HER2Δ16: A Tumor-specific Oncogene That Drives Tumorigenesis and Trastuzumab Resistance in HER2+ Breast Cancer

AN ABSTRACT

SUBMITTED ON THE ELEVENTH DAY OF MAY 2015 TO THE

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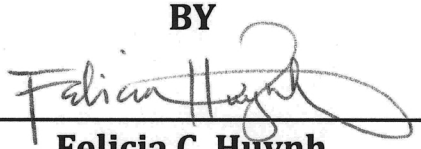
PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL

OF SCIENCE & ENGINEERING OF TULANE UNIVERSITY FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

BY

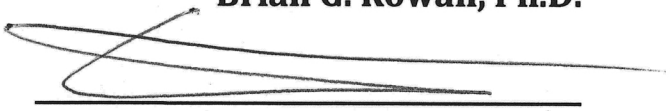


Felicia C. Huynh

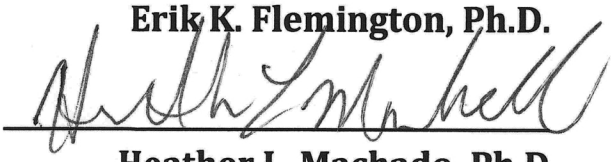
APPROVED:

Frank E. Jones, Ph.D., Advisor

Brian G. Rowan, Ph.D.



Erik K. Flemington, Ph.D.



Heather L. Machado, Ph.D.

ABSTRACT

The oncogenic isoform of HER2, HER2Δ16, is expressed with HER2 in nearly 50% of HER2 positive breast tumors where HER2Δ16 drives metastasis and resistance to multiple therapeutic interventions including tamoxifen and trastuzumab. The research carried out in this dissertation investigated the molecular mechanisms underlying HER2Δ16 activity contributing to primary trastuzumab resistance.

In recent years microRNAs have been shown to influence multiple aspects of tumorigenesis and tumor cell response to therapy. Accordingly, the HER2Δ16 oncogene alters microRNA expression to promote endocrine resistance. With the goal of identifying microRNA suppressors of HER2Δ16 oncogenic activity, we investigated the contribution of altered microRNA expression to HER2Δ16-mediated tumorigenesis and trastuzumab resistance. Using a gene array strategy to compare the microRNA expression profiles of MCF-7 to MCF-7/HER2Δ16 cells, we found that HER2Δ16 suppresses expression of the miR-7 tumor suppressor. Reestablishing miR-7 expression significantly inhibits HER2Δ16-mediated tumor cell proliferation and migration, as well as sensitizes HER2Δ16-expressing cells to trastuzumab treatment. We propose that miR-7 regulated pathways, including EGFR and Src kinase, represent targets for the therapeutic intervention of refractory and metastatic HER2Δ16-driven breast cancer.

Research in the past decade in HER2-positive breast cancer has focused on elucidating the molecular basis of primary and acquired trastuzumab resistance. Our laboratory has shown that critical and clinically important resistance pathways

may be deregulated and only revealed during drug treatment. To identify potential resistance pathways deregulated during trastuzumab treatment, we used a phosphoproteomic approach to profile a subset of phosphorylation events after HER2Δ16-overexpressing cells were treated with trastuzumab. We discovered trastuzumab treatment significantly induced activation of ribosomal p70S6 kinase 1 (p70S6K) and aberrant signaling activity of this kinase is implicated in several disease models due to its role in regulating protein synthesis, cell proliferation and survival. Our data indicates that trastuzumab activates p70S6K to promote prosurvival signaling in breast cancer cells with inherent resistance. We propose that p70S6K can be evaluated in HER2-positive breast cancer patients undergoing trastuzumab treatment as a biomarker to predict therapeutic response.

Overall, our research establishes that HER2Δ16 expression is an important genetic event in HER2 tumorigenesis and drives trastuzumab refractory breast cancer.

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A DISSERTATION

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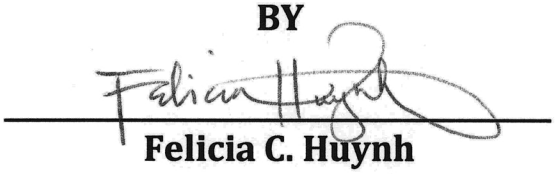

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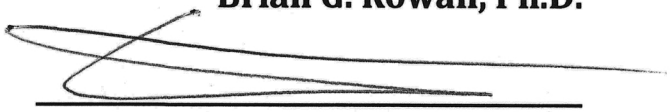

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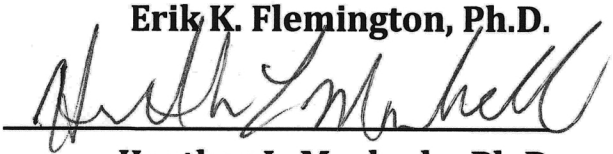

Heather L. Machado, Ph.D.

I dedicate this thesis to my loving parents:

Tho B. Huynh & Duc T. Duong

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LIST OF ABBREVIATIONS

3'-UTR – 3'-untranslated region
4EBP1 – eIF4E-binding protein-1
AI – aromatase inhibitors
Ago – Argonuate protein
ADCC – antibody-dependent cellular cytotoxicity
ALND – axillary lymph node dissection
AR – amphiregulin
BTC – betacellulin
CDK – cyclin-dependent kinase
CHO – Chinese hamster ovary
CTF – carboxy terminal fragment
CRISPR – clustered regularly interspaced short palindromic repeats
DSF – disease free survival
EGFR – epidermal growth factor receptor
Eph receptor – erythropoietin-producing receptors
EPR – epiregulin
ER – estrogen receptor
FAK – focal adhesion kinase
FDA – Food and Drug Administration
FGFR – fibroblast growth factor receptor
FISH – fluorescence *in situ* hybridization
GPCR – G-prtoein-coupled-receptors
GSK3β – glycogen synthase kinase 3β
HB-EGF – heparing-binding EGF
HER2 – human epidermal growth factor receptor 2
HRG – heregulins
IG-F1R – insulin-like growth factor-1 receptor
IHC – immunohistochemistry
ILV – itraluminal vesicles
IR – insulin receptor
JAK – Janus kinase
JM – juxtamembrane domain
KLF4 – krupple-like factor 4
LHRH – luteinizing hormone-releasing hormone

mAb – monoclonal antibody
MAPK – mitogen-activated protein kinase
miRNA/miR – microRNAs
MMP – matrix metalloproteinase
mRNA – messenger RNA
mTORC1 – mammalian target of rapamycin complex *1*
mTORC2 – mammalian target of rapamycin complex *2*
MUC4 – mucin-4
NRG – neuregulins
p70S6K1 – ribosomal p70 S6 kinase 1
PAK1 – p21-activated kinase 1
PDGFR – platelet-derived growth factor receptor
PDK1 – phosphatidylinositoll-dependent kinase 1
PH – pleckstrin-homology
PI3K – phosphatidylinositol-3-kinase
PKB – protein kinase B
PLCγ – phospholipase Cγ
PKC – protein kinase C
PR – progesterone receptor
Pre-miRNA – precursor microRNA
Pri-miRNA – primary microRNA
PTB – phosphotyrosine binding domain
Pyk2 – protein tyrosine kinase 2
RISC – RNA-induced silencing complex
rpS6 – ribosomal S6
SH2 – Src homology 2
SERM – selective estrogen receptor modulator
SLNB – sentinel lymph node biopsy
STAT – Signal Transducer and Activator of Transcription
RTK – receptor tyrosine kinase
RT-PCR – reverse transcription polymerase chain reaction
TGFα – transforming growth factor-α
TM – transmembrane domain
TNBC – triple negative breast cancer
wt-HER2 – wild-type HER2

CHAPTER 1 – INTRODUCTION AND BACKGROUND

1.1 BREAST CANCER

Breast cancer is a disease where malignant cells develop within breast tissue, most often in milk ducts or lobules. It is the most commonly diagnosed cancer in North American women and the second leading cause of cancer related deaths (1). One in eight women will develop breast cancer in her lifetime and one-third of these patients will succumb to the disease. Education of the female population on the importance of self-screening, annual mammograms and the development of new therapeutic strategies has helped to decrease breast cancer mortality rates since the 1990s. Still, about 20 to 30% of patients experience recurrent, metastatic disease that is incurable with a median survival between 2 and 4 years depending on the subtype (2).

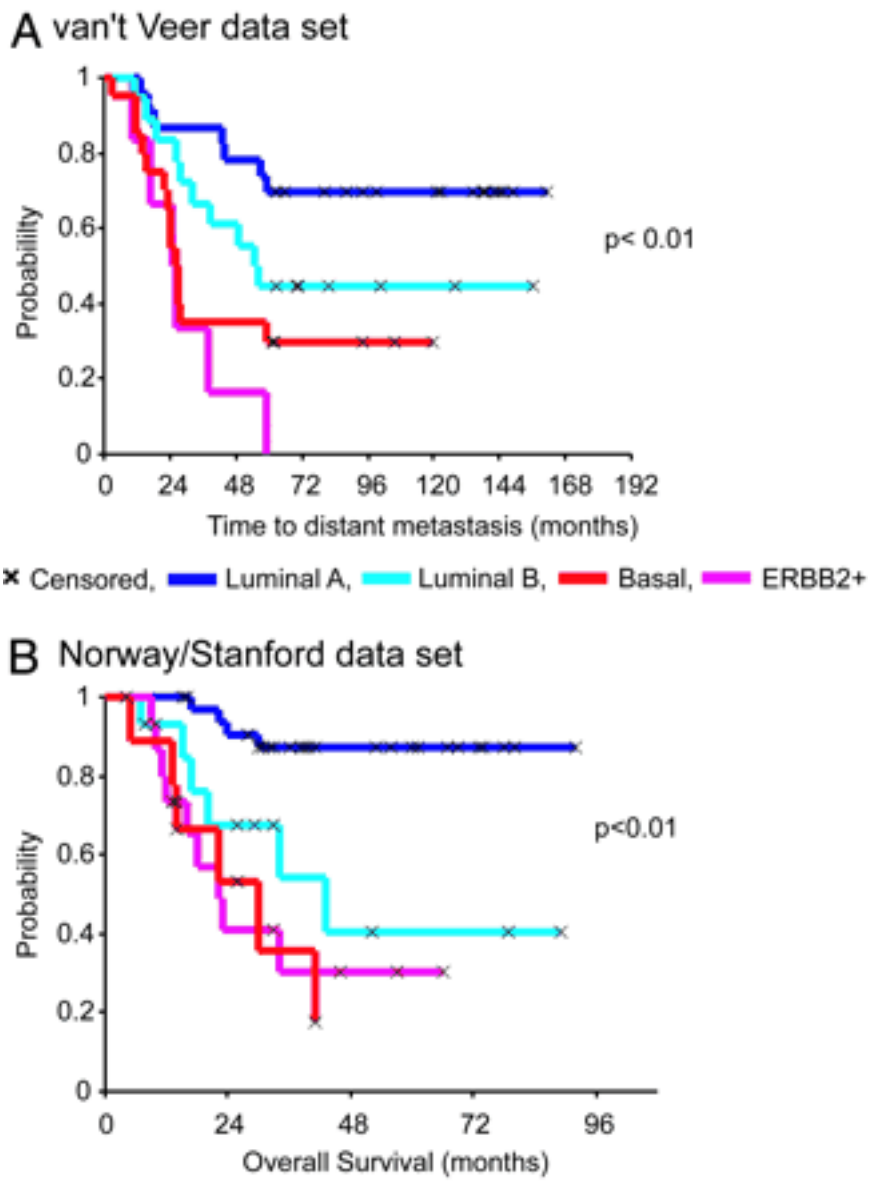
Breast cancer is a heterogeneous disease that displays distinct characteristics at the histological, clinical, genetic and genomic level. Importantly, the complexity of the disease is not fully reflected in the clinical parameters of age, node status, tumor size, histological grade, biomarkers status, which are common factors used to stratify patients for prognostic predictions and treatment plans (3). Over the past

decade, research has focused on the molecular biology of the disease. Perou *et al* analyzed 65 human breast tumor samples using complementary DNA microarrays representing over eight thousand genes with the goal of developing a system for classifying tumors on the basis of their gene expression patterns (4). Hierarchical clustering of the gene expression patterns revealed distinctive 'molecular portraits' of each tumor and the tumors could be classified into subtypes distinguished by pervasive differences in their gene expression patterns. Based on this study, the researchers proposed that breast cancer be categorized into four main molecular subtypes: luminal A, luminal B, basal-like and HER2-enriched (4). Importantly, these molecular subtypes revealed critical differences in incidence (5), survival (4,6-8) and response to treatment (9-14), leading clinicians to reconsider how they diagnose and treat patients (Figure 1.1.1). As scientists continue to expand these genomic studies, we anticipate further sub-classification of breast tumors into new molecular entities that hopefully can predict patient outcome.

Luminal A breast cancer represents 50-60% of total breast cancer subtypes and is characterized by the expression of ER and PR, the absence of HER2 expression, low proliferation rate as measured by Ki67 and low histological grade. Patients in this subgroup have overall good prognosis with significantly lower relapse rate (27.8%), and survival from the time of relapse (median 2.2 years) is longer than the other subtypes (4,6,15). Due to its positive ER status, luminal A tumors are usually treated with endocrine therapy including tamoxifen and the aromatase inhibitor letrozol (16).

Figure 1.1.1 A Kaplan-Meier analysis of disease outcome in two patient cohorts (17). Genomic studies have identified four intrinsic subtypes of breast cancer: luminal A, luminal B, basal-like and HER2-enriched. The molecular differences between the tumor subtypes are accompanied by differences in clinical features including time to development of distant metastasis (A) and overall survival (B) (17). Patients with luminal A type tumors live the longest before developing metastatic disease and have the overall best prognosis. Basal-like and HER2 tumors show much shorter disease-free time intervals and have the worst prognosis.

FIGURE 1.1.1


A van't Veer data set
Probability
1
0.8
0.6
0.4
0.2
0
0 24 48 72 96 120 144 168 192
Time to distant metastasis (months)
p< 0.01
Censored, Luminal A, Luminal B, Basal, ERBB2+
B Norway/Stanford data set
Probability
1
0.8
0.6
0.4
0.2
0
0 24 48 72 96
Overall Survival (months)
p<0.01

The luminal B tumor subtype makes up 10-20% of all breast cancers and expresses ER, PR and variable levels of HER2. Compared to luminal A tumors, women with luminal B tumors are often diagnosed at a younger age and tumors are characterized by higher histological grade, high proliferation rates, larger tumor size, lymph node-positive status and *p53* gene mutations (18,19). Luminal B tumors have worse prognosis and higher risk of relapse compared to luminal A tumors and benefit from neoadjuvant chemotherapy in combination with hormone therapy, despite its expression of ER (20).

The basal-like subtype represents 10-20% of all breast carcinomas. Basal-like tumors can be further divided into two subsets: basal-like (comprising 20% of this subtype) and triple negative breast cancer (TNBC) (comprising the remaining 80%). Basal-like tumors have cell features similar to those of the outer (basal) cells surrounding the mammary ducts and both basal-like and TNBC tumors lack expression of ER, PR and HER (21). Basal-like and TNBC are often used interchangeably in clinic, but they are not equivalent terms. Basal-like/triple-negative tumors tend to impact younger women, predominantly women of African origin, have large tumor size at time of diagnosis, high histological grade and high frequency of lymph node metastasis (22). This tumor subtype is aggressive and has poor prognosis compared to the luminal subtypes. The treatment strategy for basal-like/triple negative tumors involves a combination of surgery, radiation therapy and chemotherapy (21,22).

The HER2-enriched subtype accounts for 15-20% of all breast cancers and is characterized by HER2 gene amplification and protein overexpression and the

absence of ER and PR. HER2 positivity confers more aggressive biological and clinical behavior. Morphologically, HER2 tumors are highly proliferative, 75% have high histological and nuclear grade and more than 40% have *p53* mutation (23). Interestingly, HER2-positive tumors have variable prognoses: one subset with a clearly poor prognosis of 12% 10-year survival compared to 50-55% in the other group (24). HER2-positive tumors exhibit increased chemosensitivity, relative resistance to hormonal agents and a propensity to metastasize to the brain and visceral organs. Recent development of HER2-targeted therapies has significantly improved the outlook for patients with HER2-positive breast cancer.

Resistance to HER2 targeted therapies remains a challenging clinical problem with up to 70% of women with HER2-positive tumors developing recurrent disease. Identifying mechanisms of HER2-mediated therapy resistance is the topic of this thesis.

1.2 ERBB FAMILY

1.2.1 An Overview of Receptor Tyrosine Kinases

HER2 is a member of the EGFR-family of receptor tyrosine kinases which have notoriously complex mechanisms of signaling. Receptor tyrosine kinases (RTKs) are single-pass cell surface receptors that mediate cell-to-cell communication in response to growth factors, cytokines and hormones. RTKs regulate key cellular processes including growth, differentiation, adhesion, motility, metabolism and death (25). Sequencing of the human genome identified 90 tyrosine kinase genes, of which 58 are receptor types (contains a transmembrane domain)

and 32 are non-receptor types (do not contain a transmembrane domain) (26). The RTK family is subdivided into several subfamilies including the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), insulin receptor (IR), erythropoietin-producing (Eph) receptors and others (26). While the Eph subfamily is the largest RTK family, the ERBB family is the best-characterized and general principles of RTK signaling are founded from studies of ERBB signaling.

Signal transduction relies on tyrosine phosphorylation where tyrosine kinases catalyze phosphoryl transfers from ATP to tyrosine residues in a protein substrate. RTKs are activated by cognate ligand binding, which induces lateral receptor dimerization and cross phosphorylation of specific tyrosine residues in the cytoplasmic tail by its partner receptor (27). The phosphorylated residues serve as docking sites for adaptor or effector proteins, which in turn activate multiple downstream signaling pathways (27). Importantly, RTKs as monomers are inactive and dimerization serves as a regulator of their activity.

Due to their roles as growth factor receptors, tyrosine kinases have been demonstrated to play significant roles in the development of many disease states, including cancer (28). Recent advances in proteomics and functional genomics technology has expanded RTK signaling research from the individual components to system-wide analyses of entire cascades to provide a global view of tyrosine phosphorylation events in tumor samples and the effects of kinase inhibitors on cellular signaling networks.

1.2.2 The EGFR or ERBB Family

The ERBB (also known as HER or EGFR) subfamily of RTKs includes ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). ERBB members play essential roles in cardiac and nervous system development, as well as regulate cell cycle progression, survival and differentiation in mammary epithelial cells during puberty and pregnancy (29). In humans, insufficient ERBB signaling is associated with the development of neurodegenerative diseases such as multiple sclerosis and Alzheimer's Disease (30). Excessive ERBB signaling is implicated in multiple human pathologies; the contributions of EGFR and HER2 overexpression and activation are well characterized in breast cancer (31,32).

All four receptors share the same basic structure: an extracellular ligand binding domain, a hydrophobic transmembrane-spanning domain and an intracellular tyrosine kinase domain responsible for receptor autophosphorylation and tyrosine phosphorylation of RTK substrates (Figure 1.2.1) (32). The extracellular region is glycosylated and consists of four sub-domains: sub-domains I and III adopt a β-helical structure and mediate ligand binding in a bivalent manner, whereas the cysteine-rich domains II and IV participate in receptor dimerization (33). The transmembrane (TM) domain contributes to stabilization of full-length receptor dimers and to maintaining a signaling-competent structure (34). The intracellular domain is distinguished by three regions: the juxtamembrane (JM) domain, the non-catalytic domain and the highly conserved tyrosine kinase domain. Phosphorylation of the tyrosine residues in the JM domain is essential for ligand-dependent kinase activity and facilitates the interaction between the catalytic kinase

FIGURE 1.2.1

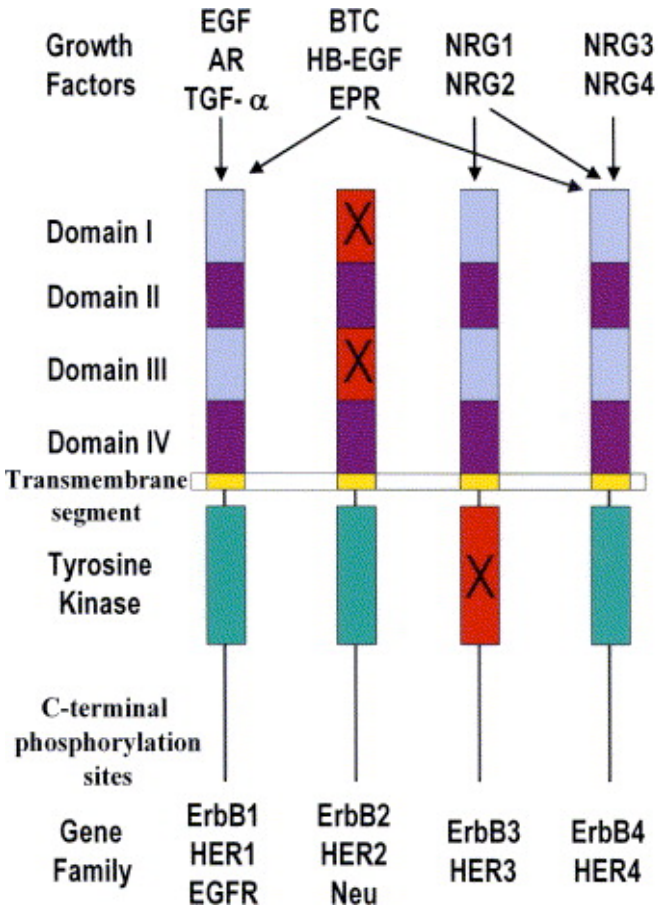


Figure 1.2.1 Epidermal growth factor family of receptors and ligands. The topology of the receptor proteins is indicated. The inactive ligand-binding domains of HER2 and the inactive kinase domain of HER3 are denoted with an X (35).

domain with their substrate tyrosine residues (36). The non-catalytic carboxy-terminal tail harbors the autophosphorylation sites required for the recruitment of adaptor proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, as well as the motifs necessary for receptor internalization and degradation (34). The tyrosine kinase domain functions to activate various downstream signaling pathways by phosphorylation of other tyrosine kinases and associated signaling molecules to produce a physiological outcome (34). In contrast to the other ERBB family members, HER3 contains substitutions of critical amino acids within the tyrosine kinase domain and thus lacks kinase activity (37). Nonetheless, the kinase-defective receptor is transphosphorylated at tyrosine residues when heterodimerized with one of its family members and is capable of recruiting phosphatidylinositol-3-kinases (PI3K) to six distinct sites and Src-homology-2 (Shc) containing to one site (38).

ERBB receptors are activated by high-affinity ligands belonging to the epidermal growth factor (EGF) family of peptide growth factors and possess an EGF-like domain (39). These peptides are produced as transmembrane precursors and are proteolytically processed to release soluble forms that function in autocrine and paracrine signaling (40,41). The soluble forms of the EGF peptides share a domain homology composed of 50 amino acids and feature a characteristic six cysteine residues that form three intramolecular disulfide-bonded loops essential for ERBB-family receptor binding and activation (31). The EGF family peptides can be divided into three distinct functional groups based on binding specificity to each ERBB receptor. Group one consists of EGF, transforming growth factor-α (TGFα) and

amphiregulin (AR), which all bind specifically to EGFR. Group two includes betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which exhibit dual specificity to EGFR and HER4. Neuregulins (NRG, also known as heregulins (HRG)) 1 to 4 comprise the third ligand group and consists of more than 15 isoforms. NRG-1 and NRG-2 both bind to HER3 and HER4, whereas NRG-3 and NRG-4 binds to HER4 but not HER3 (42). Despite the numerous ligands identified for EGFR, HER3 and HER4, no direct ligand has been discovered for HER2. Instead, HER2 is the preferred heterodimerization partner for the other ERBB receptors and plays a role in the potentiation of ERBB receptor signaling (43-45).

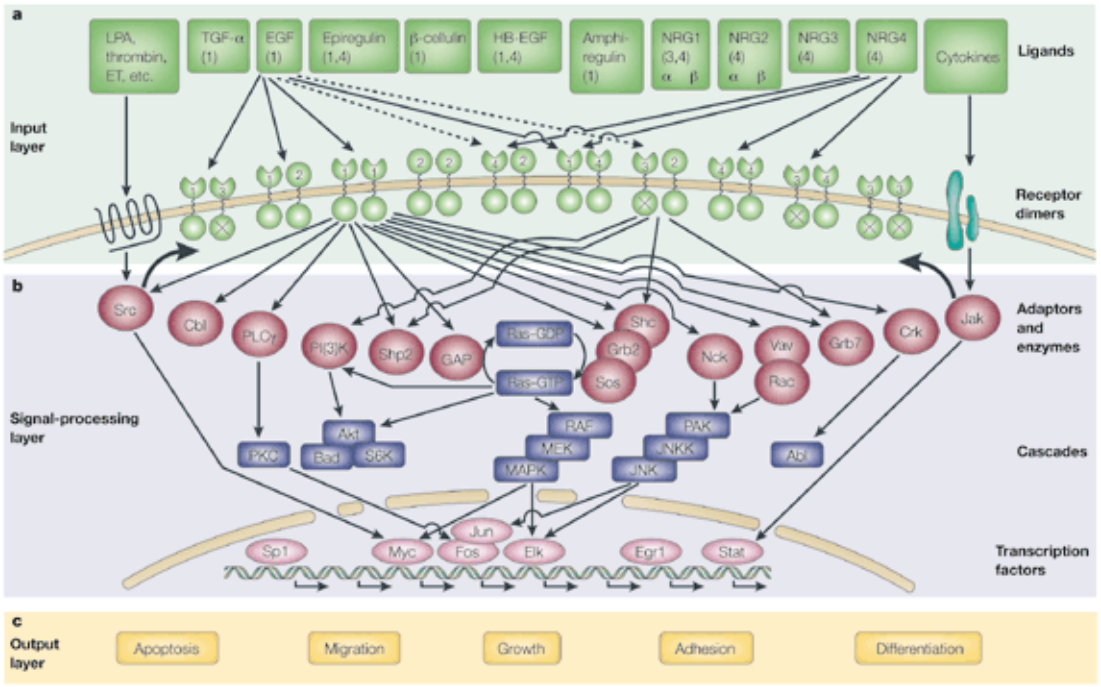
1.2.3 A Layered ERBB Signaling Network

Due to extensive receptor-receptor interactions, the ERBB family composes a signaling network with enormous potential for highly diverse biological messaging. The complexity of the ERBB signaling network can be divided into three layers as summarized in Figure 1.2.2.

