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Interplay Between Her2, Parp1, And Nf-Kb In Breast Cancer: Potential Therapeutic Implications

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INTERPLAY BETWEEN HER2, PARP1, AND NF-κB IN BREAST CANCER: POTENTIAL THERAPEUTIC IMPLICATIONS

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INTERPLAY BETWEEN HER2, PARP1, AND NF-κB IN BREAST CANCER: POTENTIAL THERAPEUTIC IMPLICATIONS

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CANCER BIOLOGY

ABSTRACT

We previously reported that HER2+ breast cancers are susceptible to Poly (ADP-Ribose) polymerase inhibitors (PARPi) alone, agents that are efficacious against homologous recombination (HR) deficient tumors. However, this phenomenon was determined to be independent of a HR repair deficiency but rather due to suppression of NF-κB activity and signaling by PARP inhibition. Further, HER2 overexpression itself was necessary and sufficient to confer this susceptibility. Interestingly, PARP1 and phosphorylated RelA/p65 (NF-κB) levels were found to be elevated in human HER2+ breast cancers compared to luminal breast cancers. These data suggest a possible interplay between HER2, PARP1, and NF-κB, and how this interplay influences PARPi sensitivity is the main goal of this dissertation.

We hypothesize that the elevated PARP1 protein levels observed in HER2+ breast tumors are regulated at the post-transcriptional level via a microRNA. Due to the fact, that resistance to HER2 agents may activate compensatory mechanisms to maintain downstream signaling we predict HER2+ trastuzumab resistant (TR) breast cancer cells will retain sensitivity to PARPi. Our data indicates that HER2+ breast tumors with high PARP-1 protein levels expressed lower levels of let-7a. We also discovered that PARP1's was regulated at the post-transcriptional level by the let-7a microRNA in HER2+ breast cancer cells. It was also observed that both pharmacological and genetic inhibition of

PARP1 decreased NF-κB activity. Specifically, we observed that suppression of PARP1 levels reduced binding of the p65 transcription factor to the promoter of a NF-κB target gene, interleukin 8 (IL-8). Finally, we detected that HER2+ trastuzumab resistant breast cancer cells remained sensitive to PARPi.

In conclusion, our data suggest that an interplay between the HER2, PARP1, and NF-κB signaling pathways exists which may be a determinant behind PARPi susceptibility in HER2+ breast cancer cells. Ultimately, the data from these studies will lead to better understanding of PARP biology and broaden the use of PARPi in the clinic.

Keywords: HER2, breast cancer, PARP1, NF-κB, let-7a, microRNA, PARP inhibition

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CHAPTER 1

RESEARCH OVERVIEW

One third of breast cancer patients exhibit amplification or overexpression of the HER2 gene or protein and this phenotype is referred to as the HER2+ subtype of breast cancer (1). We recently discovered that HER2+ breast cancer cells are sensitive to Poly (ADP-ribose) polymerase inhibition (PARPi) alone despite being homologous recombination (HR) proficient (2). However, the mechanism behind PARPi sensitivity in this subtype of breast cancer is not well understood. Thus, the overarching goal of this dissertation is to uncover the DNA repair independent determinants of PARPi sensitivity in HER2+ breast cancer cells.

We previously found that HER2 overexpression correlated with increased levels of PARP1 and phosphorylated RelA/p65 (NF-κB) compared to non-HER2 overexpressing tumors (3). Additionally, it has been reported that PARP1 has been shown to act as a co-activator of NF-κB (4). Inhibition of PARP1 has also been shown to attenuate NF-κB activity and signaling in HER2+ breast cancer cells (2). These findings suggest a potential crosstalk between the HER2, PARP1, and NF-κB pathways. Based on these data, we hypothesize that PARP1's non-DNA repair roles maybe an important determinant behind PARPi sensitivity. Specifically, we postulate that HER2 elevates PARP1 to drive sensitivity dependencies on PARP1 in HER2+ breast cancer cells and

this could be through a post-transcriptional mechanism by a let-7a microRNA. We also hypothesize that enhanced PARP1 expression subsequently fosters downstream NF-κB activation in HER2+ breast cancer cells. Next because therapeutic resistant tumors often activate compensatory pathways to circumvent upstream events in order to maintain downstream signaling, we also hypothesize that HER2+ trastuzumab resistant breast cancer cells retain sensitivity to PARPi. To test our hypotheses, we formulated the following three objectives and they are illustrated in Figure 1.1.

Our first objective was to determine the mode of regulation of the increased PARP1 protein observed in HER2+ breast cancer cells (Chapter 2). We hypothesize that PARP1 protein levels are regulated by a miRNA, based on previous findings showing that PARP1 is targeted by a microRNA in esophageal adenocarcinoma and that microRNAs are altered in HER2-overexpressing breast carcinomas (5,6). To assess the association between HER2 and PARP1 protein expression we used both knockdown and overexpression models. We also utilized the Nanostring platform to investigate the role of microRNAs in regulation of PARP1 in HER2+ breast cancers. Our studies identified *let-7a* as a suppressor of PARP1 protein levels in both HER2+ breast cancer cells and tumors. By western blot analysis and firefly luciferase assays we determined that the let-7a miRNA acts at the 3'UTR of *PARP1*. Ectopic let-7a expression also reduced cell proliferation in HER2+ breast cancer cells.

Our second objective was to further assess the role of PARP1 on NF-κB-mediated transcription in HER2+ breast cancer cells (Chapter 3). We hypothesize that the elevated PARP1 levels enhance NF-κB activation, because we previously showed that NF-κB activity and signaling were inhibited by PARPi. To test our hypothesis, we used

NanoString to investigate the effect of PARPi on the expression of NF-κB target genes. The most differentially regulated gene by PARPi was IL-8. This was verified with qRT-PCR and ELISA assays. To validate that PARP1 regulates NF-κB at the promoter of the IL-8 gene, we used a chromatin immunoprecipitation (ChIP) assay to measure recruitment of the p65 transcription factor to the IL-8 promoter. Consistent with our hypothesis, PARP1 knockdown resulted in reduced p65 binding to the IL-8 promoter.

Our third objective was to evaluate whether PARPi susceptibility is retained in trastuzumab resistant breast tumors (Chapter 3). We hypothesize that tumors resistant to HER2 targeting agents will retain sensitivity to PARPi, because resistant cell lines can activate the same downstream pathways as parental cell lines by upregulating compensatory upstream pathways. Similar to parental cells, HER2+ trastuzumab resistant breast cancer cell lines were sensitive to PARPi both *in vitro* as well as *in vivo* in mice bearing HER2+ breast xenografts. Together, these studies will improve our understanding of PARP1 biology in the context of PARPi susceptibility.

INTRODUCTION

HER2+ Breast Cancer

According to the American Cancer Society, breast cancer is the second leading cause of cancer death in women and a very heterogeneous disease (7). Tumors are classified into three breast cancer subtypes as well as a normal group based on different patterns of gene expression between each subtype. The four subtypes identified by the

genomic studies are identified as basal, ER+/luminal, human epidermal growth factor receptor positive (HER2+), and normal tumors (8).

This dissertation focuses on the 20-30% of breast cancer patients that are diagnosed with the HER2+ subtype of breast cancer (1). Patients with this subtype contain higher expression levels of genes located within the HER2 amplicon (8). In the late 1980's scientists determined that HER2 was an important gene in the disease process of breast cancer. It was also determined that there was an association between greater HER2 copy numbers and poor prognosis in breast cancer patients (9). Based on these studies, it is recommended that HER2 overexpression should be used as a predictive marker in breast cancer due to its strong correlation with HER2 amplification. In addition to breast cancer, other forms of cancers also overexpress HER2, including: gastric, esophageal, ovarian, endometrial, and non-small-cell lung cancer (10).

Clinical detection of HER2 tumors

The two most frequently used pathology tests for HER2 are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) (11). The IHC test is initially used to measure the amount of HER2 protein expression detected on the membrane of cancer cells. HER2 protein expression is scored on a 0-3 scale as defined by the parameters below (11):

1. Negative HER2 protein expression: 0 and 1+
2. Borderline expression (nonuniform or weak intensity of membrane staining): 2+
3. Positive or HER2 overexpression (uniform intensity of membrane staining):3+

FISH testing is used to further validate breast cancer tumors with indeterminate IHC scores (12). This pathology test evaluates *HER2* gene amplification in breast tumors, by measuring the HER2 to CEP17 ratio and copy number of the *HER2* gene. CEP17 is also used as centromeric probe for chromosome 17 in this test (11).

1. Negative HER2 amplification: FISH ratio <1.8, gene copy number <4.0
2. Borderline HER2 amplification: FISH ratio 1.8-2.2, gene copy number 4.0-6.0
3. Positive HER2 amplification: FISH ratio >2.2, gene copy number >6.0

HER2 Targeted Therapy

Trastuzumab (Herceptin)

One of the targeted therapies against HER2 receptors developed in the late 1990s is trastuzumab (Herceptin) (13). Trastuzumab is a humanized monoclonal antibody that binds to domain IV on HER2's extracellular segment. Consequently, this agent blocks ligand independent HER2 signaling and ultimately leads to tumor cell lysis by various mechanisms. For example; trastuzumab activates antibody-dependent cell-mediated cytotoxicity (ADCC), inhibits the downstream signaling pathways of HER2 (PI3K and MAPK), impedes angiogenesis, or endocytosis and degradation of the HER2 receptor. These mechanisms of action are demonstrated in Figure 1.2 (14). Trastuzumab has less severe effects then chemotherapy, but several deaths have been reported due to lung or heart complications experienced with its use (15). In the clinic, trastuzumab is prescribed

as an adjuvant therapy for patients with HER2+ early-stage breast cancer or advanced metastatic disease alone or in combination with chemotherapy (14).

Trastuzumab resistance mechanisms

Despite its efficacy in the clinic, resistance to trastuzumab tends to occur in metastatic HER2+ breast tumors. A small percentage of these tumors do not respond to the targeted cancer agent due to primary resistance mechanisms. It is also observed that some tumors will initially respond to treatment for twelve to eighteen months, but will then eventually acquire resistance to trastuzumab. There are numerous trastuzumab resistance mechanisms that have been identified in the literature thus far (16). The PI3KCA mutation and PTEN loss in the PI3K pathways are two resistance mechanisms found downstream of the HER2 receptor (17). Further, the laboratory of Dr. Schiff and Dr. Osborne at the Baylor College of medicine observed that HER receptors and ligands were upregulated in HER2+ trastuzumab resistant cells (18). These and other trastuzumab resistance mechanisms are exemplified in Figure 1.3 (16).

Due to the great need to address resistance to therapies, efforts have been placed in creating alternatives. In the past decade, pertuzumab, trastuzumab-DM1, and lapatinib have been developed to overcome resistance to trastuzumab. The next section discusses these other HER2 targeted therapies.

Pertuzumab (Perjeta)

Pertuzumab is a humanized monoclonal antibody targeted against HER2 receptors, much like trastuzumab. This agent binds to domain II on the extracellular

segment of HER2 and blocks ligand-dependent HER2 signaling; specifically, heterodimerization with HER1, HER3, and HER4 is impeded. The mechanisms of action of pertuzumab are inhibition of the most potent heterodimer HER2/ HER3 as well as ADCC. It has been observed that the addition of pertuzumab to trastuzumab treatment provides a more complete blockade of HER2 signaling. In a recent clinical study referred to as Cleopatra, the addition of pertuzumab to the combination therapy of trastuzumab and docetaxel improved overall survival in patients with metastatic HER2+ breast cancer as compared to patients treated with a placebo as well as the combination therapy alone (19). Pertuzumab is also prescribed as a first line therapy for patients with metastatic HER2+ breast cancer in combination with docetaxel and trastuzumab (19).

Trastuzumab-DM1

The second-line therapy recommended for patients whose cancer returned a year after completing treatment with trastuzumab and taxane is T-DM1, also known as Ado-trastuzumab emtansine. T-DM1 consists of a monoclonal antibody, trastuzumab, which is stably linked to a cytotoxic microtubule inhibitor called emtansine. The mechanism of action behind this agent is endocytosis of the HER2 receptor and the release of DM1 into the tumor cell to interrupt HER2 trafficking in the cell, which ultimately results in cell death (20).

Lapatinib

Lapatinib is another HER2-targeted agent used in the clinic and is a dual tyrosine kinase inhibitor targeting both HER1 (EGFR) and HER2. Specifically, this small-

molecule enters into the cell and prevents adenosine triphosphate (ATP) from binding to the tyrosine kinase domains located on these two receptors. This then blocks cell proliferation and inhibits the PI3K or MAPK pathways (21). It is prescribed as a third-line treatment in combination with a chemotherapeutic agent that inhibits cell division called capecitabine for patients that already received pertuzumab and T-DM1 treatment (22). This combination therapy was observed to improve progression-free survival in metastatic HER2+ breast cancers that have not responded to either the first or second line therapy.

However, resistance to these HER2 targeted agents also occurs. An example of a mechanism behind resistance to lapatinib in HER2+ breast cancer cells and xenografts is the activation of the NF-κB signaling pathway (23). The lack of therapeutic strategies for metastatic HER2+ breast cancer tumors that develop resistance to HER2 targeted agents emphasize the dire need of novel treatments.

One such strategy, we believe, is the use of PARP inhibitors, which will be discussed more extensively in chapter 3. Our laboratory previously discovered that HER2+ breast cancer cells are susceptible to the PARPi (ABT-888 (veliparib), AZD-2281 (olaparib)) alone, independent of a basal or induced HR deficiency (2). We hypothesize that PARPi sensitivity will also be retained in HER2+ trastuzumab resistant breast cancers cells.

Poly (ADP-Ribose) Polymerase Inhibition

PARP is a family of seventeen enzymes that function as ADP-ribosyl transferases. PARP1 is the most studied member of this family of enzymes and is responsible for 90%

of PARP activity in the cell (24). The expression of PARP1 is also enhanced in both basal and HER2+ breast tumors compared to their normal counterparts (25,26). This enzyme is involved in the base excision repair pathway and has the capability of sensing both single strand breaks (SSBs) and double strand breaks (DSBs) (27). Furthermore, PARP2 is another member of the PARP family that is responsible for 10% of PAR formation in the nucleus, in response to DNA-damage (28). The ADP-ribose polymers formed by PARP1 and PARP2 carry a negative charge and affect the target protein function or cause an electrostatic repulsion between DNA and histones. PARP 3, 4, 5a and 5b are also poly (ADP-ribosyl transferases). PARP 6-8, 10-12, and 14-16 are mono (ADP-ribosyl) transferases. However, PARP 9 and 13 do not contain ADP-ribosyl transferase activity because they lack a substrate binding site (29).

PARP inhibition (PARPi) in the context of HR deficient tumors has gained a lot of press in the last couple of years. In 2014, the FDA approved the first PARPi, Olaparib for use in ovarian cancer patients with germline *BRCA* mutations and a previous treatment history of 3 or more lines of chemotherapy (30). The SSB replication run-off model explains one mechanism behind cytotoxicity induced by PARPi in HR deficient tumors. In particular, PARPi induces a SSB that is then converted into a DSB. In a normal cell, the HR pathway repairs this DSB. However, in a tumor cell with an HR deficiency, such as a BRCA1/2 mutation, the DNA lesion is left unrepaired and leads to cell death (31). Based on the synthetic lethality concept, we embarked on exploiting this theory by targeting DNA repair in different tumor cell types. We discovered that inhibiting DNA repair in head and neck as well as triple negative breast tumors stimulated synthetic lethality with PARPi (32,33). Next, we hypothesized that

cytotoxicity would also be induced in HER2+ breast cancer cells if we generated a DNA repair defect and used a PARPi. However, to our surprise we found that PARPi alone produced a similar reduction in survival as the combination therapy in HER2+ breast cancer cells. The cytotoxicity produced by PARPi alone was independent of a DNA repair deficiency (2). Consequently, these results led us to question whether PARPi was affecting the non-DNA repair roles of PARP1 in these breast cancer cells.

Non-DNA Repair Roles of PARP1

PARP1 has many roles in the cell besides DNA repair. It has been shown to function in different cell death mechanisms, angiogenesis, and gene transcription. Based on our previous data that PARPi attenuates NF-κB activity and signaling in HER2+ breast cancer cell lines, we decided to focus on PARP1's role in the regulation of gene transcription in this thesis.

PARP1's role in the regulation of gene transcription

PARP1 contains numerous roles in the regulation of gene transcription. One well-studied mechanism is its role in modulating chromatin structure. PARP1 has been observed to PARylate one of the histone proteins found in nucleosomes, H1, by using nicotinamide adenine dinucleotide (NAD+) as a substrate. This modification results in the dissociation of histones from chromatin and increases transcriptional activity (34). Another important factor of chromatin, DEK, has also been shown to be released from chromatin by PARylation (35). PARP1 also plays a role in preventing DNA methylation,

by controlling the activity of a methyltransferase called DNA (cytosine-5)-methyltransferase 1 (DNMT1). The DNMT1 protein is unable to bind to DNA after being PARylated by PARP1, ultimately blocking methylation (36). Additionally, PARP1 acts as an enhancer-binding factor by binding directly to gene promoter regions. It has shown that the PARP1 protein binds to the promoter of the CXC ligand 1 (CXCL1) chemokine and enhances its expression in melanoma cells. On the other hand, PARPi decreased the expression of CXCL1 in these cell lines (37). PARP1 can also act to co-activate or corepress several transcription factors. For instance, PARP1 acts as a co-activator for the E2F transcription factor 1 (E2F1). Specifically, the transcription activation of E2F1 is enhanced after PARP1 binds to this protein (38). The transcription factor NF-κB is also regulated by PARP1. Binding of PARP1 to the NF-κB activates the expression of NF-κB target genes. This interaction has been observed on the promoter of the *HER2* gene (39). PARP1 also acts as corepressor for the Forkhead box O (FOXO1) transcription factor. The binding of PARP1 to FOXO1 at the *p27* promoter, a FOXO1 target gene, reduced gene expression (40). The enzyme activity of PARP1 for its coregulatory function is also context dependent and controversial in many cases.

Crosstalk between PARP1 and NF-κB

There are several pieces of evidence presented in the literature that suggest a potential crosstalk between PARP1 and NF-κB. For example, our group had previously observed that both NF-κB activity and signaling were decreased after PARPi treatment (32). PARP1 has been previously reported to act as a co-activator of NF-κB in inflammatory disorders(4,41). PARP1's expression, and not enzymatic activity was also

determined to be required for NF-κB-mediated transcription. Conversely, PARP1's enzymatic activity is essential for the DNA binding ability of the p65 subunit and the activation of NF-κB, after irradiation (42). Furthermore, DNA damage-induced NF-κB activation is triggered by PARP1-mediated PAR formation. In detail, PARylation recruits the NEMO (a regulatory subunit of the NF-κB signaling pathway), PIASγ, and ATM to DNA strand breaks. ATM and PIASγ activate the NF-κB signaling pathway by SUMOylating the NEMO subunit (43).

Canonical NF-κB signaling pathway

The NF-κB signaling pathway mediates cellular proliferation, resistance to apoptosis, angiogenesis, invasion and metastasis in cancer cells. This family consists of five transcription factors: p65, p50, p52, c-REL, and RELB. Dimerization, nuclear-localization, and DNA-binding domain are three conserved domains amongst all five transcription factors, including(44). The canonical pathway is activated by numerous ligands that bind to a specific receptor on the cell membrane. One classic ligand that initiates this pathway is tumor necrosis factor alpha (TNFα) and it serves as a great model to illustrate NF-κB activation(45). Specifically, the TNFα binds to the Tumor necrosis factor receptor 1 (TNFR1); subsequently, leading to the recruitment of several adaptor proteins to TNFR1. TRADD, FADD, TRAF2, and RIP1 are the adaptor proteins that are recruited to the TNFR1 receptor. The highly regulated IκB kinase complex (IKK) is then recruited, phosphorylated and activated by the ubiquinated RIP1 protein This complex consists of three subunits. Two of these subunits are catalytic: IKKα and IKKβ, and NEMO (IKKγ). is a regulatory subunit. Once IKK is phosphorylated, it will

phosphorylate the endogenous NF-κB inhibitor called IκBα. The IκBα protein binds to and inactivates NF-κB complex in the cytoplasm, by masking the nuclear localization sequences of NF-κB transcription factors (i.e. p65-p50). Next, IκBα is poly-ubiquitinated and then degraded by a proteasome. This event triggers the release of the p65-p50 dimers from IκBα. The dimers are then allowed to freely translocate to the nucleus to bind κB sites found on promoters of their target genes, such as *IL-8* (45-47). This NF-κB canonical pathway is demonstrated in Figure 1.4.

