Mechanisms of Cisplatin Resistance in Triple Negative Breast Cancer

By

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CHAPTER I

Introduction

Triple-negative breast cancer (TNBC) is defined by the lack of expression of the estrogen receptor (ER) and progesterone receptor (PR), and a lack of amplification of the HER2 gene. TNBC is a heterogeneous, highly aggressive type of breast cancer that has a worse prognosis in patients that do not achieve a pathologic complete response (pCR) with neoadjuvant treatment worse when compared to hormone receptor positive or HER2 amplified breast cancer[1]. Targeted therapies for TNBC are not yet standard of care and a few targeted therapies are just now being explored in the context of clinical trials. The difficulty in treating TNBC likely stems from the heterogeneity of the disease, which was first reported in a study from our laboratory[2]. Using gene expression microarray data, 587 TNBC cases were analyzed and six distinct subtypes of TNBC were identified based on their gene expression signatures and other genetic features[2]. TNBC was divided into six subgroups: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal/androgen receptor (LAR). In addition, key signaling pathways were identified that are altered and importantly targetable in the various TNBC subtypes, including the DNA repair, PI3K signaling, and androgen receptor (AR) signaling pathways[2].

In contrast to the poor prognosis most TNBC patients face, a subset of patients have tumors that respond well to neoadjuvant chemotherapy regimens and thus have improved outcomes. Results from several clinical trials highlight the success of treating early-stage TNBC in the neoadjuvant setting. A multi-center clinical trial of neoadjuvant anthracycline/taxane treatment found that 30% of TNBC patients achieved pathologic complete response (pCR) and that pCR was an indicator of event-free survival and overall survival[3]. In addition to anthracyclines and taxanes, the

intrastrand cross-linking agents cisplatin and carboplatin have proven to be efficacious[5,4]. A recent clinical trial showed that 22% of TNBC patients achieved a pCR when treated with single agent cisplatin[5]. A meta-analysis published earlier this year showed that the addition of a platinum agent to a more conventional anthracycline/taxane regimen raised the pCR rate from 32% to 48%[6].

There is increasing use of platinum agents for TNBC in the clinical setting and determining which patients have tumors with sensitivity to these agents is critical[3-5]. The heterogeneity of TNBC is likely one factor behind differential sensitivities. The BL1 subgroup is genomically unstable and exhibits high proliferation rates[2]. In a prior study, Lehmann et al. hypothesized that this subgroup would have highest sensitivity to cytotoxic chemotherapy and showed that cell lines representing this subtype were highly sensitive to cisplatin *in vitro* and when grown as xenograft tumors *in vivo*[2]. This hypothesis was also tested using clinical data in a study published by Masuda *et al.* in collaboration with our laboratory[7]. In collaboration with Dr. Steven Chen of the Vanderbilt Department of Biostatistics, the laboratory developed TNBCtype, an algorithm that uses gene expression profiles to assign TNBC tumors to one of the six subtypes[2,8]. Using TNBCtype, gene expression data and measurable pathologic response data were analyzed in a total of 130 patients from two clinical trials[6,9]. These patients had stage II/III TNBC and received an anthracycline and/or a taxane based therapy in a nedoadjuvant setting. It was determined that 52% of BL1 patients achieved a pCR while none of the BL2 patients had a clinical response. Similar response rates in the BL1 subtype were observed after partial analysis of the clinical data from a trial developed and led by Drs. Ingrid Mayer and Pietenpol and run through the Translational Breast Cancer Research Consortium (VICC-BRE-0904). TNBC patients with stage II/III TNBC were treated with a treatment regimen containing cisplatin and paclitaxel or cisplatin, paclitaxel, and everolimus. The pCR rate in the cisplatin/paclitaxel arm was 32% while the pCR rate in the cisplatin/paclitaxel/everolimus arm was 36%. Gene expression analysis of the pretreatment biopsies of the first twelve patients was

performed by a fellow graduate student in the lab (Bojana Jovanovic) and she discovered that patients with a basal-like subtype were more likely to respond to therapy with 4/4 basal-like tumors achieving a pCR or pathologic partial response (pPR).

Based on the increasing adoption of platinum based therapy in TNBC standard of care and the acquired resistance to this drug seen amongst many "recalcitrant" cancers, mechanisms of acquired cisplatin resistance merits study in order to provide subsequent lines of therapy for TNBC patients[10-12]. In ovarian cancer patients, the initial response rate to platinum based treatments is approximately 70%, but the five-year survival rate is only 15-20%[11]. Acquired resistance to cisplatin is also high in non-small cell lung cancer patients with 95% experiencing relapse following treatment[10]. Thus, understanding mechanisms of resistance in the TNBC setting is an important area of research investigation. We hypothesized that TNBC cell lines that exhibit sensitivity to cisplatin could be used to develop cisplatin resistant cell lines and these lines could be used to uncover mechanisms of cisplatin resistance in TNBC.

Chapter II

Materials and Methods

Cell Lines and Culture Conditions

MDA-MB-468 (American Type Culture Collection) and 293FT (American Type Culture Collection) cells were maintained in Dulbecco's Modified Eagle's Media [DMEM] (Gibco) and HCC-1806 (ATCC) cells were maintained in Roswell Park Memorial Institute [RPMI] + Glutamax (Gibco) medium. Each medium was supplemented with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin. Cells were maintained at 37 ºC in atmosphere containing 5% CO_2. Cell lines were routinely monitored for mycoplasma contamination.

Cisplatin resistant (CR) lines were created from the parental MDA-MB-468 and HCC-1806 cell lines. Normal growth medium was supplemented with 0.1 μM cisplatin (APP Pharmaceutical). Cells were passaged normally until growth rate was not impeded by the addition of cisplatin in the medium and the concentration of cisplatin was increased. Cells lines were determined to be cisplatin resistant when growth was unimpeded in at least 3 μM cisplatin and the IC_{50} values of the CR cell line was greater than the parental cell line at an amount that was statistically significant ($p < 0.05$ by student's t-test). CR cell lines were grown in media lacking cisplatin for 5-7 days before use in any experiment.

Flow Cytometry

Parental and CR HCC-1806 cell lines seeded in a 60 mm dish (Starstedt) at a density of 200,000 cells per dish. The cells were treated with 3 μM cisplatin and harvested at 24, 48, or 72 h

post treatment. An untreated control culture was plated at the same density and used for comparison. After the indicated incubation times in cisplatin media the cells were harvested with trypsinization and incubated in a propidium iodide cocktail (50 µg/ml propidium iodide (Sigma), 5 µg/ml RNase A, 0.1% Triton X-100, and 1 µg/ml sodium citrate for two hours. Cells were passaged through a 95 µm mesh filter and analyzed on a FACS Caliber (Becton-Dickinson) flow cytometer. Histograms were quantified using CellQuest Pro (Becton Dickinson).

