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Small molecule inhibitor of the bone morphogenetic protein pathway DMH1 reduces ovarian cancer cell growth

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Abstract

The bone morphogenetic protein (BMP) pathway belonging to the Transforming Growth Factor beta (TGF β) family of secreted cytokines/growth factors is an important regulator of cancer. BMP ligands have been shown to play both tumor suppressive and promoting roles in human cancers. We have found that BMP ligands are amplified in human ovarian cancers and that BMP receptor expression correlates with poor progression-free-survival (PFS). Furthermore, active BMP signaling has been observed in human ovarian cancer tissue. We also determined that ovarian cancer cell lines have active BMP signaling in a cell autonomous fashion. Inhibition of BMP signaling with a small molecule receptor kinase antagonist is effective at reducing ovarian tumor sphere growth. Furthermore, BMP inhibition can enhance sensitivity to Cisplatin treatment and regulates gene expression involved in platinum resistance in ovarian cancer. Overall, these studies suggest targeting the BMP pathway as a novel source to enhance chemo-sensitivity in ovarian cancer.

Keywords

BMP; BMPRIa; DMH1; ovarian cancer; platinum resistance

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The authors have no conflicts of interest to declare.

1. Introduction

Ovarian cancer is the leading cause of cancer deaths from gynecological malignancies [1]. It is estimated that in 2015, 21,290 women will be diagnosed with ovarian cancer and over 14,000 women will die from ovarian cancer [1; 2]. The five year survival rate is dismal at 45.6% and despite advances in treatment, the survival rates have not improved [3]. Ovarian cancer remains deadly due to its invasive nature leading to metastasis, late diagnosis, tumor heterogeneity and resistance to chemotherapy. Recently, the bone morphogenetic protein (BMP) pathway has been discovered to play critical roles during tumor formation and progression in ovarian cancers [4].

The BMP pathway is a complex director of development and part of the transforming growth factor beta (TGF β) family. BMP ligands, which are secreted by many cell types, bind to type I and type II serine/threonine kinase receptors. Upon ligand binding, type I receptors phosphorylate Smads 1, 5 and 8(mouse)/9(human), which then bind with Smad4. The complex is then translocated to the nucleus to regulate transcription of target genes. Canonical BMP target genes in response to ligand stimulation are *ID1* and the inhibitory Smad, Smad6, which functions in a negative feedback manner thus tightly regulating BMP signaling [5; 6; 7].

BMP signaling was originally characterized as tumor suppressive. Similar to TGF β , in cancer cells, the role of BMP signaling is becoming increasingly complex showing dual tumor suppressive and promoting roles. The basis of BMP tumor suppression originated with the discovery that patients with mutations in BMPR1a effectively inhibiting BMP signaling developed Juvenile Polyposis Syndrome associated hamartomas. Concordantly, mice with targeted deletion of BMPR1a in skin develop similar hamartomatous lesions [8; 9; 10; 11; 12]. When the type II BMP receptor, BMPR2, is expressed as a dominant negative in a mouse model of breast cancer, it enhances tumor metastasis [13]. Many studies have shown that treatment of normal and cancerous cells with BMP ligands reduces cell proliferation and growth and, similar to TGF β treatment, induces transcription of p21/27/57 to repress the MYC oncogene [14; 15; 16; 17]. In addition it has been shown that treatment of cells with BMP ligand antagonists Noggin and Coco can lead to increased cell proliferation and promote metastasis [18; 19]. Recently, it has been shown that breast cancer cells show enhanced cell migration and invasion when treated with BMP ligands [20; 21]. In addition, when BMP ligands are overexpressed in various cancer cells such as prostate cancer, they can demonstrate tumor-promoting phenotypes such as increased invasion and metastasis [22]. Recent reviews have highlighted these dual roles for BMPs in cancer [23; 24].

In ovarian cancer, BMP signaling has been shown to have dual tumor suppressive and promoting roles as well. For example, treatment of ovarian cancer cells with recombinant BMP-2 has an anti-proliferative effect [14] and constitutively active BMPR1a receptor activity limits metastasis of ovarian cancer cells through decreased adhesion [25]. Additionally, the BMP receptors BMPR1a and BMPR1b have redundant roles that can suppress ovarian tumor formation [26]. Conversely, BMP tumor promoting activity has been demonstrated in both ovarian tumor cells and the surrounding microenvironment [27; 28].