Crosstalk between PARP1 and HER2

The expression of PARP1 is enhanced in both basal and HER2+ breast tumors compared to their normal counterparts and suggest a possible crosstalk between PARP1 and HER2 (3,26). The mechanisms behind these observations are yet to be studied. In this dissertation, we elucidate the mechanism behind increased PARP1 levels in HER2+ breast cancer tumors. We hypothesize that PARP1 expression is regulated at the post-transcriptional level by a microRNA and these findings are discussed further in Chapter 2.

MicroRNA

MicroRNAs (miRNAs) are short non-coding RNAs about 20-22 nucleotides long. These RNA molecules can modulate genes involved in cell proliferation, differentiation, survival or motility (48). Specifically, they regulate expression at the post-transcriptional level by frequently binding to the 3' UTR (untranslated regions) of their target genes.

However, miRNA target prediction databases have also shown that they can bind to the 5'UTR or the open reading frame (ORF) (49). The seed region of the miRNA (positions 2-8) can also bind completely or partially to complementary mRNAs; subsequently, inhibiting translation or promoting mRNA degradation. One miRNA can also target multiple mRNAs, due to partial complementary binding. Multiple miRNAs can also cooperate with one another to regulate gene expression.

Biogenesis of miRNAs involves multiple steps and two maturation phases in both the nucleus and cytoplasm. MiRNAs are transcribed in the nucleus by RNA polymerase II into primary miRNA (pri-miRNA) transcripts. The microprocessor complex consisting of Dicer and Dgcr8 cleaves the miRNA into a 70-nucleotide hairpin-like pre-miRNAs. The pre-miRNA is then transported into the cytoplasm where it undergoes a second cleavage into a 22-nucleotide miRNA by a Dicer protein. After this final cleavage step, one strand of the mature miRNA is incorporated into a protein complex called the RNA-induced silencing complex (RISC). This strand also guides RISC to its target mRNA and ultimately cleaves mRNA or inhibits translation (50).

MicroRNA and cancer

The expression levels of miRNAs are altered in many different cancers. This was first discovered in chronic lymphocytic leukemia. Scientists showed that miRNA-15 and -16 were deleted at one specific region on chromosome 13, which confirmed susceptibility to this cancer (51). MiRNAs can function as oncogenes or tumor suppressors. An association between miRNAs and HER2+ breast cancer also exists. Studies have reported that expression levels of various miRNAs are altered among the

different subtypes of breast cancer, specifically HER2+ breast cancer. These miRNAs can be upregulated by HER2, such as mir-21. The upregulation of this miRNA resulted in the downregulation of an important suppressor protein involved in metastasis called PDCD4 (programmed cell death 4) (52). HER2 has also been shown to downregulate miRNA expression, for instance mir-205 (53). Conversely, multiple miRNAs have been reported to regulate the expression levels of all four HER tyrosine kinase receptors. For instance, estrogen regulated miRNA-125b downregulates HER2 expression in luminal A breast cancer patients (54). Further, miRNAs can modulate response or resistance to HER2 targeted therapy. For example, miRNA-221 was observed to be upregulated in HER2+ breast cancer and promoted trastuzumab resistance by directly binding to and repressing PTEN levels (55). MiRNAs are easily detectable both in the tissue or blood and can potentially be used as biomarkers. In the future, we hope to identify miRNA that could be possible biomarkers for PARPi treatment.

Let-7a

A gene expression analysis system used in one of our experiments revealed that the *let-7a* miRNA is expressed at lower levels in HER2 overexpressing breast cancer cells as compared to HER2- breast cancer cells (56). This correlation has also been observed by others and supports our results (56). These miRNAs are also expressed in cancer stem cells. *Let-7a* was one of the first miRNAs that was discovered in *C.elegans*. It was shown to regulate the life cycle of *C.elegans* from the fourth larval stage into the adult stage (57). The consensus sequence of *let-7* family is highly conserved among different species. There are nine different *let-7* family members that exist in humans, including *let-7a, -7b, -7c, -7d, -7e, -7f, -7g, -7i, and mir-98.* These family members also

have different sequences. However, nematodes and flies consist of one *let-7* family member. Further, the family members are encoded on chromosomes 3, 9, 11, 12 19, 21, 22, and X. The *let-7a* family member is also expressed on different chromosomes but contains the same sequence and is represented by numbers *(let-7a-1, let-7a-2,* and *let-7a-3). Let-7* members can be located on a chromosome by itself or in clusters with other *let-7* family members or miRNAs. The *let-7* family represses multiple genes such as *RAS*, *cyclin A, cyclin D*, and a transcription factor called c-*myc (58).*

Crosstalk between NF-κB and HER2

It has also been shown that the overexpression of HER2 activates the NF-κB signaling pathway, suggesting that the oncogenic signals driven by HER2 go through the NF-κB pathway(59). The canonical pathway activates the NF-κB signaling pathway by the HER2 oncoprotein. This activation was observed to be primarily dependent on the IKKα instead of the dominant subunit IKKβ, found in the canonical NF-κB signaling pathway. Knockdown of IKKα via an siRNA also decreased the expression of IL-8 and IL-6, two NF-κB regulated genes(60). NF-κB activation has been reported to be essential for mammary carcinogenesis in the HER2+ mouse model where IKKα kinase activation was required. This group observed that wildtype IKKα mice developed tumors 13 weeks earlier than $IKK^{AA/AA}$ mutant mice (61). Interestingly, our preliminary data reveal increased PARP1 and phosphorylated p65 protein levels in HER2+ as compared to HER2- breast cancer tissue specimens (3). However, the mechanism behind the crosstalk between PARP1 and NF-κB in HER2+ breast tumors is still unknown. We hypothesize that cytotoxicity induced by inhibition of PARP1 may be, in part, due to inhibition of

PARP1's role as a co-activator of NF-κB signaling in HER2+ breast cancer cell lines and subsequent reduction of key NF-κB regulated genes involved in tumorigenesis. This mechanism is further investigated in chapter 3.

In summary the HER2 targeted agent, trastuzumab, has increased the survival rate in this subtype of breast cancer but resistance eventually occurs in metastatic HER2+ breast cancer tumors. We have previously reported that in HER2+ breast cancer cells, attenuation of PARP-1 via chemical inhibition suppresses NF-κB transactivation and induces cellular cytotoxicity. In the proceeding chapters we will describe the use of PARPi as an alternative treatment strategy for HER2+ trastuzumab resistant breast tumors. We will also further discuss the function of a microRNA in the regulation of PARP1 levels as well as the role of PARP1 expression on NF-κB mediated transcription in HER2+ breast cancer cell lines.

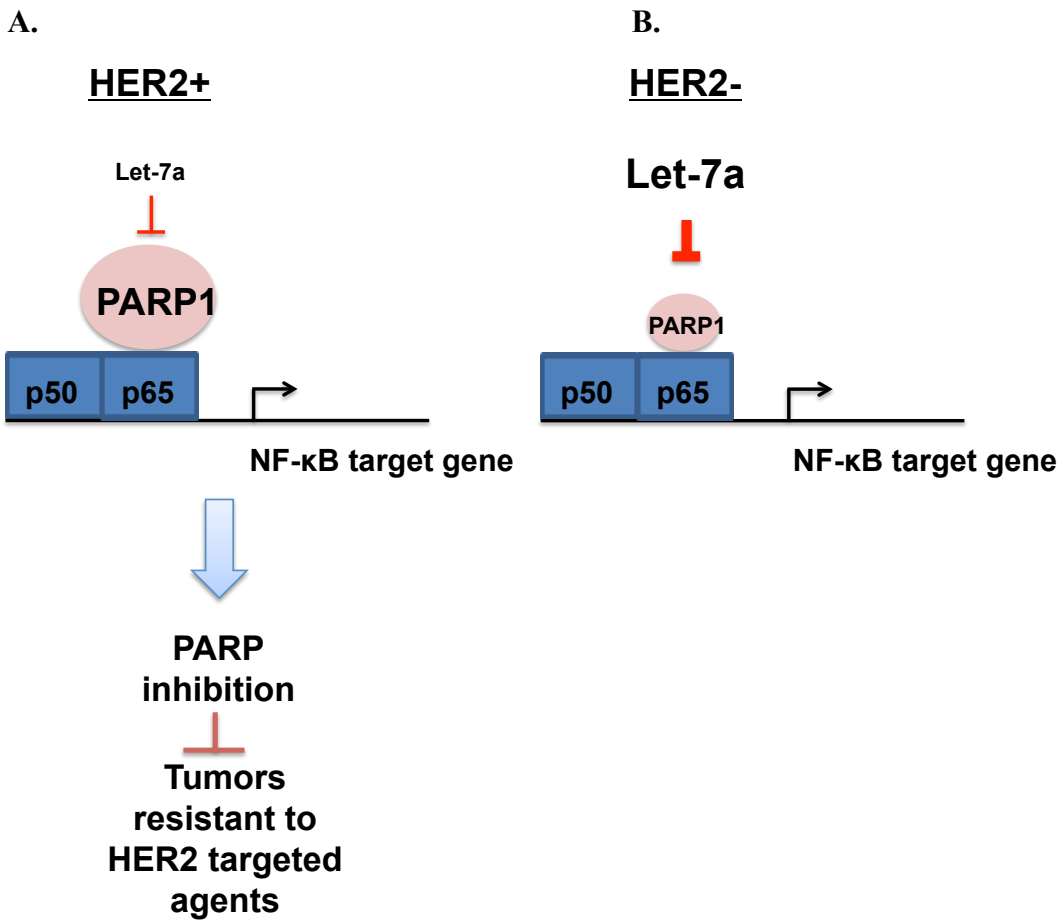

Figure 1.1 An illustrative figure depicting the interplay between HER2, PARP1, and NF-κB signaling pathways in HER2+ and HER2- breast cancer cells.

(A) HER2+ breast cancer cells have low levels of the let-7a microRNA and elevated PARP1 protein expression. These elevated levels of PARP1 enhance NF-κB mediated transcription in HER2+ breast cancer cells. PARPi disrupts this interplay in the HER2+ parental and trastuzumab resistant breast cancer cells and induces cytotoxicity. (B) In HER2- breast cancer cells, PARP1 protein expression is downregulated by increased levels of let-7a.

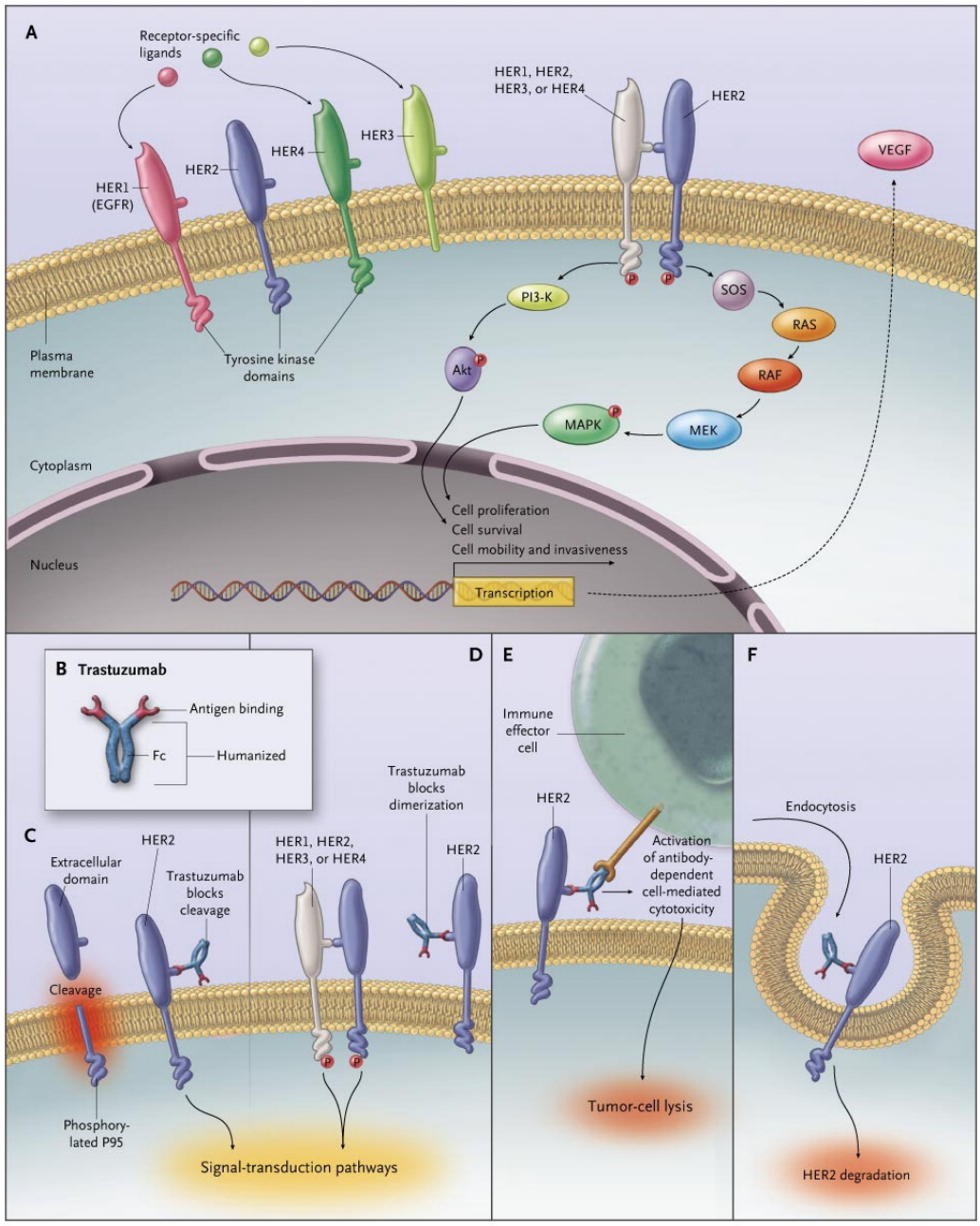

Figure 1.2 Human epidermal growth factor receptor (HER) family and the mechanisms of action of trastuzumab (Herceptin).

(A) EGFR/HER1, HER2, HER3, and HER4 are the four receptors included in the HER family of tyrosine kinase receptors. The receptors can homo- or hetero-dimerize allowing for the activation of two downstream signaling pathways, including: the P13K and MAPK pathway. (B-F) Trastuzumab is a humanized monoclonal antibody against HER2 and has several mechanisms of action, including: blocking dimerization, activating the

antibody dependent cell-mediated cytotoxicity (ADCC), and endocytosis of the HER2 receptor subsequently leading to the degradation of HER2.

From "Trastuzumab-Mechanism of Action and Use in Clinical Practice"

by C.A. Hudis, 2007, *The New England Journal of Medicine, 357,* pp.39-51.

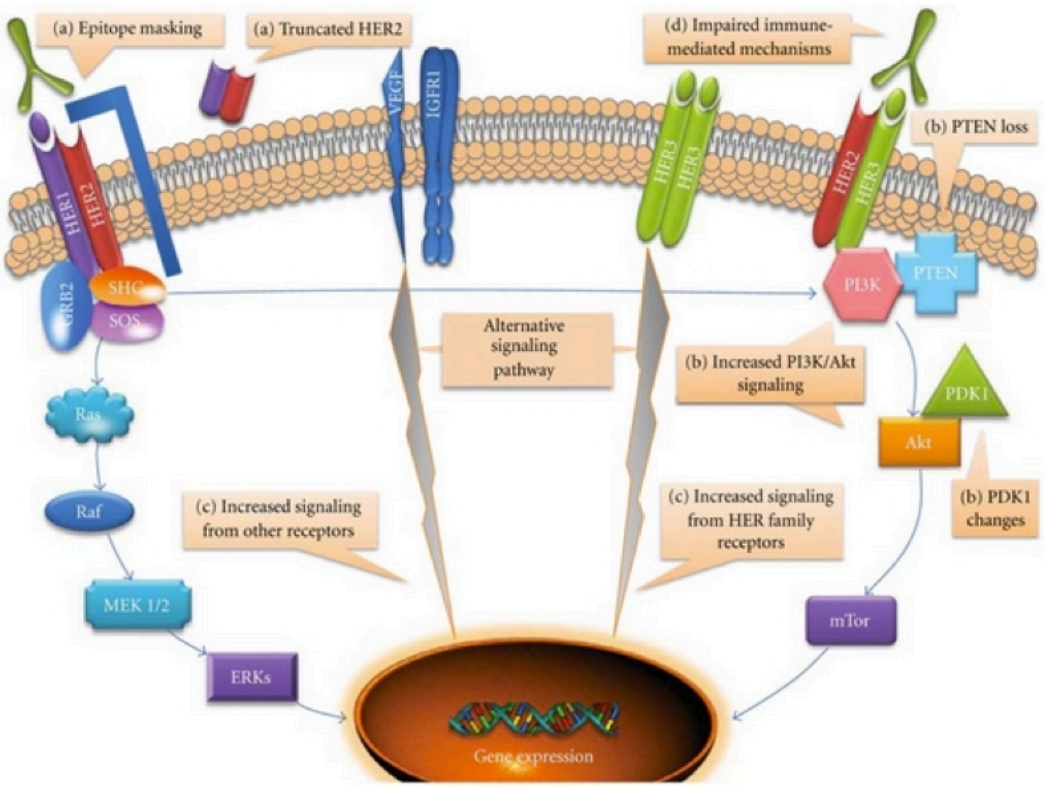

Figure 1.3 Trastuzumab Resistant Mechanisms

(A) Trastuzumab is blocked from binding to the extracellular domain of HER2 because of a truncated HER2 receptor (p95Her2) or epitope masking by different proteins such as Mucin 4 (MUC4). (B) Mutations in HER2 downstream signaling pathways such the P13K pathway cause resistance to trastuzumab. (C) Augmented levels of other HER receptors and ligands impede trastuzumab. (D) Polymorphism in the Fcγ receptor block ADCC response and suggest that impaired immune-mediated mechanism could reduce the effect of trastuzumab.

From "Molecular Mechanisms of Trastuzumab Resistance in HER2 Overexpressing Breast Cancer"
by G.L. Fiszman and M.A. Jasnis, 2011, *International Journal of Breast Cancer, 2011,* p.11

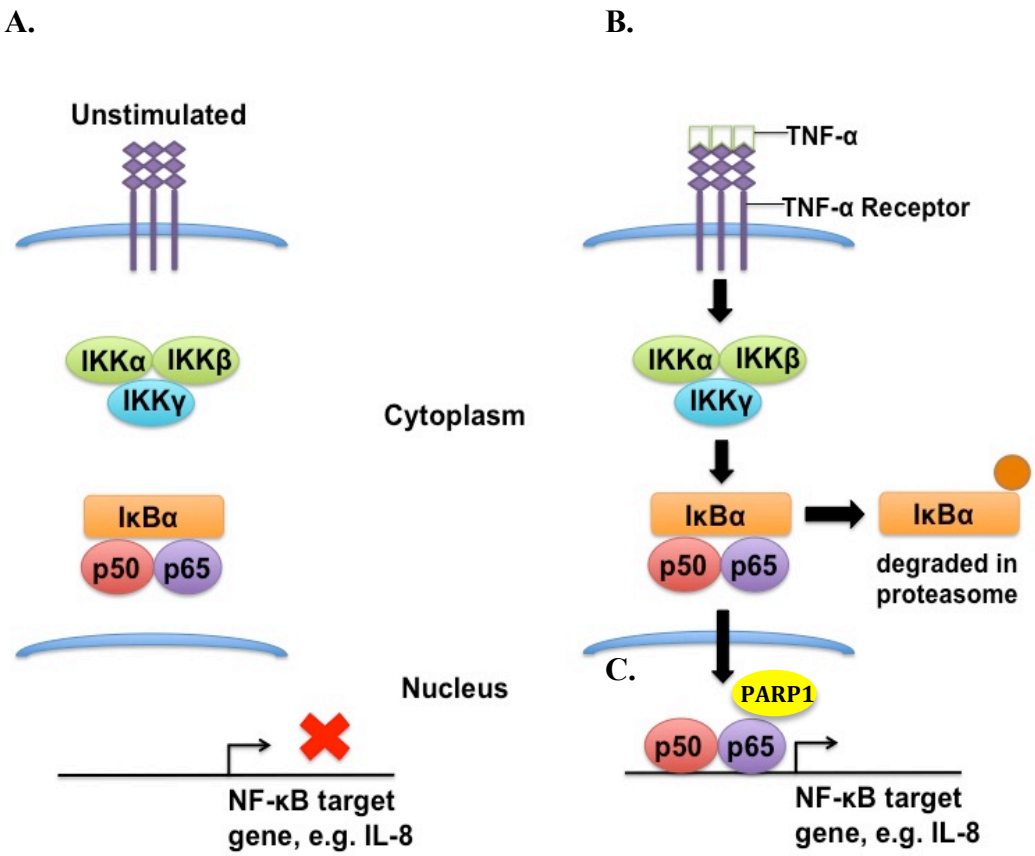

Figure 1.4 Canonical NF-κB signaling pathway

(A) The NF-κB subunits (p65 and p50) are sequestered in the cytoplasm by an endogenous inhibitor called IκBα. (B) The NF-κB pathway is activated by a ligand such as IL-8. The IκBα protein is degraded by a proteasome following the activation and phosphorylation of the IκB kinase complex (IKK). The bound NF-κB dimers are released from IκBα and then allowed to bind to the promoters of targeted genes, such as IL-8. (C) PARP1 has been previously shown to act as a co-activator of the p65 subunit to ultimately influence NF-κB mediated transcription.