Immunofluorescence

Parental and CR MDA-MB-468 and HCC-1806 cells were plated in 96-well plates at a density of 6,000 cells/well and 5,000 cells/well respectively. Following overnight attachment, media was changed to media containing cisplatin or normal growth media as a negative control. Following cisplatin treatment the cells were fixed in 4% formaldehyde (Thermo Scientific) for 10 minutes at room temperature. Cells underwent three phosphate-buffered saline (PBS) washes at room temperature. Cells were incubated in blocking buffer containing 1X PBS/5% normal goat serum (Gemini Bio Products)/0.3% Triton X-100 (EMD Millipore). Cells were incubated overnight in Anti-phospho H2AX (Ser139) primary antibody (JBW301, Millipore) overnight at 4ºC. Cells were washed three times in PBS and incubated in Alexa-Fluor 488 goat anti-mouse secondary antibody (Life Technologies) for 1h. Following three PBS washes, the cells were mounted SlowFade Gold Antifade Mountant with DAPI (Life Technologies). At least 100 nuclei were photographed at 40X magnification and quantification of γH2AX foci was performed using the CellProfiler program[13]. Integrated intensity is an arbitrary value that is produced using the number and intensity of foci.

Cell Viability Assays

Parental and CR MDA-MB-468 and HCC-1806 cells were plated in triplicate into a 96-well plate at densities of 1,000 cells/well and 3,000 cells per well respectively. Following overnight incubation media was replaced with media containing half-log dilutions of cisplatin from ranging 0.03-30 μM cisplatin. Following a 72 h incubation the media was replaced with media containing 10% Alamar Blue (Life Technologies). Cell viability was determined according to manufacturer's protocol using a Synergy Mx plate reader (Biotek). Graphical representations were generated using GraphPad Prism 4.0c. Values presented represent mean ± standard error or mean ± standard deviation as noted in figures.

Western Blot Analysis

Cells were pelleted via centrifugation following trypsinization. Cells were washed in PBS and pelleted with centrifugation. Protein lysates were harvested with a thirty minute incubation in Radioimmunoprecipitation assay (RIPA) buffer. Protein concentration was determined using the Bio-Rad Protein Assay kit according to the manufacturer's protocol. 30 μg of protein was resolved using sodium dodecyl sulfate polyacrylamide gel electrophorersis (SDS-PAGE) on a 10% (MEK/ERK experiments) or 12% (Caspase-14 experiments) gel. Following transfer to apolyvinylidene fluoride (PVDF) membrane, the membranes were blocked for 30 minutes in 5% milk diluted into tween-20/tris-buffered saline (TTBS). The following primary antibodies were used with a 4º C overnight incubation: β-Actin (I-19, Santa Cruz), Raf-B [BRAF] (C-19, Santa Cruz), Caspase-14 (8519, Cell Signaling), Phospho-p44/42 MAPK [Erk1/2] [Thr202/Tyr204] (9101 Cell Signaling), Phospho-p44/42 MAPK [Erk1/2] [Thr202/Tyr204] (L34F12, Cell Signaling), MEK1/2 (L38C12, Cell Signaling), Phospho-

MEK1/2 [Ser217/221], (9121, Cell Signaling) and Parp (9542, Cell Signaling). Following three TTBS washes the cells were incubated in the appropriate horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. After three additional TTBS washes the membranes were exposed to electrochemiluminescence buffer and protein was visualized with autoradiography.

Cell Transfection/Infection and RNAi

Parental and CR MDA-MB-468 cells were reverse-transfected with 20 nM Caspase-14 Silencer Select siRNA (s24128 and s24189, Life Technologies) or Silencer Select Negative Control No. 2 complexed with Dharmafect 3 lipid (Dharmacon). Parental and CR HCC-1806 CR were reverse-transfected with 5 nM Silencer Select siRNA against ERK1 (s11140), ERK2 (s11137) or Silencer Select Negative Control No. 2 complexed with Lipofectamine 2000 (Life Technologies) lipid. Cells were incubated in the media containing siRNA overnight before being replaced with normal growth media. Cells were harvested for Western blot analyses 72h post transfection.

Parental and CR MDA-MB-468 cell lines stably expressing a shRNA against Caspase-14 (Mission shRNA - TRC000425663, Sigma) or a non-targeting control (Mission shRNA, SHC202) were created as previously described. 48h post-infection cells were selected with 1 μg/mL puromycin (Sigma). After a 48h selection period the cells were maintained in growth media containing 0.5 μg/mL puromycin.

Chapter III

Results

Creation of cisplatin-resistant (CR) TNBC cell lines

Two cisplatin resistant (CR) TNBC cell lines were developed to study acquired cisplatin resistance. We chose cell lines representing the BL1 (MDA-MB-468) and BL2 (HCC-1806) TNBC subgroups, as these subtypes have shown the highest sensitivity to cisplatin *in vitro*[2]. A cisplatin resistance phenotype was achieved through growth of the cells over time in the presence of increasing concentrations of cisplatin (0.1μM to 3μM cisplatin) and establishment of IC_{50} values that had increased in a statistically significant manner (Figure 1).

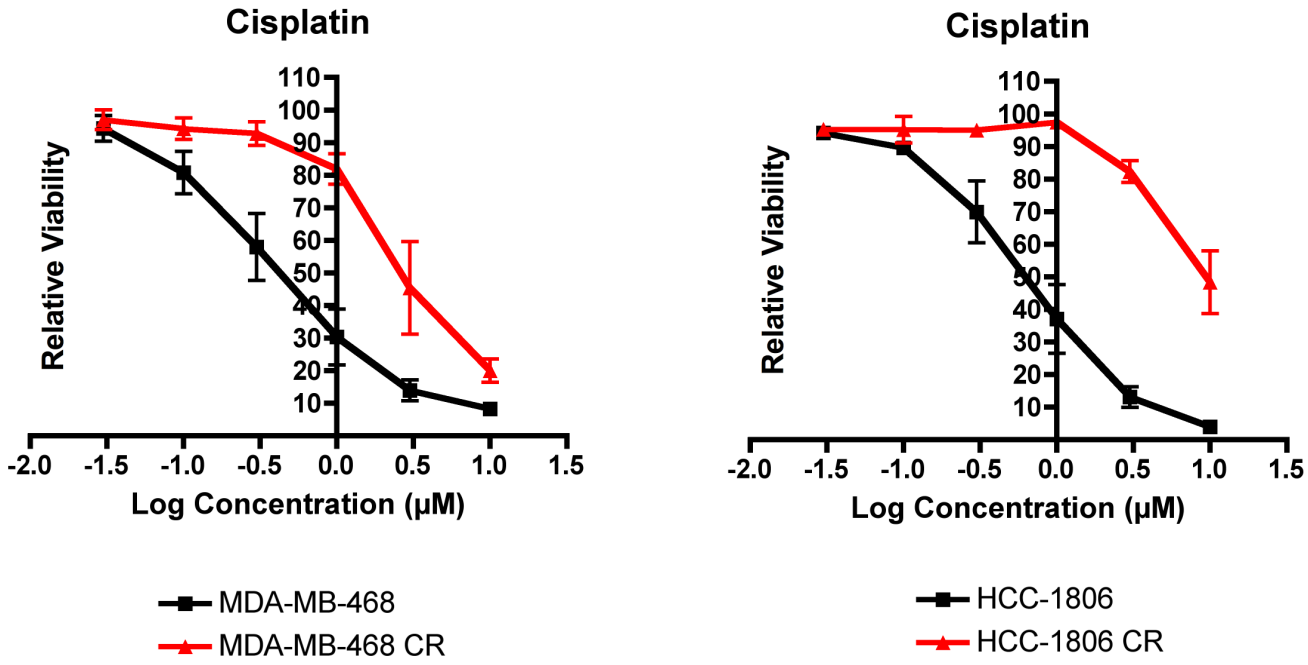
CR cell Lines Recover from Cisplatin Treatment and Do Not Undergo Apoptosis