BMP stimulation can enhance the proliferation of ovarian cancer cells [4; 29; 30; 31]. Furthermore, BMP signaling has been shown to increase ovarian cancer cell motility and migration via EMT and other pathways such as AKT [32; 33; 34]. Ovarian cancer is known to be heterogeneous at the cellular and molecular level and it remains unknown, which contexts dictate BMP function as tumor suppressive or promoting. We find that BMP activity is endogenously high in human ovarian cancers and in our model of ovarian cancer, BMP inhibition restricts growth and cooperates with standard platinum based chemotherapy.

2. Materials and Methods

2.1 Cell culture and tumor sphere assay

Cells were grown in DMEM medium with 10% Fetal Bovine Serum (FBS) and Triple antibiotic/antimycotic (Life Technologies). Cells grown as spheres were placed in ultra-low adhesion culture 6-well plates (Corning) and after five days treated with drug and allowed to grow for five additional days. Sphere media was comprised of DMEM/F12 with triple antibiotic/antimycotic, 20ng/ml EGF and 1× B27 supplement (Life Technologies). Spheres were analyzed using the Gelcount automated colony counting system. Equal sized masks were used in analysis and performed in triplicate. Routine mycoplasma testing was performed and cells were treated for 24 hours prophylactically with ciprofloxacin for 24 hours (GeneHunter). DMH1 was used at 20uM for all experiments and Cisplatin was used at 1uM, and equal quantities of DMSO were used as vehicle control.

2.2 Immunohistochemistry

A Tissue microarray comprised of 209 samples from patients diagnosed with ovarian cancer was constructed at Vanderbilt University Medical Center (VUMC) as previously described [35]. All samples consisted of tumor tissue of various grades and histologies.

Tumor spheres were formalin fixed, placed into Histogel (Harlan Scientific), were embedded and sectioned at 5 µM and dewaxed in xylene and rehydrated in alcohol with citrate antigen retrieval as previously described [13]. Standard Mayer's hematoxylin and eosin (H&E) was performed. The sections were immunostained using antibodies for Cleaved Caspase-3 (Cell Signaling Cat#9661, 1:200) and Ki-67 (Novostra Cat#ki67-mm1, 1:200) Paraffin derived sections were counterstained with hematoxylin (Vector Labs QS) and mounted with Cytoseal. Quantification of IHC and IF was performed using NIH ImageJ as previously described [36].

2.3 RNA isolation, cDNA synthesis, qPCR and primer selection

RNA isolation was performed with RNeasy purification including DNaseI treatment (Qiagen). Equal amounts of RNA were synthesized into cDNA using the VILO cDNA synthesis kit (Invitrogen). LuminoCt (Sigma) 2× SYBR mastermix was combined with 1 µM of both a forward and reverse primer sequence (primer sequences are listed in Supplemental Table 1) into 20 µl reactions and cycled for 95°–10 s to 60° for 30 s for 40 cycles followed by a melting curve. BioRad CFX96 was used and instrument provided software was used to determine relative normalized expression to *GAPDH* expression.

2.4 Western blot

Total protein was isolated using Complete LysisM Buffer (Roche). Protein was diluted to equal concentrations and equally loaded on 10% polyacrylamide gels prior to transfer to a nitrocellulose membrane. Protein concentration was determined using micro plate BCA assay (Pierce). Blots were incubated overnight with Smad1 (Cell Signaling Cat#6944 1:1000), pSmad1/5 (Cell Signaling Cat#9516 1:1000), and Actin (Sigma Cat#A2066 1:4000) antibodies. HRP-conjugated secondary antibodies were used to visualize band intensity via x-ray film exposure using ECL western substrate (Perkin Elmer).