The Input Layer: This layer involves the cross-interaction between ligands and the ERBB receptors. Complexity at this layer is derived from the multiplicity of the ERBB ligands and their distinct biochemical properties. The bivalent nature of ERBB ligands determines how receptors are paired, thus influencing activation of signaling pathways (46). Experiments using a recombinant chimeric EGF/NRG-1 ligand revealed that the amino acid sequence in the N-terminus of the ligand determines binding to the primary ERBB receptor while the C-terminus recruits the partner receptor (47). A second biochemical property of ERBB ligands is their differential binding affinity to the receptors. Experiments using virally encoded,

Figure 1.2.2 Layers of the ERBB signaling network (48). The multi-layered signal transduction network is highly complex allowing for extensive signaling diversity. a) The input layer involves the cross-interaction between over 30 ligands and ten dimeric ERBB receptor combinations. HER2 is an orphan receptor with no known ligands. b) Following receptor dimerization, activation of the signal transduction cascade can involve adaptor proteins, enzymes, second messenger and transcription factors. c) The cellular responses, which include apoptosis, proliferation, differentiation, cell motility and survival, depend on the signaling pathways induced, which are determined by receptor pairing and ligand identity.

FIGURE 1.2.2


a
LPA, thrombin, ET, etc.
TGF-α (1)
EGF (1)
Epiregulin (1,4)
β-cellulin (1)
HB-EGF (1,4)
Amphi-regulin (1)
NRG1 (3,4) α β
NRG2 (4) α β
NRG3 (4)
NRG4 (4)
Cytokines
Ligands
Input layer
Receptor dimers
b
Src
Cbl
PLCγ
PI(3)K
Shp2
GAP
Ras-GDP
Ras-GTP
Shc
Grb2
Sos
Nck
Vav
Rac
Grb7
Crk
Jak
Adaptors and enzymes
Signal-processing layer
PKC
Akt
Bad
S6K
RAF
MEK
MAPK
PAK
JNKK
JNK
Abl
Cascades
Jun
Sp1
Myc
Fos
Elk
Egr1
Stat
Transcription factors
c
Output layer
Apoptosis
Migration
Growth
Adhesion
Differentiation

low-affinity ERBB ligands results in the disruption of normal receptor down-regulation and degradation (49). This suggests that low-affinity ligands may be more potent signal inducers compared to their high-affinity counterparts. A third factor of ERBB ligands is the pH stability of the ligand-receptor interaction, which impacts receptor trafficking. The EGF-EGFR interaction is relatively pH resistant and directs EGFR to the lysosome, whereas TGFα and NRG-1 readily dissociate from their respective receptors at endosomal pH, resulting in receptor recycling (50). Thus, the pH stability of ligand binding variably controls the rate of receptor endocytosis, endocytic trafficking and receptor recycling to diversify signal strength and duration.

It is important to note that ligand binding is not always required for receptor dimerization and activation. EGFR has been shown to spontaneously dimerize independently of ligand binding and these dimers were primed for ligand binding and signaling (51). Additionally, while HER2 has no known ligand, it adopts a configuration where its dimerization arm is exposed allowing HER2 homodimerization and kinase activation to occur, particularly when the receptor is overexpressed (52,53).

While ERBB ligands can mediate homo- and heterodimer receptor activation, heterodimerization provides more diverse and potent signaling. Since each activated ERBB receptor displays a distinct pattern of C-terminal autophosphorylation sites, heterodimerization of receptors increases the number of available phosphotyrosine residues allowing for signal diversification through recruitment of multiple adaptor proteins. Interestingly, HER2-containing

heterodimers exhibit the most potent signaling activity by increasing the affinity and specificity of its partner receptor to its ligand, decrease the rate of ligand-receptor dissociation, as well as, receptor endocytosis and increase the frequency of receptor recycling to the cell surface (54,55). HER2 also recruits a larger subset of phosphotyrosine-binding proteins compared the other ERBB receptors.

There are also positive and negative feedback circuits that act on the input layer. Activation of the ERBB network triggers transcription of multiple ERBB ligands including TGFα and HB-EGF, which then contribute to ERBB signaling in an autocrine and paracrine manner, creating a positive feedback circuit (56). Activation of G-protein-coupled-receptors (GPCR) can also participate in autocrine activation of ERBB signaling by initiating cleavage of HB-EGF to its mature form (57).

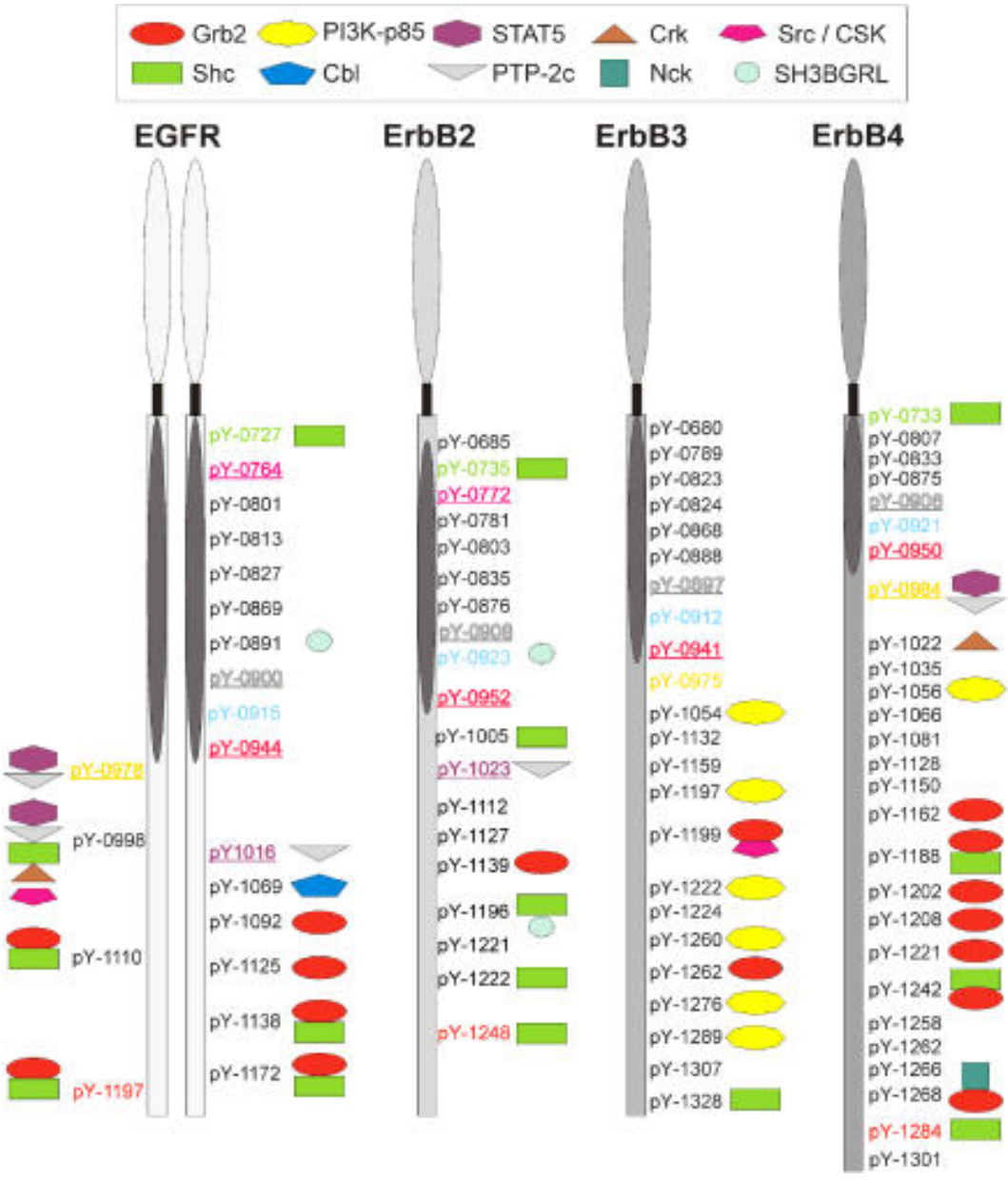
The major negative feedback loop is endocytosis. Active ligand-receptor complexes at the cell membrane undergo invagination in clathrin-coated pits, which become endocytic vesicles. These vesicles fuse with early endosomes where the receptor is sorted for recycling or to intraluminal vesicles (ILVs) destined for lysosomal degradation (58). This model of RTK endocytosis is based on the studies focused on EGFR trafficking, which is rapidly internalized and degraded following activation (59). Interestingly, HER2 avoids downregulation by using a low basal rate of internalization (mechanism unknown) or efficient recycling of endocytosed HER2 back to the plasma membrane (59,60). HER3 and HER4 are also endocytosis impaired, and instead are ubiquitinated and degraded by the lysosome and proteasome (61).

Signal-Processing Layer: This layer primarily involves the wide array of adaptor and effector proteins that are recruited to specific phosphorylated C-terminal tyrosine residues in response to ligand induced receptor dimerization and activation (34). These include the adaptor proteins Shc, Crk, Grb2, Grb7 and Gab1, the kinases Src, Chk and PI3K (via the p85 regulatory subunit), and the protein tyrosine phosphatases SHP1 and SHP2 (34). Using high-throughput technology and quantitative proteomic approaches to simultaneously analyze the 89 potential tyrosine-phosphorylation sites of the four ERBB receptors enabled mapping of effector proteins that couple to specific ERBB phosphotyrosine residues (40,62). Twenty-five of the 89 investigated tyrosine residues showed a single interaction partner and 13 additional tyrosine residues had more than one interacting protein (62). Over half (49 of 89) of the tyrosine residues had no interaction partner and are located in and around the kinase domain.

ERBB receptors signal through several well-known signaling cascades such as the Ras-mitogen-activated protein kinase (Ras-MAPK) pathway, phosphatidylinositol 3' kinase-protein kinase B (PI3K-PKB/Akt) pathway, phospholipase C-protein kinase Cγ (PLCγ-PKC) pathway and Signal Transducer and Activator of Transcription (STAT) signaling (27,28). All ERBB ligands and receptors couple to activate the Ras-MAPK pathway, either directly through Grb2 or indirectly via the Shc adaptor (Figure 1.2.3). The PI3K-Akt pathway is also downstream of active ERBB dimers, but the potency and kinetics of activation differ since PI3K activation couples directly with HER3 and HER4 and indirectly with EGFR and HER2. HER3 contains six binding sites for p85 in its C-terminus and thus is the most

Figure 1.2.3 A summary of phosphotyrosine residues and interaction partners of the different members of the ERBB receptor family (62). The interactions between scaffolding and adaptor proteins with specific tyrosine residues of the ERBB receptors are central events in cell signal transduction. All known cytosolic residues of the ERBB receptors are shown and the kinase domain is designed as an oval. Underlined and colored tyrosine residues indicate identical sequence regions between the different receptors. The majority of interaction partners bind to tyrosine residues at the C-terminal tail outside of the kinase domain. EGFR has the most interaction partners and the highest percentage of tyrosine residues that have more than one binding partner. HER2 has the fewest binding partners with Shc as the most common adaptor protein. HER3 is characterized by six binding sites for PI3K. HER4 has diverse interaction partners, with several binding sites for Grb2.

FIGURE 1.2.3


Grb2
PI3K-p85
STAT5
Crk
Src / CSK
Shc
Cbl
PTP-2c
Nck
SH3BGRL
EGFR
ErbB2
ErbB3
ErbB4
pY-0727
pY-0764
pY-0801
pY-0813
pY-0827
pY-0869
pY-0891
pY-0900
pY-0915
pY-0944
pY-0978
pY-0998
pY1016
pY-1069
pY-1092
pY-1110
pY-1125
pY-1138
pY-1172
pY-1197
pY-0685
pY-0735
pY-0772
pY-0781
pY-0803
pY-0835
pY-0876
pY-0908
pY-0923
pY-0952
pY-1005
pY-1023
pY-1112
pY-1127
pY-1139
pY-1196
pY-1221
pY-1222
pY-1248
pY-0680
pY-0789
pY-0823
pY-0824
pY-0868
pY-0888
pY-0897
pY-0912
pY-0941
pY-0975
pY-1054
pY-1132
pY-1159
pY-1197
pY-1199
pY-1222
pY-1224
pY-1260
pY-1262
pY-1276
pY-1289
pY-1307
pY-1328
pY-0733
pY-0807
pY-0833
pY-0875
pY-0908
pY-0921
pY-0950
pY-0984
pY-1022
pY-1035
pY-1056
pY-1066
pY-1081
pY-1128
pY-1150
pY-1162
pY-1188
pY-1202
pY-1208
pY-1221
pY-1242
pY-1258
pY-1262
pY-1266
pY-1268
pY-1284
pY-1301

efficient activator of PI3K (63). The SH2 domain of PLCγ mediates its binding to activated EGFR and HER2 receptors and becomes activated by subsequent phosphorylation by the RTKs (64).

EGFR is also known to directly phosphorylate STATs 1, 3 and 5. Once phosphorylated, these transcription factors dimerize through phosphotyrosine-SH2 domain interactions and translocate to the nucleus where they activate gene transcription involved in cell proliferation (65). Furthermore, proteolytic fragments of EGFR, HER3 and HER4 have been found in the nucleus where they may function as transcription factors (66).

The ERBB network can also be activated in a growth factor independent manner by integrating signals from cellular stimuli such as hormones, neurotransmitters, lymphokines and stress signals (67). Phosphorylation of C-terminal residues on ERBB receptors by non-receptor tyrosine kinases such as focal adhesion kinase (FAK), Src, protein tyrosine kinase 2 (Pyk2) or Janus kinases (JAKs) can enable the recruitment of effector molecules to affect signal activity or endocytic transport (67). These interconnections between the ERBB receptors to other signaling modules help to coordinate cellular responses to extracellular stimuli.

ERBB receptor trafficking also plays a role in signal processing. For example, EGFR interacts with Cbl, an E3 ubiquitin ligase that tags the receptor with ubiquitin and its homodimers become rapidly internalized and eventually degraded, resulting in signal suppression (38,48). Since the other ERBB receptors do not bind Cbl (HER3) or do so less effectively (HER2, HER4), they are endocytosis impaired and are more often recycled to the cell surface (59,68). Receptor pairing also determines

degradation: EGFR homodimers are targeted primarily to the lysosome while HER3 molecules are constitutively recycled as heterodimerization with HER2 decreases the rate of HER3 endocytosis and increases recycling of its partners (69).

Taken together, the complexity of the ERBB signaling network arises from ligands with distinct binding properties, multiplicity of scaffold and adaptor proteins and homo- and hetero- oligomerization of the receptors to couple specific downstream pathways with differing efficiencies, thus allowing for greater signaling possibilities.

The Output Layer: The physiological response of ERBB signaling is cell growth, differentiation, migration, adhesion and apoptosis, all of which are dependent on the cellular context, ligand identity and ERBB dimer pairing (48).

1.2.4 ERBB Receptors in Development

The importance of ERBB signaling in development has been demonstrated by studies on genetically modified mice. Homozygous null mutations of each individual ERBB receptor lead to a lethal phenotype during early development. The heart is the first organ to form during development since it is required to support the rapidly growing embryo. ERBB signaling is critical for the normal development and function of the heart (40). During mid-gestation, HER2-HER4 signaling is involved in regulating cardiac trabeculae, a finger-like extension of the ventricular mycocardium, and maintenance of cardiomyocytes. HER2 and HER4 knockout mice die at E10.5 due to trabeculae defects in the heart, in addition to observed defects in the hindbrain, sensory ganglia, and motor neurons (70,71). Myocardial re-expression of HER2 using a transgene can rescue heart development in HER2 null

mice, demonstrating the role of HER2 in cardiac development (72). HER2-HER3 signaling is responsible for cardiac cushion formation and EGFR signaling regulates cardiac valve remodeling following cardiac cushion formation. HER3-deficient mice survive to E13.5 and suffer from abnormal heart valve formation but have normal trabeculation. These animals also display a broad neural crest defect and lack Schwann cell precursors (73,74). Depending on the host, loss of EGFR results in embryonic or perinatal lethality with mice exhibiting abnormalities in the brain, skin, lung and gastrointestinal tract (75-77).

Importantly, ERBB heterodimers signaling play a significant role in various developmental processes that cannot be performed by homodimers. For example, HER2-HER4 heterodimer signaling is critical in myocardium development. In HER2-null mice, NRG-1-induced HER4 dimers cannot substitute the function of the HER2-HER4 heterodimer to achieve proper cardiac development (78). Ligand-induced HER2-HER3 heterodimer signaling is crucial in nervous system development, where mice lacking HER2, HER3 or NRG-1 have a severely underdeveloped sympathetic ganglion chain, likely due to defective migration of neural progenitors from the neural crest (79). ERBB receptor cooperation is also imperative in the hypothalamus. Before puberty commences, NRG-mediated activation of HER2 and HER4 is required for the release of prostaglandin E2, which then controls the secretion of luteinizing hormone-releasing hormone (LHRH) from specific neuroendocrine sites. LHRH is responsible for sexual development and adult reproductive function. Loss of HER2 function prevents LHRH secretion despite intact HER4 function and presence of NRG, resulting in the delay of puberty (80).

Thus, ERBB heterodimer signaling is central to developmental processes and cannot be substituted by signal transduction from ERBB homodimers.

The mammary gland is an unusual organ in that most of its development and onset of full functional capacity occurs at puberty, stimulated by steroid hormones. The contribution of the ERBB receptors in mammary gland development is during puberty, pregnancy and lactation. EGFR-HER2 signaling promotes ductal outgrowth into the fat pads during puberty (81). During breast maturation, HER2 and HER3 regulate ductal branching density and morphology and signal primarily through the PI3K pathway (82). During pregnancy, HER2 and HER4 function in alveolar and lobuloalveolar differentiation, respectively, and heterodimer cooperation of HER2, HER3 and HER4 are required for lactation (40,81).

1.2.5 ERBB Receptors in Breast Cancer

Aberrant ERBB signaling has been observed in a variety of human cancers, where ERBB receptors can promote multiple properties of neoplastic cells including proliferation, migration, angiogenesis, stromal invasion and survival. Hyperactive signaling can occur via an autocrine secretory loop involving the overproduction of ligands, gene amplification and receptor overexpression or constitutive kinase activation. All components of the ERBB signaling network including the ligands, receptors and adaptor proteins are implicated in the development and progression of cancer. The following is a brief review of the role of each ERBB family member in breast cancer.

EGFR: EGFR was discovered by Stanley Cohen and his team at Vanderbilt University in 1978 (83) and was the first tyrosine kinase to be directly linked with human

cancer. EGFR is an oncogene with a greatly varied overexpression rate (14-91%) depending on the method of assessment, occurring as a result of increased receptor synthesis (84,85). Additionally, it seems that EGFR overexpression is a late stage event in breast cancer development, predominantly occurring in invasive ductal carcinomas (86). Numerous studies have reported that elevated EGFR expression is a poor prognostic factor in breast cancer, correlates with shorter disease-free and overall survival and failure to respond to endocrine therapy (84,87-89). Higher EGFR expression was observed patients with nodal or distant metastases compared to those without metastases (90). Furthermore, expression of the estrogen receptor (ER), a positive prognostic marker in breast cancer, is inversely correlated to EGFR expression (88). Mutations of EGFR may also contribute to breast cancer development. The most common mutation, EGFRvIII, results from intragene rearrangement or mRNA alternative splicing and contains an 801-bp in-frame deletion that lacks exons 2-7, impacting a large portion of the extracellular domain. This mutant receptor is constitutively active in the absence of ligands (91,92). Overproduction of the EGFR ligand TGF-α can promote autocrine signaling to enhance transformation and lead to independent tumor cell growth (93,94). EGFR has also been implicated as a key player in the mitogenic and motility effects mediated by the HGF-c-MET signaling axis in breast cancer. Increased expression of HGF and/or c-MET is observed in tumor progression and each is independently associated with poor prognosis. There is evidence of cross-talk between these RTKs in several tumor types where HGF is able to activate EGFR and conversely, EGFR ligands can activate c-MET via intracellular signaling pathways (95-97). The

identification of EGFR as an oncogene has led to the development of therapeutic strategies to disengage EGFR signaling using ligand-based strategies, monoclonal antibodies and tyrosine kinase inhibitors (98).

ERBB2/HER2: HER2 gene amplification and protein overexpression occurs in 20-30% of breast cancers and is involved in breast cancer development, progression and response to therapy (54,99-102). HER2 was discovered in the early 1980s and found to be closely related to EGFR (103). Sometimes referred to as *neu*, a constitutively active form of HER2 first identified in rodent glioblastoma (103). It is important to distinguish that the oncogenic properties of *neu* observed in the early rat studies involved an activating mutation in the transmembrane domain, whereas HER2 is oncogenic in humans in the absence of mutations (104,105). HER2 is commonly, and inaccurately, referred to as HER2/*neu,* in deference to the work of the discovering scientists.

The initial breast cancer study by Slamon et al. in 1987 linked HER2 to adverse prognosis. Additional studies confirmed that HER2 status was an independent predictor of time to disease relapse and overall survival in node-positive breast cancer patients (101,106-108). HER2 overexpression is an early event in breast cancer development, occurring during the *in situ* stage and is thought to remain fixed for the duration of the progression of the invasive tumor. HER2-positive tumors are poorly differentiated high grade tumors, have high percentage of S-phase cells, aneuploidy and lack ER expression (106).

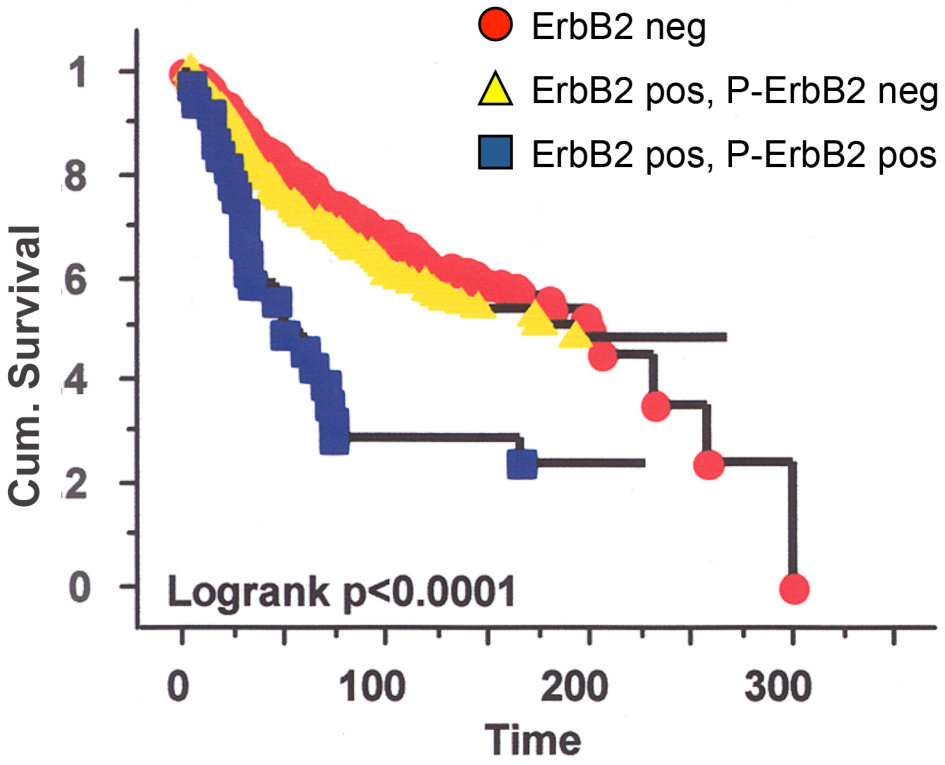
Consistent with its role in breast cancer, transgenic mouse models overexpressing activated HER2/*neu* promotes malignant transformation and tumor

formation in the mouse mammary gland with lung metastasis (109-113). In cell culture, ectopic expression of HER2 can result in phosphorylation and activation of the receptor, even in the absence of activating ligands (54,104,114). Given these observations, the number of activated (phosphorylated) receptors may be more biologically relevant than the number of total receptors present. To address this question, Thor *et al.* performed a retrospective analysis of 816 primary breast cancer cases to determine if phosphorylated HER2 (P-HER2) provided more significant or additional prognostic marker data for breast cancer patients (115). Using correlation coefficients, HER2 overexpression and P-HER2 positivity were associated with younger patients, higher tumor grade, larger tumor size, ER negativity and p53 immunopositivity. Of the 96 cases that showed strong HER2 staining (≥ 80% cells stained for HER2), 35 (36%) cases showed P-HER2 staining, indicating that high HER2 expression cannot be reliably used to determine P-HER2 positivity. Interestingly, the authors found that HER2-negative patients had marginally better survival than HER2-positive/P-HER2-negative patients (Figure 1.2.4). Whereas patients with P-HER2 expression had the worst overall survival indicating that HER2 expression alone fails to predict patient outcome.

Coexpression of EGFR and HER2 is known to occur in at least 33% of breast tumors and EGFR/HER2 heterodimers have been shown to increase the metastatic potential of breast cancer cell lines and promote endocrine resistance in ER-positive breast cancer cell lines (89,116,117). In a retrospective study of 807 breast cancer patients, immunohistochemical assays for HER2, P-HER2 and EGFR were performed and results were correlated back to the clinical outcome (118). The study found that

Figure 1.2.4 Kaplan-Meier survival curves for HER2 and phosphorylated-HER2 (115). Disease free survival (DSF) is defined as diagnosis date to recurrence date (referred to as cumulative survival in this graph). In this study, HER2 negative patients survive only marginally better than the HER2-positive/P-HER2-negative patients. The HER2-positive/P-HER2-positive patients have the worst survival rates. This data suggests that P-HER2 expression can be used to stratify HER2-positive patients and identify patients with the worst prognosis, thereby aiding in clinical management and therapeutic decisions for the patient.

Figure 1.2.4


ErbB2 neg
ErbB2 pos, P-ErbB2 neg
ErbB2 pos, P-ErbB2 pos
Cum. Survival
1
8
6
4
2
0
Logrank p<0.0001
0
100
200
300
Time

P-HER2 expression was associated with lymph node metastasis, younger patient age at diagnosis, larger tumor size, higher histologic tumor grade, ER negativity and higher mitotic and apoptotic indices. Similar associations were observed for samples coexpressing HER2 and EGFR. Survival analysis showed that patients with tumors positive for EGFR, HER2 and P-HER2 demonstrated the worst outcome. Furthermore, the researchers suggest that EGFR-dependent activation is the major mechanism for HER2 signaling in human breast cancer. These two studies suggest that assays for EGFR, HER2 and P-HER2 would be more useful than the current single assay for HER2 and could improve therapeutic decisions for each patient.