Cell death from cisplatin often occurs in S-phase when the interstrand crosslinks produced from cisplatin-DNA adducts are encountered during DNA replication[14]. A differential in the cell cycle profiles of the CR cell lines may provide insight into the mechanism of cisplatin resistance in these cell line models. The parental and CR MDA-MB-468 and HCC-1806 cell lines were treated with cisplatin and their cell cycle profiles were assayed using flow cytometry. CR cells were grown in media without cisplatin for 5-7 days prior to the beginning of all experiments so that data could properly interpreted. At 24 h after treatment, parental MDA-MB-468 cell lines exhibited a decrease in the number of cells in G1 and an increase in cells in the S-phase of the cell cycle (Figure 2A). At the

48 h and 72 h timepoints an increasing number of cells have less than 2N DNA content (sub-G1), suggesting that cells are unable to complete enter into or complete S-phase and undergo apoptosis following cisplatin treatment. The MDA-MB-468 CR cell lines also exhibited an increase in the number of S-phase cells at 24h post treatment, but without a significant fraction of sub-G1 cells. After 72 h of cisplatin treatment, the MDA-MB-468 CR cell line had a cell cycle profile similar to the untreated sample, indicating that the cells had recovered from cisplatin treatment. The parental HCC-1806 cell line exhibits a cell cycle profile similar to the parental MDA-MB-468 cell line (Figure 2B). Surprisingly, The HCC-1806 CR cell line exhibits no alterations in the cell cycle profile following cisplatin treatment and a lack any accumulation of cells in the sub-G1 fraction (Figure 2B). To confirm that the CR cell lines do not undergo apoptosis, Western blot analysis was performed using Parp cleavage as a marker of apoptosis[15]. Similar to results obtained from the flow cytometry analyses, the parental cell lines undergo apoptosis following cisplatin treatment as evidenced by the accumulation of the cleaved form of Parp (Figure 3). The CR cell lines show minimal levels of cleaved Parp after cisplatin treatment, indicating a lack of apoptosis (Figure 3). We can conclude that the apoptotic response is diminished in the CR cell lines.

MDA-MB-468 CR Cells Exhibit Diminished DNA Damage After Cisplatin Treatment

Cisplatin has previously been shown to cause DNA double strand breaks that can be assayed using the phosphorylation of H2AX (γH2AX)[16]. We used immunofluorescence to determine if the accumulation of γH2AX foci was altered in the CR cell lines following cisplatin treatment. The parental and resistant MDA-MB-468 and HCC-1806 cell lines were treated with 1 or 3 μM cisplatin for 24h and immunofluorescence was used to measure γH2AX as a marker of DNA double stranded breaks. Compared to its parental counterpart, the MDA-MB-468 CR cell exhibited a significant decrease in



Cell Line	TNBC Subgroup	Parental Cisplatin IC_{50} (μM)	CR Cisplatin IC_{50} (μM)	p-value
MDA-MB-468	BL1	0.56 ± 0.38	5.29 ± 1.26	0.038
HCC-1806	BL2	1.81 ± 0.77	26.8 ± 5.73	0.004

Figure 1. MDA-MB-468 CR and HCC-1806 CR display a cisplatin resistant phenotype
The indicated parental and CR cell lines were untreated or treated with 0.03 - 10 μM cisplatin. Alamar blue was used to measure cellular viability 72 h later. Graphs represent three independent experiments with error bars representing standard error of the mean. IC_{50} values were calculated and a student's t-test was performed to determine statistical significance.