2.5 Human breast cancer databases and statistical analysis

For analysis of the TCGA data set, we used the cBio portal (<http://www.cbioportal.org/>) [31; 37]. We queried the TCGA provisional serous ovarian dataset which was comprised of 594 patients and 609 tumors and the CCLE dataset which contained 50 ovarian cancer cell lines. Human gene symbols were queried and accessed on 11 March 2015. RNA expression cutoff was adjusted to 2.0. Analysis of gene expression correlating with RFS was performed using the kmplotter (<http://kmplot.com>). Human gene symbols were entered into ovarian (GSE14764), and JetSet probe selection was used to determine optimal representative microarray probe [38]. Automatic cutoff scores were selected during queries and PFS were selected. Statistical analysis was performed using Excel (Microsoft, Redmond, WA, USA), Prism (Graphpad, La Jolla, CA, USA), and FlowJo (TreeStar, Ashland, OR, USA) software. Statistical significance was deemed for any comparison where $P < 0.05$.

3. Results

3.1 BMP expression is amplified and active in ovarian cancer and correlates with poor relapse free survival

The BMP pathway has many ligands and their expression in cancer has only recently been addressed. We queried The Cancer Genome Atlas (TCGA) using the [cBIO.org](http://www.cbioportal.org/) portal from Memorial Sloan Kettering Cancer Center and specifically selected the serous ovarian cancer data set. We found that BMP ligands were rarely deleted or downregulated and were commonly upregulated, with BMP7 upregulation or amplification in 17% of patients with serous ovarian cancer (Figure 1A). The datasets provided from the TCGA are from whole tumor tissue samples and can represent gene expression from the tumor microenvironment in addition to the tumor cells. To assess BMP signaling specifically in tumor cells, we analyzed BMP ligand expression in ovarian cancer cell lines, which have been profiled in the Cancer Cell Line Encyclopedia (CCLE) and are available through the [cBIO.org](http://www.cbioportal.org/) portal. Upon analysis of the cell lines, we found that most cell lines showed amplification or upregulation of BMP ligands. BMP7 was the most frequently altered, upregulated or amplified in 32% of the ovarian cancer cell lines (Figure S1A). In addition to investigating BMP ligands, we looked at the expression of TGF β family receptors and co-receptors in ovarian cancer patients in the TCGA data set and found all receptors to be altered in only 10% or less of patients. The majority of these alterations were amplifications or upregulations (Figure S1B).

Next, we looked at the association between gene expression of BMP signaling molecules and survival using [kmplot.com](https://www.kmplot.com). The gene expression of two type I receptors, ACVR1 (also known as ALK2) and BMPRIa (also known as ALK3) is correlated with poor progression free survival (PFS) in human ovarian cancer (Figure 1B). To examine active BMP signaling within ovarian cancer, we analyzed the phosphorylation of Smads 1/5/9 by immunohistochemistry using a TMA comprised of 209 samples of ovarian cancer of various grades, stages and histologies. Our results revealed heterogeneous, yet positive staining throughout all grades and stages of ovarian cancers. p-Smad1/5/9 was expressed in both the tumor epithelium and the surrounding stroma (Figure 1C).

3.2 Human ovarian cell lines display cell endogenous BMP activity that when inhibited decreases sphere size

We investigated three commonly used human ovarian cancer cell lines, OVCAR3, OVCAR8 and SKOV3 and found that all untreated cells displayed active BMP signaling indicated by phosphorylation of Smads1,5 and 9 in a cell autonomous fashion (Figure 2A). Using DMH1, a small molecule kinase inhibitor for the type I BMP receptors, at 20uM for 24 hours we could reduce the canonical phosphorylation of Smads 1,5 and 9. Interestingly, we found that acute treatment of these cells with Cisplatin at 1uM for 24 hours also resulted in a reduction of BMP Smad phosphorylation, and when combined with DMH1 eliminated the remaining detectable phosphorylated Smads 1,5 and 9 (Figure 2A). When ovarian cancer metastasizes, it can form spheroids that can invade and travel to distant sites [39; 40]. To mimic this phenomenon *in vitro*, cancer cells can be grown as spheroids using ultra-low adhesion cell culture plates. We grew OVCAR3, OVCAR8 and SKOV3 cells in spheroid conditions for ten days and found that OVCAR8 had the most efficient sphere forming ability and produced the largest number of spheres from an initial seeding of 50,000 cells (Figure S2A). OVCAR8 cells were also the largest in size and made well-defined spheres within ten days of growth (Figure S2B). Interestingly, when we closely examined the spheres, we noticed that OVCAR3 cells were mostly hollow and created cyst-like structures (Figure S2B). SKOV3 cells formed the fewest number of spheres and tended to grow in aggregates and made poor cell-cell connections (Figure S2B).

