Trastuzumab resistant HER2+ breast cancer cells retain sensitivity to poly (ADP-ribose) polymerase (PARP) inhibition

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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, silencing 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

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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.

Keywords

Human epidermal growth factor receptor 2; breast cancer; trastuzumab resistance; PARP inhibitor; IL-8

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 (4).

In the last decade, poly (ADP-Ribose) polymerase inhibitors (PARPi) have been shown to be a promising therapeutic agent, especially in patients with homologous 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 (9). 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. 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 (10). 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 silencing of PARP-1 decreased p65 recruitment to the IL-8 promoter, which resulted 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, UACC-812, and SKBR3 parental and trastuzumab resistant HER2+ breast cancer cell lines were used in this study and were previously characterized in two other studies (11,12). The BT-474 and UACC-812 trastuzumab resistant breast cancer cells were also cultured with 50 μg/ ml of trastuzumab and were kindly donated by Dr. Rachel Schiff and C Kent Osborne (Department of Medicine, Baylor College of Medicine). The SKBR3 trastuzumab resistant cells were cultured with 10 μg/ ml of trastuzumab and were kindly donated by Dr. Francisco J. Esteva (Department of Medicine, NYU). All cells tested negative for mycoplasma and were validated by western analysis for HER2. However, cell authentication was not conducted. Further, the cell lines were cultured for no more than 20 passages after thawing the frozen cells. All three trastuzumab resistant breast cell lines were also verified for resistance to trastuzumab. Veliparib (ABT-888) was obtained from Enzo Life Sciences (catalog #ALX-270-444) and olaparib (AZD-2281) was purchased from LC Laboratories (catalog #O-9201). Both drugs were 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). Another PARP-1 siRNA from Sigma-Aldrich(#NM_001618, SASI_Hs01_00159524) was utilized to confirm siRNA studies. 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 PARP1 knockdown. After four days of treatment, the cells were washed with 1× 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 triplicate 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 mg/kg of ABT-888 by oral gavage for four weeks. At this dose in mice, it has been reported to result in plasma Cmax of 35μM and is considered to be the monotherapy maximum tolerated dose (MTD) dose in mice (18). Tumor size was measured triweekly during the course of the treatment with a caliper. The tumor volume was calculated with the following formula: $[(width)^2 \times length]/2$ (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 and 7.0b (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 (12) 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. Similarly, the PARPi MK-4827(Niraparib) also reduced the survival fraction of HER2+ trastuzumab resistant and parental breast cancer cell lines (Supplementary Figure S1).

To validate these results, we next established 3D microtumors (Vivo Biosciences, Supplemental Methods) using the BT-474 TR cells. As shown in Supplementary Figure S2, ABT-888 reduced the tumor viability of BT-474 TR microtumors cultured with trastuzumab. We also 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. There was no significant difference in body weight observed in the ABT-888 treated group compared to the vehicle control group(Supplementary Figure S3C). To confirm that the decrease in tumor growth was due to the inhibition of PARP-1's enzymatic activity, we examined PAR protein levels in tumors harvested from both control and ABT-888 treated animals. As shown in Supplemental Figure S3A-B, PAR levels were decreased in ABT-888 treated animals compared to control animals (Supplemental Methods, Supplementary Figure S3A-B). These results demonstrate 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, which reduced PARP-1 but not PARP2 levels (Figures 2A and 2B). Ninety-six hours after PARP-1 knockdown, cell proliferation was reduced by more than 50% in both the BT-474 TR (Figure 2C) and UACC-812 TR (Figure 2D) breast cancer cell lines. These results were confirmed with another PARP-1 siRNA from Sigma-Aldrich (Supplementary Figure S4). Next, we tested whether silencing PARP-1 induced cellular apoptosis. As shown in figures 2E and 2F, 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 (Supplemental Methods, Supplementary Figures S5).