REDUCED LET-7A MICRORNA LEVELS INCREASE PARP1 PROTEIN EXPRESSION IN HER2-OVEREXPRESSING BREAST TUMORS

by

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CHAPTER 2

REDUCED LET-7A MICRORNA LEVELS INCREASE PARP1 PROTEIN EXPRESSION IN HER2-OVEREXPRESSING BREAST TUMORS

ABSTRACT

We previously reported that HER2+ breast tumors express elevated levels of PARP1 protein. In this study, we examined the mechanism by which PARP1 is upregulated in HER2+ breast cancers. Knockdown of HER2 in HER2+ breast cancer cell lines resulted in a reduction in PARP1 protein levels. Conversely, ectopic overexpression of HER2 in a non-HER2 overexpressing cell line resulted in an increase in PARP1 protein levels. Alterations in HER2 expression had no significant effect on PARP1 mRNA levels. Instead, we found that HER2 status was inversely correlated with let-7a microRNA (miRNA) levels in breast cancer cell lines. Mechanistic studies revealed that ectopic expression of let-7a miRNA downregulated PARP1 protein levels while expression of the let-7a anti-miRNA increased PARP1 protein. Further, luciferase assays implicate let-7a in regulating PARP1 via its 3'UTR. Importantly, in human breast tumors, let-7a was expressed at significantly lower levels in human HER2+ breast tumors compared to HER2- breast tumors and inversely correlated with PARP1 protein levels. Lastly, HER2+ breast cancer cells exhibited similar cytotoxicity to ectopic let-7a expression as the PARP inhibitor veliparib (ABT-888). Collectively these results suggest that increased PARP1 expression in HER2+ breast cancers is regulated by the let-7a miRNA, and let-7a may be a potential strategy to suppress PARP1 activity.

Introduction

Poly (ADP-ribose) polymerase inhibitors (PARPi) are a novel targeted therapy for homologous recombination (HR) deficient tumors, and are extremely well tolerated (1). These agents target PARP1, an enzyme that has multiple cellular functions (2). Its most studied roles include DNA repair and transcription (3,4). PARP1 is also overexpressed in many cancer cells compared to normal cells (5).

We previously reported that ectopic overexpression of HER2 itself was sufficient to confer susceptibility to PARPi while HER2 knockdown in HER2+ breast cancer cells induced resistance to PARPi (6). Our laboratory also recently reported that PARP1 protein expression is elevated in HER2+ human breast tumor tissues as compared to HER2- breast tumor tissues (7). However, the mechanism behind the increased PARP1 expression in this particular breast cancer subtype is unknown.

Recently, multiple studies have shown that microRNAs (miRNAs) regulate gene expression at the post-transcriptional level (8). These short non-coding RNAs repress protein synthesis by binding to mRNA to inhibit translation or promote their degradation (9). The expression levels of several miRNAs are also altered in HER2-overexpressing breast carcinomas and can be up- or down-regulated by HER2 (10,11). A recent study demonstrated that PARP1 is targeted by miR-223 in Barrett's esophagus-associated esophageal adenocarcinoma (12).

In this study, we report for the first time that elevated HER2 expression correlated with reduced let-7a levels and enhanced PARP1 protein expression. Ectopic expression of the let-7a miRNA downregulated PARP1 protein levels while expression of the let-7a

anti-miRNA increased PARP1 protein. Further, luciferase assays implicate let-7a in regulating PARP1 via its 3'UTR. Importantly, in human breast tumors, let-7a was expressed at significantly lower levels in human HER2+ breast tumors compared to HER2- breast tumors and inversely correlated with PARP1 protein levels. Lastly, HER2+ breast cancer cells exhibited similar cytotoxicity to ectopic let-7a expression as the PARP inhibitor veliparib (ABT-888). Altogether, these data suggested that a novel connection between let-7a and PARP1 exists in human breast cancer cells, and altering this association may have therapeutic potential in HER2+ breast cancer patients.

Materials and Methods

Cell culture

The HER2+ breast cancer cell lines BT-474 and SKBR3 were generously donated by Dr. Donald Buchsbaum (University of Alabama at Birmingham). The BT-474 was maintained in RPMI medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 10 μg/mL insulin. SKBR3 were grown in McCoy's 5A medium supplemented with 15% FBS. MCF7 were maintained in EMEM medium supplemented with 10% FBS and 10 μg/mL insulin.

HER2 stable cell lines

MDA-MB-231 cells were transfected with 4 μg of the pcDNA3-HER2-WT plasmid (Addgene plasmid 16257) (231 HER2) or pcDNA3-vec (231 NEO) using the Lipofectamine 2000 regent. Briefly, 250,000 cells were seeded in a 6 well dish and then transfected the following day. Cells were selected and grown in selection media with 700 μg/mL G418 (Mediatech, Inc., catalog #: 30-234-CR). Individual G418 resistant-clones were then picked and analyzed for HER2 expression via Western blot analysis. The clones were then expanded and maintained in DMEM with 700 μg/mL G418.

Reagents

Control siRNA (Cell Signaling Technology, catalog # 6568) and HER2 siRNA II (Cell Signaling Technology, catalog # 6283) were purchased from Cell Signaling. Let-7a mimics, let-7a antagomiRs, and negative control miRNAs were purchased from Ambion/Life Technologies. For let-7a mimic studies, we transfected 40,000 cells plated

in a 6 well dish with hsa-let-7a-5p (Thermo Fisher, catalog #: AM17100, ID: PM10050) as well as its Negative Control #1 (Thermo Fisher, catalog #: AM17110). The let-7a antagomiR used in this study was anti-hsa-let-7a-5p (Thermo Fisher, catalog #: AM17000, ID: PM10050) as well as its Negative Control #1 (Thermo Fisher, catalog #: AM17010). Both siRNA and microRNA constructs were transfected with the Lipofectamine RNAiMAX reagent (Thermo Fisher, catalog # 13778150), according to the manufacturer-supplied protocol. Co-transfections were performed with the Lipofectamine 2000 reagent (Thermo Fisher, catalog #: 11668019).

Western Blot

Cell lysates were harvested in MPER buffer containing phosphatase and protease inhibitors. Approximately 10-25 μg of protein was separated on polyacrylamide SDS-page gels and then transferred to PVDF membranes, as previously described (13). The following are the primary antibodies used in this study: anti-human HER2 (2165S; Cell Signaling, Danvers, MA, USA), PARP1 (9532S; Cell Signaling, Danvers, MA, USA), PARP2 (ab176330; Abcam, Cambridge, MA, USA), c-Myc (5605S; Cell Signaling, Danvers, MA, USA), and β-actin HRP (sc-47778 HRP; Santa Cruz, Dallas, TX, USA). The secondary antibody used in this study was the rabbit horseradish peroxidase-conjugated (sc-2004; Santa Cruz, Dallas, TX, USA).

Luciferase assay

The PARP1 3'-UTR clone (SC209859, OriGene Technologies, Rockville, MD, USA) or the vector control (PS100062, OriGene Technologies, Rockville, MD, USA) were co-

transfected with the let-7a mimic or let-7a antagomiR along with their respective controls. Forty-eight hours after transfection luciferase expression was measured using the britelite™ Plus system (6066769, Perkin Elmer, Waltham, MA, USA) and the TopCount NXT luminescence counter.

RNA extraction and real-time RT-PCR

Total RNA was isolated from cell lines using the PureLink RNA Mini Kit (12183018A; Thermo Fisher Scientific, Waltham, MA, USA) or from FFPE slides using the High Pure FFPET RNA isolation kit (06650775001; Roche Applied Science, Penzberg, Germany). A few RNA samples were cleaned using the Zymo RNA Clean and Concentrator kit (R1015; Zymo Research, Irvine, CA, USA). The *mir*Vana miRNA Isolation kit with phenol (AM1560; Thermo Fisher Scientific, Waltham, MA, USA) was used to extract miRNAs from cell lines.

cDNA was generated using the SuperScript III First-Strand Synthesis System kit (Invitrogen; catalog # 18080-051) or the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific; catalog #4366596). Real-time PCR was done with TaqMan primer probes for PARP1, HER2, and hsa-let-7a. GAPDH or U6 primers were used as the endogenous controls and relative gene expression levels were calculated using the following formulas: $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$. Expression levels of 800 miRNAs were measured using the Human v3 nCounter miRNA Panel in the 231 NEO and HER2 cell lines, as previously described (13).

Patient samples

The Institutional Review Board at University of Alabama at Birmingham approved all the protocols and procedures used on patient samples, before this study was initiated (IRB #: X101214005). The patient and tumor features of the UAB patients used in this study are thoroughly described in our recent paper (7). Briefly, HER2- patient tissues with low PARP1 levels and HER2+ patient tissues with high PARP1 levels were obtained from the UAB Department of Pathology. Tumor tissues were demarcated by a UAB pathologist and then total RNA was extracted from these tissues. Let-7a gene expression levels were measured via qRT-PCR analysis and calculated using the following formula: $2^{-\Delta Ct}$.

Cell Proliferation

Cell proliferation experiments were used to examine the therapeutic potential of let-7a. Briefly, 1.0×10^5 cells per well were plated in a 6-well plate. Cells were then transfected with the let-7a mimic. Twenty-fours hours after transfection, the cells were harvested and then re-plated in a 24-well plate. The following day, cells were treated with DMSO or 10μM of ABT-888. Following four days of treatment, the cells were removed by trypsin and counted using a cell counter (Beckman Coulter, Fullerton, CA).

Statistical analysis

Our data was analyzed using the one-way Anova followed by a Bonferroni post-test or the two-tailed, student t-test using GraphPad Prism version 4.02 (GraphPad Software). Correlation between let-7a gene expression and PARP1 protein expression levels were assessed using a two-tailed, nonparametric Spearman's correlation test. Data were

considered significant if $p<0.05$ and presented as average ± standard error of mean (SEM).

Results

PARP1 protein expression positively correlates with HER2 in breast cancer cells.

We previously reported a significant correlation between HER2 and PARP1 protein expression in human breast tumor samples (7). To examine the effect of altering HER2 expression on PARP1 protein levels, we silenced HER2 via HER2 siRNA in two HER2 overexpressing breast cells lines, BT-474 and SKBR3. We observed that HER2 knockdown decreased PARP1 protein (Figure 1A-B). We also generated 231 HER2 cells, which are MDA-MB-231 cells (a non-HER2 overexpressing cell line) that overexpress HER2. In contrast to HER2 silencing, transient HER2 overexpression alone resulted in elevated protein levels of PARP1 as compared to cells transfected with the vector alone (Figure 1C). Similar results were also observed after stable HER2 overexpression (Figure 1D). PARP2 levels were not significantly altered after HER2 overexpression or knockdown (Figure 1A-D). All together, these data suggest that HER2 protein levels positively correlate with and may regulate PARP1 levels.

Let-7a expression inversely correlates with HER2 status

Previously, we found that PARP1 mRNA expression was increased in HER2+ breast cancer patients from the TCGA PAM-50 breast cancer data set (7). To address whether that HER2 could be regulating PARP1 at the transcriptional level, we suppressed *HER2* levels via a HER2 siRNA in the BT-474 and SKBR3 breast cancer cells and then

measured *PARP1* mRNA levels. We observed that *PARP1* mRNA expression was not significantly altered after HER2 knockdown (Supplementary Figure S1).

Next, we evaluated whether PARP1 levels were regulated at the post-transcriptional level via a miRNA. To identify miRNAs that are differentially expressed in HER2+ versus HER2- breast cancers, we compared expression levels of 800 miRNAs simultaneously using the NanoString nCounter system in 231 NEO compared to 231 HER2 breast cell lines. We observed that miR-223, which was previously shown to regulate PARP1 levels in esophageal adenocarcinoma, was not significantly altered between these two breast cancer cells (Supplementary Table S1). However, the expression levels of the let-7 family of miRNAs were significantly decreased in the 231 HER2 versus the 231 NEO cells (Table 1). One of the most significantly altered miRNA from this family was let-7a, which was reduced by 2 fold in 231 HER2 compared to its isogenic control 231 NEO (Table 1). These results were also validated via qRT-PCR analysis (Figure 2A). Further, let-7a levels also negatively correlated with HER2 and PARP1 status in human breast cancer cell lines MCF7, BT-474 and SKBR3 (Figure 2B-C).

Let-7a regulates PARP1 protein

Having observed a difference in expression levels of let-7a between HER2- versus HER2+ breast cancer cell lines, we hypothesized that diminished let-7a may be one mechanism for the elevated PARP1 expression observed in HER2+ breast cancer cells. To test this hypothesis, we first investigated the effects of let-7a on PARP1 protein. As shown in Figure 3A-C, ectopic expression of let-7a reduced the levels of PARP1

protein in all three HER2 overexpressing cell lines as compared to cells treated with the negative control miRNA (neg control). We also validated these results by assessing the effect of the let-7a mimic on the expression of c-myc, a known target of let-7a. Ectopic let-7a expression decreased the protein levels of c-myc in the BT-474 cell line (Supplementary Figure S2A) (14). Conversely, inhibiting the expression of let-7a using an anti-let-7a miRNA in the 231 NEO or MCF7 cell lines increased the levels of the PARP1 protein (Figure 3D and Supplementary Figure S2B). PARP2 levels were not altered in the BT-474, SKBR3, 231 HER2, and 231 NEO cells lines (Figure 3A-D).

To investigate the direct role of let-7a in regulating PARP1 levels, we assessed the effects of let-7a at the PARP1 3'-UTR. Let-7a had a high predicted binding score to the 3' UTR of PARP1, according to the microRNA.org database (Figure 4A). To validate direct effects of let-7a on PARP1 3' UTR, a let-7a mimic was co-transfected with the 3' UTR of PARP1 downstream of a firefly luciferase gene in HER2 overexpressing cell lines. Following 48 hours of transfection, firefly luciferase activity was decreased in the BT-474 and 231 HER2 cell line transfected with let-7a (Figure 4B-C). In contrast, co-transfection with the anti-let-7a miRNA and 3'UTR of PARP1 in the 231 NEO cells increased luciferase activity (Figure 4D). Together, these data suggest that let-7a directly targets PARP1 in breast cancer cells.

Inverse relationship between let-7a and PARP1 levels in breast cancer patients

To further validate let-7a as a regulator of PARP1 protein expression, we evaluated let-7a expression in patient breast tumors by qRT-PCR analysis. We observed a significant decrease in let-7a expression in HER2+ breast cancer patients, while higher

levels of let-7a were observed in HER2- breast cancer patients (Figure 5A). Moreover, PARP1 expression was elevated in HER2+ patients with low let-7a expression and decreased in HER2- patients with high let-7a expression (Figure 5B). Spearman's test was used to determine the association between the expression of PARP1 and let-7a expression. As shown in Figure 5B a significant inverse relationship between PARP1 and let-7a expression was observed (r_s=-0.6937, p=0.002). Collectively, these results suggest that there is an inverse correlation between let-7a and PARP1 expression in human breast tumor samples.

Let-7a reduces cell viability of HER2+ breast cancer cells

We previously reported that HER2+ breast cancer cells were sensitive to PARP inhibitors (PARPi) or PARP1 siRNA. Because let-7a suppresses PARP1 levels, we next examined whether ectopic let-7a expression could alter cell viability by diminishing PARP1 expression in HER2+ breast cancer cell lines. In both the BT-474 and 231 HER2 cells, transient transfection of let-7a significantly reduced cell proliferation to a similar extent as control transfected cells treated with 10μM of the PARP inhibitor, ABT-888 (Figure 6A-B). These results demonstrate let-7a is as efficacious as ABT-888 at reducing cell proliferation.

Discussion

Multiple studies have recently focused on determining the clinical significance of PARP1 gene and protein expression in breast cancers (5,15). We previously reported that PARP1 protein expression was elevated in HER2+ breast tumors as compared to luminal breast tumors (7). Additionally, Rojo et al. observed that the nuclear PARP1 protein was overexpressed in HER2+ breast cancers and correlated with worse outcomes (16). The

purpose of this study was to investigate the mechanism behind elevated PARP1 protein levels in the HER2+ breast cancer subtype, a concept that has been left unexplained in many studies. Here, we demonstrated that HER2 levels positively correlate with PARP1 protein expression through suppression of the let-7a miRNA.

To better understand the mechanism behind PARP1 upregulation, we examined whether PARP1 expression was regulated at either the transcriptional or post-transcriptional level. We observed that HER2 knockdown did not affect *PARP1* mRNA levels, suggesting that regulation may not be occurring at the transcriptional level. We previously reported that *PARP1* mRNA expression was altered in the 24% of 58 patients with HER2 enriched breast cancer from the TCGA PAM50 data set (7). A possible explanation behind these contradicting results could be due to the cellular heterogeneity observed in patient tumor samples versus the cellular homogeneity created with passaging established cell lines (17). Furthermore, HER2 regulation of PARP1 levels is likely multifactoria, which is reflected by the tissue results and not fully tested in our cell line models. We are currently investigating the possible factors that differ between both model systems that could produce the differences in mRNA expression. Nevertheless, alterations observed in PARP1 protein expression were consistent in both our cell lines and human tissues.

Next, due to the emerging role of miRNA in gene regulation and altered expression in cancer cells, we examined whether miRNAs might regulate PARP1 expression. Nanostring miRNA profiling revealed differential regulation of the let-7 family. We focused on one of the let-7 family members, whose expression was significantly lower in HER2+ breast cancer cell lines. We found an inverse correlation

between PARP1 and let-7a expression levels in human breast tumor samples. This is consistent with another study where HER2+ breast cancers were found to express low levels of the let-7a miRNA compared to normal breast tissue samples (18). However, we also discovered that let-7a expression from two HER2- and one HER2+ breast cancer patients did not act in accordance with this trend. These results exemplify that breast cancer is a heterogeneous disease and patients within the same subtype can also exhibit differences in molecular expression. The data also emphasize the importance of personalized medicine in the field of breast cancer research. Altogether, our research suggests that specifically HER2+ breast cancer tumors with elevated PARP1 protein and low expression levels of let-7a may benefit from PARPi versus the complete HER2+ subtype of breast cancer.

The mechanism by which HER2 inhibits let-7a remains to be deciphered. One possible mechanism is that Lin28A or Lin28B, repressors of let-7a expression, may be altered in HER2+ breast cancer tumors. Interestingly, one study detected an increase in Lin28A in HER2+ breast tumors and an increase in Lin28B in triple negative breast tumors (18). Another study showed that Lin28B inhibited let-7a gene expression levels and resulted in elevated levels of IL-6 and ultimately STAT3 (19). A second possible mechanism is c-myc transcriptionally represses let-7a levels (20). Our future studies are working to address this mechanism.

