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Eduardo Sanabria-Figueroa Date

Mechanisms of IGF-1R-mediated trastuzumab resistance in HER2-overexpressing breast cancer

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Doctor of Philosophy

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in

Graduate Division of Biological and Biomedical Sciences
Molecular & Systems Pharmacology

2017

Abstract

Mechanisms of IGF-1R-mediated trastuzumab resistance in HER2-overexpressing breast cancer
By Eduardo Sanabria-Figueroa

Breast cancer is one of the most common types of cancer among American women. Approximately 20% of metastatic breast cancer cases are classified as the HER2 subtype, characterized by HER2 gene amplification or protein overexpression. Trastuzumab, a humanized monoclonal antibody against HER2 extracellular domain IV, has been used for the treatment of HER2-positive breast cancer. However, resistance to trastuzumab develops within a year of treatment. Increased expression or compensatory signaling through IGF-1R has been associated with resistance to trastuzumab, but the molecular and biological mechanisms through which IGF-1R promotes resistance or disease progression remain unclear. Furthermore, IR has also been suggested to contribute to trastuzumab resistance, by crosstalk with IGF-1R or the formation of hybrid receptors. This dissertation identified cell invasion as the main biological effect elicited by the crosstalk of IGF-1R and HER2 in two HER2-overexpressing cells lines. Src and FAK kinases emerged as regulators of invasion downstream of IGF-1R signaling in HER2-overexpressing cells, as demonstrated by decreased invasion of cells treated with Src and FAK kinase inhibitors in the presence of IGF-1 stimulation. Transcription factor FoxM1 was also identified as an important mediator of invasion in trastuzumab-resistant cells, for FoxM1 knockdown decreased invasion of resistant cells stimulated with IGF-1, and FoxM1 overexpression blocked the anti-invasive effect of IGF-1R knockdown and trastuzumab treatment. Our results also support that IR is a mediator of invasion in HER2-overexpressing cells, similarly to IGF-1R. We demonstrate for the first time that insulin-mediated activation of IGF-1R/IR phosphorylates HER2 in breast cancer cells with primary resistance to trastuzumab, similar to IGF-1-mediated phosphorylation of HER2. Additionally, IGF-1 stimulation blocked the anti-invasive effect of transient IR knockdown and trastuzumab treatment, while insulin stimulation overcame the anti-invasive effect of stable IGF-1R knockdown and trastuzumab treatment. All together, our findings confirm that IGF-1R and IR contribute to trastuzumab resistance in HER2-positive breast cancers. Our results strongly suggest that co-targeting IR, IGF-1R, and HER2 is a rational approach for patients whose breast tumors demonstrate IGF-1R/IR activation and HER2 overexpression and have progressed on prior trastuzumab treatment.

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Acknowledgements:

It is with the utmost gratitude that I thank all the people involved in my path towards the doctoral degree. Thanks to my Dissertation Committee members: my advisors Lily Yang and Rita Nahta, and Sumin Kang and John Hepler. Your guidance, support, and genuine interest in my development as a scientist and well-rounded human being have had a profound impact in my character and intellectual formation. To the MSP program, including the faculty and administrative personnel, thank you for this amazing opportunity. Thank you to all the people I met during my graduate career. My sisters from "Team ERBS": Rachel, Bridgette, and Siobhan. Thanks to the incredibly talented, smart, and reliable "SD Crew". You all have been a role model and a motivation to continue the pursuit of excellence, even when it didn't seem achievable. Thanks to my church, Iglesia Metro Atlanta, for being my home away from home. Lastly, I will forever be grateful to my parents, Eduardo and María. Thank you for teaching me the value of family and the importance of self-respect. Thank you for teaching me that I have to give the best of me and persevere through any circumstances; even when I think I don't deserve to succeed. Everything I am today is the result of your sacrifices and unconditional love… los amo hoy, y los amaré por siempre.

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List of Abbreviations:

In alphabetical order:

4EBP1: Eukaryotic translation initiation factor 4E binding protein

Akt: Protein kinase B

ADCC: Antibody-dependent cellular cytotoxicity

AR: Amphiregulin

ATCC: American Type Culture Collection

BMK-1: Big MAP kinase-1

BSA: Bovine Serum Albumin

CREB: cAMP response element binding protein

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EphA2: Ephrin type-A receptor 2

ER: Estrogen receptor

Elk1: Erythroblastosis virus E26 oncogene homolog 1-like gene 1

Erk: Extracellular signal regulated kinases

FACS: Fluorescence-Automated Cell Sorting

FAK: Focal adhesion kinase

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FISH: Fluorescence in situ hybridization

FOXM1: Forkhead box protein M1

FOXO3a: Forkhead box protein O3a

GDF15: Growth differentiation factor 15

GEF: Guanine-nucleotide exchange factor

GRB2: Growth factor receptor-bound protein 2

GSK-3β: Glycogen synthase kinase-3β

HER2: Human Epidermal Growth Factor Receptor 2

IgG: Immunoglobulin G

IGF-1: Insulin-like growth factor 1

IGF-1R: Insulin-like Growth Factor Receptor 1

IGFBP: Insulin-like Growth Factor binding protein

IHC: Immunohistochemistry

IR: Insulin Receptor

IRS-1: Insulin receptor substrate-1

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

MEK: Mitogen-activated protein kinase kinase

MNK: MAPK-interacting serine/threonine kinase

mTOR: Mechanistic target of rapamycin

MUC4: Mucin 4

PBMC: Peripheral blood mononuclear cell

PDK1: 3-phosphoinositide-dependent kinase 1

PH: Pleckstrin homology

PI3K: Phosphoinositide 3-kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol 3,4,5-triphosphate

PR: Progesterone receptor

PTB: Phosphotyrosine binding domains

PTEN: Phosphatase and tensin homolog

RIPA: Radioimmunoprecipitation assay

S6K: Ribosomal protein S6 kinase

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SH2: Src homology 2 domain

shRNA: Small hairpin ribonucleic acid

siRNA: Small interfering ribonucleic acid

SOS: Son of sevenless

Src: Sarcoma family kinases

TBS-T: Tris-Buffered Saline, Tween 20

TCGA: The Cancer Genome Atlas

TGF-β: Transforming Growth Factor beta

TNBC: Triple negative breast cancer

WT: Wild type

Chapter 1:

Background

Chapter 1: Background

1.1 Cancer:

According to the National Cancer Institute Dictionary of Cancer Terms, cancer is a term used to describe a variety of diseases in which abnormal forms of our own bodies' cells divide uncontrollably and can invade neighboring tissues. The process of normal cells becoming abnormal is known as cancer development, and it is a multistep process that involves mutations and selection for cells with increased capacity to proliferate, survive, metastasize, and invade other tissues. As described by G.M. Cooper in the chapter "The Development and Causes of Cancer" (The Cell: A Molecular Approach, 2nd Edition, 2000), cancer development is divided into two main stages: cancer initiation, and cancer progression. Cancer initiation results from genetic alterations leading to abnormal proliferation of a single cell. Abnormal cell proliferation results in the expansion of a specific population of clonally derived highly proliferating cells, forming tumors. Many agents, including ionizing radiation, chemicals, and viruses, have been found to induce the mutations that lead to cancer initiation. These agents are known as carcinogens. Other agents, known as cancer promoters (e.g. hormones, activation of proto-oncogenes), stimulate cell proliferation rather than inducing mutations. Cancer progression follows when additional mutations occur within cells of the tumor population. Sometimes these mutations result in a set of characteristics giving a selective advantage to the cell, such as faster growth. Therefore, cells that originate from these mutated cells will become dominant within the tumor population. This process is called clonal selection. Clonal selection continues throughout the process of tumor development, allowing

tumors to grow rapidly and become increasingly malignant. The set of characteristics that allow normal cells to become cancerous are known as the hallmarks of cancer.

The publication "Hallmarks of Cancer" by Hanahan and Weinberg in 2000 has revolutionized the field of cancer biology. The six hallmarks of cancer described as changes in normal cell physiology that enable malignant growth and metastasis are: self-sufficiency in growth signaling, evasion of growth suppressive signals, unlimited replicative potential, induction of angiogenesis, evasion of apoptosis, and tissue invasion and metastasis (1). These behaviors are opposite to normal cell activity where cells do not typically produce their own growth signals, respond to growth suppressive signals, have a limited number of cell divisions, angiogenesis is highly regulated and reserved for specific scenarios (e.g. repair of injured tissues), cells respond to apoptotic signals, and cells tend to remain attached to its tissue of origin. The work of Hanahan and Weinberg has served as a guide for many researchers across the globe, helping to develop a better understanding of cancer as a disease. The hallmark relating to tissue invasion and metastasis is relevant to malignant (not benign) tumors, which are responsible for most cancer-related mortalities (2). The primary focus of this dissertation is to understand the mechanisms of drug resistance in HER2-overexpressing metastatic breast cancer.

1.2 Breast cancer:

Breast cancer is one of the most frequent and lethal types of cancer in women, affecting 10% - 12% of the worldwide female population (3). The American Cancer Society predicted that more than 246,660 women would be diagnosed with invasive breast cancer in the United States in 2016, with approximately 40,450 breast cancer-

related deaths expected (4). Breast cancer is a complex disease, where heterogeneous subpopulations of cancerous cells can be found within the same tumor. Nevertheless, most tumors exhibit a predominant subpopulation, allowing classification of tumors according to the most prevalent cell phenotype (5).

Gene expression studies have helped to identify discrete subtypes of breast tumors (6; 7). These breast cancer molecular subtypes can be classified as luminal A, luminal B, HER2-positive, and basal-like or Triple Negative Breast Cancer (TNBC). Table 1.1 summarizes these subtypes and corresponding receptor expression profiles.

The luminal A subtype encompasses 40% of breast cancers and is characterized by expression of hormone receptors: the estrogen and progesterone receptors. The luminal B subtype, present in approximately 20% of breast cancers, is also characterized by expression of hormone receptors. Contrary to the luminal A subtype, cells of the luminal B subtype may also express the HER2 receptor and higher levels of the cellular marker for proliferation Ki-67 (8; 9). The TNBC subtype correlates with poorer prognosis and lacks overexpression of the estrogen receptor, progesterone receptor, and HER2 receptor; therefore the designation “triple negative”. The incidence of TNBC is approximately 15% – 20%. However, TNBC tumors may express or depend on other receptors or agents, like the epidermal growth factor receptor (EGFR), to promote cancer development (9).

The expression of hormone receptors in the luminal A and luminal B subtypes allow us to target these types of malignancies with endocrine therapy. For example, using antiestrogen agents (e.g. tamoxifen) or aromatase inhibitors (e.g. exemestane and

Table 1.1: Hormone receptor and HER2 expression in breast cancer subtypes			
Luminal A	Luminal B	HER2-positive	Basal-like (TNBC)
ER+ and/or PR+, HER2-, Ki-67<15%	ER+ and/or PR+, HER2+/-, Ki-67 ≥ 15%	ER-, PR-, HER2+	ER-, PR-, HER2-

Table 1: **Hormone receptor and HER2 expression in breast cancer subtypes**. The most commonly recognized breast cancer molecular subtypes (Luminal A, Luminal B, HER2-positive, and Basal-like) are summarized in terms of their hormone and HER2 receptor expression. Estrogen receptor (ER) and progesterone receptor (PR) are hormone receptors. HER2 is a receptor tyrosine kinase; IHC or FISH used to determine its expression. Triple negative breast cancers (TNBC), which lack ER, PR, and HER2 overexpression, are part of the basal-like subtype.

letrozole). Since there are no approved targeted-agents to treat TNBC, they respond best to chemotherapy. Chemotherapeutic agents include drugs that interfere with microtubule dynamics like the vinca alkaloid vinorelbine or the taxane paclitaxel, antibiotics like doxorubicin, and platinum-based DNA binding drugs like cisplatin. Figure 1.1 illustrates in a simplified diagram some classes of drugs used as targeted therapies for breast cancer.

The last breast cancer molecular subtype to be described is the HER2-positive subtype. HER2-positive breast cancer will be discussed in the following section.

1.3 HER2-positive breast cancer:

1.3.1 HER2 receptor:

HER2 receptor is overexpressed in approximately 20% of breast cancers (10-12) in association with poor prognosis, reduced overall survival, and resistance to chemotherapy (13). The *HER2* gene is located on the long arm of chromosome 17 at chromosomal position 17q21 (12). The HER2 receptor tyrosine kinase consists of an extracellular region, a single transmembrane domain, and an intracellular tyrosine kinase domain (14). Due to its structural composition, this receptor is considered a member of the human Epidermal Growth Factor Receptor (EGFR) family (Figure 1.2) (15). Other members of this family include HER1/EGFR, HER3 and HER4. These other EGFR receptors also consist of an extracellular region with four subdomains, a single transmembrane domain, and an intracellular domain (16). HER2 is the only member of the EGFR family with no known high-affinity ligand (14). All other receptors bind a diversity of ligands including epidermal growth factor (EGF), amphiregulin (AR), and transforming growth factor beta (TGF-β) (17).

Figure 1.1. Drugs used as targeted therapies for breast cancer. There are multiple types of targeted therapies used for the treatment of breast cancer. The most common types of targeted therapies used in the clinic include monoclonal antibodies that target the extracellular domain of the receptor tyrosine kinases (commonly used in HER2-positive subtype), small molecule inhibitors that target the intracellular tyrosine kinase domain of receptor tyrosine kinases, and endocrine agents used to inhibit hormone receptors (commonly used to treat luminal A and luminal B subtypes).

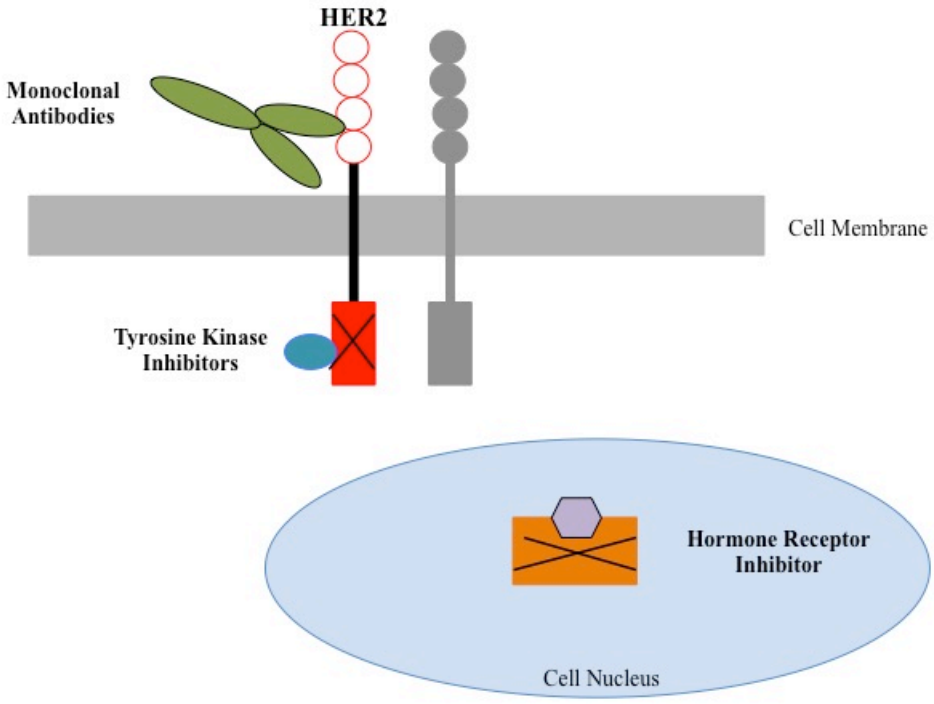
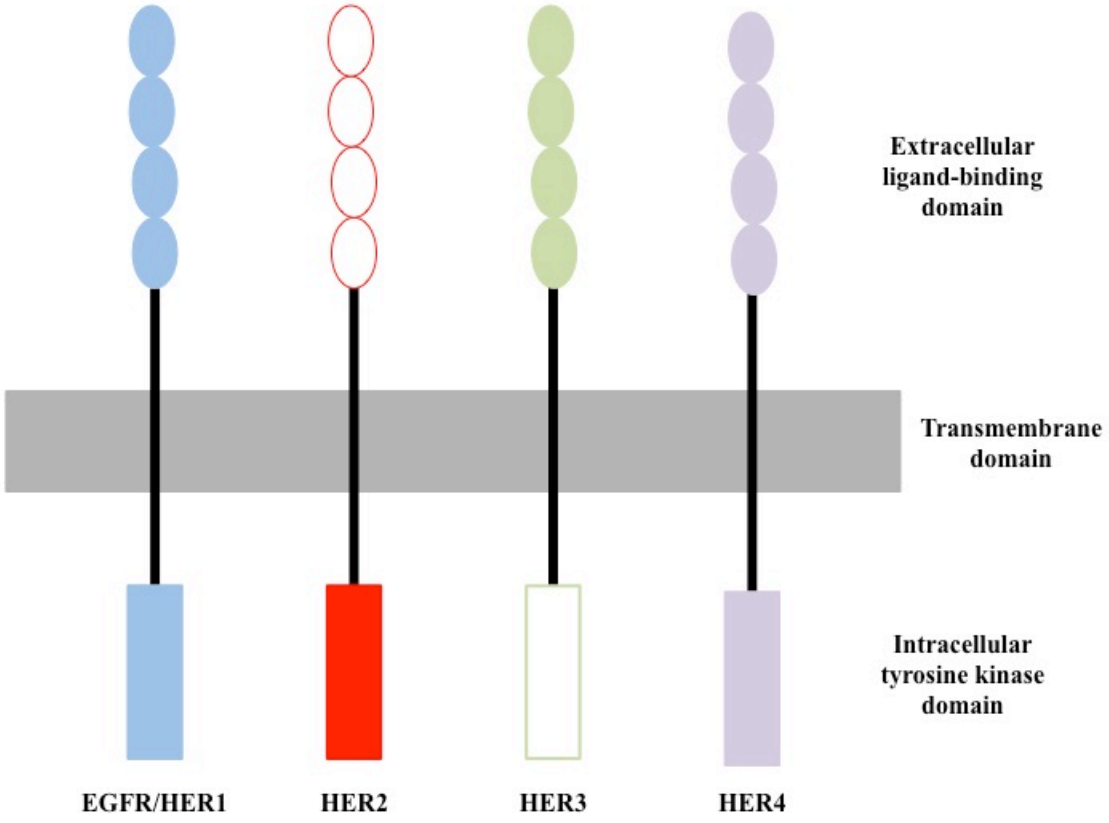

HER2
Monoclonal Antibodies
Cell Membrane
Tyrosine Kinase Inhibitors
Hormone Receptor Inhibitor
Cell Nucleus

Figure 1.2. Epidermal Growth Factor Receptor family of Receptor Tyrosine Kinases. The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases is composed of receptors HER1/EGFR (light blue), HER2 (red), HER3 (light green) and HER4 (light purple). Receptors form this family have an extracellular ligand-binding domain composed of four subdomains, a transmembrane domain, and an intracellular tyrosine kinase domain. As noted, the extracellular domain of HER2 is different from the other receptors in that it has no known ligand that binds with high affinity. HER3 is also different from the other receptors, but in this case it's the only receptor lacking kinase activity in the intracellular domain. These receptors dimerize upon ligand binding, and activate intracellular signaling cascades.


Extracellular ligand-binding domain
Transmembrane domain
Intracellular tyrosine kinase domain
EGFR/HER1
HER2
HER3
HER4

EGFR, HER2, and HER4 have an active intracellular tyrosine kinase domain, which is absent in HER3. However, HER3 can heterodimerize with other EGFR receptors to activate signaling pathways. In the specific case of HER2, receptor monomers dimerize upon ligand binding, forming homodimers or heterodimers with other receptor tyrosine kinases, including other members of the EGFR family. HER2 dimerization and subsequent tyrosine kinase activation triggers signaling pathways, most commonly the PI3K/Akt/mTOR and Mitogen-activated protein kinase (MAPK) pathways, resulting in inhibition of apoptosis and increased cell growth, invasion, and metastasis (18).

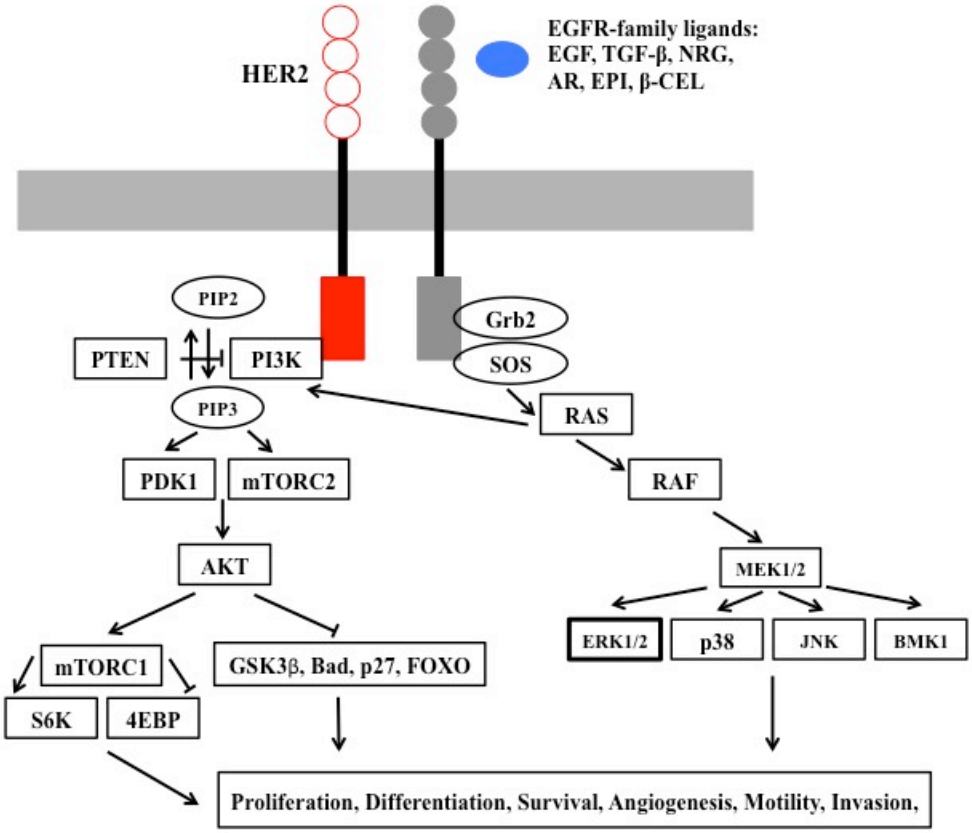
1.3.2 HER2 signaling:

The role of receptor activation and receptor-initiated signaling through members of the EGFR family in cancer development has been previously documented. However, most targeted therapies designed to inhibit EGFR activation and signaling have yielded temporary or limited clinical success in patients with breast cancer. The modest clinical benefits reported are partly due to the complex nature of EGFR signaling. The outcome of EGFR signaling cascades is determined by many factors. Mainly by the ligand identity, the identity of the receptors forming dimers, and the intracellular proteins engaged in the signaling event (19). Signaling pathways downstream of the EGFR family are widely deregulated in cancer. The activation of receptors of the EGFR family leads to the induction of signal transduction pathways, most commonly the PI3K/Akt/mTOR and MAPK pathways (Figure 1.3).

1.3.2.1 PI3K signaling:

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway is an important intracellular cascade

Figure 1.3. HER2 signaling pathways. Ligand binding (e.g. EGF, epidermal growth factor; TGF-β, transforming growth factor beta; NRG, neuregulin; AR, amphiregulin; EPI, epiregulin; β-CEL, b-cellulin) to the extracellular domain of EGFR family receptors leads to dimerization and activation of the receptors. HER2 dimerizes with other EGFR receptors and activates signal transduction pathways, most commonly the PI3K/Akt/mTOR and MAPK pathways. Signaling cascades result in the promotion of cell proliferation, differentiation, survival, angiogenesis, motility, and invasion.


HER2
EGFR-family ligands:
EGF, TGF-β, NRG,
AR, EPI, β-CEL
PIP2
PTEN
PI3K
PIP3
Grb2
SOS
RAS
PDK1
mTORC2
RAF
AKT
MEK1/2
ERK1/2
p38
JNK
BMK1
mTORC1
GSK3β, Bad, p27, FOXO
S6K
4EBP
Proliferation, Differentiation, Survival, Angiogenesis, Motility, Invasion,

commonly activated in various cancers (20; 21), where it contributes to the development of resistance to anticancer therapies (22; 23). The PI3K protein family includes at least three different classes of lipid kinases (class I, II, and III); with class IA PI3K's linked to human cancer development (24). Class IA PI3K's are heterodimers of a catalytic subunit (p110) and a regulatory subunit (p85) (25). PI3K's are activated by the stimulation of EGFR's. Constitutively activated RAS kinases are also known to activate PI3K's (26). Specifically, the p85 regulatory subunit binds to phosphorylated tyrosine residues on the catalytic domain of EGFR's or to adaptor proteins like the insulin receptor substrate-1 (IRS-1), through Src homology 2 (SH2) domains (27; 28). Binding of the p85 regulatory subunit to a receptor or adaptor protein relieves the inhibition of the p110 catalytic subunit by p85. Active PI3K is moved to the plasma membrane, eventually leading to phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (29). This step is negatively regulated by the tumor suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates PIP3 to PIP2 (24). PIP3 contributes to the localization of signaling proteins with pleckstrin homology (PH) domains to the plasma membrane, where they become activated and spread the signal (30). For example, serine/threonine Akt kinase has a PH domain that is recruited to the plasma membrane, where it can be activated by 3-phosphoinositide-dependent kinase 1 (PDK1) and second mechanistic target of rapamycin complex (mTORC2) (26). Akt's major downstream effector involves the first mechanistic target of rapamycin complex (mTORC1) (31). Activated mTORC1 inactivates the eukaryotic translation initiation factor 4E binding protein (4EBP1) and activates the ribosomal protein S6 kinase (S6K), increasing protein synthesis (25; 31). Akt also promotes inhibition of apoptosis by

targeting proteins Bad, Bim and caspase 9, the metabolic regulator glycogen synthase kinase-3β (GSK-3β), cell cycle regulators p21 and p27, and the family of transcription factors forkhead box O (FOXO) (26).