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 (19). 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 PARP1 knockdown. Western blot analysis following PARP-1 knockdown via two different PARP-1 siRNAs revealed that the level of IKKα protein was decreased in the BT-474 TR and SKBR3 TR HER2+ breast cancer cell lines (Figure 3B and C, Supplementary Figure S6). These results indicate that PARP-1 suppression inhibits 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 (20,21). 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, 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).

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 qRT-PCR 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 (22,23). 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, ABT-888 and AZD-2281 also decreased the gene expression levels of *IL-8* in the BT-474 TR cells (Figure 4D and Supplementary Figure S7D).

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-D).

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 (24). PARP-1 has been shown to promote chromatin decondensation by PARylating histones or binding directly to nucleosomes (25,26). Several other studies have shown that PARP-1 is involved in angiogenesis (27). Recently there has been an increased interest in targeting transcriptional factors with PARPi in various types of cancer models (28–30). 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.

Various mechanisms of resistance to trastuzumab have been described in the literature (30). Examples include overexpression of insulin-like growth factor I receptor (11), compensatory activation of other HER receptors (12), and reactivation of the HER2 signaling pathway. Since these pathways activate similar downstream effectors, such as NF-kB, it is not surprising that TR cells retained sensitivity to PARP inhibition.

PARP inhibitors such as ABT-888 target both PARP-1 and PARP-2 members of the PARP family that are involved in DNA repair (32). 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 (33). In this study, we focused mainly on the PARP-1 protein since we recently discovered that HER2+ breast cancers express increased levels of nuclear PARP-1 and phospho-p65 compared to non-HER2 overexpressing breast cancers (34). No significant differences were found with PARP-2 protein levels (34). 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 (35). 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 (20), androgen receptor (28), and estrogen receptor (36). 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 enzymatic activity in NF-κB dependent gene expression is controversial. Multiple studies have reported that PARP-1's enzymatic activity is independent of its co-activator function of NF-κB (21,37). However, we observed a reduction in the gene expression levels of a NF-κB target gene, *IL-8*, after PARPi in TNF-α stimulated cells, suggesting here that PARP-1 enzymatic activity may indeed play a role in the its coactivation function of NF-κB (Figure 4D and Supplementary Figure S7D). 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 (21,37,38). 