Nanostring analysis of microRNA expression also identified other let-7 family members. These let-7 family members also have high binding scores to the 3'UTR of PARP1 (data not shown). Therefore, we cannot exclude these miRNAs as essential factors in the regulation of PARP1 expression.

Importantly, ectopic let-7a expression decreased cell proliferation to the same extent as ABT-888. Recent evidence suggests that PARP trapping is a mechanism of PARPi cytotoxicity (21,22). Because let-7a reduces PARP1 levels, these results suggest that cytotoxicity may be independent of a PARP1 trapping mechanism in HER2+ breast cancer cells but due to alternative functions of PARP1, such as its role in coactivation of transcription factors (6,23).

As more PARP inhibitors are being tested in the clinical setting, there seems to be an enhanced interest in the development of new predictive biomarkers for these targeted agents. Ultimately, let-7a or PARP1 could be potential biomarkers for PARPi therapy and should be investigated further in other preclinical and clinical studies. In summary, we are the first group to correlate let-7a and PARP1 expression with HER2 status to ultimately decipher the mechanism behind elevated PARP1 protein expression observed in many studies.

Disclosure of Potential Conflicts of Interest

Eddy Yang has served on the advisory board of NanoString Technologies and has received honorarium from them. He also has a Materials Transfer Agreement with AbbVie, Inc. The other authors have no conflicts of interest to disclose.

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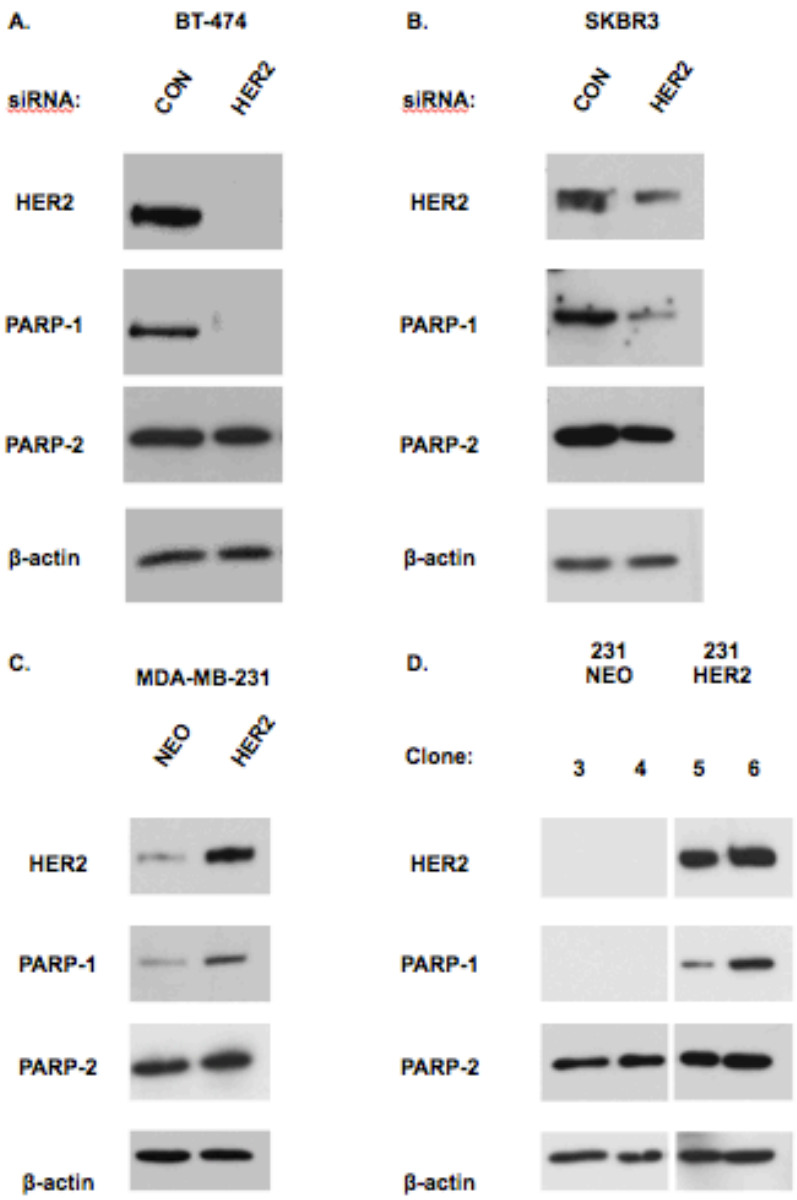


Figure 1. Altering HER2 expression changes PARP1 protein levels but not PARP2 protein levels.

(A, B) Cells were transfected with HER2 or control (CON) siRNA for 72 hours and then analyzed by Western blot analysis for HER2, PARP1, and PARP2 protein levels. β-actin was used as a loading control. MDA-MB-231 cells that (C) transiently or (D) stably express the HER2 vector (231 HER2) as compared to cells expressing the control plasmid (231 NEO) were subjected to Western blot analysis for HER2, PARP1, PARP2, and β-actin. Data shown are representative immmunoblots from one of two independent experiments.

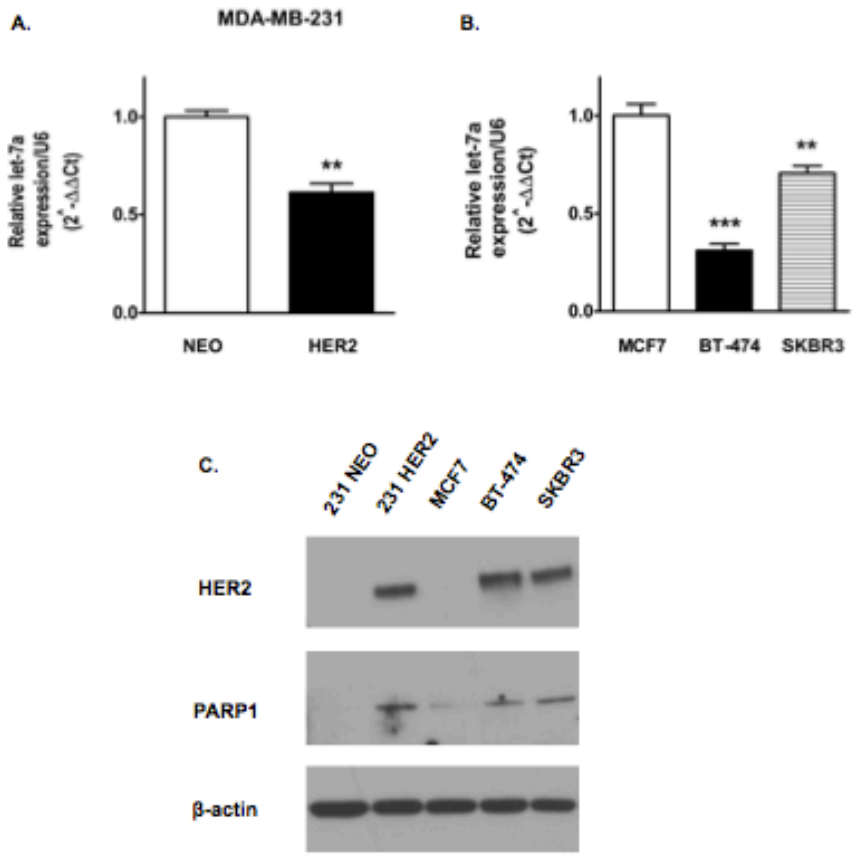


Figure 2. Let-7a levels are inversely correlated with HER2 status in breast cancer cells.

Ten nanograms of isolated miRNA from 70% confluent (A) 231 NEO and 231 HER2 or (B) MCF7, BT-474, and SKBR3 human breast cancer cells were reverse transcribed to cDNA and then analyzed by qRT-PCR for let-7a and U6 expression. The figures are representative from (A, B) one of two independent experiments performed in triplicate. A t-test or one-way ANOVA test was performed. (C) Western blot analysis of HER2, PARP1, PARP2 and β-actin levels in untreated 231 NEO, 231 HER2, MCF7, BT-474, and SKBR3 cell lines. **$p<0.01$ and ***$p<0.001$.

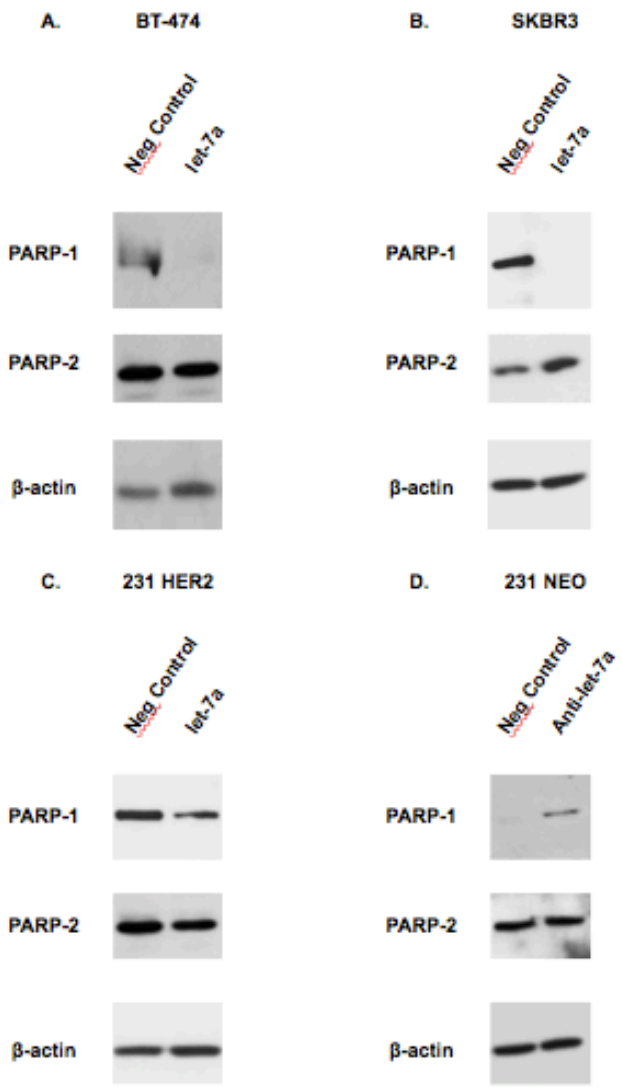


Figure 3. Let-7a regulates PARP1 expression in human breast cancer cells.

(A-C) BT-474, SKBR3 and 231 HER2 cells were transiently transfected with a let-7a miRNA (hsa-let-7a-5p) or its neg control miRNA (negative control). Following transfection, cells were analyzed by Western blot analysis for PARP-1, PARP-2, and β-actin. (D) The 231 NEO cell line was transiently transfected with an anti-let-7a miRNA (let-7a antagomiR) or a neg control miRNA (negative control). Following transfection, cells were analyzed by Western blot analysis for PARP-1, PARP-2, and β-actin. Results shown are from one of two independent experiments performed duplicate.

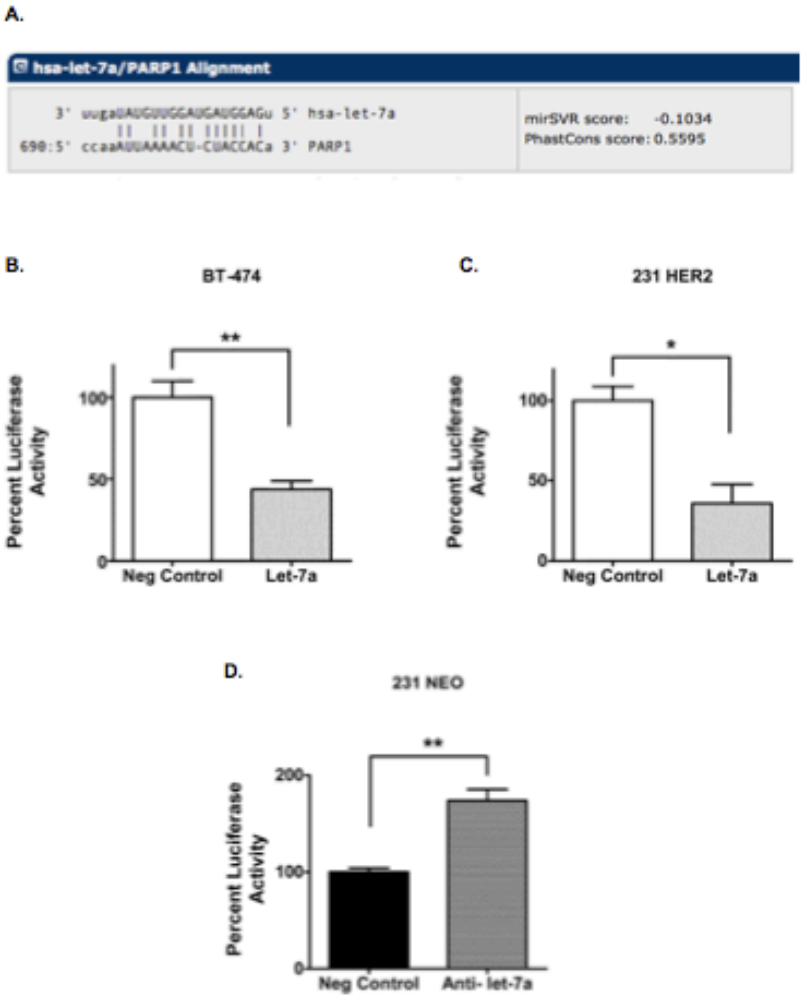


Figure 4. Let-7a targets the PARP1 3'UTR

(A) The PARP1 3'UTR was aligned with the seed sequence of the let-7a miRNA. (B, C) BT-474 and 231 HER2 cells were co-transfected with a let-7a miRNA or its neg control miRNA and a plasmid containing a firefly luciferase gene and the 3'UTR of PARP1 or a control 3'UTR. (D) 231 NEO cell line was co-transfected with an anti-let-7a miRNA or its neg control miRNA and a plasmid containing a firefly luciferase gene and the 3'UTR of PARP1 or a control 3'UTR. Luciferase activity was normalized to cells co-transfected with the respective neg control miRNA and the plasmid containing the control 3'UTR. The fold change values were calculated back to the cells co-transfected with the respective neg control miRNA and the plasmid containing the 3'UTR of PARP1. Results shown are from one of two independent experiments performed in quadruplicate. *p<0.05, **p<0.01

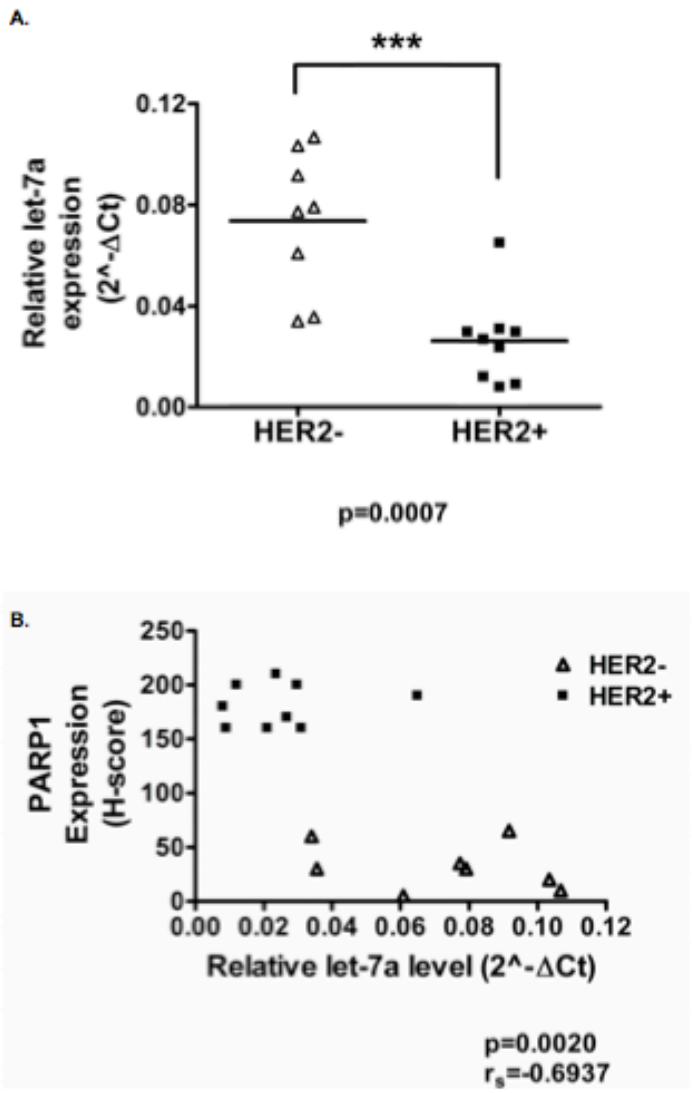


Figure 5. HER2+ breast cancer patients express low let-7a levels and high PARP1 levels.

(A) Let-7a levels were measured by qRT-PCR analysis in HER2+ and HER2- breast cancer specimens. U6 was used to normalize data. (B) Let-7a gene and PARP1 protein expression levels were correlated in 17 UAB HER2- and HER2+ breast cancer patients (r_s=-0.6937, p=0.0020). ***p<0.001