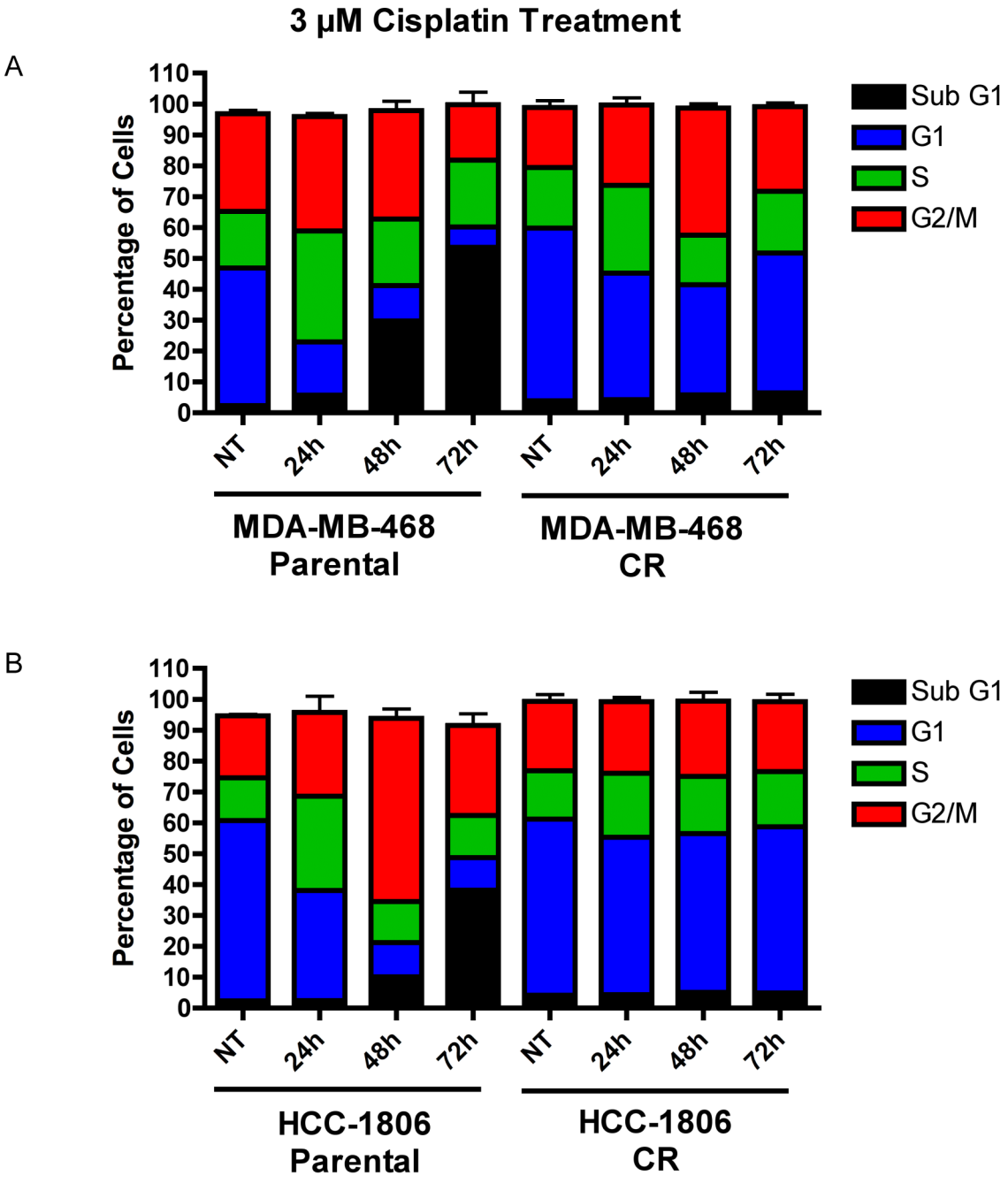


Figure 2. CR cell lines exhibit altered cell cycle profiles after cisplatin treatment. Parental and (A) MDA-MB-468 CR and (B) HCC-1806 CR cell lines were untreated (NT) or treated with 3 µM cisplatin for 24-72 h. The cells were trypsinized and incubated in propodium iodide and subjected to flow cytometry. Graphs represent three independent experiments with error bars representing standard error of the mean.

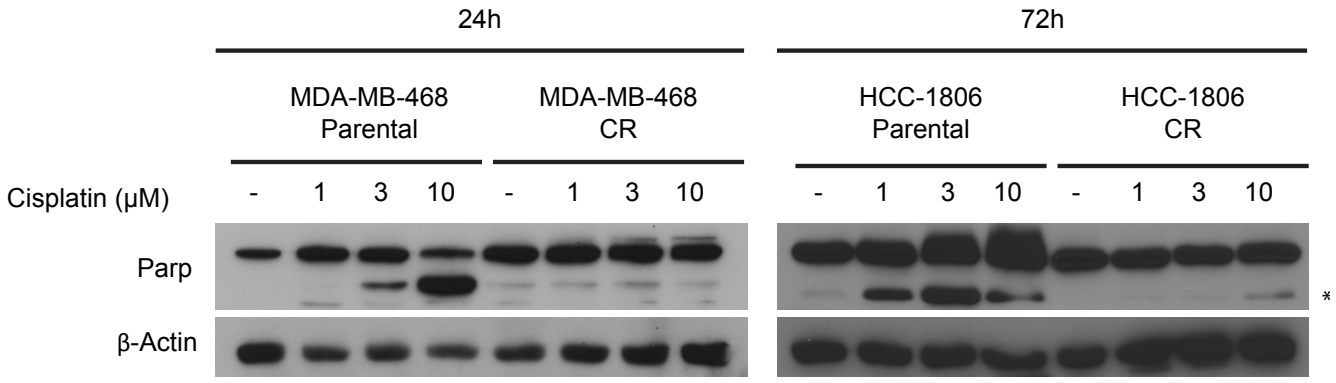


Figure 3. CR cell lines exhibit decreased apoptosis after cisplatin treatment.
The indicated parental and CR cells were untreated (-) or treated with 1, 3, or 10 μM cisplatin for 24 h (MDA-MB-468 parental and CR) or 72 h (HCC-1806 parental and CR). Western blot analysis was performed with the indicated antibody. Results are representative of three separate experiments. Cleaved Parp is denoted with (*).

γH2AX staining following a 24 h incubation in media containing either 1 or 3 μM cisplatin compared (Figure 4A). The parental cell line had a significant increase in γH2AX staining following treatment with 1 μM and 3 μM cisplatin while the CR cell line only exhibited a significant increase in DNA damage at the 3 μM concentration. To determine the kinetics of DNA damage after cisplatin treatment the parental and CR cell lines were treated with 10 μM cisplatin and γH2AX levels were quantified at 1, 2, 4, and 8 h post treatment. Similar to what was seen in the 24h treatment, the MDA-MB-468 CR cells exhibited diminished γH2AX staining (Figure 4B). A significant increase in γH2AX staining was seen at 4 h in the parental cell line and while there is an increase in γH2AX staining, no significant increase was observed in the CR cell line in any of the timepoints. In contrast to what was seen with the MDA-MB-468 parental and CR cell lines, the HCC-1806 parental and CR cells displayed nearly equal levels of γH2AX staining at 24 h following cisplatin treatment (Figure 4C). Similar levels of γH2AX were also seen between the parental and CR cell line in the 1-8 h timepoints (Figure 4D). Based on the diminished DNA damage accumulation, as measured by γH2AX staining, In the MDA-MB-468 CR cell lines cisplatin influx/efflux cannot be ruled out as a potential mechanism of cisplatin resistance. The robust γH2AX staining seen in the HCC-1806 CR cell line suggests that cisplatin is able to enter the nucleus and cause double-strand breaks in the DNA.

RNA-seq Analysis Reveals Caspase-14 as a Potential Mediator of Cisplatin Resistance

We performed whole transcriptome sequencing (RNA-seq) with the intent of identifying gene expression or genetic changes between the parental and CR cell lines that could provide insights to potential pathways that may contribute to the CR phenotype. We identified an increase in the expression of caspase-14 (data not shown). A member of the caspase family of cysteine-aspartic proteases, caspase-14 is thought to be involved in epidermal maturation[17,18]. An anti-apoptotic effect

