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BRCA2 inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis

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ABSTRACT

Tumor cells have unstable genomes relative to non-tumor cells. Decreased DNA integrity resulting from tumor cell instability is important in generating favorable therapeutic indices, and intact DNA repair mediates resistance to therapy. Targeting DNA repair to promote the action of anti-cancer agents is therefore an attractive therapeutic strategy. BRCA2 is involved in homologous recombination repair. BRCA2 defects increase cancer risk but, paradoxically, cancer patients with BRCA2 mutations have better survival rates. We queried TCGA data and found that BRCA2 alterations led to increased survival in patients with ovarian and endometrial cancer. We developed a BRCA2-targeting second-generation antisense oligonucleotide (ASO), which sensitized human lung, ovarian, and breast cancer cells to cisplatin by as much as 60%. BRCA2 ASO treatment overcame acquired cisplatin resistance in head and neck cancer cells, but induced minimal cisplatin sensitivity in non-tumor cells. BRCA2 ASO plus cisplatin reduced respiration as an early event preceding cell death, concurrent with increased glucose uptake without a difference in glycolysis. BRCA2 ASO and cisplatin decreased metastatic frequency *in vivo* by 77%. These results implicate BRCA2 as a regulator of metastatic frequency and cellular metabolic response

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following cisplatin treatment. BRCA2 ASO, in combination with cisplatin, is a potential therapeutic anti-cancer agent.

1. Introduction

A high rate of mutation and genetic instability can be considered fundamental hallmarks of cancer that distinguish tumor cells from their normal counterparts (Lee et al., 2010; Pleasance et al., 2010; Russnes et al., 2011). Lack of integrity in the cancer genome is often precipitated by compromised or incomplete DNA repair, reliance on error-prone repair, and/or defects in cell cycle control (Hanahan and Weinberg, 2011). This renders cancer cells susceptible to DNA damage-induced toxicity, and is at least partly responsible for the difference between non-tumor cells and cancer cells in sensitivity to DNA-damaging therapeutic agents.

However, genomic heterogeneity and mutability also underlie tumor cell adaptability and acquired resistance to chemo- and/or radiotherapy (Burrell et al., 2013). Thus, the same traits that render cancer cells more susceptible to DNA-damaging therapeutics can also lead to therapeutic failure.

Tumor cells must therefore preserve a level of DNA repair sufficient to maintain survival in the face of selection pressures posed by treatment, but sufficiently limited to permit enhanced adaptability (Aguilera and Garcia-Muse, 2013). However, this can be exploited for therapeutic benefit: inhibiting DNA repair may push tumor cells past a tolerable threshold of DNA damage and induce cell death, thus preventing further heterogeneity and the development of acquired drug resistance.

BRCA2 is a protein directly involved in homologous recombination repair (HRR) of DNA double stranded breaks (DSBs). Individuals with inactivating mutations in BRCA2 exhibit a strong predisposition to cancers of the breast, ovary, and other tissues. However, patients with tumors harboring BRCA2 mutations have an improved prognosis following treatment with platinum-containing and other drugs, compared to patients with tumors with intact BRCA2 genes (Maxwell and Domchek, 2012; Yang et al., 2011). In addition, BRCA2 mutation status leads to differences in metastatic frequency in patients (Bayraktar et al., 2013; James et al., 2009). This suggests that actively targeting BRCA2 in cancer cells with functional HRR may render anti-tumor treatments more effective.

Cisplatin is commonly used to treat various types of solid malignancies (Monneret, 2011). However, the associated toxicities and both inherent and acquired resistance can limit the effectiveness of these drugs in the clinic (Shen et al., 2012). Strategies to render cancer cells more sensitive to platinum agents are of high clinical importance.

Based on proof-of-concept studies (Rytelewski et al., 2013), we developed a second-generation BRCA2-targeting antisense oligodeoxynucleotide (ASO). We investigated the potential therapeutic value of the BRCA2 ASO in a setting with concomitant cisplatin treatment by determining the effects on tumor cell proliferation, metabolic response, and metastasis. We show here that BRCA2 ASO plus cisplatin treatment is potentially valuable clinically, and describe a new role for BRCA2 in the maintenance of human tumor cell metabolic response and metastasis frequency following cisplatin treatment.

2. Materials and methods

2.1. cBioPortal analysis of TCGA data

The cBioPortal website client (http://www.cbioportal.org/public-portal/) was utilized to analyze the survival differences in patients with wild type or mutated BRCA2 or BRCA1, in both ovarian and endometrial cancer. The analysis was performed using the Ovarian Serous Cystadenocarcinoma (Cancer Genome Atlas Research Network, 2011) dataset and the Uterine Corpus Endometrioid Carcinoma (Kandoth et al., 2013) dataset.

2.2. Cell lines

Human BRCA2 WT (A549, SKOV-3, MDA-MB-231, HK-2) and BRCA2-mutated (CAPAN-1) cells were obtained from ATCC (Manassas, Virginia, USA) and maintained in AMEM, DMEM, or Iscove's medium (Wisent, St. Bruno, Quebec, Canada) supplemented with 10 or 20% fetal bovine serum (FBS) (GIBCO – Life Technologies, Burlington, Ontario, Canada), or Keratinocyte Serum Free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor (GIBCO) under standard conditions (37 °C in a humidified 5% CO_2 atmosphere). Parental human head and neck squamous carcinoma HN-5a cells and cisplatin-resistant HN-5a/carbo-15a cells (Ferguson et al., 1999) were maintained in AMEM medium with 10% FBS under standard conditions.

2.3. Antisense oligodeoxynucleotide (ASO) transfection

Oligonucleotide transfection was performed according to the following protocol (Rytelewski et al., 2013): BRCA2 ASO or control ASO were diluted in serum-free medium and incubated with Lipofectamine 2000 (Invitrogen – Life Technologies) for 20 min, then added to cells to a final concentration of 20 nM. After 4 h, transfection medium was replaced or cells were harvested for subsequent treatment as described.

2.4. mRNA quantification

Total cellular RNA was isolated from cells 24 h after transfection, and mRNAs analyzed by qPCR as described previously

(Cresce et al., 2012). Trizol reagent (Ambion – Life Technologies) was used to isolate RNA, which was reverse-transcribed to generate cDNA using M-MLV-RT enzyme (Invitrogen – Life Technologies). qPCR was conducted using a Taqman master mix (Applied Biosystems – Life Technologies) and performed in a Perkin Elmer ViiA 7 Real-time PCR system (Life Technologies).