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 (Figures 4–6). 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 (39). IL-8 has also been observed to play a role in influencing trastuzumab resistance (40). For example, an inflammatory feedback loop in HER2+ breast cancers cells has also been reported to activate cancer stem cell activity and trastuzumab resistance by increasing IL-8 levels (41). 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 (42). Previous studies have shown that genetic and pharmacologic inhibition of PARP-1 reduced expression of BRCA1 and Rad51, two other proteins involved in HR. These results were also associated with a decrease in HR (43). Our results show that PARP-1 knockdown decreased the levels of *BRCA2* mRNA, but did not alter BRCA2 protein levels (Supplementary Figures S8A and S8B). We have also previously shown that HER2+ breast cancer cells do not harbor a basal or induced HR deficiency (9). 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 (44,45). A possible explanation behind these findings could be NF-κB's complex role in multiple cellular pathways (46). Several studies have also indicated that in a single cell, NF-κB can have both pro- and anti-apoptotic functions (47). These results will be investigated in future studies.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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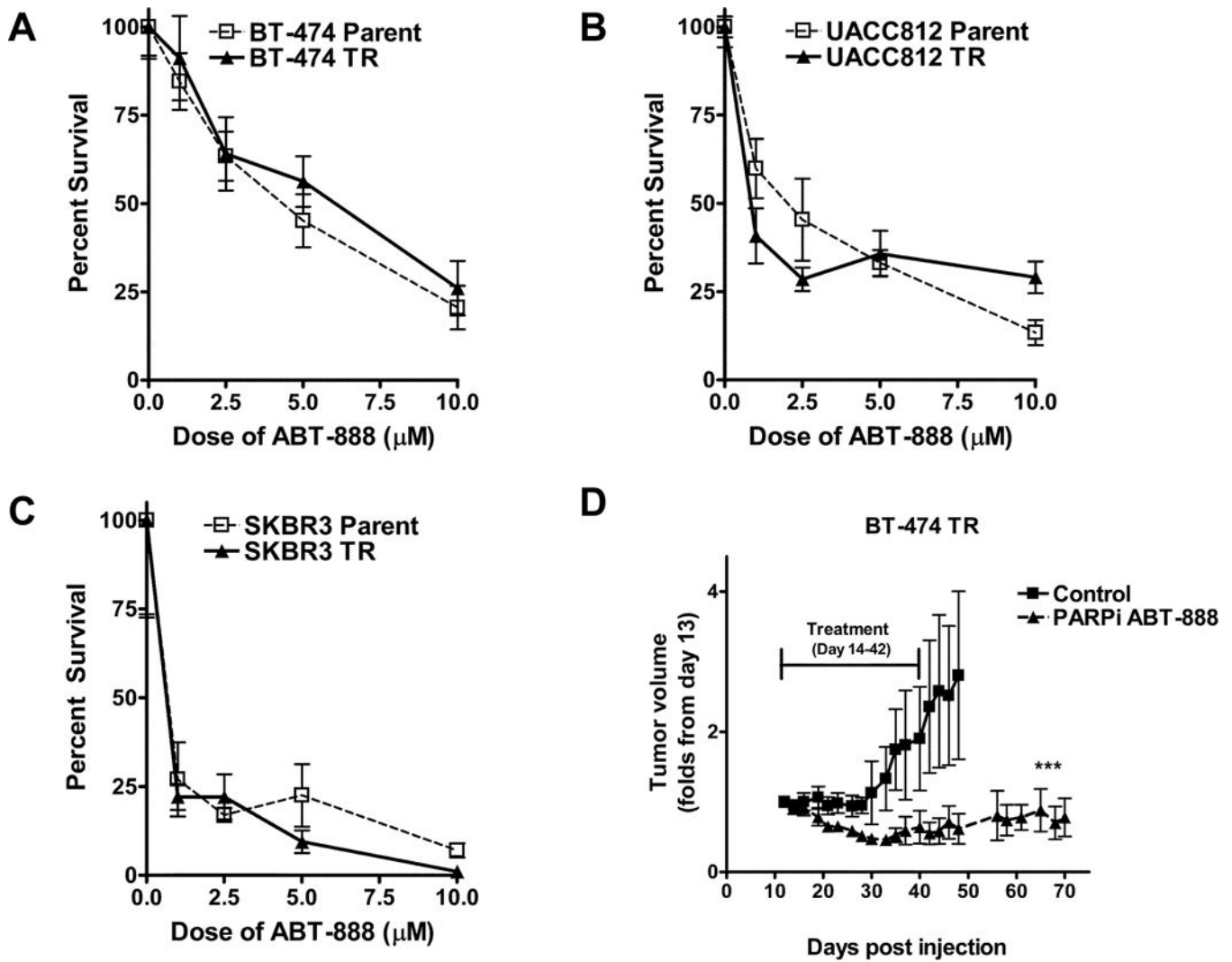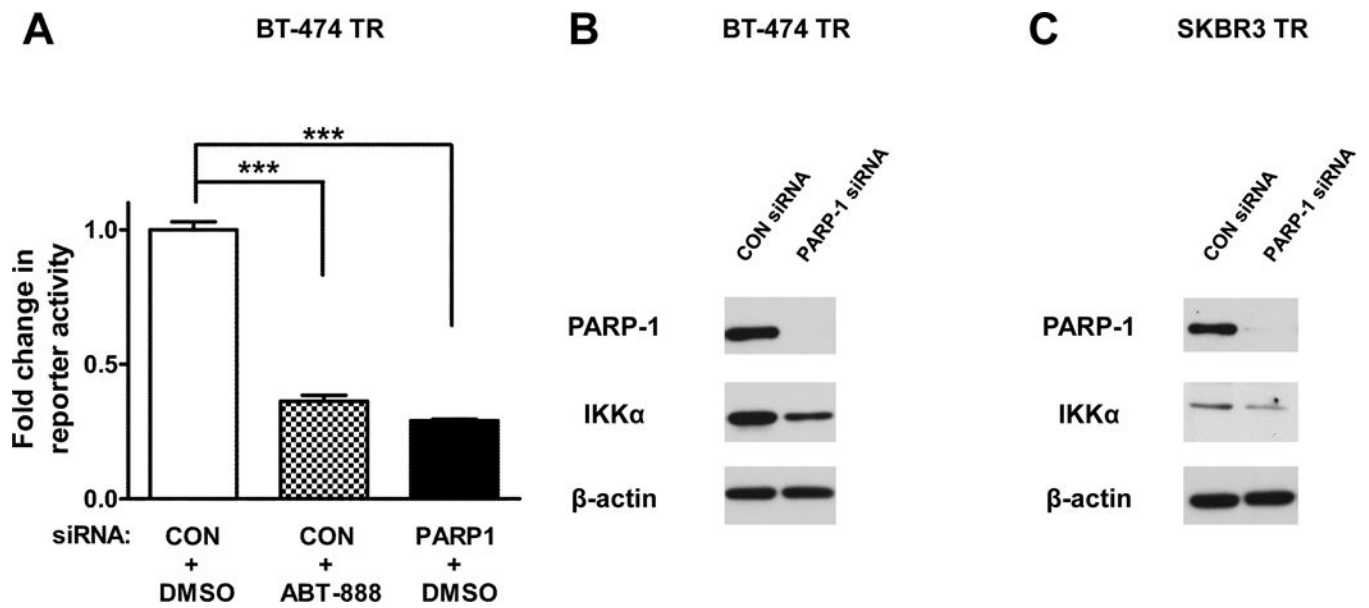
Figure 1. HER2+ trastuzumab resistant breast cancer cells are sensitive to PARP inhibition (ABT-888) *in-vitro* and *in-vivo*