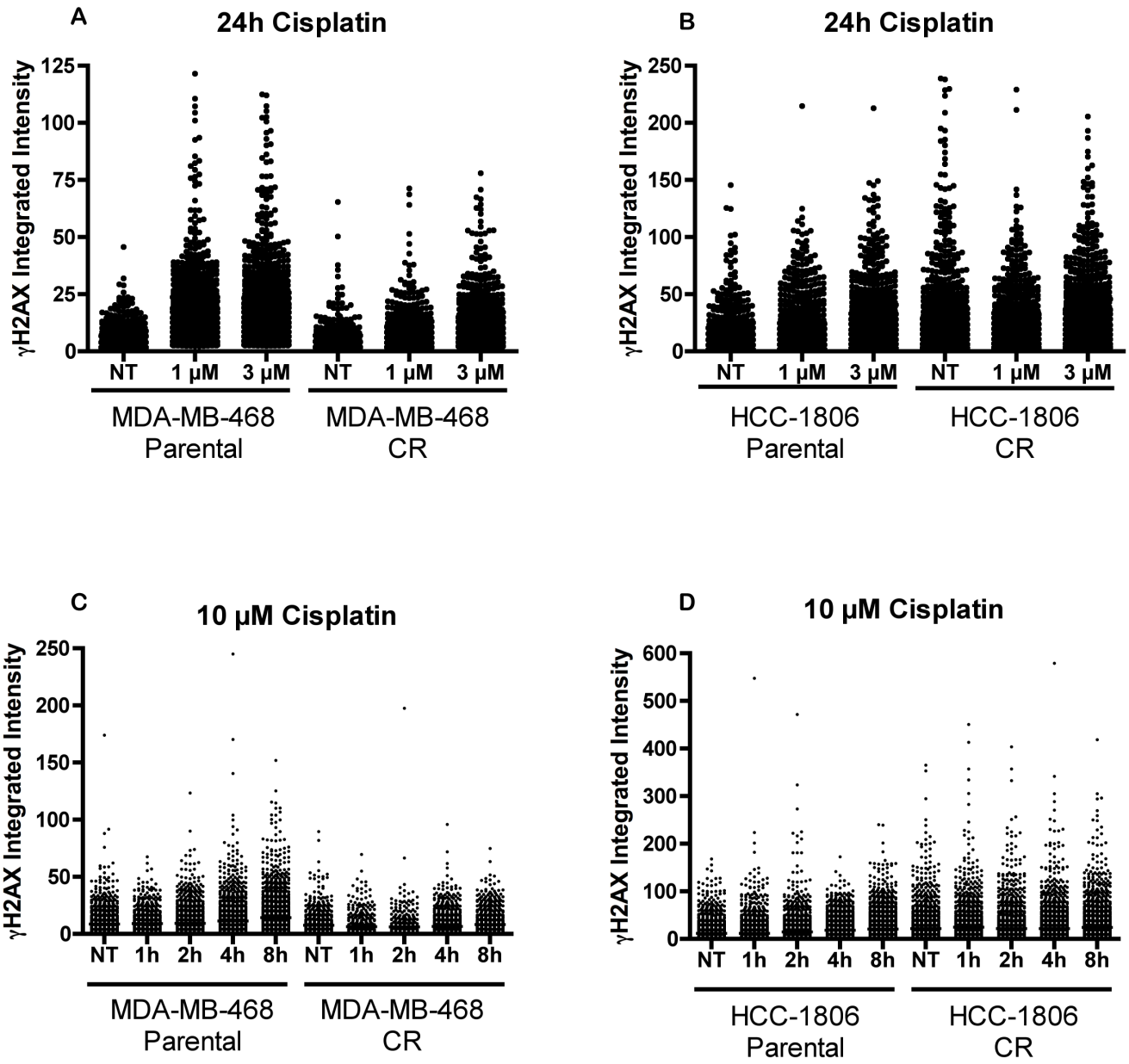


Figure 4. Cisplatin treated MDA-MB-468 CR cells have diminished levels of DNA damage
The indicated parental and CR cells were untreated (NT) or treated with cisplatin for 24 h (A & B) or from 1-8 h (C & D). Cells were incubated with antibodies against DAPI to visualize nuclei and YH2AX as a marker of DNA double stranded breaks. Quantification of YH2AX immunofluorecence is shown.

of caspase-14 has been described in lung adenocarcinoma cells, as it has been shown to Interact with apoptosis initiating factor (AIF) and prevent apoptosis in in response to cisplatin treatment[19]. Caspase-14 is highly upregulated in the MDA-MB-468 CR cells (Figure 5). Knockdown of caspase-14 was also found to sensitize U2OS cells to cisplatin in a whole-genome RNAi screen, making caspase-14 an attractive candidate for further exploration (Dr. David Cortez, personal communication). Based on these observations, we targeted caspase-14 was targeted with siRNA in the MDA-MB-468 parental and CR cell lines and the cells treated with cisplatin 24 h later. Minimal knockdown of caspase-14 in the CR cell line led to a small rescue in cisplatin sensitivity (Figure 6). Repeated attempts to improve the efficiency of caspase-14 knockdown with siRNA-based protocols proved to be unsuccessful (data not shown), thus a different approach was pursued. The parental and CR MDA-MB-468 cell lines were infected with a lentiviral-based vector containing a shRNA against caspase-14. While caspase-14 expression was diminished to a greater degree, the reduction in the protein did not rescue cisplatin sensitivity, nor was there any dose-dependent change in sensitivity (Figure 7). It is unclear if caspase-14 is necessary or sufficient for cisplatin sensitivity in the MDA-MB-468 CR cell line.

The MEK/ERK Signaling Axis is Lost in the HCC-1806 CR Cell Line

RNA-seq analysis revealed a novel mutation in the BRAF gene in the HCC-1806 CR cell line. The mutation led to a G21V amino acid change. This mutation does not fall in any characterized domains so the predicted effect on the protein is unknown. To determine a potential effect of this mutation, Western blot analysis was used to measure the expression of BRAF and two of its downstream effectors, MEK and ERK. While BRAF expression was not altered in the HCC-1806 CR cell line, there was a complete abrogation of the phosphorylated active forms of the MEK and ERK

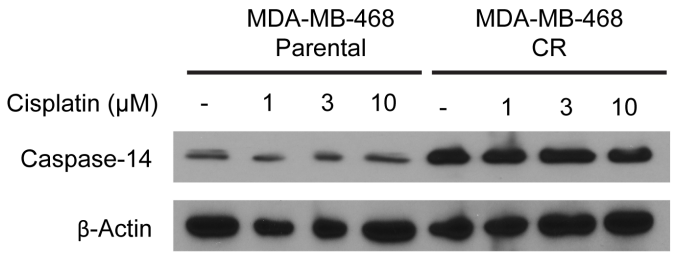


Figure 5. Caspase-14 expression is increased in MDA-MB-468 CR cells.
MDA-MB-468 parental and CR cells were untreated (-) or treated with 1, 3, or 10 μM cisplatin for 24 h. Western blot analysis was performed with the indicated antibodies. The results are representative of three independent experiments.