HER2-positive breast cancers may express hundreds of copies of the ERBB2 gene resulting in up to 2 million receptors per cell (101,119). This differential receptor expression in tumor cells compared to normal cells made HER2 an ideal target for tumor-selective therapeutics and lead to the development of the humanized monoclonal antibody trastuzumab (Herceptin). The history of HER2 and trastuzumab treatment is a successful narrative of "bedside to bench to bedside" research where the overexpression of a gene was found in breast cancer that contributed to poor prognosis, giving rise to the discovery of a novel antibody therapy, which ultimately established a new standard of treatment for breast cancer. Early clinical investigation found that trastuzumab combined with chemotherapy showed enhanced efficacy and dramatically improved disease-free survival among of women with HER2-positive breast cancer (120,121). However, only a subset of HER2+ breast cancers responds to trastuzumab, suggesting additional levels of tumor biology complexity. Furthermore, the scientific basis for

the effects of trastuzumab remains uncertain and resistance to trastuzumab continues to be a major clinical concern.

ERBB3/HER3: The kinase-deficient HER3 receptor has variable expression in breast cancer and its contribution is not clearly characterized. Literature data concerning the clinical significance of HER3 in breast cancer is conflicting; one group reported that high HER3 expression was positively associated with the presence of lymph node metastases (122) but two others failed to confirm this observation (123,124). The work of Naidu *et al.* suggested that HER3 overexpression could promote tumor progression from non-invasive to invasive (125) whereas Knowlden *et al.* demonstrated that elevated HER3 expression correlated with ER positivity and longer overall survival (126).

HER3 expression is mostly linked to HER2 overexpression, attesting to the effective association of this specific dimerization partnership. In a panel of six HER2-overexpressing human tumor cell lines, RNAi-mediated knockdown of HER3 was as effective as HER2 knockdown at inhibiting proliferation *in vitro* and xenograft tumor growth *in vivo* (82). Furthermore, preferential phosphorylation of HER3 was also observed in HER2-positive breast cancer (82,127) and inhibition of HER2 led to loss of HER3 phosphorylation (82,128,129). These observations suggest that HER3 plays a pivotal role in HER2-driven tumorigenesis.

ERBB4/HER4: Expression of HER4 is uncommon or found at low levels in breast carcinomas; in fact, its expression is reduced or lost in about 70% of breast tumors (130,131). In contrast to the other ERBB members, HER4 expression is associated with improved overall patient survival, well-differentiated tumors, low tumor grade

and ER positivity (89,131-133). A histological study of breast tumor tissue showed HER4 nuclear localization in almost half of breast tumor samples as compared to normal breast epithelial tissues, which significantly correlated with good physiological grade, implying that HER4 presence in the nucleus is related to tumor differentiation (133). Interestingly, HER4 has been shown to have tumor suppressor activity. Cell culture studies using HER4 expressing breast cancer cell lines demonstrate that HER4 induces HRG dependent antiproliferative responses in these cells (134,135). The cell culture studies together with existing clinical data suggest an anti-tumor role for HER4 in breast cancer.

1.3 BREAST CANCER THERAPY

The global burden of breast cancer exceeds all other cancers and incidence rates continue to rise, likely due to increased human lifespan and greater exposure to UV radiation, pollution and unhealthy lifestyles (obesity, poor diet, smoking, etc) (136). The heterogeneity of breast cancers makes them a challenging tumor to diagnose and treat. Thus, it is fundamentally important to understand and recognize the individuality of the disease in each breast cancer patient and design a tailored treatment approach to produce the most effective therapeutic outcome. In recent years, advanced treatment strategies for breast cancer have dramatically improved the care and prognosis of patients diagnosed with breast cancer as well as bringing new hope and excitement in the field of drug discovery. Current therapeutic decisions are based on breast cancer type and stage, histological grade of tumor

cells, age of the patient, oncogene (EGFR, HER2) activation, status of hormone receptors (ER and PR) and lymph node metastasis (101,137-139).

Breast cancer therapy includes primary and adjuvant therapy. Primary treatment typically consists of surgery or radiation. It is also known as local therapy because it specifically targets the removal of the primary tumor and surrounding breast tissue and leaves the rest of the body unaffected. Adjuvant therapy is additional therapy administered after the initial treatment. The goal of adjuvant therapy is to eliminate any cancer cells that survived primary treatment to reducing the risk of cancer recurrence. Adjuvant therapy may include radiation therapy, chemotherapy, hormone therapy and targeted therapy.

Treatment can also involve a combination of the above-mentioned techniques. The multi-modality treatment is increasingly recognized as the best approach for improving the patient's chance of a cure or prolonging survival. Ultimately, the treatment plan should be based on the patient's unique circumstance and must be carefully balanced with their potential treatment risks.

1.3.1 Surgery

The majority of patients with breast cancer have surgery to remove the cancer from the breast. Breast-conserving surgery aims to remove the tumor but not the entire breast itself and involves a *lumpectomy*, the removal of the tumor lump or a *segmentectomy/partial mastectomy* where the surrounding healthy breast tissue and chest muscle are removed along with the lump. Patients with more advanced stage breast cancer may undergo a *total mastectomy,* which involves surgical removal of the whole breast that has cancer (140). A lumpectomy alone is

associated with a higher rate of cancer recurrence compared to a mastectomy, thus patients who elect to have a lumpectomy are also treated with radiation therapy to reduce the risk of local recurrence (141).

Often lymph nodes under the arm are also removed and biopsied for metastasis of cancer cells. Axillary lymph node status is the strongest predictor of long-term prognosis in breast cancer patients. Sentinel lymph nodes are the first of the lymph nodes to be infiltrated by invading breast cancer cells; these nodes are assessed by a diagnostic procedure called a *sentinel lymph node biopsy (SLNB)* (142,143). SLNB is now accepted as the standard of care for axillary staging in early breast cancer instead of full nodal assessment. Positive SLNB results indicate metastasis to the lymphatics and necessitate the clearance of axillary lymph nodes by surgery called *axillary lymph node dissection (ALND)* (144,145). This helps to avoid local recurrence and further spreading of cancer cells. Negative sentinel lymph nodes indicate a less than 5% chance of cancer in the axillary lymph nodes and negates the need for ALND, thus patients avoid the risks of complications and risks associated with surgery.

1.3.2 Radiation

Following a lumpectomy, patients undergo post-operative radiotherapy to remove residual cancer cells and reduce the chance of cancer recurrence in and around the conserved breast tissue (141). If a patient elects to have a mastectomy, radiation therapy is not typically administered unless positive axillary nodes were detected (146). Several studies have validated the efficacy of radiotherapy in reducing the risk of recurrent breast cancer and improving long-term survival (146-

148). Over the years, the approach to radiation therapy has focused on minimizing radiation exposure and reducing toxicity by accurate targeting of the affected tissue.

Radiation therapy utilizes a technique called *external beam radiation therapy* to administer a high-energy x-ray beam to the site of cancerous tissue; this often encompasses the entire breast and treatment is typically given daily over a five to six week period (149). An additional concentrated radiation treatment, called a boost, can be given to a smaller area of the breast afflicted with cancer. The side effects from radiation therapy may include swelling or heaviness in the breast, sunburn-like damage to the skin and fatigue, all of which may disappear in six to twelve months.

Partial breast irradiation is a newer approach where radiation is applied specifically to the affected tissue rather than the whole breast and overall treatment time is shortened to five days instead of six weeks (148,149). Partial breast irradiation can be performed using a multi-catheter *brachytherapy* or a *mammosite* device. Brachytherapy is an internal treatment using catheters placed inside the breast close to the cancerous tissue where radiation can be introduced directly though the catheters (150,151). The mammosite device uses a single catheter with a balloon attached at one end that is placed where the tumor existed prior to the lumpectomy and radiation is administered into the balloon through the catheter for five days (152,153). Partial breast irradiation prevents exposure of the skin, lungs, heart, ribs, remaining healthy breast tissue and the body as a whole to radiation, thus reducing overall toxicity to the patient.

The timing or sequence of radiation therapy, such as before or after chemotherapy following breast-conserving surgery, may be important. A clinical study found that patients who had chemotherapy followed by radiation were more likely to survive to five years post-treatment than patients who had radiation, then chemotherapy. Patients who had chemotherapy first survived longer and were less likely to experience system (metastatic) recurrence of their cancer. However, patients who had radiation first were less likely to experience a local recurrence of their cancer (154).

Radiation therapy can also provide symptomatic relief in patients with advanced breast cancer (155). Patients with metastatic cancer to the bone, brain, selected lymph nodes and other sites can benefit from radiotherapy to the site of cancer recurrence. Radiation can relieve symptoms from the disease and prevent bone fractures.

1.3.3 Chemotherapy

Chemotherapy is recommended for patients with tumors that are hormone receptor negative (undetectable ER and PR expression), endocrine non-responsive and HER2-overexpression (156). Traditional chemotherapeutic agents are cytotoxic, which means they kill rapidly dividing cancer cells at the primary site as well as in circulation. It can be administered prior to surgery (*neoadjuvant therapy*) to shrink the tumor so a lumpectomy can be performed instead of a mastectomy, or it may be given after the surgery (*adjuvant therapy*) to target cancer cells that escaped surgical removal or metastasized (157). Additionally, chemotherapy may be used as

primary treatment in women with advanced disease, as well as those with triple-negative cancers (158).

The main types of chemotherapy include alkylating agents, platinum compounds, antimetabolites, anti-microtubule agents, topoisomerase inhibitors and cytotoxic antibiotics (anthracyclines) (159). Chemotherapy treatment may consist of single agents or combinations (polychemotherapy) of drugs that can be delivered intravenously (most common) or orally. Clinically, drug combinations are more effective than single drug treatments due to the synergistic effect of multiple drugs to maximize benefit, reduce toxicity and minimize drug resistance; since each drug has a different mechanism of action, they can inhibiting tumor growth by targeting various stages of the cell cycle (160). The standard chemotherapy drug combination to treat individuals with node-negative breast cancer is CMF: cyclophosphamide (Cytoxan), methotrexate (Amethopterin, Mexate, Folex) and 5-fluorouracil (Fluorouracil, 5-FU, Adrucil) (161). Importantly, optimal treatment for each patient requires careful consideration of the individual's detailed circumstance as some drug combinations may work better for certain individuals than others and blanket application of published guidelines is usually not feasible.

The non-specific action of chemotherapy drugs results in a number adverse effects. Cancer cells are rapidly undergoing cell division and have a high multiplication rate. Chemical drugs are designed to target these fast growing cells. However, certain normal tissues in the body such as hair follicles, bone marrow, the epithelial lining of the mouth, nose and intestine also have actively multiplying cells that are inadvertently attacked by these drugs resulting in various side effects such

as immunosuppression, gastrointestinal distress, nausea and vomiting, fatigue, anemia, weight loss, hair loss, mouth sores, decrease in appetite, reduction in platelet count, infertility, changes in the menstrual cycle and early menopause.

Unfortunately, chemotherapy is not always effective and may not completely destroy the cancer. The efficacy of chemotherapy depends on the type and stage of the cancer. While it may not offer a permanent cure, chemotherapy can be useful in reducing symptoms like pain or reduce the size of an inoperable tumor in hopes that it will become operable.

1.3.4 Endocrine Therapy

Steroid hormones such as estrogen and progesterone play a significant role in the development and progression of ER positive breast cancer (162). The ovaries are the main source of estrogen in pre-menopausal women. After menopause, smaller amounts of estrogen can be made in the body's fat tissue. The objective of hormone therapy is to reduce the amount of estrogen in a woman's body and block the downstream signaling from the estrogen and progesterone receptors to prevent tumor growth. Researchers have found that adjuvant hormone therapy benefits women with ER-positive, node-negative breast cancer by prolonging life and reducing recurrences. Additionally, hormonal therapy also prevents new cancers in the opposite breast (163).

Current classes of anti-estrogen drugs include aromatase inhibitors, selective estrogen receptor modulators (SERMs) and estrogen receptor antagonists (164). Historically, tamoxifen is the gold standard of hormonal therapy and functions by inhibiting estrogen from binding to its receptor in breast cancer cells. Tamoxifen is

an SERM because while it behaves as an anti-estrogen drug in breast cells, it acts like estrogen in other tissues like the uterus and bones. Aromatase inhibitors (AIs) prevent the enzyme aromatase in fat tissue from making small amounts of estrogen but cannot block estrogen production from the ovaries, so it is only effective in women whose ovaries are not functional due to surgical removal or use of luteinizing hormone-releasing hormone (LHRH) analogs or women who are post-menopausal. For this reason premenopausal women will be treated with tamoxifen. The current standard for AI treatment is five years with or without tamoxifen. ER antagonists work similarly to tamoxifen by binding ER to block estrogen but additionally downregulate ER expression.

Estrogen expression is important in both bone growth and cardiovascular health; thus lower estrogen levels can have adverse effects such as decreased bone density, joint pain and heart disease (165). Tamoxifen, while effective, has side effects similar to symptoms of menopause including hot flashes, irregular menstrual periods and vaginal dryness, discharge or bleeding. Long term use of tamoxifen can have more serious side effects such an increased blood clots, development of cataracts, as well as endometrial cancer. Tamoxifen can cause bone thinning in pre-menopausal women but contributes to bone strength in post-menopausal women. The benefits of hormone therapy outweigh the risks for almost all women with receptor-positive invasive breast cancer.

1.3.5 Targeted Therapy

Over the last two decades, the paradigm for cancer treatment has evolved from relatively nonspecific cytotoxic agents to selective, mechanism-based

therapeutics. Targeted therapy utilizes drugs or other substances to identify and attack cancer cells but not compromise normal cells, tissues and organs (166). It is distinguished from standard chemotherapy because the drugs are deliberately designed to identify molecular targets associated with cancer cells whereas chemotherapy acts on all rapidly dividing cells. Targeted therapy is the cornerstone of personalized medicine and is based on an individual's genes and proteins to prevent, diagnose and treat disease.

Targeted therapeutics are rationally developed based on the identification of molecular targets that play a key role in cancer growth and survival. The basic approach to determine potential targets is to compare normal cells to cancer cells: are there chromosomal abnormalities, mutant proteins or overexpressed proteins in the cancer cells compared to normal cells? One such differentially expressed protein is the human epidermal growth factor receptor 2 (HER2) protein. HER2-positive tumors often have gene amplification and result in receptor overexpression at the cell surface. Trastuzumab is a humanized monoclonal antibody targeted against the extracellular domain of HER2 and is the first HER2-targeting agent approved by the United States Food and Drug Administration (FDA) for the treatment of both early and metastatic HER2-positive breast cancer (121,167).

Once a candidate target has been identified, scientists attempt to develop a therapy that will interfere with its ability to promote cancer growth or survival. The drug could interfere with a ligand from binding to the receptor, promote downregulation of expression, and inhibit effector proteins from propagating a signal cascade, among other possible mechanisms (168). Most targeted therapies

are small molecules or monoclonal antibodies. Small-molecule compounds are designed to easily traverse the cell membrane and aim for targets inside the cell. Antibodies are relatively large and generally cannot enter the cell, so they are used to identify targets on the cell surface. Monoclonal antibodies are often developed in mice and then "humanized" (replacing the mouse antibody with the corresponding portions of human antibodies) to prevent the human immune system from attacking and destroying the antibody as foreign substance (169).

A number of different targeted therapies have been approved for cancer treatment including hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors, immunotherapies and toxin delivery molecules. In 2010, there were twenty-two FDA-approved targeted cancer therapies, nine of which were monoclonal antibodies (mAbs), twelve were small-molecule drugs and one was a fusion protein. Among these drugs, four mAbs and one small-molecule drug constituted 86% of the 2009 US sales. (170).

One limitation of targeted therapy is resistance: the target itself develops mutations to evade detection from the targeted therapy or the tumor utilizes an alternative pathway to achieve tumor growth and becomes independent from the target. Thus targeted therapies may perform best in combination with another targeted therapy or chemotherapy to target different parts of the cell signaling pathway (171,172). For example, trastuzumab has been used in combination with docetaxel, a traditional chemotherapy, to treat women with HER2-posiitve metastatic breast cancer (173). Another challenge faced by scientists developing

targeted therapies is the difficulty of designing drugs due to the target's structure or function. Ras mutations occur in up to one-fourth of all cancers and to date, it has not been possible to develop inhibitors of Ras signaling. Side effects such as skin problems, issues with blood clotting and wound healing, high blood pressure and gastrointestinal perforation have been observed in patients treated with targeted therapies. However, some of these side effects have also been linked with improved patient response to treatment (174,175).

In conclusion, the main goal of systemic adjuvant treatment is to control any micrometastatic disease, reduce the recurrence rate and improve the long-term overall survival. The sequence of treatment is undergoing continued evaluation. The current standard treatment of breast cancer should include definitive surgery first, followed by systemic chemotherapy and lastly radiation. Hormone therapy can begin during or following radiation. Patients with advanced breast cancer may have chemotherapy prior to surgery (neoadjuvant) to reduce the tumor burden prior to surgery and allow for greater breast conservation.

1.4 TRASTUZUMAB

In 1987, Slamon and colleagues examined 86 node-positive patients and reported that HER2 overexpression was a strong independent adverse prognostic factor (101). In a follow up study, they found that 27% of 345 node-positive patients had HER2 gene amplification and this was a significant predictor of disease-free and overall survival in a multivariate analysis (102). In a subsequent retrospective analysis of 1506 patients from the Ludwig International Breast Cancer Study Group,

HER2 overexpression was again significantly associated with the prognoses of node-positive but not node-negative patients (176). The discovery that elevated HER2 expression occurs in a significant percentage of breast tumors and confers a more aggressive behavior with poor clinical outcome prompted investigators to develop agents using HER2 as the target for treatment.

Researchers at Genentech developed a panel of mouse antibodies directed at HER2 that were capable of inhibiting the growth of HER2 overexpressing cell lines and xenograft tumors (177-179). The most potent of the monoclonal antibodies was muMab 4D5 and thus selected for further clinical development. Trastuzumab, the humanized version of murine 4D5, showed three times greater binding affinity to HER2 than the parental antibody and demonstrated a marked anti-proliferative effect on HER2 overexpressing cell lines as well as breast cancer xenografts (180,181). Furthermore, chemotherapy alone in the xenograft experiments showed only modest anti-tumor activity, whereas the trastuzumab combination resulted in distinct enhancement of the effect of chemotherapy (181).

The mechanism of action of trastuzumab is still unclear. It has been shown that trastuzumab downregulates HER2 expression by inducing endocytic degradation, which can inhibit signaling due to fewer HER2 homodimers and heterodimer coupling (182). Inhibited growth and cell division signals observed in trastuzumab treatment could be a direct effect of the antibody or reflect reduced expression of HER2 receptors (183). Trastuzumab can also trigger the host tumor response via antibody-dependent cell cytotoxicity (ADCC) mechanisms, impede the

cell's ability to repair DNA damage after radiation or chemotherapy treatment and antagonize HER2-induced expression of angiogenic factors (184-186) (Figure 1.4.1).

In 1992, three open-label, phase I clinical trials enrolled patients with refractory HER2-positive metastatic breast cancer to assess the safety, maximum to lerated dose and pharmokinetics of trastuzumab (187). Group one received a single fixed dose of 10-500 mg trastuzumab and the remaining two groups received either a weekly schedule of trastuzumab alone or in combination with cisplatin. Results suggested that trastuzumab was well tolerated at all doses as well as in combination with cisplatin. Pharmacokinetic data showed that trastuzumab has a half-life of 8.3 days, and was unaffected by coadministration with cisplatin (187). Thus, the phase II trials were recommended to use a weekly dose of 100 mg trastuzumab.

In the first of three phase II clinical trials, 46 patients with metastatic breast cancer were recruited for a single-agent study. Of the 43 assessable patients, five (12%) had clinical response, sixteen (37%) had minimal response and the median time to disease progression was 5.1 months (188). The second phase II study evaluated trastuzumab with cisplatin combination on heavily pretreated patients. Of the 37 patients evaluated, nine (24%) patients showed partial response and zero patients showed complete response (189). The median response duration was 5.3 months.

In parallel with the two previously mentioned phase II clinical trials, the third trial tested trastuzumab treatment as a single agent and involved the multinational participation of 222 women with HER2-positive metastatic breast cancer with one or two prior chemotherapy treatments (190). The primary efficacy

Figure 1.4.1

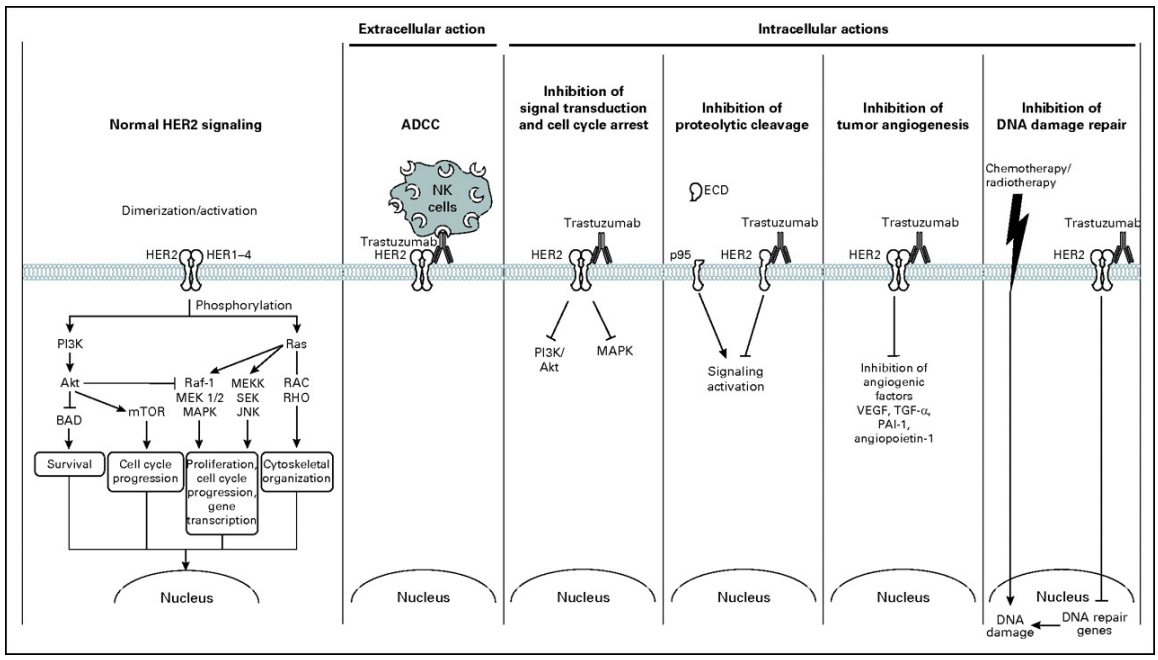


Figure 1.4.1 Potential mechanisms of action of trastuzumab. Depicted are the potential extracellular and intracellular mechanisms of action of trastuzumab (191).

endpoint was overall response rate (complete response and partial response) and secondary endpoints were duration of response, time to disease progression, time to treatment failure and survival. The overall objective response rate based on intent-to-treat analysis was 15%, which included eight complete responses and twenty-six partial responses. The median duration of response was 9.1 months, median time to progression was 3.1 months, median time to treatment failure was 11 months and median survival was 13 months. Interestingly, the subgroup of patients whose tumors had HER2 IHC staining of 3+ had an 18% response rate and median survival of 16.4 months, suggesting that trastuzumab was more effective in patients with greater HER2 overexpression. While treatment was generally well-tolerated, retrospective analysis found that 4.7% of patients experienced cardiac dysfunction. This pivotal study confirmed that trastuzumab was active as a single agent in heavily pretreated patients.

A pivotal phase III clinical trial involved a large multinational study that investigated the efficacy of chemotherapy in combination with trastuzumab in first-line therapy in 469 patients with HER2-positive (IHC 2+ or 3+) metastatic breast cancer (167). The addition of trastuzumab to chemotherapy was associated with longer time to disease progression (median 7.4 vs 4.6 months), higher objective response rate (50% vs 32%), longer duration of response (median 9.1 months vs 6.1 months), lower rate of death at 1 year (22% vs 33%), longer survival (median 25.1 months vs 20.3 months) and a 20% reduction in the risk of death. As observed previously in the phase II trial, the benefit of trastuzumab was only seen in patients whose tumors had HER2 amplification as determined by fluorescence *in situ*

hybridization (FISH). Patients who did not have gene amplification did not show improved response rates with the addition of trastuzumab.

Subsequent to the two pivotal studies discussed above, a phase II study examined trastuzumab as a monotherapy in 114 HER2 overexpressing patients (192). The objective response rate was 26%, with seven complete and 23 partial responses. As observed in the previous studies, higher response rates were seen in IHC 3+ patients and FISH+ patients (48% and 34% respectively). This study found that single-agent trastuzumab is active and provided overall improvement in global quality of life and fatigue scores in the treatment of women with HER2-positive metastatic breast cancer.

HER2 gene amplification can be detected by FISH where multiple copies of the HER2 gene can be observed in the nuclei of affected cells. Increased mRNA expression corresponding to gene amplification can be measured by Northern blot and receptor overexpression can be assessed by Western blot or immunohistochemistry (IHC) analysis. The above-discussed clinical studies have shown a strong correlation between the efficacy of trastuzumab and the strength of IHC staining and gene amplification. It is becoming the practice of many clinicians to select patients for trastuzumab therapy based on IHC 3+ reactivity and if staining is 2+, then FISH analysis is required to confirm gene amplification.

In terms of clinical safety, the large studies of trastuzumab have shown it to be generally well-tolerated, with the most common side effects being mild to moderate infusion reactions. Cardiac dysfunction had not been observed in the preclinical studies but occurred in 6 to 8.8% of pretreated patients in the single-

agent phase II study (190) and 2% of the first-line monotherapy phase II study (192). In the trastuzumab and chemotherapy combination study, 26-28% of patients experienced cardiac dysfunction compared to 6 -9.6% of patients treated with anthracycline regimen alone (167). These retrospective analyses of trastuzumab treatment suggest that cardiac toxicity is related to concurrent use of anthracyclines and patients with pre-existing risk factors to heart failure need to be closely monitored. The mechanism of cardiotoxicity is unclear and is unlikely due to trastuzumab binding to HER2 expression on heart muscle as only faint membrane staining is observed in cardiac biopsies and there is no evidence of gene amplification by FISH (193).