The most common mutations that alter normal PI3K signaling are observed in the tumor suppressor PTEN, activating mutations or amplification of the PIK3CA gene (p110 catalytic subunit), activating mutations of the PIK3R1 gene (p85 regulatory subunit), and somatic mutations or amplification of the AKT genes (26). Mutations of the PIK3CA gene are observed in 25% of breast cancers, and amplification of the PIK3CA gene are found in 9% of breast cancers (24; 25). Deletions in the AKT1 and AKT2 genes have been identified in breast cancer (32). Amplification of AKT genes have also been described in several tumor types, including breast cancers (21; 32), with 3% AKT2 amplification present in breast tumors (33). In the case of PTEN, mutations may affect one or both alleles of the gene. Mutations in one allele are found in approximately 40% of breast cancers, and mutations of both alleles in about 5% of breast cancers (34). Mutations like these are partially responsible for the promotion of several types of cancer, by promoting cell survival, protein synthesis, and cell migration.

1.3.2.2 MAPK signaling:

There are four distinct MAPK signaling cascades: the MAPK/ERK pathway, the Big MAP kinase-1 (BMK-1), the c-Jun N-terminal kinase (JNK), and the p38 signaling cascade (35). The MAPK/ERK cascade is considered the classical pathway and is one of the most deregulated pathways in human cancer (36). The basic organization of the classic MAPK signaling cascade includes two serine/threonine kinases and one threonine/tyrosine kinase (37). Kinases involved in this pathway have generic names

based on their location in the signaling pathway. From upstream to downstream, these are the MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK (24).

Ligand binding to the extracellular domain of an EGFR receptor induces dimerization and activation of the kinase activity in the intracellular domain. Activation of the kinase domain leads to phosphorylation of tyrosine residues, providing docking sites for adaptor proteins with SH2 domains or phosphotyrosine binding (PTB) domains, like the growth factor receptor-bound protein 2 (GRB2) (38). Adaptor proteins recruit other effector proteins, like the guanine-nucleotide exchange factor (GEF) son of sevenless (SOS). For example, SOS is recruited to the cell membrane where small GTPase RAS proteins (KRAS, NRAS, and HRAS) are localized and function as molecular switches of the signaling cascade (39). SOS proximity to RAS promotes the exchange of GDP for GTP on RAS, activating the protein. Activated RAS induces activation of RAF serine/threonine protein kinases (a type of MAPKKK): ARAF, BRAF, CRAF, and RAF-1 (36). Activated RAF kinase phosphorylates MAPKK's MEK1 and MEK2, which are serine/threonine/tyrosine protein kinases. MEK1/2 recognize and phosphorylate tyrosine and threonine residues of MAPK's: extracellular signal-regulated kinases (ERK) ERK1 and ERK2 (39). Activated ERK1/2 phosphorylates several substrates including transcription factors, such as the erythroblastosis virus E26 oncogene homolog 1-like gene 1 (Elk1) and cAMP response element binding protein (CREB), and protein kinases, such as P90 ribosomal S6 kinase (RSK) and MAPK-interacting serine/threonine kinase (MNK) (24).

Apart from somatic mutations, gene amplification and/or increased autocrine or paracrine signaling through the EGFR receptors, mutations in the genes of the different

components of the MAPK pathway might lead to the constitutive activation of the signaling cascade (39). The most commonly mutated components are the RAS and RAF genes. Activating mutations in the RAS genes occur nearly in 30% of human cancers, with KRAS mutations reported as the most common (24). However, somatic mutations of KRAS are rarely found in breast cancer (24). Mutations of the BRAF isoform are the most prevalent within the RAF family, with over 40 mutations already described (40). A single-nucleotide missense mutation that substitutes valine for glutamic acid at codon 600 (V600E) of the kinase domain of BRAF is the predominant mutation found in this protein (41). Nonetheless, RAF mutations are not very frequent in breast cancers (between 1% and 3% of cases) (24). Current and future drug development efforts will be important to assess the effects of mutations and deregulation of the MAPK signaling cascade.

1.4 Targeted therapies for the treatment of HER2-positive breast cancer:

Overexpression of HER2, specifically in breast cancer cells, provides a selective target for anticancer drugs. Several drugs have been developed for the treatment of HER2-overexpressing metastatic breast cancers, including HER2-targeted antibodies and tyrosine kinase inhibitors.

1.4.1 Trastuzumab:

Trastuzumab (Herceptin™, Genentech) is a humanized IgG_1 monoclonal antibody that targets extracellular domain IV of HER2 with high affinity (42; 43). This was the first HER2-targeted therapeutic approved by the Food and Drug Administration (FDA) for the treatment of HER2-overexpressing metastatic breast cancers (44). In the United States, trastuzumab is approved for the adjuvant treatment of HER2-positive early or

metastatic breast cancer as a single agent and in combination with chemotherapy (10). Phase III clinical trials established that trastuzumab, concurrent with anthracycline- or non-anthacycline-based chemotherapy, prolongs disease-free and overall survival, and is well tolerated by patients with HER2-positive early breast cancer (45; 46). The main toxicity concern noted in clinical trials was related to cardiomyopathies, especially when patients received concurrent anthracycline therapy. However, trastuzumab cardiotoxicity was reversible in most cases (10; 11).

Although the mechanisms of anti-cancer action triggered by trastuzumab remain to be fully characterized, preclinical and clinical studies suggest the following potential mechanisms of action. Trastuzumab has been shown to inhibit cleavage of the extracellular domain of HER2 (47), resulting in lower levels of circulating HER2 extracellular domain. Reduced serum levels of HER2 extracellular domain are associated with longer progression-free survival in patients that responded to trastuzumab (48). Trastuzumab inhibits PI3K/Akt signaling and proliferation (49; 50) and induces apoptosis in HER2-overexpressing breast cancer cells (51). The conserved Fc portion of trastuzumab interacts with the Fc receptor on natural killer cells and other immune cells (11), promoting antibody-dependent cellular cytotoxicity. Activation of tumor cell lysis through this immune response has been observed in response to trastuzumab treatment in multiple cell line and mouse models of HER2-overexpressing breast cancer and in patients with primary HER2-positive breast cancer (52-54).

Trastuzumab has dramatically improved survival in patients with HER2-overexpressing breast cancer. However, resistance to trastuzumab often arises within a year of treatment initiation (12; 55). Various mechanisms of resistance have been

proposed in the literature. Increased proteolytic cleavage of HER2 produces a constitutively active HER2 kinase fragment to which trastuzumab cannot bind (56). In addition, the presence of membrane-bound proteins, such as MUC4, sterically hinders the interaction between trastuzumab and the extracellular domain of HER2 (57; 58). Overexpression of other receptor tyrosine kinases, including insulin growth factor -1 receptor (IGF-1R), epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 3 (HER3), leads to activation of alternative signaling pathways compensating for HER2 inhibition (49; 59-63). Constitutive activation of downstream signaling through PI3K and MAPK has also been proposed as mechanisms of trastuzumab resistance (64-66). Finally, inhibition of the antibody-dependent cellular cytotoxicity response to trastuzumab may also represent a mechanism of resistance (67; 68).

1.4.2 Lapatinib:

Lapatinib (Tykerb^{TM}, Glaxo-SmithKline) is a reversible small molecule tyrosine kinase inhibitor of EGFR and HER2 (69). Lapatinib selectively inhibits EGFR and HER2 versus other kinases, and selectively inhibits the growth of tumor cells versus normal cells (70). Lapatinib is used as a first-line therapy in combination with letrozole for ER-positive/HER2-positive metastatic breast cancer (44) and is approved in combination with capecitabine as a second-line therapy for HER2-overexpressing breast cancer that no longer responds to trastuzumab (71). Clinical trials suggest that progression-free and overall survival rates are higher for patients with HER2-positive breast cancer when lapatinib is combined with chemotherapy or trastuzumab (72; 73). Lapatinib was well

tolerated when administered orally at a single dose, with skin rash and diarrhea as the most common side effects (74).

Similar to the clinical experience with trastuzumab, resistance to lapatinib develops rapidly. Several mechanisms have been proposed, including the development of mutations in the intracellular kinase domain of HER2, preventing lapatinib-induced inhibition (75). Upregulation of HER3, resulting in sustained PI3K signaling through HER2/HER3 heterodimers has also been proposed as a putative mechanism of lapatinib resistance (76). Increased β1 integrin signaling with activation of the downstream kinases FAK and Src (77), and upregulation of estrogen receptor signaling via activation of transcription factors FOXO3a and caveolin-1 (78) have also been reported in lapatinib-resistant cells.

The development of resistance to HER2-targeted therapies necessitates efforts to understand mechanisms of resistance and develop new therapeutic strategies for patients with HER2-overexpressing metastatic breast cancer.

1.5 Insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (IR) signaling as mechanisms of resistance to trastuzumab in breast cancer:

1.5.1 IGF/Insulin system:

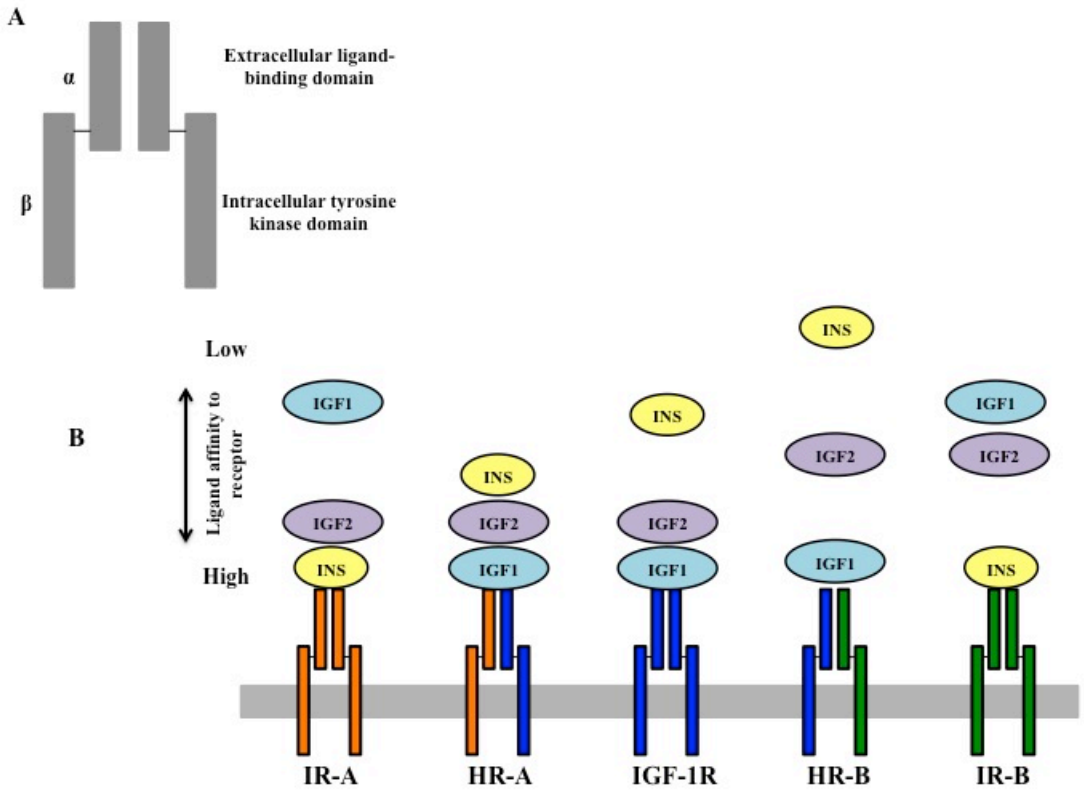
The IGF/Insulin system is not only critical for normal developmental processes, but is also implicated in tumor development and progression (79). As part of its physiological role, the IGF/Insulin system plays a necessary role in the stimulation and conservation of normal mammary gland development and function (80). During ductal development and early pregnancy, the IGF system promotes cell cycle progression and

cell survival, stimulating the initiation and maintenance of lactation (80). This important signaling cascade includes three ligands (IGF-1, IGF-2, and insulin), corresponding receptors (IGF-1R, IGF-2R, and IR), and six IGF-binding proteins (IGFBPs), which regulate ligand bioavailability and receptor signaling (81). As observed in Figure 1.4.B, each receptor has different affinities for each ligand. IGF-1 has higher affinity for IGF-1R compared with other receptors, but can still bind to IR with lower affinity (82). By contrast, IGF-2 binds to IGF-2R and IGF-1R with similar affinity (82). Insulin binds primarily to IR, and has lower binding affinity for IGF-1R (82). Ligand-receptor binding induces conformational changes and kinase activation, resulting in the recruitment and activation of adaptor proteins. Subsequently, signaling cascades, including MAPK and PI3K/Akt pathways, are activated, leading to cell survival, proliferation, and migration (83). IGF-1R and IR mediate the physiological actions related to this system, while IGF-2R serves mostly as a regulator of IGF-2 bioavailability (84). Based on evidence linking IGF-1R and IR to cancer development and progression, we will discuss the IGF-1R and IR and their role in breast cancer, especially the HER2-positive subtype, and trastuzumab resistance.

1.5.2 IGF-1R in breast cancer:

The IGF-1R gene is located at chromosome 15q25-q26 (85). The IGF-1R receptor tyrosine kinase is expressed in most normal tissues and its expression is developmentally regulated (86). IGF-1R is synthesized as a single precursor polypeptide that undergoes glycosylation and cleavage, generating one alpha and one beta subunit (85). The mature, functional IGF-1R consists of two alpha and two beta subunits joined by disulfide linkages (87). The alpha subunits are ligand-binding extracellular domains, whereas the

Figure 1.4. Ligand binding affinity to the receptors of the IGF-1/Insulin system. (A) The general structure of the Insulin-like Growth Factor 1 Receptor (IGF-1R) and the Insulin Receptor (IR). Each monomer is composed of an extracellular ligand-binding domain (alpha) and an intracellular tyrosine kinase domain (beta) linked by a disulfide bond. **(B)** Different conformations of IGF-1R and IR dimers: IGF-1R (blue) homodimer, IR isoform A homodimer (IR-A, orange), IR isoform B homodimer (IR-B, green), and hybrid receptors (HR). Ligands closer to the alpha domain of the receptor have higher affinity for the receptor, while ligands distanced from the receptor exhibit lower binding affinity. Based on work by Pandini et. al., 2002.


A
α
β
Extracellular ligand-binding domain
Intracellular tyrosine kinase domain
B
Low
High
Ligand affinity to receptor
IGF1
IGF2
INS
IR-A
HR-A
IGF-1R
HR-B
IR-B

beta subunits are transmembrane with a cytoplasmic tyrosine kinase domain (Figure 1.4.A) (88).

The primary function of IGF-1R is to regulate cell proliferation, survival, differentiation and motility (89). IGF-1R has been the focus of extensive research due to its potential implication in the development of breast cancer and other diseases (90). Analysis of breast cancers in The Cancer Genome Atlas (TCGA) database indicates that 9% of breast cancers exhibit IGF-1R overexpression, amplification or somatic mutations (84). Several studies have shown that activation of IGF-1R stimulates invasion of breast cancer cells *in vitro* (91; 92). Inhibition of IGF-1R was associated with reduced cell proliferation and increased apoptosis in precancerous breast lesions (93).

IGF-1R was first implicated in trastuzumab resistance in a study where stable IGF-1R overexpression reduced the ability of trastuzumab to induce cell cycle arrest and growth inhibition of HER2-positive breast cancer cell lines (61). In another study, IGF-1R expression was increased in trastuzumab-resistant SKBR3 cells relative to parental cells (94). Clinically, IGF-1R overexpression is significantly associated with HER2-positivity and poor disease-free survival among premenopausal women with breast cancer (95). Multiple studies, including from our group, have reported that crosstalk from IGF-1R to HER2 results in sustained HER2 phosphorylation in the presence of trastuzumab (60; 62; 96; 97). These findings support the hypothesis that IGF-1R promotes trastuzumab resistance in HER2-positive breast cancer. In addition, IGF-1R inhibition has been shown to improve response to trastuzumab in HER2-positive breast cancer cells (94), suggesting that IGF-1R-targeted therapy may be beneficial for patients who have progressed on trastuzumab.

1.5.3 IR in breast cancer:

IR is a receptor tyrosine kinase with high structural and functional similarity to IGF-1R, mainly within the tyrosine kinase domain (98; 99). IGF-1R and IR share approximately 70% overall homology and 84% homology within the kinase domain (98; 100). The IR gene is located on chromosome 19 (101). The mature IR is a heterotetrameric receptor consisting of two extracellular alpha subunits and two transmembrane beta subunits with intrinsic tyrosine kinase activity in the cytoplasmic tail (Figure 1.4.A) (102). Monomers of alpha/beta domains from each receptor can homodimerize or heterodimerize to form hybrid receptors (103; 104). There are two IR isoforms: IR-A, obtained by skipping exon 11 of the IR sequence, and IR-B, expressed in most normal tissues (105). Although it is expressed in many different cell types, IR is expressed at high levels in adult muscle, adipose tissue, and the liver, where it regulates glucose uptake and metabolism in response to insulin (89; 105).

An increase in IR content has been observed in breast cancer tissue compared with normal breast tissue (106). Increased IR levels were also found in three different transgenic mouse models of breast cancer, including one initiated by the *her2* oncogene (107). Another study found that the relative abundance of the IR-A isoform in human breast cancer tissue was significantly higher than in normal tissue (108). Moreover, they found that IGF-2 could bind to IR-A with an affinity close to that of insulin, leading to mitogenic effects (108). Given the high degree of homology between IGF-1R and IR, the relationship between high receptor expression and increased risk of cancer, and their ability to form hybrid receptors, the role of hybrid receptors is of interest to breast cancer researchers.

1.5.4 IGF-1R/IR hybrid receptors in breast cancer:

There is evidence of hybrid receptor formation in cells and tissues that overexpress both IGF-1R and IR (109-111). Studies suggest that the formation of IGF-1R/IR hybrid receptors, consisting of one alpha/beta half from the IR and the other half from IGF-1R, allows the hybrid receptors to bind all ligands of the IGF/insulin system (84; 112) with preference for IGF-1 (105; 113). Cells expressing hybrid receptor IGF-1R/IR-A are more sensitive to the biological effects, including proliferation and migration, induced by IGF-1, IGF-2, and insulin compared with cells transfected with hybrid receptors containing the IR-B isoform (114). Further, a nested case-control study found a positive relation between elevated serum IGF-1 and a higher risk of developing invasive breast cancer in premenopausal women (115). Immunohistochemical staining of invasive breast tumor samples also show that phosphorylated IGF-1R/IR and total IR are indicative of poor survival (116). Evidence suggesting that hybrid receptors bind and react to all three ligands of the IGF/insulin system, mainly those with IGF-1R and IR-A domains, might help explain why there is crosstalk between the signaling pathways activated downstream of IGF-1 and insulin in cancer.

1.6 Targeting IGF-1R and IR in breast cancer:

Preclinical results provide compelling rationale for evaluating IGF-1R/IR-targeted therapies as potential treatments for cancer. However, mixed outcomes in clinical trials of IGF-1R-targeted agents have been reported, questioning the feasibility of successfully targeting the IGF/insulin system in breast cancers.

A phase I clinical trial showed that treatment of a subset of patients with ER-positive, highly proliferative disease using the IGF-1R antibody dalotuzumab plus the mTOR inhibitor ridaforolimus resulted in anti-tumor activity in 55% of patients (117). Results from this study motivated a recently completed phase II trial including patients with advanced luminal B breast cancer treated with dalotuzumab plus ridaforolimus plus hormone therapy (117). However, another phase I study using the IGF-1R antibody cixutumumab plus the mTOR inhibitor temsirolimus in 26 patients with metastatic breast cancer (mostly ER-positive subtype) demonstrated a complete lack of response (no partial or complete responses) (118). In a phase II clinical trial, IGF-1R antibody ganitumab plus exemestane or fulvestrant were administered to postmenopausal women with hormone receptor-positive advanced or metastatic breast cancer. However, this regimen did not yield significant differences in median progression-free survival, and overall survival was higher in the placebo group compared with the experimental group (119). Generally, therapies that target IGF-1R have been well tolerated, with hyperglycemia as the main side effect (120). All together, these studies demonstrate somewhat conflicting results regarding the suitability of targeting IGF-1R for the treatment of breast cancers.

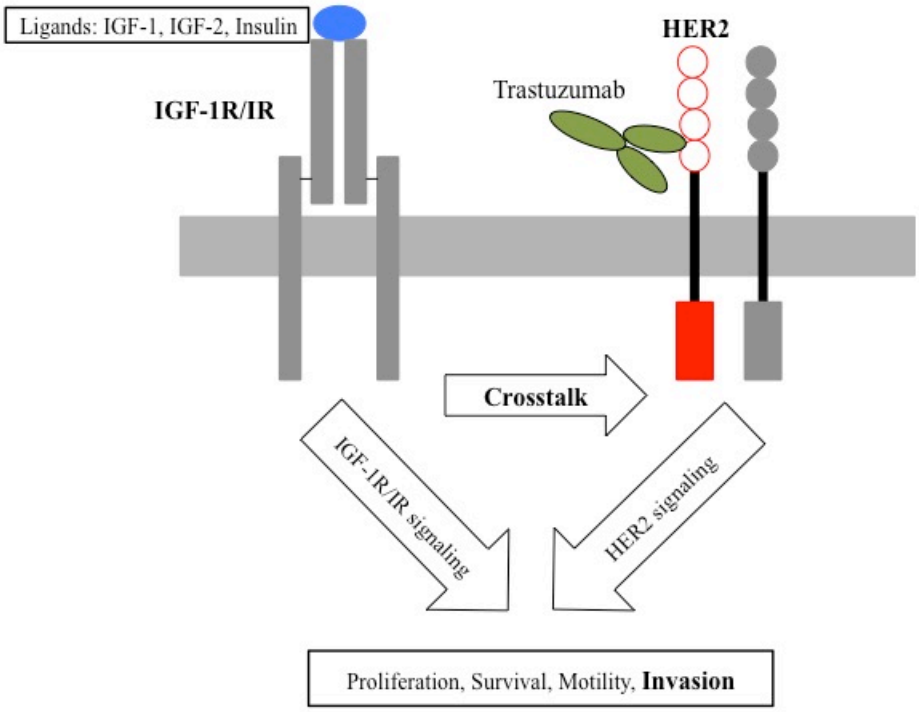
Some of the disappointing results might be due to IR signaling compensation after IGF-1R inhibition (121). Therefore, dual targeting of IGF-1R and IR might be appropriate in order to observe significant results in the clinical setting. In a phase I study, patients with advanced treatment-refractory tumors received monotherapy with the dual IGF-1R/IR inhibitor linsitinib (OSI-906); overall disease stabilization was achieved

in 46% of patients (122). Phase I/II trials using linsitinib with paclitaxel or erlotinib have been recently completed, and results should be available soon (122).

1.7 Scope of this dissertation:

In this dissertation, the role of IGF-1R and IR in trastuzumab resistance and the impact of IGF-1R/IR-targeting on trastuzumab response are examined in HER2-positive breast cancers (Figure 1.5). Protein expression and phosphorylation, ligand stimulation, cell proliferation, and cell invasion are widely assessed. We also examine mediators downstream of IGF-1R/IR and HER2 responsible for the observed behavior of these cells. Here we decipher the molecular mechanism by which IGF-1R and IR signaling stimulates the invasive phenotype of HER2-positive trastuzumab-resistant breast cancer cells, and evaluated the therapeutic efficacy of co-targeting IGF-1R and IR in the pre-clinical setting. Our findings support further preclinical evaluation of therapies that co-target IGF-1R and IR in breast cancers that have progressed on HER2-targeted treatment.

Figure 1.5. HER2 and IGF-1R/IR signaling contribute to trastuzumab resistance. Based on the literature, signaling initiated by activation of receptor tyrosine kinases IGF-1R, IR, and HER2 leads to cell proliferation, survival, migration, and invasion in HER2-positive cells. Furthermore, crosstalk between the IGF-1R and HER2 has been previously reported as a potential mechanism of trastuzumab resistance.


Ligands: IGF-1, IGF-2, Insulin
IGF-1R/IR
HER2
Trastuzumab
Crosstalk
IGF-1R/IR signaling
HER2 signaling
Proliferation, Survival, Motility, Invasion

Chapter 2:

Insulin-like growth factor-1 receptor signaling increases the invasive potential of HER2-overexpressing breast cancer cells via Src-FAK and FoxM1

Portions of this chapter were previously published: E Sanabria-Figueroa, SM Donnelly, KC Foy, MC Buss, RC Castellino, E Paplomata, L Taliaferro-Smith, PTP Kaumaya, and R Nahta (2015) Insulin-Like Growth Factor-1 Receptor Signaling Increases the Invasive Potential of Human Epidermal Growth Factor Receptor 2– Overexpressing Breast Cancer Cells via Src-Focal Adhesion Kinase and Forkhead Box Protein M1, *Mol Pharmacol*, 87(2):150-161; DOI: https://doi.org/10.1124/mol.114.095380

Chapter 2: Insulin-like growth factor-1 receptor signaling increases the invasive potential of HER2-overexpressing breast cancer cells via Src-FAK and FoxM1

2.1 Introduction:

Breast cancer is the most commonly diagnosed cancer among women in the United States (123). Multiple subtypes of breast cancer have been identified through gene profiling studies (6). Breast cancers that show amplification and overexpression of the human epidermal growth factor receptor 2 (*her2*) gene represent approximately 20-30% of metastatic cases (12). Over-expression of the HER2 receptor tyrosine kinase is associated with poor prognosis, reduced overall survival, and the development of resistance to some types of chemotherapy (12). The specific overexpression of HER2 in breast cancers serves as a selective target for anti-cancer drugs. Trastuzumab (Herceptin; Genentech, South San Francisco, CA), a humanized monoclonal antibody against an extracellular epitope on domain IV of HER2 (42), was the first HER2-targeted therapy approved by the Food and Drug Administration for the treatment of HER2-overexpressing metastatic breast cancer (44). The mechanisms through which trastuzumab promotes anti-tumor activities include blockade of downstream signaling, reduced cleavage of the extracellular domain, inhibition of angiogenesis, and induction of immune activity, primarily antibody-dependent cellular cytotoxicity (ADCC) (124). Trastuzumab plus chemotherapy improves overall response rates, time to progression, and the overall survival of patients with HER2-positive metastatic breast cancer beyond that achieved with chemotherapy alone (46). However, clinical trials demonstrate that the

median duration of single-agent trastuzumab or trastuzumab-containing chemotherapeutic regimens is less than one year (46; 125-127). Further, single-agent trastuzumab achieves moderate response rates among HER2-overexpressing metastatic breast cancers (125). These data indicate that acquired resistance and primary resistance to trastuzumab are clinical concerns in the treatment of HER2-positive metastatic breast cancer.