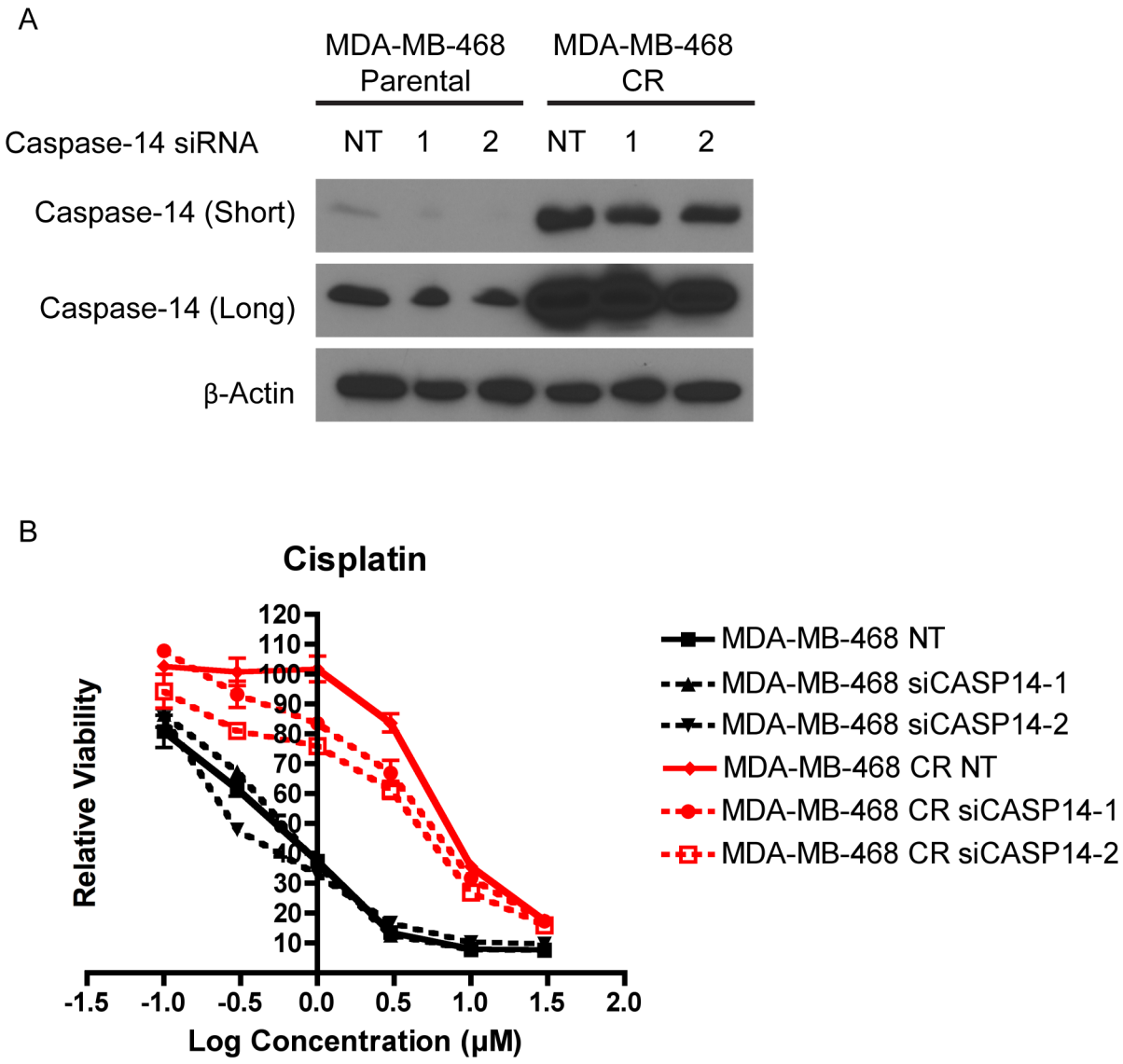


Figure 6. Inefficient caspase-14 knockdown confers minimal cisplatin sensitivity in MDA-MB-468 CR cells

A. MDA-MB-468 parental and CR cells were transfected with a non-targeting (NT) control or siRNA against caspase-14. Lysates were collected 72 h post-transfection and Western blot analysis was performed with the indicated antibodies. Results are representative of three independent experiments. Short and long exposures are shown to show difference in expression levels of caspase-14 in parental and CR cells. B. MDA-MB-468 parental and CR cells were transfected in triplicate with a non-targeting (NT) control or siRNA against caspase-14. Cells were untreated or treated with 0.1 - 30 μM cisplatin for 72 h followed by incubation in Alamar Blue to measure cellular viability. Error bars represent standard deviation of the mean.

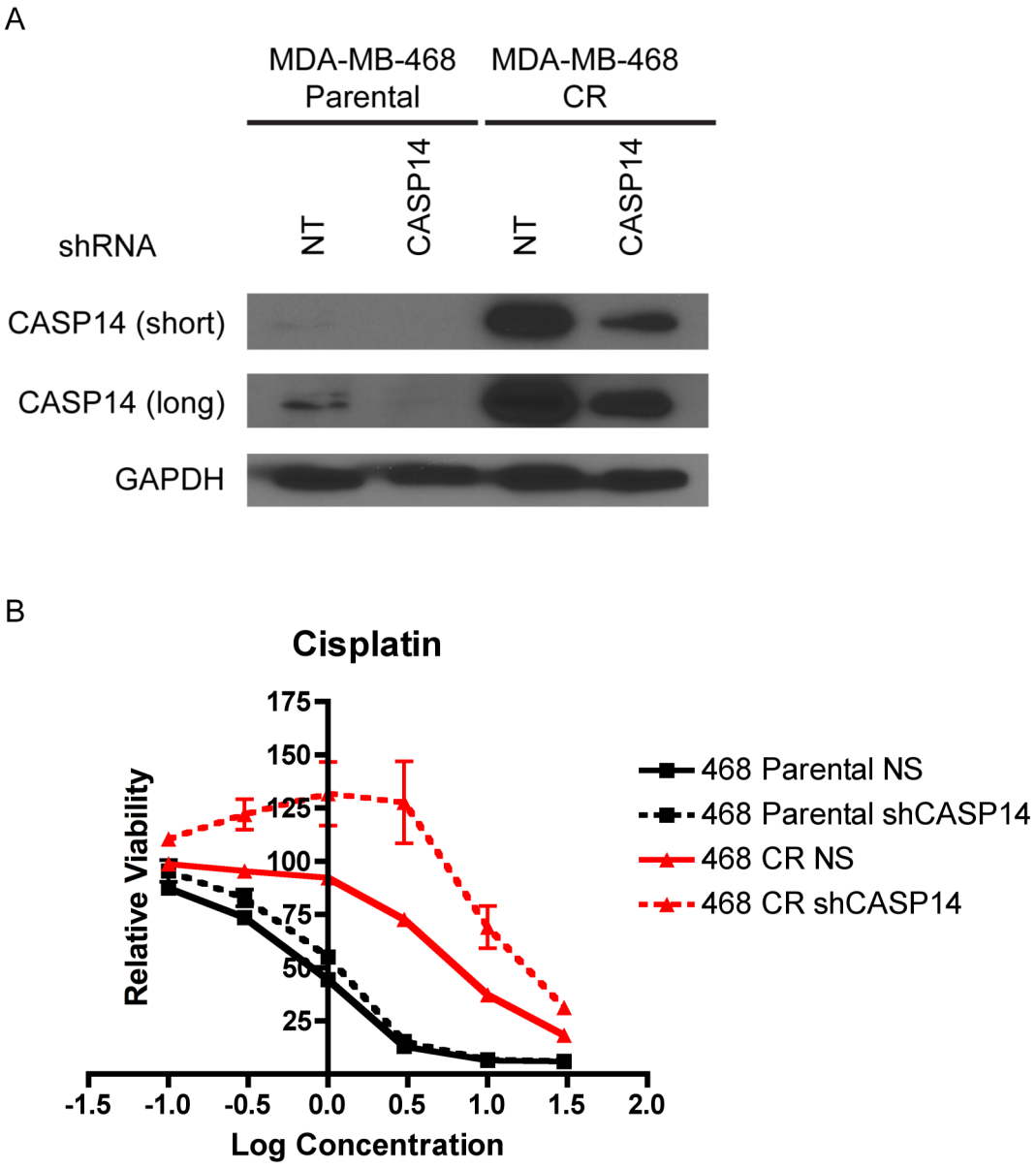


Figure 7. Caspase-14 knockdown does not correlate with cisplatin sensitivity in MDA-MB-468 CR cells

A. MDA-MB-468 parental and CR cells were infected with lentiviral particles expressing a non-targeting (NT) control or shRNA against caspase-14. Cells were treated with puromycin for 48 h to select for infected cells. Lysates were collected and Western blot analysis with the indicated antibodies is shown. Short and long exposures are shown to show difference in expression levels of caspase-14 in parental and CR cells. Results shown are representative of three independent experiments. B. MDA-MB-468 parental and CR cells expressing a non-targeting (NT) control or shRNA against caspase-14 were plated in triplicate. Cells were treated with 0.1 - 30 μM cisplatin for 72 h followed by incubation in Alamar Blue to measure cellular viability. Error bars represent standard deviation of the mean.

proteins (Figure 8). ERK has been shown to mediate cell death following cisplatin treatment in a large number of studies[20]. To determine if the ERK pathway is necessary for cisplatin-mediated cell death we used siRNA to knockdown ERK1 and ERK2 expression in parental HCC-1806 cells and treated with cisplatin. The reduction in ERK1 and ERK2 led to an increase in viable cells when compared to HCC-1806 cells transfected with a non-targeting control (Figure 9). These data suggest that the MEK/ERK signaling axis is important in mediating the apoptotic response after cisplatin treatment.