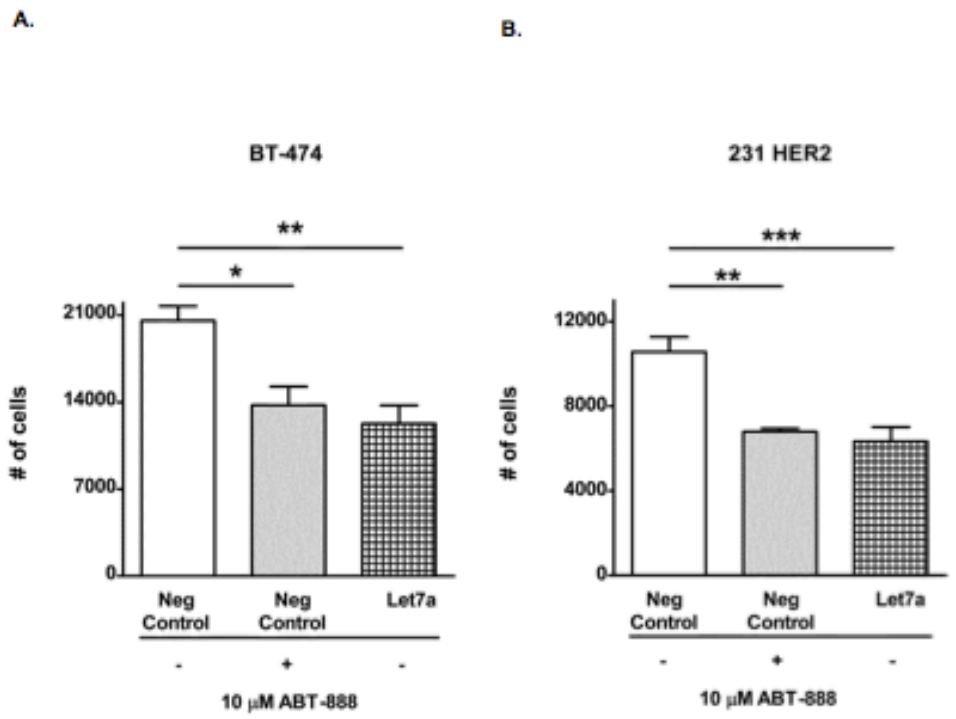


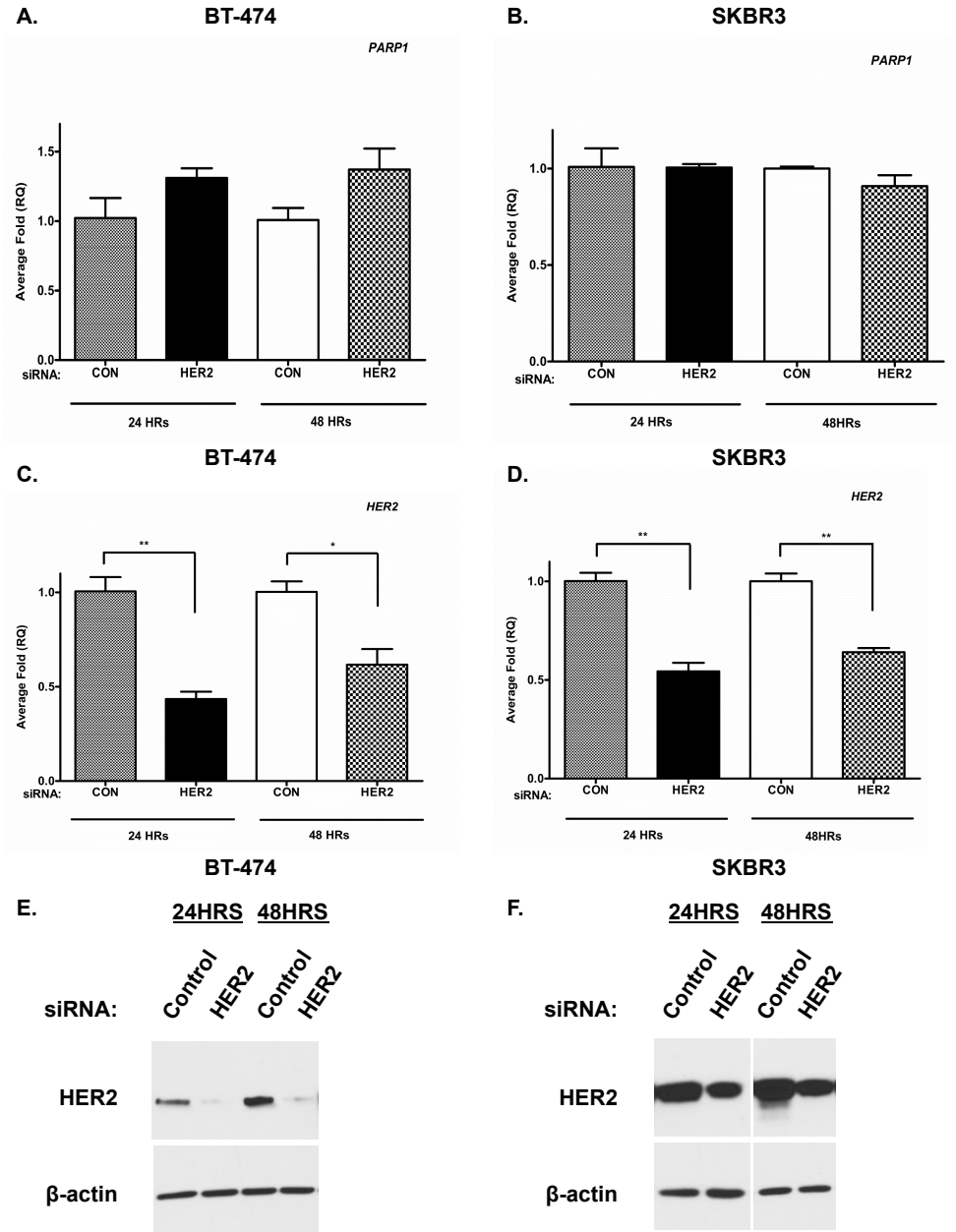
Figure 6. Let-7a reduces cell proliferation of HER2+ breast cancers similar to the PARP inhibitor ABT-888.

(A) BT-474 and (B) 231 HER2 cells were transiently transfected with a let-7a miRNA or its negative control. Forty-eight hours after transfection, the cells were treated with DMSO or 10μM ABT-888 for 96 hours and then analyzed by a cellular proliferation assay. Results shown are from one of two independent experiments performed in quadruplicate. *$p<0.05$, **$p<0.01$, and ***$p<0.001$

	MDA-MB-231		
	NEO	HER2	Fold Reduction:
let-7a	15250	7402.24	-2.06
let-7b	1788.33	997.22	-1.79
let-7c	59.11	32.97	-1.79
let-7d	530.39	515.65	-1.03
let-7e	500.86	382.19	-1.31
let-7f	94.54	61.88	-1.53
let-7g	655.6	473.39	-1.38
let-7i	1529.65	730.3	-2.09

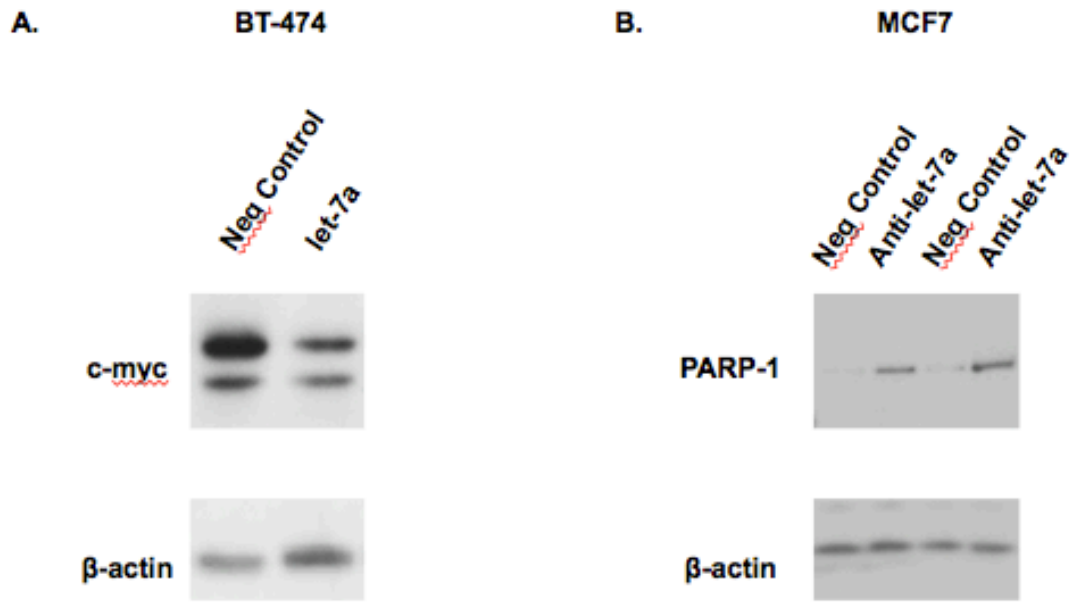
Table 1. Expression of the Let-7 microRNA family is altered after HER2 overexpression in breast cancer cells lines.

Normalized miRNA counts after ectopic HER2 overexpression in the MDA-MB-231 cell line (231 HER2). Fold change values were determined based on respective controls for each cell line.



Supplementary Figure S1. HER2 knockdown does not significantly alter PARP1 mRNA levels.

(A-D) BT-474 and SKBR3 breast cancer cell lines were transiently transfected with control (CON) or HER2 siRNA for 48 hours. (A, B) PARP1 and (C, D) HER2 mRNA levels were evaluated by qRT-PCR analysis. (E, F) Western blots of HER2 and β-actin levels 24 or 48 hours after HER2 knockdown in the BT-474 and SKBR3 cell line



Supplementary Figure S2. Let-7a regulates c-myc and PARP1 protein expression levels.

The protein expression levels of (A) c-myc in BT-474 cells and (B) PARP1 in MCF7 cells and were measured by western blot analysis, 72 hours after transfection with a pre-let-7a microRNA or its negative control.

	MDA-MB-231		
	NEO	HER2	Fold Reduction
miR-223	1.18	1.11	↓1.15

Supplementary Table S1. Expression of miR-223 is not significantly altered between HER2- and HER2+ breast cancer cells.

The 231 NEO and 231 HER2 breast cancer cell lines were subjected to NanoString Analysis using the microRNA panel. The NanoString miR-223 counts for the two cell lines are presented in the table and were normalized to expression levels of housekeeping genes included in the panel.

Tumor ID	HER status	ER status	PR status	T & N stage	PARP1 expression (0-300)	*Let-7a* expression (0-0.11)
UAB PA1	-	+	+	T_2, N_0	30	0.079
UAB PA2	-	+	+	T_2, N_0	35	0.077
UAB PA3	-	+	+	T_1, N_0	20	0.103
UAB PA4	-	+	+	T_3, N_1	65	0.092
UAB PA5	-	+	+	T_1, N_1	5	0.061
UAB PA6	-	+	+	T_3, N_2	10	0.107
UAB PA7	+	-	-	T_3, N_1	170	0.027
UAB PA8	+	+	-	T_3, N_3	160	0.031
UAB PA9	+	+	+	T_1, N_0	180	0.008
UAB PA10	+	-	-	T_3, N_0	200	0.030
UAB PA11	+	-	-	T_4, N_3	210	0.024
UAB PA12	+	+	+	T_1, N_0	190	0.065
UAB PA13	+	+	+	T_2, N_0	160	0.021
UAB PA14	+	+	+	T_2, N_1	200	0.012
UAB PA15	+	+	+	T_2, N_1	160	0.009
UAB PA16	-	+	+	T_2, N_3	30	0.036
UAB PA17	-	+	+	T_2, N_0	60	0.034

Supplementary Table S2. Patient characteristics for breast tumors analyzed for *let-7a* and PARP1 expression.

TRASTUZUMAB RESISTANT HER2+ BREAST CANCER CELLS RETAIN SENSITVITY TO POLY (ADP-RIBOSE) POLYMERASE (PARP) INHIBITION

by

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CHAPTER 3

TRASTUZUMAB RESISTANT HER2+ BREAST CANCER CELLS RETAIN SENSITVITY TO POLY (ADP-RIBOSE) POLYMERASE (PARP) INHIBITION

Abstract

HER2-targeted therapies, such as trastuzumab, have increased the survival rates of HER2+ breast cancer patients. However, despite these therapies, many tumors eventually develop resistance to these therapies. Our lab previously reported an unexpected sensitivity of HER2+ breast cancer cells to poly (ADP-Ribose) polymerase inhibitors (PARPi), agents that target homologous recombination (HR) deficient tumors, independent of a DNA repair deficiency. In this study, we investigated whether HER2+ trastuzumab resistant (TR) breast cancer cells were susceptible to PARPi and the mechanism behind PARPi induced cytotoxicity. We demonstrate that the PARPi ABT-888 (veliparib) decreased cell survival *in-vitro* and tumor growth *in vivo* of HER2+ trastuzumab resistant breast cancer cells. PARP-1 siRNA confirmed that cytotoxicity was due, in part, to PARP-1 inhibition. Furthermore, PARP-1 silencing had variable effects on the expression of several NF-κB-regulated genes. In particular, suppression of PARP-1 inhibited NF-κB activity and reduced p65 binding at the *IL-8* promoter, which resulted in a decrease in IL-8 mRNA and protein expression. Our results provide insight in the potential mechanism by which PARPi induces cytotoxicity in HER2+ breast cancer cells and support the testing of PARPi in patients with HER2+ breast cancer resistant to trastuzumab.

Introduction

Overexpression of the human epidermal growth factor receptor 2 (HER2) is observed in 20-30% of breast cancer patients and associated with poor patient survival (1). One FDA-approved agent targeted against HER2 receptors is trastuzumab (Herceptin) (2). Trastuzumab blocks HER2 signaling and ultimately leads to tumor cell lysis by multiple mechanisms (3). Despite its efficacy in the clinic, trastuzumab resistance remains a clinical challenge thus requiring novel therapeutic strategies (4).

In the last decade, poly (ADP-Ribose) polymerase inhibitors (PARPi) have been shown to be a promising therapeutic agent, especially in patients with recombination repair (HR) deficient tumors (5-8). Interestingly, we have previously found that HER2+ breast cancer cells are sensitive to the PARPi (ABT-888 (veliparib) or AZD-2281 (olaparib)) alone, independent of a basal or induced HR deficiency. However, cell survival was not affected after treatment with PARPi in non-HER2 overexpressing breast cancer cell lines (MCF7 and T47D). We also reported that PARPi suppressed NF-κB activity and HER2 overexpression alone conferred sensitivity to PARPi (9).

In this study, we hypothesized that trastuzumab resistant (TR) breast cancers may also be sensitive to PARPi as these tumors may rely on compensatory mechanisms to activate similar downstream effectors such as NF-κB (10). Indeed, we found that trastuzumab resistant HER2+ breast cancer cells are sensitive to pharmacological or genetic inhibition of PARP-1. Furthermore, we show that PARPi diminishes NF-κB (p65/RelA) transcriptional activity. Using the PanCancer Pathway Panel on the NanoString platform, which measures the expression of 770 genes involved in the 13 canonical cancer pathways, we analyzed the effects of PARPi on expression of NF-κB

target genes(11). We discovered that knockdown of PARP-1 had differential effects on the 82 NF-κB-target genes included in the PanCancer Panel, such as *IL-8, BRCA2, NFKBIZ, VEGFC, PIM1,* and *FASLG*. We also validated that PARP-1 knockdown strongly inhibited the mRNA and protein expression of IL-8, a gene involved in inflammation and angiogenesis. Furthermore, using chromatin immunoprecipitation assays, we found that PARP-1 suppression decreased p65 recruitment to the IL-8 promoter, resulting in reduced IL-8 mRNA and protein expression. In summary, these results provide evidence that PARP inhibitors may be used as a novel therapeutic strategy for HER2+ breast cancer patients and uncover PARP-1 /NF-κB (p65) signaling as a potential mechanism behind PARPi sensitivity.

Materials and Methods

Ethics statement

The animal protocol was approved by the University of Alabama at Birmingham at Birmingham Institutional Animal Care and Use Committee (APN#: 10129). Ketamine and xylazine anesthesia was used to minimize suffering before performing surgery on the mice.

Cell culture, drugs, and reagents

BT-474 and UACC-812 parental and trastuzumab resistant breast cancer cell lines were kindly donated by Dr. Rachel Schiff and C Kent Osborne (Department of Medicine, Baylor College of Medicine) and previously characterized (10). SKBR3 parental and trastuzumab resistant breast cancer cell lines were kindly donated by Dr. Francisco J. Esteva (Department of Medicine, NYU) and previously characterized (12). The BT-474 and UACC-812 trastuzumab resistant breast cancer cells were also cultured with 50 μg/

ml of trastuzumab and the SKBR3 trastuzumab resistant cells were cultured with 10 μg/ml of trastuzumab. All cells tested negative for mycoplasma and were validated by western analysis for HER2. One of the three trastuzumab resistant breast cell lines (the BT-474 TR) was also verified for resistance to trastuzumab. We observed that the BT-474 TR cell line had reduced sensitivity to trastuzumab as compared to treatment with lapatinib or a combination of lapatinib and trastuzumab (data not shown). Veliparib (ABT-888) was obtained from Enzo Life Sciences (catalog #ALX-270-444) and reconstituted in dimethyl sulfoxide (DMSO) at 10 mmol/L. ABT-888 was also obtained from AbbVie Oncology for *in-vivo* testing and reconstituted every five days in 0.9% saline at 100 mg/kg. Trastuzumab (Herceptin) was purchased from Besse Medical (catalog #: 23961). Recombinant human TNF-α was obtained from R&D systems (catalog #: 210-TA).

Clonogenic survival assay

The colony formation assay was utilized to determine the percent survival in both the parental and trastuzumab resistant breast cancer cell lines as previously described (13,14).

PARP-1 knockdown

PARP-1 siRNA was obtained from Santa Cruz Biotechnology and contains three to five siRNA pools specifically targeting the *PARP-1* gene (sc-29437; Santa Cruz Biotechnology). Control siRNA was used as a negative control (sc-37007; Santa Cruz Biotechnology). The siRNAs were transfected with Lipofectamine2000 or Lipofectamine RNAiMax according to the manufacturer's instructions. PARP-1 knockdown was confirmed by Western Blot or Real-Time PCR analysis.

Immunoblotting

Protein expression levels were analyzed via a standard immunoblotting protocol using the M-PER Mammalian Protein Extract Reagent with protease and phosphatase inhibitors as described previously (15). The PVDF membranes were immunoblotted overnight with the following primary antibodies according to the manufacturer's instructions: PARP-1 (Cell Signaling Technology, catalog # 9542), PARP-1 (Santa Cruz, catalog # sc-8007), PARP-2 (Abcam, catalog #ab176330), IKKα (Cell Signaling Technology, catalog #2682), and BRCA2 (Abcam, catalog #ab27976). The immunoblots were then incubated with a rabbit or mouse horseradish peroxidase-conjugated secondary antibody for an hour. β-actin expression levels were evaluated as a loading control (Santa Cruz Biotechnology, catalog # sc-47778 HRP).

Cell proliferation

Cell proliferation was also assessed after PARP-1 knockdown. After four days of treatment, the cells were washed with 1X ice-cold PBS and then removed with trypsin. Subsequently, the number of cells was counted using a cell counter (Beckman Coulter, Fullerton, CA).

Apoptosis analysis

Apoptosis was measured using the Annexin V-FITC Apoptosis Detection kit (Biovison Research Products; catalog #K101-400), 96 hours after transfection with control or PARP-1 siRNA and as previously described (14).

NF-κB Luciferase Reporter Assay

The NF-κB Secreted Luciferase Reporter System was used to analyze NF-κB activity.

Specifically cells were co-transfected with the NFκB-driven luciferase plasmid NFκB-MetLuc2 or its vector control MetLuc2 (Clontech; catalog # 631728) and control or PARP-1 siRNA using the Lipofectamine2000 reagent, according to the manufacturer-supplied protocol and as previously described(9).

mRNA expression

Total RNA was isolated using the Ambion PureLink RNA mini kit (catalog #12183018A) according to the manufacturer's recommendations. Gene expression was measured using the PanCancer Pathways Panel after PARP-1 knockdown, as previously described (16). One μg of total RNA was also reverse transcribed using the SuperScript III First-Strand Synthesis System kit (Invitrogen; catalog # 18080-051) and the resulting cDNA was analyzed by semiquantitative PCR using the following primer purchased from Applied Biosystems: *PARP-1* (Hs00242302_m1), *IL-8* (Hs00174103_m1), *BRCA2* (Hs00609073_m1). mRNA levels were determined with the ABI Prism 7000 Sequence Detection System (Applied Biosystems) as per manufacturer's instructions. Samples were run in triplicate and then normalized to the endogenous control, *GAPDH* (Hs02758991_g1) relative gene expression levels was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed in duplicate as previously published(17). Control or PARP-1 siRNA treated cells were sonicated and lysates were immunoprecipitated using four μg of p65 (Santa Cruz; catalog # sc-372) or normal rabbit IgG (Santa Cruz; catalog #: sc-2027) antibodies.

ELISA

Supernatants were analyzed after PARP-1 knockdown or PARPi using the Human IL-8 enzyme-linked immunosorbent assay (ELISA) (BioLegend; catalog #431504).

In-vivo studies

Ten 4-6 week old female BALB/c nude mice were obtained from Charles River. The mice were allowed to acclimatize for 1 week and then supplemented with 0.36-mg 60-day-release estradiol pellets from Innovative Research. Following two to three days of recovery, BT-474 TR cells were collected and then suspended in 200 µl of growth factor-reduced Matrigel from BD Biosciences before injection. 5×10^6 cells were injected subcutaneously in the BALB/c nude mice. After the tumors were palpable or reached ~5-6mm in diameter, we randomized the mice into two treatment groups *(n*=5): saline (vehicle control) or ABT-888. The mice were treated twice daily with 100 kg/mg of ABT-888 by oral gavage for four weeks. Tumor size was measured triweekly during the course of the treatment with a caliper. The tumor volume was calculated with the following formula: $[(\text{width})^2 \text{ x length}]/2$ (Burd and Wachsberger, 2007). The animals were measured for 68 days and then sacrificed. The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved all the animal procedures described above.

Statistical analysis

The data were analyzed via a two-tailed, student t-test or analysis of variance (ANOVA) followed by a Bonferroni post test using GraphPad Prism version 4.02 (GraphPad

Software). Data are presented as average ± standard error of mean (SEM). P-values ≤ 0.05 were considered significant.

Results

Trastuzumab resistant HER2+ breast cancer cells exhibit in vitro and in vivo sensitivity to the PARPi ABT-888

To examine whether HER2+ trastuzumab resistant (TR) breast cancer cell lines were sensitive to PARPi, we assessed PARPi-induced cellular cytotoxicity using colony formation assays. As shown in Figure 1A-C, the BT-474 TR, UACC-812 TR, and SKBR3 TR cells all demonstrated reduced colony formation in response to increasing, clinically achievable concentrations of ABT-888 and exhibited a similar profile of sensitivity as their parental counterparts. Specifically, at 10μM of ABT-888 (Veliparib), there was more than 70% reduction in the survival in all three trastuzumab resistant breast cancer cell lines.