Multiple mechanisms of trastuzumab resistance have been described in the literature. Constitutive activation of downstream PI3K/Akt signaling through PTEN downregulation or *PIK3CA* hyper-activating mutations has been reported to significantly abrogate response to trastuzumab (65; 128). In addition, the lack of an effective ADCC immune response has been shown to result in trastuzumab resistance (52; 67; 68). Increased expression or compensatory signaling through other receptor tyrosine kinases, including the insulin-like growth factor-1 receptor (IGF-1R), EGFR, or HER3, and/or crosstalk of receptor kinases to HER2 have also been reported as mechanisms of acquired resistance to trastuzumab (49; 59-63).

The first study to implicate IGF-1R in trastuzumab resistance showed that stable over-expression of IGF-1R reduces the ability of trastuzumab to induce G1 arrest and growth inhibition of HER2-overexpressing breast cancer cell lines (61). Further, among cases of HER2-overexpressing breast cancer, high IGF-1R expression or phosphorylation correlates with worse response to preoperative trastuzumab and chemotherapy (129) and reduced progression-free survival (130). We and others have reported that crosstalk from IGF-1R to HER2 results in sustained HER2 phosphorylation in the presence of trastuzumab (60; 62; 96). However, the specific mechanisms through which IGF-1R

activates HER2 and the major downstream molecular and biological effects remain poorly defined.

In this study, we found that Src activity maintained HER2 phosphorylation in trastuzumab-resistant cells. Further, we showed that the major biological effect promoted by IGF-1R was cellular invasion mediated by both Src-FAK and HER2-FoxM1 signaling. Co-targeting IGF-1R and HER2 suppressed the invasiveness of trastuzumab-resistant cells and appeared to depend in part on FoxM1 and Src inhibition, as overexpression or activation of these molecules blocked the anti-invasive effect of IGF-1R/HER2 co-targeting. These results suggest that therapeutic combinations that block IGF-1R and HER2 may reduce the invasive potential of cancer cells that are resistant to trastuzumab.

2.2 Materials and methods:

Materials. Trastuzumab (Genentech; South San Francisco, CA) was obtained from the Emory Winship Cancer Institute pharmacy and dissolved in sterile water to a stock concentration of 20 mg/ml. Lapatinib ditosylate (Santa Cruz Biotechnology; Dallas, TX) was dissolved in DMSO to a final concentration of 10 mM. The IGF-1R antibody alpha IR3 (Calbiochem; San Diego, CA) was provided at a stock concentration of 1 mg/mL. The IGF-1R antibody IGF-IR-56-81 was developed by Dr. Pravin Kaumaya from The Ohio State University (131). Briefly, rabbits were immunized with 1 mg of IGF-1R peptide, Ac-LLFRVAGLESLGDLFPNLTVIRGWKL- NH2; antibodies were purified from rabbit sera by affinity chromatography using protein A/G columns. IGF-1 ligand

(Sigma; St Louis, MO) was dissolved in sterile water at a stock concentration of 1 mg/mL. The IGF-1R kinase inhibitor NVP-AEW541 (Cayman Chemical; Ann Arbor, MI) was dissolved in DMSO to a final concentration of 10 mM. In-Solution Src kinase inhibitor PP2 (Calbiochem; San Diego, CA) was provided at a stock concentration of 10mM in DMSO. PF573228, FAK Inhibitor II (Santa Cruz Biotechnology; Dallas, TX), was dissolved in DMSO to a stock concentration of 20 mM. The pLKO.1-IGF1R- / short hairpin RNA (shRNA) plasmid and pLKO.1 empty vector plasmid (negative control) were purchased from Open Biosystems (Huntsville, AL, USA). FoxM1 siRNA (sc-270048) and control siRNA (sc-37007) (Santa Cruz Biotechnology; Dallas, TX) were resuspended in RNAse-free water. FoxM1 expression plasmid was purchased from Origene.

Cell culture. JIMT1 cells were purchased from DSMZ, Germany; all other cell lines were purchased from American Type Culture Collection (Manassas, VA). HCC1954 cells were maintained in RPMI with glutamine (Corning; Manassas, VA), which was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. JIMT1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, glutamine, and sodium pyruvate (Corning; Manassas, VA) with 10% FBS and1% penicillin/streptomycin. JIMT1 and HCC1954 cells have previously been shown to have reduced response to trastuzumab compared to other models of HER2-overexpressing breast cancer and are considered models of primary trastuzumab resistance. All cells were cultured in humidified incubators at 37°C with 5% CO2.

Creation of stable IGF-1R knockdown clones. HEK-293T cells (1.5×10^6) were seeded in 100-mm dishes for 24 h and co-transfected with 3 μg shRNA construct (pLKO.1-IGF1R- / shRNA or pLKO.1 empty vector control plasmid), 3 μg pCMV-dR8.2, and 0.3 μg pCMV-VSV-G helper constructs using TransIT-LT-1 Transfection according to the manufacturer's instructions (Mirus Bio LLC, Madison, WI, USA). Viral stocks were harvested from culture media by centrifugation 48 h after transfection and were syringe-filtered. JIMT1 cells were seeded at sub-confluent densities and infected with lentiviral vectors (1:20 dilution) in fresh culture media. Culture media was replaced with media containing 5 μg/ml puromycin 48 h after lentiviral infection to select for cells stably expressing the shRNAs. Stable knockdown was confirmed by Western blotting for IGF-1R. The IGF1R shRNA and control shRNA cells are routinely maintained on 5 μg/ml puromycin in DMEM.

Stimulation experiments. Cells were plated and serum starved for 24 h. During serum starvation, cells were treated with 500 nM PF573228 (FAK inhibitor), 100 nM lapatinib, varying concentrations of NVPAEW541, vehicle control, or untreated. Cells were then either lysed for protein or stimulated with vehicle control or 100 ng/mL IGF-1 for varying time points. Experiments were repeated at least three times with reproducible results.

Trypan blue exclusion assay. For growth inhibition assays, cells were plated in complete DMEM at 2×10^4 per well in 12-well plates. The next day, media were aspirated and replaced with media containing control mouse IgG, alpha IR3 (0.25 μg/mL),

trastuzumab (20 μg/mL), or alpha IR3 plus trastuzumab in triplicate. After 72 h, viable cells were counted under a light microscope by trypan blue exclusion. Assays were repeated at least three times with reproducible results.

Anchorage-independent cell culture growth. Cells were plated in matrigel (BD Biosciences; Franklin Lakes, NJ) at a 1:1 dilution (media:matrigel). The matrigel-cell suspension was allowed to solidify, and media containing control mouse IgG, alpha IR3 (0.25 μg/mL), trastuzumab (20 μg/mL), or alpha IR3 plus trastuzumab were added to cells in triplicate cultures. Media and drugs were renewed twice a week for 3-4 weeks. Photographs were taken with an Olympus IX50 inverted microscope at 4× magnification. Matrigel was digested using dispase (BD Biosciences), and viable cells were counted by trypan blue exclusion. The average cell viability of triplicates and standard deviation were calculated. Experiments were performed at least twice with reproducible results.

Transfection. Cells were plated in antibiotic-free media at a concentration of 2×10^5 cells/mL. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) with 10 μg/mL of one of the following plasmids (kind gifts from Dr. Sumin Kang, Emory): constitutively active (A) Src, kinase-dead (KD) Src, wild-type (WT) Src, or pcDNA3.1 empty vector control. Media was changed after 6 h of transfection and replaced with complete media; cells were harvested after 48 h.

Spheroid migration assays. JIMT1 (4.0×10^4) cells were suspended in complete media containing one of the following treatments: control IgG, 0.25 μg/mL alpha IR3, 20

μg/mL trastuzumab (tras), alpha IR3 plus trastuzumab, or untreated. Cells were seeded on 1% agar-coated 96- well plates and cultured for 24 h in a humidified atmosphere containing 5% CO2 at 37°C. Intact tumor spheroids were carefully transferred to a 96-well plate and cultured in complete media containing respective inhibitors or control vehicle for 48 h. Spheroids and migrated cells were fixed with 100% methanol, stained with 0.05% crystal violet, and observed using a normal light microscope (20×) and Olympus DP-30BW digital camera. Experiments were repeated three times with reproducible results; representative images are shown for all groups.

Invasion chamber assays. Cells were plated in serum-free media in BD BioCoat Matrigel Invasion Chambers (BD Biosciences; Franklin Lakes, NJ) (1×10^5 cells/mL) with 0.75 mL of chemoattractant (culture media containing 10% FBS) in the wells. Depending on the experiment, cells were pre-treated with 500 nM FAK inhibitor or 10 μM PP2 for 24 h or were transfected with control siRNA, FoxM1 siRNA, empty vector, or FoxM1 expression plasmid overnight prior to placing cells in invasion chambers, at which point they were treated with control or IGF-1 (100 ng/mL) for 24 h. In other experiments, control mouse IgG, alpha IR3 (0.25 μg/mL), IGF1R- 56-81 (400 μg/mL), trastuzumab (20 μg/mL), alpha IR3 plus trastuzumab, IGF1R-56-81 plus trastuzumab, or IGF-1 (100 ng/mL) were added. Treatments were added directly to chambers in all experiments. Non-invading cells were removed from the interior surface of the membrane by scrubbing gently with a dry cotton-tipped swab. Each insert was then transferred into 100% methanol for 10 minutes followed by Crystal Violet staining for 20 minutes. Membranes were washed in water and allowed to air dry completely before being

separated from the chamber. Membranes were mounted on slides with permanent mounting medium Permount (Fisher Scientific). Multiple photographs of each sample were taken at 20× magnification, with triplicates performed per treatment group. The number of cells was counted in each field; the sum total of the fields was calculated for each sample. Experiments were performed at least twice with reproducible results.

Cell cycle analysis. Cells were treated with control mouse IgG, alpha IR3 (0.25 μg/mL), trastuzumab (20 μg/mL), or alpha IR3 plus trastuzumab for 48 h. Cells were harvested, washed twice with DPBS+10% FBS, fixed in ice-cold 80% ethanol, and stored at −20°C for at least 24 h. Fixed cells were incubated in 50 μL of propidium iodide buffer (20 μg/mL PI (Sigma), 0.1% Triton-X 100, 200 μg/mL RNaseA (Promega) in DPBS) for 30 minutes in the dark. The cells were then resuspended in 400 μL DPBS for flow cytometric analysis. Samples were analyzed using a BD FACS Canto II cytometer (BD Biosciences; San Jose, CA) and BD FACS Diva software; experiments were performed in triplicate and repeated twice with reproducible results.

Western blotting. Cells were lysed in RIPA buffer (Cell Signaling; Danvers, MA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Total protein extracts were run on SDS-PAGE and blotted onto nitrocellulose. Blots were probed overnight. The following antibodies were purchased from Cell Signaling: rabbit anti-phospho-IGF-1 receptor (Tyr1135/1136) (#3024, 1:200); rabbit anti-phospho-IGF-1 receptor (Tyr1131) (#3021, 1:200); rabbit anti-IGF-1 receptor (#3018, 1:250); rabbit anti-phospho-FAK (Tyr397) (#8556, 1:250); rabbit anti-FAK (#3285); rabbit anti-phospho-

p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101, 1:1000); rabbit anti-p44/42 MAPK (Erk1/2) (#9102, 1:1000); rabbit anti-phospho-Src (Tyr416) (#2101, 1:1000); rabbit anti-Src (#2123, 1:1000); and rabbit anti-FoxM1 (#5436, 1:200). The following antibodies were purchased from AbCam (Cambridge, MA): rabbit anti-phospho-ErbB2 (Y877) (ab47262, 1:200); rabbit anti-phospho-ErbB2 (Y877) (ab108371, 1:200); and mouse anti-ErbB2 (ab16901, 1:200). Mouse anti-β-actin was purchased from Sigma-Aldrich (AC-15, 1:15,000). All primary antibodies were diluted in 5% BSA/TBS-T. Goat anti-mouse secondary IRDye 800 antibody (#926-32210, 1:10,000) was purchased from Li-Cor Biosciences (Lincoln, NE). Goat anti-rabbit Alexa-fluor 680 secondary antibody (#1027681, 1:10,000) was purchased from Invitrogen (Grand Island, NY). Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE). All blots were repeated at least 3 times with reproducible results.

Antibody-dependent cellular cytotoxicity (ADCC) assays. The ADCC assay was performed as previously described (132) using effector PBMCs, which were obtained from normal human donors and isolated by density-gradient centrifugation in Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). The cells were washed twice in RPMI 1640 with 5% FCS and then serially diluted in 96-well plates to give effector to target ratios of 100:1, 20:1, and 4:1. The following day 1×10^6 target cells (JIMT1) were treated with trastuzumab, IGF-1R antibody, combination, normal rabbit IgG (control for IGF-1R antibody), normal human IgG (control for trastuzumab), or a combination of control IgGs. Cells were incubated for 2 to 4 h at 37°C, after which cell death was measured with

a non-radioactive assay using the aCella-TOX reagent kit according to the instructions from the manufacturer; experiments were performed in triplicate.

Statistics. P-values were determined for experimental versus control treatments by two-tailed student's t-test, *$p < 0.05$, **$p < 0.005$.

2.3 Results:

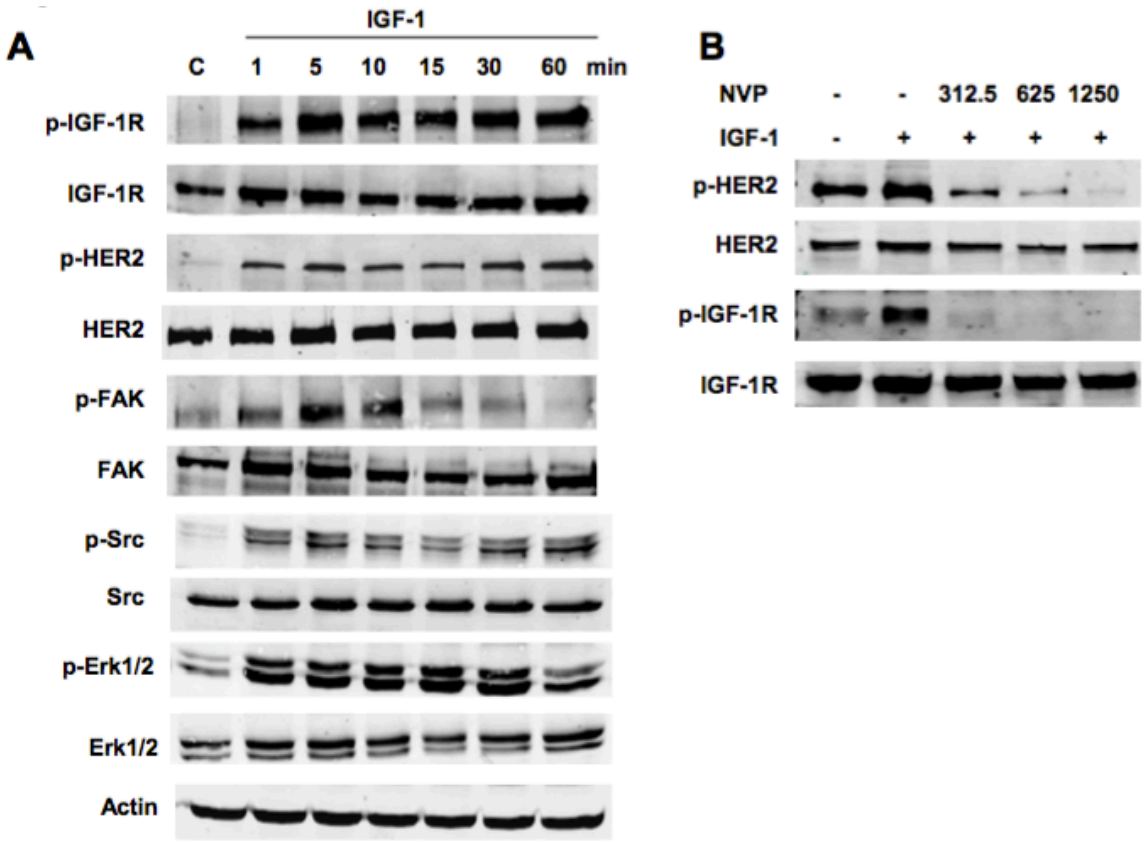
IGF-1 stimulates crosstalk from IGF-1R to HER2.

The JIMT1 cell line represents a model of HER2-overexpressing breast cancer that exhibits intrinsic resistance to trastuzumab (133). Cells were serum-starved overnight and stimulated with IGF-1 at intervals ranging from 0 to 60 minutes. IGF-1 not only induced phosphorylation of IGF-1R but also promoted phosphorylation of HER2 (Figure 2.1.A). Phosphorylation of Src, FAK, and ERK1/2 were also induced by IGF-1 stimulation. Pre-treatment of JIMT1 cells with the IGF-1R tyrosine kinase inhibitor NVPAEW541 abrogated IGF-1-mediated phosphorylation of HER2 in a concentration-dependent manner (Figure 2.1.B). These results indicate that IGF-1 stimulates phosphorylation of HER2 through IGF-1R kinase activation.

Src kinase regulates phosphorylation of HER2 in resistant cells.

Increased Src kinase activity has been linked to trastuzumab resistance; further, Src induces phosphorylation of receptor tyrosine kinases, including EGFR and HER2 (134). We found that Src phosphorylation was increased in response to IGF-1 stimulation

Figure 2.1. IGF-1 stimulates IGF-1R crosstalk to HER2. (A) JIMT1 cells were serum-starved overnight and then treated with IGF-1 (100 ng/mL) for 1, 5, 10, 15, 30, or 60 minutes (min). Western blots of total protein lysates were performed for p-Tyr1135/1136 IGF-1R, total IGF-1R, p-Tyr877 HER2, total HER2, p-Tyr397 FAK, total FAK, p-Tyr416 Src, total Src, p- Thr202/Tyr204 p42/p44 Erk1/2, total Erk1/2, or actin; lysates from serum-starved control (C) are included on the blot. Blots were repeated more than three times, and representative blots are shown. **(B)** JIMT1 cells were serum-starved overnight; cells were pre-treated with the IGF-1R tyrosine kinase inhibitor NVPAEW541 (NVP) where indicated (312.5, 625.0, or 1250.0 nM). After 24 hours, cells were stimulated with IGF-1 (100 ng/mL) for 5 minutes or left unstimulated; NVP remained present in media where indicated. Western blots of total protein lysates were performed for p-Tyr1135/1136 IGF-1R, total IGF-1R, p-Tyr877 HER2, or total HER2; blots were repeated three times, and representative immunoblots are shown.


A
IGF-1
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p-IGF-1R
IGF-1R
p-HER2
HER2
p-FAK
FAK
p-Src
Src
p-Erk1/2
Erk1/2
Actin
B
NVP - - 312.5 625 1250
IGF-1 - + + + +
p-HER2
HER2
p-IGF-1R
IGF-1R

in trastuzumab-resistant cells (Figure 2.1). Transfection of wild-type (WT) or constitutively active (A) Src constructs resulted in increased levels of phosphorylated HER2 and FAK in JIMT1 cells, in contrast to transfection of kinase-dead (KD) Src (Figure 2.2.A). Further, pharmacological inhibition of Src with the Src kinase inhibitor PP2 showed a dose-dependent decrease in HER2 phosphorylation (Figure 2.2B). These results suggest that Src activity regulates HER2 phosphorylation in trastuzumab-resistant breast cancer cells.

Effects of pharmacological inhibition of IGF-1R plus trastuzumab on cell growth.

Next, we examined the effects of co-targeting IGF-1R and HER2 in trastuzumab-resistant cells. FACS analysis of propidium-iodide-stained cells indicated that combination treatment with the IGF-1R-targeted antibody, alpha IR3, plus trastuzumab did not have major effects on the cell cycle distribution of JIMT1 cells after 48 hours (Figure 2.3). Trypan blue exclusion demonstrated modest, statistically significant reductions in the growth of trastuzumab-resistant JIMT1 and HCC1954 cells in response to the combination of alpha IR3 and trastuzumab when administered for a slightly longer treatment time period than that used in the FACS assays (Figure 2.4.A). Approximately 20% fewer cells were present in the treatment groups after 72 hours. Longer-term (2-3 weeks), matrigel-based cultures of JIMT1 cells showed higher levels of growth inhibition in response to the combination treatment, with approximately 50% growth inhibition (Figure 2.4.B). As a single agent, trastuzumab reduced anchorage-independent growth of JIMT1 cells by approximately 30% compared to a complete lack of growth inhibition by

Figure 2.2. Src kinase mediates HER2 phosphorylation in trastuzumab-resistant cells. (A) JIMT1 cells were transfected with empty-vector pcDNA3.1 (C), or constructs expressing wild- type Src (WT), constitutively active Src (A), or kinase-dead Src (KD). Western blots of total protein lysates were performed for p-Tyr1131 IGF-1R, total IGF-1R, p-Tyr877 HER2, total HER2, p-Tyr397 FAK, total FAK, p-Tyr416 Src, and total Src. Individual bands representing phosphorylated proteins were quantified and normalized to the band in the control lane. Quantification was performed directly on the Odyssey imaging machine with LI-COR software, and background was subtracted. **(B)** JIMT1 cells were treated with DMSO (-) or the Src kinase inhibitor PP2 at doses ranging from 10 nM to 5000 nM for 24 h. Western blots of total protein lysates were performed for p-Tyr877 HER2, total HER2, p-Tyr416 Src, total Src, or actin; blots were repeated at least twice, and representative sets of blots are shown.

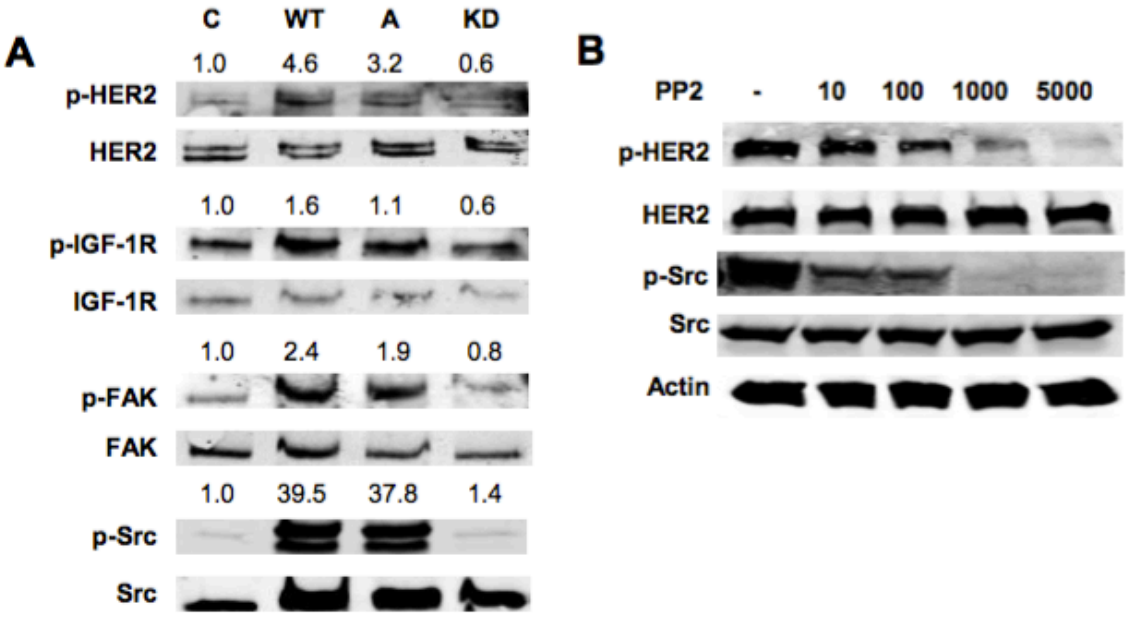

A
C
WT
A
KD
1.0
4.6
3.2
0.6
p-HER2
HER2
1.0
1.6
1.1
0.6
p-IGF-1R
IGF-1R
1.0
2.4
1.9
0.8
p-FAK
FAK
1.0
39.5
37.8
1.4
p-Src
Src
B
PP2
-
10
100
1000
5000
p-HER2
HER2
p-Src
Src
Actin

Figure 2.3. Inhibition of IGF-1R plus trastuzumab does not affect cell cycle distribution. JIMT1 cells were treated with control IgG, IGF-1R monoclonal antibody alpha IR3 (aIR3, 0.25 μg/mL), trastuzumab (Tras, 20 μg/mL), or the combination of alpha IR3 and trastuzumab (aIR3 + Tras) for 48 hours. Cells were fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Triplicate cultures were included per group, and the experiment was performed twice; a representative set of cell cycle histograms is shown.