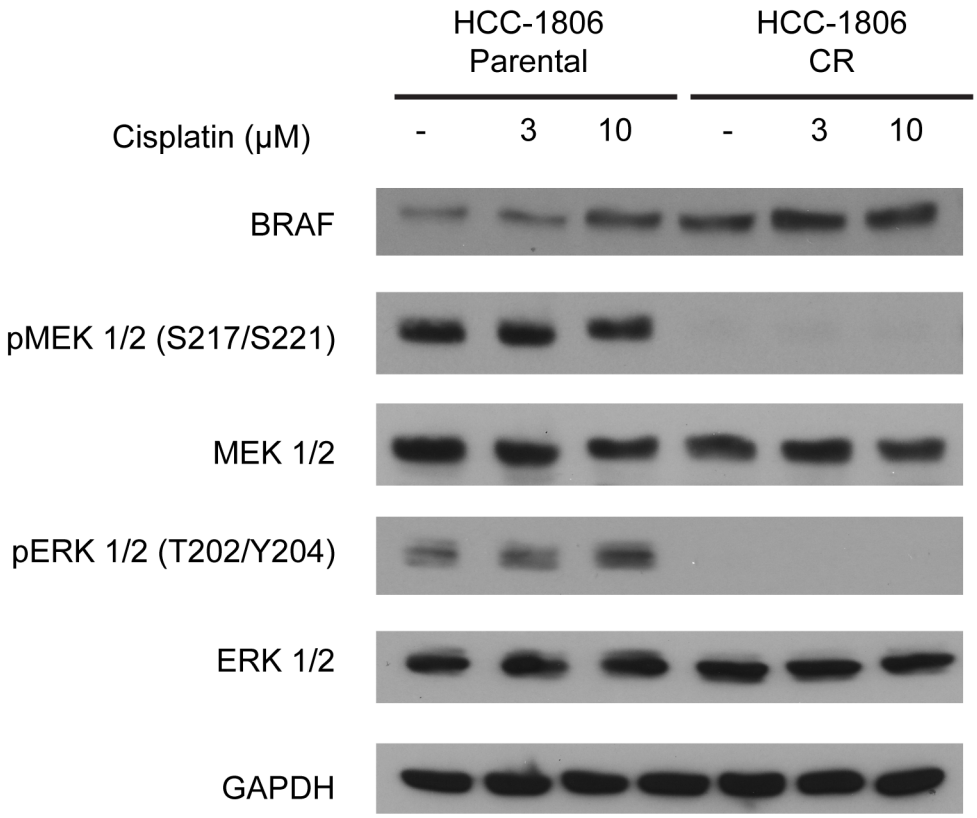


Figure 8. MEK/ERK signaling is lost in HCC-1806 CR cells
Parental and CR HCC-1806 cells were untreated (-) or treated with 1 μM or 3 μM cisplatin for 72 h. Western blot analysis was performed with the indicated antibodies. Results are representative of three independent experiments.

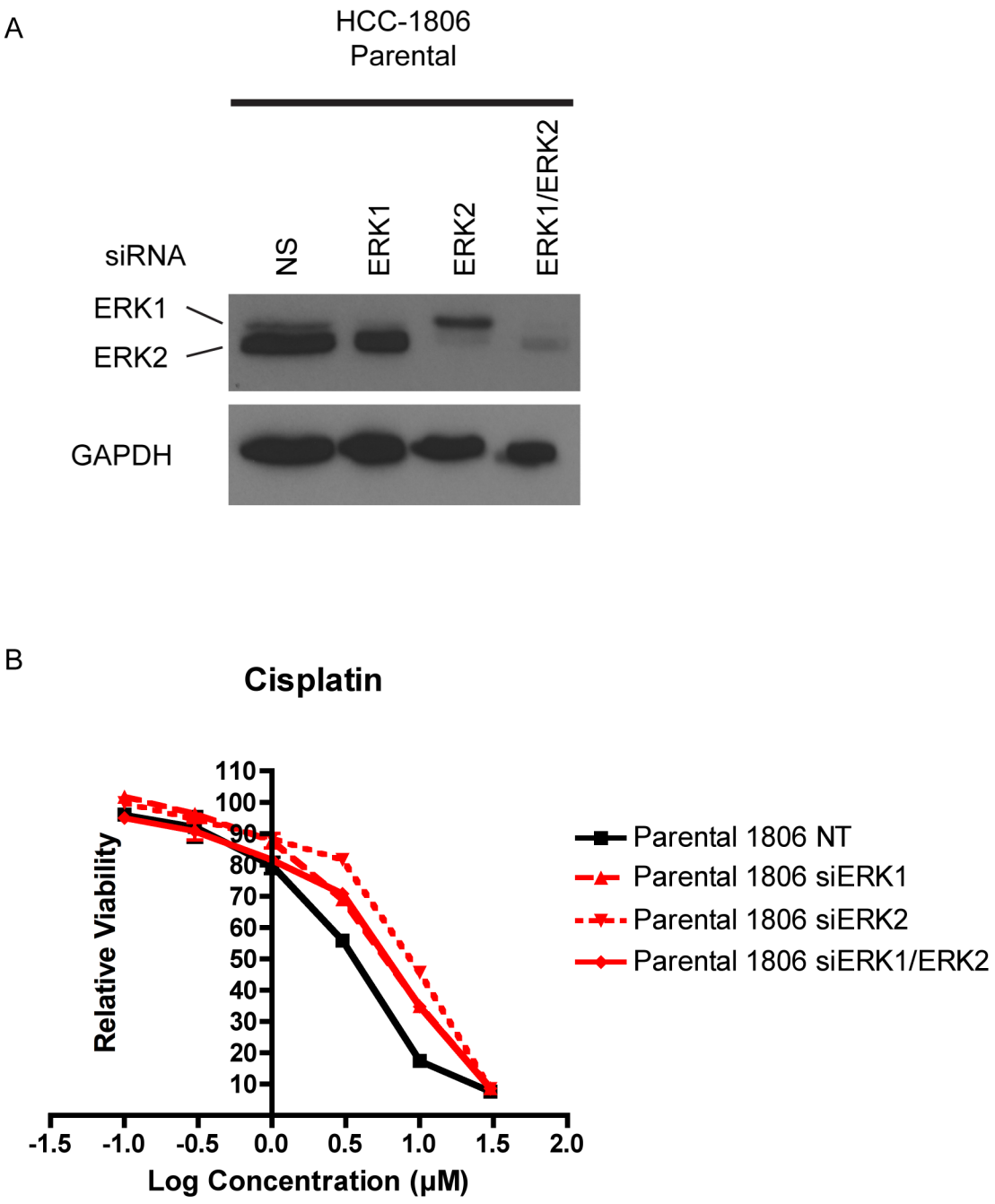


Figure 9. ERK1/ERK2 knockdown confers cisplatin resistance in parental HCC-1806 cells