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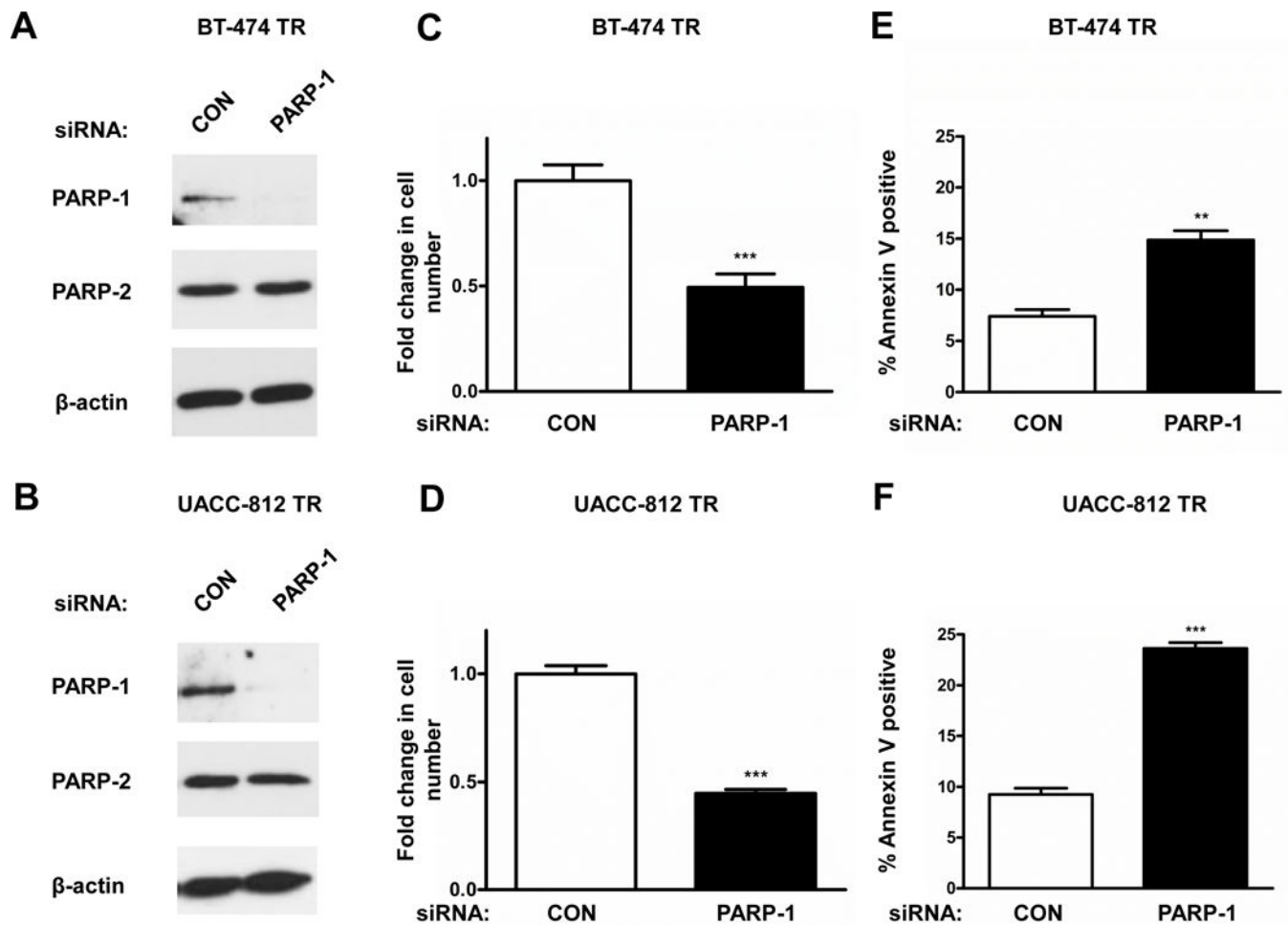
Figure 2. PARP-1 siRNA reduces cell proliferation and induces apoptosis in HER2+ trastuzumab resistant breast cancer cell lines