As a result of these early trials, trastuzumab (marketed as Herceptin) received FDA approval in September 1998 for use in women with metastatic breast cancer who have tumors that overexpress the HER2 protein. Trastuzumab can be given with paclitaxel to women who have not previously received chemotherapy for metastatic disease or as a stand-alone treatment for women who have received prior chemotherapy. Herceptin was the first FDA approved therapeutic antibody targeted to a specific cancer-related molecular marker. In November 2006, the FDA expanded the use of trastuzumab to include treatment of HER2-positive breast cancer in combination with chemotherapy following primary treatment for early stage breast cancer (194).

While trastuzumab has been heralded as a major success story in targeted therapy, 76-88% of HER2-positive patients display inherent resistance to trastuzumab treatment (195) and 70% of initial responders progress to metastatic

disease within one year of treatment, suggesting rapidly acquired resistance to trastuzumab (167,188,190,192,196). A *de novo* mechanism of resistance to HER2-targeted therapy is mutation of the target itself. Truncated isoforms of HER2, resulting from matrix metalloproteinase (MMP) cleavage or alternative translational start sites, have shown reduced response to trastuzumab (197,198). P95-HER2 (648-carboxy terminal fragment (CTF)) is formed by MMP cleavage and remains bound to the cell membrane, but releases the extracellular domain of the HER2 receptor, which can act as a decoy for trastuzumab binding, thus diminishing the effect of trastuzumab on membrane-bound HER2 (199). P110-HER2 (611-CTF) is a naturally occurring isoform that arises from alternative translation (200) and is associated with increased migration, invasion and transformation of normal breast epithelial cells, causing *in vivo* tumor formation (201). A splice variant of HER2 that lacks exon 16, which impacts the extracellular domain of the receptor, has been identified in HER2-positive breast cancers and cell lines and confers trastuzumab resistance (202). Point mutations or small insertions in the HER2 gene have been identified in other cancers, including breast cancer in the absence of HER2 gene amplification (203), but HER2 mutations in HER2-overexpression breast tumors have not been reported to date (204). A unique mechanism of resistance specific to trastuzumab is the overexpression of a membrane-associated glycoprotein, mucin-4 (MUC4), which can decrease accessibility to HER2 and mask the trastuzumab-binding site (205,206).

Acquired resistance to trastuzumab has been shown to involve compensatory signaling from other receptor tyrosine kinases. Increased expression

of ligands for EGFR and HER3 results in the activation of these receptors as well as increased EGFR/HER2 heterodimers in trastuzumab-resistant cells (207). Cross-talk with receptors outside the ERBB family has also been implicated in trastuzumab resistance. Overexpression of the insulin-like growth factor-1 receptor (IGF-1R) can result in IGF-1R/HER2 heterodimers that can potently activate PI3K-Akt signaling and confer resistance to trastuzumab *in vitro* (208). Several studies have observed increased IGF-1R or IGF-1R/HER2 complexes only after the development of acquired resistance to trastuzumab (209-211). Furthermore, IGF-1 stimulation resulted in the downregulation of $p27^{kip1}$ in breast cancer cells (212) and reduced $p27^{kip1}$ levels have been associated with trastuzumab resistance (213). The RTK Met has been shown to interact with HER3 to engage PI3K signaling independently of HER2 activity and HGF-induced signaling through Met has been shown to abrogate the action of trastuzumab (214).

Intracellular alterations downstream of HER2 activation are also implicated in trastuzumab resistance. Mutations in components of the PI3K-Akt pathway are the most frequent tumor somatic alterations including gain of function mutation in the catalytic subunit of PI3K, PIK3CA, as well as loss of PTEN, a negative regulator of the Akt pathway (215,216). Recently, Src has been established as the central mediator of all resistance pathways in both primary and acquired resistance to trastuzumab and its signaling cannot be inhibited when PTEN is deficient (195).

Trastuzumab has been used to treat over 420,000 women with HER2-positive breast cancer worldwide. The history of trastuzumab is a demonstration of how time consuming and tedious the process of drug validation can be. A critical

hurdle is our failure to understand how trastuzumab will benefit different subgroups of HER2-positive patients and the mechanisms of resistance. There are currently seven large ongoing clinical trials investigating the survival benefit, optimal timing, optimal dose and how trastuzumab behaves when combined with other treatments. Trastuzumab resistance, both primary and acquired, remains a serious clinical concern in the treatment of HER2-overexpressing metastatic breast cancer and demands further research to elucidate the molecular mechanisms contributing to this resistance as well as validating new biomarkers that can predict patient response to specific anti-HER2 therapy.

1.5 THE HER2 SPLICE VARIANT: HER2Δ16

Although clinical evidence confirms an important role for HER2 in breast cancer, preclinical models utilizing wild-type HER2 (wt-HER2) fail to recapitulate the aggressive nature of HER2-positive tumors observed in patients. Only when HER2 has been experimentally manipulated with activating mutations (point mutation or deletions of the extracellular domain) does it increase cellular transformation (105,110,217,218). Studies using transgenic mouse models provided direct evidence that mammary-specific expression of the rat HER2/*neu* gene induced tumor formation only when accompanied by activating deletions in the extracellular domain of the receptor (218-220). Reverse-transcription PCR (RT-PCR) analysis of RNA from the mammary tumors and adjacent mammary epithelium found no evidence of point mutations but rather the tumor PCR products possessed deletions (219). Furthermore, the altered *neu* cDNA could induce transformed foci

that expressed elevated tyrosine-phosphorylation. These observations suggested that activation of the rat HER2/*neu* receptor in these mammary tumors occurred due to mutations in the extracellular domain.

Interestingly, an alternatively spliced HER2 variant with an in-frame deletion of the same region observed in the mutated HER2/*neu* transgenic mice has been observed in human breast carcinoma (221). The splice variant, referred to here as HER2Δ16, encodes an aberrant receptor that is similar to full-length HER2, but lacks exon 16, which eliminates sixteen amino acids and disrupts cysteine residue pairing in the extracellular domain of the receptor. This deletion of residues 691 to 707 impacts the region immediately preceding the transmembrane domain and is thought to induce a conformational change in the HER2 receptor where the imbalance of cysteine residues promotes intermolecular disulfide bonding, resulting in the formation of active stable HER2 homodimers with enhanced mitogenic signaling activity capable of transforming cells (202,221). Other studies have demonstrated that alternative splicing can create deletions within the extracellular domain of growth factor receptors resulting in constitutively activated signaling molecules with increased transformation abilities including Met (222), Ron (223) and the fibroblast growth factor receptor 2 (FGFR2) (224).

HER2Δ16 was first identified in 1998 by researchers at the University of Texas M.D. Anderson who were studying the expression of HER2 in human cancer cell lines using RT-PCR analysis (221). They unexpectedly obtained additional smaller bands in their RT-PCR product and utilized DNA sequencing to compare the smaller products to full-length HER2. The unknown bands matched the wt-HER2

sequence except for the deletion of 48 base pairs in the extracellular domain. While previous groups recognized that deletions in the extracellular domain of HER2 could enhance oncogenic signaling, Kwong *et al.* were the first to identify this deletion in human breast tumor cells as exon 16. They proposed that HER2Δ16 was a result of alternative mRNA splicing, resulting in deletion of exon 16, since their PCR primers could produce the same product in three different cell lines and they did not observe point mutations. Furthermore, they found that HER2Δ16 activated the MAPK pathway six-fold more strongly and formed significantly more foci in a transformation assay than wt-HER2. The difference between wt-HER2 and HER2Δ16 was so dramatic that the authors concluded that the small deletion in the extracellular domain in HER2Δ16 receptor robustly enhanced the transformation capacity of HER2.

Additional studies examining the expression of HER2Δ16 in breast cancer found that HER2Δ16 mRNA expression made up ~9% of total HER2 expression in 46 human breast carcinoma samples (225). Cell lines transfected with a HER2Δ16 expression vector showed elevated levels of receptor dimers stabilized by disulfide bonds, which could induce transformation of human cells when at 10-fold lower concentrations than wt-HER2 (225). This suggests that the transformation associated with HER2 overexpression is a reflection of the increase in absolute levels of HER2Δ16 and its constitutive signaling activity. Interestingly, not all HER2Δ16 receptors were dimerized in the transfected cells, suggesting that disulfide bonding depends on the redox conditions of the tumor microenvironment (225). Importantly, the authors found that cells expressing HER2Δ16 failed to

respond to the therapeutics ZD1839, an EGFR tyrosine kinase inhibitor and trastuzumab, an anti-HER2 monoclonal antibody. Clinically, not all patients with HER2-positive tumors respond to HER2-targeted therapies. Non-responsive patients may be those whose HER2-overexpressing tumors also co-express high levels of stable HER2Δ16. It is thought that the deletion of exon 16 alters the extracellular domain of the HER2 receptor in such a way that the antibody binding epitope is hidden, thus limiting the efficacy of trastuzumab (Figure 1.1.1).

Research from our lab further established the contribution of HER2Δ16 in HER2-driven breast tumorigenesis. Analysis of HER2Δ16 expression in a panel of eighteen different normal tissues revealed that HER2Δ16 is undetected in normal tissues, including normal breast tissue (202). HER2Δ16 is also not expressed in HER2-negative tumors. In the primary invasive breast tumors analyzed, HER2Δ16 is present in 52% (27 of 52) of HER2-positive breast tumors. Thus HER2Δ16 appears to be tumor-specific and coexpressed in HER2 amplified breast tumors. Significantly, 89% of patients with tumor expression of HER2Δ16 presented with disseminated lymph node positive or metastatic disease. (202). In contrast, breast tumors that overexpress wt-HER2, but lack detectable HER2Δ16 expression, display favorable clinicopathological markers including lymph node negative cancer. Diagnosis of positive lymph nodes is a reliable clinical predictor of negative prognosis in breast cancer. The significant association between HER2Δ16 expression with positive lymph nodes implicates HER2Δ16 in promoting aggressive metastatic breast cancer. We also confirmed the previous findings that HER2Δ16 expression enhances cell tumorigenicity, forms stable dimers, activates of multiple

Figure 1.5.1

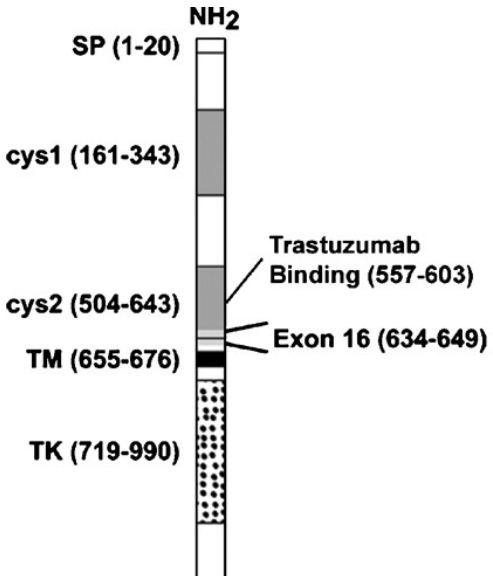


Figure 1.5.1 A schematic of HER2Δ16 (202). The binding epitope of trastuzumab does not overlap the region of exon 16. However, it is thought that the deletion of exon 16 alters the three-dimensional structure of the HER2 receptor in such a way that the antibody binding epitope is hidden.

oncogenic signaling cascades that result in increased tumor cell migration and invasion and promotes trastuzumab resistance. Our preclinical data revealed that HER2Δ16 facilitates the recruitment and activation of Src kinase, a critical upstream effector of AKT and FAK, through direct interaction at the cell membrane while suppression of Src kinase resulted in a dramatic reduction in HER2Δ16-induced cell invasion. Examination of the primary breast tumors found that 44% of HER2Δ16-expressing tumors also expressed activated Src kinase. Lastly, pharmacologic or RNAi suppression of Src resulted in loss of HER2Δ16 protein and disengages HER2Δ16-mediated cell invasion (202).

In addition to promoting resistance to trastuzumab, HER2Δ16 expression is also implicated in endocrine resistance in HER2/ERα-positive breast carcinomas. Tamoxifen is the most commonly prescribed therapy for patients with ERα-positive breast cancer (226). Patients with HER2 overexpression account for approximately half of the ERα-positive population and the majority of these patients experience *de novo* tamoxifen resistance (227). Preclinical studies demonstrate that HER2Δ16 expression, but not wt-HER2, promotes tamoxifen resistance and estrogen independence of ERα-positive breast cancer cells *in vivo* and *in vitro* (228). There are two mechanisms used by HER2Δ16 to evade tamoxifen therapy. In the first, HER2Δ16 transfected breast cancer cells treated with tamoxifen up-regulated BCL-2 protein expression by suppression of miR-15a/16 compared to tamoxifen-sensitive cell lines. Normally, BCL-2 translation is inhibited by binding of miR-15a or miR-16 to the 3'-UTR of the BCL-2 mRNA and loss of miR-15a/16 in several cancer cell lines and tumors are associated with elevated expression of BCL-2 (228-230). Tamoxifen

inhibits tumor cell growth by activating the intrinsic apoptotic pathway and BCL-2 is a potent inhibitor of the intrinsic or mitochondrial cell death pathway (231). Overexpression of the anti-apoptotic BCL-2 can convert tamoxifen-sensitive breast tumor cells to a tamoxifen-resistant phenotype (232). The second mechanism involves the down-regulation of miR-342 observed in HER2Δ16-expressing breast cancer cells and in primary breast tumors of patients who failed tamoxifen therapy (233). MiR-342 regulates expression of genes associated with tumor cell death and cancer pathways. Restored miR-342 expression sensitizes resistant breast tumor cells to tamoxifen. Thus, HER2Δ16 expression can modulate microRNA expression and contribute to therapeutic evasion by breast tumor cells.

The first HER2Δ16 transgenic mouse model was reported in 2011 (234). Analyses of these genetically engineered mice demonstrated that HER2Δ16 homodimerizes on the tumor cell plasma membrane and is capable of transforming mammary epithelium *in vivo* and promoting extensive metastatic disesae. The researchers reported higher tumor incidence, rapid tumor growth and significantly shorter latency period in the HER2Δ16 mice compared to the wt-HER2 transgenic mice (234). Furthermore, the oncogenic properties of HER2Δ16 signaling were mediated through activation of Src kinase. Notably, quantitative PCR analysis revealed that only 5 copies of the transgene were needed to drive neoplastic transformation of the mammary epithelium of the HER2Δ16 mice, compared to 30-50 wt-HER2 transgene copies required to induce breast cancer in wt-HER2 transgenic mice (235). This transgenic mouse model will be invaluable for future

studies to evaluate the role of HER2Δ16 in tumor progression as well as its response to chemotherapy and targeted therapies in HER2-positive breast cancer.

1.6 MICRORNAS

MicroRNAs (miRNAs) are a class of short (19 to 25 nucleotides) non-coding single-stranded RNAs that regulate gene expression post-transcriptionally. The first identified miRNA, *Lin-4,* was discovered in *C. elegans* in 1993 and since then, miRNAs have been identified in animals, plants and viruses (236). They are localized throughout the entire genome in intronic or exonic regions of protein encoding or non-coding genes. Half of all human miRNAs are interconnected in genomic clusters and transcribed from a single polycistronic transcription unit (237-239). It is estimated that the human genome harbors over 1,000 miRNAs and they regulate over half of protein encoding-genes (240,241). MiRNAs play key roles in a variety of biological processes including cell cycle regulation, development, differentiation, metabolism, neuronal patterning, apoptosis and aging (242).

The biogenesis of miRNAs begins in the nucleus, where an RNA polymerase II or III transcribes the primary miRNA (pri-miRNA), a long, capped, polyadenylated hairpin structure (243). The pri-miRNA is then cleaved by the microprocessor complex composed of the ribonuclease III, Drosha and the double-stranded DNA binding protein, DGCR8/Pasha (244). This cleavage step produces a ~65 nucleotide (nt) precursor miRNA (pre-miRNA) that is exported into the cytoplasm by Exportin-5 and RanGTP (245). The pre-miRNA is further trimmed by the ribonuclease III Dicer into ~22-nt imperfect duplexes (246). One strand of the duplex is

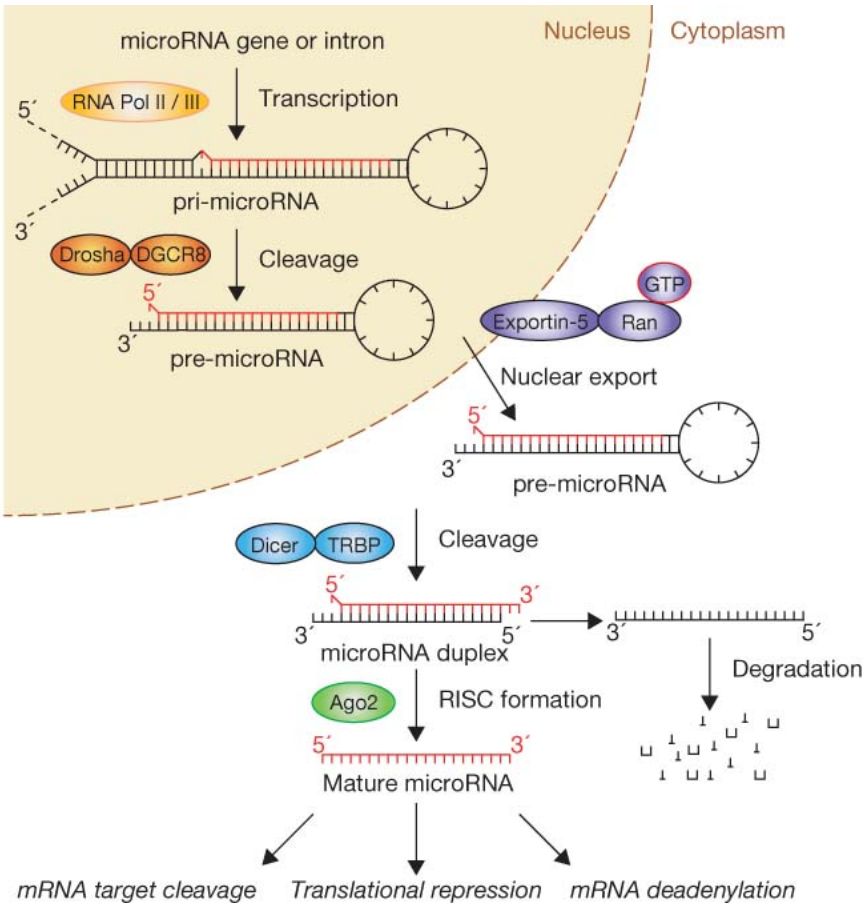
incorporated into the RNA-induced silencing complex (RISC) and one of the Argonaute (Ago) proteins (247,248). Both strands of the miRNA duplex has the potential to be incorporated into RISC (guide strand) or to be degraded (passenger strand), thus one miRNA duplex can produce two individual mature miRNAs (249). RISC can bind to the 3' untranslated region (3'UTR) of the target gene messenger RNA (mRNA) and silences gene expression by either degradation, destabilization or translational inhibition of the target gene mRNA (Figure 1.6.1) (248,250).

MiRNAs bind to their target mRNA through imperfect base pairing. The critical region of binding is characterized by Watson-Crick pairing of the miRNA "seed" region (positions 2-7 of the mature sequence) and the target mRNA (249). The majority of validated conserved targets show canonical binding sites, which have strict complementarity to the miRNA seed region, although bulges and mismatches have been observed in almost all known miRNA-mRNA interactions in animals (249,251). Imperfect binding between miRNA and target mRNA means an individual miRNA can affect a broad range of mRNAs and conversely, a single mRNA can be targeted by multiple miRNAs (252).

Biogenesis of miRNAs is a tightly regulated process but deregulation of miRNA expression has been noted in several human diseases, including cancer. It has been observed that miRNAs are frequently located in cancer-associated genomic regions such as minimal regions of amplification, loss of heterozygosity, fragile sites and common breakpoint regions in or near oncogenes or tumor suppressor genes (253). Furthermore, miRNAs can be controlled by epigenetic mechanisms (254). Investigating miRNA expression using miRNA microarray, deep sequencing,

Figure 1.6.1 Canonical pathway of microRNA biogenesis (255). The production of mature microRNAs (miRNA) starts in the nucleus where the primary miRNA is transcribed by RNA polymerase II or III and subsequently cleaved by the microprocessor complex Drosha-DGCR8/Pasha. The resulting precursor miRNA is exported into the cytoplasm by Exportin-5 and RanGTP where it is further cleaved by the RNase Dicer into ~22-nucleotide imperfect duplexes. The functional strand (red) of the mature miRNA is loaded into RISC along with Argonaute (Ago) proteins and directs RISC to silence target messenger RNA (mRNA) through mRNA cleavage, translational repression or deadenylation. The passenger strand (black) is degraded.

FIGURE 1.6.1


microRNA gene or intron
Nucleus
Cytoplasm
RNA Pol II / III
Transcription
5´
3´
pri-microRNA
Drosha
DGCR8
Cleavage
5´
3´
pre-microRNA
GTP
Exportin-5
Ran
Nuclear export
5´
3´
pre-microRNA
Dicer
TRBP
Cleavage
5´
3´
3´
5´
microRNA duplex
3´
5´
Degradation
Ago2
RISC formation
5´
3´
Mature microRNA
mRNA target cleavage
Translational repression
mRNA deadenylation

quantitative real-time PCR, flow cytometry and other tools have permitted the analysis of the entire known miRNANome (defined as the full spectrum of miRNAs expressed in a particular cell type). To date, altered miRNA expression has been associated in almost all types of cancer where miRNAs have been shown to have both oncogenic and tumor suppressor functions (256-258). Current research is focused on using miRNAs as clinical markers for cancer diagnosis and prognosis since miRNA expression patterns strongly correlate with tumor type and stage, as well as using miRNAs as therapeutic agents by inhibiting or re-expressing miRNAs to regulate a particular gene of interest (253,259).

Although miRNAs have been heavily studied in many cancers, miRNA involvement in breast cancer has not been researched extensively. In particular, little is known about miRNAs in the HER2 signaling network. Thus, characterizing the role of miRNAs in HER2 tumorigenesis and therapeutic response is critically needed.

1.7 THESIS AIMS

The purpose of this dissertation is to demonstrate that HER2Δ16 represents the transforming form of the HER2 oncoprotein and to decipher the molecular mechanisms underlying HER2Δ16 activity contributing to therapeutic evasion. Currently, the contribution of HER2Δ16 signaling in HER2-driven breast tumor progression and therapeutic response is underappreciated. My studies suggest that there is a critical need to develop clinical assays that can detect HER2Δ16 expression in breast cancer tissues so subsequent clinical management decisions

can be based on the presence or absence of HER2Δ16 expression. Most importantly, this work underscores the need for more novel therapeutic strategies to disengage HER2Δ16 oncogenic signaling contributing to metastatic disease and trastuzumab resistance.

Specific Aim 1: To investigate the role of microRNAs in HER2Δ16 tumorigenesis.

HER2-positive breast cancer is a complex, heterogeneous disease involving a variety of changes in gene expression. Traditionally, research has focused on alterations in protein-coding genes but in recent years, microRNAs (miRNAs) have been shown to influence multiple aspects of tumorigenesis and tumor cell response to therapy (256). With the goal of identifying microRNA suppressors of HER2Δ16 oncogenic activity, we used a gene array strategy to compare the microRNA expression profiles of MCF-7 to MCF-7/HER2Δ16 cells. We found that expression of HER2Δ16 significantly altered expression of 16 microRNAs by 2-fold or more including a 4.8 fold suppression of the tumor suppressor miR-7. We hypothesize that HER2Δ16 promotes breast tumor cell proliferation and invasion, in part, through suppression of miR-7 and the subsequent upregulation of miR-7 targets.

Specific Aim 2: Identify trastuzumab-induced molecular alterations contributing to resistance

Given that intrinsic and acquired trastuzumab resistance is a major clinical problem, it is crucial to identify and validate new biomarkers that can predict patient response to specific anti-HER2 therapy. We have shown that breast cancer

cells expressing HER2Δ16 are refractory to trastuzumab treatment and activate multiple oncogenic signaling pathways (202). Our data shows that in response to trastuzumab treatment, HER2Δ16 phosphorylates p70S6 kinase 1 (p70S6K1) leading to its activation. We hypothesize that HER2Δ16 activates p70S6K1 to promote prosurvival signaling via the phosphoinositide 3-kinase (PI3K)/AKT pathway and evade trastuzumab therapy.

CHAPTER 2 – MATERIALS AND METHODS

2.1 CELL LINES

The human breast cancer cell lines MCF-7, SKBR3, BT474, SUM190PT and SUM225CWN were purchased from the American Type Culture Collection and cultured according to their instructions. The stable MCF-7 cell line expressing pcDNA3 or the two independent cell lines expressing pcDNA3-HER2Δ16, referred to here as MCF-7/pcDNA, MCF-7/HER2Δ16H, and MCF-7/HER2Δ16M1, respectively, have been described elsewhere (232). For stable expression of miR-7 to generate the pooled MCF-7/HER2Δ16H/miR-7 cell line, MCF-7/HER2Δ16H cells were transfected with the miR-7 expression vector MI0000263 (Origene) using the NEON Transfection System as described by the manufacturer (Invitrogen). At two days post-transfection all pooled cell lines were selected for two days in 1 μg/ml puromycin (Gibco) and then maintained at 0.2 μg/ml puromycin.

2.2 MICRORNA EXPRESSION PROFILING

Four independent total RNA samples from MCF-7/pcDNA and MCF-7/HER2Δ16H cells were purified using the miRVANA RNA Isolation System (Life

Technologies) according to the manufacturer's instructions and RNA integrity was confirmed using a bioanalyzer (Agilent). Microarray assay was performed and analyzed using a service provider (LC Sciences) and LC-Science microRNA array miRNAHuman_11.0, which detects miRNA transcripts, listed in Sanger miRNABase Release 11.0.

2.3 QUANTITATIVE REAL-TIME PCR

Triplicate total RNA samples were purified using the miRVANA RNA Isolation System (Life Technologies) according to the manufacturer's instructions and RNA integrity was confirmed using a bioanalyzer (Agilent). First-strand complementary DNA (cDNA) was synthesized from 1.0 µg of total RNA in a 20 µl reaction volume using the Superscript III First-Strand Synthesis System (Life Technologies). 7500 Fast Real-Time PCR system (conditions as follows: 40 cycles of 50°C for 2 min, 95°C for 10 min, and 60°C for 15 sec followed by 60°C for 1 min), exactly as described by the manufacturer (Applied Biosystems). The C_T analysis for each reaction was performed using the supplied 7500 Software v2.0.5 (Applied Biosystems).