We next assessed the *in vivo* tumor growth effects of PARPi in the BT-474-TR xenografts. As shown in figure 1D, tumor growth was significantly inhibited after PARPi treatment in the HER2+ trastuzumab resistant xenografts. These results confirm that HER2+ trastuzumab resistant breast cancer cells are sensitive to PARPi alone both *in vitro* and *in-vivo.*

PARP-1 knockdown inhibits cell proliferation and induces apoptosis in HER2+ trastuzumab resistant breast cancer cells.

To verify that our observations were indeed due to the inhibition of PARP-1 and not caused by an off-target effect, we examined cellular proliferation after silencing PARP-1 using a pooled PARP-1 siRNA. Ninety-six hours after PARP-1 knockdown, cell proliferation was reduced by more than 40% in both the BT-474-TR (Figure 2A) and UACC-812-TR (Figure 2B) breast cancer cell lines. Next, we tested whether silencing PARP-1 induced cellular apoptosis. As shown in figures 2C and 2D, increased apoptosis was observed after 96 hours of PARP-1 suppression. Furthermore, inhibition of PARP by ABT-888 or PARP-1 siRNA treatment did not alter cell cycle progression (Supplementary Figures S1A and S1B) and the pooled PARP-1 siRNA reduced PARP-1 but not PARP-2 levels (Figure 2E and 2F).

NF-κB signaling is attenuated by inhibition and suppression of PARP-1

Previously, we reported that PARPi susceptibility correlated with inhibition of the NF-κB signaling pathway in the HER2+ parental breast cancer cells. We also observed that resistance to PARPi was stimulated after p65 overexpression while sensitivity was induced after overexpression of the NF-κB endogeneous inhibitor, IκBα (9). Subsequently, we tested NF-κB activity in the trastuzumab resistant cell lines with or without PARP inhibition using an NF-κB driven luciferase reporter assay. Consistent with our previous findings, pharmacological or genetic modulation of PARP-1 attenuated NF-κB transcriptional activity in the BT-474 TR (Figure 3A) cell line.

It has also been reported that HER2 overexpression activates the canonical NF-κB signaling pathway via the IKKα, catalytic subunit of the IKK complex, without the

stimulation of an inflammatory stimuli such as TNF-α(18). Since we observed an inhibition of NF-κB activity, we hypothesized that the expression of the IKKα protein, an NF-κB activator, would be reduced by PARP inhibition. Western blot analysis following PARPi or PARP-1 knockdown treatment revealed that the level of IKKα protein was decreased in two HER2+ breast cancer cell lines (Figure 3B and C). These results indicate that both PARP-1 inhibition and suppression inhibit NF-κB signaling in HER2+ trastuzumab resistant breast tumors.

PARP-1 inhibits the expression of NF-κB regulated target genes

Hassa, Hottiger et al. previously reported that PARP-1 is a coactivator of NF-κB(19,20). To further examine the role of PARP-1 as a regulator of NF-κB activity, we assessed the gene expression of NF-κB target genes using the NanoString nCounter Analysis System after PARP-1 knockdown in the BT-474 TR cell line. After normalizing the data to housekeeping genes included in the panel, the suppression of PARP-1 significantly altered the expression levels of a number of NF-κB regulated genes (Table 1). The gene most strongly impacted by PARP-1 knockdown was IL-8 (13 fold reduction). These results were also validated in an *in-vivo* model. Specifically, there was a 1.37 fold reduction in *IL-8* expression after seven days of treatment with ABT-888 in MCF7 HER2 xenografts (data not shown).

PARP-1 knockdown inhibits IL-8 gene and protein expression

To validate our NanoString data, we assessed changes in *IL-8* gene expression following PARP-1 siRNA using qPCR analysis. *IL-8* is also an excellent gene candidate and read-out to further study the role of PARP-1 in NF-κB-mediated transcription, because unlike other genes, NF-κB plays a dominant role in its expression. Specifically, the human *IL-8* promoter is highly accessible to the NF-κB transcription factor, p65

(21,22). Consistent with our NanoString data, *IL-8* mRNA was significantly reduced after PARP-1 knockdown in both the BT-474 TR and UACC-812 TR breast cancer cells (Figure 4A and B). Moreover, PARP-1 siRNA significantly reduced *IL-8* mRNA levels induced by TNF-α, which robustly activates the NF-κB signaling pathway and thus can be used as a positive control for NF-kB activation**.** *IL-8 mRNA* levels were similarly attenuated in the parental BT-474 breast cancer cell line (Figure 4C) after PARP-1 knockdown. Finally, PARPi using ABT-888 also decreased the gene expression levels of *IL-8* in the BT-474 TR cells (Figure 4D).

To determine whether these changes in mRNA expression directly correlated with similar changes in protein expression, we subjected the BT-474 TR and UACC-812 TR breast cancer cell lines to ELISA analysis after PARP-1 inhibition or knockdown. Indeed, IL-8 protein expression levels were decreased by both pharmacological or genetic modulation of PARP-1 (Figure 5A-C).

PARP-1 knockdown decreases p65 binding at the IL-8 promoter

To further investigate PARP-1 's role in regulating NF-kB activity in the context of IL-8 expression, we assessed the recruitment of the NF-κB subunit p65 to the *IL-8* promoter using chromatin immunoprecipitation (ChIP). As shown in Figure 6A, TNF-α stimulation increased p65 binding at the *IL-8* promoter in the BT-474 TR cell line, and this effect was significantly reduced with PARP-1 knockdown. p65 and IgG (negative control) were also immunoprecipitated in untreated or TNF-α treated BT-474 TR cells. We observed that p65 was associated with the *IL-8* promoter in TNF-α treated cells, and this was not detected using IgG (Figure 6B). These ChIP experiments further demonstrate that PARP-1 regulates the NF-κB subunit p65 at the *IL-8* promoter.

Discussion

PARP-1 's role in DNA damage repair, specifically the base excision repair pathway, has been studied extensively. In addition to its DNA repair functions, PARP-1 also has a role in several other cellular processes(23). PARP-1 has been shown to promote chromatin decondensation by PARylating histones or binding directly to nucleosomes(24,25). Several other studies have shown that PARP-1 is involved in angiogenesis(26). Recently there has been an increased interest in targeting transcriptional factors with PARPi in various types of cancer models (27-29). In this study, we report that the susceptibility to PARPi observed in HER2+ breast cancers (sensitive or resistant to the HER2-targeted agent trastuzumab) may be dependent on the inhibition of the NF-κB signaling pathway. We also elucidated the effects of PARP-1 on the NF-κB signaling pathway in HER2+ breast cancer cells by investigating effects on IL-8 expression.

PARP inhibitors such as ABT-888 target both PARP-1 and PARP-2 members of the PARP family that are involved in DNA repair(30). PARP-1 is the most abundant nuclear member of this family and is responsible for a larger percentage of PARP activity compared to PARP-2(31). In this study, we focused mainly on the PARP-1 protein since we recently discovered that HER2+ breast cancers express increased levels of PARP-1 compared to non-HER2 overexpressing breast cancers (32). No significant differences were found with PARP-2 protein levels (32). However, we cannot exclude PARP-2 as an essential target in this mechanism as it is still responsible for ten percent of PARylation produced in the nucleus(33). We are currently investigating the role of PARP-2 in this process but it is beyond the scope of this study.

PARP-1 has also been shown to regulate gene transcription by acting as a co-

activator. Some transcription factors that are regulated by PARP-1 include the following: NF-κB (19), androgen receptor(27), and estrogen receptor(34). Our results suggest that PARP-1 may be acting as a coactivator of NF-κB (Figure 4 and 6). However, the role of PARP-1 's enzyme activity in NF-κB dependent gene expression is very controversial. Multiple studies have reported that PARP-1 's enzymatic activity is independent of its co-activator function of NF-κB (20,35). However, we observed a slight reduction in the gene expression levels of a NF-κB target gene, *IL-8*, after PARPi in TNF-α stimulated cells (Figure 4D). These contradictory results suggest that the role of PARP-1 's enzymatic activity may be context dependent.

Conversely, the expression of PARP-1 is required and sufficient in various disease models (20,35,36). Our PARP-1 knockdown studies further confirm these results and suggest that PARP-1 regulates NF-κB activity. Specifically, PARP-1 knockdown reduced p65 binding to the *IL-8* gene promoter, leading to decreased gene and protein expression in untreated or TNF-α treated cells. All together these results suggest that PARPi attenuates NF-κB signaling in HER2+ trastuzumab resistant breast cancer cells. However, we do not propose that the sole mechanism behind PARPi sensitivity is the inhibition of IL-8 expression. We would like to further stress that *IL-8* is merely an excellent gene candidate to study the role of PARP-1 in NF-κB-mediated transcription.

Nevertheless, *IL-8* is a HER2 regulated gene and associated with metastasis in breast cancer patients. Trastuzumab has also been shown to downregulate *IL-8* gene expression levels in BT-474 breast cancer cells (37). Another study observed that IL-8 has a role in influencing trastuzumab resistance(38). These data suggest that the potential targeting of the *IL-8* gene in HER2+ breast cancer cells that develop resistance to

trastuzumab may be useful.

We also observed that other NF-κB-targeted genes were also regulated by PARP-1 besides *IL-8*. However, their expression levels were not as strongly influenced by PARP-1 suppression. This could be due to the fact that their levels are regulated by several transcription factors, whereas, *IL-8* is highly dependent on NF-κB. In particular, the expression levels of *BRCA2* were reduced after PARP-1 knockdown (Table 1). BRCA2 is a protein involved in the homologous recombination (HR) DNA repair pathway(39). Previous studies have shown that genetic and pharmacologic inhibition of PARP-1 reduced expression of BRCA1 and Rad51, two other proteins involved in HR, but not BRCA2. These results were also associated with a decrease in HR (40). Our results show that PARP-1 knockdown decreased the levels of *BRCA2* mRNA, but did not alter BRCA2 protein levels (Supplementary Figures S4A and S4B). This suggests that the effects observed after PARP-1 knockdown were independent of an HR deficiency. Conversely, PARP-1 knockdown upregulated *PIM1* and *FASLG* expression. PIM1 is involved in survival and cell proliferation whereas FASLG is involved in apoptosis(41,42). A possible explanation behind these findings could be NF-κB's complex role in multiple cellular pathways(43). Several studies have also indicated that in a single cell, NF-κB can have both pro- and anti-apoptotic functions (44). These results will be investigated in future studies.

Various mechanisms of resistance to trastuzumab have been described in the literature(45). Clearly, more novel therapeutic strategies are needed for these patients. This study supports further clinical testing of PARPi in patients with HER2 positive breast tumors that are sensitive or resistant to trastuzumab. It also suggests the use of

PARPi to suppress PARP-1 's transcriptional functions besides solely inhibiting its DNA repair roles.

Disclosure of Potential Conflicts of Interest

Eddy Yang has served on the advisory board of NanoString Technologies and has received honorarium from them. He also has a Materials Transfer Agreement with AbbVie, Inc. The other authors have no conflicts of interest to disclose.

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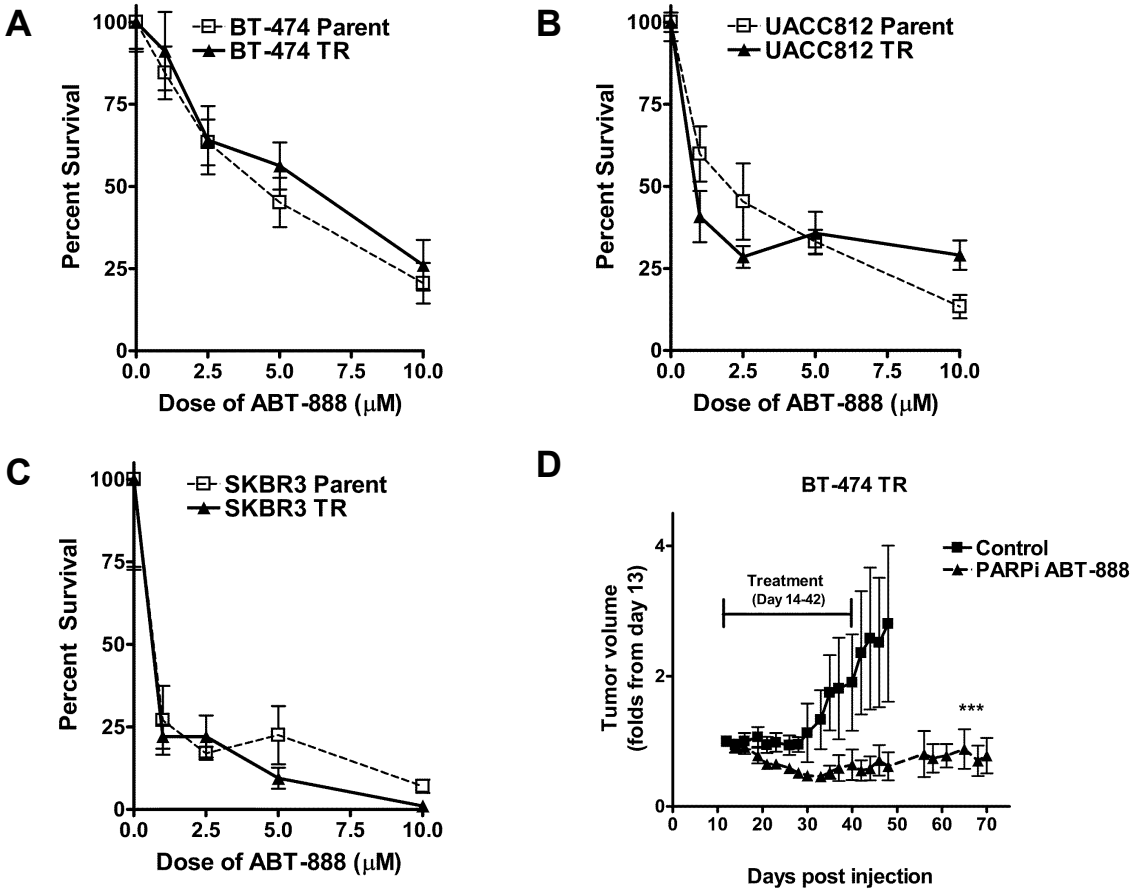


Figure 1. HER2+ trastuzumab resistant breast cancer cells are sensitive to PARP inhibition (ABT-888) *in-vitro* and *in-vivo.*

(A-C) HER2+ parental and trastuzumab resistant breast cancer cell lines were subjected to a colony formation assay after being treated with vehicle control and increasing concentrations of the PARPi ABT-888 (1-10μM). Shown is the mean percent survival (+/- SEM) from one of two independent experiments performed in triplicate. (D) BT-474 TR were injected subcutaneously in the flank of BALB/c nude mice. The mice were treated twice daily with vehicle or 100 mg/kg of ABT-888 once the tumors were palpable. Tumors were measured three times per week. Shown is the mean fold change in tumor volume +/-SEM. ***$p<0.0005$

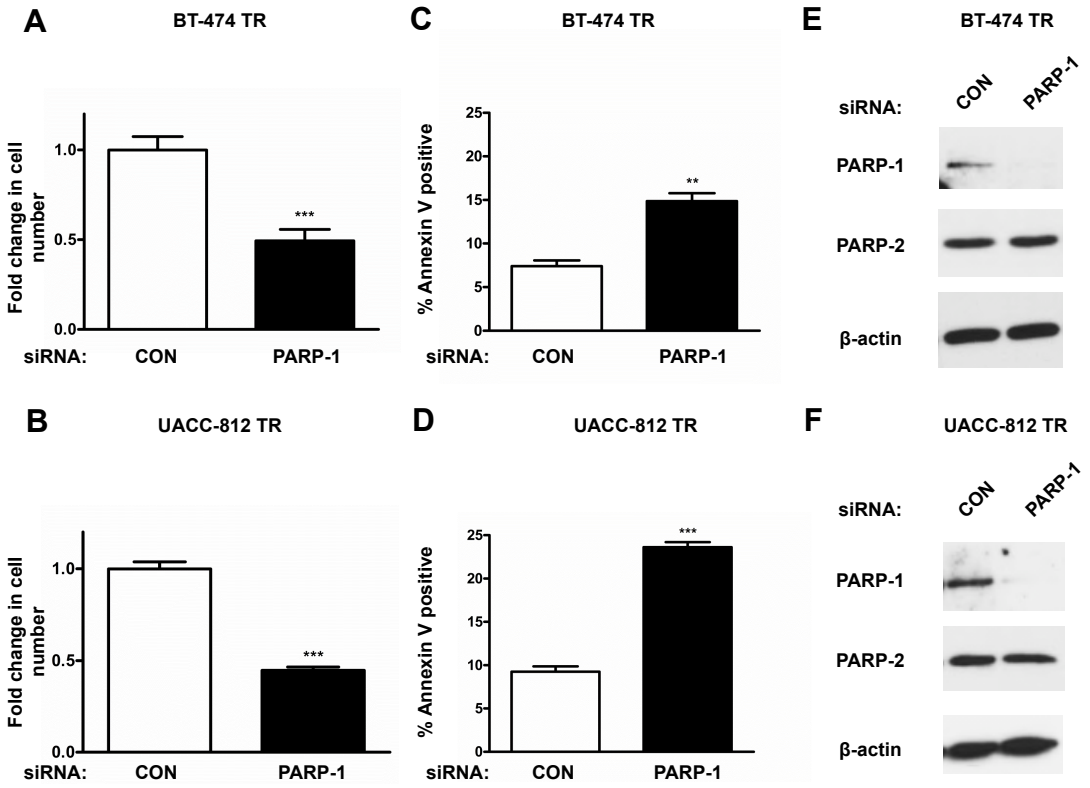


Figure 2. PARP-1 siRNA reduces cell proliferation and induces apoptosis in HER2+ trastuzumab resistant breast cancer cell lines.

BT-474 TR and UACC-812 TR were transfected with 20 nM of control (CON) or PARP-1 siRNA for 96 hours and then analyzed by a cellular proliferation assay (A and B) or FACS analysis using propidium iodide and Annexin V staining (C and D). The representative figures shown are from one of two independent experiments performed in (A and B) quadruplicate or (C and D) triplicate. Knockdown of PARP-1 protein expression levels was verified via Western Blot analysis. β-actin was used as a loading control (E and F). Data shown are representative immmunoblots from one of two independent experiments. ***$p \leq 0.0005$, ** $p < 0.005$

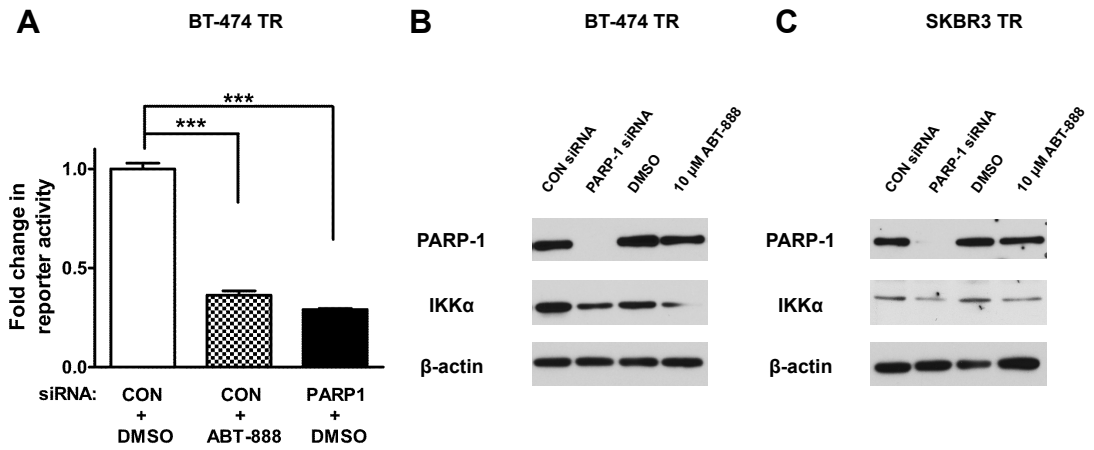


Figure 3. PARPi attenuates NF-κB activity and signaling in HER2+ trastuzumab resistant cell lines.