2.5. Protein quantification

BRCA2 immunoblot was performed according to the following protocol: cells were transfected with ASO, and cell lysates collected 72 h post-transfection. Total cellular protein (40 μg per well) was loaded on a 4–15% gradient gel (Bio-Rad, Hercules, California, USA) and electrophoresed (1.5 h, 100 V), then transferred to PVDF membrane using a Bio-Rad Turbo Transfer Pack (Bio-Rad). The membrane was blocked with TBS-T + 5% BSA for 1 h and then incubated with BRCA2 MAb (1:500, 16 h) (Cell Signaling #9012S) and actin MAb (1:1000, 1 h) (Sigma–Aldrich).

2.6. Cell proliferation assay

Cells were trypsinized, counted, and re-plated in triplicate at a density of 5.0×10^4 cells per well in a six well plate 4 h post-transfection. Cytotoxic drugs (cisplatin [Sigma–Aldrich], melphalan [Sigma–Aldrich]) were added 24 h post-transfection in triplicate wells for each drug concentration. Cells were trypsinized 76 h after addition of drug and counted using a Coulter Particle Counter (Beckman–Coulter, Mississauga, ON). Relative proliferation was calculated using the starting cell number at seeding, and normalized to the no-drug ASO treatment condition (e.g., control ASO + drug treatment was normalized to control ASO alone).

2.7. Colony formation assay

Forty-eight hours post-transfection the cells were exposed to different concentrations of drug for a total of 6 h. The cells were collected and plated in triplicate at a density of 500 cells per well in a 6 well dish. In the case of irradiation treatment, cells were harvested immediately post treatment and plated at the same density. Colonies were fixed with a Protocol® Hema-3 kit (Fisher Scientific) 7–10 d later and counted.

2.8. Chicken embryo chorioallantoic membrane (CAM) metastasis assay

Forty-eight hours post-transfection cells were exposed to 6 μM cisplatin for 6 h. The cells were harvested, washed three times, and adjusted to a final concentration of 1.0×10^6 cells per ml in PBS. 1.0×10^5 cells (100 μl) were injected into the venous circulation of 9 day old chicken embryos. Seven to 9 days post-injection the metastatic foci were counted using an Axio Zoom V16 (Carl Zeiss AG, Germany) microscope at 20× magnification.

2.9. Bionas Discovery 2500 metabolic measurement

Four hours post-transfection cells were collected and seeded onto Bionas biosensor chips (Bionas GmbH, Rostock, Germany) at a density of 180,000 cells per chip. The medium was changed to AMEM + 0.2% FBS and the biosensor chips were loaded into the Bionas system (Bionas GmbH) 24 h later. Using a 4 μl/min flow rate, the cells were exposed to AMEM + 0.2% medium for 6 h and then 6 μM cisplatin for 24 h. The cells were exposed to medium for 48 h, at which point the cells were lysed with 0.1% Triton-X to determine baseline readings.

2.10. Glucose uptake assay

Twenty-four hours post-transfection cells were treated with 6 μM cisplatin. Cells were washed with PBS and then incubated with glucose free DMEM (GIBCO) + 10% FBS for 20 min, 48 h post drug treatment. The cells were incubated in glucose free DMEM + 10% FBS + 29 μM 2-NBD fluorescent glucose (Molecular Probes, Life Technologies) for 1 h. Cells were collected, washed with PBS, and glucose uptake was assayed using flow cytometry (BD Biosciences).

2.11. Mitotracker assay

Twenty-four hours post-transfection cells were treated with 6 μM cisplatin. Cells were harvested and stained with 75 nM MitoTracker Red CMXROS (Life Technologies) according to manufacturer's instructions, 48 h post drug treatment. MitoTracker staining was enumerated using flow cytometry and expressed as mean fluorescence intensity (geometric mean).

2.12. AlamarBlue proliferation assay

Cells were transfected with ASO as described and re-plated in 96-well plates (7.5×10^3 cells per well). Twenty-four hours post-transfection cells were treated with different concentrations of cisplatin, melphalan, 5-FUdR, or pemetrexed. Seventy-two hours later medium was removed and a 1:12 dilution of AlamarBlue (Life Technologies) was added for 3 h. Quantitated by fluorescence on a Wallac Victor plate reader (Perkin Elmer, Boston, MA).

2.13. Rad51 focus formation assay

Cells were transfected with ASO as described previously, except that they were grown on microscope slide cover slips in 12-well plates prior to transfection. Cells were treated with cisplatin (6 μM, 6 h) starting 48 h post-transfection, washed with PBS and fixed (4% paraformaldehyde, 30 min), permeabilized (TBS + 0.3% Triton-X-100, 10 min), and blocked (TBS + 3% BSA, 3% goat serum, 1 h). Anti-Rad51 MAb (Calbiochem – EMD Millipore, Germany, 1:500) in blocking solution was added and incubated at 4°C for 16 h. Cells were washed in TBS and incubated with secondary antibody (Cell Signaling, 1 h, 25°C), washed again, then stained and mounted with DAPI SlowFade Gold anti-fade reagent (Molecular Probes, Life Technologies).

3. Results

3.1. Alterations in the BRCA2 gene positively impact patient prognosis

Using the cBioPortal for Cancer Genomics we analyzed the effect of alterations in the BRCA2 gene on cancer patient

survival (Gao et al., 2013). Both endometrial and ovarian cancers were investigated to assess the consequences of naturally occurring BRCA2 mutations on survival in patient populations, as BRCA2 mutations were reasonably common in these tumor types. A query of the TCGA Uterine Endometrial Carcinoma (UEC) dataset (Kandoth et al., 2013) revealed a higher disease-free survival (logrank $p = 0.032$) and a trend to higher overall survival (logrank $p = 0.10$) in patients with BRCA2 alterations (Figure 1a). However, survival of endometrial carcinoma patients with wild type BRCA2 was relatively high, diminishing the power to detect survival differences.