BT-474 TR and UACC-812 TR were transfected with 20 nM of control (CON) or PARP-1 siRNA for 96 hours. Knockdown of PARP-1 protein expression levels was verified via Western Blot analysis. β-actin was used as a loading control (**A and B**). Data shown are representative immmunoblots from one of three independent experiments. Following PARP-1 knockdown, cell counts were obtained via cellular proliferation assays (**C and D**). Apoptosis was assessed with FACS analysis using propidium iodide and Annexin V staining (**E and F**). The representative figures shown are from one of three independent experiments performed in (**C and D**) quadruplicate or (**E and F**) triplicate. ***p 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 three independent experiments performed in triplicate. **(B and C)** Western blot of PARP-1, IKKα, and β-actin after knockdown 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.
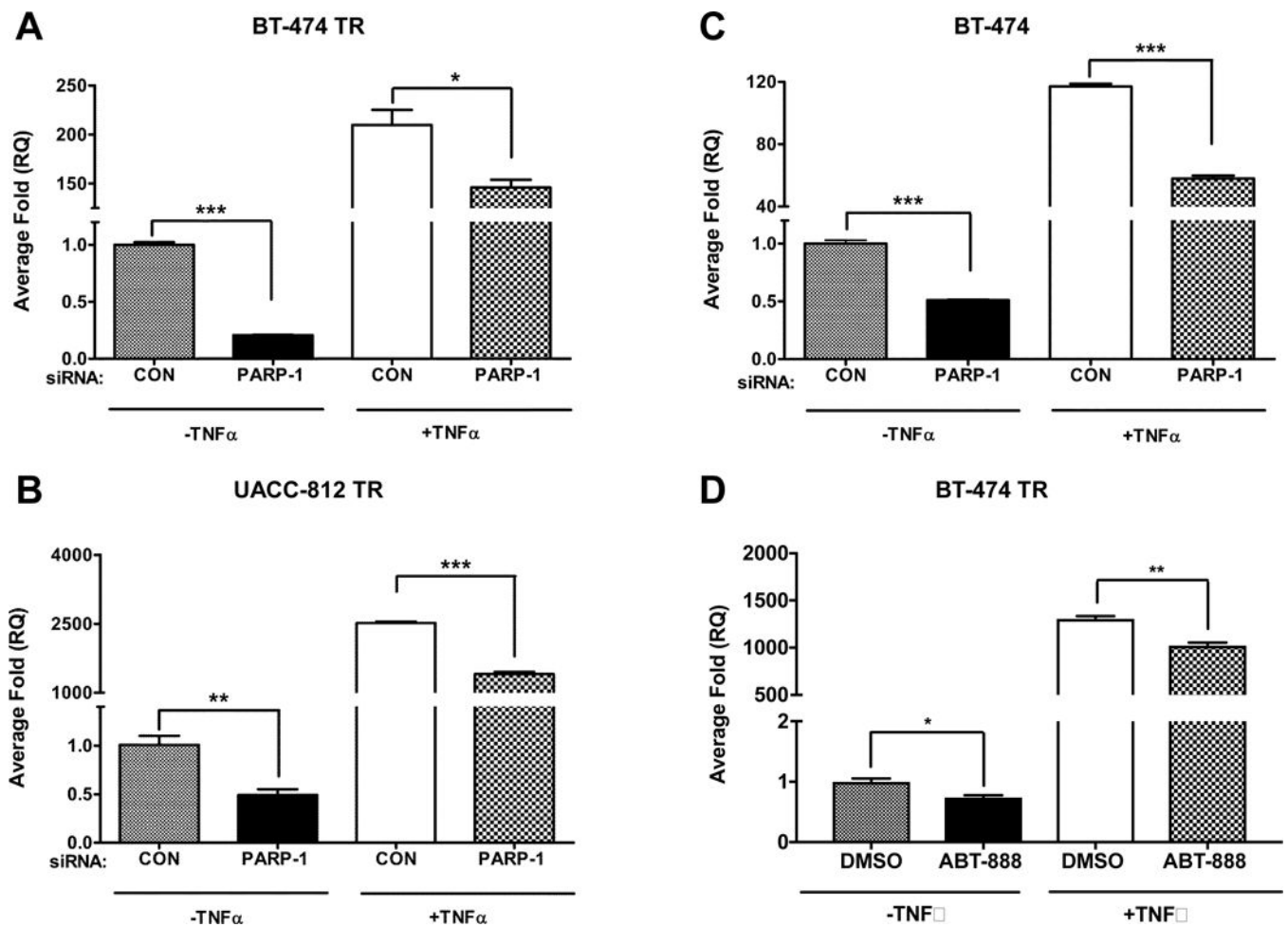
Figure 4. PARP-1 knockdown inhibits IL-8 expression in both HER2+ parental and trastuzumab resistant breast cancer cells

