulted in death of human squamous carcinoma cells in culture. The toxin gained entry into the cell cytosol where it inhibited protein synthesis (Shaw et al., 1991). Diphtheria toxin-EGF is undergoing testing for use in EGFR-overexpressing brain glioblastoma, a tumor that is extremely resistant to existing treatments. Pre-clinical studies with brain glioma cell lines showed that diphtheria toxin-EGF was toxic to the cells because they exhibited reduced [<sup>3</sup>H] thymidine incorporation. This is an ErbB therapeutic that may have use only in the brain because the blood brain barrier may protect the rest of the body from toxic side effects of the diphtheria toxin (Liu et al., 2003).

#### **II.** Tyrosine Kinase Inhibitors

Since the activation of ErbBs culminates in phosphorylation of receptor cytoplasmic tyrosines by the kinase domain, restricting kinase domain activity has been explored as a therapeutic strategy. Tyrosine kinase inhibitors (TKIs) have been developed that bind to ErbB kinase domains. TKIs are small molecules that act intracellularly by competing with ATP for binding to the nucleotide binding site of receptor tyrosine kinases (Rabindran, 2005). A clinical advantage of TKIs is that they may be given orally (Toffoli et al., 2007), in contrast to intravenous administration of MAbs. The anti-EGFR TKIs available for patient use are erlotinib (Tarceva<sup>TM</sup>) which is FDA approved, and gefitinib (Iressa<sup>TM</sup>). Anti-EGFR TKIs have shown limited clinical success. Since the ErbBs can transphosphorylate each other within dimers, TKIs targeting multiple ErbBs are more likely to be successful. Screening of many compounds identified lapatinib as a dual TKI of EGFR and HER2, and CI-1033 as a multiple TKI of EGFR, HER2, and HER4. Whether HER3 can bind TKIs has not been explored, possibly because it is deemed irrelevant due to biochemical data showing HER3 is not capable of intrinsic kinase activity.

Erlotinib and gefitinib were characterized as selective inhibitors of EGFR, and lapatinib as a dual inhibitor of EGFR and HER2. Biochemical data suggested that lapatinib might be more effective clinically. Enzyme inhibition experiments were performed to measure phosphorylation of a peptide substrate in the presence of ATP, TKI, and recombinant ErbB intracellular domain. Results revealed that similar concentrations of the TKIs gefitinib, erlotinib, and lapatinib inhibited EGFR activity. Lapatinib inhibited HER2 activity effectively, but erlotinib and gefitinib required very high concentrations to inhibit HER2 (Wood et al., 2004).

Lapatinib appeared to have potent binding properties that might make it a more effective therapeutic than erlotinib and gefitinib. Lapatinib dissociated from EGFR much more slowly than erlotinib or gefitinib, based on time elapsed until the receptor was able to phosphorylate a product. Lapatinib had a slow off-rate for HER2 as well. There was also slower recovery of tyrosine phosphorylation in cancer cells with lapatinib, compared to erlotinib and gefitinib. While erlotinib, gefitinib, and lapatinib are released from the kinase nucleotide binding site over time and allow recovery of phosphorylation, there was no recovery of kinase activity when TKI CI-1033 was bound. Mass spectrometry showed that CI-1033 makes a covalent bond through a cysteine residue with EGFR that renders it an irreversible inhibitor (Wood et al., 2004).

Structural studies showed that lapatinib blocked the ATP binding cleft of the EGFR kinase domain more effectively than erlotinib. These structural differences could account for the more potent biochemical activity of lapatinib compared to erlotinib. Significant differences were seen in the orientation of the kinase domain in crystal structures of lapatinib bound to EGFR, compared to erlotinib bound to EGFR. The ATP binding cleft appeared more closed when bound to lapatinib. When lapatinib was bound, the C-terminal of EGFR formed a helix that partially blocked the ATP binding cleft. This feature was not seen with bound erlotinib. The activation loop of EGFR bound to erlotinib was similar to that found in active kinases. When lapatinib was bound, the activation loop structure was characteristic of inactive kinases (Wood et al., 2004).

TKIs have shown limited clinical efficacy. The EGFR TKIs have shown some favorable results in EGFR-overexpressing non-small cell lung cancer (NSCLC). In NSCLC, a 9% response rate to erlotinib and small differences in overall survival resulted in FDA approval for erlotinib (Mendelsohn and Baselga, 2006). Due to the broader range of lapatinib's anti-ErbB activity compared to erlotinib, attention has turned to lapatinib in the clinic.