BRCA2 mutations were queried against survival using the TCGA Ovarian Serous Cystadenocarcinoma dataset (Cancer Genome Atlas Research Network, 2011) in which overall 5-year survival was 40% or less. As opposed to analyzing the combined effect of BRCA1 and BRCA2 mutations on outcome (Cancer Genome Atlas Research Network, 2011), our analysis correlated changes in BRCA2 alone with improved overall (72.9 vs. 43.3 months, logrank $p = 0.002$) and disease-free (26.2 vs. 15.4 months, logrank $p = 0.014$) survival (Figure 1b). No such correlation was found for BRCA1 gene mutations (Figure 1c). Overall, naturally occurring BRCA2 mutations in at least two tumor types (of the endometrium and ovary) trend toward or are significantly associated with increased survival. That association supports the hypothesis that BRCA2 may be a therapeutic target to enhance anti-cancer therapy.

3.2. BRCA2 ASO treatment decreases target mRNA, protein and RAD51 focus formation

Transfection of A549 non-small cell lung cancer cells with BRCA2 ASO decreased BRCA2 mRNA by 78% and reduced BRCA2 protein levels (Figure 2a and b). BRCA2 ASO also negatively impacted BRCA2 function on a cellular level, as shown by decreased RAD51 focus formation induced by subsequent cisplatin treatment (Figure 2c). Thus, the degree of BRCA2 ASO-induced target downregulation was sufficient to reduce at least one BRCA2 function important for HRR.

3.3. BRCA2 maintains tumor cell proliferative capacity following cisplatin treatment

To determine the effect of BRCA2 knockdown on tumor cell response to cisplatin, we transfected A549 cells with BRCA2 ASO and treated with cisplatin. The effectiveness of cisplatin was increased as much as 50 ± 3% ($p < 0.05$) in cells transfected with BRCA2 ASO compared with control (Figure 3a and Supplementary Figure 1a).

BRCA2 ASO sensitized A549 cells to melphalan, another DNA cross-linking agent, by 45 ± 2% ($p < 0.05$) compared to cells transfected with control ASO (Figure 3b and Supplementary Figure 1b). BRCA2 ASO treatment also rendered cisplatin more effective in SKOV-3 ovarian cancer cells (55% ± 3%, $p < 0.05$) and in MDA-MB-231 breast cancer cells (26% ± 8%, $p < 0.05$), confirming that this phenomenon is reproducible in human tumor cell lines of differing origin (Figure 3a and b).

Somatic BRCA2 mutations predispose human cells to malignant transformation (Wooster et al., 1995). In addition, since BRCA2 knockdown may enhance toxicity of DNA-damaging drugs to normal, non-tumor cells, we investigated the effect of BRCA2 ASO on cisplatin toxicity in human non-tumor HK-2 kidney cells. Treatment of HK-2 cells with BRCA2 ASO induced only a small increase in sensitivity to cisplatin (12% ± 6%) (Figure 3e).

Human pancreatic CAPAN-1 cells, which lack a functional BRCA2 protein (Holt et al., 2008), were subjected to the same BRCA2 ASO/cisplatin treatment. There was no increase in cisplatin sensitivity in CAPAN-1 cells following BRCA2 ASO transfection (Figure 3f), suggesting that the effects of BRCA2 knockdown with BRCA2 ASO seen in tumor cell lines are due to BRCA2 downregulation and not off-target events.

3.4. BRCA2 downregulation reverses acquired cisplatin resistance in head and neck tumor cells

To assess the effectiveness of BRCA2 downregulation in reversing acquired cisplatin resistance in human tumors, we tested cisplatin sensitivity in three human head and neck squamous carcinoma cell lines (Ferguson et al., 1999): HN-5a, HN-5a/carbo-10a, and HN-5a/carbo-15a (Figure 4a).

BRCA2 ASO treatment potentiated cisplatin in parental HN-5a cells by 63 ± 5% and cisplatin-resistant HN-5a–carbo-15a cells by 57 ± 2% (Figure 4b and c). The resulting sensitivity generated by BRCA2 ASO transfection was similar to that of parental HN-5a cells (Figure 4c). Although HN-5a/carbo-15a cells exhibit decreased intracellular accumulation of cisplatin compared to the parental HN-5a cell line (Ferguson et al., 1999), BRCA2 downregulation was still able to effectively sensitize cells to cisplatin.

An assay of *in vitro* colony formation was used as a more stringent measure of the long term effects of treatment on seeding potential (Hao et al., 2012). Treatment with BRCA2 ASO and cisplatin decreased colony-formation ability in both HN-5a and HN-5a/carbo-15a cells (Figure 4d and e), suggesting that BRCA2 inhibition can enhance the ability of cisplatin to limit cancer cell proliferation and colony forming potential.

3.5. BRCA2 modulates tumor cell metabolism following cisplatin treatment

Given the dependence of DNA maintenance and repair on functional metabolic processes (Jeong et al., 2013), it was possible that part of the BRCA2 ASO-mediated increase in cisplatin cytotoxicity was due to alterations in cellular metabolism. In addition, cisplatin has been shown to preferentially target mitochondrial DNA in tumor cells (Yang et al., 2006). To investigate, we measured changes in cell impedance, acidification and oxygen consumption associated with BRCA2 ASO and cisplatin treatment to determine monolayer integrity, cellular glycolytic activity, and respiration (Alborzinia et al., 2011).

After 24-h exposure to cisplatin, A549 tumor cells pre-treated with BRCA2 ASO had 39% less respiratory activity than cells pre-treated with control ASO. Furthermore, in BRCA2 ASO-treated cells, the respiration decrease was evident 10 h after addition of cisplatin and 15 h earlier than in cells treated with control ASO (Figure 5a). Respiration began to decrease in response to cisplatin in BRCA2-treated cells 10 h prior to observable reduction in adhesion, suggesting that respiration reduction occurred independent of changes in cell number or viability (Figure 5b). However, no difference in