Quantitative real-time RT-PCR analysis was performed with total RNAs isolated from **(A)** BT-474 trastuzumab resistant, **(B)** UACC-812 trastuzumab resistant, and **(C)** BT-474 parent 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 three 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 $***p<0.001$
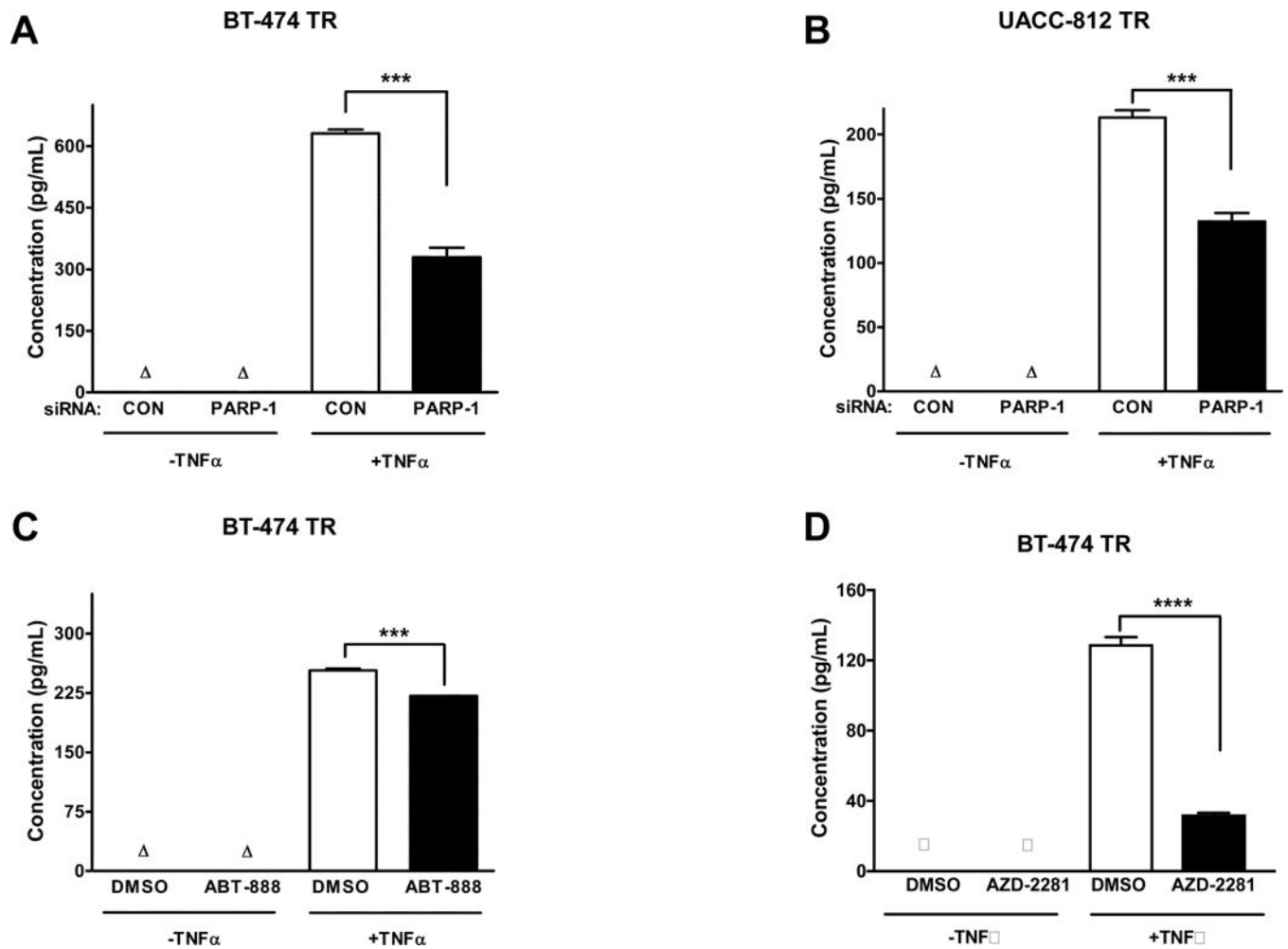
Figure 5. PARP-1 knockdown inhibits IL-8 protein expression
(A) BT-474 TR cells were transfected with control (CON) or PARP-1 siRNA for 48 hours and (B) UACC-812 TR cells were transfected for 24 hours. Cells were serum-starved for 18 hours, and then stimulated for 24 hours with 10 ng/mL of TNF-α. (C) BT-474 TR cells were serum-starved and treated with DMSO or 10 μM ABT-888 