Studies have shown that lapatinib is most effective in cancer patients whose tumors overexpress HER2. In a Phase I trial, heavily pretreated patients who had solid tumors that overexpressed HER2 were treated with lapatinib. Of 59 patients, four trastuzumab-resistant advanced breast cancer patients had a partial response for a median of 5.5 months. Twenty-four patients had stable disease, sometimes for greater than 10 months (Montemurro et al., 2007). In a Phase II trial of advanced breast cancer patients who had failed trastuzumab, lapatinib resulted in partial response or stable disease for some patients. Evaluation of biomarkers showed that patient response to lapatinib was more likely if the tumor was HER2 positive (Montemurro et al., 2007). Results are awaited for large Phase III trials in progress with lapatinib compared to placebo for early stage HER2-overexpressing breast cancer after chemotherapy (Ito et al., 2007).

Whether lapatinib is effective in patients with EGFR-overexpressing tumors is unclear. There was a greater likelihood of survival if patients with EGFRoverexpressing renal cell carcinoma were treated with lapatinib. On the other hand, a Phase II study of head and neck cancer driven by EGFR showed no responders, only stable disease in some patients, in response to lapatinib (Montemurro et al., 2007).

The reason for the limited clinical success of TKIs is under investigation. *In vitro* studies done with TKIs in cancer cell lines show they reduce ErbB phosphorylation and cell proliferation. Lapatinib inhibited the growth of EGFR-overexpressing and HER2-overexpressing human tumor xenografts (Rusnak et al., 2001). However, a recent study showed that TKI reduction of ErbB phosphorylation

was transient. This result was traced to residual HER2 activity during TKI treatment, resulting in transphosphorylation of HER3 (Sergina et al., 2007).

TKI treatment resulted in initial reduction of EGFR and HER2 phosphorylation, and reduction in phosphorylation of downstream targets such as MAPK in breast cancer cells, based on immunoblotting results. However, after a brief reduction, phosphorylation of HER3 and its downstream target Akt recurred, both in cell lines and tumor xenografts. HER3 phosphorylation could be suppressed by anti-HER2 siRNA, indicating that resurgent HER3 phosphorylation was due to incomplete blockade of HER2 activity. These data suggest that for a TKI to be effective, it must irreversibly inactivate HER2. To assess the efficacy of TKIs, the investigators recommend determining the phosphorylation status of HER3 and Akt (Sergina et al., 2007).

Another possible reason for failure of TKIs is that mutated EGFR found in cancer patients exhibits structural differences that influence the response of EGFR to the TKI. A subset of non-small cell lung cancer NSCLC patients, accounting for 10-15% of patients with NSCLC (Dassonville et al., 2007), benefited more from the TKI gefitinib than others. These patients were more likely to have EGFR somatic mutations in the kinase domain. It was hypothesized that these mutations result in longer EGFR activation upon ligand binding, and are therefore more sensitive to the inhibitory effects of the TKI. In contrast, patients with other types of somatic mutations of the EGFR kinase domain were resistant to gefitinib due to substitution of a bulky amino acid in the ATP-binding pocket, which presumably interfered with gefitinib binding (Tortora et al., 2007).

Further research may reveal additional EGFR mutations that modulate response to the TKIs. Only certain regions around the EGFR kinase domain were sequenced in the NSCLC studies (Shelton et al., 2005), leaving other regions unexplored. Knowledge of patient EGFR mutations may assist in making treatment decisions for patients with NSCLC, a deadly cancer in which few patients have responded to TKI treatment in clinical trials (Mendelsohn and Baselga, 2006).