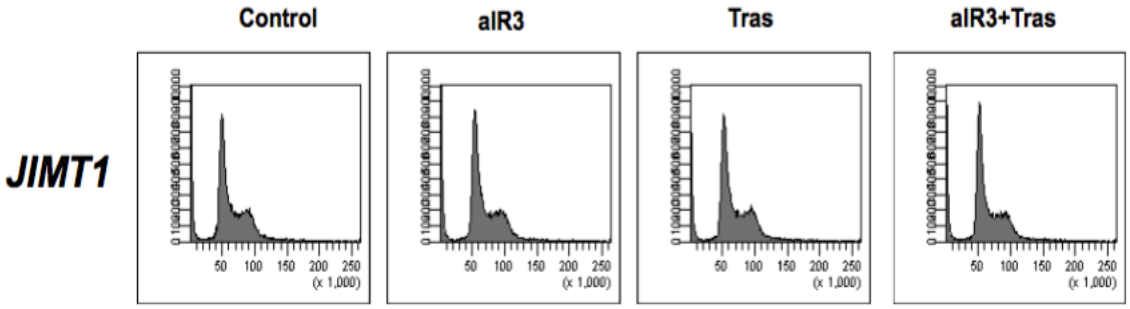
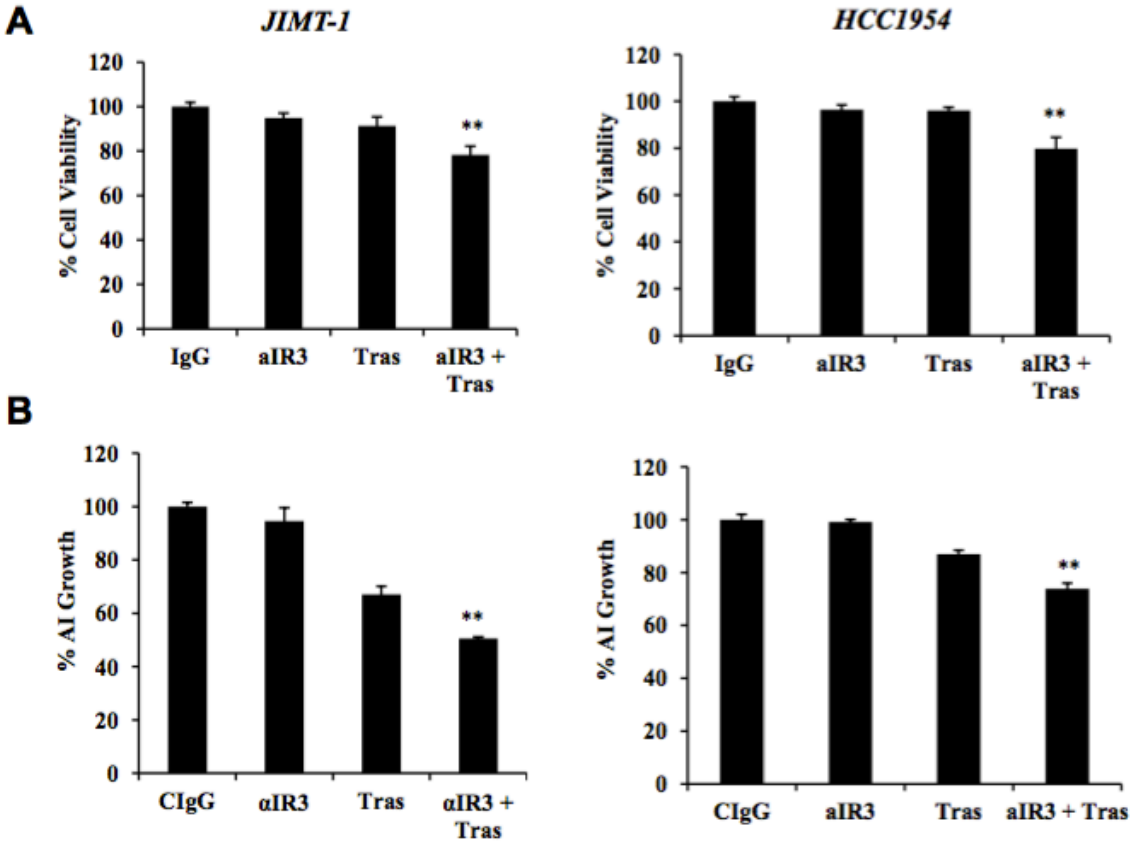

Control
aIR3
Tras
aIR3+Tras
JIMT1
50 100 150 200 250
(x 1,000)

Figure 2.4. Co-targeting IGF-1R and HER2 inhibits growth. (A) JIMT1 or HCC1954 cells were treated with control IgG, IGF-1R monoclonal antibody alpha IR3 (aIR3, 0.25 μg/mL), trastuzumab (Tras, 20 μg/mL), or the combination of alpha IR3 and trastuzumab (aIR3 + Tras). After 72 h, cells were counted by trypan blue exclusion. Data are reported as a percentage of the control IgG group; results represent the average of triplicate cultures per group. The experiment was performed three times with reproducible results; standard deviations between replicates are shown; student's t-test, $^{**}p \leq 0.005$. **(B)** JIMT1 or HCC1954 cells were plated in matrigel and maintained in media containing control IgG (CIgG), IGF-1R monoclonal antibody alpha IR3 (aIR3, 0.25 μg/mL), trastuzumab (Tras, 20 μg/mL), or the combination of alpha IR3 and trastuzumab (aIR3 + Tras). Media was changed twice a week for two to three weeks. Matrigel was dissolved with dispase, and cells were counted by trypan blue exclusion. Percent change in anchorage-independent (AI) cell survival is shown relative to the control IgG group (CIgG) (lower panels). The experiment was repeated three times with reproducible results; students t- test, $^{**}p \leq 0.005$; error bars represent standard deviation.


A
JIMT-1
HCC1954
% Cell Viability
120
100
80
60
40
20
0
**
IgG
aIR3
Tras
aIR3 + Tras
B
% AI Growth
CIgG
αIR3
aIR3 + Tras

trastuzumab in anchorage-dependent cultures. This is likely due to prolonged exposure of matrigel cultures to treatment. These results suggest that JIMT1 cells retain a low level of trastuzumab sensitivity, although they are relatively resistant compared to accepted models of sensitivity, such as BT474 and SKBR3 cells (not shown). In contrast to JIMT1, long-term, matrigel-based cultures of HCC1954 showed a similar level of growth inhibition as that observed in trypan blue exclusion assays, with ~20% fewer cells in the combination group compared to controls, and no inhibition by single agents. Overall, these results indicate that the combination of alpha IR3 and trastuzumab modestly reduces the growth of intrinsically resistant HER2- positive breast cancer cells to a greater extent than achieved with single-agent alpha IR3 or trastuzumab.

Pharmacological inhibition of IGF-1R in combination with trastuzumab suppresses invasion of resistant cells.

In contrast to effects on cell growth, the combination of alpha IR3 and trastuzumab showed dramatic effects on the invasive potential of JIMT1 and HCC1954 cells. Although neither of the antibodies reduced invasion when administered as single agents, the combination of IGF-1R and HER2 antibodies almost completely suppressed the abilities of JIMT1 (Figure 2.5.A) and HCC1954 (Figure 2.2.B) to invade across matrigel-coated Boyden chambers. In contrast, the combination of alpha IR3 and trastuzumab did not reduce the invasiveness of IGF-1R-expressing MDA-MB-231 breast cancer cells (Figure 2.6), which lack overexpression of HER2. These results reduce the likelihood that off-target effects mediate the anti-invasive effect of this antibody

Figure 2.5. Co-targeting IGF-1R and HER2 suppresses invasiveness of trastuzumab-resistant breast cancer cells. (A) JIMT1 and **(B)** HCC1954 cells were pre-treated for 48 h with control IgG (CIgG), 0.25 μg/mL alpha IR3 (aIR3), 20 μg/mL trastuzumab (Tras), or alpha IR3 plus tras. Cells were then seeded into Boyden chambers in the presence of 10% FBS and respective drugs. After 24 h of invasion, photos were taken, and the number of invaded cells were counted in 10 random fields and added together; results represent the average of triplicate cultures per group. Representative photos are shown. The experiment was performed twice with reproducible results; students t-test **$p \leq 0.005$; error bars represent standard deviation. **(C)** Spheroid migration assay of JIMT1 cells untreated, treated with control IgG, 0.25 μg/mL alpha IR3 (aIR3), 20 μg/mL trastuzumab (Tras), or alpha IR3 plus trastuzumab (aIR3+Tras). Representative images (magnification, 4×) of spheroids are shown.

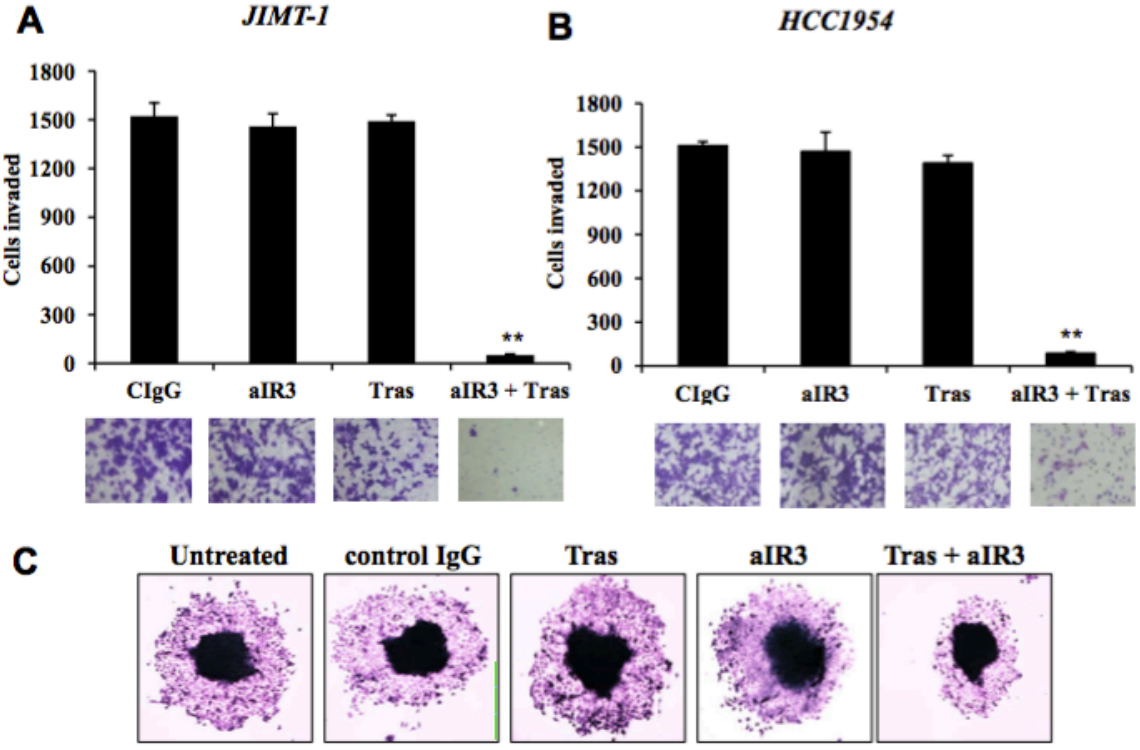
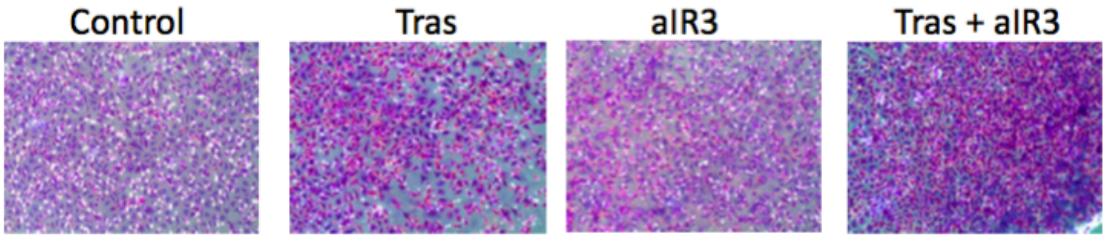
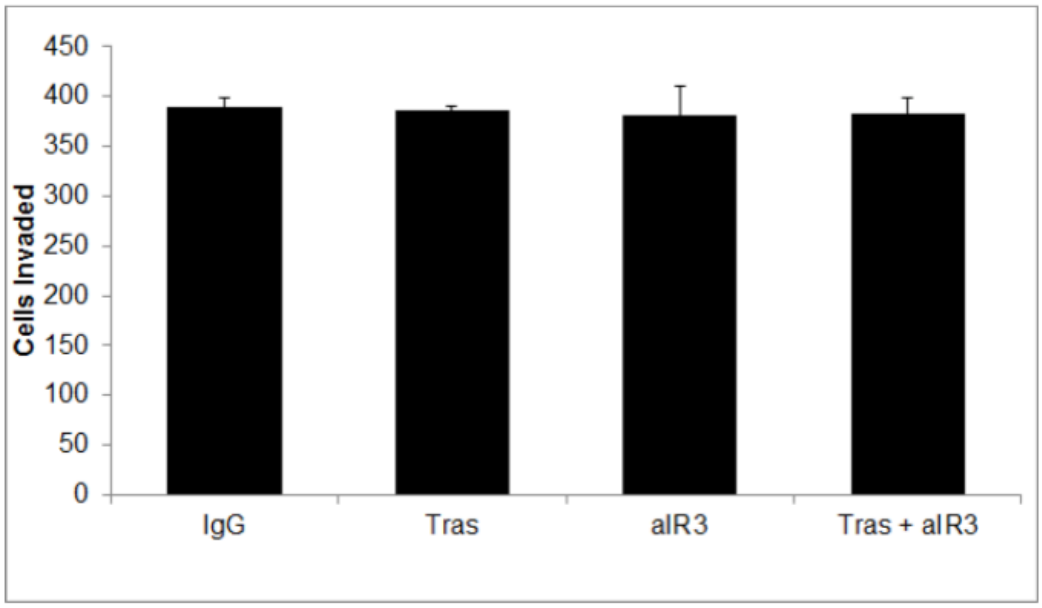

A
JIMT-1
Cells invaded
1800
1500
1200
900
600
300
0
**
CIgG
aIR3
Tras
aIR3 + Tras
B
HCC1954
Cells invaded
1800
1500
1200
900
600
300
0
**
CIgG
aIR3
Tras
aIR3 + Tras
C
Untreated
control IgG
Tras
aIR3
Tras + aIR3

Figure 2.6. Co-targeting IGF-1R and HER2 in triple negative breast cancer cells has no effect on cell invasion. MDA231 cells were seeded into Boyden chambers in serum-free media with control IgG (IgG), 0.25 μg/mL alpha IR3 (aIR3), 20 μg/mL trastuzumab (Tras), or the combination of alpha IR3 plus tras. After 24 h of invasion, photos were taken, and the number of invaded cells were counted in 10 random fields and added together; results represent the average of triplicate cultures per group. Representative photos are shown. No statistically significant differences were found between groups.


Control
Tras
aIR3
Tras + aIR3


Cells Invaded
450
400
350
300
250
200
150
100
50
0
IgG
Tras
aIR3
Tras + aIR3

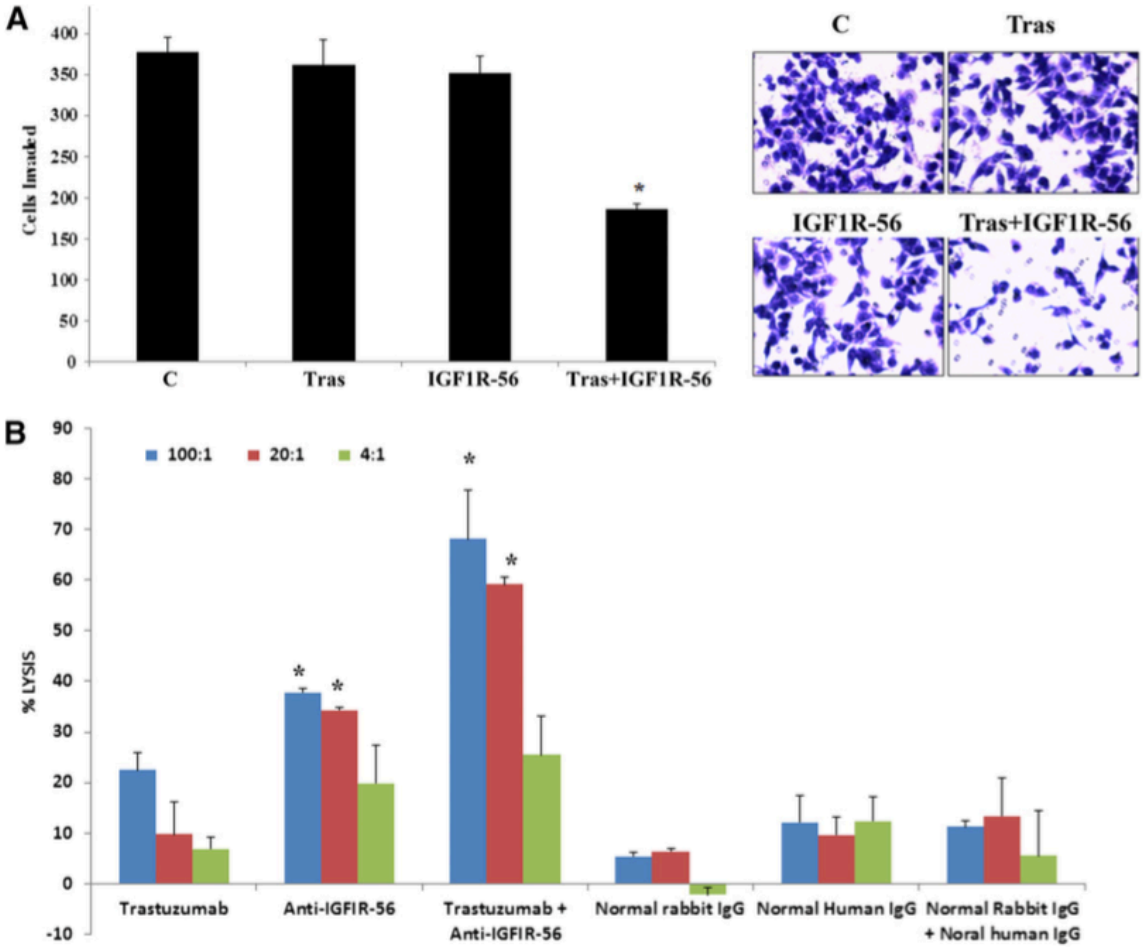
combination and suggest that endogenous HER2 overexpression may be required to elicit this effect. Similar to the Boyden assays, the combination of alpha IR3 and trastuzumab reduced the migration of JIMT1 cells in spheroid assays (Figure 2.5C). These results indicate that a major effect of co-targeting IGF-1R and HER2 in trastuzumab-resistant cells is the suppression of invasion.

To gain additional evidence that co-targeting IGF-1R and HER2 suppresses invasion, we treated JIMT1 cells with a different IGF-1R antibody, IGF-IR-56-81 (131); this antibody is directed against a different epitope of IGF-1R than alpha IR3. Similar to alpha IR3 plus trastuzumab, the combination of IGF-IR-56-81 plus trastuzumab significantly reduced JIMT1 cell invasion, whereas neither antibody alone affected invasion (Figure 2.7.A). In addition, the combination of IGF-1R and HER2 antibodies induced significant antibody-dependent cellular cytotoxicity of JIMT1 cells compared to controls and compared to either of the single agents (Figure 2.7.B). These data provide further evidence that targeting IGF-1R improves response to trastuzumab. Further, these data suggest that two major biological effects of co-targeting IGF-1R and HER2 with selective antibodies are blockade of invasion and induction of ADCC.

Combination knockdown of IGF-1R plus trastuzumab reduces growth and invasion.

In addition to pharmacological inhibition of IGF-1R, we examined effects of knocking down IGF-1R by stably infecting JIMT1 cells with lentiviral shRNA against IGF-1R versus control shRNA. Knockdown of IGF-1R improved the sensitivity of cells

Figure 2.7. IGF-1R peptide mimic-induced antibody plus trastuzumab suppresses invasion and induces ADCC of trastuzumab-resistant cells. (A) JIMT1 cells were seeded in serum-free media in Boyden chambers in the presence of the following treatments: control, rabbit antibody generated against IGF1R-56-81 peptide (400 g/mL IGF1R-56), 20 μg/mL trastuzumab (tras), 400 μg/mL IGF-IR antibody IGF1R-56-81 (IGF1R-56) plus tras. Media containing 10% FBS was used in the well as the chemo-attractant. After 24 h of invasion, photos were taken, and the number of invaded cells were counted in 12 random fields and added together; results represent the average of triplicate cultures per group. Representative photos are shown. The experiment was performed twice with reproducible results; students t-test *$p \leq 0.05$; error bars represent standard deviation. **(B)** Antibody-dependent cellular cytotoxicity (ADCC) assays were performed using 100:1, 20:1, or 4:1 PBMC:JIMT1 cell ratios. Treatment groups included 20 μg/mL trastuzumab, 400 μg/mL IGF-IR antibody IGF1R-56-81, combination, normal rabbit IgG (control for IGF-1R antibody), normal human IgG (control for trastuzumab), or combination of control IgGs. Cells were incubated for 2 to 4 h at 37°C, after which cell death was measured with a non-radioactive assay using the aCella-TOX reagent kit. The percentage of JIMT1 cell lysis is shown; experiments were performed in triplicate; students t-test, *$p \leq 0.05$ for combination versus trastuzumab alone, anti-IGF-1R-56-81 alone, or controls, *$p < 0.05$ for anti-IGF-1R-56-81 versus control; error bars represent standard deviation.


A
Cells Invaded
400
350
300
250
200
150
100
50
0
C
Tras
IGF1R-56
Tras+IGF1R-56
*
C
Tras
IGF1R-56
Tras+IGF1R-56
B
% LYSIS
90
80
70
60
50
40
30
20
10
0
-10
100:1
20:1
4:1
*
*
*
*
Trastuzumab
Anti-IGFIR-56
Trastuzumab + Anti-IGFIR-56
Normal rabbit IgG
Normal Human IgG
Normal Rabbit IgG + Noral human IgG

to trastuzumab, as demonstrated by reduced cell counts in a trypan blue exclusion assay (Figure 2.8.A). Similar to pharmacological targeting with the IGF-1R antibodies, IGF-1R knockdown in combination with trastuzumab showed an even more significant reduction in cellular invasion (Figure 2.8.B).

Src activity regulates IGF-1-mediated invasive effects in resistant cells.

Based on our data suggesting that Src regulates HER2 phosphorylation in trastuzumab-resistant cells (Figure 2.2), we examined the role of Src as a regulator of the invasive phenotype of resistant cells. Transfection of constitutively active (CA) Src into JIMT1 cells completely abrogated the significant anti-invasive effect of alpha IR3 plus trastuzumab co-treatment (Figure 2.9). Further, Src inhibition with the PP2 compound blocked IGF-1-mediated invasion in resistant cells, similar to FAK inhibition (Figure 2.10). These results suggest that IGF-1-mediated invasion in trastuzumab-resistant breast cancer cells depends in part on Src-FAK signaling. Further, these data indicate that Src inhibition may be essential in order to achieve an anti-invasive effect with a combination approach that co-targets IGF-1R and HER2.

FoxM1 contributes to IGF-1-stimulated invasion of trastuzumab-resistant cells

In order to determine if HER2 signaling is required for IGF-1-stimulated phosphorylation of Src and FAK, we treated cells with the HER2 kinase inhibitor lapatinib. Lapatinib blocked HER2 phosphorylation in JIMT1 cells but did not reduce IGF-1 signaling to Src or FAK (Figure 2.11), suggesting that crosstalk to HER2 may not be necessary for IGF-1-stimulated Src-FAK signaling. However, lapatinib

Figure 2.8. IGF-1R knockdown combined with trastuzumab treatment reduces growth and invasion. (A) JIMT1 shPLKO.1 or shIGF1R cells were treated with trastuzumab (Tras, 20 μg/mL) or vehicle control. After 72 h, cells were counted by trypan blue exclusion. Data are reported as a percentage of the control group; results represent the average of triplicate cultures per group; students t-test, *$p < 0.05$; error bars represent standard deviation. Western blots of total protein lysates were performed on the remaining cells for total IGF-1R to confirm knockdown; experiments were repeated at least three times. **(B)** JIMT1 control shRNA stables (shPLKO.1) or IGF-1R shRNA stables (shIGF1R) were seeded and treated with control or 20 μg/mL trastuzumab (Tras) in Boyden chambers in serum-free media. Media containing 10% FBS was placed in the well as the chemoattractant. After 24 h of invasion, photos were taken, and the number of invaded cells was counted in 12 random fields and added together; results represent the average of triplicate cultures per group. Representative photos are shown. The experiment was performed at least twice with reproducible results; students t-test, *$p < 0.05$; error bars represent standard deviation. Western blots of total protein lysates were performed on the remaining cells for total IGF-1R to confirm knockdown.