We continued with a focus on OVCAR8 cells because they showed the greatest ability to be able to form spheres and spheroid cells have been associated with increased tumor cell aggressiveness [41]. We sought to see if BMP inhibition and Cisplatin treatment could affect the growth of tumor spheres. Using ultra-low-adherent culture 6-well culture plates we seeded 50,000 OVCAR8 cells in triplicate for five days. After five days, small spheres had begun to form and we treated the tumor spheres once with DMH1 (20uM), Cisplatin (1uM), or both for five additional days. We measured sphere volume and diameter and following tumor sphere measurements we Formalin-Fixed Paraffin Embedded (FFPE) spheres into Histogel and perform Hematoxylin and Eosin (H&E) staining (Figure 2B, C). We found that DMH1 was sufficient to inhibit the growth of tumor spheres by total volume and sphere size (Figure 2B). Interestingly, Cisplatin treatment alone did not affect sphere size or volume; however in combination with DMH1 we observed a significant reduction in sphere size and volume (Figure 2B).

3.3 Cisplatin resistant cell lines respond to BMP inhibition

Subsequently we wanted to examine BMP inhibition in the context of chemotherapy resistance. NCI-RES is a chemo-resistant cell line derived from OVCAR8 cells [42]. We repeated our treatments of DMH1 and Cisplatin on OVCAR8 cells and NCI-RES cells grown as spheres and observed that treatment with DMH1 as a single agent was able to inhibit sphere size in OVCAR8 cells, but not in NCI-RES cells. However, when used in combination, treatment with DMH1 (20 μ M) and Cisplatin (1 μ M) for five days resulted in a significant reduction in sphere volume and size in NCI-RES resistant cells (Figure 3A). To investigate if the changes in sphere volume and size were due to proliferation, we performed IHC for the proliferation marker Ki-67 on FFPE tumor spheres from the various treatment groups after ten days of growth in suspension (Figure 3B). We found that Ki-67 positive staining was significantly decreased in response to combination therapy of DMH1 and Cisplatin in the OVCAR8 cells (Figure S3A). However, there was no significant change in proliferation, measured by the presence of Ki-67, after combination therapy in the NCI-RES cells. We next investigated cell death in the spheres in response to treatment by analyzing the expression of cleaved Caspase-3 by IHC (Figure 3C). We found no significant changes in the expression of cleaved Caspase-3 in any tumor spheres in response to treatment (Figure S3B).

3.4 BMP inhibition regulates genes associated with Cisplatin resistance

The regulation of gene expression has recently been associated with resistance in ovarian cancer cells. Recent large genomic studies have begun to identify gene expression patterns which are altered in ovarian Cisplatin resistant cells [43; 44; 45]. We treated OVCAR8 and NCI-RES cells with acute 24-hour treatment of DMH1 (20 μ M), Cisplatin (1 μ M), or both and isolated RNA. Next we analyzed the panel of genes shown to be up or downregulated in chemo-resistant ovarian cancer cells by Li et al by qPCR. We found that BMP inhibition regulates the expression of 4 genes shown to be associated with resistance: *ROBO2*, *JAG1*, *CY1B1* and *HAPLN1*. *ROBO2* and *JAG1*, which were shown to be downregulated in Cisplatin resistant ovarian cancer cells, were significantly increased in response to combination therapy of DMH1 and Cisplatin in both Cisplatin sensitive OVCAR8 cells as well as insensitive NCI-RES cells [43]. *ROBO2* mRNA was significantly increased only when treated with both DMH1 and Cisplatin. *JAG1* however was significantly upregulated after DMH1 treatment alone or in combination with Cisplatin (Figure 4A). Previously *CY1B1* and *HAPLN1* were shown to be upregulated in Cisplatin resistant cells [43]. We found that *CYP1B1* was reduced with single agent DMH1 treatment in both OVCAR8 and NCI-RES cells. *HAPLN1* expression showed a trending increase in expression with single agent exposure to either DMH1 or Cisplatin, but when combined, gene expression was decreased significantly in both OVCAR8 and NCI-RES cells (Figure 4B).