Transient responses to TKIs were due not only to incomplete inhibition of ErbB signaling but also to ErbB cross-talk with non-ErbB pathways. A study analyzing the cause of acquired resistance to lapatinib found that, while lapatinib retained its effectiveness over time in blocking HER2 downstream signaling, it promoted estrogen receptor signaling. Breast cancer cells initially underwent apoptosis in response to lapatinib, but resistant cells began to grow again within three weeks despite continued exposure to lapatinib. When cells resistant to lapatinib were analyzed by DNA microarray, estrogen receptor signaling pathway genes were found to be upregulated. Combining lapatinib with treatment to induce estrogen receptor proteolysis prevented the growth of resistant cells for at least six weeks. Therefore, blocking HER2 and estrogen receptor pathways simultaneously prevented resistance to lapatinib *in vitro* (Xia et al., 2006). These data suggest that ErbB TKIs may need to be combined with therapeutics to additional dysregulated molecules in a tumor to be successful. EGFR TKI treatment failure correlated with the absence of PTEN in glioblastoma tumor tissue (Mellinghoff et al., 2007). The absence of PTEN is also correlated with trastuzumab MAb treatment failure (Nagata et al., 2004). PTEN deficiency may exist prior to TKI treatment or occur over time during treatment. When PTEN is absent, anti-apoptotic Akt signaling becomes constitutive. The investigators suggested that tumors be tested for PTEN status by IHC or by sequencing of PTEN for mutations (Mellinghoff et al., 2007). If PTEN is nonfunctional, TKI treatment may be unsuccessful.

Contributing to the transient effect of TKIs is their inability to downregulate ErbBs. Presumably, the ErbBs remain on the cell surface and begin to signal again once the TKI treatment is removed. There was no reduction in total levels of EGFR in head and neck squamous cell carcinoma cells when exposed to erlotinib, gefitinib, or lapatinib (Wood et al., 2004). Lapatinib did not reduce HER2 protein levels in head and neck squamous cell carcinoma or in breast cancer cells (Rusnak et al., 2001). It has been suggested that when TKIs shut down kinase activity, this blocks inhibitory feedback loops (Mosesson and Yarden, 2004). It is possible that a loss of EGFR phosphorylation prevents recruitment of Cbl and Cbl-mediated receptor downregulation.

The irreversible TKI CI-1033 looks more promising than the reversible TKIs in its ability to induce downregulation of ErbBs. High concentrations of the irreversible TKI CI-1033 enhanced ubiquitylation and downregulation of EGFR in epidermoid cancer cells. Treatment with CI-1033 also resulted in increased ubiquitylation of HER2 and partial downregulation of HER2 in gastric carcinoma cells based on immunoprecipitation /immunoblot data. Viewed by immunofluorescence microscopy, CI-1033 treatment induced aggregation of HER2 into sub-membrane clusters (Citri et al., 2002). How CI-1033 is able to stimulate ubiquitylation and downregulation of EGFR and HER2 will be important to elucidate.

Results from trials with patients selected for ErbB driven tumors treated with the irreversible pan-inhibitor CI-1033 will be an important test for the future of TKIs in the clinic. Disappointing results were obtained from the use of CI-1033 as a single agent in patients who had failed chemotherapy in a phase II advanced ovarian cancer trial. No responses were observed. However, patient tumors were not pre-screened for ErbB overexpression. IHC analysis of ErbB levels in archival tumor tissue from the ovarian cancer patients revealed that none of the tumors overexpressed EGFR or HER2 (Campos et al., 2005). Although this study does not shed light on the effects of CI-1033 against ErbB-overexpressing patient tumors, it underscores that not all cancers are driven by ErbBs. Therefore, use of ErbB therapeutics in tumors driven by other aberrant molecular pathways is not likely to be successful.

#### III. Additional therapeutic strategies

Existing therapeutics seek to terminate ErbB activation and downstream signaling. A different therapeutic strategy focuses on preventing ErbB expression at the protein level. A study in which HER2 expression was turned off with antisense oligonucleotides in cancer cell lines showed cell death by apoptosis. Antisense oligonucleotides bind to complementary mRNA sequences and activate their degradation. When breast cancer cells were treated with the 15 base HER2 antisense oligonucleotide, there was almost complete reduction of HER2 mRNA seen by Northern blot, and significant reduction in protein seen by immunoblot (Roh et al., 2000).

In response to HER2 antisense treatment, cell lines that overexpressed HER2 experienced reduced cell proliferation, as determined by trypan blue exclusion. Flow cytometry results showed that HER2 antisense treatment resulted in accumulation of cells in the G0/G1 gap phase of the cell cycle. Apoptotic cell death was observed by assay of the activity of the apoptosis inducing protease caspase 3 in HER-2 overexpressing breast cancer cells (Roh et al., 2000). When HER2 antisense treatment was injected intraperitoneally into tumors, the size of breast tumor xenografts was reduced (Roh et al., 1999). HER2 antisense treatment could be combined with chemotherapy, producing a synergistic effect that increased apoptosis. The addition of chemotherapy resulted in the disappearance of breast tumor xenografts in some animals (Roh et al., 1999).