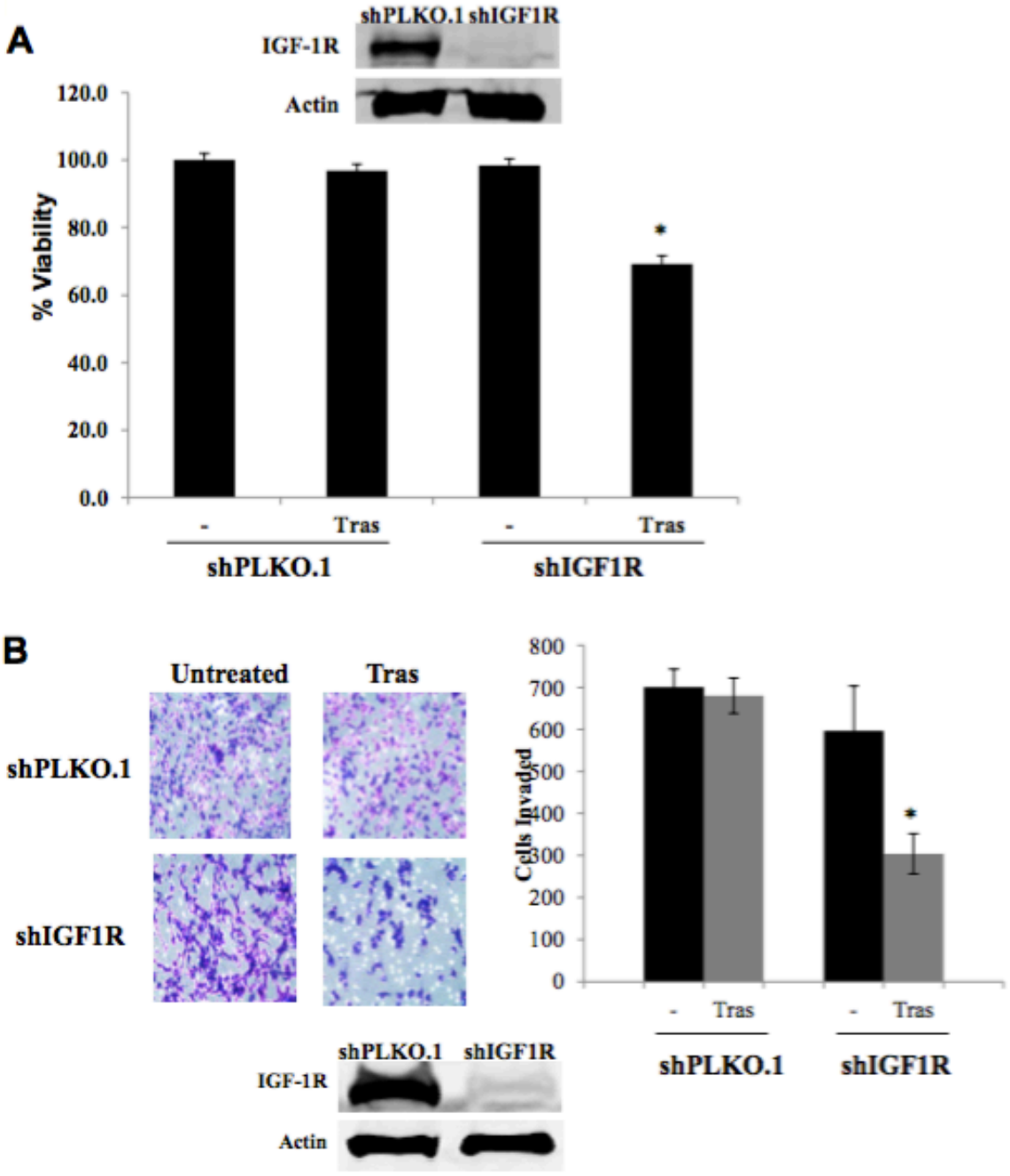

A
shPLKO.1 shIGF1R
IGF-1R
Actin
120.0
100.0
80.0
60.0
40.0
20.0
0.0
% Viability
*
-
Tras
-
Tras
shPLKO.1
shIGF1R
B
Untreated
Tras
shPLKO.1
shIGF1R
800
700
600
500
400
300
200
100
0
Cells Invaded
*
-
Tras
-
Tras
shPLKO.1
shIGF1R
shPLKO.1 shIGF1R
IGF-1R
Actin

Figure 2.9. Constitutively active Src blocks anti-invasive effect of IGF-1R/HER2 co-targeting. JIMT1 cells were transfected for 24 h with empty-vector pcDNA3.1 or constitutively active (CA) Src. **(A)** Western blots of total protein lysates were performed for p-Tyr416 Src, total Src, or actin. **(B-C)** Transfected cells were seeded in Boyden chambers in serum-free media with 10% FBS in the chamber as the chemo-attractant plus indicated treatments with control IgG (IgG), 20 μg/mL trastuzumab (Tras), 0.25 μg/mL alpha IR3 (aIR3), or alpha IR3 plus Tras. After another 24 h, photos were taken; **(B)** representative photos are shown. **(C)** The number of invaded cells was counted in 10 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with similar results; students t-test *$p \leq 0.05$; error bars represent standard deviation.

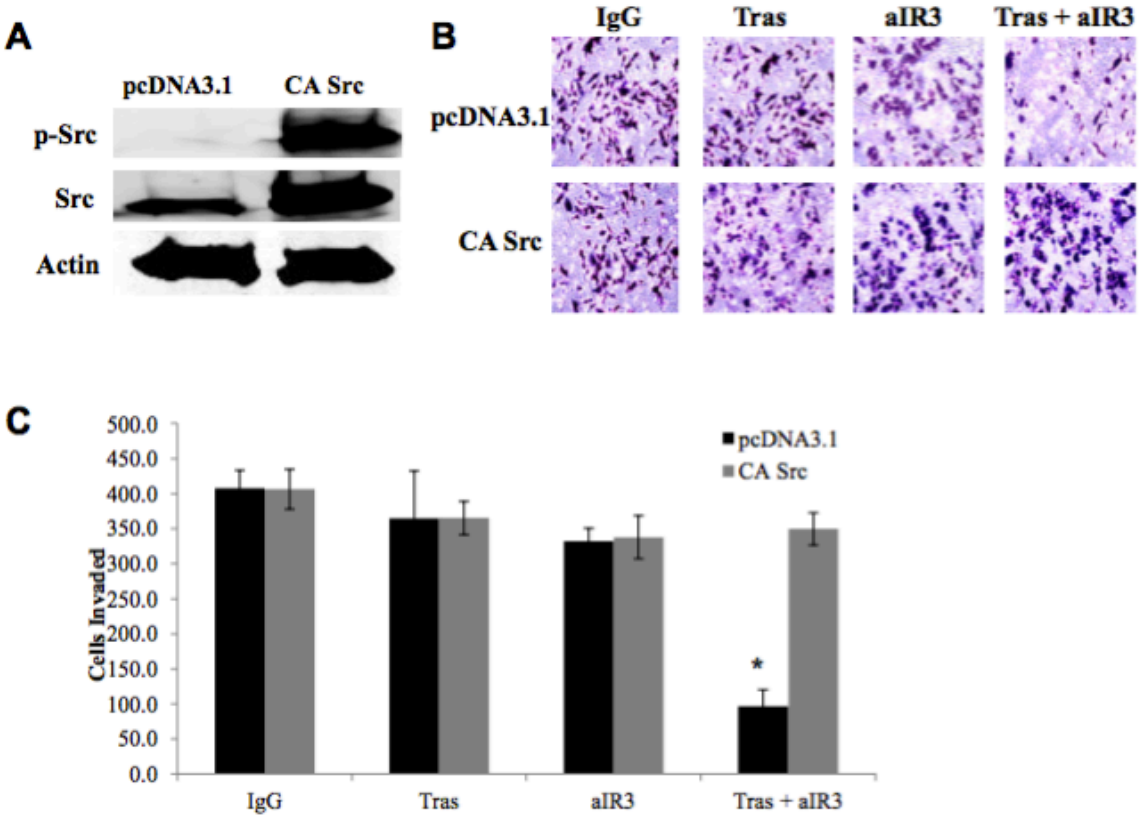

A
pcDNA3.1
CA Src
p-Src
Src
Actin
B
IgG
Tras
aIR3
Tras + aIR3
pcDNA3.1
CA Src
C
Cells Invaded
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
pcDNA3.1
CA Src
*
IgG
Tras
aIR3
Tras + aIR3

Figure 2.10. Inhibition of Src or FAK suppresses IGF-1-stimulated invasion of resistant cells. JIMT1 cells were pre-treated for 24 h with DMSO, 10 μM PP2, or 500 nM FAK inhibitor II (PF573228) in serum-free media. Cells were then seeded in Boyden chambers in serum-free media with 10% FBS in the chamber as the chemo-attractant. Drug treatment was continued, and IGF-1 ligand was added to the chambers of treatment groups where indicated. **(A)** After 24 h of invasion, photos were taken; representative photos are shown. **(B)** Western blots of total protein lysates were performed for p-Tyr416 Src, total Src, p-Tyr397 FAK, or total FAK to ensure inhibition of the target; representative blots are shown. **(C)** The number of invaded cells was counted in 12 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with reproducible results; students t-test *$p \leq 0.05$; error bars represent standard deviation.

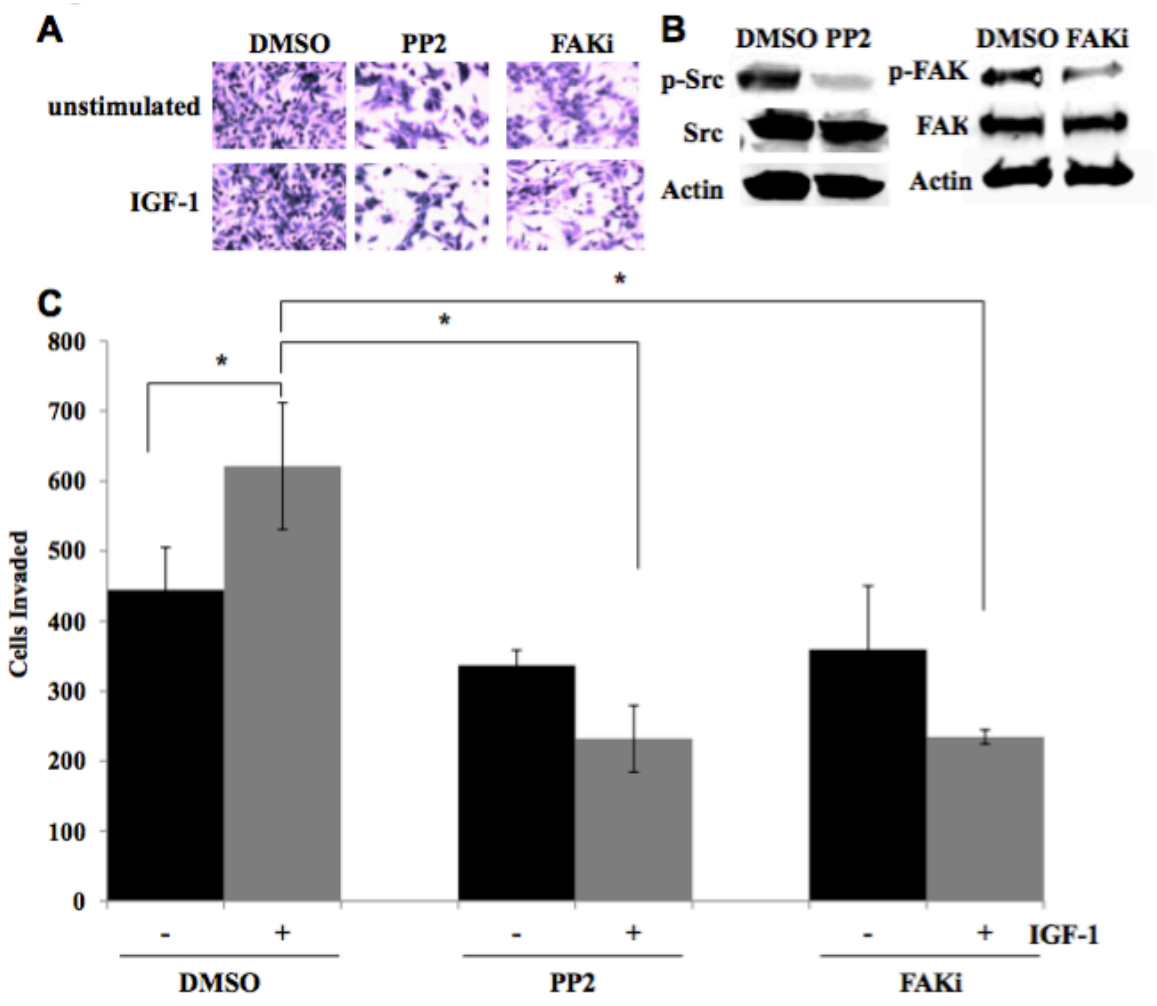
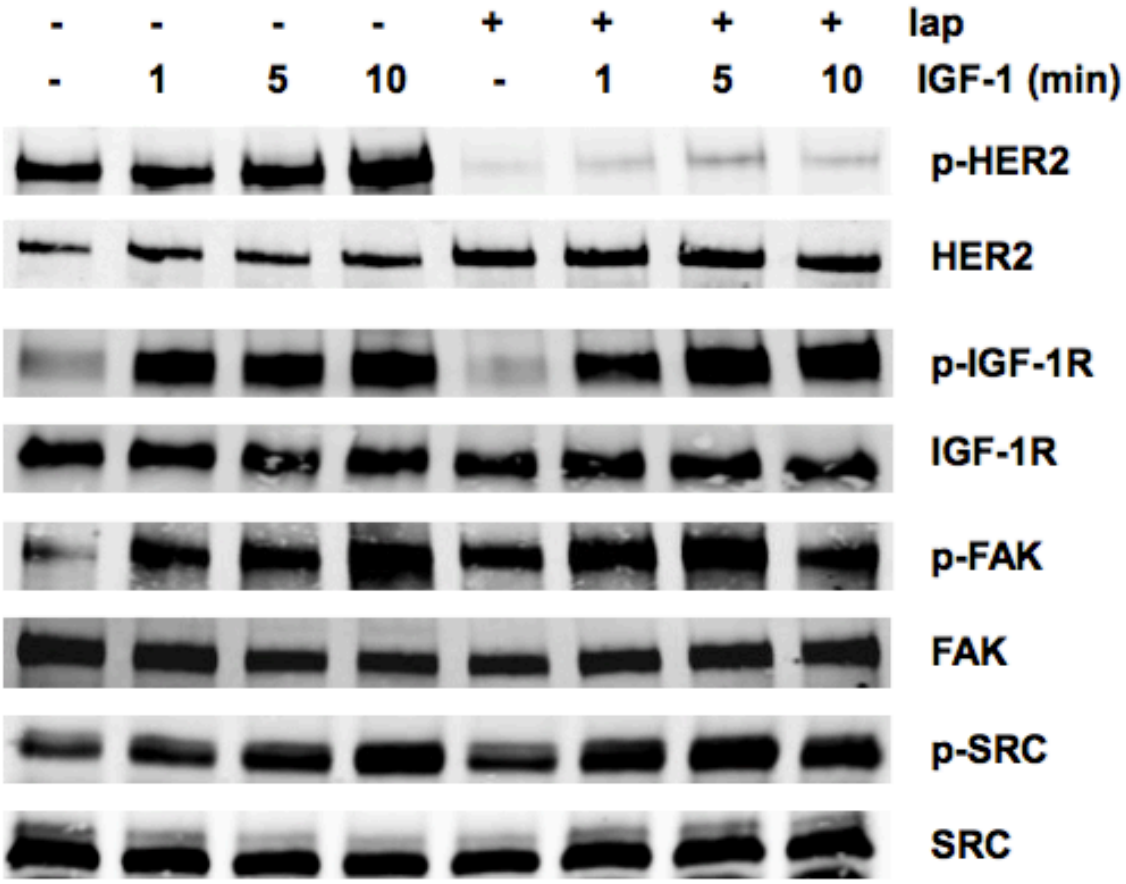

A
DMSO
PP2
FAKi
unstimulated
IGF-1
B
DMSO PP2
DMSO FAKi
p-Src
Src
Actin
p-FAK
FAK
Actin
C
Cells Invaded
800
700
600
500
400
300
200
100
0
*
*
*
- + - + - + IGF-1
DMSO
PP2
FAKi

Figure 2.11. IGF-1 signaling is not reduced by HER2 inhibition. JIMT1 cells were pre-treated overnight with DMSO control or 100 nM lapatinib, and then stimulated with IGF-1 (100 ng/mL) for 0, 1, 5, or 10 min. Protein lysates were blotted for p-Tyr877 HER2, total HER2, p-Tyr1131 IGF-1R, total IGF-1R, p-Tyr397 FAK, total FAK, p-Tyr416 Src, and total Src; representative blots are shown.


- - - - + + + + lap
- 1 5 10 - 1 5 10 IGF-1 (min)
p-HER2
HER2
p-IGF-1R
IGF-1R
p-FAK
FAK
p-SRC
SRC

blocked IGF-1-mediated invasion (Figures 2.12.A-B), indicating that HER2 kinase activity was required for IGF-1-stimulated invasion in JIMT1 cells.

We previously showed that the transcription factor FoxM1 promotes resistance to lapatinib via MEK signaling in JIMT1 cells, whereas knockdown of FoxM1 improves lapatinib sensitivity (135). Because FoxM1 functions are known to promote cancer cell invasion, we examined the role of FoxM1 in IGF-1-mediated invasion of JIMT1. Importantly, knockdown of FoxM1 blocked the ability of IGF-1 to promote cellular invasion (Figures 2.12.A-B). Further, stable IGF-1R knockdown plus trastuzumab treatment down-regulated FoxM1 expression and reduced Erk1/2 phosphorylation, whereas IGF-1R knockdown alone did not (Figure 2.13.A). These results suggest that IGF-1R and HER2 may cooperatively regulate the expression of FoxM1 in JIMT1 cells. Importantly, re-expression of FoxM1 restored invasion to stable IGF-1R-knockdown cells treated with trastuzumab (Figure 2.13.B-C). Thus, FoxM1 expression blocked the anti-invasive effect of combination IGF-1R knockdown plus trastuzumab. Together with the FoxM1 knockdown results (Figure 2.12), these data suggest that FoxM1 expression affects the anti-invasive effect of IGF-1R/HER2 co-targeting, such that FoxM1 suppression may be necessary for this approach to be effective.

Figure 2.12. HER2 kinase and FoxM1 contribute to IGF-1-stimulated invasion. (A) JIMT1 cells were pre-treated for 24 hours with DMSO or 100 nM lapatinib in serum-free media or were transfected with 100 nM control siRNA or FoxM1 siRNA (siFoxM1). Cells were then seeded in Boyden chambers in serum-free media with 10% FBS in the well as the chemo-attractant. Drug treatment was continued, and IGF-1 ligand was added to the chambers of treatment groups where indicated. Western blots of total protein lysates were performed for FoxM1, p-Tyr877 HER2, and total HER2 to ensure inhibition of the target; representative blots are shown. After 24 hours of invasion, photos were taken; representative photos are shown. **(B)** The numbers of invaded cells were counted in 12 random fields and added together; results represent the average of triplicate cultures per group; black bars, no IGF-1 stimulation; grey bars, plus IGF-1 stimulation. The experiment was performed twice with reproducible results; students t-test $*p \leq 0.05$; error bars represent standard deviation.

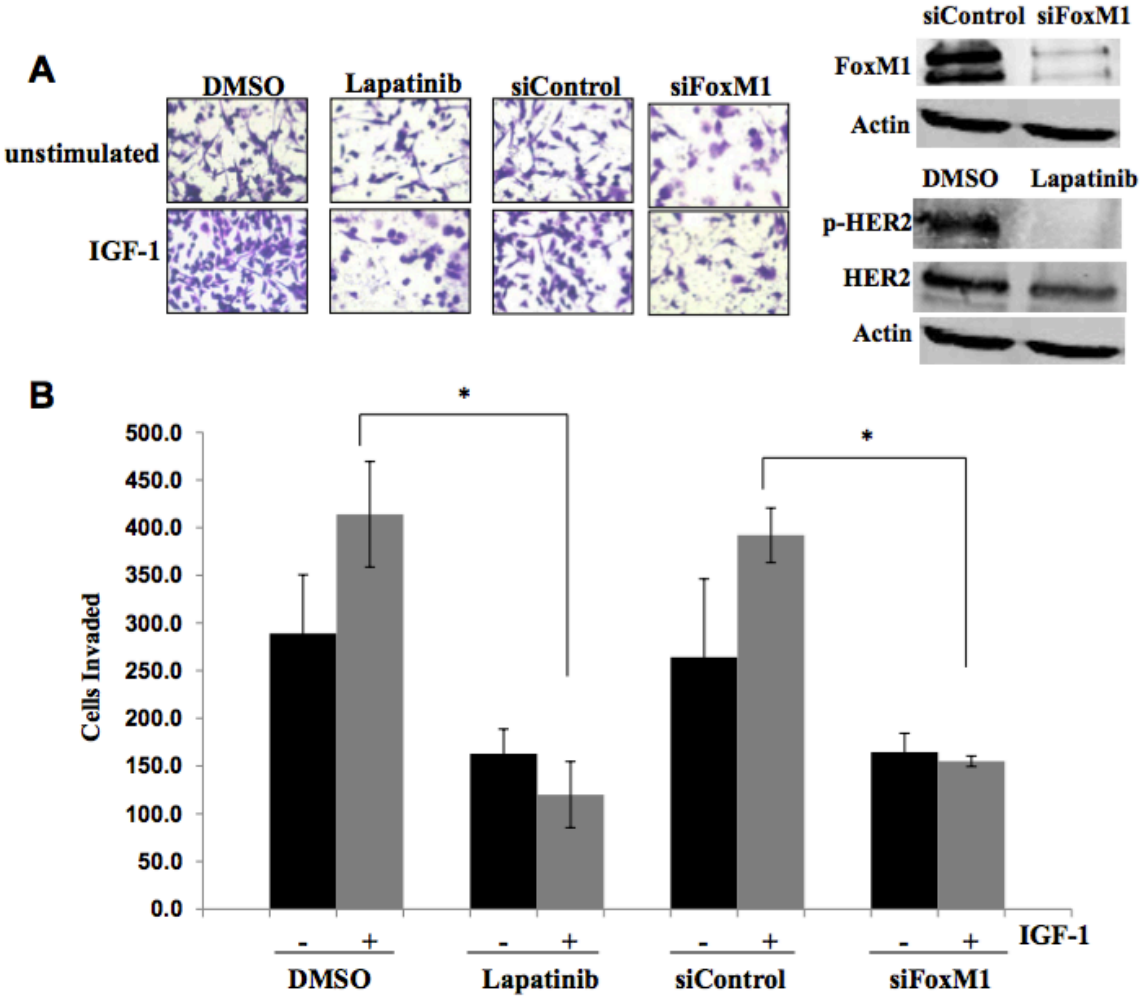
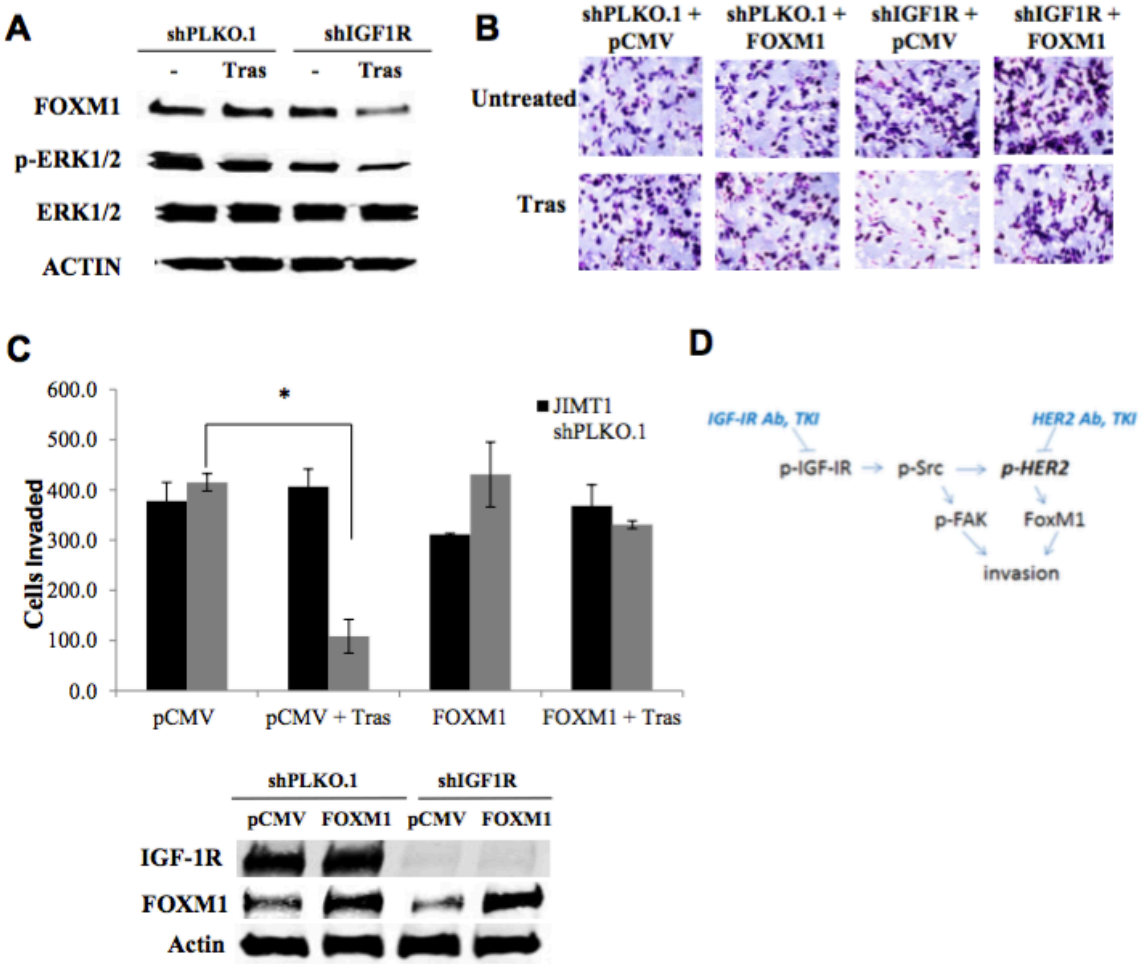

A
DMSO
Lapatinib
siControl
siFoxM1
unstimulated
IGF-1
siControl siFoxM1
FoxM1
Actin
DMSO Lapatinib
p-HER2
HER2
Actin
B
Cells Invaded
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
*
*
- + - + - + - +
IGF-1
DMSO
Lapatinib
siControl
siFoxM1

Figure 2.13. FoxM1 expression overcomes the anti-invasive effect of IGF-1R knockdown plus trastuzumab. (A) JIMT1 shPLKO.1 or shIGF1R cells were treated with trastuzumab (20 μg/mL) or vehicle control. After 72 h, protein lysates were blotted for FoxM1, p42/p44 Erk1/2, total Erk1/2, or actin; blots were repeated at least three times, and representative sets of blots are shown. **(B)** JIMT1 shPLKO.1 or shIGF1R cells were transfected for 24 h with 10 μg/mL of empty vector pCMV control plasmid or FoxM1 overexpressing plasmid. Cells were then seeded in Boyden chambers in serum-free media with 10% FBS in the well as the chemo-attractant. Trastuzumab (Tras, 20 μg/mL) was added to the chambers of treatment groups where indicated. Western blots of total protein lysates were performed for FoxM1 to ensure overexpression of the target; representative blots are shown. After 24 h of invasion, photos were taken; representative photos are shown. **(C)** The number of invaded cells was counted in 12 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with reproducible results; students t-test *$p \leq 0.05$; error bars represent standard deviation. **(D)** Overall model: IGF-1 stimulates Src-mediated crosstalk from IGF-1R to HER2, resulting in the activation of FAK downstream of Src and FoxM1 downstream of HER2. Co-inhibition of IGF-1R and HER2 is required to overcome the pro-invasive effects of Src-FAK signaling and FoxM1 in trastuzumab-resistant cells.