for 24 hours or (D) 10 μM AZD-2281 for 72 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 three independent experiments. ***p 0.001
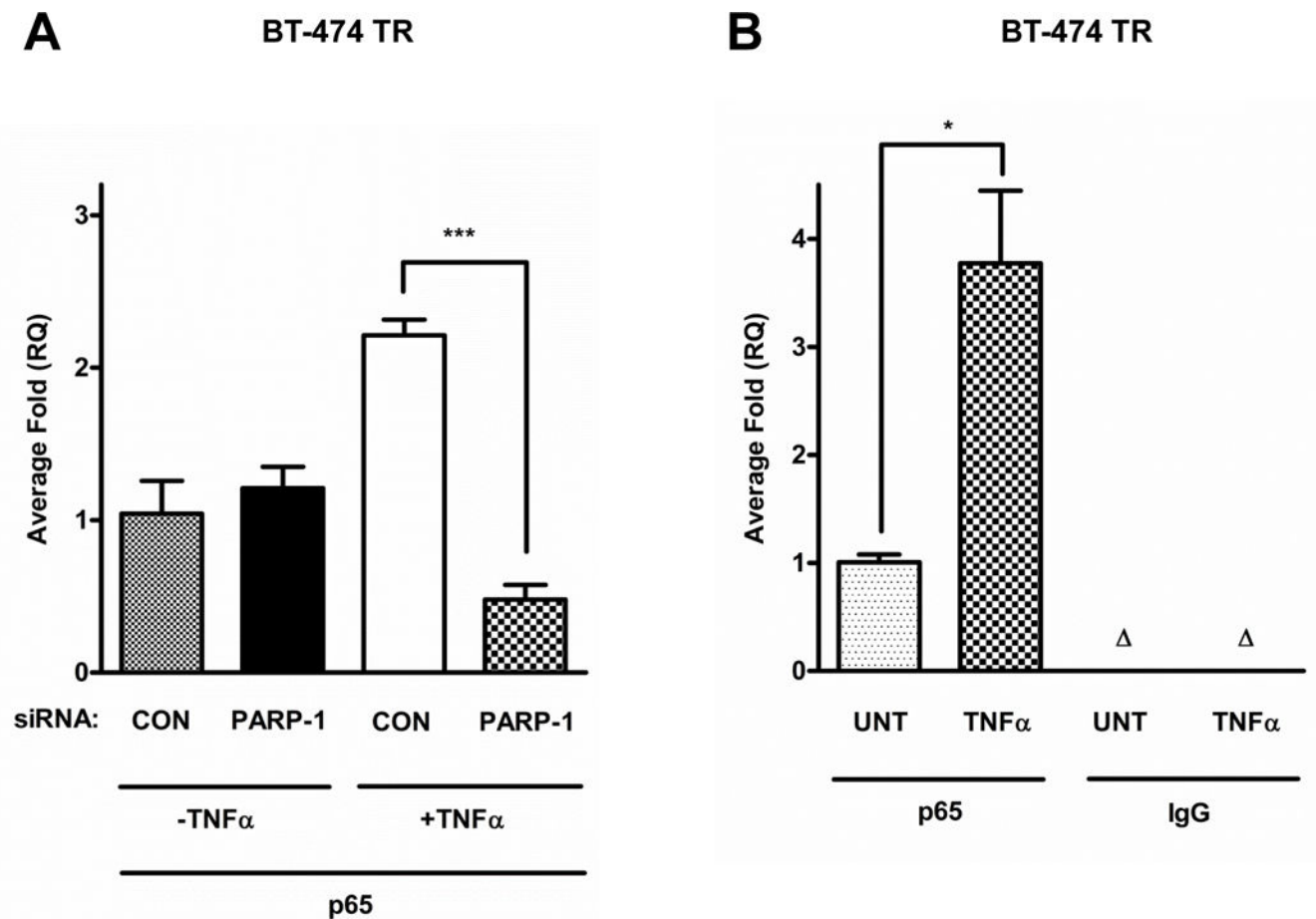
Figure 6. PARP-1 knockdown decreases p65 recruitment to the IL-8 promoter in HER2+ trastuzumab resistant cell lines

(**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 three 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

Table 1

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

NF-κB target genes:	CON siRNA:	PARP1 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

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 probe set. Shown in the table are the normalized NanoString counts along with the fold change values