The prevention of EGFR expression at the protein level has also been accomplished in cancer cell lines. Similar to the results from preventing HER2 protein expression, apoptosis was also observed in response to blocking EGFR protein expression, as well as synergy with chemotherapy. Efficacy of EGFR RNA interference was tested in a human metastatic colon cancer cell line which has high EGFR expression and resists chemotherapy, like many patients with colon cancer. Small interfering RNA (siRNA) to EGFR transfected into the cells significantly reduced both EGFR mRNA and EGFR protein levels based on PCR and immunoblot data respectively. Flow cytometry data showed increased apoptosis. RNA interference plus chemotherapy was better at inhibiting cell proliferation than either treatment alone, based on tetrazolium salt assay (Wu et al., 2007).

Since regulatory molecules have been discovered for HER2, there has been interest in blocking their ability to stabilize HER2. Hsp90 is an attractive therapeutic target given that HER2 and many signal transduction proteins involved in cancer require Hsp90 for stabilization (Zhang and Burrows, 2004). The antibiotic geldanamycin blocks the ATP binding pocket of Hsp90, preventing the ATPdependent conformational change to mature Hsp90 (Zhang et al., 2007b). In response to geldanamycin, HER2 was degraded, and reduction of cell proliferation was observed. Geldanamycin treatment resulted in the downregulation of HER2 in breast cancer cells, based on flow cytometry data. Electron microscopy showed that HER2 was localized in internal membranes of multivesicular bodies, suggesting that geldanamycin directs HER2 to the lysosomal degradative pathway (Austin et al., 2004). Treatment of growth factor ligand stimulated gastric carcinoma cells with geldanamycin resulted in reduced cell proliferation, analyzed by MTT assay (Citri et al., 2002).

Geldanamycin also showed some success in tumor xenografts, particularly when redesigned in a dimeric format. Lung epithelial carcinoma xenografts treated with geldanamycin exhibited a 36% inhibition of growth. Since Hsp90 is a dimer, a geldanamycin therapeutic was engineered to bind to a dimer. In this therapeutic, two geldanamycin molecules are joined by a flexible linker, with the goal of binding two Hsp90 molecules simultaneously. The geldanamycin dimer was almost two-fold better at inhibiting tumor growth than the geldanamycin monomer (Zhang et al., 2007b).

In light of data showing that Muc4 interacts with and regulates HER2 in cancer cells (Carraway et al., 1999), more research into anti-Muc4 therapeutics is needed. In support of this intervention, use of a mucin inhibitor resulted in reduced phosphorylation of HER3 in HER2-overexpressing stomach signet ring carcinoma cells (Yokoyama et al., 2007).

Another indirect inhibitor of HER2 is INCB3619 which inhibits the metalloprotease that proteolytically cleaves HER2 extracellular domain. Prevention of HER2 extracellular domain cleavage is important because the remaining membrane fragment may have constitutive kinase activity. INCB3619 was administered to a human breast cancer xenograft in mice. It was found to decrease HER2 extracellular domain in plasma and to decrease tumor growth. It also improved the effects of trastuzumab in reducing tumor growth (Yao et al., 2007).

INCB3619 could also inhibit the action of metalloproteases that activate ErbB ligands. This is significant because increased ErbB ligand expression is common in many cancers and in patients who become resistant to MAb or TKI therapies. Cell based assays showed that INCB3619 could inhibit the activation of the ErbB ligands transforming growth factor  $\alpha$ , HB-EGF, amphiregulin, and neuregulin. The mechanism of INCB3619 action is not known. In a head and neck squamous cell

carcinoma xenograft, INCB3619 was more effective as a single agent than chemotherapy, and had a synergistic effect with chemotherapy. IHC showed that phosphorylated Akt expression was decreased in the tumors, and apoptosis was observed by TUNEL staining (Fridman et al., 2007). These data suggest that depletion or inactivation of ErbB ligands could prevent ligand binding to ErbBs, circumventing the need for therapeutic intervention further downstream of receptor activation.