A
shPLKO.1
shIGF1R
- Tras - Tras
FOXM1
p-ERK1/2
ERK1/2
ACTIN
B
shPLKO.1 + pCMV
shPLKO.1 + FOXM1
shIGF1R + pCMV
shIGF1R + FOXM1
Untreated
Tras
C
Cells Invaded
600.0
500.0
400.0
300.0
200.0
100.0
0.0
*
JIMT1 shPLKO.1
pCMV
pCMV + Tras
FOXM1
FOXM1 + Tras
shPLKO.1
shIGF1R
pCMV FOXM1 pCMV FOXM1
IGF-1R
FOXM1
Actin
D
IGF-IR Ab, TKI
HER2 Ab, TKI
p-IGF-IR
p-Src
p-HER2
p-FAK
FoxM1
invasion

2.4 Discussion:

Trastuzumab remains a primary first-line treatment administered for HER2-overexpressing metastatic breast cancer. Primary and acquired resistance to trastuzumab occur in many patients; thus, a clear understanding of molecular mechanisms that drive resistance are required to improve therapeutic approaches for resistant tumors. We previously demonstrated that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (62). This finding was further corroborated by another study showing that IGF-1R and HER2 form a larger complex that includes HER3, with crosstalk occurring among all three receptors (60). Despite these findings, the mechanisms facilitating crosstalk and the downstream molecular and biological effects mediated by IGF-1R in HER2-overexpressing breast cancers remain poorly defined.

In this study, we found that IGF-1 stimulated phosphorylation of HER2 in trastuzumab-resistant cells. Src phosphorylation was also activated by IGF-1 and appeared to be critical for maintaining HER2 phosphorylation, as a small-molecule Src kinase inhibitor achieved dose-dependent inhibition of HER2 phosphorylation in the HER2-overexpressing JIMT1 cell line, which exhibits primary resistance to trastuzumab. Further, wild type and constitutively active Src induced phosphorylation of HER2 and FAK, indicating that Src regulates baseline phosphorylation of HER2 and FAK in resistant cells. In addition to regulating HER2 phosphorylation status, Src proved to be an important mediator of invasion in resistant cells. Src kinase inhibition blocked IGF-1-

mediated invasion, and constitutively active Src overcame the anti-invasive effect of the trastuzumab/alpha IR3 combination.

Src has previously been shown to have multiple important roles in the development of resistance. For example, although Src is inhibited by trastuzumab in sensitive cells (65), resistant cells show increased activation of Src (134). Inhibition of Src normally results in PTEN dephosphorylation with subsequent membrane relocalization and phosphatase activation of PTEN (65); in contrast, Src activity in resistant cells blocks PTEN activity and increases PI3K signaling (134). Src activation has been reported to occur downstream of multiple mechanisms of trastuzumab resistance, including increased signaling from growth factors and receptors, such as TGF-beta (136), EphA2 (137), and GDF15 (138). As a result, Src inhibition has been shown to improve trastuzumab response in multiple models (134; 139; 140). Thus, our data that Src contributes to the regulation of HER2 phosphorylation and the invasive potential of resistant cells are consistent with previous reports supporting a central role for Src in trastuzumab resistance.

The contribution of Src to the invasive potential of resistant cells may be partially due to the activation of FAK, as Src kinase activation increased FAK phosphorylation, and small-molecule inhibitors of both Src and FAK reduced IGF-1-stimulated invasion. The role of FAK in the invasiveness of HER2-overexpressing breast cancer and trastuzumab resistance is supported by previous studies. Recruitment of FAK to HER2 has been reported to occur in response to heregulin stimulation (141); further, phosphorylation of Src, FAK, and HER2 correlate in clinical breast cancer samples (142). Similar to our results with IGF- 1 stimulation, TGF-beta has been shown to induce FAK

phosphorylation downstream of Src (136). In addition, phase I investigation of the Src kinase inhibitor, saracatinib, in patients with advanced solid tumors, including 13 metastatic breast cancers, showed that FAK phosphorylation is a useful surrogate marker for Src activity (143). FAK inhibitors, including a dual IGF-1R/FAK inhibitor, have been shown to induce apoptosis in models of HER2-overexpressing breast cancers. Our data suggest that these agents may have additional utility in the setting of IGF-1-driven trastuzumab resistance.

The recruitment and activation of intracellular kinases, such as FAK, by IGF-1R has been shown to occur through integrins in some cell systems (144). Thus, the possibility that IGF-1 promotes Src-FAK signaling and invasion through integrins in the context of trastuzumab resistance should be considered in future studies. For example, HER2 function and resistance to HER2-targeted therapies has previously been associated with integrin-mediated adhesion to the extracellular protein laminin-5 in association with increased FAK signaling (145). Overexpression of β_1 integrin has also been shown to mediate trastuzumab resistance (146). Further, the erbB growth factor heregulin has been shown to regulate $\alpha_v\beta_3$ integrin levels in invasive breast cancers to affect downstream MAPK signaling (147). The potential importance of integrins to resistance is further reflected by the finding that cancer cells that overexpress both HER2 and the integrin receptor $\alpha_6\beta_4$ exhibit a highly aggressive and malignant phenotype (148). Thus, there is a clear body of literature supporting a link between integrins, HER2 signaling, and resistance. The role of IGF-1R in this context is supported by the finding that IGF-1 stimulation disrupts the v integrin/E-cadherin/IGF-1R ternary complex, leading to integrin redistribution to focal contact sites and increased invasion (149). Thus, future

studies should examine the role that integrins play in IGF-1-mediated trastuzumab resistance and the impact of integrin signaling on the efficacy of IGF-1R/HER2 combination approaches, particularly as they relate to invasion.

Another important mediator of invasion activated downstream of HER2 is the FoxM1 transcription factor; expression of FoxM1 correlates with poor prognosis and HER2 overexpression in breast cancer (150-152). We previously reported that FoxM1 expression levels and cellular localization are heavily regulated by MEK signaling in trastuzumab-resistant cells, including JIMT1 cells (135). Co-inhibition of HER2 and MEK down-regulated FoxM1 expression and blocked the growth of trastuzumab-resistant cancer cell xenografts (135). In addition to our previous results showing that HER2-MEK signaling regulates FoxM1 expression, the results of our current study indicate that FoxM1 expression is co-dependent on IGF-1R and HER2 in resistant cells. Stable knockdown of IGF-1R alone did not alter FoxM1 expression; however, IGF-1R knockdown plus trastuzumab reduced Erk1/2 phosphorylation, down-regulated FoxM1 expression, and reduced the invasive potential of resistant cells. This is likely due to the co- dependence of these cells on IGF-1R and HER2, such that both receptors must be inhibited to achieve meaningful downstream signaling blockade. Re-expression of FoxM1 restored the invasive ability of resistant cells in the context of IGF-1R knockdown plus trastuzumab treatment. Further, we found that FoxM1 was an important mediator of the invasive potential of resistant cells, such that knockdown of FoxM1 blocked IGF-1-mediated invasion. IGF-1R and HER2 signaling co-regulated FoxM1 expression in resistant cells, such that co-inhibition of both receptor kinases was required to reduce FoxM1 expression. These results support an important function for FoxM1 in

IGF-1-mediated resistance, and suggest that reduced expression of FoxM1 may be necessary to achieve the anti-invasive effect of co-targeted IGF-1R/HER2 therapy.

Past studies have shown somewhat conflicting results regarding the association between overall IGF-1R expression levels and response to trastuzumab (129; 130; 153-155). However, there is strong evidence to suggest that co-targeting IGF-1R and HER2 has increased benefit against HER2-positive breast cancers. Blockade of IGF-1R signaling with antibodies (62), tyrosine kinase inhibitors (62; 96; 156), genetic knockdown (60), or expression of IGF-1-sequestering proteins (61; 157) has been shown to improve sensitivity to trastuzumab in multiple models of trastuzumab resistance; sensitivity was primarily assessed by proliferation, apoptosis, and xenograft tumor growth in these reports. An important finding of our study was that co-targeting IGF-1R and HER2 had modest, although significant effects on the growth inhibition of cells with primary trastuzumab resistance, but almost completely suppressed cellular invasion. FoxM1 down-regulation appeared to be an essential downstream mediator of the anti-invasive effect of co-targeting IGF-1R and HER2. In addition to blocking invasion, co-inhibition of IGF-1R and HER2 induced ADCC of resistant cells, which is believed to be a major mechanism through which antibody-based therapies promote tumor regression. These results support strategies to simultaneously block both signaling pathways and support FoxM1 as a fundamental regulator of cellular invasion in trastuzumab-resistant cancers.

Overall, our results indicate that the invasiveness of resistant cells is co-dependent on IGF- 1R and HER2 signaling, such that co-inhibition of both receptors is required to suppress invasion and overcome downstream signaling (Figure 2.13.D). Constitutively

active Src and FoxM1 overexpression overcame the anti-invasive effects of dual IGF-1R/HER2 inhibition. These results lend additional support to the growing concept that Src represents a potential therapeutic target in trastuzumab-resistant breast cancer, and demonstrates that FoxM1 is an important target worthy of further investigation, particularly in the context of cancers that co-express IGF-1R and HER2. Future experiments will investigate the effects of co-targeting IGF-1R and HER2 on the local invasion and metastasis of resistant tumors in vivo, and the overall contributions of Src-FAK and FoxM1 to the in vivo progression of HER2-positive breast cancers.

Chapter 3:

Insulin receptor and Insulin-like growth factor-1 receptor signaling promotes HER2 phosphorylation and invasion of trastuzumab-resistant breast cancer cells

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3.1 Introduction:

Breast cancer is one of the most commonly diagnosed and second deadliest types of cancer among American women (158). Different molecular subtypes of breast cancer have been identified, based on gene-profiling studies and response to targeted treatments (6; 159). The HER2-enriched subtype, which represents approximately 15-20% of breast cancers, is characterized by amplification and overexpression of the human epidermal growth factor receptor 2 (her2) gene (12). HER2-positive metastatic breast cancer is associated with poor prognosis, reduced overall survival, and the development of resistance to chemotherapeutic drugs and certain targeted therapies (13).

Overexpression of HER2 in the cell membrane serves as a selective target for anticancer drugs. Trastuzumab (Herceptin^{TM}; Genentech, South San Francisco, CA), a recombinant humanized monoclonal antibody against the extracellular domain IV of the HER2 receptor tyrosine kinase (14; 42; 43), was the first HER2-targeted therapy approved by the US Food and Drug Administration for the treatment of HER2-positive metastatic breast cancer (44). Although trastuzumab has improved the duration of response to treatment, some patients exhibit *de novo* resistance and others develop resistance within a year of treatment (46; 55; 125; 160).

Multiple mechanisms of trastuzumab resistance have been proposed during the last decade. General potential mechanisms contributing to a lack of response to trastuzumab include: the inability to bind to HER2, failure to inhibit HER2-mediated downstream signaling pathways, and failure to induce antibody-dependent cellular cytotoxicity (161). An increase in the circulating extracellular domain of HER2 by proteolytic cleavage of the receptor generates a constitutively active fragment of HER2 kinase leading to resistance (56). The presence of membrane-expressed proteins like MUC4 might obstruct and reduce the interaction between trastuzumab and the extracellular domain of HER2, decreasing response to treatment (57; 58). Constitutive activation of downstream signaling pathways like PI3K and/or MAPK might also affect response to trastuzumab (64-66).

Crosstalk with other receptor tyrosine kinases, like the Insulin-like Growth Factor Receptor 1 (IGF-1R), has also been proposed as a mechanism of resistance (62). IGF-1R positivity has been correlated with poor prognosis, specifically in HER2-enriched breast cancer tumors (155). In a clinical study, high IGF-1R expression was also associated with reduced response to treatment with trastuzumab plus vinorelbine in HER2-overexpressing tumors (129). Furthermore, we have shown that IGF-1R signaling increases the invasive potential of HER2-overexpressing breast cancer cells, through pathways dependent on Src-FAK signaling and overexpression of the pro-invasive transcription factor FoxM1 (97). However, the potential involvement of the Insulin Receptor (IR), a closely IGF-1R-related receptor that has also been shown to be important for cancer development, has not been well characterized as a mechanism of trastuzumab resistance in HER2-positive breast cancer.

The IR and the IGF-1R are homologous receptor tyrosine kinases with structural and functional similarities, mainly within the catalytic domain (98; 99). These heterotetrametric receptors consist of two extracellular ligand-binding alpha domains, and two transmembrane beta domains with tyrosine kinase activity (93; 102). Monomers of alpha/beta domains from each receptor can homodimerize or heterodimerize to form hybrid receptors (103; 104). The resulting dimers undergo auto-phosphorylation upon insulin, IGF-1, and/or IGF-2 ligand binding, leading to the activation of signaling pathways like the PI3K/AKT and the MAPK pathways (162). Activation of such pathways can promote cellular proliferation, survival, migration, and invasion. Furthermore, multiple studies have linked the IR with a relevant role in cancer progression. For instance, increased IR content has been observed in breast cancer tissue, in comparison to normal breast tissue (106). Immunohistochemical staining of invasive breast tumor samples also show that phosphorylated IGF-1R/IR and total IR correlate with poor survival (116). Due to its similarities and direct interactions with IGF-1R signaling, we hypothesize that IR signaling can promote invasion of trastuzumab-resistant HER2-positive breast cancer cells.

In this study, we demonstrate that IGF-1 and insulin can stimulate HER2 phosphorylation in HER2-positive cells. We also show that co-targeting IR and HER2 in trastuzumab-resistant cells significantly decreases cellular invasion. Additionally, we present evidence that IGF-1 stimulation blocks the anti-invasive effect of transient IR knockdown plus trastuzumab in HER2-positive cells. Insulin stimulation also blocks the anti-invasive effect of stable IGF-1R knockdown plus trastuzumab in HER2-positive cells. Moreover, dual targeting of IR and IGF-1R decreases total FoxM1 protein

expression while increasing E-cadherin protein expression. These results suggest that a multi-targeted approach inhibiting IGF-1R, IR, and HER2 may be necessary to diminish the invasive potential of HER2-positive breast cancer cells.

3.2 Materials and Methods:

Reagents. Trastuzumab (Herceptin™, Genentech) was obtained from the Emory Winship Cancer Institute pharmacy (Atlanta, GA) and dissolved in sterile water to a stock concentration of 20 mg/mL. The IGF-1R antibody alpha-IR3 (Calbiochem, San Diego, CA) was provided at a stock concentration of 1 mg/mL. IGF-1 (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water at a stock concentration of 1 mg/mL. Insulin (Tocris Bioscience) was dissolved in 0.01M HCl at a stock concentration of 20 mg/mL. The pLKO.1-IGF-1R-a/b short hairpin RNA (shRNA) plasmid and pLKO.1 empty vector plasmid (negative control) were purchased from Open Biosystems (Huntsville, AL). Insulin receptor small interfering RNA (siRNA) (sc-29370) and control siRNA (sc-37007) (Santa Cruz Biotechnology) were resuspended in RNAase-free water.

Cell Culture. JIMT1 cells were purchased from DSMZ (Braunschweig, Germany), and HCC1954 cells were purchased from American Type Culture Collection (Manassas, VA). JIMT1 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, glutamine, and sodium pyruvate (Corning) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HCC1954 cells were cultured and maintained in RPMI 1640 medium with glutamine (Corning, Manassas, VA) and

supplemented with 10% FBS and 1% penicillin/streptomycin. JIMT1 and HCC1954 cells have previously been shown to exhibit a reduced response to trastuzumab compared with other models of HER2-overexpressing breast cancer, and are considered models of primary trastuzumab resistance (77; 133). All cells were cultured in humidified incubators at 37°C with 5% CO_2.

Creation of Stable IGF-1R Knockdown Clones. As previously described (97), HEK-293T cells (1.5 x 10^6) were seeded in 100-mm dishes for 24 hours (hrs) and co-transfected with 3 mg shRNA construct (pLKO.1-IGF-1R-a/b shRNA or pLKO.1 empty vector control plasmid), 3 mg pCMV-dR8.2, and 0.3 mg pCMV-VSV-G helper constructs, using TransIT-LT1 Transfection Reagent according to the manufacturer instructions (Mirus Bio LLC, Madison, WI). Viral stocks were harvested from culture media by centrifugation 48 hrs after transfection and were syringe-filtered. JIMT1 parental cells were seeded at sub-confluent densities and infected with lentiviral vectors (1:20 dilution) in fresh culture media. Culture media were replaced with media containing 5 mg/mL puromycin 48 hrs after lentiviral infection to select for stably infected cells. Stable IGF-1R knockdown was confirmed by Western blotting. The IGF-1R shRNA (shIGF1R) and control shRNA (shCntl) cells are routinely maintained on DMEM with 5 mg/mL puromycin, 10% FBS and 1% penicillin / streptomycin.

Western Blot Analyses. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Total protein extracts were run on 10% SDS-

PAGE gels and blotted onto nitrocellulose membranes. Blots were probed overnight in primary antibody at a temperature of 4ºC, and for an hour in secondary antibody at room temperature. The following antibodies were purchased from Cell Signaling Technology: rabbit anti-phospho-IGF-1Rb (Tyr1135/1136; no. 3024, 1:200), rabbit anti- phospho-IGF-1Rb (Tyr1131; no. 3021, 1:200), rabbit anti-IGF-1Rb (no. 3018, 1:250), rabbit anti-IR beta (no. 3025, 1:500), and rabbit anti-FoxM1 (no. 5436, 1:200). The following antibodies were purchased from AbCam (Cambridge, MA): rabbit anti-phospho-erythroblastic leukemia viral oncogene homolog 2 (erbB2) ErbB2 (Y877; no. ab108371, 1:200), and mouse anti-ErbB2 (no. ab16901, 1:200). Mouse anti-E-cadherin (610181, 1:1000) was purchased from BD Biosciences (San Jose, CA). Mouse anti-beta-actin was purchased from Sigma-Aldrich (AC-15, 1:15,000). All primary antibodies were diluted in 5% bovine serum albumin/Tris Buffered Saline and 0.01% Tween 20. Goat anti-mouse secondary IRDye 800 antibody (no. 926-32210, 1:10,000) was purchased from Li-Cor Biosciences (Lincoln, NE). Goat anti-rabbit Alexa Fluor 680 secondary antibody (no. 1027681, 1:10,000) was purchased from Invitrogen (Grand Island, NY). Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences). All blots were repeated at least two times with reproducible results.

Stimulation Experiments. Cells were plated and later serum starved for 24 hours. During serum starvation, cells were either untreated or treated with trastuzumab 20 µg/mL. Cells were then either lysed for protein, or stimulated with 100 ng/ml of IGF-1 ligand, or 10 nM insulin for varying time points. Experiments were repeated at least twice with reproducible results.

Cell Transfection. Cells were plated in 100-mm plates using antibiotic-free media at a concentration of 2×10^5 cells/mL. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 10 mg/mL of control siRNA or insulin receptor siRNA. Media were changed after 6 hrs of transfection and replaced with complete media. Cells were harvested after 48 hrs or seeded for experiments 24 hrs after transfection.

AlamarBlue Cell Viability Assays. JIMT1 or HCC1954 cells were transfected with control siRNA or insulin receptor siRNA, following the transfection protocol previously discussed. Cells were then seeded in a 96-well plate at a concentration of 8,000 cells per well, 24 hrs after transfection. In the case of JIMT1 shCntl or JIMT1 shIGF1R cells, they were passed directly to the 96-well plate, also at a concentration of 8,000 cells per well. Then cells were treated, in serum free media, with trastuzumab, IGF-1, insulin, trastuzumab plus IGF-1, or trastuzumab plus insulin. After 20 hrs, the media was replaced with a 10% solution of AlamarBlue in serum-free media. Corresponding treatments were maintained throughout the experiment. Fluorescence measurements (Excitation – 530 nm, Emission – 590 nm) were taken at different time points: 0, 4, 5, 6, and 8 hrs after exposure to the AlamarBlue solution. Results shown correspond to measurements collected 5 hrs after exposure to 10% AlamarBlue. The percentage of cell growth compared to the untreated control was calculated using the following formula: % cell growth compared to control = (Fluorescence Intensity at 590 nm of the Test Reagent / Fluorescence Intensity at 590 nm of the Untreated Control) ×100.

Invasion Chamber Assays. Cells were plated in serum-free media in BD BioCoat Matrigel Invasion Chambers (BD Biosciences) (1×10^5 cells/mL) with 0.75 mL of chemoattractant (culture media containing 10% FBS) in the wells. Depending on the experiment, cells were pretreated with trastuzumab (20 mg/mL) for 24 hrs or transfected with control siRNA or IR siRNA overnight prior to placing cells in invasion chambers, at which point they were either untreated, treated with IGF-1 (100 ng/ml), or treated with insulin (10 nM) for 24 hrs. Treatments were added directly to chambers in all experiments. After invasion, each chamber was transferred into 100% methanol for 10 minutes, followed by crystal violet staining for 20 min. Membranes were washed in water and allowed to air dry completely before being separated from the chamber. Membranes were mounted on slides using Cytoseal XYL xylene-based mounting medium (Richard-Allan Scientific, Kalamazoo, MI). Multiple photographs of each sample were taken at 20× magnification, with triplicates performed per treatment group. The number of cells was counted in each field; the sum total of the fields was calculated for each sample. Experiments were performed at least twice with reproducible results.

Statistical Analyses. *P* values were determined for experimental versus control treatments by two-tailed *t* tests. $P < 0.05$ was considered significant.

3.3 Results:

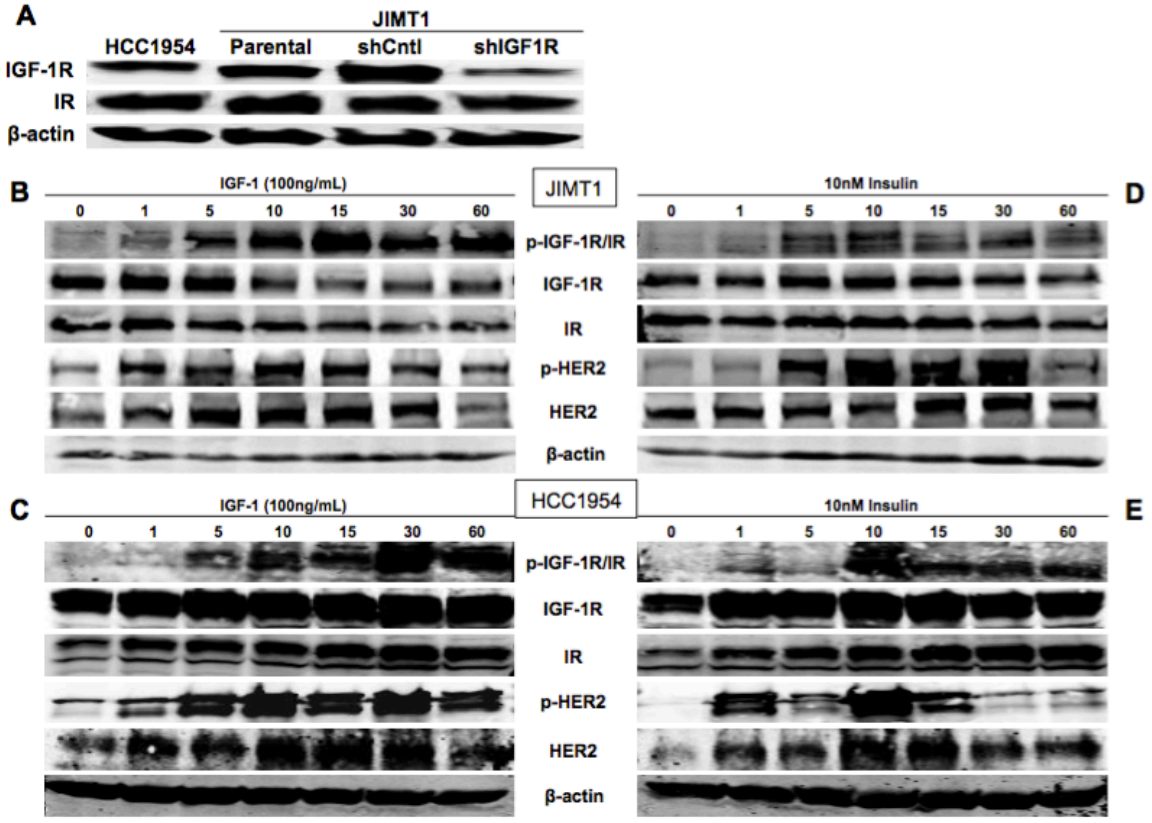
IGF-1 and insulin stimulate phosphorylation of HER2 in HER2-positive breast cancer cells.

HCC1954 and JIMT1 cell lines are representative models of HER2-positive breast cancer and *de novo* resistance to trastuzumab (77; 133). We also created stable IGF-1R knockdown clones derived from parental JIMT1 cells, giving origin to the JIMT1 shCntl (infected with an empty vector pLKO.1) and the JIMT1 shIGF1R (infected with lentiviral shRNA against IGF-1R) cells. Western blot analysis was performed to determine the baseline levels of IGF-1R and IR total protein expression in all cell lines. Results show consistent levels of IR across cell lines. As expected, IGF-1R expression is decreased in the JIMT1 shIGF1R cells when compared to the IGF-1R levels in the parental JIMT1 and JIMT1 shCntl cells (Figure 3.1.A).

IGF-1 stimulation is known to induce HER2 phosphorylation in HER2-positive breast cancer cells (60; 62; 97). IGF-1R-mediated activation of HER2 signaling is considered a potential mechanism of resistance to trastuzumab. However, insulin-mediated phosphorylation of HER2 in HER2-positive breast cancer cells has not been examined. We stimulated JIMT1 and HCC1954 cells with IGF-1or insulin at different time points, from 0 to 60 minutes. Our results demonstrate HER2 activation in response to insulin and IGF-1 stimulation in both cell lines (Figure 3.1.B-E). These results suggest that IGF-1 and insulin can both stimulate HER2 phosphorylation in trastuzumab-resistant HER2-positive cells.

Co-targeting IR and HER2 in HER2-positive cells does not affect cell growth.