(A) Cells were co-transfected with the NFκB-driven luciferase plasmid NFκB-MetLuc2 or its vector control MetLuc2 and control or PARP-1 siRNA. The cells were then treated with vehicle or 10 μM ABT-888 four hours post transfection. NF-κB transcriptional activity was analyzed 48 hours after drug treatment using the NF-κB Secreted Luciferase Reporter System. Results shown are from one of two independent experiments performed in triplicate. (B and C) Western blot of PARP-1 , IKKα, and β-actin after knockdown or inhibition of PARP-1 in BT-474 TR and SKBR3 TR cells. The representative images shown are from one of two independent experiments. ***$p<0.001$.

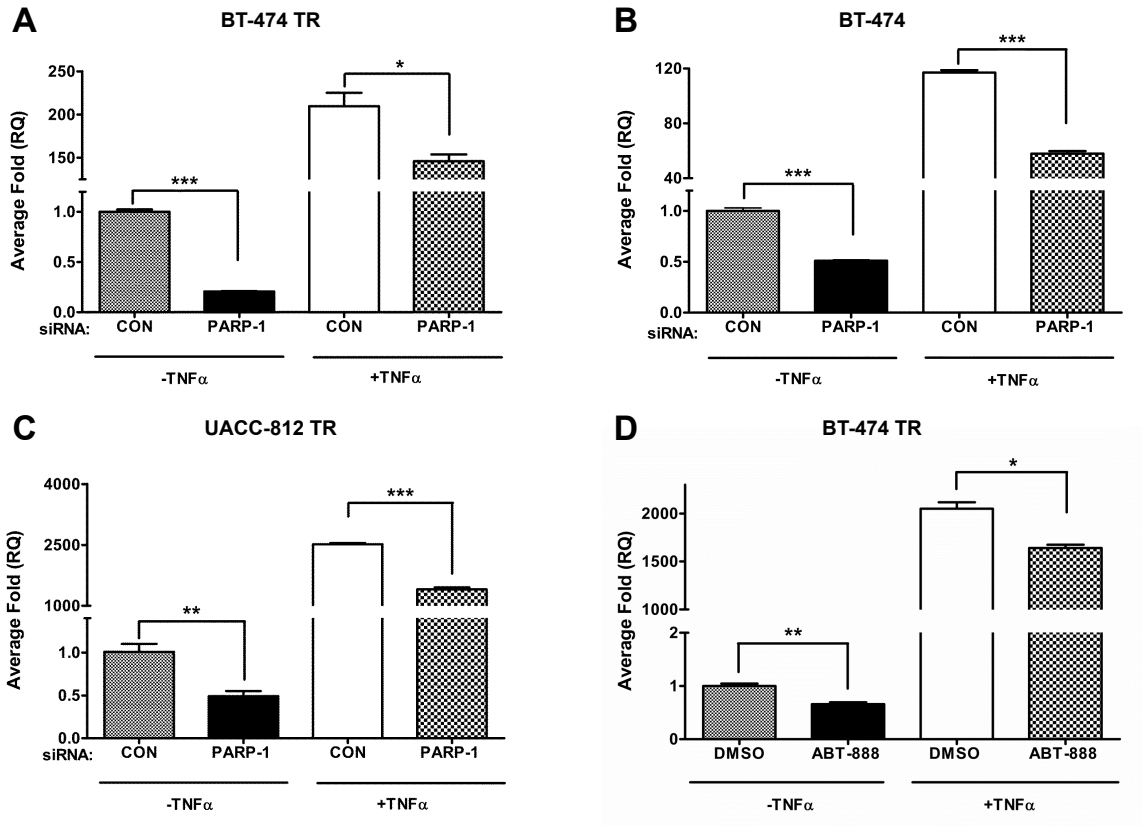


Figure 4. PARP-1 knockdown inhibits IL-8 expression in both HER2+ parental and trastuzumab resistant breast cancer cells.

Quantitative real-time PCR analysis was performed with total RNAs isolated from both (A) BT-474 trastuzumab resistant, (B) BT-474 parent, and (C) UACC-812 trastuzumab restistant cells that were transfected with 20 nM of control (CON) or PARP-1 siRNA for 48 hours, serum-starved for 18 hours, and then stimulated with 10 ng/ml of tumor necrosis factor-α (TNF-α) for 2 hours. (D) BT-474 TR were serum starved and treated for four hours with DMSO or 10 μM ABT-888 and then treated with TNF-α for an additional 2 hours. One μg of isolated RNA was reverse transcribed to cDNA and then analyzed by qRT-PCR for *IL-8* and *GAPDH* expression. The figures are representative images from one of (A-C) three or (D) two independent experiments performed in triplicate. A one-way ANOVA test was performed followed by a t-test to calculate the significance between groups. *$p<0.05$ and ***$p<0.001$

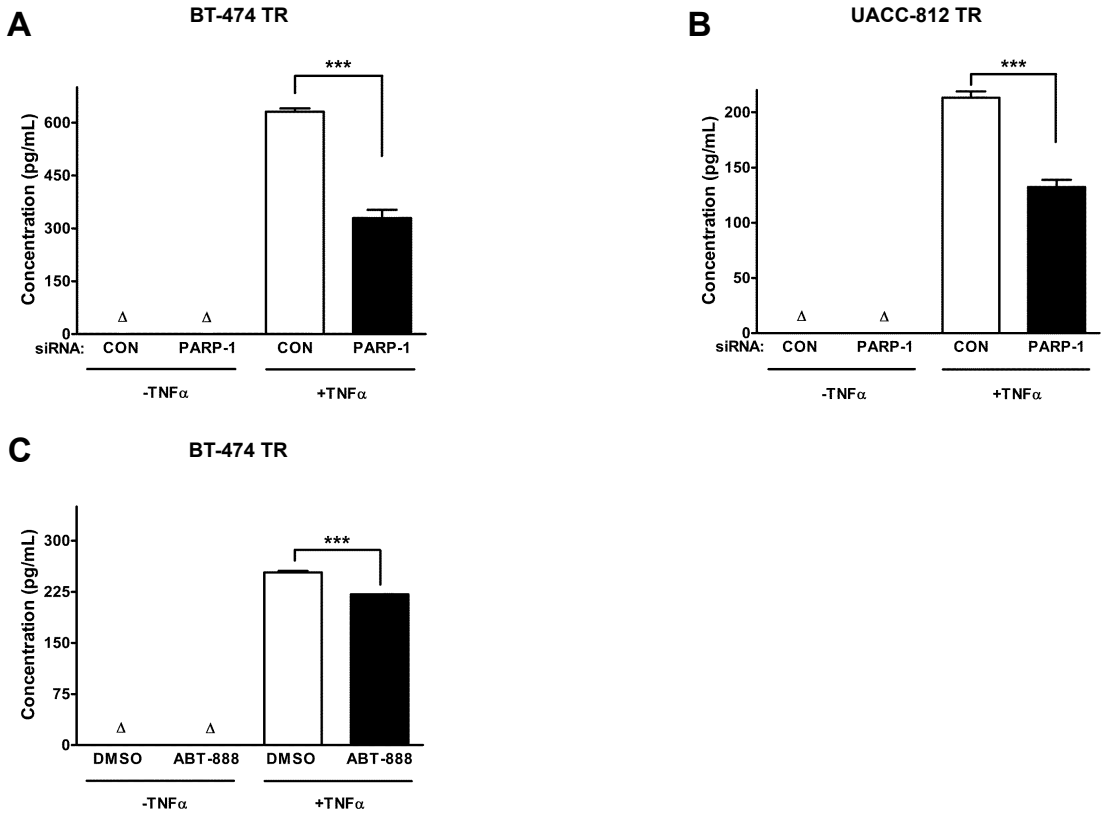


Figure 5. PARP-1 knockdown inhibits IL-8 protein expression.

(A) BT-474 TR cells were transfected with control (CON) or PARP-1 siRNA for 48 hours and (B) UACC-812 TR cells were transfected for 24 hours, serum-starved for 18 hours, and then stimulated for 24 hours with 10 ng/mL of TNF-α. (C) BT-474 TR cells were serum-straved and treated with DMSO or 10 μM ABT-888 for 24 hours after seeding and then stimulated for an additional 24 hours with TNF-α. The supernatant was then collected and IL-8 protein expression was analyzed by an enzyme-linked immunosorbent assay (ELISA). Results shown are from one of (A and B) three or (C) two independent experiments performed in triplicate. ***$p \leq 0.001$

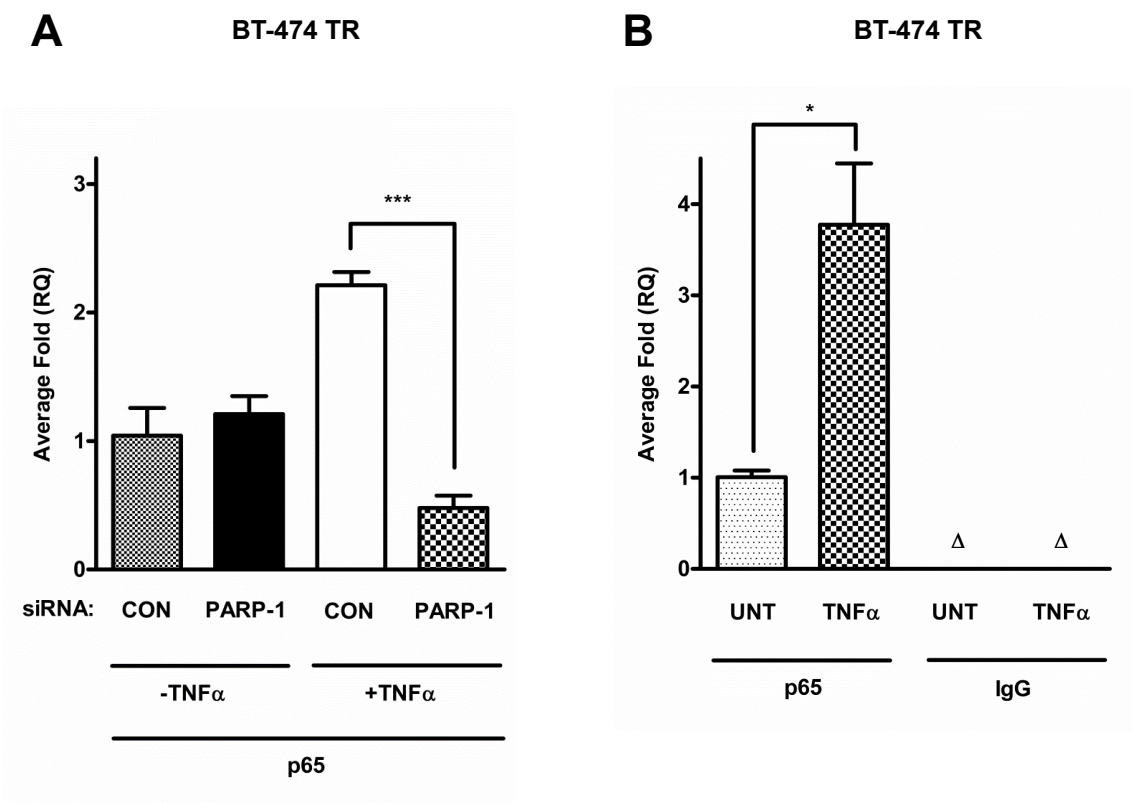


Figure 6. PARP-1 knockdown decreases p65 recruitment to the IL-8 promoter in HER2+ trastuzumab resistant cell lines.

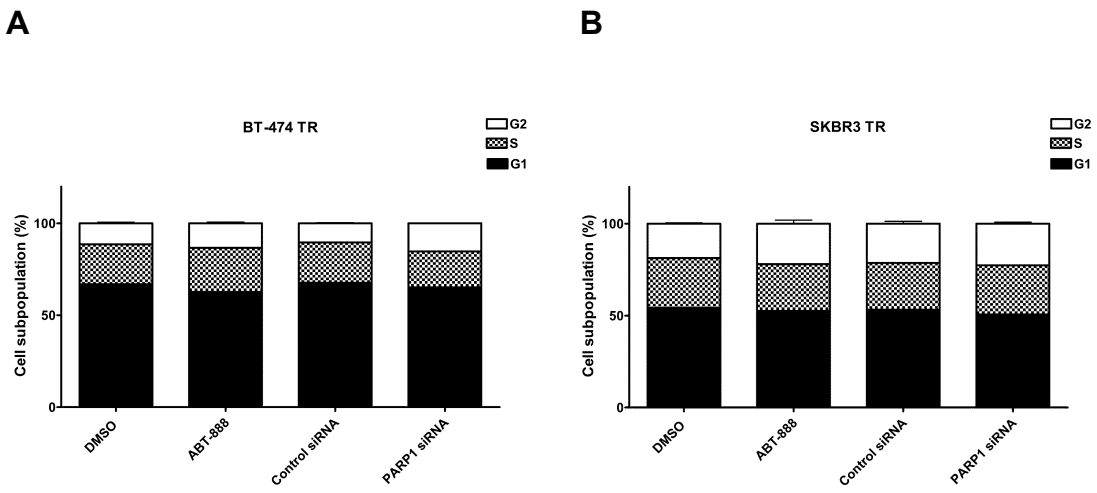
(A) BT-474 TR were transfected with control (CON) or PARP-1 siRNA for 48 hours or (B) left untreated, then serum-starved for 18 hours and stimulated without or with 10 ng/ml of TNF-α for 2 hours. The cells were then subjected to chromatin immunoprecipitation (ChIP) assay, using p65 or immunoglobulin G (IgG) control antibodies. Immunoprecipitated DNA was analyzed by qRT-PCR using human IL-8 primers. Non-immunoprecipitated DNA (input) was used as a control for total DNA levels. Data shown are from one of two independent experiments. A t-test or a one-way ANOVA test was performed followed by a t-test to calculate the significance between groups. *$p<0.05$ and ***$p<0.001$

BT-474 TR:

NF-κB target genes:	CON siRNA:	PARP-1 siRNA:	Fold Change:
IL-8	1180.83	89.72	↓13.16
BRCA2	42.3	19.7	↓2.15
NFKBIZ	51.5	27.36	↓1.88
VEGFC	44.14	24.07	↓1.83
PIM1	37.71	73.31	↑1.95
FASLG	9.2	22.98	↑2.5

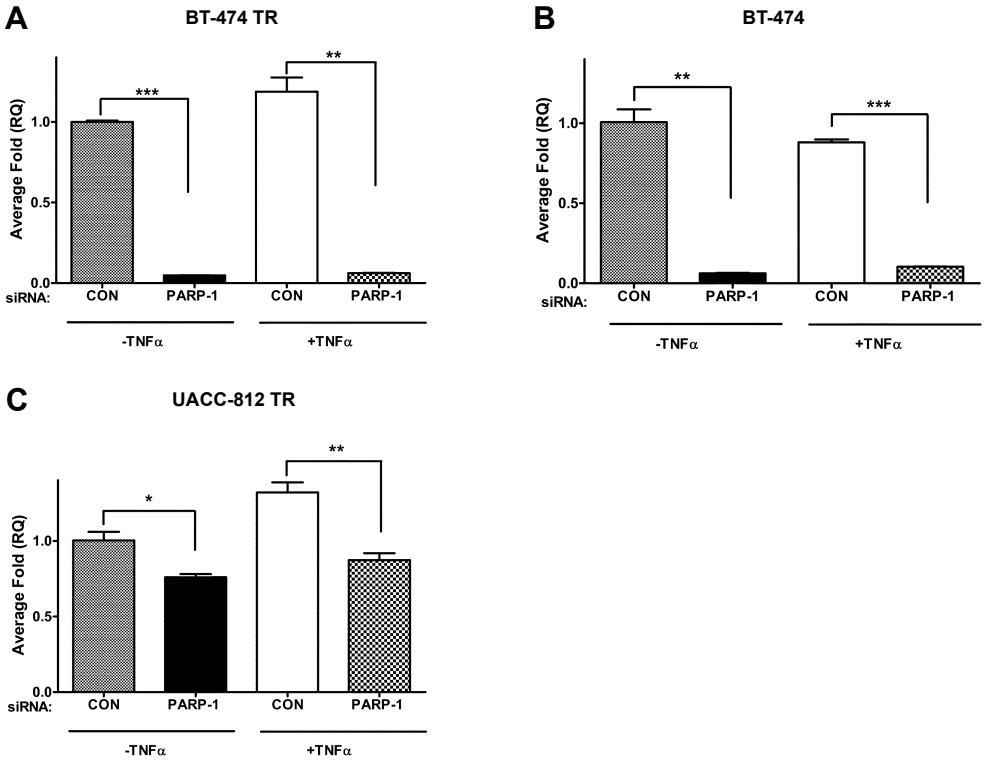
Table 1. Significant NF-κB target genes influenced by PARP-1 knockdown.

The BT-474 TR cell line was treated with control (CON) or PARP-1 siRNA for 72 hours and then subjected to NanoString Analysis using the PanCancer Pathway panel. The counts were normalized to expression levels of housekeeping genes included in the probeset. Shown in the table are the normalized NanoString counts along with the fold change values.



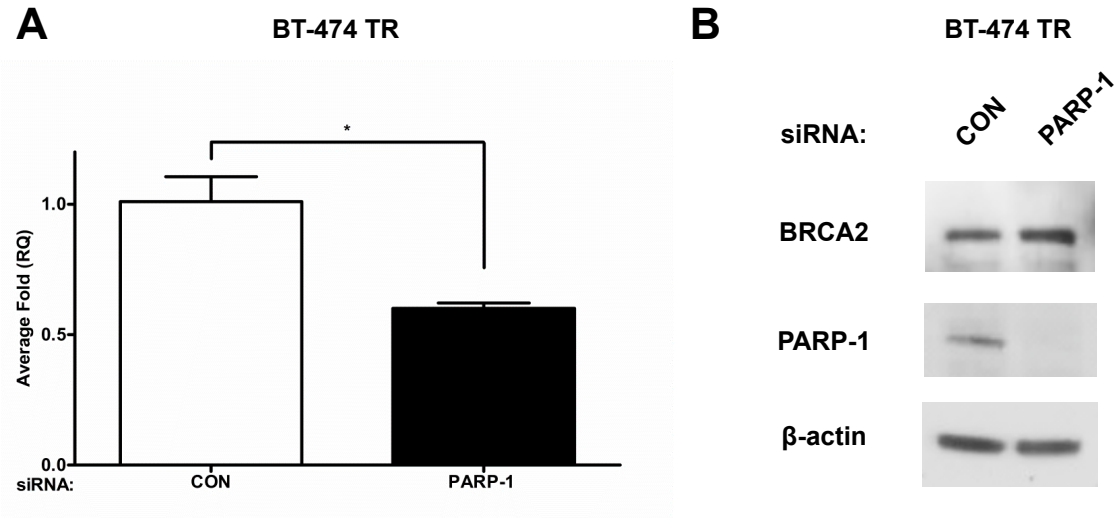
Supplementary Figure S1. Cell cycle distribution is not affected by PARPi in HER2+ trastuzumab resistant cell lines.

(A) BT-474 TR and (B) SKBR3 TR were treated with DMSO or 10 μM ABT-888 or transfected with control (CON) or PARP-1 siRNA for 72 hours and then subjected to FACS analysis after propidium iodide staining. The representative images shown are from one experiment performed in triplicate.



Supplementary Figure S2. PARP-1 gene expression levels were reduced after PARP-1 knockdown in HER2+ parental and trastuzumab resistant breast cancer cell lines.

(A) BT-474 trastuzumab resistant, (B) BT-474 parental, and (C) UACC-812 trastuzumab resistant were transfected with control (CON) or PARP-1 siRNA for 48 hours, serum-starved for 18 hours, and then treated with TNF-α for an additional 2 hours. Total RNA was isolated, reverse transcribed, and analyzed by qRT-PCR for *PARP-1* and *GAPDH* expression. Shown is the average fold change from untreated TNF-α cells treated with control siRNA (+/- SEM) from one of three independent experiments performed in triplicate. *p<0.05, and ***p<0.001



Supplementary Figure S3. BRCA2 gene and protein expression levels in BT-474 trastuzumab resistant breast cancer cell lines after PARP-1 knockdown.