Restoring negative regulatory mechanisms for ErbB downregulation may be a fruitful therapeutic strategy. However, little pre-clinical data is available. It is possible that the ubiquitin ligases Cbl and Nrdp1 have become non-functional in tumors that show ErbB overexpression. Restoration of Cbl and Nrdp1 function could assist in downregulating ErbBs in tumor cells (Sweeney and Carraway, 2004). The EGFR inhibitor LRIG1 gene is often deleted in human cancers, and reduced expression of LRIG1 protein is a feature of some transformed cell lines. Epidermoid tumor xenograft studies showed that sLRIG1 slowed tumor growth (Goldoni et al., 2007).

The negative regulator HER2 extracellular domain fragment known as herstatin was tested in EGFR driven human glioblastoma tumors in a rat model and shown to have inhibitory activity (Staverosky et al., 2005). This suggests that herstatin, an incomplete HER2 extracellular domain, could bind to an EGFR extracellular domain, possibly creating a non-functional EGFR heterodimer that is unable to produce downstream signals.

90

Herstatin was transfected into human glioblastoma cells that overexpress either EGFR or the constitutively active EGFRvIII extracellular domain truncation mutant, and the cells were inoculated into rat brain. Rats injected with the herstatin transfected wild type EGFR cells survived for more than eight weeks, and no tumor formation was seen. Immunoblot revealed a decrease in the anti-apoptotic factor p-Akt in EGF stimulated glioblastoma cells treated with exogenous herstatin. Rats did not survive when injected with the herstatin transfected EGFRvIII extracellular domain truncation mutant cells (Staverosky et al., 2005). These results show that herstatin may be an effective inhibitor of wild type EGFR in tumors but not of EGFR extracellular domain mutant tumors. It is probable that herstatin cannot bind to the mutant because the mutant lacks the extracellular domain.

An overall conclusion about ErbB therapeutic development is that there are few therapeutics that target HER3 and HER4. This is despite HER3's overexpression in multiple types of cancer, and in almost one third of invasive breast carcinomas. Furthermore, HER3 overexpression is associated with poor prognosis (Karamouzis et al., 2007). It is likely that therapeutic development has focused on ErbBs other than HER3 because of HER3's known impaired kinase activity (established by Guy, et al. in 1994). However, it is possible that weak kinase activity by HER3 could drive tumor growth. No TKI has been developed against HER3.

Even if intrinsic HER3 kinase activity is insignificant, the other ErbBs have the ability to transphosphorylate HER3 within a dimer. HER3 can bind PI3K directly and activate Akt, resulting in cell proliferation and preventing apoptosis (Hsieh and Moasser, 2007). If HER3 can be prevented from activation by its neuregulin ligands, or from dimerizing, this may prevent HER3 from binding to other ErbBs and being transphosphorylated by them. Currently, there is one study that investigated an RNA aptamer against HER3 (Chen et al., 2003). The effect of a MAb or other inhibitor generated against HER3 ligand binding sites or dimerization sites is worth studying. Prevention of HER3 expression at the protein level could also be attempted.

Research into therapeutics against HER4 is hampered by contradictory findings regarding the role of HER4 in cancer. TKIs targeting HER4 may be counterproductive due to the possible beneficial effects of HER4 signaling in cancer (Karamouzis et al., 2007). Surprisingly, the presence of HER4 correlates with reduced primary breast tumor progression and improved patient prognosis (Vidal et al., 2007). This finding seems to contradict existing basic research which shows that HER4 has mitogenic consequences for cells. Early ErbB cell biology research established that HER4 heterodimers were mitogenic (Wang et al., 1998). A proteomic study showed that HER4 coupled to the mitogenic MAPK pathway and to the transcription factor STAT5 (Schulze et al., 2005). Supporting its role in cancer, HER4 promotes neurite outgrowth in rat pheochromocytoma cancer cells in response to Ras (Tal-Or et al., 2006). High levels of nuclear HER4 have been shown to correlate with reduced cancer patient survival (Lo and Hung, 2006).

In a recent study, HER4 was tested to determine whether it might inhibit tumor growth. A constitutively active HER4 was generated by making a substitution mutation in the transmembrane domain. When the mutant HER4 was transfected into breast and prostate epithelial cancer cell lines, the level of apoptosis increased, as determined by counting of cells with condensed Hoechst staining (Vidal et al., 2007). While upregulating HER4 seems risky as a therapeutic strategy, further understanding of its correlation with good prognosis in cancer will be valuable. Perhaps HER4 competes with other ErbBs as a dimer partner, producing dimers with less potent signaling capacities.