Figure 3.1. IGF-1 and Insulin stimulate phosphorylation of HER2 in HER2-positive breast cancer cells. (A) Baseline IGF-1R and IR expression in HER2-positive trastuzumab resistant cell lines: HCC1954, parental JIMT1 (Parental), JIMT1 pLKO.1 control (shCntl), and JIMT1 shIGF-1R (shIGF1R) cells were grown in complete media. Western blots of total protein lysates were performed for total Insulin-like growth factor receptor 1 (IGF-IR), total Insulin Receptor (IR), and beta-actin (β-actin) protein expression. **(B – E)** IGF-1 and Insulin stimulation of JIMT1 and HCC1954 cells: JIMT1 and HCC1954 cells were serum starved for 24 hours. Then, cells were stimulated with 100 ng/mL of IGF-1 or 10 nM of insulin for 0, 1, 5, 10, 15, 30, or 60 minutes. Western blots of total protein lysates were performed for phospho-Insulin-like growth factor receptor 1/Insulin Receptor (p-IGF-IR/IR), total Insulin-like growth factor receptor 1 (IGF-1R), total Insulin Receptor (IR), phospho-Human Epidermal Growth Factor Receptor 2 (p-HER2), total Human Epidermal Growth Factor Receptor 2 (HER2), and beta-actin (β-actin) protein expression.


A
JIMT1
HCC1954
Parental
shCntl
shIGF1R
IGF-1R
IR
β-actin
B
IGF-1 (100ng/mL)
0 1 5 10 15 30 60
JIMT1
p-IGF-1R/IR
IGF-1R
IR
p-HER2
HER2
β-actin
D
10nM Insulin
0 1 5 10 15 30 60
C
IGF-1 (100ng/mL)
0 1 5 10 15 30 60
HCC1954
p-IGF-1R/IR
IGF-1R
IR
p-HER2
HER2
β-actin
E
10nM Insulin
0 1 5 10 15 30 60

To test the effect of co-targeting IR and HER2 in HER-2 positive cells, AlamarBlue Cell Viability Assay was performed using trastuzumab-resistant cell lines JIMT1 and HCC1954, after transfection with IR siRNA (siIR) or control siRNA (siC). In both cell lines, no difference was observed between the untreated cells and the cells treated with trastuzumab, stimulated with IGF-1, or those receiving a combination of trastuzumab plus IGF-1 (Figures 3.2.A and 3.2.B). These results were consistent in the cells with IR knockdown and the control group. Western blot analysis confirmed IR knockdown in cells transfected with IR siRNA, with sustained expression of IGF-1R in both cell lines. These results suggest that co-targeting IR and HER2 does not alter cell growth in HER2-positive breast cancer cells.

Co-targeting IR and HER2 in HER2-positive cells significantly inhibits invasion.

In contrast with the results observed in the cell growth assays, a combination of IR knockdown with trastuzumab treatment resulted in significant inhibition of invasion in JIMT1 and HCC1954 cells (Figures 3.3.A and 3.3.B). The invasive potential of cells transfected with control siRNA remained unaffected even when treated with trastuzumab. Western blot analysis was performed to confirm IR knockdown in cells transfected with IR siRNA. IGF-1R expression was maintained after IR knockdown in both cell lines (Figure 3.3.C). Our results suggest that dual targeting of IR and HER2 significantly reduces invasion of trastuzumab-resistant HER2-positive breast cancer cells.

IGF-1 stimulation blocks the anti-invasive effect of IR knockdown in HER2-positive cells.

Figure 3.2. Co-targeting IR and HER2 in HER2-positive cells does not affect cell viability. **(A-B)** Cell viability of HER2-positive cells treated with trastuzumab, IGF-1, and siRNA to IR. JIMT1 **(A)** and HCC1954 **(B)** cells were transfected with control siRNA (siControl, siC) or IR siRNA (siIR). After 24 hrs, cells were seeded in a 96-well plate at a density of 8,000 cells per well. The next day, cells were treated in serum free media with Control (untreated cells), 20μg/mL of trastuzumab (Tras), 100 ng/mL of IGF-1, or a combination of trastuzumab plus IGF-1 (Tras + IGF-1). After 20 hours, serum free media was replaced by 10% AlamarBlue in serum free media. Corresponding treatments were maintained throughout the experiment. Fluorescence measurements were taken at various time points for each treatment; triplicates performed per treatment group. These results correspond to measurements taken 5 hrs after addition of the AlamarBlue solution. Percent of cell growth compared to control was calculated using the following formula: % cell growth compared to control= (FI 590nm Test Reagent / FI 590nm Untreated Control) x 100. Each experiment was performed twice with reproducible results. Statistical significance was determined by student's t test. Western blots of total protein lysates for total IGF-1R and total IR were performed to confirm IR knockdown, as shown in right panels.

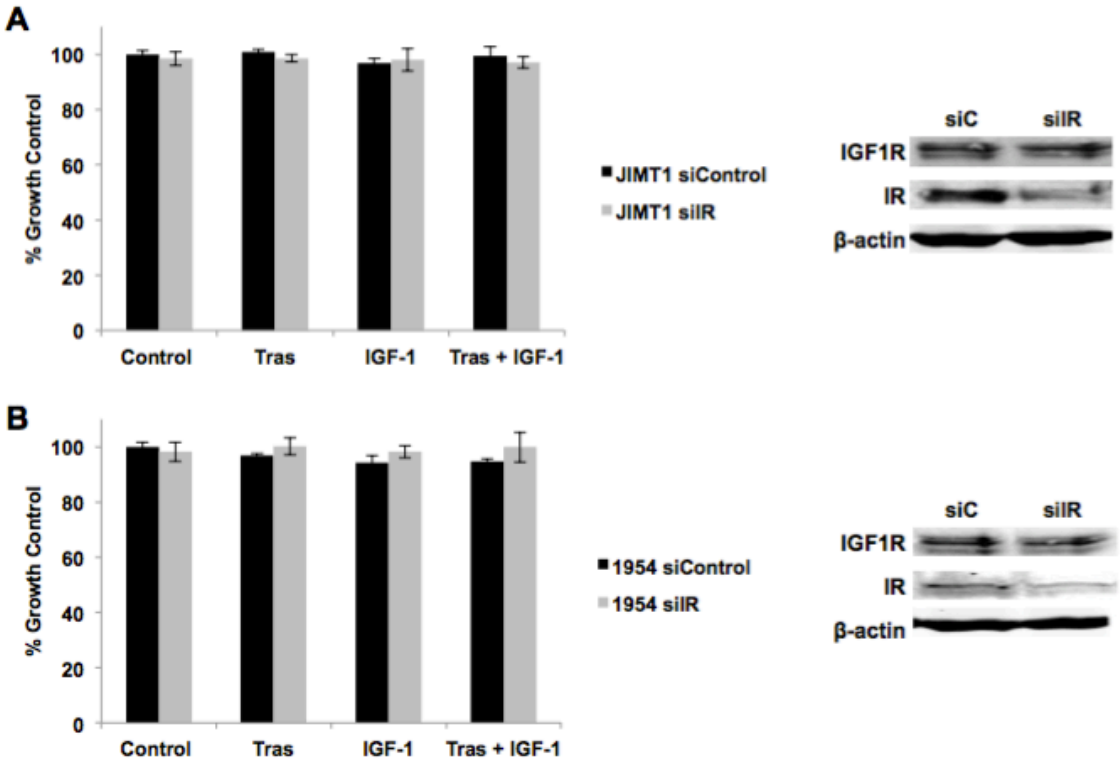
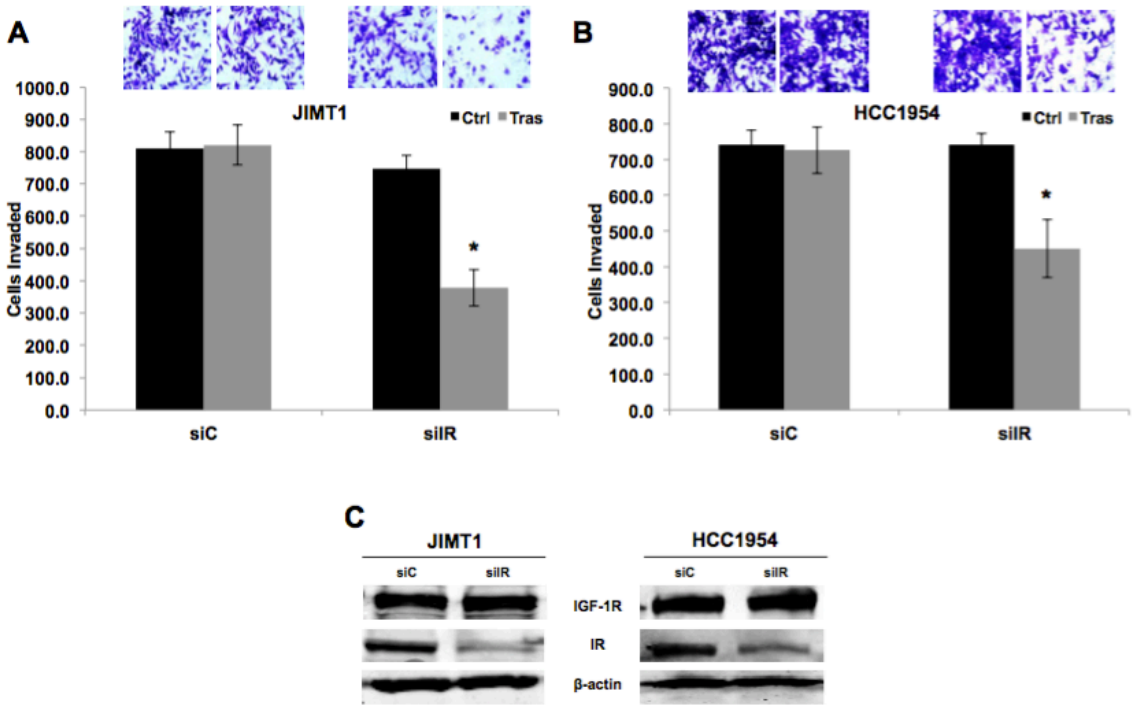

A
% Growth Control
100
80
60
40
20
0
Control
Tras
IGF-1
Tras + IGF-1
JIMT1 siControl
JIMT1 siIR
siC
siIR
IGF1R
IR
β-actin
B
% Growth Control
100
80
60
40
20
0
Control
Tras
IGF-1
Tras + IGF-1
1954 siControl
1954 siIR
siC
siIR
IGF1R
IR
β-actin

Figure 3.3. Co-targeting IR and HER2 in HER2-positive cells significantly inhibits invasion. **(A-B)** Invasive potential of HER2-positive cells treated with HER2 inhibitor and IR siRNA. JIMT1 **(A)** and HCC1954 **(B)** cells were transfected with 100nM of control siRNA (siC) or insulin receptor siRNA (siIR) for 24 h. Cells were seeded in Boyden chambers in serum-free media with 20μg/mL of trastuzumab and 10% FBS in the well as the chemo-attractant. After 24 hrs of invasion, photos were taken; representative photos are shown. The number of invaded cells were counted in 12 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with reproducible results (Student's t test, * $P < 0.05$). **(C)** Knockdown efficiency of IR is shown by Western blots. Protein lysates were detected for total Insulin-like growth factor receptor 1 (IGF-1R), total Insulin Receptor (IR), and beta-actin (β-actin) protein expression on cells that were seeded for invasion.


A
JIMT1
Ctrl Tras
Cells Invaded
1000.0
900.0
800.0
700.0
600.0
500.0
400.0
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200.0
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0.0
siC
siIR
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B
HCC1954
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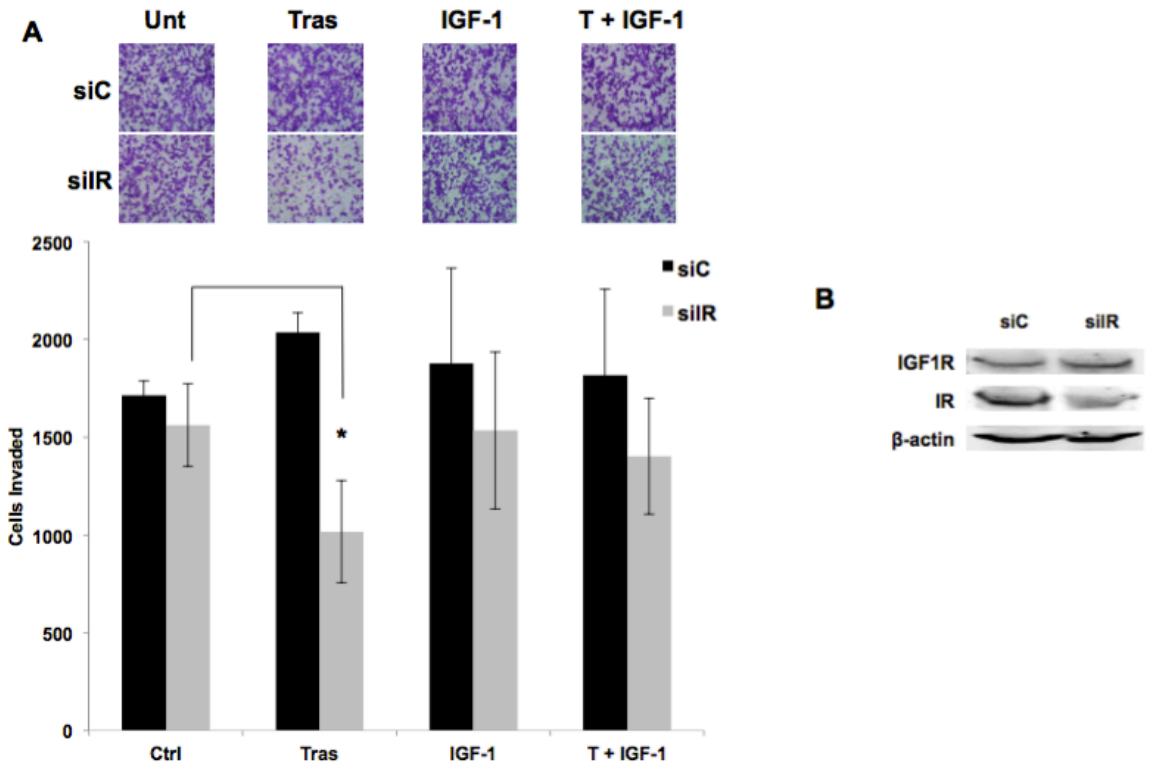
JIMT1
HCC1954
siC
siIR
siC
siIR
IGF-1R
IR
β-actin

To further study the role of IR on the invasive potential of HER2-positive cells, we seeded parental JIMT1 cells that had been transfected with IR siRNA in invasion chambers and stimulated them with IGF-1 ligand. IGF-1 preferentially stimulates IGF-1R (104), which remains expressed when IR is knocked down (Figure 3.4.B). Results from the invasion assays show that IGF-1 stimulation blocks the anti-invasive effect of combined IR knockdown plus trastuzumab in HER2-positive cells (Figure 3.4.A). These results suggest that IGF-1R stimulation can compensate for the loss of IR expression to maintain the invasive potential of HER2-positive cells.

Insulin stimulation blocks anti-invasive effect of IGF-1R knockdown in HER2-positive cells.

The AlamarBlue Cell Viability Assay was performed to detect changes in the growth of JIMT1 cells with stable IGF-1R knockdown (JIMT1 shIGF1R), compared to the JIMT1 empty vector cells (JIMT1 shCntl). Results suggest there is no evidence that trastuzumab, insulin stimulation, or a combination of both treatments would affect cell growth of either cell line (Figure 3.5.A). Conversely, treatment with trastuzumab resulted in a statistically significant decrease in invasive potential of JIMT1 shIGF1R cells (Figure 3.5.B). This observation is consistent with our published work on the role of IGF-1R signaling in the invasive potential of trastuzumab-resistant breast cancer cells (97). To further investigate the role of insulin and the IR in the invasive potential of HER2-positive cells, we stimulated JIMT1 shCntl and JIMT1 shIGF1R cells with insulin, the main ligand for the IR. Insulin stimulation of JIMT1 shIGF1R cells blocked the anti-invasive effect of IGF-1R knockdown plus trastuzumab (Figure 3.5.B). The invasiveness

Figure 3.4. IGF-1 stimulation blocks anti-invasive effect of IR knockdown in HER2-positive cells. (A) JIMT1 cells were transfected with 100nM of control siRNA (siC) or insulin receptor siRNA (siIR) for 24 h. Cells were seeded in Boyden chambers in serum-free media with 20μg/mL of trastuzumab (Tras), 100 ng/mL of IGF-1, or a combination of trastuzumab plus IGF-1 (Tras + IGF-1), and 10% FBS in the well as the chemo-attractant. After 24h of invasion, photos were taken; representative photos are shown. The number of invaded cells were counted in 12 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with reproducible results (Student's t test, * $P < 0.05$). **(B)** Knockdown efficiency of IR was determined by Western blotting. Total protein lysates were applied for Insulin-like growth factor receptor 1 (IGF-1R), total Insulin Receptor (IR), and beta-actin (β-actin) protein expression detection on cells that were seeded for invasion.


A
Unt
Tras
IGF-1
T + IGF-1
siC
siIR
2500
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1500
1000
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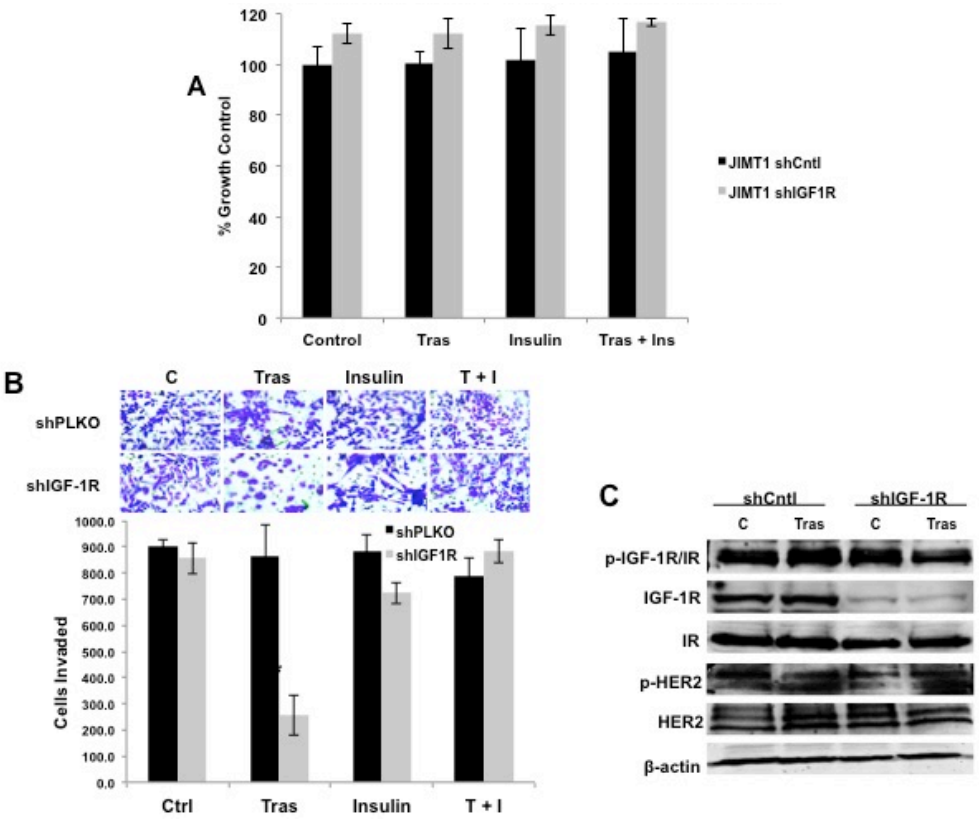
Cells Invaded
siC
siIR
*
Ctrl
Tras
IGF-1
T + IGF-1
B
siC
siIR
IGF1R
IR
β-actin

Figure 3.5. Insulin stimulation blocks anti-invasive effect of IGF-1R knockdown in HER2-positive cells. (A) JIMT1 shPLKO (shCntl) and JIMT1 shIGF1R (shIGF1R) cells were seeded in a 96-well plate. The next day, cells were treated in serum free media with Control (untreated), 20μg/mL of trastuzumab (Tras), 10 nM of insulin, or a combination of trastuzumab plus insulin (Tras + insulin). After 20 hours, serum free media was replaced by 10% AlamarBlue in serum free media. Treatments were maintained throughout the experiment. Fluorescence measurements were taken at various time points per treatment; triplicates performed per treatment group. These results correspond to measurements taken 5 hrs after addition of the AlamarBlue solution. Percent of cell growth compared to control was calculated using the following formula: % cell growth compared to control = (FI 590nm Test Reagent / FI 590nm Untreated Control) x 100. The experiment was performed three times with reproducible results. **(B)** The same cells were pre-treated with 20μg/mL of trastuzumab for 24 h in serum-free media. Cells were then seeded in Boyden chambers in serum-free media with 20μg/mL of trastuzumab, 10nM of insulin, or 20μg/mL of trastuzumab and 10nM insulin, with 10% FBS in the well as the chemo-attractant. After 24h of invasion, photos were taken; representative photos are shown. The number of invaded cells were counted in 12 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with reproducible results (Student's t test, * $P < 0.05$). **(C)** Western blots of total protein lysates were performed for phospho-Insulin-like growth factor receptor 1/Insulin Receptor (p-IGF-IR/IR), total Insulin-like growth factor receptor 1 (IGF-1R), total Insulin Receptor (IR), phospho-Her2 (p-Her2), total Her2 (Her2), and beta-actin (β-actin) protein expression on cells that were seeded for invasion.


A
% Growth Control
120
100
80
60
40
20
0
Control
Tras
Insulin
Tras + Ins
JIMT1 shCntl
JIMT1 shIGF1R
B
C
Tras
Insulin
T + I
shPLKO
shIGF-1R
Cells Invaded
1000.0
900.0
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
shPLKO
shIGF1R
Ctrl
Tras
Insulin
T + I
C
shCntl
shIGF-1R
C
Tras
C
Tras
p-IGF-1R/IR
IGF-1R
IR
p-HER2
HER2
β-actin

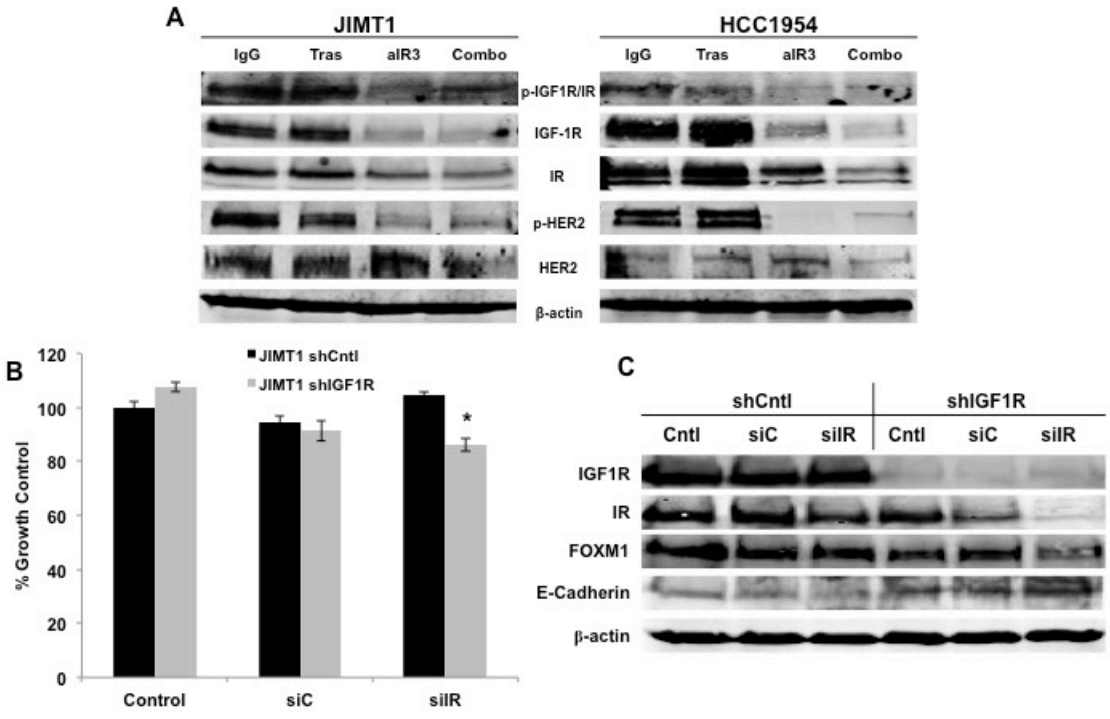
of JIMT1 shCntl cells remained unaffected at all conditions. Additionally, western blot analysis confirms knockdown of IGF-1R in the JIMT1 shIGF1R cells, while IR remains expressed in both JIMT1 shCntl and JIMT1 shIGF1R stably infected cell lines (Figure 3.5.C). These results suggest that IR stimulation can compensate for the loss of IGF-1R expression to drive invasion of HER2-positive cells.

Co-targeting IGF-1R and IR in HER2-positive breast cancer.

Our results show that both IR and IGF-1R can compensate for the lack of expression of one of the receptors. Consequently, we decided to co-target the IR and IGF-1R in HER2-positive breast cancer cells with the mouse monoclonal antibody alpha-IR3. Co-targeting IGF-1R and IR using alpha-IR3 decreased phosphorylation of HER2, total IGF-1R and total IR in both JIMT1 and HCC1954 cells (Figure 3.6.A), suggesting that HER2 activation in these trastuzumab-resistant cell lines is maintained in part through IR and IGF-1R signaling.