The expression of BRCA2 was measured by (A) qRT-PCR and (B) western blot analysis 72 hours after PARP-1 knockdown in the BT-474 TR. Results shown are from one of (A) three experiments performed in triplicate or (B) two independent experiments. *$p<0.05$.

CHAPTER 4

Discussion

Summary

HER2+ breast cancer was once the most deadly form of breast cancer. It was not until the development of trastuzumab that the overall survival rate and quality of life for these breast cancer patients improved. Despite these remarkable results, HER2+ breast tumors continue to exhibit resistance to trastuzumab. In this dissertation, we provide evidence for the use of PARPi as an alternative treatment strategy for HER2+ parental and trastuzumab resistant breast tumors. We have also thoroughly dissected the mechanism behind PARPi sensitivity in HER2+ breast cancer tumors. In chapter 2, we discovered that HER2 status positively correlated with elevated PARP1 protein expression and the mechanism behind these altered levels involved the let-7a miRNA at the PARP1 3'-UTR. In chapter 3, we determined that the mechanism by which PARPi inhibits NF-κB mediated activity is due to decreased recruitment of the NF-κB subunit p65 at a promoter of a NF-κB target gene called IL-8. Our data also revealed that PARPi susceptibility is retained in HER2+ trastuzumab resistant breast cancer cells. The culmination of this work reveals an interplay between HER2, PARP1, and NF-κB signaling that mediates the phenomenon observed in HER2+ breast tumors. The data also support the use of PARPi treatment in a non-DNA repair context to ultimately advance the knowledge of PARP1 biology.

Let-7a's role in the regulation of PARP1 protein levels

The initial objective of this study was to identify the mechanism behind the enhanced levels of PARP1 detected in HER2+ breast cancer tumors to ultimately explain the upstream mechanism behind PARPi susceptibility. HER2+ breast cancer tumors were previously reported to contain elevated levels of the PARP1 protein in our laboratory (3). First we assessed whether a correlation between HER2 and PARP1 protein expression exists in HER2+ breast cancer cells. We determined that HER2 knockdown in two native HER2+ breast cancer cells and overexpression in a non-HER2 overexpressing cell line both altered PARP1 protein levels. We also reported that HER2+ breast cancer cells and patients with HER2+ breast tumors both express low levels of the let-7a miRNA. In this study, let-7a was also determined to regulate PARP1 expression levels and ectopic let-7a expression inhibits cell proliferation in HER2+ breast cancer cells. These data suggest the let-7a could be a potential therapeutic target for HER2+ breast cancer tumors and should be investigated further. To answer this question we could stably express the let-7a mimic in HER2+ breast cancer cells and assess tumor growth *in-vivo.* It would be important to further pursue this research avenue, if we determine that restoring let-7a expression in HER2+ breast cancers inhibits tumor growth.

Several groups have reported that HER2 status alters the expression levels of the other let-7 family members (62,63). Our NanoString miRNA profiling experiment also revealed similar results. By using the publically available database on the microRNA.org website we determined that these let-7 family members also contain high predicted binding scores to the 3'UTR of PARP1. In future studies, we would like to assess the role of these microRNAs in the regulation of PARP1 expression by performing similar studies

that were used in chapter 2. It would also be interesting to test whether the regulation of PARP1 expression is more strongly impacted by the combination of two or more let-7 family members or other microRNAs. Further, we would like to test whether another let-7 miRNA targets PARP2, the other PARP protein involved in DNA repair whose expression was not altered by ectopic let-7a expression. Another experiment worth pursuing is to examine whether the loss of expression of various let-7 miRNAs promotes invasion or migration to ultimately stimulate metastasis in HER2+ breast cancer cells. This study could expand the use of let-7 as a biomarker both in early and metastatic HER2+ breast cancer patients.

One limitation of our study was the inability to identify the regulation of let-7a gene expression in HER2+ breast cancers. Specifically, it is important to consider whether HER2 is directly or indirectly modulating a downstream signaling protein to ultimately regulate let-7a expression. These findings could help narrow down possible druggable proteins or identify potential biomarkers for PARPi treatment in HER2+ breast tumors. A possible factor that may regulate let-7a is c-myc. Specifically, c-myc binds to the promoter and enhancer regions in a cluster that includes the following *let-7* family members: *let-7a, -7f, and -7d.* However, *let-7a* also targets c-myc and results in a feedback loop between c-myc and *let-7 (64).* Additionally, co-amplification of the *HER2* and c-*myc* gene is correlated with aggressive breast cancer disease (65). Future studies will be essential to determine the role of c-myc in the regulation of let-7a in HER2+ breast cancer tumors (66). Phosphorylated extracellular signal-regulated kinase (p-ERK), a downstream protein activated by the HER2 receptor, also regulates let-7a levels. Specifically, p-ERK increases the expression of the TAR RNA-binding protein (TRBP),

an enzyme that has been shown to be involved in microRNA biogenesis and RISC assembly, which then suppresses let-7a expression (67). On the other hand, the expression levels of the let-7f family member are directly regulated by p-ERK (63). Lin28A and Lin28B have also been reported to negatively regulate *let-7*. The regulation mechanisms differ among each protein, but they ultimately prevent the biogenesis of these family members. Lin28A binds to the *pri-let-7* and helps translocate it from the nucleus to the nucleolus in order to prevent Drosha mediated cleavage. Lin28A is also shown to assist terminal uridlyl transferases (TUTases) in repressing *let-7* expression. The mechanism by which Lin28B represses *let-7* expression is not fully understood and needs to be studied further; however, it is known that this mechanism is TUTase-independent(56). In addition, NF-κB has been reported to regulate let-7a by activating the repressor of this miRNA called Lin28B. The let-7a miRNA was also reported to target the NF-κB target gene, IL-6. Together, these data suggest that a positive feedback loop exists between NF-κB, Lin28B, let-7a, and IL-6 and should be investigated further in our model system because HER2+ breast cancer cells have elevated NF-κB activity (68).

The concept of therapeutic targeting of miRNAs has recently gained a lot of the attention in the field of cancer research. Therapeutic miRNA mimics, also referred to as replacement therapy, have many advantages as compared to current clinical cancer agents. One advantage of using miRNA mimics in the clinic is that they are highly specific, stable, and are well tolerated because they are not immunogenic (69). Another advantage is that miRNAs can cross the blood-brain barrier (70).

Despite several advantages of miRNA, there are some challenges that need to be considered when developing these therapeutic miRNAs. One very important challenge is

the off-target effects associated with ectopic expression. As I mentioned in the introduction, miRNA can be partially complementary to more than one mRNA. This allows a miRNA to repress the expression of multiple genes at once. For this reason, the increase in let-7a levels could not only impact the expression of PARP1 but could also alter the expression levels of other proteins (69). Regardless of these challenges, there is currently an open phase 1 clinical trial that is recruiting patients to address the safety of a let-7 mimic in non-small cell lung cancer patients and draws hope for the advancement of this treatment strategy.

PARP inhibition and the HER2 amplicon

Additionally, our previous data suggest that HER2 itself was sufficient to confer sensitivity to PARPi. One fact to consider is that patients with the HER2+ subtype of breast cancer contain elevated expression levels of genes located within the HER2 amplicon and these genes may also confer sensitivity to PARPi (8). Twenty-seven other genes were determined to be co-amplified along with HER2, including: StAR-related lipid transfer domain 3 (*STARD3),* growth factor receptor-bound 7*(GRB7),* proteasome 26S subunit and non-ATPase 3 *(PSMD3),* and PER1-like domain 1 (*PERLD1)*. These genes are important in cellular proliferation in HER2+ breast cancer cells and knockdown of these gene levels along with HER2 induced a decrease in cell viability (71). GRB7 siRNA alone has been revealed to decrease cellular proliferation in the parental BT-474 and SKBR3 HER2+ breast cancer cells lines but did alter cell viability in their trastuzumab resistant counterparts (72). Topoisomerase II, a protein involved in DNA replication, is another gene amplified on this locus (73). In a preclinical study, the

combination of topoisomerase inhibitor C-1305 and a PARP inhibitor enhanced cytotoxicity in BRCA 1/2 wild-type cells (74). These results suggest that it could be efficacious to inhibit other genes located on the HER2 amplicon along with PARP1 to ultimately target HER2+ breast cancer cells. Additionally, it would be important to study whether these genes regulate protein synthesis of PARP1.

The role of PARP1 in NF-κB mediated gene expression

The second goal of this dissertation was to examine the mechanism by which PARP1 regulates NF-κB mediated activity in HER2+ breast cancer cells. In chapter 3, we report that both PARPi and PARP1 knockdown altered NF-κB activity. We also present evidence showing that both PARP1 expression and activity alters the expression levels of IL-8, a NF-κB regulated gene. However, PARPi decreased the IL-8 mRNA and protein levels to a lesser extent than treatment with PARP1 siRNA. Based on our findings presented in this dissertation, we postulate that PARP1's expression is required for its co-activator function. This topic remains very controversial in the field due to the contradicting results seen in different model systems and warrants further investigation. Our studies depict that suppression of PARP1 levels decreased the recruitment of the NF-κB subunit, p65, to the promoter of *IL-8*. However, we failed to examine the effect of p65 recruitment to the *IL-8* promoter after inhibiting the activity of PARP1 in HER2+ breast cancer cells. We are currently working on addressing this issue by using the CRISPR-Cas9 gene editing technology to inhibit PARP1's catalytic activity.

PARP trapping mechanism is another method that could explain PARPi induced cytotoxicity. It explains that PARPi traps the PARP1 protein onto the DNA lesion that is

then transformed into a more toxic lesion in the cell (75). Veliparib was recently reported as a poor PARP-trapping agent (76,77). In chapter 2 and 3, we observed that suppression of PARP1 expression by PARP1 siRNA or the let-7a microRNA both inhibited cell proliferation in HER2+ breast cancer cells to a similar extent as veliparib. These results suggest that PARP1 activity may not be required for its co-activator function of NF-κB and that PARPi could be inhibiting PARP1's other cellular functions independent of its trapping ability to ultimately induce cytotoxicity in HER2+ breast cancer cells. Further, one limitation of our study was to examine whether more potent PARP inhibitors have similar effects on NF-κB transcriptional activity. These data would help decipher whether PARP1 expression or activity are required for its co-activator function. Further, there is no clinical data that supports the trapping mechanism and should be investigated further in future clinical trials.

There are many questions left answered from this study and should be investigated further in future studies to thoroughly decipher the downstream mechanism behind this phenomenon. First, it would be beneficial to assess the role of PARP1 expression and activity in regulating NF-κB transcriptional activity in the context of other NF-κB target genes. For instance, this study identified that the expression levels of other NF-κB target genes are differently altered by PARP1 knockdown using our nCounter analysis system. Second, PARP1 has also been shown to interact with the other NF-κB transcription called p50 (41). Further, *in-vitro* studies are needed to test the role of PARP1 on the recruitment of p50 to the IL-8 promoter. Finally, PARP2 is targeted by PARP inhibitors and its role in NF-κB mediated transcription should be examined in future studies.

Another avenue worth pursuing is the combination therapy of a PARP inhibitor with a NF-κB inhibitor. HER2+ breast cancer cells have elevated PARP1 levels and increased NF-κB activity, and we question whether NF-κB activation would cause resistance to PARPi in these cells. One mechanism behind resistance to PARPi was upregulation of the NF-κB signaling and one method of overcoming this resistance was NF-κB inhibition (78). Further pre-clinical and clinical studies are necessary to address whether both PARP and NF-κB signaling pathways should be inhibited in HER2+ breast cancer cells.

PARP inhibition in HER2+ breast cancer

The final goal of this dissertation was to evaluate whether PARPi susceptibility is retained in HER2+ trastuzumab resistant tumors. We previously published that PARPi can be efficacious in HR proficient, HER2+ breast cancer cells (2). In this dissertation, we present evidence that the HER2+ trastuzumab resistant breast cancer cell lines retain sensitivity to a similar extent as the parental breast cancer cell lines. Further, both PARPi and PARP1 knockdown induce cytotoxity in HER2+ trastuzumab resistant breast cancer cells. These data introduce a novel clinical application for PARP inhibitors in a different cell context. However, it remains to be determined whether this treatment strategy would be effective in the clinic. Our group is currently working on initiating a clinical trial to test this paradigm. Prior to patient recruitment, it is important to determine if this regimen would be used as a monotherapy or combined with a HER2-targeted agent. The European School of Oncology suggests that patients who have completed one round of treatment with trastuzumab and have a progressing breast cancer disease should continue receiving

trastuzumab along with a cytotoxic agent. They also suggest that lapatinib could be used in exchange for trastuzumab (79). This guideline also supports the evidence seen in the literature where parallel resistance mechanisms are activated in trastuzumab resistant tumors. An example of this phenomenon, is upstream HER2 signaling is re-activated in trastuzumab resistant breast cancer cells to promote proliferation (18). Ultimately, we propose that reoccurring resistance mechanisms would be impeded by inhibiting HER2 signaling with a HER2-targeted agent.

It is equally important to determine the appropriate HER2-targeted agent and PARP inhibitor that could be used together in a combination therapy for this patient population. We have planned to begin testing these combination therapies in *in-vivo* studies before we translate this regimen into the clinic. Additionally, the mini-tumor bioassay system could be used as an initial experiment to assess the efficacy of these different treatment combinations. This assay platform allows for rapid and precise results in which tumor cells are allowed to grow as mini-tissues as opposed to growing on plastic, as in cell culturing methods. It is also reported that biomarker and gene expression results are very similar to those obtained from *in-vivo* studies. By using this cost-effective assay system, we hope to determine the best combination therapy to perform *in-vivo* follow-up experiments.

Moreover, it is important to determine biomarkers that assist in identifying specific HER2+ breast cancer patients that would most strongly benefit from PARPi treatment. As breast cancer is a heterogeneous disease and not all patients respond to cancer therapies in a similar manner. The HER2+ breast cancer patient tumors and cell lines we analyzed in our experiments are heterogeneous despite being classified under the

same subtype of breast cancer. For example, not all HER2+ breast cancer tumors express low let-7a levels. We also observed that there is a variable degree of PARP1 and let-7a expression among the HER2+ breast cancer patient tumors and cell lines we used in these studies. Therefore we suggest that HER2+ breast tumors that express elevated PARP1 protein levels and low let-7a gene level could benefit the most from PARPi. It will be important to determine whether a threshold expression level is needed to respond to PARPi. These types of biomarker studies are very important in translational cancer research and drug discovery.

Conclusions

The data we presented in this dissertation reveal potential non-DNA repair mechanisms behind PARPi susceptibility in HER2+ breast cancer cells. Our results demonstrate that the let-7a miRNA binds to the 3'UTR of PARP1 and regulates PARP1's expression in HER2+ breast cancer. These data explain the upstream mechanism behind the enhanced PARP1 levels observed in both HER2+ breast cancer cells and patients. In this dissertation, we also show that these elevated PARP1 protein levels activate downstream NF-κB activation in HER2+ breast cancer cells while PARP1 knockdown inhibits NF-κB mediated transcription. These results suggest that inhibition of NF-κB signaling could be a possible downstream mechanism behind PARPi sensitivity. By performing the additional mechanistic studies mentioned above, we may uncover novel targets that will influence PARPi sensitivity. Finally, these studies will ultimately help develop future clinical trials and identify novel biomarkers for PARPi treatment. Overall we hope these findings expand the patient population that could benefit from PARPi

treatment by targeting PARP1's transcriptional regulatory roles instead of solely inhibiting its DNA repair roles in cancer cells.

GENERAL REFERENCE LIST

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APPENDIX A

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 24-Nov-2015

TO: Yang, Shih-Hsin

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 24-Nov-2015.

Protocol PI: Yang, Shih-Hsin

Title: HER2 Overexpression Confers Susceptibility to PARP Inhibition

Sponsor: American Association for Cancer Research

Animal Project Number (APN): IACUC-09983

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 05-Apr-2016

TO: Yang, Shih-Hsin

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 05-Apr-2016.

Protocol PI: Yang, Shih-Hsin

Title: DNA Repair Independent Mechanisms of HER2+ Tumor Sensitivity to PARP Inhibition

Sponsor: Komen (Susan G.) Breast Cancer Foundation

Animal Project Number (APN): IACUC-10129

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | **Mailing Address:**
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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 21-Oct-2015

TO: Yang, Shih-Hsin

FROM:

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 21-Oct-2015.

Protocol PI: Yang, Shih-Hsin

Title: Targeting HER Pathways to Render HER2+ Breast Cancer Cells Susceptible to PARP Inhibition

Sponsor: National Cancer Institute/NIH/DHHS

Animal Project Number (APN): IACUC-09241

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | **Mailing Address:**
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APPENDIX B

IRB APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on January 24, 2017. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator: YANG, SHIH-HSIN

Co-Investigator(s):

Protocol Number: **X101214005**

Protocol Title: *Analysis of Molecular Markers in Breast Cancer*

The IRB reviewed and approved the above named project on 10-5-15. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 10-5-15

Date IRB Approval Issued: 10-5-15

IRB Approval No Longer Valid On: 10-5-16

HIPAA Waiver Approved?: Yes

Marie L. Daas

Expedited Reviewer
Member - Institutional Review Board
for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

PI: YANG, SHIH-HSIN
Protocol # X101214005

UAB IRB Approval of Waiver of Informed Consent and/or Waiver of Patient Authorization

☑ **Approval of Waiver of Informed Consent to Participate in Research.** The IRB reviewed the proposed research and granted the request for waiver of informed consent to participate in research, based on the following findings:

1. The research involves no more than minimal risk to the subjects.
2. The research cannot practicably be carried out without the waiver.
3. The waiver will not adversely affect the rights and welfare of the subjects.
4. When appropriate, the subjects will be provided with additional pertinent information after participation.

Check one:
- ☑ **and** Waiver of Authorization (below)
- ☐ **or** Waiver of Authorization (below)
- ☐ Waiver of Authorization not applicable

☑ **Approval of Waiver of Patient Authorization to Use PHI in Research.** The IRB reviewed the proposed research and granted the request for waiver of patient authorization to use PHI in research, based on the following findings:

1. The use/disclosure of PHI involves no more than minimal risk to the privacy of individuals
   i. There is an adequate plan to protect the identifiers from improper use and disclosure.
   ii. There is an adequate plan to destroy the identifiers at the earliest opportunity consistent with conduct of the research, unless there is a health or research justification for retaining the identifiers or such retention that is otherwise required by law.
   iii. There is an assurance that the PHI will not be reused or disclosed to any other person or entity, except as required by law, for authorized oversight of the research study, or for other research for which the use or disclosure of PHI would be permitted.
2. The research cannot practicably be conducted without the waiver or alteration.
3. The research cannot practicably be conducted without access to and use of the PHI.

—OR—

☐ **Full Review**

The IRB reviewed the proposed research at a **convened meeting** at which a majority of the IRB was present, including one member who is not affiliated with any entity conducting or sponsoring the research, and not related to any person who is affiliated with any of such entities. The waiver of authorization was approved by the majority of the IRB members present at the meeting.

Date of Meeting

Signature of Chair, Vice-Chair or Designee

Date

☑ **Expedited Review**

The IRB used an **expedited review procedure** because the research involves no more than minimal risk to the privacy of the individuals who are the subject of the PHI for which use or disclosure is being sought. The review and approval of the waiver of authorization were carried out by the Chair of the IRB, or by one of the Vice-Chairs of the IRB as designated by the Chair of the IRB.

10-5-15
Date of Expedited Review

Marilyn Davis
Signature of Chair, Vice-Chair or Designee

10-5-15
Date

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Investigator IRB Training By Project

Report Date: 07/01/2015

Protocol: X101214005 Link Number: **APPROVED as of 10/07/2014**

Training Description	Course Type	Credit Hours	Date
COOPER, TIFFINY			
DE LOS SANTOS, JENNIFER F			
FORERO-TORRES, ANDRES			
KEENE, KIMBERLY SUE			
STANLEY, JENNIFER			
WIELGOS, MONICKA E			
YANG, SHIH-HSIN			

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