Validation of the canonical BMP response genes, *ID1* and *SMAD6*, revealed that only treatments including DMH1 directly decrease the BMP response (Figure S4A). In previous reports of genes linked to Cisplatin resistance in ovarian cancer cells, there were two BMP signaling molecules that were found to be downregulated, *BAMBI* and *CHRD1* [43]. We performed qPCR for these genes and found that Cisplatin treatment decreased *BAMBI* mRNA levels in OVCAR8 cells, yet combined treatment in NCI-RES cells led to an

increase in *BAMBI* gene expression (Figure S4B). *CHRDLL1* expression was only significantly decreased in response to Cisplatin in the OVCAR8 cells and was not significantly altered in response to BMP inhibition in either cell line (Figure S4B).

4. Discussion

The BMP pathway is crucial to normal ovarian function and has been shown to regulate hormones, ovulation and oocyte development [46]. We and others have recently shown BMP signaling to be active in and regulate ovarian cancer cells as well. It has previously been shown that BMP ligands and signaling is increased in ovarian cancer cells in comparison to normal ovarian cells [46; 47]. Similar to previous studies, here we show that BMP ligands and receptors are overexpressed in more than 50% of ovarian cancer tumors and ovarian cancer cell lines (Figure 1, Supplementary Figure 1) and is endogenously active in ovarian cancer cell lines (Figure 2). Additionally we found that BMP signaling is active in ovarian cancer samples across multiple grades, stages, histology and in both chemo-sensitive and resistant cells (Figure 1).

The BMP pathway has been historically thought of as tumor suppressive, but as a large amount of genetic data from human patients becomes more available, BMPs have emerged as possible tumor promoters. Recently the BMP ligands, BMP-2 and BMP-4, have been shown to have prognostic association with a poor outcome for ovarian cancer patients [48; 49; 50]. We chose to look at the receptor partners for BMP signaling ACVR1 (ALK2) and BMPRIa (ALK3), which we determined are also correlated with poor outcome (Figure 1B). Recent work looking at the downstream mediator of canonical signaling, Smad5, has also been implicated in poor survival in ovarian cancer patients [4].

Inhibition of the BMP pathway has only recently been investigated as a putative target in cancer therapies. BMP small molecule inhibitors have radically progressed from toxic non-selective/specific inhibitors such as Dorsomorphin and LDN-193189 to more selective and specific inhibitors such as DMH1 [51; 52; 53; 54; 55]. Other groups have used Dorsomorphin and LDN-193189 for successful outcomes in many cancer types such as Lung, Prostate, Breast and acute lymphoblastic leukemia (ALL) [56; 57; 58; 59]. We have previously reported the successful use of DMH1 in metastatic breast cancer mouse model both on the tumor cells as well as the surrounding tumor microenvironment [60]. Here we show that as a neoadjuvant, DMH1 enhanced acute low doses of Cisplatin and reduced ovarian cancer sphere growth.

One of the primary assays we used to assess the efficacy of DMH1 was the sphere assay. One of the unique characteristics of ovarian cancer cells is their ability to form spheres during the process of metastasis [33, 34]. Therefore, these spheres are crucial to investigate as they play a primary role in disease progression. As shown in Figure 3, BMP inhibition reduced sphere size yet not proliferation or apoptosis, indicating that sphere growth is an important and independent factor to consider, specifically in ovarian cancer studies.

Most notably, DMH1 was able to enhance the response of chemo-resistant cells to treatment. Currently the standard of treatment for ovarian cancer patients is to receive a combination

therapy of platinum and taxane based treatments. Resistance to platinum agents, such as Cisplatin, remains a significant source of morbidity [43; 61] and overcoming resistance is a key goal in ovarian cancer treatment [62; 63; 64; 65]. The discovery of new targets mediating resistance and overcoming or reversing resistance to chemotherapy has begun to emerge [35; 44; 66; 67; 68]. Recent gene expression studies have linked not only the BMP pathway but also the Epithelial to Mesenchymal Transition (EMT) process to chemoresistance [43; 61; 69; 70]. The BMP pathway plays an important role in EMT as it not only promotes the reverse process MET, but also antagonizes TGF β directed EMT [71; 72; 73].