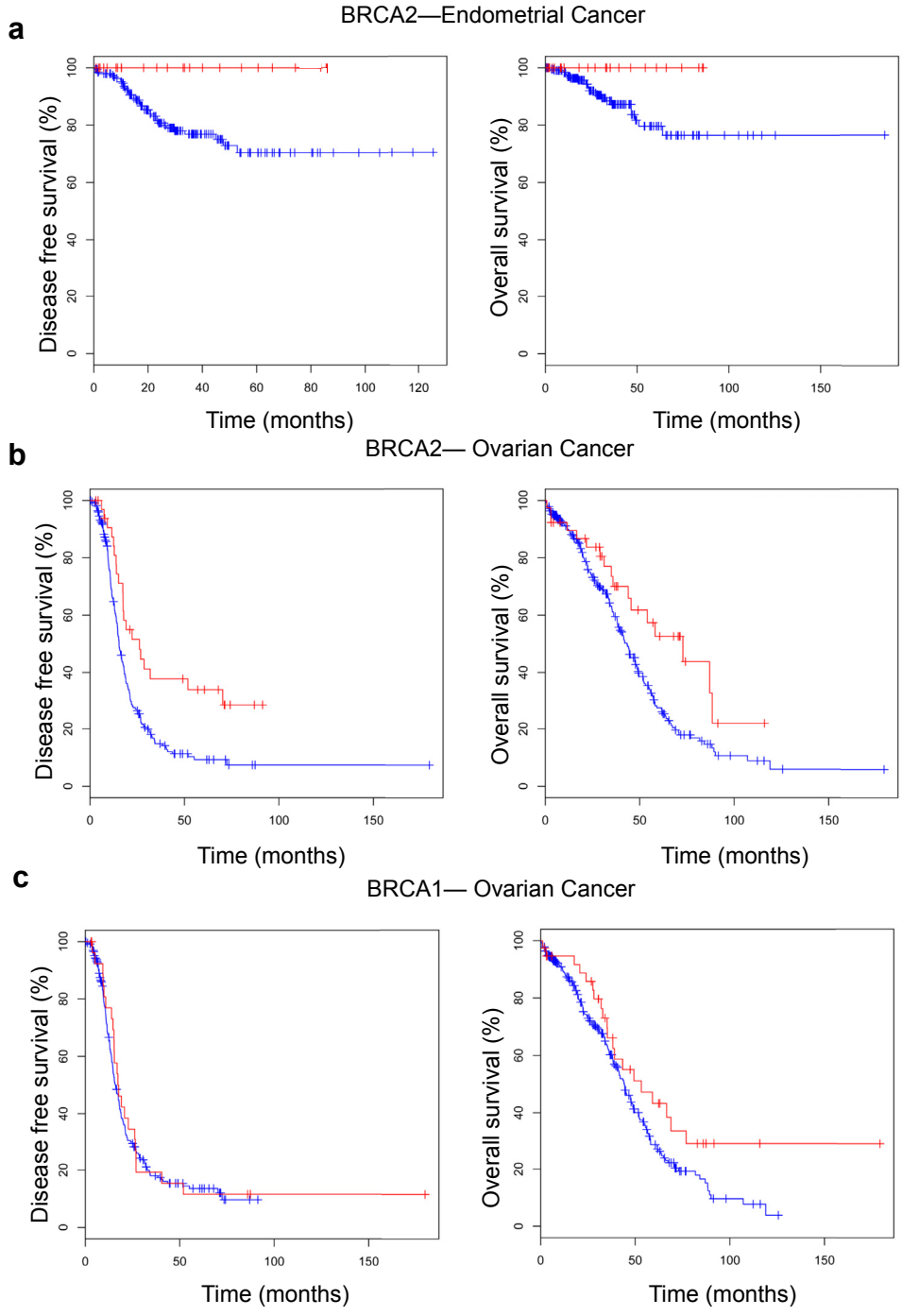

Figure 1 – Retrospective analysis of patient survival data based on BRCA2 mutation status. The TCGA Uterine Endometrial Carcinoma dataset was queried using the cBioPortal to determine changes in disease-free (a) and overall survival, (b) in patients with alterations in BRCA2 (BRCA2 WT $N = 215$ (blue), BRCA2 mut $N = 24$ (red)). The TCGA Ovarian Serous Cystadenocarcinoma dataset was queried to determine changes in disease-free and overall survival in patients with alterations in BRCA2 (BRCA2 WT $N = 276$, BRCA2 mut $N = 39$) (c and d) or BRCA1 (BRCA2 WT $N = 277$, BRCA2 mut $N = 38$) (e and f).

acidification (a measure of glycolysis) was observed between the BRCA2 ASO and control ASO groups treated with cisplatin (Figure 5c).

Changes in cellular respiration induced by BRCA2 ASO in conjunction with cisplatin suggested BRCA2 ASO-mediated inhibition of mitochondrial function. We used mitochondria-specific dye accumulation to determine the frequency of functional mitochondria in A549 and HN-5a cells, and changes in those parameters induced by BRCA2 ASO plus or minus cisplatin. Cisplatin treatment induced a 63% increase in Mitotracker staining in both A549 and HN-5a cells. There was no difference in staining between cells treated with BRCA2 ASO