MicroRNA-7 Expression: A RNU44 (Applied Biosystems) or dme-miR-7 RT-primer (Applied Biosystems) was used in the cDNA synthesis exactly as described by the manufacturer. Following reverse transcription, 180 µl of water was added to the cDNA reaction and 2 µl of the diluted cDNA was used in a 20 µl TaqMan MicroRNA Assay using the RNU44 or dme-miR-7 probe set with TaqMan Universal PCR Master Mix (Applied Biosystems) in the 7500 Fast Real-Time PCR system. MiR-

7 levels were normalized to RNU44 and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

HER2 and HER2Δ16 Expression: Reverse transcription used random primers. Following reverse transcription, 180 µl of water was added to the cDNA reaction and 2 µl of the diluted cDNA was used in a 20 µl SYBR Green Assay using the following primers: wild-type HER2 forward 5′-GTGTGGACCTGGATGACAAGGG, HER2Δ16 forward 5′-CACCCACTCCCCTCTGAC spanning the junction between HER2 exons 15 and 17, and the same reverse primer for both HER2 and HER2Δ16 5′-GCTCCACCAGCTCCGTTTCCTG, (Figure 2.3.1). HER2 and HER2Δ16 expression were normalized to GAPDH and relative expression was calculated using the $2^{-\Delta CT}$ method.

2.4 WESTERN BLOT ANALYSIS

Total cell lysates were prepared in RIPA Buffer (10 mM $NaPO_4$, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Nonidet P40) containing Complete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). Lysates were combined with NuPAGE LDS Sample Buffer (4X) (Life Technologies) and NuPAGE Sample Reducing Agent (10X) (Life Technologies). Gel electrophoresis was performed using 20 µg of protein per lane in a NuPAGE 4-12% Bis-Tris Gel (Life Technologies) and transferred to an Immobilon-FL 0.45 µm Pore Size Transfer Membrane (Millipore) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked and all antibody dilutions were performed in 5% BSA (Sigma) in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20). All washes were performed in TBST. Primary antibodies

FIGURE 2.3.1

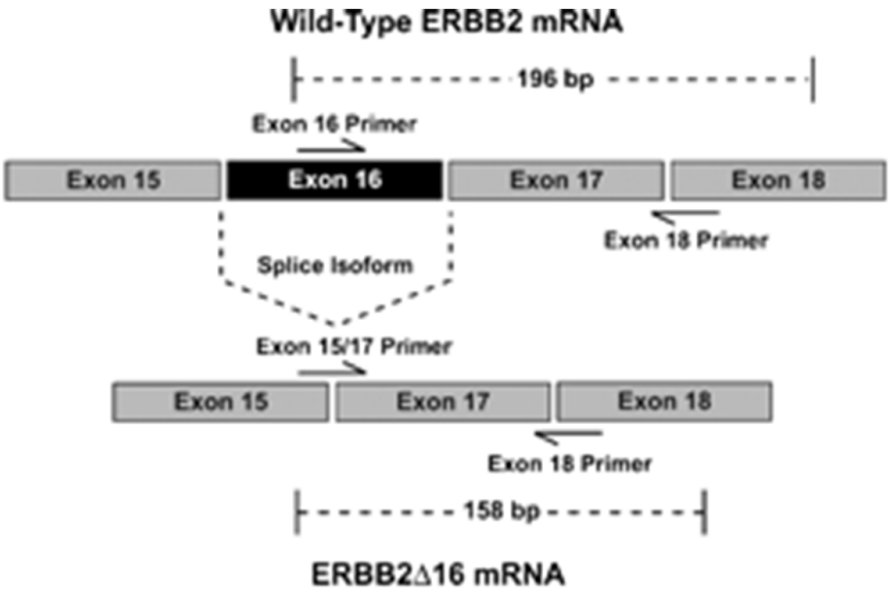


Figure 2.3.1 A schematic of mRNA of wild-type HER2 and HER2Δ16 with relative positions of RT-PCR primers (202).

included α-tubulin (Upstate Biotechnology, 05829), HER2 (Neomarkers, MS-325-P0), EGFR (Cell Signaling Technology, 4267), PAK1 (Cell Signaling Technology, 2602), Src (Cell Signaling Technology, 2110), Phospho-Src Y416 (Cell Signaling Technology, 6943), FAK (Cell Signaling Technology, 3285), FAK Y576/577 (Cell Signaling Technology, 3281), SF2/ASF (3G268) (Santa Cruz Biotechnology, sc-73026), P70S6 Kinase α T229 (Abcam, 58298), P70S6 Kinase α T389 (Cell Signaling Technology, 9205), S6 (Cell Signaling Technology, 2217) and S6 S235/S236 (Cell Signaling Technology, 4859). Secondary antibodies were Alexa Fluor Conjugated Affinity Purified Anti-Rabbit or Anti-Mouse IgG (Li-Cor, 926-68071, 926-32210) detected using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

2.5 RNAI-MEDIATED GENE EXPRESSION KNOCKDOWN

For stable suppression of EGFR to generate the pooled MCF-7/HER2Δ16/EGFRKD cell line, MCF-7/HER2Δ16H cells were transfected with either the MISSION shRNA plasmid-DNA TRCN0000121329 targeting EGFR (Sigma) or a pLKO.1 (Sigma) negative control using Fugene6 (Roche). For stable suppression of miR-7 to generate the pooled MCF-7/miR-7KD cell line, MCF-7 cells were transfected with the miRNAZip-7 anti-miR-7 microRNA construct MZIP7-PA-1 (System Biosciences) or the pGreenPuro Scramble Hairpin Control construct MZIP000-PA-1 (System Bioscience) using Fugene6. At two days post-transfection all pooled cell lines were selected for two days in 1 μg/ml puromycin (Gibco) and then maintained at 0.2 μg/ml puromycin. MISSION shRNA plasmid-DNA targeting

P70S6K1 include TRCN0000003158, TRCN0000003159, TRCN0000195109, TRCN0000003160 and TRCN0000314934 (Sigma).

2.6 COLONY FORMATION ASSAY

Cells were plated at 1,000 cells per well in a 6-well plate and media was replaced every two days for 12 days total. For trastuzumab treatments, trastuzumab was added to media at 10 μg/ml and media was replaced with fresh control or trastuzumab containing media every two days during the experiment. Colonies were fixed in 100% methanol and stained with crystal violet. Colony number and diameter were calculated using a ColCount Colony Counter (Oxford Optronix) and data was analyzed using the supplied statistical software.

2.7 CELL CYCLE ANALYSIS

Cells were synchronized in serum-free MEM for 24 hrs and then cultured in MEM with 10% FBS for an additional 24 hrs. Trypsin treated cells were fixed in 100% ethanol overnight. Fixed cells were stained with Guava Cell Cycle Reagent (Millipore) and the cell cycle was analyzed in a Guava Easy Cyte Mini Base System (Millipore) using Guava Cytosoft™ version 4.2 software (Millipore) according to the manufacturer's instructions.

2.8 CELL MIGRATION ASSAY

Cell migration was determined using the xCELLigence System (Roche) with the CIM-Plate 16 and RTCA DP Instrument (Roche) according to the manufacturer's

instructions. Briefly, 40,000 cells were added to the upper CIM-Plate 16 chamber in media containing 0.2% fetal bovine serum (FBS). Media with 10% FBS was added to the lower CIM-Plate 16 chamber and the CIM-Plate 16 was incubated in the RTCA DP Instrument for 48 hrs. Cell migration as a function of real-time changes in electrical impedance was monitored by the xCELLigence System. Cell Index (referred to here as Migration Index) and standard deviation of replicates were calculated using the supplied RTCA Software (Roche).

2.9 PROTEOME PROFILER ARRAY

The Human Phospho-kinase Array kit, (R&D Systems, ARY003) was used to profile phosphorylation events downstream of various signaling pathways. Total cell lysates were prepared in Lysis Buffer 6 (provided in array kit) and 150 ug of protein were applied to nitrocellulose membranes containing capture and control antibodies spotted in duplicate and incubated overnight on a rocker at 4°C. The arrays were washed to remove unbound proteins followed by a 2-hour incubation with a cocktail of biotinylated detection antibodies. The secondary antibody used was IRDye 800 CW Streptavidin (Li-Cor, 926-32230) and detected using an Odyssey Infrared Imaging System (LiCor Biosciences).

2.10 CELL VIABILITY ASSAY

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7570) measures the presence of ATP, an indicator of metabolically active cells. Cells were plated in white-walled, white-bottomed plates at 3,000 cells/well for the SKBR3 and

BT474 cells and 5,000 cells/well for the SUM190PT and SUM225CWN cells. The following day, cells were treated with 20 μg/ml trastuzumab, 10 nM Rapamycin, 5 μM PF-4708671 or a combination of trastuzumab and rapamycin or trastuzumab and PF-4708671. Cells were incubated for 5 days post-drug treatment. Cell plates were equilibrated at room temperature for 30 minutes and lysed with 100 μl of CellTiter-Glo enzyme/substrate mix. Luminescent signal was allowed to stabilize for 10 minutes before being recorded by a luminometer.

2.11 DRUGS

Herceptin (trastuzumab) is a monoclonal antibody targeting the extracellular domain of HER2 and was purchased from Genentech. 10 mg/ml working stock was prepared in sterile water and made fresh for each use. Rapamycin is an antifungal macrolide and immunosuppressant that specifically inhibits the mammalian target of rapamycin (mTOR)/S6 kinase (S6K)/ribosomal S6 protein (S6) pathway. Cells were treated with rapamycin (Cell Signaling, 9904, resuspended in ethanol) from 100 nM to 100 pM and assessed by Western blot for reduced S6 phosphorylation to determine the lowest concentration of rapamycin to use in the cell viability assay. PF-4708671 (Sigma, PZ0143) is a small molecule inhibitor of S6K1 and first characterized by Pearce *et al* (260). To determine the optimal treatment concentration of PF-4708671 to inhibit cell growth, a dose response treatment ranging from 10 μM to 0.1 μM was performed on the SKBR3, BT474, SUM190PT and SUM225CWN cells. Based on the cell viability assay results, 5 μM PF-4708671 was

selected for use and Western blot analysis confirmed this concentration of PF-4708671 inhibited phosphorylation of ribosomal S6 protein.

CHAPTER 3 – SPECIFIC AIM 1

Investigate the Role of MicroRNAs in HER2Δ16 Tumorigenesis.

3.1 RATIONALE AND HYPOTHESIS

Breast cancer is the most commonly diagnosed cancer in North American women and the second leading cause of cancer related deaths. At least five different molecular breast cancer subtypes have been identified and each subtype is associated with significantly different patient outcomes (4,6). The HER2 positive subtype represents 20-30% of breast cancers and patients with HER2 positive tumors have the shortest overall survival. Furthermore, patients with tumor expression of an activated and presumably highly oncogenic HER2 receptor have an even worse prognosis (115).

One tumor specific event that results in clinical activation of HER2 is expression of the alternatively spliced and constitutively active HER2Δ16 isoform. HER2Δ16 is co-expressed with HER2 in nearly 50% of HER2 positive breast tumors (261). Significantly, 90% of patients with tumor expression of HER2Δ16 present with disseminated metastatic disease. In contrast, breast tumors that overexpress wild-type HER2, but lack detectable HER2Δ16 expression, are significantly

associated with favorable clinicopathological markers including lymph node negative cancer (261). When overexpressed in breast tumor cells, HER2Δ16 promotes resistance to multiple endocrine therapies (228,233), as well as, the HER2 targeted therapy trastuzumab (261). These clinical and experimental observations suggest that HER2Δ16 expression drives HER2 positive breast cancer to an aggressive and therapeutic refractory metastatic disease. Although the molecular basis of HER2Δ16 oncogenic activity remains to be deciphered, recent studies indicate that HER2Δ16 expression alters microRNA (miRNA) expression to evade therapeutic intervention (228,233).

MiRNAs are a class of short non-coding single-stranded RNAs that regulate gene expression. Specific binding of miRNAs to the 3' untranslated region (UTR) of target gene mRNA results in suppressed target gene translation which may also be associated with degradation of the target gene mRNA. Although miRNAs play key roles during normal developmental processes, deregulation of miRNA expression has been noted in several human cancers where miRNAs have been shown to have both oncogenic and tumor suppressor functions (256-258). MiR-7 has been shown to suppress breast tumorigenesis by reducing expression of multiple target genes including epidermal growth factor receptor (EGFR) (262), p21-activated kinase 1 (PAK1) (263), focal adhesion kinase (FAK) (264), and krupple-like factor 4 (KLF4) (265). Here we show that breast tumor cells expressing oncogenic HER2Δ16 have reduced expression of the miR-7 tumor suppressor. Accordingly, reintroduced miR-7 expression suppressed HER2Δ16 oncogenic activity by inhibiting expression of EGFR and independently inactivating Src kinase.

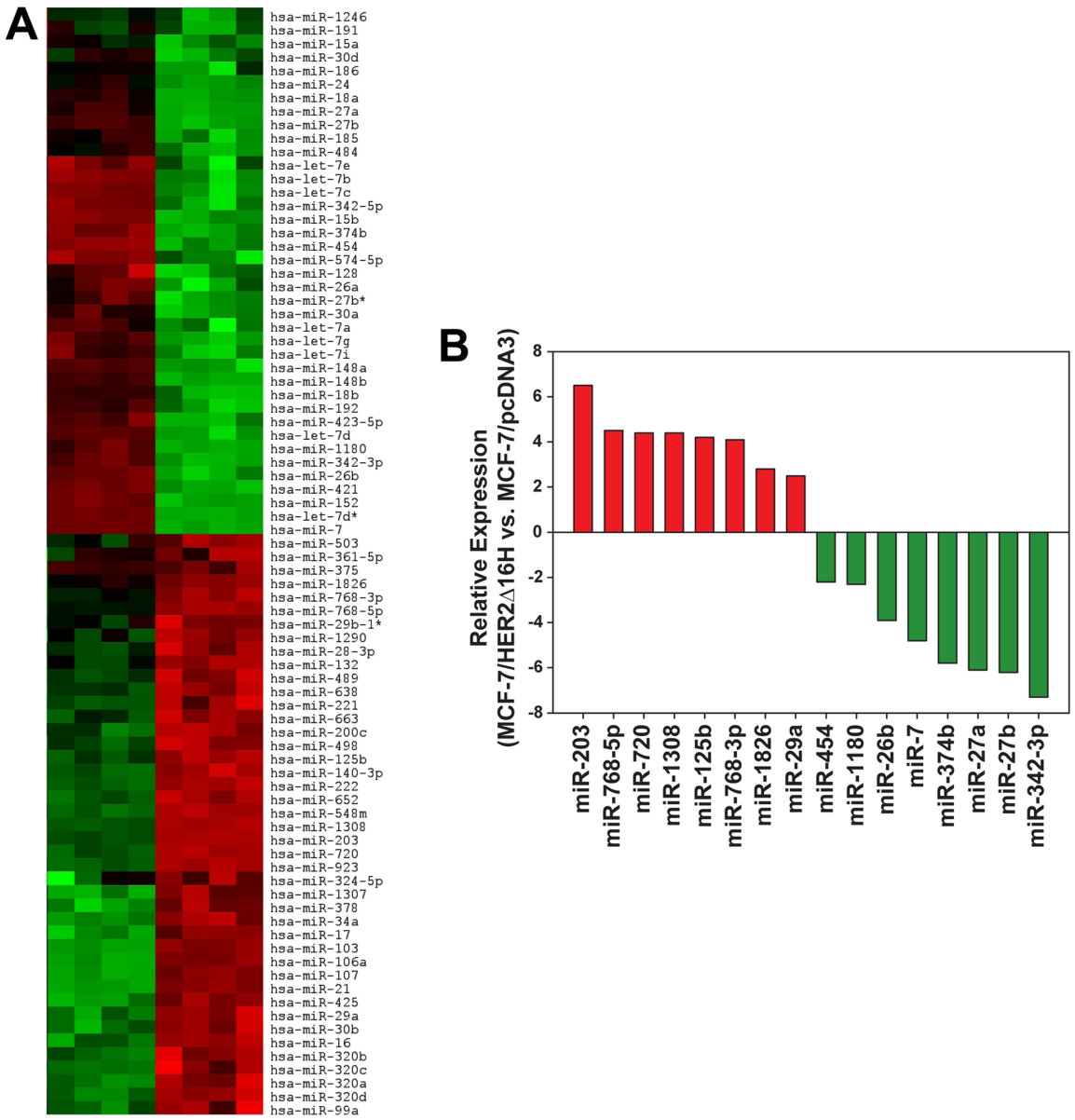
3.2 RESULTS AND DISCUSSION

3.2.1 HER2Δ16 alters expression of multiple miRNAs involved in breast tumorigenesis.

Clinical and experimental evidence suggests that wild-type HER2 is a relatively weak breast oncogene when compared to the aggressive therapeutic refractory phenotype associated with breast tumors expressing the constitutively active HER2Δ16 isoform (228,233,261,266). MiRNAs potentially regulate multiple properties of tumorigenesis and therapeutic response. Accordingly, we have recently shown that HER2Δ16 expression alters expression of miR-15a/16 and miR-342 to promote endocrine resistance of breast tumor cells (228,233). To investigate the potential role of miRNA expression contributing to HER2Δ16 driven tumorigenesis we compared global miRNA expression profiles between parental MCF-7 breast cancer cells (MCF-7/pcDNA) and a MCF-7 cell line with ectopic expression of HER2Δ16 (MCF-7/HER2Δ16H) (261). Probing a LC Sciences miRNA array containing 837 unique human miRNAs we found that HER2Δ16 expression significantly ($p < 0.01$) altered the expression of 82 miRNAs (Figure 3.1A) with 16 miRNAs altered by 2-fold or greater (Figure 3.1B). Of the 16 miRNAs altered in HER2Δ16 expressing cells at least three are consistent with a role in HER2Δ16 driven tumorigenesis. For example, we have previously shown that the suppression of miR-342-3p (the most dramatically altered miRNA) contributes to endocrine resistance of HER2Δ16 expressing breast tumor cells (233). Upregulation of miR-125b expression has been suggested to regulate chemosensitivity of breast tumor cells (267,268). For the current studies we focused our attention on the 4.8 fold

Figure 3.1 Altered miRNA expression in response to HER2Δ16 expression in MCF-7 cells (269). (A) Heat map of all microRNAs significantly ($p < 0.01$) altered by 1.2-fold or greater or (B) by 2-fold or greater in MCF-7/HER2Δ16H cells when compared to MCF-7/pcDNA cells. Data represents four independent RNA samples for each cell line.

FIGURE 3.1


A
hsa-miR-1246
hsa-miR-191
hsa-miR-15a
hsa-miR-30d
hsa-miR-186
hsa-miR-24
hsa-miR-18a
hsa-miR-27a
hsa-miR-27b
hsa-miR-185
hsa-miR-484
hsa-let-7e
hsa-let-7b
hsa-let-7c
hsa-miR-342-5p
hsa-miR-15b
hsa-miR-374b
hsa-miR-454
hsa-miR-574-5p
hsa-miR-128
hsa-miR-26a
hsa-miR-27b*
hsa-miR-30a
hsa-let-7a
hsa-let-7g
hsa-let-7i
hsa-miR-148a
hsa-miR-148b
hsa-miR-18b
hsa-miR-192
hsa-miR-423-5p
hsa-let-7d
hsa-miR-1180
hsa-miR-342-3p
hsa-miR-26b
hsa-miR-421
hsa-miR-152
hsa-let-7d*
hsa-miR-7
hsa-miR-503
hsa-miR-361-5p
hsa-miR-375
hsa-miR-1826
hsa-miR-768-3p
hsa-miR-768-5p
hsa-miR-29b-1*
hsa-miR-1290
hsa-miR-28-3p
hsa-miR-132
hsa-miR-489
hsa-miR-638
hsa-miR-221
hsa-miR-663
hsa-miR-200c
hsa-miR-498
hsa-miR-125b
hsa-miR-140-3p
hsa-miR-222
hsa-miR-652
hsa-miR-548m
hsa-miR-1308
hsa-miR-203
hsa-miR-720
hsa-miR-923
hsa-miR-324-5p
hsa-miR-1307
hsa-miR-378
hsa-miR-34a
hsa-miR-17
hsa-miR-103
hsa-miR-106a
hsa-miR-107
hsa-miR-21
hsa-miR-425
hsa-miR-29a
hsa-miR-30b
hsa-miR-16
hsa-miR-320b
hsa-miR-320c
hsa-miR-320a
hsa-miR-320d
hsa-miR-99a
B
Relative Expression
(MCF-7/HER2Δ16H vs. MCF-7/pcDNA3)
8
6
4
2
0
-2
-4
-6
-8
miR-203
miR-768-5p
miR-720
miR-1308
miR-125b
miR-768-3p
miR-1826
miR-29a
miR-454
miR-1180
miR-26b
miR-7
miR-374b
miR-27a
miR-27b
miR-342-3p

suppression of miR-7. Significantly, miR-7 is considered a potent tumor suppressor miRNA and has been shown to regulate expression of multiple target genes in breast tumor cells (262-265).

Although the exact mechanism mediating HER2Δ16 suppression of miR-7 remains to be established our previously published data suggests that the Jumonji/ARID1 B (JARID1B) transcriptional repressor may play a role. JARID1B is a breast oncogene most dramatically overexpressed in HER2 positive breast tumors (270-272). We have shown that JARID1B transcriptionally represses the expression of multiple tumor suppressor miRNAs in breast tumor cell lines (273). Significantly, over 90% of the JARID1B regulated miRNAs, including miR-7, are also similarly altered by HER2Δ16 expression. Although experimental validation is required, these observations raise the possibility that JARID1B transcriptionally represses multiple miRNAs, including miR-7, in HER2Δ16 expressing breast tumor cells.

3.2.2 MicroRNA-7 suppresses breast tumor cell proliferation and HER2Δ16 driven cell migration.

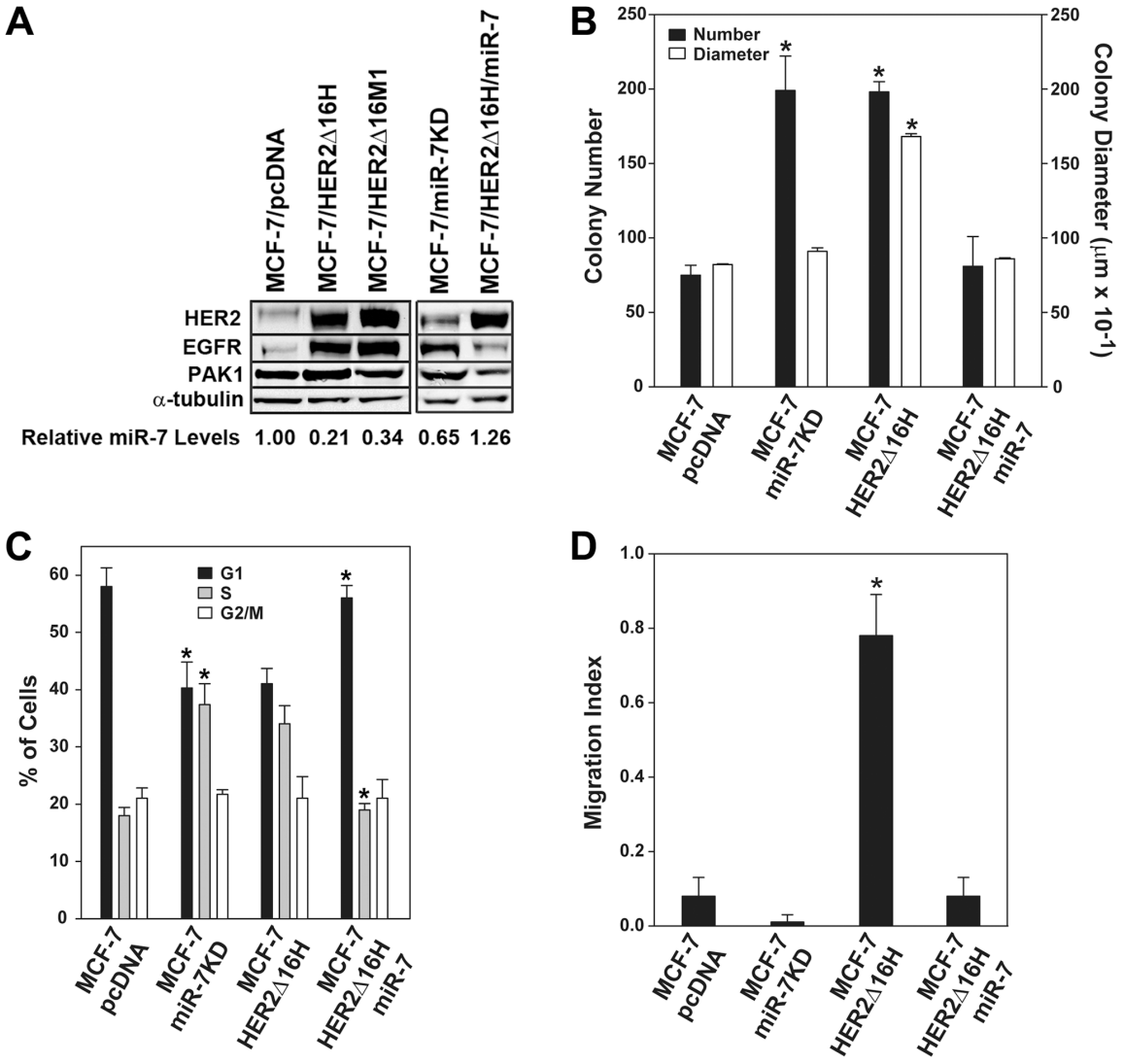
We have previously shown that HER2Δ16 expression significantly potentiates MCF-7 cell proliferation, migration/invasion, xenograft tumor formation, and resistance to multiple therapeutic interventions; whereas, expression of wild-type HER2 failed to enhance a single tumorigenic property of MCF-7 cells (228,233,261,266). Given the potent oncogenic activity of HER2Δ16 and the clinical association of HER2Δ16 with metastatic breast cancer we investigated the impact of miR-7 activity on HER2Δ16 driven tumorigenesis.

MiR-7 expression was suppressed by 3 to 5-fold in two independent HER2Δ16 expressing MCF-7 cell lines when compared to the MCF-7 parental cell line (Figure 3.2A). Modulation of miR-7 expression had a dramatic impact on the miR-7 target gene EGFR (274) with the highest levels of EGFR expression associated with reduced levels of miR-7 in the MCF-7/HER2Δ16H, and MCF-7/HER2Δ16M1 cell lines (Figure 3.2A). Consistent with these observations, knockdown of miR-7 expression in the MCF-7 cell line, MCF-7/miR-7KD, resulted in enhanced EGFR expression whereas reintroduced miR-7 expression in the MCF-7/HER2Δ16H cell line, MCF-7/HER2Δ16H/miR-7, resulted in suppressed EGFR expression. A similar but less dramatic impact on the miR-7 target gene PAK1 (263) was also observed in the modified MCF-7 cell lines. Altered miR-7 and EGFR expression was continuously monitored and remained stable in the MCF-7/miR-7KD and MCF-7/HER2Δ16H/miR-7 cell lines for greater than 20 cell passages. These results suggest that selective suppression of miR-7 and subsequent restoration of EGFR expression occurs in response to ectopic HER2Δ16 expression in MCF-7 cells.