The near future of ErbB therapeutics lies in treating patients with multiple ErbB therapies, ErbB therapies in conjunction with chemotherapy, or ErbB therapies in conjunction with therapies directed at additional oncogenic molecules. Only in rare cases in which a tumor is driven by one pathway, will a single ErbB-targeted cancer therapeutic sustain long term survival of a patient (Mendelsohn and Baselga, 2006). Most likely, an ErbB therapeutic will be one component of a multifaceted therapeutic program for a patient. Long term advances in ErbB therapeutics will depend on new insights from the study of basic biology of cancer cells, such as ways in which ErbBs are activated, signal to downstream molecules, and are downregulated.

Success with ErbB therapeutics will hinge on the development of appropriate diagnostics to tailor the therapeutic to the patient's molecular profile. It is important for clinical studies to be designed such that tumor tissue prior to therapy and post therapy may be evaluated to identify cancer biomarkers (Mendelsohn and Baselga, 2006). For determination of ErbB involvement in the tumor, potential biomarkers are ErbBs in their inactive and phosphorylated states, cleaved HER2 extracellular domain, and, as suggested by Riese et al. (2007), ErbB cDNA sequenced for

mutations. New diagnostic methods might include MAbs that could bind to and identify the presence of specific ErbB dimer pairs. This may assist in selecting a therapeutic that is optimal against the particular dimer profile present in a tumor.

While the initial clinical studies with ErbB therapeutics have been disappointing, clinical efficacy may be enhanced in the future by refining ErbB therapeutics to effectively inhibit their ErbB targets, offering ErbB therapeutics only to patients whose tumors express ErbB targets, and administering them in combination with therapeutics against other non-ErbB aberrant pathways within the tumor.

### TABLES

## Functional characteristics of ErbB receptors

Activation	EGFR	HER2	HER3	HER4
Events				
Ligand Binding	$\checkmark$	Х	$\checkmark$	$\checkmark$
Dimerization	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$
Kinase	$\checkmark$	$\checkmark$	Х	$\checkmark$
activity				

Table 1-Ligand binding, dimerization, and kinase activity are important events in initiating ErbB signal transduction, and therefore possible intervention points for therapeutic inhibition in cancer treatment. These targets are limited in HER2 because it lacks the ability to bind ligand, and in HER3 because it lacks kinase activity.

## Nuclear localization of ErbB receptors

	Found in	Nuclear	Transcriptional	Mode of
Nuclear ErbB	cancer cells	Localization	target	transport to
		Sequence		nucleus
EGFR	$\checkmark$	$\checkmark$	Cyclin D1	endocytosis
HER2	$\checkmark$	$\checkmark$	Cyclooxygenase	endocytosis
			enzyme -2	
HER3	✓	$\checkmark$	?	?
HER4 (s80)	?	?	Glial fibrillary	?
			acid protein	

Table 2-The nature of ErbB localization to the nucleus is under investigation. Endogenous nuclear ErbBs have been found in cancer cells. Data suggest that ErbBs contain a nuclear localization sequence, and may travel to the nucleus by endocytosis. ErbBs may have transcriptional targets in the nucleus that lead to mitogenic consequences for cells.

### FIGURES

#### Epidermal growth factor receptor structure



region with tyrosine phosphorylation sites. Adapted from (Linggi and Carpenter, 2006).



Figure 2: The epidermal growth factor receptor family is referred to widely as the ErbB family due to the similarity of the epidermal growth factor receptor protein sequence to that of an avian erythroblastosis viral oncogene v-ErbB. Abbreviations used to refer to the receptors are listed above.

# The biological role of ErbBs



Figure 3: The normal role of ErbBs is to control development and homeostasis. ErbB dysregulation promotes cancer.



Figure 4: Inhibition of EGF binding, dimerization, and kinase activity may prevent EGFR tyrosine phosphorylation.

HER2 heterodimers correlate with mitogenicity in cell lines



Figure 5: When HER2 was present in a dimer with other ErbBs, mitogenic consequences were observed in ErbB transfected cells. The HER2/HER3 dimer is prevalent in cancer cell lines.