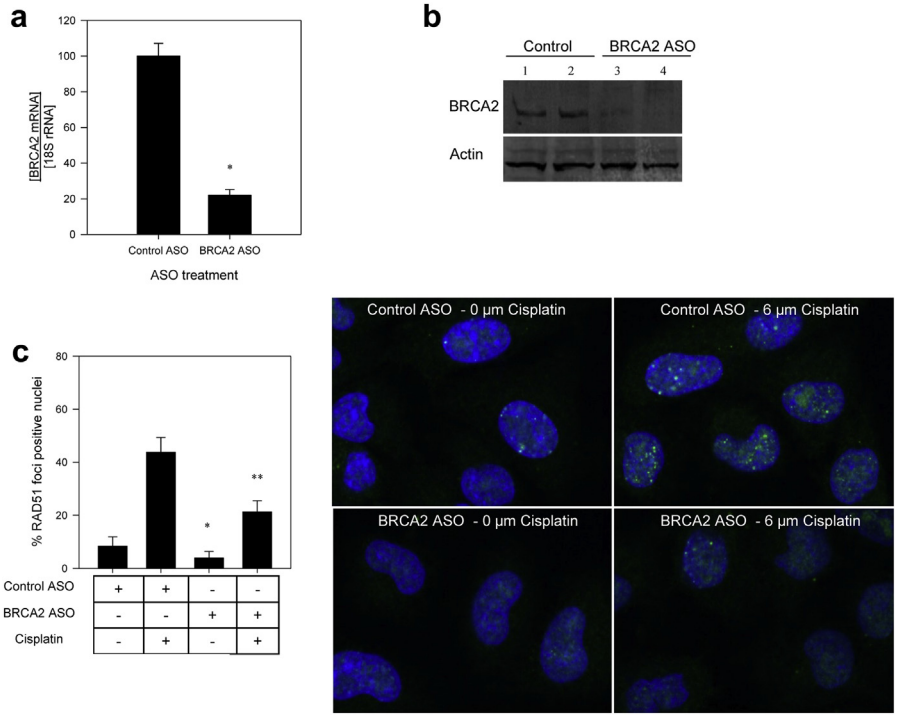

Figure 2 – BRCA2 ASO decreases BRCA2 mRNA, protein, and RAD51 foci formation frequency in A549 cells. a) A549 cells were transfected with 20 nM BRCA2 ASO and total cellular RNA was isolated 24 h later. Relative BRCA2 mRNA levels (mean ± SD) were assessed with RT-qPCR. b) BRCA2 protein levels were assessed 72 h after BRCA2 ASO transfection. c) RAD51 focus formation (mean ± SD) was quantified 48 h after BRCA2 ASO transfection. Statistical significance was determined using a Student's *t*-test ($p < 0.05$).

plus cisplatin and control ASO plus cisplatin (Figure 5d and Supplementary Figure 2a).

Cellular glucose uptake is modulated by cisplatin treatment and DNA damage (Egawa-Takata et al., 2010). We observed that cisplatin treatment of A549 cells increased glucose uptake by 60%. Pretreatment with BRCA2 ASO increased that response to cisplatin by a further 17% (Figure 5e and Supplementary Figure 2b). This raised the possibility that increased glucose entry induced by cisplatin after BRCA2 reduction might be a cellular response to generate additional energy necessary for increased DNA repair. However, decreased acidification induced by cisplatin (a measure of reduced glycolysis (Alborzinia et al., 2011)) was approximately the same regardless of ASO treatment (Figure 5c). This suggests that the BRCA2 ASO-induced increase in glucose uptake in response to cisplatin was not sufficient to enhance glycolytic activity. Furthermore, the loss of adhesion (indicative of a decrease in cell monolayer integrity (Alborzinia et al., 2011)) in response to cisplatin in cells treated with BRCA2 ASO indicates that increased glucose uptake was not capable of rescuing cells from BRCA2 ASO-mediated sensitization to cisplatin (Figure 5b).

3.6. *BRCA2 inhibition decreases metastatic frequency following cisplatin treatment*

The majority of cancer patients do not die from primary disease, but instead succumb to metastatic tumors. We therefore investigated whether concomitant BRCA2 ASO and cisplatin treatment would decrease the number of metastatic foci to a higher degree than cisplatin alone. In addition, there is some evidence linking BRCA2 mutation status to metastasis site and frequency in cancer patients (Bayraktar et al., 2013; James et al., 2009).

To ensure that the long term effects of treatment were reproducible in A549 cells before investigating metastasis directly, we repeated the *in vitro* colony formation assay in these cells. Colony formation by A549 cells treated with BRCA2 ASO and cisplatin (6 μM) was reduced by 48 ± 4% ($p < 0.05$) compared to cells treated with control ASO and cisplatin (Figure 6a). BRCA2 downregulation also decreased the ability of A549 cells to form colonies following gamma irradiation (Figure 6b), in accord with previous reports (Egawa-Takata et al., 2010).

To extend our findings to metastasis and increase their clinical relevance we utilized the chicken embryo chorioallantoic membrane (CAM) cell invasion assay to model the extravasation and invasion steps of the metastatic process *in vivo* (Arpaia et al., 2012; Cvetkovic et al., 2013). A549-GFP cells were treated with control or BRCA2 ASO in the presence or absence of cisplatin, and then injected i.v. into CAM veins. Treatment of A549-GFP cells with BRCA2 ASO alone produced a trend toward decreased metastatic focus frequency, but combined BRCA2 ASO and cisplatin treatment reduced the frequency of metastatic focus formation by 77 ± 7% ($p < 0.05$) (Figure 6c). These data indicate that intact BRCA2 function limits the ability of cisplatin to modulate *in vivo* tumor cell metastatic frequency.