We next determined the impact of altered miR-7 expression levels on breast tumor growth in a colony formation assay. Consistent with previous reports (261) expression of HER2Δ16 in the MCF-7 cell line had a significant impact on colony number and diameter (Figure 3.2B). In concordance with its role as a tumor suppressor, reestablished miR-7 expression to generate the MCF-7/HER2Δ16H/miR-7 cell line significantly reduced the number and diameter of MCF-7/HER2Δ16H colonies to levels equivalent to the MCF-7/pcDNA cell line (Figure 3.2B). Conversely, suppression of MCF-7 miR-7 expression to generate the

Figure 3.2 Altered miR-7 expression regulates breast tumor cell proliferation and migration (269). MiR-7 expression was stably suppressed in the MCF-7 cell line to generate the MCF-7/miR-7KD cell line and a miR-7 expression plasmid was stably introduced into the MCF-7/HER2Δ16H cell line to generate the MCF-7/HER2Δ16H/miR-7 cell line. (A) Western blot analysis of the MCF-7 vector control, MCF-7/pcDNA and the HER2Δ16 expressing MCF-7/HER2Δ16H and MCF-7/HER2Δ16M1 and cell lines with altered miR-7 expression. Relative miR-7 expression levels are indicated below each lane as determined by qRT-PCR. (B) Colony formation assay where colony number and diameter were calculated using a ColCount Colony Counter with supplied statistical software. Asterisk indicates cell lines significantly greater than MCF-7/pcDNA and MCF-7/HER2Δ16H/miR-7 as determined by paired Student's *t* test ($p < 0.006$). (C) Cell cycle analysis was performed in a Guava Easy Cyte Mini Base System with supplied statistical software. Asterisk indicates cell cycle phase significantly different from parental cell line as determined by paired Student's *t* test ($p < 0.01$). (D) Cell migration was determined in an xCELLigence CIM-Plate 16 with the RTCA DP Instrument for 48 hrs. Cell Index (referred to here as Migration Index) was calculated using the supplied RTCA Software. Asterisk indicates that MCF-7/HER2Δ16H is significantly greater than the other tested cell lines as determined by paired Student's *t* test ($p < 0.005$). (B-D) The data represents the mean +/- SE of at least three independent experiments.

FIGURE 3.2


A
MCF-7/pcDNA
MCF-7/HER2Δ16H
MCF-7/HER2Δ16M1
MCF-7/miR-7KD
MCF-7/HER2Δ16H/miR-7
HER2
EGFR
PAK1
α-tubulin
Relative miR-7 Levels 1.00 0.21 0.34 0.65 1.26
B
Number
Diameter
Colony Number
Colony Diameter (μm x 10-1)
MCF-7 pcDNA
MCF-7 miR-7KD
MCF-7 HER2Δ16H
MCF-7 HER2Δ16H miR-7
C
G1
S
G2/M
% of Cells
D
Migration Index

MCF-7/miR-7KD cell line resulted in a significant increase in colony number to levels equivalent to ectopic expression of HER2Δ16 (Figure 3.2B). We investigated the mechanistic basis of miR-7 tumor suppressor activity by performing cell cycle analysis. We observed a significant increase in cells arrested at G1 of the cell cycle with lower levels of S phase cells in the MCF-7/pcDNA and MCF-7/HER2Δ16/miR-7 cell lines both with enhanced expression of miR-7 (Figure 3.2C). In contrast, G1 arrest is released in the MCF-7/miR-7KD and MCF-7/HERΔ16 cell lines with suppressed miR-7 expression and these cells exhibit an increase in S phase cells (Figure 3.2C). Cell cycle analysis suggests that miR-7 suppresses tumor cell growth by inducing a G1 arrest with a concomitant reduction in proliferating S phase cells. This result corroborates a recent study that described a similar G1 arrest when miR-7 was expressed in Chinese hamster ovary (CHO) cells (275). Taken together our results indicate that suppression of miR-7 is both necessary and sufficient to promote cell cycle progression and significantly enhance colony formation of breast tumor cells.

We have previously shown that ectopic expression of HER2Δ16 induces a dramatic migration/invasion phenotype in the non-invasive MCF-7 cell line (261). To determine if altered miR-7 expression regulates MCF-7 cell migration we performed an xCELLigence migration assay on cell lines with altered miR-7 expression. Our results indicate that suppression of miR-7 expression in the MCF-7/miR-7KD cell line is not sufficient to promote MCF-7 cell migration (Figure 3.2D). Consistent with our previous observations HER2Δ16 expression in the MCF-7/HER2Δ16H cell line caused a significant increase in MCF-7 cell migration (Figure

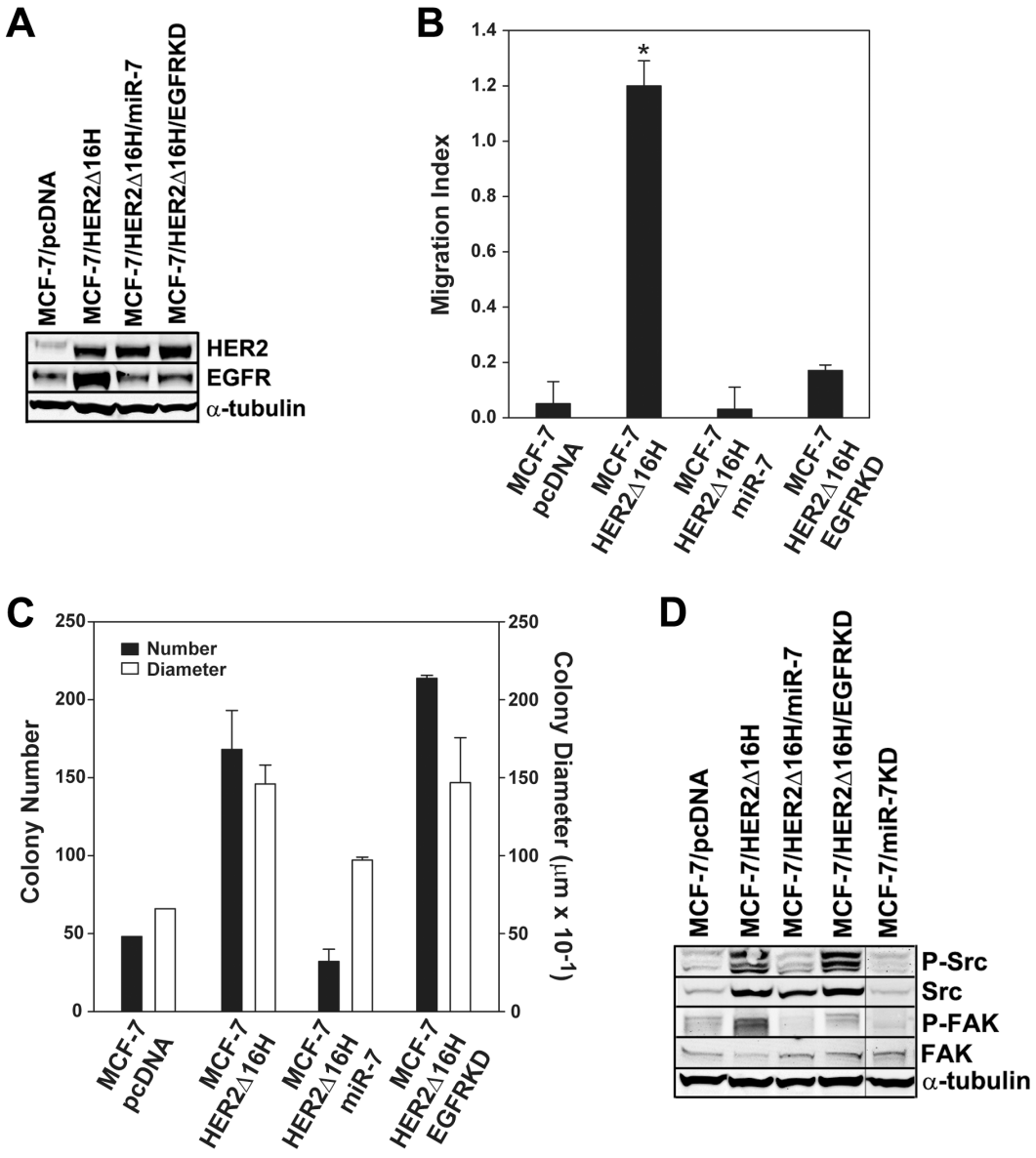
3.2D). Interestingly, reestablished expression of miR-7 in the MCF-7/HER2Δ16H/miR-7 cell line completely abolished cell migration and reduced the migration index to levels observed for parental MCF-7 cells (Figure 3.2D). These results indicate that suppression of miR-7 is necessary but, in contrast to tumor cell proliferation, not sufficient to promote breast tumor cell migration.

3.2.3 MiR-7 regulates multiple oncogenic pathways that influence HER2Δ16 driven cell proliferation and migration

We investigated the impact of altered miR-7 expression on multiple reported gene targets including FAK (264), insulin-like growth factor 1 receptor (IGF1R) (276), PAK1 (263), and EGFR (262), however, in our experimental system, EGFR was the only target that was consistently altered in response to modulated miR-7 expression (Figure 3.2A, Figure 3.3D). We therefore determined if EGFR is the miR-7 target gene that contributes to HER2D16 oncogenic activity. Using shRNA we inhibited EGFR expression in the MCF-7/HER2Δ16H cell line (MCF-7/HER2Δ16H/EGFRKD) (Figure 3.3A) and determined the impact of suppressed EGFR expression on HER2D16 mediated colony formation and migration. Suppression of EGFR expression in the MCF-7/HER2Δ16H/EGFRKD cell line essentially abolished cell migration of MCF-7/HER2Δ16H cells reducing their migration activity to levels similar to parental MCF-7/pcDNA and MCF-7/HER2Δ16H/miR-7 cells (Figure 3.3B). Surprisingly, suppression of EGFR failed to impact colony formation activity in the MCF-7/HER2Δ16H/EGFRKD cell line (Figure 3.3C). EGFR therefore appears to be an essential component of the HER2Δ16 cell migration pathway; however, EGFR signaling is dispensable for HER2Δ16 colony

Figure 3.3 MiR-7 regulates HER2Δ16 induced cell migration and proliferation through different oncogenic pathways (269). A miR-7 expression plasmid was stably introduced into the MCF-7/HER2Δ16H cell line to generate the MCF-7/HER2Δ16H/miR-7 cell line and EGFR was stably knocked down by shRNA to generate the MCF-7/HER2Δ16H/EGFRKD cell line. (A) Altered expression of EGFR in the indicated cell lines was confirmed by western blot analysis. (B) Cell migration was determined in an xCELLigence CIM-Plate 16 with the RTCA DP Instrument for 48 hrs. Cell Index (referred to here as Migration Index) was calculated using the supplied RTCA Software. Asterisk indicates that MCF-7/HER2Δ16H is significantly greater than the other tested cell lines as determined by paired Student's *t* test ($p < 0.001$). (C) Colony formation assay where colony number and diameter were calculated using a ColCount Colony Counter with supplied statistical software. Differences between MCF-7/HER2D16H and MCF-7/HER2Δ16H/EGFRKD were insignificant as determined by paired Student's *t* test. (D) Western blot analysis of the indicated cell lines probed for Src kinase (Src), Src kinase activated phosphorylated at Y416 (P-Src), and Src kinase activity through phosphorylation of FAK at Y576/577 (P-FAK). (B,C) The data represents the mean +/- SE of at least three independent experiments.

FIGURE 3.3


A
MCF-7/pcDNA
MCF-7/HER2Δ16H
MCF-7/HER2Δ16H/miR-7
MCF-7/HER2Δ16H/EGFRKD
HER2
EGFR
α-tubulin
B
Migration Index
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
MCF-7 pcDNA
MCF-7 HER2Δ16H
MCF-7 HER2Δ16H miR-7
MCF-7 HER2Δ16H EGFRKD
C
Number
Diameter
Colony Number
Colony Diameter (μm x 10-1)
250
200
150
100
50
0
MCF-7 pcDNA
MCF-7 HER2Δ16H
MCF-7 HER2Δ16H miR-7
MCF-7 HER2Δ16H EGFRKD
D
MCF-7/pcDNA
MCF-7/HER2Δ16H
MCF-7/HER2Δ16H/miR-7
MCF-7/HER2Δ16H/EGFRKD
MCF-7/miR-7KD
P-Src
Src
P-FAK
FAK
α-tubulin

formation activity. These results indicate that although miR-7 regulation of EGFR expression significantly impacts cell migration, a different miR-7 regulated pathway(s) influences MCF-7/HER2Δ16H colony formation activity.

We have previously shown that Src kinase is an important effector of multiple HER2Δ16 oncogenic activities including cell migration/invasion and colony formation (261). Accordingly, RNAi suppression of Src kinase expression or dasatinib inhibition of Src kinase activity results in the complete loss of HER2Δ16 oncogenic activity in multiple biological assays (261). We therefore examined the influence of miR-7 on Src kinase expression and activity. A slight decrease in Src protein was observed in the miR-7 expressing cell line MCF-7/HER2Δ16H/miR-7 (Figure 3.3D). Src kinase is not a predicted direct target of miR-7 (www.targetscan.org) suggesting that the decrease in expression is due to indirect effects of miR-7 activity.

Interestingly, although miR-7 fails to directly target Src kinase, Src activation through phosphorylation of the regulatory Y416 was completely abolished in the MCF-7/HER2Δ16H/miR-7 cell line (Figure 3.3D). Src activation was however retained in the MCF-7/HER2Δ16H/EGFRKD cell line (Figure 3.3D) indicating that the loss of Src activation in the MCF-7/HER2Δ16H/miR-7 cell line was not due to miR-7 suppression of EGFR. Likewise, Src activation and expression levels remained relatively low in the MCF-7/miR-7KD cell line indicating that expression of EGFR is not sufficient to activate Src kinase (Figure 3.3D).

We examined the impact of miR-7 on Src kinase activity. An important target of Src kinase activity is phosphorylation of FAK at Y576/577 (277) and as predicted

this phosphorylation site is enhanced in the MCF-7/HER2Δ16H cell line (Figure 3.3D; P-FAK) indicating that Src is active in this cell line. Loss of Src activation in the MCF-7/HER2D16H/miR-7 cell line also resulted in abolished Src activity as demonstrated by the loss of FAK Y576/577 phosphorylation (Figure 3.3D). Loss of EGFR resulted in intermediate levels of FAK Y576/577 (Figure 3D) indicating that EGFR is required for maximum Src activity and FAK phosphorylation in the MCF-7/HER2D16 cell line. The reduced levels of FAK phosphorylation may explain the loss of migration associated with EGFR suppression in the MCF-7/HER2Δ16H/EGFRKD cell line.

The mechanistic basis of miR-7 inactivation of Src kinase remains unclear. Common miR target prediction software (MiRanda, PicTar, and TargetScan) failed to detect a consensus miR-7 binding site in the *SRC* gene; however, imperfect miR-7 binding sites identified using RNAhybrid (278) were predicted to be located in the 3'-UTR and 5'-UTR of *SRC*. Although we detected robust suppression of the miR-7 target sequence using the MIR-REPORT (Applied Biosystems) reporter system, miR-7 failed to regulate the imperfect *SRC* miR-7 binding sites (data not shown) in the same experiment. The lack of direct Src kinase regulation by miR-7, suggests that miR-7 indirectly inactivates Src kinase in HER2Δ16 expressing cells through a novel miR-7 target gene or pathway. Src is activated through the actions of multiple different receptor tyrosine kinases, integrins, as well as, G-protein coupled receptors (277,279). It is possible that miR-7 suppresses Src activation by targeting one of these Src regulating pathways.

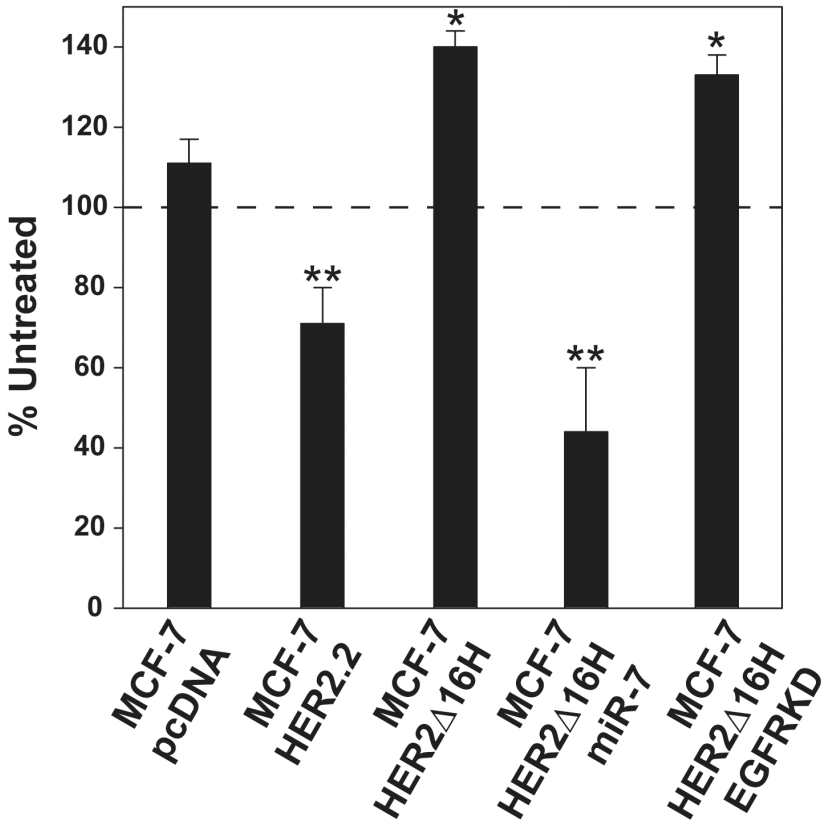
Taken together our results suggest that miR-7 inhibits HER2Δ16 induced cell migration through multiple pathways including suppression of EGFR expression and loss of Src kinase activity. MiR-7 inhibition of HER2Δ16 mediated cellular proliferation, on the other hand, was independent of EGFR suppression but likely involves a miR-7 regulated pathway that drives Src inactivation.

3.2.4 MiR-7 sensitizes refractory HER2Δ16 expressing cells to trastuzumab

We have previously demonstrated that ectopic expression of HER2Δ16 in the MCF-7 cell line promotes trastuzumab resistance (261). In fact we consistently observe enhanced growth of HER2Δ16 expressing cells in response to trastuzumab, implicating trastuzumab as a HER2Δ16 agonist (261). We therefore determined if trastuzumab resistance of HER2Δ16 expressing cells is influenced by altered expression of miR-7 or EGFR. Consistent with our previously published results (261), trastuzumab significantly suppressed colony formation of MCF-7/HER2.2 cells expressing wild-type HER2; whereas trastuzumab significantly enhanced colony formation of the HER2D16 expressing MCF-7/HER2Δ16H cell line (Figure 3.4). Suppression of EGFR expression failed to influence the response of MCF-7/HER2Δ16H/EGFRKD cells to trastuzumab as these cells also exhibited significantly enhanced colony formation activity in response to trastuzumab (Figure 3.4). In contrast, the MCF-7/HER2Δ16H/miR-7 cell line with reestablished expression of miR-7 responded to trastuzumab treatment with a significant reduction in colony formation (Figure 3.4). Importantly, miR-7 not only functions as a potent suppressor of HER2Δ16 tumorigenesis but also reverses HER2Δ16 induced trastuzumab resistance.

Figure 3.4 MiR-7 reverses trastuzumab resistance of HER2Δ16 expressing cells (269). Colony formation of each indicated cell line calculated using a ColCount Colony Counter with supplied statistical software. The MCF-7/HER2.2 cell line expresses wild-type HER2 and is included as a positive control. The data represents the percentage difference in colony number of trastuzumab treated compared to the untreated control +/- SE of at least three independent experiments. Single asterisk or double asterisks indicate trastuzumab treated cell lines with significantly enhanced ($p < 0.04$) or reduced ($p < 0.02$) colony formation, respectively. Significant differences were determined by paired Student's *t* test.

FIGURE 3.4


% Untreated
0
20
40
60
80
100
120
140
*
**
**
*
MCF-7 pcDNA
MCF-7 HER2.2
MCF-7 HER2Δ16H
MCF-7 HER2Δ16H miR-7
MCF-7 HER2Δ16H EGFRKD

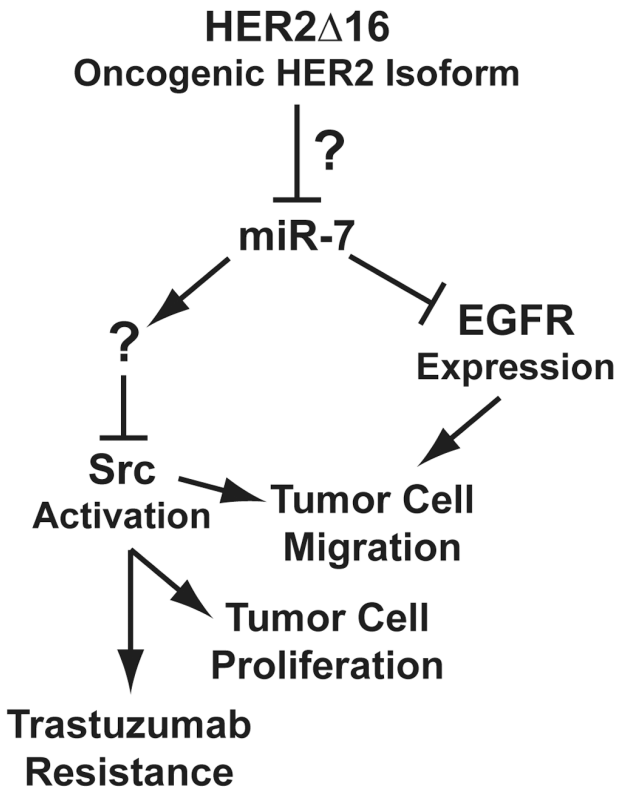
3.3 CONCLUSIONS

Despite the clinical use of the HER2 targeted therapy, trastuzumab, patients with HER2 positive breast tumors have the lowest disease specific survival and a significant percentage of HER2 positive patients fail to benefit from trastuzumab therapy (167,196). We have shown that 90% of patients with tumor expression of the HER2 isoform, HER2Δ16, also present with metastatic disease (261). Furthermore, breast tumor cell expression of HER2Δ16 promotes trastuzumab resistance. We contend that successful treatment of HER2 positive metastatic breast cancer requires a strategy to disengage HER2Δ16 oncogenic signaling. To this end we show that HER2Δ16 suppresses expression of the miR-7 tumor suppressor and reestablished miR-7 expression significantly inhibits HER2Δ16 mediated tumor cell proliferation and migration and miR-7 sensitizes HER2Δ16 expressing cells to trastuzumab treatment (Figure 3.5).

Although tumor delivery of miRs remains a significant clinical challenge, deciphering miR regulated pathways may identify suitable targets for therapy. Our findings that miR-7 suppression of HER2Δ16 oncogenic activity is mediated through inactivation of Src kinase and suppression of EGFR expression (Figure 3.5) implies that targeting these pathways would also suppress HER2Δ16 tumorigenesis. Consistent with a potential role for EGFR in HER2Δ16 tumorigenesis, coexpression of EGFR in breast tumors with activated HER2 is associated with significantly shorter patient survival than patients with tumor expression of activated HER2 or EGFR alone (118). In addition, we and others have demonstrated that targeting Src kinase sensitizes trastuzumab resistant tumors (195,261) independent of EGFR

Figure 3.5 The influence of miR-7 regulated pathways on HER2Δ16 oncogenic activity. Expression of the HER2 oncogenic isoform, HER2Δ16, in the MCF-7 breast tumor cell line results in reduced expression of the miR-7 tumor suppressor. Reintroduced miR-7 expression results in direct repression of EGFR protein expression and indirect suppression of Src activation through a novel intermediate pathway(s). EGFR expression is required for HER2Δ16 driven cell migration, whereas activated Src is an obligate effector of multiple HER2D16 activities including trastuzumab resistance. We propose that reactivation of miR-7 expression would represent an efficacious therapeutic strategy to suppress HER2Δ16 driven metastatic disease and reverse trastuzumab resistance.

FIGURE 3.5


HER2Δ16
Oncogenic HER2 Isoform
?
miR-7
?
EGFR
Expression
Src
Activation
Tumor Cell
Migration
Tumor Cell
Proliferation
Trastuzumab
Resistance

expression. Moreover, we have shown that the Src kinase inhibitor dasatinib is a potent inhibitor of HER2Δ16 mediated breast tumorigenesis (261). In conclusion, our current results showing miR-7 inactivation of Src kinase further implicates Src kinase as an obligate effector of trastuzumab resistance and HER2Δ16 oncogenic activity (Figure 3.5).

CHAPTER 4 – Specific Aim 2

Identify trastuzumab-induced molecular alterations contributing to resistance

4.1 RATIONALE AND HYPOTHESIS

Patients whose breast cancer cells overexpress the HER2 receptor tyrosine kinase are treated with trastuzumab, a humanized recombinant monoclonal antibody that binds to the extracellular domain of the receptor (180). Unfortunately, as with many targeted cancer therapies, not all HER2-positive breast cancer patients respond to trastuzumab and all patients eventually have progressive disease. Research in the past decade in HER2-positive breast cancer has focused on elucidating the molecular basis for trastuzumab resistance, either *de novo* or acquired. As a result, over 600 articles addressing this issue now appear on PubMed. The primary model systems used to study mechanisms of resistance have been cell culture based and utilize HER2-amplified breast cancer cells lines that display sensitivity or primary resistance to trastuzumab. Chronic exposure of clinically relevant doses of trastuzumab to trastuzumab-sensitive breast cancer cell lines has resulted in cell culture models of acquired resistance. The significant

limitation of this model is the lack of contributions of the natural tumor environment, tumor-stroma interactions and host factors. Furthermore, elucidating the molecular basis of trastuzumab resistance has been challenging due to difficulty obtaining tumor samples after tumor progression and in part, due to the multiple modes of pharmacologic action of trastuzumab (280).