A. Parental HCC-1806 cells were transfected with a non-targeting (NT) control or siRNA against ERK1, ERK2, or a combination of both. Lysates were collected 72 h post-transfection and Western blot analysis was performed with the indicated antibodies. Results shown are representative of three independent experiments. B. Parental HCC-1806 cells were transfected in triplicate with a non-targeting (NT) control or siRNA against ERK1, ERK2, or a combination of both. Cells were untreated or treated with 0.1 - 30 μM cisplatin for 72h followed by incubation in Alamar Blue to measure cellular viability. Error bars represent standard deviation of the mean.

Chapter III

Discussion

The use of cisplatin against TNBC is increasing in the clinic[3-5]. Cisplatin has a long history of acquired resistance in other tumor types[10-12]. The lack of pre-clinical data exploring cisplatin resistance in the TNBC setting led us to develop two TNBC cell lines in order to identify mechanisms of cisplatin resistance in TNBC. The cell lines we generated as part of the research described in this thesis represent the only reported models of acquired cisplatin resistance in TNBC. The relatively recent addition of cisplatin to TNBC treatment regimens has created the necessity to study cisplatin in the setting of TNBC. The two CR cell lines created represent two similar but distinct subgroups of TNBC (BL1 and BL2). The MDA-MB-468 CR cell line representing the BL1 subgroup, displays a differential accumulation of cells in S-phase at 24 h after cisplatin treatment. However, after 72 h after drug treatment, the CR cells display a cell cycle profile that is nearly identical to the non-treated sample (Figure 2). While the parental cell line also accumulates in S-phase post treatment, it is unable to recover from the treatment and exhibits a large population of sub-G1 DNA content, indicating a large apoptotic population. The MDA-MB-468 parental and CR cell line exhibit a cell cycle pattern that is characteristic of cells that have been treated with interstrand crosslinking agents[14]. The recovery from cisplatin treatment coupled with the presumed delayed accumulation of DNA double-strand breaks as assessed by γH2AX staining (Figure 4) could indicate an increased capacity of repair. Alterations in the homologous recombination pathway have been reported to confer cisplatin resistance in other models [21,22]. Measuring the accumulation of Rad51 as a marker of homologous recombination following cisplatin treatment would be informative to determine if the increased ability to repair cisplatin induced lesions is responsible for the cisplatin resistant phenotype

seen in the MDA-MB-468 CR cell line. Alternatively, these data could be interpreted as evidence for alterations in the cisplatin influx or cisplatin efflux pathways, which have been described in detail to confer cisplatin resitance[23-25]. In order to directly measure DNA damage caused by cisplatin antibodies against specific cisplatin induced adducts could be employed[26]. Directly measuring the ability of cisplatin to reach DNA and form adducts would allow for conclusions to be drawn about any alterations in the cisplatin influx/efflux pathways in the CR cell lines. In addition to increased repair capacity, and altered cisplatin influx/efflux, the evasion of apoptosis after cisplatin treatment is commonly seen in cisplatin resistant models[27,28]. The MDA-MB-468 CR cell line exhibits elevated expression of the anti-apoptotic protein caspase-14 (Figure 5). The contribution of elevated levels of caspase-14 expression to the cisplatin-resistant phenotype of the MDA-MB-468 CR was unable to be elucidated due to an inability to modulate the expression of caspase-14 (Figures 6 and 7). The RNAi techniques employed to knockdown caspase-14 were likely unsuccessful due to the elevated expression. The use of the CRISPR/Cas9 system could be an alternative method to employ to silence the expression of caspase-14. The high expression level of caspase-14 would be irrelevant as the CRISPR/Cas9 system relies on DNA editing to remove the gene of interest[15,29]. Efficient reduction of caspase-14 expression would allow for further exploration into the potential caspase-14/AIF interaction and its role in the cisplatin-resistant phenotype of the MDA-MB-468 CR cell line.

The HCC-1806 CR cell line represents the BL2 subgroup of TNBC. In contrast to what was seen with the MDA-MB-468 CR cell line, the cell cycle profile of the HCC-1806 CR was not changed after cisplatin treatment (Figure 2). The lack of an accumulation in S-phase may indicate that the S-phase checkpoint and subsequent repair that occurs during this checkpoint is not as important in allowing the HCC-1806 CR cells to recover from cisplatin when compared to the MDA-MB-468 CR cell line. The HCC-1806 CR cell line also exhibited a different DNA damage phenotype when compared to the MDA-MB-468 CR cell line. The HCC-1806 CR cell line had similar levels of baseline

DNA damage as its parental counterpart (Figure 4). The DNA double-strand breaks as analyzed by γH2AX staining increased at a similar rate in the parental and CR HCC-1806 CR cell lines. These data indicate that altered neither cisplatin influx/efflux nor increased repair capacity are responsible for the cisplatin-resistant phenotype. Further exploration into the modulation of apoptosis following cisplatin treatment is warranted as the MEK/ERK signaling axis has been disrupted in the CR cell line (Figure 7). The contribution of MEK/ERK signaling to the cisplatin-resistant phenotype was not fully elucidated, but preliminary data suggest that knockdown of ERK1 or ERK2 confers a cisplatin-resistant phenotype in parental HCC-1806 cells. Pharmacologic inhibition of MEK and ERK followed by cisplatin treatment would provide more evidence linking a loss of MEK/ERK signaling and evasion of apoptosis incurred by cisplatin. The downstream effectors of ERK leading to apoptosis have been well characterized and include signaling through caspase-8 to induce cytochrome c release through regulation of members of the Bcl-2 protein family such as Bax and Bak [30-33]. The loss of MEK/ERK signaling in the HCC-1806 CR cell line may provide some insight into TNBC therapies in the clinic. This signaling axis has been proposed as a target for therapy in TNBC[34]. These data suggest that the MEK/ERK signaling axis may not be an attractive target in combination with cisplatin treatment.

In conclusion, the studies presented in this thesis describe the first cell line models of cisplatin resistance in TNBC. In our studies, these cell line models were used to explore two potential mechanisms for cisplatin resistance in TNBC. Both mechanisms involve some aspect of attenuation of apoptosis after cisplatin treatment. The data provide strong preliminary evidence for the continued study of caspase-14 and the MEK/ERK signaling axis as mechanisms of cisplatin resistance in TNBC.

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