We also used siRNA to transiently knockdown IR expression in the JIMT1 shIGF1R cells to generate cells with double IGF-1R and IR knockdown. We observed that transient knockdown of IR in JIMT1 shIGF1R cells results in a modest but statistically significant decrease in cell growth when compared to JIMT1 shIGF-1R cells treated with control, whereas cell growth of JIMT1 shCntl cells remained unaffected by IR knockdown (Figure 3.6.B). Furthermore, transient IR knockdown in JIMT1 shIGF1R cells (double IGF-1R/IR knockdown) decreased expression of the pro-invasive transcription factor FoxM1, and increased expression of the epithelial marker E-cadherin

Figure 3.6. Co-targeting IGF-1R and IR in HER2-positive breast cancer. (A) Effect of co-targeting IGF-1R and IR on expression and phosphorylation of IGFR-1R and IR. JIMT1 (left) or HCC1954 (right) cells were treated with control IgG, 20 μg/mL trastuzumab, 0.25 μg/mL aIR3, or a combination of trastuzumab plus aIR3 for 48 hrs. Western blots of total protein lysates were performed for phospho-Insulin-like growth factor receptor 1/Insulin Receptor (p-IGF-1R/IR), total Insulin-like growth factor receptor 1 (IGF-IR), total Insulin Receptor (IR), phospho-Human Epidermal Growth Factor Receptor 2 (p-HER2), total HER2, and β-actin protein expression. **(B)** JIMT1 shCntl and JIMT1 shIGF-1R cells were transfected with control siRNA (siC) or IR siRNA (siIR) or remained untransfected. After 24 hrs, cells were seeded in a 96-well plate at a density of 8,000 cells per well. After 20 hours, complete media was replaced by 10% AlamarBlue in complete media. Fluorescence measurements were taken at various time points; triplicates performed per treatment group. These results correspond to measurements taken 5 hrs after addition of the AlamarBlue solution. Percent of cell growth compared to control was calculated using the following formula: % cell growth compared to control = (FI590nm Test Reagent / FI590nm Untreated Control) x 100. The experiment was performed twice with reproducible result. **(C)** FoxM1 and E-cadherin expression in HER2 positive cells with IGF-1R and IR inhibition. JIMT1 shCntl and JIMT1 shIGF-1R cells were transfected with control siRNA (siC) or IR siRNA (siIR) or remained untransfected. After 24 hrs, cells were collected and western blots of total protein lysates were performed for total Insulin-like growth factor receptor 1 (IGF-1R), total Insulin Receptor (IR), total FoxM1, E-Cadherin, and β-actin) protein expression. Experiment was performed twice with reproducible results.


A
JIMT1
IgG
Tras
aIR3
Combo
HCC1954
IgG
Tras
aIR3
Combo
p-IGF1R/IR
IGF-1R
IR
p-HER2
HER2
β-actin
B
JIMT1 shCntl
JIMT1 shIGF1R
% Growth Control
120
100
80
60
40
20
0
*
Control
siC
siIR
C
shCntl
shIGF1R
Cntl
siC
siIR
Cntl
siC
siIR
IGF1R
IR
FOXM1
E-Cadherin
β-actin

(Figure 3.6.C). All together, these results suggest that dual targeting of IR and IGF-1R inhibits invasion of HER2-positive breast cancer cells.

3.4 Discussion:

Multiple studies implicate IGF-1R in the development of trastuzumab resistance. However, the preclinical activity reported for anti-IGF-1R therapies has not been replicated in the clinical setting. The lack of clinical efficacy may be due to compensatory IR signaling. We previously demonstrated that IGF-1R signaling promotes invasion of HER2-positive breast cancer cells, with a dramatic decrease in cell invasion when IGF-1R and IR are co-targeted using the IGF-1R antibody alpha IR3 (97). Since crosstalk exists between IGF-1R and IR, and hybrid IGF-1R/IR receptors are expressed in cells and tissues overexpressing IGF-1R and IR (109-111), we hypothesized that IR contributes to the invasion of HER2-positive breast cancer cells. Here, we examine the effects of IR and HER2 co-inhibition in the growth and invasion of HER2-positive models of trastuzumab resistance.

Co-inhibition of IR and HER2 reduces invasion of HER2-positive cells; IGF-1 stimulation blocks this anti-invasive effect. Co-inhibition of IGF-1R and HER2 also reduces invasion of HER2-positive cells, with insulin stimulation blocking the anti-invasive effect. These results indicate that stimulation of IGF-1R or IR with its respective ligand (IGF-1 or insulin) compensates for the loss of expression of IR or IGF-1R, respectively, restoring invasiveness of trastuzumab-resistant cells.

Overexpression of IGF-1R and IR in breast cancer has been documented across the literature. Increased IR expression was reported in breast cancer tissue compared with normal breast tissue (106). Similarly, high IGF-1R expression has been observed in breast cancer cells, including models of trastuzumab resistance (61; 94). Evidence shows that increased IGF-1R signaling abrogates the effects of therapeutic antibodies targeting HER2, with IGF ligands stimulating Akt signaling in breast tumor cells pre-treated with trastuzumab (163; 164). Here, we provide evidence indicating that IR signaling is important for the regulation of trastuzumab-resistant breast cancer cell invasion, and confirm inhibition of invasion in response to co-targeting IGF-1R and HER2. We also show that ligand stimulation overcomes inhibition of invasion. Several studies support these findings. In premenopausal women, elevated serum IGF-1 levels increase the risk of developing invasive ductal carcinomas (115). This might explain the restored invasive potential of JIMT1 and HCC1954 cells treated with IGF-1, when IR expression was suppressed and HER2 was targeted with trastuzumab, but IGF-1R was still expressed. Another study shows that IGF-1R knockdown increases the sensitivity of breast cancer cell lines to insulin (165), which is consistent with our findings that insulin restores the invasiveness of JIMT1 shIGF1R cells treated with trastuzumab. Additionally, crosstalk between receptor tyrosine kinases has been shown to contribute to resistance toward agents targeting a single receptor tyrosine kinase. For instance, a study showed that increased signaling from HER2 and HER3 conferred resistance to the EGFR-specific antibody cetuximab (166). IGF-I signaling has also been shown to confer resistance to trastuzumab in HER2-overexpressing cells (61).

Studies suggest that IGF-1R and IR signaling promote cell growth and proliferation of cancer cells. Insulin and IGF-2 stimulate cell proliferation through the IR in cells devoid of IGF-1R expression (167). Similarly, IGF-2 stimulated cell proliferation in an IR-dependent manner in cells with IGF-1R knockdown (108). Furthermore, a study showed that IGF-1R inhibition significantly decreases tumor cell invasion and metastasis without affecting cell growth (168). Our results demonstrate that IGF-1 stimulation of cells with IR knockdown, and insulin stimulation of cells with IGF-1R knockdown, does not alter cell growth of HER2-positive cells, even when co-treated with trastuzumab. These results might be explained by IGF-1R/IR signaling crosstalk. Signaling through both IGF-1R and IR can mediate activation of cell survival in the presence of anti-tumor agents, including molecular targeted therapies like trastuzumab (169).

In other results, we found that similar to IGF-1 stimulation, insulin stimulation of trastuzumab-resistant breast cancer cells leads to HER2 phosphorylation. Numerous studies have shown IGF-1-mediated activation of HER2 (60; 62; 97). However, to the best of our knowledge, this is the first report of insulin-mediated activation of HER2 in trastuzumab-resistant breast cancer cells. Insulin-mediated phosphorylation of HER2 might occur by insulin binding to the IR and/or IGF-1R/IR hybrid receptors, which have been shown to bind all three ligands of the IGF/insulin system (84; 112). IGF-1R/IR hybrid receptors, especially those with the IR isoform A (IR-A), exhibit affinity in the low nanomolar range for IGF-1, IGF-2, and insulin (114). This suggests that the predominant composition of hybrid receptors in JIMT1 and HCC1954 cells might be IGF-1R/IR-A. It has been shown that IR-A isoform is largely expressed in breast cancer cells and breast cancer tissue specimens, in comparison to non-malignant cells and

normal tissue specimens (170). We also report that co-inhibition of IGF-1R and IR in a trastuzumab-resistant cell line decreases HER2 phosphorylation, providing more evidence that IGF-1R and IR are mediating HER2 activation in resistant cells. HER2 activation via insulin stimulation suggests that insulin might induce HER2-mediated activation of signaling pathways that promote invasion and metastasis of cancerous cells. We also observed a decrease in FoxM1 expression as a consequence of IGF-1R and IR co-inhibition. FoxM1 was found to be overexpressed, both in RNA and protein levels, in breast cancer tissue (150). In the same study, FoxM1 expression correlates with poor prognosis and HER2 overexpression in breast cancer, as determined by immunohistochemistry (150). Another study showed that stable overexpression of FoxM1 promoted metastasis of human breast cancer cells *in vivo* (171). Moreover, FoxM1 overexpression confers resistance to trastuzumab in HER2-positive breast cancer cell lines (151). We have previously studied FoxM1 in HER2-positive cells, as well. In trastuzumab-resistant cells, FoxM1 expression was heavily regulated by HER2-mediated MEK/ERK1/2 signaling (135). Treatment of IGF-1R-deficient cells with trastuzumab (co-targeting IGF-1R and HER2) decreased ERK1/2 phosphorylation, FoxM1 expression, and reduced the invasive potential of trastuzumab-resistant cells (97). Re-expression of FoxM1 in these cells restored their invasive ability. Furthermore, knockdown of FoxM1 blocked IGF-1-mediated invasion of trastuzumab-resistant cells (97). All together, results from this work and evidence from the literature indicate that FoxM1 plays an important function in IGF-1R/IR-mediated resistance to trastuzumab in breast cancer cell lines.

Another important finding of this study shows that IGF-1R/IR co-inhibition leads to an increase in the expression of the tumor suppressor E-cadherin in a HER2-positive

breast cancer cell line. The opposite effect was observed in a transgenic mouse model of hyperplastic pancreatic islet cell cancer, where a decrease in E-cadherin was detected in association with high IGF-1R expression (172). In another study, stable transfection of human E-cadherin in a cancer cell line that lacked expression of E-cadherin decreased phosphorylation of IGF-1R/IR and ERK1/2 (173). IGF-1 and insulin stimulation increased the invasion of cells with stable E-cadherin expression (173). Our results suggest that E-cadherin is regulated by IGF-1R/IR signaling in HER2-positive breast cancer cell lines.

These results provide additional support for the idea of bidirectional crosstalk between IGF-1R and IR and its importance in the development and promotion of human cancer, especially metastatic breast cancers. Future experiments will investigate IR-A expression in HER2-positive cells lines to determine the specific contribution of this isoform in trastuzumab resistance and invasion of HER2-positive cells. The study of secreted insulin and IGF-2 levels in HER2-positive cell lines is relevant, as well. Especially since IGF-2 is known to activate IR, IGF-1R, and hybrid receptors signaling, and high levels of insulin are positively associated with increased risk for breast cancer (174).

In summary, our results indicate that the invasiveness of trastuzumab-resistant cells is dependent on IR, IGF-1R and HER2 signaling. IGF-1 stimulation blocked the anti-invasive effect of IR knockdown in cells treated with trastuzumab, while insulin did the same in cells with IGF-1R knockdown treated with trastuzumab. Future preclinical in vivo studies should evaluate co-targeting of all three receptors as an approach for inhibiting invasion and metastasis of HER2-positive breast cancers.

Chapter 4:

Conclusions

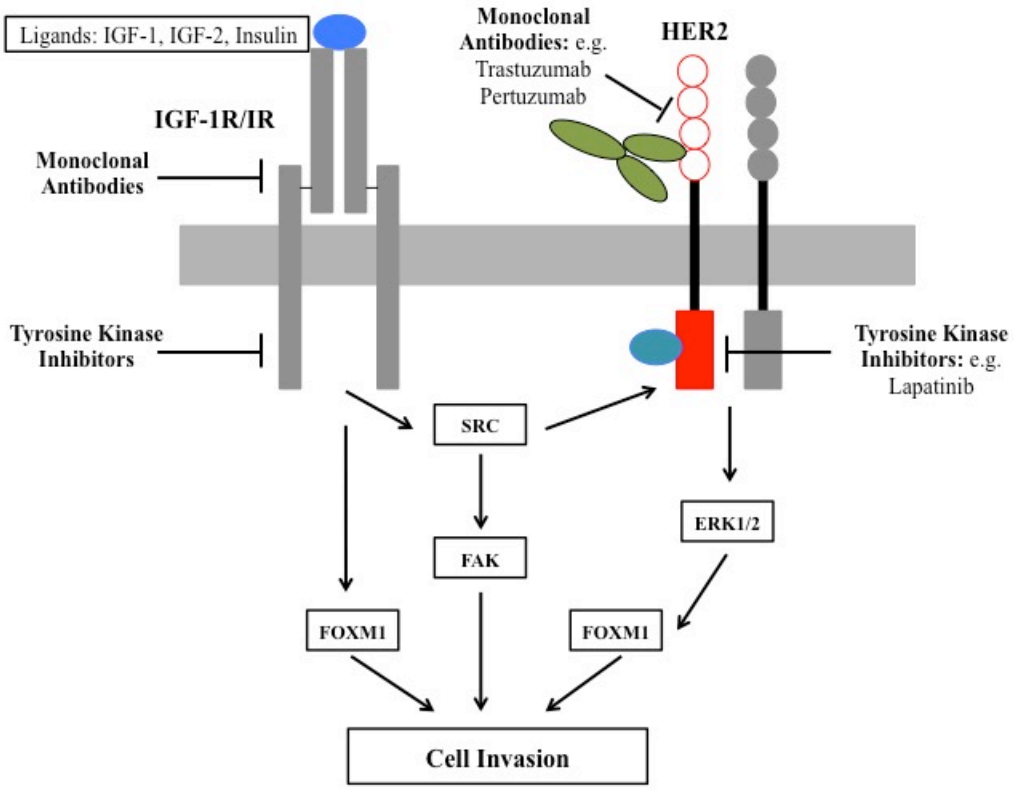
Chapter 4: Conclusions

4.1 Summary

Metastatic breast cancer is a prevalent disease that affects thousands of lives every year. Trastuzumab remains a primary component of the approved first-line treatment regimen for patients with HER2-positive metastatic breast cancer. However, *de novo* and acquired resistance to trastuzumab occurs in many patients. Therefore, a clear understanding of molecular mechanisms driving resistance is required to improve therapeutic approaches for patients with resistant tumors. The work presented in this dissertation provides evidence that IGF-1R and IR signaling promotes invasion of trastuzumab-resistant breast cancer cells (Figure 4.1). We propose that inhibition of IGF-1R, IR, and HER2 is required to suppress invasion of HER2-positive breast cancer cells. Further, we found that stimulation of IGF-1R activates Src/FAK signaling, promoting invasion. Concomitantly, HER2-mediated activation of MAPK signaling and IGF-1R/IR signaling regulates FoxM1 expression to promote invasion.

In Chapter 2, our results demonstrated that IGF-1R signaling was crucial to promote invasion of HER2-overexpressing breast cancer cells. We were very interested to study the IGF-1R/HER2 interaction, since previous research had shown that HER2 forms a complex with receptor tyrosine kinases, like IGF-1R and HER3, enabling crosstalk between the receptors in models of trastuzumab resistance (60; 62). Our goal was to dissect the mechanisms enabling crosstalk and the downstream molecular and biological effects mediated by IGF-1R in HER2-positive breast cancer.

Figure 4.1. Proposed model illustrating IGF-1R/IR and HER2 signaling promoting invasion of trastuzumab resistant breast cancer cells. Co-inhibition of IGF-1R and HER2 in trastuzumab resistant breast cancer cells resulted in suppressed invasion, while cell proliferation was moderately altered. Src kinase emerged as a mediator of the IGF-1R/HER2 crosstalk, and as a promoter of invasion, as well as FAK kinase. FoxM1 was identified as another mediator of invasion, particularly downstream of the MAPK signaling pathway. Moreover, co-inhibition of IR and HER2 resulted in a significant decrease of cell invasion, suggesting that IR is also important for the invasiveness of trastuzumab resistant cells. IGF-1R/IR signaling also regulates FoxM1 expression, since double knockdown of IGF-1R and IR was required to decrease total FoxM1 protein levels.


Ligands: IGF-1, IGF-2, Insulin
IGF-1R/IR
Monoclonal Antibodies
Tyrosine Kinase Inhibitors
Monoclonal Antibodies: e.g. Trastuzumab Pertuzumab
HER2
Tyrosine Kinase Inhibitors: e.g. Lapatinib
SRC
FAK
ERK1/2
FOXM1
FOXM1
Cell Invasion

Our data showed that IGF-1 stimulated phosphorylation of HER2, Src, FAK, and Erk1/2 in trastuzumab-resistant cells. Src activation appeared to be critical for maintaining HER2 phosphorylation, as a small-molecule Src kinase inhibitor decreased HER2 phosphorylation in a dose-dependent manner in trastuzumab-resistant JIMT1 cells. Co-inhibition of IGF-1R and HER2 was crucial to decrease the invasion of HER2-positive cells, demonstrated by pharmacological and stable genetic inhibition of IGF-1R in the presence of trastuzumab. Src and FAK emerged as regulators of invasion downstream of IGF-1R signaling in HER2-overexpressing cells, as demonstrated by decreased invasion of cells treated with Src and FAK kinase inhibitors in the presence of IGF-1. Furthermore, expression of constitutively active Src kinase blocked the anti-invasive effect of IGF-1R/HER2 co-inhibition.

We also found that FoxM1 is an important mediator of invasion in trastuzumab-resistant cells, for FoxM1 knockdown decreased invasion of resistant cells stimulated with IGF-1. Overexpression of FoxM1 overcame the anti-invasive effect of IGF-1R knockdown and trastuzumab treatment. Moreover, IGF-1R and HER2 co-inhibition was required to decrease phosphorylated Erk1/2 and FoxM1 expression, suggesting that both receptors contribute to the regulation of FoxM1 expression in trastuzumab-resistant cells. Additionally, combination of IGF-1R- and HER2-targeted antibodies induced significant ADCC of JIMT1 cells compared with the use of either single agent, suggesting that targeting IGF-1R improves response to trastuzumab in resistant cells.

Collectively, our results demonstrated that the invasiveness of trastuzumab-resistant cells is codependent on IGF-1R and HER2 signaling, and inhibition of both receptors was required to block invasion. Furthermore, our data supports the idea that

Src, FAK, and FoxM1 represent potential therapeutic targets in trastuzumab-resistant cells, particularly in patients that co-express IGF-1R and HER2.

In Chapter 3, we studied the role of IR in trastuzumab-resistant breast cancer cells. Our results showed that IR was a mediator of invasion in HER2-overexpressing cells, similarly to IGF-1R. We were interested in the study of IR in the context of trastuzumab resistance since IR expression and the formation of hybrid IGF-1R/IR receptors have been linked to compensatory signaling and decreased response to anti-IGF-1R therapies (84; 112). Our goal was to determine the contribution of IR to the invasion of trastuzumab-resistant HER2-positive breast cancer cells.

Our data demonstrates for the first time that insulin-mediated activation of IGF-1R/IR phosphorylates HER2 in trastuzumab-resistant breast cancer cells, similar to IGF-1-mediated phosphorylation of HER2. While cell growth remained unaffected, invasion of trastuzumab-resistant cells was significantly inhibited by IR and HER2 co-inhibition. IGF-1 stimulation of IGF-1R blocked the anti-invasive effect of IR knockdown and trastuzumab treatment. Furthermore, insulin stimulation of IR overcomes the anti-invasive effect of stable IGF-1R knockdown and trastuzumab treatment in JIMT1 cells. Co-targeting IGF-1R and IR with the monoclonal antibody alphaIR3 reduces total expression of IGF-1R and IR, and decreases HER2 phosphorylation, providing support to the concept that HER2 activation is mediated by IGF-1R/IR signaling in HER2-overexpressing breast cancer cells. Finally, genetic knockdown of IGF-1R and IR in JIMT1 cells decreased FoxM1 expression and increased E-cadherin expression, suggesting that IGF-1R and IR are important for maintaining an invasive phenotype.

Our results indicate that IR is a key mediator of invasion in trastuzumab-resistant cells, and inhibition of IR and HER2 was imperative to significantly inhibit invasion. These results propose that co-targeting IR and IGF-1R may be critical for successfully achieving an anti-invasive response in HER2-positive breast cancer cells.

4.2 Future directions

The contributions of downstream signaling molecules, including Src, FAK, and FoxM1, to the progression of HER2-positive breast cancer must be considered in future experiments. Phosphorylation levels of Src, FAK, and HER2 correlate in clinical breast cancer samples (142), providing evidence that supports co-targeting of these kinases. Trastuzumab-resistant cells demonstrate increased activation of Src compared with sensitive cells (65; 134). We have similarly reported that resistant models exhibit sustained Src activity due to heightened upstream signaling in contrast to sensitive cells (97; 138). Dual inhibition of IGF-1R and FAK using a single small molecule inhibitor resulted in significant inhibition of cell viability and decreased phosphorylation of Erk and Akt in cancer cells (175). Monotherapy with a FAK kinase inhibitor prevented migration of HER2-positive/ER-positive cells, and a combination of the FAK inhibitor plus trastuzumab synergistically inhibited cell proliferation (176).

Further evaluation of FoxM1 regulation should also be considered. Our studies revealed that FoxM1 downregulation is critical for the anti-invasive effect of co-targeting IGF-1R and HER2. Moreover, the function of FoxM1 seems to be much more complicated than predicted. A study suggested that FoxM1 might display not only

oncogenic properties, but also tumor-suppressive properties, a dual effect previously described for other proliferation-associated transcription factors (177).

Our results argue expression levels of IR isoforms and secreted IGF/insulin system ligands may affect resistance. IGF-1R/IR hybrid receptors, particularly those expressing IR-A, exhibit affinity in the low nanomolar range for IGF-1, IGF-2, and insulin (114). This ability to bind IGF-1, IGF-2, and insulin might prove advantageous for tumors expressing high levels of IR-A (178). The study of secreted insulin and IGF-2 levels in HER2-positive cells lines is of great importance. IGF-2 is known to activate IR-A, IGF-1R, and hybrid receptors. Studies have shown that many malignancies exhibit IR-A overexpression and IGF-2 production, leading predominantly to mitogenic and pro-invasive effects (178-180). High levels of circulating insulin are positively associated with increased risk for breast cancer (174). Therefore, we also propose further investigation of the relationship between type-two diabetes and the incidence of breast cancer (181), especially in HER2-overexpressing metastatic breast cancer. We also recognize the need for better biomarkers to predict clinical response to agents targeting IGF-1R, IR, and HER2. Further study of the IGF/insulin system in the context of HER2-positive breast cancer might help to identify these biomarkers. For example, we propose that a better understanding of the relationship between expression versus activation of a receptor tyrosine kinase will help determine the use of these parameters as a predictor of response. Analysis of IGF-1, IGF-2, and insulin secretion might help identify ligand expression as a better clinical biomarker than total receptor expression. IGFBPs also regulate ligand bioavailability and receptor activation, suggesting that a better understanding of how levels of IGFBPs might affect HER2-positive cancer development

and progression should also be considered in future studies. The use of more than one method to detect the activation and expression of receptor tyrosine kinases and other biomarkers is also important. Using different approaches will corroborate results and reduce the likelihood of incorrect classification of tumors, and increase the chances of patients receiving appropriate therapy.

Trastuzumab was the first HER2-targeted drug to improve the outcome of metastatic breast cancers. However, new HER2-targeted therapies have been studied and others remain under scrutiny. For example, the Clinical Evaluation of Pertuzumab and Trastuzumab (CLEOPATRA) study evaluated the efficacy and safety of a dual antibody regimen as first-line treatment for patients with HER2-positive metastatic breast cancer: pertuzumab plus trastuzumab (182). Pertuzumab is a humanized monoclonal antibody that prevents HER2 dimerization with other receptors, most notably HER3 (183; 184), by targeting the extracellular domain of HER2 at a different epitope (subdomain II) than trastuzumab (185). The experimental group in this study received pertuzumab plus trastuzumab plus docetaxel, as compared with placebo plus trastuzumab plus docetaxel in the control group. Results showed that median progression-free survival was extended by 6.1 months, from 12.4 months in the control group to 18.5 months in the experimental group (182). These findings suggest that dual targeting of HER2-positive tumors with monoclonal antibodies that have different mechanisms of action might improve response to treatment and prevent acquisition of resistance. We propose further investigation of pertuzumab plus trastuzumab therapy in HER2-positive cancers with high IGF-1R/IR activation and/or expression. This might highlight a potential role for pertuzumab to prevent or reduce direct interaction and crosstalk between IGF-1R/IR and HER2.

However, it is possible that sustained IGF-1R/IR activation or receptor overexpression might serve as a compensatory mechanism to bypass the clinical action of pertuzumab. We also propose a potential benefit from the examination of treatments combining IGF-1R/IR-targeted antibodies and HER2-targeted antibodies *in vivo*, to corroborate the advantage of targeting multiple receptors using monoclonal antibodies with different mechanisms of action and the outcome of IGF-1R/IR and HER2 co-inhibition in trastuzumab resistant tumors.

Our work is completely based on *in vitro* models. Consequently, it is important to recognize such limitations, for the results might not be representative of clinical samples. Our studies assume a collection of homogeneous cells and do not consider the role of the tumor microenvironment and how interactions with surrounding normal tissues, matrix-degrading enzymes, hormones, and immune cells might affect cancer progression. Therefore, we recommend *in vivo* studies to assess the translational potential of the results obtained *in vitro*. The lack of a specific antibody to distinguish between IGF-1R and IR phosphorylation was another drawback of our studies. Availability of such phospho-antibodies will significantly benefit the study of the IGF/insulin system and its role in tumorigenesis and cancer development. The fact that genetic knockdown, using either siRNA or shRNA, does not ensure complete inhibition of IGF-1R or IR expression may impact our results. Nevertheless, we argue that residual expression of IGF-1R and IR after knockdown might be more representative of actual tumor behavior. IGF-1R and IR are ubiquitously expressed and it's receptor overexpression/activation what drives IGF-1R/IR contribution to trastuzumab resistance in HER2-overexpressing breast cancer.

Finally, our data support the development of therapeutic approaches that co-target IGF-1R, IR, and HER2 to delay or treat local invasion and metastasis of trastuzumab-resistant tumors. Additional *in vivo* studies are required to evaluate the translational potential of our *in vitro* findings and determine the side effects of co-targeting IGF-1R, IR, and HER2. In the clinic, although therapies targeting IGF-1R have been well tolerated, with hyperglycemia as the main toxicity (120), the lack of efficacy of this approach has been disappointing. Potential explanations include previous trial designs, which did not select patients based on IGF-1R expression or IGF-1R activation status. Identification of surrogate endpoints, such as biomarkers indicating inhibition of IGF-1R signaling, were also lacking in past study designs. Lastly, most clinical trials did not co-inhibit compensatory pathways, such as IR, HER2, or Src, potentially allowing sustained signaling in the presence of IGF-1R inhibition.

In conclusion, the results of our dissertation studies suggest that co-targeting IR, IGF-1R, and HER2 is a rational approach for patients whose breast tumors demonstrate IGF-1R/IR activation and HER2 overexpression and have progressed on prior trastuzumab treatment. Further investigation of these receptors and the pathways they activate will provide significant knowledge to identify novel therapeutic targets, better treatment options, and predict potential mechanisms of resistance that might emerge in the future.

Chapter 5:

References

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