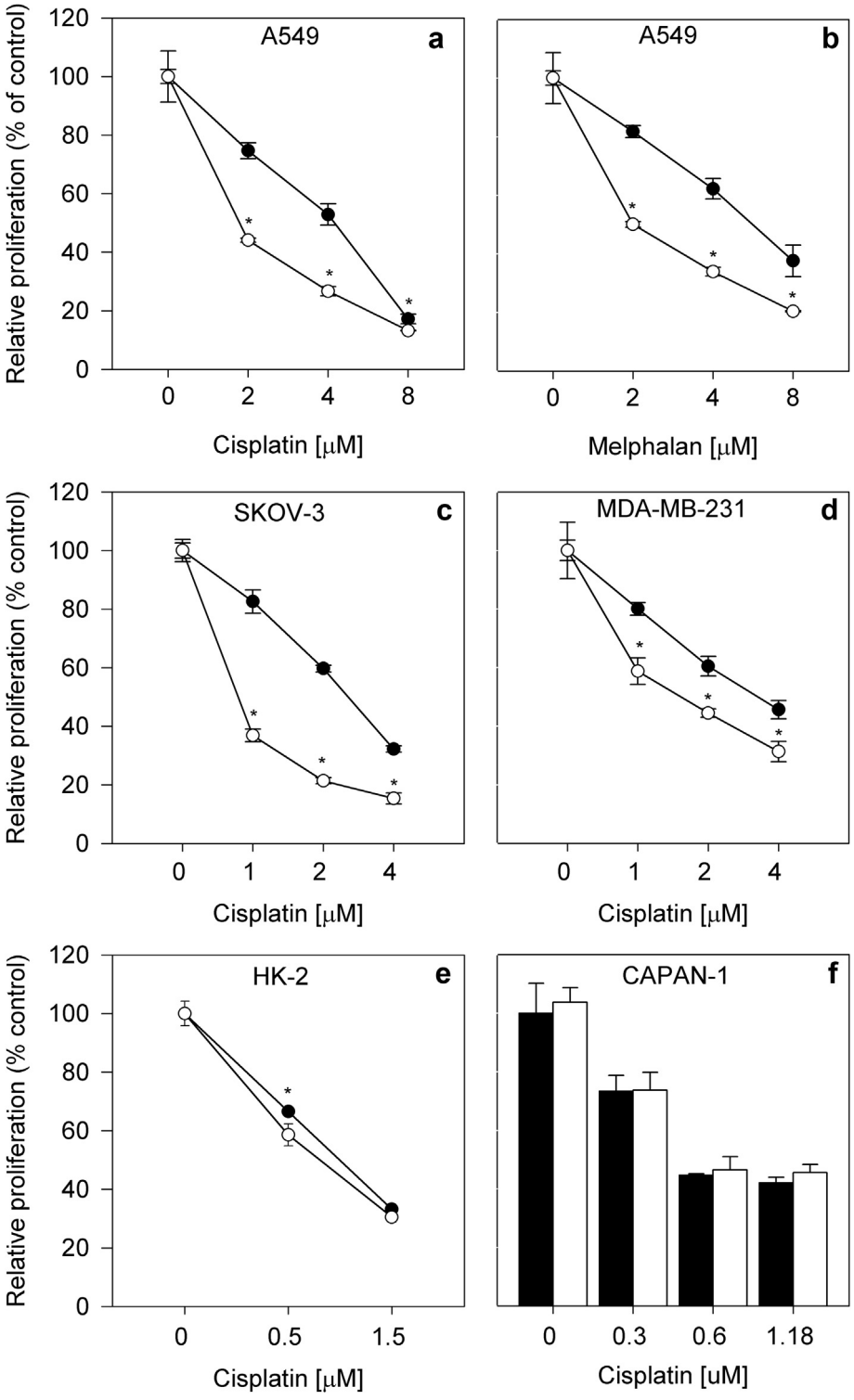

Figure 3 – BRCA2 regulates cisplatin-induced reduction of lung, ovarian, and breast tumor cell proliferation. A549 cells were treated with varying concentrations of cisplatin (a) or melphalan (b) and control (black circles) or BRCA2 (white circles) ASO. Cells were counted 96 h post-transfection to assess effects of BRCA2 knockdown. SKOV-3 cells (c) and MDA-MB-231 cells (d) were treated with different concentrations of cisplatin and control or BRCA2 ASO, and counted 96 h post-transfection. e) Non-tumor HK-2 kidney cells were treated with two concentrations of cisplatin and control or BRCA2 ASO, and relative proliferation was quantified using cell counting 96 h post-transfection. f) BRCA2 deficient CAPAN-1 cells were exposed to varying concentrations of cisplatin and control or BRCA2 ASO, and counted 96 h post-transfection. *Different from cells treated with control ASO ($p < 0.05$).

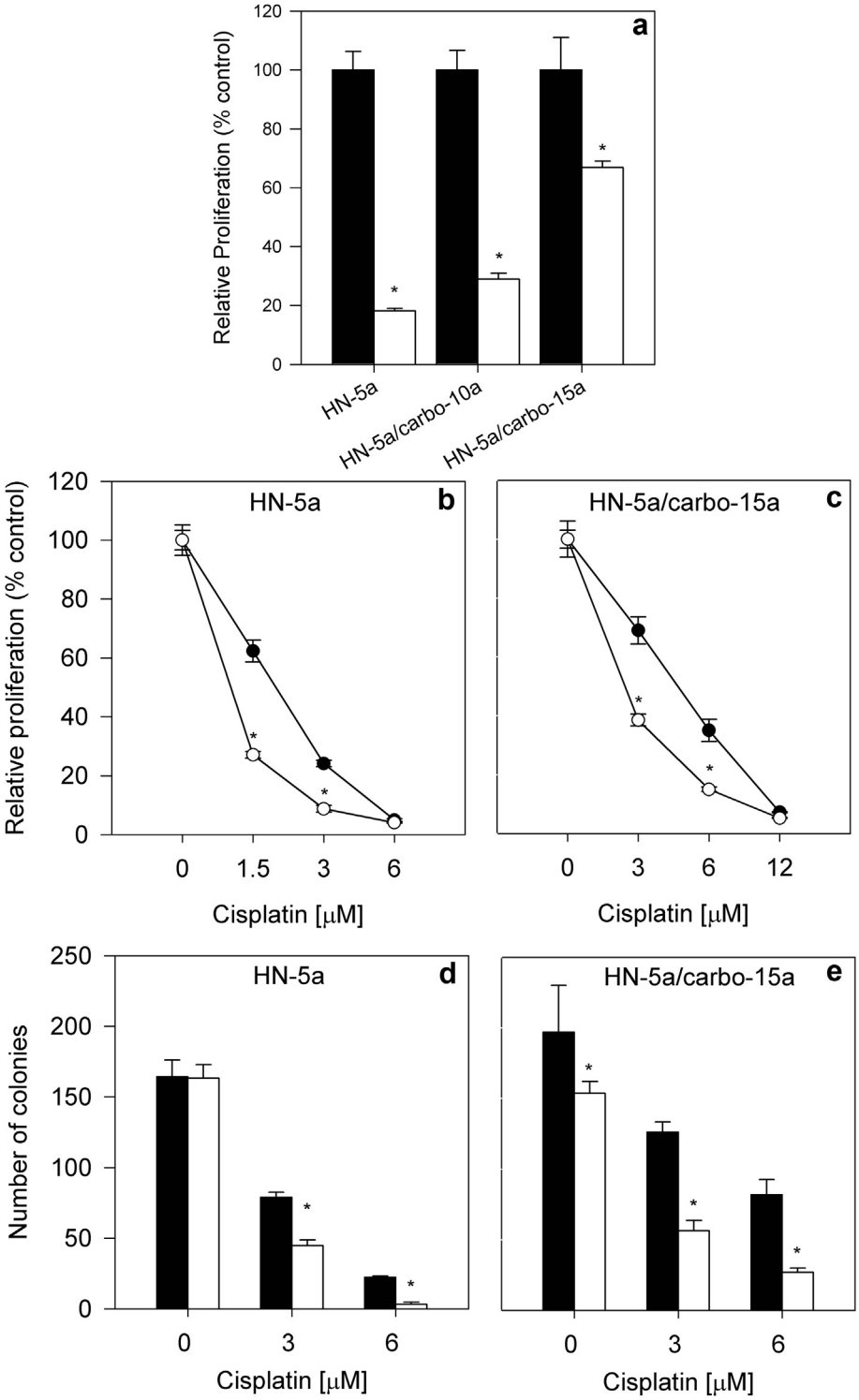

Figure 4 – BRCA2 ASO reverses acquired cisplatin resistance in human head and neck cancer cells. a) HN-5a, HN-5a/carbo-10a, and HN-5a/carbo-15a cells were exposed to cisplatin and effects on proliferation were assessed 72 h after drug treatment. HN-5a cells (b) and HN-5a/carbo-15a cells (c) were transfected with control (black circles) or BRCA2 (white circles) ASO, treated with varying concentrations of cisplatin and then counted 96 h post-transfection. HN-5a cells (d) and HN-15a cells (e) were transfected with control or BRCA2 ASO, treated with different concentrations of cisplatin for 6 h, and then re-plated at a density of 500 cells per well to determine colony forming ability. *Different from cells treated with control ASO using a Student's *t*-test ($p < 0.05$).