4.1.1 Activation of the PI3K/Akt Pathway in Trastuzumab Resistance

Numerous mechanisms of trastuzumab resistance of HER2-positive breast cancer have been reported, including activation of the phosphoinositide 3-kinase (PI3K)-Akt pathway (Figure 4.1), which regulates diverse cellular functions including cell proliferation, differentiation, migration, angiogenesis, apoptosis and survival (281). In its active form, PI3K is composed of a regulator p85 subunit and a catalytic p110 subunit. When activated, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) on the cell membrane to generate phosphatidylinositol (3,4,5)-triphosphate (PIP_3), which serves as a docking site for proteins that harbor pleckstrin-homology (PH) domains. PIP_3 recruits both phosphatidylinositol-dependent kinase 1 (PDK1) and Akt to the membrane and activates PDK1. Akt becomes activated through dual phosphorylation by PDK1 and mammalian target of rapamycin complex 2 (mTORC2) (282). Activated Akt phosphorylates a large number of downstream substrates, including mTOR complex 1 (mTORC1), which subsequently phosphorylates its downstream substrates eIF4E-binding protein-1 (4E-BP1) and ribosomal p70 S6 kinase (p70S6K), resulting in the inactivation of the former and activation of the latter, thus initiating protein translation (283,284). Akt also controls cell cycle progression by phosphorylating and inactivating p27 and

FIGURE 4.1

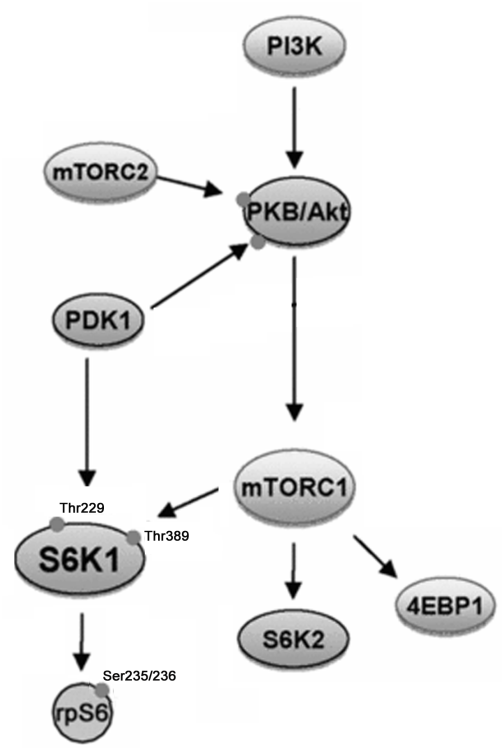


Figure 4.1 PI3K/Akt/mTOR signaling pathway (285).

glycogen synthase kinase 3β (GSK3β) (281). p27 is a cyclin-dependent kinase (CDK) inhibitor protein that controls cell cycle progression at G1. Phosphorylation of p27 by Akt results in its inactivation and translocation from the nucleus to the cytoplasm for degradation, thus allowing for cell cycle progression (281). One function of GSK3β is to phosphorylate cyclin D1 to promote its proteasomal degradation. Inactivation of GSK3β results in stable cyclin D1 expression and drives the G1/S phase transition of the cell cycle (286).

Hyperactivation of the PI3K/Akt pathway frequently occurs in response to HER2 overexpression, loss of function of PTEN or an activating mutation of PIK3CA. PTEN is a tumor suppressor and negatively regulates PI3K by dephosphorylating PIP3 to PIP2. Loss of PTEN occurs in 50% of breast cancers (287) and is associated with poor prognosis and resistance to trastuzumab (216,288). Retrospective studies have shown that activating mutations of the gene encoding the catalytic p110α/PIK3CA subunit of PI3K can induce constitutive activation of the PI3K/Akt pathway and reduce benefit from trastuzumab (289,290). Increased Akt phosphorylation and activity, as well as PDK1 overexpression have also been implicated with trastuzumab resistance (291-293). Several studies have investigated whether PTEN loss, PIK3CA mutations, phosphorylated AKT and phosphorylated p70S6K could predict trastuzumab response in HER2-positive patients, but only PTEN loss was identified as a modulator of trastuzumab sensitivity in retrospective studies (216,288,294,295). Although these studies would implicate PI3K as an important therapeutic target to overcome trastuzumab resistance, drugs targeting PI3K have limited clinical value due to high levels of

toxicity. We hypothesize that the PI3K pathway may be disengaged by eliminating critical downstream effectors such as mTOR.

While multiple signaling molecules that have been proposed as mediators of resistance, there are currently no clinically validated markers predictive of resistance to HER2-targeted therapy. HER2 and the estrogen receptor (ER) are the only validated biomarkers that are used to determine therapeutic decisions in patients with HER2-positive breast cancer (296). There is overwhelming need to understand mechanisms of trastuzumab resistance in order to develop strategies to overcome it, as well as identifying relevant biomarkers that could serve as a tool for patient selection for therapies that mitigate that resistance and to reduce unwanted side effects from overtreatment.

Most studies on trastuzumab resistance have focused on the mechanisms underlying acquired trastuzumab resistance and less attention has been given to primary resistance. We have shown that breast cancer cells expressing HER2Δ16 are refractory to trastuzumab treatment and activate multiple oncogenic signaling pathways (202). We propose that the coexpression of HER2Δ16 in HER2-overexpressing tumors is one mechanism of *de novo* resistance. Thus we set out to investigate how HER2Δ16 expression induces molecular alterations contributing to trastuzumab resistance, Interestingly, in response to trastuzumab treatment, HER2Δ16-expressing cells show hyperactivation of p70 S6 kinase 1 (p70S6K). We hypothesize that breast cancer cells with intrinsic resistance to trastuzumab activate p70S6K to promote prosurvival signaling via the PI3K/Akt pathway. We

predict that inhibition of the PI3K/Akt/mTOR/p70S6K signaling pathway will sensitize trastuzumab-refractory cells to trastuzumab treatment.

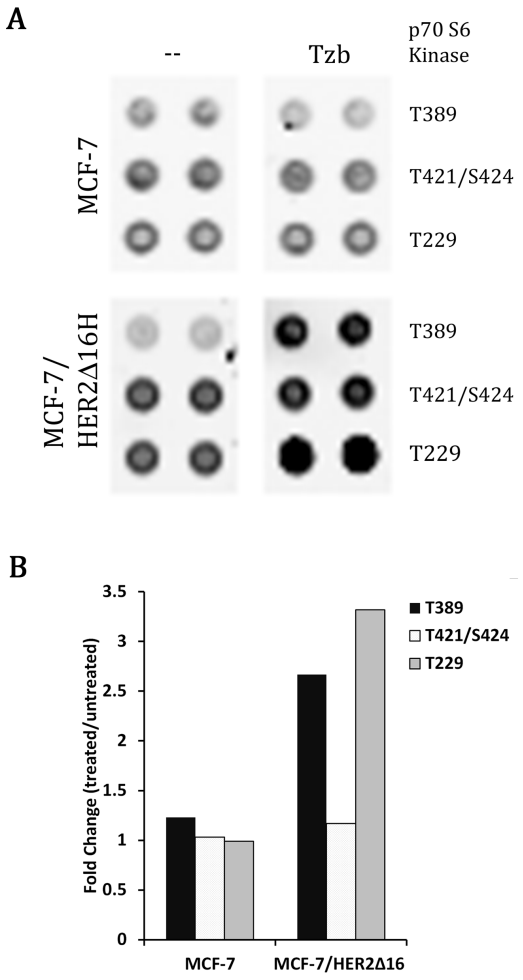
4.2 RESULTS AND DISCUSSION

4.2.1 Trastuzumab treatment of MCF-7/HER2Δ16H cells activates p70 S6 Kinase, a downstream signaling molecule in the PI3K/Akt pathway.

The predominant experimental strategy for identifying drug resistance pathways is to identify differences between sensitive and resistant cell lines, modulate expression or inhibit potential resistance pathways and monitor the impact of pathway loss of function on drug response. However, our laboratory has shown that critical and clinically important resistance pathways may in fact be deregulated and therefore only revealed during drug treatment. To identify potential resistance pathways deregulated during trastuzumab treatment, we used a phosphoproteomic approach to profile a subset of phosphorylation events after the trastuzumab resistant HER2Δ16-overexpressing cells were treated with trastuzumab. Kinase phosphorylation is fundamental in cellular signaling and among the 46 phospho-antibodies investigated, we discovered trastuzumab treatment significantly induced ribosomal p70S6 kinase 1 (p70S6K) phosphorylation in MCF-7/HER2Δ16H stable cells compared to parental MCF-7 cells (Figure 4.2A). Specifically, we observed a 5-fold increase in phosphorylation of p70S6K after 2 hours of trastuzumab treatment at sites T229, T421/S424 and T389 compared to the untreated cells (Figure 4.2B). Phosphorylation at T421/S424 is believed to induce conformational changes allowing for sequential phosphorylation

Figure 4.2 p70S6K hyperactivation in MCF-7/HER2Δ16 cells in response to 20 μg/ml Trastuzumab 2-hour treatment. The dysregulation of RTK expression and phosphorylation is associated with the development of cancer as well as contributing to therapeutic resistance. Using a phospho-kinase array composed of 46 antibodies spotted in duplicate on nitrocellulose membranes, we investigated how trastuzumab treatment impacts kinase signaling in MCF-7 and MCF-7/HER2Δ16H cells treated with trastuzumab for 2 hours. (A) p70S6K showed the most dramatic increase in phosphorylation in MCF-7/HER2Δ16H cells treated with trastuzumab. MCF-7 control cells showed no phosphorylation changes in p70S6K between untreated and treated samples. (B) Quantification and analysis of the signals from the kinase array showed a 5-fold increase in phosphorylation of p70S6K at T229, T421/S424 and T389 in MCF-7/HER2Δ16H cells after 2 hours of trastuzumab treatment compared to the untreated MCF-7/HER2Δ16H cells.

FIGURE 4.2

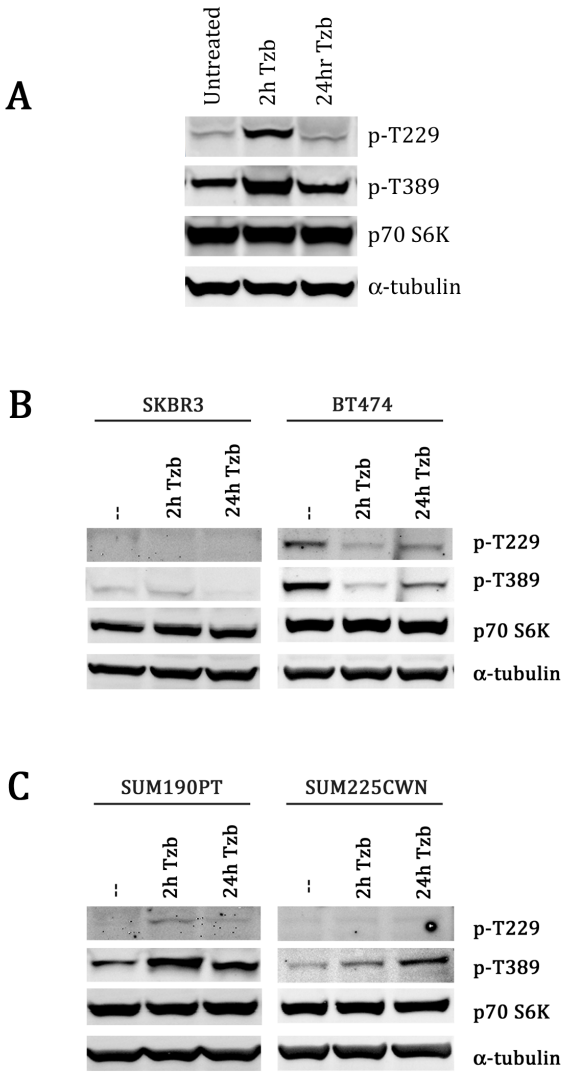

A
--
Tzb
p70 S6 Kinase
MCF-7
T389
T421/S424
T229
MCF-7/ HER2Δ16H
T389
T421/S424
T229
B
Fold Change (treated/untreated)
3.5
3
2.5
2
1.5
1
0.5
0
T389
T421/S424
T229
MCF-7
MCF-7/HER2Δ16

of p70S6K, including site T389 (297). Phosphorylation at T389 by mTORC1 is a precursor for subsequent phosphorylation at T229 by PDK1, resulting in maximal activation of p70S6K (298,299). Phosphorylation at the T389 site most closely reflects the activated state of p70S6K (300). Importantly, aberrant S6 kinase activity is implicated in several disease models due to its role in regulating protein synthesis (301), cell proliferation (302) and survival (303,304). Although the antitumor mechanism(s) of trastuzumab action remains unclear, evidence suggests that trastuzumab mediates its anti-proliferative effects in HER2-positive cells by downregulating the PI3K/AKT signaling pathway (293); thus one strategy for HER2Δ16-expressing tumor cells to evade trastuzumab therapy could involve the activation of p70S6K.

To confirm the phospho-kinase array results, we preformed a Western blot that showed unchanged endogenous levels of p70S6K in the MCF-7/HER2Δ16 cells during trastuzumab treatment, but acute stimulation of the T229 and T389 sites occur after 2 hours of trastuzumab treatment (Figure 4.3A). The dephosphorylation observed at 24 hours is expected since peak activation of p70S6K is known to occur within one hour of stimulation (305). To further investigate if activation of p70S6K is associated with trastuzumab response, we evaluated the phosphorylation of p70S6K in the HER2-overepxressing cell lines SKBR3, BT474, SUM190PT and SUM225CWN after treatment with trastuzumab (Figure 4.3B). SKBR3 and BT474 cells are trastuzumab sensitive while the SUM190 and SUM225 cell lines are trastuzumab resistant (306). Phosphorylation of p70S6K in SKBR3 cells was undetected in both untreated and trastuzumab treated samples (Figure 4.4A). We

Figure 4.3 Western blot validation of p70 S6 kinase phosphorylation observed in the phospho-kinase array. Cells were treated with 20 μg/ml trastuzumab for 2 and 24 hours, followed by collection of whole cell lysate and Western blot assessment for p70S6K and its phosphorylation at T229 and T389. (A) p70S6K phosphorylation at T229 and T389 in MCF-7/HER2Δ16H cells treated with trastuzumab confirms the observations from the phosphokinase array. (B) Trastuzumab-sensitive cell lines SKBR3 and BT474 show no activation or reduced activity of p70S6K, respectively, in response to trastuzumab treatment. (C) Trastuzumab-resistant cell lines SUM190PT and SUM225CWN cells show increased phosphorylation of p70S6K after trastuzumab treatment, but at differing rates. The SUM190PT cells show a dramatic increase of T389 and subtle increase in T229 phosphorylation of p70S6K after 2 hours of trastuzumab exposure whereas the SUM225CWN cells show a gradual increase in phosphorylation of T389 after longer exposure to trastuzumab.

FIGURE 4.3


A
Untreated
2h Tzb
24hr Tzb
p-T229
p-T389
p70 S6K
α-tubulin
B
SKBR3
BT474
--
2h Tzb
24h Tzb
--
2h Tzb
24h Tzb
p-T229
p-T389
p70 S6K
α-tubulin
C
SUM190PT
SUM225CWN
--
2h Tzb
24h Tzb
--
2h Tzb
24h Tzb
p-T229
p-T389
p70 S6K
α-tubulin

observed basal levels of phosphorylation of p70S6K at T389 in the untreated BT474 cells, which is acutely reduced in response to trastuzumab treatment at 2 hours and maintained at 24 hours (Figure 4.3B). Interestingly, similar to what we observed in the MCF-7/HER2Δ16H cells, the trastuzumab resistant SUM190PT cell line showed a significant increase in phosphorylation at T389 in response to trastuzumab treatment for 2 hours and remains elevated at 24 hours. A minor increase in T229 phosphorylation is also detected at 2 hours of trastuzumab treatment in the SUM190PT cells (Figure 4.3C). The SUM225CWN cells display increasing levels of p70S6K T389 phosphorylation over longer exposure to trastuzumab, but T229 phosphorylation was not observed. These initial results support our hypothesis that phosphorylated p70S6K is a trastuzumab regulated pathway in HER2-positive breast cancer cells that may drive resistance.

We have shown that HER2Δ16 expression promotes trastuzumab resistance *in vitro* and is present in 90% of patients with HER2-positive metastatic breast cancer. Unpublished data from our lab has shown that SKBR3 co-express HER2 and HER2Δ16. Trastuzumab-resistant SKBR3 cells, developed from continuous exposure to trastuzumab, showed a 3-fold increase in HER2Δ16 expression, suggesting that increased HER2Δ16 expression is associated with trastuzumab resistance. We evaluated the HER2 and HER2Δ16 expression in our four HER2-overexpression cell lines by real-time qRT-PCR and found that HER2Δ16 expression is coexpressed with HER2 in all four cell lines (Figure 4.4). Interestingly, higher HER2 expression corresponds to higher HER2Δ16 expression. The SUM190PT and SUM225CWN cells show two and three-fold, respectively, more HER2 expression than the SKBR3 and

FIGURE 4.4

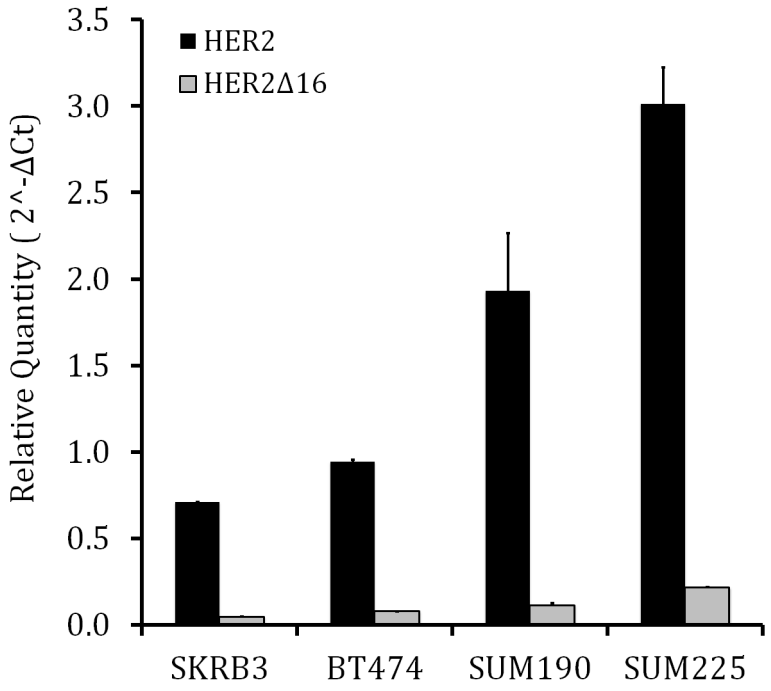


Figure 4.4 HER2 and HER2Δ16 are coexpressed in these HER2+ positive breast cancer cell lines. Triplicate RNA samples were collected for each cell line and 1 µg was used to synthesize cDNA, followed by real-time qRT-PCR analysis of HER2 and HER2Δ16 expression.

BT474 cells and have at least three-fold greater expression of HER2Δ16. The higher expression of HER2Δ16 in the SUM190PT and SUM225CWN cells may explain their primary resistance to trastuzumab. These data suggest that breast tumor cells with high levels of HER2 expression will have correspondingly higher HER2Δ16 expression, which may impact the tumor cells' response to trastuzumab therapy.

4.2.2 Inhibition of the Akt signaling pathway using rapamycin and PF-4708671

The trastuzumab-resistant MCF-7/HER2Δ16H, SUM190PT and SUM225CWN cells demonstrate low basal levels of p70S6K phosphorylation (untreated samples, Figure 4.3A and C). Once exposed to trastuzumab, these cells quickly respond by activating p70S6K, which is known to promote cell survival and enhance tumor aggressiveness (307). Thus, inhibition of this pathway may be required to overcome trastuzumab resistance.

Rapamycin is a macrocyclic antibiotic produced by bacteria found in the soil of Easter Island. It was originally developed as an antifungal agent until it was discovered to have potent immunosuppressive and antiproliferative properties useful for organ transplant and cancer treatment, respectively (283). Rapamycin is an mTORC1 specific inhibitor. It functions by first binding to immunophilin FK506-binding protein 12 (FKBP12) and together this complex interacts with the FKBP12-rapamycin-binding domain in mTORC1 to inhibit mTORC1-mediated signal transduction pathways, including p70S6K phosphorylation (Figure 4.5) (308). Inhibition of mTORC1 by rapamycin results in decreased translation of the RNAs involved in cell cycle progression and proliferation leading to growth arrest and

FIGURE 4.5

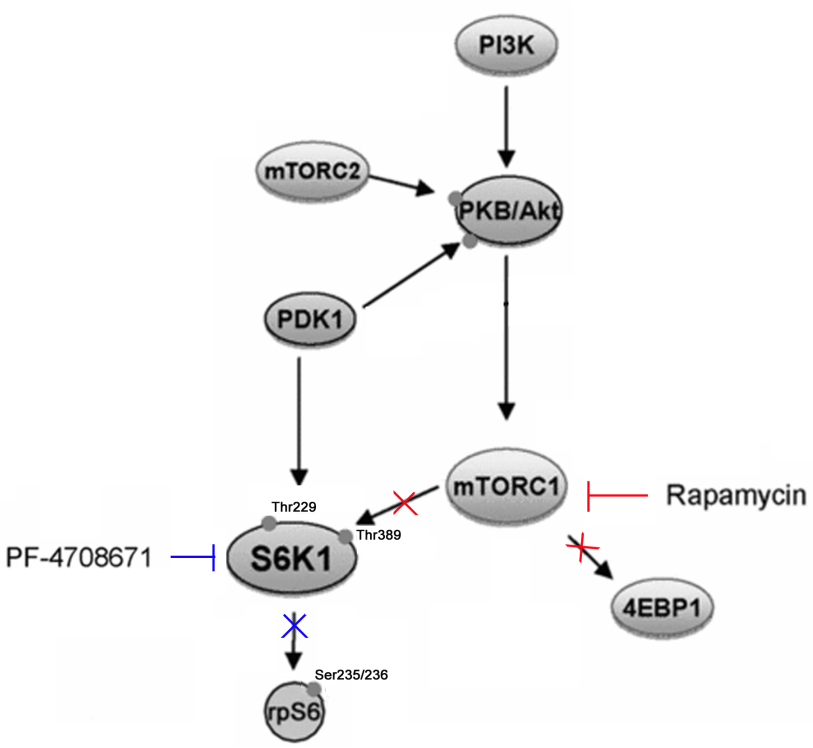


Figure 4.5 Inhibition of mTORC1 by rapamycin and p70S6K by PF-4708671 to disrupt downstream signaling to rpS6 (285).

apoptosis (309-311). To evaluate the concentration of rapamycin required to overcome p70S6K hyperactivation in response to trastuzumab in SUM190PT cells, we performed a 2-hour dose response treatment of rapamycin in combination with 20 μg/ml trastuzumab and evaluated the phosphorylation status of ribosomal S6 (rpS6) protein, a direct substrate of p70S6K (Figure 4.6). Our results show that 10 nM rapamycin was sufficient to inhibit mTORC1 activation of p70S6K in SUM190 cells, which prevented rpS6 phosphorylation, indicating that rapamycin was capable of inhibiting the mTORC1 downstream signaling pathway in the presence of trastuzumab.

Rapamycin inhibition of mTORC1 inhibits activation of p70S6K, but also prevents the phosphorylation of other mTORC1 substrates such as 4EBP1 and the autophagy kinase ULK1 (312). Thus, we analyzed the impact of the compound PF-4708671, a novel, cell permeable and highly specific p70S6K inhibitor. Previous characterization of PF-4708671 found that phosphorylation of rpS6 protein, a direct substrate of p70S6K, was inhibited in a dose-dependent manner so that rpS6 phosphoryaltion was significantly reduced at 3 μM and almost abolished at 10 μM (260). Based on this information, we use 5 μM PF-4708671 in the subsequent experiments.

To evaluate if rapamycin or PF-4708671 treatment could overcome trastuzumab resistance in the SUM190PT and SUM225CWN cells, we tested these drugs individually and in combination with 20 μg/ml trastuzumab in a cell proliferation assay for a 5-day time period (Figure 4.7). This luminescent cell viability assay determines the number of viable cells in culture based on the

Figure 4.6 Rapamycin inhibition of mTORC1 signaling prevents downstream phosphorylation of rpS6. SUM190PT cells were treated with 20 μg/ml trastuzumab alone or in combination with rapamycin for 2 hours, followed by collection of whole cell lysate and Western blot assessment for rpS6 and its phosphorylation at S235/S236. 10 nM rapamycin is selected for follow-up experiments due to the observed inhibition of rpS6 phosphorylation, the downstream substrate of p70S6K, after 2 hours of treatment.