In our studies, we found that dual therapy of DMH1 and Cisplatin altered sphere size and gene expression of chemo-resistant cells in a distinct manner from single-agent therapies (Figure 3A, Figure 4). Sphere size of these cells was only significantly decreased in response to dual treatment. Interestingly, inhibition of BMP signaling in NCI-RES cells was able to upregulate genes that were shown to be downregulated during chemo-resistance and downregulate genes shown to be upregulated during chemo-resistance (Figure 4) indicating the ability of BMP inhibition to affect drug resistance. We saw unique effects of adjuvant therapy specifically on the expression of *ROBO2* and *HAPLN1* highlighting the distinct, synergistic effect of dual therapy on resistant cells (Figure 4). This may also indicate that *ROBO2* and *HAPLN1* could act as markers of overcoming resistance due to the fact that only combined significantly affects the gene expression. Our findings suggest that the addition of DMH1 to the current chemotherapeutic regimens may enhance the ability of chemo-resistant cell lines to respond.

We are optimistic that based on our findings, the BMP/TGF β pathway is a targetable pathway in all ovarian cancer stages, grades and histology and that inhibition of the BMP pathway could positively affect patient response to the current chemotherapeutic agents used. More broadly, our findings show that as large datasets become available, we will be able to more accurately define pathways and signaling molecules that are important to each cancer subtype. As a result we will be able to effectively combine multiple drugs, making substantial improvements to the current therapies. Ultimately we hope that our findings will set the stage for advanced studies with additional cell lines and drug treatments to elucidate the role of BMP signaling in ovarian cancer cells and chemo-resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Bone morphogenetic protein (BMP) ligands are overexpressed in ovarian cancer.

BMP receptors increased expression correlate with poor progression-free-survival (PFS).

Small molecule BMP receptor inhibitor DMH1 reduces ovarian cancer cell growth.

DMH1 enhances effect of cisplatin chemotherapy and in cisplatin resistant cell lines.

Figure 1. BMPs are widely expressed in human ovarian cancer and BMP receptors correlate with poor survival

A. OncoPrint from cBio.org indicating the percent change in BMP ligands expressed in the provisional TCGA human serous ovarian cancer dataset. B. Progression Free Survival (PFS) based on gene expression of BMP receptors. C. IHC for pSmad1/5/9 in primary human ovarian cancers grade 1 and grade 3. Black arrows indicate positive staining. Scale bars indicate 50 μ m.

Figure 2. Human ovarian cell lines have active BMP signaling

A. Western blot for three human ovarian cancer cell lines OVCAR3, OVCAR8 and SKOV3 reveals active BMP signaling by the presence of phospho-Smad1/5/9. Cells were treated with DMH1 (20μM), Cisplatin (1μM), or both for 24 hours as indicated by a plus symbol. B. OVCAR8 cells grown in tumor sphere conditions for ten days were treated with DMH1 (20μM), Cisplatin (1μM), or both on day 5 after seeding. C. Tumor spheres embedded in histogel were stained for H&E. Error bars indicate SEM. *p<0.05, **p<0.01. Scale bars indicate 100μm.

Figure 3. Cisplatin resistant cell lines respond to BMP inhibition

A. OVCAR8 (white bars) and NCI-RES (black bars) cells were grown as tumor spheres and were treated after five days of culture with DMH1 (20μM), Cisplatin (1μM), or both for five days. B. Tumor spheres were FFPE and IHC for proliferation marker Ki-67 was performed. C. IHC for apoptotic marker cleaved Caspase-3 was performed on FFPE tumor spheres. Error bars indicate SEM. *p<0.05, **p<0.01. Scale bars indicate 100μm.

A Genes downregulated during chemo-resistance

B Genes upregulated during chemo-resistance

Figure 4. BMP inhibition regulates gene expression associated with ovarian Cisplatin resistance mRNA was collected from OVCAR8 (white bars) and NCI-RES (black bars) cells treated with DMH1 (20 μ M), Cisplatin (1 μ M), or both for 24 hours. to measure expression of genes A. qPCR analysis of *ROBO2* and *JAG1* which are downregulated in Cisplatin resistant cells. B. qPCR analysis of *CYP1B1* and *HAPLN1* which are upregulated in Cisplatin resistant cells. mRNA is normalized to *GAPDH* levels and relative to DMSO treated cells. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$.