## HER2 regulatory molecule Hsp90 and Muc4 binding sites



Figure 6: Hsp90 binds to the HER2 kinase domain. Muc4 binds to the HER2 extracellular domain. Therapeutics may modulate regulatory molecules to inhibit HER2 activation

### Crystal structure of the EGFR dimer

Crystal Structure of EGF:EGF Receptor Complex



Figure 1. Crystal Structure of the 2:2 EGF-EGFR Complexes

(A) Ribbon diagram with the approximate two-fold axis oriented vertically. One EGF chain in the 2:2 EGF•EGFR complex is pale green, and the other EGF chain is pink. Domains I, III, III, and IV in one receptor in the dimer are colored yellow, orange, red, and gray, respectively. Domains I, II, III and IV in the other receptor are colored cyan, dark blue, pale blue, and gray, respectively. Most of domain IV is disordered. The disulfide bonds are shown in yellow. The intervening parts that were not assigned are transparent. (B) The top view of (A).

(C) A surface model corresponding to (A).

Figure 7- Crystal structure from (Ogiso et al., 2002) of the EGFR dimer with two bound EGF ligands. The structure identifies the ligand binding site as between Domain I and Domain III on each monomer, and the dimerization site in Domain II MMDB ID:20809

### EGFR dimer extracellular domain inhibition sites



Figure 8: Each EGFR monomer contains extracellular domains I, II, III, and IV. EGF binds to domains I and III within each monomer, enabling domain II of each monomer to contact each other, forming the EGFR homodimer. To prevent dimerization, EGF binding to domains I and III could be inhibited, or contact between domain II of each monomer could be inhibited. These inhibition sites are designated by a red X.

Adapted from a figure in (Dawson et al., 2005).





Figure 9: Potential inhibition sites to prevent EGFR dimerization may include the transmembrane domain (Duneau et al., 2007) and cytoplasmic regions (Clayton et al., 2007) (Zhang et al., 2006) (Yu et al., 2002). These sites are designated by a red X.

ErbB heterodimer and homodimer combinations



Figure 10: All ErbB heterodimer combinations have been observed in transfected cells, and some combinations have been found in cancer cells. An ErbB heterodimer with IGF-1R was observed in breast cancer cells. HER2 and HER3 homodimers are found under certain conditions.

ErbB downstream binding partners that couple to cell proliferation and survival pathways

EGFR	HER2	HER3	HER4	
P-Tyrosines	P-Tyrosines	P-Tyrosines	P-Tyrosines	
Grb2	Grb2	Grb2	Grb2	
Shc	Shc	Shc	Shc	
PLCγ	Abl	PI3K	Abl	
Abl	CrkL	Abl	STAT5	
STAT5		CrkL		

Grb2/Shc $\rightarrow$ MAPK pathway (activates transcription factors that promote mitogenicity)

PLC $\gamma \rightarrow$  activates the transcription factor Protein kinase C

PI3K $\rightarrow$  recruits the Akt kinase which promotes cell cycle progression and cell survival

STAT5  $\rightarrow$  transcription factor that promotes cell proliferation Abl/CrkL $\rightarrow$  promote leukemia

Figure 11: Downstream binding partners couple directly to ErbB phosphorylated tyrosine residues, initiating signal transduction pathways leading to cell proliferation and survival. Well characterized interactions are in blue, while newer findings are in green.
## ErbB negative regulatory molecules



Figure 12: Negative regulators of ErbBs may inhibit tyrosine phosphorylation of receptors, reduce downstream signaling, and promote endocytosis. Negative regulators are often lost in cancers, suggesting that restoration of these molecules may be therapeutic interventions.

## Therapeutic interventions for the ErbB family



Figure 13-Overview of ErbB targeted cancer therapeutics ranging from FDA approved to those still in pre-clinical development. Negative regulatory molecules to be restored are in green italics. Inhibition of signal transduction molecule binding to phosphorylated C-terminal tyrosines is indicated by a red X. Inhibition of ErbB nuclear translocation is indicated by a red X.

## ErbB monoclonal antibody binding sites



Figure 14-Cetuximab binds to Domain III of EGFR (Mendelsohn and Baselga, 2006). Trastuzumab binds to Domain IV of HER2 (Cho et al., 2003). Pertuzumab binds to Domain II of HER2 (Franklin et al., 2004). No therapeutic antibodies have been developed for HER3 or HER4.

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