FIGURE 4.6

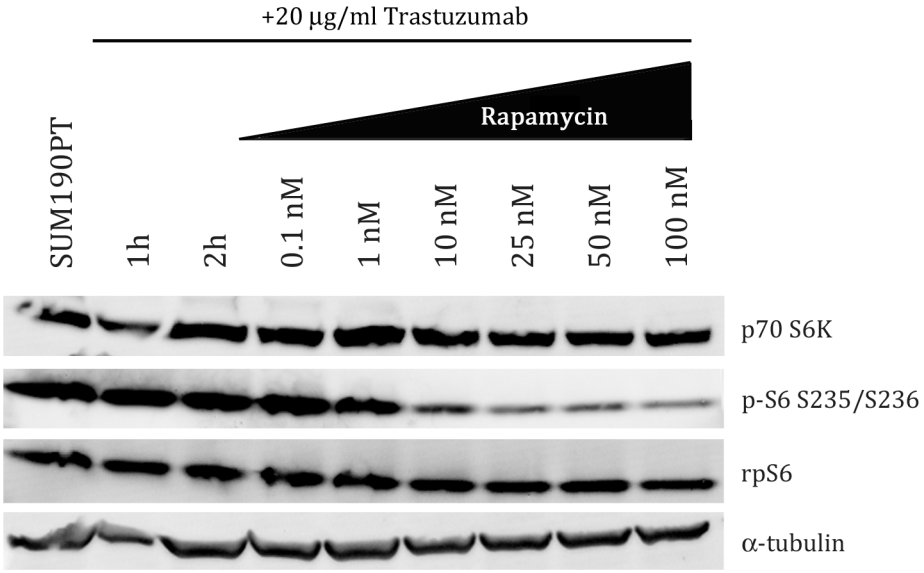
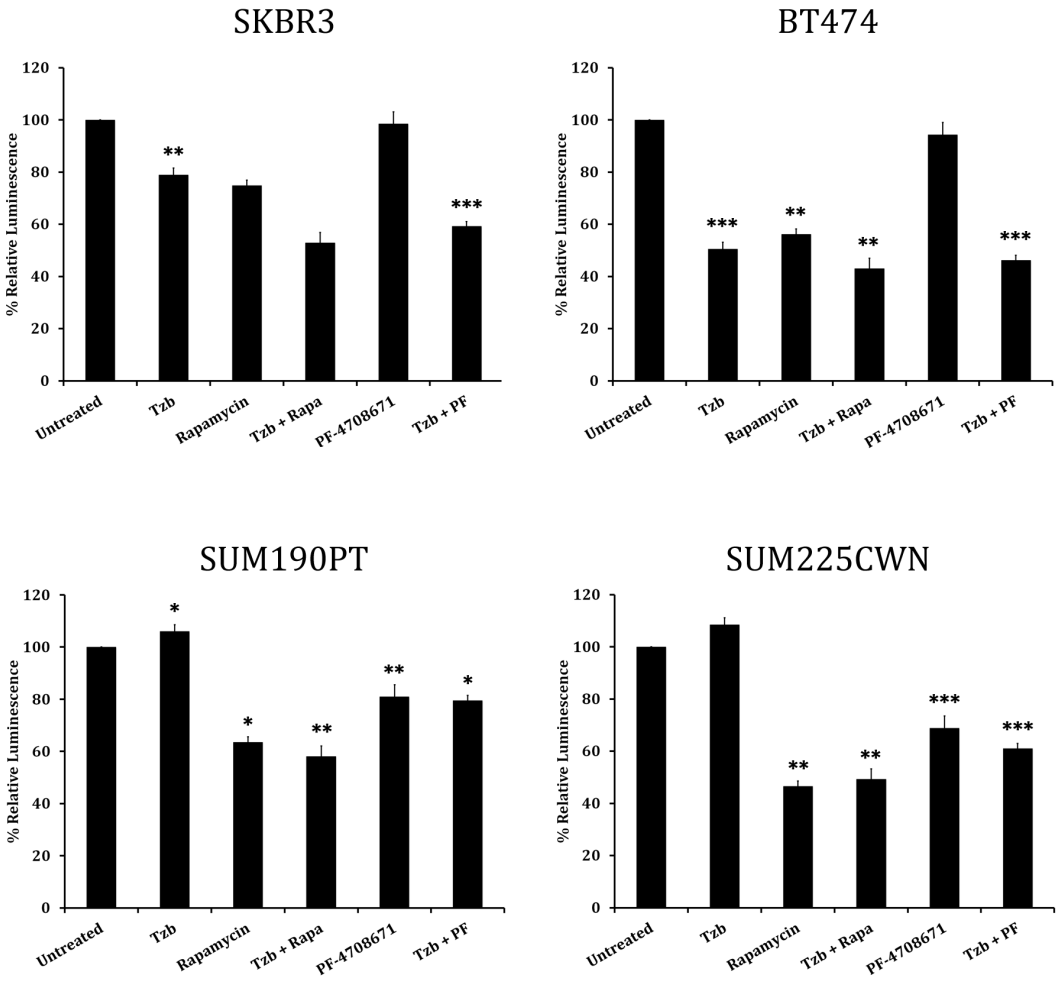

+20 µg/ml Trastuzumab
Rapamycin
SUM190PT
1h
2h
0.1 nM
1 nM
10 nM
25 nM
50 nM
100 nM
p70 S6K
p-S6 S235/S236
rpS6
α-tubulin

Figure 4.7 Cell viability assay on HER2+ cell lines treated with 20 µg/ml trastuzumab, rapamycin and PF-4708671 as single agents or in combination. Cells were seeded in 96-well plates, drug treated the following day and incubated for 5 days total. SKBR3 and BT474 cells are trastuzumab sensitive while the SUM190PT and SUM225CWN cells are trastuzumab resistant. Treatment of the trastuzumab-resistant cells with rapamycin, a specific mTORC1 inhibitor, or PF-4708671, a p70S6K-specific inhibitor, obstructs PI3K/Akt signal transduction and allows trastuzumab-mediated inhibition of cell proliferation. The data represents the amount of luminescence from the luciferase reaction to ATP presence, a global indicator of cellular metabolism. All drug treatment samples are compared to the untreated control +/- SE of at least three independent experiments. Single asterisk ($p < 0.05$), double asterisks ($p < 0.01$) and triple asterisks ($p < 0.001$) indicate that the particular drug treated cell lines show significantly reduced cell proliferation, respectively. Significant differences were determined by unpaired Student's *t*-test.

FIGURE 4.7


SKBR3
BT474
SUM190PT
SUM225CWN
% Relative Luminescence
0
20
40
60
80
100
120
Untreated
Tzb
Rapamycin
Tzb + Rapa
PF-4708671
Tzb + PF
*
**
***

quantity of ATP present, which is directly proportional to the number of metabolically active cells (313). As expected, the trastuzumab-sensitive cells SKBR3 and BT474 cells respond to trastuzumab-mediated growth inhibition while the trastuzumab-resistant cells SUM190PT and SUM225CWN cells show an increase in cell proliferation in response to trastuzumab. The 10 nM rapamycin treatment inhibited cell proliferation in all four cell lines to varying degrees. It has been shown that HER2-overexpressing cells with high levels of Akt/mTORC1 signaling have higher sensitivity to rapamycin (314). In our data, the SKBR3 cells showed the lowest Akt/mTORC1 signaling based on the phosphorylation status of p70S6K and this corresponded to the lowest growth inhibition in response to rapamycin. We expected the rapamycin and trastuzumab combination treatment to have a synergistic impact on growth inhibition of trastuzumab sensitive cells, however the effects appear to be additive in the SKBR3 and BT474 cells. It is thought that the inability of trastuzumab to completely inhibit mTOR signaling can permit synergy of trastuzumab with mTORC1 inhibitors to prevent the growth of HER2 overexpressing cancer cells. However, in our resistant cell lines, the single-agent rapamycin treatment performed similarly to the combination treatment. These results suggest that even in the presence of a p70S6K inhibitor, the SUM190PT and SUM225CWN cells retain resistance to trastuzumab. Although a growth inhibiting interaction between rapamycin and trastuzumab was not observed, inhibition of p70S6K does appear to be an effective therapeutic option for breast tumor cells with *de novo* trastuzumab resistance and this is consistent with reports from numerous studies (315-318). Interestingly, the PF-4708671 single-agent treatment had

minimal impact on the proliferation of SKBR3 and BT474 cells but in combination with trastuzumab, significant growth inhibition was observed. PF-4708671 significantly inhibited the SUM190PT and SUM225CWN cell proliferation as a single-agent treatment, however, consistent with our findings with rapamycin, trastuzumab failed to decrease cell viability when used in combination with PF-4708671. Based on these cell proliferation results, rapamycin is better at inhibiting tumor cell growth compared to PF-4708671. However, it may be beneficial to selectively target p70S6K directly to potentially avoid the side effects linked to systemic inhibition of mTORC1, such as hyperglycemia, hypercholesterolemia and hyperlipidemia (319).

In response to promising preclinical data (320-322) supporting the use of mTOR inhibitors combined with trastuzumab, rapamycin and its analogs have been developed into therapeutic drugs for various human malignancies and are currently undergoing clinical evaluations. Currently there are five ongoing clinical trials investigating mTOR inhibitors in combination with trastuzumab and chemotherapy, as well as, eight clinical studies recruiting patients. Results from two completed phase I studies found that rapamycin and trastuzumab together were well tolerated and showed a promising clinical benefit in patients with metastatic HER2-positive breast cancer who have progressed on prior trastuzumab therapy (315,317).

4.3 CONCLUSION

Although trastuzumab treatment remains the standard of care for patients with HER2 overexpressing breast cancer, primary and acquired resistance is a

serious concern. Understanding the molecular mechanisms of resistance may lead to novel therapeutic approaches to overcome or prevent this resistance. Our work examined the molecular response and signaling of HER2-overexpressing breast cancer cells with primary trastuzumab resistance subjected to trastuzumab treatment. Treatment of the trastuzumab-resistant MCF-7/HER2Δ16 cells with trastuzumab showed hyperactivation of p70S6K, a principle kinase downstream of mTORC1. The trastuzumab-resistant SUM190PT and SUM225CWN cells also showed similar activation of p70S6K in response to trastuzumab treatment. We demonstrated that trastuzumab resistance in these cell lines can be overcome by targeting mTOR using two different specific inhibitors, rapamycin and PF-4708671. Based on these data, we propose that phosphorylated p70S6K can be evaluated in HER2-positive breast cancer patients undergoing trastuzumab treatment as a biomarker to predict therapeutic response to rapamycin.

Boulay *et al* analyzed the mTORC1 effectors 4EBP1 and p70S6K in tumor, skin and peripheral-blood mononuclear cell (PBMC) extract to evaluate the performance of everolimus, an mTOR inhibitor (315,323). They showed that suppression of tumor growth was correlated with inactivation of p70S6K and that p70S6K could be measured reliably in human PBMCs. This method is currently in use to monitor mTOR inhibition in clinical studies (315,323,324). Since primary tumor samples can be difficult to attain and even more difficult to acquire during and post-treatment, blood sample collection is a more feasible and less invasive method for monitoring drug response. We believe this is a valuable method that

could be implemented to test our hypothesis that patients with *de novo* resistance to trastuzumab will show elevated levels of phosphorylated p70S6K during treatment.

Furthermore, current biomarker studies are investigating the phosphorylation status of Akt, mTORC1, p70S6K and 4E-BP1 or loss of PTEN in tumors as an indicator of PI3K/Akt/mTOR pathway activated signaling in HER2-overexpressing breast cancer (294,314,325). Unfortunately clinical samples are limited; available samples are either untreated primary tumors or are treated with combination therapy, which adds further complexity for data analyses since the relative contribution of each drug can be difficult to assess. Importantly, our data suggests that the PI3K/Akt/mTOR pathway may be inactive in tumor cells with inherent trastuzumab resistance and do not display activation until exposure to treatment. Thus performing IHC staining of p70S6K on untreated primary tumor samples may fail to predict patient response to trastuzumab. Furthermore, these studies have found that analysis of single biomarkers did not significantly correlate to trastuzumab response, therefore evaluation of multiple targets may identify patients likely to benefit from trastuzumab-based therapy.

In conclusion, our study has shown that trastuzumab treatment of HER2-positive breast cancer cells have varying molecular responses. We have shown that phosphorylated p70S6K is elevated in response to trastuzumab treatment in cells that are trastuzumab refractory and could be developed into a biomarker to facilitate the selection of appropriate patients who can benefit from trastuzumab therapy.

CHAPTER 5 – SUMMARY

5.1 SUMMARY AND CONCLUSIONS

HER2 gene amplification and overexpression occurs in 20-30% of invasive human breast cancers and confers a more aggressive phenotype with poor clinical outcome (101). The selective overexpression of HER2 in cancer tissues makes it an ideal target for cancer therapeutics and led to the development of trastuzumab, a monoclonal antibody that targets the extracellular region of HER2 (180). Despite positive preclinical data, only 11 to 26% of patients responded to trastuzumab monotherapy and 70% of initial responders progressed to metastatic disease within one year of treatment, suggesting inherent and rapidly acquired resistance to trastuzumab (188,190,192,196,288). Trastuzumab combined with chemotherapy showed enhanced efficacy and is the foundation of care for patients with HER2-positive breast cancer (326). Unfortunately the therapeutic action tends to be short lived, so trastuzumab resistance remains a serious clinical problem and underscores the need for novel targeted therapies and validated biomarkers to improve diagnosis, as well as, predict patient response to trastuzumab.

Although clinical evidence confirms an important role for HER2 in breast cancer, preclinical models utilizing wild-type HER2 fails to recapitulate the

aggressive nature of HER2-positive tumors observed in patients. Only when HER2 has been experimentally manipulated with activating mutations does it increase cellular transformation (110,217). One tumor specific event that results in clinical activation of HER2 is expression of a HER2 splice variant lacking exon 16, referred to here as HER2Δ16 (202). Deletion of exon 16 disrupts cysteine residues and induces a conformational change in the HER2 receptor extracellular domain that promotes intermolecular disulfide bonding to form stable receptor dimers that constitutively activate intrinsic tyrosine kinase signaling (202,221). Our preclinical data shows that HER2Δ16 is present in nearly 50% of HER2 positive breast cancer and promotes increased activation of multiple oncogenic pathways including PI3K/Akt, MAP kinase, FAK and Src signaling compared to wild-type HER2 (202). Consistent with our *in vitro* data, which showed that HER2Δ16 expression dramatically potentiates cell migration and invasion, we also found that 89% of 27 patients with HER2-positive tumors coexpressing HER2Δ16 presented with positive lymph nodes and have higher-grade ER-negative tumors (202). While others have demonstrated lower binding of trastuzumab to HER2Δ16 (225), we observed equivalent levels of trastuzumab binding to both wild-type HER2 and HER2Δ16 (202). However, HER2Δ16-expressing cells are resistant to trastuzumab (202,269). We propose that HER2Δ16 expression increases as HER2 expression is amplified in human breast tumors and once HER2Δ16 expression reaches a critical threshold, the splice variant can contribute to breast cancer progression and primary resistance to trastuzumab. Patients with HER2-positive breast tumors and do not respond to trastuzumab may have tumors expressing high levels of HER2Δ16.

Given the diversity of proposed mechanisms of trastuzumab's therapeutic action, it is not surprising that our understanding of primary and secondary trastuzumab resistance is incomplete. Modes of acquired resistance have been thoroughly studied while the number of studies exploring the mechanism of *de novo* resistance is quite limited. The research carried out in this dissertation investigated the molecular mechanisms underlying HER2Δ16 activity contributing to trastuzumab resistance.

Using a gene array strategy to compare microRNA expression profiles of MCF-7 to MCF-7/HER2Δ16H cells, we found that expression of HER2Δ16 significantly altered expression of 16 microRNAs by 2-fold or more including a 4.8 fold suppression of the miR-7 tumor suppressor. Reestablished expression of miR-7 in the MCF-7/HER2Δ16H cell line caused a G1 cell cycle arrest and reduced both colony formation and cell migration activity to levels of parental MCF-7 cells. Suppression of miR-7 in the MCF-7 cell line resulted in enhanced colony formation activity, but not cell migration, indicating that miR-7 suppression is sufficient to drive tumor cell proliferation but not migration. MiR-7 inhibited MCF-7/HER2Δ16H cell migration through a mechanism involving suppression of the miR-7 target gene EGFR. In contrast, miR-7 inhibition of MCF-7/HER2Δ16H cell proliferation involved a pathway where miR-7 expression resulted in the inactivation of Src kinase independent of suppressed EGFR expression. Also independent of EGFR suppression, reestablished miR-7 expression sensitized refractory MCF-7/HER2Δ16H cells to trastuzumab. Our results demonstrate that reestablished miR-7 expression abolishes HER2Δ16-induced cell proliferation and migration while

sensitizing HER2Δ16-expressing cells to trastuzumab therapy. We propose that miR-7 regulated pathways, including EGFR and Src kinase, represent targets for the therapeutic intervention of refractory and metastatic HER2Δ16-driven breast cancer.

We used a phosphoproteomic approach to identify potential downstream signaling pathways activated by trastuzumab in trastuzumab-resistant HER2Δ16-expressing cells. We identified p70S6K, an mTORC1 effector, as a potential mediator of trastuzumab resistance. P70S6K activity regulates the cell cycle, cell growth and cell survival. Treatment of MCF-7/HER2Δ16H cells with trastuzumab resulted in acute phosphorylation of p70S6K at T229 and T389. We showed similar trastuzumab activation of p70S6K in the trastuzumab-resistant HER2-overexpressing cells, SUM190PT and SUM225CWN. Trastuzumab treatment did not activate or reduced p70S6K activity in the trastuzumab-sensitive HER2-amplified cells SKBR3 and BT474, respectively. Furthermore, all four HER2-positive cell lines coexpressed HER2Δ16, with the SUM190PT and SUM225CWN showing the highest levels of HER2 splice variant expression. Lastly, we showed that targeted inhibition of the PI3K/Akt/mTOR pathway using rapamycin or PF-4708671 could sensitize the trastuzumab-resistant cells to trastuzumab mediated growth inhibition. This data shows that trastuzumab treatment induces changes in protein phosphorylation in cells with inherent trastuzumab resistance and identified p70S6K as a mediator of pro-survival signaling. It also underscores the disconnect between failure of trastuzumab to inhibit tumor cell growth and the ability of this drug to induce other important phenotypic changes in tumor cells.

In summary, trastuzumab is unquestionably effective for some patients with HER2-positive breast cancer, but for non-responders, there is compelling need to identify and characterize mediators of primary trastuzumab resistance. The differential response to trastuzumab in patients with HER2-positive tumors suggests a genetic difference in tumor populations. Evidence from this work demonstrates that the HER2 isoform HER2Δ16 represents the transforming form of the HER2 oncoprotein. The covert influence of HER2Δ16 oncogenic activity on metastatic progression and trastuzumab resistance provides a mechanistic explanation for the clinically unpredictable prognosis and therapeutic response of patients with HER2-positive tumors. Thus, there is a critical need to develop clinical assays that can detect HER2Δ16 expression in breast cancer tissues so subsequent clinical management decisions can be based on the presence or absence of HER2Δ16 expression. Furthermore, this work emphasizes the need for novel therapeutic strategies to disengage HER2Δ16 oncogenic signaling contributing to metastatic disease and trastuzumab resistance.

5.2 FUTURE DIRECTIONS

5.2.1 Regulation of HER2Δ16 expression

Investigation of dysregulated downstream signaling of HER2 in the development of secondary trastuzumab resistance is already extensively studied, while there is still much to be learned regarding the basis of primary trastuzumab resistance. We propose to continue examining the contribution of HER2Δ16 as an intrinsic mechanism of trastuzumab resistance. Limited studies on the HER2 splice

variant suggest its expression is generated by alternative splicing rather than mutation. Alternative splicing occurs in 95% of human multiexonic genes and contributes to proteome complexity (327). Deregulation of splicing is associated with cancer and we propose to study the role of serine/arginine-rich splicing factor 1 (SRSF1, SF2/ASF), which is upregulated in several human neoplasias including breast cancer (328). We have identified a partial RNA recognition sequence for SRSF1 in exon 17 of HER2 suggesting a potential role for SRSF1 mediated splicing of HER2. We hypothesize that SRSF1 is the splice factor responsible for production of the HER2Δ16 spice variant from wild-type HER2.

To investigate if SRSF1 plays a role in HER2 splicing, we propose to modulate its expression using an expression plasmid or clustered regularly interspaced short palindromic repeats (CRISPR) technology to overexpress and silence SRSF1 expression, respectively. SRSF1 protein analysis in HER2-amplifed breast cancer cell lines indicates higher SRSF1 expression in the trastuzumab-resistant cells than trastuzumab-sensitive cells (Figure 5.1). We hypothesize that overexpression of this splice factor in the presence of HER2 overexpression will increase expression of HER2Δ16. Using RT-PCR, we will examine if increased SRSF1 expression results in increased HER2Δ16 expression and if this will impact tumor cell proliferation and trastuzumab sensitivity in the trastuzumab sensitive cells SKBR3 and BT474. Furthermore, we will eliminate HER2Δ16 expression using CRISPR technology in the SUM190PT and SUM225CWN cells and evaluate if HER2Δ16-induced trastuzumab resistance is reversed.

FIGURE 5.1

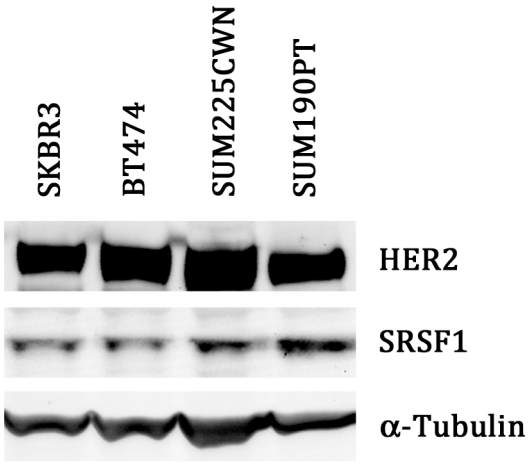


Figure 5.1 SRSF1 protein expression is higher in trastuzumab-resistant SUM190PT and SUM225CWN cells than in trastuzumab-sensitive SKBR3 and BT474 cells. We hypothesize that SRSF1 is the splice factor responsible for generating the HER2Δ16 spice variant from wild-type HER2. Increased expression of SRSF1 in the SUM190PT and SUM225CWN cells are consistent with our data showing increased HER2Δ16 expression in these cell lines (Figure 4.4).

5.2.2 Disengage HER2Δ16 dimerization and signaling

Pertuzumab is a humanized anti-HER2 monoclonal antibody similar to trastuzumab, but binds to the extracellular domain II of the HER2 receptor, preventing HER2 heterodimerization with other HER family receptors (329). Pertuzumab's mechanism of action is complementary to that of trastuzumab in that it also inhibits the PI3K/Akt pathway and also triggers the ADCC response with comparable efficacy (330). Since it is thought that the deletion of exon 16 alters the three-dimensional structure of the HER2 receptor in such a way that the trastuzumab binding epitope is hidden, it would be worth investigating if pertuzumab can bind more effectively to HER2Δ16 and suppress its oncogenic signaling.

We have also shown that HER2Δ16 receptors are expressed as stable homodimers (127,202,234). It is thought that the loss of exon 16 disrupts the balance of cysteine residues in the extracellular domain of the HER2 receptor, thus favoring the formation of disulfide bonds resulting in HER2Δ16 homodimerization. Disulfide bonds are usually formed through the oxidation of sulfhydryl (–SH) groups. S-acetomidomethyl (Acm) is a chemical compound used in peptide synthesis to inhibit disulfide bond formation, but retains the cysteine and preserves the primary structure of the protein (331). If Acm could be developed to inhibit free sulfhydryl groups of proteins on the cell membrane, we could test its ability to disrupt HER2Δ16 dimerization.

5.2.3 HER2Δ16-specific antibody

While we have shown that our PCR primers can specifically detect and distin-

FIGURE 5.2

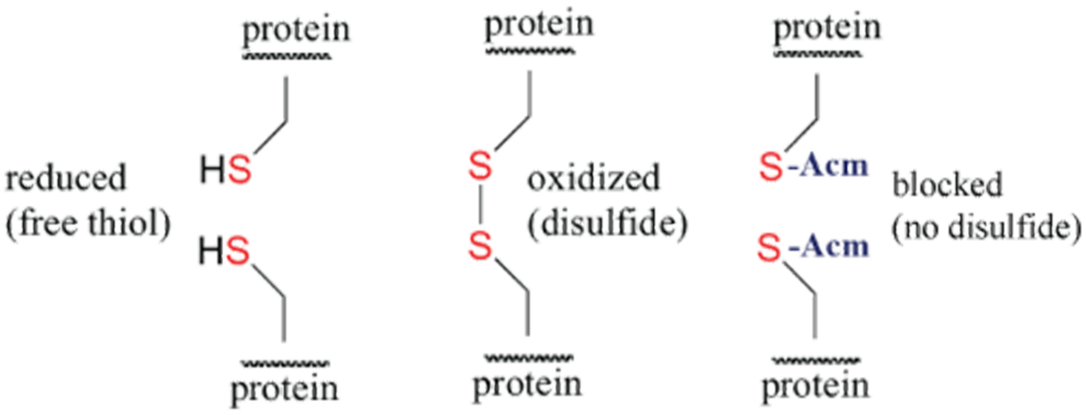


Figure 5.2 S-acetomidomethyl (Acm) blocks disulfide bond formation (331). Disulfide bonds are integral for providing structure and stability to the three-dimensional conformation of many proteins. Acm is a compound that can prevent disulfide bond formation without disrupting the primary structure of the protein.

guish between HER2 and HER2Δ16 (202), obtaining RNA from patient tumor samples is not feasible. In order to screen for HER2Δ16 expression in HER2-overexpressing tumors by IHC, we need to develop an antibody specific for HER2Δ16. It is unclear how the loss of exon 16 impacts the overall three-dimensional structure of the receptor and protein crystallization would be able to elucidate this question. Comparing the structural differences between HER2 and HER2Δ16 will increase our chances of developing antibodies that can discriminate between the two receptors. Furthermore, the crystallized structure of the HER2Δ16 protein can also be useful in the formulation of small molecule inhibitors.

5.2.4 Standardize clinical sample collection

While the scope of this dissertation has focused on trastuzumab and its resistance for the treatment of HER2-positive breast cancer, there are numerous strategies undergoing clinical investigation to overcome trastuzumab resistance including the development of vaccines, antibody-drug conjugates and tyrosine kinase inhibitors, dual HER2 inhibition strategies, inhibition of the PI3K/Akt/mTOR signaling pathway and development of modulators of immune checkpoints (332). Since several different drug targets are under investigation, there is a need to identify and validate biomarkers that may provide information about the type, state and progression of the tumor, as well as biomarkers predictive of tumor response to particular therapeutic treatments. Retrospective studies have shown that the assessment of single biomarkers does not significantly correlate to patient response, thus diagnostic assays would be more useful if they assessed multiple signaling proteins. We propose that clinical trials include tumor sample collection in all of

their studies, as the greatest limitation in biomarker research is lack of clinical samples. Furthermore, these collections should be standardized to include a matched tumor and normal tissue sample before and after treatment.

Overall, the development of trastuzumab represents a major advance in targeted therapy. However, the prevalence of primary and secondary resistance in HER2-positive breast cancer patients demand improved therapeutic development that can mediate trastuzumab resistance. We and others have demonstrated that HER2Δ16 could actually represent the transforming and oncogenic form of HER2 and warrants further studies to elucidate the biological involvement of HER2Δ16 in human breast cancer progression and therapeutic resistance.

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BIOGRAPHY

Felicia Cam Huynh was born in Vietnam on March 23, 1983. Her family immigrated to Olympia, WA in July 1988. She graduated in the top 20 students in her class at Capital High School and earned a bachelor's degree in Biology at Whitman College in Walla Walla, WA. After her undergraduate studies, she worked as a research scientist for the pharmaceutical company Merck for five years before attending Tulane University in New Orleans, LA to pursue a Ph.D. in Cell and Molecular Biology. She is the first member of her family and extended family, all first generation immigrants, to earn a doctorate degree. In August 2015, she will marry her love of nine years in Seattle, WA and plans to pursue a career in teaching.