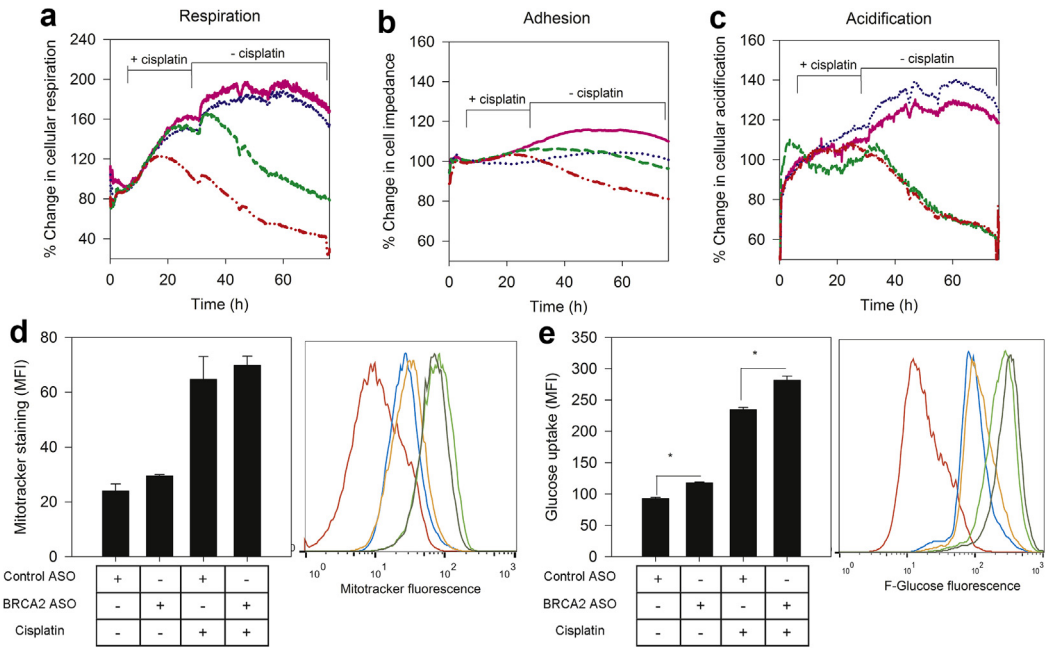

Figure 5 – BRCA2 modulates tumor cell metabolic response following cisplatin treatment. A549 cells were exposed to cisplatin (6 μM, 24 h) following 6 h of incubation in medium to determine baseline metabolic levels. At 24 h after addition of cisplatin, medium was exchanged for medium without cisplatin. Measurements of oxygen consumption (a), impedance (b), and changes in medium pH (c) were conducted. Magenta = Control ASO, Blue = BRCA2 ASO, Green = Control ASO + Cisplatin, Red = BRCA2 ASO + cisplatin. A549 cells were transfected with control or BRCA2 ASO and then treated with cisplatin (6 μM). Mitotracker staining (d) and glucose uptake (e) were determined using flow cytometry. Unstained control = Red, Control ASO = blue, BRCA2 ASO = orange, Control ASO + Cisplatin = light green, BRCA2 ASO + cisplatin = dark green. *Different from cells treated with control ASO using a Student's *t*-test ($p < 0.05$).

4. Discussion

A high level of genomic instability is a cancer-specific feature that fundamentally distinguishes tumor cells from non-cancerous cells. Chemotherapeutic agents can be used to exploit this difference in DNA fidelity by damaging DNA in an effort to preferentially push tumor cells over the edge of survival. However, tumor cells with intact DNA repair pathways are less sensitive to this strategy, and capable of acquiring treatment resistance by outgrowth of clonal populations. Therefore, DNA repair mediators represent targets for therapeutic intervention, and such a strategy may selectively affect tumor cells due to the inherent level of DNA damage relative to normal cells.

TCGA database analysis revealed that BRCA2 mutations in endometrial and ovarian cancer provide a better survival benefit than BRCA1 mutations. Although the lack of correlation does not rule out possible therapeutic advantages after inhibition of BRCA1 in patients with functional BRCA1 genes, these data suggest that BRCA2 may be a more useful target. Our analysis of human tumor sample data is in accord with previous reports that BRCA2 mutations may be beneficial for patient prognosis following drug treatment and/or radiotherapy (Maxwell and Domchek, 2012; Vencken et al., 2011) and supports the potential of BRCA2 reduction to enhance patient survival.

We developed a BRCA2-targeting ASO candidate drug to specifically sensitize cancer cells to the effects of DNA-damaging agents such as cisplatin. Our rationale was based on the known role of BRCA2 in DNA repair and clinical data that individuals with BRCA2-mutated tumors respond more favorably to chemotherapy. In addition, high levels of BRCA2 mRNA have been associated with poor patient prognosis (Egawa et al., 2002). Retrospective analysis of RNA-seq data revealed that BRCA2 mRNA is highly expressed in different types of human cancers (Figure 7), suggesting that BRCA2 may be a good target for an antisense-based drug directly targeting BRCA2 mRNA.

We showed that combining BRCA2 ASO and cisplatin treatment is an effective strategy to render different types of cancer cells more sensitive to the effects of DNA-damaging drugs. In particular, the ability of BRCA2 ASO treatment to reduce acquired cisplatin resistance in head and neck tumor cells is an important finding from a clinical perspective, since a proportion of cancer patients eventually develop resistance to platinum-based chemotherapy (Martin et al., 2008).

The fact that BRCA2 ASO did not greatly potentiate cisplatin treatment in non-cancerous HK-2 cells suggests that tumor cells may be more sensitive to this type of treatment regimen. We hypothesize this is due to differences in DNA integrity. However it remains to be determined whether this is the case, or if specific, common mutations in tumor cells predispose them to BRCA2 ASO-mediated sensitization to cisplatin.

Furthermore, an emerging phenomenon is the development of treatment resistance in previously treatment-responsive BRCA2-mutated tumors. Such tumors can

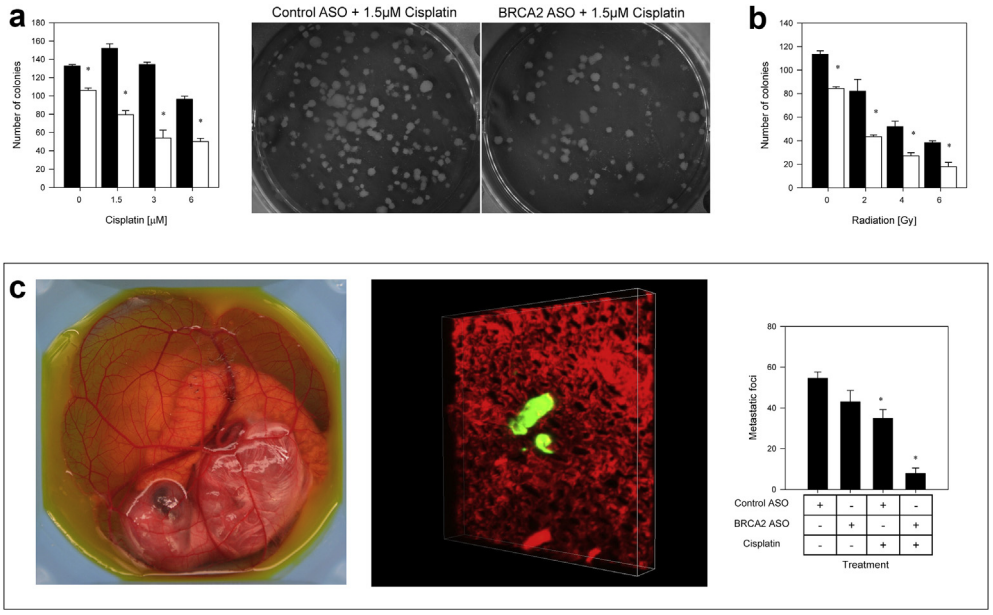

Figure 6 – BRCA2 regulates tumor cell metastatic frequency following cisplatin treatment *in vivo*. A549 cells were transfected with control (black) or BRCA2 (white) ASO and then exposed to varying concentrations of cisplatin (a) or different doses of ionizing radiation (b). Six hours after treatment, the capacity of cells to form colonies *in vitro* was determined. c) A549-GFP cells were transfected with control or BRCA2 ASO, treated with 6 μM cisplatin for 6 h, then injected i.v. into the CAM (Left and Center panel). Metastatic foci were counted 7–9 days following injection (Right panel) ($N = \geq 6$). *Different from cells treated with control ASO ($p < 0.05$).

undergo reversion mutations that re-activate BRCA2 function and lead to treatment failure (Ashworth, 2008; Sakai et al., 2009). The implications are two-fold. First, it highlights the importance of BRCA2 function for tumor cell survival in the face of strong selection pressures such as cytotoxic drug treatment. Second, it suggests that BRCA2 ASO treatment could be of benefit even in individuals with tumors composed primarily of cells harboring inactivating BRCA2 polymorphisms.

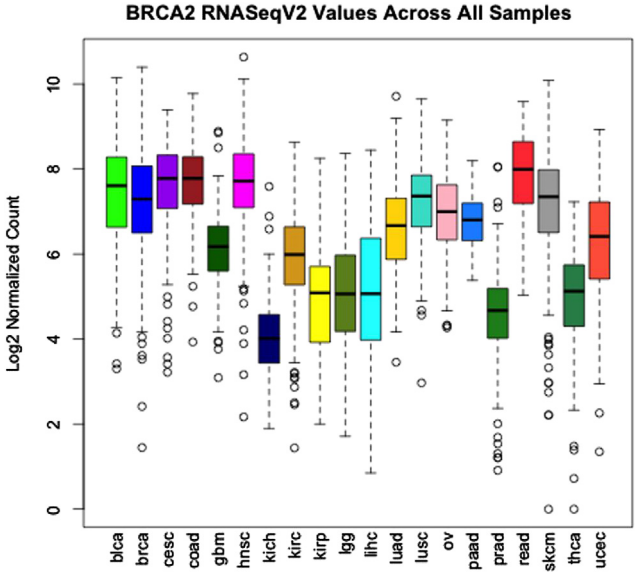

Figure 7 – BRCA2 mRNA expression levels in different human tumors. The TCGA database was queried for BRCA2 mRNA expression levels across different human cancer types.

For the first time, we linked BRCA2 with cellular metabolic response following cisplatin treatment and showed that cellular respiration is negatively affected in cells treated with BRCA2 ASO and cisplatin. We were unable to detect a difference in mitochondrial function by MitoTracker staining, suggesting that the defect in respiration is either mitochondria-independent, or not related to changes in the number of functioning mitochondria per cell. Furthermore, we identified an increase in glucose uptake that did not result in increased glycolysis, nor did it prevent a decrease in cell adhesion. Therefore, this may be a marker of cell stress and a response by affected cells to generate additional energy necessary to deal with the results of this stress. Overall, these data suggest that BRCA2 helps to maintain cellular metabolic processes following cisplatin treatment and, after reduction of BRCA2, cisplatin induces respiratory collapse and a non-productive increase in glucose uptake.

Due to the clinical importance of metastatic disease, we determined the effect of our treatment regimen on metastatic frequency. We found that ASO-mediated reduction of BRCA2 led to decreased human tumor cell metastatic frequency *in vivo* after cisplatin treatment. This suggests that intact BRCA2 function during cisplatin treatment contributes to tumor invasion. Cisplatin may be particularly effective at preventing metastases in cells without active BRCA2, presumably due to an enhanced capacity to reduce tumor cell viability. Therefore, inhibition of BRCA2 may be a useful strategy to decrease metastatic burden in patients treated with cisplatin.

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Conflict of interest

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.05.017.

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